The Evolutionarily Conserved Mediator Subunit MDT-15/MED15 Links Protective Innate Immune Responses and Xenobiotic Detoxification

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Abstract

Metazoans protect themselves from environmental toxins and virulent pathogens through detoxification and immune responses. We previously identified a small molecule xenobiotic toxin that extends survival of Caenorhabditis elegans infected with human bacterial pathogens by activating the conserved p38 MAP kinase PMK-1 host defense pathway. Here we investigate the cellular mechanisms that couple activation of a detoxification response to innate immunity. From an RNAi screen of 1,420 genes expressed in the C. elegans intestine, we identified the conserved Mediator subunit MDT-15/MED15 and 28 other gene inactivations that abrogate the induction of PMK-1-dependent immune effectors by this small molecule. We demonstrate that MDT-15/MED15 is required for the xenobiotic-induced expression of p38 MAP kinase PMK-1-dependent immune genes and protection from Pseudomonas aeruginosa infection. We also show that MDT-15 controls the induction of detoxification genes and functions to protect the host from bacteria-derived phenazine toxins. These data define a central role for MDT-15/MED15 in the coordination of xenobiotic detoxification and innate immune responses.

Introduction

In nature, organisms encounter environmental insults, such as chemical toxins, secreted microbial virulence factors and invasive pathogens, that threaten their ability to survive and reproduce. As a result, metazoans have evolved protective pathways to counter these challenges. For example, gene families such as cytochrome P450s (CYPs), glutathione-s-transferases (GSTs), and UDP-glucuronosyltransferases (UDPs) detoxify xenobiotic small molecule toxins and are conserved from nematodes to humans [1]. Likewise, innate immune defenses provide protection from invasive pathogens [2]. Recent publications have suggested that recognition of xenobiotic toxins is involved in the activation of immune response pathways [3,4]. From an evolutionary perspective, it is logical that hosts respond to threats encountered in the wild at least in part through surveillance pathways that monitor the integrity of core cellular machinery, which are often the targets of xenobiotic small molecules or microbe-generated toxins. These studies predict that organisms may integrate detoxification and immune responses as a means to respond rapidly to such challenges, but the mechanisms underlying this coordinated host response have not been reported.

Our research group and others use bacterial and fungal pathogenesis assays in the nematode Caenorhabditis elegans to investigate mechanisms of immune pathway activation in intestinal epithelial cells [2]. Genetic analyses of C. elegans that are hypersusceptible to bacterial infection have revealed that the nematode mounts defense responses through evolutionarily conserved innate immune pathways. For example, the C. elegans NSY-1/SEK-1/PMK-1 Mitogen Activated Protein (MAP) kinase pathway, orthologous to the ASK1 MAP kinase kinase/MKK3/6 (MAP kinase kinase)/p38 (MAP kinase) pathway in mammals, is required for protection against pathogens [5]. C. elegans animals carrying loss-of-function mutations in this pathway have defects in the basal and pathogen-induced expression of immune effectors and are hypersusceptible to killing by bacterial and fungal pathogens [5–7].

We previously used a C. elegans pathogenesis assay as a means to identify small molecules that protect the host during bacterial infection [8]. One of the compounds identified in this screen, a small molecule called RPW-24, extended the survival of nematodes infected with the human bacterial pathogen Pseudomonas aeruginosa by stimulating the host immune response via the p38 MAP kinase PMK-1 pathway [9]. A genome-wide microarray analysis of animals exposed to RPW-24 revealed that, in addition
to inducing the transcription of putative immune effectors, this molecule also strongly upregulated Phase I and Phase II detoxification enzymes (CYPs, GSTs and UDPs), suggesting that RPW-24 is a xenobiotic toxin to *C. elegans*. Consistent with this hypothesis, RPW-24 caused a dose dependent reduction of nematode lifespan on nonpathogenic food and delayed development of animals that were exposed starting at the first larval stage.

Here we sought to use RPW-24 as a tool to characterize mechanisms of p38 MAP kinase PMK-1 pathway activation in *C. elegans*. We found that activation of PMK-1-regulated pathogen response genes is genetically linked to the induction of genes involved in the detoxification of small molecule toxins. We show that the evolutionarily conserved Mediator subunit MDT-15 function has important physiological effects on the ability of an animal to mount protective immune responses, resist bacterial infection and survive challenge from lethal bacterial toxins.

**Results**

**RNAi Screen Identifies Regulators of p38 MAP Kinase PMK-1-Dependent Genes**

To investigate mechanisms of immune activation in *C. elegans*, we generated a GFP transcriptional reporter for the immune response gene *F08G5.6*. *F08G5.6* is a putative immune effector that contains a CUB-like domain [6] and is transcriptionally induced by exposure of *C. elegans* to several bacterial pathogens, including *P. aeruginosa* [6,10]. We chose *F08G5.6* for these studies because it is upregulated more than 100-fold by RPW-24 in a manner that requires the p38 MAP kinase PMK-1 [9]. *pF08G5.6::GFP* was induced in the *C. elegans* intestine during *P. aeruginosa* infection and GFP expression was also robustly upregulated in *pF08G5.6::GFP* animals following exposure to RPW-24 when animals were feeding on nonpathogenic *E. coli* (Figure 1A). When three components of the p38 MAP kinase PMK-1 signaling cassette were individually knocked down by RNAi [tir-1 [11], pmk-1 [6] and *orf-7* [12]], *pF08G5.6::GFP* induction by RPW-24 was entirely abrogated (Figure 1A).

The level of *F08G5.6* induction during bacterial infection is dependent upon the virulence of the invading pathogen (Figure S1). We exposed *C. elegans* to several *P. aeruginosa* strains, each of which was previously shown to have a different pathogenic potential toward nematodes [13] and used qRT-PCR to determine the expression levels of *F08G5.6* in these animals. In general, more pathogenic *P. aeruginosa* strains caused significantly greater induction of *F08G5.6*, suggesting that some aspect of *P. aeruginosa* virulence, rather than a structural feature of the bacteria itself, causes the activation of *F08G5.6*. Consistent with these data, it was previously shown that *C. elegans* primarily responds to virulence-related cues to mount its innate immune defenses towards *P. aeruginosa* [14–16].

To identify genes that regulate the p38 MAP kinase PMK-1 pathway in response to RPW-24, we screened a library of RNAi clones corresponding to 1,420 genes expressed in *C. elegans* intestinal epithelium (approximately 9% of the genome, Table S1B) for gene inactivations that abrogated the RPW-24-mediated induction of *pF08G5.6::GFP*. We specifically focused on intestinally expressed genes because of the recognized role for intestinal cells in coordinating the host response to ingested pathogens [2,17], and because *P. aeruginosa* and RPW-24 induce *F08G5.6* expression in the intestine (Figure 1A). Our initial screening effort identified 153 genes that, when inactivated by RNAi, diminished or abrogated the induction of *pF08G5.6::GFP* by RPW-24.

We took several steps to identify specific regulators of p38 MAP kinase PMK-1-dependent immune effectors among these 153 gene inactivations. First, we noticed that knockdown of many of these genes markedly slowed nematode growth. To eliminate genes that simply reduced GFP reporter expression as a consequence of pleiotropic effects on worm growth and development, we determined if these 153 gene inactivations also affected induction of the *C. elegans* immune reporter *irg-1::GFP* [14]. *irg-1* is strongly upregulated in intestinal epithelial cells during *P. aeruginosa* infection or by an *E. coli* strain that expresses the bacterial virulence factor Exotoxin A (ToxA), but via a pathway independent of p38 MAP kinase PMK-1 signaling [14–16]. Moreover, RPW-24 does not cause the induction of *irg-1::GFP* and mutation of the *zip-2* gene, which encodes the transcription factor that regulates *irg-1* expression, does not affect the RPW-24-mediated induction of *F08G5.6* (data not shown) or alter the ability of RPW-24 to extend the survival time of nematodes infected with *P. aeruginosa* [9]. We therefore discarded the genes that, when inactivated, reduced the induction of *irg-1::GFP* by *E. coli* expressing ToxA, reasoning that they were unlikely to be specific regulators of the p38 MAP kinase PMK-1-dependent pathogen response genes. Using this approach, we selected 56 of the 153 genes for further study.

In a tertiary screen, we determined the effects of these 56 gene inactivations on *pF35E12.5::GFP*, a second immune reporter that is also strongly induced in the intestine by RPW-24 in a PMK-1-dependent manner [9,10]. 29 of the 56 RNAi clones reduced or eliminated the induction of both the *pF35E12.5::GFP* and *pF08G5.6::GFP* reporters by RPW-24 (Table S1A). Validating the screen, the 29 clones we identified as putative regulators of the p38 MAP kinase PMK-1-dependent genes included the three known components of the p38 MAP kinase PMK-1 pathway that were present in the screening library, which suggested that the screen could identify additional, unrecognized components of this signaling pathway.

To confirm further the results of the screen, we used RNAi to knockdown the expression of a representative sample of the 29 genes identified, and tested the induction levels of *F08G5.6* and
To determine if MDT-15 is required for the induction of other RPW-24-induced genes, we used NanoString nCounter gene expression analysis to generate transcription profiles of 118 C. elegans genes with known involvement in immune, stress and detoxification responses (Table S2). As in our microarray analysis [9], we found that RPW-24 caused robust transcriptional changes in wild-type nematodes. 40 of the 118 genes in the NanoString codeset were induced at least 4-fold or greater. 25 of these 40 genes are putative immune effectors upregulated during pathogen infection (shown in Figure 2) and 13 are genes putatively involved in the detoxification of small molecule toxins (discussed below). Of note, we had previously observed that 31 of these 40 genes, including 28 of the 31 most strongly induced, were also upregulated in whole genome Affymetrix GeneChip microarray analysis of wild-type animals exposed to RPW-24 versus DMSO [9]. Of the 25 pathogen-induced genes upregulated by RPW-24, 21 have been shown to be induced during P. aeruginosa infection [6,7,22,23]. The RPW-24-induced expression of 13 of the 25 putative immune effectors was significantly reduced in pmdt-15(RNAi) animals compared to wild-type controls (Figure 2 top panel) in accord with the previously determined role for p38 MAP kinase PMK-1 in regulating both pathogen-induced [6] and RPW-24-induced [9] expression of putative immune effector genes.

Consistent with a role for MDT-15 in the expression of PMK-1-activated genes, the NanoString analysis revealed that the RPW-24-dependent induction of 10 of the 13 PMK-1-dependent genes was abrogated in mdt-15(RNAi) animals compared to controls (Figure 2 bottom panel). We used qRT-PCR to show that mdt-15 was knocked down by RNAi in this experiment and to confirm that mdt-15 depletion caused a dramatic reduction in the RPW-24-induced expression of three pmk-1-dependent immune genes (Figure S2A). Further, we found that the RPW-24-mediated induction levels of these three immune genes was reduced in the mdt-15(tm2182) mutant, which recapitulated our findings in mdt-15(RNAi) animals (Figure S2B).

Knockdown of mdt-15 reduced the expression of the top five most strongly upregulated p38 MAP kinase PMK-1-dependent immune effectors by several orders of magnitude (C32H11.1, F33E12.5, F08G5.6, F49F1.7, and F49F1.1) (Figure 2 top panel). These five genes were still induced by RPW-24 in mdt-15(tm2182) animals (Figure 2 top panel), but their induction was entirely abrogated by knockdown of mdt-15 (Figure 2 bottom panel). These data suggest that MDT-15 coordinates inputs from PMK-1 and other immune signaling pathway(s) to modulate the expression of these p38 MAP kinase PMK-1-dependent putative immune effectors. We also identified a requirement for mdt-15 in the induction of five putative immune effectors of the 246, C29F7.2, K03B2.4, C32B4.1, T28C12.4) that are not transcriptional targets of the PMK-1 pathway (Figure 2, compare top and bottom panels). Thus, MDT-15 is required for the induction of p38 MAP kinase PMK-1-dependent immune genes and a second group of defense effectors that are independent of the p38 MAP kinase PMK-1 signaling pathway.

The p38 MAP kinase PMK-1 pathway plays an important role in the regulation of putative immune effectors during P. aeruginosa infection [6]. To determine whether MDT-15/MED15 is also involved in the regulation of these genes during bacterial infection, we infected C. elegans with P. aeruginosa PA14 for 8 hours and used qRT-PCR to compare the induction levels of several immune response genes in mdt-15(RNAi) and control animals. The basal and pathogen-induced expression of three p38 MAP kinase PMK-1-dependent immune effectors (C32H11.1,
MDT-15 (MED15) Links Immunity and Detoxification

One possible explanation for these observations is that the Mediator subunit MDT-15 is a general regulator of transcription that non-specifically affects the expression of a large number of genes. In the NanoString experiment, however, we identified 10 genes that were induced 4-fold or greater in wild-type N2 animals by RPW-24 are presented in Figures 2 and 6. The 13 genes that were dependent on the p38 MAP kinase PMK-1 for their induction in the top panel are grouped and indicated in the bottom panel. Data are the average of two replicates each of which was normalized to three control genes with error bars representing standard deviation and are presented as the value relative to the average expression from the replicates of the indicated gene in the baseline condition [N2 animals (top) or vector control (L4440) animals (bottom) exposed to DMSO]. We confirmed that mdt-15 expression was significantly knocked down by mdt-15(RNAi) in each of these experiments (p<0.001)(see Figure S2A). * p<0.05 for the comparison of the RPW-24-induced conditions.

Figure 2. The Mediator subunit MDT-15 regulates p38 MAP kinase PMK-1-dependent and independent immune genes in response to RPW-24. The expression of 118 C. elegans genes was analyzed using NanoString nCounter gene expression analysis in wild-type N2 and pmk-1(km25) animals (top) and in vector control (L4440) and mdt-15(RNAi) animals (bottom) exposed to either 70 μM RPW-24 or the solvent control DMSO. The 40 genes that were induced 4-fold or greater in wild-type N2 animals by RPW-24 are presented in Figures 2 and 6. The 13 genes that were dependent on the p38 MAP kinase PMK-1 for their induction in the top panel are grouped and indicated in the bottom panel. Data are the average of two replicates each of which was normalized to three control genes with error bars representing standard deviation and are presented as the value relative to the average expression from the replicates of the indicated gene in the baseline condition [N2 animals (top) or vector control (L4440) animals (bottom) exposed to DMSO]. We confirmed that mdt-15 expression was significantly knocked down by mdt-15(RNAi) in each of these experiments (p<0.001)(see Figure S2A). * p<0.05 for the comparison of the RPW-24-induced conditions.

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F08G5.6 and F35E12.5) was reduced in mdt-15(RNAi) animals by one to three orders of magnitude (Figure 3). We also tested the induction levels of three genes whose transcription is activated during P. aeruginosa infection in a manner independent of PMK-1 (irg-1, irg-2 and F01D5.5) and found that PMK-1-independent immune effectors were induced in mdt-15(RNAi) animals during P. aeruginosa infection to levels comparable to that observed in wild-type animals (Figure 3). The expression levels of irg-1 and irg-2 were higher under basal conditions in mdt-15(RNAi) animals compared to L4440 controls (Figure 3). Together, these data support the observations from our NanoString experiments that MDT-15 is required for the expression of some, but not all, immune genes that are activated in response to an environmental insult.

One possible explanation for these observations is that the Mediator subunit MDT-15 is a general regulator of transcription that non-specifically affects the expression of a large number of genes. In the NanoString experiment, however, we identified 10 genes that were induced by RPW-24 in mdt-15(RNAi) animals to levels similar to the wild-type controls (Figure 2 bottom panel). Also of note, our secondary screen demonstrated that mdt-15(RNAi) had no effect on the induction of ng-1::GFP by ToxA. We further wondered if the defects in expression of the PMK-1 targets in mdt-15(RNAi) animals were due to direct transcriptional regulation of the p38 MAP kinase PMK-1 pathway components by MDT-15. However, we found that the mRNA levels of pmk-1, tir-1 and sek-1 in mdt-15(RNAi) animals were not different from the wild-type control (Figure S2C).
The experiments in the preceding sections show that the Mediator subunit MDT-15/MD15 is necessary for the induction of the p38 MAP kinase PMK-1-regulated genes, both in response to RPW-24 and during *P. aeruginosa* infection. We therefore reasoned that mutation or RNAi-mediated knockdown of *mdt-15* would result in enhanced susceptibility to *P. aeruginosa* infection. Initial *P. aeruginosa* pathogenesis assays showed a modest, but significant and reproducible, enhanced susceptibility to infection in *mdt-15(RNAi)* (Figure S3A) and *mdt-15(tm2182)* (Figure S3B) animals compared to controls. However, both *mdt-15(tm2182)* and *mdt-15(RNAi)* animals have reduced brood sizes and varying degrees of sterility. Sterile animals are more resistant to *P. aeruginosa* infection than wild-type animals, due in part todaf-16-dependent induction of stress response genes [25]. To eliminate this potentially confounding effect, we made all animals in the *P. aeruginosa* pathogenesis assay sterile by knocking down daf-16, a technique that has been used previously in *C. elegans* bacterial pathogenesis assays [26]. Under these conditions, we found that *mdt-15(tm2182)* animals were markedly hypersusceptible to *P. aeruginosa* infection compared to control animals (Figure 5). Moreover, injection of the *mdt-15* gene under control of its own promoter partially rescued the enhanced susceptibility to *P. aeruginosa* phenotype of *mdt-15(tm2182)* animals (Figure 5). Knockdown of *mdt-15* by RNAi also caused a hypersusceptibility to *P. aeruginosa* phenotype in *C. elegans fer-15(hb26);fem-1(hc17)* sterile animals [6,27], and as predicted, to a greater degree than wild-type animals that were not made sterile by *dcd-25.1(RNAi)* (Figure S3C).

We also found that the ability of RPW-24 to extend survival of *P. aeruginosa*-infected wild-type and sterile nematodes was significantly attenuated in *mdt-15(RNAi)* animals compared to controls, suggesting that MDT-15 is required for the immunostimulatory activity of RPW-24 (Figure S3A and S3C). The degree of lifespan extension by RPW-24 during *P. aeruginosa* infection was reduced in *mdt-15(tm2182)* compared to controls, but this difference did not reach statistical significance (*p = 0.09*; Figure S3B). As discussed above, the gene expression defects of *mdt-15* targets were more severe in *mdt-15(RNAi)* animals than in the hypomorphic *mdt-15(tm2182)* allele [19,21], which may account for this observation.

One caveat concerning the observation that *mdt-15* depleted animals are hypersusceptible to *P. aeruginosa* infection is that *mdt-15(RNAi)* and *mdt-15(tm2182)* animals have a reduced lifespan when grown on the normal laboratory food source *E. coli* OP50 compared to wild-type controls [18,19]. Several observations indicate, however, that MDT-15 is an important modulator of nematode survival during bacterial infection. First, we have shown
above that mdt-15(RNAi) animals fail to upregulate p38 MAP kinase PMK-1-dependent immune effectors both in response to a xenobiotic toxin and during P. aeruginosa infection, but retain the ability to induce other immune genes in response to pathogens and following exposure to the bacterial toxin ToxA. In addition, mdt-15(RNAi) animals do not respond to the immunostimulatory effects of RPW-24. We have shown previously that C. elegans with mutations in the ZIP-1 and FSHR-1 immune pathways, which act in parallel to the p38 MAP kinase PMK-1 cassette, are hypersusceptible to P. aeruginosa infection, but retain the ability to respond to RPW-24 [9]. That mdt-15(RNAi) animals are blind to the immunostimulatory effects of RPW-24 suggests a specific role of MDT-15 in regulating p38 MAP kinase PMK-1 pathway activity. It is also important to note that despite their reduced lifespan, mdt-15(RNAi) animals are not sensitive to all environmental insults. For example, animals deficient in mdt-15 are sensitive to the toxin fluoranthene, but not β-naphthoflavone, and are not more sensitive to high temperatures than wild-type animals [19].

Figure 4. The Mediator subunit MDT-15 acts downstream of the p38 MAP kinase PMK-1 to regulate the induction of F08G5.6 and F35E12.5. (A) Wild-type or mdt-15(tm2182) mutant synchronized L1 animals containing the pf08G5.6::GFP immune reporter were grown on vector control (L4440), vhp-1(RNAi) or a combination of vhp-1(RNAi) and pmk-1(RNAi) bacteria and then transferred as L4 animals to PA14 for 18 hours. Animals were photographed under the same imaging conditions. (B) qRT-PCR was used to examine the expression levels of F08G5.6, F35E12.5 and C32H11.1 in wild-type N2 and mdt-15(tm2182) mutant animals exposed to vhp-1(RNAi) or the vector control (L4440) under basal conditions (as described above) and 8 hours after exposure to P. aeruginosa. Knockdown of vhp-1 caused significant induction of F08G5.6 and F35E12.5 in wild-type N2 animals (p<0.001), but not in mdt-15(tm2182) animals (p>0.05), under baseline (E. coli) and pathogen-induced conditions. The expression of C32H11.1 was significantly induced by vhp-1(RNAi) (p<0.001) in an mdt-15-dependent manner under baseline conditions (p<0.001), but not following exposure to P. aeruginosa. Data are the average of two biological replicates each normalized to a control gene with error bars representing SEM and are presented as the value relative to the average expression of the indicated gene in the baseline condition (L4440 animals exposed to E. coli).

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Figure 5. MDT-15 is required for defense against P. aeruginosa infection. A P. aeruginosa pathogenesis assay with wild-type N2, mdt-15(tm2182) mutant worms and mdt-15(tm2182) animals carrying pmdt-15::mdt-15 (three independent lines agEx116, agEx117 and agEx118) is shown. The difference in P. aeruginosa susceptibility between mdt-15(tm2182) animals and each of the three transgenic lines carrying pmdt-15::mdt-15 is significant, as is the survival difference between N2 and mdt-15(tm2182) animals (p<0.001). For sample sizes, see Table S3.

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Mediator Subunit MDT-15/MED15 Regulates the Induction of p38 MAP Kinase PMK-1-Independent Detoxification Genes and is Required to Resist the Toxic Effects of the Xenobiotic RPW-24

We previously demonstrated that RPW-24 is a xenobiotic toxin [9]. MDT-15 is known to coordinate protection from the toxin fluoranthene and regulate the transcriptional induction of CYPs [19]. We found that the RPW-24-mediated induction of the 13 detoxification genes in the NanoString codeset was nearly entirely abrogated by RNAi knockdown of mdt-15 (Figure 6A top panel and Table S2). This result was confirmed for three detoxification genes by qRT-PCR (Figures S2B and S2D). To determine if MDT-15 is required to protect C. elegans from the toxic effects of RPW-24, we studied the development of wild-type, pmk-1(RNAi) and mdt-15(RNAi) in the presence of the xenobiotic RPW-24, an
MDT-15/MED15 Links Immunity and Detoxification

MDT-15/MED15 is not required for the avoidance behavior induced by RPW-24

Many xenobiotic toxins, including RPW-24, induce an avoidance response wherein C. elegans leave a lawn of bacterial food, to which they are otherwise attracted, if it contains a toxic compound [3,9]. We therefore wondered if the detoxification machinery in C. elegans is regulated by the p38 MAP kinase PMK-1 pathway. We found, however, that 12 of the 13 RPW-24-induced detoxification genes were upregulated in pmk-1(km25) null mutants to levels comparable to that in wild-type animals exposed to RPW-24 (Figure 6A bottom panel). We used qRT-PCR to confirm this observation for three cytochrome P450 genes (Figure S2D). Thus, the MDT-15-dependent xenobiotic detoxification program is induced in a manner independent of the p38 MAP kinase PMK-1.

Figure 6. Protection from the toxic effects of the xenobiotic RPW-24 requires MDT-15, but not PMK-1. (A) The thirteen xenobiotic detoxification genes that were induced 4-fold or greater by RPW-24 in the NanoString nCounter gene expression analysis are presented. The top panel compares the RPW-24-mediated induction of these genes in vector control (L4440) and mdt-15(RNAi) animals, and the bottom panel shows these data for wild-type N2 versus pmk-1(km25) animals, as described in the legend for Figure 2. * p<0.05 for the...
MDT-15/MED15 Links Immunity and Detoxification

Comparison of the RPW-24-induced conditions. (B) Vector control (L4440), mdt-15(m2182) and pmk-1(km25) animals were exposed to 70 μM RPW-24 or the solvent control DMSO from the L1 stage and photographed after 70 hours of development at 20°C. See Figure S4 for the quantification data from this experiment.

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Reduced ability to kill both wild-type and mdt-15(m2182) animals (Figure S6A). In contrast to L4 animals, we observed that almost no young adult, wild-type animals were killed after six hours of exposure to the phenazine toxins compared with 98% death of L4 staged animals (Figures 7A and S6A), which reproduces the findings of others [28,29]. In contrast to wild-type animals, young adult mdt-15(m2182) animals were dramatically susceptible to P. aeruginosa in this assay and this pathogenesis required the secretion of phenazine toxins, as P. aeruginosa Δphz was markedly less pathogenic toward mdt-15(m2182) young adults (Figure 7A). Moreover, mdt-15(m2182) animals were not simply hypersusceptible to the high osmolarity conditions of this assay because we observed no mortality over the course of the assay in mdt-15(m2182) mutants exposed to “fast kill” media containing the normal nematode food source E. coli OP50 (Figure 7A).

We confirmed that phenazine toxins are lethal to mdt-15-depleted animals by supplementing “fast kill” growth media with both phenazine-1-carboxylic acid and 1-hydroxyphenazine in the absence of pathogen [28]. These two particular phenazines are toxic to wild-type nematodes [28] and, as expected, the mixture of phenazine-1-carboxylic acid and 1-hydroxyphenazine rapidly killed L4 animals (Figure S6B). Young adult wild-type animals were resistant to the lethal effects of these molecules (Figure 7B). However, mdt-15(RNAi) young adult animals were dramatically susceptible to phenazine-mediated killing (Figure 7B).

We also found that pmk-1(km25) loss-of-function mutants were more susceptible to P. aeruginosa in the “fast killing” assay than wild-type animals, although were less susceptible than mdt-15(m2182) animals. This lethality, however, was mediated by factors other than phenazine toxins, since there was no difference in pathogenicity of the wild-type and Δphz P. aeruginosa toward pmk-1(km25) mutants (Figure 7A). Likewise, pmk-1(RNAi) animals were not more susceptible to phenazines-1-carboxylic acid and 1-hydroxyphenazine than L4440 RNAi control animals (Figure 7B).

Together, these data define a role for MDT-15, but not PMK-1, in the protection from bacterial-derived phenazine toxins.

Discussion

Detecting and countering environmental threats is central to the ability of organisms to survive and reproduce in the wild. We examined the C. elegans response to the xenobiotic RPW-24, which is able to induce a host immune response that is protective for animals infected with the lethal bacterial pathogen P. aeruginosa [9]. In an RNAi screen with RPW-24, we identified a number of genes, including mdt-15/MED15, which are required for induction of p38 MAP kinase PMK-1-dependent immune effectors. mdt-15 encodes a subunit of the highly conserved Mediator complex that controls the activation of a variety of genes involved in the response to external stress. We demonstrate that: (i) MDT-15 is required for the induction of p38 MAP kinase PMK-1-dependent immune effectors following exposure to a xenobiotic toxin, as well as during infection with P. aeruginosa. (ii) MDT-15 controls the expression of some p38 MAP kinase PMK-1-independent immune effectors, but not all defense genes, (iii), MDT-15 functions downstream of the p38 MAP kinase PMK-1 cascade to control the induction of at least two immune effectors, (iv) the induction of xenobiotic detoxification genes and protection from the toxic

![Figure 7. Resistance to P. aeruginosa phenazine toxins requires MDT-15.](image-url)
immune pathways involving the transcription factor ZIP-2 and the p38 MAP kinase PMK-1. Several observations suggest, however, that MDT-15-mediated regulation of immune activation and detoxification gene induction by RPW-24 does not occur via a mechanism involving translation inhibition. First, we previously found that a loss-of-function mutation in the zip-2 gene did not affect the ability of RPW-24 to extend the survival of animals infected with P. aeruginosa and genome-wide transcriptional analysis of animals exposed to RPW-24 did not suggest that this compound is an inhibitor of translation [9]. Second, the transcriptional analysis of animals exposed to ToxA did not show an abundance of Phase I and II detoxification genes [15]. Finally, we show here that the ToxA-responsive, ZIP-2-regulated genes, vg-1 and vg-2, are induced in mdt-15(RX4) animals during P. aeruginosa infection to levels comparable to that observed in wild-type animals.

Melo et al. recently studied the behavioral response of C. elegans to xenobiotic toxins known to disrupt the function of the mitochondria, the ribosome, and the endoplasmic reticulum [5]. Inhibiting these essential processes by the action of small molecules or through targeted gene disruptions triggered an avoidance response, which required serotonergic and JNK signaling pathways. They proposed that organisms monitor disruption of core metabolic processes as a means to detect pathogen invasion and challenges from xenobiotic toxins. Based on the data reported here, however, it is not clear, whether immune signaling is an integral part of xenobiotoxic-elicited avoidance behavior, at least with respect to RPW-24. We found that the function of neither MDT-15 nor PMK-1 was required for the avoidance of RPW-24, indicating that the behavioral component of this protective response occurs upstream of MDT-15, or via separate mechanism altogether.

It will be interesting to determine the mechanism by which MDT-15 activates immune and detoxification responses in C. elegans. In our genetic screen for regulators of the p38 MAP kinase PMK-1 pathway, we identified a number of genes that are involved in fatty acid biosynthesis, including mdt-15, fat-6, fat-7, elo-5, acs-19 and C25A1.5. Indeed, MDT-15 is known to control the expression of fat-6 and fat-7 [18–20]. It is therefore possible that a fatty acid signaling molecule or membrane component is required for p38 MAP kinase PMK-1 activity. We found, however, in epistasis analyses with the MAP kinase phosphatase VHP-1 that MDT-15 functions downstream of PMK-1 to coordinate the expression of F08G5.6 and F33E12.5. Thus, for at least a subset of immune genes, MDT-15 likely also physically interacts with sequence-specific regulators, such as ATF-7, a transcription factor that is the downstream signaling target of the p38 MAP kinase PMK-1 pathway [12], to coordinate protective host responses mounted following exposure to xenobiotoxic toxins.

Materials and Methods

**C. elegans and Bacterial Strains.**

* C. elegans were grown on standard NGM plates with *E. coli* OP50 [37] unless otherwise noted. The previously published *C. elegans* strains used in this study were: N2 Bristol [37], pmk-1(km25) [5], AY101 [nlsI01[pDB09.1[pF35E12.5::GFP]; prF4[rol-6(sa11006)]] [10], XA7702 mdt-15(tm2182) [19,21], C512 fer-15(R262)j fem-1(hc17) [38], and AU0135 [agCh1(psy-1::GFP; pmyo-2::mCherry)] [14]. The *C. elegans* strains created for this study were: AU0307 [agls44(pF08G5.6::GFP::unc-54:3’LTR; pmyo-2::mCherry)], AU0316 [mdt-15(tm2182); agIs44; AU0325 [mdt-15(tm2182); agEx116 (mdt-15;pmyo-3::mCherry)], AU0326 [mdt-15(tm2182); agEx117 (mdt-15;pmyo-3::mCherry)], AU0327 [mdt-15(tm2182); agEx118 (mdt-15;pmyo-3::mCherry)].
The strain carrying \textit{ag44} was constructed by PCR amplification from 
\textit{N2} genomic DNA of an 851 bp region upstream of the start codon of the 
\textit{P}FOG3.6 gene (primers GACGGTTACAAT-GAACATTTATTCAATCTCA 
and CGGCAGGTTGC-CATTGATAAGTGA) and ligated to the GFP coding region, and
\textit{unc-54-3'UTR} sequences amplified from pPD95.75 using published primers, and a previously described protocol [39]. 
The \textit{ag44} construct was transformed into \textit{N2} animals with the co-
injection marker \textit{pmyo-2::mCherry} using established methods [40]. A 
strain carrying the \textit{pFOG5::GFP;unc-54-3'UTR} and \textit{pmyo-2::mCherry} transgenes in an extrachromosomal array was irradiated, and strains carrying the integrated array \textit{agB44} were isolated. 
\textit{AC0307} was backcrossed to \textit{N2} five times.

The \textit{mdt-15} rescuing arrays \textit{agEx16}, \textit{agEx17} and \textit{agEx18} contain a 4.8 kb \textit{mdt-15} genomic fragment, which includes 707 bp upstream and 1075 bp downstream of the \textit{mdt-15} coding region, 
amplified from \textit{N2} genomic DNA (primers GACGGTTACAAT-GAACATTTATTCAATCTCA 
and CGGCAGGTTGC-CATTGATAAGTGA) and ligated to the GFP coding region, and
\textit{unc-54-3'UTR} sequences amplified from pPD95.75 using published primers, and a previously described protocol [39]. A strain carrying the \textit{pFOG5::GFP;unc-54-3'UTR} and \textit{pmyo-2::mCherry} transgenes in an extrachromosomal array was irradiated, and strains carrying the integrated array \textit{agB44} were isolated. 
\textit{AC0307} was backcrossed to \textit{N2} five times.

\textbf{RNAi clones presented in this study were from the Ahringer [41] or Vitali [42] RNAi libraries unless otherwise stated. The \textit{atf-7} [12] and the \textit{pmk-1} [5] RNAi clones have been previously reported. All RNAi clones presented in this study have been confirmed by sequencing. The \textit{P. aeruginosa} strain \textit{PA14} were used for all studies, unless otherwise indicated. The \textit{P. aeruginosa} strains used in Figure S1 have been previously described [13] and were (in order of descending virulence toward \textit{C. elegans}): \textit{CF18, PA14, MSH10, S54405, PA01, PAK, 19660}, and \textit{E2}. The \textit{P. aeruginosa} \textit{PA14} phenazine null mutant (\textit{aph2}) lacks both the \textit{phzA1-G1} and \textit{phzA2-G2} operons and has been previously described [43]. The \textit{BL21. E. coli} strain that expresses the bacterial toxin Exotoxin A (ToxA) has been previously described [15].

\section*{Feeding RNAi Screen}

1,420 RNAi clones that correspond to genes expressed in the \textit{C. elegans} intestine based on their annotation in Wormbase (www.
\textit{wormbase.org}) in April, 2008 were selected from the Ahringer [41] or Vitali [42] RNAi libraries (see Table S1B). RNAi clones were 
pinned into 1.2 ml of LB plus 100 \mu g/ml carbenicillin in 96-well 
plates (Corning Incorporated) and grown overnight at 
\textit{37°C} with shaking at \textit{950 RPM} in a Multitron II Shaking 
Incubator (Appropriate Technical Resources). 40 \mu L of the \textbf{10 \times} concentrated overnight culture were added to each well of a 
24-well plate containing \textit{RNAi} agar medium and grown overnight at
room temperature. The following day, 50–100 L1 staged 
\textit{AC0307} animals, which carry the \textit{agEx44} transgene, were added to each well 
and allowed to grow until they were at the \textit{L4} or young adult stage.

\textit{Worms} were then transferred to new 24-well screening plates 
containing 1 mL of “slow kill” media supplemented with 70 \mu M 
\textit{RPW}-24 and seeded with \textit{E. coli OP50} food. Animals were 
dried on the screening plates for several hours at room temperature and 
then incubated overnight at 20\textdegree C. The \textit{L4440} vector and \textit{pmk-1} RNAi 
clones were included on each of the screening plates as the 
negative and positive controls, respectively. Animals were scored 
for GFP expression and rated on a subjective scale from 0 (no 
\textit{GFP} expression in response to \textit{RPW}-24) to 5 (\textit{RPW}-24-mediated 
induction of \textit{GFP} expression equivalent to \textit{L4440}). Exposure of the 
\textit{C. elegans} transcriptional reporter \textit{atg-1-GFP} to an \textit{E. coli} strain that 
expresses ToxA was performed as previously described [15].

\section*{C. elegans Bacterial Infection and Other Assays}

“Slow killing” \textit{P. aeruginosa} infection were performed as 
previously described [9,44]. In all of these assays, the final 
concentration of \textit{DMSO} was 1% and \textit{RPW}-24 was used at a concentration of 70 \mu M, unless otherwise indicated. The propen-
sity of wild-type \textit{C. elegans} to leave a lawn of bacteria supplemented 
with \textit{RPW}-24 was assayed using a previously described protocol 
[39] with minor modifications. Rather than adding the toxin on 
top of the small lawn of food, 20 \mu g of \textit{RPW}-24 was mixed with \textit{E. coli OP50}, which was spotted onto NGM plates. To assess the 
toxicity of \textit{RPW}-24, we assayed the development of animals 
exposed to vector control (\textit{L4440}), \textit{pmk-1(RN4)} and \textit{mdt-15(RN4)} in the 
presence of 70 \mu M \textit{RPW}-24, as previously described [9]. \textit{P. aeruginosa} “fast kill” 
pathogenesis assays were conducted with late \textit{L4} and early young adult animals (picked 1–3 hours after the \textit{L4} molt) obtained from timed egg lays as described [28,29]. For the 
killing assay using toxic phenazines, 50 \mu g/ml phenazine-1-
carboxylic acid (PCA) and 5 \mu g/ml 1-hydroxyphenazine in 
\textit{DMSO} were added to modified “fast kill” media (1% bacto-
peptone, 1% glucose, 1% NaCl, 150 mM sorbitol, 1.7% bacto 
agar, 5 \mu g/ml cholesterol and 50 mM sodium citrate, pH 5) [29]. 
These phenazine concentrations correspond to the amount of 
PCA and 1-hydroxy-phenazine that are produced under “fast kill” 
conditions [28]. \textit{E. coli OP50} was used as the food source. The 
modeled “fast kill” media pH 5.0 plus 1% \textit{DMSO} was used as the 
control condition. These assays were incubated at 21–23\textdegree C.

\section*{Quantitative RT-PCR (qRT-PCR) and NanoString nCounter Gene Expression Analyses}

Synchronized \textit{L1} staged \textit{C. elegans \textit{N2} animals were grown to \textit{L4/young adult stage on the indicated \textit{RNAi} strain, transferred to 
assay plates and incubated at 25\textdegree C for 24 hours. To prepare the 
assay plates, 70 \mu M \textit{RPW}-24 or \textit{DMSO} was added to 20 mL 
“slow killing” media [44] in 10 cm petri dishes seeded with \textit{E. coli OP50}. \textit{N2} and \textit{pmk-1(km25)} animals were raised on \textit{E. coli OP50} and 
extposed to the above conditions for 18 hours at 20\textdegree C. For 
\textbf{qRT-PCR studies of nematodes infected with \textit{P. aeruginosa PA14} or the 
indicated strain of \textit{P. aeruginosa}}, 20 \mu L of “slow killing” media 
containing either \textit{DMSO} or 70 \mu M \textit{RPW}-24 was added to 10 cm 
petri dishes. Plates were seeded with either 75 \mu L of \textit{E. coli OP50} or 
\textit{P. aeruginosa}, each from cultures grown for 15 hours at 37\textdegree C.
The plates were incubated for 24 hours at 37\textdegree C and 24 hours at 
25\textdegree C. \textit{L4/young adult animals were added to the assay plates and 
incubated at 25\textdegree C for eight hours. RNA was isolated using 
\textit{TriReagent} (Molecular Research Center, Inc.) and analyzed by 
\textit{NanoString nCounter Gene Expression Analysis} (NanoString Technologies) using a “codest” designed by NanoString that 
contained probes for 118 \textit{C. elegans} genes (Table S2). \textbf{Probe 
hybridization, data acquisition and analysis were performed 
according to instructions from NanoString with each RNA sample 
normalized to the control genes \textit{snb-1}, \textit{ama-1} and \textit{act-1}. For the 
\textbf{qRT-PCR studies, RNA} was reverse transcribed to \textit{cDNA} using the 
\textit{Retroscript} kit (Life Technologies) and analyzed using a 
\textit{CFX1000} machine (Bio-Rad) with previously published primers [6,18]. The \textbf{qRT-PCR primers for the \textit{mdt-15}, \textit{pmk-1}, \textit{atf-7} and \textit{okr-1} genes were designed for this study and are available upon request. All values were normalized against the control gene \textit{snb-1}. Fold change was calculated using the \textit{Pfaff} method [45].

\section*{Microscopy}

Nematodes were mounted onto agar pads, paralyzed with 
10 mM levamisole (Sigma) and photographed using a \textit{Zeiss AXIO 
Imager Z1 microscope with a Zeiss AxioCam HRm camera and
Axiovision 4.6 (Zeiss) software. For comparisons of GFP expression in the F08G5.6::GFP transgenic animals, photographs were acquired using the same imaging conditions.

Statistical Analyses
Differences in survival of C. elegans animals in the P. aeruginosa pathogenesis assays were determined with the log-rank test in each of two biological replicates. Differences were considered significant only if the p value was less than 0.05 for both replicates. In the manuscript, data from one experiment that is representative of both replicates is shown and the sample sizes for these experiments are given in Table S3. To determine if the increase in survival conferred by RPW-24 treatment was significant in one population compared to another, we examined the difference in the effect of RPW-24 treatment on the hazard in each group using a Cox proportional hazard model (Stata13, Stata, College Station, TX) and a two-tailed student t-tests.

Accession Numbers
Accession numbers for genes and gene products are given for the publically available database Wormbase [http://www.wormbase.org]. The accession numbers for the principal genes mentioned in this paper are: af-7 (C07G2.2), C32H11.1, cyp-35A1 (C03G6.14), cyp-35B2 (K07G6.3), cyp-35G1 (C06G3.3), F35E12.5, F08G5.6, F01D5.5, fshr-1 (C50H2.1), ig-1 (C07G3.2), ig-2 (C49G7.5), mdt-15 (R12B2.5), npr-1 (C07G2.1), pmk-1 (B0218.3), sek-1 (R03G5.2), skn-1 (T19E7.2), tir-1(F13B10.1), and zip-2 (K02F3.4).

Supporting Information
Figure S1 P. aeruginosa strains with greater virulence towards C. elegans cause more robust induction of the immune response gene F08G5.6. (A) Wild-type N2 C. elegans were exposed for eight hours to eight P. aeruginosa strains, each with a different virulence potential toward C. elegans [13]. Data are the average of two biological replicates, each normalized to a control gene with error bars representing SEM and are presented as the fold induction of F08G5.6 compared to its expression in C. elegans exposed to E. coli OP50. *p<0.05 for the difference from the F08G5.6 induction in the most virulent P. aeruginosa strain (CF18). (B) Worms carrying the pF08G5.6::GFP reporter were exposed to P. aeruginosa PA14 and PAK for 18 hours, and photographed under the same conditions.

Figure S2 The Mediator subunit MDT-15 regulates immune and detoxification genes in response to RPW-24. (A) The expression of putative C. elegans immune effector genes was analyzed by qRT-PCR in vector control (L4440) and mdt-15(RNAi) animals exposed to either 70 μM RPW-24 or the solvent control DMSO, as indicated. The difference in expression of each of the genes in both mdt-15(RNAi) conditions was significantly different from vector control (L4440) (p<0.001). (B) The RPW-24-induced expression levels for the indicated genes were compared in mdt-15(tm2182) mutant animals and wild-type controls. Data are presented as the average expression value of the indicated gene following RPW-24 exposure in mdt-15(tm2182) animals versus wild-type animals. (C) The expression levels of three components of the p38 MAP kinase PMK-1 pathway were compared in animals exposed to 70 μM RPW-24 or DMSO. (D) RPW-24-mediated induction levels of cyp-35B1, cyp-35B2 and cyp-35G1 were assessed in pmk-1(km25) and wild-type N2 animals as described above. There was no significant difference in the induction of these genes (p>0.05). All data in this figure are the average of two or three replicates, each normalized to a control gene with error bars representing SEM. For the analyses in A and C, data are presented as the value relative to the average expression from all replicates of the indicated gene in the baseline condition [L4440 (A) or N2 (C) animals exposed to DMSO].

Figure S3 The Mediator subunit MDT-15 is required for defense against P. aeruginosa infection. C. elegans wild-type N2 (A) and fer-15(hb26)jft-1/che17) sterile (C) animals were exposed to vector control (L4440) or mdt-15(RNAi) and infected with P. aeruginosa in the presence of the solvent control DMSO or 70 μM RPW-24. There is a significant difference in susceptibility to P. aeruginosa infection between the L4440/DMSO and mdt-15(RNAi)/DMSO conditions in A and C (p<0.001). In addition, the magnitude of lifespan prolongation conferred by RPW-24 during P. aeruginosa infection was significantly reduced in mdt-15(RNAi) animals compared to control animals for both assays (p<0.001). (B) C. elegans wild-type N2 or mdt-15(tm2182) animals were infected with P. aeruginosa in the presence of the solvent control DMSO or 70 μM RPW-24. There is a significant difference between the N2/DMSO and mdt-15(tm2182)/DMSO conditions, which was also significant in a biological replicate of this experiment (p<0.001). The degree of lifespan extension conferred by RPW-24 exposure during P. aeruginosa infection was reduced in mdt-15(tm2182) animals compared to wild-type animals, but the difference did not reach statistical significance (p = 0.09). In A, B and C, data at each time point are the average of three plates per strain, each with approximately 30–40 animals per plate (see Table S3 for sample sizes). Each assay presented in this figure is representative of two independent experiments.

Figure S4 Protection from the toxic effects of the xenobiotic RPW-24 requires MDT-15, but not PMK-1. The percentage of the population of animals that were at the indicated development stage after 70 hours of incubation at 20°C is presented. Adult animals all had a fully formed vulva and were divided into three categories based on the number of eggs carried in the animal. Larval stages were determined based on microscopic examination of the gonad. See Table S3 for the sample sizes.

Figure S5 Neither MDT-15 nor PMK-1 are required for the behavioral avoidance response to RPW-24. (A) The percentage of wild-type N2, pmk-1(km25) and mdt-15(tm2182) animals that were off a lawn of E. coli OP50 supplemented with either DMSO or 20 μg RPW-24 (average of three replicates) 16 hours after exposure is presented with error bars representing standard deviation. There is no significant difference in the percentage of animals off a lawn containing RPW-24 among the conditions in this experiment (p>0.05). See Table S3 for the sample sizes. (B) Representative photographs of each condition are shown.

Figure S6 Resistance to P. aeruginosa phenazine toxins requires MDT-15. The controls for the experiment in Figure 7 are presented. (A) C. elegans wild-type N2 and mdt-15(tm2182) animals at the L4 stage are sensitive to killing by P. aeruginosa in the “fast killing” toxic phenazine assay. (B) Likewise, L4 staged nematodes are rapidly killed by toxic phenazines added to assay
media in the absence of pathogen. These experiments were conducted in parallel with and as described for the data presented in Figure 7.

(EPS)

Table S1 Genes identified in an RNAi screen for regulators of the p38 MAP kinase PMK-1 pathway. (A) Presented are the 29 genes that were identified in a screen of 1,420 RNAi clones, the complete list of which is given (B), because they reduce or abrogate the induction of the p38 MAP kinase PMK-1-dependent immune reporters F08G5.6::GFP and F35E12.5::GFP. Knockdown of these genes does not affect the induction of mg-1::GFP by an E. coli strain engineered to express ToxA. qRT-PCR of three replicates was used to confirm that the RNAi-mediated knockdown of the indicated gene in wild-type animals reduced the induction of F08G5.6 and F35E12.5 by RPW-24. We previously showed that aft-7 and pmk-1 are required for the RPW-24-mediated induction of F08G5.6 and F35E12.5 [9]. These 29 genes were identified in an RNAi screen of 1,420 clones, which are presented in (B). Of note, the 29 genes presented in (A) were sequenced confirmed.

(XLSX)

Table S2 Relative expression of the 118 genes in the nCounter Gene Expression Analysis. Nanostring nCounter Gene Expression Analysis data for (A) N2 wild-type and pmk-(kn25), and (B) Vector control (L4440) and mdt-15(RNAi), each exposed to DMSO or RPW-24, are presented. Expression values are the average of two independent replicates each of which was normalized to three control genes and are presented as the value relative to the average expression of the indicated gene in the baseline condition [N2 animals (A) or vector control (L4440) animals (B) exposed to DMSO]. p values for the comparison of the indicated conditions are presented.

(XLSX)

References


