WWP-1 Is a Novel Modulator of the DAF-2 Insulin-Like Signaling Network Involved in Pore-Forming Toxin Cellular Defenses in Caenorhabditis elegans

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Abstract

Pore-forming toxins (PFTs) are the single largest class of bacterial virulence factors. The DAF-2 insulin/insulin-like growth factor-1 signaling pathway, which regulates lifespan and stress resistance in Caenorhabditis elegans, is known to mutate to resistance to pathogenic bacteria. However, its role in responses against bacterial toxins and PFTs is as yet unexplored. Here we reveal that reduction of the DAF-2 insulin-like pathway confers the resistance of Caenorhabditis elegans to cytotoxic crystal (Cry) toxins produced by Bacillus thuringiensis. In contrast to the canonical DAF-2 insulin-like signaling pathway previously defined for aging and pathogenesis, the PFT response pathway diverges at 3-phosphoinositide-dependent kinase 1 (PDK-1) and appears to feed into a novel insulin-like pathway signal arm defined by the WW domain Protein 1 (WWP-1). In addition, we also find that WWP-1 not only plays an important role in the intrinsic cellular defense (INCED) against PFTs but also is involved in innate immunity against pathogenic bacteria Pseudomonas aeruginosa and in lifespan regulation. Taken together, our data suggest that WWP-1 and DAF-16 function in parallel within the fundamental DAF-2 insulin/IGF-1 signaling network to regulate fundamental cellular responses in C. elegans.

Introduction

Pore-forming toxins (PFTs) are bacterial toxins that damage the plasma membrane of host cells and play important roles in the pathogenesis of many important human pathogens including Staphylococcus aureus, Streptococcus pyogenes, Clostridium perfringens, and Enterococcus faecalis [1,2,3]. Crystal (Cry) toxins produced by the Gram-positive spore-forming soil bacterium Bacillus thuringiensis (Bt) are a large family of PFTs [4]. Over 100 phylogenetically related three-domain Cry toxins are known [4]. Although known primarily for their ability to kill insects [5], we have reported that some Cry toxins, including Cry5B and Cry21A, can intoxicate a wide range of plant-parasitic, animal-parasitic, and free-living nematodes including the standard laboratory nematode species, Caenorhabditis elegans [6,7,8].

This C. elegans–Cry toxin interaction system opened up the first, and to date the only, whole-animal genetic model for studying PFTs in vivo and led to the discovery of signal transduction pathways that protect cells against PFTs, including the p38 mitogen-activated protein kinase (MAPK) pathway and the unfolded protein response (UPR) pathway, which are also protective against and/or activated by PFTs in mammalian cells [9,10,11]. More recently, we have shown that the hypoxia response pathway also protects C. elegans against PFTs [12]. Although significant and extensive responses to PFTs at the molecular level (e.g., modulation of signal transduction cascades) have been recorded, the roles of these responses in coping with PFTs as yet poorly understood [13]. Thus, functional analyses of innate immune responses to the single largest class of bacterial protein virulence factors remains a largely understudied area.

The DAF-2 insulin/insulin-like growth factor 1 (IGF-1) receptor pathway is part of a neuroendocrine system that regulates longevity, metabolism, and development in C. elegans and is homologous to the mammalian insulin and IGF-1 signaling pathway [14,15]. The C. elegans daf-2 gene encodes the worm homolog of the insulin/IGF-1 receptor. C. elegans strains that carry reduction-of-function or loss-of-function mutations in daf-2 are long-lived age-1 and daf-2 are long-lived age-1. These age-1 daf-2 gene mutations confer resistance to Bt Cry PFTs in C. elegans [16,17,18].

Here we demonstrate that reduction of the DAF-2 insulin/IGF-1 signaling pathway confers resistance to Bt Cry PFTs in C. elegans. Unexpectedly, this resistance does not solely rely on the canonical DAF-2 insulin/IGF-1 signaling pathway through AKT/PKB and DAF-16 but that rather, at least in part, deviates from the main pathway at PDK-1 and includes WWP-1. We demonstrate that WWP-1 may be a novel signaling arm that diverges from the downstream phosphoinositide 3-kinase (PI3K) age-1 are long-lived and are also resistant to a variety of stresses and bacterial pathogens [16,17,18].
pathogenic attacks since loss of this pathway leads to animals hypersensitive to PFTs and pathogenic bacteria *Pseudomonas aeruginosa* in *C. elegans*.

## Results

### Reduction of the DAF-2 Insulin-Like Receptor Signal Confers Resistance to Bt Cry PFTs in *C. elegans*

To address whether the DAF-2 insulin/IGF-1 signaling pathway plays a role in *C. elegans* against the PFTs, *C. elegans daf-2* reduction-of-function mutant *daf-2(e1370)* were qualitatively compared to wild-type N2 animals in their susceptibilities to the nematocidal PFT, Cry5B (Figure 1A). Fourth larval (L4) stage worms were fed for 48 hours either on control plates with *E. coli* strain JM103 that did not express Cry5B or plates prepared with *E. coli* JM103 expressing Cry5B. Specifically, the relative health of each worm was evaluated qualitatively by comparing body size, darkness of the intestine as an indicator of feeding, and activity, including pharyngeal pumping and whether the worm demonstrated spontaneous movement. In the absence of Cry5B, the wild-type and mutant worms are healthy adults with similar appearance. In the presence of the Cry5B, wild-type N2 worms were severely intoxicated compared to those found on control nontoxin plates, as evidenced by their smaller sizes and paler appearances (Figure 1A). However, under the same conditions, the *daf-2(e1370)* mutant animals were qualitatively healthier and appeared resistant to Cry5B.

The sensitivity to Cry5B PFT of animals mutant for the DAF-2 insulin/IGF-1 receptor was also quantitatively assessed using a dose-dependent mortality assay (Figure 1B) [19]. Form these data, LC₅₀ (lethal concentration at which 50% of the animals die) values were obtained (Table 1). These quantitative results confirm that *daf-2(e1370)* mutant animals are at least an order of magnitude more resistant to Cry5B than wild-type N2 animals (*P* < 0.01). In order to test whether *daf-2(e1370)* mutant animals were also resistant to another Cry PFT, we also quantitatively analyzed the LC₅₀ values of *daf-2(e1370)* mutants to the nematocidal Cry PFT, Cry21A [7,12]. These data show that *daf-2(e1370)* animals are 7X more resistant to Cry21A than wild-type N2 animals (Figure 1C and Table 1; *P* < 0.01).

To independently confirm the importance of DAF-2 in PFT responses, DAF-2 function was reduced using RNA interference (RNAi) in the RNAi-sensitive strain ref-3(pk1426) (Figure 1D). The resistant to Cry5B and Cry21A resulting from knockdown of *daf-2* was also seen, confirming the Cry PFT resistant phenotype is caused by reduction of DAF-2 function. Interestingly, knock down of *bre-3* gene induced resistance to Cry5B but not to Cry21A at the level detectable by this qualitative assay, although more quantitative data indicate that mutants lacking *bre-3* that functions in the same pathway as *bre-3* show partial resistance to Cry21A (data not shown). This result implies that Cry21A at least partly might require a different receptor than Cry5B for intoxication of *C. elegans*.

### Resistance of *daf-2* Mutant to Cry Toxins Is, in Part, through a *daf-16*-Independent Manner

In fertile animals, the extended lifespan and enhanced stress and pathogen resistance phenotypes associated with reduction-of-function mutations in *daf-16* are totally suppressed by loss-of-function mutations in *daf-16*, which encodes a Forkhead transcription factor [17,20,21,22,23]. To test whether DAF-16 is also functionally downstream of DAF-2 with regards to Cry PFT resistance, animals containing the partial loss-of-function *daf-2(e1370)* mutant, the *daf-16* null mutant, *mu86* [24], and the *daf-2(e1370);daf-16(mu86)* double mutant were exposed to Cry5B and Cry21A and scored for viability at the single dose of 40 μg/ml purified Cry3B [8]. Under these conditions, *daf-2(e1370)* animals are significantly more resistant (3.3X more alive animals) to Cry5B than wild-type N2 animals (*P* < 0.01) whereas loss-of-function *daf-16(mu86)* mutant animals are as sensitive as N2 (Figure 2A, Table 2; *P* = 0.683), *daf-2(e1370);daf-16(mu86)* double mutant animals had an intermediate phenotype that was statistically more sensitive than *daf-2(e1370)* animals (*P* < 0.001) but statistically more resistant (respectively 4.3X and 3.7X more alive animals) than *daf-16(mu86)* or wild-type animals in response to Cry5B (*P* < 0.001 and *P* < 0.001). Similar results were also seen in the animals treated with 8 μg/ml of the Cry21A, *daf-2(e1370);daf-16(mu86)* double mutant animals also had an intermediate phenotype that was statistically more sensitive than *daf-2(e1370)* animals (*P* < 0.05) but statistically more resistant (respectively 2.3X and 2.4X more alive animals) than *daf-16(mu86)* or wild-type animals in response to Cry21A (*P* < 0.05 and *P* < 0.05) (Figure 2B, Table 2). These results indicate that DAF-16 is required for some, but not all, of the PFT resistance conferred by reduction of DAF-2 function.

The Resistance of Cry Toxins Signal in DAF-2 Signaling Pathway in Part Deviates from PDK-1

In the canonical DAF-2 insulin/IGF-1 signaling pathway for the regulation of lifespan, energy metabolism, and dauer development in *C. elegans*, DAF-2 regulates DAF-16 through the activation of P3K, encoded by *age-1* and *age-2* for the catalytic subunit and the regulatory subunit respectively. P3K potentiates the activity of DAF-1, which in turn activates the three downstream serine/threonine kinases: the Akt/PKB homologs AKT-1 and AKT-2, and the serum- and glucocorticoid-inducible kinase homolog, SGK-1. These three serine/threonine kinases inhibit the nuclear translocation and transcriptional activity of DAF-16 by phosphorylating DAF-16 at different serine/threonine residues [25]. In order to identify where in the canonical DAF-2 insulin-like signaling pathway a DAF-16-independent arm might branch off for responding to Cry PFTs, we obtained available mutants in this pathway from the Caenorhabditis Genetic Center (CGC) and exposed them to *E. coli* expressing Cry5B in liquid medium. In this Cry5B toxicity assay, the *daf-2(e1370), age-1*(hx546), *aap-1*(m889), *pdk-1*(sa560), *pdk-1*(sa709), and *daf-2(e1370);daf-16(mu86)* animals are all statistically resistant to Cry5B compared to the wild-type N2 animals (Figure 2C, Table 2). Consistent with these results, *daf-18(e1375)* mutant animals [DAF-18 encodes the mammalian PTEN lipid phosphatase homolog that antagonizes P3K], are hypersensitive to Cry5B compared to wild-type animals. Thus, animals from the insulin-receptor down to PDK-1 behave as expected from what is known about the pathway. However, animals with mutations in the three serine/threonine kinases immediately downstream of PDK-1, including *akt-1*(sa573), *akt-1*(ok525), *akt-1*(mg144), *akt-2(ok393)*, *akt-1*(mgl142) were not resistant to Cry5B (Figure 2C, Table 2), and in fact in two cases (*akt-1*(ok325) and *akt-2*(ok393)) were hypersensitive to the PFT.

To independently test these results, the sensitivity to Cry5B of animals with mutations in the *pdk-1*, *akt-1*, *akt-2* and *sgk-1* genes were quantitatively assessed using the Cry5B dose-dependent mortality assay (Figure 2D and 2E). Our quantitative results comparing LC₅₀ values confirmed that animals with reduction-of-function mutations in *pdk-1*, including *pdk-1*(sa560) and *pdk-1*(sa709), were statistically more resistant to Cry5B than wild-type N2 animals. A gain-of-function mutation in *pdk-1*(mg142) was, as predicted, sensitive to Cry5B. As above, we found animals with the loss-of-function mutations in *akt-1*, *akt-2* and *sgk-1*, including
Figure 1. Reduction of the DAF-2 insulin-like receptor signal confers resistance to Cry toxins. (A) Comparison of the reduction-of-function 
\( \text{daf-2(e1370)} \) mutant animals to wild-type N2 animals and the known Cry5B resistant 
\( \text{bre-3(ye28)} \) animals on Cry5B-expressing \( \text{E. coli} \) plates indicates 
\( \text{daf-2(e1370)} \) animals are resistant to Cry5B. The experiment was performed three times. Two representative worms are shown for each strain 
48 hours after feeding either on \( \text{E. coli} \) with (Cry5B) or without Cry5B (No Toxin). (B) A dose-dependent mortality assay was performed using purified 
Cry5B toxin to quantitatively compare sensitivities of wild-type N2 to the 
\( \text{bre-3(ye28)} \) and \( \text{daf-2(e1370)} \) mutants. Lethality was determined after 6 days. 
This semi-log graph represents three independent experiments, and each data point is the mean and standard deviations of the experiments. Note, 
that although \( \text{daf-2} \) mutant animals are highly resistant based on their ability to stay alive, they clearly are not as resistant as \( \text{bre-3} \) animals since the 
latter are alive and robustly active and healthy whereas the former are alive but sickly at the end of this 6 day assay. (C) The dose-dependent mortality 
assay for Cry21A spore-crystal lysates. The fact that spores are present in these assays is due to the fact we currently do not have purified Cry21A. The 
presence of spores increases the toxicity of the Cry protein. Hence, the curves in (B) and (C) cannot be directly compared. (D) RNA interference \( \text{E. coli} \)-expressed Cry5B toxin plate assay. RNAi sensitive \( \text{rrf-3(pk1426)} \) worms after feeding on \( \text{pL4440} \) (empty vector control), \( \text{bre-3} \), or \( \text{daf-2} \) genes dsRNA expressing \( \text{E. coli} \) were transferred to the \( \text{E. coli} \)-expressing either Cry5B (Cry5B), Cry21A (Cry21A) or without toxin (No Toxin) bacterial lawns for 48 
hours. Knock down of the \( \text{daf-2} \) gene leads to resistant to both Cry5B and Cry21A toxins. The experiment was performed three times and two 
representative worms are shown for each RNAi knock down strain.

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akt-1(sa573), akt-1(ok525), akt-2(ok393), and sgk-1(ok538) were either as sensitive or hypersensitive to Cry5B compared to N2 animals (Figure 2D and Table 1). Since there is some redundancy between akt-1, akt-2, and sgk-1 mutant animals, it is possible that the triple mutant (which is lethal and therefore not possible to test [26]) might be partly resistant. However, it should be noted that sgk-1 mutant animals on their own have a long-lived phenotype [27]. The fact that sgk-1 animals were not resistant to Cry5B and that no level of resistance was seen with akt-1 or akt-2 suggested that there is a divergence of the insulin pathway with regards to Cry5B response upstream of these genes and branching off at PDK-1.

WWP-1 Is a Novel PDK-1 Interacting Protein Involved in Cry5B Defense

In order to identify the novel signaling arm that branches off from PDK-1, we searched the C. elegans Interactome database (http://vidal.dfci.harvard.edu/interactomeDb/i-View/interactomeCurrent.pl) for novel PDK-1 interacting proteins [28]. Two novel PDK-1 interacting proteins, WWP-1 (WW domain Protein) and PCM-1 (Protein CarboxyMethyltransferase), were identified from high-throughput yeast two-hybrid screens using H42K12.1 (PDK-1) as the bait (Figure 3A). The wwp-1 gene encodes a putative E3 ubiquitin ligase orthologous to budding yeast Rsp5, Drosophila Su(dx), and human WWP1 and WWP2 [29] (Figure 3B), and the pcm-1 gene encodes an L-isoaspartate O-methyltransferase orthologous to human PCMT1 [30]. We requested all available mutants of these two genes from CGC and exposed them to Cry5B. The sensitivity to Cry5B of animals with mutations in the wwp-1 and pcm-1 genes were quantitatively assessed using the Cry5B dose-dependent lethality assay and LC50 values were obtained (Figure 3C and Table 1). The results showed that wwp-1(ok1102) loss-of-function mutant animals are significantly hypersensitive to Cry5B PFT compared to wild type N2 animals (6.6 fold; P<0.01), but the sensitivity to Cry5B PFT of pcm-1(qa201) mutant animals are statistically indistinguishable from N2 animals. Two additional wwp-1 mutant alleles, wwp-1(gk372) and wwp-1(gk397) (Figure 3B), were also tested. All three wwp-1 mutant alleles are significantly hypersensitive to Cry5B PFT (Figure 3D, Table 1).

| Table 1. Data analysis of the quantitative Crystal toxins lethal concentration assays. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Toxin and Strain               | LC50 (µg/ml)¹ | Standard Deviation | p value relative to N2 | Relative sensitivity (LC50 mutant/LC50 N2) |
| Cry5B LC50 assay (Figure 1B)  |                 |                   |                               |                                      |
| N2 wild-type                   | 11.24           | 1.86              |                               |                                      |
| bre-3(ye28)                    | >120¹           | ¹                 | <0.01                         | >10                                 |
| daf-2(e1370)                   | >120¹           | ¹                 | <0.01                         | >10                                 |
| Cry21A LC50 assay (Figure 1C)  |                 |                   |                               |                                      |
| N2 wild-type                   | 3.73            | 1.03              |                               |                                      |
| daf-2(e1370)                   | 26.10           | 8.03              | p<0.01                        | 7.00                                |
| Cry5B LC50 assay (Figure 2D)  |                 |                   |                               |                                      |
| N2 wild-type                   | 10.42           | 1.09              |                               |                                      |
| daf-2(e1370)                   | >120¹           | ¹                 | <0.01                         | >10                                 |
| pdk-1(iso680)                  | >120¹           | ¹                 | <0.01                         | >10                                 |
| pdk-1(sa709)                   | >120¹           | ¹                 | <0.01                         | >10                                 |
| pdk-1(mg142)                   | 7.19            | 0.76              | p<0.05                        | 0.69                                |
| Cry5B LC50 assay (Figure 2E)  |                 |                   |                               |                                      |
| N2 wild-type                   | 20.55           | 6.64              |                               |                                      |
| akt-1(ok525)                   | 10.21           | 1.40              | p<0.05                         | 0.50                                |
| akt-1(sa573)                   | 5.77            | 0.82              | p<0.01                         | 0.28                                |
| akt-2(ok393)                   | 18.30           | 1.96              | 0.589                          | 0.89                                |
| sgk-1(ok538)                   | 13.95           | 1.49              | 0.133                          | 0.68                                |
| Cry5B LC50 assay (Figure 3C)  |                 |                   |                               |                                      |
| N2 wild-type                   | 7.44            | 1.45              |                               |                                      |
| wwp-1(ok1102)                  | 1.33            | 0.15              | p<0.01                         | 0.18                                |
| pcm-1(qa201)                   | 6.46            | 1.92              | 0.428                          | 0.87                                |
| Cry5B LC50 assay (Figure 3D)  |                 |                   |                               |                                      |
| N2 wild-type                   | 8.33            | 1.09              |                               |                                      |
| wwp-1(gk372)                   | 2.52            | 1.03              | p<0.01                         | 0.30                                |
| wwp-1(gk397)                   | 1.90            | 0.77              | p<0.01                         | 0.23                                |
| wwp-1(ok1102)                  | 1.30            | 0.26              | p<0.01                         | 0.16                                |

¹The range of the LC50 values for Cry5B in N2 animals varies from 8.33 to 20.55 µg/ml, because the absolute quality and measured quantity of toxins varies from different fermentation batches. We performed each experiment using the same batch of the purified toxins.

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akt-1(sa573), akt-1(ok525), akt-2(ok393), and sgk-1(ok538) were either as sensitive or hypersensitive to Cry5B compared to N2 animals (Figure 2D and Table 1). Since there is some redundancy between akt-1, akt-2, and sgk-1 mutant animals, it is possible that the triple mutant (which is lethal and therefore not possible to test; [26]) might be partly resistant. However, it should be noted that sgk-1 mutant animals on their own have a long-lived phenotype [27]. The fact that sgk-1 animals were not resistant to Cry5B and that no level of resistance was seen with akt-1 or akt-2 suggested that there is a divergence of the insulin pathway with regards to Cry5B response upstream of these genes and branching off at PDK-1.
Figure 2. The resistance to Cry toxin is only partly dependent upon DAF-16 FOXO and forks at PDK-1 in the DAF-2 insulin-like network. (A, B) Comparisons of the daf-2(e1370), daf-16(mu86), and daf-2(e1370);daf-16(mu86) mutant animals to wild-type N2 animals in 40 μg/ml purified Cry5B or 8 μg/ml Cry21A indicate daf-2(e1370) mutant and daf-2(e1370);daf-16(mu86) double mutant are all statistically resistant to Cry5B and Cry21A compared with N2. * indicates P<0.001 (P=0.05 in B) relative to wild-type N2. # indicates P<0.001 (P<0.05 in B) relative to daf-2(e1370);daf-16(mu86) mutant. (C) Comparisons of the mutants in the canonical DAF-2 insulin-like signaling pathway, including daf-2(e1370), age-1(hx546), aap-1(m889), daf-18(e1375), pdk-1(sa680), pdk-1(sa709), akt-1(sa733), akt-1(ok525), akt-2(ok393), sgk-1(ok538), daf-16(mu86), daf-2(e1370);daf-16(mu86), to wild-type N2 animals in E. coli-expressed Cry5B liquid toxicity assay. * indicates P<0.01 relative to wild-type N2. (D, E) Dose-dependent mortality were performed using purified Cry5B toxin to quantitatively compare sensitivities of wild-type N2 to the daf-2(e1370), pdk-1(sa680), pdk-1(sa709), pdk-1(mg142), akt-1(ok525), akt-1(sa733), akt-2(ok393), and sgk-1(ok538) mutants. This semi-log graph represents three independent experiments, and each data point shows the mean and standard deviations from three experiments.

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To independently test that our results stem from loss of function phenotypes, RNAi sensitive rrf-3(pk1426);glp-4(bn2) mutant worms were used to knock down the wwp-1 genes by two independent E. coli RNAi feeding clones (8A5 and 8A15) from the Ahringer RNAi library [31]. rrf-3(pk1426);glp-4(bn2) animals were used because the rrf-3(pk1426) mutant is hypersensitive to RNAi and the glp-4(bn2) mutant does not produce progeny that would otherwise complicate the assay (via internal hatching of larvae that sometimes occurs when adult C. elegans are intoxicated with Cry proteins). We have demonstrated that both mutants have roughly normal response to Cry5B [10]. DNA sequencing results demonstrated that these two wwp-1 RNAi clones target different sequence regions of the wwp-1 gene (Figure 3B). The resistant and hypersensitive phenotypes to Cry5B result from knockdown of daf-2 and wwp-1 respectively were seen, confirming the phenotypes are caused by loss or reduction of function of these genes (Figure 3E, Table 3). Overall, these data suggest that WWP-1 is also involved in innate immunity against Cry5B PPT.

### WWP-1 Is also Involved in Innate Immunity and Aging Regulation

The DAF-2 signaling network in C. elegans is well documented for essential in innate immunity against various pathogens, including the human Gram-negative bacterial pathogens Pseudomonas aeruginosa [17,18]. If WWP-1 is in the DAF-2 pathway and is antagonistic to DAF-2 function as it is relative to the Cry protein response (i.e., DAF-2 animals are resistant whereas WWP-1 mutant animals are hypersensitive), we predict WWP-1 would also play a role in response to P. aeruginosa and be antagonistic to DAF-2. We exposed wwp-1 mutants to the pathogenic bacteria P. aeruginosa strain PA14 (Figure 4 and Table 4). As we predicted, animals lacking WWP-1 are significantly hypersensitive to the killing by P. aeruginosa PA14 (P<0.01; the opposite phenotype of DAF-2 mutant animals). These data also demonstrate that WWP-1 is involved in promoting innate immunity against pathogenic bacteria.

The DAF-2 insulin/IGF-1 signaling network as aforementioned also plays important roles in regulation of lifespan in C. elegans. It also has been suggested that signal transductions in the DAF-2 insulin/IGF-1 signaling pathway for longevity and innate immunity can be interrelated [16,18]. If WWP-1 is in the DAF-2 pathway, it might also have a role in C. elegans lifespan, again antagonistic to DAF-2. As predicted, we found that wwp-1(ok372) and wwp-1(ok1102) mutant animals had statistically significant shorter lifespan compared with wild-type N2 animals (Figure 5A, Table 4). These data indicate that wwp-1 is also a positive regulator of lifespan in C. elegans.

To confirm that the hypersensitivity of WWP-1 mutant animals to aging, P. aeruginosa, and Cry proteins is not due to general ill health of these animals, we tested whether wwp-1 mutant animals are hypersensitive to two other toxic chemical compounds, the heavy metal CuSO4 (a toxic insult that kills with kinetics similar to

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**Table 2.** Data analysis of the toxins toxicity assays.

| Cry21A 40 µg/ml (Figure 2A) | Survival (%) | Standard Deviation | p value relative to N2 | p values relative to daf-2;daf-16 |
|-----------------------------|--------------|--------------------|------------------------|----------------------------------|
| N2 wild-type                | 11.52        | 9.47               | p<0.001                | p<0.001                          |
| daf-2(e1370)                | 97.91        | 3.45               | p<0.001                | p<0.001                          |
| daf-16(mu86)                | 9.81         | 7.72               | p=0.663                | p<0.001                          |
| daf-2(e1370);daf-16(mu86)   | 42.16        | 10.69              | p<0.001                | p<0.001                          |

To independently test that our results stem from loss of function phenotypes, RNAi sensitive rrf-3(pk1426);glp-4(bn2) mutant worms were used to knock down the wwp-1 genes by two independent E. coli RNAi feeding clones (8A5 and 8A15) from the Ahringer RNAi library [31]. rrf-3(pk1426);glp-4(bn2) animals were used because the rrf-3(pk1426) mutant is hypersensitive to RNAi and the glp-4(bn2) mutant does not produce progeny that would otherwise complicate the assay (via internal hatching of larvae that sometimes occurs when adult C. elegans are intoxicated with Cry proteins). We have demonstrated that both mutants have roughly normal response to Cry5B [10]. DNA sequencing results demonstrated that these two wwp-1 RNAi clones target different sequence regions of the wwp-1 gene (Figure 3B). The resistant and hypersensitive phenotypes to Cry5B result from knockdown of daf-2 and wwp-1 respectively were seen, confirming the phenotypes are caused by loss or reduction of function of these genes (Figure 3E, Table 3). Overall, these data suggest that WWP-1 is also involved in innate immunity against Cry5B PPT.

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The DAF-2 insulin/IGF-1 signaling network as aforementioned also plays important roles in regulation of lifespan in C. elegans. It also has been suggested that signal transductions in the DAF-2 insulin/IGF-1 signaling pathway for longevity and innate immunity can be interrelated [16,18]. If WWP-1 is in the DAF-2 pathway, it might also have a role in C. elegans lifespan, again antagonistic to DAF-2. As predicted, we found that wwp-1(ok372) and wwp-1(ok1102) mutant animals had statistically significant shorter lifespan compared with wild-type N2 animals (Figure 5A, Table 4). These data indicate that wwp-1 is also a positive regulator of lifespan in C. elegans.

To confirm that the hypersensitivity of WWP-1 mutant animals to aging, P. aeruginosa, and Cry proteins is not due to general ill health of these animals, we tested whether wwp-1 mutant animals are hypersensitive to two other toxic chemical compounds, the heavy metal CuSO4 (a toxic insult that kills with kinetics similar to...
Figure 3. WWP-1 is a novel PDK-1 interacting protein involved in Cry5B defense. (A) Two novel PDK-1 interacting protein identified by the Worm Interactome Database. Two bait-prey interactions, including H42K12.1(pdk-1)-Y65B4BR.4(wwp-1) and H42K12.1(pdk-1)-C10F3.5(pcm-1), were identified by using H42K12.1(pdk-1) as the bait in the high-throughput yeast two hybrid screens [28]. (B) Predicted genomic structure of wwp1. Boxes and lines denote exons and introns respectively. The predicted functional domains of WWP1, including a C2 domain, four WW domains and a HECT domain, are indicated. The regions corresponding to the interfering dsRNA clones, 8AS and 8A15 are underlined. The mutation regions of the three wwp1 mutant alleles used in this study are indicated as gray boxes. (C) A Cry5B dose-dependent mortality assay was performed to quantitatively compare sensitivities of wild-type N2 to the wwp-1(ok1102) and pcm-1(qa201) mutants. Only wwp-1(ok1102) mutant animals showed statistically (p<0.01) hypersensitivity to Cry5B compared to N2. (D) A Cry5B dose-dependent mortality assay was performed to quantitatively compare sensitivities of wild-type N2 to three wwp1 mutants, including wwp-1(gk372), wwp-1(gk397), wwp-1(ok102). All wwp1 mutants showed statistically hypersensitive to Cry5B. (E) RNAi sensitive rrf-3(pk1426);glp-4(bn2) mutant worms after feeding on pL4440 (empty vector control), daf-2, or two wwp-1(8AS and 8A15) dsRNA expressing E. coli were exposed to purified Cry5B at 25°C. Lethality was determined after 6 days. Animals knocked down for the daf-2 gene were statistically resistant to Cry5B whereas animals knocked down for the wwp-1 gene by two independent wwp-1 RNAi clones were statistically hypersensitive to Cry5B.

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Cry5B [11] and the oxidative stress agent H2O2 (a toxic insult that kills much more rapidly). All wwp-1 mutants, including wwp-1(gk372), wwp-1(gk397), and wwp-1(ok1102), have the similar sensitivity as wild type to killing by either CuSO4 or H2O2 (Figure 5B and 5C; Table 4). These data argue against the supposition that these mutants are hypersensitive to the PFT and P. aeruginosa tested above merely because they are generally unhealthy. Taken together, the above results suggest that WWP-1 is not only a positive regulator in the longevity regulation but also specifies in the intrinsic cellular defense (INCED) against PFTs and the innate immune response against pathogenic bacteria in C. elegans.

Table 3. Data analysis of the RNA interference assays.

| RNAi in rrf-3(pk1426); ghp-4(bn2) (Figure 3E) | Survival (%) | Standard Deviation | p value relative to RNAi empty vector control (pL4440) |
|-----------------------------------------------|--------------|--------------------|---------------------------------------------------|
| pL4440 (vector control)                        | 22.55        | 3.00               |                                                   |
| daf-2 RNAi (pAD48)                             | 47.91        | 4.11               | p<0.01                                           |
| wwp-1 RNAi (8A5)                               | 7.97         | 0.98               | p<0.01                                           |
| wwp-1 RNAi (8A15)                              | 5.43         | 2.15               | p<0.01                                           |

Table 4. Data analysis of the lifespan and general stresses assays.

| Life span assay (Figure 5A) and strain | Median survival (days) | p value relative to N2 |
|----------------------------------------|------------------------|------------------------|
| N2 wild-type                           | 17.00                  |                        |
| wwp-1(gk372)                           | 12.00                  | p<0.01                 |
| wwp-1(ok1102)                          | 10.50                  | p<0.01                 |

| CuSO4 (Figure 5B) and strain | LC50 (mM) | Standard Deviation | p value relative to N2 |
|-----------------------------|----------|--------------------|------------------------|
| N2 wild-type                | 5.95     | 0.20               |                        |
| wwp-1(gk372)                | 7.57     | 0.18               | p<0.01                 |
| wwp-1(gk397)                | 7.35     | 0.18               | p<0.01                 |

| H2O2 (Figure 5C) and strain | LC50 (mM) | Standard Deviation | p value relative to N2 |
|-----------------------------|----------|--------------------|------------------------|
| N2 wild-type                | 5.62     | 0.15               |                        |
| wwp-1(gk372)                | 6.52     | 0.15               | p<0.01                 |
| wwp-1(gk397)                | 6.32     | 0.15               | p<0.01                 |
| wwp-1(ok1102)               | 6.12     | 0.15               | p<0.01                 |

| PA14 killing assay (Figure 4) and strain | Median survival (hours) | p value relative to N2 |
|------------------------------------------|-------------------------|------------------------|
| N2 wild-type                             | 75                      |                        |
| wwp-1(gk372)                             | 50                      | p<0.01                 |
| wwp-1(gk397)                             | 50                      | p<0.01                 |
| wwp-1(ok1102)                            | 50                      | p<0.01                 |

Cry5B [11] and the oxidative stress agent H2O2 (a toxic insult that kills much more rapidly). All wwp-1 mutants, including wwp-1(gk372), wwp-1(gk397), and wwp-1(ok1102), have the similar sensitivity as wild type to killing by either CuSO4 or H2O2 (Figure 5B and 5C; Table 4). These data argue against the supposition that these mutants are hypersensitive to the PFT and P. aeruginosa tested above merely because they are generally unhealthy. Taken together, the above results suggest that WWP-1 is not only a positive regulator in the longevity regulation but also specifies in the intrinsic cellular defense (INCED) against PFTs and the innate immune response against pathogenic bacteria in C. elegans.

Figure 4. WWP-1 is involved in the innate immunity against P. aeruginosa PA14. A lifespan assay was used to compare the wwp-1 mutants to slow killing by P. aeruginosa PA14. This graph represents combined data from three experiments. The lifespan of wwp-1 mutants, including wwp-1(gk372) and wwp-1(ok1102), feeding on P. aeruginosa PA14 bacteria are statistically shorter than wild-type N2 animals. doi:10.1371/journal.pone.0009494.g004
WWP-1 Works Downstream of DAF-2 and in Parallel to DAF-16 in the DAF-2 Insulin/IGF-1 Signaling Network

The two-hybrid interactome data and the Cry5B/aging/P. aeruginosa results are consistent with WWP-1 acting in the DAF-2 pathway antagonistic to and downstream of DAF-2. If true, then we predict that knock-down of WWP-1 should suppress a DAF-2 mutant phenotype. We therefore knocked down wwp-1 in daf-2(e1370) and daf-2(e1370);daf-16(mu86) mutant animals and exposed these wwp-1 knockdown animals (and control “no knock down” animals in which the animals were fed empty RNAi vector) to purified Cry5B (Figure 6, Table 3). Our results showed that wwp-1 RNAi can partly suppress the daf-2(e1370) resistant phenotype and that the combination of daf-16 knock out (via mu86 mutation) and wwp-1 knock down (via RNAi) can completely suppress daf-2(e1370) resistance back to a response similar to wild-type (p = 0.12 daf-2;daf-16/wwp-1 RNAi vs. N2/L4440 vector control; note, although the response of daf-2;daf-16/wwp-1 RNAi animals looks less hypersensitive than that of N2/wwp-1 RNAi animals, the two are actually statistically similar; P = 0.06). These data are consistent with both daf-16 and wwp-1 acting in parallel daf-2-dependent pathways to mediate resistance to Cry5B PFT. Furthermore, that wwp-1 RNAi can not further sensitize the wwp-1(ok1102) worms in this experiment also confirmed that this mutant allele is a total loss-of-function mutant.

Discussion

Here we demonstrate for the first time that the DAF-2 insulin/IGF-1 pathway is involved in intrinsic cellular defenses (INCED) against PFTs. Furthermore, our data suggest that the daf-2 PFT defense pathway bifurcates at PDK-1 into DAF-16-dependent and DAF-16-independent branches. This is the first report of a bifurcation of the daf-2 insulin/IGF-1 pathway at this junction. We furthermore find a protein, WWP-1, that appears to be involved in the DAF-16 –independent branch of the INCED/
Innate immune response. Loss of WWP-1, a protein in the ubiquitin E3 ligase family, leads to hypersensitivity to the PFT Cry5B and the pathogen *P. aeruginosa* and that, based on double and triple knock out/knock down analyses with *daf-2* and *daf-16* mutants, appears to work in parallel with DAF-16. A model summarizing our findings is shown in Figure 7.

The assignment of WWP-1 as downstream of the DAF-2 insulin/IGF-1 pathway is based on two-hybrid interactome data with PDK-1, on phenotypic analyses of responses to PFT, *P. aeruginosa*, and aging, and on our genetic pathway data. A similar inference is evidenced from a previous genome-wide RNAi screen in which it was shown that knockdown of *wwp-1* gene decreased median lifespan by 24% in *daf-2(e1370)* mutants, 5% in *daf-2(e1370);daf-16(n3274)* mutants, and 9% in wild-type N2 animals [32]. The knockdown of *wwp-1* gene more significantly shortened the lifespan of *daf-2* animals but still decreased the lifespan of *daf-2;daf-16* mutant and wild-type N2 animals. This suggested that *wwp-1* functions in parallel to *daf-16* and converging within the DAF-2 insulin/IGF-1 signaling network to regulate longevity, which is reminiscent of our data. In addition, recently ubiquitin ligase activity has been shown for WWP-1, as well as demonstration that WWP-1 is involved in a *daf-16*-independent life span extension in response to diet restriction [33]. Taken together, our and others’ results imply that WWP-1 functions in parallel to DAF-16 and converging within the fundamental DAF-2 insulin/IGF-1 signaling network to regulate the INCED against PFTs attack, innate immune responses against *P. aeruginosa*, as well as longevity in *C. elegans*.

Our results also show that regulation of PFT defense and lifespan can clearly be decoupled. First, we note that lifespan extension of *daf-2* mutants is completely dependent upon *daf-16* [34] but response to PFT is not. Second, we note that where *sgk-1* mutants are long-lived [27], mutation in this gene alone is not resistant to PFTs. The hypersensitivity of akt-1 and possibly akt-2 mutant animals is intriguing and suggests that they might also have other roles outside of the DAF-2 insulin/IGF-1 pathway in PFT defenses. Interestingly along these lines, we note that in mammalian cells a role of AKT in response to PFTs has already been noted [35].

That DAF-2 signal can be decoupled from the conical pathway is reminiscent of several recent reports. Firstly, it has been demonstrated that EAK-3 (Enhancer of AKT-1 null) functions in parallel to AKT-1 to inhibit the expression of Forkhead transcription factor DAF-16 target genes involved in *C. elegans* dauer development. That Eak-3 mutants have normal lifespan indicates EAK-3 decouples insulin-like regulation of development and longevity [36,37]. Secondly, it has been reported that long-lived mutants of genes downstream of *daf-2*, such as *pdk-1* and *gsk-1*, show wild-type resistance to the human opportunistic pathogen, *Pseudomonas aeruginosa* strain PA14. However, mutants of *akt-1* and *akt-2* show enhanced resistance to *P. aeruginosa* PA14 [26]. Thirdly, it has also been reported that some *akt-1* and *pdk-1* alleles can uncouple the dauer arrest, adult longevity and stress resistance phenotypes of *age-1(n9109)* mutants [38]. They also demonstrated reproductive development in *age-1(n9109)*; *mg227* animals required only *akt-1*, and *pdk-1* activity was dispensable in this background. The expression of *age-1* and *pdk-1* genes is associated with the other possible PDK-1 interacting protein identified in the interactome database, PCM-1, has also been demonstrated to participate in the repair of age-damaged proteins and overexpression of PCM-1 increases adult life span [39,40]. However in our results, PCM-1 is not involved in Cry5B INCED. All of these data suggest that dauer formation, lifespan regulation, stress response, and pathogen resistance signals can be intertwined and in some cases decoupled within the DAF-2 insulin/IGF-1 signaling network in *C. elegans*.

In summary, we have identified specifically WWP-1 and the DAF-2 insulin/IGF-1 signaling network as components of INCED against PFTs. The biological functions of the DAF-2/DAF-16 signaling pathway in longevity and pathogens resistance have been extensively studied for years [15,18,41]. Our current hypothesis is that the DAF-2 signaling network has split off a separate arm at PDK-1/WWP-1 not only involved in longevity regulation and the innate immunity against *P. aeruginosa* PA14 but also the INCED against PFTs as well as perhaps some yet unknown responses.

### Materials and Methods

**C. elegans** and Bacterial Strains

Some *Caenorhabditis elegans* strains used in this work were provided by the *Caenorhabditis* Genetics Center (CGC), which is funded by the NIH National Center for Research Resources (NCRR). *C. elegans* strains were maintained on NG plates using *Escherichia coli* strain OP50 as the food source [42]. Strains used in this study were wild-type Bristol strain N2, *daf-2(e1370)*, *age-1(hb546)*, *aap-1(m389)*, *daf-18(e1375)*, *pdk-1(sa680)*, *pdk-1(ka709)*, *pdk-1(n9142)*, *akt-1(ok523)*, *akt-1(sa573)*, *akt-1(mg144)*, *akt-2(ok393)*.

![Figure 7. Schematic illustrating relationship between WWP-1 and DAF-2 insulin-like signal network.](image)
sgk-1(ok338), daf-16(mu86), daf-2(e1370);daf-16(mu86), bre-3(ce28), rgy-3(pk1426), rgy-3(pk1426); ghp-4(b2), pcm-1(e2091), wasp-1(ok1102), wasp-1(gk372), and wasp-1(gk397) were each backcrossed at least 4 times. Bacteria expressing dsRNA directed against br-3 and wasp-1 were part of a C. elegans RNAi library in E. coli strain HT115 (Geneservice, Cambridge U.K.). All RNAi clones have been confirmed by plasmid DNA sequencing. Escherichia coli HT115 transformed with the pAD48 construct, which expresses dsRNA targeting the daf-2 gene, was kindly provided by A. Dillin (Salk Institute, San Diego) [43]. All bacterial strains were cultured under standard conditions.

Cry Toxins Toxicity Assays and Microscopy

All assays were performed at 25°C unless indicated elsewhere. Qualitative toxicity assays based on visual comparison of worm intoxication were performed on plates with E. coli-expressed Cry5B as described [19]. L4 stage worms were fed for 48 h on control plates with E. coli that did not express Cry5B (pQE9 vector control) or on plates prepared with E. coli expressing Cry5B (pQE9-Cry5B) [7]. The relative health of each worm was determined qualitatively by comparing body size, darkness, and activity. Images were acquired using an Olympus SZ60 compound microscope using the 3X magnification linked to a Canon PowerShot A620 digital camera. Quantitative mortality assays were performed as described [19]. Concentrations of each toxin were set up in triplicate for each assay with approximately 20–30 worms per well, and each assay was performed independently at least three times. The purified Cry5B and the crystal-spore toxin were used as negative control of RNAi. Synchronized L1 animals were cultured on the NG-IC plates to quantitative scored the survival. Similar conditions were used as the quantitative mortality assays described above, except that E. coli strains expressing bacteria at 0.6 OD600 in steady purified Cry5B and E. coli OP50 were used in this assay. The survival rate of each well was scored after incubating at 25°C for 6 days.

RNA Interference (RNAi)

For figures 1D, RNAi assays were carried out on E. coli-expressed Cry5B Plates. E. coli strain HT115 transformed with RNAi plasmids were spread on NG-IC plates [NG plates with 25 µg/ml carbenicillin and 0.1 mM isopranyl-β-D-1-thiogalactopyranoside (IPTG)] and incubated at 25°C overnight to induce the dsRNA expression. E. coli strain HT115 with pL4440, an empty vector, was used as negative control of RNAi. Synchronized rga-3(pk1426) L1 larvae were obtained using standard protocols [19] then cultured on pL4440, br-3, or daf-2 gene dsRNA expressing RNAi-plates at 20°C until L4 stage. These L4 stage worms were transferred to either control plates with E. coli HT115 that did not express Cry toxins (empty vector) or plates prepared with E. coli strain HT115 expressing either Cry5B or Cry21A (using our standard expression vector; [7]) together with E. coli strain HT115 either carrying RNAi plasmids or the pL4440 plasmid and then incubated at 25°C for 48 h. The relative health of each worm was determined qualitatively by its appearance as described above.

For figures 3E, synchronized L1 rga-3(pk1426); ghp-4(hn2) animals (mutant is hypersensitive to RNAi and does not produce progeny that would otherwise complicate the assay; both mutants have roughly normal response to Cry5B; [10]) were cultured on the NG-IC daf-2 or wasp-1 RNAi-plates at 20°C until L4 stage. L4 stage rga-3(pk1426); ghp-4(hn2) RNAi knock down animals were washed out by S medium and transferred to the wells of 48-well plate with S medium containing E. coli HT115 RNAi bacteria at 0.6 OD600 and 20 µg/ml of purified Cry5B. For incubating at 25°C for 6 days, the survival rate of each well was scored.

For figure 6, the daf-2(e1370), daf-16(mu86), daf-2(e1370);daf-16(mu86) and wasp-1(ok1102) mutant worms were used. Synchronized L1 animals were cultured on the NG-IC wasp-1 RNAi or pL4440 E. coli strain HT115 plates at 20°C until L4 stage. L4 stage RNAi knock down animals were washed out by S medium and transferred to the wells of 48-well plate with S medium containing with either wasp-1 RNAi or pL4440 E. coli strain HT115 bacteria at 0.6 OD600 and 20 µg/ml of purified Cry5B. After incubating at 25°C for 6 days, the survival rate of each well was scored.

Lifespan Assay

Lifespan analysis was conducted according to standard protocols [34,45]. All life span experiments were performed in the absence of 5-fluoro-2′-deoxyuridine. Briefly, to obtain a synchronously growing population, eggs were prepared by treating a population of C. elegans with hypochlorite/NaOH solution and transferring the resulting eggs to NG agar plates covered with E. coli strain OP50. When these had reached the young adults, ~150 nematodes were transferred to fresh plates, which also represents the first day of life span analysis. Nematodes were transferred to fresh plates daily during the progeny production period and after that were transferred every second to third day but monitored daily for dead animals. Nematodes that did not respond to gentle prodding and displayed no pharyngeal pumping were scored as dead. Animals that crawled off the plate or died due to internal hatching or protrusion of the gonads through the vulva were censored. Censoring describes an event where partial information on the life span of an individual animal is lost as a consequence of premature death. Thus, censored animals were included in statistical analysis only until the day of the censoring event. Survival analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA). The Mantel-Cox logrank test was used to assess statistical significance of difference in survival. Only p-values<0.01 were considered significant to minimize type I errors.

Pseudomonas aeruginosa PA14 Killing Assay

The P. aeruginosa killing assay was performed on slow-killing plates as described [46], with the following modifications: PA14 was cultured overnight in tryptic soy broth instead of King’s broth and then spread on slow-killing plates complemented with 75 µM 5-fluoro-2′-deoxyuridine. The experiment was performed three times with approximately 100–150 worms total per strain at 25°C. The Mantel-Cox logrank test was used to assess statistical significance of difference in survival. Only p-values<0.01 were considered significant to minimize type I errors.

General Stressors Analysis

CuSO₄ assay. Assays were carried out as described [11]. In brief, a serial doses of CuSO₄, E. coli OP50 at an optical density of 0.2–0.25 OD600, and ~30 L4 larvae were used per well in 48-well plates. Lethality was determined after 6 days of CuSO₄ exposure at 25°C.

H₂O₂ assay. Assays were carried out as described [11]; lethality was determined after 4 hours of H₂O₂ exposure at 25°C.

Data Analysis

All experiments were performed a minimum of three times. LC₅₀ values were determined by PROBIT analysis [47]. The
lethal concentration assays are represented graphically using nonlinear regression performed with the software GraphPad Prism 5.0. Statistical analysis between two values was compared with a paired t-test. Statistical analysis among three or more values was compared with one-way ANOVA with Dunnett adjustment. All data analysis was performed using SPSS, ver 13.0 (SPSS, Chicago, IL). Statistical significance was set at p<0.05.

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Author Contributions

Conceived and designed the experiments: CSC RVA. Performed the experiments: CSC AB CYK YLY HDC FCO. Analyzed the data: CSC RVA. Wrote the paper: CSC RVA.

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