The Pseudokinase NIPI-4 Is a Novel Regulator of Antimicrobial Peptide Gene Expression

Sid ahmed Labeled, Shizue Omi, Martha Gut, Jonathan J. Ewbank, Nathalie Pujol

Abstract

Hosts have developed diverse mechanisms to counter the pathogens they face in their natural environment. Throughout the plant and animal kingdoms, the up-regulation of antimicrobial peptides is a common response to infection. In C. elegans, infection with the natural pathogen Drechmeria coniospora leads to rapid induction of antimicrobial peptide gene expression in the epidermis. Through a large genetic screen we have isolated many new mutants that are incapable of upregulating the antimicrobial peptide nlp-29 in response to infection (i.e. with a Nipi or ‘no induction of peptide after infection’ phenotype). More than half of the newly isolated Nipi mutants do not correspond to genes previously associated with the regulation of antimicrobial peptides. One of these, nipi-4, encodes a member of a nematode-specific kinase family. NIPI-4 is predicted to be catalytically inactive, thus to be a pseudokinase. It acts in the epidermis downstream of the PKCδ-TPA-1, as a positive regulator of nlp antimicrobial peptide gene expression after infection. It also controls the constitutive expression of antimicrobial peptide genes of the cnn family that are targets of TGFβ regulation. Our results open the way for a more detailed understanding of how host defense pathways can be molded by environmental pathogens.

Introduction

Pathogenic microorganisms represent one of the most ubiquitous and powerful sources of selection for higher eukaryotes including humans [1]. Different pathogens have specific natural host tropisms, sometimes broad, as in the case of Pseudomonas aeruginosa [2,3], and in other cases, such as HIV, very narrow. Part of this tropism reflects the divergent mechanisms of host resistance, as exemplified by cultivar-specific resistance in plants [4]. The evolution of adaptive immunity is often cited as an extreme example of immune system evolution. But even among invertebrates that rely on their innate immune systems, there is evidence for considerable variation from the phylum to the species level. For example, in contrast to most other animal species, nematodes, for considerable variation from the phylum to the species level. For

* E-mail: pujol@ciml.univ-mrs.fr

Copyright: © 2012 Labeled et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Citation: Labeled Sa, Omi S, Gut M, Ewbank JJ, Pujol N (2012) The Pseudokinase NIPI-4 Is a Novel Regulator of Antimicrobial Peptide Gene Expression. PLoS ONE 7(3): e33887. doi:10.1371/journal.pone.0033887

Editor: François Leulier, French National Centre for Scientific Research - Université Aix-Marseille, France

Received January 6, 2012; Accepted February 23, 2012; Published March 21, 2012

Copyright: © 2012 Labeled et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was funded by institutional grants from INSERM and CNRS, program grants from the Agence Nationale de la Recherche - French National Research Agency (ANR FUNGENOMICS) and the Fondation pour la Recherche Médicale - Medical Research Foundation (FRM ING20091217918). S.L. is supported by a fellowship from the French Ministry of Research. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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

* E-mail: pujol@ciml.univ-mrs.fr
characterized. Wounding and infection require G-protein signaling, involving the Gα protein GPA-12 and the Gβ RACK-1, while infection specifically involves the Tribbles-like kinase NPI-3 [18,20].

In addition to provoking the increased expression of AMPs, wounding also triggers a rise in intracellular Ca²⁺. This is controlled by an epidermal signal transduction pathway that includes the Gα(q) EGL-30. This pathway is required for actin-dependent wound closure, but not for injury-induced AMP expression [22]. On the other hand, the Death-associated protein kinase DAPK-1 negatively regulates wound repair and AMP gene expression [23]. Many, but not all, of the elements that act in the epidermis also mediate the innate defenses against intestinal pathogens and toxins [24,25,26,27,28,29,30,31,32]. Conversely, certain genes that participate in p38 MAPK signaling in the intestine, including dkh-2 [28] are not required for the induction of nlp-29 [20].

Our current understanding of both epidermal and intestinal innate immunity is far from complete. In the current study, we therefore undertook a large genetic screen for components of the signaling pathways that control AMP gene expression in the epidermis. We isolated and mapped 26 mutant alleles, uncovering 6 new genes required for AMP gene induction after D. coniosispora infection. We cloned one of these genes, nipi-4 (npi for “no induction of peptide after Droschmeria infection”). We show here that nipi-4 encodes a nematode-specific protein with a kinase-like domain that is predicted to be a pseudokinase. It acts downstream of PKCβ/TPA-1, which was previously shown to modulate the activity of a conserved p38 MAPK cassette [20]. This provides an illustration of an animal family-specific modulation of an innate immune signaling pathway.

**Results**

**A genetic screen for Nipi mutants**

We undertook a large-scale genetic screen for mutants that prevented the normal induction of a Pulp-29::GFP reporter transgene after infection with D. coniosispora. From 130,000 mutagenized haploid genomes, we isolated 57 candidate mutant strains. These were then subjected to a confirmatory round of screening and outcrossing. We retained 44 mutant strains that had a sufficiently penetrant phenotype. All behaved as if they were carrying simple recessive alleles. To characterize these in further detail, we first quantified reporter gene expression in uninfected and infected worms. All mutants showed a reduction of Pulp-29::GFP induction with the most penetrant alleles showing essentially a complete block of the reporter (Figure 1A).

Three strains exhibited resistance to the phorbol ester PMA. As the only gene known to provoke PMA-resistance in C. elegans is tsp-1 [33,34], we sequenced this gene in one mutant and thus identified a G334E mutation. We presume that the other two mutants are also tsp-1 alleles, but did not characterize them as there are already more than 50 available tsp-1 alleles. For the other 41 strains, we performed classical SNP mapping to assign alleles to individual chromosomes, which was unambiguous for 26 of them. We then performed targeted complementation tests, between alleles, and with candidate genes on the appropriate chromosome. When a new allele failed to complement a candidate gene, the corresponding gene from the mutant was sequenced. This allowed the identification of new alleles for 4 previously characterized Nipi genes, 6 for npi-12, 3 for npi-1-1, 2 for npi-1-1 and 1 for sta-2. These numbers give an indication of the degree of saturation of the screen. The remaining alleles appear to correspond to previously uncharacterized genes. They fall into 6 complementation groups, some represented by multiple alleles (Figure 1B, Table 1).

**Molecular identification of nipi-4**

One complementation group was given the name nipi-4 and characterized in detail. Whole-genome resequencing of pooled recombinants [35] between nipi-4(fr106) and the polymorphic Hawaiian strain CB4856 clearly delineated a candidate region for the mutation on the center of chromosome V (Figure 2A). Within this region, only one nonsense mutation was found, in the gene F40A3.5, which is predicted to encode a 396 amino acid membrane-bound protein with a tyrosine kinase domain [36] (Figure S1). Sequencing this gene from the other nipi-4 alleles revealed 3 independent mutations, a different nonsense mutation in fr71, an alteration of a splice acceptor site in fr99 that would be predicted to lead to a severely truncated protein, and a missense mutation in the kinase domain in fr68 (Figure 2B, Table 1). This very strongly suggests that nipi-4 corresponds to F40A3.5. In contrast to fr68, which has a milder phenotype, the alleles fr71, fr99 and fr106 all provoke similarly penetrant phenotype and are predicted to correspond to null alleles (Figure 2C). Transformation rescue confirmed the identity of nipi-4 as F40A3.5 (Figure 2D).

Interestingly, we have only identified Nipi-4/F40A3.5 orthologs in nematodes. The Camorhabditis species, from elegeans, briggsae, breneria, japonica and renan species, are predicted to be kinase-dead, since they lack the essential aspartic acid active site residue. In contrast, predicted Nipi-4 orthologs from non-Camorhabditis species like Ascaris suum and Pseustonchus pacificus are expected to be functional kinases. Conversely, only the Camorhabditis species have a tyrosine in the predicted activation loop that could potentially be the target of phosphorylation (Figure S1). We discuss the significance of these observations below.

nipi-4 acts cell autonomously in epidermal cells

To identify the cells in which nipi-4 is expressed, we generated transgenic animals carrying a GFP transcriptional reporter construct (Figure 2B). We observed expression in the epidermis of C. elegans throughout development (Figure 3A–E). This pattern overlaps with that of the previously characterized components of the PKCβ/p38 MAPK pathway, including snf-12 and sta-2 [21] and suggests that nipi-4 may act in a cell-autonomous manner. To evaluate this directly, we generated transgenic animals in which the expression of nipi-4 was under the control of the col-19 promoter, which is expressed specifically in epidermal cells as animals enter adulthood [37]. In these worms we observed an essentially normal expression of Pulp-29::GFP upon infection (Figure 3F–G). On the other hand, expression of nipi-4 in the intestine under the control of the cha-6 promoter [30] did not give any rescue (Figure S2). Together, this indicates that nipi-4 acts cell-autonomously in the epidermis to regulate antimicrobial peptide gene expression.

nipi-4 regulates AMP gene expression after infection and wounding

To define further the function of nipi-4, we assayed the expression of the Pulp-29::GFP reporter transgene in the nipi-4 mutant background under other conditions that normally lead to its expression, including injury, exposure to PMA and osmotic stress [16,18,20]. In a nipi-4(fr71) mutant, in addition to a nearly complete block of Pulp-29::GFP expression after infection, there was no induction of the reporter gene upon needle wounding or exposure to PMA. There was, however, a strong induction of Pulp-29::GFP expression upon exposure to high salt, comparable to that...
seen in a sta-2 mutant. Similar results were obtained with nipi-4(fr99) and nipi-4(fr106) (Figure 4A, and results not shown). This suggests that nipi-4 acts downstream of the PKCa TPA-1 to regulate nlp-29 expression specifically after wounding and infection.

We also analyzed the expression of other genes that have been shown to be induced upon D. coniospora infection [16]. We could confirm by qRT-PCR that in the nipi-4(fr106) mutant, just as in sta-2 or snf-12 mutants [21], the induction of nlp-29 after infection was essentially abrogated. Two other genes of the nlp-29 cluster, nlp-31 and nlp-34 were similarly affected (Figure 4B). It is interesting to note that in the nipi-4 and sta-2 mutants the constitutive expression of nlp-34 was reduced by 10 fold whereas it was not greatly changed for nlp-29 and nlp-31. The genes of the cnc-2 cluster are regulated in a manner distinct from nlp-29 as their induction after D. coniospora infection is p38 MAPK independent. Rather their induction requires signaling via a non-canonical TGFβ/DBL-1 pathway [19]. We found by qRT-PCR that loss of function of nipi-4 strongly affected the constitutive expression of cnc-1 and cnc-2 and to a lesser extent cnc-4. This parallels the phenotype due to loss of sta-2 function (Figure 4B), as well as snf-12 [21]. Indeed, as discussed below, the constitutive expression of
these genes was reduced to such a degree that it is technically
difficult to evaluate the extent of gene induction after infection.
Thus, like snf-12 and sta-2, nipi-4 plays a role in innate immune
signaling and influences targets of both the PKC
h/p38 MAPK/PMK-1 and TGF
b/DBL-1 pathways.

Modulation of AMP gene expression by
gpa-12 requires nipi-4

Our previous dissection of the innate immune signaling
pathways that govern AMP expression in the epidermis relied
on the use of PMA to activate TPA-1/PKC
h and an active form of
the G
a protein GPA-12 (GPA-12* [34]), produced under the
control of a heat-shock promoter [20,21]. As both PMA and heat-
shock have pleiotropic effects on the physiology of
C. elegans, we
developed a more refined tool, with GPA-12* under the
control of the
col-19 promoter, driving its expression in the adult epidermis
[37]. We injected this construct into worms carrying an integrated
P
nlp-29::GFP reporter. In uninfected transgenic worms carrying
the P
col-19::GPA-12* construct, we observed a very marked
increase in the expression of P
nlp-29::GFP in the epidermis from
the late L4 stage onwards. The level of reporter gene expression
was even further increased upon infection with
D. coniospora(Figure 5A & B). As expected, increased
P
nlp-29::GFP expression
was totally abrogated in a tpa-1 mutant (results not shown). We
found by qRT-PCR that the transgenic strain exhibited an

Table 1. New Nipi alleles.

| Allele | Genomic position | Gene | Mutation | Protein modification |
|--------|------------------|------|----------|----------------------|
| fr103  | II:5,023,842     | nsy-1| C to T   | nonsense             |
| fr98   | II:5,026,498     | nsy-1| C to T   | nonsense             |
| fr96   | IV:105,994       | tpa-1| G to A   | G384E (B0545.1a)    |
| fr76   | IV:5,026,945     | nipi-4| C to T  | G313E                |
| fr68   | V:7,869,401      | nipi-4| G to A  | G313E                |
| fr99   | V:7,869,753      | nipi-4| G to A  | Splice acceptor      |
| fr106  | V:7,870,474      | nipi-4| C to T  | T          |
| fr71   | V:7,874,675      | sta-2| G to A  | D212N                |
| fr101  | X:7,818,617      | sek-1| G to A  | G194R                |
| fr75   | X:9,030,736      | snf-12| G to A | E472K                |
| fr72   | X:9,031,202      | snf-12| G to A | G391R                |
| fr74   | X:9,031,495      | nipi-4| G to A  | G391R                |
| fr81   | X:9,032,001      | snf-12| T to G  | nonsense             |
| fr102  | X:9,032,231      | snf-12| TA insertion | Frame shift |
| fr70   | X:9,032,686      | snf-12| G to A  | R792K                |
| fr73   | II:5             | nipi-5|         |                      |
| fr77   | II:6             | nipi-6|         |                      |
| fr97   | III:7            | nipi-7|         |                      |
| fr108  | III:7            | nipi-7|         |                      |
| fr110  | III:7            | nipi-7|         |                      |
| fr89   | X:8             | nipi-8|         |                      |
| fr92   | X:9             | nipi-9|         |                      |

1WormBase Release WS228.

doi:10.1371/journal.pone.0033887.t001

Figure 2. nipi-4 encodes a pseudokinase required for the
induction of nlp-29. (A) SNP mapping with WGS. The positions of SNP
loci on Chromosome V for the fr106 allele are depicted as a XY scatter
plot, where the ratio ‘Hawaiian/total number of reads’ for each SNP is
represented, as in [35]. The region without Hawaiian SNPs contains the
mutation (red arrow). (B) Exon-intron structure of nipi-4, adapted from
WormBase (WS220), with the positions of the fr68, fr71, fr99 and fr106
mutations indicated. Also shown is the structure of the p
nipi-4::GFP &
p
nipi-4::NIPI-4 constructs. (C) Biosort quantification of the normalized
fluorescence ratio in wild type, sta-2(ok1860) and the 4
nipi-4 alleles
fr68, fr71, fr99 and fr106 following infection. For this and
subsequent figures, see Materials and Methods for details of the data
processing and the number of worms analyzed. The results are
representative of 3 independent experiments. (D) Biosort quantification of the normalized fluorescence ratio in wild type, nipi-4(fr106) and nipi-4(fr106) with a rescuing transgene p
nipi-4::NIPI-4, carrying frIs7 following infection.
doi:10.1371/journal.pone.0033887.g002
The form of GPA-12 was abrogated, to a similar degree as in the expression of the other mutant background (Figure 5A). The effect of GPA-12* on the P cells (arrowhead in A), scale bar 10 μm. (F–G) nipi-4(fr71) and nipi-4(fr71):frEx496 (Pcol-19:niPI-4) worms strains carrying an integrated Nlp-29::GFP reporter (fris7) following infection. The expression of nipi-4 in epidermal cells in the adult rescues the nipi-4 phenotype. Green and red fluorescence is visualized simultaneously with a GFP long pass filter.

Discussion

To characterize the molecular pathways that underpin anti-fungal innate immunity in C. elegans, we previously undertook a small-scale genetic screen for genes required for the induction of an AMP reporter gene after infection. We isolated and characterized 5 alleles that fall into 4 complementation groups, of which 2 corresponded to genes that were hit in the previous screen, and 3 to genes previously duplicated. Certain defense mechanisms have been lost in parasitic nematodes, and are never found in free-living nematodes. Interestingly, while orthologs in A. suum and Pristionchus pacificus possess the characteristic catalytic aspartate residue, this residue is absent in all Caenorhabditis species, so these proteins are predicted to be catalytically inactive pseudokinases. This suggests that NIPI-4 has evolved a kinase-independent function in Caenorhabditis species. NIPI-4 might compete with one or more active kinases for substrates or binding partners. In such a scenario, the putative kinase(s) would need to play a negative regulatory role. The loss of catalytic activity in NIPI-4 has, however, been mirrored by the acquisition of a potential activation loop phosphorylation site not seen in other species. It is therefore tempting to speculate that NIPI-4 in Caenorhabditis species is able to donate its activation loop to another kinase following heterodimerization, as is seen for example with STRAD and LKB1 [41]. NIPI-4 and all its identified orthologs possess a predicted transmembrane segment, N-terminal to the kinase domain that could allow its association with the membrane of one or more classes of intracellular vesicles. We previously showed that SNF-12 is found in endosome-like vesicles, and that endocytosis is indispensable for the transcriptional response to infection [21]. One could conjecture that NIPI-4, SNF-12 and STA-2 form a signaling complex on endosomes that is activated following physical association with the MAPK PMK-1. Understanding the function of NIPI-4 at the biochemical and cellular level, an elevated constitutive expression of nlp-29, nlp-31 and nlp-34, and cnc-1, cnc-4, and to a lesser extent cnc-2 (Figure 5C). The results mirrored to a striking degree the pattern of gene expression changes induced by infection (Figure 4B). When we crossed the Pcol-19::GPA-12* transgene into nipi-4(fr106) mutant, the elevated expression of the Pnlp-29::GFP reporter provoked by the active form of GPA-12 was abrogated, to a similar degree as in the sta-2 mutant background (Figure 5A). The effect of GPA-12* on the expression of other npc and nlp genes was also abolished in the nipi-4(fr106) mutant, as judged by qRT-PCR (Figure 5C). Together, these results confirm the role of nipi-4 as a novel regulator of AMP gene expression during the infection of the worm, acting genetically downstream of PKCe TPA-1.

Figure 3. The nipi-4 gene acts cell autonomously in the epidermis. (A–E) Expression of nipi-4 is seen throughout the epidermis (A & B), in larvae (C) and adults (A,B,D,E), from head (D) to tail (E), in vulval cells (arrow in B), in rectal cells (arrow in E), but not in the seam cells (arrowhead in A), scale bar 10 μm. (F–G) nipi-4(fr71) and nipi-4(fr71):frEx496 (Pcol-19:niPI-4) worms strains carrying an integrated Nlp-29::GFP reporter (fris7) following infection. The expression of nipi-4 in epidermal cells in the adult rescues the nipi-4 phenotype. Green and red fluorescence is visualized simultaneously with a GFP long pass filter.
objective of future studies, will give insights into how a species-specific host defense pathway can be molded by the natural pathogens found in a particular environmental niche.

Materials and Methods

Nematode strains

All strains were maintained on nematode growth media (NGM) and fed with *E. coli* strain OP50, as described [42]. In addition to the wild-type strain N2 and CB4856 that were obtained from the *Caenorhabditis* Genetics Center (CGC), the following mutants were used for complementation tests all carrying the frIs7 transgene containing the P$nlp$-29::GFP and P$col$-12::DsRed reporters [18]: snf-12(tm692) X, sek-1(km4) X, hsp-3(ok1083) X, nsy-1(age3) II, tir-1(tm3036) III, tpa-1(k530) IV, egl-8(n488) V and sta-2(ok1860) V. The 4 nipi-4 alleles were outcrossed twice with N2.

Mutants Isolation

We mutagenized IG274 wild type worms carrying the frIs7 transgene with EMS using standard procedures [43]. 130,000 genomes were screened using the same criteria described in [18]. Briefly, synchronized F2 worms were infected at the L4 stage with *D. coniospora*. After 24 h at 25°C, we screened for worms that failed to show an elevated level of GFP expression after *D. coniospora* infection and transferred them onto nystatin containing NGM plates. Mutant alleles were mapped through standard genetic and bulk SNP mapping by analysis of 20 to 30 recombinants with the strain CB4856 [44]. Genetic complementation tests were done between mutants located on the same chromosome, defining 6 new independent complementation groups.

Whole Genome Sequencing

nipi-4(fr106) mutation was further mapped and identified using a whole genome sequencing-SNP mapping protocol [35]. Briefly, nipi-4(fr106) was crossed with Hawaiian CB4856 males and 20 F2 mutant recombinant lines were isolated. The DNA of these pooled lines was prepared using a standard protocol with proteinase K lysis, RNAse A treatment and phenol/chloroform extraction. The pooled DNA was subjected to whole genome sequencing in multiplexed run with 4 samples in one sequencing lane of a v1.5 flowcell on HiSeq 2000 instrument, generating paired 100 nucleotide reads. The results were analyzed using Mappgen [45].

Figure 4. The nipi-4 gene is required for the response to infection and wounding. (A) nipi-4 mutants do not block the induction of nlp-29 expression upon osmotic stress. Biosort quantification of the normalized fluorescence ratio in wild type, sta-2(ok1860) and nipi-4(fr71) worms carrying frIs7 following infection by *D. coniospora*, wounding, PMA treatment and osmotic stress. (B) Quantitative RT-PCR analysis of gene expression levels in non-infected and infected wild type, sta-2(ok1860) and nipi-4(fr106) worms. The columns show the average expression level (arbitrary units) and SEM from 4 experiments. The level of nlp-34 expression in control animals is set at 1024 (see Materials and Methods). doi:10.1371/journal.pone.0033887.g004
A Pseudokinase in *C. elegans* Immunity

29:GFP reporter, with or without a transgene carrying an activated form of GPA-12 under the control of an epidermis promoter (Pcol-19::GPA-12*). (B) Images of the wild type strain carrying frs7 with (+GPA-12*) or without (−GPA-12*) Pcol-19::GPA-12* in control animal (−Dc) or worm infected by *D. coniospora* (−Dc). Green and red fluorescence is visualized simultaneously. (C) Quantitative RT-PCR analysis of gene expression levels in wild type and nipi-4(fr106) worms with or without Pcol-19::GPA-12*. The columns show the average expression level (arbitrary units) and SEM from 3 experiments. The level of *nlp-34* expression in control animals is set at 1024 (see Materials and Methods).

doi:10.1371/journal.pone.0033887.g005

Infection, wounding, exposure to high salt and PMA

Infections with *D. coniospora* and wounding were carried out at 25°C as described [18]. Briefly, animals were infected with *D. coniospora* at the L4 stage or exposed to high salt and incubated at 25°C. After 18 h, age-matched non-infected animals were used for wounding assays, exposure to PMA, or kept as control. Exposure of worms to high salt (350 mM NaCl) and PMA (1 µg/ml) were done on NGM plates as previously described [20].

Constructs and transgenic lines

*Pnipi-4::GFP* was obtained by Gateway cloning ([Invitrogen]™). A 2,521 bp fragment upstream of the *nipi-4* start site was amplified (with primers JEP1974–JEP1975), cloned into the pDONRP4-P1R vector, then transferred into the destination vector pDEST-DD04-Neo a generous gift from D. Dupuy [46] so that it was cloned upstream of the GFP::unc-54::3’UTR cassette. The *Pnipi-4::GFP* was injected at 20 ng/µl together with Ptxx-3:DsRed2 at 70 ng/µl into N2 worms. Two independent lines were generated showing the same expression pattern IG1341 wt; frEx483 and IG1342 wt; frEx484.

*Pnipi-4::NIPI-4* (pMS18) was obtained by multisite recombination Gateway cloning ([Invitrogen]™). A *nipi-4* genomic fragment comprising the entire ORF with the ATG but without the stop codon was amplified (JEP1964–JEP1965) and cloned into pDONR/Zeo ([Invitrogen]™). The promoter and gene entry clones were used together with a *unc-54::3’UTR* entry clone in a multi-partite LR reaction into the pJPDest R4R3 vector, to produce *Pnipi-4::NIPI-4*. This construct was injected at 20 ng/µl together with *Punc-53::GFP* [47] at 70 ng/µl into *nipi-4(fr106); frIs7*. One line was generated IG1343 wt; frEx485.

*Pcol-19::NIPI-4* was obtained by Gateway cloning ([Invitrogen]™). The *nipi-4* gene entry clone described above was recombined into the destination vector pCZGY1434 that contains the promoter of *col-19* (Pcol-19), a generous gift from A. Chisholm [22]. This construct was injected at 30 ng/µl together with *Pbnc-53::GFP* [47] at 70 ng/µl in IG1352 *nipi-4(fr71); frIs7*. Two lines were generated IG1404 wt; frEx496 and IG1405 wt; frEx497.

*Pnha-6::NIPI-4::GFP* (pMS21) was obtained by multisite recombinational Gateway cloning ([Invitrogen]™). A 1,255 bp genomic fragment upstream of the *nha-6* start site was amplified (with primers JEP1982–JEP1983), cloned into the pDONRP4-P1R gene entry clone described above was used together with *Punc-53::GFP* [47] at 70 ng/µl in IG1352 *nha-6::GFP* (fr71); frIs7. Two lines were generated IG1410 wt; frEx498 and IG1411 wt; frEx499.

*Ppol-19::GPA-12* was obtained by Gateway cloning ([Invitrogen]™). The DNA encoding an activated form of GPA-12 (with the Q205L mutation) was amplified from the construct pRPP205 a generous gift from R. Korswagen [34] with the primers JEP1976–JEP1977, inserted into a Gateway pDONR/Zeo ([Invitrogen]™) then recombined into the destination vector pCZGY1434 [22].
This construct was injected at 30 ng/µl together with pBabe-53::GFP [47] at 70 ng/µl in IG274 act; frIs7. One line was generated IG1363 wt; frEx486 and then subsequently integrated using Gamma rays and outcrossed several times with N2 generating IG1309 act; frIs7 IV; frEs9.

Analysis with the COPAS Biosort

Analysis of Pnlp-29::GFP induction in the strain carrying the frIs7 integrated array for the different treatments were all done at the same time on worms 24 h after the L4 stage with the COPAS Biosort (Union Biometrica™) [18]. The frIs7 integrated array consists of two reporter transgenes, Pnlp-29::GFP and Pcol-12::DsRed2. As the latter exhibits a constitutive expression in the epidermis that is unaffected by infection or other tested conditions, the fluorescence ratio green/red represents the variation in Pnlp-29::GFP expression normalized for the size of individuals [18]. The mean values are shown normalized to the wild type control that is set to one. The number of animals used in each experiment is given below. As previously described [18], due to the nature of the distribution, standard deviations are not always an informative parameter when measuring fluorescent reporter gene expression using the Biosort. Data are, however, in all cases representative of at least 3 independent experiments.

Number of animals quantified with the COPAS Biosort

Figure 2C: 231, 242, 197, 158, 91, 118, 139, 90, 122, 139, 67, 95

Figure 2D: 202, 182, 153, 123, 219, 120 (Combined 3 experiments)

Figure 4A: 104, 111, 98, 103, 158, 171, 160, 54, 72, 110, 139, 97, 85, 64, 81

Figure 5A: 286, 234, 542, 296, 515, 256 (Combined 3 experiments)

qRT-PCR

L4 worms were infected for 6 h at 25°C with D. constricta. 1 µg of total mRNA from infected and non-infected worms were used for reverse transcription (Applied Biosystems™). Quantitative real-time PCR was performed using 1 µl of cDNA in 10 µl of SYBERgreen Applied Biosystems™ and 0.1 µM of primers on a 7500 Fast Real-Time PCR System using act-1 [JEP538–JEP539] as a control, with nlp-29 [JEP948–JEP952], nlp-31 [JEP550–JEP553], nlp-34 [JEP969–JEP970], cnc-1 [JEP1087–JEP1088], cnc-2 [JEP944–JEP549] and unc-4 [JEP1124–JEP1125], for primer sequences see [21]. Results were normalized to act-1, and then relative expression calculated using 2((A+b)−x). A being the normalized cycle number for nlp-34 in the non-infected sample and x the value of interest. Control and experimental conditions were tested in the same run. Means and SEMs were calculated from a minimum of 3 independent experiments.

Primer sequences

JEP1966 ggggacagctcttcttgacaaggtgaatgctgattcagttgatcgcggcct
JEP1967 ggggacactttgtagaacggtagcgagcagcggattc
JEP1974 ggggacactttgtagaacggtagcgagcagcggattc
JEP1975 ggggacactttgtagaacggtagcgagcagcggattc
JEP1982 ggggacactttgtagaacggtagcgagcagcggattc
JEP1983 ggggacactttgtagaacggtagcgagcagcggattc

Supporting Information

Figure S1 Alignment of the predicted NIPI-4 proteins. Accession numbers for the different proteins are the following: C. elegans NP_505028, C. remanei XP_003115465, C. brenneri EGT43601, C. briggsae CAP37545, C. japonica JA58647. The Ascaris and Pristionchus proteins present in Genbank (ADY47863, PP1134) appear to have been mis-predicted. The figure presents more plausible predictions based on manual editing, respecting splice consensus sequences, of the output from tblastn using the C. elegans NIPI-4 protein against the relevant genomic sequence. All included sequences were found as significant matches with a smallest sum probability of at least e-25. For Meloidogyna hapla, Oncocera volvulus, Strongyloides ratti only partial sequences are presented, no attempt to reconstruct complete sequences was made (*). Alignments were produced with Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Thanks to G. Manning for the annotation of the different domains.(DOC)

Figure S2 Intestinal expression of NIPI-4 does not rescue the Nipi phenotype. (A–C) wild type (A, nipi-4(fr71) (B) and nipi-4(fr71);Pshrl-6::NIPI-4 (C) worm strains carrying frIs7 following infection. The expression of nipi-4 in the intestinal cells in the adult does not rescue the nipi-4 phenotype. Green and red fluorescence is visualized simultaneously. The green fluorescence at the level of the head and vulva in C is due to the co injection marker Punc-53::GFP [47]. (EPS)

Acknowledgments

We thank J. Belougne for worm sorting, G. Yuen, R. Duhecquet Deraveulle and C. Kergourlay for their help in the genetic screen, M. Dossidou, Z. Jin Tu, F. Montañana-Sanchis and S.Jaeger for their help in installing Maqgene, L. Agueda and M. Bayes for whole genome sequencing, A. Chisholm, D. Dupuy and R. Korschagen for sharing constructs, G. Manning for insight on the structure of the NIPI-4 kinase and for proposing its possible heterodimerization, members of the lab for helpful discussion and A. Chisholm and P. Golstein for critical reading of the manuscript. Some nematode strains were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR), or by the National Bioresource Project coordinated by S. Mitani.

Author Contributions

Conceived and designed the experiments: NP. Performed the experiments: SL, SO MG NP. Analyzed the data: SL, NP JJE. Contributed reagents/materials/analysis tools: MG. Wrote the paper: NP JJE.

References

1. Barreiro LB, Quintana-Murci L (2010) From evolutionary genetics to human immunology: how selection shapes host defence genes. Nat Rev Genet 11: 17–30.
2. He J, Baldini RL, Deziel E, Sauzier M, Zhang Q, et al. (2004) The broad host range pathogen Pseudomonas aeruginosa strain PA14 carries two pathogenicity islands of type b-acting plant and animal virulence genes. Proc Natl Acad Sci U S A 101: 2530–2535.
3. Rahme LG, Stevens EJ, Tompkins RG, et al. (1995) 4. Eitas TK, Dangl JL (2010) NB-LRR proteins: pairs, pieces, perception, partners, and pathways. Curr Opin Plant Biol 13: 472–477.
5. Rubin GM, Yardim MD, Worman JR, Gabor Miklos GL, Nelson CR, et al. (2000) Comparative genomics of the caykuate biosynthesis operons. Science 287: 2204–2215.

A Pseudokinase in C. elegans Immunity

PLoS ONE | www.plosone.org 8 March 2012 | Volume 7 | Issue 3 | e33887
24. Kim DH, Feinbaum R, Alloing G, Emerson FE, Garsin DA, et al. (2002) A reverse genetic analysis of components of the Toll signalling pathway in Caenorhabditis elegans. Curr Biol 11: 809–821.

23. Tong A, Lynn G, Ngo V, Wong D, Moseley SL, et al. (2009) Negative regulation of C. elegans immunity by novel viruses related to novel viruses. Proc Natl Acad Sci U S A 106: 1457–1461.

22. Xu S, Chisholm AD (2011) A Galpha(q)-Ca(2+) signaling pathway regulates migration of the nematode Caenorhabditis elegans. PLoS Biol 6: 2736–2752.

21. Dierking K, Polanowska J, Omi S, Engelmann I, Gut M, et al. (2011) Unusual actin-mediated epidermal wound closure in C. elegans. Curr Biol 10: 1615–1618.

20. Schulenburg H, Ewbank JJ (2004) Diversity and specificity in the interaction between Caenorhabditis elegans and the pathogen Seratia marcescens. BMC Evol Biol 4: 49.

19. Zugasti O, Ewbank JJ (2009) Neuroimmune regulation of antimicrobial peptide expression by a noncanonical TGF-beta signaling pathway in C. elegans. PLoS Pathog 4: e1000105.

18. Engelman I, Griffon A, Tschui L, Montanana-Sanchis F, Wang G, et al. (2011) A comprehensive analysis of gene expression changes provoked by bacterial and fungal infection in C. elegans. PLoS One 6: e19055.

17. Pujol N, Cypowyj S, Ziegler K, Millet A, Astrain A, et al. (2008) Distinct innate immune responses to infection and wounding in the C. elegans epidermis. Curr Biol 18: 481–489.

16. Pujol N, Link EM, Liu LX, Kurz CL, Alloing G, et al. (2001) A reverse genetic antibiotic selection marker for nematode transgenesis. Nat Methods 7: 721–723.

15. Jansson HB, Jeyaprakash A, Zuckerman BM (1985) Differential adhesion and expression of pseudokinases. Trends in cell biology 16: 443–452.

14. Engelmann I, Pujol N (2010) Innate immunity in C. elegans. PLoS Genet 6: e15435.

13. Dijksterhuis J, Veenhuis M, Harder W (1990) Ultrastructural study of adhesion to the nematode pathogen Microsporidia are natural intracellular parasites of the nematode Caenorhabditis elegans. Microbacterium nematophilum, induces morphological change in the nematode C. elegans. Curr Biol 10: 1615–1618.

12. Coleman JJ, Mylonakis E (2009) The tangled web of signaling in innate immunity. Cell Host Microbe 5: 341–352.

11. Hodgkin J, Kuwabara PE, Corneliussen B (2007) Detection and avoidance of a natural product from the pathogenic bacterium Microbacterium nematophilum by Caenorhabditis elegans. Proc Natl Acad Sci U S A 104: 2295–2300.

10. Hodgkin J, Kuwabara PE, Corneliussen B (2007) Mos1 mutagenesis reveals a diversity of mechanisms affecting response of Caenorhabditis elegans to bacterial pathogens. Microbacterium nematophilum. Genetics 175: 681–697.

9. Troemel ER, Felix MA, Whiteman NK, Barriere A, Ausubel FM (2008) Phosphorylation of the conserved transcription factor ATF-7 by PMK-1 p38 MAPK regulates innate immunity in Caenorhabditis elegans. PLoS Genet 4: e1000892.

8. Felix MA, Ashe A, Pilettini T, Wu G, Niez I, et al. (2011) Natural and experimental infection of Caenorhabditis elegans by novel viruses related to novel viruses. Proc Natl Acad Sci U S A 108: 6393–6398.

7. Huffman DL, Abrami L, Sasik R, Corbeil J, van der Goot FG, et al. (2004) Mitogen-activated protein kinase pathways defend against bacterial pore-forming toxins. Proc Natl Acad Sci U S A 101: 10995–11000.

6. Pujol N, Link EM, Liu LX, Kurz CL, Alloing G, et al. (2001) A reverse genetic antibiotic selection marker for nematode transgenesis. Nat Methods 7: 721–723.

5. van der Linden AM, Moorman C, Cuppen E, Korswagen HC, Plasterk RH (2003) Hyperactivation of the G12-mediated signalling pathway in Caenorhabditis elegans induces a developmental growth arrest via protein kinase C. Curr Biol 13: 516–521.

4. Doutsidou M, Poole RJ, Sarin S, Bigelow H, Hobert O (2010) C. elegans mutant identification with a one-step whole-genome-sequencing and SNP mapping strategy. PLoS One 5: e15435.

3. Manning G (2005) Genomic overview of protein kinases. WormBook. 2007/12/01 ed. http://www.wormbook.org: The C. elegans Research Community ed. pp 1–19.

2. Cox GN, Fields C, Kramer JM, Rosenzweig B, Hirsh D (1989) Sequence comparisons of developmentally regulated collagen genes of Caenorhabditis elegans. Gene 76: 331–344.

1. Pujol N, Bonnerot C, Ewbank JJ, Kohara Y, Thierry-Mieg D (2001) The Caenorhabditis elegans unc-32 gene encodes alternative forms of a vasopressin-like protein in the nematode C. elegans. Proc Natl Acad Sci U S A 98: 10995–11000.