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Evolution of host innate defence: insights from *C. elegans* and primitive invertebrates

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Preface

The genetically tractable model organism *Caenorhabditis elegans* was first used to model bacterial virulence *in vivo* a decade ago. Since then, great strides have been made in the identification of host response pathways that are involved in the defence against infection. Strikingly, *C. elegans* seems to detect and respond to infection without the involvement of its Toll-like receptor homologue, in contrast to the well-established role for these proteins in innate immunity in mammals. What, therefore, do we know about host defence mechanisms in *C. elegans*, and what can they tell us about innate immunity in higher organisms?

As metazoans evolved from simple multicellular organisms to mammals, they evolved increasingly sophisticated immune systems1. Consequently, mammalian innate immune systems are presumably composed of elements of those systems that evolved in their now extinct invertebrate predecessors2. Conversely, pathogens have evolved sophisticated mechanisms to defend themselves against metazoan antimicrobial mechanisms and to take advantage of flaws in this defence[3]. Therefore, to understand the evolution and function of human innate immunity, a reasonable place to start is the study of invertebrate (and, likely, more ancient) immunity. Similarly, the study of invertebrate pathogenesis models should provide new insights into conserved virulence strategies that have been successful for pathogens, irrespective of the host.[3]

Nematodes are the most abundant animals on Earth[4]. Microbes and nematodes have interacted and co-evolved for over 600 million years[3]. Some free-living soil nematodes, such as *Caenorhabditis elegans*, are bacterivores. From a bacterial perspective, the evolution of both defensive and offensive mechanisms to avoid predation by nematodes and other invertebrates may be the origin of virulence-related traits3, 5–7. Conversely, the evolution of defence mechanisms in nematodes (in the form of an immune system) became essential for surviving the effects of ingesting potential pathogens.

An important discovery in evolutionary biology during the past few decades was that many of the signalling pathways involved in human development, homeostasis, and disease pathogenesis had already evolved by the time of the last common ancestor between humans and nematodes, approximately 1 billion years ago[8]. It is often observed that Nature re-uses pathways that work, assigning new additional functions to them and creating variations on a theme by molecular evolution. This is also believed to apply to innate defence mechanisms: the most successful immune strategies have probably been conserved over the ensuing billion years of separate evolution of humans and nematodes because of their high defensive value. By understanding host defence systems at the genetic and molecular levels in the simple,

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genetically amenable organism *C. elegans*, we can learn about the fundamental pathways that are conserved in nematodes and humans. What are the underlying themes, and what can we learn about human immunity from the study of nematodes?

In this Review, we aim to discuss recent advances in the field of *C. elegans* host defence, with particular emphasis on a survey of the signalling pathways involved and the novel concepts that have emerged from integrated genetic, genomic and cell-biology approaches. Because other recent reviews cover the subjects of immune effectors and coordination among different tissues in detail[9–11], we attempt to integrate recent information into a broader perspective of innate host defence and emphasize some parallels with mammalian innate immunity.

C. elegans as a model host

C. elegans has been a workhorse of biological exploration since its initial use in the 1970s as a model system to study the genetic control of development[12]. Several features of *C. elegans* make this nematode a premier genetic model (Box 1). Many human pathogens — bacterial and fungal — cause potent intestinal infections in *C. elegans* that result in death[13–17]. The adult *C. elegans* intestine comprises 20 intestinal epithelial cells (IECs) that are non-renewable — that is, they are not shed and do not proliferate as mammalian IECs do (Figure 1). These cells share similar morphological features with mammalian IECs, or enterocytes, thereby making *C. elegans* a powerful model in which to study the interaction of microbial pathogens with primordial epithelial cells. *C. elegans* can be infected by simply replacing the normal food source (*E. coli*; Box 1) with the pathogen of choice[13]. Because *C. elegans* is transparent, one can follow infection and disease progress in real time. Several readouts can be used to monitor pathogenesis, including quantifying nematode survival18, observing morphological and behavioural changes19–21, measuring bacterial persistence and/or accumulation in the intestine[22], and monitoring changes in gene expression by using microarrays, quantitative reverse transcription polymerase chain reaction (qRT-PCR)[23] and reporter constructs in transgenic worms24. These methods allow the detailed study of host–pathogen interactions at the cellular, genetic and molecular levels in the context of the whole organism.

Box 1

C. elegans as a premier genetic model

In the laboratory, *C. elegans* is propagated and maintained on agar plates with lawns of non-pathogenic *Escherichia coli* as a food source[13]. With a 3-day life cycle, each animal produces ~300 genetically identical progeny, facilitating the establishment and maintenance of large populations of animals that can be housed in the laboratory in refrigerator-sized incubators. *C. elegans* is diploid and hermaphroditic, although males are easy to generate and readily mate with hermaphrodites for use in genetic analysis. Gene expression in *C. elegans* can conveniently be knocked down by double-stranded (ds)RNA-mediated RNA interference (RNAi) by feeding worms live non-pathogenic *E. coli* expressing dsRNA corresponding to the appropriate *C. elegans* genes (almost 90% of the genome is available as a dsRNA expression library97). Transgenic *C. elegans* can also be easily generated by microinjection or bombardment of DNA. *C. elegans* is transparent, greatly facilitating characterization of gene expression patterns. Adult organisms have exactly 959 somatic nuclei, corresponding to cells whose developmental lineages have been completely traced to the fertilized egg.

Since the initial description of *C. elegans* as a model for bacterial infection in 1999[25–27], great strides have been made in identifying the genetic components that mediate the host response at the level of intestinal and epidermal epithelia, as well as understanding the virulence mechanisms used by pathogens to defeat these defences. These studies have provided important

insights into the evolution of innate immunity. The emerging picture is one of several parallel signalling pathways being involved in host defence, in many cases depending on the route of infection and the pathogen that is causing the infection. Through incompletely understood mechanisms, these pathways are likely to interact extensively in a signalling network encompassing different tissues to integrate host physiological information with the antimicrobial defence response.

Signaling pathways in *C. elegans* innate immunity

Signalling pathways that are important during early development are frequently used again in the adult for innate immune signalling. This realization highlights links between processes such as cell growth and differentiation during tissue repair, metabolic regulation and innate immune signalling, or the suspected relationship between chronic infection (and inflammation) and dysregulated tissue repair leading to neoplastic transformation[28, 29].

Toll-like receptors

Work in *Drosophila melanogaster* identified the key role of Toll receptors in development and pathogen detection30. The Toll receptor was originally described as a developmental gene[31], but in the adult organism, Toll functions upstream of an evolutionarily conserved signalling cascade that is involved in innate immunity. Toll is activated by Spaetzle, a cytokine produced downstream of detection of pathogen-associated molecular patterns (PAMPs) in insects. In vertebrates, Toll-like receptors (TLRs) are activated directly by PAMPs. In both insects and vertebrates, these events lead to the downstream activation of transcription factors of the nuclear factor-κB (NF-κB; also known as Rel) family via signalling pathways involving Mal and MyD88-class scaffold proteins (Figure 2)[32]. NF-κB proteins regulate several aspects of cell differentiation during development, as well as the transcription of host response genes that encode proteins involved in the innate immune response, such as antimicrobial peptides and vertebrate pro-inflammatory cytokines[33]. In *D. melanogaster*, the Toll-dependent pathway mediates activation of NF-κB transcription factors primarily in response to fungal and Gram-positive bacterial pathogens, whereas the parallel IMD (immune deficiency) pathway (which is related to the mammalian tumor necrosis factor receptor [TNFR] pathway) does so primarily in response to Gram-negative pathogens[30, 34].

Analysis of the *C. elegans* genome identified a single TLR (TOL-1) and other components related to those of the insect and mammalian TLR signalling pathways, including TRF-1 (which is related to TRAF [TNFR-associated factor]), PIK-1 (related to Pelle and IRAK [IL-1R-associated kinase]), and IKB-1 (related to IκB [inhibitor of NF-κB]) (Figure 2)[20]. However, the *C. elegans* genome does not appear to encode homologues of the MyD88 scaffold protein or NF-κB-like transcription factors (Figure 2). Moreover, *C. elegans* does not appear to carry a clear IMD homologue, suggesting that the IMD pathway is also not present.

In addition to TOL-1, the only other *C. elegans* protein that contains a Toll/IL-1R (TIR) protein–protein interaction domain (a salient feature of some TLR-pathway signalling components) is the scaffold protein TIR-1, which is homologous to human sterile α- and armadillo-motif-containing protein (SARM). Human SARM functions as a negative regulator of TIR-domain-containing adaptor protein inducing IFNβ (TRIF)-dependent signalling downstream of TLR3 and TLR4 (Figure 2)[35]. Because TOL-1 and TIR-1 are the only two *C. elegans* proteins with TIR domains, it seemed likely that TOL-1 might function upstream of TIR-1 in an immune signalling cascade. Surprisingly, whereas knockdown[36, 37] or mutation[38] of *tir-1* results in hypersusceptibility to all pathogens tested to date, partial loss of function of *C. elegans tol-1* does not cause enhanced susceptibility to most pathogens[20]. Two alleles of *tol-1* have been analyzed to date. The first allele represents a deletion of part of the extracellular domain, and causes severe recessive developmental abnormalities. The second

allele represents a deletion of the intracellular TIR signaling domain, lacks developmental phenotypes (thus considered a hypomorph), and is commonly considered null for conventional immune signaling.[20] Furthermore, reduction of *tol-1* function does not cause defects in the induction of host response genes during infection with *Staphylococcus aureus* or *Pseudomonas aeruginosa* (J. Irazoqui, E. Troemel and F. Ausubel, unpublished observations) or *Drechmeria coniospora*[39]. *tol-1* mutants do, however, exhibit susceptibility to infection by *Salmonella enterica*, possibly owing to the decreased expression of antimicrobial peptides or developmental effects of the mutation[40]. With the proviso that the *tol-1* allele used and knockdown result in partial loss of function, these studies imply that TOL-1 is not a central component of the nematode innate immune response, as it is in flies and mammals. The receptor (s) that functions upstream of TIR-1 remain unknown.

p38 mitogen-activated protein kinase pathways

An important signalling pathway downstream of TLRs in insects and mammals involves a p38 mitogen-activated protein kinase (MAPK) cascade[41–44] (Figure 2). By screening for mutants with shortened longevity on infection with *P. aeruginosa* (but not with non-pathogenic *E. coli*), a NSY-1–SEK-1–PMK-1 p38 MAPK cascade was identified as a key component of the *C. elegans* immune response[45–47]. *nsy-1*, *sek-1* and *pmk-1* encode the *C. elegans* orthologues of human ASK-1 (a MAPK kinase kinase [MAPKKK]), MKK3/MKK6 (a MAPKK), and p38 (a MAPK), respectively, all of which have been directly implicated in the mammalian cellular immune response[48]. NSY-1, SEK-1 and PMK-1 appear to function in a linear phosphotransfer cascade36,45. Intriguingly, TIR-1, but not TOL-1, functions upstream of the NSY-1–SEK-1–PMK-1 p38 MAPK cascade, as knockdown of *tir-1* blocks phosphorylation of PMK-1[36]. By analogy with other systems, PMK-1 is likely phosphorylated by SEK-1, promoting its translocation into the nucleus, where it presumably phosphorylates target transcription factors to control gene transcription. Consistent with this view, PMK-1 phosphorylation also depends on NSY-1 and SEK-1[45].

The phosphorylation state of PMK-1 is modulated by MEK-1 (a MAPKK), a component of a parallel MAPK cassette involving KGB-1/Jun N-terminal kinase (JNK), which is activated in response to heavy metal stress[46]. The phosphatase VHP-1 negatively regulates PMK-1 and KGB-1 phosphorylation, but whether VHP-1 activity is regulated remains unknown46. The downstream phosphorylation targets of PMK-1 also remain to be elucidated, but might include the *C. elegans* programmed cell death pathway[49].

The evolution of TLR, MAPK signalling pathways

In *C. elegans*, the presence of a TIR-1–NSY-1–SEK-1–PMK-1 signalling pathway (known as the PMK-1/p38 MAPK cassette) that does not involve TOL-1 led to the hypothesis that the cassette might be an ancient ancestral immune pathway that evolved before the recruitment of NF-κB, TLRs and MyD88 as key components of immune signalling[50], and that TIR-1 (SARM) was the ancestral founding member of the TIR-domain-containing family of scaffold proteins. This was previously deemed consistent with the traditional phylogenetic placement of *C. elegans* on an evolutionary branch that diverged before the appearance of coelomates, including flies and mammals[50]. Also, because a *C. elegans tol-1* null allele exhibits lethal developmental defects, it was proposed that a role for TOL-1 in immune signalling evolved more recently than its role in development. Thus, nematodes were thought to have diverged before the co-option of TLRs for immune signaling and the evolution of NF-κB and MyD88[50].

However, recent sequencing of primitive metazoan genomes has since challenged this view. Unexpectedly, the genome of the cnidarian *Nematostella vectensis* (sea anemone)[51, 52] includes genes encoding TLRs, MyD88 and NF-κB (Figure 3)[51]. Furthermore, additional

sequencing work favours the phylogenetic placement of nematodes alongside other moulting animals, such as insects (Figure 3)[51]. Consequently, the favored conclusion is that the MyD88 and NF-κB genes have been lost in the *C. elegans* lineage, which is consistent with the higher rates of evolution observed in *C. elegans* compared to vertebrates and insects[51]. With respect to innate immune pathways, *C. elegans* seems to be representative of nematodes in general, as analyses of other nematode genomes essentially recapitulate the findings in *C. elegans*[53] (J. Urbach and F. Ausubel, unpublished observations).

The presence of MyD88 and NF-κB in *N. vectensis* does not necessarily imply a role in immunity and, until functional analysis is carried out on the cnidarian MyD88 and NF-κB homologues, it is still possible that the immune function of TLRs evolved after nematodes branched off from the rest of bilateria. Nonetheless, the fact that the single TLR in *C. elegans* does not have a major function in immune signaling, in combination with the lack of MyD88 and NF-κB, implies that other signalling components assume their function. It is therefore of great interest to identify these functional equivalents and to determine whether they also function as immune signalling components in other lineages, including vertebrates.

Analysis of the *Monosiga brevicollis*, *Hydra magnipapillata*, *N. vectensis* and *C. elegans* genomes has uncovered the presence of homologues of lipopolysaccharide (LPS)-binding protein (LBP). In mammals, LBP is an adaptor for LPS binding by TLR4 that is involved in sensing Gram-negative bacteria[52]. In *C. elegans*, there are at least nine putative LBP homologues (F55B12.5, T19C3.5, C06G1.1b, C05C9.1, C55C3.1, F10D11.6, F44A2.3, F01G10.10 and C06E8.5; J. Urbach and F. Ausubel, unpublished). Intriguingly, although none of these genes have been shown to be involved in immune defense in *C. elegans*, F44A2.3 is induced during infection with several pathogenic bacteria[54]. *D. melanogaster* lacks LBP homologues, and instead uses peptidoglycan recognition protein L_C and -L_E (PGRP-L_C and -L_E) to activate the IMD pathway and thereby detect Gram-negative peptidoglycan[30]. Since *C. elegans* lacks homologues of IMD pathway components, the prevailing view is that an IMD-like pathway does not function in bacterial sensing in nematodes.

Of these, only p38 MAPK signalling pathways are present in *M. brevicollis*, the sequenced single-cell eukaryote that is most closely related to metazoans (Figure 3). MAPKs are also present in unicellular fungi, in which they are involved in responding to cell-wall stress, among other functions[55]. This connection with stress at the cell cortex is conserved in *C. elegans*, as the PMK-1/p38 MAPK cassette is also involved in the host response to pore-forming toxins produced by the soil pathogen *Bacillus thuringiensis*[56–58]. Consistent with a putative role in cortical stress, the PMK-1/p38 MAPK cassette is required for defence against every intestinal bacterial pathogen that has been tested to date, most of which are known to secrete pore-forming toxins, which disrupt the cell membrane integrity of the host[59]. Likewise, the cassette is required for defense against *D. coniospora*, which punctures the epidermis during infection[60, 61].

Regulating the PMK-1/p38 MAPK cassette

Upstream regulation of the PMK-1/p38 MAPK cassette in *C. elegans* varies according to the site of action (intestine versus epidermis) and type of cellular stress (fungal invasion versus wounding).

In the intestine

In the intestine, the protein kinase Cδ (PKCδ) TPA-1 activates the protein kinase D (PKD) DKF-2 upstream of the cassette[62] (Figure 4). The mechanism of action of these upstream components is unknown; presumably, it involves phosphorylation events. However, whether DKF-2 directly phosphorylates any of the components of the PMK-1/p38 MAPK cassette

remains unknown. The upstream signals that control TPA-1 activity also remain unknown, although a likely candidate is diacylglycerol (DAG), suggesting that upstream regulation of this pathway may also require the activity of an unidentified phospholipase C acting in the intestine[63] 64. The pathogen-derived signals that trigger TPA-1/PMK-1 activation in the intestine remain unknown.

In the epidermis

Wounding or infection by the natural fungal pathogen *Drechmeria coniospora* triggers a host epidermal response that involves the expression of antimicrobial genes encoding neuropeptide-like peptides (NLPs) and caenacins (CNCs)[61, 64, 65]. The PMK-1/p38 MAPK cassette is required for NLP but not CNC expression.[39, 64] The proximal upstream signals that are sensed to activate the PMK-1 cassette during wounding are unknown; however, upstream regulation involves repression by the death-associated protein kinase DAPK-1 acting in the epidermis (Figure 4)[66].

Surprisingly, during infection by *D. coniospora*, *nlp* gene activation in the epidermis by the PMK-1/p38 MAPK cassette involves a different upstream pathway to that involved in wounding. Upstream regulation of PMK-1 for the induction of *nlp*s in the epidermis involves protein kinase C paralogs TPA-1 (identified as No Induction of Peptide after Drechmeria Infection 1, or *nipi-1)* and PKC-3, the phospholipase C paralogs EGL-8 and PLC-3, the heterotrimeric G-protein subunits GPA-12 and RACK-1, and a protein related to human Tribbles-like kinase, NIPI-3[61, 64]. Not all steps in this complex pathway are clearly delineated yet, although NIPI-3 seems to function in parallel with GPA-12 and RACK-1, the PLC proteins and the PKC proteins to activate the PMK-1/p38 cassette during infection but not wounding (Figure 4). Thus, NIPI-3 is involved in the transduction of an infection-specific signal to the PMK-1/p38 MAPK cassette. Although NIPI-3 activity is required in the epidermis for PMK-1 regulation, whether the other upstream components also function cell-autonomously in the epidermis is unknown. The same study showed that DKF-2, which functions downstream of TPA-1 to regulate PMK-1 in the intestine, is not required in the epidermis for the induction of intestinal genes, and neither is its paralogue DKF-1[64]. Thus, it is possible that TPA-1 regulates PMK-1/p38 MAPK in the epidermis directly, or through an unidentified kinase other than DKF-1 and DKF-2.

The induction of genes encoding CNCs during *D. coniospora* infection requires a non-canonical TGFβ signalling pathway that is composed of the TGFβ receptor subunits SMA-6 and DAF-4, as well as the downstream signalling component SMA-3 (Figure 4). These gene products function cell-autonomously in the epidermis, responding to a DBL-1/TGFβ signal emanating from the nervous system[39]. Importantly, these observations highlight the role of the nervous system in the induction of host defenses. Similar observations involve insulin-mediated signaling (see below). Human epithelial cells secrete TGFβ in response to bacterial stimulation, priming lymphocytes to adopt a tolerigenic phenotype[67]. Human TGFβ has dual effects on innate immunity and inflammation, having both pro- and anti-inflammatory activity, presumably depending on poorly understood contextual influences68. Thus, *C. elegans* provides a valuable opportunity to study the role of TGFβ in the induction of epidermal host responses to infection via neuroendocrine mechanisms.

Insulin signalling

The highly conserved DAF-2–DAF-16 insulin-like signalling pathway regulates metabolism, reproduction, development, lifespan and resistance to a variety of environmental stresses in *C. elegans*[69], and also has an important role in innate immunity[70]. The insulin receptor DAF-2 inhibits the downstream transcription factor DAF-16 by phosphorylation through a kinase cascade that includes the phosphoinositide 3-kinase AGE-1, the phosphoinositide-dependent

kinase PDK-1, AKT-1/2 and the serum/glucocorticoid-regulated kinase SGK-1[71] (Figure 4). Mutations in *daf-2* or *age-1* constitutively activate DAF-16, prolonging lifespan, delaying ageing, and enhancing resistance to killing by both Gram-positive and Gram-negative bacterial pathogens[70]. It is clear that the enhanced resistance of these mutants is not simply a consequence of their enhanced longevity. First, DAF-16 upregulates the transcription of a variety of genes with homology to antimicrobial defence genes[72, 73]. Second, the extent of the increase in longevity of *daf-2* and *age-1* mutants does not correlate with the degree of increase in pathogen resistance[70, 74] (for example, *daf-2* mutants live about twice as long as their wild-type counterparts on an *E. coli* diet but are five-fold more resistant to *S. aureus*70). And, finally, lifespan regulation and stress resistance have been shown to be genetically separable from antimicrobial resistance[75]. Thus, the prevailing view is that inactivation of DAF-2 induces several parallel responses, including stress resistance, metabolic regulation, and antimicrobial defense, all cumulatively resulting in enhanced longevity.

Transcriptional profiling identified a large number of DAF-16 target genes with predicted functions in stress resistance and host defence[73, 76], leading to the hypothesis that DAF-16 must be an important component of host defence during infection. However, *daf-16* null mutants do not exhibit the phenotype that would be expected if this hypothesis were correct — that is, they are not significantly more susceptible than wild-type to pathogen-mediated killing by a broad array of pathogens[23, 24, 70]. Moreover, even at late stages of infection in wild-type *C. elegans*, when host damage is apparent by light and electron microscopy, DAF-16 remains localized to the cytosol (not the nucleus, where it is active)[24, 74]. Furthermore, *daf-16* mutants are not defective in the induction of host response genes during infection with *P. aeruginosa* or *S. aureus*[23, 24, 74]. These observations led to the prevailing view that, although constitutive activation of DAF-16 enhances pathogen resistance, DAF-16 is not normally activated during pathogen infection in wild-type *C. elegans*. This implies that DAF-16-independent pathways may be involved in the host response to intestinal infection and that the considerable cellular damage observed during infection does not result in DAF-16 activation. The molecular mechanism(s) responsible for this phenomenon are currently unclear, but might involve active repression of DAF-16 by crosstalk with other pathogen-induced host defence signalling pathways or physiological pathways, such as reproduction[74]. For example, infection with *P. aeruginosa* leads to the induction of insulin-like INS-7 in the nervous system, which activates DAF-2 and downregulates DAF-16 in the intestine[77]. In contrast to these observations, it was recently shown that infection with enteropathogenic *E. coli* causes DAF-16 activation, leading to the expression of a “conditioned” phenotype that protects *C. elegans* from subsequent pathogenic challenge.[78] In summary, the involvement of DAF-16-mediated immune defense appears to be triggered by unknown pathogen-specific signals.

Mutations in *daf-2* and *pmk-1* cause opposing phenotypes (pathogen resistance and pathogen susceptibility, respectively). Genetic epistasis analysis shows that *daf-2;pmk-1* double mutants exhibit the *pmk-1* phenotype[23]. The formal interpretation of this analysis is that *pmk-1* functions downstream of, or parallel to, *daf-2.* Additionally, transcriptional profiling shows there to be essentially no overlap between those genes regulated downstream of DAF-2 by PMK-1 and those regulated by DAF-16[23]. Thus, the simplest interpretation is that PMK-1 and DAF-16 function in parallel pathways; most likely, the PMK-1/p38 MAPK pathway functions in parallel to insulin signalling[23]. The reason that *daf-2;pmk-1* mutants exhibit a *pmk-1* phenotype must therefore reflect the fact that PMK-1 signalling is indispensable for the establishment of immunity, irrespective of the activation of other immune pathways. Similar observations have been made in regard to a recently identified pathway involving BAR-1/β-catenin[24] (see below).

P. aeruginosa and *S. aureus* activate distinct responses

Global microarray analysis has identified host response genes that are activated when *C. elegans* is exposed to various pathogens, including the human pathogens *P. aeruginosa* and *S. aureus*23, 24, [79]. During early infection (at 4–8 hours), *P. aeruginosa* activates some of the same immune effectors identified downstream of PMK-1 and DAF-16, but most *P. aeruginosa*-induced genes do not include those induced by PMK-1 or DAF-16[23]. This observation implies that *P. aeruginosa* activates previously unidentified signalling pathways that function in parallel to the PMK-1/p38 MAPK and DAF-2–DAF-16 pathways. Similarly, *S. aureus* response genes are also activated independently of PMK-1 and DAF-16 at the same early stage of infection.[24] Moreover, there seems to be minimal overlap, in terms of induction of gene expression, between the early host responses to *S. aureus* and *P. aeruginosa*, indicating that *C. elegans* discriminates between different pathogens and mounts pathogen-specific responses (J. Irazoqui, E. Troemel and F. Ausubel, unpublished observations). Alternatively, the bacterial pathogens may be able to manipulate the host response to their advantage, resulting in differences at the level of transcript abundance of host response genes. Using a different set of pathogens, similar observations were made at both early and late stages of infection (24 hours) [54, 80]. In addition, a set of genes encoding proteins involved in necrosis is commonly induced at late stages by several different pathogens54. These transcriptional profiling data, combined with the genetic data described above, indicate that *C. elegans* has several parallel immune response pathways that activate similar categories of defence response genes, but that there is relatively little overlap among the particular genes activated by each of the pathways. One major focus in the field has been to identify additional *C. elegans* signalling pathways involved in the host response to infection, some of which are outlined below.

β-catenin signalling and host defence

A reverse genetic approach identified the β-catenin gene, *bar-1*, a component of the *C. elegans* canonical Wnt signalling pathway (Box 2), as a critical determinant of susceptibility to *S. aureus*24. BAR-1 (β-catenin) is involved in post-embryonic vulva formation and neuroblast migration, and in oxidative stress resistance, in addition to host defense81–83. EGL-5, a homeobox (HOX) transcription factor, is a downstream mediator of BAR-1, and is also involved in the regulation of the host response to *S. aureus* infection[24]. Some *S. aureus*-induced genes require BAR-1 and EGL-5 for proper regulation. Others require only BAR-1, and yet others are BAR-1 and EGL-5 independent. These results hint at the existence of additional host defence pathways that are a) BAR-1-dependent but EGL-5-independent, and b) BAR-1-independent[24].

Box 2

A role for β-catenin signaling in epithelial immunity?

In human epithelial cells, β-catenin and EGL-5/HOX homologues modulate nuclear factor κB (NF-κB)-dependent transcription downstream of Toll-like receptor (TLR)2 and inhibitor of NF-κB kinase (IKK) activation[24, 98, 99], implying that a putative β-catenin-dependent host defence pathway might crosstalk with TLR-dependent NF-κB function100–104. It is incompletely understood how β-catenin is regulated during bacterial infection in human epithelial cells, although reactive oxygen species and Wnt signalling appear to be involved upstream[105–110].

Are β-catenin target genes involved in host defence in humans? Human antimicrobial peptides called defensins, produced in the intestinal epithelium, are direct targets of β-catenin signalling[111, 112]. Reduced defensin expression owing to defective β-catenin-dependent transcription correlates with increased susceptibility to Crohn's disease[109, 113]. Furthermore, it is possible that β-catenin regulates the expression of human EGL-5-like

homeobox genes that have roles in immunity, as in *C. elegans*. Supporting this view, the human homeobox transcription factors Cdx1 and Cdx2 are transcriptional targets of β-catenin in intestinal epithelia, where they control the expression of antimicrobial factors[114–117]. The *D. melanogaster* homologue *Caudal* positively drives the constitutive, NF-kB-independent production of antimicrobial peptides in barrier epithelia such as the salivary glands[118]. In contrast, *Caudal* also negatively regulates NF-κB in the fly intestinal epithelium, where it modulates the expression of antimicrobial agents and, thereby, the composition of the microbiota[119]. Thus, β-catenin and HOX seem to have significant roles in epithelial host defence in worms, flies and humans.

Epistasis analysis has shown that BAR-1 and EGL-5 function in parallel to the PMK-1/p38 MAPK cassette during host defence[24]. Similar to PMK-1, loss of *bar-1* in *daf-2* mutants causes the *bar-1* susceptibility phenotype; *daf-16* mutation does not affect the host response to *S. aureus*. These observations suggest that BAR-1 functions downstream of, or parallel to, DAF-2 in a DAF-16-independent manner. The upstream signals that regulate BAR-1 function during the host response remain unknown; it is also unclear whether other components of the canonical Wnt signalling cascade are involved in host defence. In this regard, EGL-5 was independently shown to be required for the host rectal epithelial swelling response to infection by *M. nematophilum*; this response might constitute the worm equivalent to tissue inflammation[84, 85]. EGL-5 functions during normal development of the hindgut, specifying cellular competence to respond to unknown bacterial signals triggering the swelling response mediated by an ERK-like MAPK signaling cascade that involves MPK-1/ERK[86]. It is unknown whether EGL-5 also functions post-developmentally as a component of immune-response pathways[84]. These studies spurred the identification of β-catenin and HOX proteins as important modulators of innate immune signalling downstream of TLR2 signalling in humans (see Box 2)[24].

Interestingly, *S. aureus* primarily activates genes regulated by the BAR-1 signalling pathway, whereas *P. aeruginosa* activates genes regulated by the PMK-1/p38 MAPK pathway[24]. This observation suggests that the BAR-1 pathway might be more important in conferring resistance to *S. aureus* than the PMK-1 pathway and *vice versa* for *P. aeruginosa*. Indeed, *pmk-1* mutants are relatively more susceptible to *P. aeruginosa* than to *S. aureus*, and *bar-1* mutants are relatively more susceptible to *S. aureus* than to *P. aeruginosa*[24]. These results indicate that pathogen specificity of the host response may be achieved through pathogen-specific signalling pathways in *C. elegans*. These distinct pathways might potentially be regulated by different upstream receptors, and considerable effort is being invested in their identification. Two putative upstream transmembrane receptors that regulate the *C. elegans* response to infection have been identified[87, 88].

FSHR-1 as a putative immune receptor

Cell-surface molecules that contain extracellular leucine-rich repeat (LRR) domains are good candidates for immune receptors on the basis that this domain is present in major classes of pathogen receptors in plants and animals (for example, TLRs, NLRs, the plant flagellin receptor FLS2). As discussed above, TOL-1, the single TLR homologue in *C. elegans*, does not have a central role in innate immunity. However, loss of function of FSHR-1, one of 14 candidate transmembrane LRR receptors in *C. elegans*, results in a high degree of susceptibility to killing by *P. aeruginosa*, *S. aureus* and *Enterococcus faecalis*, but not in a reduced lifespan with non-pathogenic *E. coli*[87]. This putative immune receptor is the sole *C. elegans* LGR (LRR-containing G-protein-coupled receptor). Genetic analysis indicates that FSHR-1 functions in the intestine in a separate pathway from PMK-1 and DAF-2[87]. Further, qRT-PCR analysis showed that FSHR-1 and the PMK-1/p38 MAPK cassette regulate the induction of overlapping, but nonidentical, sets of *P. aeruginosa*-induced genes. Thus, FSHR-1 and PMK-1/

p38 MAPK pathways function in parallel to each other but converge on a common set of target effector genes in response to infection by *P. aeruginosa*.

How is FSHR-1 involved in mediating the *C. elegans* host response? We currently lack evidence that FSHR-1 can sense infection directly. The canonical ligand for this class of GPCR in mammals is the heterodimeric glycopeptide hormone follicle-stimulating hormone (FSH)α/β. Worms do not have an identifiable FSHα subunit, and the gene that most closely resembles an FSHβ subunit does not seem to have an important role in *C. elegans* immunity[87]. However, it still remains a possibility that FSHR-1 is activated by as-yet-unidentified non-canonical host ligands. Because FSHR-1 has important roles in the development of the nervous system and the germline, it is likely that endogenous ligands exist.[89, 90]

CED-1 and the unfolded protein response

In their efforts to identify upstream immune receptors in *C. elegans*, Aballay and coworkers found that *ced-1* mutants are hypersusceptible to *S. enterica* infection[88]. These mutants are also hypersusceptible to the fungal pathogen *Candida albicans*[91]. CED-1 functions as a receptor during phagocytic uptake of apoptotic cells in *C. elegans*[92]. Microarray analysis of *ced-1* mutants showed that CED-1 functions upstream of presumptive signalling pathways that regulate a non-canonical unfolded protein response (UPR), thereby implicating this response in host defence against *S. enterica*[88]. Consistent with this idea, mutants defective in components of this UPR are hypersusceptible to *S. enterica*[88]. It is possible that the *Salmonella*-derived trigger for CED-1-dependent regulation of the UPR is a pore-forming toxin; these virulence factors are well known to be secreted by bacterial pathogens. In support of this hypothesis, Cry5B, a pore-forming toxin made by *Bacillus thuringiensis* that targets *C. elegans*, triggers a canonical UPR-mediated response that is required for survival[56]. Importantly, the UPR in this case is activated downstream of the PMK-1/p38 MAPK cassette during attack by Cry5B[56]. It remains to be determined whether CED-1 functions upstream of the PMK-1/p38 MAPK cassette during *S. enterica* and Cry5B-mediated *B. thuringiensis* attack to trigger these distinct UPRs. These and other results[93, 94] suggest that monitoring cell damage or damage-associated molecular patterns[95] (such as membrane integrity and protein homeostasis) is a key component of *C. elegans* immunity.

Conclusions and perspectives

It is clear that *C. elegans* mounts pathogen-specific host defence responses upon pathogenic attack, as other animals do. In other systems, TLR-mediated control of NF-κB activity has a central role in this defence; it is therefore surprising to find that *C. elegans* has the capacity to mount specific host responses in the absence of NF-κB and independently of TLR. How does this happen? Several signalling pathways (some identified, some not) are involved. Presumably, during the evolutionary divergence of nematodes from the basal bilaterian ancestor, the NF-κB gene was lost owing to functional redundancy with some unknown factor (s). Likewise, although we do not know yet whether TLRs perform immune functions in basal metazoans like the cnidarians, it is possible that additional pathways superseded TLR signalling in the worm as central immune pathways. It is also possible that these additional immune pathways evolved in primitive metazoans such as cnidarians and have been conserved throughout metazoan evolution, including mammalian lineages, acting in concert with TLR pathways. *C. elegans* seems to have several parallel pathways that can carry out such roles; these different pathways might be important for mediating the appropriate host response to distinct pathogens. It is tantalizing to think that by studying *C. elegans*, which does not rely on NF-κB for innate immunity, it might be possible to identify as-yet-unknown mammalian host defence pathways that function in addition to the canonical TLR pathways.

Further work is needed to elucidate the bacterially-derived signals that trigger morphological and transcriptional host responses to infection in *C. elegans*. Important areas that require further study include extensive characterization of the signalling networks that influence the outcome of infection and host response, and the cell types in which they function. At the whole organism level, different tissues and organ functions are likely to be coordinated during infection through systemic endocrine signals that remain to be fully delineated. Further insight will be gained by precise examination of the actual mechanisms involved in pathogenesis for each pathogen type and infection process. How many of these important answers will apply to mammals as well, and to what extent? What pathways have been conserved, which have been modified, and which have been lost altogether in the splits and radiations from the common eumetazoan ancestor?

Insights gained from the study of host defence strategies can be applied to the design of better therapeutic approaches, which are desperately needed in view of human overpopulation and antibiotic resistance. Conversely, it is imperative that the molecular mechanisms used by pathogens to exploit their hosts be fully understood. The use of model hosts will be instrumental in understanding the molecular functions of virulence factors and their regulation during infection *in vivo*. *C. elegans* provides a means to quickly test hypotheses related to fundamental features of host epithelial cells in the whole organism context, and identify points of weakness that bacteria have evolved to so expertly exploit. The ability to carry out automated high-throughput chemical screens on whole infected nematodes[96] provides an opportunity to identify new ways to deprive pathogenic bacteria of their highly evolved weapons.

At A Glance

- The nematode *C. elegans* was developed as a model for studying bacterial virulence and innate immunity in 1999. *C. elegans* does not have circulating cells and appears to rely almost exclusively on epithelial immunity to combat pathogen attack. Several parallel immune response pathways have been identified that activate distinct but partially overlapping sets of immune effectors. Despite its simplicity, the *C. elegans* immune response is highly pathogen specific and different pathogens activate distinct immune response pathways.
- Although *C. elegans* has a single Toll-Like-Receptor (TLR), MyD88 and NF-kB are not encoded in the *C. elegans* genome or in the genomes of other nematode species. Moreover, the single *C. elegans* TLR does not appear to play an important role in the immune response. Because some cnidarians (e.g., the sea anemone *Nematostella vectensis)* have TLRs, MyD88 and NF-kB, it appears that TLR signaling has been lost in the nematode lineage.
- A highly conserved p38 MAPK signalling cascade plays a central role in the *C. elegans* immune response as it does in mammals. The p38 pathway is required for the activation of a set of immune effectors that are required to maintain a basal level of immune function.
- The p38 MAPK signalling pathway is active during both infection and wounding and functions in at least the intestine, neurons, and epidermis in response to pathogen attack.
- Several highly conserved metazoan signalling pathways play dual roles, functioning as important components of the *C. elegans* immune response. It is of interest to determine whether the same pathways function in immune signalling throughout metazoan evolution, including acting in concert with TLR pathways in mammals.

- In addition to its role in stress resistance, lifespan extension, and metabolic regulation in *C. elegans*, the DAF-2/DAF-16 insulin signalling pathway confers resistance to a wide variety of pathogens when DAF-16 is constitutively activated.
- The *C. elegans*β-catenin homologue BAR-1 and the downstream EGL-5/HOX protein play central roles in activating the *C. elegans* immune response to infection by *Staphylococcus aureus* but not *Pseudomonas aeruginosa*. Roles for β-catenin and HOX in immune signaling in flies and mammals has also been recently demonstrated.
- The G protein coupled receptor FSHR-1 is the first candidate immune receptor to be identified in *C. elegans*.

GLOSSARY

Hermaphrodite	An organism that has both male and female reproductive organs
RNA interference	The silencing of gene expression by the introduction of double-stranded RNA that triggers the specific degradation of a homologous target mRNA, often accompanied by a concomitant decrease in production of the encoded protein
Microarrays	A technique for measuring the transcription of genes. It involves hybridization of fluorescently labelled cDNA prepared from a cell or tissue of interest to glass slides or other surfaces dotted with oligonucleotides or cDNA, representing all genes in the species
Quantitative reverse transcription polymerase chain reaction (qRT-PCR)	A quantitative PCR method that is used to measure relative or absolute mRNA concentrations
Scaffold protein	A protein that functions as a support to assemble a multiprotein complex
Gram-negative pathogens	Bacteria that present as pink-red after exposure to Gram stain. The Gram stain is useful to distinguish faintly staining bacteria (Gram-negative), which possess an extracellular cell wall composed of an outer membrane and a periplasmic space separating it from the plasma membrane. A relatively thin layer of peptidoglycan resides in the periplasmic space and provides structural stability to the cell wall. In contrast, darkly staining bacteria (Gram-positive) lack an outer membrane, but are surrounded by a thick layer of peptidoglycan
Coelomates	Animals that possess an internal body cavity derived from the mesoderm
Epistasis	An interaction between non-allelic genes, such that one gene masks, interferes with or enhances the expression of the other gene
Reverse genetic approach	A genetic approach that proceeds from genotype to phenotype by gene-manipulation techniques, such as homologous recombination in embryonic stem cells and RNA interference

Homeobox genes	Genes that contain a 180-base-pair sequence coding for the homeodomain and are involved in the regulation of animal and plant development. This sequence encodes a DNA-binding helix–turn–helix motif (the homeodomain), indicating that homeobox-containing gene products function as transcription factors
Leucine-rich repeat (LRR) domains	Domains that contain LRRs have a conserved solenoid structure, typically of 20–29 residues and containing an 11 amino-acid consensus sequence, LXXLXLXX(N/C)XL, where X denotes any amino acid. These domains lack considerable identity or similarity in the amino acids surrounding this structure, both between and among families. Sequence substitutions in LRR-containing proteins are associated with changes in specificity and relative affinity towards LRR domain binding partners
Neuropeptide-like peptides	Family of short peptides, with sequence homology to YGGWamide neuropeptides, which can be induced during infection
Caenacins	Family of peptides related to neuropeptide-like peptides, which can be induced during infection
Apical junctions	Junctional multiprotein complexes localized to the apical junctions of intestinal epithelial cells in *C. elegans.* The *C. elegans* apical junctions can be viewed as functionally equivalent to the cell junctions from Drosophila epithelia and the tight junctions, zonula adherens and desmosomes from vertebrate epithelia

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Biographies

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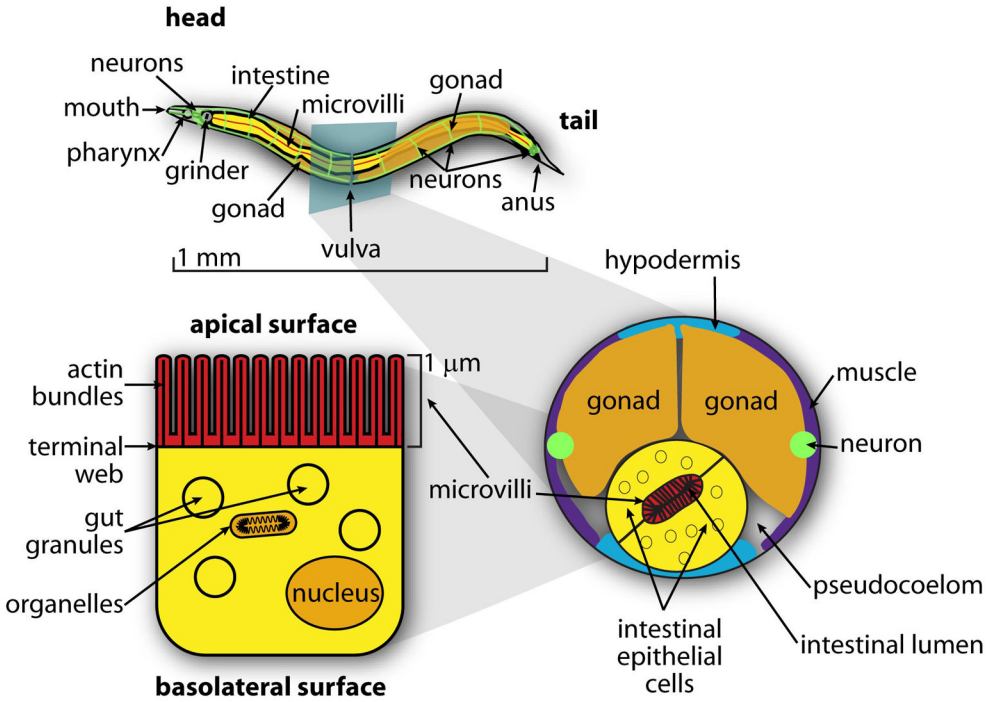

Figure 1. Schematic representation of the *C. elegans* intestine

The *C. elegans* intestine is composed of 20 intestinal epithelial cells. These cells are organized in 9 rings: ring 1 contains four cells and rings 2–9 contain two cells each. The apical surface of each of the intestinal epithelial cells forms the microvillar brush border and faces the intestinal lumen. The intestinal epithelium is the major interface of interaction between *C. elegans* and ingested microbes.

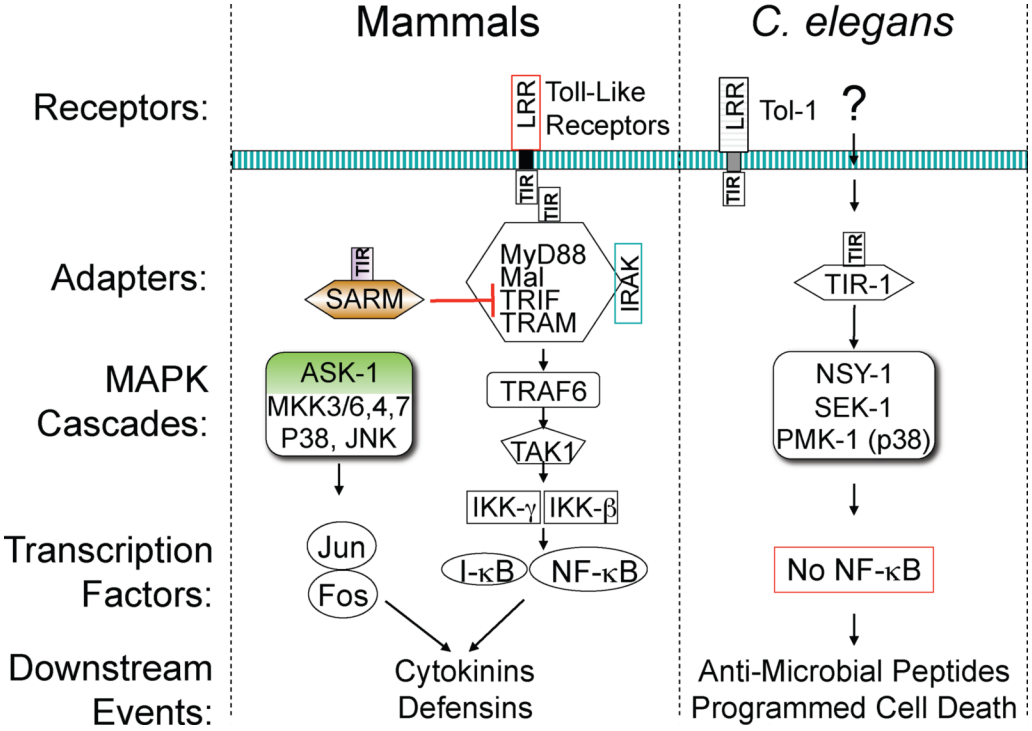

Figure 2. Parts of mammalian TLR signalling are conserved in *C. elegans*

a | model of Toll-like receptor (TLR)-dependent signalling. Engagement of TLRs by cognate ligands such as lipopolysaccharide (LPS) trigger the recruitment of scaffolding proteins, such as MyD88. MyD88 and interleukin-1 receptor-associated kinase (IRAK) recruit and activate the scaffold tumor necrosis factor receptor-associated factor (TRAF)6, which, in turn, activates transforming growth factor-β (TGFβ)-activated kinase 1 (TAK1). TAK1 activates inhibitor of nuclear factor κB (NF-κB) kinase (IKKβ/γ), which phosphorylates inhibitor of NF-κB (IκB) resulting its degradation, thus releasing NF-κB from inhibition. Free NF-κB translocates into the nucleus and drives the transcription of inflammatory host responses, including cytokines and defensins. TAK1 also activates the p38 mitogen-activated protein kinase (MAPK) cascade, which comprises ASK-1/MAPK kinase kinase (MAPKKK), MKK3/6/4/7/MAPKK, and p38 and Jun N-terminal kinase (JNK). p38 and JNK target the activator protein-1 (AP-1) transcription factor to drive the transcription of host response genes. **b** | *C. elegans* has homologues for some components of TLR signalling, but notably lacks MyD88, IKKβ/γ and NF-κB homologues. MOM-4 (TAK1) functions during embryonic development in non-canonical Wnt signalling. The NSY-1–SEK-1–PMK-1 cassette is a central regulator of host defence, and depends on the upstream scaffold TIR-1 for activity. In mammals, the TIR-1 homologue sterile α- and armadillo-motif-containing protein (SARM) negatively regulates TLR signalling, showing how the same protein can have opposite roles in different organisms.

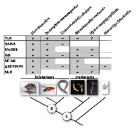

Figure 3. Components of Toll-like receptor signalling in bilaterians and cnidarians

Cnidarians and bilaterians diverged from the common eumetazoan ancestor (**E**) ~600–630 million years ago (2). One cnidarian branch, represented by *N. vectensis*, conserves nuclear factor κB (NF-κB), whereas a second branch, represented by *H. magnipapillata*, does not. Deuterostomes (including vertebrates) and protostomes (including nematodes and arthropods) diverged from the common bilaterian ancestor (**B**) ~570–582 million years ago.[91, 92] Because *D. melanogaster* and *N. vectensis* both possess NF-κB and MyD88, the most likely scenario is the loss of NF-κB and MyD88 from the nematode lineage. The presence or absence of other components of Toll-like receptor (TLR) signalling, including sterile α- and armadillo-motif-containing protein (SARM), inhibitor of NF-κB (IκB) and p38 mitogen-activated protein kinase (MPAK), is indicated.

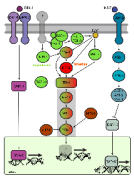

Figure 4. Parallel signalling pathways in the induction of *C. elegans* host defence

The transforming growth factor (TGF)-β homologue DBL-1 signals to SMA-3 in the epidermis through the heterodimeric receptor DAF-4–SMA-6 for the induction of caenacin (CNC) genes during *D. coniospora* infection. Unknown upstream signals induce the transcription of neuropeptide-like peptide (NLP) genes in the epidermis through a signalling cascade involving the heterotrimeric G-protein subunits GPA-12 and RACK-1, the phospholipase C proteins PLC-3 and EGL-8, the protein kinase C proteins TPA-1 and PKC-3 and PMK-1 during *D. coniospora* infection. During wounding, death-associated protein kinase (DAPK-1) functions as an upstream negative regulator of the PMK-1 cassette in the epidermis; however, the exact point of input is unknown. In the intestine, TPA-1 and DKF-2 (protein kinase D) activate the PMK-1 cassette for the induction of host defence genes during infection by *P. aeruginosa*. The MAPKK MEK-1 increases PMK-1 phosphorylation. The phosphatase VHP-1 downregulates PMK-1 by dephosphorylation. Insulin (such as INS-7, which is upregulated during *P. aeruginosa* infection) acitvates the insulin receptor DAF-2, which sequentially activates AGE-1 (phosphoinositide 3-kinase), PDK-1 (phosphoinositide-dependent kinase-1), AKT-1, AKT-2, and SGK-1 (protein kinase B) to phosphorylate, and thereby inhibit, the FOXO transcription factor DAF-16.