Genetic analysis of p38 mitogen-activated protein kinase signaling in innate immunity and stress physiology of *Caenorhabditis elegans*

by

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ABSTRACT

Host-microbe interactions play an important role in the physiology and evolution of animals. Interactions with microbes can generally be considered beneficial or pathogenic to the host. The ability of an organism to mount an immune response to infection by pathogenic microbes is critical to its survival, and basic mechanisms of innate immunity are conserved in evolutionarily diverse species. A pivotal signaling pathway in the evolutionarily conserved innate immune responses of multicellular organisms is the stress-activated p38 mitogen-activated protein kinase (MAPK) pathway.

This thesis focuses on the physiological role of p38 MAPK signaling in the host defense of Caenorhabditis elegans. In Chapter Two, I report the identification and characterization of the conserved ATF/CREB bZIP protein ATF-7 as the key transcriptional regulator of the PMK-1 p38 MAPK-dependent immune response to pathogens. These data suggest a model in which the PMK-1 p38 MAPK phosphorylates ATF-7 and switches it from a transcriptional repressor to a transcriptional activator of effector genes of the innate immune response of C. elegans. In Chapter Three, I characterize the roles of PMK-1 and a second p38 MAPK ortholog, PMK-2, which are encoded in an operon, in tissue-specific signaling mechanisms involved in host defense. I show that PMK-2 functions redundantly with PMK-1 in the nervous system to mediate neurobehavioral responses to pathogens. Furthermore, I demonstrate a role for the miR-58/80-82 family of microRNAs in regulating the tissue expression of pmk-2, which suggests a role for microRNAs in the establishment of tissue-specific expression of co-operonic genes. The work described in this thesis establishes the ancient evolutionary origins of the p38 MAPK-CREB/ATF pathway in innate immunity, and establishes a role for microRNAs in defining the tissue-expression pattern of co-operonic p38 MAPK genes. New directions for further understanding the ancient evolutionary mechanisms of p38 MAPK signaling and their tissue-specific regulation are discussed in Chapter Four.

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Chapter One

Introduction
Introduction

Microbes play a critical role in host organismal physiology. The interactions between microbes and their host organism can be innocuous or even beneficial for the host, as in the cases of commensal and mutualistic microbes, respectively. Pathogenic microbes, however, present a danger to the host organism. The ability of the host to mount a proper response and defend against pathogen attack is crucial to its survival and fitness. This chapter will review primitive host defense mechanisms, focusing in particular on stress-activated mitogen-activated protein kinase (MAPK) signaling and its conserved role in the physiology of host defense in phylogenetically diverse organisms. A theme that will be present in this chapter is host responses to pathogens and stress are conserved and ancestral in origin, with the implications that the study of these responses in the physiological context of simple, genetically tractable organisms can provide insights into understanding the stress and host defense responses of evolutionarily diverse host organisms, including humans.

Host defense

The initial line of host defense is the innate immune response. The innate immune response is an immediate and inducible defense against pathogen attack, functioning to detect pathogens and activate a neutralizing response. Cells of the innate immune system use pattern recognition receptors (PRRs) to discriminate self from nonself though the detection of conserved pathogen-associated molecular patterns (PAMPs) that are present on some microbes. These cells then function to eliminate the pathogens, through mechanisms such as phagocytosis or secretion of antimicrobial peptides.
In addition to the innate immune response, host defense of vertebrates has evolved adaptive immune responses to pathogen that differ from the innate immune response in a number of ways. First, the innate response to pathogen is nonclonal and recognizes self from nonself through conserved patterns associated with microbes (PAMPs), whereas the adaptive response is clonal and has complete antigen specificity. Clonal selection leads to immunological memory, a hallmark of the adaptive response in which prior infection with a pathogen protects against reinfection. The innate response lacks such immunological memory. Over the past two decades, it has become increasingly appreciated that in addition to its role in the detection and neutralization of pathogens, the innate immune response serves to activate the adaptive response of vertebrates through the processing and presentation of antigens and secretion of immunostimulatory cytokines.

At the molecular level, the innate immune response consists of signaling pathways that transmit signals from external stimuli, such as PAMPs, to targets proteins, such as transcription factors, which function to elicit the appropriate responses. Of particular importance is Toll-like receptor (TLR) signaling. TLR signaling was originally discovered for its role in dorsoventral patterning in fruit fly development. Subsequently, TLR signaling was shown to play a prominent role in the fly’s response to fungal pathogen, specifically in the regulation of antibacterial gene expression. Two years after the discovery showing the importance of TLR signaling in the innate immune response of Drosophila, the mouse Lpr locus, which is involved in the detection of lipopolysaccharide and the mouse immune response, was cloned and the causative gene determined to be a Toll-like receptor. The Drosophila Toll homolog was identified in humans and shown to regulate the expression of cytokines in macrophages.
conserved across phylogenetically diverse organisms and may have ancestral origins. This paradigm set the stage for the study of the innate immune responses in simple organisms, as advances in the understanding of the responses of these organisms will certainly provide insight into the innate immune responses of more complex organisms, such as humans.

TLR-signaling converges on a number of key regulators of the immune response, including NF-κB and the stress-activated mitogen-activated protein kinases (MAPKs) p38 and JNK. Recently, it has become evident that mammalian stress-activated MAPKs are activated in response to infection with certain microbes in a TLR-independent manner. For instance, the p38 and JNK MAPKs are activated in TLR-deficient macrophages in response to Legionella pneumophila to increase the expression of proinflammatory cytokines. However, little is known about the TLR-independent mechanisms regulating stress-activated MAPK signaling in mammals.

The fundamental basis of innate immunity, in that PRRs recognize conserved PAMPs, fails to explain how cells of the innate immune system differentiate pathogenic from non-pathogenic microbes that present the same molecular patterns. Taken together with the importance of TLR-independent activation of immune responses in mammals, this suggests that the host may recognize other features of infection. Damage to cells and tissues during the infection process causes the release of intracellular molecules which can activate the immune response and have been termed danger-associated molecular patterns (DAMPs). In addition, modifications to host proteins and processes by microbes can be detected by "surveillance pathways" and trigger an immune response. In the case of the TLR-independent activation of stress-activated MAPKs during L. pneumophila infection, L. pneumophila effector proteins inhibit host translation, which activates p38 MAPK. Like TLR signaling, the ability of
organisms to detect DAMPs and survey core processes appear to be conserved features of immune responses in evolutionarily diverse organisms.

**C. elegans-pathogen interactions**

Since its adoption by Sydney Brenner as a simple multicellular animal in which to study mechanisms of development\(^4\), *C. elegans* has become a well-established model for the study of a number of diverse processes ranging from programmed cell death to aging. Recent advances in genome editing, sequencing technologies, and reverse genetic approaches, combined with decades of classical genetics, greatly facilitate the genetic analysis of *C. elegans* and the study of a number of conserved aspects of organismal physiology, including host defense.

*C. elegans* is a microbivorous nematode that is found in the wild primarily in microbial-rich environments, such as rotting fruit and compost heaps\(^5\). In addition to being a source of nutrition, microbes may cause lethal infection of *C. elegans*. The lifestyle of wild *C. elegans*, migrating from one microbe-rich environment to another, necessitates the ability of the nematode to detect and mount a proper defense response to the pathogenic microbes it undoubtedly encounters in the wild.

The study of *C. elegans*-pathogen interactions began in the late 1990s when *C. elegans* was used as a host organism for the study of *Pseudomonas aeruginosa* virulence as part of a broader “multi-host” pathogenesis system approach, starting with the infection of plants with *P. aeruginosa*\(^6,17\). *P. aeruginosa* is a human opportunistic pathogen that is of particular threat to immunocompromised individuals or patients with cystic fibrosis, making the study *P. aeruginosa* virulence factors as well as components of host defense against infection especially interesting. *P. aeruginosa* transposon insertion libraries were screened for mutants with impaired
ability to infect and kill *C. elegans*. This early work showed that the same factors regulating the virulence of *P. aeruginosa* in the *C. elegans* infection model were important for the virulence of *P. aeruginosa* in other host organisms.

Shortly afterward, focus shifted onto *C. elegans* and its response to infection with *P. aeruginosa*. In addition to *P. aeruginosa*, it was determined that a number of diverse microbes could infect and be pathogenic to *C. elegans*, including fungal, viral, and other bacterial pathogens. The host range of these pathogens can vary widely. For instance, the Orsay virus, which is a natural virus of *C. elegans*, fails to infect the closely related nematode species *C. briggsae*, while other pathogens, such as the aforementioned Gram-negative bacterium *P. aeruginosa*, have broader host ranges including plants and humans.

Host defense in *C. elegans* involves both an innate immune response and a learned pathogen avoidance behavior. *C. elegans* lacks dedicated immune cells, instead, it employs a mucosal immune response at the site of infection. Over the last decade, a number of approaches have been used to identify factors regulating host defense in *C. elegans*. One such approach, which involves comparative genomics followed by reverse genetics has had success, albeit somewhat limited. The *C. elegans* genome contains two genes, *tol-1* and *tir-1*, which encode proteins with Toll/interleukin-1 receptor (TIR) domains, which are ancestral domains that facilitate protein-protein interactions in host defense of diverse organisms and are essential for TLR signal transduction pathways. *tol-1* encodes a Toll-like receptor whereas *tir-1* encodes an adapter protein which typically serve to transmit signals from TLRs to effector proteins. Mutants that carry a deletion in *tol-1* die during embryogenesis, suggesting that Toll signaling is required for *C. elegans* development paralleling its role in *Drosophila*. However, mutants that carry a deletion in the *tol-1* gene that results in a truncated receptor without its TIR domain are viable.
These mutants are not more susceptible to bacterial or fungal pathogens compared to wild type worms, although it has been reported that they are defective in avoidance behavior to certain pathogens. This result suggested that immunity in *C. elegans* is independent of Toll signaling, which is further corroborated by the lack of a homolog to the Toll-like receptor-dependent transcriptional regulator NF-κB. Somewhat surprisingly, the TIR-domain containing protein, TIR-1, was shown to be required for immunity in *C. elegans*, as RNAi of the *tir-1* gene led to increased susceptibility to pathogens. TIR-1 is orthologous to the mammalian protein SARM, which is the most ancient TLR signaling adaptor protein. Its role in the immune response of mammals has remained puzzling, although recent studies have suggested a rather unique role for this adaptor in the inhibition of NF-κB.

A forward genetic approach to the study of *C. elegans* host defense has revealed evolutionarily conserved mechanisms of innate immunity. A simple screen for mutants with enhanced susceptibility to pathogen identified the requirement for p38 MAPK signaling, which functions in TLR-independent innate immunity of *C. elegans*.

**Stress-activated MAPKs**

Mitogen-activated protein kinases are conserved signaling proteins of ancestral origin that are central to a number of distinct processes in phylogenetically diverse organisms ranging from yeast to humans. MAPKs are the third component of a three-part protein phosphorylation cascade, consisting of a MAPK kinase kinase (MAPKKK), which phosphorylates and activates a MAPK kinase (MAPKK), which in turn, phosphorylates and activates a MAPK. MAPK cassettes respond to external and environmental stimuli, and activate downstream targets to elicit appropriate responses. There exist three classes of MAPKs in metazoans: extracellular signal-
regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 kinases. The ERK MAPKs plays important roles in organismal growth and development. The JNK and p38 MAPKs comprise a subgroup referred to as stress-activated MAPKs that, in addition to roles in development, play important roles in stress physiology.

The JNK and p38 MAPKs are homologous to the yeast stress-activated MAPK Hog1, which regulates the yeast response to osmotic stress. During conditions of hyperosmolarity, Hog1 MAPK is quickly activated by Pbs2 MAPKK and phosphorylates transcriptional regulators, such as Hot1 and Sko1, resulting in the expression of genes that constitute the osmotic stress response, some of which encode proteins that are important for glycerol metabolism and uptake. Interestingly, Hog1 has been reported to switch the Sko1 transcription factor from a repressor to an activator of effector genes. It appears that the Hog1 MAPK pathway duplicated and gave rise to the p38 and JNK stress-activated signaling pathways.

In mammals, the p38 and JNK MAPK pathways respond to various stress stimuli, such as oxidative stress, heat, and ultraviolet light. In addition to regulating stress physiology, these MAPKs play an important role in the innate immune response. p38 and JNK MAPKs are targets of TLR-dependent and TLR-independent immune signaling and are required for production of immunostimulatory cytokines. Genetic studies of stress-activated MAPK signaling in the mammalian innate immune response have been limited due the to lethality associated with mutations in these pathways. Investigating the physiological roles of stress-activated MAPK signaling in simple, genetically tractable organisms will provide insight into the conserved roles for these MAPKs in diverse organisms, including mammals.
Stress-activated MAPKs in C. elegans

The p38 and JNK stress-activated MAPKs of C. elegans have roles in development, stress and immune responses, and behavioral physiology (Figs. 1 and 2). The C. elegans genome contains three gene that encodes JNK MAPK and three genes that encode p38 MAPK. Importantly, loss of function of any p38 or JNK MAPK is viable, allowing for the study of the physiological roles of these MAPKs in host defense. Interestingly, the three genes encoding p38 MAPK – *pmk-1*, *pmk-2*, and *pmk-3* – form an operon and are co-transcribed from a shared upstream promoter. The *pmk-1* and *pmk-2* genes are extremely similar at the nucleotide level and the proteins they encode are both activated by the mammalian MKK3 MAPK kinase and phosphorylate mammalian ATF2 *in vitro*, consistent with their high homology with the mammalian p38 MAPK28. The *pmk-2* gene is likely a recent duplication of the *pmk-1* gene, as the genomes of related nematode species only contain *pmk-1* and *pmk-3* genes. The *pmk-3* gene appears to have diverged from *pmk-1* as it encodes a protein with a TQY motif in its activation domain, as opposed to the canonical TGY motif of p38 MAPKs. The functional diversification of PMK-3 and PMK-1 is evident in the differential specificity of activation by upstream MAPK kinases and phosphorylation of target proteins (Fig. 1). The JNK MAPKs JNK-1 and KGB-1 also appear to have undergone functional diversification in the worm, as they function downstream of separate MAPK kinases, forming two distinct JNK MAPK pathways (Fig. 2).
Figure 1. p38 MAPK signaling in *C. elegans*. *C. elegans* p38 MAPKs function in multiple tissues in diverse processes, including stress and immune responses, neural development, and neurobehavioral responses to bacteria.

Figure 2. JNK MAPK signaling in *C. elegans*. *C. elegans* JNK MAPKs function in stress responses and the neuronal control of coordinated movement.

**In development of neural asymmetry**

The *C. elegans* nervous system is comprised of 302 neurons. Many neurons exist in bilaterally symmetric left/right pairs that are indistinguishable based on anatomy and function. A subset of these bilateral neuron pairs undergoes asymmetric cell fate decisions during development. One such pair is the AWC olfactory neuron pair in which the asymmetric cell fate is stochastic\(^\text{29}\). Either the left or right AWC neuron, but not both, expresses the G protein-coupled receptor STR-2, resulting in distinct chemosensory profiles for each AWC neuron\(^\text{30}\). A
forward genetic screen for mutants that express str-2 in both AWC neurons (Neural symmetry, Nsy phenotype) identified the requirement for NSY-1, an ortholog of mammalian ASK1 MAPKKK that functions upstream of p38 and JNK MAPKs, in the establishment of AWC asymmetry. A later study identified the requirement for SEK-1 MAPKK in AWC neural asymmetry. SEK-1 MAPKK is orthologous to mammalian MAPKKs M KK3 and M KK6, which specifically activate p38 MAPK. The upstream components of the p38 MAPK signaling pathway that regulates this decision have been elucidated and include the TIR-domain containing protein, TIR-1. The downstream components of this TIR-1-NSY-1 MAPKKK-SEK-1 MAPKK signaling pathway that regulate the expression of str-2, including the p38 MAPK, are unknown (Fig. 1).

**In stress responses and host defense**

JNK MAPK signaling in *C. elegans* has been shown to play important roles in coordinated motion, thermotolerance, lifespan, and response to heavy metal stress. There are two distinct JNK MAPK signaling pathways in *C. elegans*: the JKK-1 MAPKK – JNK-1 MAPK pathway and the MLK-1 MAPKKK – MEK-1 MAPKK – KGB-1 MAPK pathway (Fig. 2). The JKK-1-JNK-1 pathway is required in the nervous system for proper coordinated motion. In addition, this pathway appears to regulate thermotolerance and lifespan through the regulation of the conserved forkhead transcription factor FOXO/DAF-16. The MLK-1-MEK-1-KGB-1 pathway has been shown to be required for protection against heavy metal stress. Mutations in *mlk-1, mek-1*, and *kgb-1* confer increased sensitivity to heavy metals. Additionally, phosphorylation of KGB-1 MAPK was increased in the presence of heavy metals. Recent work has elucidated the mechanism of KGB-1-dependent gene activation, showing that genes involved
in the response to heavy metal stress are held transcriptionally silent by a FOS-1/HAD-1 repressor complex, which dissociates upon KGB-1-dependent phosphorylation of FOS-1, leading to the transcriptional activation of target genes.\textsuperscript{37}

The p38 MAPK pathway of \textit{C. elegans} is essential for protection against a number of stressors. PMK-1 MAPK activation is increased in the presence of UV light, heat, and oxidative stress.\textsuperscript{38,39} \textit{pmk-1} and \textit{sek-1} mutants have compromised survival when exposed to oxidative stressors such as sodium arsenite, implicating the importance of the SEK-1 MAPKK – PMK-1 MAPK pathway in response to oxidative stress. The SEK-1-PMK-1 pathway has been shown to phosphorylate and regulate the subcellular localization of the Nfr/SKN-1 transcriptional regulator, which controls the expression of detoxification enzymes.\textsuperscript{39} In addition to its role in stress physiology, the p38 MAPK pathway has important roles in two distinct modes of host defense in \textit{C. elegans}: innate immunity and aversive learning.

**Innate immunity**

The requirement for a NSY-1 MAPKKK – SEK-1 MAPKK – PMK-1 MAPK signaling pathway, orthologous to the mammalian ASK1 MAPKKK – MKK3/MKK6 MAPKK – p38 MAPK pathway, in the innate immune response of \textit{C. elegans} was identified through a simple forward genetic screen for \textit{C. elegans} mutants with increased susceptibility to the pathogen \textit{P. aeruginosa}.\textsuperscript{23} The TIR adaptor protein TIR-1, orthologous to the mammalian adaptor protein SARM, was also shown to be required for protection against infection as \textit{tir-1} mutants also conferred enhanced susceptibility to pathogens.\textsuperscript{20,21} Levels of activated PMK-1 are diminished in \textit{tir-1} mutant animals, placing TIR-1 upstream of the NSY-1-SEK-1-PMK-1 signaling pathway.
The TIR-1-PMK-1 p38 MAPK signaling pathway functions cell autonomously in both the intestine and epidermis in responses to bacterial and fungal pathogens, respectively\(^{40,41}\) (Fig 1).

The factors involved in host defense upstream of TIR-1, including pathogen recognition receptors, have remained largely elusive. A likely explanation for this is the existence of multiple redundant factors that signal through the TIR-1-PMK-1 p38 MAPK pathway. Protein kinase C signaling has been implicated in host defense upstream of the TIR-1-PMK-1 p38 MAPK pathway\(^{42,43}\); however, its contribution to the immune response in minimal, supporting the idea of redundancy upstream of TIR-1. A reverse genetic screen of transmembrane proteins containing leucine-rich repeat (LRR) domains, which bind to PAMPs and are present on Toll-like receptors, identified the requirement for the G protein-coupled receptor FSHR-1 in *C. elegans* host defense\(^{44}\). While FSHR-1 functions in the intestinal immune response to *P. aeruginosa* infection, it does so in parallel to the TIR-1-PMK-1 p38 MAPK pathway. Recently, it has been proposed that the p38 MAPK pathway responds indirectly to pathogen infection by surveying the inhibition of core cellular processes that pathogens target, similar to what was reported for TLR-independent activation of p38 MAPK through inhibition of translation during *L. pneumophila* infection of macrophages\(^{13,45-47}\).

**Aversive learning**

A second mode of host defense in *C. elegans* that is distinct from the immune response is a learned pathogen avoidance behavior\(^{48}\). *C. elegans* can learn to avoid odors associated with pathogens, a trait that was likely selected for in the wild where *C. elegans* encounters both nutritional and pathogenic microbes. This learning response is regulated by the ADF chemosensory neurons and is dependent on the neurotransmitter serotonin. *P. aeruginosa*
induces the expression of \(\textit{tph-1}\), the rate limiting enzyme in the biosynthesis of serotonin, in the ADF neuron resulting in the increased secretion of serotonin and consequent uptake by interneurons, culminating in the learning behavior. The TIR-1-NSY-1-SEK-1 module of the TIR-1-p38 MAPK signaling pathway is required for the pathogen-induced upregulation of \(\textit{tph-1}\) expression in the ADF chemosensory neuron, suggesting that this innate immune pathway also functions in the nervous system of \(\textit{C. elegans}\) to regulate an aversive learning behavioral defense\(^{40}\) (Fig. 1).

In addition to its role in aversive learning behavior, the TIR-1-NSY-1-SEK-1 signaling module functions in the nervous system of \(\textit{C. elegans}\) to regulate reproductive egg laying\(^{40}\) (Fig. 1). Notably, both neuronal functions of the TIR-1-NSY-1-SEK-1 signaling module are independent of PMK-1 p38 MAPK, implicating the function of a different p38 MAPK. The dual tissue specific roles of the TIR-1-NSY-1-SEK-1 signaling module have led to the idea that this innate immune pathway was co-opted in the evolution of interactions with both nutritional and pathogenic microbes.

**Summary**

The ability to mount a proper response to infection is crucial for organismal survival. Mechanisms of host defense, including the innate immune response, are evolutionarily conserved and ancestral in origin. Of particular importance are stress-activated MAPK signaling pathways. Our understanding of these pathways and the physiological roles they play in mammalian immune responses has been limited. The study of p38 MAPK signaling in \(\textit{C. elegans}\) physiological responses to pathogens can provide insight into the conserved roles of p38 MAPK signaling in host defense responses of diverse organisms, including humans.
The focus of this thesis is to further define and understand the physiological role of p38 MAPK signaling in *C. elegans* host defense. In Chapter Two, the conserved ATF/CREB transcriptional regulator ATF-7 is identified as a mediator of PMK-1 p38 MAPK signaling in the *C. elegans* immune response to pathogens. In Chapter Three, the distinct tissue-specific roles of PMK-1 and PMK-2 p38 MAPKs in *C. elegans* host defense are characterized and a role for the miR-58/80-82 family of microRNAs in establishing the tissue specific expression of co-operonic p38 MAPKs is demonstrated. Finally, in Chapter Four, additional studies of *C. elegans* host defense and stress physiology are presented and future directions for further understanding of p38 MAPK signaling in host defense are discussed.
References


Chapter Two

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


* These authors contributed equally to this work

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

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Summary

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

Studies of innate immunity in phylogenetically diverse organisms have revealed the conservation of key signaling pathways mediating pathogen defense [1,2]. In mammals, the initial encounter between cells of the immune system and pathogenic bacteria triggers the activation of the innate immune response to infection, which is under the control of the transcription factor NF-kB and stress-activated mitogen-activated protein kinases (MAPKs) p38 and JNK [3]. Multiple phosphorylation targets for p38 and JNK MAPKs have been identified in mammalian systems, including members of the cyclic AMP-responsive element binding (CREB)/activating transcription factor (ATF) family such as ATF2 [4], activating protein 1 (AP-1), transcription factors Fos and Jun [5], and multiple kinases including the MAPK-activated protein kinase MK2 [6]. Genetic analysis of MK2 knockout mice is suggestive of a role for p38 MAPK regulation of MK2 in the post-transcriptional regulation of TNF-α production [7]. However, genetic analysis of transcription factor targets of p38 and JNK MAPKs has been limited by lethality of knockouts and possible redundancy [8], and thus the identification and characterization of the physiologically relevant targets of MAPK signaling in innate immunity remains a major challenge [9].

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

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

In this paper we report the results of a forward genetic screen for mutants deficient in immune signaling through the PMK-1 p38 MAPK pathway. We report the identification of ATF-7, a putative ortholog of the mammalian ATF2 family of basic-region leucine zipper (bZIP) transcription factors, as a key downstream target of the PMK-1 p38 MAPK pathway in *C. elegans*. Our data establish a pivotal role for ATF-7 as a transcriptional regulator of the PMK-1-mediated innate immune response in *C. elegans*. 

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Results

Isolation and characterization of mutant alleles of atf-7

We modified our prior screen for mutants with enhanced susceptibility to pathogens (Esp phenotype) [10] to focus on the identification of genes encoding components of PMK-1 p38 MAPK-dependent innate immunity in C. elegans. We used as our starting strain a wild-type (WT) N2-derived strain carrying the agls219 transgene, which is comprised of the promoter of a PMK-1-regulated gene, T24B8.5, encoding a ShK-like toxin peptide, fused to green fluorescent protein (GFP) and provides an in vivo sensor of PMK-1 pathway activity [21]. Mutagenized animals with diminished GFP expression were enriched using the COPAS worm sorter, and this enriched population was subsequently transferred to Pseudomonas aeruginosa PA14 for isolation of mutants with diminished PMK-1-dependent reporter expression and an Esp phenotype. From an initial round of high-throughput screening of worms derived from 140 000 mutagenized genomes, we isolated 33 mutants representing five complementation groups using a cutoff for Esp screening that required mutants to be dead prior to the death of any unmutagenized worms treated in parallel (Table 1). Using a combination of complementation testing and sequencing of candidate genes, we determined that four of the complementation groups correspond to genes encoding the established TIR-1-NSY-1-SEK-1-PMK-1 pathway.

The fifth complementation group was defined by a single allele, qd22. The qd22 mutant exhibited a marked decrease in expression of the agls219 transgene (Figure 1A) and conferred a strong Esp phenotype (Figure 1B and S10). The lifespan of the qd22 mutant on relatively non-pathogenic Escherichia coli OP50 was comparable to WT (Figure S1 and S16). Using lysates from qd22 mutant worms, we carried out immunoblotting against the activated form of PMK-1. We found that unlike the other mutants isolated in the screen which carry mutations in PMK-1
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Table 1. List of isolates from a screen for mutants with diminished GFP expression from the *agls219* transgene and enhanced susceptibility to killing by *P. aeruginosa* PA14.

Mutations in *tir-1* are in reference to gene model *F13B10.1b*. The mutation in *atf-7* is in reference to gene model *C07G2.2a.*
pathway components functioning upstream of PMK-1 [21], the qd22 mutant did not exhibit diminished levels of PMK-1 activation, and in fact had increased levels of activated PMK-1 compared to WT (Figure 1C). Taken together, these data suggested that the qd22 mutation affected PMK-1-dependent reporter gene expression either at a step downstream of or parallel to PMK-1.

Using single nucleotide polymorphism (SNP)-based mapping [25,26], we narrowed the region containing the qd22 mutation to a 250 kb region of the left arm of LG III, where we identified a C→T missense mutation causing a P58S change in the open reading frame defined by the gene atf-7, encoding a basic-region leucine zipper (bZIP) domain-containing protein (Figure 2A). Phylogenetic analysis, based on the comparison of the conserved putative DNA binding domain sequence of ATF-7 with other bZIP transcription factors (A.W. Reinke and A.E. Keating, unpublished data), suggests that C. elegans ATF-7 is an ortholog of the mammalian ATF2/ATF7/CREB5 family of bZIP transcription factors [27] (Figure 2B and 2C). Injection of the fosmid 25cA04, that includes the atf-7 locus, resulted in partial rescue of the Esp phenotype (Figure S2).

We were unable to phenocopy the diminished GFP reporter gene expression of the atf-7(qd22) mutant by RNAi of the atf-7 gene in the WT background (Figure S3A), but interestingly, we observed that RNAi of atf-7 in the atf-7(qd22) mutant background resulted in reversion of the diminished GFP reporter gene expression (Figure S3A). RNAi of atf-7 in WT worms resulted in an Esp phenotype (Figure S3B and S11), but notably, RNAi of atf-7 in the atf-7(qd22) mutant conferred increased pathogen resistance and partial suppression of the Esp phenotype of the atf-7(qd22) mutant (Figure S3C and S11). These data suggested that qd22 is a gain-of-function mutant allele of the atf-7 gene, which was further corroborated by analysis of
the phenotype of the *atf-7(qd22)/atf-7(qd22 qd130)* trans-heterozygote (the *qd130* loss-of-function allele is described below), which is nearly as susceptible to *P. aeruginosa* as the *atf-7(qd22)* mutant (Figure S4 and S12). Interestingly, we observed that the *atf-7(qd22)* allele is recessive with respect to the Esp phenotype (Figure S4).

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

To confirm that the observed Esp phenotype of the *atf-7(qd22 qd130)* mutant was caused by the nonsense mutation in *atf-7*, we also analyzed a second putative null allele of *atf-7, atf-7(qd137)* (Figure 2A), which we isolated from a separate screen (described below). The *atf-7(qd137)* mutant exhibited the same Esp phenotype as that observed for the *atf-7(qd22 qd130)* mutant, as well as the trans-heterozygote, *atf-7(qd22 qd130)/atf-7(qd137)* (Figure 1D and S13). We also carried out rescue experiments using a transgene comprised of the genomic *atf-7* locus with GFP fused to the 3’ end between the *atf-7* stop codon and the 3’-untranslated region (UTR). We observed that this transgene partially rescued the Esp phenotype of the *atf-7(qd22 qd130)*
Figure 1. Characterization of *atf-7* mutants that affect signaling downstream of PMK-1 p38 MAPK. (A) Fluorescence microscopy images of GFP expression from the *agls219* transgene in wild-type, *atf-7(qd22)* and *atf-7(qd22 qd130)* one-day-old adults. (B) Pathogenesis assay of L4 larval stage wild-type worms, *atf-7(qd22)* and *atf-7(qd22 qd130)* mutant animals, on *P. aeruginosa* PA14. All strains carry the *agls219* transgene. The differences in susceptibility between *atf-7(qd22)* mutant animals and wild-type worms, *atf-7(qd22)* and *atf-7(qd22 qd130)* mutant animals, and *atf-7(qd22 qd130)* mutant animals and wild-type worms are all significant (*p*<0.0001 for each comparison). Replicate data can be seen in Figure S10. (C) Immunoblot analysis of worm lysates from *atf-7(qd22)* worms. Total PMK-1 was identified using a polyclonal antibody generated against *C. elegans* PMK-1. Activated PMK-1 levels were identified using an antibody specific for the doubly phosphorylated TGY motif of activated PMK-1. (D) Pathogenesis assay of L4 larval stage wild-type worms; *atf-7(qd22 qd130)* and *atf-7(qd137)* mutants; and *atf-7(qd22 qd130)/atf-7(+), atf-7(qd137)/atf-7(+), and atf-7(qd22 qd130)/atf-7(qd137)* trans-heterozygotes, on *P. aeruginosa* PA14. All strains carry the *agls219* transgene. The differences in susceptibility between *atf-7(qd22 qd130)* mutant animals and *atf-7(qd22 qd130)/atf-7(+)* trans-heterozygotes, and *atf-7(qd137)* mutants animals and *atf-7(qd137)/atf-7(+/+)* trans-heterozygotes are significant (*p*<0.0001 for each comparison). There is no difference in susceptibility between *atf-7(qd22 qd130)* mutant animals and *atf-7(qd22 qd130)/atf-7(qd137)* trans-heterozygotes, and *atf-7(qd137)* mutant animals and *atf-7(qd22 qd130)/atf-7(qd137)* trans-heterozygotes (*p*>0.35 for each comparison). Replicate data can be seen in Figure S13.
Figure 1

A. Wild-type, atf-7(qd22), atf-7(qd22 qdl30)

B. Fraction Alive

C. Wild-type, tir-1(qd4), atf-7(qd22), Phospho-PMK-1, PMK-1

D. Fraction Alive

Wild-type, atf-7(qd22), atf-7(qd22 qdl30), atf-7(qd130), atf-7(qd22 qdl30)/atf-7(qd137), atf-7(qd137)/atf-7(+)
Figure 2. Mutant alleles of *C. elegans atf-7*, an ortholog of the mammalian ATF2/ATF7/CREB5 family of bZIP transcription factors. (A) Identification of mutations in the *C. elegans atf-7* gene. Changes shown in amino acid residues are in reference to the coding region of gene model *C07G2.2a*. (B) Sequence alignment comparing the DNA binding domains of *C. elegans* ATF-7 and human ATF-2. (C) Phylogenetic analysis grouping *C. elegans* ATF-7 with the mammalian ATF2/ATF7/CREB5 family of bZIP transcription factors.
mutant (Figure S5). The partial degree of rescue observed for both the atf-7(qd22) and atf-7(qd22 qd130) mutants may reflect the detrimental effects of overexpression of atf-7.

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

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

Because atf-7(qd22) appeared to be a gain-of-function allele of atf-7 that exhibited the same phenotypes as observed for mutants carrying loss-of-function mutations in PMK-1 pathway components, we hypothesized that ATF-7 might be negatively regulated by the PMK-1 pathway and function as a repressor of the innate immune response. To test this hypothesis, we carried out epistasis analysis using the atf-7(qd22 qd130) and pmk-1(km25) null alleles. We observed that the atf-7(qd22 qd130) loss-of-function allele suppressed the diminished agIs219 GFP reporter gene expression phenotype of the pmk-1(km25) mutant (Figure 3A). Also, the atf-7(qd22 qd130); pmk-1(km25) double mutant had a reduced pathogen susceptibility compared to pmk-1(km25), comparable to that of the atf-7(qd22 qd130) single mutant (Figure 3B and S14). The partial suppression of the Esp phenotype of the pmk-1(km25) mutant by atf-7(qd22 qd130) was rescued by a transgene carrying wild-type atf-7 fused with GFP (Figure S7). Furthermore, we found that atf-7(qd22 qd130) also suppressed the pathogen susceptibility (Figure 3D and S15) and diminished agIs219 GFP reporter expression (Figure 3C) phenotypes of the sek-1(km4) mutant.
The Esp phenotypes of the *atf-7(qd22 qd30); pmk-1(km25) and atf-7(qd22 qd130); sek-1(km4)* double mutants are comparable to the Esp phenotype of *atf-7(qd22 qd130)* single mutant. The lack of effect of the *pmk-1(km25)* or *sek-1(km4) mutations on the Esp phenotype in the *atf-7(qd22 qd130)* mutant background is particularly noteworthy in view of the strong Esp phenotype conferred by inactivation of the PMK-1 pathway in the WT background.

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

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

We used quantitative real-time PCR (qRT-PCR) to measure changes in expression of three representative genes previously identified as being regulated by the PMK-1 pathway [22]. We observed that the *atf-7(qd22)* mutant, consistent with the observed effects on *agIs219* GFP reporter expression (Figure 1A), exhibited sharply diminished expression of PMK-1-regulated genes relative to WT on *E. coli* OP50, comparable to the levels observed in the *pmk-1(km25)* mutant (Figure 4A). These data confirm that the observed effects of the *atf-7(qd22)* mutation on *agIs219* expression reflect a change in the regulation of PMK-1-regulated genes.
Figure 3. The loss-of-function atf-7(qd22 qd130) mutation suppresses the immunodeficient phenotype caused by deficient signaling in the PMK-1 pathway. (A) Fluorescence microscopy images of GFP expression from the agls219 transgene in pmk-1(km25) and atf-7(qd22 qd130); pmk-1(km25) one-day-old adults. (B) Pathogenesis assay of L4 larval stage wild-type worms; atf-7(qd22 qd130) and pmk-1(km25) mutant animals; and atf-7(qd22 qd130); pmk-1(km25) double mutant animals, on P. aeruginosa PA14. All strains carry the agls219 transgene. The difference in susceptibility between pmk-1(km25) mutant animals and atf-7(qd22 qd130); pmk-1(km25) double mutant animals is significant (p<0.0001). Replicate data can be seen in Figure S14. (C) Fluorescence microscopy images of GFP expression from the agls219 transgene in sek-1(km4) and atf-7(qd22 qd130); sek-1(km4) one-day-old adults. (D) Pathogenesis assay of L4 larval stage wild-type worms; atf-7(qd22 qd130) and sek-1(km4) mutant animals; and atf-7(qd22 qd130); sek-1(km4) double mutant animals, on P. aeruginosa PA14. All strains except for KU25 [sek-1(km4)] carry the agls219 transgene. The difference in susceptibility between sek-1(km4) mutant animals and atf-7(qd22 qd130); sek-1(km4) double mutant animals is significant (p<0.0001). Replicate data can be seen in Figure S15.
Figure 3

A

\[ pmk-1(km25) \]

\[ att-7(qd22 qd130); pmk-1(km25) \]

B

\[ \text{Fraction Alive} \]

\[ \text{Time (h)} \]

\[ \text{Wild-type} \]

\[ att-7(qd22 qd130) \]

\[ att-7(qd22 qd130); pmk-1(km25) \]

\[ pmk-1(km25) \]

C

\[ sek-1(km4) \]

\[ att-7(qd22 qd130); sek-1(km4) \]

D

\[ \text{Fraction Alive} \]

\[ \text{Time (h)} \]

\[ \text{Wild-type} \]

\[ att-7(qd22 qd130) \]

\[ att-7(qd22 qd130); sek-1(km4) \]

\[ sek-1(km4) \]
Figure 4. **ATF-7 regulation of PMK-1-regulated genes.** qRT-PCR analysis of the expression of PMK-1-regulated genes. (A) L4 larval stage worms of the indicated genotype were propagated on *E. coli* OP50 and RNA was prepared as described in the Methods. Expression is relative to wild-type. Shown is the mean from two independent biological replicates with error bars representing SEM. (B) As in (A), except that worms of the indicated genotype were exposed to *P. aeruginosa* PA14 for 4 h. Expression is relative to wild-type on *E. coli* OP50.
Figure 4

A

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

Upon exposure to pathogen infection, a number of genes are up-regulated in a PMK-1-dependent manner [22]. We observe that genes that require PMK-1 for induction by *P. aeruginosa* PA14 also require ATF-7 for pathogen-induced expression (Figure 4B). Although the basal expression of PMK-1-regulated genes on *E. coli* OP50 in the *atf-7(qd22 qd130)* and *atf-7(qd137)* mutants is comparable to WT, no induction of expression is observed in the presence of *P. aeruginosa* (Figure 4B). These data also suggest dual switching roles for ATF-7, both as a PMK-1-regulated repressor of the basal expression of PMK-1-regulated genes as well as a PMK-1-dependent activator of PMK-1-regulated genes upon pathogenic *P. aeruginosa* infection. This requirement may contribute to the observedEsp phenotype of the *atf-7(qd22 qd130)* mutant, as
optimal regulation of the *C. elegans* innate immune response may be dependent on PMK-1 regulation of ATF-7.

**Phosphorylation of ATF-7 by PMK-1**

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

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

We used a mutated version of PMK-1 that does not have kinase activity to establish that ATF-7 and PMK-1 physically interact. Immunoprecipitation using the T7 antibody, followed by immunoblotting using anti-HA, revealed an HA-PMK-1(kinase-dead)-T7-ATF-7 interaction that
Figure 5. ATF-7 is expressed in the nuclei of intestinal cells in *C. elegans*. Fluorescence and DIC microscopy of a representative L4-staged wild-type worm carrying an *atf-7::GFP* transgene under the regulation of the endogenous genomic *atf-7* promoter and 3’-untranslated region. The red fluorescence from the pharynx is due to a *Pmyo-2::RFP* co-transformation marker. The rescuing capability of this translational fusion transgene was confirmed in both the *atf-7(qd22 qd130)* mutant (Figure S5) and *atf-7(qd22 qd130); pmk-1(km25)* double mutant (Figure S7). Scale bar, 100 μm.
Figure 5
Figure 6. Phosphorylation of ATF-7 by PMK-1. (A) Cos7 cells were transfected with T7-ATF-7 or T7-ATF-7 carrying the P58S qd22 mutation, along with HA-PMK-1 and FLAG-SEK-1 as indicated. Whole cell extracts were immunoblotted with antibodies that recognize T7 (top), HA (middle), and FLAG (bottom). (B) Cos7 cells were transfected with T7-ATF-7 or T7-ATF-7 carrying the P58S qd22 mutation, along with kinase-dead (KD) HA-PMK-1 and FLAG-SEK-1 as indicated. ATF-7 was immunoprecipitated with anti-T7 and immunoblotted with anti-HA (top). Whole cell extract were immunoblotted with antibodies that recognize T7 (middle top), HA (middle bottom), and FLAG (bottom).
Figure 6

A

<table>
<thead>
<tr>
<th></th>
<th>T7-ATF-7</th>
<th>HA-PMK-1</th>
<th>FLAG-SEK-1</th>
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<tr>
<td>+</td>
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<tr>
<td>qd22</td>
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α T7  

α HA  

α FLAG

B

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<thead>
<tr>
<th></th>
<th>T7-ATF-7</th>
<th>HA-PMK-1 (KD)</th>
<th>FLAG-SEK-1</th>
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<tr>
<td>+</td>
<td></td>
<td>+</td>
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<tr>
<td>qd22</td>
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IP:T7  

α HA  

α T7  

WCE  

α HA  

α FLAG
was dependent on the activated form of PMK-1, as determined by the requirement for co-transfection of *sek-1* cDNA (Figure 6B).

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

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

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

Interestingly, on the Gram-positive pathogen *E. faecalis* MMH594, the atf-7(qd22 qd130) mutant does not have an appreciable Esp phenotype (Figure 7B), suggestive that ATF-7 may not serve as a positive regulator of resistance to *E. faecalis*. In addition, the atf-7(qd22 qd130) mutation only partially suppresses the Esp phenotype of the pmk-1(km25) mutant (Figure 7B), suggestive that there are both ATF-7-dependent and ATF-7-independent mechanisms downstream of PMK-1 in response to *E. faecalis*. Distinct sets of genes have been observed to be induced by exposure to different bacteria, and little is known about how the transcriptional responses to Gram-positive bacteria and Gram-negative bacteria differ [28]. Of note, we observed diminished GFP expression from the agls219 transgene on Gram-positive bacteria relative to expression on *E. coli* OP50 (D.H.K., unpublished data). Gene expression studies of genes induced in *C. elegans* infection with Gram-positive bacteria, and the identification of such genes that are regulated by the PMK-1 pathway may further illuminate the differences in the role of ATF-7 observed between *E. faecalis* and *P. aeruginosa*.

The PMK-1 pathway regulates the response to arsenite and oxidative stress through regulation of the transcription factor SKN-1 [29,30]. We observed that the atf-7(qd22 qd130) mutant did not exhibit enhanced sensitivity to arsenite stress, and in addition, did not suppress the arsenite sensitivity of the pmk-1(km25) mutant or the sek-1(km4) mutant (Figure 8). In addition, we observed that skn-1 mutants did not exhibit enhanced susceptibility to *P. aeruginosa* (Figure S9). Whereas PMK-1 mediates multiple responses to environmental stressors, including oxidative stress and pathogen infection, these data suggest that the transcription factor substrates of PMK-1, ATF-7 and SKN-1, confer specificity to PMK-1-mediated responses.
Figure 7. Requirement for ATF-7 in resistance to other bacterial pathogens. (A) Pathogenesis assay of L4 larval stage wild-type worms; atf-7(qd22 qd130) and pmk-1(km25) mutant animals; and atf-7(qd22 qd130); pmk-1(km25) double mutant animals, on S. marcescens Db10. All strains carry the agls219 transgene. The difference in susceptibility between atf-7(qd22 qd130) mutant animals and wild-type worms is significant (p<0.0001). (B) Pathogenesis assay of L4 larval stage wild-type worms; atf-7(qd22 qd130) and pmk-1(km25) mutant animals; and atf-7(qd22 qd130); pmk-1(km25) double mutant animals, on E. faecalis MMH594. All strains carry the agls219 transgene. The difference in susceptibility between pmk-1(km25) mutant animals and atf-7(qd22 qd130); pmk-1(km25) double mutant animals is significant (p<0.0001). There is no difference in susceptibility between atf-7(qd22 qd130) mutant animals and wild-type worms (p>0.9).
Figure 7

A

B

Fraction Alive

Fraction Alive

Time (h)

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

![Bar graph showing fraction alive for different genotypes.]

- Wild-type
- atf-7(qd22) qd130
- pmk-1(km25)
- sek-1(km4)
- atf-7(qd22) qd130; pmk-1(km25)
- sek-1(km4)
Discussion

We have described the identification and characterization of ATF-7, a *C. elegans* ortholog of the mammalian ATF2/ATF7/CREB5 family of bZIP transcription factors, as a transcriptional regulator of PMK-1-mediated innate immunity in *C. elegans*. We isolated four mutant alleles of *C. elegans atf-7* from three different forward genetic screens. First, the gain-of-function *qd22* allele was isolated from a large-scale screen for mutants with diminished PMK-1-dependent GFP reporter gene expression and an Esp phenotype. The isolation of *qd22* served as a starting point for the characterization of ATF-7, and the analysis of this unusual gain-of-function allele provided insights into the mechanism of ATF-7 regulation by PMK-1. The increased levels of PMK-1 activation in the *atf-7(qd22)* mutant relative to WT (Figure 1C) may reflect feedback loops that serve to counteract the suppression of the PMK-1-mediated transcriptional response by increasing levels of activated PMK-1. Although *atf-7(qd22)* acts as a gain-of-function allele, we determined that *atf-7(qd22)* is recessive with regard to pathogen susceptibility (Figure S4). We suggest that whereas the *qd22* mutant ATF-7 protein cannot be phosphorylated by PMK-1 and thus functions as a constitutive repressor (Figure 6A), the resulting protein may undergo changes in structure and folding that compromise the ability of the mutant ATF-7 to compete with WT ATF-7 at corresponding promoter sites in the *atf-7(qd22)/atf-7(+) trans-heterozygote.*

Based on evidence that *atf-7(qd22)* was a gain-of-function allele, we isolated the *atf-7(qd22 qdl30)* allele as an intragenic suppressor of *atf-7(qd22)*. A third genetic screen aimed at isolating suppressors of *pmk-1(km25)* yielded two additional putative null alleles of *atf-7*, *qd137* and *qd141*. The genetic analysis of loss-of-function alleles of *atf-7* allowed us to begin to address the physiological role of ATF-7 in innate immunity. Genetic interaction analysis of *atf-7*...
mutant alleles suggests that PMK-1 negatively regulates ATF-7, which in turn functions as a negative regulator of *C. elegans* innate immunity to *P. aeruginosa* and *E. faecalis*. At the same time, the Esp phenotype of *atf-7* loss-of-function mutants and the analysis of *P. aeruginosa*-induced gene expression were suggestive of a requirement for ATF-7 in the activation of the inducible innate immune response, as ATF-7 was shown to be required for the increased expression of PMK-1-regulated genes in response to *P. aeruginosa* infection. Interestingly, the lack of an Esp phenotype of *atf-7(qd22 qd130)* on *E. faecalis* may be suggestive of the absence of a PMK-1-regulated inducible response to *E. faecalis* that is regulated by ATF-7.

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

In view of the multiple substrates for p38 MAPK that have been established in mammalian cell systems and the multiple activities of p38 MAPK in mammalian innate immunity, it is perhaps surprising that loss of activity of a single transcription factor, the *C. elegans* ortholog of mammalian ATF2, is sufficient to suppress the immunocompromised
Figure 9. Model for the function of ATF-7 in C. elegans innate immunity. (A) Repression of PMK-1-regulated immune effector gene expression by ATF-7 in the absence of PMK-1 activation. (B) Activation of basal and pathogen-induced immune effector gene expression by PMK-1 phosphorylation of ATF-7, which switches ATF-7 from a repressor to an activator of transcription.
Figure 9

A

PMK-1

ATF-7

Immune Response

B

E. coli

P. aeruginosa

PMK-1-P

ATF-7

Immune Response
phenotype caused by loss of p38 MAPK activity in C. elegans. Mice homozygous for a deletion of the ATF2 gene die shortly after birth due to the lack of pulmonary surfactant [8], although analysis of cells from mutant mice expressing low levels of ATF2 are suggestive of a role for ATF2 in the modulation of cytokine expression [32]. Interestingly, these studies also indicate a role for mammalian ATF2 in both activating and inhibitory activities on the immune response [32]. Whether ATF2 plays a correspondingly homologous role in mammalian innate immunity downstream of p38 MAPK, paralleling the activity of C. elegans ATF-7, awaits the further molecular genetic analysis of the ATF2/ATF7/CREB5 family in mammalian systems. Our data establish the SARM-p38 MAPK-ATF-7 pathway as a major pathway of C. elegans innate immunity and suggest that the regulation of ATF2/ATF7 family of bZIP transcription factors by p38 MAPK may represent a key feature of innate immunity in ancestral organisms that was retained even as Toll-dependent NF-κB immune signaling was lost [33]. The mechanism of regulation of ATF-7 activity by PMK-1 may also provide insights into conserved mechanisms by which p38 MAPK modulates the activity of ATF2/ATF7 in mammalian innate immunity.
Material and Methods

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

Previously isolated and characterized mutants used: LG II: *nsy-1*(ag3) [10], *nsy-1*(ky397) [35]. LG III: *tir-1*(qd4) [21]. LG IV: *pmk-1*(km25) [36], *skn-1*(zu67), *skn-1*(zu135) [37]. LG X: *sek-1*(km4) [10,38].

Mutants described in this study: ZD442 [agIs219* atf-7*(qd22) III] was isolated as described below and backcrossed three times to its parental strain, AU78. ZD318 [agIs219* atf-7*(qd22 qd130) III] was isolated as described below and outcrossed four times to wild-type strain N2. ZD39 [agIs219 III; *pmk-1*(km25) IV] was made by crossing the agIs219 transgene from strain AU78 into *pmk-1*(km25). ZD395 [agIs219 III; *sek-1*(km4) X] was made by crossing the agIs219 transgene from strain AU78 into *sek-1*(km4). ZD332 [agIs219* atf-7*(qd137) III; *pmk-1*(km25) IV] and ZD402 [agIs219* atf-7*(qd141) III; *pmk-1*(km25) IV] were isolated as described below. ZD332 was backcrossed three times to ZD39. ZD350 [agIs219* atf-7*(qd137) III] was made by removing *pmk-1*(km25) from agIs219* atf-7*(qd137); *pmk-1*(km25), which had been previously backcrossed twice to ZD39, by outcrossing to N2. ZD326 [agIs219* atf-7*(qd22 qd130) III; *pmk-1*(km25) IV] was made by crossing *pmk-1*(km25) into agIs219* atf-7*(qd22 qd130) and was outcrossed to *pmk-1*(km25) an additional three times. ZD340 [agIs219* atf-7*(qd22 qd130) III; *sek-1*(km4) X] was made by crossing *sek-1*(km4) into agIs219* atf-7*(qd22 qd130) and was outcrossed to *sek-1*(km4) an additional three times.

Pathogenesis assays. Pathogenesis assays with *P. aeruginosa* PA14 [39], *S. marcescens* Db10 [40] and *E. faecalis* MMH594 [41,42] were performed as described previously with the
following modifications. Single colonies of *P. aeruginosa* PA14 and *S. marcescens* Db10 were used to inoculate 3 ml cultures of Luria-Bertani (LB) broth, which were then incubated overnight at 37°C. Five microliters of the *S. marcescens* Db10 culture was used to seed standard 35-mm slow-kill assay plates, whereas five microliters of the *P. aeruginosa* PA14 culture was used to seed 35-mm slow-kill assay plates containing 0.05 mg/ml 5-fluorodeoxyuridine (FUDR), used to prevent eggs from hatching. Seeded plates were incubated at 37°C overnight and then incubated at room temperature overnight. A single colony of *E. faecalis* MMH594 was used to inoculate a 3 ml culture of brain heart infusion (BHI) broth containing 80 μg/ml of kanamycin, which was then incubated at 37°C for 5 hours. Seven microliters of culture was used to seed 35-mm BHI agar plates containing 80 μg/ml of kanamycin, which were incubated at 25°C overnight. In all pathogenesis assays, the size of the bacterial lawn was small, meaning that the culture was seeded in the middle of the plate and was not spread to the edge. For each assay, approximately 20-40 L4-staged worms were picked over to prepared plates, with 3-5 plates per strain. The sample sizes for each assay are provided in Table S1. All pathogenesis assays were conducted at 25°C. Plates were checked at regular intervals for survival and worms that did not respond to gentle prod from a platinum wire were scored as dead. Worms on *S. marcescens* plates were transferred to new plates on days 1, 2, and 3 of the assay. All *S. marcescens* plates in a single assay were seeded on the same day. Worms on *P. aeruginosa* PA14 plates containing FUDR and *E. faecalis* MMH594 plates did not require transferring. Statistical analyses of survival curves were performed in Prism 5 (GraphPad) using the log-rank test function, which computes the Mantel-Haenszel method.

**Isolation of mutants with diminished agIs219 reporter expression and enhanced susceptibility to killing by *P. aeruginosa* PA14.** Mutagenesis using ethyl methanesulfonate
(EMS) was carried out following standard methods [43]. The synchronized F2 generation L1 stage larvae were plated onto NGM plates seeded with E. coli OP50 and incubated for 55 hours at 20°C and subsequently sorted with a Union Biometrica COPAS Biosorter. Worms with diminished fluorescence compared to wild-type worms were directly plated onto a plate seeded with P. aeruginosa PA14, and incubated at 25°C. The plates were screened at 24 hours for dead worms. Following the rationale of our previously reported screen for Esp mutants [10], carcasses of dead worms were picked to individual NGM plates seeded with E. coli OP50, allowing the fertilized eggs inside each carcass to hatch so that the mutant strains could be recovered. In three separate screens a total of 140 000 haploid genomes were mutagenized. We note that the yield of the screen is strongly dependent on the time at which the Esp screening takes place, and that the 24 h time point represented a particularly stringent time such that siblings were rarely isolated among the mutant isolates. The early time of screening also accounts for the relatively low yield of mutants from the number of genomes mutagenized and the high specificity of isolated mutations for the PMK-1 pathway.

Single-nucleotide polymorphism (SNP)-based mapping using the C. elegans isolate CB4856 was performed as reported [26] with modifications utilizing SNPs that were analyzed by the Dral restriction enzyme for the rapid rough mapping of mutant isolates [25]. Once chromosomal linkage was determined, complementation testing was performed using previously isolated mutant alleles (tir-1(qd4), nsy-1(ag3) and nsy-1(ky397), sek-1(km4), and pmk-1(km25)). After assignment of the isolated alleles into complementation groups, the open reading frame of the affected gene was sequenced to identify the causative mutation in each allele. Isolates from the screen and the identified mutations are shown in Table 1. Using this approach, a single mutant allele not corresponding to previously identified mutants, qd22, was isolated. Fine
mapping of qd22 was carried out using CB4856 SNP-based mapping. The location of qd22 on
the left arm of LG III was in the vicinity of the agls219 integrated transgenic array, and thus a
strain carrying qd22 without agls219 was generated and SNP mapping was carried out using the
Esp phenotype of qd22. In order to facilitate interpretation of the pathogen killing assays with
recombinants, a strain carrying the qd22 mutation (without the agls219 transgene) and the
CB4856-derived allele of npr-1, 215F, was utilized for crossing with CB4856 because of the
enhanced susceptibility conferred by the 215F allele of npr-1 relative to the Bristol N2
background [44,45] in which qd22 was initially isolated.

**Isolation of atf-7(qd22 qd130).** A forward genetic screen to identify suppressors of the
atf-7(qd22) diminished agls219 GFP fluorescence phenotype was carried out similarly as above.
Briefly, *C. elegans atf-7(qd22) hermaphrodites carrying agls219* were mutagenized with EMS
and synchronized larvae of the F2 generation were plated onto NGM plates seeded with *E. coli
OP50* and incubated for 55 hours at 20°C. The F2 worms were screened for GFP expression
from the agls219 transgene using a Zeiss Stereo V12 Discovery microscope with a GFP wide-
band fluorescence cube. Any F2 worm with increased fluorescence compared to atf-7(qd22) was
singled to a NGM plate seeded with *E. coli OP50*. Isolates with increased fluorescence were
then tested for suppression of the atf-7(qd22) Esp phenotype to *P. aeruginosa PA14* using the
PA14 pathogenesis assay described above. The atf-7 coding region was then sequenced in
isolates that had both increased fluorescence and diminished pathogen susceptibility.

**Isolation of atf-7(qd137) and atf-7(qd141).** A genetic screen for suppressors of the pmk-
l(km25) mutant was carried out using ZD39 as the starting strain and following a procedure as
outlined for the isolation of atf-7(qd22) suppressor mutants.
Generation of transgenic animals. *C. elegans* genomic fosmids (Geneservice) were isolated using Qiagen Miniprep Kits following the standard protocol. Fosmid 24cA04 was injected into ZD442 at a concentration of 20 ng/µl, along with 25 ng/µl of *Pmyo-2::RFP* as a co-injection marker and 55 ng/µl of pBlueScript (Stratagene) as carrier DNA. The fluorescently-tagged *atf-7* construct was generated by yeast-mediated ligation of genomic fragments generated by PCR using fosmid 25cA04 as template DNA and Phusion high-fidelity DNA polymerase (New England Biolabs). A 22794 bp genomic region, from 7474 to 30267 with respect to fosmid 25cA04, was covered in the fluorescently-tagged *atf-7* construct. This construct was injected into ZD326 at a concentration of 20 ng/µl, along with 32 ng/µl of *Pmyo-2::RFP* as a co-injection marker and 10 ng/µl of pBlueScript (Stratagene) as carrier DNA. Two independent lines carrying this construct were crossed into strain ZD318 using standard genetic techniques.

Yeast-mediated ligation construction of *atf-7::GFP*. Yeast-mediated ligation of *atf-7::GFP* was performed as previously described [46]. Briefly, the 22794 bp operonic region containing the *atf-7* gene was amplified in fragments ranging in size from ~1 kb to 4 kb in 8 separate PCR reactions with at least a 50 bp overlap between adjacent fragments. The gene encoding GFP was amplified from the Fire vector pPD95.75 [47]. The 9 PCR products, along with destination vector pRS426 (ATCC) digested with BamH1 and Xho1 restriction enzymes (New England Biolabs), were transformed into yeast strain FY2 following standard procedures. Phenol-chloroform extraction was used to isolate yeast DNA, which was then transformed into DH5-α electrocompetent cells (Invitrogen) and isolated using Qiagen Miniprep Kits following the standard protocol.

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

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

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

**Visualization of *agls219* GFP reporter gene expression and fluorescently-tagged atf-7.** To visualize expression of the *agls219* reporter, L4-staged worms, grown at 20°C, were picked over to normal maintenance plates and placed at 20°C overnight. After approximately 18
h, worms were mounted on 2% agarose pads and immobilized in 10 mM sodium azide. Slides were viewed using an Axiolmager Z1 fluorescence microscope (Zeiss) with an A-Plan 10X/0.25 objective (Zeiss) and pictures were taken using an AxioCam HRm camera. To visualize expression of fluorescently-tagged *atf-7*, L4-staged worms, grown on NGM agar plates seeded with *E. coli* OP50 at 20°C, were mounted and imaged as described above with a Plan-Apochromat 20X/0.8 objective (Zeiss). Background intestinal autofluorescence was removed by taking a picture with the DAPI filter and subtracting the resulting picture from the image taken with the GFP filter.

**Expression in Cos7 Cells, immunoprecipitation, and immunoblotting.**

Cos7 cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 μg/ml penicillin G and 100 μg/ml streptomycin at 37°C and 5% CO2. Cos7 cells (1x10⁶) were plated in 6-cm dishes and transfected with a total of 6 μg DNA containing various expression vectors by using FuGENE6 (Roche). The ATF-7 expression vector contained the *atf-7c* isoform. After 48 h, cells were collected and washed once with ice-cold phosphate-buffered saline (PBS) and lysed in 0.6 ml of extraction buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1.7 μg/ml aprotinin and 0.5% Triton X-100). Cellular debris was removed by centrifugation at 10 000 x g for 5 min. Small aliquots of each cell lysate were boiled with SDS-sample buffer and were used as whole cell extracts. Remaining cell lysates were divided into 200 μl and each cell lysate was incubated with 0.5 μg of various antibodies and 10 ml protein G-Sepharose (Amersham Biosciences, Piscataway, NJ). The immune complexes were washed five times with wash buffer (20 mM HEPES, pH 7.4, 150 mM NaCl) and then boiled with SDS-sample buffer. Phosphatase treatment
was performed on immunoprecipitated samples with Lambda protein phosphatase (New England Biolabs) at 30°C for 10 minutes. Immunoblotting was performed as described previously [51].

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

**Lifespan assays.** Strains used in the lifespan assays were maintained on *E. coli* OP50 at 20°C. Approximately 40 L4-staged worms (Day 0) were picked over to NGM plates containing 0.05 mg/ml of FUDR, seeded with *E. coli* OP50. Four to five plates for each strain were used in each experiment and plates that had become contaminated or plates in which the worms had borrowed were excluded upon the appearance of contamination/borrowing. Worms that had protruding/exploding vulvas and worms that crawled off the plate were censored. The sample sizes for each assay are provided in Table S1. Lifespan assays were performed at 20°C. Animals were considered dead when they no longer responded to a gentle prod with a platinum wire.
Supplementary Figures

Figure S1. Lifespan of \textit{atf-7(qd22)} and WT worms on \textit{E. coli OP50}. Lifespan assay of L4 larval stage wild-type worms and \textit{atf-7(qd22)} mutant animals on \textit{E. coli} OP50. Both strains carry the \textit{agIs219} transgene. Replicate data can be seen in Figure S16.
Figure S1

Fraction Alive vs Time (d)

- Wild-type
- atf-7(qd22)
Figure S2. Rescue of *atf-7(qd22)* Esp phenotype. Pathogenesis assay of L4 larval stage wild-type worms, *atf-7(qd22)* mutant animals, and three independent transgenic lines (*qdEx14*, *qdEx15*, and *qdEx16*) of *atf-7(qd22)* mutant animals carrying fosmid 25cA04. All strains carry the *agls219* transgene. The difference in susceptibility between *atf-7(qd22)* mutant animals and each transgenic line carrying fosmid 25cA04 is significant (p<0.0001).
Figure S2

- Wild-type
- atf-7(qd22)
- atf-7(qd22); qdEx14
- atf-7(qd22); qdEx15
- atf-7(qd22); qdEx16
Figure S3. *atf-7(qd22)* is a gain-of-function allele. (A) Fluorescence microscopy images of GFP expression from the *agls219* transgene in wild-type worms and *atf-7(qd22)* mutant worms each exposed to both control RNAi and RNAi of *atf-7*. (B) Pathogenesis assay comparing the effects of control RNAi and RNAi of *atf-7* on survival of wild-type worms on *P. aeruginosa* PA14. The difference in susceptibility between wild-type worms treated with control RNAi and *atf-7* RNAi is significant (*p*<0.0001). Replicate data can be seen in Figure S11. (C) Pathogenesis assay comparing the effects of control RNAi and RNAi of *atf-7* on survival of *atf-7(qd22)* mutant animals on *P. aeruginosa* PA14. The difference in susceptibility between *atf-7(qd22)* mutant animals treated with control RNAi and *atf-7* RNAi is significant (*p*<0.0001). Replicate data can be seen in Figure S11.
Figure S3

A

Wild-type

atf-7(qd22)

Control RNAi

atf-7 RNAi

B

Fraction Alive

0.0

0.5

1.0

Time (h)

0

50

100

150

Wild-type

Control RNAi

atf-7 RNAi

C

Fraction Alive

0.0

0.5

1.0

Time (h)

0

50

100

150

atf-7(qd22)

Control RNAi

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

- **Wild-type**
- **atf-7(qd22)**
- **atf-7(qd22 qd130)**
- **atf-7(qd22)/atf-7(+)**
- **atf-7(qd22)/atf-7(qd22 qd130)**
Figure S5. Expression of atf-7::GFP rescues the atf-7(qd22 qd130) Esp phenotype.

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

- Wild-type
- \textit{atf-7(qd22 qd130)}
- \textit{atf-7(qd22 qd130); qdEx17}
- \textit{atf-7(qd22 qd130); qdEx19}
**Figure S6. Lifespan of atf-7 loss-of-function mutants and WT worms on *E. coli* OP50.**

Lifespan assay of L4 larval stage wild-type worms, *atf-7(qd22 qd130)* and *atf-7(qd137)* mutant animals on *E. coli* OP50. All strains carry the *agIs219* transgene. Replicate data can be seen in Figure S16.
Figure S6

- Wild-type
- \textit{atf-7(qd22 qd130)}
- \textit{atf-7(qd137)}
Figure S7. Expression of *atf-7::GFP* rescues the *atf-7(qd22 qd130)* suppression of *pmk-1(km25)*. Pathogenesis assay of *pmk-1(km25)* mutant animals and *atf-7(qd22 qd130); pmk-1(km25)* double mutant animals, along with three independent transgenic lines (*qdEx17, qdEx18, and qdEx19*) of *atf-7(qd22 qd130); pmk-1(km25)* double mutant animals carrying the *atf-7::GFP* construct under the control of the endogenous *atf-7* genomic promoter and 3'-untranslated region, on *P. aeruginosa* PA14. All strains carry the *agIs219* transgene. The difference in susceptibility between *atf-7(qd22 qd130); pmk-1(km25)* double mutant animals and each transgenic line carrying the *atf-7::GFP* transgene is significant (*p*<0.0001 for each comparison).
Figure S7

Fraction Alive vs. Time (h)

- $\text{atf-7(qd22 qd130); pmk-1(km25)}$
- $\text{pmk-1(km25)}$
- $\text{atf-7(qd22 qd130); pmk-1(km25); qdEx17}$
- $\text{atf-7(qd22 qd130); pmk-1(km25); qdEx18}$
- $\text{atf-7(qd22 qd130); pmk-1(km25); qdEx19}$
Figure S8. PMK-1 phosphorylation of ATF-7 is sensitive to phosphatase. Cos7 cells were transfected with T7-ATF-7, HA-PMK-1, and FLAG-SEK-1 as indicated. ATF-7 was immunoprecipitated with anti-T7, treated with phosphatase where indicated, and immunoblotted with anti-T7 (top). Whole cell extracts were immunoblotted with antibodies that recognize HA (middle) and FLAG (bottom).
Figure S8

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IP:T7

- α T7

WCE

- α HA

- α FLAG
Figure S9. *skn-1* mutants do not exhibit an Esp phenotype. Pathogenesis assay of L4 larval stage wild-type worms, *skn-1(zu67)* and *skn-1(zu135)* mutant animals on *P. aeruginosa* PA14.
Figure S9

Fraction Alive

Time (h)

- Wild-type
- \textit{s}kn\textit{-}1(zu67)
- \textit{s}kn\textit{-}1(zu135)
Figure S10. Replicate of pathogenesis assay shown in Figure 1B. Chart and bar graphs showing the LT$_{50}$ means, LT$_{50}$ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms, *atf-7*(qd22) and *atf-7*(qd22 qd130) mutant animals.
Figure S10

### Experiment #1

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### Experiment #2

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![Bar chart showing Mean $LT_{50}$ (h) for Experiments #1 and #2](chart.png)

- **Wild-type**
- **$aff-7(qd22)$**
- **$aff-7(qd22 \ qd130)$**
Figure S11. Replicate of pathogenesis assay shown in Figures S3B and S3C. Chart and bar graphs showing the LT$_{50}$ means, LT$_{50}$ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms treated with control RNAi and *atf-7* RNAi, and *atf-7(qd22)* mutant animals treated with control RNAi and *atf-7* RNAi.
Figure S11

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Figure S12. Replicate of pathogenesis assay shown in Figure S4. Chart and bar graphs showing the LT$_{50}$ means, LT$_{50}$ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms; *atf-7(qd22)* and *atf-7(qd22 q130)* mutant animals; and *atf-7(qd22)/atf-7(+), atf-7(qd22 q130)/atf-7(+), and atf-7(qd22)/atf-7(qd22 q130)* trans-heterozygotes.
### Experiment #1

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<td>atf-7(qd22 qd130)/atf-7(+)</td>
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<tr>
<td>atf-7(qd22)/atf-7(qd22 qd130)</td>
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### Experiment #2

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Figure S13. Replicate of pathogenesis assay shown in Figure 1D. Chart and bar graphs showing the LT₅₀ means, LT₅₀ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms; *atf-7(qd22 q130)* and *atf-7(qd137)* mutant animals; and *atf-7(qd22 qd130)/atf-7(+), atf-7(qd137)/atf-7(+),* and *atf-7(qd22 qd130)/atf-7(qd137)* trans-heterozygotes.
### Figure S13

**Experiment #1**

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**Experiment #2**

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Figure S14. Replicate of pathogenesis assay shown in Figure 3B. Chart and bar graphs showing the LT$_{50}$ means, LT$_{50}$ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms; *atf-7(qd22 q130)* and *pmk-1(km25)* mutant animals; and *atf-7(qd22 qd130); pmk-1(km25)* double mutant animals.
Figure S14

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**Figure S15. Replicate of pathogenesis assay shown in Figure 3D.** Chart and bar graphs showing the LT$_{50}$ means, LT$_{50}$ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms; *atf-7(qd22 q130)* and *sek-1(km4)* mutant animals; and *atf-7(qd22 qd130); sek-1(km4)* double mutant animals.
Figure S15

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Figure S16. Replicate of lifespan assay shown in Figures S1 and S6. Chart and bar graphs showing the LT50 means, LT50 standard deviations (S.D.), and sample sizes from two independent lifespan assays with wild-type worms, *atf-7(qd22), atf-7(qd22 q130)*, and *atf-7(qd137)* mutant animals.
### Experiment #1

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### Experiment #2

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<td>$atf-7(qd137)$</td>
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**Figure S16**

![Bar chart showing Mean $LT_{50}$ (d) for Wild-type, $atf-7(qd22)$, $atf-7(qd22 \ qd130)$, and $atf-7(qd137)$ across Experiment #1 and #2.](chart.png)

- **Wild-type**
- **$atf-7(qd22)$**
- **$atf-7(qd22 \ qd130)$**
- **$atf-7(qd137)$**
Supplementary Tables

Table S1. Sample sizes for each pathogenesis, arsenite stress, and lifespan assay. Chart showing the sample sizes for each pathogenesis, arsenite stress, and lifespan assay presented in this work. Sample size does not include censored worms.
<table>
<thead>
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<th>Genotype</th>
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Acknowledgments

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References


Chapter Three

The miR-58 Family Restricts Expression of a C. elegans Operonic p38 MAPK to Neurons

Daniel J. Pagano, Elena R. Kingston, and Dennis H. Kim

Conceived and designed the experiments: DJP DHK.
Performed the experiments: DJP ERK DHK.
Analyzed the data: DJP.
Wrote the paper: DJP DHK.

Manuscript submitted for publication
Summary

An operon is a cluster of two or more genes that are transcribed into a single polycistronic mRNA. Bacterial genes functioning in a particular physiological process are transcriptionally coregulated in operons. Operonic gene organization has also been found in a number of eukaryotic species, including trypanosomes, parasitic and free-living nematodes, and ascidians, where in contrast to bacteria, genes in an operon need not share similarity in function, and the polycistronic pre-mRNA undergoes processing involving trans-splicing to generate monocistronic mRNAs. How co-operonic genes in eukaryotes exhibit variation in expression, and in particular, if and how co-transcribed genes are expressed in distinct tissues of multicellular organisms is not understood. Here, we report the mechanism underlying the different tissue expression of two paralogous genes, pmk-1 and pmk-2, each of which encodes a p38 mitogen-activated protein kinase (MAPK) in a C. elegans operon. PMK-1 functions in the intestine to regulate innate immunity, whereas we show that PMK-1 and PMK-2 function redundantly in the nervous system to regulate neuronal development and behavioral responses to pathogenic bacteria. We demonstrate that this tissue-specific genetic redundancy between pmk-1 and pmk-2 is a consequence of the restriction of pmk-2 expression to the nervous system through the redundant activities of miR-58, miR-80, miR-81, and miR-82 microRNAs, which function to destabilize the trans-spliced pmk-2 mRNA transcribed from the operon promoter in non-neuronal cells. Our data establish a role for microRNAs in directing the distinct tissue expression of genes in an operon, which may facilitate the evolution and maintenance of operonic organization in diverse multicellular organisms. Moreover, taken together with prior reports of the abundance and constitutive expression of mir-58 (approximately 50% of microRNAs in C. elegans) and enrichment for predicted binding sites for miR-58 in neuronally-expressed genes, our findings
may reflect to a more general role for the miR-58/80-82 family of microRNAs in restricting the expression of genes to neurons of *C. elegans*.
Results and Discussion

Three *C. elegans* genes with homology to mammalian p38 MAPK—*pmk-1, pmk-2, and pmk-3*—are in a single operon along with an additional upstream gene, *islo-1*, encoding a putative transmembrane protein that is required for the proper localization of the BK channel SLO-1 (Fig. 1a). PMK-1 and PMK-2 are highly homologous, sharing a 62% amino acid sequence identity and have the signature TGY motif found in the activation loop of p38 MAPKs. PMK-1 regulates innate immunity in the intestine of *C. elegans* and is activated by a MAPK signaling cassette comprised of p38 MAPK kinase SEK-1 and the MAPKKK NSY-1, homologous to mammalian MKK3/6 and ASK1, respectively. Functioning upstream of NSY-1 and required for activation of PMK-1 in *C. elegans* is TIR-1, a conserved Toll-Interleukin-1 Receptor domain adaptor protein orthologous to mammalian SARM. TIR-1-NSY-1-SEK-1 functions in the nervous system to regulate the specification of neuronal asymmetry in the AWC neuron pair, reproductive egg laying behavior, and the upregulation of serotonin biosynthesis in the ADF chemosensory neurons in response to infection by *Pseudomonas aeruginosa*, but the MAPK targeted in the nervous system for these processes had not been defined, with *pmk-1* loss-of-function not affecting these neuronal phenotypes.

We used mutant alleles of *pmk-1* and *pmk-2* to confirm that PMK-1 alone is required for expression of an intestinal reporter for p38 MAPK activity and innate immunity to infection by *P. aeruginosa* in the intestine (Fig. 1b, c, and Fig. S1). To evaluate the roles of PMK-1 and PMK-2 in mediating the activities of the TIR-1-NSY-1-SEK-1 signaling module in the nervous system, we utilized two assays of neuronal signaling processes that are dependent on TIR-1-NSY-1-SEK-1. First, the establishment of asymmetry in the AWC neurons during development is a stochastic process for which expression of a transgenic reporter, *str-2::GFP*, in
Figure 1. Co-operonic *pmk-1* and *pmk-2* exhibit genetic redundancy in the nervous system but not the intestine. a) The *pmk* operon showing mutations utilized and isolated in this study. Gray fill, corresponding unspliced transcript; white fill, corresponding 5' and 3' UTRs. *pmk-2* mutations: *qd284*, 10 bp deletion, frameshift; *qd287*, 7 bp insertion, frameshift; *qd279* and *qd280*, as indicated in reference to isoform *pmk-2b*; *qdl71*, 913/184 bp insertion/deletion. b-e) Phenotypic analysis of mutants deficient in p38 MAPK signaling. b) Bright field and fluorescence microscopy images of 1-day-old adult worms carrying the *agIs219[P_{T24B8.5}::GFP]* transgene. c) Pathogenesis assay of L4 larval stage worms on *P. aeruginosa* PA14. All strains carry the *agIs219[P_{T24B8.5}::GFP]* transgene. d) Expression of *str-2::GFP* in the AWC olfactory neurons of L3 and L4 larval stage and young adult worms. e) Quantification of GFP expression from the *nls145[P_{pho-1}::GFP]* transgene in 1-day-old adult worms after a 6 hr exposure to *E. coli* OP50 or *P. aeruginosa* PA14. Shown is a representative experiment. Error bars, ± standard deviation. (n.s. not significant, *** P<0.001, two-way ANOVA with Bonferroni post-test).
Figure 1

**a**

Promoter

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<th>pmk-3</th>
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q274 f d279 (H105Y)

q280 (R211K)

q171

km25

**b**

Wild type

sek-1(km4)

pmk-1(km25)

pmk-2(qd284)

pmk-2(qd287)

pmk-2(qd279 qd171) pmk-1(km25)

pmk-2(qd280 qd171) pmk-1(km25)

**c**

Fraction alive

Time (h)

**d**

<table>
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<th>Genotype</th>
<th>Percentage of animals</th>
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<th>2 AWC&lt;sup&gt;ON&lt;/sup&gt;</th>
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**e**

P<sub>pmk</sub>:CFP fluorescence (AU)

E. coli

P. aeruginosa

n.s.
one AWC neuron or the other serves as a readout\textsuperscript{15}. TIR-1-NSY-1-SEK-1 signaling represses the expression of \textit{str-2::GFP} in one AWC neuron such that in a wild type animal, only one AWC neuron expresses \textit{str-2::GFP}. Strains carrying loss-of-function mutations in the TIR-1-NSY-1-SEK-1 signaling module express \textit{str-2::GFP} in both AWC neurons\textsuperscript{13-15}. We observed expression of \textit{str-2::GFP} in only one AWC neuron in \textit{pmk-1} and \textit{pmk-2} loss-of-function single mutants (Fig. 1d). In contrast, using two strains carrying loss-of-function mutations in both genes—\textit{pmk-2(qd279 qd171) pmk-1(km25)} and \textit{pmk-2(qd280 qd171) pmk-1(km25)}—we observed expression of \textit{str-2::GFP} in both AWC neurons, similar to what is observed in the \textit{sek-1(km4)} mutant (Fig. 1d). A second process that is dependent on TIR-1-NSY-1-SEK-1 activity in the nervous system is the increased expression the serotonin biosynthetic enzyme TPH-1 in the ADF chemosensory neurons in response to pathogenic \textit{P. aeruginosa}\textsuperscript{10,16}. Upregulation of serotonin levels in the ADF neuron pair has been implicated in aversive learning behavior to pathogenic bacteria\textsuperscript{16}. We observed that PMK-1 and PMK-2 also function redundantly in the \textit{P. aeruginosa}-induced expression of a \textit{P_{ph-1}:GFP} transgene reporter in the ADF neurons (Fig. 1e). These data establish that PMK-1 alone is required to regulate intestinal innate immunity, but that PMK-1 and PMK-2 function redundantly in the nervous system downstream of the TIR-1-NSY-SEK-1 signaling module in neuronal developmental and pathogen-dependent responses (Fig. S1).

We reasoned that the genetic redundancy of \textit{pmk-1} and \textit{pmk-2} in neurons, but not in the intestine, might be the result of differences in tissue expression of these genes. We proceeded to examine the tissue-expression patterns for PMK-1 and PMK-2 by constructing a translational reporter for the \textit{pmk} operon consisting of upstream promoter sequence and the entire length of the operon with a GFP tag engineered onto the C-terminus of PMK-2 and a mCherry tag engineered onto the C-terminus of PMK-1 (Fig. 2a). Expression of PMK-1 was observed in the
intestine, hypodermis, muscle, and nervous system of the worm, whereas expression of PMK-2 was restricted to the nervous system (Fig. 2b). These data suggest that the tissue-specific genetic redundancy of p38 MAPK signaling in C. elegans is a consequence of distinct tissue expression patterns of the co-transcribed operonic pmk-1 and pmk-2 genes.

Insight into the post-transcriptional regulatory mechanism underlying the restricted tissue expression of PMK-2 came from a genetic screen for suppressors of the immunocompromised phenotype of a pmk-1 deletion mutant\(^{17}\), in which we serendipitously isolated a gain-of-function mutant of pmk-2, qd171, containing a 913 bp insertion/184 bp deletion located in the 3' untranslated region (UTR) of pmk-2 (Fig. 1a). The starting strain for the screen carried the km25 deletion allele of pmk-1 (Fig. 1a) and the agIs219 integrated GFP transgene that serves as a reporter of p38 MAPK activity in the intestine\(^{10,17}\) (Fig. 3a). The pmk-2(qd171) deletion suppressed the diminished intestinal GFP expression from the agIs219 reporter transgene (Fig. 3a) and the enhanced pathogen susceptibility of the pmk-1(km25) mutant (Fig. 3b). RNAi of sek-1 and RNAi of pmk-2, reverted the pmk-2(qd171) pmk-1(km25) pathogen resistance and agIs219 intestinal GFP expression phenotypes associated with suppression of pmk-1 loss-of-function (Fig. 3a, b). These data suggest that the ability of the pmk-2(qd171) mutation to suppress pmk-1 loss-of-function is dependent on PMK-2.

Because the location of the pmk-2(qd171) insertion/deletion in the 3'UTR of pmk-2 might be anticipated to influence mRNA stability, we proceeded to measure levels of pmk-2 mRNA in the pmk-2(qd171) pmk-1(km25) mutant. We detected a 5.7-fold increase in pmk-2 mRNA levels in the pmk-2(qd171) pmk-1(km25) mutant compared to wild type worms (Fig. 3c). The effect on pmk-2 mRNA levels is not due to an increase in transcription of the pmk operon containing pmk-2, as pmk-3 mRNA levels did not change in the pmk-2(qd171) pmk-1(km25) mutant relative to
Figure 2. Co-transcribed pmk-1 and pmk-2 exhibit different tissue expression patterns. a) The pmk operon translational reporter. Green fluorescent protein was engineered onto the C-terminal end of PMK-2. The red fluorescent protein mCherry was engineered onto the C-terminal end of PMK-1. b, c) Fluorescence and differential interference contrast (DIC) microscopy of a representative wild type worm carrying the pmk operon translational reporter (b) or a mutated pmk operon translational reporter (c) with specific mutations (indicated in Fig. 4a) engineered into the second and third miR-58 family seed match sites in the 3’UTR of pmk-2. Scale bars, 25 µm.
Figure 2

a

Promoter [iso-1] pmk-3 - pmk-2 - GFP - pmk-1 - RFP

b

qdEx86

[P::Isso-1::pmk-3::pmk-2::GFP::pmk-1::mCherry]

DIC

PMK-2::GFP

PMK-1::mCherry

Merge

c

qdEx87

[P::Isso-1::pmk-3::pmk-2::GFP::pmk-1::mCherry]

DIC

PMK-2::mCherry

PMK-1::mCherry

Merge
Figure 3. A deletion in the 3'UTR of pmk-2 confers an increase in PMK-2 expression that can substitute for PMK-1 activity in the intestine. a, b) Phenotypic analysis of the pmk-2(qd171) pmk-1(km25) mutant. Bright field and fluorescence microscopy images (a) and P. aeruginosa pathogenesis assays (b) of worms treated with RNAi as indicated for two generations. c) qRT-PCR analysis of pmk-2 and pmk-3 mRNA levels in L4 larval stage animals. Levels of pmk-2 and pmk-3 mRNA are normalized to the levels of snb-1 mRNA. Values plotted are the fold changes relative to wild type. Show is the mean ± SEM (n = 4 independent biological replicates, *** P<0.001, two-way ANOVA with Bonferroni post-test). d) Immunoblot analysis of lysates from RNAi-treated mixed stage animals using an antibody recognizing the doubly phosphorylated TGY motif of activated PMK-1 and PMK-2 p38 MAPKs and an antibody that recognizes β-tubulin. a-d) All stains carry the agls219[P_j2488.5::GFP] transgene.
RNAi Gonotype

---

pmk-l (kmm25) pmk-2(qdl71) pmk-1(km25)

pmk-2 pmk-2(qd171) pmk-1(km25)

sek-1 pmk-2(qd171) pmk-1(km25)

25 50 75 100

Tirm(h)

Figure 3

a

RNA: pffnk-2 sek-i

phospho-PMK-2 phospho-PMK-1

B-tubulin

b

RNAi Genotype

- pmk-1(km25)
- pmk-2(qd171) pmk-1(km25)
- pmk-2 pmk-2(qd171) pmk-1(km25)
- sek-1 pmk-2(qd171) pmk-1(km25)

Fraction alive

0.0 0.5 1.0

Time (h)

0 25 50 75 100

c

Relative mRNA levels

Wild type pmk-1(km25) pmk-2(qd171) pmk-1(km25)

***

0 2 4 6

d

RNAI:

phospho-PMK-2 phospho-PMK-1

β-tubulin
wild type. We next determined levels of activated PMK-2 protein in the pmk-2(qd171) pmk-1(km25) mutant using an antibody that recognizes the dually phosphorylated TGY motif in the activation domain of activated mammalian p38 MAPK and that is cross-reactive with C. elegans PMK-1 and PMK-2. Corroborating the increase in mRNA levels, we detected at least a 5.7-fold increase in activated PMK-2 protein levels in the pmk-2(qd171) pmk-1(km25) mutant (Fig. 3d). Taken together, these data suggest that a deletion in the 3'UTR of the gene encoding PMK-2 p38 MAPK can suppress the immunodeficient phenotype conferred by loss-of-function of pmk-1 through increased stability of pmk-2 mRNA and levels of activated PMK-2 protein.

We next sought to determine the critical cis-regulatory features of the pmk-2 3'UTR that function to repress PMK-2 expression. We performed 3' RACE on pmk-2 mRNA isolated from wild type and pmk-2(qd171) pmk-1(km25) mutant animals to determine the effect of the pmk-2(qd171) mutation on the length of the 3'UTR of pmk-2 mRNA. Sequencing of the 3' RACE products revealed a 206 bp truncation in the 3'UTR of pmk-2 mRNA in the pmk-2(qd171) pmk-1(km25) mutant (Fig. 4a). We examined the truncated region for cis-regulatory elements conserved among Caenorhabditis species and identified four seed match sites for the miR-58/80-82 family of microRNAs, three of which are conserved among other Caenorhabditis species and absent from the 3'UTR of pmk-2 in the pmk-2(qd171) pmk-1(km25) mutant (Fig. 4a).

The miR-58/80-82 family consists of miR-58, miR-80, miR-81, miR-82, and miR-1834. Mutants carrying deletions in mir-58, mir-80, and mir-81-82, were used to assess whether the miR-58/80-82 family of microRNAs functions to repress the expression of pmk-2. Loss of any individual mir-58/80-82 family member had no effect on pmk-2 mRNA levels relative to wild type (Fig. 4b). However, loss of both mir-58 and mir-80 resulted in a 3-fold increase in pmk-2 mRNA levels relative to wild type, and loss of mir-58/80-82 led to an even further increase in
pmk-2 mRNA to a level 6.3-fold greater than wild type (Fig. 4b), without altering levels of pmk-3 mRNA that served as a measure of transcription of the pmk operon. Corroborating the mRNA analysis, we observed at least similar increases in activated PMK-2 protein levels in both mir-80; mir-58 and mir-80; mir-58; mir-81-82 mutants, but not in any single mutant or other combinations of mutants of mir-58/80-82 family microRNAs (Fig. 4d). These data suggest that the miR-58/80-82 microRNA family acts redundantly to repress the expression of PMK-2.

The observation that the pmk-2(qdl71) mutation suppresses the loss of pmk-1 function in the intestine suggested that not only are PMK-2 levels increased in the pmk-2(qdl71) pmk-1(km25) mutant, but that the site of expression had also changed. To determine the effect of releasing pmk-2 from regulation by the miR-58/80-82 family, we engineered our pmk operon translational reporter (Fig. 2a) to carry mutations in the second and third miR-58/80-82 seed match sites in the 3'UTR of pmk-2 (Fig. 4a). Expression of PMK-1 was unchanged, whereas expression of PMK-2 was detected in the intestine and overlapped with PMK-1 expression (Fig. 2c). These data suggest that the miR-58/80-82 family of microRNAs restricts expression of PMK-2 p38 MAPK to the nervous system through the post-transcriptional regulation of pmk-2 mRNA (Fig. 4d).

Genes found in operons of multicellular organisms do not appear to necessarily share similarity in function, and thus co-regulation in expression, temporally and spatially, might be anticipated to be an unfavorable constraint evolutionarily. Internal promoters that direct transcription independently of the operon promoter, giving a so-called “hybrid operon,” may contribute to observations of variation in expression among genes of an operon. Our data suggest a role for microRNAs in the establishment and maintenance of distinct patterns of tissue expression of genes of multicellular organisms that have evolved operonic genome organization,
Figure 4. The miR-58/80-82 family of microRNAs functions redundantly to restrict expression of PMK-2 to the nervous system. a) A schematic of the 3’UTR of pmk-2 mRNA in wild type and pmk-2(qd171) pmk-1(km25) mutant animals as determined by 3’ RACE analysis. Gray fill, exon; white fill, 3’UTR. Conservation of sequence among Caenorhabditis species by PhastCons in shown. miR-58/80-82 seed match sites are annotated. Vertical line, gap in alignment > 5 bp. Mutations engineered into the pmk operon translational reporter (Fig. 2c) are indicated. PAS, polyadenylation site. b) qRT-PCR analysis of pmk-2 and pmk-3 mRNA levels in L4 larval stage wild type worms and mir-58/80-82 family mutants. Levels of pmk-2 and pmk-3 mRNA are normalized to the levels of snb-1 mRNA. Values plotted are the fold changes relative to wild type. Show is the mean ± SEM (n = 3 independent biological replicates, *** P<0.001, two-way ANOVA with Bonferroni post-test). c) Immunoblot analysis of lysates from L4 larval stage wild type worms and mir-58/80-82 family mutants using antibodies that recognize activated p38 MAPK and β-tubulin. d) A model for the function of the miR-58/80-82 family of microRNAs in defining the tissue-specific expression of operonic PMK-2 p38 MAPK.
**Figure 4**

**a**

![Diagram showing seed match and site type](image)

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<thead>
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<th>Site type (miR-58)</th>
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Engineered mutations:
- C. elegans
- C. briggsae
- C. remani
- C. brenneri
- C. japonica

**b**

![Bar graph showing relative mRNA levels](image)

**c**

![Western blots showing phosphorylated PMK-2 and PMK-1](image)

**d**

![Schematic showing transcription and translation](image)
acting where trans-splicing mechanisms have generated monocistronic mRNAs that can be regulated independently.

A functional role for microRNAs in establishing specific cell type expression was demonstrated by the identification and characterization of the *lsy-6* microRNA, which regulates the development of left/right asymmetry through the specific repression of its target in one of two ASE chemosensory neurons. In contrast to the highly selective expression and function of the *lsy-6* microRNA in regulating neuronal patterning, the miR-58 family functions in a large number of cells to restrict expression of its target *pmk-2* in non-neuronal tissues. The miR-58 microRNA is the most abundant microRNA in *C. elegans*, equal to over 50% of the total microRNAs, and is present at all developmental stages. The role of the miR-58/80-82 family in targeting *pmk-2* mRNA in the intestine is consistent with the reported anatomical expression pattern of *mir-58* in the intestine. Both the abundance and reported tissue expression pattern of *mir-58*, as well as the presence of multiple miR-58 binding sites in the 3'UTR of *pmk-2*, may contribute to the large increase of *pmk-2* mRNA and protein levels when miR-58 family regulation is inhibited. The approximately six-fold difference we observe in *pmk-2* mRNA levels and comparable difference in PMK-2 protein levels reflect quantitation from whole worm lysates in which PMK-2 is detectably expressed in the nervous system. Thus, the magnitude of PMK-2 repression in the intestine is likely greater than this change would indicate. The switch-like “off” state of PMK-2 expression imposed by the miR-58/80-82 family in the intestine in regulating the spatial expression of PMK-2 is reminiscent of the magnitude of target repression exhibited by *lin-4* and *let-7* microRNAs in the temporal control of developmental timing, with the notable difference that *mir-58* expression appears to be constitutive. The dramatic decrease in *pmk-2* mRNA from miR-58/80-82 regulation is particularly notable in the context of the genome-wide
analysis of tissue-specific gene expression in *C. elegans*, which revealed enrichment for miR-58 binding sites among neuronal genes\(^9\).

Our data, in the context of these additional considerations, suggest a more general role for the miR-58 family in the maintenance of nervous system-specific expression for some neuronally expressed genes. Paralleling the relative abundance of miR-58 and its role in the tissue-specific regulation of gene expression, the characterization of microRNAs expressed in specific tissues of mice revealed the presence of a single microRNA and/or microRNA family in high abundance\(^27\), raising the possibility that such microRNAs might function to establish tissue expression patterns. Our data on the regulation of *pmk-2* tissue expression by the miR-58 family, defined in the context of operonic gene expression that excludes the possibility of transcriptional mechanisms conferring tissue-specific expression, provide strong experimental support for this hypothesis.

**Materials and Methods**

**Strains.** All *C. elegans* strains were maintained and propagated on *E. coli* OP50 as described previously\(^30\). N2 was the wild-type strain. The following mutations were used in this study:

LGI: kyIs140[\textit{str-2::GFP, lin-15(+)}]

LGIII: agls219I[\textit{P}_{12,488.5}::GFP, \textit{P}_{\text{lin-3}}::GFP}, \textit{tir-1(qd4)}, \textit{mir-80(nDf53)}

LGIV: \textit{mir-58(n4640), pmk-2(qd284), pmk-2(qd287), pmk-2(qd279), pmk-2(qd280), pmk-2(qd171), pmk-1(km25)}

LCX: \textit{mir-81(nDf54), mir-82(nDf54), sek-1(km4), nIs145[P_{ph-1::GFP, lin-15(+)}]}

Extrachromosomal arrays: \textit{qdEx86[P_{operon::islo-1::pmk-3::pmk-2::GFP::pmk-1::mCherry}], qdEx87[P_{operon::islo-1::pmk-3::pmk-2::GFP::pmk-1::mCherry]}
A list of all strains used in this study is provided in Table S1.

**Pathogenesis assays.** Cultures of *P. aeruginosa* PA14 were grown in Luria-Bertani (LB) broth overnight at 37°C. Five microliters of the overnight culture was used to seed 35-mm slow-kill assay plates containing 0.05 mg/ml 5-fluorodeoxyuridine (FUDR), used to prevent eggs from hatching. The culture was seeded in the middle of the plates and was not spread to the edges, meaning the resulting lawn would be “small.” The seeded plates were incubated overnight at 37°C and then overnight at room temperature. Roughly 40 L4 larval stage worms were placed onto a plate containing the prepared *P. aeruginosa* bacterial lawn with four plates per strain. The assay was carried out at 25°C. Plates were checked at the indicated times and worms that did not respond to a gentle prod from a platinum wire were scored as dead. Worms that crawled off of the plate or burrowed were censored.

**Ptpo-1::GFP imaging and quantification of GFP fluorescence.** Adult worms (specifically, 12-16 hr post L4 larval stage at 25°C) were transferred to plates containing either a lawn of non-pathogenic *E. coli* OP50 or pathogenic *P. aeruginosa* PA14 and incubated at 25°C for 6 hr at which time the worms were immobilized with 50 mM sodium azide and mounted on 2% agarose pads. Immobilized worms were viewed using an AxioImager Z1 fluorescence microscope (Zeiss) with an EC Plan-Neofluar 40x/1.3 Oil DIC objective and the focal plane with the strongest GFP signal in the ADF neuron was used to take a 30 ms exposure picture with an AxioCam HRm camera. The images were analyzed in Fiji, where the ADF neuron was located and the pixel intensity values were examined. The maximum pixel intensity value in the ADF neuron was used as the *Ptpo-1::GFP* fluorescence value for each worm. In each experiment, 7-10 worms of the indicated genotype were imaged for each condition (OP50, PA14). For the experiment shown in Figure 1e, one outlier was identified (in the *tir-1(qd4)* mutant, *P. aeruginosa* exposure dataset).
using Grubbs’ test and excluded from the graph and analysis. Statistical analyses of changes in fluorescence were performed in Prism 5 (GraphPad) using a two-way ANOVA and Bonferroni post-test.

**Isolation of pmk-2(qd284) and pmk-2(qd287).** The qd284 and qd287 alleles of pmk-2 were isolated by CRISPR-Cas9-mediated genome editing as described previously. Two separate pmk-2 sgRNA expression vectors derived from pUC57 were constructed following the published protocol. Both sequences targeting the designed pmk-2 sgRNAs were located in what corresponds to the first exon of the pmk-2 transcript and overlap with restriction enzyme recognition sites facilitating the screening for mutations. Germline transformation was performed as described previously using the following plasmids: 50 ng/μl P_{ef-3}::Cas9-SV40 NLS::tbb-2 3’UTR, 45 ng/μl P_{U6}::pmk-2 sgRNA, 5 ng/μl pCFJ104[P_{myo-3}::mCherry]. Screening for mutations was performed as outlined.

**Isolation of pmk-2(qd171).** The qd171 allele of pmk-2 was isolated from a screen for suppressors of the enhanced susceptibility to pathogen (Esp) conferred by pmk-1 loss-of-function. pmk-1(km25) mutant L4 larvae carrying the agIs219 reporter transgene for p38 activity in the intestine were mutagenized with ethyl methanesulfonate (EMS) as described previously. Synchronized F2 progeny from the mutagenized animals were screened for an increase in expression of GFP from the agIs219 reporter transgene using a dissecting microscope equipped to detect GFP fluorescence. Mutants with increased expression of GFP were clonally, secondarily screened for the ability to suppress the Esp conferred by loss of pmk-1 function. Mutants that suppressed the Esp conferred by loss of pmk-1 function were then subsequently screened for suppression of the diminished expression of pmk-1 target genes conferred by loss of pmk-1 function. Single-nucleotide polymorphism (SNP)-based mapping using the *C. elegans*
isolate CB4856 was performed as described previously\textsuperscript{34,35}. One of the suppressors from this screen, \textit{qd171}, mapped to a region on LGIV containing the \textit{pmk} operon. Sequence determination of \textit{pmk-1} and \textit{pmk-2} revealed that \textit{qd171} was an allele of \textit{pmk-2}.

**Isolation of \textit{pmk-2(qd279 qd171)} and \textit{pmk-2(qd280 qd171)}.** The \textit{qd279} and \textit{qd280} alleles of \textit{pmk-2} were isolated from a screen for suppressors of the \textit{pmk-2(qd171)} suppressor phenotype of \textit{pmk-1} loss-of-function. \textit{pmk-2(qd171) pmk-1(km25)} mutant L4 larvae carrying the \textit{agls219} reporter transgene for p38 activity in the intestine were mutagenized with EMS as described previously\textsuperscript{33}. Synchronized F2 progeny from the mutagenized animals were screened for a decrease in expression of GFP from the \textit{agls219} reporter transgene using a dissecting microscope equipped to detect GFP fluorescence. The sequence of \textit{pmk-2} was determined in mutants with diminished GFP expression from the \textit{agls219} transgene. We identified three alleles of \textit{pmk-2}: \textit{qd279} and \textit{qd280} (which are missense mutations in conserved residues and were utilized in this study), as well as \textit{qd281}.

**RNA isolation, 3' RACE, and quantitative RT-PCR.** Hypochlorite-synchronized populations of L4 larval stage worms were flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction using TRI reagent (Ambion). Strain MT15563 carrying mutations in \textit{mir-58,80-82} grew slower than the other strains and therefore was harvested \textasciitilde10 hr after the wild type strain. For 3' RACE experiments, cDNA was prepared using the FirstChoice® RLM-RACE Kit (Ambion). Two successive rounds of PCR were performed using nested primers specific to \textit{pmk-2} and an adaptor at the 3' end. Sequence determination was performed by direct Sanger sequencing of 3' RACE products. For quantitative RT-PCR experiments, cDNA was prepared with the RETROscript® Kit (Ambion) using oligo dT primers. qRT-PCR was performed with a Mastercycler Realplex (Eppendorf) with SYBR Green detection (Roche) in triplicate 20 µl.
reactions. pmk-2 and pmk-3 mRNA levels were normalized to the control gene snb-1. Fold change relative to wild type was determined using the Pfaffl method\textsuperscript{36}. Primer sequences are available in Table S2.

**Immunoblotting.** For the experiments with pmk-1(km25), pmk-2(qd171) pmk-1(km25), and pmk-2(qd279 qd171) pmk-1(km25) strains with RNAi treatment (Figure 3d), mixed stage populations of worms subjected to RNAi for multiple generations were used for Western analysis. For experiments with mir-58/80-82 family microRNA deletion strains (Figure 4c), hypochlorite-synchronized populations of L4 larval worms were used for Western analysis. Strain MT15563 carrying mutations in mir-58,80-82 grew slower than the other strains and therefore was harvested \textasciitilde 10 hr after the wild type strain. Worms were collected, washed twice with M9, incubated in M9 while rotating at 20°C to clear the gut of bacteria, washed again twice with M9, and then pelleted. An equal volume of 2x lysis buffer (4% SDS, 100mM Tris HCl pH 6.8, and 20% glycerol) was added to the worm pellets, which were then boiled for 15 minutes with occasional vortexing and then centrifuged to pellet the debris. The protein concentration of the lysates (supernatant from the previous step) was determined using the BCA Protein Assay Kit (Pierce). For each sample, 50 \( \mu \)g of total protein was separated on a 10% SDS-PAGE gel (Bio-rad) and then transferred to a nitrocellulose membrane (GE Healthcare). Blots were blocked with TBST supplemented with 5% skim milk power and then probed with either a 1:1,000 dilution of rabbit anti-ACTIVE p38 MAPK pAb (Promega), which recognizes the dually phosphorylated TGY motif of activated p38 MAPK, or a 1:10,000 dilution of mouse anti-\( \beta \)-tubulin (E7 Developmental Hybridoma Bank, Iowa City) in TBST with 5% skim milk powder. Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG secondary antibodies
(Cell Signaling Technology) were used followed by detection with ECL reagents (GE Healthcare).

**RNA interference.** Feeding RNAi was performed as previously described\(^{37}\). NGM agar plates supplemented with 2 mM isopropylthio-β-galactoside (IPTG) and 25 μg/ml of carbenicillin were seeded with *E. coli* HT155 bacterial cultures carrying the control plasmid pL4440 or carrying specific plasmids derived from pL4440 designed to target either *sek-1* or *pmk-2* for RNAi. For pathogenesis assays and visualization of GFP expression from the *agls219* reporter, 3-6 L4 larval stage worms were placed onto seeded RNAi plates and their progeny were assayed. For Western analysis, ~10 L4 larval stage worms were placed onto seeded RNAi plates and the worms were harvested before being deprived of food.

**Plasmids.** The *pmk* operon translational reporter was constructed through ligation of overlapping PCR amplicons by homologous recombination in *Saccharomyces cerevisiae* strain FY2 as previously described\(^{38}\). A ~20.3 kb region containing the *pmk* operon and ~4.4 kb of upstream sequence was amplified from fosmid 27cB07 in adjacent fragments with at least 50 bp of overlap between fragments. For the *pmk* operon translational reporter carrying mutations in the 3'UTR of *pmk-2*, 90 bp reverse complement primers containing the desired mutations were designed and used to amplify the 3'UTR of *pmk-2*. The gene encoding GFP was amplified from pPD95.75 (Addgene). The gene encoding mCherry was amplified from pCFJ90 (Addgene). The PCR amplicons and were transformed into *S. cerevisiae* strain FY2 along with destination vector pNP30 (gift of N. Paquin, pNP30 a pRS426-derived plasmid compatible with MosSCI integration at locus *ttTi5605* on LGII) digested with XhoI and AvrII (New England Biosciences). Yeast DNA was extracted with phenol-chloroform and transformed into DH5-α electrocompetent cells (Protein Express). Plasmids were prepped using a Miniprep Kit (Qiagen).
and their sequence was verified. Germline transformations were performed as described previously\textsuperscript{32}.

**Microscopy.** To visualize expression of GFP from the \textit{agls219} reporter transgene, adult worms (16-24 hr post L4 larval stage at 20°C) were picked over to an unseeded NGM agar plates and immobilized with 50 mM sodium azide. Worms were viewed using a Stereo V12 fluorescence microscope (Zeiss) and pictures were taken with an AxioCam MRC camera. To visualize expression of GFP and mCherry from the \textit{pmk} operon translational reporter, transgenic animals were immobilized with 50 mM sodium azide and mounted on 2\% agarose pads. Worms were viewed using an AxioImager Z1 fluorescence microscope (Zeiss) and pictures were taken with an AxioCam HRm camera. All images were prepared in Photoshop (Adobe).
Supplementary Figures

Figure S1. Neuronal-specific genetic redundancy of p38 MAPK signaling in *C. elegans.*

PMK-1 p38 MAPK functions independently of PMK-2 p38 MAPK in the intestine downstream of the TIR-1-NSY-1-SEK-1 signaling module in the regulation of immune effector gene expression in response to pathogenic microbes. PMK-1 and PMK-2 p38 MAPKs function redundantly in the nervous system downstream of TIR-1-NSY-1-SEK-1 in the regulation of AWC neural asymmetry and pathogen-induced upregulation of *tph-1* expression in the ADF neurons.
Figure S1

INTESTINE

NERVOUS SYSTEM

AWC olfactory neurons

NSY-1 → TIR-1

SEK-1 → PMK-1

Immune response

ADF chemosensory neurons

NSY-1 → TIR-1

SEK-1 → PMK-1

Asymmetric cell fate

PMK-1, PMK-2

Aversive learning
Supplementary Tables

**Table S1.** Strains used in this study.

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Table S2. Sequences of primers used for qRT-PCR

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Acknowledgments

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References


Chapter Four

Additional Studies and Future Directions
Introduction

The discovery of the PMK-1 p38 MAPK-dependent transcriptional regulator ATF-7 gives rise to a number of future directions for the study of host defense in *C. elegans*. ATF-7 adds specificity downstream of PMK-1 p38 MAPK signaling in the intestine, as it appears to be dispensable for protection against oxidative stress, and suggests that the identification of the direct transcriptional targets of ATF-7 will define the transcriptional output of the PMK-1 p38 MAPK-dependent immune response. In addition, the model for ATF-7 function in the immune response of *C. elegans* suggests that ATF-7 acts as a transcriptional repressor of immune effector genes, until phosphorylation by PMK-1 p38 MAPK, at which time ATF-7 switches from a repressor to a transcriptional activator of immune effector genes. This switch-like model of function appears to have been conserved, as the yeast Hog1 MAPK-dependent Sko1 transcriptional regulator also undergoes a switch from repressor to transcriptional activator. Identification of additional mediators of the PMK-1 p38 MAPK-dependent transcriptional immune response may provide insight into this conserved mode of function. The additional studies presented here summarize the results of a genetic approach to identify mediators of the PMK-1 p38 MAPK immune response.

The identification of mir-58/80-82 function in the regulation of the tissue-specific expression of operonic PMK-2 p38 MAPK has revealed a novel role for microRNA-mediated gene regulation. The future direction of this work will focus on determining the role of microRNAs in the global regulation of tissue-specific expression of co-operonic genes.
Additional studies

A screen to identify mediators of PMK-1 p38-dependent immunity

Other factors likely cooperate with ATF-7 to regulate the expression of immune effector genes, as the requirement for corepressors and chromatin-remolding complexes in the Sko1-mediated regulation of transcription in the osmotic stress response of yeast have been identified. To identify additional proteins with function downstream of PMK-1 in the regulation of immune effector gene expression, I performed a large-scale EMS-based screen for suppressors of the pmk-1 immunocompromised phenotype as outlined in the Material and Methods section of Chapter Three. In addition to isolating additional loss-of-function alleles of atf-7, I anticipated this screen would identify novel components required for the proper function of ATF-7 and others factors that may function downstream of PMK-1 independently of ATF-7.

I isolated 47 suppressors of the pmk-1 immunocompromised phenotype. Only three of the pmk-1 suppressors were specific to the PMK-1 p38 MAPK-dependent immune response. Mutant qd171 formed the foundation for the work presented in Chapter Three. Mutants qd153 and qd146 carry mutations in atf-7. qd146 is a particularly interesting allele of atf-7 that confers a semi-dominant suppression of the pmk-1 immunocompromised phenotype. The qd146 allele of atf-7 carries a deletion that likely results in a truncated protein lacking the bZIP domain, which is critical for dimerization and DNA binding. Although unlikely, it is possible that there exist domains outside of the bZIP domain that are required for dimerization. If true, the qd146 allele of atf-7 would work in a dominant negative fashion by encoding a truncated protein that binds wild type ATF-7 protein, leading to a nonfunctional dimer and the semi-dominant phenotype observed in the qd146 mutant. Perhaps more likely is the existence of domains or critical residues outside of the bZIP domain in ATF-7 that are important for interactions with other
proteins required for the repressor activity of ATF-7. The truncated ATF-7 protein may bind to these proteins, forming nonfunctional repressor complexes and leading to the semi-dominant phenotype observed in the qd146 mutant. This unique allele of atf-7 sets a framework for the study of the switch-like mechanism of ATF-7 activity, much like the qd171 allele of pmk-2 set the framework for the discovery of the mir-58/80-82 family of microRNAs in the tissue-specific regulation the PMK-2 p38 MAPK expression.

The remaining suppressors of the pmk-1 immunocompromised phenotype appear to function independently of PMK-1 p38 MAPK pathway. These suppressors either specifically regulate the expression of the agls219 reporter transgene or the endogenous T24B8.5 gene, but not other PMK-1 p38 MAPK-dependent genes. In addition, these suppressors have a slow growth pleiotropy that likely accounts for their ability to suppress the enhanced susceptibility to infection conferred by pmk-1 loss-of-function, as these suppressors spend more time in the L4 larval stage, a stage at which susceptibility to infection is not as severe compared to adults\(^2\). A number of these suppressors carry loss- or reduction-of-function mutations in the tatn-1 and hpd-1 genes, which encode tyrosine aminotransferase and 4-hydroxyphenylpyruvate, respectively, and function in the degradation of tyrosine. A general trend in the strength of impairment of tyrosine degradation on overall growth and reproductive physiology, along with lifespan, is observed in these mutants as it appears that a small increase in tyrosine levels may have a hormetic effect on stress physiology and lifespan, whereas large increases in tyrosine levels appear to be toxic to \(C.\) \(elegans\). Further work aimed at understanding the mechanisms underlying these tyrosine-mediated effects may provide insight into the stress physiology and lifespan of \(C.\) \(elegans\).
Future directions

Identify the direct transcriptional targets of ATF-7

Microarray studies on sek-1 loss-of-function mutants have revealed candidate immune effector genes, such as C-type lectins and antimicrobial peptides, as being regulated by the PMK-1 pathway. However, validated transcriptional targets of this pathway that function in pathogen defense have remained largely elusive due to the lack of a known PMK-1-regulated transcription factor that functions in the immune response. The discovery of ATF-7 and its both prominent and specific role in PMK-1-dependent immunity suggests that analysis of ATF-7 transcriptional targets will identify the genes that comprise the immune response. An in-depth transcriptome analysis of the genes negatively regulated by ATF-7 in a pmk-1 mutant background and the genes positively regulated by ATF-7 after exposure to P. aeruginosa in a wild type background will test our switch-like model of ATF-7 function on a genome-wide scale and set the groundwork for subsequent chromatin-immunoprecipitation (ChIP) studies aimed at identifying the direct transcriptional targets of ATF-7. The physiological relevance of these transcriptional targets of ATF-7 can then easily be tested by RNA interference or mutant analysis.

To facilitate ATF-7 ChIP-Seq, I have cloned the operon containing the atf-7 gene, which was engineered to carry a 6xHis::v5 or 3xHSV tag on the C-terminus of ATF-7, along with the upstream operon promoter and placed it into a vector allowing for single-copy insertion into the C. elegans genome. Alternatively, these plasmids can be used to add the sequence encoding the 6xHis::v5 or 3xHSV tag onto the endogenous atf-7 gene using CRISPR-Cas9-mediated genome editing technology. Both v5 and HSV tags have been shown to work well in ChIP experiments in yeast.
Identifying interactors of the TIR-1 – PMK-1 p38 MAPK – ATF-7 pathway

While genetic screens aimed at identifying additional components of PMK-1 p38 MAPK-dependent immunity in *C. elegans* have been successful, such as in the discovery of the downstream transcriptional regulator ATF-7, still little is known about the components that function upstream of the SARM ortholog TIR-1. As suggested elsewhere, this may be the result of many functionally redundant pathways signaling to the TIR-1-NSY-1-SEK-1-PMK-1 p38 MAPK pathway. Another forward genetic screen, designed to identify factors that function with ATF-7 to mediate the transcriptional output of the PMK-1 p38 MAPK-dependent immune response, failed at identifying additional factors with function downstream of PMK-1 p38 MAPK. This result suggests that co-repressors and chromatin remodeling complexes that are likely required for the switch-like function of ATF-7 may be essential. Considering the caveats of these screens, a combined biochemical and reverse genetic approach may be more fruitful than genetic approaches at identifying additional components both upstream and downstream of TIR-1-NSY-1-SEK-1-PMK-1 p38 MAPK in *C. elegans* host defense.

To facilitate co-immunoprecipitation experiments aimed at identifying the interactors of TIR-1, NSY-1, and ATF-7, I have cloned the genes encoding the aforementioned proteins and engineered a 2xStrep::3xFLAG tag onto their C-termini. These plasmids can be used for single-copy insertion into the *C. elegans* genome or as templates for the addition of the tag onto the endogenous gene using CRISPR-Cas9 technology. Following immunoprecipitation, potential interactors can be identified by mass spectrometry analysis and physiological relevance assessed by RNAi-mediated knockdown or mutant analysis.
Investigating the role of microRNAs in the global regulation of operonic gene expression

The regulation of PMK-2 p38 MAPK expression by the miR-58/80-82 family of microRNAs is a clear example of microRNAs regulating the tissue-specific expression of co-operonic genes in an on/off switch-like manner. Perhaps the most interesting question to arise from this work is whether microRNAs regulate the tissue-specific expression of operonic genes on a genome-wide scale. Another future direction might include investigation into whether paralogous genes are more likely to be regulated by microRNAs. A global analysis of trends in microRNA seed match sites in operonic and paralogous genes would be a start to answering these questions.
References


