Immunity in *Caenorhabditis elegans*: A Tale of Two Transcription Factors

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Molecular Genetics and Microbiology in the Graduate School
of Duke University

2009

ABSTRACT

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Abstract

Recently, the study of invertebrate innate immunity has garnered considerable attention after the discovery that mammalian homologues of the *Drosophila melanogaster* Toll pathway play a role in mammalian innate immunity. One invertebrate model system that has begun to be intensely studied is the nematode *Caenorhabditis elegans*. Immunity in *C. elegans* has been shown to be inducible in that it responds uniquely to different pathogens. These changes in gene expression require transcription factors in order for certain genes to be transcribed. We utilized an RNA interference screen of potential transcription factors to identify the GATA transcription factor ELT-2 as a possible transcription factor involved in immunity. We then demonstrated that ELT-2 was required for resistance to a wide range of pathogens and was responsible for regulating expression of the C-type lectin *clec-67*, a marker of immunity.

We also studied another transcription factor known to play a role in *C. elegans* immune function, the FOXO transcription factor DAF-16. We specifically focused in on the role of DAF-16 in germline-deficient mutants, and we demonstrated that such mutants are resistant to many different pathogens. This led to further investigation of the germline-deficient mutant *glp-4*, which should also show broad range resistance to pathogens but fails to do so. Through whole genome sequencing, we identified mutations that may be responsible for the *glp-4* phenotype. We also demonstrated that

DAF-16 was active in *glp-4* mutants, leading to us proposing a model where *glp-4* plays a role in influencing *C. elegans* immunity besides its involvement in germline development.

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1. Introduction

1.1 Infection and Immunity

All organisms are continuously confronted with challenges posed by pathogens. Consequently, all life forms have been forced to develop mechanisms to repel pathogens. The most basic of these defenses are physical barriers (Kurz and Ewbank 2003). However, no physical barrier can be perfect, and thus, multicellular organisms have developed an immune system to eliminate the threat posed by these invading pathogens.

Immune systems can be broken into two major groups: the innate immune system and the adaptive immune system (Janeway, Travers et al. 2001). Vertebrates feature both systems while most other organisms only feature an innate immune system (Mylonakis and Aballay 2005). The adaptive immune system features incredible specificity, and the immunogical memory of the adaptive immune system extends the resistance to the specific pathogen (Alberts, Johnson et al. 2002). These traits of the adaptive immune response have enabled the development of vaccines against specific pathogens, a process whose study first began in earnest with Edward Jenner in the late eighteenth century (Kurz and Ewbank 2003). Perhaps, as a result of these interesting qualities of adaptive immunity, the historical focus of immunologists has been in seeking to understand the adaptive immune system (Kurz and Ewbank 2003).

Innate immunity—in contrast with the delayed response of adaptive immunity—features a rapid, non-specific response to pathogen invasion (Alberts, Johnson et al. 2002). This innate immune response also fails to generate immunogical memory. Relatively recently though, interest in studying innate immunity has increased as a number of factors have come to light. These factors are that, even in vertebrates, the innate immune system plays a critical role in stemming the tide of an infection, often successfully blocking infections in an early stage, and if having failed to fully block the infection, the innate immune response plays a crucial role in the activation and function of the adaptive immune response (Medzhitov and Janeway 1998; Barton and Medzhitov 2002).

1.2 Host-Pathogen Interactions

Once a pathogen has successfully made it past the physical barriers of the host, the host must recognize the pathogen and begin an immune response. In 1989, Charles Janeway proposed a model in which innate immune receptors recognized conserved microbial structures prior to molecular identification of such a system (Janeway 1989). Since then multiple classes of pattern recognition receptors (PRRs) have been identified which recognize a wide variety of pathogen-associated molecular patterns (PAMPs) (Mogensen 2009). Upon recognition of PAMPs, these PRRs induce a number of signaling pathways, which include adaptor molecules, kinases, and transcription factors (Akira and Takeda 2004). These pathways and the transcription factors they activate

result in gene expression changes leading to the production of a wide range of pro-immunity molecules (Akira, Uematsu et al. 2006). So far three major classes of PRRs have been identified (Mogensen 2009). Two of these classes are present in the cytosol: the retinoid acid inducible gene I (RIG-I)-like receptors (RLRs) (Yoneyama, Kikuchi et al. 2004) and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Kanneganti, Lamkanfi et al. 2007). The most well studied class of PRRs though is the membrane-bound Toll-like receptor (TLR) family.

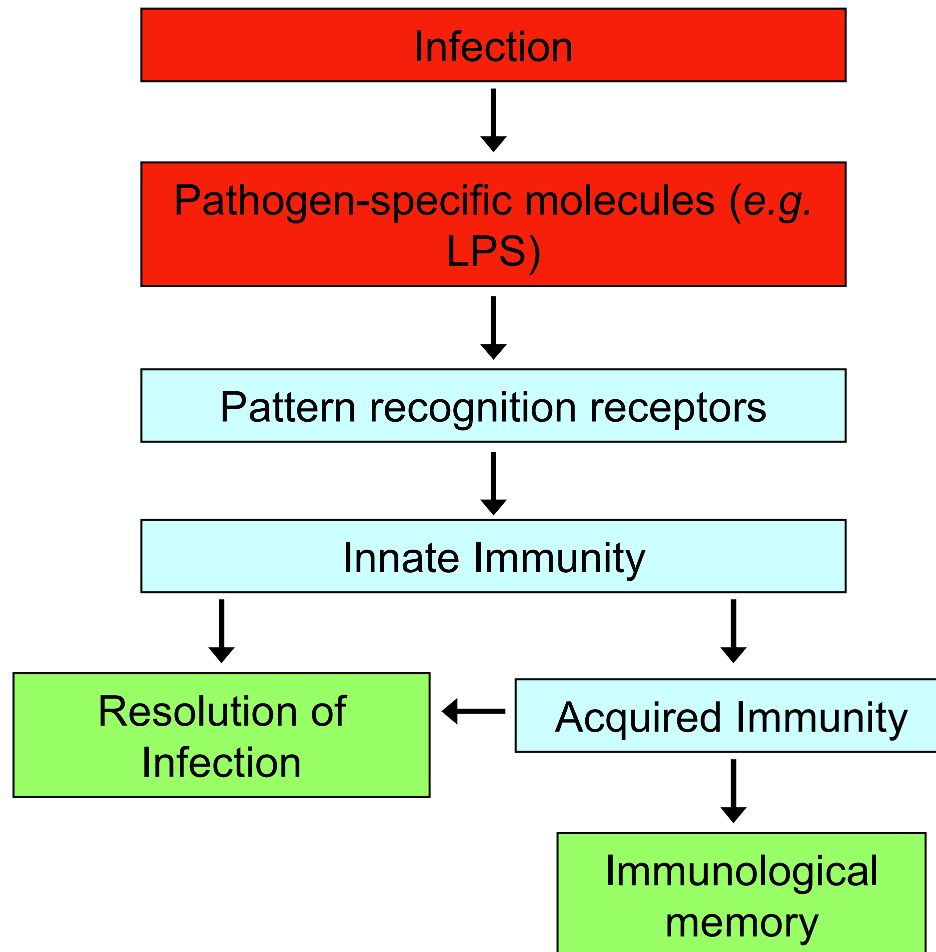

Figure 1: An Overview of Innate Immune Recognition.

During the course of infection, pathogen-specific molecules known as PAMPs are recognized by PRRs thereby activating innate immunity. This allows for direct resolution of the infection by the innate immune system or through activation of the acquired immune system. Activation of the acquired immune system also leads to immunological memory.

TLRs derive their name from the *Drosophila melanogaster* protein Toll. Toll was initially identified as a transmembrane receptor required for proper dorsoventral patterning of the developing embryo in *Drosophila* (Hashimoto, Hudson et al. 1988).

However, Toll demonstrated remarkable similarity to the mammalian IL-1 receptor, and the cytosolic domains of the two proteins are so conserved that they are referred to as the Toll/IL-1 receptor (TIR) domain (Takeda, Kaisho et al. 2003). Furthermore, other components of the pathway including the Rel-type transcription factors Dorsal and NF-κB showed much similarity (Takeda, Kaisho et al. 2003). As IL-1 leads to the activation of the transcription factor NF-κB, which results in the activation of many immune responses, it was proposed that the Toll pathway may play a role in regulating *Drosophila* immunity (Belvin and Anderson 1996). The role of the Toll-mediated pathway in immunity was then demonstrated when mutant flies lacking the components of the pathway were shown to be highly susceptible to fungal infection (Lemaitre, Nicolas et al. 1996). A second Rel-type transcription factor called Dorsal-related immunity factor (Dif) was discovered in *Drosophila*, which did not participate in dorsoventral patterning, but instead appeared to become activated in response to infection (Ip, Reach et al. 1993). Subsequent studies indicated that Dorsal is involved in embryogenesis whereas Dif is mainly involved in the production of antifungal peptides (Meng, Khanuja et al. 1999; Rutschmann, Jung et al. 2000). Further research indicated that the Toll-mediated pathway played a role in resisting infection by Gram positive bacteria as well as fungal pathogens (Rutschmann, Kilinc et al. 2002). A second pathway in *Drosophila*, first identified by a mutation in the *immune deficiency* (*imd*) gene, was described that played a critical role in immunity to Gram negative bacteria (Lemaitre,

Kromer-Metzger et al. 1995). This pathway also leads to a Rel-type transcription factor, Relish (Takeda, Kaisho et al. 2003).

Shortly after the discovery of the role of the Toll pathway in *Drosophila* immunity, a mammalian homologue of Toll was identified (Medzhitov, Preston-Hurlburt et al. 1997). Since then, twelve total members of the TLR family have been identified (Akira 2009). As PRRs, the TLRs can recognize a wide range of PAMPs including many conserved pathogen markers such as peptidoglycan from bacteria, lipopolysaccharide (LPS) from Gram-negative bacteria, envelope glycoproteins from viruses, flagellin from flagellated bacteria, zymosin from fungi and ssRNA and dsRNA from viruses (Mogensen 2009). This broad range of activation both across the family and by individual TLRs allows any specific pathogen to likely activate multiple TLRs while a broad range of unrelated pathogens can all activate any specific TLR (Mogensen 2009). Ultimately, these TLRs play a profound role in mobilizing the innate immune system to fend off infection as well as in helping connect the immediate, rapid response of the innate system to the delayed, longer-lasting response of the adaptive immune system (reviewed in Takeda, Kaisho et al. 2003; Akira 2009; Mogensen 2009).

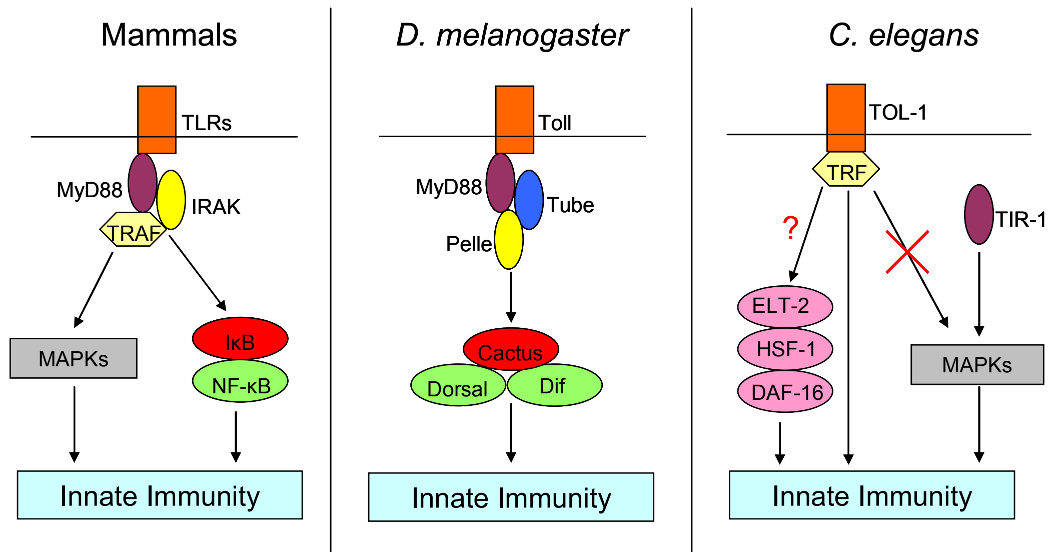

Figure 2: An Overview of Toll Signaling Across Species.

In mammals and *Drosophila*, Toll signaling leads to the activation of a Rel-type transcription factor (NF-κB, Dorsal, Dif) leading to activation of innate immunity. In *C. elegans*, the toll ortholog, TOL-1 leads to innate immune activation, but the method remains unknown; it may work through one of the known transcription factors involved in *C. elegans* immunity (ELT-2, HSF-1, DAF-16). The MyD88 ortholog TIR-1 also activates innate immunity through MAP kinases, but TOL-1 and TIR-1-induced immunity have not yet been connected. Figure is adapted from Mylonakis and Aballay (2005) to reflect changes in the understanding of *C. elegans* immunity.

1.3 Utilizing C. elegans *as a Model for Host-Pathogen Interactions*

Following the discoveries of the Toll-mediated pathway and TLR function in mammalian systems, interest in studying invertebrate innate immunity grew. The nematode *Caenorhabditis elegans* provided an appealing model organism for doing this type of research. *C. elegans* first began being utilized as a model system by Sydney Brenner in the late 1960s to study cell division and differentiation during development

(Hoffenberg 2003). Currently over 400 labs throughout the world are utilizing *C. elegans* not only to study development, but also neurobiology, aging, and innate immunity (Hoffenberg 2003).

C. elegans features a number of tremendous advantages as a model organism. The first of these advantages is the size of the organism, which is about 1 mm in length; this small size greatly reduces the cost of working with the organism as well as allowing work in minimal lab space with easy handling (Riddle, Blumenthal et al. 1997). The relatively short life span—from egg to gravid adult in 3 days at 25°C, and a total life span of 2-3 weeks—combined with its ability to self-fertilize allows for the development of large clonal populations in short periods of time as well as allowing experiments carried out over the entire life span of the animal to be conducted in a timely manner (Riddle, Blumenthal et al. 1997). The presence of males, in addition to self-reproducing hermaphrodites, allows for the utilization of genetic crosses between strains (Riddle, Blumenthal et al. 1997). The small and completely sequenced genome of approximately 100 Mb allows for the utilization of genomic tools (Hodgkin 2005). The ease of with which RNA interference can be utilized in *C. elegans* allows for the selective knockdown of any specific gene of choice (Fire, Xu et al. 1998). Finally, the transparent nature of the nematode allows direct observation of development and the course of an infection as well as gene function through the utilization of transgenic lines carrying green fluorescent protein (GFP)-labeled reporters (Hutter 2006).

Those general advantages of *C. elegans* as a model organism can also be combined with other specific qualities to make it a tremendous resource for studying host-pathogen interactions. One such specific advantage is the fact that *C. elegans* lives and feeds on a lawn of microorganisms (Riddle, Blumenthal et al. 1997). Typically, a strain of *Escherichia coli* is utilized as food for the nematode but this can be switched with another pathogen of interest (Tan, Mahajan-Miklos et al. 1999; Tan, Rahme et al. 1999). Another advantage is that the combination of qualities mentioned earlier (small size, completely sequenced genome, short life span, low cost, etc.) allows for massive screens looking both for virulence factors present in pathogens and immune genes present in the host (Kurz and Ewbank 2003; Gravato-Nobre and Hodgkin 2005). Finally, as an invertebrate, *C. elegans* lacks any sort of adaptive immunity. Furthermore, *C. elegans* appears to lack any specialized immune cells such as macrophages or neutrophils that are capable of bacterial phagocytosis (Gravato-Nobre and Hodgkin 2005). There are scavenger cells called coelomocytes, which are capable of endocytosis, but their involvement in bacterial phagocytosis seems unlikely (Tahseen 2008). Thus, without adaptive or cellular immunity, *C. elegans* allows for the study of immune function at its most basic level.

Unfortunately, *C. elegans* does have some major drawbacks as a model for host-pathogen interactions. The first of these is that although the lack of adaptive and cellular immunity allows the study of basic immune function, it also means that

interactions between those immune systems cannot be studied in *C. elegans*. Another drawback is that *C. elegans* cannot survive at the normal human temperature of 37°C (Gravato-Nobre and Hodgkin 2005); thus, some temperature-dependent virulence factors will not be expressed in the pathogens to which *C. elegans* are exposed. Finally, although some preliminary work has been done in utilizing *C. elegans* as a model for viral infections (Shaham 2006), for the most part, *C. elegans* has yet to be successfully utilized as a model for viral, intracellular, or multicellular pathogens (Gravato-Nobre and Hodgkin 2005).

1.3.1 Pathogens Investigated with the *C. elegans* Model

The first validation of the *C. elegans* host-pathogen interaction model came when research utilizing the worm identified virulence factors in *Pseudomonas aeruginosa* that were also required for pathogenesis in mammalian systems (Mahajan-Miklos, Tan et al. 1999; Tan, Mahajan-Miklos et al. 1999; Tan, Rahme et al. 1999). While these results were compelling, it raised the possibility that the use of the *C. elegans* model may be limited to opportunistic pathogens with a broad host range such as *P. aeruginosa*. However, this fear was abated when it was demonstrated that the highly specialized pathogen *Salmonella enterica* infected and killed *C. elegans* (Aballay, Yorgey et al. 2000). Again, virulence factors important in mammalian pathogens were shown to play a role in causing pathogenesis in *C. elegans* (Aballay, Yorgey et al. 2000). These findings set off a wave of discoveries demonstrating the utility of the *C. elegans* model in studying a

variety of pathogens including other Gram-negative bacteria such as *Serratia marcescens* (Kurz and Ewbank 2000; Mallo, Kurz et al. 2002; Kurz and Ewbank 2003), *Yersinia pestis* (Darby, Hsu et al. 2002; Joshua, Karlyshev et al. 2003; Styer, Hopkins et al. 2005), a variety of *Burkholderia* species (O'Quinn, Wiegand et al. 2001; Gan, Chua et al. 2002; Kothe, Antl et al. 2003), and *E. coli* (Garsin, Sifri et al. 2001); Gram-positive bacteria such as *Staphylococcus aureus* (Garsin, Sifri et al. 2001; Sifri, Baresch-Bernal et al. 2006), *Streptococcus pneumoniae* (Garsin, Sifri et al. 2001; Jansen, Bolm et al. 2002), *Streptococcus pyogenes* (Jansen, Bolm et al. 2002), and *Enterococcus faecalis* (Garsin, Sifri et al. 2001; Sifri, Mylonakis et al. 2002); and the fungal pathogens *Cryptococcus neoformans* (Mylonakis, Ausubel et al. 2002) and *Candida albicans* (Breger, Fuchs et al. 2007). Furthermore, two nematode-specific pathogens, the Gram-positive bacteria *Microbacterium nematophilum* (Hodgkin, Kuwabara et al. 2000) and the fungus *Drechmeria coniospora* (Jansson 1994).

For many of these pathogens, the primary mode of infection is the establishment of an intestinal infection (Gravato-Nobre and Hodgkin 2005). In these instances, as the nematode feeds on the pathogen, some of the microbes survive passage through the pharyngeal grinder of the nematode, and once in the intestine, these pathogens are able to establish an infection, leading ultimately to the death of the nematode. Examples of pathogens that are able to establish such an infection include *S. enterica* (Aballay, Yorgey et al. 2000), *S. marcescens* (Mallo, Kurz et al. 2002), *E. faecalis* (Garsin, Sifri et al. 2001), and *C. neoformans* (Mylonakis, Ausubel et al. 2002). Other infections such as *Bacillus*

thuringiensis kill primarily in a toxin-mediated method (Devidas and Rehberger 1992; Marroquin, Elyassnia et al. 2000). Finally, a very limited number of pathogens can bind to the exterior of the worm; the fungus *D. coniospora* binds to the mouth and vulva of the nematodes (Jansson 1994). Some pathogens are able to infect *C. elegans* through a mix of methods. *P. aeruginosa* can establish an intestinal infection as well as kill through the production of toxins (Mahajan-Miklos, Tan et al. 1999; Tan, Mahajan-Miklos et al. 1999) while *Y. pestis* can kill either by establishing an intestinal infection or through biofilm formation (Darby, Hsu et al. 2002; Joshua, Karlyshev et al. 2003; Styer, Hopkins et al. 2005).

1.3.2 *C. elegans* Immune System

Although *C. elegans* has been a tremendous model system for examining the pathogen side of host-pathogen interactions, it has also been incredibly useful in studying host immunity. As in all multicellular organisms, the first impediments to infection that *C. elegans* produces are physical barriers. The exterior surfaces of the nematode are protected by the cuticle, an exoskeleton consisting of multiple layers of collagen (Gravato-Nobre and Hodgkin 2005). Only a very small number of pathogens such as *D. coniospora* are able to penetrate this exterior defense at its weak points such as the mouth, vulva , and anus (Gravato-Nobre and Hodgkin 2005). Those pathogens that are ingested must survive passage through the pharyngeal grinder of the nematode, which is made of cuticle, reinforced with chitin, and designed specifically to disrupt live

microbes prior to entry into the intestine (Gravato-Nobre and Hodgkin 2005). The importance of both of these barriers can be seen in animals with mutations in key components. Mutant animals with altered exterior surface antigenicity differ in their susceptibility to *M. nematophilum* (Hodgkin, Kuwabara et al. 2000) and to *Yersinia* species (Joshua, Karlyshev et al. 2003; Hoflich, Berninsone et al. 2004). Nematodes with mutations to the gene *phm-2* have defective pharyngeal grinders and also show increased susceptibility to *P. aeruginosa* and *S. enterica* (Labrousse, Chauvet et al. 2000; Kim, Feinbaum et al. 2002; Smith, Laws et al. 2002; Tan 2002).

A secondary defense response by the nematode is avoidance of the pathogen. Nematodes have the ability to respond to chemical cues allowing avoidance while allowing for attraction to nutritious sources (Troemel 1999). These cues allow the nematode to distinguish between different types of bacteria (Andrew and Nicholas 1976). This behavior has been established in response to *B. thuringiensis* (Schulenburg and Muller 2004), *P. aeruginosa* (Tan, Mahajan-Miklos et al. 1999), *S. enterica* (Aballay, Yorgey et al. 2000), and *Burkholderia pseudomallei* (O'Quinn, Wiegand et al. 2001). Interestingly, mutations in the one TLR found in *C. elegans*, *tol-1*, indicate it may play a role in helping the nematode avoid undesirable pathogens as mutants lacking functional *tol-1* failed to avoid *S. marcescens* (Pujol, Link et al. 2001).

Pathogens that are unable to be avoided and that get past the physical barriers of *C. elegans*, then activate the innate immune system of the nematode. The immune

system of *C. elegans* is inducible; in other words, the nematode recognizes the pathogen and responds in a specific manner. Thus, it has been shown that different combinations of genes are regulated by exposure to *S. marcescens* (Mallo, Kurz et al. 2002), *M. nematophilum* (O'Rourke, Baban et al. 2006), *P. aeruginosa* (Shapira, Hamlin et al. 2006), *D. coniospora* (Pujol, Zugasti et al. 2008), *Y. pestis* (unpublished data from our group), and *S. enterica* (unpublished data from our group). Thus, each pathogen can be said to produce its own antimicrobial fingerprint of gene regulation as observed when gene expression was compared between *S. marcescens*, *P. aeruginosa*, and *S. aureus* (Alper, McBride et al. 2007). Genes that are strongly upregulated by pathogen infection include a wide variety of antimicrobial proteins including C-type lectins, lysozymes, proteases, and lipases, as well as a number of proteins containing unknown functions (Shapira, Hamlin et al. 2006; unpublished data from our group).

These responses to pathogens are induced in *C. elegans* by a number of different but often interconnected signaling pathways. In the last decade, eight of these pathways have been identified: a transforming growth factor β (TGF-β)-like pathway (Mallo, Kurz et al. 2002), an insulin-like receptor pathway (Garsin, Villanueva et al. 2003), a programmed cell death pathway (Aballay, Yorgey et al. 2000; Aballay and Ausubel 2001), a HSF-1/heat shock factor pathway (Singh and Aballay 2006; Singh and Aballay 2006), a TOL-1/Toll-like receptor signaling pathway (Tenor and Aballay 2008), and three MAP kinase pathways—p38 MAP kinase (Kim, Feinbaum et al. 2002; Aballay, Drenkard

et al. 2003; Couillault, Pujol et al. 2004; Huffman, Abrami et al. 2004; Liberati, Fitzgerald et al. 2004), c-Jun amino terminal kinases (JNK) (Kim, Liberati et al. 2004), and an extracellular signal-related kinase (ERK) MAP kinase (Nicholas and Hodgkin 2004). These pathways appear to protect the nematode in unique ways, and they seem to respond differently to specific pathogens (Alper, McBride et al. 2007; Coolon, Jones et al. 2009). Knowledge of the components of these pathways remains incomplete and their activation and interactions are just beginning to be studied. However, it does appear that many of these pathways play additional roles besides immune function in the nematode; the roles include regulating dauer-formation (a long-lived phase of the nematode life cycle where essential features such as germline development are switched off in response to stress) and other responses to stress be it from heavy metals, oxidative stress, osmotic stress, or heat stress (Gravato-Nobre and Hodgkin 2005). Also, although the pathways feature unique components and appear that they may respond differently to specific pathogens, many of the same effectors appear to be regulated by multiple pathways (Mochii, Yoshida et al. 1999; Murphy, McCarroll et al. 2003; Troemel, Chu et al. 2006; Alper, McBride et al. 2007).

According to Janeway's hypothesis, the inducible nature of *C. elegans* immunity suggests the presence of PRRs that respond to PAMPs. As of now, these PRRs remain undiscovered; this leads to the possibility that the different immune responses observed in *C. elegans* could be due to different kinds of stress induced by different infections.

Studies have begun to address this. For example, it was shown that some mutants of *S. enterica* with altered LPS failed to elicit germline programmed cell death in spite of high intestinal titers suggesting LPS recognition may be occurring (Aballay, Drenkard et al. 2003). Furthermore, in spite of initial studies failing to show an immune effect of the one TLR present in *C. elegans*, *tol-1*, our group has recently demonstrated that *tol-1* plays a key role in protecting the nematode from pharyngeal invasion and premature death (Tenor and Aballay 2008).

Regardless of whether the immune response in *C. elegans* is induced by PRRs responding to PAMPs or whether it is due to responses to generalized stress, the different gene expression profiles seen in response to different pathogens must be due to the effect of transcription factors. Although TOL-1 is a Toll homologue, *C. elegans* appears to lack a Rel-type transcription factor such as NF-κB, Dif, or Relish (Kanzok, Hoa et al. 2004). Thus, although TOL-1 may play a key role in *C. elegans* immunity, it remains unclear through what transcription factor TOL-1 may be acting. Indeed, this is true for almost all of the *C. elegans* immune pathways listed earlier; thus far only DAF-16, a FOXO transcription factor in the insulin-like receptor pathway, and HSF-1, a transcription factor in the heat shock pathway, are the only known transcription factors in the *C. elegans* immune pathway (Garsin, Villanueva et al. 2003; Murphy, McCarroll et al. 2003; Singh and Aballay 2006; Singh and Aballay 2006). The research covered in this study will focus on DAF-16 as well as another transcription factor, ELT-2, which was

identified as playing a role in *C. elegans* immunity (Kerry, TeKippe et al. 2006; Shapira, Hamlin et al. 2006).

1.4 GATA Transcription Factors

One of the classes of transcription factors that may play a role in mediating immunity in *C. elegans* is the GATA family of transcription factors. GATA transcription factors are so named because they bind to the consensus DNA sequence (A/T) GATA (A/G) (Patient and McGhee 2002). The DNA binding domain of each GATA transcription factor consists of one or two distinctive zinc-finger domains combined with an adjacent conserved highly basic region (Patient and McGhee 2002). This family has been found throughout eukaryotic life from plants to fungi to animals where they have been shown to play critical roles in development (Lowry and Atchley 2000). The size of the GATA family is relatively small when compared to that of other transcription factor families: six GATA factors have been identified in vertebrates while eleven have been found in *C. elegans* (Patient and McGhee 2002).

In mammalian systems, GATA-1, GATA-2, and GATA-3 have all been shown to play a key role in regulating hematopoietic stem cells and those cells that derive from them (Molkentin 2000). A similar function seems to also be found in the most closely related GATA transcription factors found in *Drosophila* (Rehorn, Thelen et al. 1996). Meanwhile, GATA-4, -5, and -6 are expressed in a number of mesoderm- and endoderm-derived tissues including the heart, lung, gonad, and intestine where they

play a role in proper development and maintenance of the tissue (Molkentin 2000). In *C. elegans*, a cascade of GATA transcription factors help guide the developing embryo from the four cell stage through mesoderm and endoderm differentiation, gastrulation, intestine formation, and intestinal differentiation (Maduro and Rothman 2002). Additional GATA factors in *C. elegans* play a key role in specifying cell fate in the hypodermis where in the absence of the GATA transcription factor ELT-1, the hypodermal precursor cells switch their fate to that of their non-hypodermal neighbor (Page, Zhang et al. 1997).

Besides playing a role in cellular development and differentiation, GATA transcription factors appear to play a role in modulating a number of other effects, including influencing immunity. In mammalian systems, GATA-3 has been shown to modulate the commitment of T cells to Th-1 or Th-2 cells (Zheng and Flavell 1997; Mullen, Hutchins et al. 2001). It also appears that GATA-3 and TGF-β signaling are connected in Th-2 cells (Blokzijl, ten Dijke et al. 2002). GATA-4 has also been shown to regulate cardiac stress responses (Suzuki 2003; Aries, Paradis et al. 2004; Tenhunen, Sarman et al. 2004). Additional data have suggested that GATA transcription factors play a role in influencing innate immunity in invertebrates as the promoters of genes important in defense responses have been shown to contain GATA-binding elements in *D. melanogaster* (Petersen, Kadalayil et al. 1999; Bernal and Kimbrell 2000; Wertheim, Kraaijeveld et al. 2005), the silkworm *Bombyx mori* (Cheng, Zhao et al. 2006), and the

mosquito *Aedes aegypti* (Cheon, Shin et al. 2006). Research in *C. elegans* has shown that the GATA transcription factors ELT-3, ELT-5, and ELT-6 play a key role in regulating a number of genes involved with aging (Budovskaya, Wu et al. 2008).

1.5 FOXO Transcription Factors

FOXO transcription factors are a subgroup of the Forkhead family of transcription factor, a family defined by its conserved DNA-binding domain known as the "Forkhead box" or FOX. In humans, the Forkhead family is very large, consisting of more than 100 members. The FOXO subfamily is considerably smaller though, consisting of only four members in mammals (FOXO1, FOXO3, FOXO4, and FOXO6) while only having one member in each of *C. elegans* and *Drosophila* (DAF-16 and dFOXO respectively) (Carter and Brunet 2007).

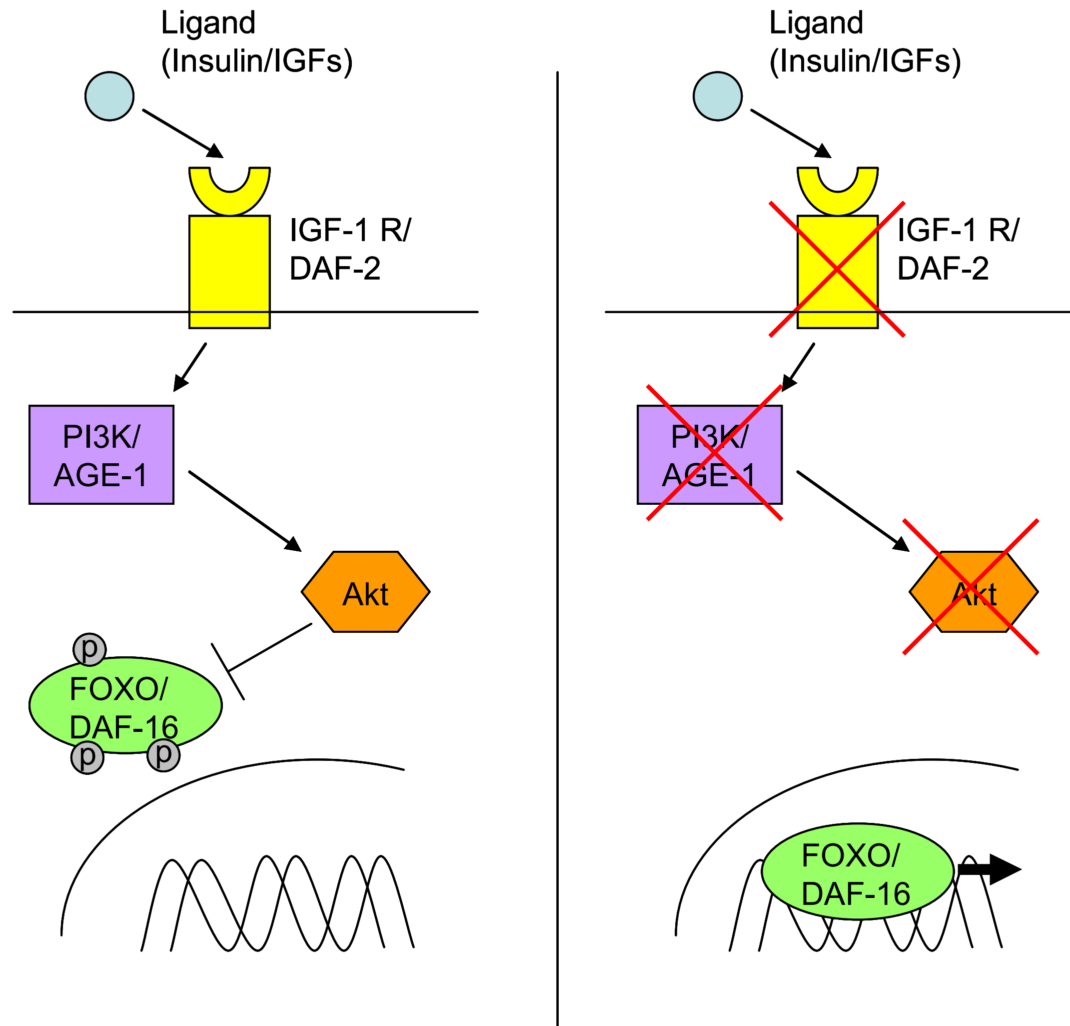

Figure 3: The Insulin receptor/PI3K/Akt pathway.

This pathway is conserved between mammals and *C. elegans*. The name of the protein in *C. elegans* is listed second; also IGF-1 R is just one of the receptors of this pathway found in mammals. On the left, the intact pathway responds to a ligand, leading to Akt phosphorylation of the FOXO transcription factor, keeping FOXO in the cytosol. On the right, mutations in any of the three components of the pathway, prevent phosphorylation of FOXO by Akt, leading to transcription of FOXO-mediated genes.

One signaling pathway that is responsible for the regulation of FOXO transcription factors and is highly conserved is the insulin/growth factor receptor

pathway (Figure 3). In this pathway, insulin or insulin-like growth factors are recognized by receptors at the cell surface (Carter and Brunet 2007). This leads to activation of the insulin-phosphatidylinositol 3 kinases (PI3K)-Akt pathway, resulting in Akt-dependent phosphorylation of three specific sites on the FOXO transcription factors (Brunet, Bonni et al. 1999; Kops, de Ruiter et al. 1999; Brunet, Park et al. 2001; Brunet, Kanai et al. 2002). Akt-dependent phosphorylation of FOXO promotes nuclear export of the FOXO transcription factor, thereby repressing FOXO transcriptional function (Salih and Brunet 2008). This PI3K/Akt pathway has been shown to play key roles in regulating cell proliferation, differentiation, and apoptosis with dysfunction of the pathway has been shown to play a role in the pathogenesis of many diseases including heart and vascular diseases, diabetes, auto-inflammatory diseases, and cancer (Sedding 2008).

In addition to the insulin-PI3K/Akt signaling pathway, a number of other pathways and conditions have been demonstrated to regulate FOXO function. Many different stress stimuli such as oxidative stress, DNA damage, nutrient deprivation, cytokines, and hypoxia have all been shown to influence FOXO activation (Seoane, Le et al. 2004; Gomis, Alarcon et al. 2006; Gomis, Alarcon et al. 2006; Huang, Regan et al. 2006; Bakker, Harris et al. 2007; Greer, Dowlatshahi et al. 2007; Greer, Oskoui et al. 2007). These factors influence FOXO function through a tremendous range of post-transcriptional modifications including phosphorylation, acetylation, and ubquitination

at a number of regulatory sites, and these modifications occur through the action of many different signaling pathway, including the JNK, AMP-dependent kinase (AMPK), the deacetylase Sirt1, and the mammalian Ste20-like kinase (MST1) (Salih and Brunet 2008).

One of the key roles that FOXO transcription factors appear to play is the regulation of aging and longevity (Kenyon 2005). This influence on longevity is best understood in its involvement with the insulin/PI3K/Akt pathway, and that pathway has been shown to influence life span in nematodes, flies, and mammals (Tatar, Bartke et al. 2003). The role of this pathway was first discovered in *C. elegans*, where it was shown that mutations in *daf-2*, an insulin/IGF-1 receptor ortholog (Kimura, Tissenbaum et al. 1997) led to dramatically increased life spans in the worm (Kenyon, Chang et al. 1993). Furthermore, this lifespan extension is also observed when the *C. elegans* ortholog of a catalytic subunit of PI3K called AGE-1 is removed (Friedman and Johnson 1988; Friedman and Johnson 1988; Malone, Inoue et al. 1996). It was also discovered that this long-lived phenotype could be completely suppressed by knocking out the gene *daf-16* (Kenyon, Chang et al. 1993; Gottlieb and Ruvkun 1994; Dorman, Albinder et al. 1995; Larsen, Albert et al. 1995; Malone, Inoue et al. 1996; Tissenbaum and Ruvkun 1998). DAF-16 was then discovered to be the *C. elegans* FOXO transcription factor (Lin, Dorman et al. 1997; Ogg, Paradis et al. 1997).

In addition to its role in longevity, DAF-16 has also been shown to play a role in regulating *C. elegans* immunity (Garsin, Villanueva et al. 2003; Lee, Kennedy et al. 2003; Murphy, McCarroll et al. 2003). Nematodes that overexpress DAF-16 from a transgenic source (data not shown) or have increased activation of DAF-16 through the mutation of *daf-2* have greater resistance to a variety of pathogens (Garsin, Villanueva et al. 2003; Anyanful, Dolan-Livengood et al. 2005; Kerry, TeKippe et al. 2006; Singh and Aballay 2006). Further evidence for the role of DAF-16 in immunity comes from the fact that many of the effector molecules regulated by DAF-16 appear to play key roles in antimicrobial defense (Murphy, McCarroll et al. 2003).

1.6 Reproduction and Immunity

One of the most difficult challenges for an organism is balancing finite energy resources between a number of costly activities in an effort to maximize fitness (Klein and Nelson 1999). Two of the costly traits an organism must balance are reproduction and immunity. Studies in a variety of organisms have illustrated that reproduction extracts a cost in terms of the longevity of the organism (Westendorp and Kirkwood 1998; Davies, Kattel et al. 2005; Partridge, Gems et al. 2005). Additional studies have linked successful reproduction with reductions in immunocompetence (McKean and Nunney 2001; Fedorka, Zuk et al. 2004; Gwynn, Callaghan et al. 2005; Fedorka, Linder et al. 2007).

C. elegans has long been utilized as a model to study the costs of balancing reproduction, longevity, and immune function (reviewed in Mukhopadhyay and Tissenbaum 2007). In *C. elegans*, the gonad arises from four precursor cells called Z1, Z2, Z3, and Z4; Z1 and Z4 give rise to the somatic gonad, which includes the sheath, spermatheca, and uterus while Z2 and Z3 give rise to the germ cells (Hubbard and Greenstein 2005). Removal of the germ cell precursors Z2 and Z3 leads to a dramatic increase in life span for the nematode (Hsin and Kenyon 1999). A similar long-lived effect can be produced by utilizing mutations such as a mutation to the gene *glp-1*, which encodes a Notch family receptor critical to germline development, that eliminate the presence of the germline (Arantes-Oliveira, Apfeld et al. 2002). This life span extension upon removal of the germline precursor cells occurs in another nematode species, *Pristionchus pacificus*, which diverged from *C. elegans* 100 million years ago indicating a link between germline function and longevity in nematodes that is very old and still conserved (Hsin and Kenyon 1999). Interestingly, removal of all four germline precursor cells fails to lead to an extended life span (Hsin and Kenyon 1999). This suggests that it is not simply a matter of sterility that leads to longevity in the nematodes but that instead communication between the somatic gonad and the rest of the animal is required to produce the long-lived phenotype (Hsin and Kenyon 1999).

The life span extension observed in *C. elegans* lacking a germline is dependent on the FOXO transcription factor DAF-16 (Lin, Hsin et al. 2001; Arantes-Oliveira, Apfeld et

al. 2002; Libina, Berman et al. 2003). Interestingly, despite the fact that knocking out DAF-2, the *C. elegans* insulin/insulin-like growth factor, also leads to increased longevity (Kenyon, Chang et al. 1993), the life span extension observed in germline-ablated nematodes appears to be largely independent of the insulin/PI3K/Akt pathway (Mukhopadhyay and Tissenbaum 2007). This is indicated by the fact that *daf-2* mutant nematodes lacking a germline live longer than either *daf-2* mutant nematodes with a germline or germline-deficient nematodes with a functional *daf-2* gene (Hsin and Kenyon 1999; Arantes-Oliveira, Berman et al. 2003). Furthermore, mutations in *daf-2* induce DAF-16 nuclear localization throughout the nematode while DAF-16 nuclear localization in germline-deficient animals is limited to the intestine (Lin, Hsin et al. 2001). The importance of this intestinal activation of DAF-16 was shown through the use of intestinal-specific promoters to express transgenic DAF-16, which demonstrated a restoration of the long-lived phenotype in *daf-16* mutants that lacked a germline (Libina, Berman et al. 2003).

Some components of the pathway responsible for inducing intestinal DAF-16 activation in nematodes lacking a germline have been identified. DAF-18 is a PTEN phosphatase that antagonizes PI3K signaling downstream of DAF-2 (Ogg and Ruvkun 1998; Gil, Malone Link et al. 1999). Expression of DAF-18 is required for the increased longevity and nuclear localization of DAF-16 both in *daf-2* mutants and germline-deficient animals (Dorman, Albinder et al. 1995; Larsen, Albert et al. 1995; Berman and

Kenyon 2006). As such DAF-18 appears to be critical in activating DAF-16 in both the insulin/PI3K/Akt pathway and the germline-ablation pathway.

Two other proteins that play a role in DAF-16 activation and life span extension in germline-deficient nematodes are DAF-9 and DAF-12. Unlike DAF-18, knocking out DAF-9 or DAF-12 in a *daf-2* mutant fails to eliminate DAF-16 activation and the long-lived phenotype (Berman and Kenyon 2006). In germline-deficient animals though, DAF-9 and DAF-12 function are required for DAF-16 nuclear localization in the intestinal cells as well as for the long-lived phenotype (Hsin and Kenyon 1999; Antebi, Yeh et al. 2000; Gerisch, Weitzel et al. 2001; Berman and Kenyon 2006). The *daf-12* gene encodes for a nuclear hormone receptor related to the vitamin D and pregnane X receptors while *daf-9* encodes for a cytochrome P450 postulated to make a lipophilic ligand for DAF-12 (Berman and Kenyon 2006). Interestingly, although constitutively active DAF-16 rescues the long-lived phenotype in germline-deficient *daf-9* mutants, it fails to completely do so in germline-deficient *daf-12* mutants (Berman and Kenyon 2006). This suggests a parallel function of DAF-12 in helping to increase life span in germline-deficient animals separate from its role in DAF-16 activation. Further support for this can be found in the fact that germline-deficient nematodes lacking both DAF-16 and DAF-12 have shorter life spans than germline-deficient animals with only a *daf-16* mutation (Berman and Kenyon 2006).

Recent evidence has indicated that the ligand produced by DAF-9 for DAF-12 may be pregnenolone (PREG). Studies demonstrated that PREG levels were increased in germline-deficient *glp-1* mutant animals, but decreased in *daf-9*; *glp-1* double mutants. Meanwhile, treating the nematodes with PREG caused increased life spans in *glp-1*; *daf-9* double mutants but failed to increase longevity in *glp-1*; *daf-12* double mutants (Broue, Liere et al. 2007).

Another protein that has been shown to play a role in the pathway from germline-ablation to DAF-16 activation is KRI-1. A mutation in *kri-1* causes a suppression of life span extension in *glp-1* mutants but fails to suppress life span both in wild-type and *daf-2* mutant animals. Currently, the only known function of *kri-1* is in promoting DAF-16 translocation to the nucleus as a constitutively nuclear DFA-16 bypasses the need for *kri-1* (Berman and Kenyon 2006).

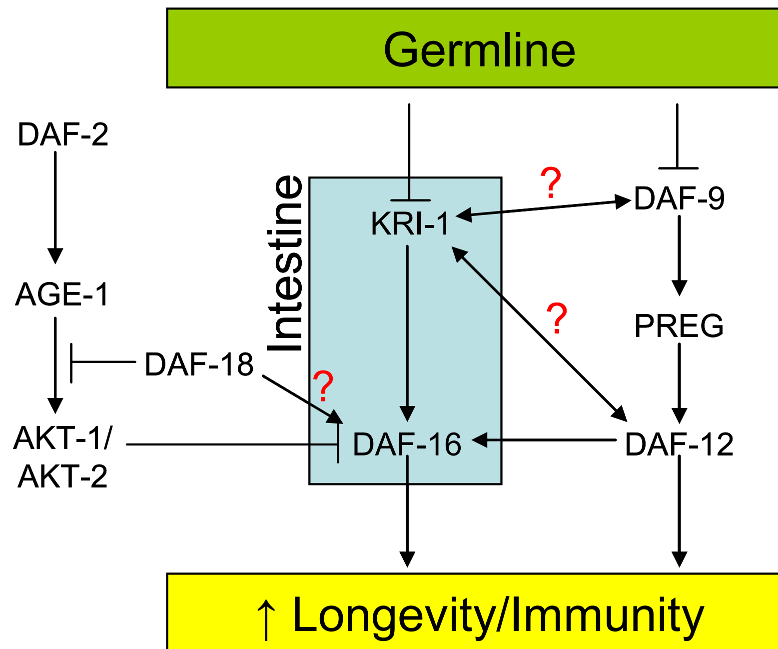

Figure 4: Proteins involved in germline-deficient immunity

Lack of the germline leads to increased longevity and immunity by causing the activation of a number of proteins, ultimately culminating in intestinal DAF-16 activation. At this time, it is unclear through exactly what manner KRI-1 interacts with the pathway. It is also unclear if DAF-18 plays a second role in activating DAF-16 in germline-deficient nematodes or if the inhibition of AGE-1 signaling by DAF-18 is simply required for the long-lived phenotype of germline-deficient animals. This figure is adapted from Mukhopadhyay and Tissenbaum (2007).

It may be expected that a significant reason for the longevity observed in germline-deficient nematodes is a result of increased immune function. This is due to the fact that DAF-16 is known to play a prominent role in regulating nematode immunity (Garsin, Villanueva et al. 2003; Lee, Kennedy et al. 2003; Murphy, McCarroll et al. 2003). Furthermore, these longevity studies have been performed on live *E. coli*, which has been shown to be pathogenic (Garsin, Villanueva et al. 2003) and intestinal bacterial overgrowth is a leading cause of death in nematodes (Garigan, Hsu et al. 2002).

A recent study indicated that germline-deficient animals were more resistant to *P. aeruginosa* (Miyata, Begun et al. 2008). Research presented in this study will also provide additional support for this claim.

Finally, it remains unclear exactly how interplay between the reproductive system and immune function occurs in wild-type nematodes with a germline. A new study, though, has demonstrated differing levels of expression of the gene *gld-1* when exposed to different species of bacteria expressed in grassland soil (Coolon, Jones et al. 2009). GLD-1 functions to limit proliferation of germ cells (Francis, Barton et al. 1995; Francis, Maine et al. 1995), and as such, it is situated to help integrate signals from the nematode, including immune responses, and control reproduction (Coolon, Jones et al. 2009). Additionally, since targets of DAF-16 have been shown to suppress *gld-1*-induced tumors (Pinkston, Garigan et al. 2006; Pinkston-Gosse and Kenyon 2007), it seems likely that immune responses may help influence reproduction (Coolon, Jones et al. 2009). Further support for the interaction of immunity and reproduction can be seen in response to *S. enterica* infection, which induces the programmed cell death pathway and germ cell apoptosis (Aballay and Ausubel 2001).

1.7 Introduction to Thesis Work

The basis of the thesis work presented here has been focused on understanding the role of transcription factors and immunity in *C. elegans*. As such, we identified a GATA transcription factor called ELT-2, which we demonstrated plays a role, not only

in *C. elegans* development, but also in *C. elegans* immunity (Kerry, TeKippe et al. 2006). Our investigations of *elt-2* demonstrated that it was required for immunity to many different pathogens including *S. enterica*, *P. aeruginosa*, *E. faecalis*, and *C. neoformans*. We also demonstrated that *elt-2* was important in regulating a known marker of immunity, the C-type lectin *clec-67*. We also demonstrated that *elt-2* immune function appears to be independent to DAF-16-mediated immunity and the insulin/insulin-like growth factor pathway. This work was begun in our lab by Sam Kerry, as she initially identified the involvement of ELT-2 in *C. elegans* immunity. I followed up on this work confirming her initial results and demonstrating the independence of ELT-2 and the insulin/PI3K pathway. We wrote the paper which demonstrated this research together (Kerry, TeKippe et al. 2006), and we are listed as equal contributors.

Interest in DAF-16 led us to investigate germline-deficient mutants. We showed that the germline-deficient mutant *glp-1* demonstrates resistance to a wide variety of pathogens. However, another germline-deficient mutant *glp-4* failed to show increased resistance to pathogens in the same manner as *glp-1* mutants. As the *glp-4* mutation is unknown, we utilized high-throughput methods developed by our group to identify the possible mutations that may be responsible for the *glp-4* phenotype. We also demonstrated that *glp-4* mutants, like other germline-deficient nematodes, show increased levels of DAF-16 activation. These results point us to a secondary role for *glp-*

4 in influencing *C. elegans* immunity, independent of its role in germline development or the activation of DAF-16.

2. The conserved GATA transcription factor *elt-2* is required for *C. elegans* immunity to pathogens

2.1 Introduction

As described earlier, the study of innate immunity, especially in invertebrate systems, has received renewed attention in recent years when the first parallels between mammalian and *Drosophila melanogaster* immunity were discovered (reviewed in Hoffmann 2003; Kim and Kim 2005; Mylonakis and Aballay 2005). Since then, various invertebrate model systems have been used to dissect highly-conserved immune responses without the complications of adaptive immunity. The genetically tractable nematode *Caenorhabditis elegans*, which has been used for decades to study the mechanisms of a number biological processes, has become a well-established invertebrate model for the study of microbial pathogenesis and innate immunity (reviewed in Kurz and Ewbank 2003; Gravato-Nobre and Hodgkin 2005; Kim and Ausubel 2005; Mylonakis and Aballay 2005; Sifri, Begun et al. 2005). *C. elegans* is a free-living, non-pathogenic nematode that lives in the soil, where it uses bacteria as a food source. As in mammals, peristalsis, lytic enzymes, and antimicrobial peptides prevent the colonization of potential microbial pathogens in the *C. elegans* intestine. However, some pathogens are capable of escaping these defenses and proliferating in the *C. elegans* intestine, thereby killing the nematode by an infectious process. *C. elegans* has evolved mechanisms to recognize and respond to potential pathogens using an inducible immune system that contains many highly-conserved effectors, including anti-bacterial

proteins, lysozymes, lipases, and C-type lectins (Nicholas and Hodgkin 2004). Additionally, many conserved signaling pathways have been linked to *C. elegans* immunity, including the TGF-β, insulin-PI3K, and multiple MAP kinase pathways (reviewed in Kurz and Tan 2004; Gravato-Nobre and Hodgkin 2005; Mylonakis and Aballay 2005), suggesting that despite the vast evolutionary gulf between nematodes and mammals, some of the underlying mechanisms of immunity may be similar.

Despite the similarities, there are also characteristics of the immune response of *C. elegans* that distinguish it from mammals and other invertebrates. Although the Toll-mediated pathway, first studied in *Drosophila melanogaster* and subsequently studied in mammals, is highly conserved across species, it does not appear to function identically in *C. elegans*. The single nematode Toll receptor homolog, TOL-1, has been shown to play a role in providing immune defense in *C. elegans* both directly and through bacterial avoidance behaviors (Pujol, Link et al. 2001; Tenor and Aballay 2008). However, unlike in *Drospholia* and mammalian the Toll-mediated response appears to lack a Rel-type transcription factor such as NF-κB (Kanzok, Hoa et al. 2004). NF-κB-like transcription factors are critical in the regulation of Toll immune responses as they control the expression of key genes involved in innate immunity in many organisms (reviewed in Moynagh 2005). Thus, there is a distinct need to determine exactly which transcription factors are involved in modulating nematode immune responses, some of which may also play important immunity roles in mammals. Prior to the beginning of

this thesis research, only two transcription factors, DAF-16 and HSF-1, had been linked to *C. elegans* immunity (Garsin, Villanueva et al. 2003; Singh and Aballay 2006; Wolff, Ma et al. 2006) and only DAF-16 has been shown to regulate the expression of immune effectors in the nematode (Murphy, McCarroll et al. 2003).

Here we characterize the role of the intestinal GATA transcription factor ELT-2 in *C. elegans* immunity. We show that disruption of ELT-2 expression causes nematodes to have increased susceptibility to *Salmonella enterica*-mediated killing and that ELT-2 upregulates the expression of *clec-67*, which is a marker of *S. enterica* infection in *C. elegans*. Our results also demonstrate that ELT-2 is part of a multi-pathogen defense pathway that regulates innate immunity not only to *S. enterica,* but also to *Pseudomonas aeruginosa, Enterococcus faecalis,* and *Cryptococcus neoformans*. We also show that ELT-2 functions in *C. elegans* immunity independently of the DAF-2/DAF-16 signaling pathway. These data indicate that ELT-2 is an important regulator of a conserved *C. elegans* immune response to pathogens.

2.2 Results

2.2.1 *elt-2(RNAi)* animals are more susceptible to *S. enterica*-mediated killing

C. elegans consumes bacteria as its primary food source, thus the digestive tract is a primary interface between the *C. elegans* immune system and potential bacterial pathogens. This is especially important in the case of bacteria such as the human pathogen *S. enterica,* which is capable of establishing a persistent infection in the *C.*

elegans intestine (Aballay, Yorgey et al. 2000; Labrousse, Chauvet et al. 2000). In addition, several known effectors of the *C. elegans* immune system are expressed in the intestinal cells. Because of this link between the intestine and immunity, we sought to identify intestinal transcription factors which might modulate *C. elegans* immune responses.

Over 900 functional transcription factors are predicted to be encoded in the *C. elegans* genome (Reece-Hoyes, Deplancke et al. 2005). To identify genes encoding transcription factors involved in the regulation of immune effectors expressed in the *C. elegans* intestinal cells, candidates were selected based on the following criteria: 1) genes known to be expressed in the intestine (141 candidates), 2) genes expressed during adulthood (10 of the 141 candidates), and 3) genes which only regulate intestinal functions or for which a function in adulthood has not been reported (7 of the 10 candidates). Of the seven candidates, five were chosen for further analysis based on the availability of RNAi clones (Kamath, Fraser et al. 2003). Thus, to address whether the selected candidate genes play a role in *C. elegans* immunity, we used RNAi to inhibit their expression and tested the susceptibility of these gene ablated animals to *S. enterica*. The results shown in Table 1 demonstrate that only RNAi ablation of *elt-2* results in consistent increased susceptibility of *C. elegans* to *S. enterica*.

Table 1: ELT-2 is required for the *C. elegans* immune response to *S. enterica*

RNAi treatment	$TD_{50} \pm SEM$	Events/Obs	p-value vs control
control	5.90 ± 0.23	149/210	
elt-2	1.91 ± 0.06	114/120	< 0.0001
F42G2.6	5.24 ± 0.22	97/120	0.1483
F23F12.9	5.59 ± 0.15	97/120	0.3375
ZK632.2	5.28 ± 0.19	93/120	0.1900
C18G1.2	5.69 ± 0.20	62/90	0.8779

Wild type nematodes grown on *E. coli* carrying a vector control or on *E. coli* expressing double-stranded RNA of the gene listed in the RNAi treatment column were exposed to *S. enterica* SL1344. $TD_{50} \pm SEM$ and p-values were calculated using PRISM as described in Section 2.4.7.

The increased susceptibility of *elt-2(RNAi)* animals to *S. enterica* indicates that ELT-2 may be involved in the regulation of an immune response to this pathogen. However, it is also possible that *elt-2(RNAi)* animals are sickly due to a malfunctioning intestine. ELT-2 is a crucial GATA transcription factor involved in intestinal differentiation (Fukushige, Hawkins et al. 1998; Fukushige, Hendzel et al. 1999; Moilanen, Fukushige et al. 1999; Fukushige, Goszczynski et al. 2003; Fukushige, Goszczynski et al. 2005) and a knockout mutation in *elt-2* is lethal (Fukushige, Hawkins et al. 1998). *elt-2* knockouts exhibit a gut-obstructed (Gob) phenotype that results in arrest at L1 stage larvae and subsequent death, apparently by starvation (Fukushige,

Hawkins et al. 1998). Therefore, to study whether *elt-2* ablation by RNAi makes the animals sickly, we first assayed the life span of *elt-2(RNAi)* nematodes. Figure 5B shows that although *elt-2(RNAi)* animals exhibit a reduced life span compared to control animals, they survive at least eight days, indicating that starvation is not a cause of death.

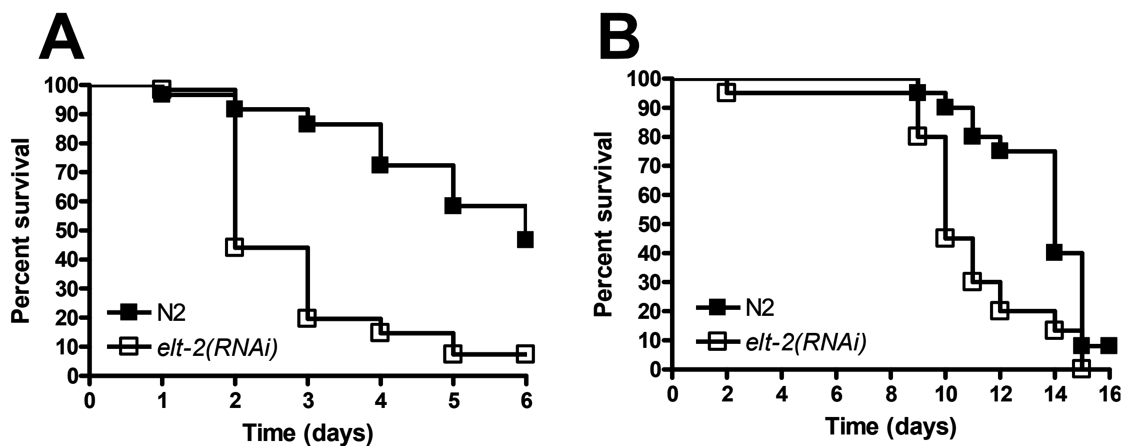

Figure 5: Survival of wild-type nematodes versus *elt-2(RNAi)* animals on *S. enterica* and heat-killed *E. coli*

Wild-type nematodes grown on *E. coli* carrying vector control or on *E. coli* expressing *elt-2* dsRNA were exposed to **(A)** *S. enterica* ($P < 0.0001$) or **(B)** heat-killed *E. coli* on plates containing FUdR ($P = 0.0011$) (B). Results are representative of at least 3 independent experiments.

To test whether *elt-2(RNAi)* animals are more susceptible to *S. enterica*-mediated killing when taking into account their reduced life span on *E. coli*, the "relative mortality" of *elt-2(RNAi)* animals feeding on *S. enterica* [defined as: (TD_{50} of control worms on *S. enterica*/TD_{50} of *elt-2(RNAi)* worms on *S. enterica*)/(TD_{50} control worms on *E. coli*/TD_{50} of *elt-2(RNAi)* on *E. coli*)] was used to assess the susceptibility to *S. enterica*-

mediated killing of *elt-2(RNAi)* animals. The relative mortality measure has the advantage of normalizing any observed change in longevity of the *elt-2(RNAi)* animals feeding on *S. enterica* to any change in longevity when feeding on *E. coli* and, therefore, takes into account changes in life span due to a general modification in fitness rather than a specific defect in innate immunity against *S. enterica*. The relative mortality of *elt-2(RNAi)* animals was calculated using the time for 50% of the nematodes to die (TD_{50}) from various independent experiments and determined to be 2.4 (Table 2). The relative mortality obtained is comparable to the relative mortality of animals lacking CED-3-mediated immunity (Aballay and Ausubel 2001) and greater than the relative mortality of animals lacking p38/PMK-1 (Tenor, McCormick et al. 2004), which is crucial for *C. elegans* immunity (Kim, Feinbaum et al. 2002; Aballay, Drenkard et al. 2003; Huffman, Abrami et al. 2004; Kim, Liberati et al. 2004), indicating that the shortened life span of the *elt-2(RNAi)* animals infected by *S. enterica* is not simply a consequence of being sickly.

Table 2: Relative Morality of *elt-2(RNAi)* animals on *S. enterica*

Bacteria	RNAi treatment	Experiment	$TD_{50} \pm SEM$	Events/Obs[a]
S. enterica	control	1[b]	6.11±0.24	26/60
	elt-2		2.06±0.17	40/60
	control	2	8.99±0.35	9/20
	elt-2		2.53±0.22	16/20
	control	3	7.46±0.38	5/20
	elt-2		3.98±0.36	12/20
	control	4	6.67±0.25	30/60
	elt-2		1.97±0.08	50/60
	control	5	5.12±0.15	42/60
	elt-2		2.81±0.09	46/60
	control	6	7.17±0.27	20/40
	elt-2		1.10±0.27	32/40
	control	7	6.20±0.09	98/120
	elt-2		0.82±0.12	56/60
E. coli	control	1[c]	13.48±0.15	16/20
	elt-2		10.25±0.16	19/20
	control	2	15.33±0.35	6/20
	elt-2		10.96±0.16	18/20
	control	3	12.15±0.13	13/20
	elt-2		8.03±0.23	13/20
Bacteria	**RNAi treatment**	**Mean±SD**		
S. enterica	control	6.82±1.14		
S. enterica	*elt-2*	2.17±0.99		
E. coli	control	13.65±1.30		
E. coli	*elt-2*	9.75±1.25		
Relative Mortality = 2.24				

Wild-type nematodes grown on *E. coli* carrying a vector control or on *E. coli* expressing *elt-2* dsRNA were exposed to *S. enterica* or heat-killed *E. coli*. [a]Animals that were not found or that died as a result of getting stuck on the wall of the plate were censored. [b]Indicates experiment depicted in Figure 5A. [c]Indicates experiment depicted in Figure 5B.

2.2.2 The intestine of *elt-2(RNAi)* animals appears to be normal but is persistently colonized by *E. coli*

RNAi-mediated ablation of *elt-2* results in fewer viable adult progeny when compared to vector control, suggesting that many animals do not survive past the L1 stage, consistent with the results obtained using animals carrying a knockout mutation

of the *elt-2* gene (Fukushige, Hawkins et al. 1998). The *elt-2(RNAi)* animals that do survive to adulthood are typically smaller and thinner than control counterparts (data not shown). Although the life span experiments (Figure 5B) indicate that *elt-2(RNAi)* animals do not die by starvation, we decided to study whether *elt-2(RNAi)* nematodes exhibit the Gob phenotype observed in *elt-2* knockouts (Fukushige, Hawkins et al. 1998). Vector control and *elt-2(RNAi)* animals were exposed to *E. coli* expressing the fluorescence marker DSred and observed by confocal microscopy at different timepoints. No blockage in the intestine was observed within 48 hours of exposure; bacteria were seen to span the entire length of the intestinal tract (data not shown). Additionally, bacteria seemed to stay within the area of the intestinal lumen, with no evidence of escape into neighboring tissues (Figure 6B). This indicates that intestinal blockage cannot be the cause of *elt-2(RNAi)* animal death seen upon exposure to *S. enterica* (Table 1 and Figure 5A) and that there are no apparent anatomical abnormalities that allow bacteria to infect other tissues of the organism.

Interestingly, within 24 hours, *elt-2(RNAi)* animals show distended intestines full of *E. coli* that clearly contrast with the non-distended intestinal lumens of control animals (Figure 6). This intestinal distension was not seen within 20 minutes of exposure to *E. coli* expressing DSred (data not shown), which indicates that intestinal distension of *elt-2(RNAi)* animals is caused by proliferating *E. coli* rather than by an abnormal intestinal structure. This is not surprising given that proliferating *E. coli* is a cause of

death in *C. elegans* (Garigan, Hsu et al. 2002), that *E. coli* grown on rich media kills *C. elegans* (Garsin, Sifri et al. 2001), and that immunocompromised animals are killed by *E. coli* (Singh and Aballay 2006). Additionally, after 24 hours of exposure to *E. coli* expressing DSred, *elt-2(RNAi)* animals exhibited intestinal colonization by the bacteria; DSred fluorescence was present in the *elt-2(RNAi)* nematode intestine even after three one-hour-transfers onto fresh nonfluorescent *E. coli* plates (data not shown). This indicates that *E. coli* expressing DSred is able to persistently colonize *elt-2(RNAi)* animals, which is of interest as *E. coli* does not typically colonize the nematode intestine in a persistent fashion (Aballay, Yorgey et al. 2000).

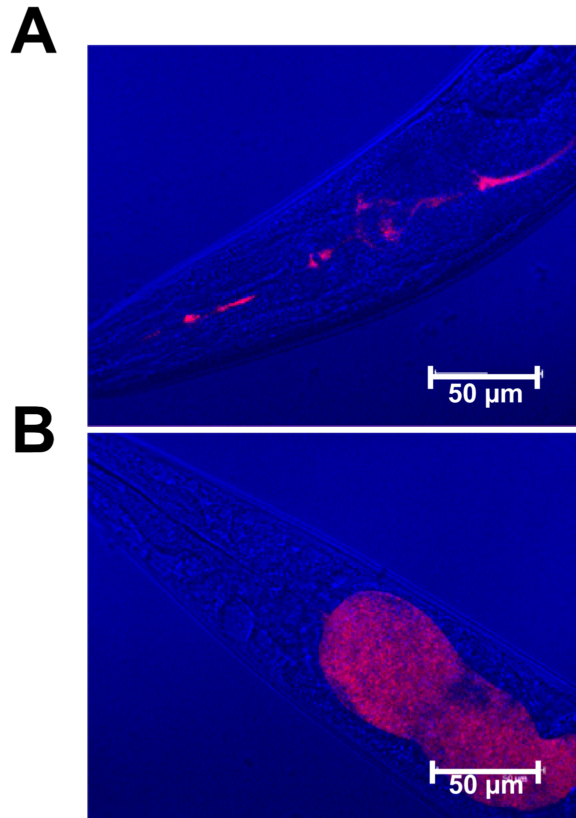

Figure 6: *E. coli* persistently colonizes the intestines of *elt-2(RNAi)* nematodes

Wild-type nematodes grown on *E. coli* carrying vector control **(A)** or on *E. coli* expressing *elt*-2 dsRNA **(B)** were exposed to *E. coli* expressing DSred for 24 hours than visualized.

To ensure that the intestinal distension and *E. coli* colonization are not caused by an unseen blockage or an inability of the intestinal muscles to move bacteria through the gut, the defecation of *elt-2(RNAi)* nematodes exposed to *E. coli* was measured. As shown in Figure 7, defecation of *elt-2(RNAi)* animals is not significantly different from vector control animals.

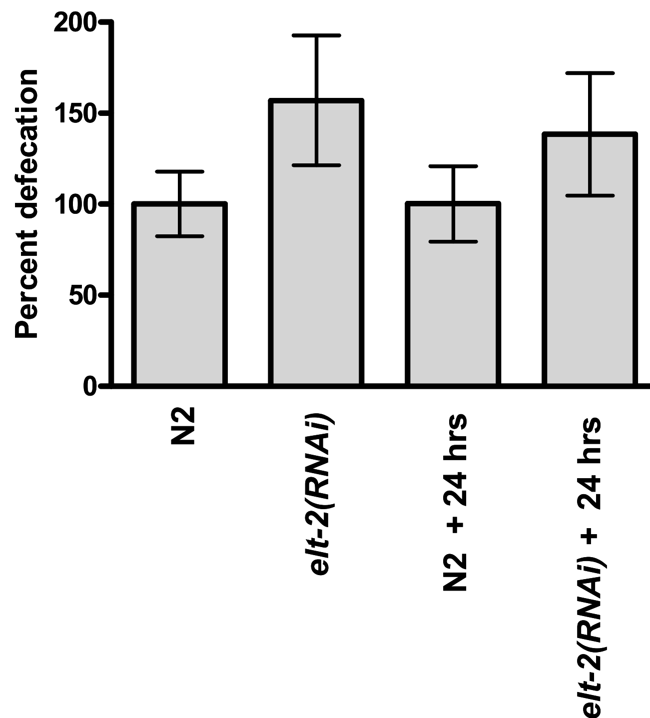

Figure 7: *elt-2(RNAi)* nematodes do not show a defecation defect

Wild-type nematodes grown on *E. coli* carrying vector control or on *E. coli* expressing *elt-2* dsRNA were either immediately or after 24 hours exposure transferred to a clean LB plate, where they were allowed to defecate for 2 hours. Plates were then placed at 37°C overnight and colonies were counted. The combined data of 10 animals are shown, and the data were normalized to the median colony count for the vector control at each timepoint.

2.2.3 ELT-2 controls expression of *clec-67* which is a marker of *C. elegans* immune response to *S. enterica*

To provide further evidence of the role of ELT-2 in innate immunity, we studied its involvement in the expression of the gene *clec-67*, which encodes a C-type lectin domain. Traditionally, C-type lectins function as soluble mediators that specifically bind carbohydrates, resulting in pathogen clearance by opsonization. However, recent evidence illustrates the importance of lectins as receptors in the immune response and it

has been proposed that they participate in the recognition of pathogen-associated molecular patterns in *C. elegans* (Nicholas and Hodgkin 2004). In addition, genes encoding C-type lectin domains have been found to be upregulated in *C. elegans* by a human opportunistic pathogen (Mallo, Kurz et al. 2002), a nematode specific pathogen (O'Rourke, Baban et al. 2006), and a bacterial toxin (Huffman, Abrami et al. 2004), indicating that they are good markers of *C. elegans* immunity.

We decided to use *clec-67* as a marker of *C. elegans* immunity to *S. enterica* because it is consistently upregulated by this pathogen in several expression profiling experiments performed in our laboratory, because it is expressed in the intestine (Figure 8A), and because its promoter region contains a GATA binding site (A/T GATA A/G). As shown in Figure 8B, the *clec-67* upregulation by *S. enterica* observed in our microarrays was confirmed by qRT-PCR. Figure 8B also shows that when *elt-2* expression is ablated by RNAi, *clec-67* is downregulated compared to vector control, indicating that ELT-2 regulates the expression of this effector of *C. elegans* immunity to *S. enterica*. To ensure that the differences in expression pattern of *clec-67* are not due to intestinal defects in *elt-2(RNAi)* animals, we measured the expression of six intestinal housekeeping genes in *elt-2(RNAi)* and vector control nematodes exposed to *S. enterica* (Figure 8C). The expression of each of these genes is less than two-fold different from vector control, well within the error range of the assay, and much less than the differences seen in expression of *clec-67*. Therefore, the change in the expression pattern

of *clec-67* is due to changes in ELT-2 levels, and not to nonspecific changes in intestinal gene expression.

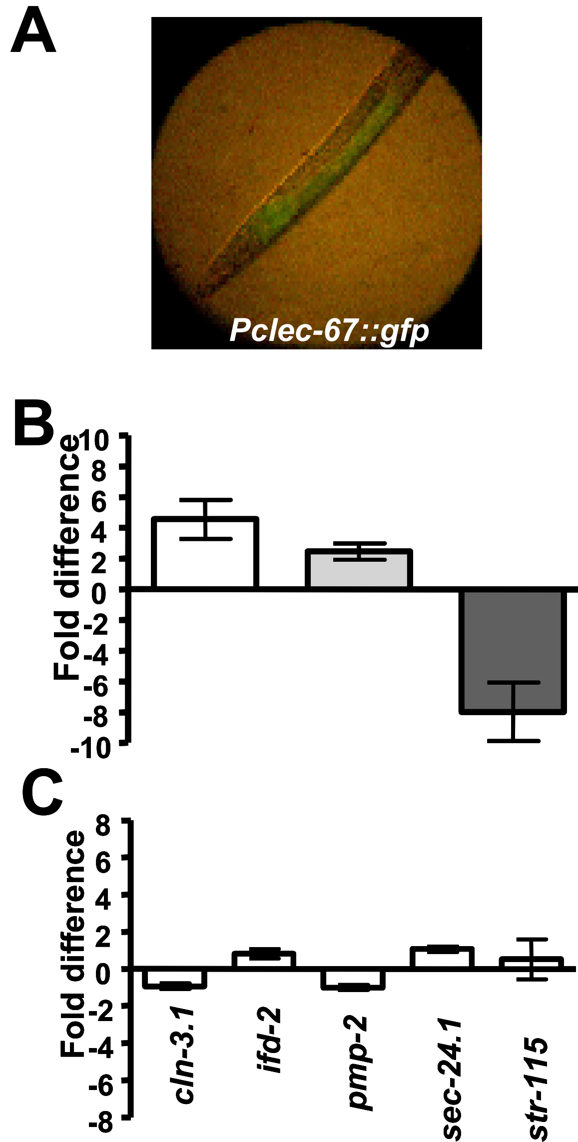

Figure 8: Expression of *clec-67* is controlled by ELT-2

(A) *Pclec-67:gfp* nematodes were visualized. **(B)** Microarray (white) and qRT-PCR comparing expression of *clec-67* in wild-type nematodes grown on *S. enterica* versus wild-type nematodes grown on *E. coli*. qRT-PCR comparing expression of *clec-67* in wild-type nematodes grown on *E. coli* expressing vector control versus *elt-2(RNAi)* nematodes (dark grey). **(C)** qRT-PCR of a variety of intestinal housekeeping genes in wild-type nematodes grown on *E. coli* expressing vector control versus *elt-2(RNAi)* nematodes. Results are averages of 5 experiments.

2.2.4 ELT-2 is required for proper *C. elegans* immunity to a variety of microbial pathogens

To determine whether ELT-2 is part of an immune system specific to *S. enterica* or whether it is required for immunity to pathogens in general, *elt-2(RNAi)* animals were infected with the Gram-negative bacterial pathogen *P. aeruginosa* (Tan, Mahajan-Miklos et al. 1999), the Gram-positive bacterial pathogen *E. faecalis* (Garsin, Sifri et al. 2001), and the fungal pathogen *C. neoformans* (Mylonakis, Ausubel et al. 2002) as described. For all of these pathogens, *elt-2(RNAi)* animals exhibited increased mortality compared to vector control animals (Figure 9). These data indicate that RNAi ablation of *elt-2* increases *C. elegans* susceptibility to different types of bacterial and fungal pathogens.

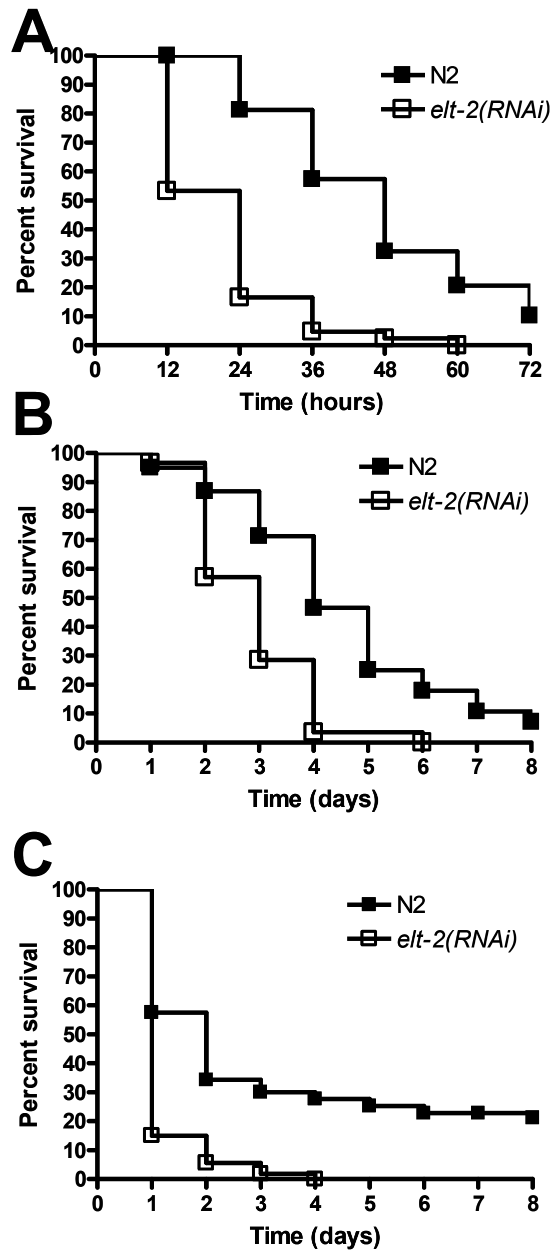

Figure 9: *elt-2(RNAi)* animals are more susceptible than wild-type nematodes to a variety of pathogens.

Wild-type nematodes grown on *E. coli* carrying a vector control plasmid or on *E. coli* expressing *elt-2* dsRNA were exposed to **(A)** *P. aeruginosa* (P = 0.0001), **(B)** *E. faecalis* (P < 0.0001), or **(C)** *C. neoformans* (P < 0.0001). 60-120 nematodes were used for each condition. Results are representative of at least 3 independent experiments.

2.2.5 ELT-2-dependent regulation of the *C. elegans* immune system is independent of the DAF-2/DAF-16 insulin-PI3K pathway

The DAF-2/DAF-16 insulin-like pathway which regulates longevity in *C. elegans* has recently been linked to immunity against bacteria. DAF-16 is a FOXO transcription factor, which regulates a wide variety of genes required for longevity, stress-response, development, and immunity (Henderson and Johnson 2001; Lee, Kennedy et al. 2003; Murphy, McCarroll et al. 2003), and it is negatively regulated by DAF-2 (Lee, Hench et al. 2001). Thus, *daf-2* mutants exhibit higher DAF-16 activity and are more resistant to different bacterial pathogens (Garsin, Villanueva et al. 2003; Anyanful, Dolan-Livengood et al. 2005; Singh and Aballay 2006). Because our data indicate that ELT-2 regulates a multi-pathogen immune response, we studied whether the GATA transcription factor is required for the effects of DAF-2 in immunity to pathogens. First, we studied whether *daf-2(e1370)* mutants were more resistant to the fungal pathogen *Cryptococcus neoformans* and then whether RNAi ablation of *elt-2* reduces the resistance phenotype of *daf-2(e1370)* animals. As shown in Figure 10, *daf-2(e1370)* mutants are more resistant not only to *S. enterica* and *E. faecalis*, as previously shown (Garsin, Villanueva et al. 2003), but also to *Cryptococcus neoformans*, indicating that DAF-2 regulates immune response to pathogens in general. Furthermore, this increased resistance to *C. neoformans* is dependent upon DAF-16 function as *daf-2(e1370);daf-16(RNAi)* nematodes have increased mortality when compared to *daf-2(e1370)* mutants (Figure 10D).

To address whether the DAF-2 pathway regulates the immune system of the nematode via ELT-2, RNAi was used to ablate *elt-2* in *daf-2(e1370)* animals which were then exposed to *S. enterica, E. faecalis*, and *C. neoformans*. Visibly, the *daf-2(e1370);elt-2(RNAi)* nematodes appear similar to *elt-2(RNAi)* animals, being smaller, thinner, and more transparent than wild-type nematodes or *daf-2(e1370)* mutants. However, the *daf-2(e1370)*; *elt-2(RNAi)* nematodes are significantly more resistant to all pathogens tested than *elt-2(RNAi)* animals (Figure 10). Interestingly, *daf-2(e1370); elt-2(RNAi)* animals have increased resistance to *E. faecalis* compared to *daf-2(e1370)* animals, indicating that ELT-2 may suppress immune defenses specific to *E. faecalis* that are typically inhibited by DAF-2. Controls of *daf-2(e1370);daf-16(RNAi)* were performed and demonstrated increased susceptibility to all pathogens when compared to *daf-2(e1370)* (Figure 10D and data not shown).

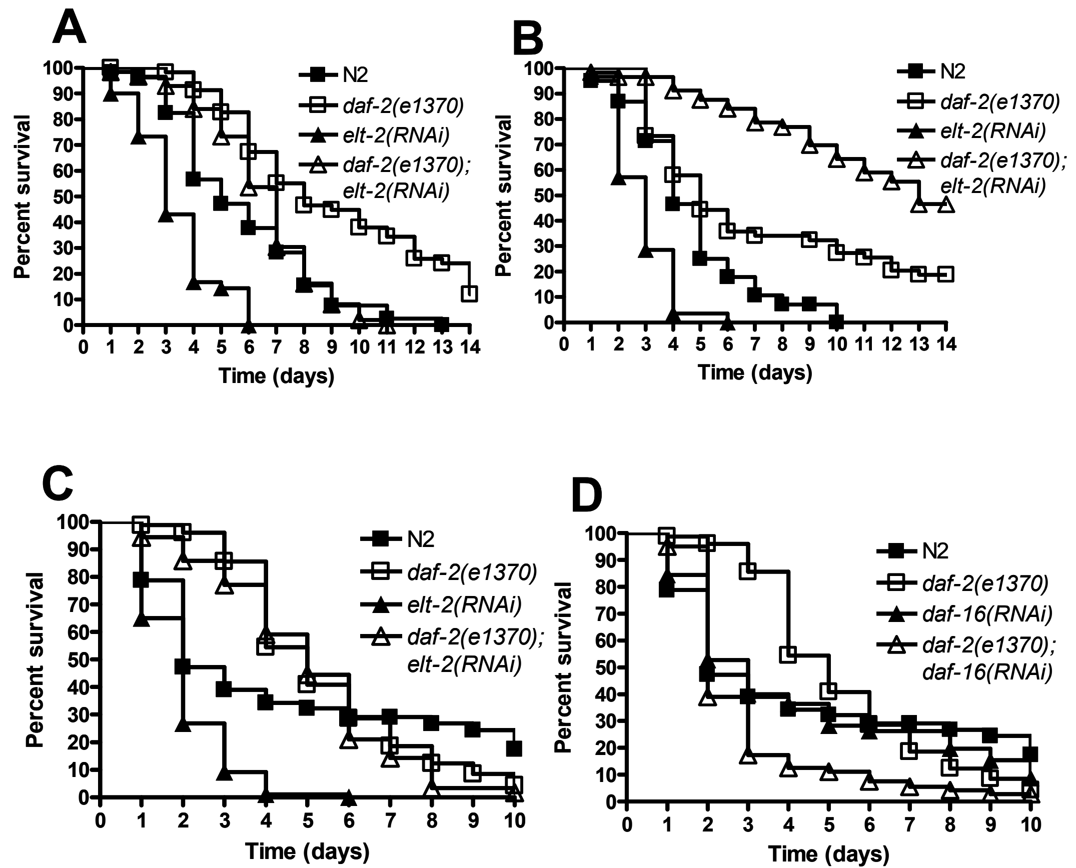

Figure 10: The *daf-2(e1370)* mutation rescues the increased susceptibility to pathogens phenotype of *elt-2(RNAi)* animals

(A) Wild-type and *daf-2(e1370)* nematodes grown on *E. coli* carrying a vector control plasmid or on *E. coli* expressing *elt-2* dsRNA were exposed to *S. enterica*. Significant differences were found when wild-type was compared to *daf-2(e1370)* ($P < 0.0001$), when *elt-2(RNAi)* was compared to *daf-2(e1370);elt-2(RNAi)* ($P < 0.0001$), and when *daf-2(e1370)* was compared to *daf-2(e1370);elt-2(RNAi)* ($P < 0.0001$). **(B)** Wild-type and *daf-2(e1370)* nematodes grown on *E. coli* carrying a vector control plasmid or on *E. coli* expressing *elt-2* dsRNA were exposed to *E. faecalis*. Significant differences were found when wild-type was compared to *daf-2(e1370)* ($P = 0.0056$), when *elt-2(RNAi)* was compared to *daf-2(e1370);elt-2(RNAi)* ($P < 0.0001$), and when *daf-2(e1370)* was compared to *daf-2(e1370);elt-2(RNAi)* ($P < 0.0001$). **(C)** Wild-type and *daf-2(e1370)* nematodes grown on *E. coli* carrying a vector control plasmid or on *E. coli* expressing *elt-2* dsRNA were exposed to *C. neoformans*. Significant differences were found when wild-type was compared to *daf-2(e1370)* ($P = 0.0174$) and when *elt-2(RNAi)* was compared to *daf-2(e1370);elt-2(RNAi)* ($P < 0.0001$). No significant difference was found when *daf-2(e1370)*

was compared to *daf-2(e1370);elt-2(RNAi)* (P = 0.1666). **(D)** Wild-type and *daf-2(e1370)* nematodes grown on *E. coli* carrying a vector control plasmid or on *E. coli* expressing *daf-16* dsRNA were exposed to *C. neoformans*. A significant difference was found when *daf-2(e1370)* was compared to *daf-2(e1370);daf-16(RNAi)* (P < 0.0001), but no significant difference was found when wild-type was compared to *daf-16(RNAi)*. 60-240 nematodes were used for each condition. Results are representative of at least 3 independent experiments.

2.3 Discussion

Our results suggest that the GATA transcription factor ELT-2 regulates a *C. elegans* innate immunity independently of DAF-2/DAF-16 and that it regulates the expression of *clec-67* which is a marker of *C. elegans* immunity to *S. enterica* infection. Previously, ELT-2 had been linked to intestinal development and the regulation of genes involved in intestinal homeostasis, including *sphingosine-1-phosphate lyase*, *pho-1*, *mtl-1*, *mtl-2*, and *pha-4* (Fukushige, Hawkins et al. 1998; Kalb, Lau et al. 1998; Fukushige, Hendzel et al. 1999; Moilanen, Fukushige et al. 1999; Fukushige, Goszczynski et al. 2003; Fukushige, Goszczynski et al. 2005; Oskouian, Mendel et al. 2005), but its role in innate immunity was unknown. Since DAF-16 does not appear to be required for immunity to bacterial (Garsin, Villanueva et al. 2003) and fungal pathogens (Figure 10D), the ELT-2 requirement for proper immunity to bacterial and fungal pathogens described here provides the first direct evidence indicating that a *C. elegans* transcription factor is required not only for the expression of immunity-related genes but also for survival in the presence of pathogens. These results are further supported by a paper that was

published while this research was ongoing which also indicated that ELT-2 was required for immunity to *P. aeruginosa* (Shapira, Hamlin et al. 2006).

This work shows the importance of GATA transcription factors in *C. elegans* immunity. The most well-known mammalian GATA transcription factor is GATA-3, which regulates the development of CD4+ T cells to a Th2 phenotype (Zheng and Flavell 1997). However, since *C. elegans* does not have adaptive immunity, our data indicate that GATA transcription factors are also involved in innate immune responses. This is supported by other invertebrate models, where GATA binding elements have been found in the promoters of genes important in defense responses of *D. melanogaster* (Petersen, Kadalayil et al. 1999; Bernal and Kimbrell 2000; Wertheim, Kraaijeveld et al. 2005), the silkworm *Bombyx mori* (Cheng, Zhao et al. 2006), and the mosquito *Aedes aegypti* (Cheon, Shin et al. 2006). GATA transcription factors are evolutionarily conserved in a wide variety of eukaryotes and are most often involved in cellular development and differentiation (Patient and McGhee 2002). Human GATAs 4-6, which are the most similar to *C. elegans* ELT-2, are involved in development of gut and cardiac tissue. Additionally, human GATA-4 has been shown to regulate cardiac stress responses (Suzuki 2003; Aries, Paradis et al. 2004; Tenhunen, Sarman et al. 2004), indicating that GATA-4 may have more than a developmental role in mammals. Further evidence for this was found in a recent study that suggests that GATA-6 helps protect

A549 lung cells in tissue culture conditions from damage from *P. aeruginosa* (Shapira, Hamlin et al. 2006).

Since *C. elegans* does not appear to have functional NF-κB-like transcription factors, ELT-2 may be an ancestral transcription factor required for surviving microbial infections in a common ancestor of nematodes and vertebrates. The only other *C. elegans* transcription factor, DAF-16, known to regulate the expression of classical immune effectors is not required for defense to bacterial pathogens. We show here that like DAF-16, ELT-2 appears to regulate aging and the expression of immune effectors. In addition, it seems to be required for survival in the presence of Gram-negative bacteria, Gram-positive bacteria, and fungal pathogens. Increased DAF-16 activation, as found in the *daf-2(e1370)* mutant rescues, fully or partially, the increased susceptibility to pathogens phenotype of *elt-2(RNAi)* animals suggesting that ELT-2 acts upstream or independently of the DAF-2/DAF-16 pathway. The facts that RNAi ablation of ELT-2 results in an increased susceptibility to pathogens and that RNAi ablation of DAF-16 (Figure 10) or mutations in *daf-16* (Garsin, Villanueva et al. 2003) does not affect the susceptibility to pathogens argue in favor of the second possibility. Taken together, our results indicate that DAF-16 and ELT-2 are two key transcription factors that regulate two independent pathways required for *C. elegans* innate immunity to microbial pathogens.

2.4 Materials and Methods

2.4.1 Microbial and Nematode Strains

The following strains were used: *Escherichia coli* OP50 (Brenner 1974), *Salmonella enterica* serovar *typhimurium* SL1344 (Wray and Sojka 1978), *Enterococcus faecalis* OG1RF (Murray, Singh et al. 1993), *Cryptococcus neoformans* H99 (Franzot, Salkin et al. 1999), and *Pseudomonas aeruginosa* PA14 (Tan, Mahajan-Miklos et al. 1999). *C. elegans* strains utilized were wild-type N2 and *daf-2(e1370)*. These strains were originally obtained from the Caenorhabditis Genetics Center and maintained in our laboratory.

2.4.2 Transgenic Animals

To generate the *Pclec-67::gfp* construct, the genomic region 520 to 20 bp upstream of *clec-67* was PCR amplified using the following primers generated by PCR Primer Design for *C. elegans* promoter::gfp fusions (http://elegans.bcgsc.bc.ca/promoter_primers/): TCGTTTCTAATGCTCTCGGAA and TGGGTCCTTTGGCCAATCCCGGGGCGTTTTATGCGGGTTTGTTTA. The *gfp* sequence was amplified from pPD95.79 (Addgene, Cambridge, MA) by PCR with the following primers: CCCGGGATTGGCCAAAGGACCCAAAG and CCGCTTACAGACAAGCTGTGACCG. These PCR products were mixed and an additional PCR with the outside primers was performed to fuse the promoter and *gfp* sequences. The linear PCR product of this reaction was injected at 10 ng/μl into N2 animals to create the transgenic line. The coinjection marker pRF4 was injected at 50

ng/μl. Transgenic animals were anesthetized with 10% sodium azide solution and visualized using a Leica MZ FLIII fluorescence stereomicroscope.

2.4.3 *C. elegans* Killing Assays

C. elegans wild-type N2 animals and *daf-2* mutants were maintained as hermaphrodites at 15°C, grown on modified NG agar plates and fed with *E. coli* strain OP50 as described (Brenner 1974). Cultures were grown in Luria-Bertani (LB) broth and agar plates, except *C. neoformans* H99 and *E. faecalis* OG1RF which were grown in yeast peptone dextrose (YPD) and brain-heart infusion (BHI) medium, respectively. All pathogens were grown at 37°C except *C. neoformans* which was grown at 30°C. Pathogen lawns used for *C. elegans* killing assays were prepared by spreading 10-20 μl of an overnight culture of the bacterial strains on modified NG agar medium (0.35% peptone) in 3.5 cm diameter Petri plates. *C. neoformans* and *E. faecalis* were plated on BHI with 50 μg/ml gentamycin selection. Plates were incubated overnight before seeding them with young adult RNAi animals, created as described above. The killing assays were performed at 25°C and animals were transferred once a day to fresh plates, until no more progeny were evident. Animals were scored at the times indicated and considered dead upon failure to respond to touch.

2.4.4 *C. elegans* Defecation Assays

RNAi animals were transferred individually either directly from RNAi plates (time 0) or after 24 hours of exposure to *E. coli* OP50 on modified NGM plates with

0.35% peptone. Animals were initially individually transferred to clean LB agar plates for 10 minutes to remove excess bacteria, then transferred to a new LB agar plate and allowed to defecate for 2 hours at 25°C. Animals were then removed, and the plates incubated overnight at 37 °C. Amount of defecation was determined by counting colonies of *E. coli* on each plate. Data were normalized to the median colony count of vector control for each experiment.

2.4.5 *C. elegans* aging assays

Modified NGM plates containing 0.35% peptone and 100 ug/ml 5-fluorodeoxyuridine FUdR (Gandhi, Santelli et al. 1980) were plated with *E. coli* OP50 lawns and allowed to incubate at 37°C overnight. Young adult RNAi animals were seeded onto these plates, and scored as indicated for *C. elegans* killing assays. As FUdR inhibits growth of progeny, daily transfer of nematodes was unnecessary.

2.4.6 RNA interference

We used RNA interference to generate loss-of-function RNAi phenotypes by feeding worms with *E. coli* strain HT115(DE3) expressing double-stranded RNA that is homologous to a target gene (Timmons and Fire 1998; Fraser, Kamath et al. 2000). Briefly, *E. coli* harboring the appropriate vectors were grown in LB broth containing ampicillin (100 μg/ml) and tetracycline (10 μg/ml) at 37°C overnight. Bacteria were plated onto NGM plates containing 100 μg/ml ampicillin and 10 mM Isopropyl β-D-

thiogalactoside (IPTG) to induce double-stranded RNA expression, and were allowed to grow overnight at 37°C.

Gravid adults were allowed to lay eggs on RNAi-expressing lawns of bacteria for 6-12 hours. The eggs were allowed to develop into young adults on RNAi or vector control plates at 25°C or at 15°C in experiments involving *daf-2(e1370)*. *Unc-22* RNAi was used as a positive control for the creation of loss-of-function phenotypes. Bacterial strains expressing double-stranded RNA to inactivate the *C. elegans* genes have been described (Kamath, Fraser et al. 2003). The clone identity was confirmed by sequencing.

2.4.7 Statistical analyses

Animal survival was plotted as a staircase curve using the PRISM (version 4.00) computer program. Survival curves are considered significantly different than the control when P values are <0.05. Prism uses the product limit or Kaplan-Meier method to calculate survival fractions and the logrank test, which is equivalent to the Mantel-Heanszel test, to compare survival curves. The time for 50% of the nematodes to die (time to death 50, TD_{50}) was calculated using Prism software (version 4.01) using a non-linear regression analysis of survival proportions utilizing the equation: Y=Bottom + (Top-Bottom)/(1 + $10^{(LogEC_{50}-X)*Hill\ Slope)}$), where Top is set at 100, Bottom is set at 0, X is the time in days and Y is the percentage of nematodes alive at time X. In this instance, TD_{50} is equivalent to $LogEC_{50}$. The relative mortality of *elt-2* RNAi animals was calculated

using the equation: (TD_{50} of control worms on *S. enterica*/TD_{50} of *elt-2* RNAi worms on *S. enterica*)/(TD_{50} control worms on *E. coli*/TD_{50} of *elt-2* RNAi on *E. coli*).

2.4.8 Confocal microscopy

Modified NGM plates with 0.35% peptone were plated with overnight cultures of *E. coli* OP50 carrying the pDSred Express plasmid (Invitrogen) and allowed to incubate overnight. Vector control or *elt-2* RNAi young adult nematodes were seeded onto these plates and allowed to feed at 25°C. At the times indicated, the animals were removed from the plates and placed on microscope slides with 2% agarose pads in 10% sodium azide solution. A coverslip was placed on top of the agar pad, and sealed with clear nail polish. Images were captured at the indicated magnifications using a Leica TCS SL confocal microscope and Leica Confocal software (version 2.61 Build 1537) (Leica Microsystems Heidelberg GmbH). Images were resized and adjusted for brightness and contrast in Adobe Photoshop.

2.4.9 RNA isolation

Gravid adult N2 nematodes were lysed using a solution of sodium hydroxide and bleach, washed, and the eggs synchronized overnight in S basal liquid medium at room temperature. Synchronized L1 animals were seeded onto modified NGM plates with 0.35% peptone containing *S. enterica* or *E. coli* OP50, and incubated at 25°C until the nematodes attained young adult stage (approximately 24-30 hours). The animals were then collected by washing the plates with M9 buffer, and RNA extracted using Trizol

reagent. Samples were further purified using a QIAGEN RNeasy kit and residual genomic DNA removed using DNase treatment (DNA-free kit Ambion Inc Austin TX). RNA concentration was determined by spectrophotometer and samples were diluted to 10ng/ul in RNase free water. A minimum of two individual RNA isolations was performed for each experiment.

2.4.10 Quantitative Real-time PCR (qRT-PCR)

qRT-PCR was conducted using the Applied Biosystems Taqman One-Step Real-time PCR protocol using SYBR Green fluorescence (Applied Biosystems) on an Applied Biosystems 7900HT real-time PCR machine in 96 well plate format. Primers were designed using Aceprimer version 1.2 (http://elegans.bcgsc.bc.ca/aceprimer/aceprimer.shtml). Each independent RNA preparation was measured at least twice by qRT-PCR. Within each experiment, a minimum of duplicate wells was run for every primer set. The data for each gene were normalized to the housekeeping gene, *ama-1*, the large subunit of *C. elegans* RNA polymerase II. Once normalized, the fold difference between SL1344 and OP50 samples was determined and plotted using PRISM.

3. The *C. elegans* gene *glp-4* plays a critical role both in germline development and pathogen-specific immunity

3.1 Introduction

As described earlier, *Caenorhabditis elegans* has proven to be an excellent model system to study innate immunity (reviewed in Kurz and Ewbank 2003; Gravato-Nobre and Hodgkin 2005; Kim and Ausubel 2005; Mylonakis and Aballay 2005; Sifri, Begun et al. 2005). As *C. elegans* has been demonstrated as having an inducible immune system (Mallo, Kurz et al. 2002; O'Rourke, Baban et al. 2006; Shapira, Hamlin et al. 2006; Alper, McBride et al. 2007; Pujol, Zugasti et al. 2008), there must be transcription factors involved with immunity to induce changes in gene expression. Thus far, only three transcription factors involved in *C. elegans* immunity have been identified: DAF-16 (Garsin, Villanueva et al. 2003), HSF-1 (Singh and Aballay 2006; Singh and Aballay 2006), and ELT-2 (Kerry, TeKippe et al. 2006; Shapira, Hamlin et al. 2006).

In addition to serving as a model for the study of innate immunity, *C. elegans* has also been used to study the relationship between reproduction and life span (reviewed in Mukhopadhyay and Tissenbaum 2007). Studies in a variety of species ranging from insects to mammals have demonstrated that reproduction extracts a cost in terms of lifespan (Westendorp and Kirkwood 1998; Davies, Kattel et al. 2005). Additional studies have indicated that sexual development and activity have an adverse effect on immunity (Klein and Nelson 1999; McKean and Nunney 2001). It appears likely that reproduction

competes with somatic cells for critical resources, which play a role in maximizing immune function and general stress responses.

The interaction of reproduction and longevity has been studied extensively in *C. elegans* through the use of animals lacking a germline. Nematodes that have had their germline removed, either by laser ablation or by mutation to *glp-1*,which encodes the Notch family receptor, have a much longer life span than wild-type nematodes (Hsin and Kenyon 1999; Arantes-Oliveira, Apfeld et al. 2002). This life span extension is dependent on the FOXO transcription factor DAF-16 (Berman and Kenyon 2006). In *glp-1* loss-of-function mutants, DAF-16 localizes to the nuclei of the intestinal cells, and the lifespan extension observed in these mutants is dependent on this localization (Lin, Hsin et al. 2001; Libina, Berman et al. 2003; Berman and Kenyon 2006).

As DAF-16 had been shown to play a role in immunity when it was activated by knocking out the insulin/PI3K/Akt pathway (Garsin, Villanueva et al. 2003), we were interested in determining if DAF-16 also played a role in enhancing immunity when it was activated through germline removal. This hypothesis was supported by considerable evidence. Most of the studies which have demonstrated lifespan extension in nematodes lacking a germline have been performed on live *Escherichia coli*. Although *E. coli* is the food source for the nematodes in the lab and is often considered nonpathogenic, the facts that (1) intestinal bacterial overgrowth is a leading cause of death in nematodes (Garigan, Hsu et al. 2002), (2) *E.coli* on rich media kills *C. elegans*

(Garsin, Sifri et al. 2001), and (3) *E. coli* kills immunocompromised worms (Kerry, TeKippe et al. 2006; Singh and Aballay 2006) all suggest that increased immunity to *E. coli* plays a significant role in the life span extension observed in germline-deficient animals. Consequently, we expected to find increased resistance to a wide range of pathogens in germline-deficient animals. This broad range resistance was further supported by a recent study demonstrated increased resistance to *Pseudomonas aeruginosa* in sterile mutants that was dependent on DAF-16 (Miyata, Begun et al. 2008).

In this study, we show the germline-deficient mutant *glp-1* indeed does demonstrate increased resistance to the fungal pathogen *Cryptococcus neoformans*, the Gram positive bacterial pathogen *Enterococcus faecalis*, and the Gram negative pathogen *Salmonella enterica* as well as *E. coli* and *P. aeruginosa*. However, we also demonstrate the germline-lacking mutant strain *glp-4* fails to demonstrate increased resistance to *E. coli* and *S. enterica* while showing increased resistance to *C. neoformans*, *E. faecalis*, and *P. aeruginosa*. We also demonstrate that the resistance to *C. neoformans* observed in *glp-4* mutants is caused by more than the lack of matricide, a process of internal hatching of progeny leading to the death of the adult nematode. Furthermore, we show that DAF-16 activation occurs in the intestine of *glp-4* mutant nematodes in a manner very similar to that found in *glp-1* mutants, leading to what should be an increase in resistance to a broad range of pathogens. These results lead us to suggest a model in which the *glp*-4

gene plays a role in mediating *C. elegans* immunity independent of its effects on germline development.

3.2 Results

3.2.1 *glp-4* mutant nematodes are resistant to specific pathogens in a pattern different from the resistance observed in *glp-1* mutant animals

Nematodes lacking a germline due to mutation have been shown to have an increased resistance to the quick-killing pathogen *P. aeruginosa* and the slower-killing pathogen *E. coli* (Arantes-Oliveira, Apfeld et al. 2002; Miyata, Begun et al. 2008). These studies have utilized a temperature-sensitive mutation to the gene *glp-1* (Arantes-Oliveira, Apfeld et al. 2002; Miyata, Begun et al. 2008). In order to determine if the increased immune response observed in germline-deficient animals conferred increased resistance across a range of pathogens, we tested *glp-1* mutant nematodes against a wide array of pathogens including the Gram negative bacteria *E. coli*, *P. aeruginosa*, and *S. enterica*, the Gram positive bacteria *E. faecalis*, and the fungal pathogen *C. neoformans*. The *glp-1* mutants had increased resistance to all five of these pathogens (Figure 11). This indicates that the loss of the germline leads to an increase in the general immune function of the nematode.

We also tested the temperature-sensitive mutant *glp-4* against the same array of pathogens. Similarly to *glp-1* mutants, *glp-4* mutants lack a germline (Beanan and Strome 1992). As the increased longevity and resistance observed in *glp-1* mutants has

been attributed to the absence of the germline and not a specific function of the *glp-1* gene (Arantes-Oliveira, Apfeld et al. 2002; Miyata, Begun et al. 2008), one might expect *glp-4* mutant nematodes to show a similar pattern of broad-range resistance as seen with the *glp-1* mutants. However, previous studies with *glp-4* mutant nematodes failed to show an increased resistance to *E. coli* and *S. enterica* (Tenor, McCormick et al. 2004). We observed the same results with *glp-4* mutants, which unlike the *glp-1* mutants, failed to show increased resistance to *E. coli* and *S. enterica* (Figures 11A and 11C). However, the *glp-4* mutants did demonstrate increased resistance to *P. aeruginosa*, *E. faecalis*, and *C. neoformans* (Figures 11B, 11D, and 11E).

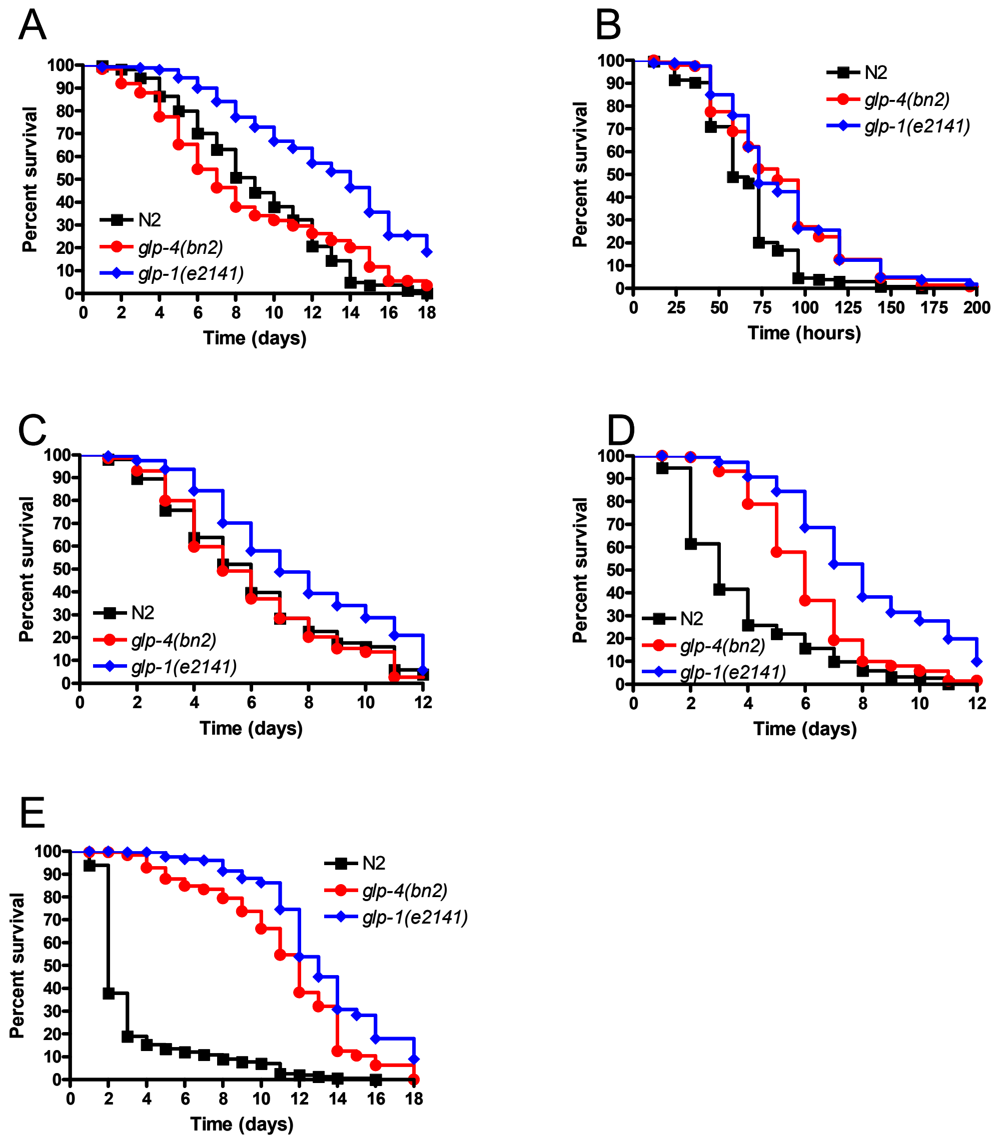

Figure 11: *glp-1* mutant nematodes are more resistant to a wide array of pathogens while *glp-4* mutants only show resistance to specific pathogens

Wild-type, *glp-4(bn2)* mutant, and *glp-1(e2141)* mutant nematodes were exposed to **(A)** *E. coli*, **(B)** *P. aeruginosa*, **(C)** *S. enterica*, **(D)** *E. faecalis*, and **(E)** *C. neoformans*. Significant differences were found when wild-type nematodes were compared to *glp-1(e2141)* mutants on all five pathogens ($P < 0.0001$). Significant differences were also found when wild-type nematodes were compared to *glp-4(bn2)* mutants on *P. aeruginosa* ($P < 0.0001$), *E. faecalis* ($P < 0.0001$), and *C. neoformans* ($P < 0.0001$) but not on *E. coli* ($P = 0.9433$) nor *S. enterica* ($P = 0.1485$). 160-300 nematodes were used for each condition.

It remained a possibility that the increased resistance to pathogens observed in *glp-1* and *glp-4* mutants was simply due to a lack of matricide, the process of internal hatching of progeny leading to the death of the adult nematode. To test this, we compared the survival of N2 wild-type, *glp-4* mutant, and *glp-1* mutant nematodes to that of *fer-1* and *fer-15* mutant nematodes. Both *fer-1* and *fer-15* mutant nematodes have defects in sperm production; thus, although those strains still contain the bulk of the germline, *fer-1* and *fer-15* mutants do not suffer from matricide due to the lack of fertilization (Ward and Miwa 1978; Roberts and Ward 1982). On *E. coli*, a pathogen that induces very little matricide, the *fer-1* and *fer-15* mutants have life spans very similar to that of N2 wild-type animals (Figure 12B). Furthermore, although the *fer-1* and *fer-15* mutants live longer than N2 wild-type animals on *C. neoformans*, a pathogen that very high rates of matricide, the *fer-1* and *fer-15* mutants do not live as long as *glp-4* or *glp-1* mutant nematodes (Figure 12A). These results indicate that the increased resistance observed in *glp-4* mutants, at least to *C. neoformans*, is not simply due to a lack of matricide.

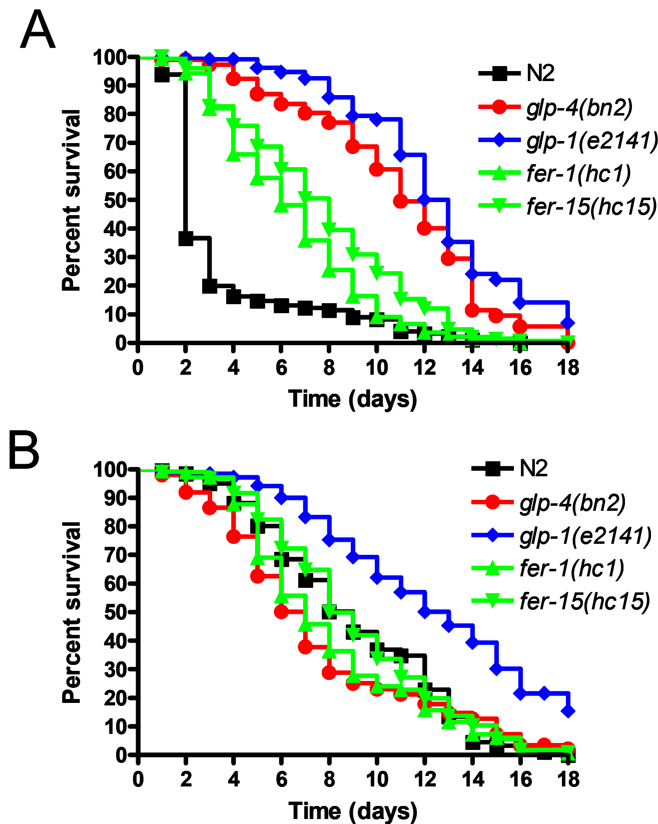

Figure 12: The resistance to pathogens found in germline-deficient mutants is not due simply to a lack of matricide

(A) Wild-type, *glp-4(bn2)* mutant, *glp-1(e2141)* mutant, *fer-1(hc1)* mutant, and *fer-15(hc15)* mutant nematodes were exposed to *C. neoformans*. When compared to wild-type nematodes, all four mutants showed significant differences ($P < 0.0001$). Significant differences were also found when *glp-4(bn2)* mutants or *glp-1(e2141)* mutants were compared to *fer-1(hc1)* ($P < 0.0001$; $P < 0.0001$ respectively) or to *fer-15(hc15)* ($P < 0.0001$; $P < 0.0001$ respectively) mutants. **(B)** Wild-type, *glp-4(bn2)* mutant, *glp-1(e2141)* mutant, *fer-1(hc1)* mutant, and *fer-15(hc15)* mutant nematodes were exposed to *E. coli*. When compared to wild-type nematodes, only *glp-1(e2141)* mutants showed significant increases in resistance ($P < 0.0001$). 260-300 nematodes were used for each condition.

The increase in resistance to *P. aeruginosa* found in *glp-1* mutants was found to be due to increased intestinal DAF-16 activity (Miyata, Begun et al. 2008). Additionally, DAF-16 activation has been shown to be critical in promoting longevity (Hsin and

Kenyon 1999; Arantes-Oliveira, Apfeld et al. 2002; Berman and Kenyon 2006). Typically, these longevity studies have been performed on live *E. coli* (Hsin and Kenyon 1999; Arantes-Oliveira, Apfeld et al. 2002; Berman and Kenyon 2006). By these standards, it appears that *glp-4* mutants do not have an increase in longevity as they do not live longer on *E. coli* (Figure 11A). To test this further, we performed a survival assay with both *glp-4* and *glp-1* mutants on heat-killed *E. coli*; we found that both *glp-4* and *glp-1* mutant nematodes live considerably longer than N2 wild-type animals (Figure 13). This suggests that both *glp-1* and *glp-4* mutant strains have extended life spans, and that *glp-4* mutants are susceptible to live *E. coli* as they do not live proportionally as long on live *E. coli* as they do on heat-killed *E. coli*.

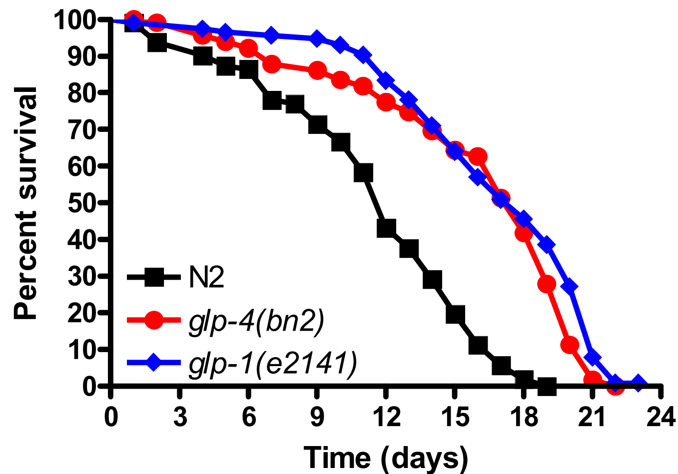

Figure 13: Both *glp-4* and *glp-1* mutant animals show increased life spans on heat-killed *E. coli*

Wild-type, *glp-4(bn2)* mutant, and *glp-1(e2141)* mutant nematodes were placed on lawns of heat-killed *E. coli* and survival was measured. When compared to wild-type nematodes, both *glp-4(bn2)* mutants ($P < 0.0001$) and *glp-1(e2141)* mutants ($P < 0.0001$) showed significant differences. 120 nematodes were used in each condition.

3.2.2 Mapping the location of the *glp-4* gene and mutation

The *glp-4* gene and the mutation responsible for the *glp-4* phenotype had not been previously identified. Previous efforts to map the location of the mutation had placed it on Chromosome I (www.wormbase.org). These efforts in mapping had utilized multi-point mapping techniques by crossing the *glp-4* mutant to marker strains. Members of our group worked on developing high speed methods of mapping mutations in *C. elegans*, and they utilized a high-throughput single nucleotide

polymorphism (SNP) called Ampliflour® to confirm the location of the *glp-4* mutation (Fuhrman, Shianna et al. 2008).

Recently, whole genome sequencing has been shown to be an efficient method identifying mutations of interest in *C. elegans* (Hillier, Marth et al. 2008; Sarin, Prabhu et al. 2008; Shen, Sarin et al. 2008). To take advantage of this method, we sequenced the genome of the *glp-4* mutant utilizing a Solexa Sequence Analyzer (Illumina). Paired end reads of 36 base pairs were obtained, and the reads were aligned to the wild-type reference genome. We then examined a 500 kb region surrounding the mapped location of the *glp-4* mutation for homozygous SNPs or small insertions or deletions (InDels). We limited our interest in the SNPs to homozygous SNPs as the recessive quality of the *glp-4* phenotype strongly suggested that mutation involved must be homozygous to produce an effect. We failed to identify any InDels in the region of interest, but ten SNPs that were possible mutations responsible for the phenotype were identified (Table 3). We then performed PCRs of the areas around the SNPs of interest and sequenced these PCR products to confirm the whole genome sequencing results. Of the ten SNPs, five were confirmed to not be potential causes for the *glp-4* mutation, either because sequence of the PCR products failed to confirm the whole genome sequence results (instead, showing the same result as the wild-type reference strain) or in one instance, because sequencing of the wild-type nematode demonstrated the same result as the

whole genome sequence indicating either an error in the reference strain or a potential polymorphism at location (Table 3).

Table 3: Potential SNPs responsible for *glp-4* phenotype identified by whole genome sequencing

Position	Gene	Location	Reference Sequence	Whole Genome Sequence	PCR sequence confirmation
13,749,980	*Y6B3B.9*	intron	A	T	?
13,773,177	*Y37H9A.1*	exon	G	A	Yes
13,853,849	*W04A8.6*	intron	G	A	Yes
13,860,805	*taf-1*	upstream	C	T	No
13,860,836	*taf-1*	upstream	G	C	No
13,950,663	*Y71A12B.9*	intron	A	T	Yes
13,985,037	*Y71A12B.17*	exon	A	G	Yes*
14,049,138	*smd-1*	intron	T	C	?
14,083,381	*fbxb-93*	intron	A	C	No
14,097,923	*Y63D3A.4*	intron	T	C	No

For PCR sequence confirmation, "Yes" indicates that we have confirmed that the *glp-4* mutant strain matches that of the whole genome sequence, "No" indicates that the *glp-4* mutant matches the reference strain, and "?" indicates that successful PCR sequence confirmation has not yet been completed.

*During PCR sequencing, it was discovered that the N2 wild-type strain in our lab along with the *glp-4* mutant strain demonstrated the SNP found in the whole genome sequence rather than that found in the reference sequence.

We chose to further focus on the mutation located at 13,773,177 on Chromosome I. This SNP is located in the first exon of the gene *Y37H9A.1* where it induces a shift in the amino acid sequence from glycine to a glutamic acid at position 40. Of the potential SNPs identified by the whole genome sequence, this SNP is the only one that would induce an amino acid change. Additional evidence that the SNP at 13,773,177 may be responsible for the *glp*-4 phenotype comes from the fact that this SNP represents a shift

in the DNA sequence from a G to an A. This is an important fact because the *glp-4* strain was created through mutagenesis with ethylmethanesulfonate (EMS) (Beanan and Strome 1992), and the primary effect of EMS mutagenesis is the production of point mutations as a result of G/C to A/T transitions (Anderson 1995).

In order to confirm that this SNP in *Y37H9A.1* was the cause of the phenotype, we attempted to compliment the *glp-4* mutation with a Y37H9A.1 transgene. This transgene was produced by a PCR, which included the entire Y37H9A.1 gene as well as the 1.6 kb upstream of the gene to serve as a promoter. We chose to utilize the 1.6 kb upstream of the gene as this was the distance in the genome between *Y37H9A.1* and the nearest upstream gene. An HA tag was also attached to this transgene to serve as a marker. The transgene was then injected into nematodes, along with the co-injection marker, *rol-6*, which encodes for a cuticle collagen protein that causes the nematodes to "roll" when overexpressed. This allows for easy identification of worms containing the transgene following injection. We were able to obtain rollers following these injections, and upon DNA sequencing, these animals were shown to contain the *Y37H9A.1:HA* transgene. Unfortunately, these transgenics failed to compliment the *glp-4* phenotype. However, Western blot demonstrated that these transgenic animals failed to produce any proteins containing the HA tag. Thus, it remains likely that *Y37H9A.1* is the *glp-4* gene and the SNP at 13,773,177 is the mutation responsible for the *glp-4* phenotype.

3.2.3 *glp-4* mutants fail to respond to the lack of GLP-1

In order to investigate the causes of the pattern of resistance to different pathogens found in *glp-4* mutants, we sought to knockdown both *glp-4* and *glp-1* in the same nematode. We chose to knockdown GLP-1 levels through the use of RNA interference. We then performed survival assays on *E. coli* and *C. neoformans*. On both pathogens, knocking down *glp-1* gene expression via RNAi in N2 wild-type nematodes caused an increased resistance to the pathogen when compared to the N2 wild-type animals treated with vector control (Figure 14). However, *glp-4; glp-1(RNAi)* nematodes showed no difference in resistance to either *E. coli* or *C. neoformans* when compared to *glp-4* nematodes treated with vector control (Figure 14). These assays were also performed by treating *glp-1* nematodes with *glp-1* RNAi to serve as a control for any extraneous effects of the RNAi treatment; we observed no difference in survival on either *E. coli* or *C. neoformans* between *glp-1* mutants treated with *glp-1* RNAi or those treated with vector control (data not shown).

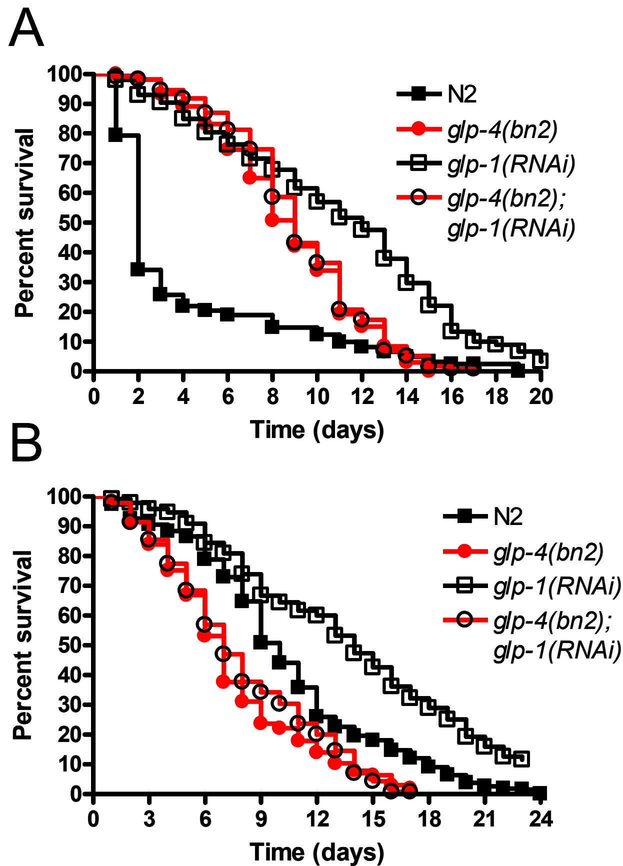

Figure 14: *glp-4* fail to respond to treatment with *glp-1* RNAi.

Wild-type and *glp-4(bn2)* mutant nematodes were grown on *E. coli* carrying a vector control plasmid or expressing *glp-1* dsRNA were exposed to **(A)** *C. neoformans* or **(B)** *E. coli*. When compared to wild-type, *glp-1(RNAi)* showed significant differences on both *C. neoformans* ($P < 0.0001$) and *E. coli* ($P < 0.0001$). No significant differences were observed when *glp-4(bn2)* mutants were compared to *glp-4(bn2);glp-1(RNAi)* animals on either *C. neoformans* ($P = 0.2257$) or *E. coli* ($P = 0.2525$). 240-420 nematodes were used for each condition.

3.2.4 *glp-4* mutants demonstrate intestinal DAF-16 activation across multiple pathogens

As mentioned earlier, the increased longevity and immune function found in *glp-1* mutants is due to increased activation of DAF-16 in the intestinal cells (Arantes-

Oliveira, Apfeld et al. 2002; Berman and Kenyon 2006; Miyata, Begun et al. 2008). In order to determine the involvement DAF-16 may have in *glp-4* mutant immune function, we knocked down DAF-16 through the use of RNA interference in N2 wild-type nematodes as well as *glp-1* and *glp-4* mutant animals. When *daf-16* is knocked down in N2 wild-type animals, we saw no effect on either *C. neoformans* (Figure 15A); this corresponds with results found in other studies (Kerry, TeKippe et al. 2006). Interestingly, *daf-16(RNAi)* nematodes had a slight but significant reduction in life span on *E. coli* (Figure 15B); this contrasts with previously reported results (Garsin, Villanueva et al. 2003). This reduction in life span on *E. coli* in *daf-16(RNAi)* nematodes seems less likely to be due to a loss of immune function than due to a loss of resistance to aging. This is suggested by the fact that no difference is seen between wild-type and *daf-16(RNAi)* nematodes throughout the first six days on *E. coli*; additional support comes from the previously unreported fact that on heat-killed *E. coli*, DAF-16 is required for a proper life span (Figure 16). However, with both *glp-1* and *glp-4* mutants, the knockdown of DAF-16 via RNAi leads to a reduction in life span when compared to vector control-treated nematodes (Figure 15). Furthermore, this reduction in life span occurs on both *C. neoformans* and *E. coli*, and on *E. coli*, the reduction in life span with both *glp-4* and *glp-1* mutants happens much sooner than in wild-type nematodes as the curves begin to separate on the third day of the experiment as opposed to the sixth day with wild-type nematodes (Figure 15). Since knocking down DAF-16 does not seem to

affect the immune function of the nematode unless DAF-16 is overexpressed (Garsin, Villanueva et al. 2003; Kerry, TeKippe et al. 2006), these results suggest that DAF-16 has increased activation in both *glp-1* and *glp-4* mutant animals.

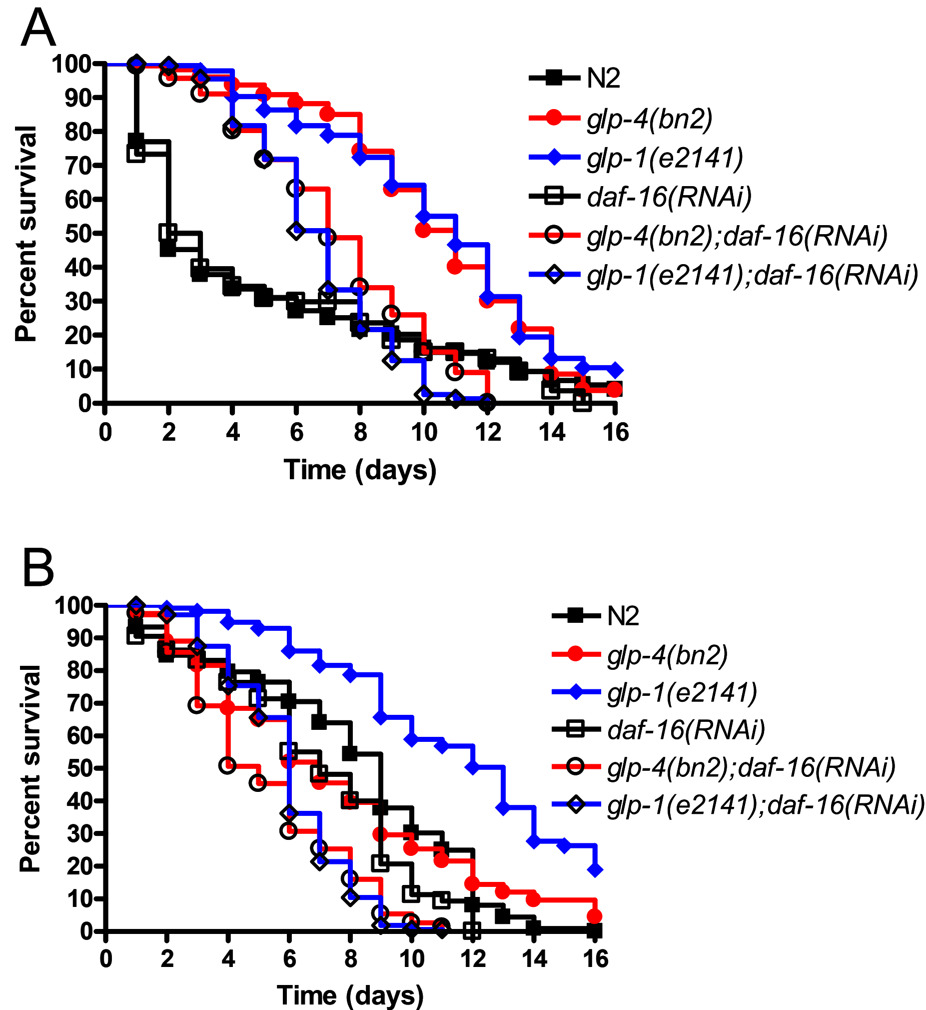

Figure 15: Life span extension observed in both germline-deficient mutants *glp-1* and *glp-4* is dependent on DAF-16

Wild-type, *glp-4(bn2)* mutant, and *glp-1(e2141)* mutant nematodes grown on *E. coli* carrying a vector control plasmid or expressing *daf-16* dsRNA were exposed to **(A)** *C. neoformans* or **(B)** *E. coli*. Significant differences were found when *glp-4(bn2);daf-16(RNAi)* worms were compared to vector control-treated *glp-4(bn2)* nematodes on both *C. neoformans* ($P < 0.0001$) and *E. coli* ($P < 0.0001$). Likewise, significant differences were found when *glp-1(e2141);daf-16(RNAi)* nematodes were compared to vector control-treated *glp-1(e2141)* nematodes on both *C. neoformans* ($P < 0.0001$) and *E. coli* ($P < 0.0001$). When wild-type nematodes were compared to *daf-16(RNAi)* animals, significant differences were seen on *E. coli* ($P < 0.0001$) but not on *C. neoformans* ($P = 0.7084$). 160-300 nematodes were used for each condition.

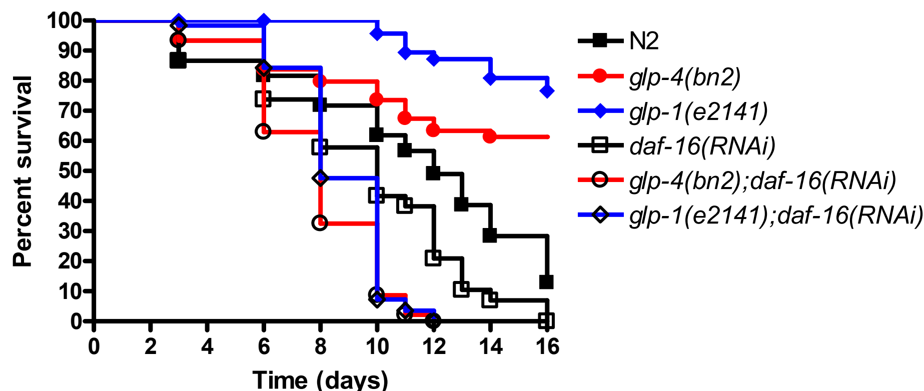

Figure 16: DAF-16 is critical for longevity in *C. elegans*

Wild-type, *glp-4(bn2)* mutant, and *glp-1(e2141)* mutant nematodes were grown on *E. coli* carrying a vector control plasmid or expressing *daf-16* dsRNA were placed on lawns of heat-killed *E. coli* and survival was measured. Significant differences were found when wild-type nematodes were compared to *daf-16(RNAi)* (P = 0.0122), when *glp-4(bn2)* mutants were compared to *glp-4(bn2);daf-16(RNAi)* (P < 0.0001), and when *glp-1(e2141)* mutants were compared to *glp-1(e2141);daf-16(RNAi)* (P < 0.0001). 60 nematodes were used for each condition.

To measure the level of DAF-16 activation directly, we crossed both *glp-1* and *glp-4* mutant nematodes to a strain containing a transgene that creates a DAF-16:GFP fusion protein under the regulation of the intestinal-specific promoter of *gly-19* (Gami, Iser et al. 2006). We chose to utilize a DAF-16:GFP fusion protein regulated by an intestinal promoter since DAF-16 is specifically activated in the intestinal cells of *glp-1* mutants and that activation is required for life span extension in *glp-1* mutants (Lin, Hsin et al. 2001; Libina, Berman et al. 2003; Berman and Kenyon 2006). We then scored worms as having predominately nuclear localization of DAF-16 (Figure 17A) or as diffusely cytoplasmic (Figure 17B). We found that although fewer *glp-4* mutant

nematodes showed DAF-16 intestinal activation than *glp-1* mutant nematodes, *glp-4* mutant animals were significantly more likely to demonstrate DAF-16 activation in the intestinal cells than N2 wild-type animals regardless of whether they were exposed to *E. coli* or *C. neoformans* (Figure 18).

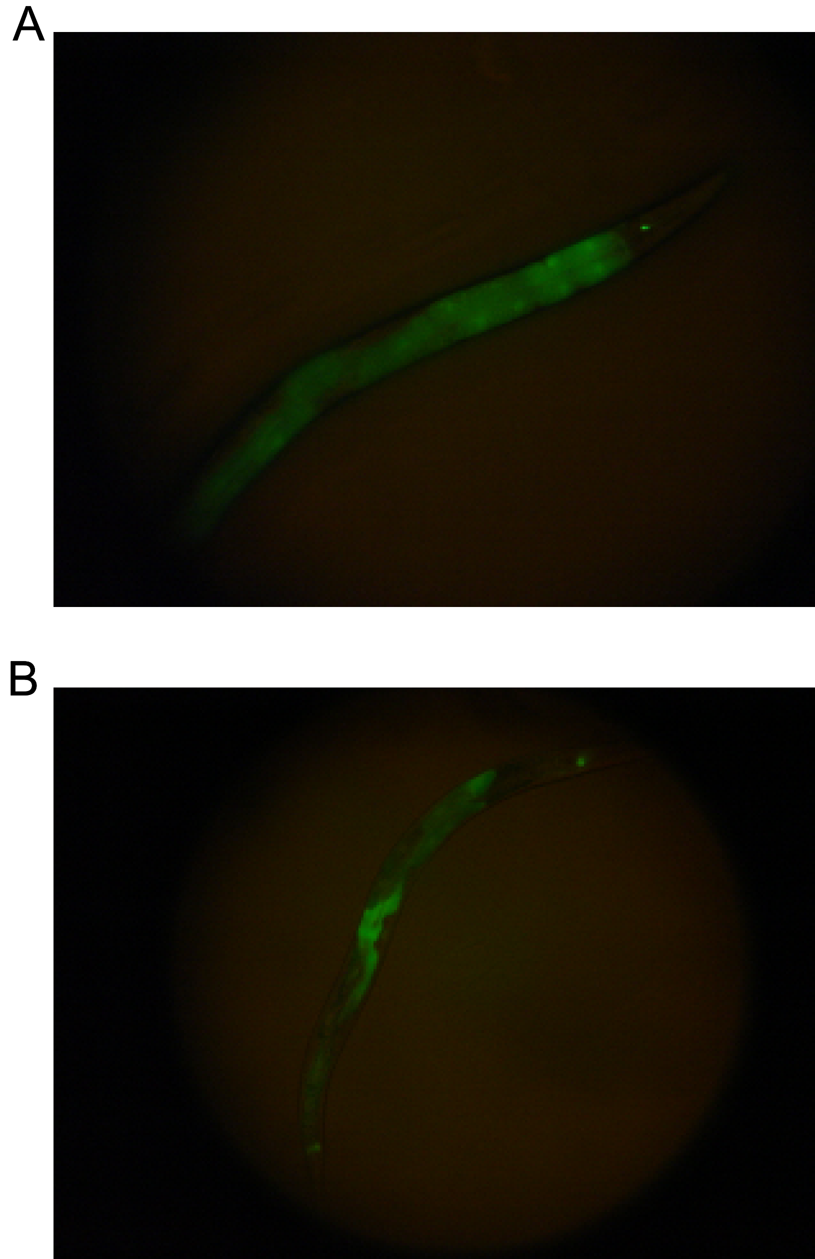

Figure 17: DAF-16:GFP transgenics

(A) A *glp-1(e2141)* mutant nematode expressing a *daf-16:gfp* transgene under control of *Pgly-19* after exposure to *E. coli*. **(B)** A wild-type nematode expressing a *daf-16:gfp* transgene under control of *Pgly-19* after exposure to *E. coli*.

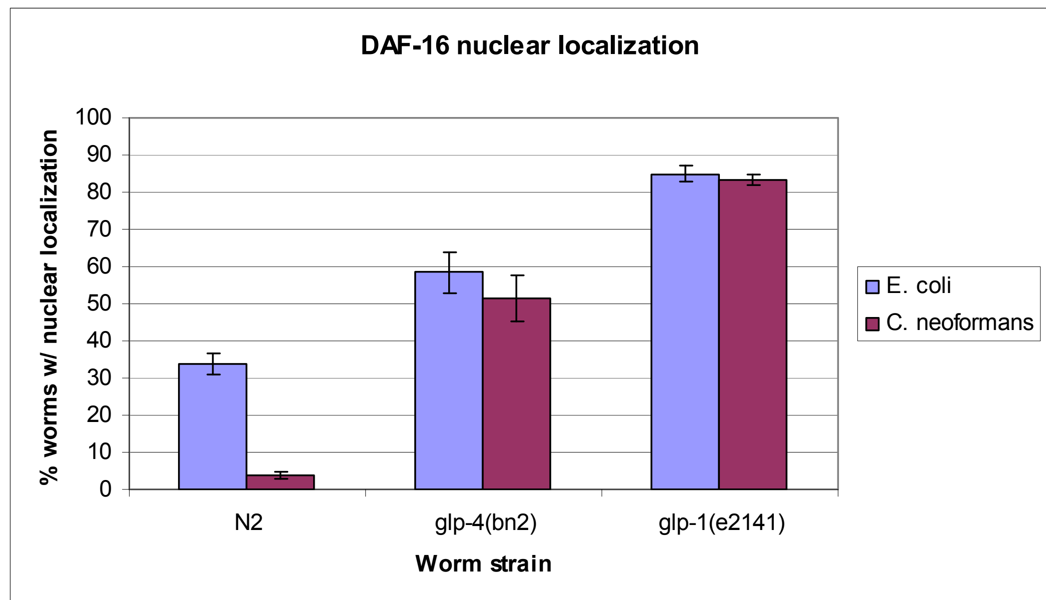

Figure 18: DAF-16 intestinal activation is more prevalent in *glp-4* and *glp-1* mutants than in wild-type nematodes regardless of pathogen

Wild-type, *glp-4(bn2)* mutant, and *glp-1(e2141)* nematodes expressing transgenic DAF-16:GFP under control of *Pgly-19* were exposed to either *E. coli* or *C. neoformans* and categorized as predominately nuclear or cytoplasmic as described in Section 3.4.8. Significant differences were found when *glp-4(bn2)* mutants were compared to wild-type on both *E. coli* (P = 0.0003) and *C.neoformans* (P < 0.0001). Likewise, significant differences were also found when *glp-1(e2141)* mutants were compared to wild-type on both *E. coli* (P < 0.0001) and *C. neoformans* (P < 0.0001). No significant differences were found when comparing the two *glp-4(bn2)* groups (P = 0.4363) nor with the two *glp-1(e2141)* groups (P = 0.7802), but there were significant differences in DAF-16 localization between the wild-type nematodes on *E. coli* and *C. neoformans* (P = 0.0002).

3.2.5 *glp-4* mutant nematodes show increased expression levels of both pro-immunity and anti-immunity genes

A microarray comparing expression profiles between *glp-4* and N2 wild-type animals has already been published (Jiang, Ryu et al. 2001). We looked to compare genes that demonstrated overexpression or underexpression from that microarray to

genes that have been shown to be differentially expressed in other microarrays related to immunity. To do this, we calculated the representation factors comparing the different groups of genes. Representation factors indicate how over- or under-represented one group of genes is in another group of genes from a different microarray. Factors greater than one indicate that the overlap between the two groups of genes is greater than expected by random chance while representation factors less than one indicate a smaller overlap than expected (further description of representation factor and the web application used in the study can be found at http://elegans.uky.edu/MA/progs/overlap_stats.html).

In the microarray comparing *glp-4* mutant nematodes to N2 wild-types, there are 407 genes which are overexpressed by at least threefold in *glp-4* mutants when compared to N2 wild-type animals (Jiang, Ryu et al. 2001). Furthermore, there are 1545 genes that are underexpressed by at least threefold when comparing *glp-4* mutants to N2 wild-type nematodes (Jiang, Ryu et al. 2001). We compared those groups of genes to genes that are upregulated and downregulated by DAF-16 (Murphy, McCarroll et al. 2003), by *P. aeruginosa* (Shapira, Hamlin et al. 2006), and by *S. enterica* (microarrays performed by our lab). We found that the genes overexpressed in *glp-4* mutants contained over-representations from all three groups that would be expected to contain pro-immunity genes: genes upregulated by DAF-16, by exposure to *P. aeruginosa*, and by exposure to *S. enterica* (Figure 19A). Interestingly, the genes that were overexpressed

in *glp-4* mutants also were overrepresented in the three groups that could be expected to contain anti-immunity genes, namely those genes downregulated by DAF-16, *P. aeruginosa*, or *S. enterica* (Figure 19A). Meanwhile, the genes that were underexpressed in *glp-4* mutants showed under-representation of the genes upregulated by DAF-16, the genes upregulated by exposure to *P. aeruginosa*, and the genes downregulated by *S. enterica*; however, the genes underexpressed in *glp-4* mutants did show an over-representation of the genes upregulated by *S. enterica* exposure (Figure 19B).

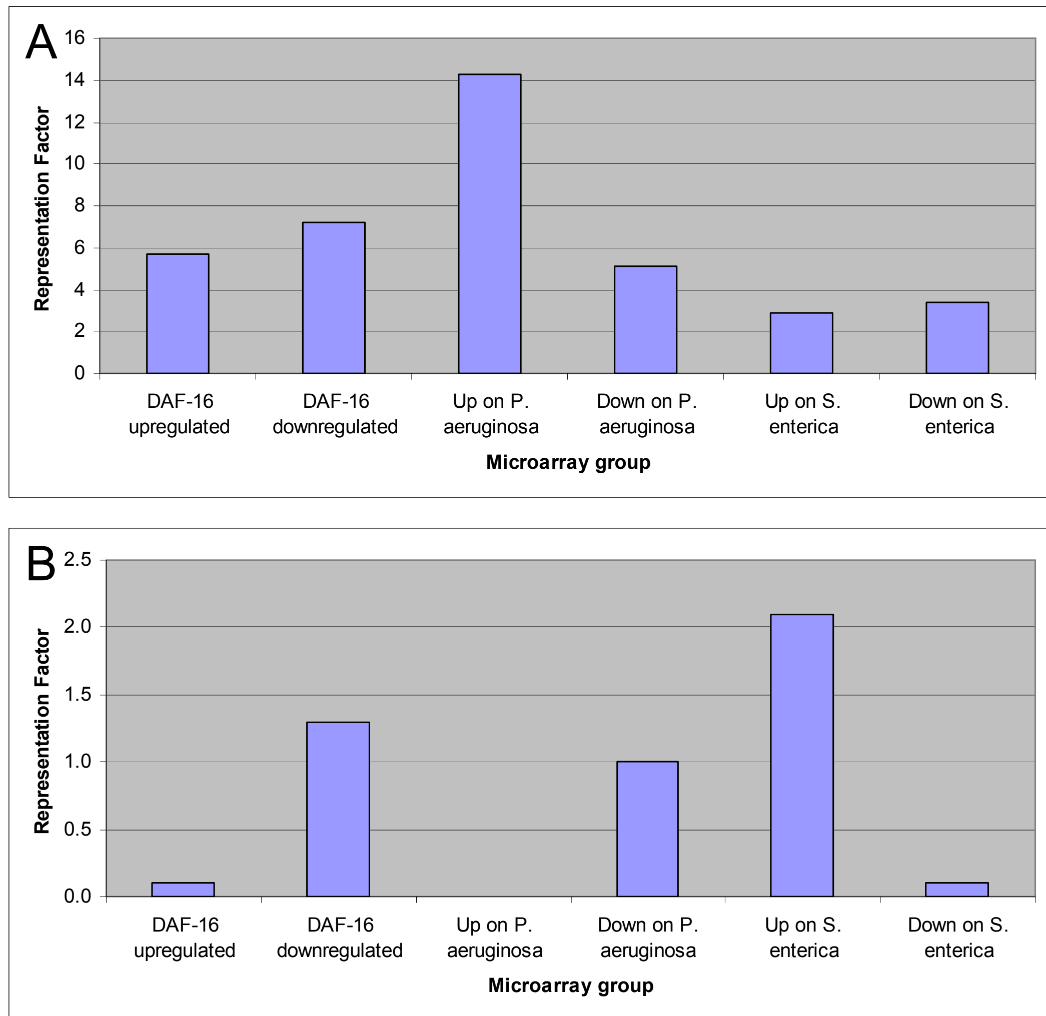

Figure 19: Representation factors comparing genes regulated by *glp-4* with genes regulated by other conditions

Representation factors were calculated as described in Section 3.4.9 comparing the overlap of genes that are **(A)** overexpressed or **(B)** underexpressed in *glp-4(bn2)* with genes that were regulated by DAF-16 (Murphy, McCarroll et al. 2003), by exposure to *P. aeruginosa* (Shapira, Hamlin et al. 2006), or by exposure to *S. enterica* (unpublished data from our group). All groups in **(A)** have a representation factor that is significantly different from 1 ($P < 0.01$) as due all the groups in **(B)** ($P < 0.01$) with the exception of the DAF-16 downregulated ($P = 0.077$) and downregulated by *P. aeruginosa* ($P = 0.417$) groups.

3.3 Discussion

3.3.1 Loss of the germline leads to increased immunity to a broad range of pathogens

The development of the germline in *C. elegans* extracts a cost in terms of the longevity and immune function; previous work had demonstrated this cost in terms of extended life spans of *E. coli* and *P. aeruginosa* for animals lacking a germline (Hsin and Kenyon 1999; Arantes-Oliveira, Apfeld et al. 2002; Miyata, Begun et al. 2008). Our results indicate that the increased immunity of the germline-deficient mutants is indiscriminate as the animals without germlines have an enhanced resistance to a wide range of pathogens including Gram positive bacteria, Gram negative bacteria, and fungi. Furthermore, it appears this increased immune function is the result of an improvement in the overall health of the nematode as seen in the extended life span of *glp-1* mutant nematodes on heat-killed *E. coli*.

3.3.2 DAF-16 plays a critical role in longevity and immunity in wild-type nematodes

DAF-16 is known to have roles both in immunity (Garsin, Villanueva et al. 2003) and longevity (Kenyon, Chang et al. 1993). However, in these studies, no effect has ever been observed when DAF-16 is knocked out in a wild-type background; instead, for effects to be observed, DAF-16 must be knocked down in a background where it is overexpressed such as a *daf-2* mutant or a germline-deficient nematode (Kenyon, Chang et al. 1993; Hsin and Kenyon 1999; Garsin, Villanueva et al. 2003). In this study, we

show that DAF-16 appears to be crucial for maintaining longevity in a wild-type background as *daf-16(RNAi)* worms have slightly shorter life spans on live *E. coli* and dramatically shorter life spans on heat-killed *E. coli* than wild-type nematodes. This finding is the first to demonstrate an affect on life span due to the loss of DAF-16 in wild-type nematodes; prior studies on a wide range of pathogens had not shown an effect (Garsin, Villanueva et al. 2003; Kerry, TeKippe et al. 2006; Singh and Aballay 2006). A recent study has proposed that DAF-16 plays a role in maintaining a basal level of immunity, but that it does not appear to be induced by pathogen; instead other immune signaling pathways such as the p38 MAP kinase pathway are induced by pathogen-exposure (Shivers, Youngman et al. 2008). Thus, a potential explanation for the shortened life span of *daf-16(RNAi)* nematodes on heat-killed *E. coli* but not on other pathogens is that the presence of live pathogens activates other signaling pathways that help extend the lifespan of the nematode.

It also appears that DAF-16 may play a key role in mitigating immune response to *C. neoformans*. In wild-type nematodes, about one third of the animals showed DAF-16 activation after 24 hour exposure to *E. coli* as adults. This figure drops by approximately 10-fold if the worms are exposed to *C. neoformans* for 24 hours instead of *E. coli.* This drop in DAF-16 activation is not observed in the two germline-deficient nematodes we tested, the *glp-1* and *glp-4* mutants, and the lack of a drop in DAF-16 activation may help explain the dramatic difference in resistance to *C. neoformans*

between wild-type nematodes and *glp-1* or *glp-4* mutant nematodes. It is unclear how or why the drop in DAF-16 activation occurs in wild-type nematodes. One possibility is that *C. neoformans* is some how able to down-regulate DAF-16 expression in wild-type nematodes, but that this process is somehow avoided in the germline-deficient nematodes. A recent study has demonstrated that *P. aeruginosa* is able to deactivate DAF-16 in the intestine of the nematode through the activation of the insulin-signaling pathway (Evans, Kawli et al. 2008); a similar process may be induced by *C. neoformans*.

3.3.3 The *glp-4* mutation has been narrowed down to a number of possible SNPs

Using whole genome sequencing, we have narrowed the number of potential mutations responsible for the *glp-4* phenotype down to 5 SNPs. Of these 5 SNPs, the one that is by far the most likely is the one located at position 13,773,177 on Chromosme I, in an exon of the gene *Y37H9A.1*. This is the most likely mutation responsible for the phenotype for a number of reasons. First, as mentioned earlier, the DNA shift is from a G to an A, which is one of the two point mutations commonly induced by EMS mutagenesis. Only one of the other SNPs that have been confirmed or have not yet been ruled out is one of the preferred point mutations. Secondly, the SNP at 13,773,177 is the only SNP we identified that causes a change in an amino acid. Finally, the amino acid shift is from a glycine, which is the smallest amino acid, neutral, and can function either as polar or nonpolar, to a glutamic acid, which is much larger, negatively charged, and highly polar.

Currently, not much is known about the gene *Y37H9A.1*. It appears to be conserved across *Caenorhabditis* species, but no orthologs outside of the nematode system have been identified. The protein does seem to contain a weakly conserved C-terminal spinocerebellar ataxia type 10 protein domain (Marz, Probst et al. 2004). The only reference to *Y37H9A.1* in the literature is that it appeared in an RNAi screen looking to identify genes involved with proper neuronal development (Schmitz, Kinge et al. 2007). As such, should *Y37H9A.1* be demonstrated to be the gene responsible for the *glp-4* phenotype, it likely plays a key role in the development of multiple systems in the nematode. Currently, we are working on creating a *Y37H9A.1* transgene with a 3' untranslated region (UTR) from the gene *unc-54*, which has been shown to increase transcriptional activity in transgenes (Etchberger and Hobert 2008).

3.3.4 *glp-4* appears to play a role in *C. elegans* immunity independent of its role in germline development

As a mutant lacking a germline, the *glp-4* mutant should show a similar enhancement of longevity and immune function. However, previous studies had failed to show an extended life span of *glp-4* mutants on *E. coli* or on *S. enterica* (Tenor, McCormick et al. 2004); these results were confirmed in this study. This fact leads to three possible hypotheses. The first such hypothesis is that for some reason, the germline defect caused by the *glp-4* mutation fails to induce the DAF-16-dependent pathway responsible for increased longevity and immunity. Under this hypothesis, the life span extension seen on some pathogens could be explained by a lack of matricide. Indeed,

prior studies that had reported a life span extension of *glp-4* mutants on *C. neoformans* had attributed that extension to a lack of matricide (Mylonakis, Ausubel et al. 2002; van den Berg, Woerlee et al. 2006). However, we demonstrated that matricide cannot explain the extended life span of *glp-4* mutants on *C. neoformans* as the *glp-4* mutants live considerably longer than both the *fer-1* and *fer-15* mutants which similarly lack matricide. Additional evidence to this can be found in the extended life span of *glp-4* mutants on heat-killed *E. coli*, which is a food source that causes almost no matricide.

A secondary hypothesis was that in *glp-4* mutants, the pathway leading to increased immunity and life span was only activated on certain pathogens, namely *C. neoformans*, and not on others such as *E. coli*. The inability of the *glp-1* knockdown to synergize with the *glp-4* mutation provided additional evidence that DAF-16 intestinal activation was likely responsible for the increased life span of *glp-4* mutants on certain pathogens. However, the lack of an increased life span in the *glp-4; glp-1(RNAi)* nematodes on *E. coli* suggested that the intestinal DAF-16 pathway was also activated in the *glp-4* mutants on *E. coli*. The increased activation of intestinal DAF-16 in *glp-4* mutants, regardless of pathogen to which the worm had been exposed, was then demonstrated through both the utilization of a survival assay with DAF-16 interference and the direct observation of intestinal DAF-16 activation with a DAF-16:GFP fusion protein.

Consequently, our results led us to propose the following model (Figure 20). In this model, *glp-4* mutants—through their lack of a germline—have increased intestinal DAF-16 activation leading to increased immune function and longevity. However, we hypothesize that the *glp-4* gene plays a second role in directly promoting immunity to certain specific pathogens. Thus, on certain pathogens such as *E. coli* and *S. enterica*, the loss of this pro-immune function balances out the gains found in the increased intestinal DAF-16 activation. This model is further supported by the comparison of the *glp-4* microarrays to other microarrays involving immunity which suggests an overexpression of both pro-immune and anti-immune genes in *glp-4* mutants. Additional support for this hypothesis comes from the fact that, the only gene group that showed an over-representation when compared to genes underexpressed in *glp-4* mutants was the group of genes which were upregulated by exposure to *S. enterica*—a group which should consist largely of pro-immune genes responsible for helping respond to an *S. enterica* infection.

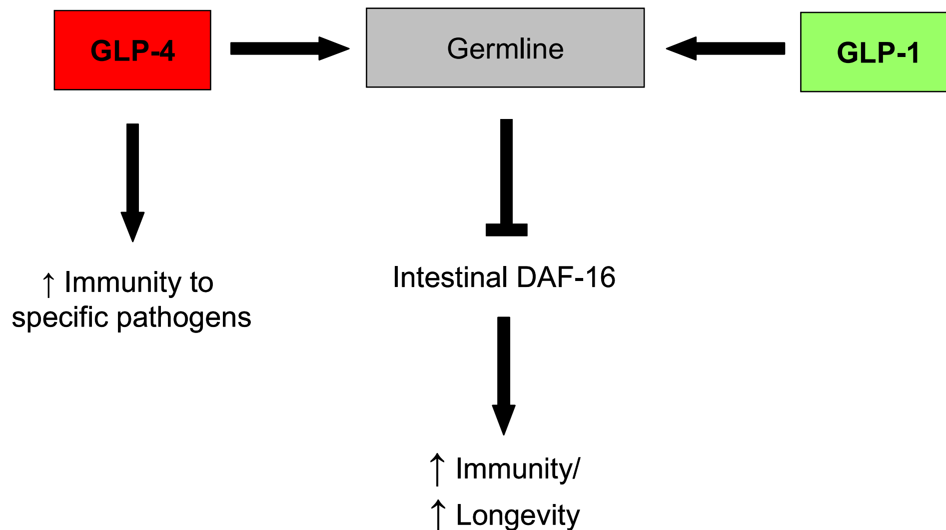

Figure 20: Model for *glp-4* involvement in immunity

In this model, knocking out either *glp-1* or *glp-4* function leads to failure of the germline to develop. This in turn removes the inhibition of intestinal DAF-16, leading to increased immunity and longevity. However, GLP-4 plays a separate role in promoting immunity to specific pathogen, which is also lost when *glp-4* is knocked out.

3.4 Materials and Methods

3.4.1 Microbial and Nematode Strains

The following strains were used: *Escherichia coli* OP50 (Brenner 1974), *Salmonella enterica* serovar *typhimurium* SL1344 (Wray and Sojka 1978), *Enterococcus faecalis* OG1RF (Murray, Singh et al. 1993), *Cryptococcus neoformans* H99 (Franzot, Salkin et al. 1999), and *Pseudomonas aeruginosa* PA14 (Tan, Mahajan-Miklos et al. 1999). *C. elegans* strains utilized were wild-type N2, *glp-1(e2141)*, *glp-4(bn2)*, *fer-1(hc1)*, *fer-15(hc15)*. These strains were originally obtained from the Caenorhabditis Genetics Center and were maintained as hermaphrodites at 15°C, grown on modified NG agar plates and fed with *E. coli* strain OP50 as described (Brenner 1974).

3.4.2 Transgenic Animals

The *Pgly-19:daf-16:gfp* transgenic animal was obtained from the Wolkow lab (Gami, Iser et al. 2006). A *gcy-7:gfp* transgene, which is expressed in one or two head neurons, was also present in this strain as a co-injection marker (Wolkow, CA personal communication). This strain was then backcrossed to our lab's strain of wild-type N2 animals three times to standardize the genomic background. The *glp-1* and *glp-4* strains were generated by crossing the backcrossed transgenic animal *Pgly19:daf-16:gfp* to the *glp-1* and *glp-4* strains in our lab.

To generate the *Y37H9A.1* transgene, a primer was created approximately 1.6 kb upstream of the *Y37H9A.1* gene: ATCAATTGGAAAACATGCGTCACGATTAGTGCCAACAGTTGC. A second primer was created at the stop codon of the *Y37H9A.1* that included the sequence for the HA tag positioned prior to the stop codon: TTAAGCGTAATCTGGAACATCGTATGGGTAATTGTCCACGTGGACTCCGAGCTCTTCGAGAAGTCGTTTTCGATCG. A PCR using these primers was then performed to generate the transgene. This linear PCR product was injected at a concentration of 20 ng/μl into *glp-4(bn2)* nematodes to create the transgenic strain. The co-injection marker pRF4, which contains the *rol-6* gene, was also injected at a concentration of 75 ng/μl.

3.4.3 *C. elegans* killing assays

Cultures for the killing assays were grown in Luria-Bertani (LB) broth, except for *C. neoformans* H99 and *E. faecalis* OG1RF which were grown in yeast peptone dextrose (YPD) and brain-heart infusion (BHI) broth, respectively. All pathogens were grown at 37°C except *C. neoformans,* which was grown at 30°C. The pathogen lawns for the *C. elegans* killing assays were prepared by spreading 10-20 μl of an overnight culture of the bacterial strains on modified NG agar medium (0.35% peptone) in 3.5 cm or 6 cm diameter Petri plates. *C. neoformans* and *E. faecalis* were plated on BHI with 50 μg/ml gentamycin. Plates were incubated overnight before seeding them with young adult animals. These young adult animals were generated by placing gravid adults on NGM plates with lawns of *E. coli* OP50 and letting them lay eggs at 15°C for 6-10 hours. The gravid adults were then removed, and the eggs on the plate were allowed to develop at 25°C for 2.5 days to produce the young adults. The killing assays were performed at 25°C and animals were transferred once a day to fresh plates, until no more progeny were evident. Additional transfers were done after that point as needed to replenish food sources and to prevent the plates from drying out. The germline-deficient mutants were transferred at the same time as the N2 wild-type to maintain consistency. Animals were scored at the times indicated and were considered dead upon failure to respond to touch.

3.4.4 *C. elegans* aging assays

E. coli OP50 was cultured in 50 ml of LB broth overnight at 37°C. The bacteria were then spun down and resuspended in 5 ml of LB broth. The *E. coli* were then heat-killed by placing the resuspended culture at 70°C for 3 hours. Twenty μl of this culture of heat-killed bacteria were then spread on modified NGM plates containing 0.35% peptone, 100 μg/ml 5-fluorodeoxyuridine (FUdR) (Gandhi, Santelli et al. 1980), and 50 μg/ml gentamycin and allowed to incubate at 37°C overnight. Young adult animals were seeded onto these plates, and scored as indicated in Section 3.4.3.

3.4.5 RNA interference

RNA interference was used to generate loss-of-function RNAi phenotypes by feeding nematodes with *E. coli* strain HT115(DE3) expressing dsRNA that is homologous to a target gene (Timmons and Fire 1998; Fraser, Kamath et al. 2000). Briefly, *E. coli* with the appropriate vectors were grown in LB broth containing ampicillin (100 μg/ml) at 37°C overnight. RNAi plates were then generated by spreading these *E. coli* onto NGM plates containing 100 μg/ml ampicillin and 10 mM Isopropyl β-D-thiogalactoside (IPTG) to induce dsRNA expression, and the *E. coli* were allowed to grow on these plates overnight at 37°C.

L4 animals were placed on RNAi plates generated as described above and were allowed to develop into gravid adults at 15°C. Once these animals were gravid, they were transferred to fresh RNAi plates where they were allowed to lay eggs for 6-10

hours at 15°C. The gravid adults were then removed, and the eggs and plates were transferred to 25°C. The eggs were allowed to develop at 25°C for 2.5 days at which time they were seeded onto experimental plates and used as described in Sections 3.4.3 and 3.4.4. *unc-22* RNAi was used as a positive control for the creation of loss-of-function phenotypes.

3.4.6 Statistical analyses

Animal survival was plotted as a staircase curve using the PRISM (version 4.00) computer program. Survival curves are considered significantly different than the control when P values are less than 0.05. Prism uses the product limit or Kaplan-Meier method to calculate survival fractions and the logrank test, which is equivalent to the Mantel-Heanszel test, to compare survival curves.

3.4.7 Mapping and Whole Genome Sequencing

Ampliflour® mapping of the *glp-4* mutation was performed primarily by Laura Fuhrman as described in Fuhrman, *et al*. (2008). Whole genome sequencing was performed by utilizing DNA obtained from *glp-4(bn2)* mutants in the standard Illumina Genome Analyzer genomic sample preparation protocol to create a DNA library, which was then sequenced. The data was then analyzed with the Mapping and Assembly with Quality (MAQ) software which performs read alignment and SNP prediction. These SNPs were then examined to determine if they were homozygous. PCRs of 500 bp to 1000 bp in length that contained the presumed SNP were then performed using DNA

from both N2 wild-type and *glp-4* mutants as a template, and standard sequencing was then performed on these PCR products to confirm the reference strain in the N2 wild-type animals and the presence of the SNP in the *glp-4* mutants.

3.4.8 DAF-16 localization assays

Experimental plates featuring lawns of *E. coli* OP50 or *C. neoformans* H99 were generated as described in Section 3.4.3. The transgenic animals expressing *Pgly-19:daf-16:gfp* were generated as described in Section 3.4.2, and young adults were generated as described in Section 3.4.3. These young adult transgenic animals were then transferred to the experimental plates and left at 25°C for 24 hours. The animals were then visualized using a Leica MZ FLIII fluorescence stereomicroscope where they were categorized as predominately nuclear (as seen in Figure 17A) or predominately cytoplasmic (as seen in Figure 17B) as described in Berman, *et al*. (2006). Eight to ten plates of nematodes were scored over multiple, independent days, and the percentage of nematodes that were predominately nuclear was determined for each plate. Means and standard deviations were then calculated for each condition. Different conditions were compared using a two-tailed Mann-Whitney test (calculated by PRISM software) with a $p < 0.05$ being considered significant.

3.4.9 Representation Factor

The representation factor is a measure of how many genes two groups have in common. It is calculated by dividing the number of genes overlapping in the two

groups by the number of expected overlapping genes. Thus, a representation factor of one is equivalent to the overlap that would be expected due to random overlap, while a representation factor greater than one indicates a larger overlap than expected and a representation factor less than one indicates a smaller overlap than expected. The number of expected overlapping genes is calculated by multiplying the number of genes in one group to the number of genes in the second group, and then dividing that product by the total number of genes in the microarray. Further description of representation factor and the web application used in the study can be found at http://elegans.uky.edu/MA/progs/overlap_stats.html.

For these comparisons, we created the groups of genes overexpressed or underexpressed in the *glp-4* mutant by looking at those genes either upregulated or downregulated threefold in Jiang, *et al*. (2001). The groups of genes that were upregulated by DAF-16 (which were called Class I) and downregulated by DAF-16 (which were called Class II) came from Murphy, *et al*. (2003) while the genes that were overxpressed or underexpressed on *P. aeruginosa* came from Shapira, *et al.* (2006). The genes that were overexpressed or underexpressed on *S. enterica* demonstrated at least a two-fold difference when compared to nematodes that were raised on *E. coli*.

4. Conclusions and Perspectives

4.1 Identification of Genes Involved in C. elegans *Immunity is a Key Point of Research*

In this work, we have identified a GATA transcription factor ELT-2 that plays a role in immune response to a broad range of pathogens as well as an additional gene, *glp-4,* that appears to play a role in immune function against a smaller set of specific pathogens. The identification of new genes involved in *C. elegans* immunity is currently an explosive field of research. Since 2006, our research group has identified numerous such genes that indicate a complex multisystem approach to immunity in *C. elegans* (Table 4). As other groups have also identified new genes involved with immunity, the number of pathways involved in the *C. elegans* immune response has increased dramatically.

Table 4: *C. elegans* Immune Genes Identified by the Aballay Lab

Gene	Description	Reference
hsf-1	Heat shock factor-1	Singh, *et al*. 2006
hsp90	Heat shock protein 90	Singh, *et al*. 2006
elt-2	GATA transcription factor	Kerry, *et al.* 2006
tol-1	*C. elegans* Toll ortholog	Tenor, *et al*. 2008
npr-1	Neuropeptide G-coupled protein receptor	Styer, *et al.* 2008
ced-1	Phagocytic receptor	Haskins, *et al*. 2008
Multiple *pqn/abu* genes	Genes involved with unfolded protein response	Haskins, *et al*. 2008
nol-6	nucleolar RNA-associated protein	Fuhrman, *et al.* 2009
cep-1	p53 protein	Fuhrman, *et al.* 2009
glp-4	unknown protein (Y37H9A.1?), involved with germline development	Chapter 3 of this thesis

The importance of these discoveries is threefold. First, they offer the possibility of gaining a basic understanding of immune function in a much simpler system than mammalian immunity, as the *C. elegans* system lacks cellular immunity but still appears to mobilize a unique response to specific pathogens. Secondly, the study of immunity in *C. elegans* allows us to gain a better understanding of the evolutionary history of immunity. Studies in *C. elegans* have demonstrated that many pathways important in modulating immunity are also important in development (Nicholas and Hodgkin 2004; Schulenburg, Kurz et al. 2004). ELT-2, described in this thesis, is one such protein critical for development and immunity (Kerry, TeKippe et al. 2006; Shapira, Hamlin et al. 2006), and in *D. melanogaster*, the Toll pathway is critical for both immunity and

development (Ip, Reach et al. 1993; Lemaitre, Nicolas et al. 1996). *C. elegans* provides an excellent model for studying these evolutionary links. Finally, it remains likely that some of the findings in *C. elegans* will pertain more directly to mammalian immunity. The most concrete example of research in invertebrate immunity leading to better understanding of mammalian immunity is the discovery of the Toll pathway in *Drosophila*. With *C. elegans*, one example of a potential insight into mammalian immunity comes from work with ELT-2, as Shapira, *et al*. (2006) showed GATA-6 may play a role in resistance to pathogens. Other work along this line includes studies with TGF-β which has been shown to play a role in immune function both in *C. elegans* (Mallo, Kurz et al. 2002) as well as in mammalian systems (reviewed in Wan and Flavell 2007; Taylor 2009).

Undoubtedly, more genes involved with *C. elegans* immunity will be identified in the near future. These genes may include PRRs which have not yet been identified, despite increasing evidence that suggests pathogen-specific inducible responses (Alper, McBride et al. 2007). Additionally, connections between the pathways have yet to be fully determined. For example, the heat shock pathway has been demonstrated to interact with the DAF-2/DAF-16 insulin/PI3K signaling pathway (Singh and Aballay 2006), but the molecular mechanisms of that interaction are still unknown. Consequently, *C. elegans* immunity remains a rich area of research.

4.2 Transcription Factors such as ELT-2 and DAF-16 Play Critical Roles in Modulating **C. elegans** *Immunity*

One type of protein that must play a role in immune function are transcription factors as the changes in gene expression in response to pathogen must be induced by transcription factors. Of the over 900 predicted transcription factors in *C. elegans* (Reece-Hoyes, Deplancke et al. 2005), thus far only three transcription factors have been identified as playing a role in immunity: *elt-2* (Kerry, TeKippe et al. 2006; Shapira, Hamlin et al. 2006), *hsf-1* (Singh and Aballay 2006; Singh and Aballay 2006), and *daf-16* (Garsin, Villanueva et al. 2003; Murphy, McCarroll et al. 2003). This thesis work has studied two of these three transcription factors. Work done in this study helped lead to the identification of the involvement of *elt-2* in immunity. We also examined the role of DAF-16 in immunity, focusing specifically on its role in germline-deficient animals. It seems likely that more transcription factors involved in *C. elegans* immunity remain to be found. These proteins will likely play key roles in modulating gene expression changes in response to pathogens.

Additional work must also be done to improve our understanding of the transcription factors currently known to be involved with *C. elegans* immunity. For example, at this time, it remains unknown with which signaling pathways *elt-2* may interact; we presented data in this thesis that suggests that *elt-2* functions independently of the DAF-2/DAF-16 pathway, but little else is known at this time. The pathway through which germ cell loss induces DAF-16 activation is just beginning to be explored,

and additional components such as PREG and *kri-1* continue to be identified (Berman and Kenyon 2006; Broue, Liere et al. 2007). Furthermore, we are just beginning to identify those immune effectors that are influenced by these transcription factors. A number of genes that are regulated by DAF-16 have been identified by microarray (Murphy, McCarroll et al. 2003), but the extent to which these genes influence immunity remains largely unknown.

4.3 Aging and Immunity Are Tightly Connected in C. elegans

Differentiating between effects on longevity and effects on immune function in *C. elegans* has proven difficult. This is partly because the primary method for studying effects both on longevity and on immunity is the survival assay, which obtains quantitative data by compiling a large number of qualitative results (is this worm alive today, yes or no?). As a result, the survival assay is not the most finely honed assay for distinguishing between aging and immunity.

To deal with this issue, a number of additional assays and measures can be utilized. One assay that is often performed involves utilizing heat-killed bacteria to create a situation where general stress will affect life span, but immune function will not be involved. Another such measure that has been utilized is relative mortality (Aballay and Ausubel 2001; Tenor, McCormick et al. 2004; Kerry, TeKippe et al. 2006). This ratio of TD_{50} on pathogen to TD_{50} on heat-killed bacteria attempts to standardize the effects of immunity versus effects on aging (Aballay and Ausubel 2001; Tenor, McCormick et al.

2004; Kerry, TeKippe et al. 2006). Another method for determining if a gene affects longevity or immunity is to see if that gene regulates known antimicrobial peptides. We demonstrated that ELT-2 regulates *clec-67*, a C-type lectin shown to be overexpressed in response to pathogen exposure (Kerry, TeKippe et al. 2006). DAF-16 has been shown to be responsible for promoting expression of a number of C-type lectins, lysozymes, proteases, and other antimicrobial peptides (Murphy, McCarroll et al. 2003). An additional method for studying longevity versus immunity is testing the nematodes against a variety of other nonpathogenic stresses. For example, *elt-2(RNAi)* nematodes were shown to have a similar resistance to oxidative, heavy metal, and heat stress as wild-type nematodes (Shapira, Hamlin et al. 2006).

Another issue in separating immunity and longevity is that they appear to be tightly linked with components influencing both outcomes. This tight linkage is perhaps best exemplified by DAF-16. DAF-16 has been demonstrated as playing as influential role in modulating *C. elegans* immunity (Garsin, Villanueva et al. 2003; Murphy, McCarroll et al. 2003; Evans, Kawli et al. 2008; Shivers, Youngman et al. 2008). When DAF-16 is overexpressed, either by *daf-2* mutation or germline removal, nematodes live longer on a number of pathogens that kill very rapidly (Garsin, Villanueva et al. 2003; Kerry, TeKippe et al. 2006; Singh and Aballay 2006; Miyata, Begun et al. 2008; Figure 11). As mentioned earlier, DAF-16 has also been shown to regulate a number of antimicrobial peptides (Murphy, McCarroll et al. 2003; Shivers, Youngman et al. 2008).

It even appears that *P. aeruginosa* may actively suppress DAF-16 function and that DAF-16 function appears critical for extending survival on *P. aeruginosa* (Evans, Kawli et al. 2008).

Yet it is apparent that DAF-16 is critical for extending longevity as well. We demonstrated that *daf-16(RNAi)* nematodes do not live as long on heat-killed *E. coli* as wild-type nematodes (Figure 16); this is not the case when *daf-16(RNAi)* nematodes are compared to wild-type on pathogens (Garsin, Villanueva et al. 2003; Kerry, TeKippe et al. 2006; Singh and Aballay 2006). Furthermore, *glp-1* and *glp-4* mutant nematodes which have increased intestinal DAF-16 activation live longer on heat-killed *E. coli* (Figure 13). DAF-16 is also known to play a role in helping deal with a number of different types of general stress (Honda and Honda 1999; Barsyte, Lovejoy et al. 2001; Honda and Honda 2002; Yanase, Yasuda et al. 2002). Finally, DAF-16, in addition to regulating immune effectors, appears to also regulate a number of general stress response proteins important in aging (Murphy, McCarroll et al. 2003; Ruzanov, Riddle et al. 2007).

Thus, as the DAF-16 example illustrates, it is very difficult to separate immune and aging functions. This is likely the result of *C. elegans* evolving a number of stress pathways which overlap and include responses to pathogens.

4.4 Immune Responses Require Integration of Multiple Systems throughout the Organism

Research presented in this thesis indicates that in addition to playing a role in germline development, the gene *glp-4* may also play a role in immune function. We also demonstrated that loss of the germline, as in the *glp-1* mutant, leads to increased immune function against a broad range of pathogens. This work helps support previous work that suggested interactions between the germline and immune function (Aballay and Ausubel 2001; Miyata, Begun et al. 2008). However, the reproductive system is only one of many diverse systems that work together to help fight off pathogens; indeed, it is becoming apparent that innate immune function in the nematode is a matter of responses throughout the whole organism. For example, the role of DAF-16 in immunity has been discussed in detail in this thesis, and it appears that endocrine/paracrine signaling in the form of insulin-like molecules play a crucial role in inhibiting DAF-16 through the insulin receptor DAF-2 (Kenyon, Chang et al. 1993).

The nervous system of *C. elegans* is ideally situated to integrate signals from different parts of the organism. As described earlier, *C. elegans* has the ability to avoid pathogenic bacteria while being attracted to nutritious sources (Troemel 1999). This sort of behavioral immune function appears to be only one type of role that the nervous system plays in *C. elegans* immunity, though. Recent studies have illustrated that mutants with defects in neurotransmission involving dense core vesicles show increased resistance to *P. aeruginosa* while mutants with increased neurosecretion demonstrated

increased susceptibility (Kawli and Tan 2008). This influence of neurosecretion on resistance was dependent on DAF-16 activation through the insulin-PI3K pathway suggesting that secretion of insulin-like peptides from neurons were negatively affecting immune function (Kawli and Tan 2008). Another recent study demonstrated that the neuropeptide G protein-coupled receptor NPR-1, which is related to the neuropeptide Y receptor and expressed in certain neurons in *C. elegans*, is also important in mounting an immune response to *P. aeruginosa* (Styer, Singh et al. 2008). Neuronal production of DBL-1, a TGF-β homologue, has recently been shown to activate a number of antimicrobial peptides in the epidermis responsible for helping fend off infection by *D. coniospora* (Zugasti and Ewbank 2009). Finally, another recent study has suggested that dopamine plays a critical role in modulating immune "conditioning," where previous exposure to the pathogenic *E. coli* strain EPEC resulted in increased survival upon re-exposure (Anyanful, Easley et al. 2009). These findings, along with those involving the reproductive system, lead to a picture of the nematode as incorporating numerous signals from all parts of the organism to find a balance for energy use between reproduction, immune function, and other energy-intensive traits with this balance being controlled by the nervous system.

4.5 Conclusions

The work done in this thesis has looked to examine the role of transcription factors in *C. elegans*. In an effort to identify new transcription factors involved with

immunity, we identified ELT-2, a GATA transcription factor that was known to play a role in intestinal development. Utilizing RNA interference, we demonstrated that ELT-2 was required for resistance to a wide range of pathogens including Gram-positive bacteria, Gram-negative bacteria, and fungi. We also demonstrated that ELT-2 was responsible for regulating *clec-67*, a C-type lectin known to be upregulated in response to *S. enterica* and *P. aeruginosa* infection. Evidence was also provided that suggested ELT-2 functioned independently of the insulin/PI3K pathway.

Again, interest in studying transcription factors led us to examine the FOXO transcription factors DAF-16, specifically in its role in promoting immunity in germline-deficient mutants. We demonstrated that the germline-deficient mutant *glp-1* showed increased resistance to a broad range of pathogens. We also showed that DAF-16 was required for complete longevity in wild-type nematodes as *daf-16(RNAi)* nematodes lived significantly shorter lives on heat-killed bacteria than wild-type N2 nematodes.

Our work with germline-deficiency brought to our attention the mutant *glp-4*. As a germline-deficient mutant, *glp-4* mutants should have an extended life span on pathogens like *glp-1* mutants, but the *glp-4* mutants do not live longer than wild-type on *E. coli* or *S. enterica*. Since the mutation responsible for the *glp-4* phenotype is unknown, we used whole genome sequencing to identify possible causes for the mutation. We have narrowed the potential mutations to five SNPs, the most likely of which induces a change in the amino acid sequence of Y37H9A.1, a protein conserved across the

Caenorhabditis genus. We also demonstrated higher levels of DAF-16 activation in both *glp-4* and *glp-1* mutants. The results have led us to propose that *glp-4* plays a role in *C. elegans* immunity besides its influence on the germline.

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Biography

Michael "Mickey" TeKippe was born and raised in the small town of West Union, Iowa were he graduated from North Fayette High School in 1998. He went to Iowa State University for his undergraduate education, where he majored in biology and mathematics, while enjoying himself in a number of extracurricular activities, especially quiz bowl competitions. While at Iowa State, he did research on retrotransposon structure and function in the laboratory of Dr. Dan Voytas, and he also studied bone marrow stem cell development in knockout mice in the lab of Dr. David Harrison at The Jackson Laboratories in Bar Harbor, Maine. Mickey graduated Iowa State in the spring of 2002, and he joined the MSTP at Duke University that summer. In the fall of 2004, he joined the lab of Dr. Alejandro Aballay where he studied host-pathogen interactions utilizing the nematode *C. elegans* as a model system. Once done with his Ph.D., Mickey plans on finishing medical school, joining a pediatric residency program, and remaining in academic medicine. He currently lives in Durham, NC with his wife (and senior prom date) Erin and their two soft-coated wheaten terriers Red and Buster.