C. elegans MAP Kinase Mutants Show Enhanced Susceptibility to Infection by the Yeast *S. cerevisiae*

A Thesis Submitted to the Faculty
of
Worcester Polytechnic Institute
In Partial Fulfillment of the Requirements for the
Master of Science Degree
In Biology and Biotechnology
By

Meijiang Yun

May, 2010

APPROVED:

Samuel Politz, Ph.D. Major Advisor WPI	David Adams, Ph.D. Committee Member WPI	Elizabeth Ryder, Ph.D. Committee Member WPI

Acknowledgements

Thanks to Charu Jain for helping me around the lab. Thanks to my lab mates Muxun Zhao and Shinya Iguchi. I thank my committee members Professor David S. Adams and Professor Elizabeth F. Ryder for their useful advice. Thanks to my husband Ye Tian, and my parents Taida Yun and Xiaolian Jiang for their support and understanding. And finally, I thank my advisor Professor Samuel M. Politz for his guidance. Without his encouragement, I could not have finished this thesis.

Abstract

C. elegans is as an extremely powerful model for the study of innate immunity. MAP kinase signaling pathways in *C. elegans* are involved in the response of *C. elegans* to infection by pathogenic bacteria. The yeast *S. cerevisiae* can infect *C. elegans*, producing pathogenic effects. In this project, we tested whether several MAP kinase pathways are important for *C. elegans'* resistance to yeast infection. We tested members of several MAP kinase pathways including *tir-1, nsy-1, sek-1* and *pmk-1* in the p38 pathway, *mek-1, jnk-1* and *kgb-1* in JNK pathway and *mek-2* and *mpk-1* in the ERK pathway. We used survival assays to compare the responses of mutants of components of these pathways to the control responses of wild-type *C. elegans*. In the survival assay, we found that mutants in all three MAP kinase pathways showed a decreased survival relative to wild type; therefore all three pathways are important for innate immunity against the yeast pathogen. With respect to the p38 pathway, mutations affected survival but not the deformed anal region (Dar) phenotype, a putative defensive response induced by yeast in wild-type *C. elegans*. This indicates that for the p38 pathway, survival depends on some other immune response besides Dar. Finally, we hypothesize that cross talk occurs between p38 and JNK MAPK pathways in the *C. elegans* immune responses.

Table of Contents

List of Figures

List of Tables

Introduction

1. Fungal Infection

Systemic fungal infections caused by nosocomial fungal pathogens are not easy to cure, and high morbidity and mortality results from such infections in immunocompromised patients (Means *et al.,* 2009). Although antifungal drugs are available, due to their lack of effectiveness, new antifungal agents and therapies are needed (Richardson and Lass-Florl, 2008). In order to design new antifungal drugs on a rational basis, it is important to understand the mechanism of immune responses to fungal infection.

Immune responses to fungal infections have been studied largely *in vitro* using cultured cells such as macrophages, and thus these responses may not reflect what happens in an *in vivo* infection. *C. elegans* has evolutionarily conserved signaling pathways that are also required for innate immunity in a large group of organisms, including humans (Kim, 2008). It has been found that the innate immune system is required for effective immune responses to pathogenic yeast (Means *et al.,* 2009). Thus, the study of host defense mechanisms in *C. elegans* provides an ancient evolutionary view on innate immunity, which may produce insights into the conserved processes in diverse host organisms, including humans (Kim, 2008). A large number of bacterial species pathogenic in humans are also pathogenic in *C. elegans*, but as yet only

a few fungal infections of *C. elegans* have been studied.

2. *Saccharomyces cerevisiae* as a Model Pathogen

Although the budding yeast *Saccharomyces cerevisiae* is best known for its ancient uses in baking, brewing, and viniculture, it is emerging as an opportunistic, albeit sporadic, pathogen in immunodeficient patients, neutropenic patients, and debilitated patients (McCusker *et al.,* 2006). Also, *S. cerevisiae* is closely related to the most common pathogenic fungi, *Candida albicans* and *C. glabrata* (McCusker *et al.*, 2006), and results from studying *S. cerevisiae* infection may, in part, be directly transferable to these common pathogens. Moreover, *S. cerevisiae* is a thoroughly studied genetic model organism. The available resource of a completely sequenced genome, mutants, and a complete genome knockout collection in *S. cerevisiae* are also important reasons for using *S. cerevisiae* as a model pathogen to investigate host-pathogen interactions (Jain *et al*., 2009). These advantages directly contrast with *Candida* species, which are genetically intractable, in spite of the availability of their genomic DNA sequences. A very strong similarity in genome sequences between *C. albicans* and *S. cerevisiae* indicates that the information obtained from using *S. cerevisiae* as a model pathogen may also be applicable to understanding *C. albicans* infections (Tzung *et al.*, 2001).

3. *C. elegans* as a Model Host

Caenorhabditis elegans has emerged as a powerful host model to study innate immunity and has been found to mount protective responses to many kinds of bacterial and a few fungal pathogens (Alegado *et al.*, 2003; Nicholas and Hodgkin, 2004a; Gravato-Nobre and Hodgkin, 2005). Several human pathogenic bacteria have been demonstrated to cause pathogenic infections in *C. elegans.* For example, *Enterococcus faecalis,* a bacterium that causes meningitis, endocarditis and urinary tract infections in humans, is pathogenic to *C. elegans* (Garsin *et al*., 2001; Kim *et al*., 2002; Darby, 2005). *E. faecalis* colonizes the *C. elegans* intestine, resulting in a shortened host life span (Darby, 2005). When pathogens infect, *C. elegans* mounts protective responses. One such response is elicited by a *Microbacterium nematophilum* infection, which can induce a defensive response characterized by a deformed anal region (DAR) phenotype, a swelling just posterior to the rectal opening accompanied by accumulation of bacteria at this site (Hodgkin *et al*., 2000). The Dar response requires the ERK MAP kinase pathway, because infection of *C. elegans* mutants encoding nonfunctional ERK pathway kinases induces no anal swelling (Nicholas and Hodgkin, 2004b; Darby, 2005; Nicholas and Hodgkin, 2009). When infected, these mutants become severely constipated and have a decreased life span. Thus, this suggests Dar is a defensive response of the nematode (Nicholas and Hodgkin, 2004b). Also, a connection between the Hox transcription factor EGL-5 and the ERK MAP kinase cascade has been confirmed, since gene *egl-5* appears to function upstream of the ERK MAP kinase pathway (Nicholas and

Hodgkin, 2009). This is consistent with *egl-5* being expressed in the hindgut cells which are enlarged during the Dar response.

There are several advantages to using *C. elegans* as host model in our study. The first one is its small size; the adult *C. elegans* male is about 0.8 mm and the adult hermaphrodite is around 1mm in length (Altun and Hall, 2009). The second reason is that the body plan of *C. elegans* is very simple. The adult hermaphrodite has 959 somatic cells and the adult male has 1031 somatic cells (Altun and Hall, 2009). The third reason is the rapid three day generation time of *C. elegans* (Altun and Hall, 2009). The fourth reason is that the *C. elegans* body is transparent throughout its life cycle, so it can be easily examined at the cellular level in the living worm under the microscope. The fifth reason is that the maximum life span of wild-type *C. elegans* grown under ordinary conditions is relatively short (21 days), which facilitates survival analysis during infections. The sixth reason is that 99.9% of *C. elegans* populations are self-fertilizing hermaphrodites, which allows homozygous worms to generate genetically identical progeny (Altun and Hall, 2009). Also, *C. elegans* is amenable to genetic crosses so double mutants can be easily obtained (Altun and Hall, 2009).

4. Life Cycle of *C. elegans*

The *C. elegans* life cycle consists of the embryonic stage, four postembryonic larval stages (L1-L4), and a reproductive adult stage (Altun and Hall, 2009) (**Fig.1**). During the embryonic stage, the first cleavage starts at approximately 40 minutes after

hatching. At the end of the embryonic stage, a *C. elegans* hermaphrodite has 558 somatic cells (Altun and Hall, 2009). Also, the main body plan of *C. elegans* is established by the time of hatching and does not transform during postembryonic development (Altun and Hall, 2009). At the end of each larval stage, the new and stage-specific cuticle is synthesized, and at the same time the old cuticle is shed (Altun and Hall, 2009). This process is called molting. Besides these four stages, there is an alternative stress-resistant dauer larva stage. If *C. elegans* experiences harsh environmental conditions such as absence of food during the L1 and L2 larval stages, it will molt into a facultative diapause stage, called the dauer larva, which is also a non-aging stage; that means time spent in this stage does not count into the postdauer life span. When favorable conditions are available, *C. elegans* exits dauer larva and molts into the L4 stage after about 10 hours. The adult stage occurs approximately at 45-50 hours after hatching at 22°C.

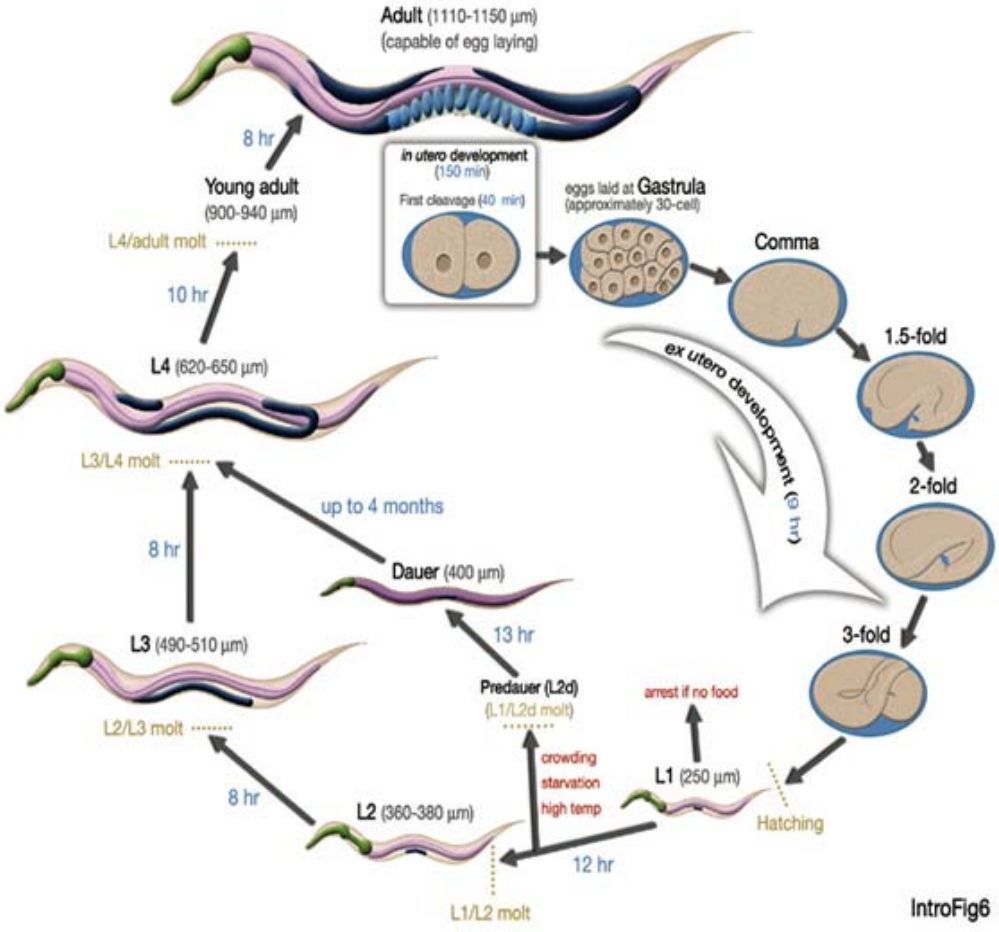


Fig.1 Developmental stages of *C. elegans* (Altun and Hall, 2009)

5. Innate Immune Responses

Because this thesis concerns the role of MAP kinase signaling pathways in *C. elegans* immunity, it is important to first review the pertinent aspects of mammalian innate immunity.

The immune defense system consists of two main parts: an innate response and an adaptive response (Schulenburg *et al*., 2004; Murphy, 2008). Innate immunity is

an evolutionarily ancient form of host defense against infection, since invertebrates, plants, and fungi rely exclusively on innate immunity, and the same molecular modules are observed, which indicates innate immunity arose before the divergence of these taxa (Janeway and Medzhitov, 2002; Schulenburg *et al*., 2004; Akira *et al*., 2006). However, the mammalian immune system relies on both an innate response and an adaptive response (Akira *et al*., 2006). The innate immune system is the critical first line of host defense against pathogens, and the long-term survival of patients with defects in innate immunity is very poor (Janeway and Medzhitov, 2002).

Three steps are involved in the vertebrate innate immune response. First is pathogen recognition; that is, the invading pathogen is recognized by host germline-encoded pattern recognition receptors (PRRs) (Murphy, 2008). Second is signal transduction or the triggering of effector responses during which the information of pathogen recognition is transmitted via the activation of signaling pathways (Murphy, 2008). Finally, these signaling cascades cause upregulation of antimicrobial effectors (Murphy, 2008). Triggering of the signaling pathways leads to the induction of transcription of a variety of immune response genes, including antimicrobial peptides and inflammatory cytokines (Medzhitov and Janeway, 2000).

6. Pathogen Recognition in Mammals

Toll-like receptors (TLRs) play an established role in pathogen recognition and immune responses (Janeway and Medzhitov, 2002). In humans, nine TLRs are known,

and each has a characteristic specificity for a particular subset of pathogen associated molecular patterns (PAMPs). For example, TLR4 recognizes bacterial lipopolysaccharide (LPS) and the fusion protein of the respiratory syncytial virus (Kurt-Jones *et al*., 2000; Janeway and Medzhitov, 2002).

G protein-coupled receptors (GPCRs) form one of the largest and most diverse protein families encoded in mammalian genomes, and their major function is to transduce extracellular stimuli into intracellular signals via activation of the intracellular second messenger, cyclic AMP (cAMP) (Kroeze *et al*., 2003; Calebiro *et al*., 2009). GPCRs signal to cAMP and then are internalized. Internalized GPCRs may inhibit signaling to second messengers like cAMP, and instead, activate mitogen-activated protein kinase (MAPK). However, recently it was found that after GPCRs internalize, cAMP may be continually stimulated, since activation of downstream components requires cAMP (Calebiro *et al*., 2009). Finally, the mammalian scavenger receptors are a group of structurally heterogeneous proteins categorized together because they recognize various anionic and acetylated lipoprotein PAMPs. These are less well characterized than TLRs or GPCRs.

Innate immunity involves many aspects of host defense against pathogenic microbes and employs evolutionarily conserved signaling pathways, since the same molecular modules are observed in both plants and animals (Janeway and Medzhitov, 2002; Nicholas and Hodgkin, 2004a). MAP kinase pathways in mammals include p38 pathways, ERK pathways, and JNK pathways. Each of these is named for a particular

MAP kinase that activates transcription directly, often by phosphorylating specific transcription factors. MAP kinase pathways are involved in all aspects of immune responses in mammals, including the activation of innate immunity and adaptive immunity, as well as the initiation of immune responses to activation induced cell death (Dong *et al*., 2002). In mammals, MAP kinase pathways transduce pathogen recognition signals into effector responses (Janeway and Medzhitov, 2002). Recognition of these PAMPs allows the immune system to destroy infectious nonself cells (Janeway and Medzhitov, 2002).

Fig 2 shows the pathway in innate immunity which activates the key transcription factor NF-κB, as well as JNK and p38 MAP kinase pathways (Murphy, 2008). In the pathway shown in figure 2, the bacterial lipopolysaccharide (LPS) PAMP (left panel, blue in the diagram) binds a circulating LPS-binding protein (LBP) (green), which in turn binds to the cell-surface protein CD14 (not shown in the diagram). The ligand-bound CD14 interacts with Toll-like receptor 4 (TLR-4) (yellow), which can recognize PAMPs on microbial organisms, and as a result, induce inflammatory responses (Medzhitov and Janeway, 1997; Dong *et al*., 2002). The adaptor protein myeloid differentiation factor 88 (MyD88) (red) possesses a Toll- IL-1- Receptor (TIR) domain at one end, by which it binds to the cytoplasmic tail of TLR-4, and a death domain (Reddy *et al*., 2009) at the other end, by which it activates the IL1-receptor associated kinase (IRAK) (second panel, green). The TIR domain in MyD88 marks it as a member of the mammalian TIR domain family, which includes several other adaptors that act as adaptor proteins to transduce

signals to downstream kinase cascades in innate immunity. In the pathway shown in Fig. 2, IRAK is a serine/threonine protein kinase and contains a death domain. The activated IRAK then binds the adaptor TNF receptor-associated factor 6 (TRAF-6, also a TIR domain containing protein) (yellow), which in turn activates TGF-beta activated kinase 1 (TAK1) (third panel, purple), a MAPK kinase kinase. Finally, TAK-1 activates two MAP kinase pathways (fourth panel, upper), and also activates the IκB kinase (IKK) complex (fourth panel, lower), which in turn liberates nuclear factor-kappa B (NF-κB) from its inhibitor, IκB. NF-κB is a key transcriptional regulator of innate and adaptive immunity (Spehlmann and Eckmann, 2009).

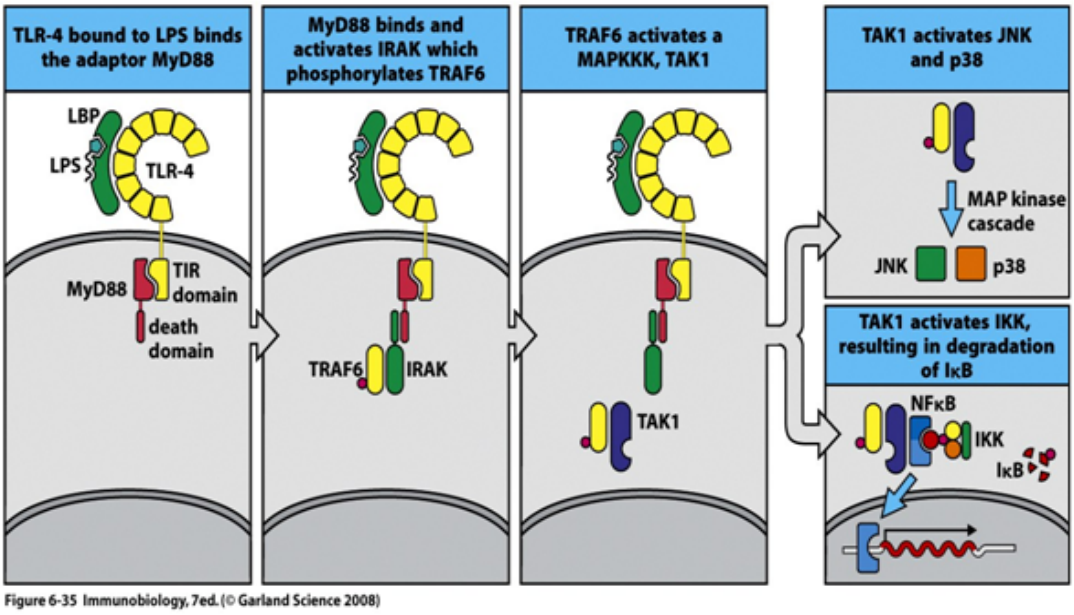


Fig.2 Toll-like receptors activate NF-κB and two MAP kinase pathways (Murphy, 2008)

The TAK1 kinase, activated by a single pathogen-recognition event, triggers activation of three different signaling pathways. Further evidence of this is that a given

upstream MAPKK may phosphorylate more than one MAPK. For example, the MEK1/MKK1 and MEK2/MKK2 groups normally phosphorylate the MAPK ERK, while MKK3 and MKK6 phosphorylate the MAPK p38, and MKK7/JNKK2 phosphorylate the MAPK JNK (Chang and Karin, 2001). However, MKK4/SEK1/JNKK1 can phosphorylate both JNK and p38 (Brancho *et al*., 2003). With respect to this thesis, it is important to remember that although serine-threonine kinases may be theoretically grouped into the same family based on sequence similarity, the ultimate test of similarity is functional (i.e., substrate specificity).

7. Signaling Pathways in *C. elegans* Immunity Include MAP Kinase Pathways

Understanding pattern recognition in *C. elegans* immunity is in its infancy compared to, for example, the extensive information available for mammalian TLRs. For example, *C. elegans* has only one gene encoding a TLR, *tol-1*. However, comprehensive studies of *tol-1* loss of function mutations or RNAi knockdown of TOL-1 expression have for the most part not detected any effect on immune function (Pujol *et al*., 2001), except for one study which described a requirement for *tol-1* in resistance to a specific bacterial pathogen (Tenor and Aballay, 2008). Interestingly, the *C. elegans* genome does not encode a homolog of NF-κB, a well known end-stage mediator of innate immunity signal transduction.

G protein-coupled receptors (GPCRs) have an early evolutionary origin, since in addition to mammals, bacteria, yeast, plants, and *C. elegans* also have GPCRs in their

genomes (Kroeze *et al.,* 2003). The GPCR FSHR-1 is required for the *C. elegans* innate immune response, since it regulates transcription of antimicrobial effectors induced by pathogen infection (Powell *et al*., 2009). FSHR-1 signals are in parallel to the p38 MAPK pathway (Powell *et al*., 2009). The *npr-1* gene, which encodes a G protein-coupled receptor related to mammalian neuropeptide Y receptors, apparently inhibits innate immune responses (Styer *et al*., 2008). However, the mechanism of NPR-1-mediated pathogen resistance does not directly regulate innate immunity, instead, such resistance occurs via oxygen-dependent behavioral avoidance (Reddy *et al*., 2009).

Scavenger receptors constitute a diverse group of pattern recognition receptors that recognize pathogen-derived ligands (Means *et al*., 2009). The *C. elegans* scavenger receptor orthologues CED-1 and C03F11.3 mediate recognition of fungal pathogens and production of antimicrobial peptide in *C. elegans* (Means *et al*., 2009).

C. elegans has been shown to mount protective responses to many fungal and bacterial pathogens, which indicates the existence of an innate immune system, relying on specific signal transduction pathways (Nicholas and Hodgkin, 2004a; Ewbank, 2006). MAP kinase signaling pathways have well established roles in innate immunity in *C. elegans* (Sakaguchi *et al*., 2004). The p38, JUN, and ERK MAP signal transduction pathways in *C. elegans* are homologous with mammalian signaling pathways. Each pathway contains three classes of protein kinases: MAPK, MAPK kinase (MAPKK) and MAPK kinase kinase (MAPKKK) (Sakaguchi *et al*., 2004). Normally, the MAPKKK phophorylates and activates the MAPKK, which in turn activates the MAPK by dual

phophorylation.

The canonical *C. elegans* MAP kinase pathways are shown in **Fig. 3**. The p38 MAP kinase pathway plays a significant role in cellular stress and immune responses in mammals as well as in insects, nematodes, and plants, indicating that it is one of the most ancient and conserved signal pathways (Schulenburg *et al*., 2004). The p38 pathway is also involved in asymmetric sensory neuronal differentiation in *C. elegans* (Chuang and Bargmann, 2005). The JNK pathway genes *jkk-1* and *jnk-1* are required for coordinated movement (Sakaguchi *et al*., 2004). Because expression of these genes is neuron-specific, this suggests that the JNK pathway affects the neuronal control of movement. The JNK pathway also plays an important role in the responses to inflammation and stress (Sakaguchi *et al.*, 2004). The ERK pathway is required in the development of hypodermal cells and the degradation of proteins (Szewczyk and Jacobson, 2003; Nicholas and Hodgkin, 2004b). The ERK pathway is also involved in development of olfaction and germline cells via the Ras/ERK MAP kinase signaling pathway (Hirotsu *et al*., 2000; Rocheleau *et al*., 2008).

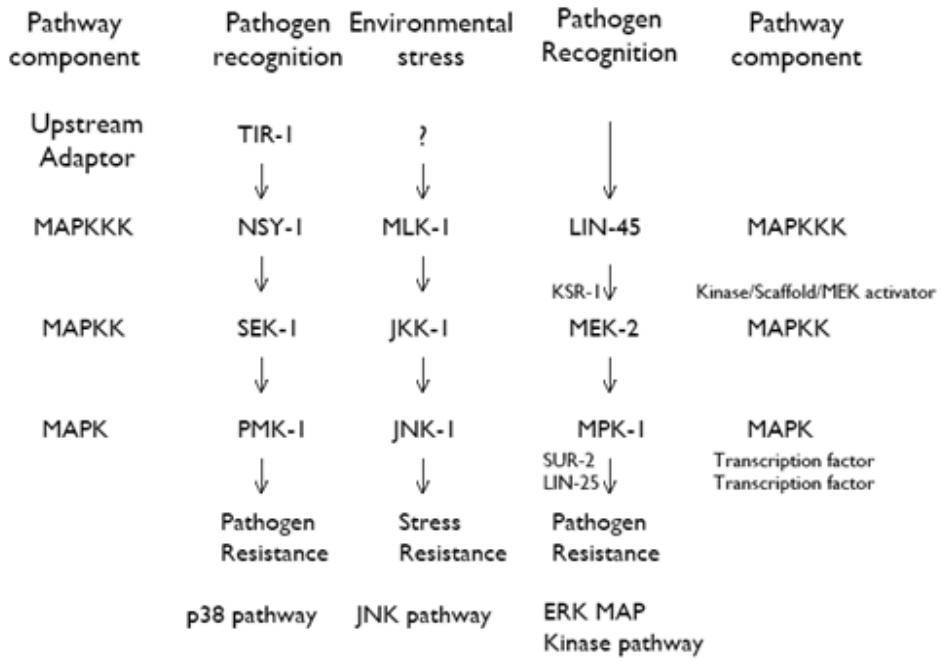


Fig. 3. Canonical MAP kinase pathways in *C. elegans.*

Compared to wild-type *C. elegans*, *sek-1* MAPKK and *nsy-1* MAPKKK mutants show an enhanced susceptibility to pathogen in the presence of *Pseudomonas aeruginosa,* which is an opportunistic pathogen of humans (Kim *et al*., 2002; Sakaguchi *et al*., 2004). There is also an enhanced susceptibility to pathogen phenotype found when *pmk-1* RNAi worms are exposed to *P. aeruginosa* (Kim *et al*., 2002; Sakaguchi *et al*., 2004). Thus, the p38 pathway is required for innate immunity of *C. elegans* (Kim *et al.*, 2002). Wild-type *C. elegans* has been found to exhibit a deformed anal region (DAR) phenotype in the presence of the bacterium *Microbacterium nematophilum*, while reduction of function mutants in the ERK MAP pathway failed to develop this phenotype (Hodgkin *et al*., 2000; Nicholas and Hodgkin, 2004b). It appears that Dar is a part of a

protective response; the swelling distorts the rectal anatomy and thereby limits the extent of infection (Nicholas and Hodgkin, 2004b). Thus, the ERK MAP pathway is also required in innate immunity of *C. elegans*.

Less is known about the JNK pathway in *C. elegans* immunity. The JNK-like MAPK JNK-1 does not appear to affect immunity to *P. aeruginosa* (Mizuno *et al*., 2004). However, both the JNK-like kinase *kgb-1* and a protein phosphatase, *vhp-1*, appear to modulate immunity to *P. aeruginosa* (Mizuno *et al*., 2004; Kim *et al*., 2004), suggesting that JNK-like MAP kinase pathways are important for immunity in *C. elegans* (Kim *et al*., 2002).

Despite the paucity of information available about pattern recognition in *C. elegans* immunity, several steps have been identified that may link the p38 pathway to pathogen recognition. It is well-established that the TIR domain adaptor protein TIR-1 is required upstream of the p38 pathway for immune function (Liberati *et al*., 2004). It has been proven that the PKC gene *tpa-1* acts upstream of *tir-1*, and two PLC genes, *plc-3* and *egl-8*, act upstream of *tpa-1* to regulate *npl-29* which encodes an antimicrobial peptide (Ziegler *et al*., 2009) (**Fig. 4**). In addition, the *C. elegans* Gα proteins GPA-12 and RACK-1 appear to act upstream of EGL-8/PLC3. Because Gα proteins are subunits of heterotrimeric G proteins that interact directly with GPCRs, these results potentially link an as yet unknown GPCR to the p38 pathway via TIR-1. Thus, in the evolution of the *C. elegans* immune response, the p38 pathway may have been recruited downstream of GPCR pathogen recognition receptors, rather than the TLRs used by

mammals. Because the *C. elegans* genome encodes > 1,000 GPCRs, identification of pathogen-recognition receptors may continue to be a daunting task.

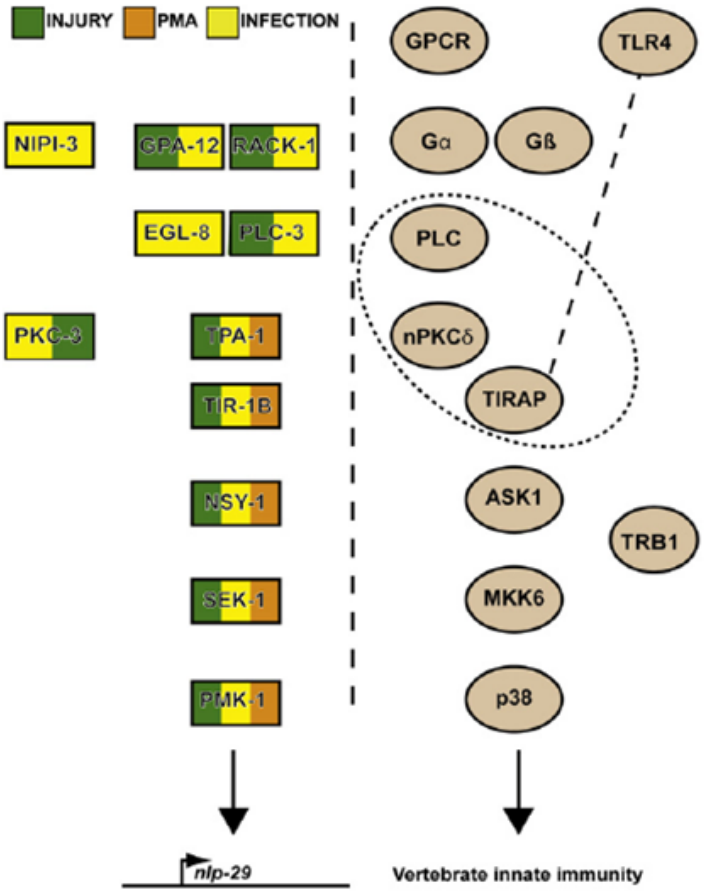


Fig.4. PLC-PKC is associated with an innate immune signaling pathway (Ziegler *et al*., 2009)

Interestingly, many of the genes reported to be important for immunity in *C. elegans* have also been determined to be important for responses to cellular stresses such as those induced by oxidants, heavy metals, and osmotic pressure. This suggests that the innate immune system may have evolved by the addition of specific recognition

and effector functions to existing signaling pathways.

Thesis Purpose

Jain *et al* (2009) undertook a preliminary study of infection of *C. elegans* by *S. cerevisiae*. It was shown that growth of *C. elegans* from hatching on a lawn composed of mixed *E. coli* and *S. cerevisiae* cells results in accumulation of intact yeast cells in the intestine, consequentially causing severe distension of the intestinal lumen. The Dar phenotype, first described for *M. nematophilum* infections of *C. elegans* (Hodgkin *et al.*, 2000) was also observed by Jain *et al* in *C. elegans* grown in the presence of *S. cerevisiae*. The ERK pathway mutant *mek-2 (n1989)* did not exhibit Dar in the presence of yeast, and *mek-2 (n1989)* also showed reduced survival in the presence of yeast (Jain *et al.*, 2009). In this project, we wanted to build on these results by testing mutants altered in several genes of each of the three *C. elegans* MAP kinase pathways for resistance to yeast infection. Our hypothesis was that all of the MAP kinase pathways are important for resistance to yeast. We tested mutations in genes *tir-1, nsy-1, sek-1* and *pmk-1* in the p38 pathway, *mek-1, jnk-1* and *kgb-1* in JNK pathway and *mek-2* and *mpk-1* in the ERK pathway for survival in the presence of yeast. We also tested *nsy-1*, *sek-1*, and *pmk-1* mutants for the Dar phenotype in the presence of yeast to determine whether the p38 pathway is required for Dar.

Methodology

Strains

Mutant *C. elegans* strains were obtained from the Caenorhabditis Genetics Center (CGC, Minnesota). The mutants used for the study included: *mek-1 (ks54)* X*, mek-2 (n1989)*I*, fer-1 (b232)* I*, jnk-1 (gk7)* IV*, kgb-1 (km21)* IV*, pmk-1 (km25)* IV*, sek-1 (km4)* X*, nsy-1 (ag3)* II*, nsy-1 (ok593)* II*, tol-1 (nr2033)* I*, tir-1 (ok1052)* III, *CB189(unc-32 (e189)* III*)* and *MH37(mpk-1 (ku1) unc-32 (e189)* III*)*. The wild-type strain used was N2, *C. elegans* var. Bristol.

Media and Growth Conditions

C. elegans stocks used for the study were grown on Nematode Growth agar medium (NGM) (Sulston and Hodgkin, 1988) on *E. coli* OP50 and transferred to new plates when *E. coli* OP50 were consumed. *E. coli* OP50 was grown overnight in LB Broth at 37°C and yeast wild type Y101 was grown overnight at 30°C in YPD broth (Rose *et al.,* 1990).

Egg Preparation

Egg preparation was performed in order to make sure that all *C. elegans* used in survival assays were at a similar developmental stage and contaminants were not

introduced from stock plates. Stock plates of the different worm strains were prepared and incubated at 20°C five days in advance. A 1:4 ratio of commercial bleach (5.25%) and dH_2O were mixed with 1g NaOH to produce 100 ml of alkaline sodium hydrochlorite solution. For each strain, four stock plates were washed with M9 buffer (Brenner, 1974) using a sterile glass Pasteur pipette, and the liquid suspension containing worms and eggs was transferred to a sterile 15 ml conical test tube, followed by an additional 2 minutes centrifugation at 900 x g. After centrifugation, the supernant was carefully removed. 10 ml of the alkaline bleach solution was added to the worm and egg suspension for a period of 2 minutes, during which the tube was mixed homogeneously by inversion, followed by an additional 2 minutes centrifugation. This procedure lyses adult worms, however, eggs are not disrupted by this brief exposure to bleach. The supernatant was quickly removed, and the eggs were washed by adding 10 ml of M9 buffer and centrifuged for two minutes at 900 x g followed by removal of supernatant. This process was repeated 3 times in order to dilute the bleach and wash the eggs.

Test Plate Preparation

E. coli OP50 was grown overnight in LB Broth at 37°C, and yeast wild type strain Y101 was grown overnight at 30°C in YPD. Liquid cultures were centrifuged at full speed in a micro-centrifuge. Cell pellets were weighed and resuspended to a final concentration of 200 mg/ml for *E. coli* OP50 and 20 mg/ml for yeast wild type strain Y101, respectively. A mixture of 10 µl of 50 mg/ml streptomycin sulfate stock, 2.5 µl *E.*

coli and 7.5 µl yeast was spotted on each NGM plate before egg preparation. For control plates, the yeast aliquot was substituted with 7.5 µl of sterile water.

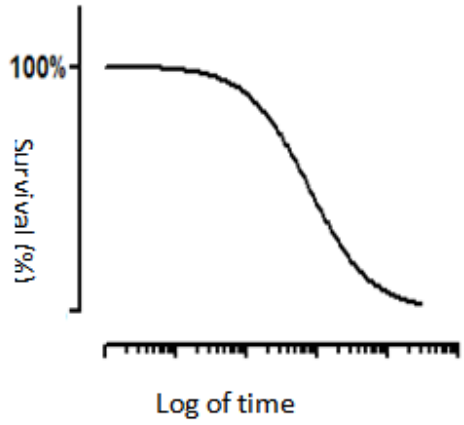
C. elegans Survival Analysis

For survival analysis, test plates and *C. elegans* eggs were prepared as described in the test plate preparation section. Each plate was started with 30 ± 5 eggs; each experiment included three yeast and three *E. coli* plates per strain. Beginning on the second day after plating eggs, the number of dead and live worms on each plate was recorded daily. Live worms were transferred to new plates as necessary to avoid confusing the original worms with their offspring. All plates were of the same composition as the original test plates. Software GraphPad Prism 5.01 (*GraphPad Software, Inc.*) was used to analyze the survival curve data. Significance, defined as $p<0.05$, was assessed using the Log-Rank test. In our experiments, worms that left the plates in the first several days were "censored", i.e., removed from the counts of subsequent days. The Log-Rank test offers equal weight to all the data. The number of censored worms was taken as the total number of worms (dead plus live worms) on that day minus total worms on the previous day. All of the data except *fer-1* were collected from four independent experiments. *fer-1* data was collected from two independent experiments.

Method of Calculating TD50

TD50 was calculated in order to present the time in days on which half of the worms died. TD50 was calculated by nonlinear regression using GraphPad Prism 5.01 (*GraphPad Software, Inc.*). In theory, either linear and nonlinear regression can be used to find the values of the parameters, such as slope and intercept for linear regression, that make the curve come as close as possible to the data. However, unlike linear regression, nonlinear regression can fit data to any equation that defines Y as a function of X and one or more parameters. Linear regression is just a special case of nonlinear regression. The equation of choice for TD50 was a dose-response model which assumes that dose is proportional to time grown on yeast.

The model for dose response curves:



% survival = 100/[1+10logT-logTD50], where T=time.

Method of Calculating Relative Mortality

The relative mortality was calculated in order to remove bias resulting from an intrinsic mutant reduction in survival, which is unrelated to yeast infection. The equation for relative mortality is TD50 WT/TD50 individual mutant.

Method of Calculating Corrected Relative Mortality

In this study, the relative mortality was corrected by using the ratio of TD50's on *E. coli* to remove bias. The equation for corrected relative mortality is:

$$\frac{[\text{TD50 WT/TD50 } \textit{kgb-1}] \text{ (yeast)}}{[\text{TD50 WT/TD50 } \textit{kgb-1}](\textit{E.coli})}$$

If the corrected relative mortality is greater than 1, the survival of the mutant is reduced by yeast. If the corrected relative mortality is less than 1, the survival of the mutant is unrelated to yeast.

Results

p38 mutants show enhanced susceptibility to infection by the yeast *S. cerevisiae*

We tested deletion mutants *nsy-1 (ok593)*, *sek-1 (km4)* and *pmk-1 (km25)*, as well as nonsense mutation *nsy-1 (ag3)* in the p38 pathway for effects on survival when exposed to *S. cerevisiae* (**Figs. 5-8**). The *nsy-1*, *sek-1*, and *pmk-1* genes encode MAPKK Kinase, MAPK kinase, and MAP kinase in the *C. elegans* p38 pathway, respectively. Survival assays indicated that mutants from the *C. elegans* p38 pathway, *nsy-1 (ok593)* (Fig. 5, P< 0.0001, Log-Rank test), *sek-1 (km4)* (Fig. 6, P< 0.0001, Log-Rank test), and *pmk-1 (km25)* (Fig. 7, P< 0.0001, Log-Rank test) showed a significant difference in survival when grown on *S. cerevisiae* compared to *E. coli* (Figs. 5-7). However, a *nsy-1* nonsense mutation *(ag3)* did not affect survival (Fig. 8, P=0.1225, Log-Rank test). Wild-type *C. elegans* showed a slight yet significant difference in survival when grown on *S. cerevisiae* compared to *E. coli* (Fig. 5, wild type results are also reproduced in Figs. 6-14 and Fig. 16 for comparison). When compared to wild type *C. elegans* grown on yeast, the survival assays also indicated that *nsy-1 (ok593)* (P< 0.0001), *sek-1 (km4)* (P< 0.0001, Log-Rank test), and *pmk-1 (km25)* (P< 0.0001, Log-Rank test) showed significant differences relative to that sample (**Figs. 5-7**). However, the *nsy-1 (ag3)* (P=0.0638, Log-Rank test) mutant was not significantly different in survival compared to wild-type *C. elegans* on yeast (**Fig. 8**).

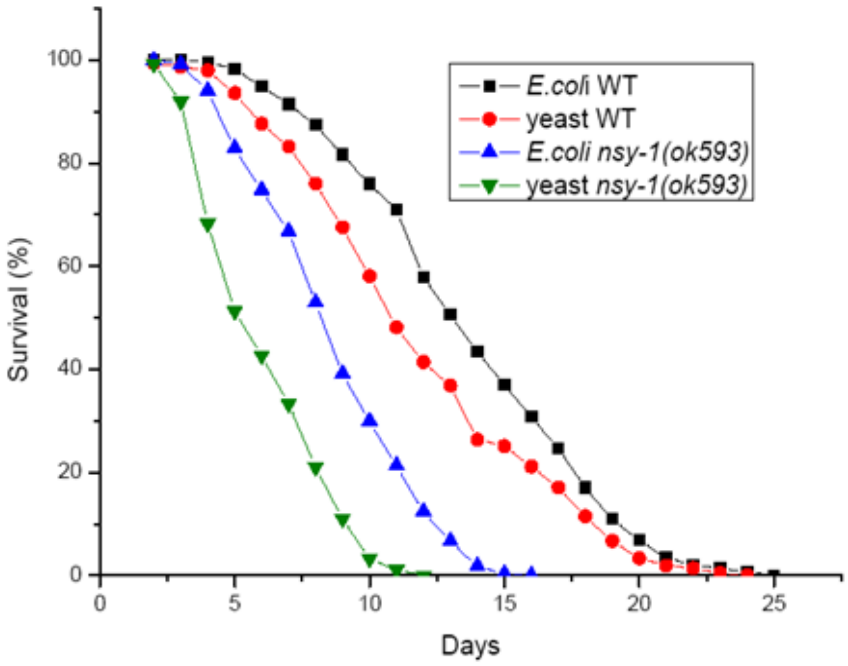


Figure 5. ***C. elegans*** **MAP kinase mutant** ***nsy-1 (ok593)*** **shows enhanced susceptibility to infection by the yeast** ***S. cerevisiae.*** Wild-type N2 *C. elegans* (red circles) and *nsy-1 (ok593)* (green triangles) were exposed to both yeast *S. cerevisiae* and *E. coli.* Cultures containing yeast also contained *E.coli* because the L1 larvae could not grow and develop without the presence of *E. coli* (Jain *et al.,* 2009). Wild-type N2 *C. elegans* (black squares) and *nsy-1 (ok593)* (blue triangles) were exposed to *E. coli* alone. Survival curves for the *nsy-1 (ok593)* mutant and wild-type N2 *C. elegans* indicate that *nsy-1 (ok593)* ($P < 0.0001$, Log-Rank test) were more susceptible to yeast than wild-type worms. The graphs represent combined results of four independent experiments, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

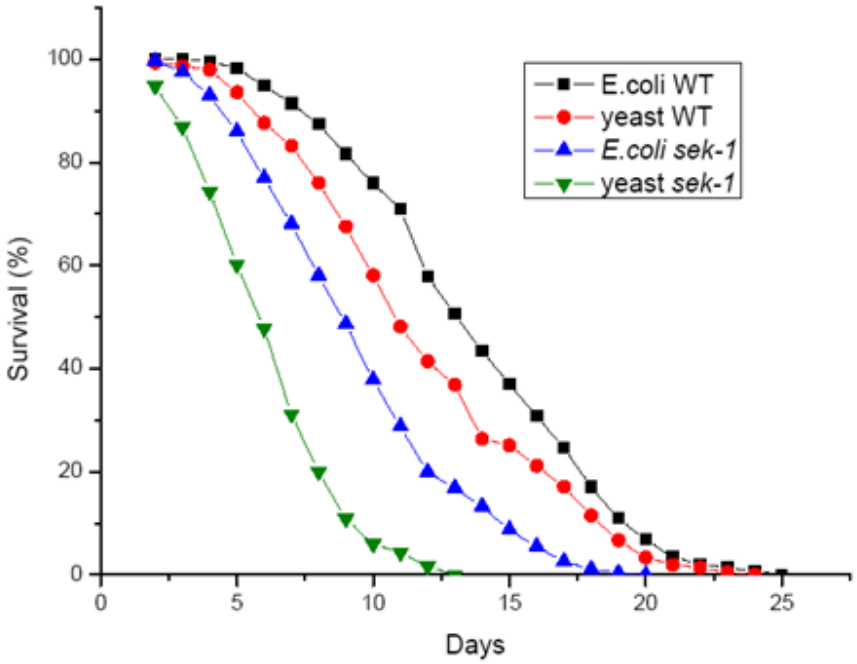


Figure 6. ***C. elegans*** **MAP kinase mutant** ***sek-1 (km4)*** **shows enhanced susceptibility to infection by the yeast** ***S. cerevisiae.*** Wild-type N2 *C. elegans* (red circles) and *sek-1 (km4)* (green triangles) were exposed to both yeast *S. cerevisiae* and *E. coli.* Wild-type N2 *C. elegans* (black squares) and *sek-1 (km4)* (blue triangles) were exposed to *E. coli* alone. Survival curves for the *sek-1 (km4)* mutant and wild-type N2 *C. elegans* indicate that *sek-1 (km4)* ($P < 0.0001$, Log-Rank test) were more susceptible to yeast than wild-type worms. The graphs represent combined results of four independent experiments, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

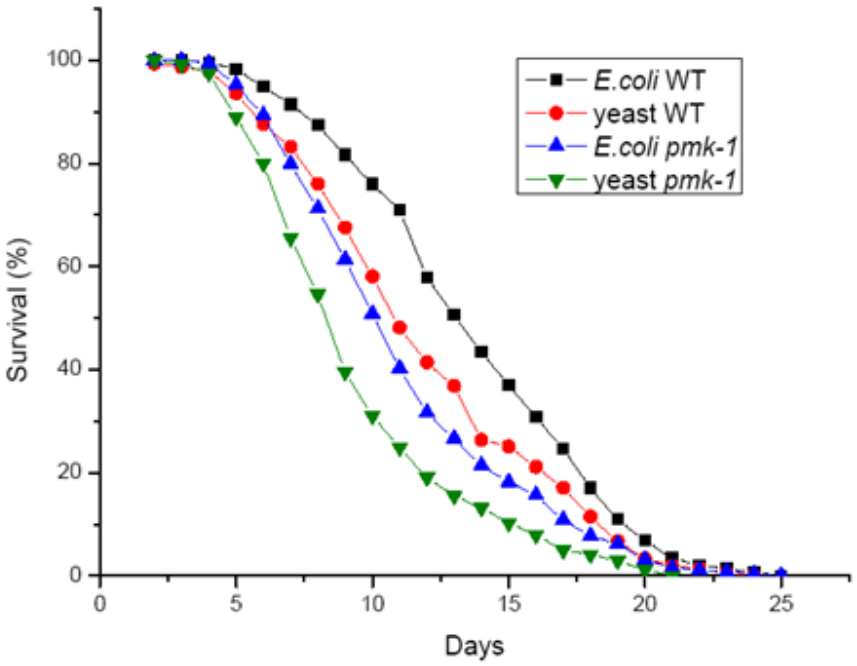


Figure 7. ***C. elegans*** **MAP kinase mutant** ***pmk-1 (km25)*** **shows enhanced susceptibility to infection by the yeast** ***S. cerevisiae.*** Wild-type N2 *C. elegans* (red circles) and *pmk-1 (km25)* (green triangles) were exposed to both yeast *S. cerevisiae* and *E. coli.* Wild-type N2 *C. elegans* (black squares) and *pmk-1 (km25)* (blue triangles) were exposed to *E. coli* alone. Survival curves for the *pmk-1 (km25)* mutant and wild-type N2 *C. elegans* indicate that *pmk-1 (km25)* ($P < 0.0001$, Log-Rank test) were more susceptible to yeast than wild-type worms. The graphs represent combined results of four independent experiments, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

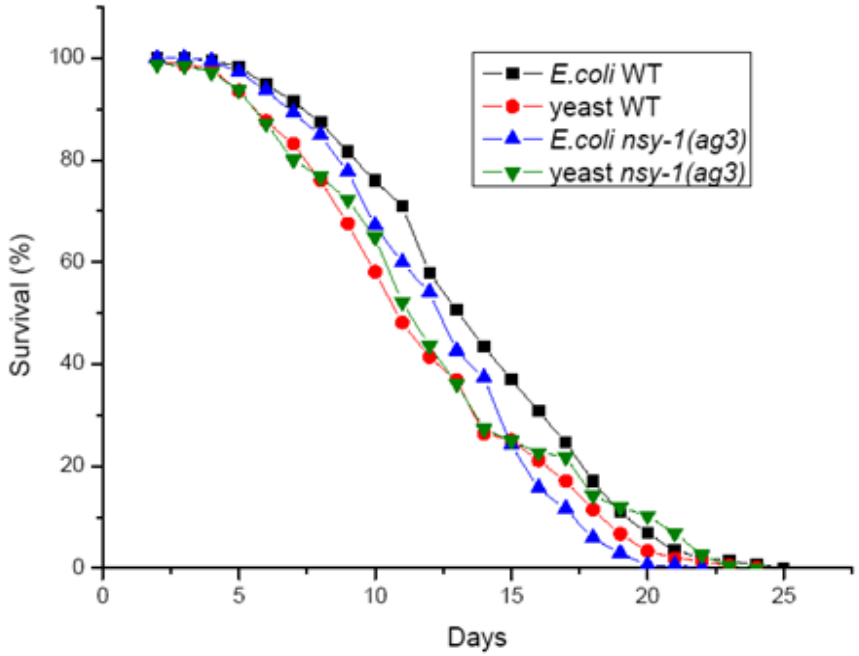


Figure. 8. ***C. elegans*** **MAP kinase mutation** ***nsy-1 (ag3)*** **did not enhance susceptibility to infection by the yeast** ***S. cerevisiae.*** Wild-type N2 *C. elegans* (red circles) and *nsy-1 (ag3)* (green triangles) were exposed to both yeast *S. cerevisiae* and *E. coli.* Wild-type N2 *C. elegans* (black squares) and *nsy-1 (ag3)* (blue triangles) were exposed to *E. coli* alone. Survival curves for the *nsy-1 (ag3)* mutant and wild-type N2 *C. elegans* indicate that *nsy-1 (ag3)* (P=0.1225, Log-Rank test) were not more susceptible to yeast than wild-type worms. The graphs represent combined results of four independent experiments, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

The *C. elegans tir-1* gene encodes a Toll Interleukin Receptor that is required for resistance to several different microbial pathogens. *Tir-1* acts upstream of the *C. elegans* p38 MAP kinase pathway (Couilliault *et al.,* 2004; Liberati *et al*., 2004). A *tir-1 (ok1052)* mutant (P< 0.0001, Log-Rank test) showed a slight difference in survival on *S. cerevisiae* compared to *E. coli* (**Fig. 9**). The result for wild-type vs *tir-1 (ok1052)* on yeast was also significant (P< 0.0001, Log-Rank test). Overall, the results from analyzing

four known members of the p38 pathway indicate that mutations in any of the four members enhances infection of *C. elegans* with *S. cerevisiae*.

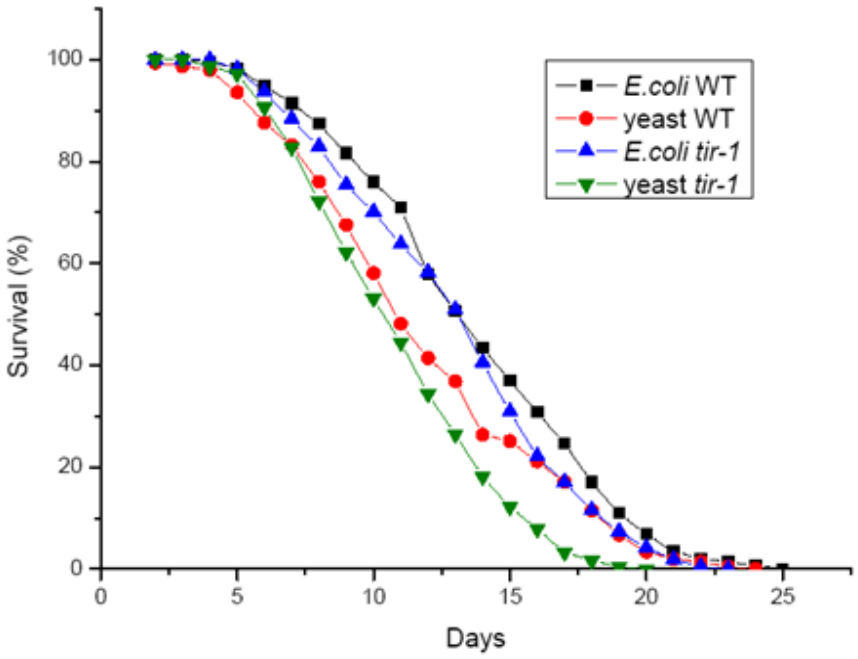


Figure 9. ***tir-1*** **slightly affects survival in the presence of** ***S. cerevisiae***. Wild-type N2 *C. elegans* (red circles) and *tir-1 (ok1052)* (green triangles) were exposed to both yeast *S. cerevisiae* and *E. coli.* Wild-type N2 *C. elegans* (black squares) and *tir-1 (ok1052)* (blue triangles) were exposed to *E. coli* alone. The *tir-1 (ok1052)* mutants were slightly more susceptible to yeast than their wild-type counterparts. The graphs represent combined results of four independent experiments, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

A JNK pathway mutant shows enhanced susceptibility to infection by the yeast *S. cerevisiae*

Several *C. elegans* genes encode apparent homologs of components of the MAP kinase pathway that activates mammalian c-Jun (Kim *et al.,* 2004). We tested

deletion mutants in *jnk-1* and *kgb-1,* which both encode JNK-like MAP kinases (Kim *et al*., 2004), for effects on survival when exposed to yeast *S. cerevisiae* (**Figs. 10-11**). Survival assays indicated that *kgb-1 (km21)* mutants showed a significant difference in survival on *S. cerevisiae* compared to *E. coli* ($P < 0.0001$, **Fig. 10**). In contrast, *jnk-1 (gk7)* ($P = 0.2045$, Log-Rank test) did not show a difference in survival (**Fig. 11**). The survival assays also indicated that *kgb-1 (km21)* ($P < 0.0001$, Log-Rank test) showed a significant difference in susceptibility to yeast killing compared to wild-type *C. elegans* grown on yeast (**Fig. 10**).

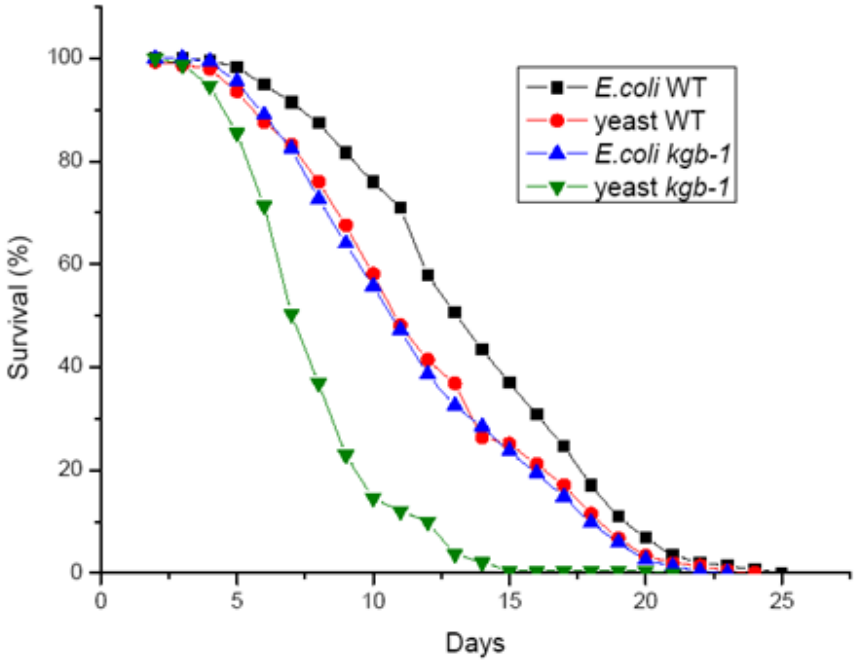


Figure 10. ***C. elegans*** **MAP kinase mutant** ***kgb-1 (km21)*** **shows enhanced susceptibility to infection by the yeast** ***S. cerevisiae.*** Wild-type N2 *C. elegans* (red circles) and *kgb-1 (km21)* (green triangles) were exposed to both yeast *S. cerevisiae* and *E. coli.* Wild-type N2 *C. elegans* (black squares) and *kgb-1 (km21)* (blue triangles) were exposed to *E. coli* alone. Survival curves for the *kgb-1 (km21)* mutant and wild-type N2 *C. elegans* indicate that *kgb-1 (km21)* ($P < 0.0001$, Log-Rank test) were more susceptible to yeast than wild-type worms. The graphs represent combined results of four independent experiments, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

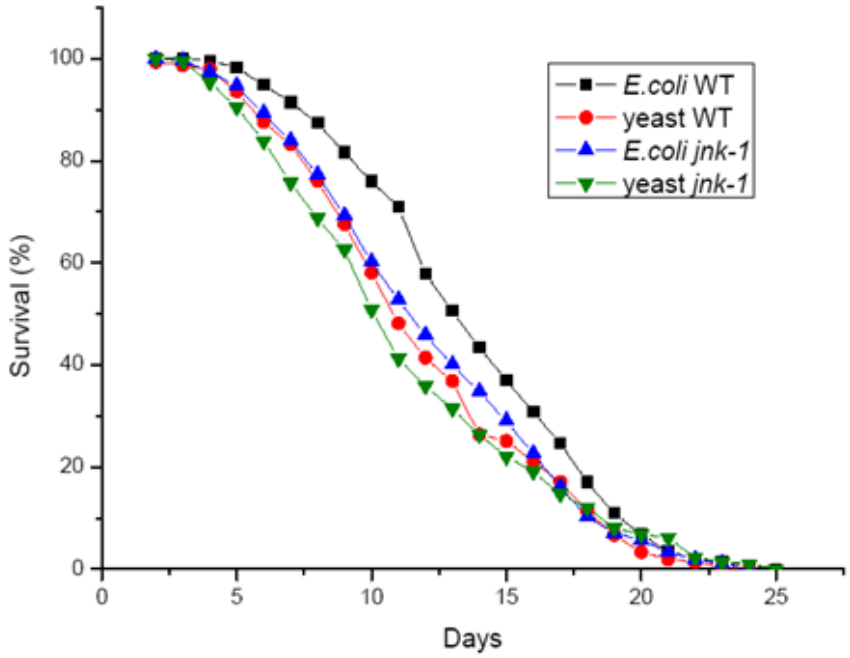


Figure 11. ***C. elegans*** **MAP kinase mutant** ***jnk-1 (gk7)*** **does not show enhanced susceptibility to infection by the yeast** ***S. cerevisiae.*** Wild-type N2 *C. elegans* (red circles) and *jnk-1 (gk7)* (green triangles) were exposed to both yeast *S. cerevisiae* and *E. coli.* Wild-type N2 *C. elegans* (black squares) and *jnk-1 (gk7)* (blue triangles) were exposed to *E. coli* alone. Survival curves for the *jnk-1 (gk7)* mutant and Wild-type N2 *C. elegans* indicate that *jnk-1 (gk7)* (P=0.2045, Log-Rank test) were less susceptible to yeast than wild-type worms. The graphs represent combined results of four independent experiments, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

We tested another MAP kinase kinase (MAPKK) mutant, *mek-1 (ks54),* for survival in the presence of yeast. The *mek-1* mutation, originally characterized as a deletion generated by imprecise excision of an inserted transposable element (Koga *et al.*, 2000) identified a gene which showed sequence similarity to mammalian MAPKK7, which specifically phosphorylates JNK MAP kinase. Mizuno *et al* (2004) demonstrated that the *C. elegans* JNK-like MAPK, KGB-1, is regulated by MEK-1. Interestingly, MEK-1 also appears to act upstream of the p38 homolog PMK-1 (Kim *et al.*, 2004). This

suggested that MEK-1 might be involved in cross-talk between the p38 and JNK pathways in *C. elegans*.

However, more recently it has been shown that the *C. elegans* strain containing *mek-1 (ks54)* carries a separate deletion extending into the coding sequence of the closely linked gene *sek-1*, which encodes the MAPKK immediately upstream of PMK-1 (Dennis Kim, personal communication). Thus the strain containing *mek-1 (ks54)* is actually a double *mek-1; sek-1* mutant. By testing the *mek-1 (ks54); sek-1* double deletion, we were able to assess the effects of simultaneously knocking out the p38 pathway and the pathway controlled by *kgb-1*. The *mek-1 (ks54); sek-1* double mutant showed significantly reduced survival compared with the wild-type control when grown on yeast (**Fig. 12**).

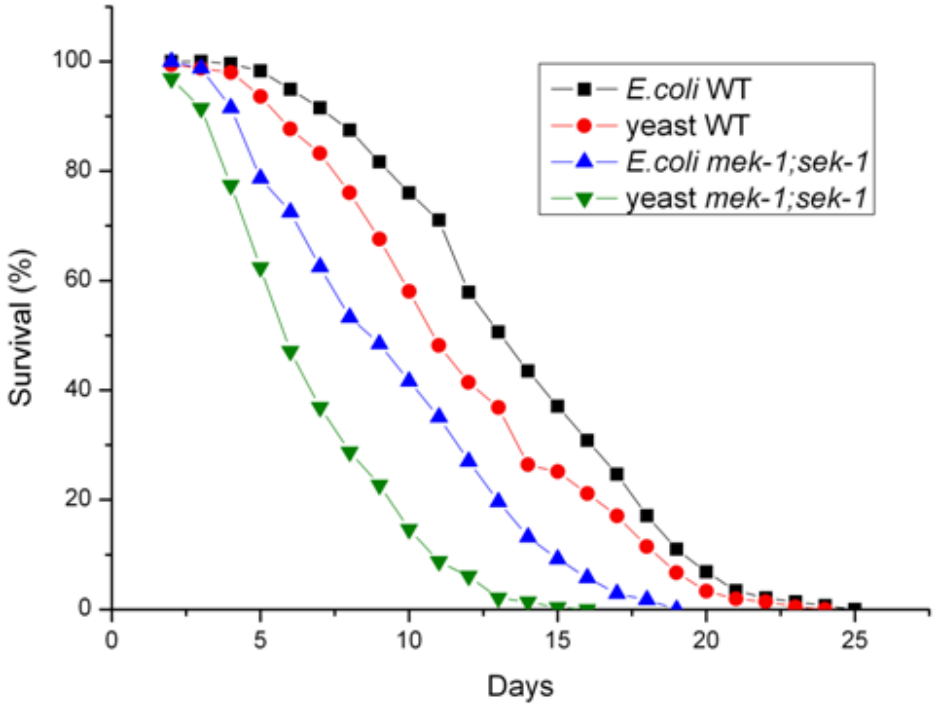


Figure 12. ***C. elegans*** **MAP kinase mutant** ***mek-1 (ks54); sek-1*** **shows enhanced susceptibility to infection by the yeast** ***S. cerevisiae.*** Wild-type N2 *C. elegans* (red circles) and *mek-1 (ks54) sek-1* (green triangles) were exposed to both yeast *S. cerevisiae* and *E. coli.* Wild-type N2 *C. elegans* (black squares) and *mek-1 (ks54) sek-1* (blue triangles) were exposed to *E. coli* alone. Survival curves for the *mek-1 (ks54) sek-1* mutant and wild-type *C. elegans* indicated that *mek-1 (ks54); sek-1* (P< 0.0001, Log-Rank test) worms were more susceptible to yeast than wild-type worms. The graphs represent combined results of four independent experiments, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

ERK mutants show enhanced susceptibility to infection by the yeast *S. cerevisiae*

C. elegans has an ERK MAP kinase pathway that has been shown to be required for the Dar response (Nicholas and Hodgkin, 2004b; Jain *et al.,* 2009). The *mek-2* and *mpk-1* genes encode MAPK Kinase and MAP kinase in the *C. elegans* ERK pathway, respectively. We tested missense mutants *mek-2 (n1989)* and *mpk-1 (ku1)* for effects on survival when exposed to yeast *S. cerevisiae* (**Figs. 13 and 14**). It was necessary to

use point mutants because most ERK pathway null mutants are lethal in *C. elegans*. Survival assays indicated that *mek-2 (n1989)* showed a significant difference in survival on *S. cerevisiae* compared to *E. coli* (P< 0.0001, Log-Rank test, **Fig. 13**). Because *mpk-1 (ku1)* was only available as a double mutant with *unc-32*, to test the contribution of *mpk-1* to survival on exposure to yeast, *unc-32 (e189)* was used as a control group instead of wild type, and *mpk-1 (ku1); unc-32 (e189)* was used as the *mpk-1* test mutant.

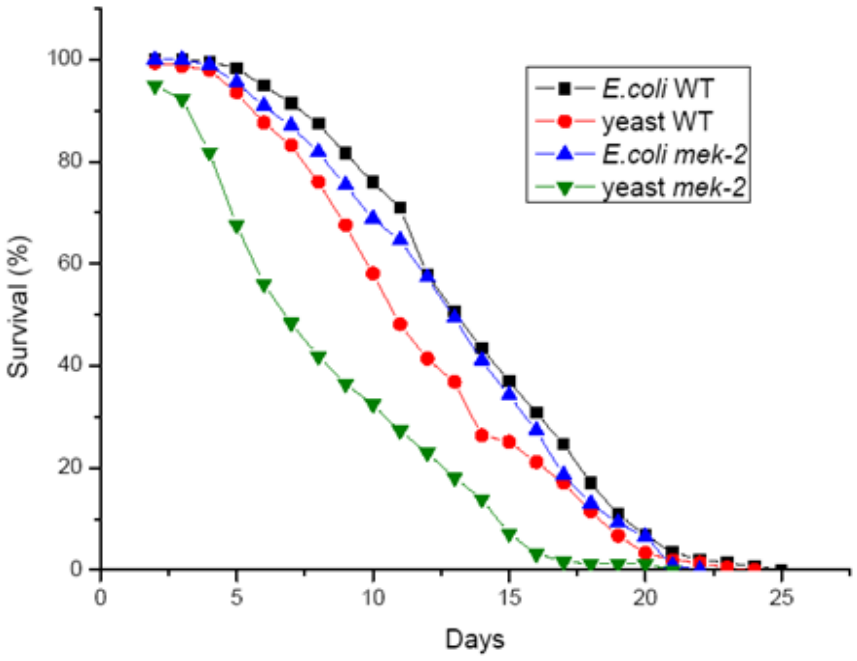


Figure 13. ***C. elegans*** **MAP kinase mutant** ***mek-2 (n1989)*** **shows enhanced susceptibility to infection by the yeast** ***S. cerevisiae.*** Wild-type N2 *C. elegans* (red circles) and *mek-2 (n1989)* (green triangles) were exposed to both yeast *S. cerevisiae* and *E. coli.* Wild-type N2 *C. elegans* (black squares) and *mek-2 (n1989)* (blue triangles) were exposed to *E. coli* alone. Survival curves for the *mek-2 (n1989)* mutant and wild-type N2 *C. elegans* indicate that *mek-2 (n1989)* (P< 0.0001, Log-Rank test) were more susceptible to yeast than wild-type worms. The graphs represent combined results of four independent experiments, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

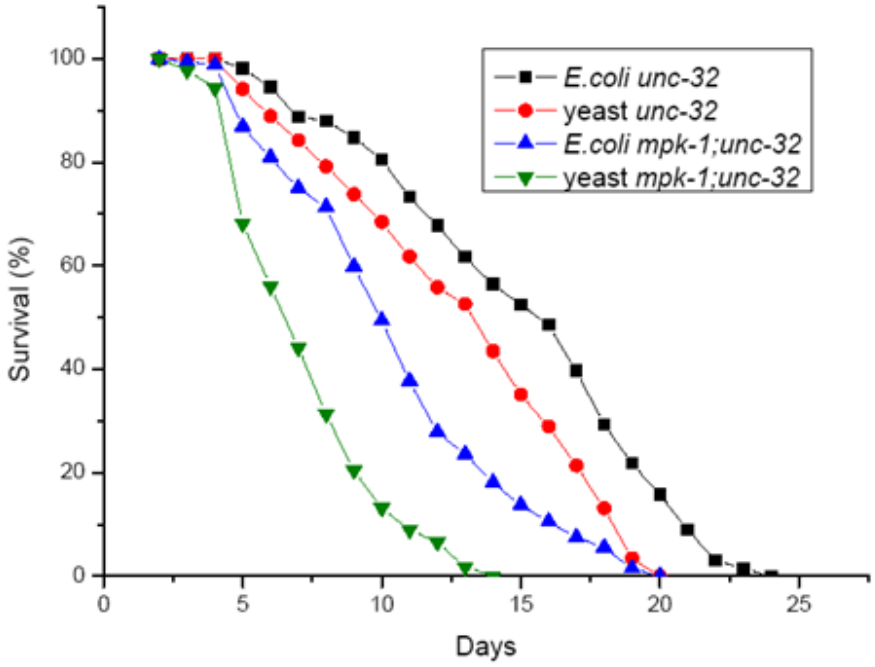


Figure 14. ***C. elegans*** **MAP kinase mutant** ***mpk-1 (ku1); unc-32 (e189)*** **shows enhanced susceptibility to infection by the yeast** ***S. cerevisiae.*** *unc-32 (e189)* (red circles) and *mpk-1 (ku1); unc-32 (e189)* (green triangles) were exposed to both *S. cerevisiae* and *E. coli.* *unc-32 (e189)* (black squares) and*(mpk-1 (ku1); unc-32 (e189)* (blue triangles) were also exposed to *E. coli* alone. Survival curves for *mpk-1 (ku1); unc-32 (e189)* and *unc-32 (e189) C. elegans* indicate that the strain containing *mpk-1 (ku1)* ($P < 0.0001$, Log-Rank test) was more susceptible to yeast than *unc-32 (e189)* control worms. The graphs represent combined results of two independent experiments, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

S. cerevisiae causes a deformity in the postanal region (Dar) in p38 mutants

Contact with the bacterial pathogen *Microbacterium nematophilum* or the yeast *S. cerevisiae* causes a swelling in the postanal region of the *C. elegans* tail (Nicholas and Hodgkin, 2004b; Jain *et al*., 2009). *Nsy-1 (ok593)*, *sek-1 (km4)*, and *pmk-1 (km25)* deletion mutants from the *C. elegans* p38 pathway, as well as wild-type control *C. elegans*, exhibited a high percentage of the Dar phenotype in response to *S. cerevisiae*.

Although *nsy-1* and *pmk-1* mutants showed a small reduction in Dar that was statistically significant, overall, these data suggest that mutations in the p38 pathway have little effect on the animal's ability to produce the Dar phenotype (**Fig. 15**).

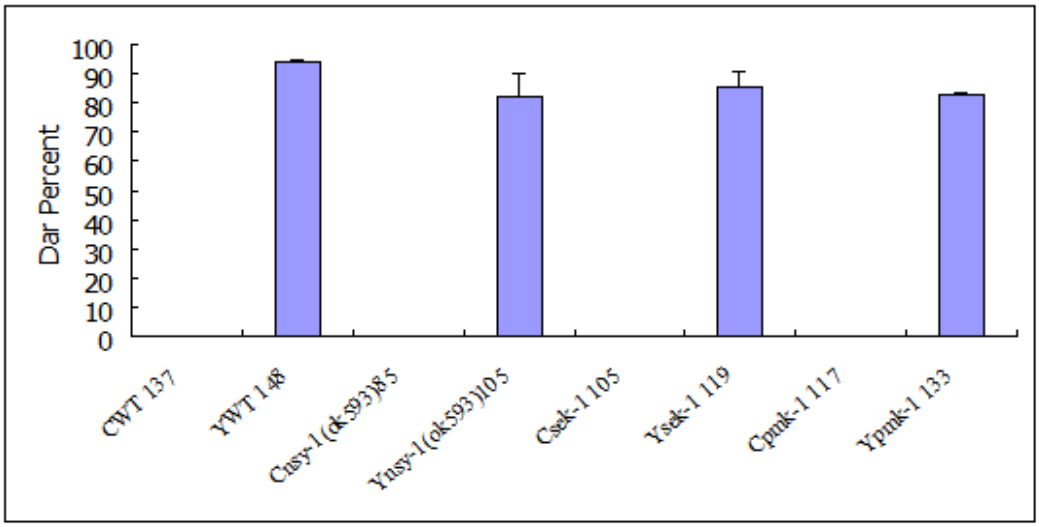


Fig. 15. The p38 pathway is not required for the DAR phenotype when exposed to yeast *S. cerevisiae*. *C. elegans* mutants *nsy-1 (ok593)*, *sek-1 (km4)* and *pmk-1 (km25) of* the p38 pathway *as well as* wild-type worms were exposed to wild-type yeast, and the DAR phenotype was scored on day 4. The *C. elegans* mutants *nsy-1 (ok593)*, *sek-1 (km4)* and *pmk-1 (km25)* as well as wild-type worms showed a high percentage of Dar when exposed to yeast. Statistical analysis showed a slight but significant reduction of the proportion of Dar animals in *nsy-1* and *pmk-1* mutants when compared with wild type animals (ANOVA, p=0.029; Dunnett's post-hoc tests, p=0.025 for each comparison). No significant difference was observed between wild type and *sek-1* mutants (Dunnett's post-hoc, p=0.07). The number of worms for each group is marked under the bar. Error bars denote standard deviation.

C. elegans tol-1 mutants show little difference in susceptibility to infection by the yeast *S. cerevisiae*

C. elegans has a single homolog of *Drosophila* and human Toll-Like Receptors, encoded by *tol-1*. Because Toll-Like Receptors are important pattern recognition receptors in human innate immune responses, we tested *C. elegans tol-1 (nr2033)* for

survival in the presence of yeast. The survival curves for the *tol-1 (nr2033)* deletion mutant and wild-type *C. elegans* indicated that *tol-1 (nr2033)* (P=0.0011, Log-Rank test) showed a slight yet significant difference in survival on *S. cerevisiae* compared to *E. coli* (**Fig. 16**).

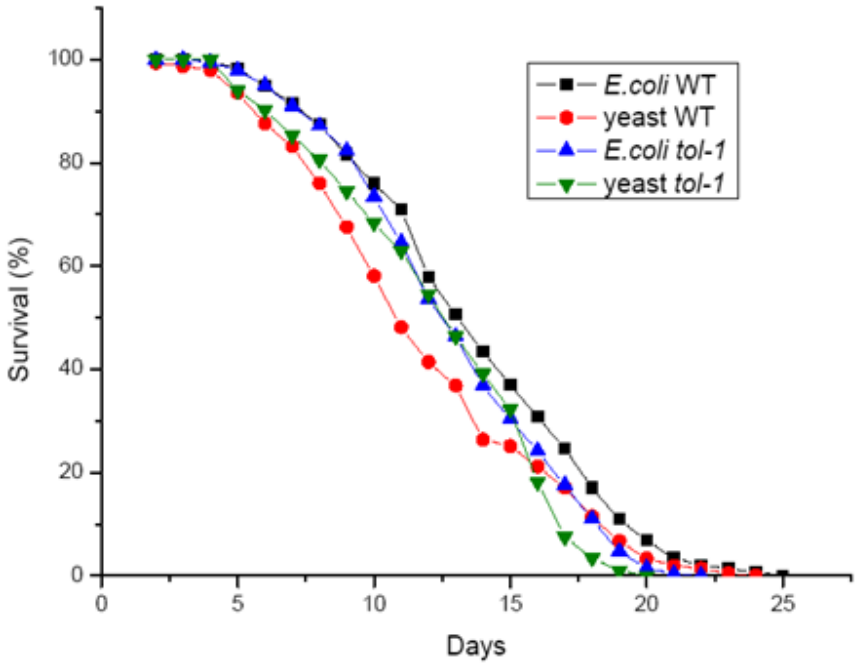


Figure 16. TOL-1-mediated immunity has little effect on survival in the presence of *S. cerevisiae*. Wild-type N2 *C. elegans* (red circles) and *tol-1 (nr2033)* (green triangles) were exposed to both *S. cerevisiae* and *E. coli.* Wild-type N2 *C. elegans* (black squares) and *tol-1 (nr2033)* (blue triangles) were exposed to *E. coli* alone. Survival curves for the *tol-1 (nr2033)* mutant and wild-type N2 *C. elegans* indicate that *tol-1 (nr2033)* (P=0.0011, Log-Rank test) showed a slight yet significant difference in survival on *S. cerevisiae* compared to *E. coli.* The graphs represent combined results of four independent experiments, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

Correction of survival results for intrinsic reduction in viability of mutants

As an alternative way to compare mutant survival to wild-type survival on yeast, the TD50 and relative mortality of mutants grown on yeast were calculated. When compared to wild type by relative mortality, most mutants tested on yeast showed a reduced TD50 compared to wild-type, indicated by a relative mortality > 1 (**Table 1**). However some mutants showed relative mortality > 1 even when grown on *E. coli* alone, suggesting that the mutants are less viable than wild type (**Table 1**). In order to remove bias resulting from a possible mutant reduction in survival, which is unrelated to yeast infection, relative mortalities were corrected for the differences in survival on *E. coli*. Even after correcting for the differential survival of mutant and wild-type *C. elegans* on *E. coli*, most of the mutants showed reduced survival when grown on yeast (**Table 1** and **Fig. 17**, corrected relative mortality > 1). Exceptions were noted in the case of *nsy-1 (ag3), pmk-1 (km25), tol-1 (nr2033),* and *tir-1 (ok1052)*, all of which showed increased survival when grown on yeast (**Fig. 17** and **Table 1**). Thus, the relative mortality results were generally consistent with P values in the survival assay (Fig. 17 and Table 1).

Strains	TD50 (*E.coli)*	TD50 (yeast)	Relative Mortality (yeast)	Relative Mortality (*E.coli*)	Corrected Relative Mortality
Wild Type (N2)	11.09	8.590			
nsy-1(ag3)	10.40	9.168	0.9370	1.0663	0.8787
nsy-1(ok593)	6.556	4.013	2.1405	1.6916	1.2654
sek-1	6.449	3.966	2.1659	1.7196	1.2595
pmk-1	7.884	6.176	1.3909	1.4066	0.9888
mek-1;sek-1	6.677	4.072	2.1095	1.6609	1.2701
jnk-1	9.149	7.656	1.1220	1.2122	0.9256
kgb-1	8.601	4.593	1.8702	1.2894	1.4504
mek-2	11.48	4.895	1.7549	0.9660	1.8167
mpk-1	7.916	5.122	2.4795	1.8115	1.3688
tol-1	11.40	11.57	0.7424	0.9728	0.7632
tir-1	10.83	8.559	1.0036	1.0240	0.9801

Table 1. Relative mortality results. TD50 is the time in days on which half of the worms died, and was calculated by nonlinear regression as described in the Materials and Methods section. If the relative mortality or corrected relative mortality is greater than 1, survival of the mutant is apparently reduced by yeast.

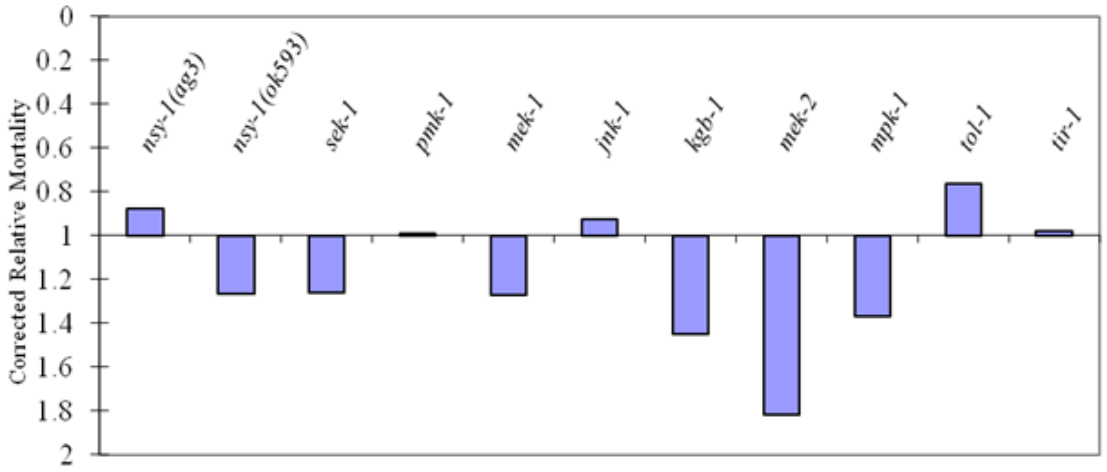


Fig. 17. Graphic summary of corrected relative mortality results. Corrected relative mortalities were calculated as described in Materials and Methods and the legend to Table 1. Bars below the x axis (corrected relative mortality > 1) represent mutants whose survival is reduced by yeast. Mutant alleles are not shown in the figure except for *nsy-1 (ag3)* and *nsy-1 (ok593)*.

Effects of fertility on survival in the presence of yeast

Jain *et al* (2009) reported that *C. elegans* hermaphrodites grown on yeast exhibited delayed egg-laying, manifested by hatching of eggs inside the gonad. This results in killing of the mother, and is a well-known consequence of growth in adverse environmental conditions, such as starvation. Jain *et al* (2009) suggested that this was a major cause of death in *C. elegans* grown on yeast. To test this hypothesis, we used *fer-1 (b232)*, a temperature-sensitive fertilization-defective mutant of *C. elegans*. *Fer-1 (b232)* hermaphrodites are fertile at 16°C, but completely sterile at 20°C because of a defect in sperm development (Argon and Ward, 1980). This phenotype allowed us to test survival of *C. elegans* in the absence of internally hatched offspring by growing *fer-1 (b232)* worms at the standard survival test temperature of 20°C.

Surprisingly, the lifespan of *fer-1 (b232)* was greatly increased compared to that of wild-type *C. elegans* when both were grown on *E. coli* at 20°C (**Fig. 18**, P< 0.0001, Log-Rank test, TD50 of *fer-1 (b232)* = 25.78 days; TD50 of wild-type = 11.09 days, **Table 2**). The lifespan of *fer-1 (b232)* at 20°C was significantly reduced when it was grown on yeast compared to its lifespan on *E. coli* (**Fig. 18**, P < 0.0001, TD50 = 16.80 days), even though no fertile eggs were visible in the adult hermaphrodite gonad under these conditions. Therefore, the killing of *fer-1 (b232)* worms grown on yeast cannot readily be explained by an internal hatching of eggs.

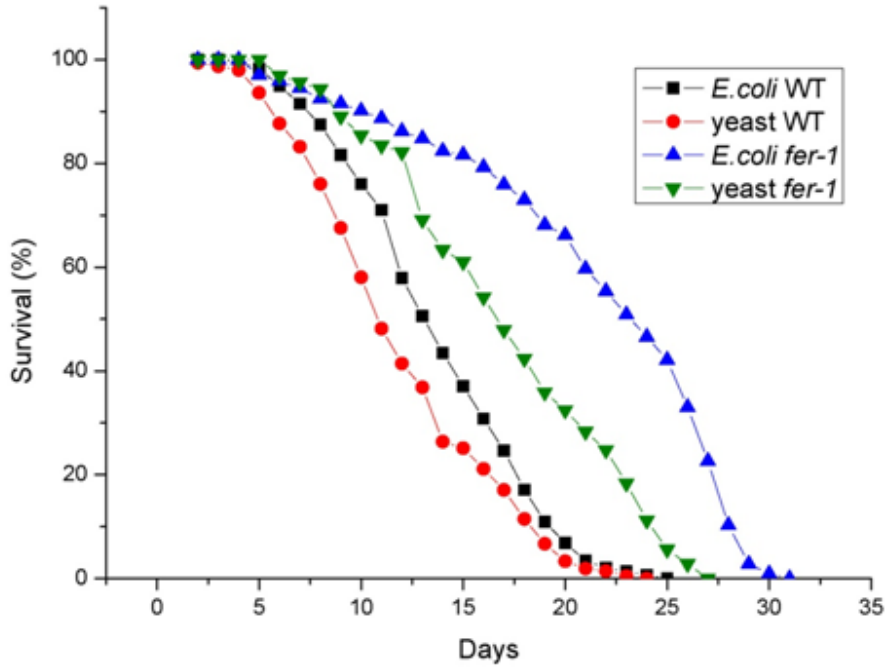


Fig. 18. *C. elegans* mutant *fer-1 (b232)* is sensitive to yeast in the absence of fertilization. Wild-type *C. elegans* (red circles) and *fer-1 (b232)* (green triangles) were exposed to both *S. cerevisiae* and *E. coli.* Wild-type *C. elegans* (black squares) and *fer-1 (b232)* (blue triangles) were also exposed to *E. coli* alone. In all survival tests, worms were grown at 20°C, the restrictive temperature for *fer-1 (b232)*. Survival curves for the *fer-1 (b232)* mutant on yeast versus *E. coli* alone indicated that *fer-1 (b232)* mutants (P< 0.0001, Log-Rank test) were susceptible to yeast even in the absence of fertilization. The graphs represent combined results of four independent experiments for wild-type *C. elegans* and two independent experiments for *fer-1*, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

Strains	TD50 (*E.coli)*	TD50 (yeast)	Relative Mortality (yeast)	Relative Mortality (*E.coli*)	Corrected Relative Mortality
Wild Type (N2)	11.09	8.590			
fer-1	25.78	16.80	0.5113	0.43018	1.1886

Table 2. Relative mortality results for *fer-1*. Relative mortality and corrected relative mortality were calculated as described in the legend to Table 1.

Nevertheless, it remained possible that the reduced survival of the MAP kinase pathway mutants, as opposed to wild-type *C. elegans*, might result from internal hatching of offspring. As a test case, we constructed a *fer-1 (b232); kgb-1 (km21)* double mutant (Muxun Zhao and S. Politz, unpublished). In the *fer-1 (b232)* mutant background, the *kgb-1* mutation reduced the lifespan significantly (P< 0.0001, Log-Rank test, Fig. 19) on yeast. However, the *fer-1* lifespan was also reduced significantly by *kgb-1* even when the strains were compared on *E. coli* (P <0.0001, Log-Rank test, **Fig. 19**). Because of this, the relative mortality corrected for the difference in lifespan on *E. coli* was very close to 1 (0.9797, **Table 3**). Thus, although *kgb-1* showed reduced survival in the presence of yeast even when fertilization was absent, the results were inconclusive.

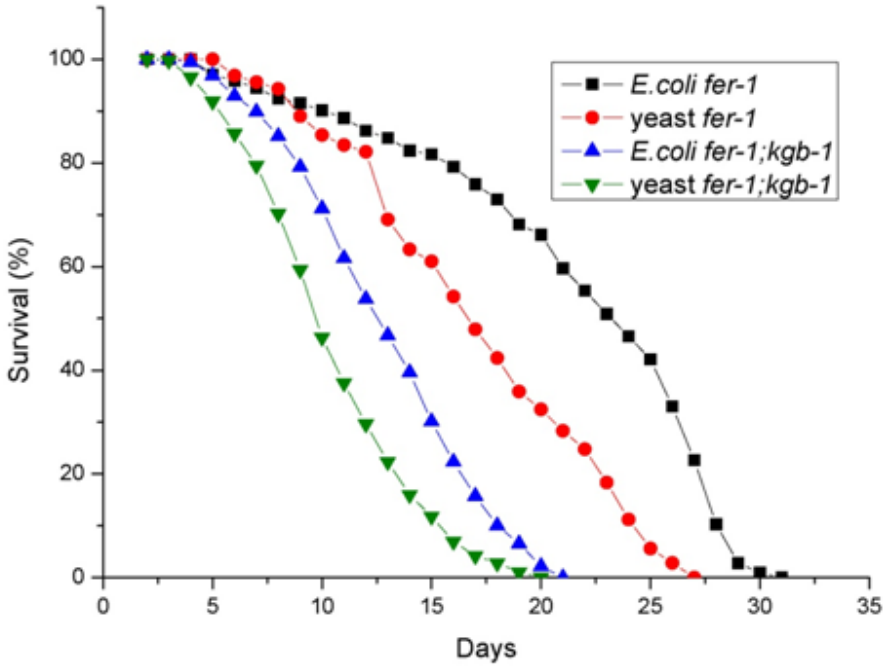


Fig. 19. A *C. elegans kgb-1* mutant is sensitive to yeast in the absence of fertilization. *C. elegans fer-1* (red circles) and *fer-1; kgb-1* (green triangles) mutants were exposed to both *S. cerevisiae* and *E. coli.* *C. elegans fer-1 C. elegans* (black squares) and *fer-1; kgb-1* (blue triangles) were also exposed to *E. coli* alone. The graphs represent combined results of four independent experiments for *fer-1; kgb-1* and two independent experiments for *fer-1*, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

Strains	TD50 (*E. coli)*	TD50 (yeast)	Relative Mortality (yeast)	Relative Mortality (*E. coli*)	Corrected Relative Mortality
fer-1	25.78	16.80			
fer-1; kgb-1	11.79	7.842	2.1423	2.1866	0.9797

Table 3. Relative mortality results for *fer-1; kgb-1*. In table 3, the equation used for relative mortality was TD50 *fer-1*/TD50 *fer-1; kgb-1*. Corrected mortality for Table 3 was calculated from the equation

[TD50 *fer-1* /TD50 *fer-1;kgb-1*] (yeast)

[TD50 *fer-1*/TD50 *fer-1;kgb-1*](*E.coli).*

fer-1 was used as the background genotype for comparison to *fer-1; kgb-1*.

C. elegans* wild type and MAP kinase pathway mutants show enhanced survival on heat-killed *E. coli* compared to live *E. coli

Studies of bacterial pathogenesis in *C. elegans* have shown that even *E. coli* can cause a reduction in survival, when compared to *C. elegans* grown on heat-killed *E. coli* (Mallo *et al.,* 2002). In our experiments, wild-type *C. elegans* showed a significant reduction in survival on live *E. coli* compared to heat-killed *E. coli* (**Figs. 20-21**, P=0.0164). Similarly, *kgb-1 (km21)* (P=0.0176, log-rank test), and *pmk-1 (km25)* (P=0.0002, log-rank test) showed a significant reduction in survival on live *E. coli* compared to heat-killed *E. coli* (**Figs. 20** and **21**, respectively). Thus, these results are consistent with the interpretation that live *E. coli* causes a reduction in survival compared with innocuous heat-killed bacteria.

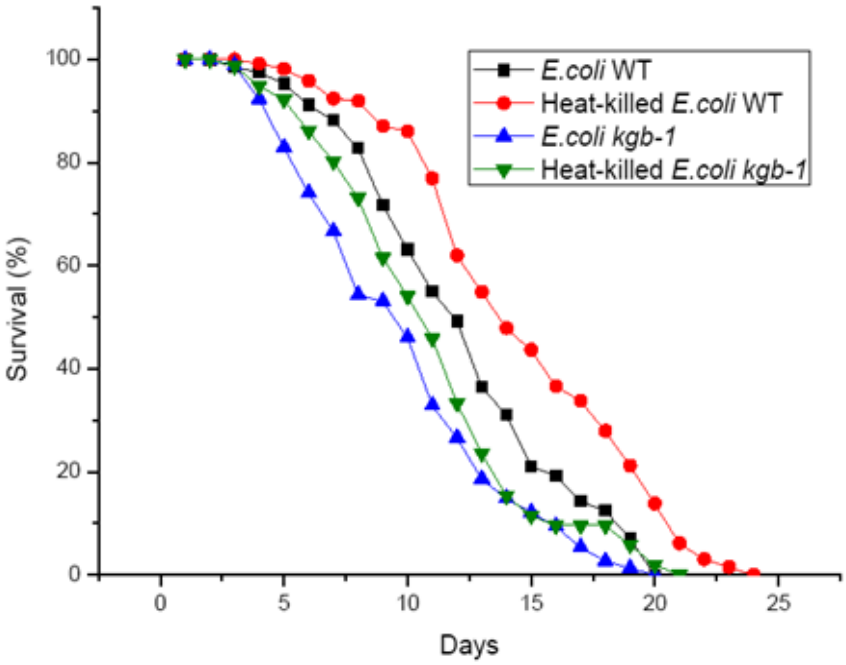


Figure 20. Survival assay of *C. elegans* wild-type and *kgb-1* mutant grown on heat-killed *E. coli.* Wild-type N2 *C. elegans* (black squares) and *kgb-1 (km21)* (blue triangles) were exposed to live *E. coli.* Wild-type N2 *C. elegans* (red circles) and *kgb-1 (km21)* (green triangles) were also exposed to heat-killed *E. coli.* All tests were performed at 20°C. Wild-type *C. elegans* (P= 0.0164, log-rank test) showed a significant reduction in survival on live *E. coli* compared to heat-killed *E. coli.* The *kgb-1 (km21)* mutant (P=0.0176, log-rank test) also showed a significant reduction in survival on live *E. coli* compared to heat-killed *E. coli.* The graphs represent combined results of two independent experiments for wild-type N2 *C. elegans* and one experiment for *kgb-1*, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

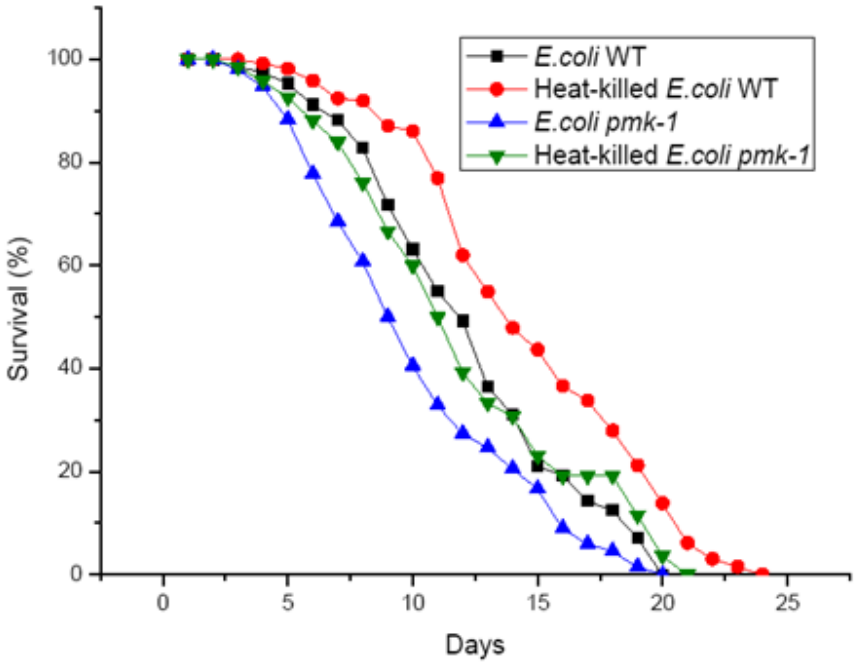


Figure 21. Survival assay of wild-type and *pmk-1* mutant *C. elegans* grown on heat-killed *E. coli.* Wild-type N2 *C. elegans* (black squares) and *pmk-1 (km25)* (blue triangles) were exposed to live *E. coli.* Wild-type N2 *C. elegans* (red circles) and *pmk-1 (km25))* (green triangles) were also exposed to heat-killed *E. coli.* Wild-type N2 *C. elegans* (P= 0.0164, log-rank test) showed a significant reduction in survival on live *E. coli* compared to heat-killed *E. coli.* *pmk-1(km25)* (P=0.0002, log-rank test) also showed a significant reduction in survival on live *E. coli* compared to heat-killed *E. coli.* The graphs represent combined results of two independent experiments for Wild-type N2 *C. elegans* and one independent experiment for *pmk-1*, each of which included three yeast and three *E. coli* plates per strain; each plate was started with 30 ± 5 eggs.

Discussion

p38 mutants show enhanced susceptibility to infection by the yeast *S. cerevisiae*

A survival assay was used to assess the relative effect of various MAP kinase pathway mutations on *C. elegans'* immunity to yeast infection. We found that two p38 MAP kinase pathway components are important for innate immunity against the yeast pathogen. Survival assays showed that *nsy-1 (ok593)* (*P*< 0.0001, Log-Rank test) and *sek-1 (km4)* (*P*< 0.0001, Log-Rank test) were affected by the yeast *S. cerevisiae* (**Figs. 5-6**), which indicated that *nsy-1* and *sek-1* play a crucial role in the immune response to yeast infection. Similar survival results have also been observed with *P. aeruginosa* infection of *C. elegans* mutants *sek-1 (km4) (*Kim *et al.*, 2002; Kim *et al.*, 2004; Troemel *et al*., 2006) *and nsy-1 (ky597) (*Kim *et al*., 2002). *Sek-1* encodes a mitogen-activated protein kinase kinase (MAPKK) that functions upstream of the p38 MAP kinase *pmk-1*. *Sek-1* mediates asymmetric sensory neuron differentiation (Tanaka-Hino *et al*., 2002) and egg laying (Tanaka-Hino *et al*., 2002) in addition to being required for responses to bacterial infection including *Pseudomonas aeruginosa* and fungal infection including *Drechmeria coniospora* (Kim *et al.*, 2002; Kim *et al*., 2004; Troemel *et al*., 2006; Pujol *et al*., 2008) and wounding (Pujol *et al*., 2008). *Nsy-1* encodes a MAP kinase kinase kinase (MAPKKK) and also belongs to the p38 Kinase pathway, upstream of *sek-1*. *Nsy-1* determines asymmetric olfactory neuron differentiation, which affects stereotypical chemotaxis

responses (Wes and Bargmann, 2001; Sagasti *et al*., 2001; Torayama *et al*., 2007). Like *sek-1*, *nsy-1* is also required for the immune response to bacterial pathogens.

In our experiments, we tested two *nsy-1* mutant alleles for effects on survival. We found that the mutant carrying nonsense mutation *nsy-1 (ag3)* was unaffected by *S. cerevisiae* (**Fig. 8**). In contrast, the deletion mutant *nsy-1 (ok593)* showed a significant reduction in survival when grown on yeast (**Fig. 5**). Because the nonsense mutation *ag3* is a late stop codon at amino acid codon 1003 of 1425 total amino acids (WormBase 2010), it is more likely to leave partial activity than the large deletion *ok593*. Therefore, we conclude from the *ok593* deletion that *nsy-1* is important for *C. elegans* resistance to yeast infection.

An exception to this pattern of involvement of p38 pathway components in resistance to yeast infection was noted in the case of *pmk-1 (km25)*. Log-rank statistics of the *pmk-1* mutant survival curve indicated that *pmk-1 (km25)* showed enhanced susceptibility to yeast (**Table 1**). However, when relative mortality of *pmk-1* was calculated and corrected for the intrinsic loss of viability of *pmk-1 (km25)*, survival of the *pmk-1* mutant did not appear to be reduced by yeast (**Table 1 and Fig. 17**). This result points out the usefulness of the correction of relative mortality in cases where the survival curves appear to show only a slight effect of yeast infection. PMK-1 encodes a p38-like MAPK that has been shown to act downstream from the *sek-1* to *nsy-1* kinase cascade. PMK-1 regulates the stress response (Pujol *et al*., 2008), programmed cell death (Aballay *et al*., 2003) and the immune response (Kim *et al*., 2002; Aballay *et al*.,

2003; Kim *et al.*, 2004; Troemel *et al*., 2006; Bolz *et al*., 2010). There was also an enhanced susceptibility to pathogen phenotype found when *pmk-1* RNAi worms were exposed to *P. aeruginosa* (Kim *et al*., 2002; Troemel *et al*., 2006)*, Y. pestis* (Bolz *et al*., 2010) and *S. enterica* (Aballay *et al.*, 2003). It was therefore surprising that *pmk-1 (km25)*, a large deletion, did not affect resistance to yeast infection. Although we cannot explain this discrepancy, it is possible that in the case of yeast infection, a different MAP kinase is responding to signaling through *sek-1* and *nsy-1*.

The *tir-1* gene encodes a Toll Interleukin Receptor similar in sequence to other members of this important family of adaptor proteins. In mammals and fruit flies, the TIR domain-containing proteins bind to Toll-like receptors and transduce signals to downstream kinase cascades that activate immune effectors (Murphy, 2008), including the p38 pathway. *C. elegans* TIR-1 is required for induction of antimicrobial peptides in response to an infection by the fungus *Drechmeria coniospora* (Couillault *et al.*, 2004), and is required for resistance to infection by several different microbial pathogens (Couillault *et al.*, 2004; Liberati *et al.*, 2004). *Tir-1* is also required for asymmetric sensory neuron development (Chuang and Bargmann, 2005). In all of these responses, TIR-1 acts upstream of the p38 pathway.

We tested the deletion allele *tir-1 (ok1052)* for survival on yeast and observed a significant reduction in survival (**Fig. 9**). Again, however, correction of relative mortality eliminated this difference (**Table 1** and **Figure 17**). This is perhaps surprising, given *tir-1*'s established role in immune responses in *C. elegans*. However, previous studies

indicated that the *ok1052* deletion may have partial activity. Upregulation of expression of an antimicrobial peptide by the phorbol ester PMA was normal in *tir-1 (ok1052)*, but blocked in another deletion mutant, *tir-1 (tm3036)* (Ziegler *et al.*, 2009). Consistent with this, the *tir-1 (ok1052)* deletion only affects the *a*, *c*, and *e* isoforms of TIR-1, whereas *tm3036* affects all TIR-1 isoforms (Chuang and Bargmann, 2005). Therefore, *tir-1 (tm3036)* should be tested for reduced survival on yeast in future studies, and we predict it will show a significant effect in the survival assays.

A *C. elegans* JNK-like MAP kinase, KGB-1, is required for resistance to infection by *S. cerevisiae*

Kgb-1 encodes a *C. elegans* MAP kinase that is involved in resistance to heavy metal stress (Mizuno *et al*., 2004), oocyte maturation (Smith *et al*., 2002), and the immune response (Kim *et al.*, 2004). *Kgb-1* was placed in the JNK-like subfamily based on its 52% sequence identity with human JNK-3. In our experiments, the survival assays indicated that *kgb-1 (km21)* ($P < 0.0001$, Log-Rank test) survival was affected by the yeast *S. cerevisiae* (**Fig. 10**). Similar survival results were also observed in *C. elegans kgb-1 (km21)* infected with *P. aeruginosa* (Kim *et al*., 2004).

An exception was noted in the case of *jnk-1 (gk7),* which showed no enhanced susceptibility to infection when grown on yeast *S. cerevisiae* (**Fig. 11**). *Jnk-1* encodes a JNK-like MAPK and is active in the JUN Kinase pathway, which is involved in movement coordination (Villanueva *et al*., 2001), adult life span (Oh *et al*., 2005) and stress

response (Oh *et al*., 2005). A similar lack of effect on survival was also observed in *jnk-1 (gk7)* mutants exposed to *P. aeruginosa* (Kim *et al*., 2002). Thus, it is possible that *jnk-1* has no function in immune defense in *C. elegans*, and that *kgb-1* is not a JNK kinase.

Three subfamilies of MAP kinases have been identified: ERK, JNK, and p38. The amino acid motif at the activation site that is phosphorylated by an upstream MAPKK distinguishes these three groups: TEY for the ERK family, TPY for the JNK family, and TGY for p38 (Kawasaki *et al*., 1999). The *C. elegans kgb-1* coding sequence, in contrast, contains the sequence SDY at the analogous site (**Fig. 22**) which matches none of the consensus sites. Therefore KGB-1 may represent a novel kinase type.

```
                  * *
Hu JNK1      FMMTPYVVTRYYRAPEVI
Hu JNK3      FMMTPYVVTRYYRAPEVI
Ce JNK1      FMMTPYVVTRYYRAPEVI
Ce KGB1      MRMSDY-VTRYYRAPEVI
```

Figure 22. Alignment of the phosphorylation activation regions of several MAP kinases, including human JNK1, human JNK3, *C. elegans* JNK1 and *C. elegans* KGB1. The Thr-X-Tyr motif is indicated by asterisks.

Mizuno et al (2004) demonstrated that KGB-1 proteins possessing the mutated phosphorylation sites SDF and ADY were incapable of phosphorylating human c-Jun substrate *in vitro*. This indicates that the SDY sequence is required for activation of the kinase activity of KGB-1. However, demonstration that KGB-1 activation *in vivo*

depended on the activity of a specific JNK pathway MAPKK was inconclusive, because the control MAPKK mutant chosen was *mek-1 (ks54)*, which carries mutation in both *sek-1* and *mek-1*, and thus affects the p38 pathway (Mizuno *et al.,* 2004; Dennis Kim, personal communication). Thus the upstream regulators of KGB-1 *in vivo* are not known. It is possible, therefore, that during yeast infection, KGB-1 substitutes for PMK-1 in the p38 pathway controlled by *sek-1* and *nsy-1*. This possibility is shown in **Fig. 23**. This model could explain why *sek-1* and *nsy-1* mutations affect the survival of *C. elegans* in the presence of yeast, but a *pmk-1* mutation does not. *Kgb-1* appears to have a stronger enhanced susceptibility phenotype than *sek-1* and *nsy-1* (**Table 1** and **Figure 17**). It is possible, therefore, that *kgb-1* is involved in more than one pathway that affects immunity. Future experiments should include making a double *sek-1; kgb-1* mutant, and testing it in the survival assay. If *sek-1; kgb-1* double mutants show an enhanced susceptibility phenotype similar to that shown by *sek-1* and *kgb-1* single mutants when exposed to yeast *S. cerevisiae*, it would suggest that *sek-1* and *kgb-1* act in the same pathway. If *sek-1; kgb-1* double mutants show a more severe enhanced susceptibility phenotype, it would suggest that *sek-1* and *kgb-1* act in different MAP kinase pathways.

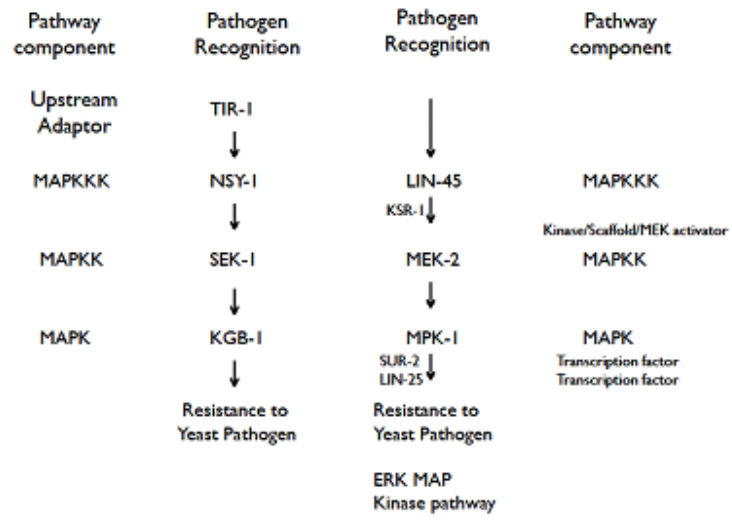


Fig.23. Hypothetical MAP kinase pathways involved in the *C. elegans* response to yeast infection based on this thesis.

We also tested survival of *mek-1 (ks54)* in the presence of the yeast *S. cerevisiae* (**Fig. 12**), and survival was reduced. Similar survival results were also observed in *C. elegans mek-1 (ks54)* when exposed to *P. aeruginosa* (Kim *et al*., 2004). *Mek-1* encodes a Mitogen-activated protein kinase kinase (MAPKK). While MEK-1 was originally proposed to function upstream of JNK-1, Kim et al (2004) proposed that it also acted upstream of PMK-1. *Mek-1* is involved in heavy metal stress (Koga *et al.,* 2000), starvation response (Koga *et al.,* 2000), regulation of growth rate (Mizuno *et al*., 2004), reproduction (Mizuno *et al*., 2004), stress responses (Pujol *et al*., 2008) and the immune response (Kim *et al*., 2004). However, *mek-1 (ks54)* is the only available mutation in this MAPKK, and this strain is actually a *mek-1; sek-1* double mutant (D. Kim, personal communication). Thus, all interpretations of all published experiments using this strain

are confounded by the fact that it is a double mutant. Future experiments should include making a single *mek-1* mutant, and testing it in the survival assay.

Cross talk between p38 and JNK MAPK pathways in immune responses

Considering the comparable enhanced susceptibility of *nsy-1 (ok593), sek-1 (km4)* and *kgb-1 (km2)* to infection by the yeast *S. cerevisiae*, and the lack of effect of *pmk-1 (km25)* and *jnk-1 (gk7)* on survival in the presence of yeast, we hypothesize that cross talk occurs between p38 and JNK MAPK pathways in immune responses (**Fig. 23**). It is possible that during yeast infection, *nsy-1* activates *sek-1*, which in turn activates *kgb-1*, rather than *pmk-1*. Consistent with this possibility, SEK-1 is similar to both human MKK3/6 (50% identity) and MKK4 (43% identity). MKK3 and MKK6 are specific activators of p38, whereas MKK4 can activate both JNK and p38 MAPK's (Derijard *et al.*, 1995; Moriguchi *et al*., 1996*;* Stein *et al*., 1996). Ichijo *et al.* (1997) also provided evidence that the human NSY-1 homolog ASK1 activated both JNK and p38 pathways (Ichijo *et al*., 1997), and Tanaka-Hino *et al.* (2002) demonstrated that *C. elegans* SEK-1 might activate both *jnk-1* and *pmk-1* in the yeast Hog pathway.

ERK mutants show enhanced susceptibility to infection by the yeast *S. cerevisiae*

The survival assays indicated that *mek-2 (n1989)* ($P < 0.0001$, Log-Rank test) was affected by the yeast *S. cerevisiae* (**Fig. 13**), which indicates that *mek-2* plays a crucial role in the *C. elegans* immune response to pathogen infection. To our knowledge, a

survival assay for *mek-2* using any other microbial pathogen has not been performed. *Mek-2* encodes a mitogen-activated protein kinase kinase (MAPKK) and is active in the ERK MAP pathway, which is involved in developmental events (Wu *et al.,* 1995) and pathogen defense (Nicholas and Hodgkin, 2004b).

The survival assays indicated that the double mutant *mpk-1 (ku1) unc-32 (e189)* ($P < 0.0001$, Log-Rank test) showed significantly reduced survival on yeast than *unc-32 (e189)* worms (**Fig. 14**), which indicates that *mpk-1* plays an important role in the *C. elegans* immune response to pathogen infection. To our knowledge, survival assays on mutants in the ERK pathway using any other microbial pathogens has not been performed previously. *Mpk-1* encodes the *C. elegans* ERK mitogen-activated protein kinase (MAPK), which is involved in genotoxic stress (Ulm *et al.*, 2001), development processes (Hsu *et al.*, 2002) and pathogen defense (Nicholas and Hodgkin, 2004b).

The *C. elegans* Dar phenotype requires the activity of ERK pathway, but not p38 pathway, components

S. cerevisiae causes a deformity in the postanal region (Dar) in *C. elegans* (Jain *et al.*, 2009), as has been observed previously in infections of *C. elegans* with the bacterium *Microbacterium nematophilum* (Nicholas and Hodgkin, 2004b). We tested the effects of ERK pathway and p38 pathway mutants on the Dar phenotype formation. The ERK pathway mutant *mek-2* affected both survival and Dar (Jain *et al.*, 2009 and **Fig. 13**), consistent with the hypothesis that Dar is a defensive inflammation response to

pathogen infection (Nicholas and Hodgkin, 2004b; Jain *et al*., 2009). It is possible that the failure of ERK pathway mutants to mediate Dar caused the reduced lifespan of mutants.

Nsy-1 (ok593) and *pmk-1 (km25)* showed a slight but significant reduction of the proportion of Dar animals when compared with wild type animals (ANOVA, p = 0.029; Dunnett's post-hoc tests, p = 0.025 for each comparison). However, no significant difference was observed between wild type and *sek-1 (km4)*. All of these mutations are deletions that are likely to be null. We conclude that the p38 pathway is not required for the Dar response of worms exposed to *S. cerevisiae*.

Tol-1 is not required for resistance to infection by the yeast _S. cerevisiae_

Tol-1 (nr2033) (P=0.0011, Log-Rank test) showed a significant reduction in survival during infection by the yeast *S. cerevisiae* (**Fig. 16**), but this difference was not confirmed by analysis of corrected relative mortality. In fact, after correction for intrinsic difference in viability, *tol-1 (nr2033)* actually showed no reduction of survival in the presence of yeast. This indicated that *tol-1* is not required for resistance to infection by the yeast *S. cerevisiae.* *Tol-1* is the only *C. elegans* gene encoding a Toll-like receptor (TLR) homolog, and has been demonstrated to be required for avoidance of the pathogen *S. marcescens* and early development (Pujol *et al*., 2001). However, Pujol *et al.* (2001) found results similar to ours, i.e., indicating that *C. elegans tol-1 (nr2033)* had no effect on susceptibility to *P. aeruginosa.*

Live *E. coli* reduces survival of wild-type *C. elegans*

Survival assay (at 20°C) showed that wild-type *C.elegans* (P=0.0164, log-rank test) showed a significant reduction in survival on live *E.coli* compared to heat-killed *E. coli* (**Figs. 20-21**). Similarly, *kgb-1 (km21) (*Fig. 20, P=0.0176, log-rank test), and *pmk-1 (km25) (*Fig. 21, P=0.0002, log-rank test) also showed a significant reduction in survival on live *E. coli* compared to heat-killed *E. coli* (**Figs. 20-21**). A similar result was also obtained by Mallo *et al.* (2002) for wild-type *C. elegans*. These data indicated that *E.coli* OP50 might be an opportunistic pathogen for *C. elegans.* They also suggest that the best negative control for future assays comparing mutant to wild-type survival might be heat-killed *E. coli*.

***C. elegans* mutant *fer-1 (b232)* is sensitive to yeast in the absence of fertilization**

To determine whether yeast-mediated killing of *C. elegans* is simply a result of internal hatching of offspring (Jain *et al*., 2009), we tested *fer-1 (b232)* and a *fer-1 (b232); kgb-1 (km21)* double mutant for survival. Since *fer-1 (b232)* and *fer-1 (b232); kgb-1 (km21)* double mutant are completely sterile at the standard survival test temperature of 20°C, we tested the survival of *C. elegans* in the absence of internally hatched offspring. The survival assay showed that the lifespan of *fer-1 (b232)* at 20°C was significantly reduced when it was grown on yeast compared to its lifespan on *E. coli* (**Fig. 18**, P < 0.0001, **Table 2**). A similar survival result is also observed in *C. elegans fer-1*

(*hc1*ts) infected with *P. aeruginosa* (Tan *et al*., 1999). Since sterile hermaphrodites were also killed, killing of worms *fer-1 (b232)* grown on yeast cannot readily be explained by internal hatching of eggs.

It was surprising that *fer-1 (b232)* lived twice as long as wild type *C. elegans* when grown on *E. coli* OP50 (**Fig. 18** and **Table 2**), because there is little evidence that mutants incapable of making viable sperm have extended lifespans (Kenyon, 1997). The sperm-defective mutant *spe-26* has extended lifespan, but this may be unrelated to its sperm defect (Kenyon, 1997). To our knowledge, our finding of increased lifespan of a *fer-1* mutant is novel, and may warrant further investigation.

Similarly, in the *fer-1 (b232)* mutant background, *kgb-1* mutation reduced the lifespan significantly ($P < 0.0001$, Log-Rank test, **Fig. 19**, **Table 3**) on yeast, which indicated that a *C. elegans kgb-1* mutant is sensitive to yeast in the absence of fertilization. Therefore, we conclude that the reduced survival of the MAP kinase pathway mutants we tested might not result from internal hatching of eggs. According to the study by Tan *et al* (1999), it is possible that proliferation of pathogenic bacteria in the *C. elegans* intestine is a major cause of death in *C. elegans* grown on pathogens, so this may also be the case with *S. cerevisiae* infection.

Correction of survival results for intrinsic reduction in viability of mutants

In this thesis, we calculated the relative mortality of mutants for all mutants tested, in order to remove potential bias resulting from apparent intrinsic mutant

reduction in survival, which is unrelated to yeast infection. For the cases of *pmk-1 (km25)* (**Fig. 7**, P< 0.0001, Log-Rank test), *tir-1 (ok1052)* mutant (P< 0.0001, Log-Rank test, **Fig. 9**), and *tol-1 (nr2033)* (P=0.0011, Log-Rank test, **Fig. 16**), if we had just used the Log-Rank P values, we would have concluded that there was an increase in susceptibility. But when we calculated the corrected relative mortality, those effects disappeared (**Table 1**). Therefore, it is useful to calculate the relative mortality.

Although the corrected relative mortality is useful, it does not directly provide a way to test significance of the difference between the mutant and wild-type survival. The log rank test itself does not allow direct consideration of other variables such as intrinsic viability (Bewick *et al*., 2004). However, Cox's proportional hazards model does consider other covariates (Bewick *et al*., 2004). Therefore, we suggest that the best statistical model for future assays comparing mutant to wild-type survival might be Cox's proportional hazards model.

References

Aballay, A., Drenkard, E., Hilbun, L.R., and Ausubel, F.M. (2003). *Caenorhabditis elegans* innate immunity response triggered by Salmonella enteric requires intact LPS and is mediated by a MAPK signaling pathway. Curr. Biol. 13, 47-52.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell, 124, 783-801.

Alegado, R. A., Campbell, M. C., Chen, W. C., Slutz, S. S. and Tan, M. W. (2003). Characterization of mediators of microbial virulence and innate immunity using the *Caenorhabditis elegans* host–pathogen model. Cell Microbiol. 5, 435–444.

Altun, Z.F. and Hall, D.H. (2009). Introduction to *C.elegans* anatomy. *WormAtlas*, doi:10.3908/wormatlas.1.1, http://www.wormatlas.org

Argon, Y. and Ward, S. (1980). *Caenorhabditis elegans* fertilization-defective mutants with abnormal sperm. Genetics, 96, 413-433.

Bewick, V., Cheek, L. and Ball, J. (2004). Statistics review 12: Survival analysis. Crit Care, 8, 389–394.

Bolz, D.D., Tenor, J.L., and Aballay, A. (2010). A conserved PMK-1/p38 MAPK is required in *C. elegans* tissue-specific immune response to *Y. pestis* infection. J Biol Chem. [Epub ahead of print]

Brancho, D., Tanaka, N., Jaeschke, A., Ventura, J.J., Kelkar, N., Tanaka, Y., Kyuuma, M., Takeshita, T., Flavell, R.A., and Davis, R.J. (2003). Mechanism of p38 MAP kinase activation in vivo. Genes Dev. 17,1969-78.

Brenner, S. (1974). The Genetics of *Caenorhabditis elegans.* Genetics, 77, 71-94.

Calebiro, D., Nikolaev, V.O., Gagliani, M.C., Filippis,D. T., Dees, C., Tacchetti, C., Persani, L., and Lohse, M.J. (2009). Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. PLoS Biol. 7, e1000172.

Chang, L. and Karin, M. (2001). Mammalian MAP kinase signalling cascades. Nature, 410, 37-40.

Chuang, C. F. and Bargmann, C. I. (2005). A Toll-interleukin 1 repeat protein at the synapse specifies asymmetric odorant receptor expression via ASK1 MAPKKK signaling. Genes Dev. 19, 270-281.

Couillault, C., Pujol, N., Reboul, J., Sabatier, L., Guichou, J.F., Kohara, Y., and Ewbank, J.J. (2004). TLR-independent control of innate immunity in *Caenorhabditis elegans* by the TIR domain adaptor protein TIR-1, an ortholog of human SARM. Nat. Immunol. 5, 488–494.

Darby, C. (2005). Interactions with microbial pathogens., WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.21.1, http://www.wormbook.org.

Derijard, B., Raingeaud, J., Barrett, T., Wu, I.H., Han, J., Ulevitch, R.J., Davis, R.J. (1995). Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science, 267, 682-5.

Dong, C., Davis, R.J., and Flavell, R.A. (2002). MAP kinases in the immune response. Annu. Rev. Immunol. 20, 55-72.

Ewbank, J. J. (2006). Signaling in the immune response. *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.83.1, http://www.wormbook.org.

Garsin, D.A., Sifi, C.D., Mylonakis, E., Qin, X., Singh, K.V., Murray, B., Capderwood, S.B., and Ausubel, F.M. (2001). A simple model host for identifying Gram-positive virulence factors. PNAS, 98, 10892-10897.

Gravato-Nobre, M. J. and Hodgkin, J. (2005). *Caenorhabditis elegans* as a model for innate immunity to pathogens. Cell Microbiol. 7, 741-751.

Hirotsu, T., Saeki, S., Yamamoto, M., and Iino, Y. (2000). The Ras-MAPK pathway is important for olfaction in *Caenorhabditis elegans*. Nature, 404, 289-293.

Hodgkin, J., Kuwawbara, P.E., and Corneliussen, B. (2000). A novel bacterial pathogen, *Microbacterium nematophilum*, induces morphological change in the nematode *C. elegans*. Curr. Biol. *10, 1615-1618.*

Hsu V, Zobel CL, Lambie EJ, Schedl T, Kornfeld K. (2002). *Caenorhabditis elegans* lin-45 raf is essential for larval viability, fertility and the induction of vulval cell. Genetics,

160, 481-492.

Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science, 275, 90-94.

Jain, C., Yun, M., Politz, S.M., Rao, R.P. (2009). A Patho-assay using *S. cerevisiae* and *C. elegans* reveals novel roles for yeast AP-1, Yap1 and host dual oxidase, BLI-3 in fungal pathogenesis. Eukaryot Cell. 8, 1218-1227.

Janeway, C. A., Jr. and Medzhitov, R. (2002). Innate immune recognition. Annu. Rev. Immunol. 20, 197-216.

Kawasaki, M., Hisamoto, N., Iino, Y., Yamamoto, M., Ninomiya-Tsuji, J., and Matsumoto, K. (1999). A Caenorhabditis elegans JNK signal transduction pathway regulates coordinated movement via type-D GABAergic motor neurons. EMBO J. 18, 3604-15.

Kenyon, C. (1997). Environmental factors and gene activities that influence the lifespan of *C. elegans.* The nematode Caenorhabditis elegans, II. Cold Spring Harbor, NY, pp. 791-813.

Kim, D.H., Feinbaum, R., Alloing, G., Emerson, F.E., Garsin, D.A., Inoue, H., Tanaka-Hino, M., Hisamoto, N., Matsumoto, K., Tan, M.W., and Ausubel, F.M. (2002). A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity. Science, 297, 623-626.

Kim, D.H., Liberati, N.T., Mizuno, T., Inoue, H., Hisamoto, N., Mastumoto, K., Ausubel, F.N. (2004). Integration of *Caenorhabditis elegans*, MAPK pathways mediating immunity and stress resistance by MEK-1 MAPK kinase and VHP-1 MAPK phosphatase. Proc. Natl. Acad. Sci. USA 101, 10990-10994.

Kim, D. (2008). Studying host-pathogen interactions and innate immunity in *Caenorhabditis elegans*. Dis Model Mech. 1, 205-208.

Koga, M., Zwaal, R., Guan, K.L., Avery, L., and Ohshima, Y. (2000). A *Caenorhabditis elegans* MAP kinase kinase, Mek-1, is involved in stress responses. EMBO J. 19, 5148-5156.

Kroeze, W.K., Sheffler, D.J., and Roth, B.L. (2003). G-protein-coupled receptors at a glance. J. Cell Sci. 116, 4867-4869.

Kurt-Jones, E.A., Popova, L., Kwinn, L., Haynes, L.M., Jones, L.P., Tripp, R.A., Walsh, E.E., Freeman, M.W., Golenbock, D.T., Anderson, L.J., and Finberg, R.W. (2000). Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat. Immunol. 1, 398-401.

Liberati, N.T., Fitzgerald, K.A., Kim, D.H., Feinbaum, R., Golenbock, D.T., and Ausubel, F.M. (2004). Requirement for a conserved Toll/interleukin-1 resistance domain protein in the Caenorhabditis elegans immune response. Proc Natl Acad Sci U S A 101, 6593-6598.

Mallo, G.V., Kurz, C.L., Couillault, C., Pujol, N., Granjeaud, S., Kohara, Y., and Ewbank, J.J. (2002). Inducible antibacterial defense system in *C.elegans*. Curr. Biol. 12, 1209–1214.

McCusker, J.H., Heitman, J., Filler, S.G., Edwards, J.E., Jr., and Mitchell, A.P. (2006). *Saccharomyces cerevisiae*: an Emerging and Model Pathogenic Fungus, pp. 245-259, in Molecular Principles of Fungal Pathogenesis, ed., ASM Press, Washington, DC.

Means, T.K., Mylonakis, E., Tampakakis, E., Colvin, R.A., Seung, E., Puckett, L., Tai, M.F., Stewart, C.R., Pukkila-Worley, R., Hickman, S.E., Moore, K.J., Calderwood, S.B., Hacohen, N., Luster, A.D., and Khoury, J.E. (2009). Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36. J Exp Med. 206, 637-53.

Medzhitov, R., and Janeway, C.A. Jr. (1997). Innate immunity: the virtues of a nonclonal system of recognition. Cell. 91**,** 295-298.

Medzhitov, R., and Janeway, C.A. Jr. (2000). Innate immune recognition: mechanisms and pathways. Immunol. Rev. 173, 89-97.

Mizuno, T., Hisamoto, N., Terada, T., Kondo, T., Adachi, M., Nishida, E., Kim, D.H., Ausubel, F.M., and Matsumoto, K. (2004). The *Caenorhabditis elegans* MAPK phosphatase VHP-1 mediates a novel JNK-like signaling pathway in stress response. Embo. J. 23, 2226-2234.

Moriguchi, T., Toyoshima, F., Gotoh, Y., Iwamatsu, A., Irie, K., Mori, E., Kuroyanagi, N., Hagiwara, M., Matsumoto, K., and Nishida, E. (1996). Purification and identification of a major activator for p38 from osmotically shocked cells.

Activation of mitogen-activated protein kinase kinase 6 by osmotic shock, tumor necrosis factor-alpha, and H2O2. J Biol Chem. 271, 26981-8.

Murphy, K. (2008). Janeway's Immunobiology. New York, Garland Science.

Nicholas, H. R. and Hodgkin, J. (2004a). Responses to infection and possible recognition strategies in the innate immune system of *Caenorhabditis elegans*. Mol. Immunol. 41, 479-93.

Nicholas, H.R., and Hodgkin, J. (2004b). The ERK MAP kinase cascade mediates tail swelling and a protective response to rectal infection in *C. elegans*. Curr. Biol. 14, 1256-1261.

Nicholas, H. R. and Hodgkin, J. (2009). The *C. elegans* Hox gene egl-5 is required for correct development of the hermaphrodite hindgut and for the response to rectal infection by *Microbacterium nematophilum*. Dev. Biol. 329, 16-24.

Oh, S.W., Mukhopadhyay, A., Svrzikapa, N., Jiang, F., Davis, R.J.,Tissenbaum, H.A. (2005). JNK regulates lifespan in *Caenorhabditis elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16. Proc Natl Acad Sci U S A, 102, 4494-4499.

Powell, J.R., Kim, D.H., and Ausubel, F.M. (2009). The G protein-coupled receptor FSHR-1 is required for the *Caenorhabditis elegans* innate immune response. Proc Natl Acad Sci U S A ,106,2782-2787.

Pujol, N., Link, E.M., Liu, L.X., Kurz, C.L., Alloing, G., Ray, K.P., Solari, R., Johnson, C.D., Ewbank, J.J., and Tan,M.W. (2001). A reverse genetic analysis of components of the Toll signaling pathway in *Caenorhabditis elegans*. Current Biology. 11, 809-821.

Pujol, N., Cypowyj, S., Ziegler, K., Millet, A., Astrain, A., Goncharov, A., Jin, Y., Chisholm, A.D., and Ewbank, J.J. (2008). Distinct innate immune responses to infection and wounding in the *C. elegans* epidermis. *Curr. Biol.* 18,481-489.

Reddy, K.C., Andersen, E.C., Kruglyak, L., and Kim, D.H. (2009). A polymorphism in npr-1 is a behavioral determinant of pathogen susceptibility in *C. elegans*. Science. 323**,** 382-384.

Richardson, M. and Lass-Florl, C. (2008). Changing epidemiology of systemic fungal infections. Clin. Microbiol. Infect. 4, 5-24.

Rocheleau, C.E., Cullison, K., Huang, K., Bernstein, Y., Spilker, A.C., and Sundaram, M.V. (2008). The *Caenorhabditis elegans ekl* (enhancer of ksr-1 lethality) genes include putative components of a germline small RNA pathway. Genetics, 178,1431-43.

Rose, M.D., Winston, F., Hieter, P. (1990). Methods in yeast genetics a laboratory course manual, Cold Spring Harbor Laboratory.

Sagasti, A., Hisamoto, N., Hyodo, J., Tanaka-Hino, M., and Matsumoto, K., and Bargmann, C.I. (2001). The CaMKII UNC-43 activates the MAPKKK NSY-1 to execute a lateral signaling decision required for asymmetric olfactory neuron fates. Cell, 105, 221-232.

Sakaguchi, A., Matsumoto, K., and Hisamoto, N. (2004). Roles of MAP kinase cascades in *Caenorhabditis elegans*. J. Biochem. 136, 7-11.

Schulenburg, H., Kurz, C.L., and Ewbank, J.J. (2004). Evolution of the innate immune system: the worm perspective. Immunol. Rev. 198, 36-58.

Smith, P., Leung-Chiu, W.M., Montgomery, R., Orsborn, A., Kuznicki, K., Gressman-Coberly, E., Mutapcic, L., and Bennett, K. (2002). The GLH proteins, *Caenorhabditis elegans* P granule components, associate with CSN-5 and KGB-1, proteins necessary for fertility, and with ZYX-1, a predicted cytoskeletal protein. Dev. Biol. 251, 333-347.

Spehlmann, M. E. and Eckmann, L. (2009). Nuclear factor-kappa B in intestinal protection and destruction. Curr. Opin. Gastroenterol, 25, 92-99.

Stein, B., Brady, H., Yang, M.X., Young, D.B., and Barbosa, M.S. (1996). Cloning and characterization of MEK6, a novel member of the mitogen-activated protein kinase kinase cascade. J Biol Chem. 271, 11427-33.

Styer, K.L., Singh, V., Macosko, E., Steele, S.E., Bargmann, C.I., and Aballay, A. (2008). Innate immunity in *Caenorhabditis elegans* is regulated by neurons expressing NPR-1/GPCR. Science, 322, 460-464.

Sulston J. and Hodgkin J. (1988). *Methods,* The Nematode *Caenorhabditis elegans.,* 587-606. W. Wood., ed. New York: Cold Springs Harbor Laboratory

Szewczyk, N. J. and Jacobson, L. A. (2003). Activated EGL-15 FGF receptor promotes protein degradation in muscles of *Caenorhabditis elegans*. Embo. J. 22, 5058-5067.

Tan, M.W., Mahajan-Miklos, S., Ausubel, F.M. (1999). Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. Proc Natl Acad Sci U S A, 96, 715-20.

Tanaka-Hino, M., Sagasti, A., Hisamoto, N., Kawasaki, M., Nakano, S., Ninomiya-Tsuji, J., and Bargmann, C.I., and Matsumoto, K. (2002). SEK-1 MAPKK mediates Ca2+ signaling to determine neuronal asymmetric development in *Caenorhabditis elegans*. EMBO Rep, 3, 56-62.

Tenor, J. L. and Aballay, A. (2008). A conserved Toll-like receptor is required for *Caenorhabditis elegans* innate immunity. EMBO Rep. 9, 103-109.

Torayama, I., Ishihara, T., and Katsura, I. (2007). *Caenorhabditis elegans* integrates the signals of butanone and food to enhance chemotaxis to butanone. J. Neurosci. 27, 741-750.

Troemel, E.R., Chu, S.W., Reinke, V., Lee, S.S., Ausubel, F.M., and Kim, D. H. (2006). p38 MAPK regulates expression of immune response genes and contributes to longevity in *C. elegans*. PLoS Genet. 2, e183.

Tzung KW, Williams RM, Scherer S, Federspiel N, Jones T, Hansen N, Bivolarevic V, Huizar L, Komp C, Surzycki R, Tamse R, Davis RW, Agabian N. (2001). Genomic evidence for a complete sexual cycle in *Candida albicans*. Proc. Natl. Acad. Sci. USA, 98, 3249–3253.

Ulm, R., Revenkova, E., Sansebastiano, G.P., Bechtold, N., and Paszkowski, J. (2001). Mitogen-activated protein kinase phosphatase is required for genotoxic stress relief in Arabidopsis. Genes Dev. 15, 699–709.

Villanueva, A., Lozano, J., Morales, A., Lin, X., Deng, X., Hengartner, M.O., and Kolesnick, R.N. (2001). jkk-1 and mek-1 regulate body movement coordination and response to heavy metals through jnk-1 in *Caenorhabditis elegans*. EMBO J. 20, 5114-5128.

Wes, P. D. and Bargmann, C. I. (2001). *C. elegans* odour discrimination requires asymmetric diversity in olfactory neurons. Nature, 410, 698-701.

WormBase web site, http://www.wormbase.org, release WS213, April 24, 2010.

Wu, Y., Han, M., and Guan, K.L. (1995). MEK-2, a *Caenorhabditis elegans* MAP kinase kinase, functions in Ras-mediated vulval induction and other developmental

events. Genes Dev 9, 742-55.

Ziegler, K., Kurz, C.L., Cypowyj, S., Couillault, C., Pophillat, M., Pujol, N., and Ewbank, J.J. (2009). Antifungal innate immunity in *C. elegans*: PKCdelta links G protein signaling and a conserved p38 MAPK cascade. Cell Host Microbe, 5, 341-352.