

Effects of *Bli-3* on Survival Rates of *Caenorhabditis elegans* in the Presence of Yeast Pathogens

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Abstract

In *C. elegans*, the gene *bli-3* codes for Duox1, a protein in the NADPH oxidase (Nox) family responsible for cuticle cross-linking. The canonical example of a Nox family protein is human Nox2, which is responsible for the respiratory burst that occurs in phagocytic leukocytes. In order to determine if Duox is important in *C. elegans* host defense, both available *bli-3* mutants and *C. elegans* subjected to RNAi knockdown of *bli-3* were subsequently exposed to yeast pathogens, and survival assays were performed. When *bli-3* mutants were exposed to *Saccharomyces cerevisiae*, a slight decrease in survival was observed, but the decrease was insignificant. However, one of the *bli-3* mutants and the whole body *bli-3* RNAi knockout worms showed a significant decrease in survival when exposed to *Candida albicans*. This indicates that *bli-3* does play an innate immune role in *C. albicans* infections. Tissue-specific RNAi knockdown was performed in order to determine where this innate immune function is localized, and knockdowns appeared to have similar effects regardless of the tissue.

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Introduction

Duox and NADPH-Oxidases

NADPH-oxidases (NOXs) couple the transfer of electrons across biological membranes to the receptor, which is usually oxygen, creating superoxides (Bedard & Krause, 2007). The reaction catalyzed is $2O_2 + NADPH \rightarrow 2O_2^{\cdot -} + NADP + H^+$. Superoxides rapidly form hydrogen peroxide, either by enzymatic activity (superoxide dismutase) or spontaneously (at low pH) (Bedard & Krause, 2007). Reactive oxygen species (ROS) are created which destroy or permanently alter the function of their target molecule (Bedard & Krause, 2007). ROS are radicals, ions, and molecules derived from oxygen that are highly unstable and reactive. They can cause damage to organisms that produce them, and have been implicated in the aging process and in cellular damage (Harman, 1956; Beckman & Ames, 1998). However, ROS is also involved in a variety of beneficial processes, including regulatory processes and host defense, in what is called the respiratory burst, which occurs in phagocytic leukocytes (Bedard & Krause, 2007). This respiratory burst is critical to killing of certain pathogens, but is unnecessary in the killing of others (Rada & Leto, 2008). NADPH oxidases in human neutrophils and monocytes are important in killing pathogens, including *Staphylococcus aureus*, *Aspergillus fumigates*, and *Candida albicans* (Rada & Leto, 2008). The NADPH oxidase respiratory burst uses the chemical reactivity of ROS to kill pathogens (Rada & Leto, 2008). The respiratory burst may also increase production of cytokines and other proinflammatory mediators (El-Benna et al., 2007). This NADPH-oxidase catalyzed reaction is absent in patients with chronic granulomatous disease (CGD), a rare, fatal syndrome in young boys (Berendes et al., 1957; Baehner & Nathan, 1967; Quie et al., 1967). These patients show increased sensitivity to Gram-positive bacteria, Gram-negative bacteria, and fungi (including *Candida albicans*) (El-Benna et al., 2007). NADPH oxidase can also be found in tumor cells (Szatrowski & Nathan, 1991), fibroblasts (Meier et al., 1991), and vascular muscle cells (Griendling et al., 2000).

Mammalian NOX family members have several conserved structures including an NADPH-binding site (at the COOH terminus), a FAD-binding region, six transmembrane domains, and four histidines capable of binding heme (Bedard & Krause, 2007). No crystal structure is currently available for NOX enzymes, but a schematic of their structure can be seen in Figure 1 (Bedard & Krause, 2007). As is shown, all families have conserved structural elements, including a NADPH-binding domain near the C terminus, an FAD-binding domain (located between the NADPH-binding domain and the most C-terminal transmembrane domain), and six transmembrane domains with four heme-binding histidines (Bedard & Krause, 2007). Heme groups are bound to the third and fifth helices within the lipid bilayer

(Groemping & Rittinger, 2005; Vignais, 2002). The canonical example of an NADPH-oxidase is human NOX2, a gene expressed in neutrophils and macrophages as well as a variety of other cells, including cardiomyocytes (Heymes et al., 2003), skeletal muscle myocytes (Javesghani et al., 2002), endothelial cells (Gorlach et al., 2000; Jones et al., 1996; Li & Shah, 2002), hepatocytes (Reinehr et al., 2005), neurons (Serrano et al., 2003), and even hematopoietic stem cells (Piccoli et al., 2005). In both humans and mice, the gene for NOX2 is located on the X chromosome. NOX2 is localized in intracellular compartments in resting neutrophils and is translocated to the surface of the cell as granules fuse with the membrane (Borregaard et al., 1983; Goldblatt & Thrasher, 2000).

NOX2 cannot be activated without five additional proteins; p22^{phox}, p47^{phox}, p67^{phox}, Rac, and p40^{phox} (Bedard & Krause, 2007). The protein p22^{phox} stabilizes the protein complex and docks the cytosolic factors and proteins (including p47^{phox} and p67^{phox}) (Bedard & Krause, 2007). The cytosolic protein p47^{phox} acts as an organizer, and upon cell stimulation is translocated, along with bound p67^{phox}, to the membrane (Bedard & Krause, 2007). Rac activates the protein complex, and p67^{phox} activates NOX2 (Takeya, et al., 2003; Quinn et al., 1993). The modulatory subunit p40^{phox} was discovered more recently, and less is known about its function (Wientjes et al., 1993).

In the first step of the NOX2 catalyzed reaction, electrons are transferred from NADPH to FAD, producing FADH₂ (Nisimoto et al., 1999). Next, a single electron is transferred from FADH₂ to the iron of the inner heme (Cross & Segal, 2004; Doussiere et al., 1996; Vignais, 2002). The iron can only accept one electron, so it donates the electron to the outer heme in order to be able to accept a second electron from the partially reduced FADH₂ (Cross & Segal, 2004; Doussiere et al., 1996; Vignais, 2002). In order for electron transfer from the inner heme to the outer heme to be energetically favorable, oxygen must be bound to the outer heme to receive the electron (Cross & Segal, 2004; Doussiere et al., 1996; Vignais, 2002). An outline of this mechanism can be seen in Figure 2 (Cross & Segal, 2004). NOX family members are found in many cell types, including the gastrointestinal system and the respiratory system (Bedard & Krause, 2007). The colon shows high levels of NOX family proteins; however, their function is not yet understood (Bedard & Krause, 2007). Two popular hypotheses include a role in host defense and regulation of cell proliferation (Bedard & Krause, 2007). In the liver, ROS derived from NOX proteins is involved in apoptosis (Lee et al., 2004; Reinehr et al., 2005). In alveolar macrophages, NOX proteins are involved in host defense (Bedard & Krause, 2007). NOX proteins are also found in airway epithelial cells (Bedard & Krause, 2007). Many functions have been proposed for NOX family members in airway epithelial cells; including regulation of gene expression (Lavigne & Eppihimer, 2005; Papaiahgari, et al.,

2004), mucin expression (Shao & Nadel, 2005), acid production (Segal et al., 1981), response to mechanical stress (Chapman et al., 2005), apoptosis (Zhang, et al., 2003), and host defense (Geiszt et al., 2003). Finally, in pulmonary vasculature, NOX enzymes play a role in cell response to changes in oxygen concentration (Chowdhury, et al., 2005; Fisher et al., 1999; Marshall et al., 1996; Weissmann, et al., 2000, 2005).

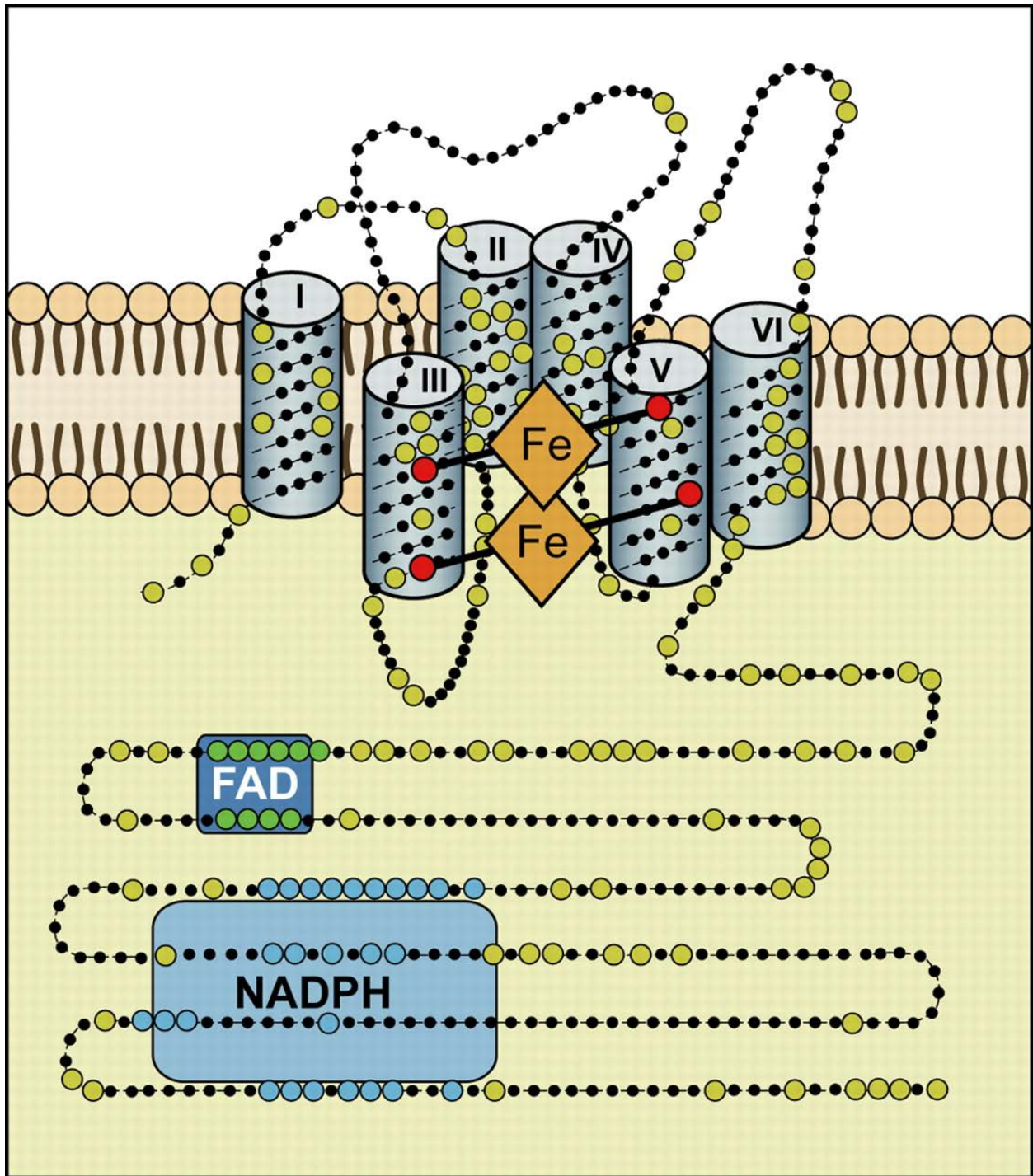


Figure 1: NOX Structure (Bedard & Krause, 2007)

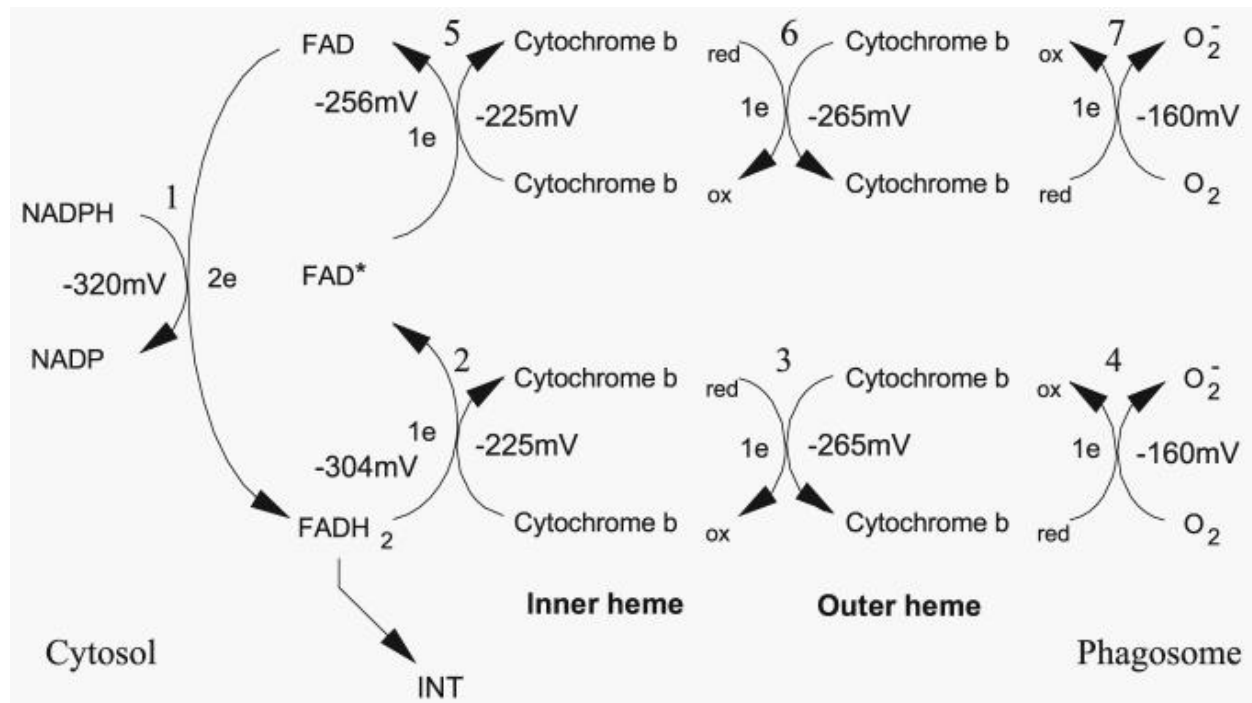


Figure 2: NOX2 Reaction Mechanism (Cross & Segal, 2004)

Another subset of the NADPH-oxidase family consists of the Duox proteins (Bedard & Krause, 2007). The Duox isoforms share about 50% identity with NOX2 (De Deken, et al., 2000). A schematic showing the main structural differences between NOX and Duox proteins can be seen in Figure 3 (Cross & Segal, 2004). These proteins have a NOX1-4 homology domain, an EF-hand domain, and an additional (seventh) transmembrane domain with a peroxidase like domain (De Deken, et al., 2000). In some examples, the EF-hand domain binds Ca^{2+} and activates the target protein (Ikura, 1996). Studies suggest that the EF-hand domain is functional, as Duox can be activated by Ca^{2+} (Ameziane-El-Hassani, et al., 2005). It is unknown whether the peroxidase homology domain functions as peroxidase. The Duox peroxidase homology domain lacks many amino acids important for peroxidase function (Colas & Ortiz de Montellano, 2003; Daiyasu & Toh, 2000; Nauseef, 2001). Furthermore, a separate peroxidase is often coexpressed in Duox systems (Bedard & Krause, 2007). However, one study did find peroxidase function in Duox peroxidase homology domains that were expressed as recombinant proteins (Edens, et al., 2001). Immature Duox2 produced superoxide, whereas the mature protein produces hydrogen peroxidase (Ameziane-El-Hassani, et al., 2005). One possible reason is that posttranscriptional modifications favor intramolecular dismutation (Ameziane-El-Hassani, et al., 2005). In *Drosophila*, humans, and many other organisms, dual oxidase is involved in reactive oxygen species production in response to gut pathogens (Ha et al., 2005).

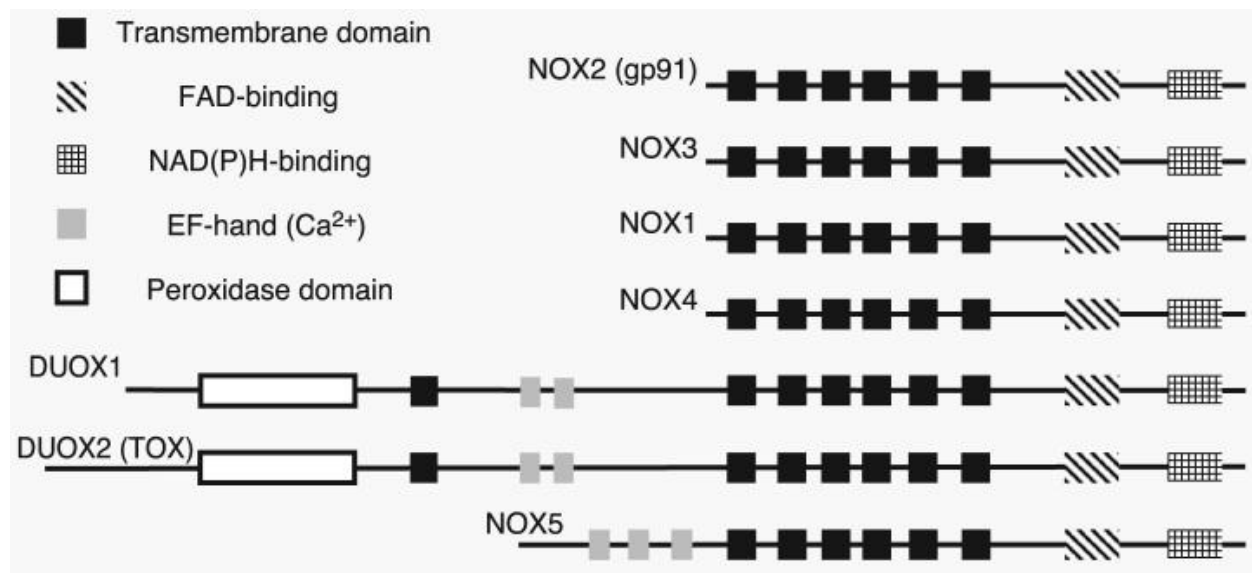


Figure 3: Structural Differences between NOX and Duox enzymes (Cross & Segal, 2004)

In humans, Duox1 and Duox2 are thyroid epithelial cell enzymes that catalyze the production of hydrogen peroxide (Bjorkman & Ekholm, 1984). The genes for Duox1 and Duox2 are located in a head-to-head configuration on chromosome 15 (Pachucki et al., 2004). Additional proteins, DuoxA1 and DuoxA2, are ER proteins called Duox maturation factors that are necessary to overcome entrapment in the ER (Grasberger & Refetoff, 2006). Duox enzymes do not require activator and organizer subunits similar to those required for NOX enzymes (Bedard & Krause, 2007). Duox1 and Duox2 are also found in epithelial cells lining airways, salivary glands, and the GI tract, and provide hydrogen peroxide to produce antimicrobial hypothiocyanite ions in a reaction catalyzed by lactoperoxidase (Gerson, et al., 2000; Klebanoff, 1999). Enzymes such as Duox1 and Duox2 that secrete ROS into the intercellular space generally produce less toxic ROS that is slightly less harmful to tissues (Rada & Leto, 2008). *Duox2* catalyzes hydrogen peroxide generation, the rate limiting step in thyroid hormone production (Dupey et al., 1999). Mutations in *Duox2* result in congenital hypothyroidism in humans (Dupey et al., 1999). Although usually not life-threatening, patients with hypothyroidism must take hormone replacement therapy all of their life (NCBI, 2010).

Model System

The following study uses the model organism *Caenorhabditis elegans*, an organism whose entire genome sequence has been determined (The C. elegans Sequencing Consortium, 1998). More than half of the genes found in *C. elegans* have human homologues, making it a valuable model organism (Kamath, et al., 2003). *C. elegans* are low in cost, easy to maintain, reproduce by self-fertilization

(creating natural clones), are transparent (allowing for easy phenotypic observation), and do not take up much laboratory space (Schulenburg et al., 2004; Gravato-Nobre & Hodgkin, 2005). They are often used to study host-pathogen interactions, and investigating how worms respond to pathogens can help to advance these studies (Chavez et al., 2009). Although they do not possess adaptive immune systems, *C. elegans* can be used to study the innate immune system, the most ancient and fast-acting type of immunity (Gravato-Nobre & Hodgkin, 2005). Interestingly, *C. elegans* lack specialized cells equivalent to macrophages, neutrophils, or scavenging phagocytes (as are found in invertebrates, such as *Drosophila*) (Schulenburg et al., 2004; Gravato-Nobre & Hodgkin, 2005). They do, however, have scavenger cells called coelomytes in the body cavity, although it seems unlikely that these cells have phagocytic functions (Gravato-Nobre & Hodgkin, 2005).

The *C. elegans* genome encodes two dual oxidases, Duox1 and Duox2 (the latter of which appears to be non-functional) (Edens et al., 2001). The predicted primary amino acid sequence of *C. elegans* Duox1 shows 30% homology with the human dual oxidase proteins and has similar size and domain organization (Edens et al., 2001). In *Caenorhabditis elegans*, Duox1 is encoded by the gene *blister-3 (bli-3)* and has two main functions. The first function, from which the gene gets its name, is cuticle cross-linking (Edens et al., 2001). This reaction is outlined in Figure 4 (Edens et al., 2001). Organisms with mutations affecting the peroxidase domain of Dual Oxidase, the protein that *bli-3* encodes, show loss of cuticle integrity, resulting in what appear to be blisters (Brenner, 1974; Edens et al., 2001). Several previous studies show that *C. elegans* Duox/BLI-3 is also a catalyst for ROS production (Edens et al., 2001; Jain et al., 2009). In *C. elegans* with Bli-3/Duox knockdown, reduced survival has been noted upon exposure to *Enterococcus faecalis*, indicating that Duox may play a role in host defense (Chavez et al., 2009).

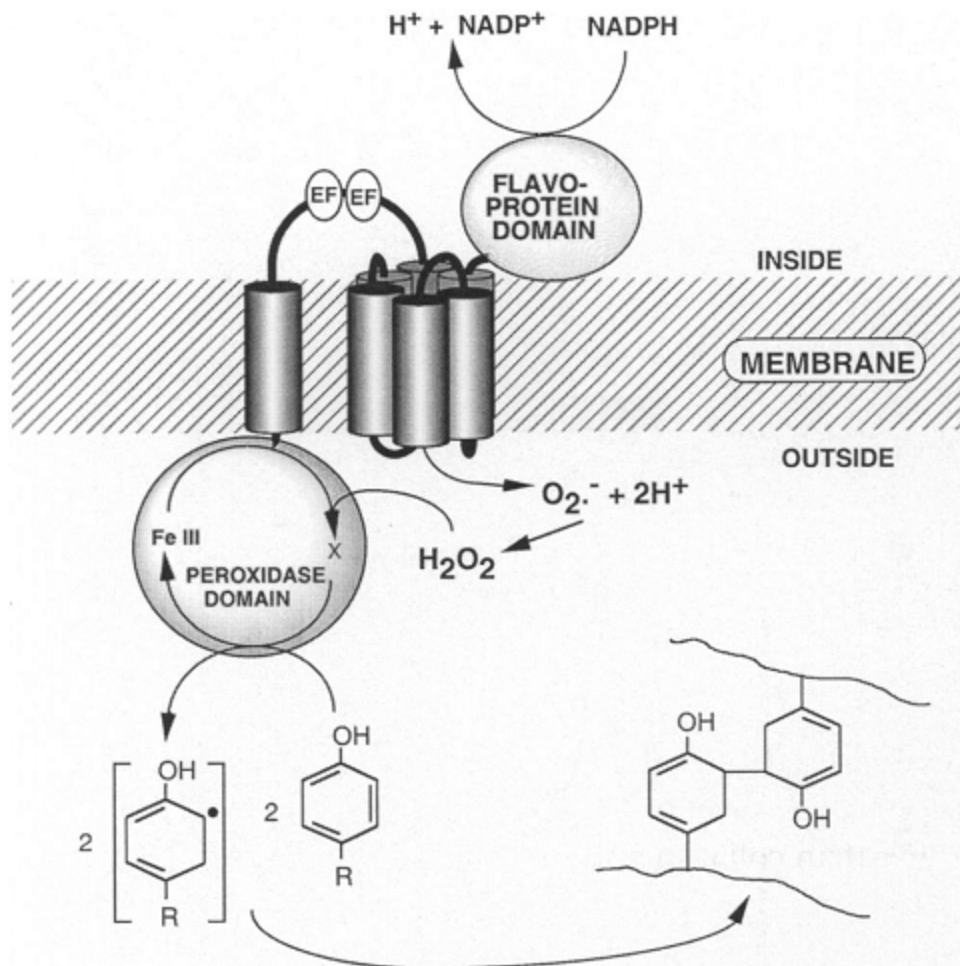


Figure 4: Topology Model of Duox Enzymatic Activity (Edens et al., 2001)

Yeast Pathogens

Several studies have been performed in our lab on *C. elegans*' response to the yeast *Saccharomyces cerevisiae*. The work described here studied the host response of *C. elegans* to the common pathogenic yeast, *Candida albicans*. *Candida* is the most frequently isolated yeast associated with human infection, and causes vaginitis (Hazen, 1995). As stated earlier, NADPH oxidases in human neutrophils and monocytes are important in killing *Candida albicans* (Rada & Leto, 2008). Duox1 may perform a similar *Candida* killing function in *C. elegans*.

Duox Regulation in *Drosophila* and *C. elegans*

Duox regulation in *Drosophila* can be broken down into two possible pathway branches; the transcriptional regulation branch, sometimes referred to as the “Duox expression pathway”, and the protein activity branch, sometimes called the “Duox activity pathway” (Ha, et al., 2009a).

Regulation of Duox Activity

The p38 MAPK cascade has been shown to act upstream of Duox transcription in *Drosophila* (Rhee, 2001; Ha, et al., 2009b). In *C. elegans*, the activation of the p38 MAPK Cascade involved in innate immunity begins with the activation of G proteins G α (GPA-12) and G β (Rack-1) (Ziegler, et al., 2009). These G proteins go on to activate PLC γ (PLC-3) and PLC β (EGL-8) (Ziegler, et al., 2009). These two PLCs, or phospholipase C's, catalyze the hydrolysis of PIP₂ (phosphatidylinositol 4,5-bisphosphate) to generate two intracellular messengers, inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) (Ziegler, et al., 2009). In a similar process in *Drosophila*, these products go on to activate the two branches of the Duox regulation pathway (Rhee, 2001; Ha, et al., 2009b). However, the importance of this pathway for Duox regulation in *C. elegans* has not been determined.

In *Drosophila*, after PIP₂ is cleaved, IP₃ goes on to increase Duox protein activity (Ha, et al., 2009b). Activation of the IP₃ receptor results in the release of Ca²⁺ (Berridge, 1993). The IP₃ receptor in *C. elegans* is encoded by the gene *itr-1* (Baylis et al., 1999). This protein has 42% conservation at the primary amino acid level with mammalian IP₃ receptors and its binding domains function analogously (Baylis et al., 1999). The *Drosophila* encoded Duox protein contains a conserved EF-hand Ca²⁺ domain, which is traditionally associated with calcium binding. Previous publications indicate that released Ca²⁺ binds this domain, activating Duox (Ha, et al., 2009b). The EF-hand domain in *C. elegans* Duox is only partially conserved and may or may not have calcium binding function (Edens et al., 2001).

Regulation of Duox Transcription

Duox activity in *Drosophila* is also regulated transcriptionally. This branch of the pathway is activated when the host requires more Duox in order to increase ROS production (Ha, et al., 2009a). *C. elegans* has been shown to respond to a number of pathogens (including gram-positive and gram-negative bacteria) through a pathway similar to the Duox transcriptional activation pathway in *Drosophila* (Hayakawa et al., 2006; Ziegler, et al., 2009). In the *C. elegans* pathway, diacylglycerol (DAG), the second product of PIP₂ hydrolysis, activates protein kinase C δ (PKC δ), which is encoded by *tpa-1* (Ren et al., 2009; Ziegler, et al., 2009). PKC δ activates Tir-1, an ortholog of the human protein SARM (Ziegler, et al., 2009).

Tir-1 is a toll-interleukin like receptor (TLR) adaptor protein (Liberati et al., 2004). TLRs are capable of activating MAPK cascades and inducing transcription (Liberati et al., 2004). Tir-1 is responsible for transducing signals from TLRs to downstream components of the p38 pathway in *C. elegans* (Liberati et al., 2004). Tir-1 has been shown to interact with NSY-1, the MAP3K in the *C. elegans* p38 MAPK pathway (Sagasti et al., 2001).

MAPK cascades are among the most conserved pathways among multi-cellular organisms and can be found in species including *Arabidopsis*, *Drosophila*, and mammals (Asai et al., 2002; Dong, 2002). In MAPK pathways, protein kinases attach phosphates to the side chains of serine, threonine, or tyrosine residues of specific proteins (Johnson & Lapadat, 2002). First the stimulus reaches the activator, which phosphorylates the MAP3K, which then phosphorylates the MAP2K, which phosphorylates the MAPK, which phosphorylates the substrate (Johnson & Lapadat, 2002). Protein phosphatases remove the phosphates that were transferred to the protein substrate by the MAPK, acting as a negative regulator for the pathway (Johnson & Lapadat, 2002).

Three different MAPK pathways intervene in response to different types of stress (Ewbank, 2006). The first is the ERK (Extracellular signal-regulated kinase) pathway, which in multicellular organisms is involved in regulation of meiosis, mitosis, and postmitotic functions, and responds to stimuli including growth factors, cytokines, viral infection, ligands of G-protein coupled receptors, transforming agents, and carcinogens (Johnson & Lapadat, 2002). In *C. elegans*, the ERK pathway also regulates responses to some gram-positive bacterial infections, including *Microbacterium nematophilum* (Nicholas & Hodgkin, 2004). The second type of MAPK pathway, the C-Jun NH₂-terminal kinase (JNK) pathway, is activated by stresses such as the inhibition of protein synthesis and is important in apoptosis (Johnson & Lapadat, 2002). In *C. elegans*, organisms with mutations in the JNK pathway show uncoordinated locomotion and hypersensitivity to heavy metals and starvation (Sakaguchi et al., 2004; Koga et al., 2000).

The third MAPK pathway, the P38 pathway, is activated by inflammatory cytokines and environmental stresses and may be involved in diseases such as asthma and autoimmunity (Johnson & Lapadat, 2002). The MAP3K in the *C. elegans* P38 pathway is *nsy-1*, the MAP2K is *sek-1*, and the MAPK is *pmk-1* (Kim, et al., 2002; Ewbank, 2006). The P38 pathway has been shown to be activated by *tir-1* during *C. elegans* innate immune response to fungi and both gram-positive and gram-negative bacteria (Gravato-Nobre & Hodgkin, 2005). TIR-1, NSY-1, and SEK-1 can be co-immunoprecipitated when co-expressed in cell culture, indicating that they probably form a physical complex (Chuang & Bargmann,

2005). Furthermore, the role of the P38 pathway may be somewhat conserved, as TIR proteins and NSY-1 have been found to be involved in invertebrate and vertebrate innate immune function (Couillault, et al., 2004; Liberati et al., 2004). PMK-1 activates a transcription factor called ATF-7, which activates many immune pathways (Shivers, et al., 2010). The proposed Duox regulation pathway is shown in Figure 5, and components are listed in Table 1.

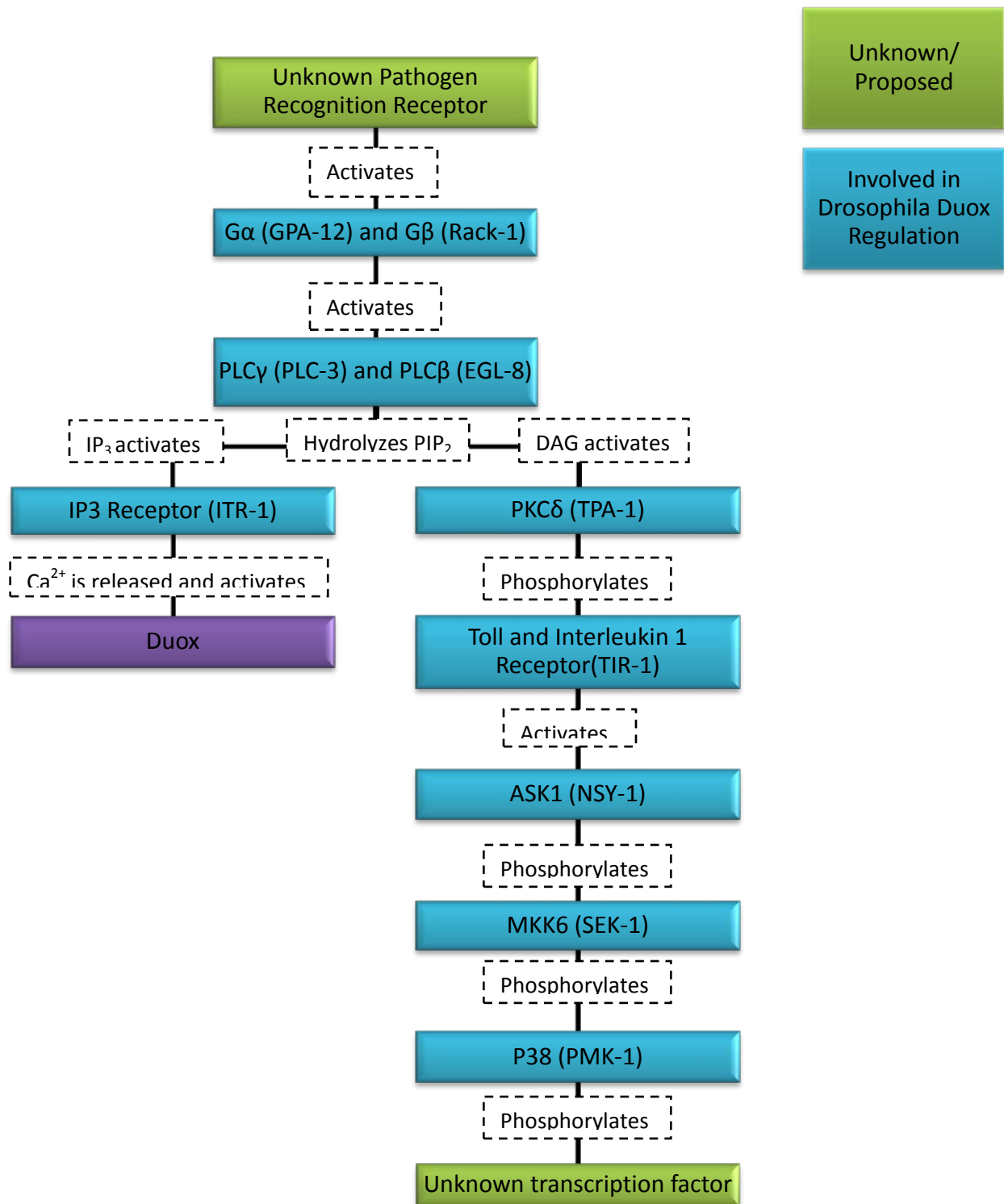


Figure 5: Proposed Duox Regulation Pathway

Table 1: Potential Duox Regulatory Proteins

| Pathway Protein | Pathway Branch | Role | Evidence for Involvement | <i>C. elegans</i> gene name | Homologous proteins in Humans and <i>Drosophila</i> (BLAST) |
|--|----------------------------|--|---|---------------------------------|---|
| Unknown Pathogen Recognition Receptor | Both | Recognizes pathogens, activates G α and G β | – | Unknown | Unknown |
| G α and G β | Both | Activates PLC γ and PLC β | Involved in <i>Drosophila</i> pathway (Ha, et al., 2009b). Shown to act upstream of <i>TPA-1</i> , <i>PLC-3</i> , and <i>EGL-8</i> in <i>C. elegans nlp-29</i> regulation (Ziegler, et al., 2009). | <i>Gpa-12</i> and <i>Rack-1</i> | Guanine nucleotide-binding protein subunit α [Homo sapiens] and G protein alpha [<i>Drosophila melanogaster</i>]; Guanine nucleotide-binding protein subunit beta [Homo sapiens] and Receptor of activated protein kinase C [<i>Drosophila melanogaster</i>] |
| PLC γ and PLC β | Both | Hydrolyzes PIP $_2$ yielding IP $_3$ and DAG | Involved in <i>Drosophila</i> pathway (Ha, et al., 2009b). <i>PLC-3</i> and <i>EGL-8</i> act upstream of <i>TPA-1</i> in <i>C. elegans nlp-29</i> regulation (Ziegler, et al., 2009). | <i>Plc-3</i> and <i>Egl-8</i> | 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase [Homo Sapiens] and Small wing [<i>Drosophila melanogaster</i>]; Phospholipase C [Homo sapiens] and [<i>Drosophila melanogaster</i>] |
| IP $_3$ receptor | Protein activation | Binds IP $_3$ and releases Ca $^{2+}$ (which binds the EF-hand domain on Duox, activating the protein) | In <i>Drosophila</i> , after PIP $_2$ is cleaved, IP $_3$ goes on to increase protein activity (Ha, et al., 2009b). Activation of the IP $_3$ receptor results in the release of Ca $^{2+}$ (Berridge, 1993). | <i>ltr-1</i> | Inositol 1,4,5-trisphosphate receptor [Homo sapiens] and [<i>Drosophila melanogaster</i>] |
| PKC δ | Transcriptional activation | Activated by DAG, phosphorylates (and therefore activates) TIR-1 | Involved in <i>Drosophila</i> pathway (Ha, et al., 2009b). In <i>C. elegans nlp-29</i> regulation, PKC δ activates TIR-1 (Ziegler, et al., 2009). | <i>Tpa-1</i> | Protein kinase C [<i>Drosophila melanogaster</i>] and [Homo sapiens] |
| TIR (Toll and Interleukin 1 Receptor) domain protein family member (tir-1) | Transcriptional activation | Activates MAPK cascades | Involved in <i>Drosophila</i> pathway (Ha, et al., 2009b). Shown to interact with NSY-1, the MAP3K in the <i>C. elegans</i> p38 MAPK pathway (Sagasti et al., 2001). | <i>Tir-1</i> | Ect4 [<i>Drosophila melanogaster</i>] and SARM [Homo sapiens] |

| Pathway Protein | Pathway Branch | Role | Evidence for Involvement | <i>C. elegans</i> gene name | Homologous proteins in Humans and <i>Drosophila</i> (BLAST) |
|---|----------------------------|--------------------------------------|--|-----------------------------|---|
| Neuronal Symmetry family member (nsy-1) | Transcriptional activation | Phosphorylates MAP2K | Involved in <i>Drosophila</i> pathway (Ha, et al., 2009b). The MAP3K in the <i>C. elegans</i> P38 pathway is <i>nsy-1</i> (Kim, et al., 2002). | <i>Nsy-1</i> | Mitogen-activated protein kinase kinase kinase 15 [Homo sapiens] and Protein kinase at 92B [<i>Drosophila melanogaster</i>] |
| SAPK/ERK kinase family member | Transcriptional activation | Phosphorylates MAPK | Involved in <i>Drosophila</i> pathway (Ha, et al., 2009b). The MAP2K in the <i>C. elegans</i> P38 pathway is <i>sek-1</i> (Kim, et al., 2002). | <i>Sek-1</i> | Dual specificity mitogen-activated protein kinase kinase [Homo sapiens] and Stress-activated MAP kinase kinase [<i>Drosophila melanogaster</i>] |
| Mitogen-activated protein kinase pmk-1 | Transcriptional activation | Phosphorylates transcription factor | Involved in <i>Drosophila</i> pathway (Ha, et al., 2009b). The MAPK in the <i>C. elegans</i> P38 pathway is <i>pmk-1</i> (Kim, et al., 2002). | <i>Pmk-1</i> | Mitogen-Activated Protein Kinases [Homo sapiens] and p38 β [<i>Drosophila melanogaster</i>] |
| Unknown transcription factor | Transcriptional activation | Activates <i>Bli-3</i> transcription | – | Unknown | Unknown |
| Dual Oxidase | | – | – | <i>Bli-3</i> | Dual oxidase [<i>Drosophila melanogaster</i>], dual oxidase 2 precursor [Homo sapiens], and dual oxidase 1 precursor [Homo sapiens] |

Other molecules, including P38 phosphatase, thioredoxin, and H_2O_2 may play a role in Duox regulation. P38 phosphatase, or VHP-1 MAPK phosphatase, a homologue of mammalian MPK7, negatively regulates the p38 pathway in *C. elegans* by dephosphorylating P38 MAPKs, including PMK-1 (Kim, et al., 2004). Regulation of ASK-1, the mammalian p38 MAP3K (encoded by *nsy-1* in *C. elegans*), has also been studied and is pictured below in Figure 6. In non-activated systems, reduced thioredoxin (Trx) is bound to two ASK-1 molecules (Fujino, et al., 2007). When reactive oxygen species are present, Trx is oxidized and unbinds from the ASK-1, enabling ASK-1 to be activated by TRAF2 and TRAF6 (Fujino, et al., 2007). The levels of free versus bound ASK-1 regulate the levels of P38 pathway activity (Hsieh & Papaconstantinou, 2006). Reactive oxygen species dissociate the Trx-ASK-1 complex, increasing P38 activity (Hsieh & Papaconstantinou, 2006). Because the p38 pathway may be an upstream activator of Duox in *C. elegans*, this would constitute a positive feedback loop that might stabilize Duox transcription during infection.

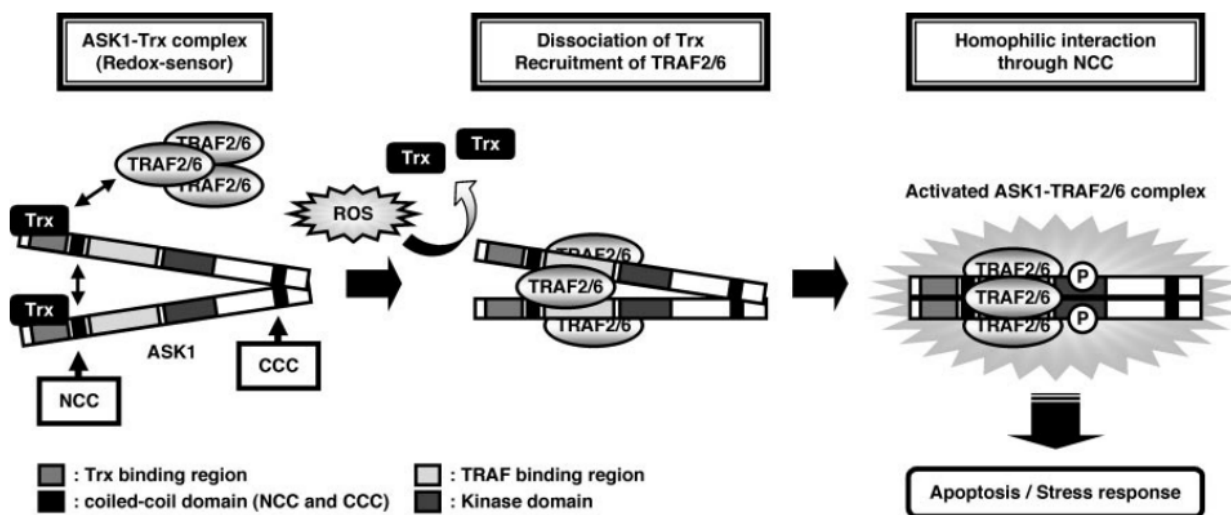


Figure 6: ASK-1 Regulation

Research Plan

In order to test the effects of *bli-3* on survival of worms exposed to *Saccharomyces* and *Candida*, survival assays were performed utilizing available mutants and RNAi knockdown. Two *bli-3* mutants were used, *bli-3 (e767)* and *bli-3 (n529)*. In both cases, the mutation is a single G to A base change (Simmer, et al., 2003). Both mutations occur in the sequence encoding the peroxidase domain, which is located from amino acids #26-590 (Simmer, et al., 2003). Preliminary observations indicate that *bli-3 (n529)* worms bear a more severe blistered phenotype than do the *bli-3 (e767)* worms. Additionally, *E.*

coli containing vectors expressing double-stranded RNA specific for Bli-3 were fed to wild type *C. elegans* in order to induce an RNA interference response and knockdown *bli-3* expression.

Experiments were aimed at testing the hypothesis that Duox/BLI-3 activity is required for optimal survival of worms exposed to yeast pathogens, and that this immune function occurs in the intestine. If this were the case, the loss of function mutants *bli-3 (e767)* and *bli-3 (n529)*, and worms subjected to knockdown of *bli-3* would show decreased survival (enhanced susceptibility) when exposed to the yeast pathogens. Furthermore, worms with Duox/BLI-3 knocked down in the intestine would show decreased survival when compared with worms with the same protein knocked down in the hypodermis or muscle cells. It was especially important that testing be performed on worms with Duox/BLI-3 knocked down in the hypodermis in order to see if the effects on the cuticle also reduce survival of worms exposed to yeast.

Previous research indicates that decreasing expression of Duox/BLI-3 reduces *Enterococcus faecalis*-induced ROS production by *C. elegans*, and that *C. elegans* exposed to *Bli-3* dsRNA produced significantly less ROS than worms fed the vector control dsRNA (Chavez et al., 2009). Furthermore, reduction of Duox in the intestine and hypodermis, but not in the muscle, causes increased sensitivity to *E. faecalis* (Chavez et al., 2009, 2007). This makes sense, given that pathogens that manage to pass through the pharyngeal grinder often exert their effect on the intestine (Schulenburg et al., 2004; Gravato-Nobre & Hodgkin, 2005). Duox has been shown to function in the intestine in *Drosophila*, further suggesting that this may be the case in *C. elegans* (Ha et al., 2005, 2009b). It is hypothesized that Duox/BLI-3 is also involved in *C. elegans*' innate immune response to yeast pathogens.

In order to determine the location of *bli-3* immune function, *rde-1* mutants will be used for tissue specific RNAi knockdown. The gene *rde-1* codes for an argonaute protein, a part of the RISC complex (Tabara, et al., 1999). Rde stands for RNAi deficient, and loss of function *rde-1* mutants render the RNAi machinery inactive (Tabara, et al., 1999). This leads to no obvious defects in growth or development (Tabara, et al., 1999). Wild-type *rde-1* can be expressed under a tissue-specific promoter in *rde-1* mutant worms, and RNAi sensitivity only occurs in tissues expressing *rde-1* (Qadota, et al., 2007). Feeding worms *E. coli* expressing dsRNA is usually sufficient to induce interference, making this a fairly simple experimental procedure (Timmons & Fire, 1998). In order to determine in which tissue *bli-3* functions, *rde-1* mutants containing the *rde-1* gene driven by a tissue specific promoter (intestine, muscle, or hypodermal) will be used. *Bli-3* activity will then be knocked down by RNAi only in the tissue expressing *rde-1*. Survival assays will be run on worms with *bli-3* activity knocked down in different

tissues in order to determine where it is required for resistance to yeast infection. This procedure is outlined in Figure 7. The current estimated frequency of false-positive RNAi phenotypes is less than 1%, meaning that positive results are very significant (Kamath, et al., 2003). However, RNAi is not completely effective, so results can be difficult to obtain and false negatives are high (Kamath, et al., 2003); (Simmer, et al., 2003). RNAi results (even from experiments done in the same laboratory) vary from 10% to 30% (Simmer, et al., 2003).

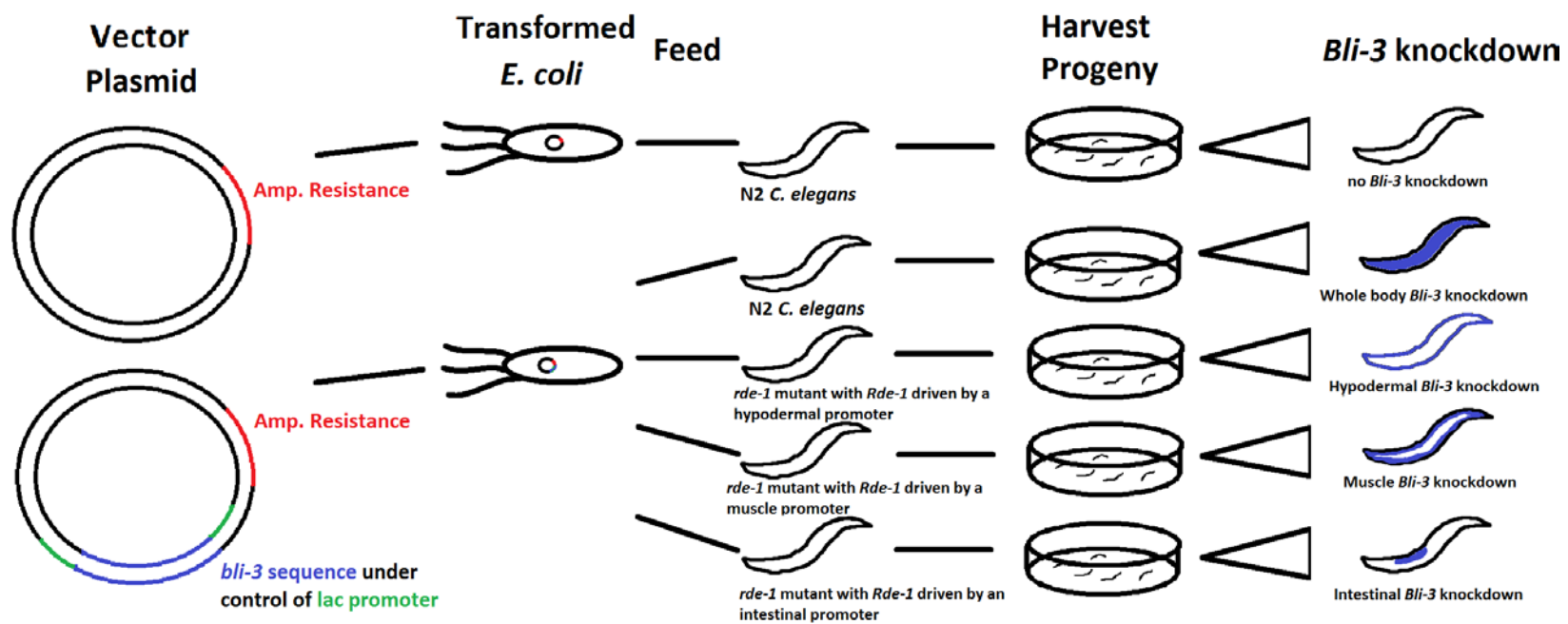


Figure 7: RNAi Experimental Procedure

Materials and Methods

Stocks

Four *C. elegans* strains were used in this experiment. The control strain was the wild type strain, N2. Two *bli-3* mutants were used, *MT 1141 bli-3 (n529)* and *CB 767 bli-3 (e767)*. Finally, *rde-1* mutants with the wild type *rde-1* gene expressed under a tissue specific promoter were used for tissue-specific RNAi *bli-3* knockdown. NR222 (*rde-1 (ne219) V; kzs9[pKK1260(lin-26p::nls::gfp), pKK1253(lin-26p::rde-1), pRF4(rol-6 marker)]*), which has a dominant *rol-6* allele as a marker, was used for hypodermal RNAi knockdown. NR350 (*rde-1(ne219) V; kzs20 [pDM#715(hlh-1p::rde-1) + pTG95(sur-5p::nls::GFP)]*), which uses GFP as a marker gene, was used for muscle specific RNAi knockdown. WP303, which also uses *rol-6(d)* as a marker and contains *rde-1* driven by the *nhx-2* intestinal specific promoter, was used for intestinal significant RNAi (K. Strange, personal communication; see also Espelt et al 2005). NR222 and NR350 were obtained from the Caenorhabditis Genetics Center. WP303 was obtained from the laboratory of Kevin Strange. These strains all contained the transgenes as integrated multi-copy arrays. All strains were kept on NGM agar plates with *E. coli* OP50 as a food source, and were incubated at 16 or 20°C. Yeast strains used were Y101 (*S. cerevisiae*) and SC5314 (*C. albicans*). Single colonies of the yeast strains were picked from YPD agar plates to a liquid culture containing 2mL YPD that was incubated overnight at 30°C (*S. cerevisiae*) or 37°C (*C. albicans*).

Survival Assays without RNAi

Saccharomyces and *E. coli*

Survival assays were first performed without the use of RNA interference. The survival of *bli-3* mutants and wild type worms was tested on both *Saccharomyces cerevisiae* and *E. coli* (control). NGM agar plates were spotted with 20µL of 1:1:2 *Saccharomyces* stock: YPD: *Streptomycin* (50mg/mL stock, used to prevent contamination) and incubated at 30°C overnight. *Saccharomyces* stock cultures were created by inoculating 2mL YPD with a single colony from a streaked plate, followed by overnight incubation at 30°C. *E. coli* plates were spotted with 40µL of *E. coli* stock solution (OP50 grown on LB broth overnight at 37°C) and incubated at 37°C overnight. More *E. coli* was added to plates than *Saccharomyces* because *Saccharomyces* lawns grow much more quickly on the plates than do *E. coli*, and 20µL of *E. coli* culture was insufficient to last to the end of the assay. Thirty L4 stage worms were individually picked from stock plates to each test plate. Three plates of each worm strain to be tested (wild type, *bli-3 (n529)*, and *bli-3 (e767)*) were started at the same time. Live and dead worms on each

plate were counted and recorded daily. Dead worms were removed after each count to avoid double counting. Worms that crawled off the agar were not counted, as their cause of death could not be determined. Adult worms were re-plated every 2-3 days to avoid confusion between the original worms and their progeny.

Candida

Survival of the *bli-3* mutants and wild type worms was also tested on *Candida albicans* lawns. Each agar plate was spotted with 20µL of 1:1:2 *Candida* stock: YPD: *Streptomycin* solution and incubated overnight at 30°C. Liquid *Candida* stock was prepared by picking single colonies from an agar plate and incubating them at 37°C overnight in 2mL YPD. Thirty L4 worms were individually picked from stock plates to each test plate. Each strain was tested on three separate plates. Live and dead worms were counted and recorded every 8-20 hours. Dead worms were removed after each count to avoid double counting. Worms that crawled off the agar were not counted, as their cause of death could not be determined. Adult worms were re-plated every 2-3 days to avoid confusion between the original worms and their progeny.

Survival Assays with RNAi

E. coli RNAi feeding strains

Two *E. coli* plasmids were utilized for RNAi experimentation. The first plasmid, L4440, is the RNAi vector without insert, was used as a control in feeding experiments (Kamath, et al., 2003). The second plasmid, F56 C11.1, contained an inserted sequence from the *Bli-3* gene expressed under control of a *lac* promoter on each strand in an anti-parallel arrangement (Kamath, et al., 2003). The *lac* promoter is expressed in the presence of IPTG (which is present in the agar on the feeding plates). Transcription occurs from both promoters, resulting in dsRNA containing a sequence from the *bli-3* gene. Both vectors contained an ampicillin-resistance gene for plate selection of transformed colonies. Tetracycline resistance was encoded by an insertion into the *E. coli* host genome. Strains were obtained from Source Biosource, Oxford, UK.

Streaking Plates

LB plates containing 10µg/mL tetracycline and 50µg/mL ampicillin were used to select for presence of plasmids, check the viability of the strains through streaking, and grow colonies to be used

to make feeding and freezing cultures. The LB formula used can be found in the “Molecular Cloning: A Laboratory Manual” (Maniatis et al., 1982).

Plates were streaked with the *E. coli* strains containing L4440 and F56 C11.1 using a sterile inoculating loop.

Freezing Stocks

A sterile inoculating loop was used to inoculate 5mL of media (LB with 10µg/mL tetracycline and 50µg/mL ampicillin) with a colony from a streaked plate. This culture was incubated overnight at 37°C. A solution of 15% glycerol was created by combining 0.85mL of the overnight culture with 0.15mL glycerol. This culture was frozen at -80°C for long term storage. The culture was re-streaked from the frozen stock to confirm viability after freezing.

Making Feeding Plates

500mL of NGM agar was made containing 25µg/mL Carbenicillin and 1mM IPTG. Fifty 60mm Petri dishes were poured, each containing 10mL of this media. Plates were allowed to sit for one week at room temperature. Liquid cultures of the feeding strains were made in 2mL LB containing 50µg/mL ampicillin. One tube was inoculated with a single colony of L4440 or F56 C11.1. Feeding stocks were incubated overnight at 37°C. Feeding plates were spotted with 150µL of the overnight culture (Ahringer, 2006).

Harvesting Progeny

Twenty L4 worms were plated to each feeding plate. The original worms were transferred to new feeding plates once progeny were observed. This process was repeated as necessary. After enough progeny had been obtained, L4 progeny were transferred to *Candida* plates and survival assays were run in the same fashion as described for the non-RNAi survival assays.

Results

Survival Assays without RNAi

Comparison of Pathogens

In the survival assay without the use of RNAi, wild type, *bli-3* (e767), and *bli-3* (n529) worms were exposed to *E. coli*, *S. cerevisiae*, and *C. albicans*. As is shown in Figure 8, all *C. elegans* strains survived longest on the control, *E. coli*, and showed the largest decrease in survival on *C. albicans*. In all worm strains, differences in survival were statistically significant between *E. coli* vs. *S. cerevisiae*, *E. coli* vs. *C. albicans*, and *S. cerevisiae* vs. *C. albicans* (see appendix 1 for tabulated p values).

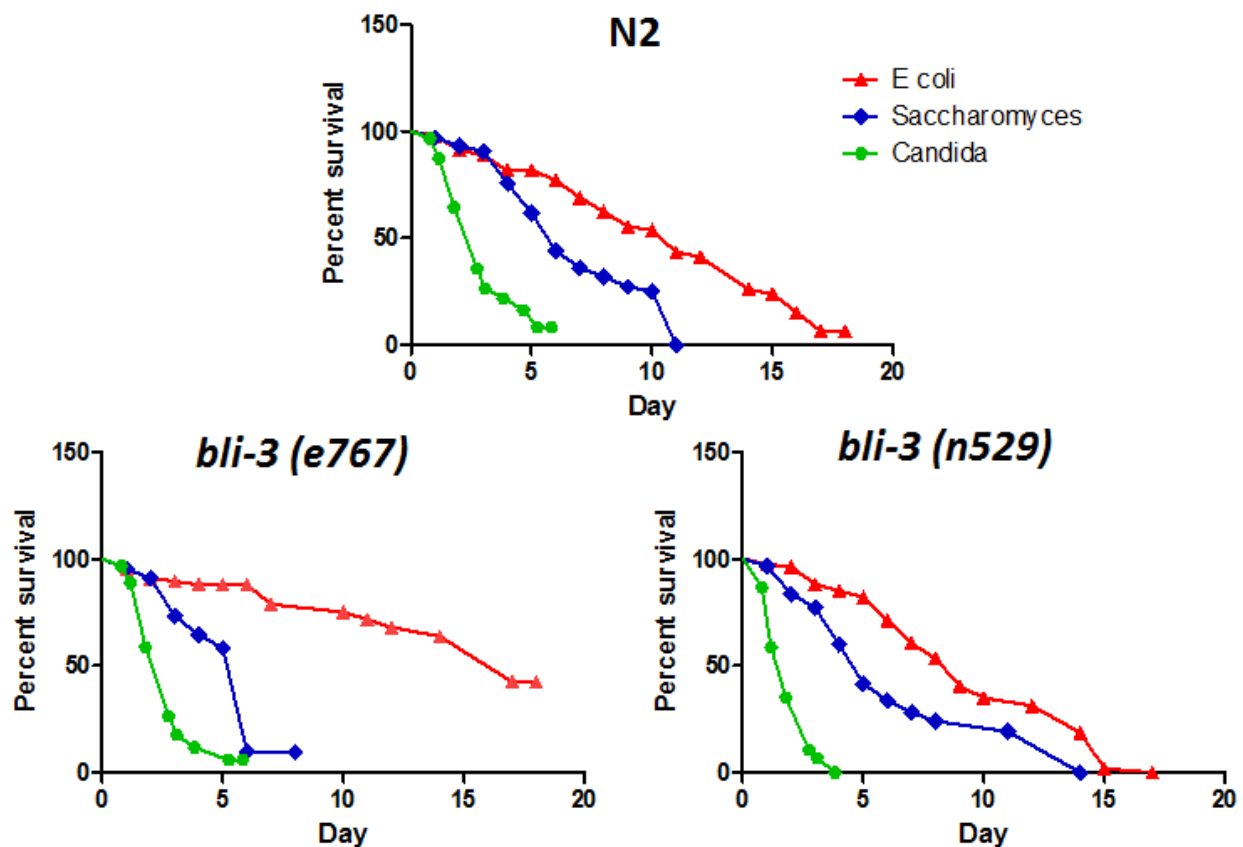


Figure 8: Yeast pathogens decreased the survival rates of all three *C. elegans* strains, and *Candida albicans* affected survival more than *Saccharomyces cerevisiae*. Wild-type (N2), *bli-3* (e767), and *bli-3* (n529) *C. elegans* were exposed to an *E. coli* control (red) and two different yeast pathogens, *Saccharomyces cerevisiae* (blue) and *Candida albicans* (green). All three worm strains survived longest on *E. coli*, and showed a significant decrease in survival when exposed to either pathogen (log-rank test, $p < 0.001$). Worms of all three strains exposed to *Candida albicans* also had significantly decreased survival when compared to worms exposed to *Saccharomyces cerevisiae* (log-rank test, $p < 0.001$ in all cases). Each survival curve represents results combined from three plates, with 30 worms per plate.

Effects of *bli-3* mutations

Differences in survival between different *C. elegans* strains plated on the same food source (*E. coli*, *S. cerevisiae*, or *C. albicans*) were also observed. Data from Figure 8 were re-plotted for comparison (Fig. 9, Fig. 10, and Fig. 11). The *bli-3* (*e767*) strain showed no significant decrease in survival compared with wild type when exposed to *Saccharomyces* or *Candida* (Fig. 10 and Fig. 11, respectively). However, surprisingly, the *bli-3* (*e767*) strain survived significantly longer than the N2 strain on *E. coli* (Fig. 9). In contrast, the *bli-3* (*n529*) strain showed a significant decrease in survival compared to wild-type when exposed to *E. coli* (Fig. 9) or *Candida* (Fig. 11). Although there appeared to be a decrease in survival of *bli-3* (*n529*) *C. elegans* exposed to *Saccharomyces* when compared to N2 and *bli-3* (*e767*) worms, the difference was not statistically significant (Fig. 10).

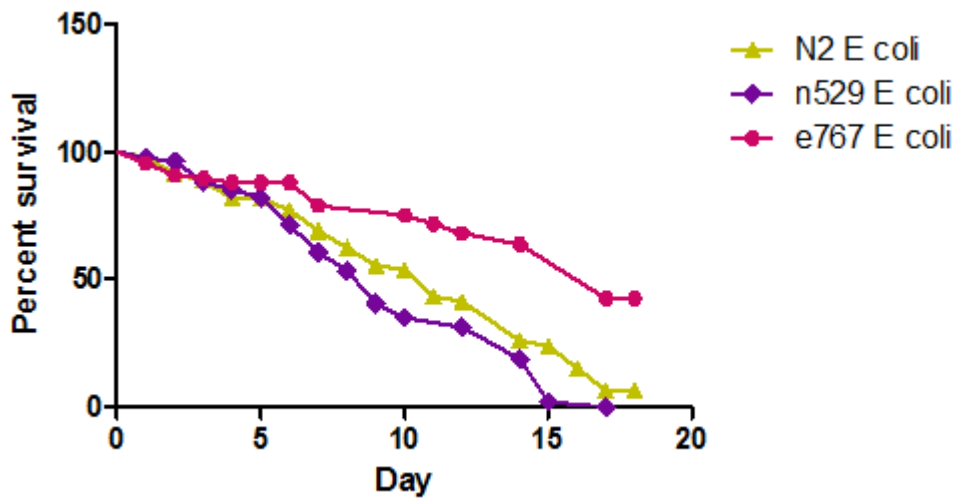


Figure 9: Differential survival of wild type and *bli-3* mutant *C. elegans* exposed to *E. coli*. Wild-type (N2) (yellow), *bli-3* (*e767*) (fuchsia), and *bli-3* (*n529*) (purple) *C. elegans* were plated on *E. coli*. Data are re-plotted from Figure 8. The *bli-3* (*e767*) worms survived longer than wild-type or *bli-3*(*n529*). When compared to wild-type using the Log-Rank test, *bli-3* (*e767*) worms had a p value of 0.0004 and *bli-3* (*n529*) worms had a p value of 0.0436. When compared to each other, the mutants had a p value of <0.0001. Results included three plates per strain, with 30 worms per plate.

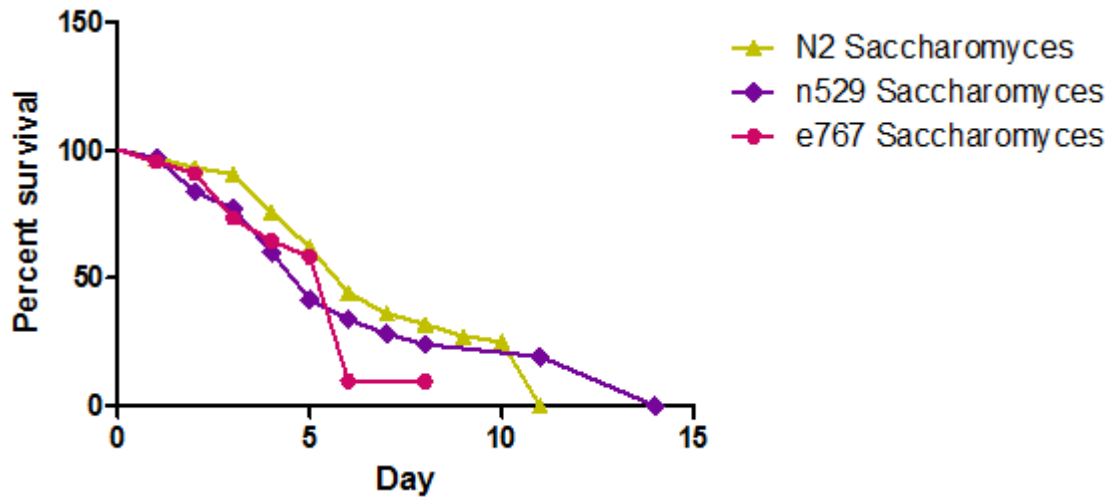


Figure 10: Differential survival of wild-type and *bli-3* mutant *C. elegans* exposed to *Saccharomyces cerevisiae*. Wild-type (N2) (yellow), *bli-3* (e767) (fuchsia), and *bli-3* (n529) (purple) *C. elegans* were exposed to *Saccharomyces cerevisiae*. The slight apparent decrease in survival between each mutant and wild-type was not significant (Log-Rank test p values were as follows: *bli-3* (e767) – 0.0994 and *bli-3* (n529) – 0.1933). Results included three plates per strain, with 30 worms per plate.

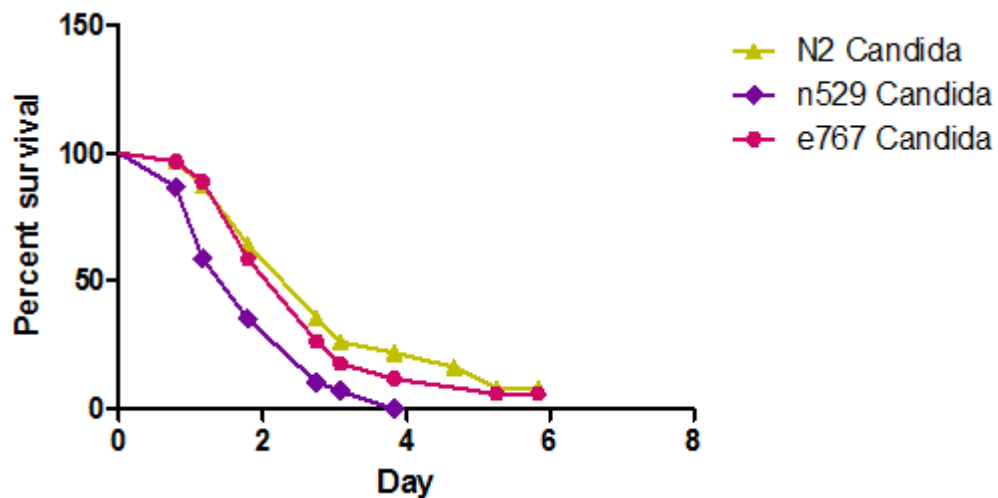


Figure 11: Differential survival of wild-type and *bli-3* mutant *C. elegans* exposed to *Candida albicans*. Wild-type (N2) (yellow), *bli-3* (e767) (fuchsia), and *bli-3* (n529) (purple) *C. elegans* were exposed to *Candida albicans*. The *bli-3* (n529) strains showed a statistically significant decrease in survival when compared to either N2 ($p < 0.0001$) or *bli-3* (e767) worms ($p < 0.0001$). Although there appeared to be a slight decrease in survival of the *bli-3* (e767) worms when compared to N2, this difference was not significant according to the Log-Rank test ($p = 0.3961$). This graph represents combined results which included three plates per strain, with 30 worms per plate.

Survival Assays with RNAi

The effects of *Bli-3* on survival were also studied using an RNAi knockdown procedure. An *E. coli* strain transformed with a vector containing a short sequence of *Bli-3* dsRNA expressed under the *lac* promoter was used to induce *Bli-3*-specific RNAi in worms. Wild type *C. elegans* were fed *E. coli* containing either an empty vector (control) or the vector expressing ds*Bli-3*. Based on the greater severity of survival reduction in worms exposed to *Candida albicans*, we chose to use *C. albicans* rather than *S. cerevisiae* as the pathogen in the RNAi tests. The progeny of worms receiving RNAi or control treatment were then exposed to *Candida albicans* and a survival assay was performed. Results of the assay are shown in Figure 12. *C. elegans* from the *Bli-3* RNAi treated sample showed a significant decrease in survival when compared with the control.

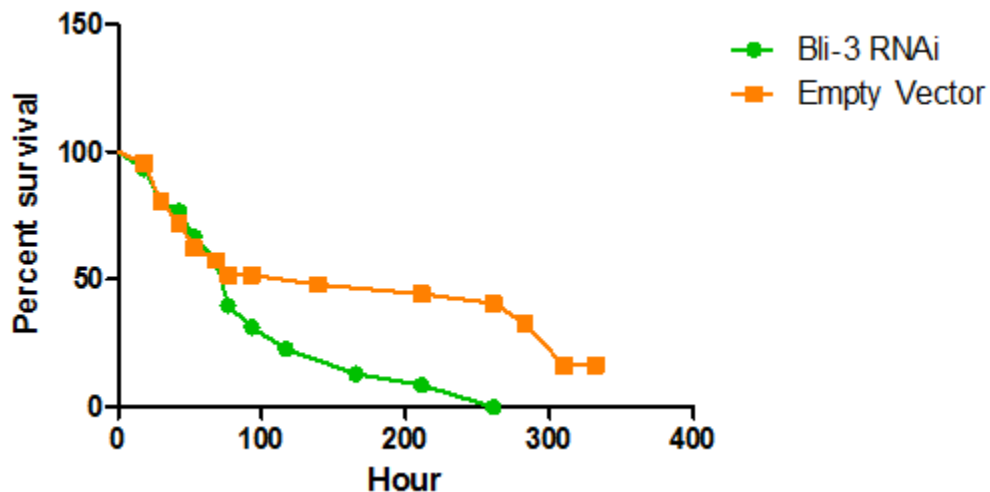


Figure 12: Reduction in survival in *C. elegans* treated with ds*Bli-3* (green) prior to exposure to *Candida albicans*. L4 *C. elegans* were fed transformed *E. coli* with either an empty vector insert (L4440, orange) or a vector insert with *bli-3* dsRNA (F56 C11.1, green). Their progeny were then plated on *Candida albicans* when they reached the L4 stage. Survival curves were generated using the Graph Pad Prism software. When compared to worms fed empty-vector transformed *E. coli*, whole body RNAi *bli-3* knockout worms show a decrease in survival. This difference is statistically significant according to the Log-Rank test ($p=0.0209$). This graph represents combined results which included three plates per strain; with 30 worms per plate.

TD50s and Relative Mortalities of Mutant and Knockdown Worms

TD50 is defined as the estimated time for 50% of the individuals in a survival test to die. Results are summarized in Figure 13 and tabulated in Table 2. It can be seen that for wild-type as well as the two *bli-3* mutants, exposure to yeast reduced TD50, with exposure to *Candida* having a more severe effect than *Saccharomyces*. As described above, the TD50 for *bli-3* (*e767*) worms on *E. coli* was surprisingly high.

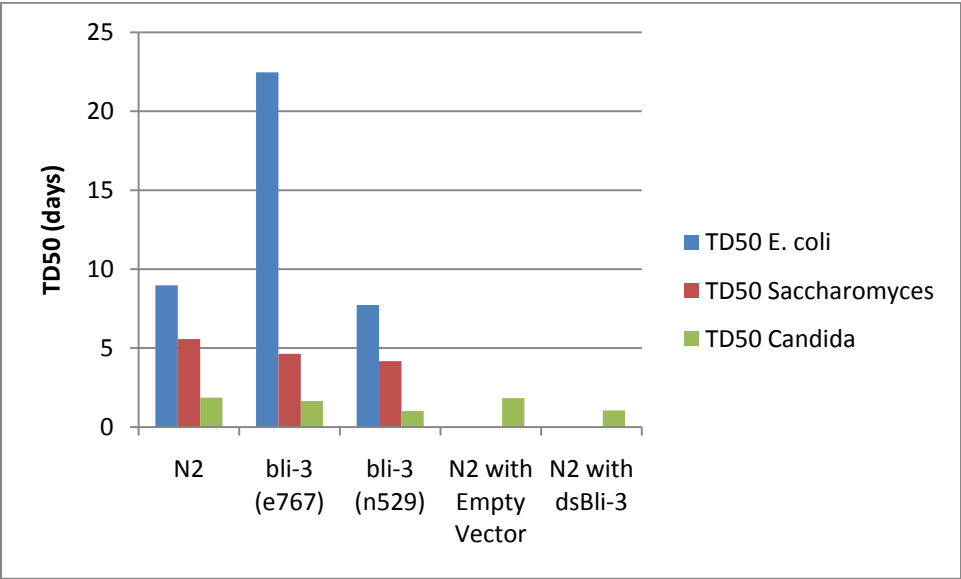


Figure 13: TD50 Values of wild-type *bli-3* mutant, and previously RNAi-treated *C. elegans* exposed to yeast. Data from the experiments shown in Figures 9-12 were used to estimate TD50 using a nonlinear regression, dose-response model (GraphPad Prism software). TD50 numerical values are also tabulated in Table 2.

TD50s were used to determine relative mortality, defined as the ratio of the TD50 of the control group to the TD50 of the test group. The equation for relative mortality can be seen in Equation 1: Relative Mortality. In survival assays without the use of RNAi, wild type *C. elegans* were used as the control and the test groups were *bli-3 (e767)* and *bli-3 (n529)*. In the whole-body RNAi survival assay, *C. elegans* exposed to empty vector were used as a control and *C. elegans* exposed to the vector carrying the dsBli-3 message were the test group. Relative mortalities greater than one indicate that the test group showed a decrease in survival, whereas relative mortalities of less than one indicate that the test group survived better. The latter was the case in the relative mortality of *bli-3(e767)* on *E. coli*. Calculated relative mortalities of the strains tested are shown in Table 2.

Equation 1: Relative Mortality

$$Relative\ Mortality = \frac{TD50_{control}}{TD50_{mutant}}$$

The final measurement of mortality calculated was corrected relative mortality. This adjusts the relative mortality to remove bias due to the fact that mutants generally show decreased survival, even under control conditions. For this calculation, relative mortality of the mutant on *E. coli* was used as a control, and the relative mortality of the mutant on the yeast was divided by this control. The equation can be seen in Equation 2: Corrected Relative Mortality. In order to use this correction, strains had to be compared after exposure to both *E. coli* and a yeast pathogen. When compared in this way, the corrected mortality for *bli-3(n529)* on both yeast pathogens was still greater than one (Table 2), indicating that changes in survival on *E. coli* did not change the interpretation of the reduction in survival as having resulted from the pathogen. In the case of the *bli-3 (e767)* mutant, corrected relative mortality may be misleading, as it is surprising that the relative mortality is less than one, and further experimentation would need to be performed in order to see if the population was in fact representative of the strain's survival under these conditions. The misleading nature of corrected relative mortality in this example led to the choice to graphically represent the relative mortality of different experimental groups on *Candida*, rather than the corrected relative mortality (Figure 14).

Equation 2: Corrected Relative Mortality

$$Corrected\ Relative\ Mortality = \frac{Relative\ Mortality_{yeast}}{Relative\ Mortality_{E.coli}}$$

Table 2: Relative Mortalities of *C. elegans* exposed to yeast

| Strain | RNAi Vector | TD50 <i>E. coli</i> | TD50 <i>Saccharomyces</i> | TD50 <i>Candida</i> | Relative Mortality <i>E. coli</i> | Relative Mortality <i>Saccharomyces</i> | Relative Mortality <i>Candida</i> | Corrected Relative Mortality <i>Saccharomyces</i> | Corrected Relative Mortality <i>Candida</i> |
|---------------------|-----------------|---------------------|---------------------------|---------------------|-----------------------------------|---|-----------------------------------|---|---|
| N2 | | 8.98 | 5.569 | 1.862 | | | | | |
| <i>bli-3</i> (e767) | | 22.47 | 4.639 | 1.652 | 0.399644 | 1.20047424 | 1.127119 | 3.003859262 | 2.820307 |
| <i>bli-3</i> (n529) | | 7.732 | 4.174 | 1.024 | 1.161407 | 1.334211787 | 1.818359 | 1.148789036 | 1.565652 |
| N2 | Empty | | | 1.83 | | | | | |
| N2 | ds <i>Bli-3</i> | | | 1.0525 | | | 1.738717 | | |

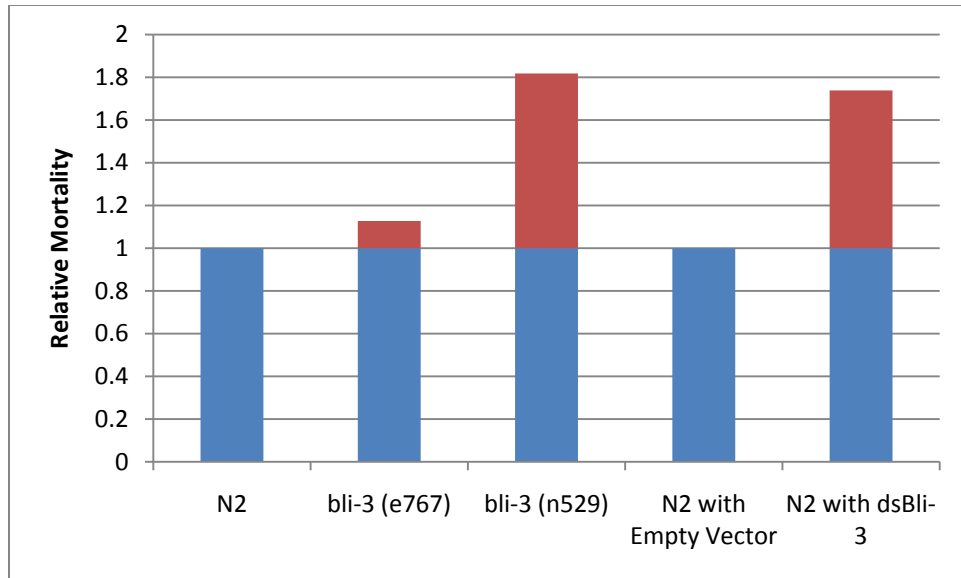


Figure 14: Relative Mortality on *Candida albicans*. Relative mortality was calculated for mutants and RNAi knockdown *C. elegans*. Relative mortalities that are greater than 1 are shown in red and indicate a decreased level of survival. *bli-3* (e767) mutants showed only a small decrease in survival, whereas N2 worms treated with *Bli-3*-specific RNAi and *bli-3*(n529) worms showed comparable decreases.

Tissue Specific RNAi

Tissue specific RNAi and survival assays on *Candida* were performed in order to identify in what tissue Duox's immune function is required. *Bli-3* knockdowns were performed in the hypodermis, muscle, and intestine. As is shown in Figure 15, differences in survival were not significant and survival was similar in all groups.

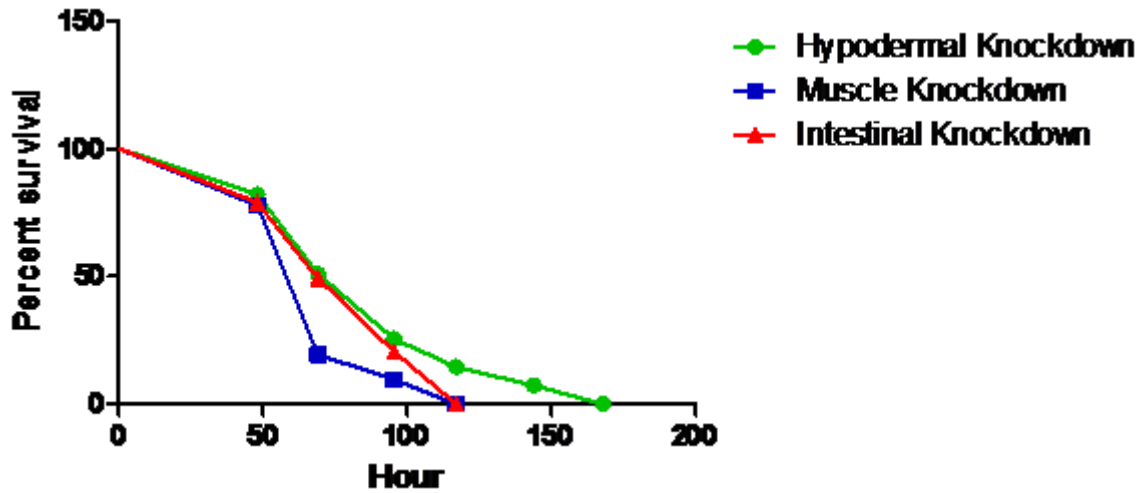


Figure 15: Survival rates are similar among *C. elegans* with *bli-3* knockdown in the hypodermis, muscle, and intestine. *C. elegans* expressing *rde-1*, a gene necessary for RNAi function, in specific tissues (hypodermis, muscle, and intestine) were exposed to dsRNA for *bli-3* and survival assays were performed on *Candida*. Differences in survival between worms with *bli-3* knocked down in certain tissues were not significant according to the Log-Rank test. P values were as follows: hypodermal v. muscle – 0.1187, muscle v. intestinal – 0.4019, and hypodermal v. intestinal – 0.3293.

Discussion

Survival assays were performed utilizing available mutants, RNA interference, and tissue specific RNAi in order to test the hypothesis that *bli-3* is required for immune function, and that this immune function is mainly localized in the intestine. Survival assays with available mutants showed that *bli-3* (*n529*) mutants had a significant decrease in survival after exposure to *Candida albicans* when compared to wild-type worms. Whole body RNAi knockdowns of *bli-3* also showed decreased survival on *Candida*, further supporting the hypothesis that *C. elegans*' Bli-3/Duox has an immune function. Tissue specific RNAi results indicated no significant differences in survival between worms with *bli-3* knocked down in different tissues, but these results were inconclusive as to *bli-3* function because the appropriate controls with the non-RNA inducing empty vector strain L4440 were not performed. The tissue-specific RNAi experiments should be repeated in future experiments, with the inclusion of these controls.

Survival assays utilizing *bli-3* (*e767*) and *bli-3* (*n529*) mutants plated on *E. coli*, *Saccharomyces cerevisiae*, and *Candida albicans* were used to assess the effects of *bli-3* mutation on survival rates of *C. elegans* exposed to different yeast pathogens. The effects of *bli-3* mutations on innate immunity were more visible and statistically significant when assays were performed with *Candida* than with *Saccharomyces*. As a result of this observation, RNAi survival assays were performed using *Candida* as a pathogen. Another advantage of using *Candida* is that the shorter survival time resulted in a shorter assay and less time for the RNA interference effect to wear off.

The results of the first survival assay gave partial support for the hypothesis that *bli-3* mutation reduces survival when exposed to yeast pathogens compared to wild type strains. The *bli-3* (*e767*) strain showed no significant decreases in survival when exposed to *Candida*. However, the *bli-3* (*n529*) strain showed a significant decrease in survival rate when exposed to *E. coli* and *Candida*. Both *bli-3* (*e767*) and *bli-3* (*n529*) showed no significant decrease in survival of when exposed to *Saccharomyces*.

The *bli-3* (*e767*) worms did not show a statistically significant decrease in survival on *Candida*, indicating that the site of mutation may play a role in the effect on the immune response. Both *bli-3* (*e767*) and *bli-3* (*n529*) are point mutations in the peroxidase domain-encoding sequence of *bli-3*. However, the mutations occur at different nucleotides.

In addition to assays using available mutants, RNAi knockdown *C. elegans* were used in order to determine the effect of loss of *bli-3* function. Worms treated with dsRNA for the *bli-3* gene showed a

significant decrease in survival when compared to worms exposed to an empty vector. The decrease in survival was slightly less than that of *bli-3* (*n529*) worms. This is likely due to the fact that RNAi knockdowns do not destroy all transcripts, and therefore small amounts of the Duox protein may still be present. Together with the observation that *bli-3* also showed decreased survival on *Candida*, these results indicated that *Bli-3* likely plays a role in host defense. RNAi knockdown assays do have pitfalls, so more should be performed. For example, knockdown may affect other genes similar in sequence to *Bli-3*. The F56 C11.1 vector may target multiple polypeptide sequences; whole body RNAi using this vector results in 10% embryonic lethality (Kamath, et al., 2003).

Worms in the whole-body RNAi survival assay lived longer than the other survival assays. However, the TD50s from this assay were similar to those of other assays. This is likely due to a large number of censored worms and a few outliers that lived significantly longer than expected. Possible confounding variables include the age of the liquid *Candida* culture, which was stored at 4°C for up to several weeks, and consequent variability of rate of growth of the *Candida* lawn.

Tissue specific RNAi results indicated no significant difference in survival among *C. elegans* with *bli-3* knockdown in different tissues after exposure to *Candida*. However, Chavez et al. observed a significant decrease in survival among *C. elegans* with *bli-3* knockdown in the intestine and the hypodermis, but not in the muscle (Chavez et al., 2007, 2009). Chavez et al (2009) recognized that the *C. elegans* strains engineered for tissue-specific RNAi had different lifespans, even when grown on the empty vector *E. coli* strain. They therefore calculated relative mortality to compare the strains. In future experiments, survival assays should also include exposure of each strain to the empty vector control, and relative mortalities should be calculated for control vs. RNAi treated in order to determine if differences in survival exist between worms with *bli-3* knocked down in different tissues.

The NADPH oxidase family of proteins is found in a variety of cell types and tissues and is implicated in aging, cellular damage and apoptosis, cellular signaling, and host defense. Mutations in Nox family proteins can result in a variety of diseases, ranging from congenital hypothyroidism to chronic granulomatous disease (a rare, fatal syndrome in young boys lacking the Nox catalyzed respiratory burst). Studies of Nox family proteins in well-understood and easily manipulated model organisms can increase the scientific communities understanding of these important proteins and lead to novel treatments for disorders related to mutations in these proteins.

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Appendices

Appendix I: Log-Rank p Values for Survival Assays without RNAi

Table 3: p Values for Survival Assays without RNAi

| | N2 Ec | N2 Sc | N2 Ca | e767 Ec | e767 Sc | e767 Ca | n529 Ec | n529 Sc | n529 Ca |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| N2 Ec | | <0.0001 | <0.0001 | 0.0004 | 0.0002 | <0.0001 | 0.0436 | <0.0001 | <0.0001 |
| N2 Sc | <0.0001 | | <0.0001 | <0.0001 | 0.0994 | <0.0001 | 0.0009 | 0.1933 | <0.0001 |
| N2 Ca | <0.0001 | <0.0001 | | <0.0001 | <0.0001 | 0.3961 | <0.0001 | <0.0001 | <0.0001 |
| e767 Ec | 0.0004 | <0.0001 | <0.0001 | | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| e767 Sc | 0.0002 | 0.0994 | <0.0001 | <0.0001 | | <0.0001 | 0.0001 | 0.5944 | <0.0001 |
| e767 Ca | <0.0001 | <0.0001 | 0.3961 | <0.0001 | <0.0001 | | <0.0001 | <0.0001 | <0.0001 |
| n529 Ec | 0.0436 | 0.0009 | <0.0001 | <0.0001 | 0.0001 | <0.0001 | | <0.0001 | <0.0001 |
| n529 Sc | <0.0001 | 0.1933 | <0.0001 | <0.0001 | 0.5944 | <0.0001 | <0.0001 | | <0.0001 |
| n529 Ca | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | |

*Red numbers indicate that the difference is insignificant