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Increased Susceptibility of C. elegans to Candida albicans Infection after Tissue-specific RNAi Knockdown of an ROS-generating NADPH Oxidase

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Increased Susceptibility of *C. elegans* to *Candida albicans* Infection after Tissue-specific RNAi Knockdown of an ROS-generating NADPH Oxidase

A Major Qualifying Project Report

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Abstract

Tissue-specific RNA interference was performed on the model organism *Caenorhabditis elegans* to cause the knockdown of *bli-3,* which is hypothesized to play an important role in immune defense through the production of reactive oxygen species. Survival assays on *C. elegans* infected by the pathogenic yeast *Candida albicans* revealed highly significant effects on the survival of the worms when the gene was knocked down in the hypodermal and intestinal tissues. There was no significant effect on the survival of worms with *bli-3* knockdown in body wall muscle.

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Introduction

It is well known that fungal infections can be very difficult to treat, as fungal cells are eukaryotic, making them difficult to target without also harming human cells (Jain, *et al.*, 2009). However, effector components of our innate immune response do target these types of infections, such as a family of enzymes important in fighting numerous types of infections, the NADPH oxidases (Nox). Homologues of these enzymes have been found in fungi and plants, as well as animals (Bedard and Krause, 2007). For example, two Nox homologues exist in the fruit fly model organism *Drosophila melanogaster*, dNox and dDuox (Ritsick *et al.*, 2007). The dNox homologue was found to play a role in the regulation of calcium flux in the ovaries, therefore affecting the fly's egg-laying abilities, while dDuox helps to maintain balance with the bacterial fauna of the gut (Ritsick *et al.*, 2007) (Ha *et al.*, 2009). A Nox enzyme also is found in the model organism *Caenorhabditis elegans* (*C. elegans*), a species of roundworm (Edens e*t al.*, 2001). We will describe here the use of this species to study the role of one of these enzymes in innate immune responses *in vivo* through RNA interference (RNAi) experiments.

Members of the Nox family all participate in electron transport in order to produce reactive oxygen species (ROS). The Nox family currently characterized consists of five Nox enzymes and two Duox enzymes (Bedard and Krause, 2007). Although these enzymes differ slightly from each other, they generally consist of a NADPH-binding domain at the carboxyl terminus, a FAD-binding region, six transmembrane domains and four heme-binding histidines (Bedard and Krause, 2007). The Duox enzymes, however, also contain a peroxidase domain and EF hand domains that allow Duox activity to be regulated by calcium ions (Edens, *et al.*, 2001; Leto and Geizst, 2004).

Although many of the Nox enzymes are involved in immune responses, they all have slightly different functions and can be found in different parts of the body. For example, Nox3 and Nox5 do not have a role in immune responses, as Nox3 is essential for gravity perception and Nox5 is found in lymphoid cells and important for calcium-dependent signaling (Leto and Geizst, 2004). Nox1 is thought to play a role in cell division, but is still activated by immune cytokines as are Nox4 and Nox2 (Bedard and Krause, 2007). Nox2, also called $gp91^{phox}$, is often considered the classic example of this family of enzymes, as it is the most familiar.

Nox2 is expressed in phagocytes, where it plays a role in innate immune defense. Along with its gp91 catalytic subunit, it also consists of subunits p22, p47, p67 and Rac, all of which contribute to its functionality (Rada, *et al,* 2008). Prior to stimulation of fMLP or C5a receptors by antigens, gp91 and gp22 subunits are located in granular membranes, while p22, p47 and p67 are in the cytosol of the phagocyte (Murphy, 2012). However, stimulation of the aforementioned receptors leads to Rac activation, causing the subunits in the cytosol to meet with gp91 and gp22, which activates Nox2 (Murphy, 2012). Similarly to all Nox enzymes, Nox2 helps to transfer electrons across cellular membranes from NADPH to oxygen, which serves as the electron acceptor (Rada, *et al,* 2008). During times of infection, activity of Nox2 enzymes results in phagocytic consumption of up to fifty times more oxygen, during what is known as the respiratory burst (Babior, 1984).

The reaction catalyzed by Nox2 and other NOX family members is: $O_2 + NADPH \rightarrow O_2^- + NADP^+ + H^+$ (Babior, 1984). Essentially, a pair of electrons is removed from NADPH to be added to the oxygen, which reduces it, forming a radical known as superoxide (Babior, 1984). Superoxide is somewhat toxic to pathogens, but more importantly, it serves as a precursor to other ROS, such as hypochlorite or hydrogen peroxide (Babior, 1984). Superoxide can accept hydrogen ions in the following reaction: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$, which is catalyzed by the enzyme superoxide dismutase (Babior, 1984). The hydrogen peroxide produced by this reaction is a powerful ROS. The movement of electrons in order to form the superoxide produces an electric potential which is essential to immune defense, as it allows the movement of other ions in reactions of the ROS with the pathogen's DNA, lipids and proteins, which inhibits the pathogen's growth and ultimately leads to its destruction (Rada, *et al,* 2008). ROS are also able to affect the pH of the phagosomes. Although phagosomes generally maintain an acidic environment in order to eliminate pathogens, the ROS in the neutrophils initially causes an increase in pH by decreasing the amount of H^+ ions in the inside of the phagosome (Lee *et al.,* 2003). Not only does the production of H_2O_2 require the consumption of H^+ ions, but the presence of ROS also reduces the amount of H^+ ions entering the phagosome through ATPases, as well as increase the amount of H^+ ions leaving the phagosomes due to overall leakiness (Lee *et al.,* 2003). Subsequent effects on the pH inside of phagosomes are not well understood (Lee *et al.,* 2003).

Duox1 and Duox2, which are most important to this research, can also be essential to an organism's survival against pathogens. The Duox enzymes were originally discovered in the thyroid glands when research was being conducted on thyroid hormone synthesis and were called the dual oxidases because they have both a NADPH oxidase domain, as well as an extracellular peroxidase domain, which is thought to, perhaps, bind hydrogen peroxide (Chavez, *et al.*, 2009) (Edens, *et al.*, 2001). These enzymes also have EF hand domains, which are not found in other Nox enzymes, that allow for activation by directly binding calcium (Leto and Geizst, 2004). In humans, Duox2 was found in the salivary glands, respiratory tract epithelium, and thyroid tissue, while Duox1 was found in many tissues, such as the lung, thyroid, placenta, testis, prostate, pancreas and heart (Lambeth, 2004). Because these enzymes are found in many important epithelial tissues, which are often the first tissues to come into contact with such bacterial or yeast pathogens, it is thought that these enzymes also play a role in immune defense.

Because Duox2 is essential to the organification of iodide, which is needed for thyroid hormones, mutations in Duox2 lead to hypothyroidism (Leto and Geizst, 2004). The Duox enzymes are also found to play an important role in saliva and mucosal secretions in the mouth and airways by creating hydrogen peroxide, which reacts with thiocyanate to form hypothiocyanite, which fights microbial infections (Leto and Geizst, 2004). Figure 1 below contains an image of the role of Duox2 in the thyroid (top) and in the secretions of epithelial tissues (bottom).

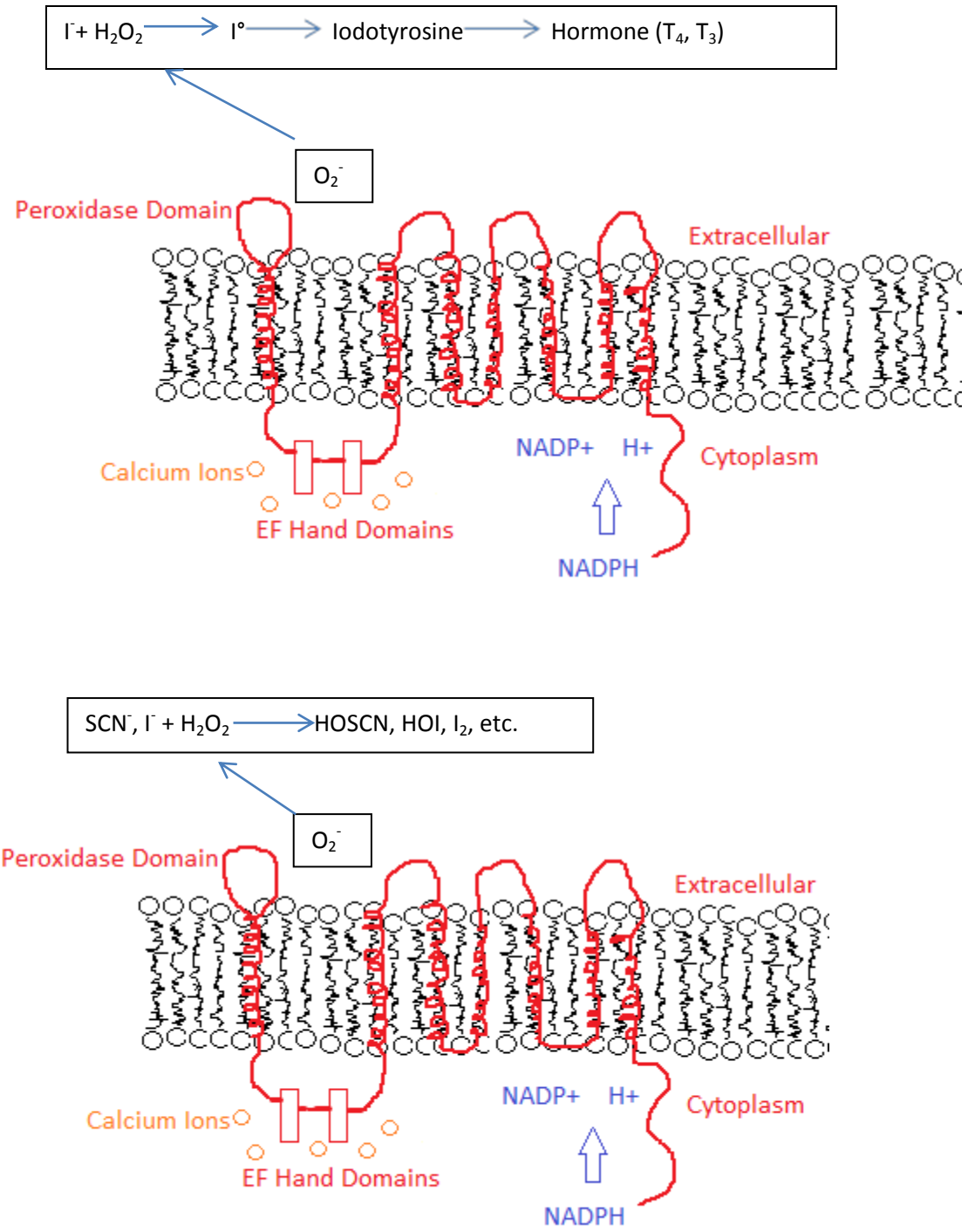

Figure 1: Essential Roles of Duox Enzymes (Adapted from Leto and Geizst, 2004). The top diagram shows the role of Duox (shown in red) in the thyroid where it is responsible for forming hormones, while the bottom diagram demonstrates the role of Duox in epithelial tissues, in which it is responsible for the secretion of microbicidal secretions that fight off pathogens.

As one can see above, the hydrogen peroxide produced from the Duox enzymes not only leads to the production of important thyroid hormones, but it also leads to the formation of important antimicrobial agents, such as hypothiocyanite in the mucus of epithelial tissues (Leto and Geizst, 2004).

In the *Caenorhabditis elegans* model organism used in this research, Duox1 and Duox2 share 30% of amino acid identity with human Duox1 and Duox2 (Lambeth, 2004). The *C. elegans* Duox2 is thought to be unrelated to the ROS immune responses, as no indication of expression has been found in previous studies (Chavez, *et al.*, 2009; Edens, *et al.*, 2001). In *C. elegans*, Duox1 is essential for the formation of the cuticle, as it is needed for the tyrosyl cross-linking within the cuticle collagens (Edens, *et al.*, 2001). However, the role of the peroxidase domain is still unknown, as well as the identity of the molecules targeted for oxidation by the Duox enzymes (Lambeth, 2004).

C. elegans is a model organism that has been useful in genetic and many other types of biological research. It is a non-parasitic roundworm that is easily cultured on *Escherichia coli (OP50)* in laboratory conditions. Because one gender of the worm is a hermaphrodite, it can self-fertilize, yielding genetically identical offspring. However, other genotypes can be introduced into offspring if a male fertilizes a hermaphrodite (Barriere and Felix, 2005). The worms have a very rapid developmental cycle and generation time, progressing from egg to adults, which can produce a number of worms weighing approximately half of the amount of bacteria they were fed (Stiernagle, 2006). Their developmental cycle consists of four larval stages, each of which ends with ecdysis, or molting, preceding an adult reproductive stage (Hubbard, 2005).

Most importantly, however, they are very useful in genetic and many other areas of biological research because adult hermaphrodites consist of less than 1,000 cells, which can be seen under the microscope due to the worms' transparency. This has allowed their developmental processes, such as embryogenesis and morphogenesis, to be understood in great detail. Sequencing of the entire *C. elegans* genome was completed in 1999 (The *C. elegans* Genome Consortium, Wilson, 1999) which indicated indicated that about 40% of *C. elegans* genes are homologous to human genes (Chang and Warb, 2001). In addition, almost every human protein domain is also present in *C. elegans*.

Not only genes, but whole pathways are conserved between mammals and the *C. elegans* model. For example, when a mammal has a pathogenic infection, MAP (mitogen-activated) kinase pathways are activated, which alerts the body that there is an infection and leads to various immune responses (Murphy *et al.*, 2012). Three MAP kinase pathways also exist in *C. elegans* (Kim *et al.*, 2002). One of these pathways, the p38 pathway, which is also present in mammals, is shown in the right side of Figure 2.

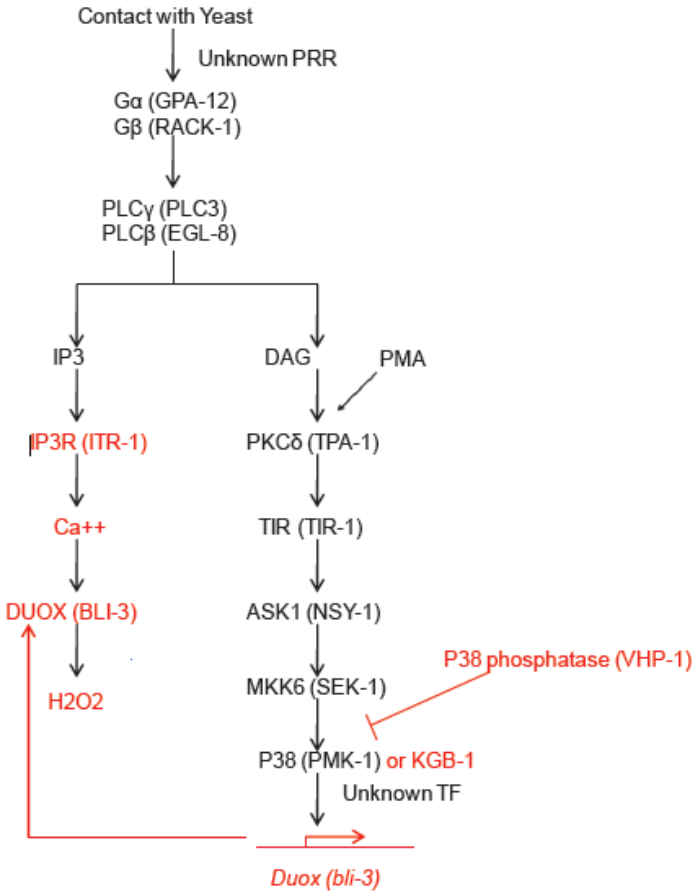

Figure 2: Hypothetical signaling pathways for regulation of Duox (*bli-3*) in *C. elegans*. Pathways known to activate other *C. elegans* immune responses are shown in black. Hypothetical pathways and components are shown in color. *C. elegans* homologues of mammalian components are shown in parentheses.

While the p38 pathway has been identified as being involved in *C. elegans* immune responses to a variety of pathogens (Figure 2, right side pathway), it is not known whether this pathway, or upstream factors which activate it, are involved in regulation of Duox.

A second pathway hypothesized to be involved in Duox regulation is downstream of the intracellular message molecule IP3 (Figure 2, left side pathway). We hypothesize that binding of IP3 to its receptor, a membrane calcium channel, releases calcium ions which activate Duox via its EF hand domains.

The effects of loss of function of Duox (*bli-3)* in *C. elegans* have been studied. The hydrogen peroxide produced by Duox allows tyrosyl radicals to cross-link in the *C. elegans* cuticle (Edens, *et al.*, 2001). When expression of *bli-3* is reduced, abnormal phenotypes, such as

cuticle blisters appear (Edens, *et al.*, 2001). However, studies performed by Chavez *et al*. (2009) and Jain *et al.*(2009) supported the idea that *bli-3* also plays an important role in immune defense. Research by Jain *et al.* showed that *bli*-3 mutants led to reduced ROS production. Additionally, research by Chavez *et al.* suggested that when expression of *bli-3* was reduced, either by mutation or RNAi, so was the production of ROS in response to pathogenic bacteria, which also reduced survival of the worms (Chavez, *et al.*, 2009). We seek to extend these results by studying the effects of *bli-3* loss of function in specific tissues on survival during infection by the pathogenic yeast *Candida albicans*.

In vivo RNAi experiments are essential to studying the role of Nox and Duox enzymes because cells containing these enzymes often cannot be preserved in culture (Leto and Geizst, 2004). RNAi involves introducing dsRNA (double-stranded RNA) into the organism under study that is capable of gene-specific inhibition of translation of the mRNA of interest (Novina and Sharp, 2004).

In *C. elegans*, feeding is often used to introduce dsRNA into the worms, which consists of literally feeding the worms bacteria that have been transformed with a plasmid that synthesizes the desired dsRNA. Figure 3 below contains a diagram of how the dsRNA acts interacts with various proteins in order to knock down the target mRNA.

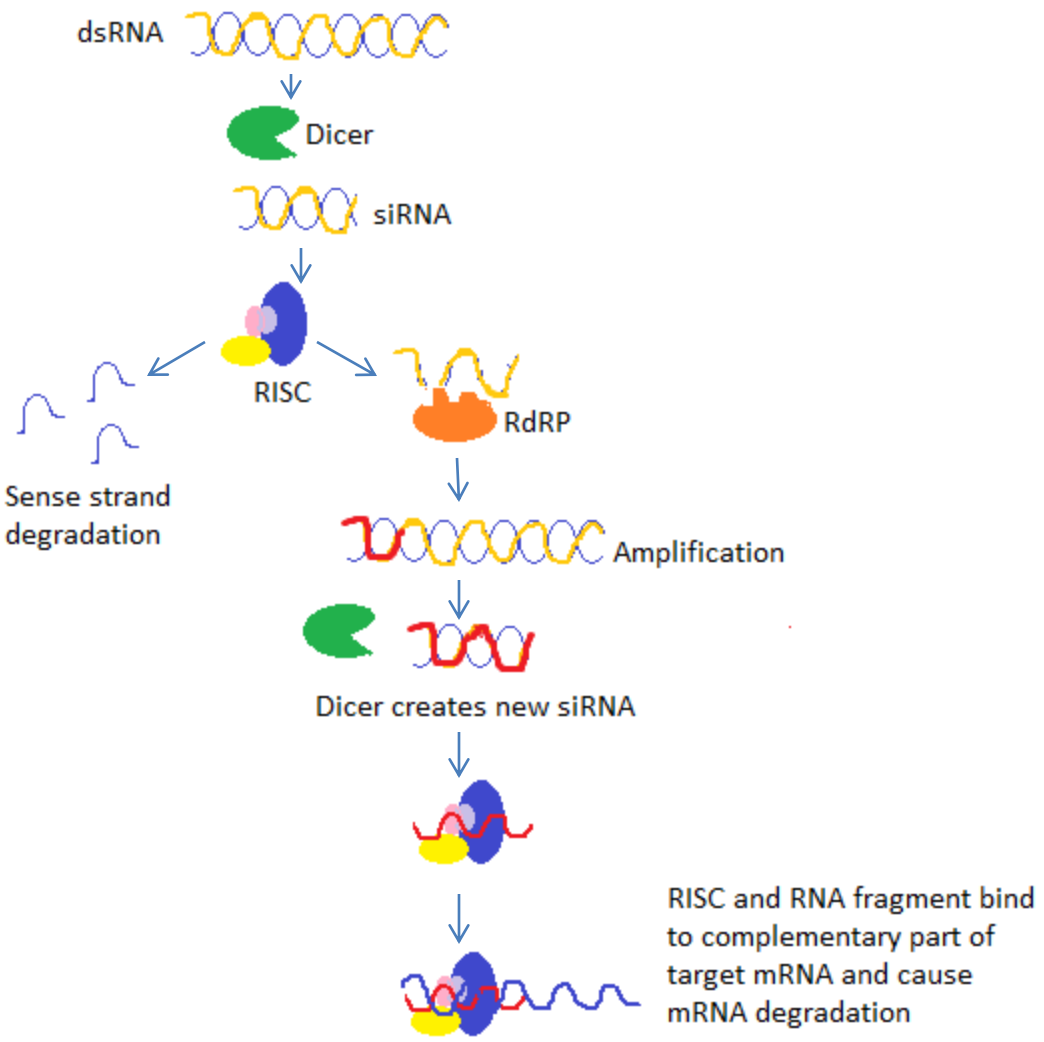

Figure 3: Processes of RNAi (Adapted from Novina and Sharp, 2004). Dicer (shown in green) cleaves the double-stranded RNA (dsRNA) to create short interfering RNA (siRNA) (Novina and Sharp, 2004). The RNA-induced silencing complex (RISC) degrades the sense strand of the siRNA (shown in blue) (Novina and Sharp, 2004). Through an RNA-dependent RNA polymerase (RdRP, which is shown in orange), the antisense strand (pictured in yellow) can be used to base pair with a complementary mRNA and serve as the template for the formation of more full-length RNA in the process of amplification (Novina and Sharp, 2004). The RISC then binds to the antisense strand and helps complementary base pairing to occur between the sense strand fragment and the target mRNA, which causes degradation (Novina and Sharp, 2004).

Once the bacteria containing the dsRNA is fed to the worms, multiple steps occur leading to the silencing of the target mRNA. The first step, which is referred to as the RNAi initiating step, involves the binding of the dsRNA to an RNA nuclease, called Dicer or DCR (Agrawal *et al.*, 2003). Dicer contains metal ion pairs, which interact with the negatively charged phosphates

of the dsRNA in order to orient the dsRNA into the active site of Dicer (MacRae, 2006). Thus, the homologous sequence of the dsRNA can correspond to the catalytic sites, allowing for cleavage to produce short interfering RNA (siRNA) (MacRae, 2006).

In what is sometimes referred to as the effector step, the siRNA then binds to the RNA induced silencing complex (RISC), a complex that includes the Argonaute2 protein. It is predicted that the RISC uses ATP in order to unwind the strands of the siRNA (Agrawal *et al.,* 2003). The RISC binds to the antisense strand, which complementarily binds to the target mRNA, targeting the desired mRNA for endonucleolytic cleavage (Agrawal *et al.,* 2003). It is thought that the Argonaute protein might help direct the siRNA to the RISC during the RNAi process, as interactions between Dicer and Argonaute were found to occur (Hannon, 2002). More importantly, however, Argonaute2 contains endonuclease activity due to its PIWI domain, which has structural similarities to an RNase domain (Song *et al.,* 2004; Diederichs and Haber, 2007). Argonaute2, therefore, can be seen as the catalytic subunit of RISC, as it is responsible for the cleaving activity of RISC and such activity cannot occur without it (Diederichs and Haber, 2007).

As pictured above, the dsRNA can also be amplified (Agrawal *et al.,* 2003). It is thought that enzymes known as RNA-dependent RNA polymerases are important in the amplification process, which are encoded by the *ego1* gene in *C. elegans*. For example, an Rdrp can bind to an antisense strand of siRNA in order to act as a template to synthesize another dsRNA, thus allowing the spread of the mRNA silencing throughout the organism when these newly formed dsRNA are also degraded (Novina and Sharp, 2004).

However, in this research, amplification and spreading were prevented through the use of tissue-specific promoters. Previous research indicated that *rde-1,* which encodes an Argonaute protein, was essential to the process of RNAi catalyzed by dsRNA (Grishok *et al*., 2000). Qadota *et al.* (2007) took advantage of this requirement for *rde*-1 to create a method for achieving tissue-specific RNAi in *C. elegans*. This method was ideal for our purposes.

In our experiments, we used a *C. elegans* strain carrying a mutation in *rde-1* which makes the worms unable to carry out RNAi. By using worm strains carrying an integrated *rde-1* gene under control of promoters that are specifically expressed in the hypodermis, intestine and muscle, wild-type expression of *rde-1* and the production of a wild-type Argonaute protein could be achieved in those respective tissues. Under these conditions, RNAi occurred only in the tissues where *rde*-1 was expressed (Qadota *et al.*, 2007). RNAi could not spread to other tissues, where RNAi remained defective.

For our experiments, we chose to perform tissue-specific RNAi in the epithelial tissues thought to be most important for immune defense, the intestine and the hypodermis. A third tissue, the body wall muscle, was chosen as a negative control (Figure 4).

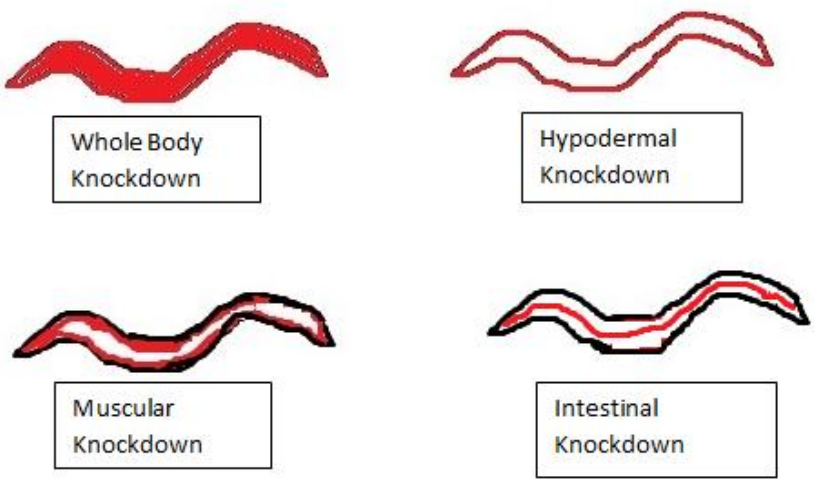

Figure 4: Areas of Tissue-Specific Knockdown of *bli-3*. Areas in red indicate expression of wild-type *rde-1*expressed under a tissue-specific promoter, which are also areas that are susceptible to *bli-3* knockdown due to RNAi.

Experimentally, when the worms are fed a bacterial strain expressing dsRNA specific for the *C. elegans* Duox gene *bli-3*, expression of *bli-3* will be knocked down only in the specific tissues where *rde-1* is expressed. In order to determine which tissues are important for immune defense against pathogenic yeast, RNAi-treated worms are to be incubated with plates containing lawns of the pathogenic yeast *Candida albicans*. Those affected by the hypodermal *bli-3* RNAi should also exhibit cuticle deformities, such as blisters, due to the role of *bli-3* in cuticle cross-

linking. However, it will also be interesting to see the effects of *bli-3* knockdown in the intestine because it is predicted that *bli-3* and Duox could be important in this organ, as it is the area infected by the yeast *Saccharomyces cerevisiae* once it is ingested by the worm.

Materials and Methods

In this project, a *C. elegans rde-1* mutant background was chosen to express a transgenic wild-type *rde-1* under control of a hypodermis-specific promoter (strain NR222), a body-wall muscle-specific promoter (NR350 strain) or an intestine-specific promoter (WP303 strain) (Qadota *et al.*, 2007). Expression under these specific promoters allowed RNAi knockdown of the *bli-3* gene to occur in those specific tissues. Worms were fed *E. coli* transformed with a plasmid containing a *bli-3* insert (F56 C11.1), which allowed the formation of *bli-3* dsRNA and, therefore, RNAi of the desired gene or *E. coli* containing the vector plasmid without the *bli-3* insert (L4440), which should not lead to *bli*-3 RNAi knockdown (Qadota *et al.*, 2007). The *E. coli* containing the L4440 plasmid served as a control in these experiments. Effects of the *bli-3* RNAi on immune defense were then explored by conducting survival assays on *Candida albicans*. The NR222 strain of *C. elegans* was obtained from the Caenorhabditis Genetics Center, NR350 was obtained from H. Qadota, and WP303 was obtained from Kevin Strange. All preparations and methods for these assays are described in this Methodology Section.

Maintenance of Bacteria

The transformed *E. coli* strains used in this project were maintained on plates containing LB media, supplemented with ampicillin and tetracycline. These plates were prepared according to the recipe below.

Recipe for LB, Ampicillin and Tetracycline Media

To prepare these plates LB media was first made by adding 10g peptone, 10gNaCl, 5g yeast extract and 15g agar with 975ml deionized H_2O in a 2 liter flask (Maniatis, 1982). Once thoroughly mixed, this medium was then autoclaved. After the autoclave cycle was complete and the media was allowed to cool, the antibiotics, 0.05g of solid ampicillin and 0.8mL of 12.5mg/ml tetracycline were added to a final concentration of 50ug/ml ampicillin and 10ug/ml tetracycline. Lastly, 30mL of this media was added to each 100mm petri dish utilizing sterilized tubing and a peristaltic pump. These plates were allowed to sit at room temperature overnight before eventually being stored in the freezer.

The transformed *E. coli* strains containing the F56 C11.1 and L4440 plasmids were streaked, utilizing sterile technique, and grown on these plates overnight at 37°C.

Preparation of Feeding Plates

The F56 C11.1 and L4440 transformed strains grown on the LB, ampicillin and tetracycline plates were then used to produce a culture for the feeding plates to which the *C. elegans* strains would be transferred to induce the RNAi effects.

Recipe for Feeding Plates

To prepare these plates, NGM media was first made by mixing 1.5g NaCl, 8.5g agar, 1.25g peptone and 487.5mL of H_2O was in a 1L Erlenmeyer flask. Once thoroughly mixed, this media was then autoclaved. After the autoclave cycle was complete, the media was allowed to cool slightly before the rest of the NGM contents were added, which include: 0.5mL 1M $CaCl_2$, 0.5mL 5mg/mL cholesterol, 0.5mL 1M $MgSO_4$, and 12.5mL 1M KPO_4. After it was allowed to cool, 0.5mL of a 25mg/mL liquid solution of carbenicillin was added, as well as 0.5mL of 1M IPTG. Sterilized tubing and a peristaltic pump were used to add 10mL of this media to each 60mm petri dish. These plates were allowed to sit at room temperature for one week.

Overnight Bacteria Culture

An overnight bacteria culture was made to be used to spot the feeding plates by utilizing a sterile inoculating loop to transfer one colony from either the F56 C11.1 and L4440 strains grown on the LB, ampicillin and tetracycline plates to a sterile tube containing 2mL of LB broth plus 50ug/mL ampicillin. These tubes were then placed in a 37°C incubator and allowed to grow overnight.

This overnight bacteria culture was then used to spot the feeding plates by transferring 150ul of the overnight culture to each feeding plate and allowing dsRNA expression to be induced by IPTG at room temperature overnight. For some survival assays, a 1:10 dilution of the overnight culture into 50ug/ml ampicillin in LB was used to spot the feeding plates.

Maintenance of *Candida albicans*

Wild type *Candida albicans* (strain SC5314) was streaked and grown on YPD agar plates at 37°C for 48 hours. These plates were stored at 2°C and used later as test plates for survival assays. *C. albicans* was obtained from Reeta Prusty Rao.

Recipe for YPD Agar

One liter of YPD agar was prepared by adding 10g of yeast extract, 20g of peptone, 20g of dextrose, and 15g of agar in about a liter of H_2O. Before autoclaving, the pH of the medium

was adjusted to 6.5, and the volume was brought up to 1000mL. Approximately 30mL of this medium was added to 100mm petri dishes.

Overnight Yeast Culture

An overnight culture to be used for spotting for the yeast plates was made by transferring a single SC5314 colony from the YPD plate to a sterile tube with a sterilized inoculating loop containing 2mL of YPD liquid medium. The culture was placed in a shaker at 37°C overnight.

This overnight yeast culture was then mixed with YPD liquid medium and 50ug/ml streptomycin in a mixture of 1 part overnight culture, 1 part YPD liquid and 2 parts streptomycin solution. In assays that were performed on a diluted SC5314 culture, the overnight yeast culture was first diluted in a 1:30 ratio with YPD liquid broth before being mixed with the YPD liquid and streptomycin in the 1: 1: 2 ratio. Next, 20ul of the mixture was added to 60mm plates containing NGM. The spotted plates were then incubated overnight at 37°C.

Survival Assays

Each *C. elegans* strain was first maintained on NGM plates containing OP50 *E. coli*. About 5-10 L4 worms were transferred to feeding plates. Worms were kept on the feeding plates until enough progeny were produced to begin the survival assay or a minimum of three days. If necessary, progeny were transferred to new feeding plates to avoid starving before the survival assays were begun.

Four *C. elegans* strains, N2 (wild type), WP303 (*rde-1* under intestinal promoter), NR350 (*rde-1* under muscular promoter) and NR222 (*rde-1* under hypodermal promoter) were observed on *C. albicans* plates after feeding on F56 C11.1 or L4440-expressing *E. coli*. For each condition, there were three yeast plates containing 30 worms each. The number of worms on each plate was counted as close to every 12 hours as possible, at which time the number of live worms, dead worms, total worms on the plate and censored worms were recorded. Censored worms were the number of worms that were not counted dead or alive since the previous counting, as they were not present on the plate. The cause of death for these worms was undetermined and they were, therefore, not taken into account for data analysis. Dead worms were removed from the plate and flamed. All data were transferred into *Graphpad Prism* Software plotting and further analysis using the log-rank test of significance.

Results

To test the hypothesis that the CeDuox protein is required for defense against the pathogenic yeast *C. albicans*, worms were grown under conditions that induced RNAi knockdown of *bli-3*, then transferred to plates containing *C. albicans*. Results were measured by monitoring survival of worms over time.

In early versions of the survival assays, overnight bacterial cultures were plated undiluted on the "feeding plates" to induce the RNAi effect. In addition, the *C. albicans* culture that was plated for the survival assays was not diluted. Figure 1 shows the survival curves from these assays. No significant effects of tissue-specific RNAi were observed for the survival assays shown below performed under undiluted conditions. All worms died rapidly under these conditions, with 100% of the worms in the hypodermal knockdown dead after less than 100 hours.

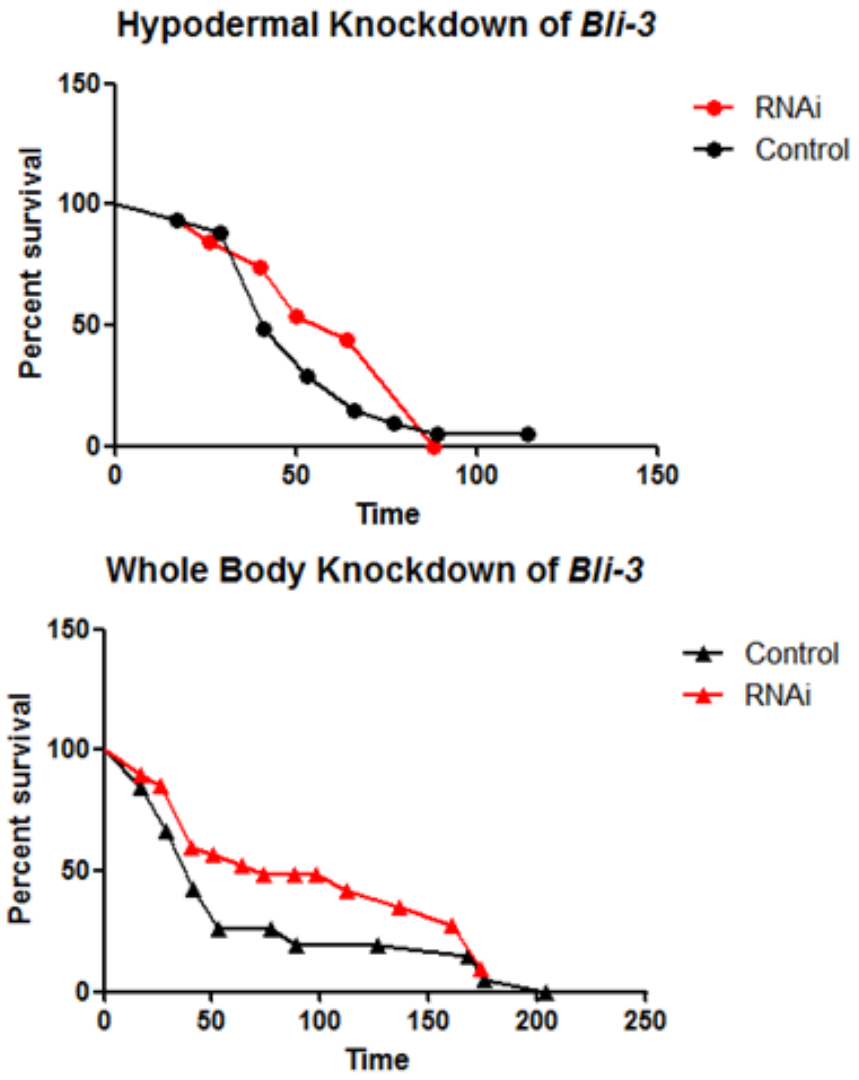

Figure 1: Survival Curves of Hypodermal and Whole-Body Knockdown of *bli-3* without Dilutions. These survival assays were performed without any dilutions to bacterial culture for the feeding plates or the yeast culture used for the survival assays. All other procedures were performed as described in the Materials and Methods Section. Survival curves for hypodermal knockdown of *bli-3* are shown (top), as well as survival curves for whole-body knockdown of *bli-3* (bottom). The log-rank p-values obtained were 0.3390 and 0.1479 for the hypodermal and whole-body knockdowns, respectively, supporting that significant RNAi effects were not observed.

Survival assays were also conducted in which a 1:10 dilution of the F56 C11.1 and control L4440 bacterial cultures was performed before they were plated on feeding plates. In addition, a 1:30 dilution of the yeast culture was performed before it was plated on the survival assay plates. These adjustments were made in hopes that RNAi effects would be more easily

observed without the severe pathogenicity of the undiluted *C. albicans*. The survival curves of each strain are displayed in Figures 3-6 below.

Results indicated that *bli-3* knockdown in the whole body significantly affected survival on *C. albicans*, suggesting that the function of Duox was important to host immune defense (Fig. 2, 0.08 level of significance). After feeding with the *E. coli* that induced the RNAi effects, blistering on many of the worms was also observed, indicating that RNAi was effective, as knockdown of *bli-3* disrupts cuticle formation.

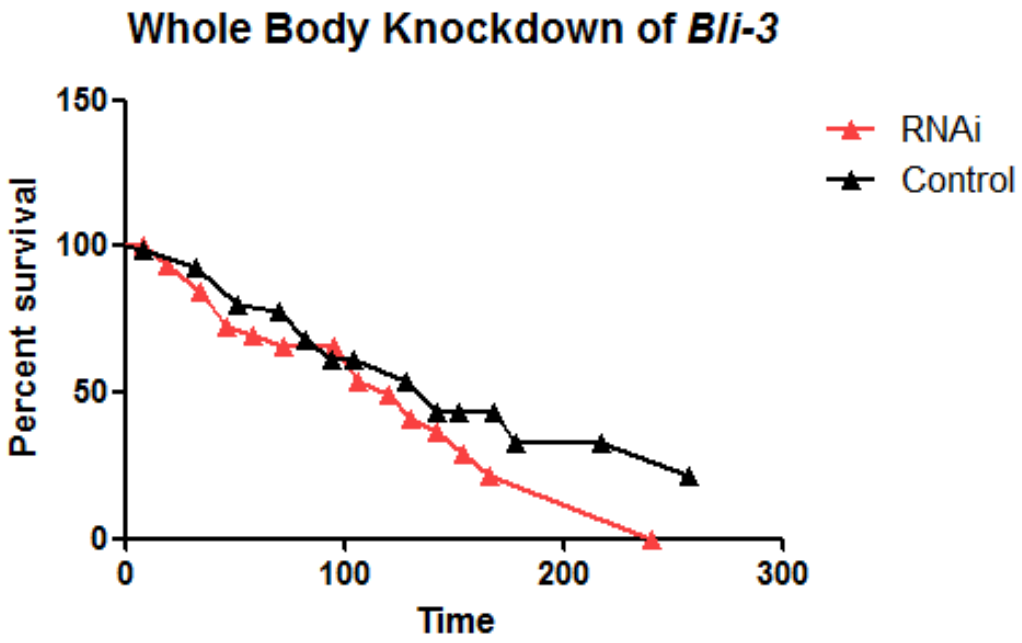

Figure 2: Survival Curves of N2 Worms After Feeding with OP50 transformed with L4440 and OP50 transformed with F56 C11.1. A 1:10 dilution was performed on both the L4440 and F56 C 11.1 before the cultures were plated on feeding plates. The yeast culture was diluted in a 1:30 solution with YPD liquid broth. All other procedures were performed as stated in the Materials and Methods Section. Feeding with OP50 transformed with the F56 C11.1 plasmid (shown in red) significantly affected survival of wild-type worms compared to those fed with OP50 transformed with the L4440 plasmid (shown in black) (p-value=0.0791). *Bli-3* knockdown in the entire body had a significant effect on the worms' survival on the pathogenic *C. albicans.*

Bli-3 knockdown in the hypodermis resulted in highly significant reduction in survival of the worms on *C. albicans*, indicating that Duox function in the hypodermis was important for survival (Fig. 3). Blistering was also observed in these worms due to *bli-3* knockdown.

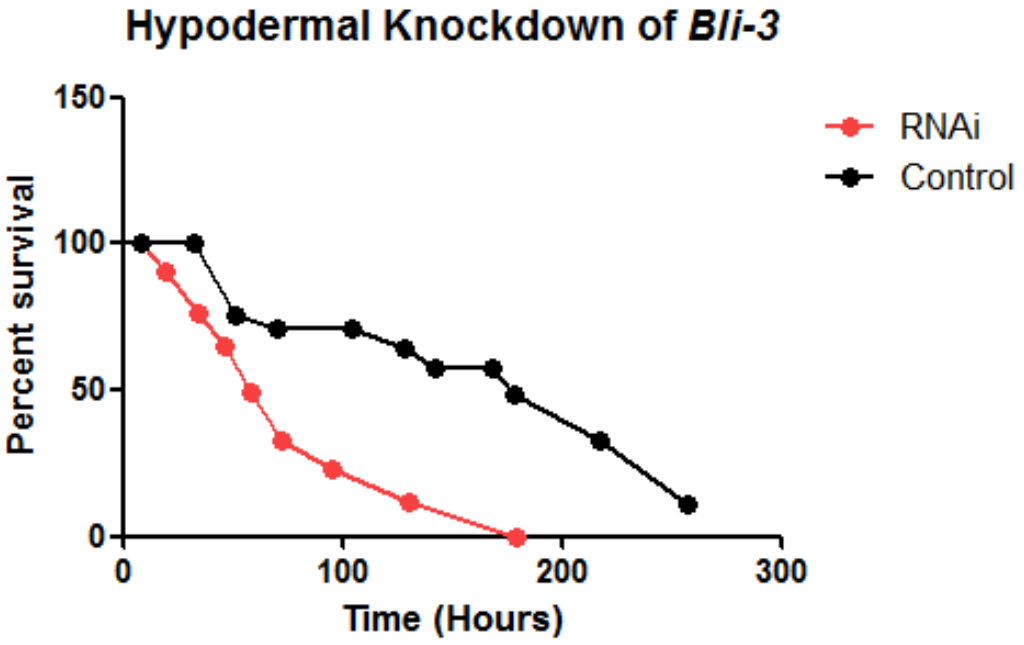

Figure 3: Survival Curves of NR222 Worms After Feeding with OP50 Transformed with L4440 and OP50 Transformed with F56 C11.1. The survival assay was performed as described in the Materials and Methods Section. The survival curves generated were significantly different (p-value<0.0001), indicating that hypodermal *bli-3* knockdown had a significantly negative effect on survival of the worms on *C. albicans.*

Similarly, *bli-3* knockdown in the intestine resulted in significant reduction in survival of worms on yeast (Fig. 4).

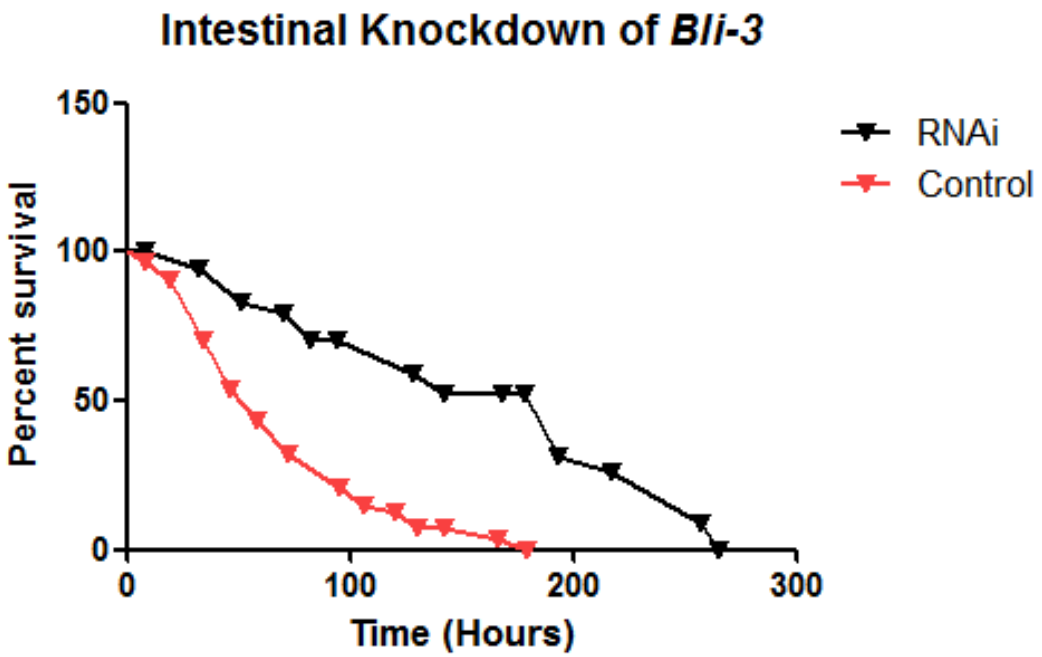

Figure 4: Survival Curves of WP303 Worms After Feeding with OP50 Transformed with L4440 and OP50 Transformed with F56 C11.1. Feeding on F56 C11.1 *E. coli* greatly reduced the ability of the worms to survive exposure to *C. albicans* (p-value<0.0001). Intestinal *bli-3* knockdown and loss of Duox function caused an increase susceptibility to *C. albicans.*

In contrast to the results of hypodermal and intestinal knockdown of *bli-3*, RNAi knockdown of *bli-3* in muscle did not significantly affect survival of the worms on *C. albicans* (Fig. 5).

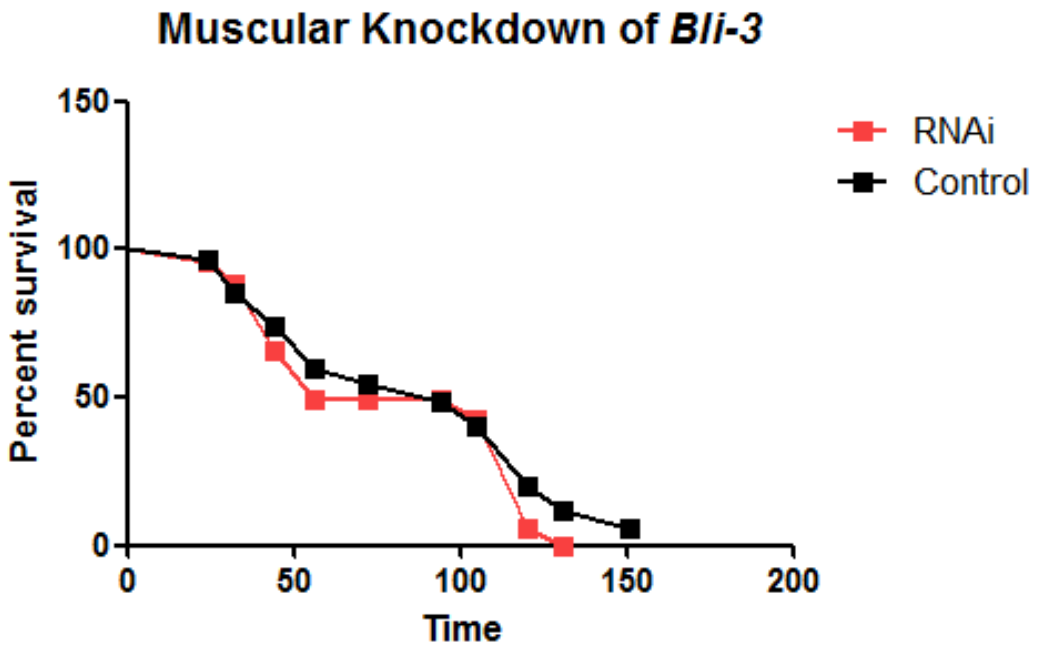

Figure 5: Survival Curves of NR350 Worms After Feeding with OP50 Transformed with L4440 and OP50 Transformed with F56 C11.1. As can be observed by the survival curve above, after feeding with F56 C11.1 transformed OP50 (shown in red) and the survival curve after feeding with L4440 transformed OP50 (shown in black), feeding on F56 C11.1 transformed bacteria did not significantly affect survival of the worms on yeast (p-value=0.5038). *Bli-3* function in the muscle was not shown to be significant to survival of *C. elegans* on *C. albicans.*

The survival assays performed allowed the effects of *bli-3* knockdown in specific tissues to be observed. Results indicated that effects of *bli-3* knockdown on survival of *Candida* exposure was significant in the hypodermal and intestinal tissues, as well as in the whole body of the worm, while it was not significant in the muscular tissue of the worm. Further implications of these results will be explored in the following Discussion section.

Discussion

The results obtained from the RNAi and survival assays performed support the hypothesis that the Duox activity of *bli-3* is important to host immune defense, as *bli-3* knockdown in the whole body of the worm significantly decreased survival on the pathogenic yeast, *C. albicans*. The finding that *bli-3* knockdown in the hypodermal and intestinal tissues significantly reduced survival, but did not reduce survival in the muscle, further support the hypothesis that *bli-3* and the function of Duox is important to host immunity, as epithelial tissues, such as the hypodermis and intestine are often the first to come into contact with pathogens. The intestine is also known to be the main site of infection during infection with the pathogenic yeast *Saccharomyces cerevisiae* (Jain *et al.*, 2009).

The results reported here are consistent with those described for the *C. elegans* response to the bacterial pathogen *Enterococcus faecalis.* In that study, RNAi knockdown of *bli-3* in the hypodermis and intestine reduced survival of worms infected with *E. faecalis* (Chavez *et al.*, 2009).

However, further research in order to support these findings is suggested by the importance of *bli-3* in cross-linking during cuticle formation (Edens *et al.*, 2001). In these assays, knockdown of *bli-3* not only affected Duox production, but worms with *bli-3* knockdown in the whole body and in the hypodermis also had blistered cuticles. We also observed blistered cuticles in worms subject to tissue-specific knockdown of *bli*-3 in the hypodermis. Thus, the increased susceptibility to *C. albicans* could have been due to effects on cuticle structure, rather than being directly caused by a requirement for Duox in host defense. In order to further investigate this issue, one could perform RNAi and survival assays as previously described with other blister genes, such as *bli-1* or *bli-2*, which are known to be involved in cuticle formation and cause blistering, but are not known to be involved with immune defenses (Wormbook, 2007). Comparison of *bli-1* or *bli-2* knockdown with *bli-3* knockdown would allow one to observe the importance of cuticle formation for survival in contrast to decreased production of reactive oxygen species.

In addition, the highly significant effects of intestinal knockdown on survival suggest that Duox is present in the intestinal tissues, although this has not been determined directly. Edens *et al* (2001) did not detect *bli-3* expression in the intestine, using a Bli-3-specific antibody. However, expression in the intestine may be induced by infection, as we hypothesized in the Introduction (Fig. 2). Further investigations, such the use of a reporter gene, would help to determine whether or not Duox is actually expressed in the intestine. For example, green fluorescence protein (GFP) could be inserted into the *C. elegans* genome at the end of the *bli-3* gene or downstream of the *bli-3* promoter, which would then fluoresce in tissues which express the *bli*-3/GFP fusion.

In conclusion, the assays performed allowed for direct comparisons to be made between worms with full *bli-3* function and without *bli-3* function in the respective tissues. It was found that the knockdown of *bli-3* and, therefore, the knockdown of reactive oxygen species production had an increased negative effect on host survivability in the epithelial tissues of the hypodermis and intestine compared with knockdown in the body wall muscle. These findings suggest that CeDuox does play a role in defense against the pathogenic yeast *C. albicans.* If explored further, the role of CeDuox could perhaps provide some insight to the role of Duox in our bodies and its possible importance in fighting off harmful and resistant fungal infections.

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