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Potential Roles of Peroxidases in Caenorhabditis Elegans Innate Immunity

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A

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by

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Potential roles of peroxidases in *Caenorhabditis elegans* innate immunity

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The production of ROS (reactive oxygen species) in response to pathogen detection is a rapid, nonspecific response that is evolutionarily conserved from nematodes to humans. ROS serve as direct and indirect effectors of innate and adaptive immunity. In *Caenorhabditis elegans*, a ROS burst is observed during infection and is mediated by the dual oxidase BLI-3, which produces H_2O_2. RNAi (RNA interference) to reduce the amount of BLI-3 results in a significant increase in susceptibility to pathogens, suggesting BLI-3 has a role in the immune response. However, H_2O_2 by itself is not a potent antimicrobial and in other systems is converted to a more potent oxidant by an affiliated peroxidase. During my work, I have characterized a group of previously unstudied peroxidases in *C. elegans* and determined their involvement in the host immune response to *Enterococcus faecalis*. In particular, I focused on SKPO-1 (SkT-containing peroxidase) and how it contributes to the host immune response with respect to BLI-3.

By RNAi and *skpo-1* mutant analysis, I determined that SKPO-1 is involved in the host immune response during *E. faecalis* infection. By tissue-specific RNAi, I determined that SKPO-1 is functionally active in the hypodermis and required for wild type resistance to infection. Additionally, by immunohistochemistry, I observed that SKPO-1 is only expressed in the hypodermis and that its protein levels do not change in response to *E. faecalis*. In support of SKPO-1 acting as a peroxidase, I observed a significant increase in H_2O_2 levels when expression of the gene was reduced by RNAi. The increased H_2O_2 was observed only during infection and was BLI-3-dependent. Thus, I have characterized a likely BLI-

3/SKPO-1 system, potentially similar to the oxidative burst systems present in higher eukaryotes.

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Chapter 1: Introduction, Background and Significance

Introduction and Background

Caenorhabditis elegans as a model for infectious diseases

In 1999, the Ausubel laboratory developed *Caenorhabditis elegans* as a model for medically relevant infectious agents in mammals. Using *Pseudomonas aeruginosa*, they demonstrated that the pathogen utilized some of the same virulence factors to cause infection in simple and complex metazoan hosts, *i.e.* nematodes and mice (TAN *et al.* 1999a; TAN *et al.* 1999b). Since then, *C. elegans* as an infectious disease model has been used to investigate both sides of host-pathogen interactions by elucidating how bacterial and fungal virulence factors cause infection in the host. Some of the pathogen models studied in *C. elegans* are *Staphylococcus aureus*, *Enterococcus faecalis*, *Yersinia pestis*, *Salmonella enterica*, *Drechmeria coniospora* and *Cryptococcus neoformans* (GARSIN *et al.* 2001; MYLONAKIS *et al.* 2002; PUJOL *et al.* 2008; STYER *et al.* 2005; TENOR *et al.* 2004). How the host defends against different pathogen insults is very important. By using *C. elegans* it is possible to genetically dissect host defense mechanisms that may be extremely difficult or even impossible to address in mammals due to increased complexity in their innate immune responses.

In *C. elegans*, the β-catenin human ortholog, *bar-1*, and a downstream homeobox transcription factor (HOX) known as EGL-5 (egg-laying defective) contribute to the host's resistance to *S. aureus* in the intestinal epithelia (IRAZOQUI *et al.* 2008). BAR-1 and EGL-5-like proteins are evolutionarily conserved in humans and play an important role in intestinal immunity (IRAZOQUI *et al.* 2008). In humans, α-defensins are modulated through the β-catenin pathway and these antimicrobial peptides are important in intestinal epithelia defense (ANDREU *et al.* 2005), (VAN ES *et al.* 2005). Like in *C. elegans,* the human HOX genes *cdx1/2* and *hoxa9* are transcriptional targets of the β-catenin pathway and these homeobox transcription factors regulate expression of antimicrobial factors in the human

intestinal epithelia thus contributing to the innate immune response (ANDREU *et al.* 2005), (VAN ES *et al.* 2005), (IRAZOQUI *et al.* 2008). Thus, the β-catenin pathway and HOX transcription factors maintain their role in modulating host intestinal defense from *C. elegans* to humans.

Another example of where humans increased our understanding of human innate immunity came from the discovery of the Toll signaling pathway's involvement in *Drosophila melanogaster* innate immunity (LEMAITRE *et al.* 1996). These TLR (Toll-like receptor)-mediated innate immune responses have been observed in vertebrates, including humans, which demonstrates TLRs are ancient, and evolutionarily conserved (COUILLAULT *et al.* 2004), (PUJOL *et al.* 2001), (TENOR *et al.* 2004); reviewed by (IRAZOQUI *et al.* 2010b). Like vertebrates, *C. elegans* has a Toll-pathway consisting of TOL-1 (Toll-like receptor), PIK-1 (Pell/IRAK kinase), TRF-1 (TNFR-associated factor), and IκB (inhibitor of NF-κB) (PUJOL *et al.* 2001). However, *C. elegans* lacks a NF-κB transcription factor homolog that canonical TLR pathways activate to mediate an innate immune response (PUJOL *et al.* 2001). In contrast to the TLR pathways present in vertebrates, the *C. elegans* Toll pathway is crucial for nematode development, but has a very minor role in mediating host resistance to pathogens, as *tol-1* mutants are only hypersusceptible to *Salmonella enterica* (TENOR *et al.* 2004), (TENOR and ABALLAY 2008). Thus, the importance of TLRs recognizing pathogen associated molecular patterns (PAMPs) resulting in host immune response modulation appears to have evolved after the last common ancestor shared by *C. elegans* and humans.

With respect to mammalian immunity, it is currently unknown if insulin-like growth factor (IGF) signaling directly affects immunity. However, the IGF/abnormal Dauer formation (IGF/DAF-2) pathway has been well-studied in *C. elegans* due to its involvement in innate immunity, various environmental stresses, reproduction, and metabolism (EVANS *et al.*

2008), (WOLKOW *et al.* 2000). DAF-2 negatively regulates DAF-16 through phosphorylation; however, in a *daf-2* mutant, where DAF-16 is constitutively active, the animals were significantly more resistant to Gram-positive pathogens like *E. faecalis* (GARSIN *et al.* 2003). Therefore, studying IGF/DAF-2 signaling in *C. elegans* may prove useful for elucidating the role of IGF signaling in vertebrate innate immunity.

Another important immune response signaling cascade in vertebrates is the p38 MAPK pathway (KIM *et al.* 2002). Due, in part, to pathogen studies conducted in *C. elegans*, the role of p38 MAPK signaling in mediating innate immune responses has been shown to be evolutionarily conserved from *C. elegans* to humans (KIM *et al.* 2002). In *C. elegans*, the conserved p38 MAPK cascade is composed of NSY-1, SEK-1 and PMK-1, which are orthologs of human ASK1 (apoptosis signal-regulating kinase 1; MAP kinase kinase kinase), MKK3 and MKK6 (MAP kinase kinase 3/6) and p38 (MAP kinase), respectively (IRAZOQUI *et al.* 2010b). The importance of the NSY-1-SEK-1-PMK-1 cassette cannot be understated as it is involved in infections that occur in the hypodermis (*D. coniospora*) and the intestine (*P. aeruginosa*, *S. aureus*, and *E. faecalis*) in *C. elegans* ((KIM *et al.* 2002; PUJOL *et al.* 2008; SHIVERS *et al.* 2009; SIFRI *et al.* 2003), unpublished). In mammals, LPS (lipopolysaccharide)-induced reactive oxygen species (ROS) production results in activation of the p38 MAPK signaling cascade (MATSUZAWA *et al.* 2005). Ultimately, host cytokines are produced that aid in combating sepsis (MATSUZAWA *et al.* 2005). A similar system is observed in *C. elegans* where an unknown signal activates BLI-3 to produce ROS. The ROS then stimulate the NSY-1-SEK-1-PMK-1 pathway to regulate a protective response (VAN DER HOEVEN *et al.* 2011), (SHIVERS *et al.* 2010). Thus, two ancient, evolutionarily conserved, and connected systems (ROS and the p38 MAPK cascade) are used by the host to affect an innate immune response in an evolutionarily

conserved manner, again implicating the usefulness of studying *C. elegans* with respect to mammalian innate immunity.

Reactive oxygen species (ROS) in innate immune responses

ROS, such as H_2O_2, are not limited to being cytotoxic effectors during a host response to injury and/or infection. ROS can serve as secondary messengers to mediate chemotaxis of leukocytes or serve as a substrate in the cross-linking of plant cell walls in response to pathogen (DESIKAN *et al.* 1998), (NIETHAMMER *et al.* 2009). Therefore, I will discuss how ROS serve as both indirect and direct effectors of the host immune response during infection.

It was demonstrated by Niethammer *et al.* that wounding the Zebrafish's, *Danio rerio*, tail fin resulted in the rapid recruitment of leukocytes to the site of injury (NIETHAMMER *et al.* 2009). During the wounding response, they observed a rapid and significant increase in H_2O_2 levels at the site of injury that coincided with leukocyte extravasation (NIETHAMMER *et al.* 2009). The monitoring of global H_2O_2 levels was accomplished by introducing HyPer mRNA, by injection, into Zebrafish embryos (NIETHAMMER *et al.* 2009). The HyPer system relies on the bacterial OxyR::YFP transcription factor fusion that allows for determination of H_2O_2 levels, as it is highly specific to H_2O_2 (NIETHAMMER *et al.* 2009). Through the use of morpholinos (antisense oligomers used for RNAi; RNA interference) against Duox and an NADPH oxidase chemical inhibitor (DPI; diphenyleneiodinium chloride) they determined that the dual oxidase, Duox, was responsible for the H_2O_2 gradient (NIETHAMMER *et al.* 2009). Additionally, through RNAi and chemical inhibition of H_2O_2 by antioxidants, they determined that leukocyte recruitment to the wounded tail fin was dependent on the Duox-produced H_2O_2 gradient (NIETHAMMER *et al.* 2009). Thus, they demonstrated for the first time that Duox-produced ROS mediated the efficient recruitment of leukocytes to an injury site in a paracrine-dependent manner (NIETHAMMER *et al.* 2009).

While ROS are important as signaling molecules in the immune response of many eukaryotic species, scientists rarely consider their importance in plants. Three plant species *Arabidopsis thaliana*, *Glycine max* (soybean), and *Nicotiana tabacum* have been paramount in the understanding of how ROS, such as H_2O_2, contribute to plant innate immunity, especially with respect to the hypersensitive response (HR)—a form of programmed cell death (PCD) (DESIKAN *et al.* 1996), (DESIKAN *et al.* 2000), (LEVINE *et al.* 1994), (LEVINE *et al.* 1996), (TENHAKEN *et al.* 1995). HR is a very specialized feature of non-host resistance in which an entire plant cultivar displays resistance to infection by a microbial pathovar (DESIKAN *et al.* 1996), (LEVINE *et al.* 1994), (TENHAKEN *et al.* 1995). The HR response is initiated by a plant disease resistance (R) protein specifically recognizing its cognate Avr (avirulence) effector molecule produced by a pathogen (ROJAS and MYSORE 2012), (ROJAS *et al.* 2012). Upon recognition of the Avr, an oxidative burst is triggered. Two major producers of the H_2O_2 in response to infection are respiratory burst oxidase homologs (RBOHs *i.e.* NADPH oxidases; NOXs) and glycolate oxidases (GOXs). Similar to NOX in animals, RBOH enzymes produce superoxide (O_2^-) that dismutates into H_2O_2—this reaction is catalyzed by a superoxide dismutase (SOD). However, GOX produces H_2O_2 directly as a byproduct of the glyoxylate reaction in which glycolate and O_2^- are the substrates (ROJAS and MYSORE 2012), (ROJAS *et al.* 2012). In response to nonhost pathogen elicitors, it was determined that the soybean reinforces its cell wall as treatment with a pectinase and cellulose mixture, used by microbes to gain entry into plant cells, displayed a 50% reduction in their ability to generate protoplasts with respect to uninfected soybean suspensions (BRADLEY *et al.* 1992), (BRISSON *et al.* 1994). The elicitor-induced increase in cell wall strength was determined to be the result of oxidative cross-linking of p100 and p33, a (hydroxyl)proline-rich protein (HRGP) and proline-rich protein (PRP), respectively (BRADLEY *et al.* 1992), (BRISSON *et al.* 1994), (TENHAKEN *et al.* 1995). Excitingly, it was determined that H_2O_2 was required for this (elicitor-induced) oxidative cross-linking, as the presence of

the antioxidants ascorbate or catalase precluded oxidative cross-linking, regardless of the elicitor being present or absent (BRADLEY *et al.* 1992), (BRISSON *et al.* 1994), (TENHAKEN *et al.* 1995). Additionally, production of tyrosine-rich proteins such as HGRPs and PRPs is upregulated during this rapid response, which is interesting as tyrosine residues serve to cross-link structural proteins such as collagens in the *C. elegans* cuticle (THEIN *et al.* 2009). It is thought that this rapid host response is meant to prevent further invasion and trap these pathogens within cells destined for HR, allowing time for the production of antimicrobials such as phytoalexin (BRADLEY *et al.* 1992), (BRISSON *et al.* 1994).

Once the H_2O_2 concentration threshold (6 mM) has been surpassed, HR-mediated PCD occurs only in the infected cells in a calcium-mediated fashion similar to apoptosis in mammalian cells (LEVINE *et al.* 1996). At a concentration of 2 mM, H_2O_2 induces transcription of glutathione S-transferase (*gst*) as well as glutathione peroxidase (*gpx*), both of which are involved in protecting the cell from oxidative stress (LEVINE *et al.* 1994), (LEVINE *et al.* 1996), (TENHAKEN *et al.* 1995). While the infected cells are destined for PCD this is not necessarily the case for the surrounding adjacent, uninfected, cells. Because H_2O_2 is a membrane permeable molecule it diffuses out of the infected cells and conditions the surrounding cells through upregulating expression of oxidative stress genes such as *gst* and *gpx* (LEVINE *et al.* 1994), (LEVINE *et al.* 1996), (TENHAKEN *et al.* 1995). It is speculated that the adjacent cells do not undergo HR because the upregulation of the oxidative stress response genes prevent toxic levels of H_2O_2 accumulation, thus ameliorating the spread of PCD (LEVINE *et al.* 1994), (LEVINE *et al.* 1996), (TENHAKEN *et al.* 1995).

Another interesting example of infection-induced ROS signaling that contributes to the host's immune response involves *Erwinia carotovora Ecc15* and *D. melanogaster* larvae (WU *et al.* 2012). Wu *et al*. elegantly demonstrated that ingestion of *Ecc15* by *D. melanogaster* larvae resulted in immunological communication between the intestine and

fat body organs (WU *et al.* 2012). The fat body is an important organ in the innate immune response of *Drosophila* larvae, akin to the liver (WU *et al.* 2012). While infection resulted in H_2O_2 production at the site of infection *i.e.* the gut, H_2O_2 was not involved in modulating expression of the antimicrobial peptides (AMPs) drosomycin (*Drs*) and diptericin (*Dpt*) in the gut, however, it did modulate expression of *Drs* and *Dpt* in the fat body (WU *et al.* 2012). In another study, in response to intestinal infection by *Ecc15*, nitric oxide synthase (NOS) expression was markedly induced causing a concomitant increase in NO (FOLEY and O'FARRELL 2003). Through chemical and genetic methods it was determined that NO acts as a signaling molecule during the larvae's immune response to activate hemocytes (FOLEY and O'FARRELL 2003). These hemocytes then act on the fat body ultimately resulting in the production of Dpt, which contributes to the host's immune response (FOLEY and O'FARRELL 2003). Additionally, it was demonstrated that intestinal H_2O_2-induced expression of *Dpt* was modulated in a NO-dependent manner; however, *Drs* expression was not significantly affected by NO (WU *et al.* 2012). Thus intestinal H_2O_2 modulated the expression of NO to affect the expression of *Dpt* in the fat body, while the same intestinal H_2O_2 was necessary and sufficient for inducing expression of *Drs* in the fat body (WU *et al.* 2012).

One final example of how ROS acts as a secondary messenger in response to infection involves NOD2 and NF-κB in the human intestine (LIPINSKI *et al.* 2009). NOD2 belongs to the NOD-like receptor (NLR) family and is well-documented in modulating innate immune signaling in response to muramyl dipeptide (MDP), a constituent of peptidoglycan, by ultimately promoting nuclear localization of NF-κB ((LIPINSKI *et al.* 2009); reviewed by (CORREA *et al.* 2012)). In this study, Lipinski *et al*. determined that activation of NOD2 by MDP or TNFα resulted in the upregulation in transcription and translation of DUOX2 in Caco-2 cells (human colonic epithelial adenocarcinoma cell line) and ultimately increased production of ROS (LIPINSKI *et al.* 2009). Like primary human intestinal epithelial cells,

Caco-2 cells produce DUOX2 (EL HASSANI *et al.* 2005b). It was demonstrated in both Caco-2 and HEK293 (human embryonic kidney) cells that cellular invasion by *Listeria monocytogenes* was significantly reduced when NOD2 or DUOX2 were present. However, the presence of both NOD2 and DUOX2 produced a greater reduction in cellular invasion suggesting a synergistic effect with respect to the host immune response (LIPINSKI *et al.* 2009). Furthermore, infected HEK293 cells expressing NOD2 that were transfected with siRNA (small inhibitory RNA) against *duox2* displayed decreased ROS levels (LIPINSKI *et al.* 2009). Knock down of *duox2* in NOD2-expressing Caco-2 cells displayed increased cellular invasion thus further supporting the NOD2-dependent increase in ROS production by DUOX2 (LIPINSKI *et al.* 2009). Because NF-κB can be turned on by NOD2-mediated signaling or through H_2O_2, Lipinski *et al*. decided to determine if DUOX2 was involved in NOD2-mediated activation of NF-κB (LIPINSKI *et al.* 2009). Through the NF-κB luciferase reporter assay they demonstrated that DUOX2-generated H_2O_2, which was enhanced by MDP, further stimulated NOD2-mediated NF-κB activation (LIPINSKI *et al.* 2009).

NADPH Oxidase (NOX)/ Dual Oxidase (DUOX) family

I have discussed how ROS, particularly H_2O_2, are important as secondary messengers in innate immune signaling by the host. However, I have yet to delve into how ROS can act as direct immune effectors against pathogens. While H_2O_2 is an oxidant, it is not as effective at killing pathogens as other ROS *e.g.* HOCl, HOI, HOBr, or HOSCN (hypochlorous, hypoiodous, hypobromous, and hypothiocyanous acid, respectively). In this part of the introduction, I will discuss how ROS are potent microbicidal effectors produced by the host in response to infection. However, I must also include how these ROS are generated which requires elaboration on NADPH Oxidases (NOX) and Dual Oxidases (DUOX) as well as the peroxidases that use the H_2O_2 to catalyze the formation of these microbicidal ROS.

gp91^{phox}, now known as NOX2, is the archetypal member of the NOX/DUOX family, as it was the first discovered protein of the NOX/DUOX family. More importantly, the gp91^{phox} subunit of the heterodimeric flavocytochrome b_{558} complex, the catalytic center, is highly conserved in the rest of the NOX/DUOX members (reviewed by (NAUSEEF 2014)). Other features of NOX2 that are conserved in the NOX/DUOX family include two membrane-embedded prosthetic heme groups, as well as the FAD and NADPH binding domains (reviewed by (RADA and LETO 2008)). The heme moieties allow for the transfer of electrons from the cytosol into the phagosomal compartment, in the case of NOX2, or the extracellular space in the case of DUOX (MEIER *et al.* 1993), (YU *et al.* 1998). In neutrophils and macrophages, activated NOX2 is comprised of membrane bound flavocytochrome b_{558} complex (gp91^{phox} and p22^{phox}), cytosolic p47^{phox}/p40^{phox}/p67^{phox} complex as well as the cytosolic GTPase Rac2. This NOX2 complex is present in the phagolysosome membrane and is responsible for the respiratory burst observed in response to engulfed bacterial and fungal pathogens (JACKSON *et al.* 1995).

The respiratory burst is an important facet of the host's innate immune response mediated by neutrophils, and macrophages, as evidenced by the X-linked and autosomal recessive forms of chronic granulomatous disease (CGD), characterized by a nonfunctional NOX2 multiprotein complex (JACKSON *et al.* 1995). Approximately 60% of patients exhibit the X-linked form of CGD which is due to a mutation in gp91^{phox}; however, mutations in genes encoding p22^{phox}, Rac2, p40^{phox}, p67^{phox} and p47^{phox} result in CGD (ELLSON *et al.* 2006), (JACKSON *et al.* 1995), (KNAUS *et al.* 1991). Phagocytosis of pathogens is not impaired in CGD neutrophils or macrophages, but there is a reduction in the consumption of O_2, which is reduced to O_2^-, which rapidly dismutates into H_2O_2 (COHEN *et al.* 1981), (HENRIET *et al.* 2012). Additionally, there is a high occurrence of granuloma formation due to macrophages being unable to efficiently kill the pathogen (COHEN *et al.* 1981). CGD

patients are also characterized by frequent, severe infections by catalase positive bacteria and fungi such as *S. aureus*, *Candida albicans* and *Aspergillus nidulans*; however, catalase negative bacteria such as Streptococci are efficiently eliminated by CGD neutrophils (COHEN *et al.* 1981).

While H_2O_2 production is necessary for the respiratory burst, it is not the main mediator of microbicidal activity in the phagolysosome. HOCl resulting from the reaction of H_2O_2 and chloride ions catalyzed by myeloperoxidase (MPO) is the major microbicidal oxidant produced in the phagolysosome. Neutrophils in MPO-deficient patients are unable to efficiently kill *C. albicans* which corresponds to findings in CGD patients thus indicating that both H_2O_2 and MPO are very important for candidacidal activity and prevention of disseminated candidiasis (BEILKE *et al.* 1989), (HAMPTON *et al.* 1998), (KLEBANOFF *et al.* 2013), (LEHRER 1970). While HOCl is a very potent antimicrobial, its method of action within the phagolysosome was unknown for the longest time until it was observed that protein bound 3-chlorotyrosine was a marker for HOCl-mediated oxidation (CHAPMAN *et al.* 2002), (ROSEN *et al.* 2002). Formation of 3-chorotyrosine in *E. coli* and *S. aureus* proteins occurred when either organism was engulfed by neutrophils that were wild type for H_2O_2 production and *hMPO* (human) expression (CHAPMAN *et al.* 2002), (KLEBANOFF *et al.* 2013), (ROSEN *et al.* 2002). When MPO-deficient or CGD neutrophils were incubated with *E. coli,* a marked decrease in 3-chlorotyrosine was observed in the recovered *E. coli* proteins (ROSEN *et al.* 2002), (KLEBANOFF *et al.* 2013). Additionally, there was a positive correlation in *E. coli* and *S. aureus* death and 3-chlorotyrosine bound protein, suggesting that in wild type neutrophil phagolyosomes the H_2O_2-MPO-Cl^- system produces HOCl, a highly microbicidal oxidant, that chlorinates the engulfed bacteria (CHAPMAN *et al.* 2002), (KLEBANOFF *et al.* 2013), (ROSEN *et al.* 2002). Another way that HOCl contributes to microbicidal killing in the phagolysosome is through the production of chloramines such as monochloramine (NH_2Cl)

(BEILKE *et al.* 1989), (HAMPTON *et al.* 1998), (KLEBANOFF *et al.* 2013). Monochloramine is a potent microbicidal agent and is able to cross cell membranes more readily than HOCl as monochloramine is highly lipophilic (BEILKE *et al.* 1989), (HAMPTON *et al.* 1998).

For years it was viewed that the purposeful production of ROS in response to infection was limited to professional phagocytes and any ROS detected in non-phagocytic cells was due to mitochondrial leakage ((IDE *et al.* 1999); reviewed by (NAUSEEF 2014)). However, in the past 15 years this view was proven to be incorrect with the discovery of several NOX isoforms and two DUOX proteins (Figure 1.1). The revolution in thought began with the discovery of NOX1 in the human colon (SUH *et al.* 1999). NOX3 was discovered to be expressed in the inner ear, while NOX4 was expressed in the kidneys (GEISZT *et al.* 2000), (PAFFENHOLZ *et al.* 2004). Interestingly, NOX5 possesses an extra N-terminal cytosolic arm that contains four Ca^{2+}-binding EF-hands which is sufficient for regulation of its activity by binding of Ca^{2+} (BANFI *et al.* 2001). NOX5 is found primarily in pachytene spermatocytes as well as the lymph nodes and spleen (BANFI *et al.* 2001). Like NOX5, DUOX activity is modulated by Ca^{2+}-binding to their two EF-hands; however, DUOX proteins possess an N-terminal, extracellular, peroxidase-like domain (AMEZIANE-EL-HASSANI *et al.* 2005), (MEITZLER and ORTIZ DE MONTELLANO 2009). Expression of the DUOX proteins has been observed in a myriad of mammalian tissues including thyroid follicle cells as well as mucosal surfaces such as those of the gastrointestinal tract, airways, and oral cavity (CONNER *et al.* 2002), (EL HASSANI *et al.* 2005a), (FORTEZA *et al.* 2005), (MORENO *et al.* 2002). DUOX2 was first observed in the apical membrane of thyroid follicle cells and was found to contribute to thyroid hormone synthesis by supplying H_2O_2 to thyroid peroxidase (TPO) as hetero- and homozygous mutations in DUOX2 resulted in mild-to-severe congenital hypothyroidism (MORENO *et al.* 2002). Congenital hypothyroidism is a severe problem for newborn children as thyroid hormone has been linked with brain

development and mental retardation (MORENO *et al.* 2002). While DUOX2's role in thyroid hormone synthesis is appreciated, DUOX2's role in mucosal defense is equally important for the host with respect to microorganisms.

While it was known that LPO (lactoperoxidase) was responsible for the production of the potent antimicrobial $OSCN^-$ in airways, saliva, milk and tears, the source of H_2O_2 for LPO was unknown (GEISZT *et al.* 2003b), (FORTEZA *et al.* 2005), (MOSKWA *et al.* 2007). Over the years with the use of several airway animal models including, sheep, rat, bovine, and humans it was determined that DUOX 1/2 were responsible for producing the H_2O_2 utilized by LPO (FORTEZA *et al.* 2005), (GEISZT *et al.* 2003b), (GERSON *et al.* 2000), (MOSKWA *et al.* 2007), (RADA *et al.* 2008), (RADA and LETO 2010). At neutral pH, hypothiocyanate ($OSCN^-$), the predominant form and conjugate base of HOSCN, is still a very potent microbicidal agent produced by the DUOX1/2-LPO-SCN^- system in the airways of humans (FORTEZA *et al.* 2005), (RADA *et al.* 2008), (RADA and LETO 2010). At the physiological concentrations present in the airway during an immune response, $OSCN^-$ is able to efficiently clear *S. aureus*, *P. aeruginosa*, and *Burkholderia cepacia*; however, $OSCN^-$ does not damage the host's mucosal epithelial cells in the process, unlike HOCl, which is an extremely non-specific and highly toxic oxidant that causes damage to both the host and the pathogen (MOSKWA *et al.* 2007), (RADA and LETO 2010). The DUOX1/2-LPO-SCN^- antimicrobial system in airway mucosal epithelial cells is constitutively active in mammals (GERSON *et al.* 2000), (MOSKWA *et al.* 2007), (RADA and LETO 2010). Additionally, upon detection of the pathogen, *P. aeruginosa*, significant increases in H_2O_2 and $OSCN^-$ are observed, which correlate with enhanced pathogen clearance (RADA and LETO 2010). This increase in H_2O_2, and ultimately $OSCN^-$, is directly attributable to DUOX activity as the H_2O_2 levels are modulated by Ca^{2+} concentration and DPI, both of which are known to

stimulate or inhibit activity, respectively (AMEZIANE-EL-HASSANI *et al.* 2005), (MOSKWA *et al.* 2007), (RADA and LETO 2010).

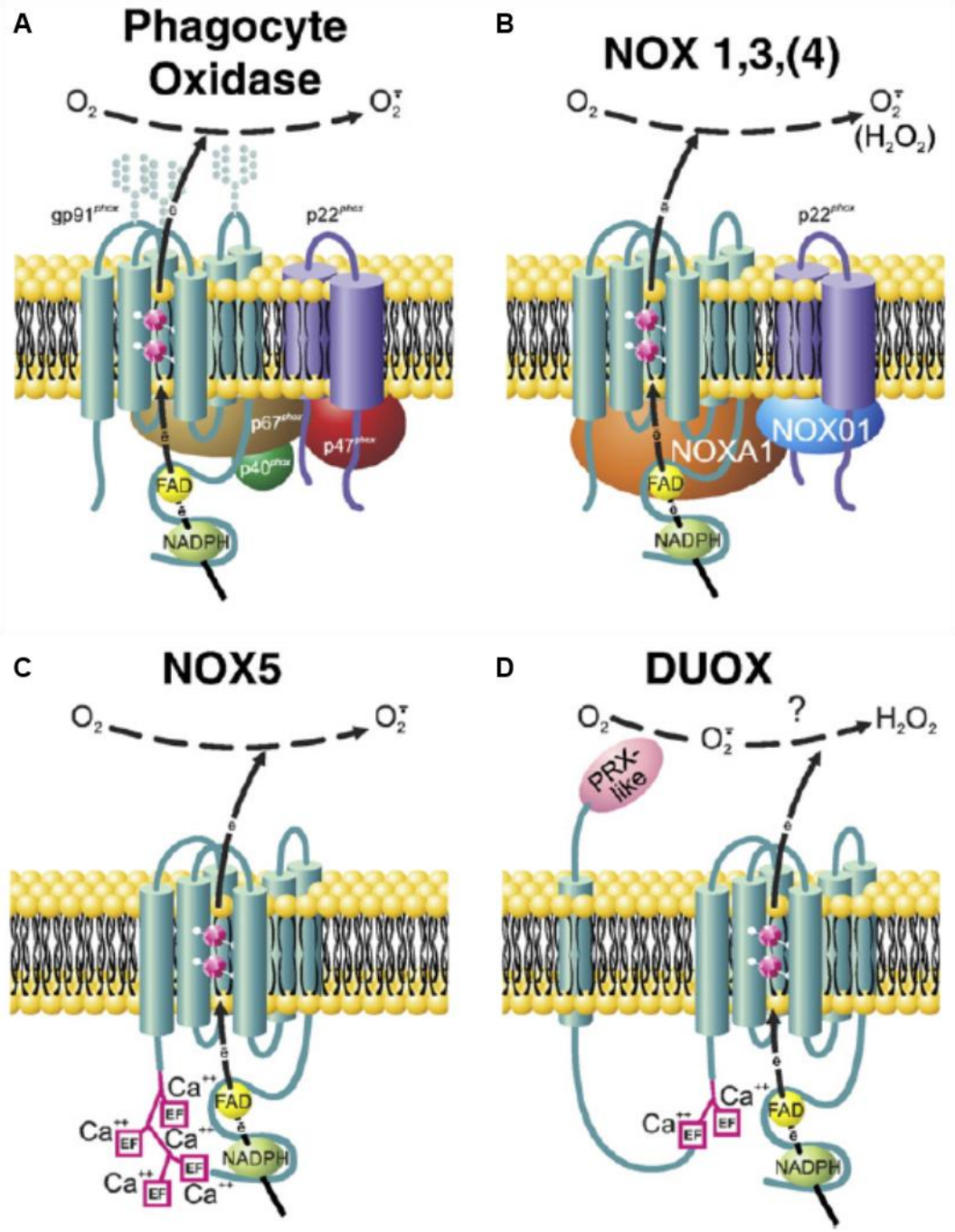


Figure 1.1 Comparison of NOX/DUOX family members A). Phagocytic oxidase (NOX2) is structurally similar to NOX1, 3, and 4. Cytosolic NADPH binds to the NADPH binding domain and two electrons are transferred from NADPH to the FAD domain. Two, single electron transfers are mediated by the FAD domain and the two membrane-embedded heme moieties that reduce two equivalents of O_2, result in the production of two O_2^- radicals. **B)**. In complexes containing NOX 1, 3, and 4, $p40^{phox}$ is absent. Additionally, NOX activating protein 1 (NOXA1) and NOX organizing protein 1 (NOXO1) replace $p67^{phox}$ and $p47^{phox}$, respectively. NOX4 is in parentheses as it produces H_2O_2 instead of O_2^-. **C)**. NOX5 possesses two pairs of calcium-binding motifs in its cytosolic N-terminus. **D)**. DUOX proteins possess one pair of calcium-binding hands as well as an extracellular peroxidase-like domain at their N-terminus (reviewed by (NAUSEEF 2014) and (VIGNAIS 2002). Figure adapted from (NAUSEEF 2014).

NAUSEEF, W. M., 2014 Detection of superoxide anion and hydrogen peroxide production by cellular NADPH oxidases. Biochim Biophys Acta 1840: 757-767.

In cystic fibrosis patients the CFTR (CF transmembrane conductance regulator) is mutated, resulting in impaired SCN^- transport into the airway surface liquid (ASL) where LPO and H_2O_2 are located (MOSKWA *et al.* 2007), (RADA *et al.* 2008), (RADA and LETO 2010). Moskwa *et al*. genetically restored CFTR in human CF airway epithelial cells (hAEC) and observed transport of SCN^- across the apical membrane of the hAECs into the ASL resulting in $OSCN^-$ production and efficient clearance of *S. aureus* (MOSKWA *et al.* 2007). Thus, CF patients have an impaired oxidative defense system that is unable to efficiently clear Gram-positive and –negative pathogens (MOSKWA *et al.* 2007), (RADA *et al.* 2008), (RADA and LETO 2010).

In *D. melanogaster*, it was observed that dDUOX was important during gut infection with *Ecc15*, but not during a systemic infection (HA *et al.* 2009a), (HA *et al.* 2009b). Restoration of dDUOX under a gut-specific promoter was necessary and sufficient to restore wild type resistance during gut infection with *Ecc15* (HA *et al.* 2009a), (HA *et al.* 2009b). Interestingly, basal levels of the dDUOX protein were important in mediating gut-microbe homeostasis as gut-specific RNAi of dDUOX resulted in uncontrolled proliferation of the nonpathogenic microbe *Saccharomyces cerevisiae* (HA *et al.* 2009a), (HA *et al.* 2009b). This uncontrolled proliferation of *S. cerevisiae*, which serves as a nutrient source, ultimately resulted in host death (HA *et al.* 2009a). However, upon gut infection by *Ecc15*, an increase in ROS production was observed following an increase in dDUOX translation (HA *et al.* 2009a), (HA *et al.* 2009b). This increase in dDUOX expression was necessary as mutants in the p38 pathway resulted in enhanced susceptibility during gut infection (HA *et al.* 2009b). Thus, basal levels of dDUOX are sufficient for maintaining host-microbe gut homeostasis in uninfected conditions, but greater amounts of dDUOX are necessary to generate an effective ROS response during infection (HA *et al.* 2009b).

In *C. elegans,* it has been documented that DUOX-1/BLI-3 is important in both the host immune response and cuticle biogenesis (CHAVEZ *et al.* 2009), (THEIN *et al.* 2009). In the latter example, it was observed through a combination of genetics and biochemistry that BLI-3 is important in di- and tri-tyrosine cross-link formation (EDENS *et al.* 2001), (THEIN *et al.* 2009). RNAi knock down of *bli-3* resulted in cuticle blistering due to reduced di- and tri-tyrosine cross-linking of essential collagens DPY-13 and COL-12 (THEIN *et al.* 2009). A slightly more severe blistering phenotype was observed in *mlt-7* RNAi animals and *mlt-7* (*tm1794*) partial deletion mutant animals, which was also due to decreased di- and tri-tyrosine cross-linking of DPY-13 and COL-12 (THEIN *et al.* 2009). Additionally, *bli-3* (*e767*); *mlt-7* (*tm1794*) double mutants displayed post-embryonic lethality due to severe cuticle blistering and were extremely dumpy (THEIN *et al.* 2009). These results suggest that BLI-3 supplied H_2O_2 to both its peroxidase-like domain and MLT-7 for the purpose of cuticle cross-linking, as neither protein was sufficient to completely compensate for loss of the other's activity (THEIN *et al.* 2009). However, upon loss of both proteins, a more significant blistered phenotype was observed, suggesting BLI-3 and MLT-7 cooperate together, but are in parallel pathways (THEIN *et al.* 2009). This work by Thein *et al*. in conjunction with the plethora of data on NOX/DUOX-peroxidase immune systems, as well as BLI-3 producing H_2O_2 in response to *E. faecalis* infection provided much of the rationale for my work (THEIN *et al.* 2009).

By *bli-3* RNAi knock down in intestinal- and hypodermal-specific strains, it was determined that BLI-3-produced H_2O_2 was required for wild type resistance to *E. faecalis* infection (CHAVEZ *et al.* 2009). Additionally, by the use of two distinct point mutations in BLI-3's peroxidase-like domain, it was observed that the peroxidase domain did not significantly contribute to host immunity during infection (CHAVEZ *et al.* 2009). With these data in mind, and the previous examples of NOX/DUOX-peroxidase immune responses, I hypothesized

that during infection BLI-3 supplies H_2O_2 to a peroxidase(s) involved in innate immunity. In this work, I identify potential immune-related peroxidases and characterize one, SKPO-1, in detail. I propose that the BLI-3/SKPO-1 oxidative immune response contributes to *C. elegans* resistance during infection with *E. faecalis*. The results of my work will be discussed in Chapter 3.

Peroxidase-cyclooxygenase superfamily

In this penultimate segment, I will discuss the relationship between peroxidases and their associated prosthetic heme group and how this defines the peroxidase with respect to substrate specificity and reactivity. Additionally, I will briefly touch on the molecular phylogeny of some peroxidase protein clades within the peroxidase-cyclooxygenase superfamily. Ultimately, these topics will further the molecular understanding of why peroxidases such as MPO, LPO and SKPO-1 are important to immunity.

Minus the oxidation of I^- and SCN^-, most peroxidases such as horseradish peroxidase (HRP) are incapable of oxidizing halide substrates Cl^- and Br^- under physiological pH (HUANG *et al.* 2005). However, at pH of 5.0, HRP is capable of oxidizing Br^- and Cl^-. While HRP is able to catalyze these reactions under acidic pH, the protein's activity is rapidly decreased resulting in inactivation of HRP by these extremely electrophilic, oxidizing agents (HUANG *et al.* 2005). These (pseudo)hypohalous oxidants react with the heme prosthetic group resulting in formation of halide adducts *i.e.* bromoheme (HUANG *et al.* 2005). Interestingly, the mammalian peroxidases LPO and MPO can catalyze the formation of antimicrobial oxidants ($OSCN^-$ and HOCl, respectively) without inactivating themselves due to the covalent linkages to the heme prosthetic group (HUANG *et al.* 2005); reviewed by (COLAS and ORTIZ DE MONTELLANO 2003), (O'BRIEN 2000), (ORTIZ DE MONTELLANO 2008). It was determined that when either Asp or Glu were mutated that LPO and MPO's ability to catalyze reactions was reduced due to modification

of the heme group during catalytic turnover ((COLAS *et al.* 2002), (HUANG *et al.* 2005); reviewed by (COLAS and ORTIZ DE MONTELLANO 2003), (ORTIZ DE MONTELLANO 2008)). Not only do these covalent ester bonds protect the heme from modification during reactions, but they aide in the peroxidase's redox potential through distortion of the heme's planarity and distribution of electron density (reviewed by (COLAS and ORTIZ DE MONTELLANO 2003), (ORTIZ DE MONTELLANO 2008)).

MPO is unique amongst the mammalian peroxidases as it contains a methionine residue that is absent in the other mammalian peroxidases. The presence of this Met residue allows for formation of a methionine sulfonium covalent linkage. The positive sulfonium bond is electron-withdrawing and is responsible for MPO's ability to utilize Cl^- as a substrate, as mutation of Met to the residue at the corresponding position in LPO or TPO (Gln or Val, respectively) results in loss of HOCl production (HUANG *et al.* 2005), (KOOTER *et al.* 1999). While these covalent bonds are important for catalyzing hypohalous acid formation they are not necessary for peroxidase activity as they are absent in non-animal peroxidases (reviewed by (COLAS and ORTIZ DE MONTELLANO 2003), (ORTIZ DE MONTELLANO 2008)). The residues that are necessary for an efficient catalytic site in peroxidases are Gln, Asn, His and Arg, all of which are present in LPO, MPO and SKPO-1. The distal side of the hMPO-associated heme has three key catalytic residues, Gln257, His261, and Arg405 while the proximal side has His503 (reviewed by (COLAS and ORTIZ DE MONTELLANO 2003)). In conjunction with H_2O_2, His261 and Arg405 are critical for formation of the heme group's reactive intermediate compound I (reviewed by (COLAS and ORTIZ DE MONTELLANO 2003), (ORTIZ DE MONTELLANO 2008)). The distal His acts as an acid-base catalyst while the Arg polarizes the peroxidic (H-O-O-H) bond, resulting in heterolytic cleavage and the release of H_2O (reviewed by (COLAS and ORTIZ DE MONTELLANO 2003), (ORTIZ DE MONTELLANO 2008)). While H_2O_2 was reduced, the heme was oxidized and the two electron oxidizing equivalents are stored as an oxyferryl moiety (Fe^{iv}=O) and a π

radical cation on the tetrapyrrole ring (reviewed by (COLAS and ORTIZ DE MONTELLANO 2003), (ORTIZ DE MONTELLANO 2008)). This reaction is observed in animal peroxidases. Depending on a peroxidase's ability to utilize a particular substrate and availability of a substrate, two different reactions might occur. All peroxidases, including the non-animal superfamily, are able to oxidize phenol-containing substrates such as tyrosine (reviewed by (COLAS and ORTIZ DE MONTELLANO 2003)). This peroxidation reaction (R1) is characterized by reduction of compound I to compound II and then reduction of compound II to ferric heme through two, single electron transfers from two tyrosine phenolic groups resulting in two tyrosyl radicals (Figure 1.2). These tyrosyl radicals can react with each other to form tyrosine cross-linkages that are essential in thyroid hormone biosynthesis and cuticle formation in humans and *C. elegans*, respectively ((EDENS *et al.* 2001), (THEIN *et al.* 2009); reviewed by (COLAS and ORTIZ DE MONTELLANO 2003), (ORTIZ DE MONTELLANO 2008)). The other possibility is the halogenation reaction (R2), where compound I is reduced to its resting state through a simultaneous transfer of two electrons from a halide ion resulting in a hypohalous acid—as a result compound II is not formed (Figure 1.2).

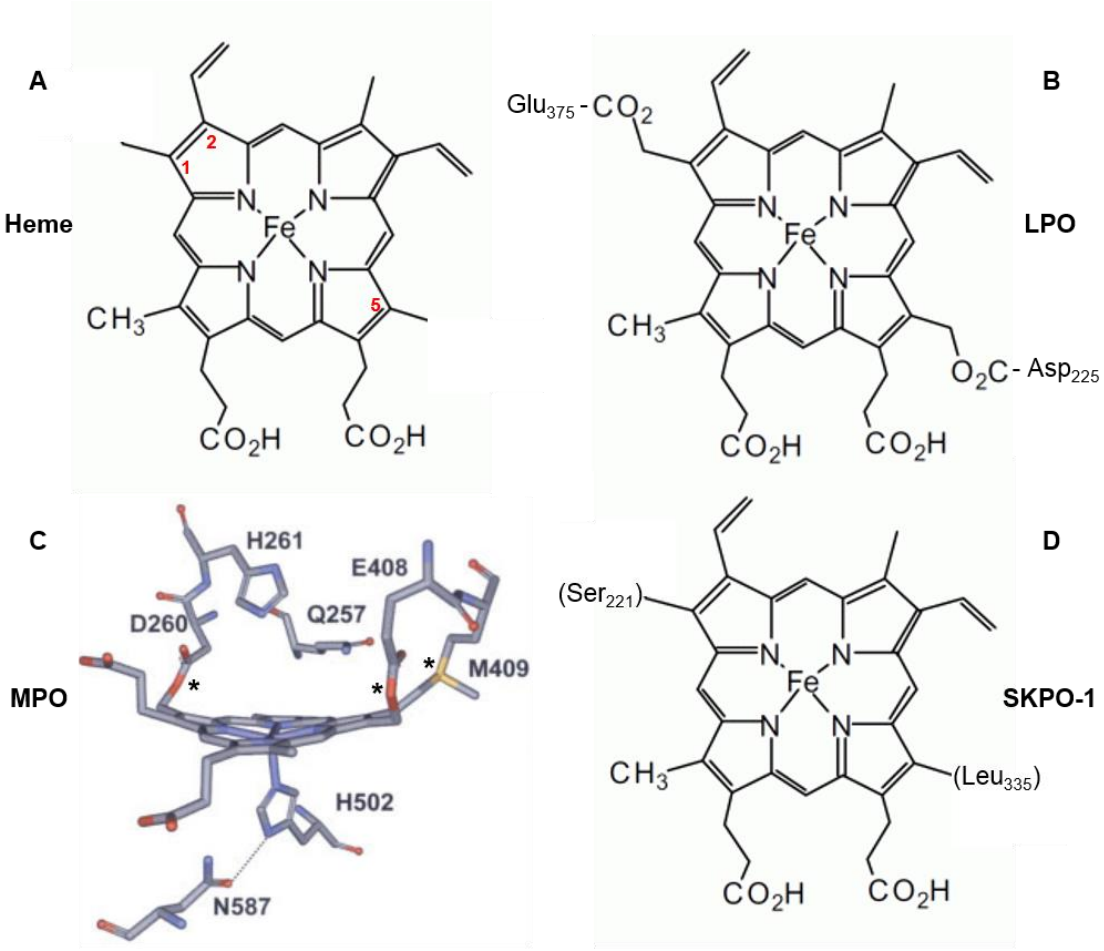
As BLI-3 lacks the distal His residue (Tyr106), its rate of turnover is very low with respect to other mammalian peroxidases (MEITZLER *et al.* 2010). Additionally, *in vitro* studies demonstrated BLI-3 possesses extreme substrate specificity, as it does not readily oxidize a general peroxidase substrate (ABTS; 2, 2'-Azino-di(3-ethylbenzthiazoline-6-sulfonate)). However, BLI-3 readily oxidizes a tyrosine substrate (L-tyrosine ethyl ester) (MEITZLER *et al.* 2010). Meitzler *et al*. demonstrated that the recombinant Y106H BLI-3 peroxidase-like domain mutant possessed significantly higher peroxidase activity compared to the wild type BLI-3 peroxidase-like domain (~7-fold increase) (MEITZLER *et al.* 2010). However, the Y106H mutant's ability to oxidize L-tyrosine ethyl ester was still 170-fold lower than bovine LPO (MEITZLER *et al.* 2010). Thus, it is highly unlikely that BLI-3 is capable of utilizing its own H_2O_2 for an antimicrobial purpose due to the inefficiency of its peroxidase-

like domain, which may explain its functional association with MLT-7 for cross-linking the cuticle (EDENS *et al.* 2001), (MEITZLER *et al.* 2010), (THEIN *et al.* 2009). These results further support the rationale that one or more additional peroxidases functionally interact with BLI-3 to contribute to the host's immune response against *E. faecalis*.

Within the peroxidase-cyclooxygenase superfamily it has been suggested, by evolutionary genetics, that the mammalian peroxidase subfamily evolved from the "traditional" nematode peroxidasin subfamily (ZAMOCKY *et al.* 2008), (SOUDI *et al.* 2012). Peroxidasins and peroxinectins are multi-domain proteins that possess multiple leucine-rich repeats (LRR) and immunoglobulin-like (Ig) domains in addition to the peroxidase domain (ZAMOCKY *et al.* 2008), (SOUDI *et al.* 2012). Interestingly, mature mammalian peroxidases are structurally similar to a different group of nematode peroxidasins termed short peroxidasins, as both mammalian peroxidases and short peroxidasins lack LRRs and Ig domains (ZAMOCKY *et al.* 2008), (SOUDI *et al.* 2012). It has been proposed that the short peroxidasin group is a link between the peroxinectin and traditional nematode peroxidasin subfamilies (ZAMOCKY *et al.* 2008), (SOUDI *et al.* 2012). As SKPO-1 lacks LRRs and Ig domains, it is classified as a short peroxidasin (ZAMOCKY *et al.* 2008), (SOUDI *et al.* 2012). However, SKPO-1 does possess a Shk toxin (ShkT)-like domain. In the sea anemone, *Metridium senile*, the ShkT protein family was characterized as a potassium channel blocker (TILLER and GARSIN 2014). However in *C. elegans*, the ShkT-like domain lacks the necessary residues to block potassium ion channels (TILLER and GARSIN 2014). Currently, it is thought that the ShkT-like domain mediates protein-protein interactions in nematodes (TILLER and GARSIN 2014). With respect to nematodes, the ShkT-like domain's function has yet to be experimentally determined (TILLER and GARSIN 2014).

In crustaceans, peroxinectins (PXT) are important in the host's innate immune response and their expression increases upon detection of PAMPs (β-1,3 glucan and LPS) and/or (opportunistic marine) pathogens (*S. aureus*, *Vibrio harveyi*), ((DONG *et al.* 2011),

(DU *et al.* 2013), (JOHANSSON *et al.* 1995), (JOHANSSON *et al.* 1999), (LIU *et al.* 2004), (VIZZINI *et al.* 2013); reviewed by (CERENIUS *et al.* 2008)). Upon detection of infection, PXTs are exocytosed from granular hemocytes to aid in hemocyte cell-adhesion and opsonization (DONG *et al.* 2011), (DU *et al.* 2013), (JOHANSSON *et al.* 1999), (LIU *et al.* 2004), (VIZZINI *et al.* 2013); reviewed by (CERENIUS *et al.* 2008). These PXTs bind to hemocytes by their integrin-binding motif and also by binding to an extracellular superoxide dismutase (EC-SOD) (DONG *et al.* 2011), (DU *et al.* 2013), (JOHANSSON *et al.* 1999), (LIU *et al.* 2004), (VIZZINI *et al.* 2013); reviewed by (CERENIUS *et al.* 2008). Upon degranulation, the PXTs are proteolytically processed and gain peroxidase activity. It has been proposed that the EC-SOD/PXT complex may produce hypohalous acids; however, this is still unknown (JOHANSSON *et al.* 1999), (VIZZINI *et al.* 2013); reviewed by (CERENIUS *et al.* 2008). It has been demonstrated that MPO readily binds to the plasma membrane of neutrophils by the CD11b/CD18 integrins resulting in enhanced degranulation, respiratory burst activity as well as increased CD11b surface presentation (LAU *et al.* 2005). Strangely, the function(s) of peroxidasins (PXN) in *D. melanogaster* is relatively unknown besides their expression in hemocytes, which is important in phagocytosis as well as deposition of ECM (GOTENSTEIN *et al.* 2010). Likewise in *C. elegans*, peroxidasins are new territory. It was demonstrated that PXN-2, but not PXN-1, was crucial for post-embryonic basement membrane integrity and muscle attachment to the hypodermis (GOTENSTEIN *et al.* 2010). Additionally, even less is known about short peroxidasins; however, my research on SKPO-1 has demonstrated its involvement in the host's immune response.



R1 $H_2O_2 + 2AH_2 \longrightarrow 2H_2O + 2\ AH^{\bullet}$

R2 $H_2O_2 + X^- + H^+ \longrightarrow H_2O + HOX$

Figure 1.2 Heme, iron protoporphyrin IX, is the prosthetic group in both the non-animal (bacterial, fungal, plant) and peroxidase-cyclooxygenase superfamilies. Within the peroxidase-cyclooxygenase superfamily, mammalian peroxidases possess covalently bound heme, which is not present in members of the non-animal superfamily. **A)**. Unincorporated heme prosthetic group. The red text denotes the three sites that are able to form a covalent linkage between the heme and peroxidase. **B)**. Most mammalian peroxidases, represented by LPO, are capable of forming two covalent bonds at the 1- and

5-methyl positions (Glu375 and Asp225, respectively). **C)**. MPO is unique because it can form a third covalent, sulfonium, bond with its heme group at the 2-vinyl position in addition to the 1- and 5-methyl covalent bonds. In the 3D model of MPO-associated heme, there are three ester linkages denoted by the red (D260 and E408) and yellow (M409) bonds—the asterisks are meant as an additional visual aide. **D)**. SKPO-1 is unable to form covalent bonds with its heme prosthetic group at 1- and 5-methyl positions due to the absence of the Asp and Glu residues (replaced with Ser221 and Leu335, respectively). As seen with MLT-7, the heme prosthetic group is noncovalently associated with SKPO-1. R1 is the peroxidation reaction that is responsible for production of organic radicals such as tyrosyl. R2 is the halogenation reaction and is responsible for antimicrobial (pseudo)hypohalous acid formation. AH = organic substrate; X^- = halide substrate ((TILLER and GARSIN 2014), (ZAMOCKY *et al.* 2008); reviewed by (COLAS and ORTIZ DE MONTELLANO 2003), (ORTIZ DE MONTELLANO 2008)).

Used with permission from John Wiley and Sons and Proteins: Structure, Function, and Bioinformatics. ZAMOCKY, M., C. JAKOPITSCH, P. G. FURTMULLER, C. DUNAND and C. OBINGER, 2008 The peroxidase-cyclooxygenase superfamily: Reconstructed evolution of critical enzymes of the innate immune system. Proteins 72: 589-605.

Used with permission from Dr. Paul Ortiz de Montellano (personal communication).

Significance

Throughout this introduction, I have conveyed the importance of studying ancient, evolutionarily conserved immune responses by using a variety of model organisms. As *C. elegans* is a genetically tractable model, it has proven to be useful for dissecting conserved features in a variety of host innate immune responses. Additionally, I have briefly covered some of the various physiological processes in which ROS are involved with respect to the host immune response. By doing so, I have shown the importance of ROS, particularly H_2O_2, in the host immune response as both a secondary signaling molecule as well as a microbicidal effector. Interestingly, for H_2O_2 to be an effective antimicrobial agent it must be converted into a more potent oxidant, such as a hypohalous acid, which requires peroxidase involvement. These NOX/DUOX-peroxidase systems are incredibly important in the war against viral, fungal, parasitic, and bacterial infections. It is notable that the use of an oxidative defense system arose prior to professional phagocytic cells and to adaptive immunity, neither of which are present in *C. elegans*.

Previously, Chavez *et al*. demonstrated that in response to infection, *C. elegans* dramatically increased H_2O_2 production in a BLI-3-dependent manner to contribute to the host immune response (CHAVEZ *et al.* 2007), (CHAVEZ *et al.* 2009). My work built upon the BLI-3-produced H_2O_2 response during *E. faecalis* infection of *C. elegans.* As I have recounted, H_2O_2 by itself, is a poor antimicrobial oxidant and serves as a co-substrate in conjunction with a halide for a peroxidase to catalyze the formation of a potent antimicrobial. In my work, I demonstrated that SKPO-1 is a peroxidase that uses BLI-3-produced H_2O_2 during *E. faecalis* infection to protect the host. Additionally, I discovered that this BLI-3/SKPO-1 system is present and active at an underappreciated host-pathogen interface, *i.e.* the hypodermis and not the intestine, in *C. elegans*. While I have determined that SKPO-1 requires BLI-3-produced H_2O_2 to contribute to host resistance during *E. faecalis* infection, I do not know how SKPO-1 utilizes the H_2O_2. One possibility is that

SKPO-1 uses H_2O_2 to produce a hypohalous acid to kill the pathogen. Another is that SKPO-1 could be using the H_2O_2 to reinforce the cuticle barrier. Finally, SKPO-1 may be acting as an antioxidant to prevent cellular damage from excessive H_2O_2 production. These possibilities are not mutually exclusive; SKPO-1 may be involved in each of these processes during infection.

Chapter 2: Materials and Methods

Portions of this chapter are based on my first author publication. The Genetics Society of America (GSA) journal of Genetics does not require me to obtain permission to reproduce data or text from an article in which I am an author. I have contributed significantly to this publication. The article is listed for reference: TILLER, G. R., and D. A. GARSIN, 2014 The SKPO-1 Peroxidase Functions in the Hypodermis To Protect *Caenorhabditis elegans* from Bacterial Infection. Genetics.

Creation of cDNA pL4440 constructs for dsRNA production in *E. coli*

A standard RNA isolation procedure was used to procure total RNA from healthy N2 adults grown on *E. coli* OP50 (VAN DER HOEVEN *et al.* 2011). I followed the first-strand cDNA synthesis protocol from the Life Technologies SuperScript® III Synthesis System to produce first-strand total cDNA. I then followed a standard PCR protocol using primers specific to *F32A52.A*, *F32A52.B* and *ZK994.3*, respectively (primers listed in Table 2.2). PCR products were digested and ligated into pL4440 and transformed into chemically competent *E. coli* HT115 to produce dsRNA to the gene-of-interest, respectively.

***C. elegans* strains and maintenance**

C. elegans strains were grown and maintained as previously described (HOPE 1999). The strains used for the hypodermal and intestinal RNAi studies are as follows: *rde-1*(*ne219*); *Is*[*wrt-2prom::RDE-1::unc54 3'utr; myo2p::RFP3*] and *sid-1*(*qt9*); *Is*[*vha-6::sid-1*], respectively. The RB1437 [*skpo-1* (*ok1640*) II] strain is a partial deletion mutant for *skpo-1* and was verified via sequencing (www.WormBase.org). The RB1437 [*skpo1* (*ok1640*) II] strain was created by the *C. elegans* Reverse Genetics Core Facility at UBC, part of the International *C. elegans* Gene Knockout Consortium and obtained through the Caenorhabditis Genetics Center. The independent lines of *skpo-1* strains, GF89, GF90 and GF91, were obtained through backcrossing the RB1437 [*skpo-1* (*ok1640*) II] strain with the wild type N2 Bristol strain five times (B.C. x 5).

Tiller and Garsin 2014

RNAi Interference

RNAi was performed by exposing L1 through L4 stage larvae to *E. coli* HT115 expressing dsRNA to target genes. RNAi clones were obtained from the *C. elegans* library (FRASER *et al.* 2000) (KAMATH *et al.* 2003). *skpo-1* worms exposed to pathogens were prone to a maternal bagging phenotype therefore *cdc-25.1* RNAi was used to induce sterility in some experiments (VAN DER HOEVEN *et al.* 2011).

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Survival and Longevity Assays

Unless otherwise indicated, these bacterial strains were used OP50 (*E. coli*) (BRENNER 1974), OG1RF (*E. faecalis*) (DUNNY *et al.* 1978), and PA14 (*P. aeruginosa*) (RAHME *et al.* 1995). Exposure to RNAi, survival assays, and longevity assays were performed as previously described (GARSIN *et al.* 2001) (GARSIN *et al.* 2003) (KIM *et al.* 2002) (VAN DER HOEVEN *et al.* 2011). Briefly, for *E. faecalis* survival assays, *E. faecalis* grown in Brain Heart Infusion (BHI) medium for 5 hours was seeded (10 ul) onto BHI plates (gentamycin 50 ug/ml) and incubated at 37˚C for 24 hours. While for *P. aeruginosa* survival assays, *P. aeruginosa* was cultured in Luria broth (LB) overnight at 37˚C, seeded (10 ul) onto slow-killing plates and incubated first for 24 hours at 37˚C and then for the duration of the experiment at 25˚C (VAN DER HOEVEN *et al.* 2011). For the *E. coli* longevity assays, 20X *E. coli* was seeded (100 ul) onto Nematode Growth (NG) medium plates supplemented with FUDR (5-Fluoro-2'-deoxyuridine; 25 ug/ml) and streptomycin (25 ug/ml). Seeded NG plates were incubated at 25˚C for 24 hours. In all assays, a total of 90 L4 larvae were transferred to three replica plates and the assay performed at 25˚C. Worms were scored as live and dead at various time points.

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Amplex Red assay for H_2O_2 measurements

The Amplex Red hydrogen peroxide/peroxidase kit (Invitrogen Molecular Probes, Eugene, OR) was previously adapted to *C. elegans* to measure pathogen-stimulated hydrogen peroxide release (CHAVEZ *et al.* 2009) (CHAVEZ *et al.* 2007). The same protocol was followed with the following modifications. L4 worms were exposed to a bacterial strain for 12 hours, and the fluorescence of 30 worms per well was measured after 30 minutes of incubation with Amplex Red (540/590 nm excitation and emission, respectively). 80 uM diphenyleneiodinium chloride (DPI) (TCI Tokyo, Japan) was added to some wells and allowed to incubate for 15 minutes prior to addition of Amplex Red, HRP and Na_2PO_4 (pH 7.4) and fluorescence was measured as above.

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Protein Expression and Purification

Standard molecular cloning was used to generate and introduce wild type *skpo-1* cDNA into pET-29b(+) and pET-28a(+) vectors. I isolated total RNA from healthy N2 adults grown on *E. coli* OP50. I followed the first-strand cDNA synthesis protocol from the Life Technologies SuperScript® III Synthesis System to produce first-strand total cDNA. I then followed a standard PCR protocol using primers specific to full-length *skpo-1* (Table 2.2). Standard transformation was used to introduce the SKPO-1::HIS6-containing, full-length and peroxidase domain versions, and empty vector constructs into *E. coli* BL21 (DE3). Expression and purification of the various constructs was carried out according to Novagen® and Clontech Laboratories TALON® protocols, respectively.

Western Blot

Using a semi-dry transfer, protein samples were transferred to a nitrocellulose membrane from a 10% SDS-PAGE gel. The nitrocellulose membrane was blocked with 5% non-fat milk in TBS-T for 30 minutes in the walk-in 4˚ C on a rocking platform. The membrane then incubated with 5% non-fat milk TBS-T and THE™ His Tag, mouse, mAb (1:5,000) for 60 minutes, rocking, at room temperature. The membrane was then washed with 5% non-fat milk TBS-T, ten minutes each, four times. The membrane was then incubated with 5% non-fat milk TBS-T and goat anti-mouse HRP-conjugated secondary (1:20,000) for 30 minutes, rocking, at room temperature. Again, the membrane was washed four times, ten minutes each, with 5% non-fat milk TBS-T. Finally, the membrane was incubated with Thermo Scientific SuperSignal® Dura Extended Duration Substrate ECL (enhanced chemiluminescence) for five minutes and then the membrane was imaged using the BioRad ChemiDoc™. This procedure was used for both *E. coli* and whole worm lysates. In the case of detecting SKPO-1 in whole worm lysates, the primary and secondary antibodies used were anti-SKPO-1 polyclonal antibody and goat anti-rabbit HRP-conjugated secondary (1:1,000 and 1:5,000), respectively.

Indirect Immunofluorescence

Immunofluorescence for *C. elegans* was performed according to Seydoux and Dunn on young adult animals (SEYDOUX and DUNN 1997). Custom peptide synthesis, rabbit immunization and affinity purification of the SKPO-1 polyclonal antibody was performed by LifeTein® (South Plainfield, NJ). The CRVGRRAFDIENGSR peptide corresponds to the C-terminus of the *C. elegans* protein SKPO-1. Adult N2 and GF89 worms were imaged using an Olympus IX81 automated inverted microscope and Slidebook (version 5.0) software along with the SKPO-1 polyclonal primary antibody and Alexa Fluor 488 goat anti-rabbit IgG secondary antibody to localize SKPO-1 in *C. elegans*.

Immunogold Labeling and Transmission Electron Microscopy

Approximately 1,000 N2 and GF89 animals were raised under standard conditions to the young adult stage. They were rinsed off of the propagation plates with M9 and spun down at 2000 RPM for 1 minute. The worms were sequentially resuspended and pelleted 3 times in 1 ml M9 (HOPE 1999). After the final wash they were resuspended in 1 ml 3% formalin, 0.15% glutaraldehyde in Millonig's buffer pH 7.45 and fixed for three days at 40°C. Then the buffer was drawn off and the samples were incubated in 0.1% fresh made sodium borohydride in Millonig's buffer (RT (room temperature), 10 min). This was followed by incubations in Millonig's buffer (RT, 10 minutes, repeated twice). The samples were then dehydrated at room temperature by incubating in 50% ethanol (10 min, twice) and 70% ethanol, (15 min, twice). The samples were then permeated with 50% LR-White resin and 50% ethanol for 60 min, followed by an overnight incubation at room temperature in 50% LR-White resin sealed on a rotator. The samples were then incubated at room temperature for four hours in 70% LR-White resin and then for five hours in 100% LR-White resin, while on a rotator. The pellets were then sealed into BEEM capsules and allowed to polymerize in a 53°C oven overnight.120nm thin sections of the LR-White blocks were cut using a DiATOME diamond knife and a Leica Ultracut R microtome. Sections were floated onto 200 mesh nickel grids. The grids were then floated in 50mM glycine (RT, 15 min) and then washed twice by floating in drops of PBS. The samples were blocked by floating them for 30 min in Aurion Blocking Solution followed by washes in incubation buffer (PBS with 0.1% Aurion BSA-C) (5 min, three times). The SKPO-1 antibody was diluted into the incubation buffer at 1:1000 and the grids were incubated for 1.5 hours at RT. This was followed by washes in incubation buffer (10 min, four times) and then incubation with the secondary antibody (EMS Goat-Anti-Rabbit IgG with 10 nm gold) diluted 1:20 in incubation buffer. The

samples were washed again in incubation buffer (10 min, five times) followed by PBS (10 min, three times). A post-fix treatment of 3% glutaraldehyde was applied for 10 min followed by a final wash with distilled water (5 min, 2 times). The grids were dried in a 70°C oven and then imaged using a JEOL 1200 transmission electron microscope at 60 kv and captured with a 2k x 2k Gatan CCD camera.

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RNA isolation and qRT-PCR

Prior to RNA isolation, wild type and *skpo-1* mutant animals were grown on *cdc-25.1* dsRNA expressing *E. coli* HT115 from the egg to L4 larval stage. L4 animals were then exposed to either *cdc-25.1* RNAi or *E. faecalis* OG1RF for 18 hours. The RNA was isolated using Trizol (Invitrogen) according to the manufacturer. RNA samples were treated with DNase I to eliminate contaminating DNA by the Turbo DNA free kit (Applied Biosystem) according to the manufacturer. qRT-PCR was performed as described in (VAN DER HOEVEN *et al.* 2011). Primers used are listed in Table 2.2 (*act-1* served as the reference gene).

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Bacterial Colonization

The CFU analysis was conducted similar to (Breger *et al*. 2007). Briefly, L4 wild type and *skpo-1* mutant animals grown on *E. coli* OP50 were exposed to 100 mm BHI gentamycin 10 ug/mL plates seeded with 100 ul of *E. faecalis* OG1RF for either 12 or 36 hours at 25˚ C. Worms were washed 3 times with M9 buffer at 1.4 rpm. Worms were then washed twice with 25 mM tetramisole hydrochloride to prevent ingestion of the antibiotic treatment. Worms were incubated at room temperature for 60 minutes in 25 mM tetramisole hydrochloride supplemented with ampicillin and kanamycin, both at 1 mg/mL, to kill surface-

attached *E. faecalis.* Worms were collected at 1.4 rpm and washed twice with 25 mM tetramisole hydrochloride prior to grinding. Ten worms in 10 ul were transferred to 200 ul of M9 and ground for 1 minute using a motorized pestle (Kontes cordless cat# K749540-0000 and pestles cat# K749521-1590. Serial dilutions were performed and 100 ul of each dilution plated onto 100 mm BHI gentamycin 10 ug/mL plates for 24 hours at 37˚ C.

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Table 2.1

Name	Description	Marker	Insert	Reference
L4440(pPD129.36)	Cloning vector with convergent T7 polymerase promoters flanking a multiple cloning site (MCS).	Ampicillin (Amp)	Empty vector	(TIMMONS and FIRE 1998)
pGT01	L4440 backbone with *F32A52.A* (*skpo-3a*) cDNA	Ampicillin (Amp)	*F32A52.A*	This study
pGT02	L4440 backbone with *F32A52.B* (*skpo-3b*) cDNA	Ampicillin (Amp)	F32A52.B	This study
pGT03	L4440 backbone with *pxn-1* cDNA	Ampicillin (Amp)	*pxn-1*	This study
L4440-*pxn-2**	L4440 backbone with genomic *pxn-2* fragment	Ampicillin (Amp)	*pxn-2*	This study
L4440-*mlt-7**	L4440 backbone with genomic *mlt-7* fragment	Ampicillin (Amp)	*mlt-7*	This study
L4440-*bli-3**	L4440 backbone with genomic *bli-3* fragment	Ampicillin (Amp)	*bli-3*	This study
L4440-*C16C8.2* (*skpo-2*)*	L4440 backbone with genomic *C16C8.2* fragment	Ampicillin (Amp)	*C16C8.2*	This study
L4440-*C46A5.4**	L4440 backbone with genomic *C46A5.4* fragment	Ampicillin (Amp)	*C46A5.4*	This study
L4440-*F09F3.5**	L4440 backbone with genomic *F09F3.5* fragment	Ampicillin (Amp)	*F09F3.5*	This study
L4440-*R08F11.7**	L4440 backbone with genomic *R08F11.7* fragment	Ampicillin (Amp)	*RO8F11.7*	This study
L4440-*F49E12.1* (*skpo-1*)*	L4440 backbone with genomic *skpo-1* fragment	Ampicillin (Amp)	*skpo-1*	This study
L4440-*K10B4.1**	L4440 backbone with genomic *K10B4.1* fragment	Ampicillin (Amp)	*K10B4.1*	This study
L4440-*T06D8.10**	L4440 backbone with genomic *T06D8.10* fragment	Ampicillin (Amp)	*T06D8.10*	This study

*gene names are listed according to the Ahringer *C. elegans* RNAi library to aid in plasmid identification

Table 2.2

Oligo name	Purpose	Sequence
F32A52.A 5F	cloning	5’ TTG CGG CCG CAA TTC ACC AGG GAA TTT ACA CC 3’
F32A52.A 3R	cloning	5’ CCT CGA GGG TGG TCT TTT CAC ATT CTG G 3’
F32A52.B 5F	cloning	5’ TTG CGG CCG CAA CAA CCA CTC ATT TCA CCA GG 3’
F32A52.B 3R	cloning	5’ CCT CGA GGC TGT AGT TGT TAC TCT TTG TGG 3’
ZK994.3 (*pxn-1*) 5F	cloning	5’ TTG CGG CCG CAA GGA CTC TGG AAG GTA CAC 3’
ZK994.3 (*pxn-1*) 3R	cloning	5’ CCT CGA GGA TAC TTG GCA CTT CCA CTC T 3’
L4440 5F	sequencing	5’ GTT TTC CCA GTC ACG ACG TT 3’
L4440 3R	sequencing	5’ TGG ATA ACC GTA TTA CCG CC 3’
skpo-1 transcriptional 5F	cloning	5’ CCC AAG CTT GTC ACT ATT TCC AAA TTC CTT TC 3’
skpo-1 transcriptional 3R	cloning	5’ GCG TCG ACC GGT CTG AAA AAA AGT TGC AG 3’
skpo-1 translational 5F	cloning	5’ CGG GAT CCA TGA AGT CTC TCC TCT TCT CC 3’
skpo-1 translational 3R	cloning	5’ GCG GTA CCG GAG GAG CTT CTC TCC AGG CTT CAA GGT 3’
F49 internal sequence 5F	sequencing	5’ GTC TTA TGC CAG CTG CTT ATG AT 3’
F49 internal sequence 3R	sequencing	5’ TTT CAG TCA CAG AAG TTG TCA AT 3’
F49 internal intergenic 5F	sequencing	5’ CAA TTC AAT TTT TCC CTC GTT 3’
F49 internal intergenic 3R	sequencing	5’ CCC TTA GAT GTC TAT GCT TCT 3’
skpo-1 pET29b 5F (full length *skpo-1*)	cloning	5’ GGG AAA CAT ATG AAG TCT CTC CTC TTC TCC 3’
skpo-1 pET29b 3R (full length *skpo-1*)	cloning	5’ GGA AAA GGT ACC TTC TCT CCA GGC TTC AAG G 3’
skpo-1 mutagenesis 5F	site-directed mutagenesis	5’ GTT AAC TCA CAA TTT CAA GAA GTT AAT AGA TAC CGG TTT ATT GAT GGA GTT A 3’
skpo-1 mutagenesis 3R	site-directed mutagenesis	5’ TAA CTC CAT CAA TAA ACC GGT ATC TAT TAA CTT CTT GAA ATT GTG AGT TAA C 3’
T7 terminator 3R	sequencing	5’ GCT AGT TAT TGC TCA GCG G 3’
RB1437 set1 back cross 5F	PCR genotyping	5’ CTC TCA GAC GTG TTC TTT AAC 3’
RB1437 set1 back cross 3R	PCR genotyping	5’ CGA CGA TAG TCA TTG TAA G 3’

skpo-1 pET29b peroxidase domain only 5F	cloning	5’ GGG AAA CAT ATG GCC CGT TTC CGG CAA CAA ATG 3’
skpo-1 pET28a peroxidase domain only 3R	cloning	5’ GGG AAA GGA TCC CTA CTA TTC TCT CCA GGC 3’
skpo-1 Max Planck fosmid 5F	sequencing	5’ CAG TTC CAT GCT CAT CGA TTA CAG GAT TGA ACC TTG AAG CCT GGA GAG AAG AAG TGC ATA CCA ATC AGG A 3’
skpo-1 Max Planck fosmid 3R	sequencing	5’ TGG CAC TTT TAA GGA ATA CGG TGA ATG AGA ATG ATT GAA ACA AAT GTC TAC TTG TCG TCG TCA TCC TTG T 3’
clec-35 F	qRT-PCR	5’ AGA TGC TGG ACA GTG GAA AAG 3’
clec-35 R	qRT-PCR	5’ GTG CGG AGT ATT GTA GCG TAG 3’
clec-42 F	qRT-PCR	5’ GTA ACT CCG TAT TGG CTG GG 3’
clec-42 R	qRT-PCR	5’ GTA AAC GCA GCT TCC AAT CTC 3’
clec-60 F	qRT-PCR	5’ TGT AAG AGA ACA GTT GGA ACC C 3’
clec-60 R	qRT-PCR	5’ TAT GTG CAT GGG TAC TGA TCG 3’
clec-71 F	qRT-PCR	5’ ACG ACA GGA AGT GAT GTA TTG G 3’
clec-71 R	qRT-PCR	5’ TTG ACG GAC TTT AGC CAC TG 3’
act-1 F	qRT-PCR	5’ ACC ATG TAC CCA GGA ATT GC 3’
act-1 R	qRT-PCR	5’ TGG AAG GTG GAG AGG GAA G 3’

Table 2.3

Strains	Genotype	References
Enterococcus faecalis		
OG1RF	Wild type strain, Fa^R, Rf^R	(DUNNY *et al.* 1978)
Pseudomonas aeruginosa		
PA14	Wild type strain	(RAHME *et al.* 1995)
Escherichia coli		
OP50	Wild type strain, Str^R	(BRENNER 1974)
HT115	Used to express dsRNA for RNAi in *C. elegans*, Amp^R, Tet^R	(GARSIN *et al.* 2001), (GARSIN *et al.* 2003), (KIM *et al.* 2002), (VAN DER HOEVEN *et al.* 2011)
BL12 (DE3)	Used to express pET-29b(+) and pET-28a(+)	(EDENS *et al.* 2001)
Caenorhabditis elegans		
N2	Wild type	(BRENNER 1974)
eri-1	Enhanced RNAi strain, *eri-1*(*mg366*)	(KENNEDY *et al.* 2004)
Hypodermal	Tissue-specific RNAi strain, *rde-1*(*ne129*); *Is*[*wrt-2prom::RDE-1::unc-54 3' utr; myo2p::RFP3*]	(MELO and RUVKUN 2012)
Intestinal	Tissue-specific RNAi strain, *sid-1*(*qt9*)*; Is*[*vha-6::sid-1*]	(MELO and RUVKUN 2012)
RB1437	Outcrossed x 0, [*skpo-1*(*ok1640*) II]	This study
skpo-1 GF89-91	RB1437 outcrossed X 5 with N2, three independent lines	This study

Chapter 3: SKPO-1 protects *C. elegans* from *Enterococcus faecalis* infection

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Introduction

The peroxidase SKPO-1 (ShkT-containing peroxidase) contributes to the host immune response during infection with *E. faecalis*

Heme-containing peroxidases play critical, wide-ranging roles in biological systems. Once called the “animal heme peroxidases,” members of the peroxidase-cyclooxgenase superfamily are actually found in all kingdoms of life (ZAMOCKY *et al.* 2008). The roles of most are poorly characterized, but some are clearly involved in immune defense. For example, the most famous and best-studied member of this group, myeloperoxidase (MPO), is found in the granulocytes of neutrophils where it catalyzes the formation of the potent oxidant HOCl from H_2O_2 and Cl^- to kill invading microbes (KLEBANOFF 2005). Another is lactoperoxidase (LPO), which is found on mucosal surfaces and generates the protective oxidant hypothiocyanite ($OSCN^-$) from H_2O_2 and thiocyanate (SCN^-). This process is impaired in the lungs of cystic fibrosis (CF) patients contributing to the poor clearance of pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, the most common causes of lung infection in CF patients (CONNER *et al.* 2002), (FORTEZA *et al.* 2005), (GEISZT *et al.* 2003a), (MOSKWA *et al.* 2007).

Many of these peroxidases are functionally associated with members of the NADPH Oxidase (NOX)/Dual Oxidase (DUOX) protein family as NOX/DUOX enzymes generate the H_2O_2 required as substrate for the peroxidases. For example, Nox2 is the source of H_2O_2 for MPO. While all of the NOX/DUOX proteins produce ROS, DUOXs differ from NOXs in that they encode an extracellular N-terminal peroxidase domain in addition to the oxidant-generating NADPH oxidase domain, which is located at the C-terminus (RADA *et al.* 2008), (SUMIMOTO 2008). Intriguingly while DUOXs possess a peroxidase domain, these proteins appear to still associate with separate peroxidases. For example, LPO utilizes H_2O_2 generated by Duox1 or Duox2 (CONNER *et al.* 2002), (FORTEZA *et al.* 2005), (GEISZT *et al.*

2003a). In fact, the peroxidase domains of human Duox1/2 are reported to lack peroxidase activity and may have other functions, such as serving as an interaction domain for separate, active peroxidases (MEITZLER *et al.* 2013), (MEITZLER and ORTIZ DE MONTELLANO 2009), (MEITZLER and ORTIZ DE MONTELLANO 2011).

The host's production of ROS is an ancient, evolutionarily conserved defense mechanism and *C. elegans* has been used as a model host to study various aspects of the innate immune response including the purposeful generation of reactive oxygen species (ROS) during infection (CHAVEZ *et al.* 2007), (CHAVEZ *et al.* 2009). The animal encodes only one functional NADPH oxidase, a dual oxidase called BLI-3 and when exposed to human pathogens such as the Gram-positive, opportunistic bacterium, *Enterococcus faecalis*, H_2O_2 is released (CHAVEZ *et al.* 2007), (EDENS *et al.* 2001). The production of H_2O_2 in response to infection appears protective, as its loss by reducing the expression of *bli-3* by RNAi (RNA interference) renders the animals more sensitive to killing by the pathogen (CHAVEZ *et al.* 2009). Using indirect immunofluorescence, BLI-3 was localized to the hypodermis, which is essentially the "skin" of *C. elegans* (EDENS *et al.* 2001). Additionally, there is speculation that BLI-3 may be present in the intestinal cells (CHAVEZ *et al.* 2007), (CHAVEZ *et al.* 2009).

In addition to playing a role in innate immunity, BLI-3 is essential to the normal development of the worm because it contributes to the generation of the tyrosine-linked collagen necessary for proper biogenesis of the cuticle, *i.e.* the exoskeleton of the animal. Its role appears two-fold. First, BLI-3 generates the necessary substrate, H_2O_2, by its NOX domain. Second, BLI-3 uses its N-terminal peroxidase domain and oxidizes the tyrosines of the collagen proteins to tyrosyl radicals that then combine to form di- and tri-tyrosines, thereby cross-linking the cuticle (EDENS *et al.* 2001). Unlike human Duox1/2, the peroxidase domain of BLI-3 has low levels of peroxidase activity that is essential to this process, and mutations in the peroxidase domain that disrupt this activity results in a "blistered" (*bli*) phenotype (BRENNER 1974), (MEITZLER and ORTIZ DE MONTELLANO 2009), (MEITZLER *et al.*

2010), (MEITZLER and ORTIZ DE MONTELLANO 2011), (SIMMER *et al.* 2003). Interestingly, recent work has demonstrated that the peroxidase domain of BLI-3 is not the only peroxidase involved in this process. A separate peroxidase, MLT-7, contributes to cuticle cross-linking and loss of this activity results in the same *bli* phenotype as loss of the BLI-3 peroxidase domain. Additionally, loss of both peroxidase activities greatly increases the severity of cuticle blistering (THEIN *et al.* 2009).

Because of the prevalent functional association of NOX/DUOX proteins with peroxidases in immune responses, we hypothesized that *C. elegans* might also utilize a peroxidase(s) in host defense, perhaps in conjunction with its NADPH oxidase, BLI-3. In support of this hypothesis, during an earlier investigation by our laboratory, it was determined that the peroxidase domain of BLI-3 is not involved in the immune response, as point mutants in this domain had wild type resistance to *E. faecalis,* despite their blistered phenotype (CHAVEZ *et al.* 2009).

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Thus is stands to reason that either the NADPH oxidase domain of BLI-3 is sufficient to provide protection during infection or that an unknown peroxidase is functionally associating with BLI-3 to contribute to the host's defense.

Results

Host resistance to *E. faecalis* is affected by *skpo-1*

To investigate if *C. elegans* possesses any peroxidases involved in host defense, I utilized an RNAi-killing assay to screen candidate peroxidase genes. The candidate genes were found using the BLI-3 peroxidase domain as the BLAST query on WormBase.org. Twelve putative peroxidase-encoding genes were identified, nine of which were available in the RNAi library (FRASER *et al.* 2000), (KAMATH *et al.* 2003), including the previously studied

mlt-7 (THEIN *et al.* 2009). Standard molecular biology techniques were used to generate the three missing RNAi constructs (Materials and Methods). Using a background in which RNAi efficiency is increased (*eri-1)* (KENNEDY *et al.* 2004), the expression of these genes was reduced in *C. elegans*. The animals were then exposed to *E. faecalis* and survival was scored over time. By these means, the genes were screened for possible roles in innate immune function. The reduced expression of three genes—*F09F3.5, R08F11.7*, and *F49E12.1*—resulted in a statistically significant susceptibility phenotype (Table 3.1). I decided to focus on *F49E12.1*, as it possessed high homology to human myeloperoxidase (hMPO, 34% sequence identity) and a deletion mutant was available through the CGC (Caenorhabditis Genetics Center). As described in more detail below, the predicted protein associated with this gene contains an N-terminal metridin Shk toxin (ShkT)-like domain and a C-terminal peroxidase domain and was therefore named SKPO-1 for ShkT-containing peroxidase.

Table 3.1 Susceptibility to *E. faecalis* following RNAi of putative peroxidase genes

RNAi Treatment	Susceptibility Phenotype	Median survival (days)	*P*-value
mlt-7	Wild type	6	0.3070
control		6	
pxn-1	Wild type	4	0.9218
control		4	
pxn-2	Wild type	6	0.8233
control		6	
C16C8.2	Wild type	9	0.1067
control		9	
C46A5.4	Wild type	10	0.7206
control		10	
F09F3.5	susceptible	6	0.0058
control		7	
F32A5.2a	Wild type	5	0.2499
control		5	
F32A5.2b	Wild type	6	0.4115
control		6	
F49E12.1 (skpo-1)	susceptible	5	0.0053
control		9	
R08F11.7	susceptible	6	0.0004
Control		7	
K10B4.1	Wild type	8	0.7904
Control		8	
T06D8.10	Wild type	8	0.6636
Control		8	

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An example of the survival of *skpo-1* RNAi *eri-1* mutant animals on *E. faecalis* compared to vector control (VC) RNAi *eri-1* mutant animals is shown in Figure 3.1A. *skpo-1* RNAi animals displayed an enhanced susceptibility phenotype relative to VC RNAi (P = 0.0011). The experiment was repeated five times and the data was tabulated in Table 3.2. I also examined the lifespan of these animals to see if *skpo-1* RNAi caused a general fitness defect. However, on *E. coli* OP50*,* lifespan of the *skpo-1* RNAi animals was not significantly different when compared to VC RNAi animals (P = 0.3772) (Figure 3.1B and Table 3.3).

To further examine the phenotype resulting from the loss of *skpo-1*, I obtained the partial deletion strain, RB1437 from the CGC. The deletion is between bases 827 and 3,202 of the 5,008 full-length transcript, which ablates a significant portion of the protein, including the critical amino acid residues necessary for peroxidase activity in classical animal heme peroxidases (Figure 3.2) (ORTIZ DE MONTELLANO 2008). Strain RB1437 was backcrossed five times into our wild type N2 strain and three lines were generated GF89, GF90 and GF91. As shown in Figure 3.3, these animals were very susceptible to *E. faecalis* compared to wild type N2 animals. Also, as significant differences in susceptibility were not observed between the strains, I continued the studies using GF89, which will be referred to as *skpo-1*.

Interestingly, I observed a significant “bagging” phenotype—hatching of embryos inside the hermaphrodite that had failed to be expelled—when the *skpo-1* mutant animals were exposed to *E. faecalis*, unlike the *skpo-1* RNAi animals. It has been postulated that a bagging phenotype in response to infection may result in an enhanced susceptibility phenotype that is an artefact. The opposite side of the argument is that in response to infection, a bagging phenotype provides protection for the developing larvae as well as nutrition.

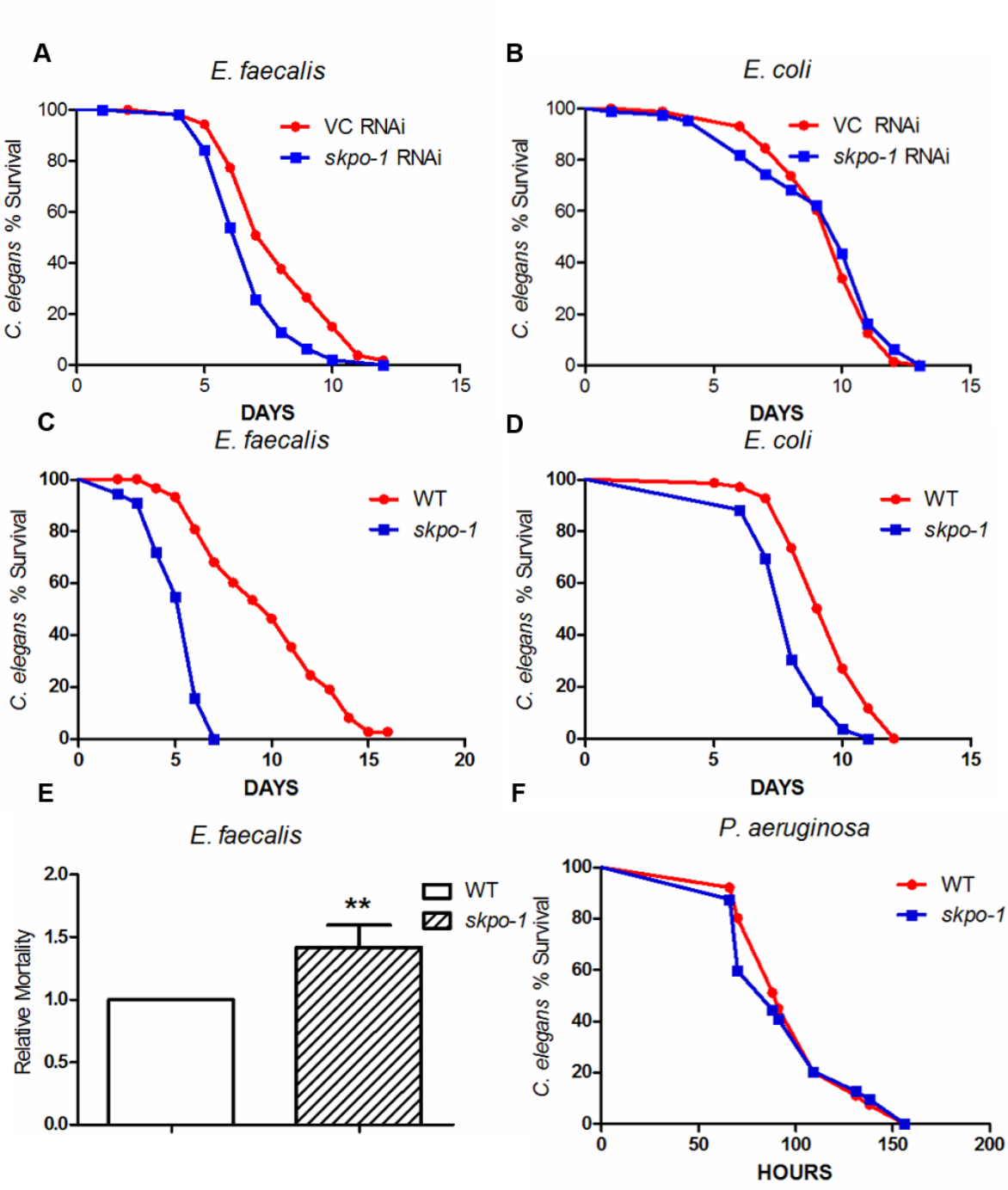

Figure 3.1 SKPO-1 contributes to *C. elegans* resistance to *E. faecalis*. (A) Survival of *eri-1* mutant worms on *E. faecalis* OG1RF following exposure to vector control (VC) RNAi and *skpo-1* RNAi ($P = 0.0011$). (B) Longevity of *eri-1* mutant worms on *E. coli* OP50 following exposure to VC RNAi, and *skpo-1* RNAi ($P = 0.3772$). (C) Survival of wild type

and *skpo-1* mutant worms on *E. faecalis* following exposure to *cdc-25.1* RNAi ($P < 0.0001$). (D) Longevity of wild type and *skpo-1* mutant worms on *E. coli* OP50 following exposure to *cdc-25.1* RNAi ($P < 0.0001$). (E) The relative mortality of *skpo-1* mutant worms exposed to *E. faecalis* (EF) is expressed as a ratio of (LT_{50} of wild type *cdc-25.1* RNAi on EF/LT_{50} of *skpo-1* mutant *cdc-25.1* RNAi on EF) over (LT_{50} of wild type *cdc-25.1* RNAi on *E. coli*/LT_{50} of *skpo-1* mutant *cdc-25.1* RNAi on *E. coli*). The average of four, independent experiments, with 90 animals each, was used to calculate the relative mortality. (F) Survival of wild type and *skpo-1* mutant worms on *P. aeruginosa* following exposure to *cdc-25.1* RNAi ($P = 0.3783$). Error bars represent standard error of the mean (S.E.M.) and the asterisk indicates a significant difference between wild type and *skpo-1* mutant worms ($P = 0.0091$). The *P*-values were calculated using Student's paired t-test. The median survival for survival and longevity assays are listed in Tables 3.2 and 3.3 along with replicates of the experiments.

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Table 3.2 Median survival and *P*-values of *E. faecalis* OG1RF killing assays

Figure number	Experiment number	Strain, exposure conditions during development	Median survival (days)	*P*-value
3.1A	1	wild type, VC RNAi	8	C
		wild type, *skpo-1* RNAi	7	=0.0404
	2	wild type, VC RNAi	7	C
		wild type, *skpo-1* RNAi	6	=0.0331
	3	*eri-1*, VC RNAi	8	C
		eri-1, skpo-1 RNAi	7	=0.001
	4	*eri-1*, VC RNAi	7	C
		eri-1, skpo-1 RNAi	5	=0.0053
	5	*eri-1*, VC RNAi	9	C
		eri-1, skpo-1 RNAi	8	=0.0037
3.1C	1	wild type, *cdc-25.1* RNAi	10	C
		skpo-1, *cdc-25.1* RNAi	6	<0.0001
	2	wild type, *cdc-25.1* RNAi	10	C
		skpo-1, *cdc-25.1* RNAi	8	<0.0001
	3	wild type, *cdc-25.1* RNAi	13	C
		skpo-1, *cdc-25.1* RNAi	8	=0.0016
	4	wild type, *cdc-25.1* RNAi	13	C
		skpo-1, *cdc-25.1* RNAi	8	=0.0005
	5	wild type, *cdc-25.1* RNAi	13	C
		skpo-1, *cdc-25.1* RNAi	7	<0.0001
3.10A	1	*wrt-2*, VC RNAi	5	C
		wrt-2, *skpo-1* RNAi	4	=0.0002
	2	*wrt-2*, VC RNAi	4	C
		wrt-2, *skpo-1* RNAi	3	=0.0176
	3	*wrt-2*, VC RNAi	5	C
		wrt-2, *skpo-1* RNAi	4	=0.0376
	4	*wrt-2*, VC RNAi	4	C
		wrt-2, *skpo-1* RNAi	4	=0.0058
3.10C	1	*vha-6*, VC RNAi	11	C
		vha-6, *skpo-1* RNAi	11	=0.6379
	2	*vha-6*, VC RNAi	11	C
		vha-6, *skpo-1* RNAi	11	=0.735
3.3	1	wild type, OP50	8	C
		*skpo-1**, OP50	3	<0.0001
	2	wild type, OP50	8	C
		*skpo-1**, OP50	3	<0.0001

C = control, *Data only shown for GF89 *skpo-1* strain.

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Table 3.3 Median survival and *P*-values of *E. coli* OP50 longevity assays

Figure number	Experiment number	Strain, exposure conditions during development	Median survival (Days)	*P*-value
3.1B	1	*eri-1*, VC RNAi	10	C
		eri-1, *skpo-1* RNAi	10	=0.3772
	2	*eri-1*, VC RNAi	10	C
		eri-1, *skpo-1* RNAi	10	=0.8948
3.1D	1	wild type, *cdc-25.1* RNAi	10	C
		skpo-1, *cdc-25.1* RNAi	8	<0.0001
	2	wild type, *cdc-25.1* RNAi	14	C
		skpo-1, *cdc-25.1* RNAi	11	<0.0001
	3	wild type, *cdc-25.1* RNAi	14	C
		skpo-1, *cdc-25.1* RNAi	11	<0.0001
3.10B	1	*vha-6*, VC RNAi	10	C
		vha-6, *skpo-1* RNAi	10	=0.9997
	2	*vha-6*, VC RNAi	11	C
		vha-6, *skpo-1* RNAi	10	=0.4484
3.10D	1	*wrt-2*, VC RNAi	11	C
		wrt-2, *skpo-1* RNAi	11	=0.6379
	2	*wrt-2*, VC RNAi	11	C
		wrt-2, *skpo-1* RNAi	11	=0.7235
3.4	1	wild type, OP50	14	C
		skpo-1, OP50	11	<0.0001
	2	wild type, OP50	14	C
		skpo-1, OP50	11	<0.0001
3.5*	1	N2, *cdc-25.1* RNAi*	16	C
		skpo-1, *cdc-25.1* RNAi*	13	<0.0001
	2	N2, *cdc-25.1* RNAi*	14	C
		skpo-1, *cdc-25.1* RNAi*	12	<0.0001

C = control, *Assayed on heat-killed OP50

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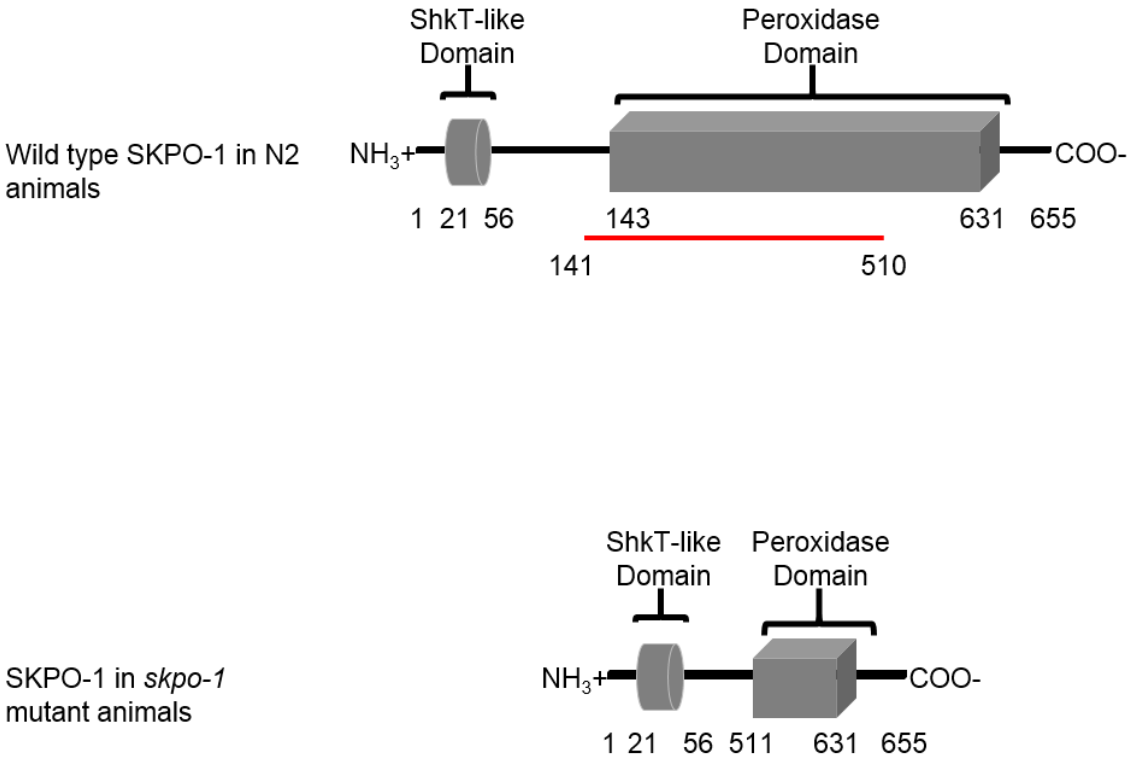

Figure 3.2 Wild type and mutant SKPO-1 protein domain cartoon. Cartoon depiction of the protein domains in both the wild type (*Top)* and mutant (*Bot.*) SKPO-1 protein. Red line indicates the amino acids (141 through 510) deleted as a result of EMS mutagenesis due to the host's double-stranded DNA break repair mechanism (unpublished).

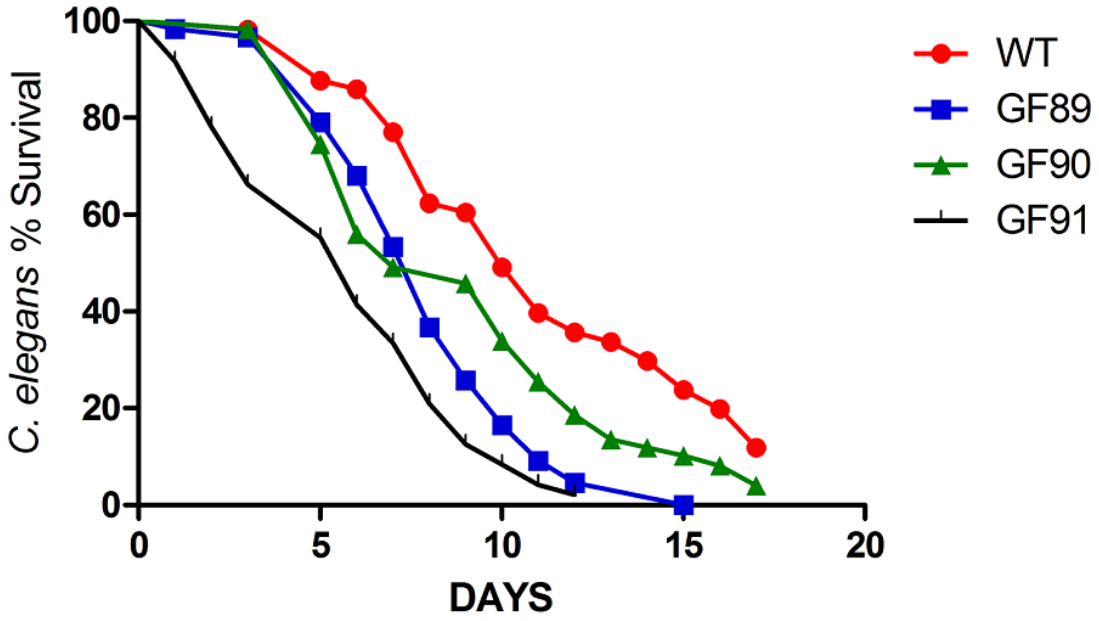

Figure 3.3 Survival of *cdc-25.1* RNAi-treated *skpo-1* mutant animal lines on *E. faecalis*. Wild type and *skpo-1* mutant worms were grown on *cdc-25.1* dsRNA expressing *E. coli* HT115 from L1 through L4 larval stage prior to exposure to *E. faecalis* OG1RF. The *skpo-1* mutant worms (GF89, GF90 and GF91) were more susceptible to *E. faecalis* relative to wild type (*P*-values < 0.0001, 0.0110, and < 0.0001, respectively).

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As I was concerned that this "bagging" was artificially enhancing their sensitivity to *E. faecalis*, thus *cdc-25.1* RNAi was employed to induce sterility, as done in previous *C. elegans* pathogenesis studies (IRAZOQUI *et al.* 2008), (SHAPIRA *et al.* 2006), (VAN DER HOEVEN *et al.* 2011) (Figure 3.1 C-E). *skpo-1* mutant animals grown on *cdc-25.1* dsRNA-expressing *E. coli* HT115 did not display this maternal bagging phenotype when they were subsequently exposed to *E. faecalis*. *cdc-25.1* RNAi-exposed *skpo-1* mutant worms retained a more pronounced susceptibility phenotype to *E. faecalis* relative to similarly exposed wild type animals ($P < 0.0001$) (Figure 3.1C and Table 3.2). This result suggests that the pathogen sensitivity is not explained by a maternal bagging phenotype.

Regardless of whether *skpo-1* mutant worms were sterile, *i.e. cdc-25.1*-treated (Figure 3.1D and Table 3.3), or fecund (Figure 3.4 and Table 3.3), they displayed a slight reduction in lifespan, relative to wild type, when exposed to live *E. coli* OP50 ($P < 0.0001$, for both). Because live OP50 has been shown to have slightly pathogenic effects (GARIGAN *et al.* 2002), I also examined lifespan on heat-killed OP50 and found that *cdc-25.1* RNAi-exposed *skpo-1* mutant worms still displayed a reduction in lifespan (Figure 3.5 and Table 3.3). To determine if the reduction in lifespan completely accounted for the pathogen sensitivity or not, the relative mortality of *skpo-1* mutant worms, compared to wild type, was calculated as in previous work (CHAVEZ *et al.* 2007), (TENOR *et al.* 2004). Relative mortality is the ratio of the LT50 of pathogen-infected animals to uninfected animals with the ratio of wild type animals normalized to one and has been used in previous work to analyze susceptibility phenotypes (CHAVEZ *et al.* 2007; TENOR *et al.* 2004). The survival defect for the *skpo-1* mutant animals on *E. faecalis* was more severe than on non-pathogenic *E. coli* compared to wild type animals ($P = 0.0091$) (Figure 3.1E and Table 3.4). These data argue against the general fitness defect fully explaining the susceptibility phenotype to *E. faecalis*.

To further examine the extent of the susceptibility-to-pathogen phenotype and determine if it was pathogen specific, I used *P. aeruginosa* instead of *E. faecalis* in the killing assay (P = 0.3783) (Figure 3.1F and Table 3.5). I observed no significant difference in survival between *cdc-25.1* RNAi *skpo-1* mutants and *cdc-25.1* RNAi wild type animals when exposed to *P. aeruginosa*. These results suggest that the function of SKPO-1 may be beneficial during infection with some, but not all pathogens.

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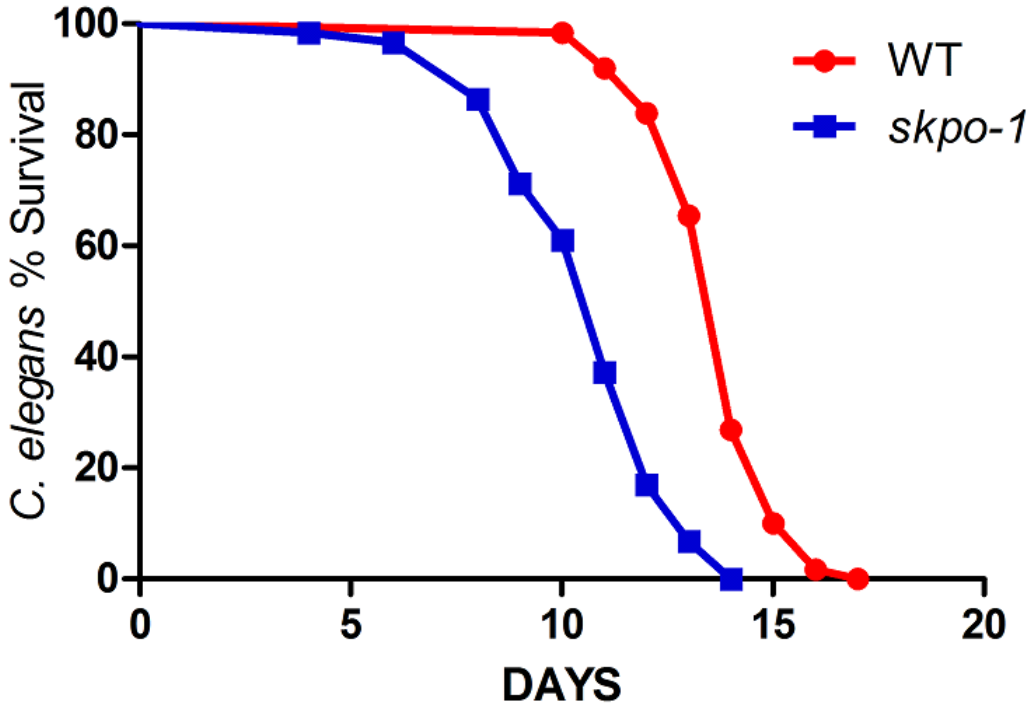

Figure 3.4 Lifespan defect of *skpo-1* mutants on *E. coli* OP50 without prior *cdc-25.1* exposure. Wild type and *skpo-1* mutant worms were grown on *E. coli* OP50 from L1 through L4 larval stage prior to the longevity assay in which they were transferred to NGM plates with FUDR (100 µg/ml) seeded with concentrated (20X) *E. coli* OP50. The average lifespan of the *skpo-1* mutant worms was significantly shorter than their wild type, counterparts (*P*-value < 0.0001).

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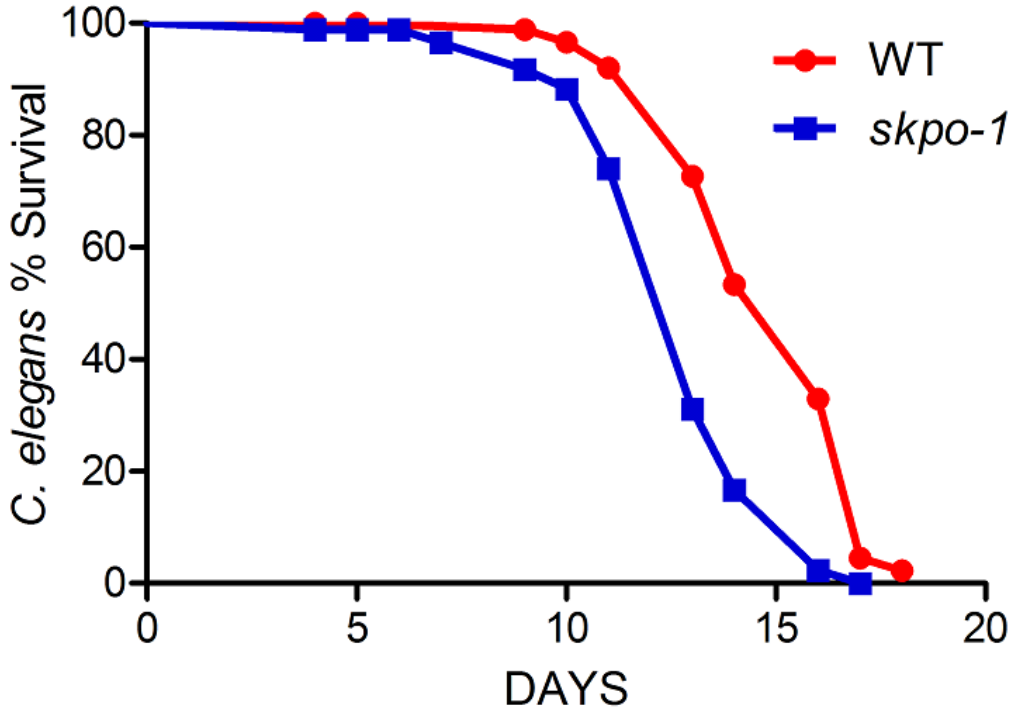

Figure 3.5 *skpo-1* mutants display a lifespan defect on heat-killed *E. coli* OP50. Wild type and *skpo-1* mutant worms were grown on *cdc-25.1* dsRNA expressing *E. coli* HT115 from L1 through L4 larval stage prior to the longevity assay. The worms were transferred to NGM plates like in Figure 3.4; however, the *E. coli* OP50 had been heat-killed. We observed that the *skpo-1* mutant worms displayed a longevity defect on heat-killed OP50, relative to wild type worms (*P*-value <0.0001).

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Table 3.4 Data for Relative Mortality Calculation

Figure number	Experiment number	Strain	Relative mortality	*P*-value
3.1E	Average of experiments 1-4	N2	1 1 1 1	
			Avg = 1	C
		skpo-1	1.312 1.452 1.654 1.253	
			Avg = 1.418	=0.0091

C = control

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Table 3.5 Median survival and *P*-values of *P. aeruginosa* PA14 killing assays

Figure Number	Experiment number	Strain, exposure conditions during development	Median survival (Hours)	*P*-value
3.1F	1	N2, *cdc-25.1* RNAi	91	C
		skpo-1, *cdc-25.1* RNAi	88	=0.3783
	2	N2, *cdc-25.1* RNAi	89	C
		skpo-1, *cdc-25.1* RNAi	69	=0.0639
	3	N2, *cdc-25.1* RNAi	72	C
		skpo-1, *cdc-25.1* RNAi	72	=0.2613

C = control

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To determine if the susceptibility phenotype on *E. faecalis* was a result of changes in pathogen burden, I assessed how many colony-forming units (CFUs) were in the intestines of the infected worms. Wild type and *skpo-1* mutant animals were raised on *E. coli* OP50 until L4 and then exposed to *E. faecalis* for 12 or 36 hours. The infected worms were washed, to remove surface bacteria, homogenized, and then serial dilutions of the homogenates were spread onto Gentamycin-containing BHI plates (thus killing *E. coli* OP50) and incubated 37˚ C. I observed no significant difference in CFUs per worm between the wild type and *skpo-1* mutant animals at either time point (Figure 3.6). The result suggests that the susceptibility phenotype of the *skpo-1* mutant cannot be explained by an increased load of bacteria in the intestine.

To test for alterations in the immune response between the *skpo-1* mutant and wild type animals a small panel of genes known to be upregulated in response to *E. faecalis* were examined, *clec-35*, *42*, *60*, and *71.* These genes encode C-type lectins (*clec*), many of which are upregulated in response to pathogens (DIERKING *et al.* 2011), (IRAZOQUI *et al.* 2010a). I investigated if these genes were differentially regulated in the *skpo-1* mutant, which could be indicative of an altered immune response. Sterile (by exposure to *cdc-25.1* RNAi) wild type and *skpo-1* mutant animals at the L4 stage were exposed to *E. faecalis* or *E. coli* for 18 hours, at which point the animals were lysed and RNA was extracted. By qRT-PCR no significant difference in fold induction for any of the *clec* genes was observed between the wild type and *skpo-1* mutants exposed to *E. faecalis* (Figure 3.7).Thus, loss of *skpo-1* does not significantly affect fold induction of the *clec* genes tested.

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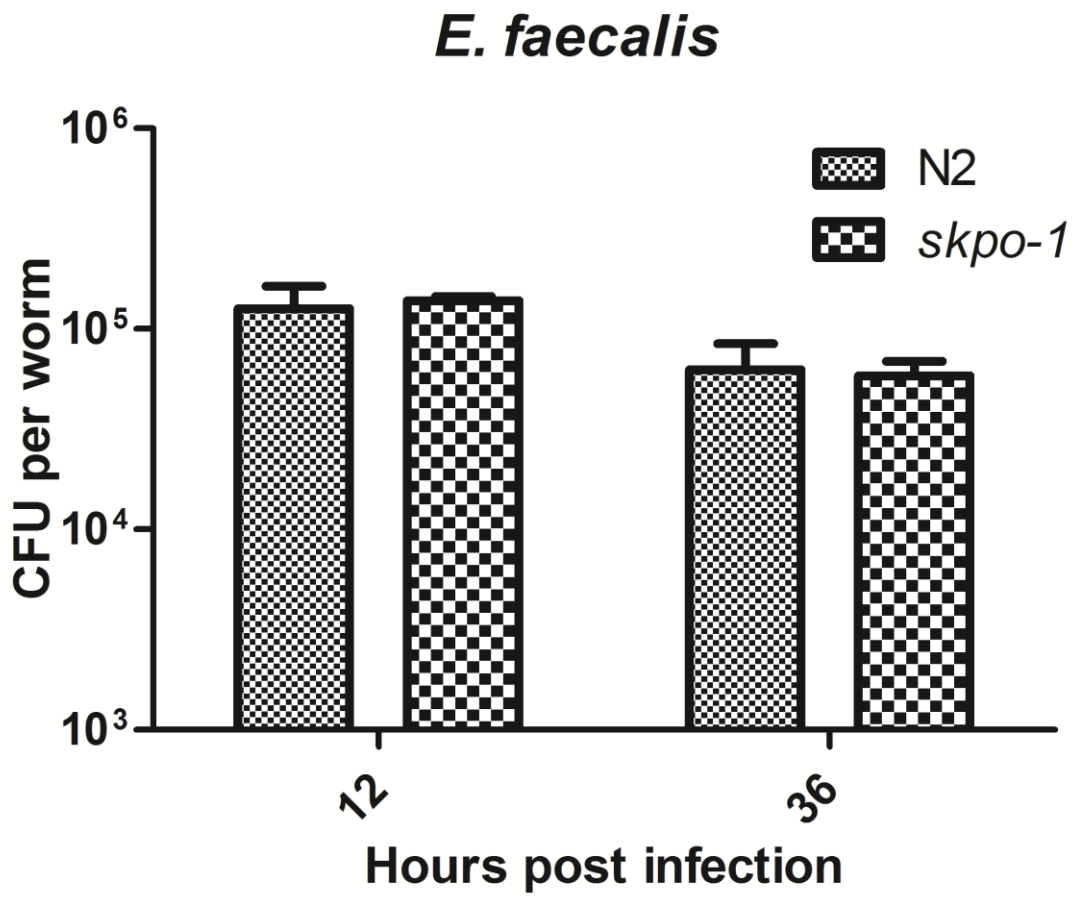

Figure 3.6 The *skpo-1* mutant does not have increased intestinal bacterial load during *E. faecalis* infection. L4 wild type and *skpo-1* mutant animals were exposed to *E. faecalis* at 25˚ C for 12 or 36 hours, respectively. CFU values for both wild type and *skpo-1* mutant animals indicated that there is no significant difference in intestinal colonization by *E. faecalis*. Three biological replicates for each strain were used per time point and the experiment repeated twice—*i.e* a total n = 60 per strain. The graph is an average of the two independent experiments. *P*-values were 0.3913 and 0.3592 for 12 and 36 hours, respectively.

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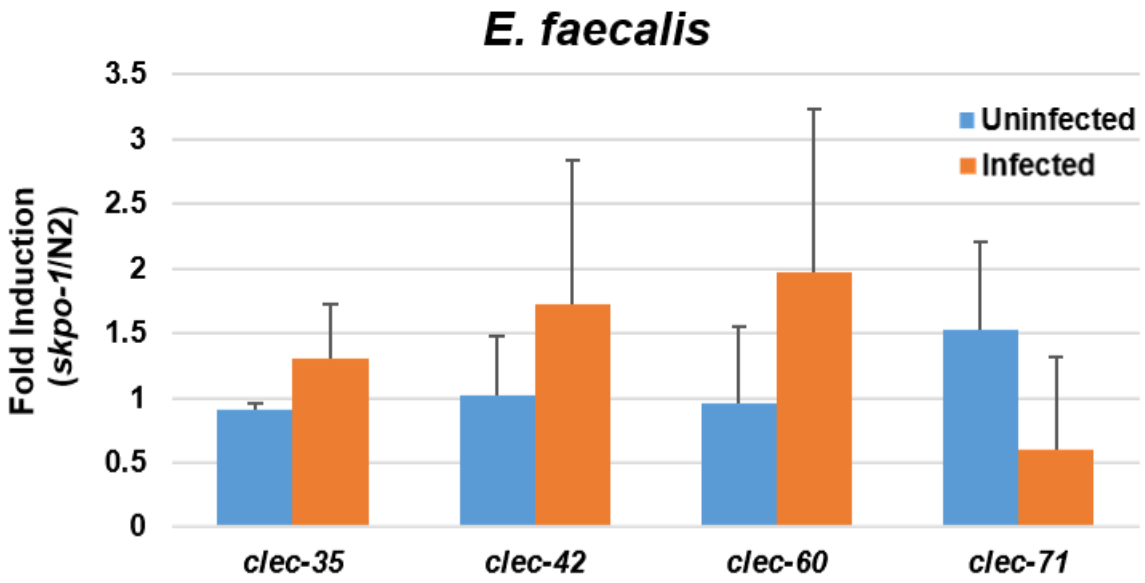

Figure 3.7 Fold induction of selected *clec* genes are not significantly altered in *skpo-1* mutants. L4 wild type and *skpo-1* mutant animals were exposed to *E. faecalis* for 18 hours at 25˚ C. Three technical replicates per strain per gene were assayed for relative expression of innate immune response genes and the experiment was repeated, independently, twice. The graph represents the average of the two independent experiments. These genes were previously found to display increased expression in wild type animals in response to *E. faecalis* (WONG *et al.* 2007). None of the *clec* genes assayed displayed altered fold induction in (un)infected *skpo-1* mutants relative to N2 animals. *clec* gene expression was normalized to *act-1. P*-values were 0.2203, 0.1868, 0.1396, and 0.4828 for *clec-35, 42, 60* and *71*, respectively (unpublished).

Morphological characterization of *skpo-1* mutant animals

Animals deficient in the peroxidase *mlt-7* displayed altered morphology, including a *bli* (blistered) phenotype, indicative of incomplete cross-linking of the cuticle (THEIN *et al.* 2009). The phenotype strongly suggests that MLT-7's peroxidase activity contributes to cuticle formation (THEIN *et al.* 2009). Interestingly, despite this rather dramatic cuticle defect, an increase in susceptibility to *E. faecalis* was not observed (Table 3.1). To determine if SKPO-1 is also involved in cuticle biogenesis I observed the morphology of *skpo-1* RNAi and mutant animals. RNAi of *skpo-1* did not result in any visible morphological change in the *eri-1* mutant animals (Figure 3.8B). However, the *skpo-1* mutant did display some morphological phenotypes. I observed young adult animals under the dissecting microscope (N = 300) and found that they ranged from very dumpy (16%), to slightly dumpy (33%), to wild type (51%) in appearance (Figure 3.8C – E). This *dpy* phenotype range is apparent from L1 onwards. Interestingly, regardless of the hermaphrodite parent's morphotype that sired the brood, I observed the aforementioned ratio of very dumpy, slightly dumpy and wild type in its progeny—which suggests incomplete penetrance. Additionally, no blistering of the cuticle was ever observed. Because a dumpy phenotype can be associated with cuticle defects (THEIN *et al.* 2009), these results suggest that SKPO-1 may have some role in cuticle biogenesis, but one that is different than MLT-7.

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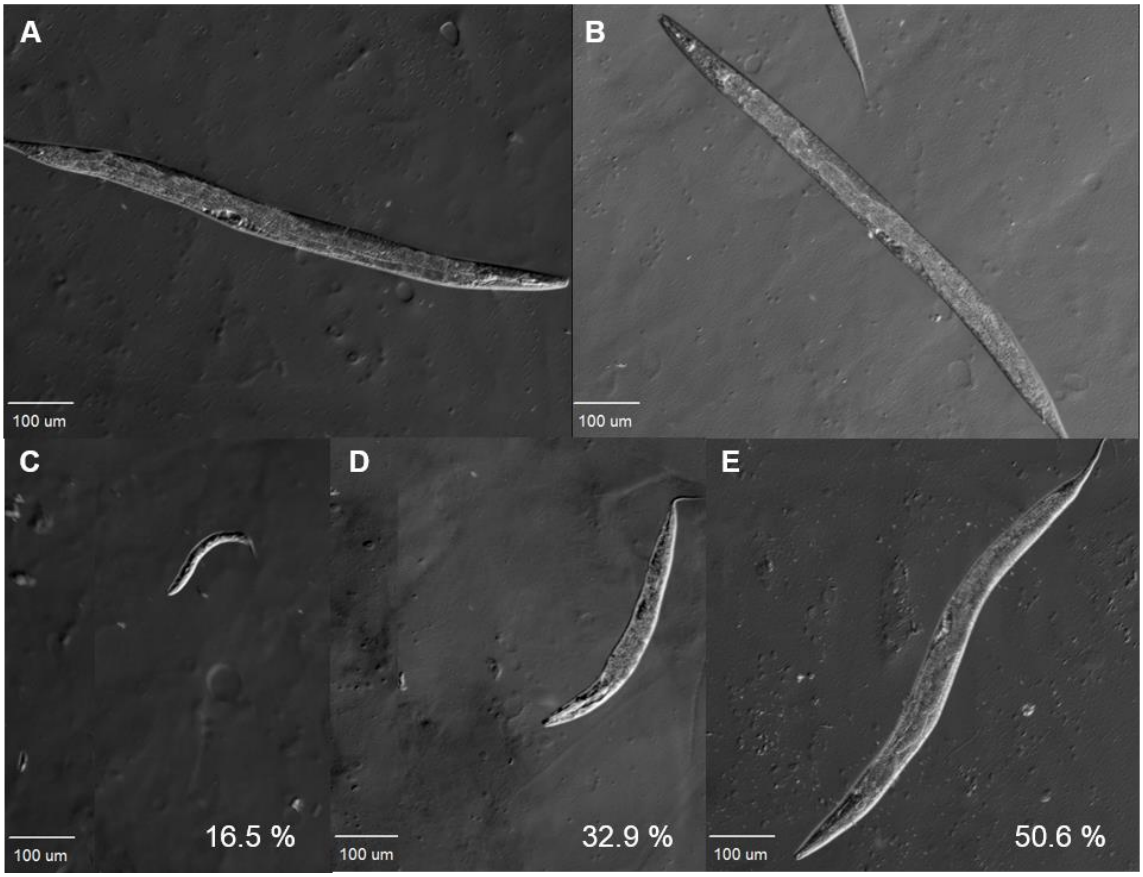

Figure 3.8 *skpo-1* mutant morphology suggests incomplete penetrance. (A) Wild type young adult representing the approximate average size and morphology of a typical *C. elegans*. (B) *skpo-1* RNAi young adult raised on *skpo-1* dsRNA expressing *E. coli* HT115 from L1 through L4 stage. (C – E) Young adult *skpo-1* mutants ranged from very dumpy to wild type in size. 10X microscopy images are representative of >100 wild type, *skpo-1* RNAi and *skpo-1* mutant worms observed, respectively.

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SKPO-1 features and activity

As mentioned, SKPO-1 contains a ShkT-like domain at its N-terminus from approximately residues 21 to 56 (www.WormBase.org) in addition to the predicted peroxidase domain (Figure 3.9A). It shares this feature with MLT-7 (THEIN *et al.* 2009), and putative peroxidases C16C8.2 and F32A5.2 (Table 3.1). For this reason I propose naming F49E12.1, SKPO-1, and C16C8.2 and F32A5.2, SKPO-2 and SKPO-3 for ShkT-containing peroxidase. In addition to the ShkT-like domain, the very 5' end contains a predicted signal sequence for secretion, and cleavage is predicted to occur at Ser19 (www.predisi.de).

To examine SKPO-1's peroxidase domain, I aligned it with other well-characterized peroxidase domains-of-interest, specifically those contained in hMPO, BLI-3 and MLT-7. Upon alignment, I observed SKPO-1 possesses the catalytic triad (distal histidine, H^{222}; arginine, R^{332}; and proximal histidine, H^{428}) necessary for peroxidase activity (ORTIZ DE MONTELLANO 2008). However, SKPO-1 lacks the residues necessary for covalent heme-binding, characteristic of the animal heme peroxidase family (ORTIZ DE MONTELLANO 2008). Interestingly, human Duox1/2 (hDuox1/2) also lack the conserved aspartic and glutamic residues required for covalent linkage of the heme prosthetic group; however, both of these recombinant peroxidase domains still bind heme, albeit weakly (MEITZLER and ORTIZ DE MONTELLANO 2009; MEITZLER and ORTIZ DE MONTELLANO 2011). This non-covalent binding may explain MLT-7's peroxidase activity even though MLT-7 lacks these covalent heme-binding residues (THEIN *et al.* 2009).

By comparison, SKPO-1 possesses all of the highlighted residues in MLT-7 (Figure 3.9A) and shares significant identity with hMPO (34% identity). Thus, I decided to indirectly assay SKPO-1's potential peroxidase activity using an Amplex Red assay, modified for whole animals, that detects H_2O_2 (CHAVEZ *et al.* 2007), (CHAVEZ *et al.* 2009). Previously, our laboratory observed that exposure to *E. faecalis* causes a significant release in H_2O_2 dependent on the NADPH oxidase, BLI-3 (CHAVEZ *et al.* 2007), (CHAVEZ *et al.* 2009). Thus,

I reasoned that loss of an important peroxidase during this release might increase the amount of H_2O_2 detected. After infecting VC and *skpo-1* RNAi *eri-1* mutant animals with *E. faecalis* for twelve hours, released H_2O_2 was measured (Figure 3.9B). As hypothesized, *skpo-1* RNAi *eri-1* mutant animals released significantly greater ($P = 0.0091$) amounts of H_2O_2, relative to VC RNAi *eri-1* mutants, consistent with the loss of a predicted H_2O_2 sink. The same result was observed with the *skpo-1* mutants compared to wild type animals ($P < 0.0001$) (Figure 3.9C). The difference in H_2O_2 detected required *E. faecalis*, and the concomitant release of H_2O_2, as no significant differences were observed between wild type and *skpo-1* deficient animals on *E. coli* (Figures 3.9B and 3.9C).

As mentioned earlier our laboratory previously observed that BLI-3 was necessary for the increase in H_2O_2 levels in response to infection by using the Amplex Red assay in conjunction with a NADPH oxidase inhibitor (CHAVEZ *et al.* 2007). Also, our laboratory demonstrated, previously, that BLI-3 is the only expressed NADPH oxidase encoded by the genome (CHAVEZ *et al.* 2009), and diphenyleneiodonium chloride (DPI) can be utilized to reduce its activity in wild type animals (CHAVEZ *et al.* 2007). Therefore to determine if the increased amount of H_2O_2 produced by the *skpo*-1 mutant during infection was dependent on BLI-3 activity, I added the NADPH oxidase inhibitor, DPI, to the assay. As seen in Figure 3.9C, DPI abrogated the enhanced H_2O_2 generation observed in *skpo-1* mutant animals. Taken together, Figure 3.9 supports my hypothesis that SKPO-1 is a peroxidase that utilizes H_2O_2 produced by BLI-3 during infection. Additionally, my attempts to purify the protein to demonstrate definitive peroxidase activity *in vitro* were not successful and will be discussed in Chapter 4.

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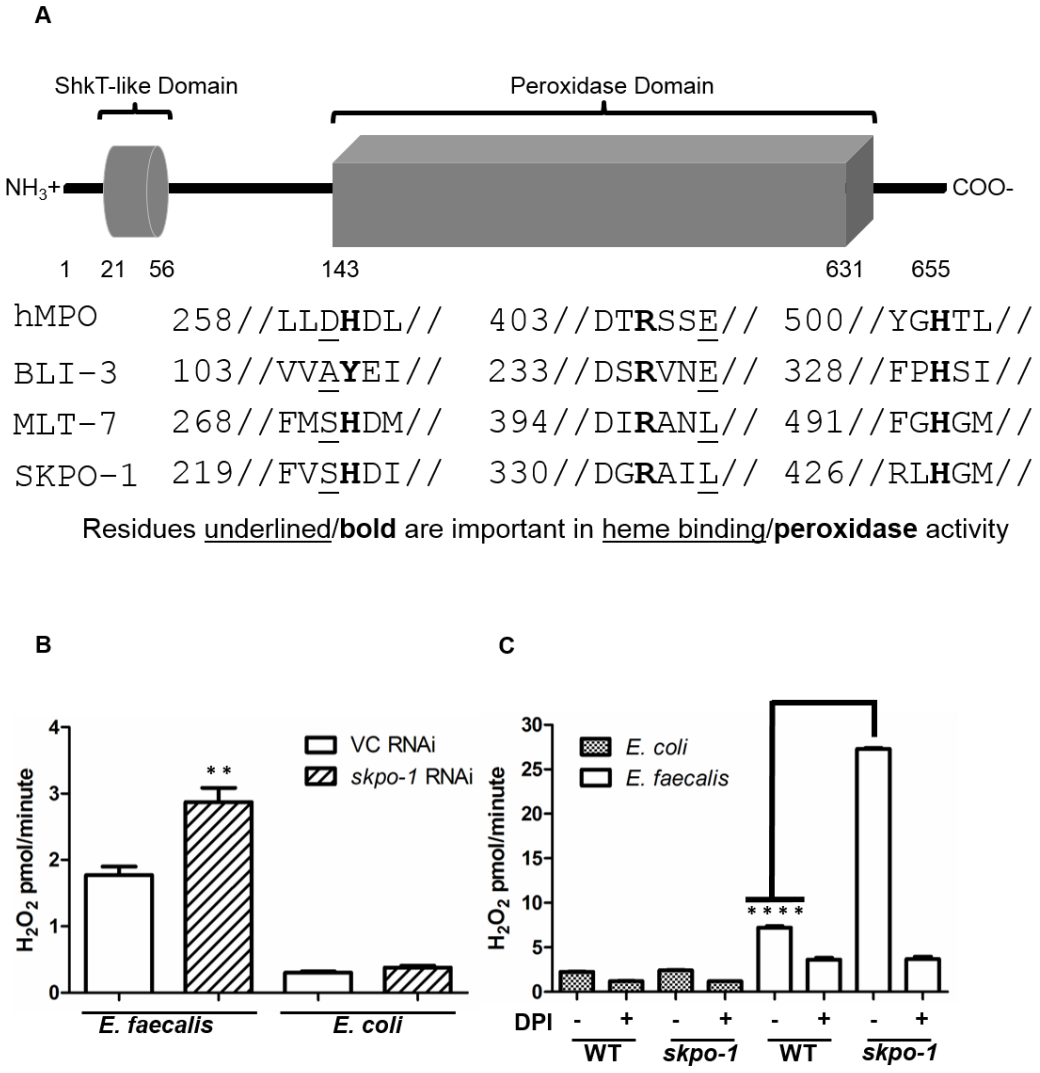

Figure 3.9 Evidence that *skpo-1* is a potential peroxidase. (A) Peroxidase domain sequences were aligned against the putative peroxidase domain of SKPO-1. SKPO-1 possesses the distal histidine (H^{222}), catalytic arginine (R^{332}), and proximal histidine (H^{428}), which are necessary for peroxidase activity. However, SKPO-1 lacks covalent heme-binding residues (S^{221} and L^{335}) that are characteristic of mammalian peroxidases (ORTIZ DE MONTELLANO 2008). (B) *eri-1* mutant worms were grown on VC RNAi or *skpo-1* RNAi prior to exposure with either *E. coli* or *E. faecalis* for twelve hours at 25˚C. (C) Wild type and

skpo-1 mutant worms were grown on *cdc-25.1* RNAi prior to exposure with either *E. coli* or *E. faecalis* for twelve hours at 25˚C. (B-C) Following exposure to *E. coli* or *E. faecalis* the amount of H_2O_2 produced per minute was determined using the Amplex Red assay. Error bars represent the S.E.M. and the asterisks indicate significant differences between *eri-1* worms exposed to VC RNAi or *skpo-1* RNAi that were infected with *E. faecalis* as well as between wild type and *skpo-1* mutant worms exposed to *cdc-25.1* RNAi prior to infection with *E. faecalis* [*P* = 0.0091 and *P* = <0.0001 (B) and (C), respectively]. Additionally, wild type and *skpo*-1 mutant worms were exposed to 80 uM DPI (diphenyleneiodinium chloride) and H_2O_2 levels were calculated for both *E. coli* and *E. faecalis* exposed animals (E.C.: *P* = 0.0752; E.F.: *P* = 0.4161, respectively) *P*-values were calculated via Student's paired t-test. Data in (B) and (C) are representative of at least two independent replicates.

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SKPO-1 localizes to the hypodermis

In the wild, *C. elegans* is found both in the soil and on fruits, therefore its cuticle is in constant contact with its surrounding environment which includes nonpathogenic and pathogenic microbes. In the laboratory setting, *C. elegans* interfaces with *E. faecalis* at the cuticle, synthesized by the underlying hypodermis, as it crawls through the pathogen lawn, and in its intestine, due to ingestion of the bacteria (GARSIN *et al.* 2001). It has previously been demonstrated that *C. elegans* mounts immune responses at these host-pathogen boundaries. Additionally, these immune responses depend on the pathogen and the nature of the infection (IRAZOQUI *et al.* 2010a), (PUJOL *et al.* 2008), (WONG *et al.* 2007). To address what tissue SKPO-1's activity is required for normal levels of resistance to *E. faecalis*, a tissue-specific RNAi approach was employed. RNAi-defective strains of *C. elegans* were used in which RNAi activity was genetically restored to specific tissues through intestinal or hypodermal specific promoters (*vha-6::SID-1* and *wrt-2::RDE-1*, respectively) (MELO and RUVKUN 2012). In Figures 3.10A and 3.10C (and Table 3.2), I tested the susceptibility of the hypodermal and intestinal-specific RNAi strains to *E. faecalis*.

As in Figure 3.1, these strains were exposed to VC and *skpo-1* RNAi prior to infection. In the hypodermal RNAi strain, I observed an enhanced susceptibility phenotype to *E. faecalis* in the *skpo-1* RNAi animals relative to VC RNAi ($P = 0.0002$); however, in the intestinal RNAi strain, no significant difference between *skpo-1* and VC RNAi animals was observed when on *E. faecalis* ($P = 0.9435$). On *E. coli*, no significant difference was observed between VC and *skpo-1* RNAi for either the hypodermal or intestinal RNAi line ($P = 0.9997$ and $P = 0.6379$; Figure 3.10B and 3.10D, respectively and Table 3.3). From these experiments I conclude that SKPO-1's functional activity during pathogen exposure is required in the *C. elegans* hypodermis.

To determine in which tissue SKPO-1 is produced, I used indirect immunofluorescence to visualize SKPO-1 localization in young adult animals raised under

standard conditions. The reason for using young adult animals is that L4 or young adults are typically used to study infectious processes. Using rabbits, a polyclonal primary antibody was raised against a chemically synthesized SKPO-1 peptide (see Materials & Methods). A freeze-cracking methodology was used to disrupt the cuticle of the animal and allow for internal fixation and staining (SEYDOUX and DUNN 1997). Following fixation, the samples were double stained with the polyclonal primary antibody to SKPO-1 and an Alexa Fluor 488 secondary antibody. Localization was clearly observed in the hypodermis of wild type animals, and an example is shown in Figure 3.11 (A-C and D-F; 40X and 10X, respectively). I observed strong fluorescent staining just under the outermost layer, which corresponds to the hypodermis. No internal organs showed evidence of staining, including the gonad and the intestine, which in this animal had become partially separated from the rest of the body. In contrast to wild type, *skpo-1* mutant animals showed no evidence of staining in any organ (Figure 3.11G-I).

By using a secondary antibody conjugated to 10 nm gold particles for immuno-gold labeling, we examined localization at higher resolution by transmission electron microscopy (TEM). As seen in Figure 3.11J, the black dots, indicating the gold particles, were located just under the cuticle layer in association with the hypodermal cells of wild type animals. Hardly any particles were observed in the *skpo-1* mutant animals (Figure 3.11K). The few observed, were randomly scattered. In total, these results demonstrate SKPO-1 is both physically present and functionally active in the *C. elegans* hypodermis.

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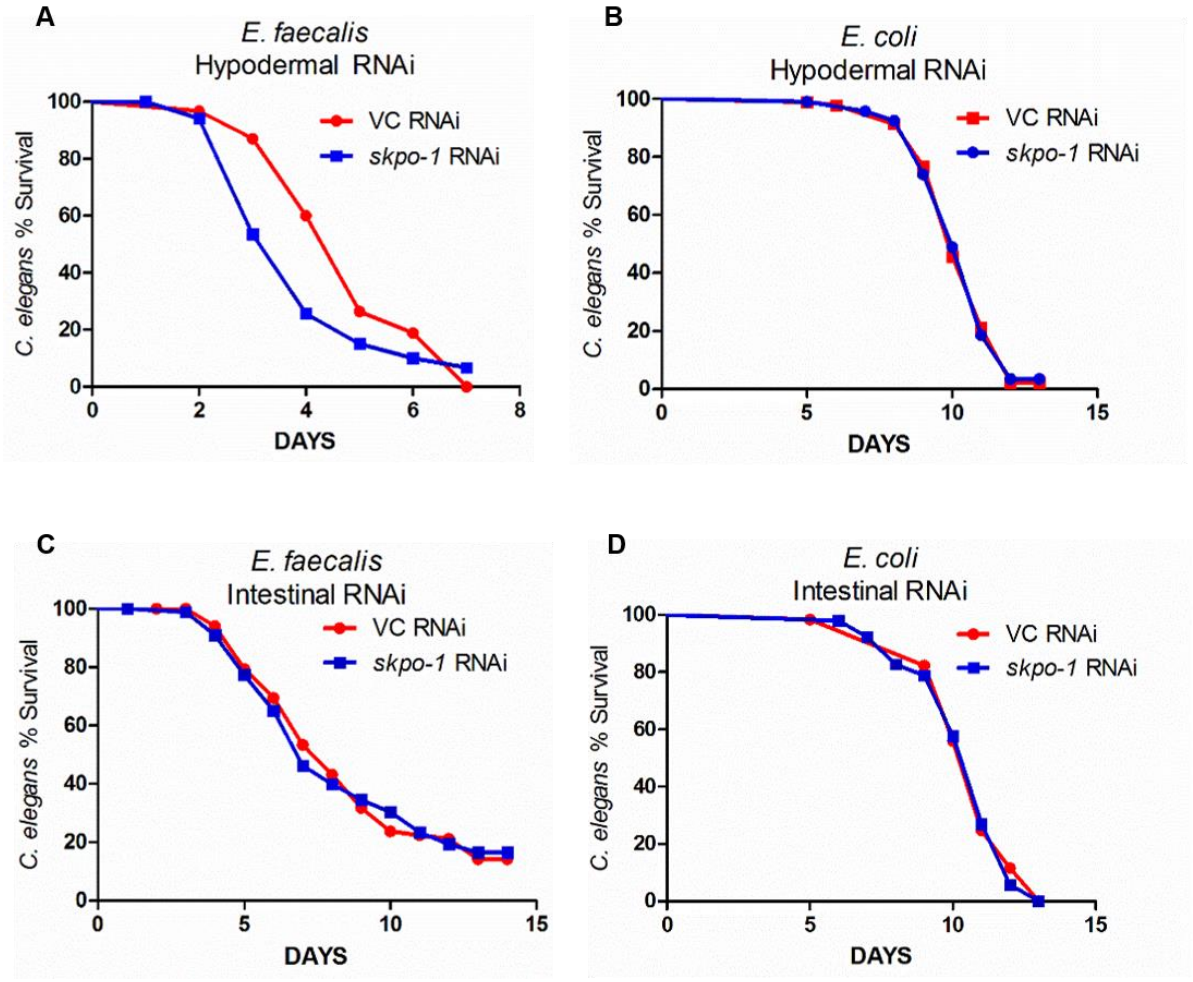

Figure 3.10 SKPO-1 is necessary in the hypodermis for resistance to *E. faecalis* infection. (A) Survival of the hypodermal RNAi strain on *E. faecalis* following exposure to VC RNAi or *skpo-1* RNAi ($P = 0.0002$). (B) Longevity assay on *E. coli* OP50 of the hypodermal RNAi strain following exposure to VC RNAi or *skpo-1* RNAi ($P = 0.9997$). (C) Survival of the intestinal RNAi strain on *E. faecalis* following exposure to VC RNAi or *skpo-1* RNAi ($P = 0.9435$). (D) Longevity assay of the intestinal RNAi strain on *E. coli* OP50 following exposure to VC RNAi or *skpo-1* RNAi ($P = 0.6379$).

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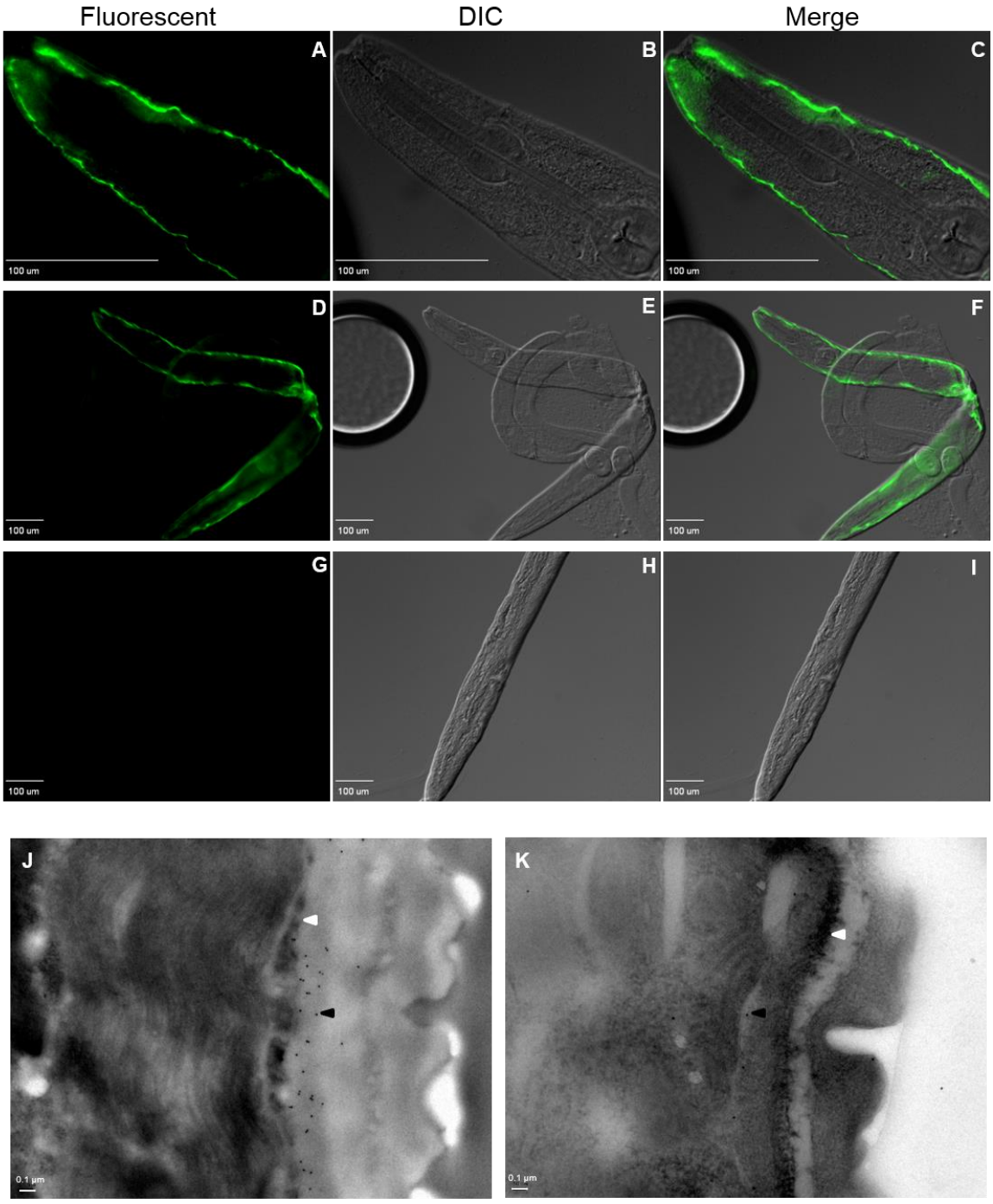

Figure 3.11 SKPO-1 localizes to the *C. elegans* hypodermis. (A-F, J) Wild type and (G-I, K) *skpo-1* mutant worms were immunostained with anti-SKPO-1 polyclonal antibodies and imaged using fluorescence (A-I) or transmission electron microscopy (J-K), respectively. (A-

C) 40X magnification of a wild type worm shows hypodermal SKPO-1 localization. (D-F) 10X magnification of a wild type worm. (G-I) 10X magnification of *skpo-1* mutant worm shows loss of SKPO-1 staining. (J) In wild type worms, the black dots, indicative of immuno-gold labeling, are localized beneath the cuticle layer, but external to the outer hypodermal cell surface. (K) In *skpo-1* mutant worms, very few black dots are observed. White arrowhead = apical hypodermal surface; black arrowhead = 10 nm gold-labeled secondary to SKPO-1). Microscopy images are representative of >100 (fluorescent) or >10 (TEM) wild type and *skpo-1* mutant worms observed. The TEM was performed by Steve Kolodziej.

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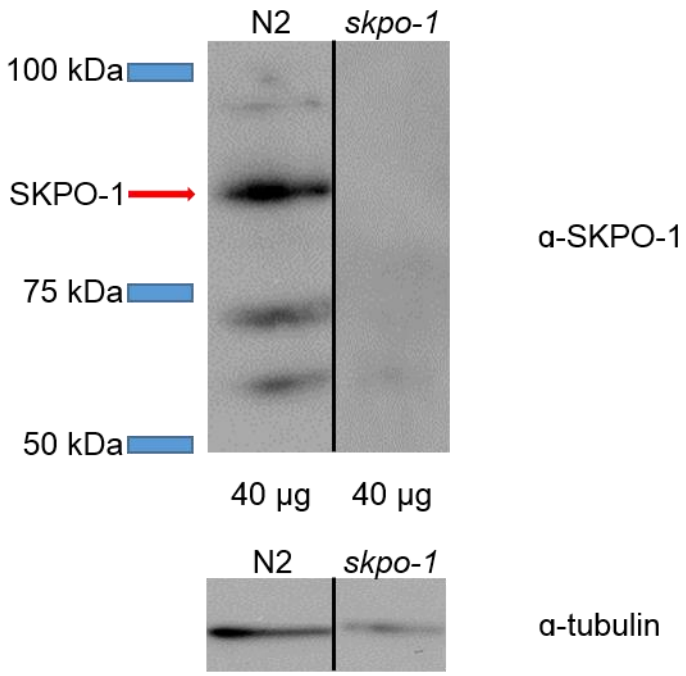

Figure 3.12 Immunoblot demonstrates SKPO-1 is absent in *skpo-1* mutant animals. L4 wild type and *skpo-1* mutant animals (grown on *E. coli* OP50 from eggs) were exposed to *E. coli* for 18 hours at 25˚ C prior to sonication. SKPO-1 was observed to between 75-100 kDa which is suggestive of post-translational modification as SKPO-1 is predicted to be 655 a.a. Black bar denotes that the lanes were cropped together from the same immunoblot. Immunoblot is representative of three independent biological replicates (unpublished data).

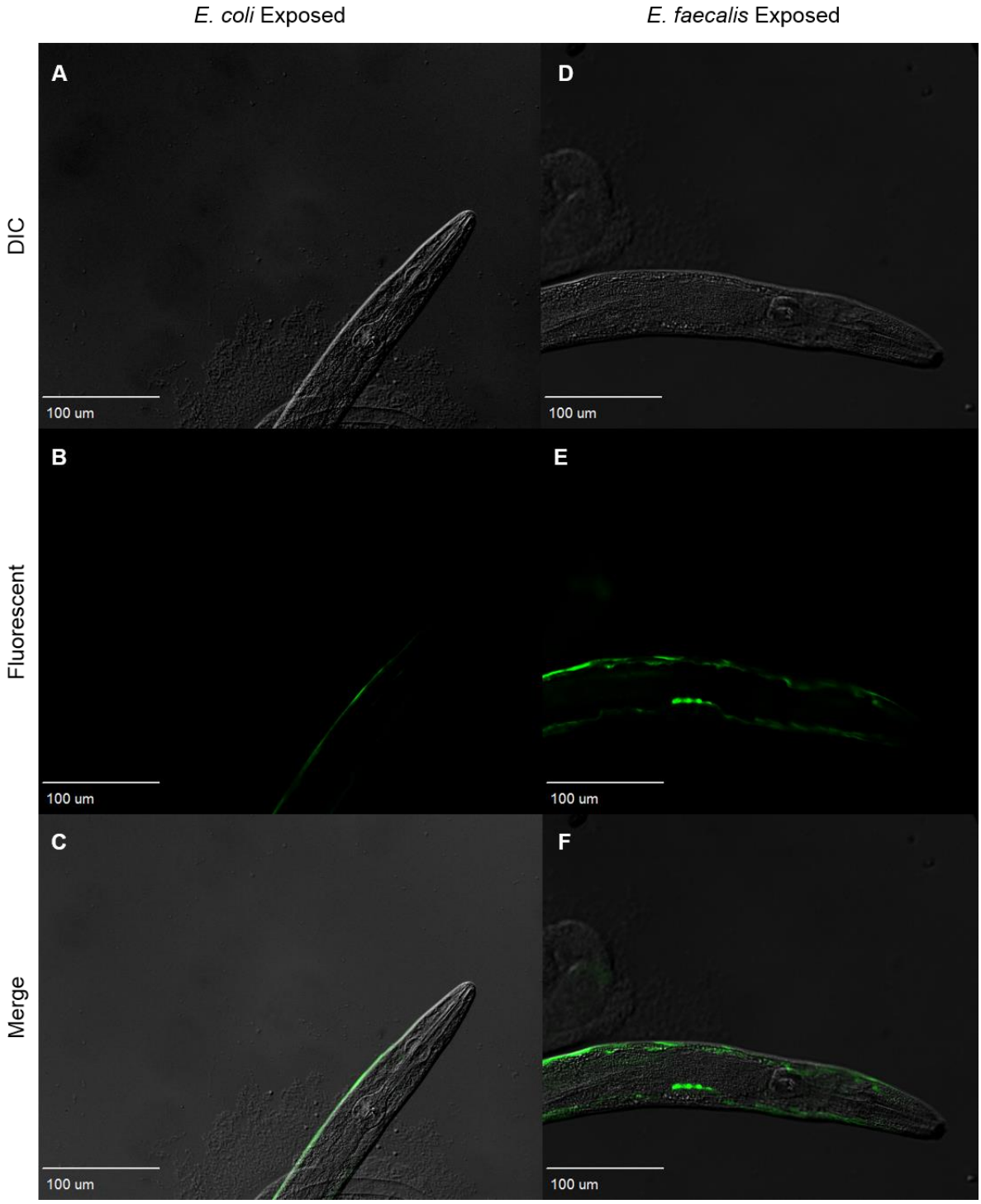

Figure 3.13 SKPO-1 hypodermal localization pattern is independent of infection. L4 wild type animals were exposed to *E. coli* or *E. faecalis* for 24 hours at 25˚ C and then

immunostained with anti-SKPO-1 to determine if SKPO-1's localization changes in response to infection. (A-C) Wild type animals exposed to *E. coli* displayed hypodermal localization of SKPO-1. (D-F) Wild type animals exposed to *E. faecalis* also revealed SKPO-1 to only localize to the hypodermis. All images are at 20X magnification. Microscopy images are representative of >100 fluorescent wild type worms observed (unpublished data).

To confirm that the α-SKPO-1 polyclonal antibody was specific to SKPO-1, I performed a Western blot on total worm lysates for wild type and *skpo-1* mutants. As shown in Figure 3.12, SKPO-1 was detected in wild type, uninfected, animals, but not in the *skpo-1* mutants. I postulate SKPO-1 may be post-translationally modified due to its molecular weight (MW) being higher than its predicted a.a. sequence (655)—*i.e.* 75-100 kDa vs. 73.5 kDa, respectively. Additionally, there are data that reveal certain animal heme peroxidases (hMPO) to be glycosylated, both experimentally and bioinformatically (hMPO; SKPO-1) ((FURTMULLER *et al.* 2006), (JOHNSON *et al.* 1987); data not shown).

Finally, to determine if the localization pattern of SKPO-1 was altered during infection I exposed L4 worms to either *E. coli* or *E. faecalis* for 24 hours, prior to immunostaining for SKPO-1. I observed that SKPO-1's hypodermal localization was independent of the host being uninfected or infected (Figure 3.13A-C and 3.13D-F, respectively). It is of note that, SKPO-1's localization to the hypodermis may also explain the dumpy phenotype, as mutations in cuticle proteins have been associated with this morphological phenotype (PAGE 2007) (Figure 3.8).

Conclusions and future directions

SKPO-1 is a functional peroxidase

In this chapter, I demonstrated that a previously unstudied *C. elegans* protein with a peroxidase domain, F49E12.1, plays a protective role during infection with *E. faecalis.* I named this protein SKPO-1 for ShkT-containing peroxidase, because it contains an N-terminal ShkT-like domain. The ShkT domain family was originally defined as a potassium channel blocker in the sea anemone (*Metridium senile*). Binding to the potassium channel requires two conserved residues that are not found in this particular ShkT-like domain of SKPO-1 or others from *C. elegans* (data not shown). It is postulated that the more general function of ShkT-like domains are as contact surfaces for protein interactions (TSANG *et al.*

2007). For this reason, SKPO-1 and other ShkT-containing peroxidases may be most closely related to the peroxidasins, subfamily 2 of the peroxidase-cyclooxygenase superfamily, and in fact, SKPO-1 was placed in this family by phylogenetic analysis (SOUDI *et al.* 2012). These peroxidase domain-containing proteins also have protein interaction domains, but they are typically type C-like immunoglobulin domains, leucine-rich repeats, or von Willebrand factor C modules. This is in contrast to the mammalian peroxidases, MPO, LPO and EPO that lack these extra domains and belong to subfamily 1 (ZAMOCKY *et al.* 2008). Additionally, it is thought that sub-family 1 evolved from sub-family 2 (ZAMOCKY *et al.* 2008). Other *C. elegans* peroxidase domain-containing proteins that have a ShkT-like domain include MLT-7 (THEIN *et al.* 2009), C16C8.2 and F32A5.2, but no study has yet addressed this domain's function in the context of a peroxidase.

An unexpected finding was that SKPO-1 is located in the hypodermis and is functionally protective in this tissue against *E. faecalis* infection, but not against *P. aeruginosa* which is another well-studied human pathogen in *C. elegans*. Infection of *C. elegans* with *E. faecalis* results in colonization of the gut, leading to distension of the intestinal lumen and clear signs of physical damage, such as effacement of the microvilli (CRUZ *et al.* 2013), (GARSIN *et al.* 2001). Though the worm is exposed to *E. faecalis* on its outer surface as it moves through the pathogen lawn, there is no notable colonization or characterized physical damage to the cuticle or hypodermis. In contrast, bacterial pathogens such as *Microbacterium nematophilum, Xenorhabdus nematophila* and *Yersinia pestis* adhere to and colonize the cuticle surface (DARBY *et al.* 2002), (HODGKIN *et al.* 2000), (MALLO *et al.* 2002). The natural fungal pathogen *Drechmeria coniospora* initially adheres to the cuticle and then penetrates the hypodermis whereas the human fungal pathogen *Candida albicans* first colonizes the intestine and eventually penetrates the cuticle from the inside out (BREGER *et al.* 2007), (JANSSON *et al.* 1985). Our laboratory previously showed that tissue-specific loss of *bli-3* in the hypodermis also increased

susceptibility of *C. elegans* to *E. faecalis* (CHAVEZ *et al.* 2009). Based on the protective effects of SKPO-1 and BLI-3 in this tissue, I postulate that a hypodermal immune response does play some role in protecting *C. elegans* during infection with *E. faecalis.* The question is by what mechanism?

Several models for how these proteins might exert their protective effects can be imagined. It could be that loss of *skpo-1* results in a weaker cuticle barrier that increases susceptibility. Alternatively, SKPO-1 could use H_2O_2 produced by BLI-3 to form more potent antioxidants, analogous to the human Duox/LPO system on mucosal surfaces (CONNER *et al.* 2002), (FORTEZA *et al.* 2005), (GEISZT *et al.* 2003a). Clearly, the amount of H_2O_2 is important. Our laboratory previously showed that loss of BLI-3 and the resulting decrease in H_2O_2 production caused an increase in susceptibility to infection (CHAVEZ *et al.* 2009), whereas in this chapter I show that loss of a peroxidase and a concurrent increase in H_2O_2 also increases susceptibility. A similar situation is apparent from studies of infection using the model host *Drosophila melanogaster* (HA *et al.* 2005a), (HA *et al.* 2005b). Loss of an intestinal DUOX enzyme or an intestinal catalase both increase susceptibility to infection, but with opposite effects on ROS levels in this tissue. It is possible that SKPO-1 is catalyzing the degradation of excess H_2O_2 to prevent host damage. Additionally, our laboratory demonstrated, by a variety of means, that infection causes oxidative stress in *C. elegans*, much of it dependent on BLI-3 activity (CHAVEZ *et al.* 2007), (MOHRI-SHIOMI and GARSIN 2008), (VAN DER HOEVEN *et al.* 2011).

Interestingly, there is evidence for a hypodermal response to several pathogens that are thought to mainly cause infection in the intestine. Microarray studies that examined the transcriptional responses of *C. elegans* to *E. faecalis, Serratia marcescens, Erwinia carotovora, Photorhabdus luminescens, S. aureus* and *P. aeruginosa* all noted a dramatic downregulation in the expression of genes related to cuticle biosynthesis, such as those encoding collagens (IRAZOQUI *et al.* 2010a), (WONG *et al.* 2007). The response is not

thought to be due to a general reduction of gene transcription in this tissue and may be indicative of several possibilities (WONG *et al.* 2007). The changes in transcription of the genes could be part of a protective response that is occurring in the hypodermis to protect against pathogens. Or the changes could be purposely caused by the pathogens as part of their virulence programs to damage the host. Alternatively, the changes in expression of the cuticle biosynthetic genes could be a neutral side effect resulting from alterations in signaling due to pathogen exposure. Overall, these studies suggest that pathogen exposure, even to those pathogens not thought to directly affect the cuticle, cause major changes in the expression of the cuticle biosynthetic genes that might be indicative of a response to the infection in the hypodermis.

In addition to the pathogen susceptibility phenotype, loss of *skpo-1* resulted in a dumpy phenotype of incomplete penetrance, suggestive of some role in cuticle biosynthesis (THEIN *et al.* 2009). In support of a functional peroxidase domain, I noted that SKPO-1 shares the same critical residues as MLT-7 in its active site (THEIN *et al.* 2009), and animals mutant for *skpo-1* produce significantly more H_2O_2 during infection. The functional and physical location for the protein was shown to be the hypodermis, which was surprising since *E. faecalis* has been characterized as infecting the intestine of *C. elegans* (CRUZ *et al.* 2013), (GARSIN *et al.* 2001). This result, along with other evidence from the literature (IRAZOQUI *et al.* 2010a), (WONG *et al.* 2007), suggests that the hypodermis plays an important role during exposure of *C. elegans* to many human pathogens that do not obviously colonize or damage the cuticle, warranting further investigation.

Chapter 4: Recombinant protein engineering, expression and purification of SKPO-1

Introduction

Recombinant protein engineering, expression, and purification of SKPO-1

In Chapter 3, I illustrated that live *skpo-1* mutants displayed increased H_2O_2 levels relative to wild type animals, when infected, by Amplex Red assay. This assay demonstrated that SKPO-1 was a peroxidase in an indirect manner. In order to directly determine if SKPO-1 possesses peroxidase activity and is capable of producing antimicrobial hypohalous acids to defend the host during infection, I decided to recombinantly express and purify SKPO-1. Traditionally, animal heme peroxidase domain-containing proteins such as MPO, LPO and DUOX are expressed in eukaryotes using a baculovirus system (MEITZLER and ORTIZ DE MONTELLANO 2009). Recently it was demonstrated that the BLI-3 peroxidase domain and the MLT-7 peroxidase displayed peroxidase activity following expression and purification from *E. coli* (EDENS *et al.* 2001), (THEIN *et al.* 2009). After conversing with members of the labs that claimed to have utilized *E. coli* to express these eukaryotic peroxidase domains, I decided to express a full-length, functional, SKPO-1 protein with a C-terminal HIS6 tag in *E. coli* BL21 (DE3) (Drs. Lambeth and Page, personal communications). If the SKPO-1 protein is insoluble, I could at least use it to generate a polyclonal antibody (pAb) and perform immunohistochemistry (IHC) in *C. elegans*. Additionally, if everything failed I would have the opportunity to express *skpo-1* in a eukaryotic system with the help of some potential collaborators.

Results

Recombinant protein expression in *E. coli*

Outlined below is the protein schema for how I expressed and purified SKPO-1::HIS6 from *E. coli* (Figure 4.1). Wild type, full-length, SKPO-1 cDNA was cloned into the

pET-29b(+) expression system using standard, molecular techniques and the primers listed in Table 2.2. For all pET constructs used in this study, I inoculated 500 mL of fresh LB media supplemented with Kanamycin (Kan; 50 ug/mL) with single isolated colonies. These cultures were allowed to incubate, shaking, for 15 hours at 37˚ C. 100 mL of each starter culture was then transferred to 900 mL of fresh LB Kan (50 ug/mL) and allowed to incubate, shaking, for 4 hours at 37˚ C. Thus, 2 liters of each plasmid was grown (uninduced and induced conditions). For induction, 1 mM IPTG (final concentration; isopropyl β-D-1-thiogalactopyranoside) was introduced into the corresponding cultures. All cultures were transferred to the 30˚ C shaking incubator and grown for an additional 3 hours prior to harvesting, chemical lysis and purification.

Cells were chemically lysed in B-PER buffer containing 80 ug/mL lysozyme, Roche® protease inhibitor cocktail tablet plus 1mM PMSF (phenylmethylsufonyl fluoride) and stored on ice when necessary to limit degradation of recombinant full-length SKPO-1::HIS6. Following ultracentrifugation and membrane pellet solubilization, I ran 5 uL of the crude extract, insoluble and soluble fractions for cells expressing SKPO-1::HIS6 pET-29b(+) and empty vector (uninduced and induced conditions). There was no difference in total protein levels between uninduced and induced conditions by Coomassie. Therefore, I omitted the uninduced samples from Figure 4.2. By Coomassie, I observed no discernible difference in total protein levels between empty vector and SKPO-1::HIS6-expressing pET-29b(+) in the crude extract, as well as the soluble and insoluble fractions (Figure 4.2). By using THE™ His Tag, mouse, monoclonal antibody (mAb) and a goat anti-mouse HRP-conjugated secondary, I was unable to detect the full-length SKPO-1::HIS6 expression product in all fractions (~74 kilodaltons; kDa). However, I detected two degradation products of SKPO-1::HIS6 running between ~37-49 kDa in the crude extract and insoluble, but not soluble, fraction.

Following further communication with Dr. Page, I decided to only express the SKPO-1 peroxidase domain with the C-terminal HIS6 tag. Dr. Page suggested that the ShkT-like domain may have impeded proper protein folding of SKPO-1 in *E. coli* (personal communication with Dr. Page). Similar to the full-length SKPO-1::HIS6 construct, I observed that the C-terminal HIS6-tagged SKPO-1 peroxidase domain was only detected in the crude extract and insoluble fraction (Figures 4.3-4.5). In addition to the previously observed degradation products, I observed a novel expression product from cells expressing the SKPO-1::HIS6 peroxidase domain as compared to the full-length version. The novel product was ~64 kDa which roughly corresponds with the predicted size of the SKPO-1::HIS6 peroxidase domain construct (~60 kDa) which possesses ~50 a.a. upstream and downstream of the peroxidase domain, but excludes the ShkT-like domain—modeled after the MLT-7 construct used by Thein *et al*. (THEIN *et al.* 2009).

I decided that the likelihood of obtaining a functionally active SKPO-1::HIS6 protein from *E. coli* was unlikely, and thus decided to purify the insoluble aggregate in order to have a polyclonal antibody raised against the SKPO-1 peroxidase domain. On the off chance that the HIS6 tag was solvent exposed, I attempted purification under native conditions as opposed to immediately attempting to purify the aggregate under denaturing conditions. The latter process would have required denaturation, as well as introducing hemin (heme precursor) in resolubilization buffers (ZONG *et al.* 1995). In order to purify the SKPO-1::HIS6 peroxidase domain, I used a TALON® purification protocol, a cobalt ion variant of the nickel-NTA (nitrilotriacetic acid) resin, and a native buffer combination previously used to purify a *C. elegans* HSP70 chaperone from *E. coli* (ODUNUGA *et al.* 2012). By Western blot, I observed that the C-terminal HIS6-tagged SKPO-1 peroxidase domain was detected in the crude extract pre- and post-TALON® incubation flow through (Figure 4.4). However, the SKPO-1::HIS6 peroxidase domain was absent in the subsequent wash and eluate fractions.

Additionally, progressive increases in the imidazole concentration did not result in elution of SKPO-1::HIS6 peroxidase domain. These results suggest that the HIS6 tag is not solvent exposed and thus cannot bind to the TALON® resin. Thus, the two alternatives were to attempt denaturation and resolubilization of the SKPO-1::HIS6 peroxidase domain or to clone the SKPO-1 peroxidase domain into the pET-28(a)+ vector, thus moving the position of the HIS6 tag to the N-terminus.

Using different primers (Table 2.2), I cloned the SKPO-1 peroxidase domain into the pEt-28a(+) vector to introduce the HIS6 tag at the protein's N-terminus and expressed the construct in *E. coli* BL21 (DE3) cells. The rationale for moving the position of the HIS6 tag was two-fold. While the HIS6 tag is small, it may have lowered the peroxidase domain's solubility at the C-terminus. Additionally, the HIS6 tag may interfere with the peroxidase domain's enzymatic activity, as the peroxidase domain is in the C-terminus of the protein. Unfortunately, like the other two attempts, the peroxidase domain was insoluble and degraded when expressed in *E. coli* as evidenced by Figure 4.5. Thus, it does not appear that the HIS6 tag's position is affecting the protein's stability in *E. coli*. Additionally, I was never able to detect SKPO-1 by Coomassie, only by Western blot, which is problematic for having a pAb raised against the insoluble SKPO-1 aggregate; most companies require 5 mg/mL of purified protein. I could have pursued denaturation and resolubilization of the protein or attempted expressing SKPO-1's peroxidase domain with a GST tag to increase solubility. However, I had already established a collaboration with Dr. Leto, who offered to express the SKPO-1 peroxidase using his baculovirus system (Dr. Leto, personal communication).

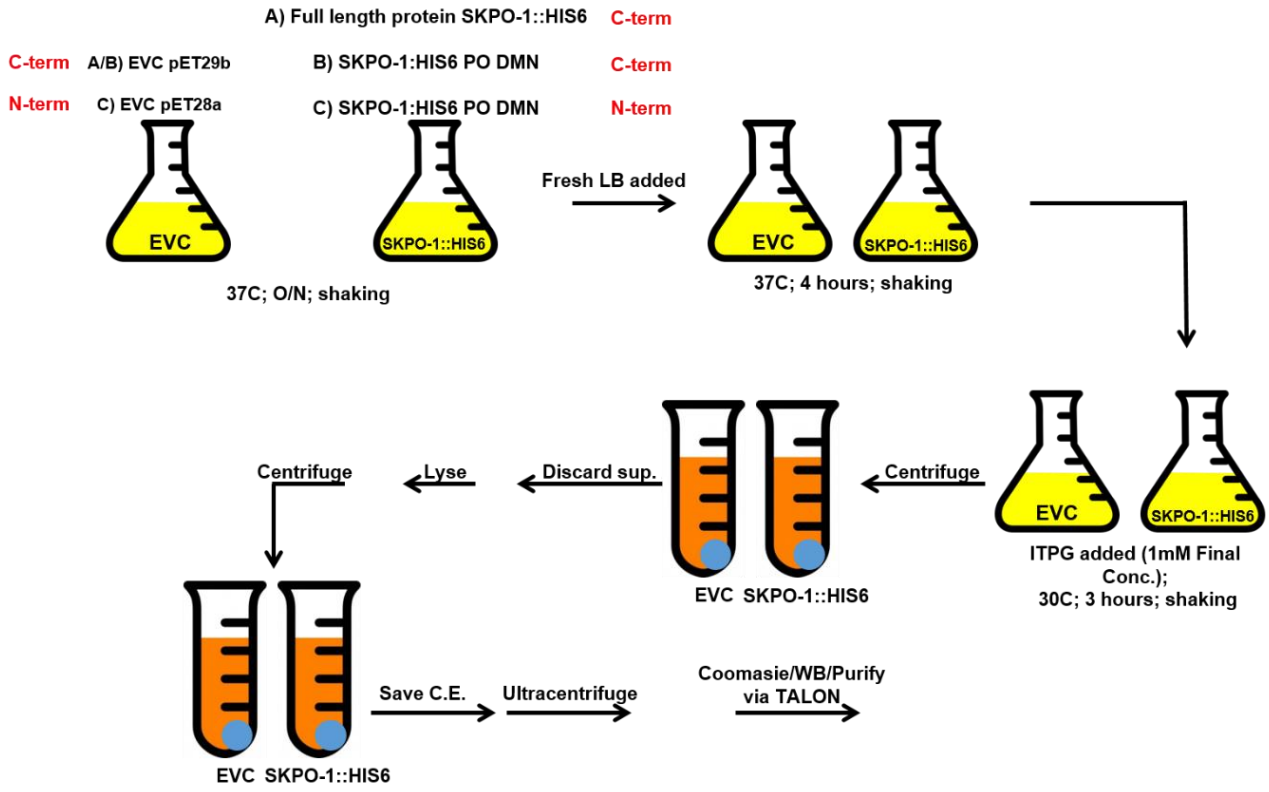

Figure 4.1 Recombinant SKPO-1 protein expression and purification scheme from *E. coli* BL21 (DE3). The protein expression process was uniform between the three different constructs tested minus the SKPO-1 cDNA content and HIS6-tag positioning. Full-length, wild type, SKPO-1 cDNA was cloned into the pET-29b(+) vector upstream of the C-terminal HIS6 tag. Wild type SKPO-1 peroxidase domain cDNA was cloned into the pET-29b(+) and pET-28a(+) vectors such that the HIS6 tag was at the C- or N-terminus of the cDNA, respectively. Empty vector pET-29b(+) served as the control in both **A** and **B**. Empty vector pET-28a(+) served as the control in **C**. C.E. = crude extract. Blue pellet = *E. coli* cell pellet or lysed cell pellet.

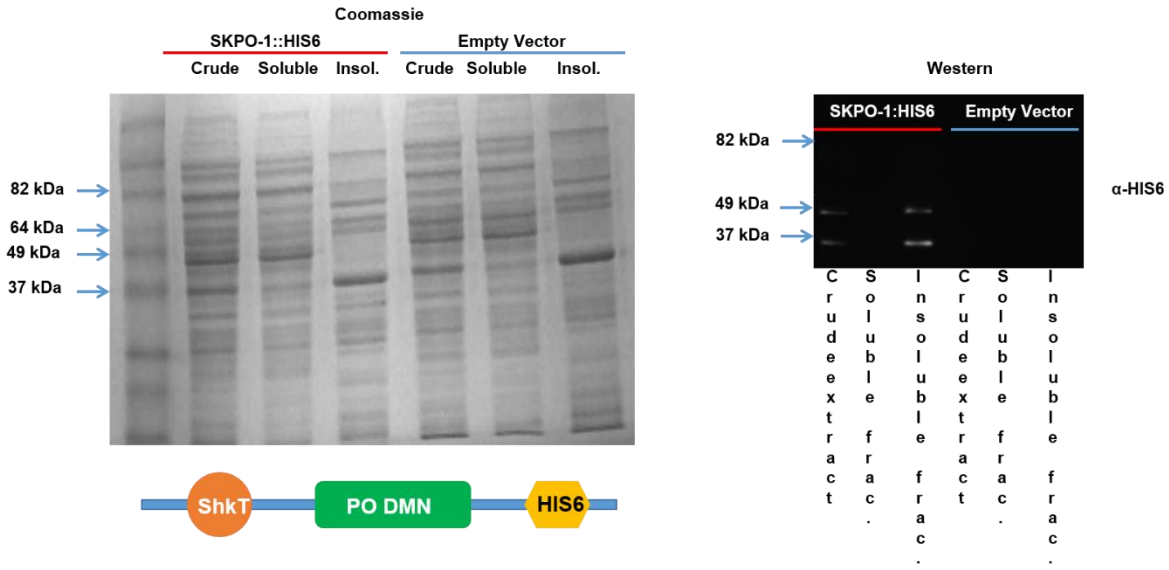

Figure 4.2 Full-length SKPO-1::HIS6 protein is degraded and present in the insoluble fraction. All lanes are of samples that were induced with 1mM IPTG. By the Coomassie brilliant blue (CBB) stained-SDS-PAGE gel, I observed no discernible difference in the protein expression level of full-length SKPO-1::HIS6 (C-terminus) in the crude extract, soluble or insoluble fractions as compared to the corresponding pET-29b(+) empty vector samples. By Western blot, SKPO-1::HIS6 degradation products were detected in the crude extract and insoluble fraction, but not the soluble fraction. No full-length SKPO-1::HIS6 was detected (unpublished data).

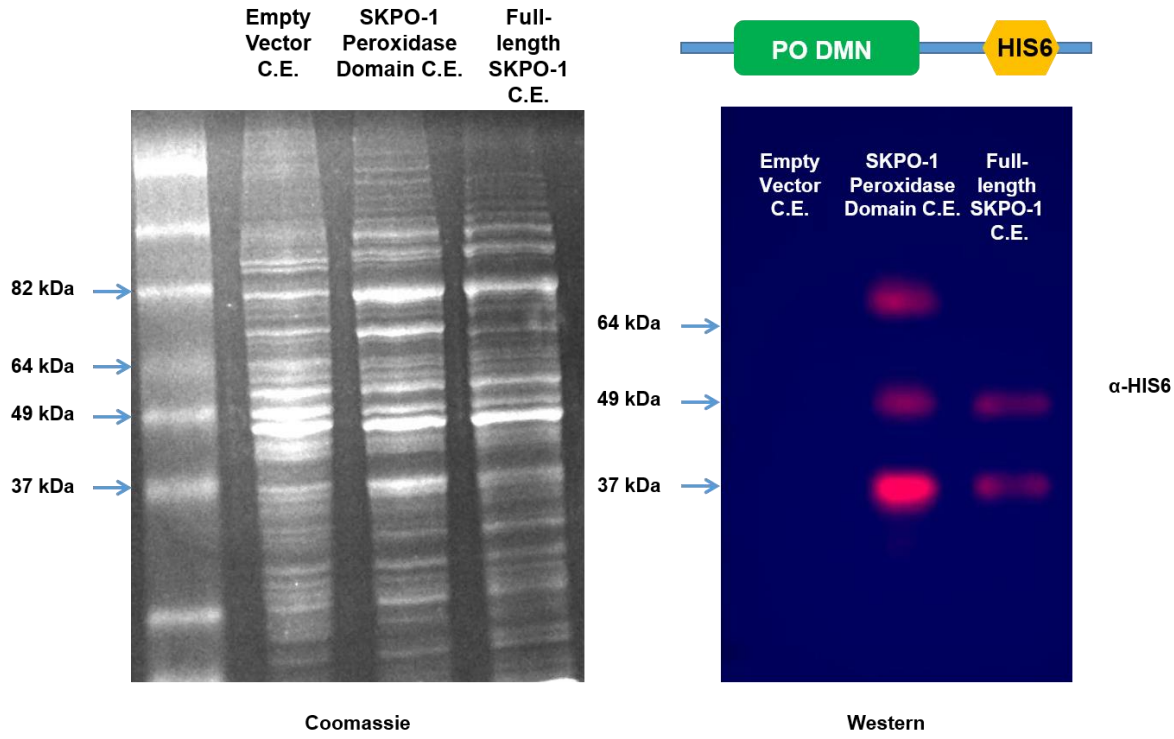

Figure 4.3 SKPO-1::HIS6 peroxidase domain pET-29b(+) construct expresses higher MW recombinant species. All lanes are of samples that were induced with 1mM IPTG. By Coomassie, I was unable to detect a difference in SKPO-1::HIS6 protein levels for the recombinant peroxidase domain variant as compared to the empty vector pET-29b(+) crude extract. By Western blot, I observed a ~64 kDa MW species was present in *E. coli* BL21 (DE3) cells expressing the SKPO-1::HIS6 peroxidase domain construct. This higher MW species is absent in the full-length SKPO-1::HIS6 expressing *E. coli* (unpublished data).

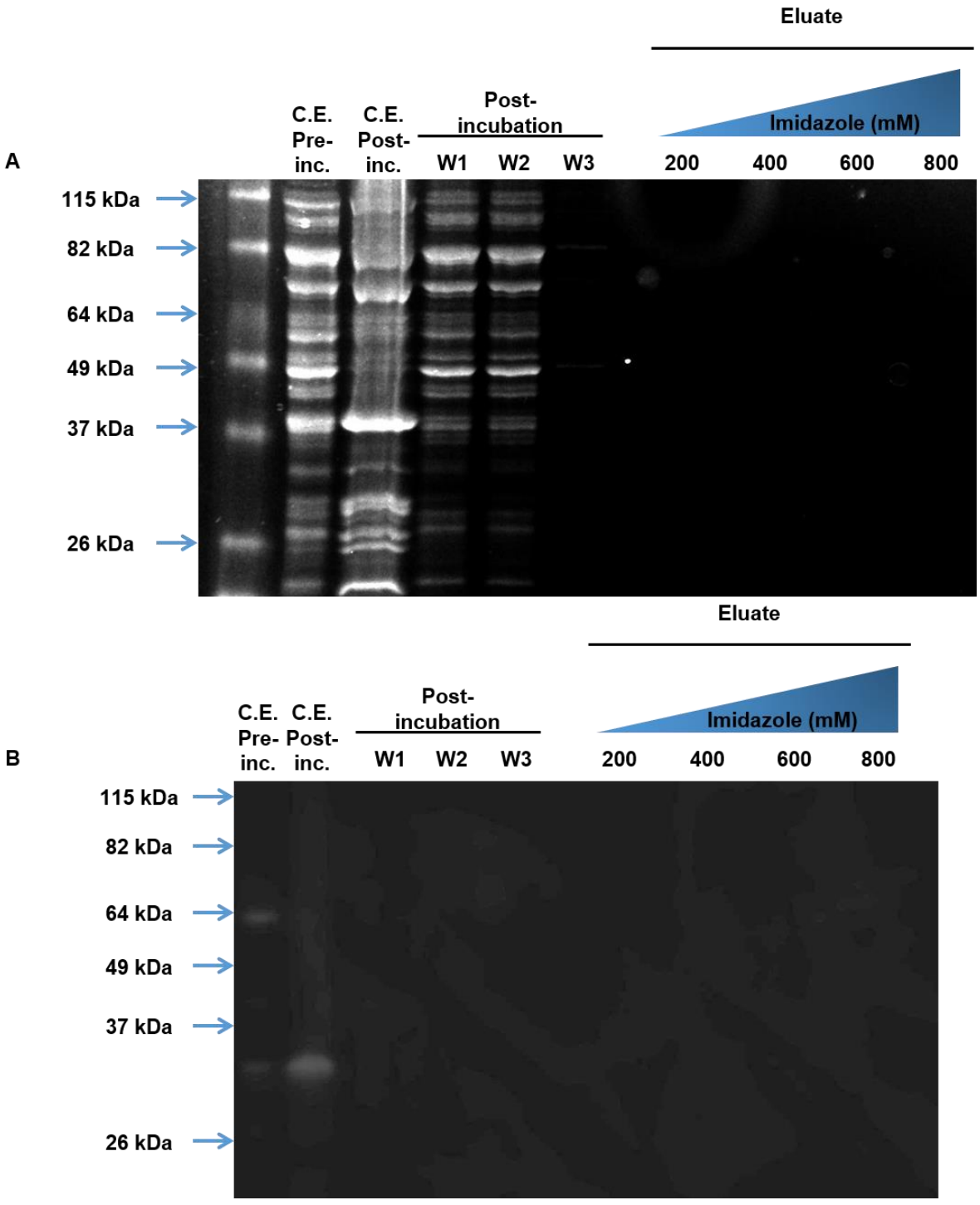

Figure 4.4 The C-terminal HIS6-tagged SKPO-1 peroxidase domain is unable to bind the TALON resin. A). 5 ul of each sample was loaded into a SDS-PAGE gel, electrophoresed and stained with CBB. I observed proteinaceous content in all lanes minus

the lanes containing eluates treated with increasing imidazole. **B)** 5 ul of each sample was used in the immunoblot. After 1˚ and 2˚ antibody, and luminol treatment, I observed α-HIS6 reactive products corresponding to 64 and 29 kDa in the C.E. pre-TALON® incubation flow through. The 29 kDa α-HIS6 reactive product is present in the C.E. post-TALON® incubation flow through. The resin was then washed three times (W1-3), but the α-HIS6 reactive product was absent in the flow through. I steadily increased the imidazole concentration (200-800 mM) in the elution buffer and collected their corresponding eluate fractions, but did not observe any α-HIS6 reactive product **B)**.., This result, in conjunction with Figure 4.5, suggests that the HIS6 tag is hidden within the insoluble SKPO-1::HIS6 protein, and thus unable to bind to the TALON resin (unpublished data).

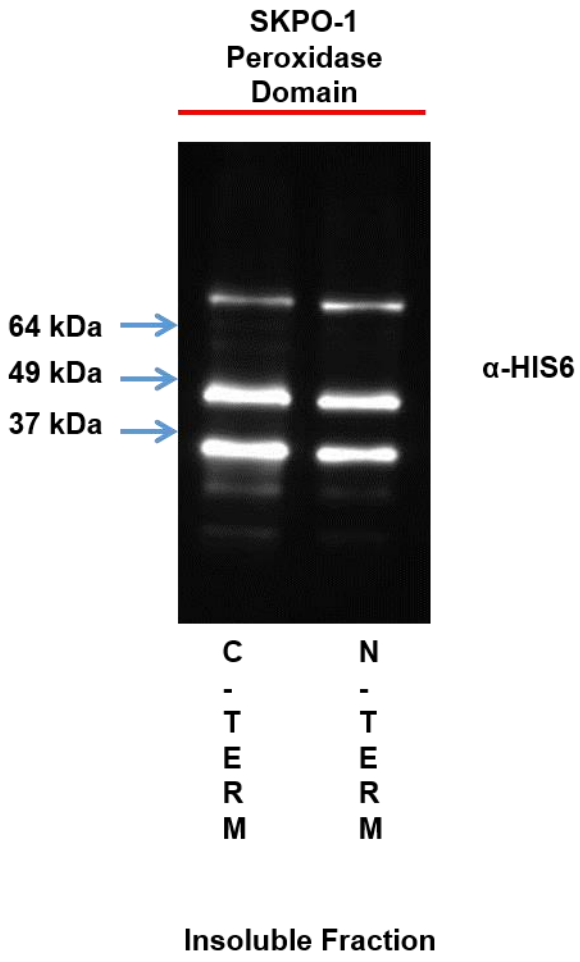

Figure 4.5 HIS6 tag position does not affect the solubility and degradation of the recombinant SKPO-1::HIS6 peroxidase domain. Wild type SKPO-1 peroxidase domain cDNA was cloned in both pET-29b(+) and pET-28a(+) and expressed in *E. coli* BL21 (DE3), left and right lanes respectively. By Western blot, I observed that the SKPO-1 peroxidase is degraded in *E. coli*. Additionally, the HIS6 tag position did not affect recombinant protein expression *i.e.* more or less SKPO-1::HIS6 recombinant species were not detected due to the tag's position (unpublished data).

Conclusions and future directions

Expression and purification of SKPO-1::HIS6 using *E. coli*

While successful expression of peroxidase-domain containing proteins is possible, expressing eukaryotic proteins in heterologous eukaryotic systems is the most reliable method (for eukaryotic heme peroxidases) ((JOHNSON *et al.* 1987), (MEITZLER and ORTIZ DE MONTELLANO 2009), (MEITZLER *et al.* 2010), (MEITZLER and ORTIZ DE MONTELLANO 2011), (SHIMOKAWA and SMITH 1991)). Unfortunately, while I successfully expressed some versions of SKPO-1 in *E. coli*, they were degraded and insoluble. *E. coli* is an important protein expression system, but it is not always ideal for expressing certain proteins. As *E. coli* protein expression is relatively quick and inexpensive it is a good system for initial troubleshooting. As for why *skpo-1* expression was unsuccessful in *E. coli* there are several possible reasons. One issue may have been rare codon bias in which *skpo-1* is improperly/poorly translated as certain tRNAs are rare or lacking in *E. coli* and can result in premature translation termination and/or amino acid misincorporation (DEL TITO *et al.* 1995). Disulfide bond formation is another important consideration when expressing proteins in *E. coli* because the cytosol is highly reducing and thus is not a favorable environment for disulfide bond formation (PRINZ *et al.* 1997). The *E. coli* Rosetta-gami™ 2(DE3) strain possesses all seven rare tRNAs on a chloramphenicol^{r} (Cam) plasmid and has mutations in glutathione reductase (*gor*) and thioredoxin reductase (*trxB*) which enhances formation of disulfide bonds in the cytoplasm (DEL TITO *et al.* 1995), (PRINZ *et al.* 1997). I used this strain to express *skpo-1* but observed SKPO-1 to be degraded and present only in the insoluble fraction similar to the construct expressed in BL21 (DE3). Therefore, I doubt rare codon bias or disulfide bond formation were the main problems. An additional problem for expressing eukaryotic proteins in *E. coli* include solubility issues which can be remedied by fusing the gene to glutathione-S-transferase (GST) or maltose-

binding protein (MBP). However, I did not attempt to fuse these tags to *skpo-1* as Thein *et al* used a HIS6 tag and claimed to have success (THEIN *et al.* 2009). Additionally, I had already formed a collaboration with Dr. Leto at the NIH to have *skpo-1* expressed using his baculovirus system (Dr. Leto, personal communication)

One final issue concerns the types of posttranslational modifications supported by *E. coli*. *E. coli* is incapable of catalyzing N- and O-linked glycosylation amongst other posttranslational modifications (BRONDYK 2009). N-linked glycosylation can occur at an Asn residue within the Asn-Xaa-Ser/Thr triplet motif of the polypeptide in the endoplasmic reticulum (ER) (HUTTMANN *et al.* 2013), (WEERAPANA and IMPERIALI 2006). Loss of N-linked glycans has been observed to have variable effects on proteins (HUTTMANN *et al.* 2013), (ZONG *et al.* 1995). In some cases, N-linked glycosylation is required for enzymatic activity of some proteins, and in others cases, loss of glycosylation does not affect enzymatic activity. α-amylase and chloroperoxidase (CPO) are respective examples (HUTTMANN *et al.* 2013), (ZONG *et al.* 1995). MPO, LPO, EPO (myeloperoxidase, lactoperoxidase, and eosinophil peroxidase, respectively) are examples of N-linked glycosylated proteins. Typically, these mammalian heme peroxidases are recombinantly expressed and purified in eukaryotic systems such as the baculovirus-*Spodoptera frugiperda Sf9* and *Sf21* expression systems (MEITZLER and ORTIZ DE MONTELLANO 2009). However, there are examples of peroxidase domain containing proteins being successfully expressed from *E. coli*—CPO, MLT-7 and BLI-3's peroxidase domain (EDENS *et al.* 2001), (THEIN *et al.* 2009), (ZONG *et al.* 1995). CPO, from *Caldariomyces fumago*, typically possesses N-glycosyl moieties; however, the CPO expressed in *E. coli* lacked these N-linked glycans, yet demonstrated wild type-like peroxidase activity (ZONG *et al.* 1995). Unfortunately, recombinantly expressed CPO from *E. coli* lacked heme and was always present in the periplasm as an insoluble protein (ZONG *et al.* 1995). Thus, Zong *et al*. had to denature and

renature CPO and introduce heme to CPO before the recombinant protein displayed peroxidase activity (ZONG *et al.* 1995). Likewise, Thein *et al*. had insolubility issues when they were expressing the MLT-7 peroxidase domain from *E. coli* (Dr. Andy Page, personal communication). By bioinformatics (www.cbs.dtu.dk/services/NetNGlyc/), MLT-7 has predicted N-glycosyl sites; however, this has not been experimentally determined. Edens *et al*. claim to have successfully expressed an active BLI-3 peroxidase domain from *E. coli* (EDENS *et al.* 2001). This result is contentious within the NOX/DUOX field, as only one lab has been able to successfully express a functional NOX/DUOX protein in a prokaryotic system. Also, BLI-3 lacks any N-glycosyl sites (EDENS *et al.* 2001). By bioinformatics, SKPO-1 is predicted to possess asparagine-derived glycosylation sites (www.cbs.dtu.dk/services/NetNGlyc/).

With all of these setbacks, I was unable to express enough of the insoluble peroxidase domain to have a pAb raised against SKPO-1. On the upside, I gained experience with protein expression and purification as well as established a collaboration to have SKPO-1 expressed in a eukaryotic system. The ultimate goal of the collaboration would involve me directly assessing, by *in vitro* biochemical assays, SKPO-1's peroxidase and microbicidal activity (HA *et al.* 2005a), (THEIN *et al.* 2009). However, while the collaboration is ongoing, I have yet to receive the purified protein and I no longer have the time to do these assays. With respect to having a pAb raised for IHC, I designed a synthetic SKPO-1 peroxidase domain peptide to be chemically synthesized and have a pAb raised in rabbits against said peptide by Lifetein®. This endeavor was successful and is covered in Chapter 3.

Chapter 5: SKPO-1 transgenic nematodes

Introduction

SKPO-1 transgenic nematodes

While assessing SKPO-1's peroxidase activity by biochemical assays is important for definitively establishing its peroxidase and/or microbicidal abilities, this does not address where SKPO-1 is expressed in *C. elegans* in non-stress and stress conditions. As I had yet to have the α-SKPO-1 polyclonal antibody made, I decided to complement my hypodermal RNAi findings from Chapter 3 by creating transgenic nematodes to observe SKPO-1 tissue localization. By creating *skpo-1::GFP* constructs and introducing them into wild type *C. elegans* I would be able to visualize SKPO-1::GFP during infected and uninfected conditions. Additionally, if this approach worked I would be able to potentially determine whether SKPO-1::GFP and BLI-3::mCherry co-localize during infected and uninfected conditions by introducing the *skpo-1::GFP* construct into *bli-3::*mCherry-expressing *C. elegans*.

Microinjection of *skpo-1::GFP* constructs

One standard method of transgenesis in *C. elegans* is microinjection. To introduce the transgene into *C. elegans* by microinjection, the DNA is introduced into the cytoplasmic syncytium of the gonads by a glass needle (EVANS 2006), (MELLO *et al.* 1991). The plasmid DNA homologously recombines into a large, heritable, extrachromosomal array that is able to be integrated into the chromosome upon UV radiation (EVANS 2006), (MELLO *et al.* 1991). Co-injecting the transgene-of-interest along with a phenotypic marker plasmid allows visual confirmation of whether the injection was successful (EVANS 2006), (MELLO *et al.* 1991). Under advisement from Dr. Kyung Park, I did not use a co-injection marker because the partial *skpo-1* ORF was translationally fused to GFP, thus bypassing the need (Figure 5.1A) (Dr. Kyung Park, personal communication). After I successfully injected ~30-60 young adult, wild type, animals, I allowed them to recover and lay eggs. I screened the

subsequent F_1 progeny by using a fluorescent dissecting microscope, but did not observe any GFP-expressing worms at any developmental stage. I requested aid from my microinjection mentor, Dr. Park, to inject the same plasmid just in case my technique was poor. Likewise, Dr. Park did not observe GFP-expressing F_1 progeny at any developmental stage. A potential problem with the first construct may have been that the required regulatory information was further upstream of the sequence I used in this translational fusion (EVANS 2006). Thus, I increased the length of the upstream regulatory sequence to include sequence from the upstream gene, *ubc-24* (Figure 5.1B). Again I injected ~30-60 young adult, wild type, worms and screened the resulting F_1 progeny for GFP-expression. However, the progeny lacked GFP-expression. Likewise, when Dr. Park injected this construct, he did not observe GFP-expression in the F_1 progeny.

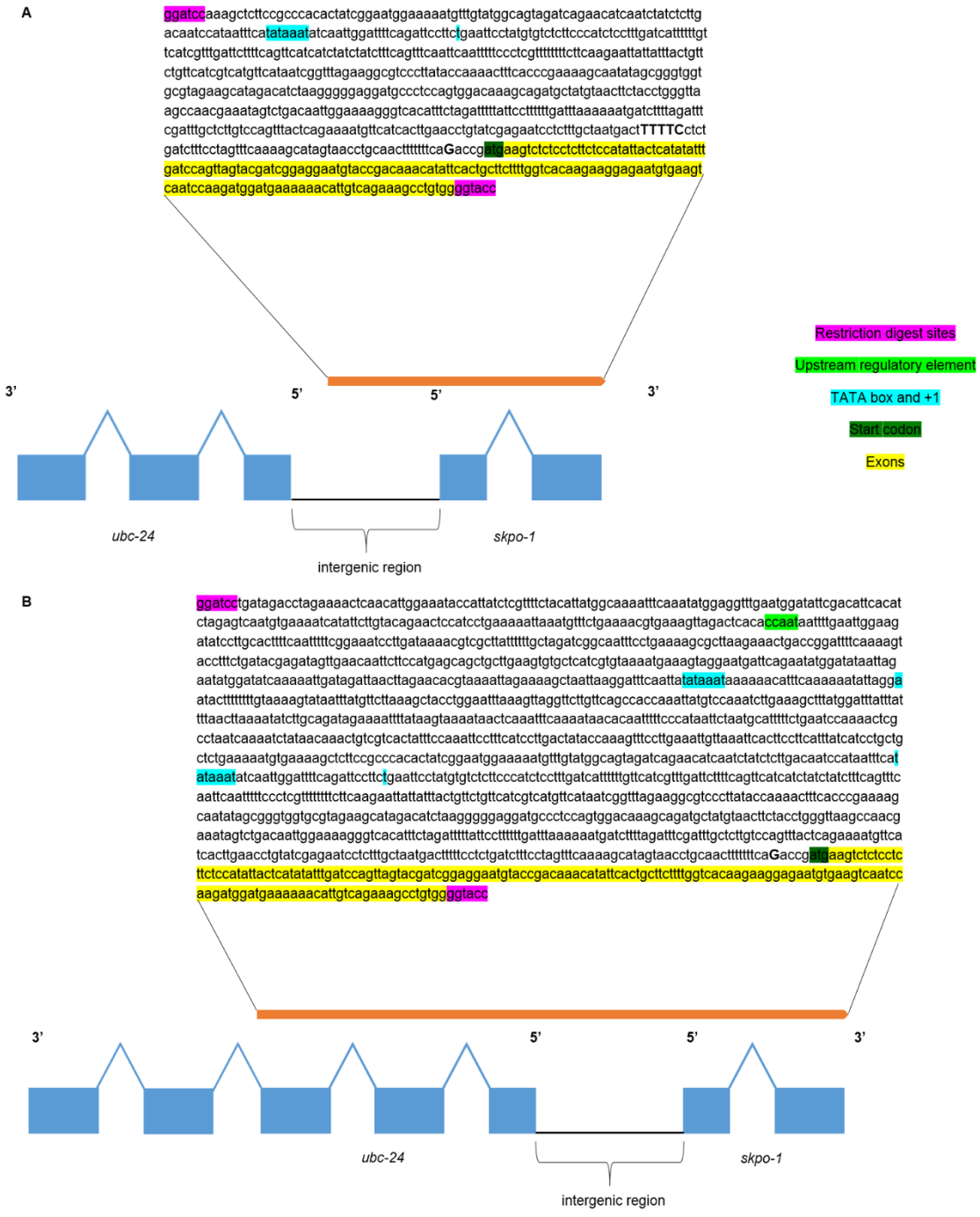

Figure 5.1 *skpo-1* sequences for *C. elegans* transgenesis. *skpo-1* sequence used to create a translational GFP fusion, in pPD95.75, for microinjection into *C. elegans* gonads (A and B, respectively). In this figure, the intron between exon 1 and 2 in *skpo-1* has been

excised for simplicity, however, it is present in the actual construct. All annotated features within the *skpo-1* sequence are hypothetical and based on motifs generally recognized as important in eukaryotic transcriptional and translational regulation. The insert in **B** possesses a putative CCAAT enhancer element as well as a second tentative TATA box as opposed to the insert in **A** (unpublished data).

Conclusions and future directions

The lack of GFP-expressing progeny suggests that the amount of regulatory sequence supplied with the *skpo-1* insert was inadequate for driving expression of GFP. It has also been observed that certain transgenes are toxic to the embryo (EVANS 2006), (MELLO *et al.* 1991). Thus, no progeny would have been observed. I designed the constructs to include the 5' transcriptional and translational elements deigned necessary for proper expression (EVANS 2006). Additionally, I included the first two exons as well as the first intron as pre-mRNA splicing is important for stability as well as translation (EVANS 2006). It has been demonstrated that synthetic introns also increase reporter gene expression and the GFP within pPD95.75 contains synthetic introns (EVANS 2006). The pPD95.75 lacks the heterologous 3' *unc-54* UTR that is present in some, but not all, Andy Fire vectors. These vectors are typically used for microinjection and/or gene bombardment in *C. elegans*. While the 3' UTR is important in translation, I decided against including the native 3' UTR in the constructs I used as many successful transgenic constructs are designed without it, as mentioned above ((AN and BLACKWELL 2003); Drs. Van der Hoeven and Park, personal communications). Unfortunately, I recently learned that 3' UTRs in *C. elegans* are very important for successful expression and proper localization of the expressed gene product (Drs Arur and Vizuete; personal communications). Therefore, I postulate the lack of further exons, introns and/or the lack of the 3'UTR may explain why expression of these *skpo-1::GFP* constructs was unsuccessful. Another consideration is that the GFP fused to the partial SKPO-1 protein may have been misfolded due to improper subcellular targeting. Therefore, the GFP would not fluoresce as the product was degraded. Both constructs possessed the first two exons of *skpo-1* fused to *gfp*. This could have been problematic as the ShkT-like domain is encoded within the second exon (www.WormBase.org). In *C. elegans*, the ShkT-like domain is purported to support protein-protein interactions; however, this has not been experimentally determined yet (TSANG *et al.*

2007). It is possible that the ShkT-like domain is not targeted to the proper subcellular areas which could result in the fusion product being degraded.

While these constructs were unsuccessful, I have one last alternative for successful transgenesis. I received a cosmid from the Max Planck Institute (MPI) that contains a large portion of chromosome II (~45 kilobases) and all of the *skpo-1* ORF. Thus, this cosmid could be introduced into *C. elegans* through microinjection or gene bombardment to create a transgenic worm. As this cosmid contains all of the upstream and downstream regulatory information of *skpo-1* there is a higher chance of proper expression. Additionally, within the *skpo-1* ORF is a *gfp* ORF near the 3'-end of the *skpo-1* ORF, thus it is possible to visually observe expression of *skpo-1*. If this approach were successful, I would suggest introducing the *skpo-1* containing cosmid into the *BLI-3::mCherry*-expressing *C. elegans* strain. This BLI-3::mCherry is a translational fusion that was constructed by Dr. van der Hoeven and the strain was created by Dr. Park. As all attempts at determining co-localization of SKPO-1 and BLI-3 in *C. elegans* by immunohistochemistry (IHC) have been unsuccessful, this method may prove otherwise.

Chapter 6: Final Conclusions and Perspectives

SKPO-1 localizes and contributes to the host's immune response in the hypodermis

In this body of work, I determined by RNAi the involvement of three previously unknown peroxidase genes in the host immune response—*F09F3.5*, *R08F11.7*, and *F49E12.1*. *F49E12.1* was characterized in detail and observed to belong to the short peroxidasin family (SOUDI *et al.* 2012). However, due to the presence of a metridin ShkT-like domain at its N-terminus, in addition to its C-terminal peroxidase domain, I renamed *F49E12.1* to *skpo-1* (ShkT-containing peroxidase). In Chapter 3, I determined that SKPO-1 is involved in the host immune response during infection with *E. faecalis*, but not *P. aeruginosa*. Additionally, I observed by immunohistochemistry SKPO-1 localizes to the hypodermis, where BLI-3 has been demonstrated to localize and supply H_2O_2 to MLT-7 for cuticle biogenesis (EDENS *et al.* 2001), (THEIN *et al.* 2009) (Figure 6.1). SKPO-1 contributes to the innate immune response at the hypodermal, not the intestinal, interface which was very surprising. This finding is interesting as a majority of host-pathogenesis studies in *C. elegans* focus on the intestinal immune response. As *C. elegans* is a bacteriovore, the intestinal tract comes into contact with the ingested microbes that have either been lysed due to pharyngeal grinding or those microbes that are still intact. The bacteria that avoid being lysed by the pharyngeal grinder are able to colonize the intestine, which is the case for human pathogens *P. aeruginosa*, *S. aureus*, and *E. faecalis* (IRAZOQUI *et al.* 2010a), (VAN DER HOEVEN *et al.* 2011). Published data thus far suggest *P. aeruginosa*, *S. aureus*, and *E. faecalis* cause disease within the *C. elegans* intestine (IRAZOQUI *et al.* 2010a), (VAN DER HOEVEN *et al.* 2011). The hypodermal-specific *skpo-1* RNAi data implicate *E. faecalis* kills *C. elegans* by causing an infection at the hypodermis presumably by damaging the cuticle. It has been demonstrated that *P. aeruginosa, S. aureus,* and *E. faecalis* downregulate the expression of genes involved in cuticle biosynthesis which could indicate these pathogens are hampering the host's ability to repair the damaged cuticle (IRAZOQUI *et al.* 2010a), (WONG *et al.* 2007). *E. faecalis* is hampering the host's ability to repair the

damaged cuticle by downregulating expression of cuticle biosynthesis genes. Thus, it is possible that *E. faecalis* causes unappreciated pathology to the hypodermis of the worms that does not occur during *P. aeruginosa* infection.

Other pathogens are well recognized for causing hypodermal pathology. For example, *D. coniospora* infects *C. elegans* by attaching to the cuticle and then penetrating the cuticle as well as the underlying hypodermis (PUJOL *et al.* 2008). Hyphae used in the colonization of *C. elegans* then develop from the appressorial structures used to puncture the cuticle and hypodermis (PUJOL *et al.* 2008). In *C. elegans* it was genetically determined that expression of the antimicrobial neuropeptide-like peptide (NLP), *nlp-29*, is upregulated in response to infection through (Toll/IL-1R) TIR-1-p38 MAPK signaling (PUJOL *et al.* 2008). However, through sterile wounding, *i.e.* wounding of the hypodermis in the absence of bacteria or fungi, it was determined that *nlp-29* expression is controlled by a different kinase, NIPI-3 (no induction of peptide after *Drechmeria* infection), upstream of the p38 MAPK cascade (PUJOL *et al.* 2008). Pujol *et al.* observed a thick layer of cuticle had been secreted forming a scar where he wound occurred in the *C. elegans* cuticle (PUJOL *et al.* 2008). As the *C. elegans* p38 MAPK ortholog, PMK-1, is extremely important in both hypodermal and intestinal immune/stress responses it comes as little surprise to see its involvement. Thus, *C. elegans* is able to differentiate between disparate stimuli whose upstream signals converge into the major immune response cascade.

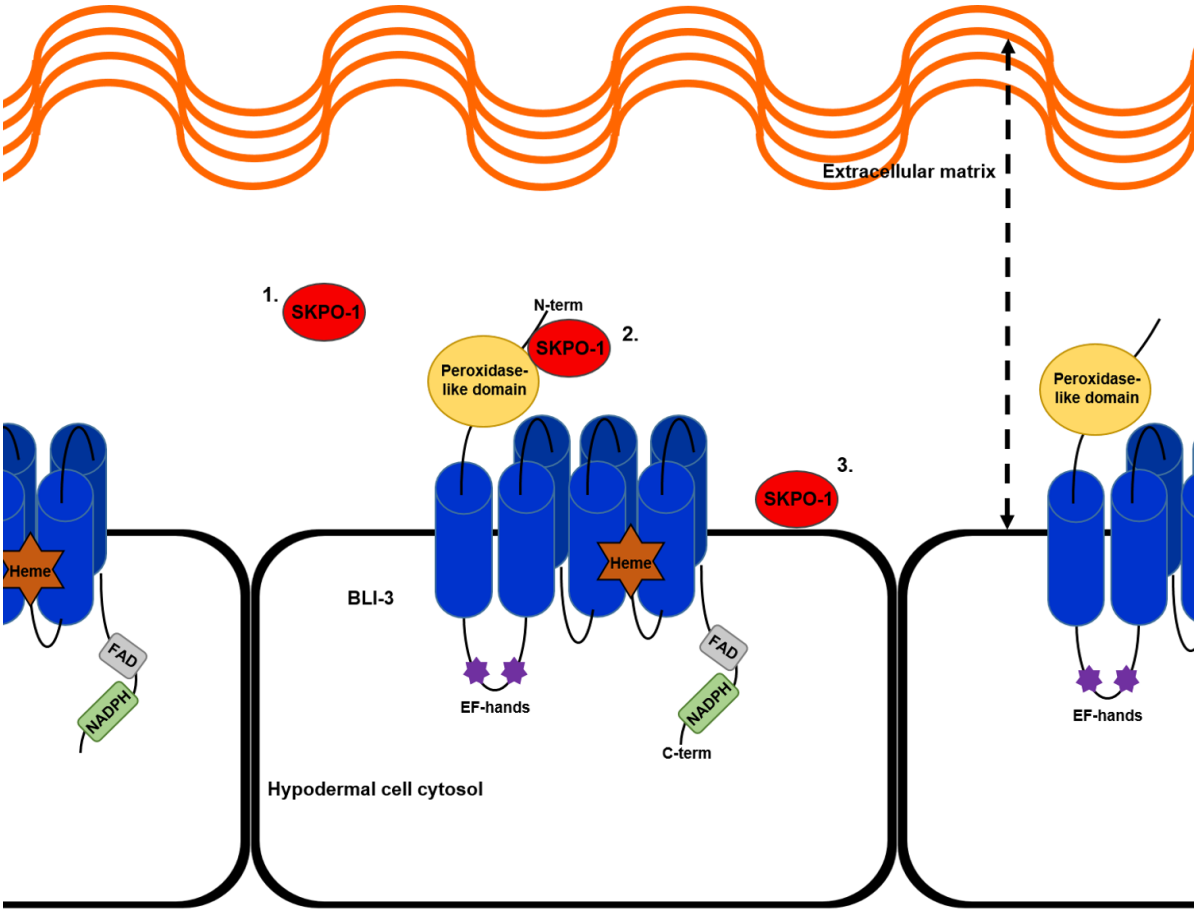

Figure 6.1 Model for BLI-3/SKPO-1 hypodermal localization. BLI-3 localizes to the apical membrane of the hypodermal cell where it supplies H_2O_2 to extracellular peroxidases for the purpose of immune defense. SKPO-1 is predicted to be a secreted, extracellular peroxidase that localizes to the apical surface of the hypodermis beneath the cuticle (extracellular matrix). Listed are three possibilities (1, 2, and 3) for SKPO-1's localization. **1)**. SKPO-1 is not physically associated with the hypodermal cell surface but is in close proximity to BLI-3. **2)**. In order to efficiently use BLI-3-produced H_2O_2 during an immune response, SKPO-1 physically interacts with BLI-3, perhaps by forming a hetero-dimer with the peroxidase-like domain. **3)**. Like in scenario 1, SKPO-1 is in close proximity to BLI-3 in

order to use its H_2O_2; however, SKPO-1 physically interacts with the apical membrane of the hypodermal cell surface or with an unknown protein. The orange waves represent the cross-linked cuticle.

As to why there was no significant difference in the susceptibility of wild type and *skpo-1* animals to *P. aeruginosa*, I hypothesize that *P. aeruginosa* utilizes its heme-scavenging proteins to subvert the BLI-3/SKPO-1 oxidative defense system. I only exposed wild type and *skpo-1* worms to the "slow killing" mode of action, wherein *P. aeruginosa* colonizes the worm, as opposed to the "fast killing" mode of action which involves toxin production. The reason I tested the slow killing phenotype is that it is easier to discover pathogen susceptibility phenotypes as the worms die over a period of 2-3 days versus fast killing, in which a LT_{50} is obtained by 10 hours (RUIZ-DIEZ *et al.* 2003). I hypothesize there are enough similarities between the slow killing and fast killing phenotypes that it is likely that *P. aeruginosa* would also nullify SKPO-1's ability to defend the host in the presence of the phenazine pyocyanin (RUIZ-DIEZ *et al.* 2003), (TAN *et al.* 1999a), (TAN *et al.* 1999b). The main difference between these modes of killing is the production, or lack of, phenazine toxins such as pyocyanin (fast vs slow killing, respectively).

In *C. elegans*, pyocyanin is necessary for fast killing (MAHAJAN-MIKLOS *et al.* 1999). Pyocyanin is able to traverse plasma membranes due to its zwitterionic nature and exerts its toxicity by redox cycling (RADA *et al.* 2008). Through its redox cycling nature, pyocyanin promotes ROS production within host cells leading to oxidative damage to the host (RADA *et al.* 2008). One method of potentiating oxidative damage to the host is by oxidizing glutathione (GSH) to GSSG (oxidized GSH) thereby depleting the pool of an available antioxidant (RADA *et al.* 2008). In combination with this method, pyocyanin also utilizes the available NADPH within the host cells resulting in $O_2^{\cdot -}$ and H_2O_2 molecules that damage the host (RADA *et al.* 2008). As observed in mammals, this subterfuge of consuming the host's available NADPH prevents the host from mounting an effective oxidative burst (RADA *et al.* 2008). Like all NOX/DUOX systems, the DUOX1/2-LPO-SCN^- antimicrobial system requires

NADPH to provide the electrons to the NOX domain of the DUOX proteins to ultimately produce H_2O_2 to aid in clearance of *P. aeruginosa* in the airway (RADA *et al.* 2008).

It is possible that in *C. elegans*, pyocyanin is able to counteract the BLI-3/SKPO-1 defense system by consuming the host's NADPH, O_2, as well as oxidize GSH and generate unwanted ROS-mediated oxidative damage. Additionally, the pyocyanin may interfere with BLI-3 trafficking and/or protein folding by preventing incorporation of the heme groups within BLI-3's NOX domain. I propose that pyocyanin interferes with heme incorporation and thus BLI-3-produced H_2O_2 being supplied to SKPO-1 when *P. aeruginosa* hemophores are present. An alternative hypothesis is that SKPO-1 is not involved in the host immune response during *P. aeruginosa* infection. *P. aeruginosa* has been characterized through electron and light microscopy to strictly colonize and affect pathology within the intestinal tract of *C. elegans* (IRAZOQUI *et al.* 2010a). As I did not use *P. aeruginosa* to screen the putative peroxidase genes, (Table 3.1) it is possible that another peroxidase may contribute to the intestinal immune response during *P. aeruginosa* infection. Therefore, SKPO-1 may be the main peroxidase responsible for the hypodermal immune response during *E. faecalis* infection, but one or more peroxidases may contribute to an intestinal immune response during *P. aeruginosa* infection. *P. aeruginosa* may also possess other ROS-detoxification mechanisms that protect it from the hypodermal immune response that involves SKPO-1.

My data preliminarily suggest that SKPO-1 contributes to the immune response by its peroxidase activity and this protective activity is dependent on BLI-3-produced H_2O_2. While SKPO-1 displays peroxidase activity, I have yet to determine how SKPO-1 is utilizing its peroxidase activity to aid the host during *E. faecalis* infection *i.e.* in a microbicidal, wound repair or antioxidant capacity. Whatever ROS-consuming function(s) SKPO-1 serves in hypodermal immunity, it is likely to be essential in defending against other cuticle damaging pathogens like *D. coniospora* as it enters *C. elegans* by puncturing the cuticle and

hypodermis. Thus, it is likely that the *skpo-1* mutant may display enhanced susceptibility to *D. coniospora*. It is also likely that a *bli-3* mutant would have a similar susceptibility phenotype as BLI-3 supplies ROS to SKPO-1 during infection.

With respect to NOX-derived ROS-mediated innate immune signaling, Matsuzawa and Park *et al*. elegantly demonstrated that host mammalian cells produced ROS upon detection of LPS by TLR4 (MATSUZAWA *et al.* 2005), (PARK *et al.* 2004). As the HEK293T (HEK293 expressing SV40 large T-antigen) cells lack the NOX2 multiprotein complex present in neutrophils and macrophages, yet produce ROS in response to LPS, it must be due to a different NOX isoform (PARK *et al.* 2004). While HEK293 cells normally express NOX4, these cells do not express TLR4 endogenously and thus must be transfected in order to express TLR4 (PARK *et al.* 2004). By exposing HEK293T cells to LPS, Park *et al.* observed a significant increase in H_2O_2 as determined by NF-κB-dependent luciferase reporter activity (PARK *et al.* 2004). However, upon introduction of diphenyleneiodinium chloride (DPI) to these LPS-stimulated cells, a drastic reduction in luciferase activity was observed (PARK *et al.* 2004). A similar reduction in LPS-induced ROS-stimulated fluorescence was observed by Matsuzawa *et al*. when mice RAW264.7 cells pre-treated with HPF (hydroxyphenyl fluorescein) were incubated with either antioxidant propyl gallate or *N*-acetyl-$_L$-cysteine (PG and NAC, respectively) (MATSUZAWA *et al.* 2005). Both of these cell-based assays suggest the involvement of NOX4 producing ROS in response to TLR4-mediated detection of LPS. Park *et al*. demonstrated direct binding of NOX4 and TLR4 in response to LPS treatment by co-IP (co-immunoprecipitation) and Y2H (yeast two-hybrid) (PARK *et al.* 2004). This PAMP-induced binding event is exciting as it demonstrates a PAMP-binding receptor directly interacting with a ROS-producing enzyme whose ROS ultimately mediates pro-inflammatory cytokine production (IL-6, IL-1β, and TNF; tumor

necrosis factor) through the mammalian p38 MAPK signaling cascade (MATSUZAWA *et al.* 2005), (PARK *et al.* 2004).

In *C. elegans*, the PAMP and/or DAMP receptor proteins are unknown. Thus, how these important primary signaling molecules are detected, and their information transmitted in the form of secondary messengers to the host cells is unknown. An interesting hypothesis put forth by van der Hoeven *et al*., is that BLI-3, through its C-terminus, may directly interact with the unknown receptor involved in detection of PAMPs and/or DAMPs resulting in ROS production, much like NOX4 (VAN DER HOEVEN *et al.* 2011). Thus, in response to *E. faecalis* infection, BLI-3 increases H_2O_2 production and SKPO-1 contributes to the hypodermal immune response by using its peroxidase activity to potentially aid in wound repair, hypohalous acid production and/or ROS detoxification (Figure 6.2).

A previously unconsidered role for SKPO-1 is detoxification of ROS by acting as a pseudocatalase (DAS *et al.* 1995), (JANTSCHKO *et al.* 2005). It was previously observed in the rat stomach that GPO (gastric peroxidase) possessed pseudocatalase activity in the presence of SCN^- (DAS *et al.* 1995). In the rat stomach, the oxidized product $OSCN^-$ cannot accumulate due to the high concentration of GSH, which in turn, is oxidized to GSSG (DAS *et al.* 1995). The GSSG is then reduced back to GSH by GR (glutathione reductase) and NADPH. Due to the hexose monophosphate shunt pathway, NADPH is produced and serves as an electron donor for GR (DAS *et al.* 1995). This is important because GPO can reduce H_2O_2 to H_2O and O_2 in the presence of SCN^- and GSH can reduce $OSCN^-$ (a potent oxidant) back to SCN^- (DAS *et al.* 1995). However, to accomplish this, GSSG needs to be reduced by GR to GSH, which requires NADPH (DAS *et al.* 1995). Similar pseudocatalase activity has been observed with LPO, MPO, and TPO (thyroid peroxidase) in the presence of high H_2O_2 concentrations (DAS *et al.* 1995), (JANTSCHKO *et al.* 2005). Thus, SKPO-1 in

response to pathogen-induced oxidative stress may aid in ameliorating H_2O_2 levels in *C. elegans* hypodermal cells.

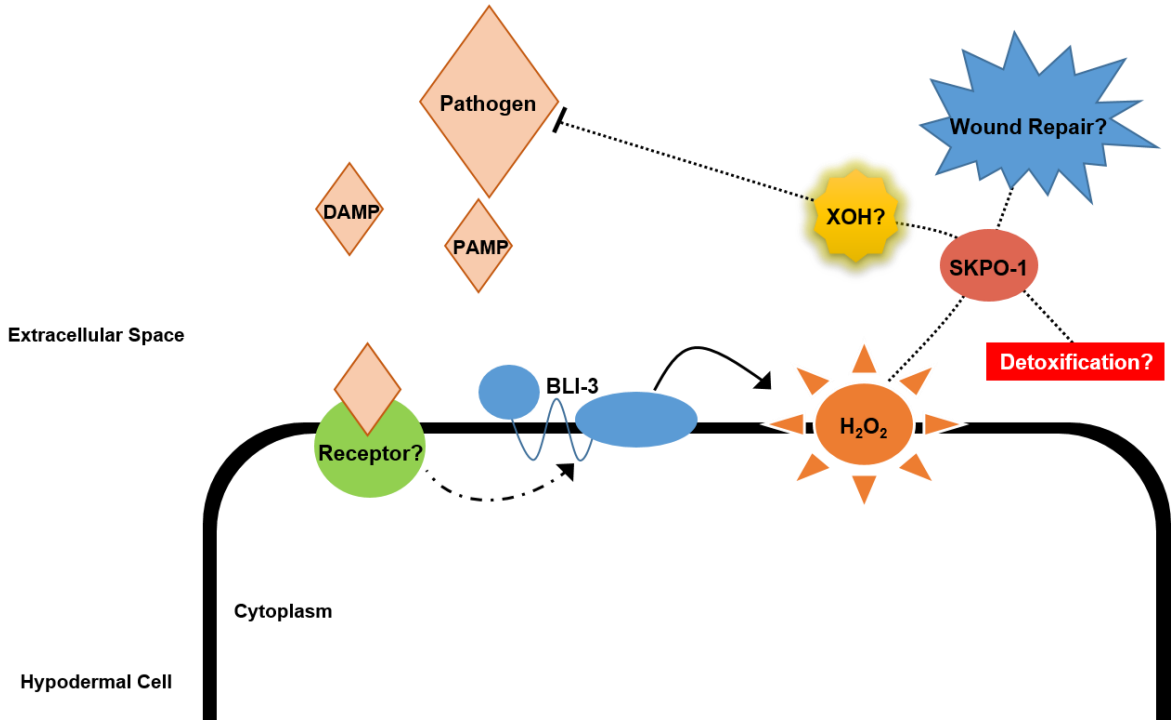

Figure 6.2 Putative roles of SKPO-1 in response to *E. faecalis* infection. SKPO-1 utilizes BLI-3-produced H_2O_2 in response to *E. faecalis* infection at the hypodermal surface. It is possible that SKPO-1 contributes to the hypodermal immune response by producing a hypohalous acid to kill *E. faecalis.* Another role could involve SKPO-1 repairing the worm's cuticle. Finally, SKPO-1 may act as pseudocatalase and prevent BLI-3-mediated oxidative damage to the host due to H_2O_2 accumulation. It is conceivable that SKPO-1 may contribute to the hypodermal immune response by fulfilling one or more of the listed possibilities. It is currently unclear how SKPO-1 utilizes BLI-3-produced H_2O_2; however, both proteins are required for SKPO-1 to contribute to the host's immune response to *E. faecalis*. XOH = unknown hypohalous acid.

Expanding our understanding of SKPO-1

While the body of work presented here has led to one role for SKPO-1 in *C. elegans*, there are other avenues that need to be explored, especially as it is the first heme peroxidase involved with the *C. elegans* immune response. While my efforts to assess whether SKPO-1 and BLI-3 co-localize have been unsuccessful, there are alternatives. As mentioned in Chapter 5, the *skpo-1::GFP* containing cosmid from the Max Planck Institute could be introduced into *C. elegans* by gene bombardment into the *pBLI-3::mCherry*-expressing *C. elegans* strain. By creating this strain, "real-time" *in vivo* imaging could be obtained with respect to how, or if, BLI-3 and SKPO-1 functionally interact during different stresses—such as infection with various pathogenic microorganisms. An additional benefit to introducing the *skpo-1::GFP* cosmid into *C. elegans* would be being able to observe *skpo-1* expression during the worm's development, especially with respect to cuticle biogenesis during the different larval stages.

Previously, I attempted to determine if BLI-3 and SKPO-1 co-localize in the hypodermis by immunofluorescence, but I was unsuccessful. Additionally, my attempt at co-localization of SKPO-1 and BLI-3 using the *pBLI-3::mCherry*-expressing strain failed, I hypothesize this may have been due to the mCherry fluorophore being inactivated due to fixation, which was necessary for exposing the hypodermis by freeze-crack immunohistochemistry (IHC). However, there are two other approaches to assess if these proteins co-localize. One approach is to perform immuno-gold electron microscopy in which secondary antibodies, specific to each protein, labeled with different sized gold-particles are used. The other approach is co-immunoprecipitation (co-IP) using whole worm lysates. Additionally, co-IP would determine if these proteins physically interact or merely coexist together in the hypodermis. If BLI-3 and SKPO-1 physically interact the next step would be to generate a SKPO-1 protein, which lacks the ShkT domain, as it is purported to be a

general protein interaction domain in *C. elegans* (TSANG *et al.* 2007). Additionally, it was demonstrated that the hDUOX1 peroxidase-like domain mediates protein-protein interactions by its solvent-exposed cysteines and this function may be conserved in BLI-3 (MEITZLER *et al.* 2013). A potential problem with the co-IP approach is that BLI-3 is an integral membrane protein, thus this approach may not be feasible.

Determining why disruption of SKPO-1 results in ~50% of the worms to display a *dpy* phenotype of incomplete penetrance is also an interesting question. The collagen protein DPY-7 is involved in maintaining wild type-like annular furrow rings in the cuticle (THEIN *et al.* 2009). Misprocessing of collagen proteins by disrupting the enzymes, like MLT-7, that act on collagen proteins often result in a *dpy* phenotype (THEIN *et al.* 2009). When these rings were disrupted in the *mlt-7* mutant, they observed that the normally circumferential annular rings displayed a mycelia-like pattern in the *dpy* worms (THEIN *et al.* 2009). By using an anti-DPY-7 collagen antibody in conjunction with an Alexa Fluor 488 secondary antibody, as Thein *et al*. did in their work on MLT-7, I would be able to determine if SKPO-1 is involved in cross-linking the cuticle similar to MLT-7 and BLI-3 (THEIN *et al.* 2009)... Another experiment would be to assess the degree of tyrosine cross-linked COL-12::Ty and DPY-13::Ty (collagen 12 and dumpy 13, tyrosine epitope-tagged proteins) by Western Blot (THEIN *et al.* 2009). This method was used by Thein *et al*. to determine that both MLT-7 and BLI-3, individually, affected assembly of these two collagens, both of which are important in cuticle biogenesis (THEIN *et al.* 2009). One other interesting experiment to conduct using *skpo-1* worms is cuticle permeability. Thein *et al*. demonstrated that in *mlt-7* worms, the cuticle was more permeable to the nuclei marker Hoescht 33258 (THEIN *et al.* 2009). This assay demonstrated that the cuticle of wild type animals excluded the Hoescht stain, while the cuticle of the *mlt-7* worms was unable to preclude nuclear staining (THEIN *et*

al. 2009). By performing this assay, I would be able to quickly assess SKPO-1's role in cuticle cross-linking versus the more biochemical methods proposed.

In Chapter 3, by IHC in uninfected and infected conditions, I did not observe an increase in SKPO-1 at the hypodermis. Therefore, it is possible that SKPO-1's peroxidase activity increases in response to *E. faecalis* infection similar to BLI-3-mediated H_2O_2 production. In order to directly determine if SKPO-1 possesses peroxidase and/or microbicidal activity, I would need to purify SKPO-1. My attempts at expression and purification of SKPO-1 from *E. coli* were unsuccessful. The possible reasons for this could be rare codon bias, improper glycosylation, improper disulfide bond formation as well as missing a necessary protein-protein interaction. However, the collaboration underway involves expression of full-length *skpo-1* cDNA in a baculovirus system by a protein-expression core facility at the NIH. Upon receiving purified SKPO-1, I would be able to biochemically assess the aforementioned properties. A standard *in vitro* peroxidase activity assay involves incubating the purified peroxidase with the tyrosine substrate L-tyrosine ethyl ester, or ABTS (2, 2'-Azino-di(3-ethylbenzthiazoline-6-sulfonate)), as mentioned in Chapter 1. In a seminal paper by Ha *et al*., they determined microbicidal activity of the *Drosophila* DUOX peroxidase homology domain (PHD) by incubating the purified PHD with NaCl, H_2O_2 and Erwinia carotovora *Ecc15* (HA *et al.* 2005a). They allowed the pathogen to incubate with this mixture and then performed a colony-forming unit (CFU) assay and observed that there was a significant decrease in CFUs when the three constituents were present (HA *et al.* 2005a). I would test different (pseudo)halides (Br^-, Cl^-, and SCN^-) with purified SKPO-1 in conjunction with *E. faecalis*, *D. coniospora*, and *P. aeruginosa* followed by a CFU assay to observe if SKPO-1 is capable of producing hypohalous antimicrobial oxidants. By performing these assays, the *C. elegans* field would potentially learn if *C.*

elegans possesses an oxidative burst defense mechanism similar to those present in vertebrates.

As mentioned in Chapter 1, ROS such as H_2O_2 are important in modulating the host innate immune response by triggering the hypersensitive response in plants or by promoting chemotaxis of leukocytes to sites of injury in *Danio rerio* (DESIKAN *et al.* 2000), (LEVINE *et al.* 1996), (NIETHAMMER *et al.* 2009). Other ROS such as HOCl and $OSCN^-$ are potent antimicrobial agents produced in response to pathogens at different locations within cells and tissues (CONNER *et al.* 2002), (KLEBANOFF *et al.* 2013). These oxidative burst defense mechanisms are important as a first line of defense against pathogens as defects in producing the potent antimicrobial oxidants can result in serious infections—chronic granulomatous disease (CGD) and cystic fibrosis (CF) (COHEN *et al.* 1981), (MOSKWA *et al.* 2007).Thus, if BLI-3 and SKPO-1 cooperated together similar to mammalian oxidative defense systems it would be an exciting discovery as it would further elucidate the innate immune response in *C. elegans*. Additionally, it would demonstrate that the antimicrobial oxidative defense system is evolutionarily conserved from *C. elegans* to humans.

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VITA

George Tiller was born in Shreveport, Louisiana, the son of Charles D. Tiller and Megyn R. Tiller. George attended and graduated from Loyola College Preparatory School in May 2005. George attended Centenary College of Louisiana where he studied Biology and graduated Magna cum Laude in May of 2009. During his time at Centenary, George used molecular genetics to determine the evolutionary relationship between cryptic *Peromyscus* species. George joined UT-Graduate School in Biomedical Science in Houston in August of 2009 and chose the Microbiology and Molecular Genetics program. He joined laboratory of Danielle A. Garsin in June 2010, where he characterized the role of unknown peroxidases in the *C. elegans* immune response. George aspires to utilize his training in the life science industry. Recently George was accepted into the Postdoctoral Professional Masters (PPM) Program at the Keck Graduate Institute for Applied Sciences where he will learn the business side of life science industry.