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Genetic Analysis of the Role of GRP170B (T14G8.3) During Toxin Induced Protein Folding Stress in Caenorhabditis elegans

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Genetic Analysis of the Role of GRP170B (T14G8.3) During Toxin Induced Protein Folding Stress in *Caenorhabditis elegans*

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Abstract of a Thesis

Genetic Analysis of the Role of the Chaperone GRP170B (T14G8.3) During Toxin Induced Protein Folding Stress in *Caenorhabditis elegans*

Chaperones maintain cellular homeostasis by facilitating protein folding. However, under standard cultured conditions, *Caenorhabditis elegans* strains genetically deficient for the ER chaperone GRP170B (encoded by the T14G8.3 locus) seems unaffected by the loss of this large chaperone. For most ER chaperones, the loss of GRP170B chaperone increases sensitivity to toxins that disrupt protein folding in the ER. I hypothesized that GRP170B's role may involve mediating the nematodes response to ER protein folding stress. To investigate a stress role for GRP170B chaperone, I analyzed the effect of toxins that are known to effect ER protein folding on nematodes deficient for the chaperone. The three toxins tested I tested were: 1. tunicamycin (TM), an ER glycosylation inhibitor, 2. dithiothreitol (DTT), a disrupter of disulfide bond formation and 3. thapsigargin (TG) an inhibitor of an ER calcium pump which disrupts protein folding by interfering with the activity of calcium dependent ER chaperones. Eggs of nematodes deficient for GRP170B (strain BSC06) and the control strain (N2) were cultured at varying doses of each toxin, and the development of the nematodes was monitored. At 80-320 μg/μl of TM, the GRP170B deficient worms were less sensitive to the toxin than the control strain. This was similar to a previous study which found that nematodes deficient for GRP170B were less sensitive to 3 μg/ml TM. At the highest dose of TM tested (640 μg/μl), development was similar in nematodes with and without GRP170B. Results showed that at low doses of the second toxin DTT (80-320 μg/μl), development was similar in nematodes with and without GRP170B. However, at highest dose (640 μg/μl) GRP170B deficient worms were more sensitive to the toxin and none of them developed to L4/adult stages. For the third toxin tested, TG there was a notable experimental variation across all treatments from 0.5 μM to 1.25 μM. The effect of TG on development of nematodes deficient for GRP170B was also tested. Thapsigargin doses (0.5 nM to 5 nM) did not affect the development of either strain. These data demonstrate that GRP170B deficient worms are not being affected specifically with one toxin versus the other. These data revealed that GRP170B has a critical role during normal *C. elegans* physiology role and during stress physiology with the use TM. However, the role of GRP170B could not be determined using DTT and TG at the tested doses.

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By

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Introduction

Overview

The production and processing of proteins in the endoplasmic reticulum (ER) depends on a suite of protein modification enzymes and protein chaperones. The ER chaperones are commonly referred to as glucose regulated proteins (GRPs) because they were initially identified as proteins induced by glucose starvation in cultured cells (Shiu, Pouyssegur, & Pastan, 1977). GRPs facilitate polypeptide folding, protein assembly, and enable the degradation of misfolded proteins (Bukau & Horwich, 1998). The role of chaperones in protein folding is supported by the observation that loss of a chaperone function typically increases sensitivity to toxins that disrupt protein folding (Aviram & Schuldiner, 2017; Ellgaard, McCaul, Chatsisvili, & Braakman, 2016; McCaffrey & Braakman, 2016). The precise role of individual components of the ER protein folding and processing machinery, the interactions of these components and the role of these components in moderating physiological stress has not been fully characterized.

One of the largest of the ER chaperones, GRP170 is the focus of this study. It is widely distributed among the eukaryotes and is structurally a member of the HSP70 superfamily of chaperones (Easton, Kaneko, & Subjeck, 2000). Although it has been characterized in vitro, GR170's role in the physiology of multicellular animals is poorly understood. The deletion alleles for the two GRP170 loci in *Caenorhabditis elegans* provide a unique tool for the genetic analysis of this chaperone (Asrani, 2009). In this study, I will use isogenic nematode strains that differ only by the presence or absence of functional alleles encoding the GRP170B chaperone to study the role of the chaperone in mediating the physiologic response to protein folding toxins.

Protein Synthesis in the ER

GRP170 is localized in the ER, a major site of protein synthesis and processing (Ni & Lee, 2007). Genomics suggest that thirty percent of eukaryotic proteins are secreted and, therefore, are synthesized on ER ribosomes (Behnke, Feige, & Hendershot, 2015). Additionally, most non-secreted proteins of the endomembrane system organelles are also synthesized on ER bound ribosomes. The ribosomes that synthesize these proteins are bound to the cytoplasmic face of the ER membranes (Behnke *et al*., 2015). As the mRNA is translated by the ribosome, the newly synthesized protein is cotranslational transported through the ER membrane into the ER lumen (Aviram & Schuldiner, 2017). The chaperones of the ER engage the newly synthesized protein as soon as it is exposed in the lumen. Within the ER, proteins can be proteolytically processed to remove the signal peptide and glycosylated (Behnke *et al*., 2015). Most ER synthesized proteins are transported to the Golgi, where they are further modified before secretion or transport to an endomembrane organelle. While most proteins enter the ER through this co-translation process, some proteins are known to be synthesized on free ribosomes and then enter the ER post-translationally (Ellgaard *et al*., 2016).

ER Chaperones System

GRP170 is just one of a complex set of ER chaperones that mediate the folding and modification of ER proteins. The ER chaperone family can be divided into three classes. These include, chaperones homologous to heat shock proteins (HSPs). These HSP70 homologues act as molecular chaperones and maintain homeostasis in the ER by facilitating proper protein folding under stressful conditions by mechanisms similar to those employed by HSPs. This involves the prevention of aggregation by binding to hydrophobic domains exposed to the solvent by

misfolding (Ni & Lee, 2007). GRP170 is a part of the heat shock protein family which acts as a structural and functional homologue although it is not heat inducible (Wang *et al*., 2015; Zuo, Subjeck, & Wang, 2016). There are also substrate specific chaperones which process specific unfolded protein substrates (Ni & Lee, 2007). Finally, there are the chaperone lectins such as calnexin assists in folding of glycosylated proteins (Ni & Lee, 2007). It is important to note that these chaperones work together as a complex system.

Endoplasmic-reticulum-associated protein degradation (ERAD)

The ER has evolved a sophisticated mechanism of protein folding quality control that depends on chaperones. Proteins that fail to fold properly are exported to the cytosol and targeted for destruction by the proteasome in a process called Endoplasmic-Reticulum-Associated protein Degradation (ERAD). These proteins are transferred to the cytosol for oligosaccharide removal and then to the proteasome for degradation. As reviewed by Alberts 2002, in order for proteins to achieve a three-dimensional shape, proteins must be glycosylated. ERAD relies on glyosidic modifications of ER proteins to monitor folding. Specifically, N-glycosylation is monitored by calnexin and calreticulin. These proteins bind to N-linked oligosaccharides which are missing a terminal glucose molecule. During the process of protein folding there is a glucose trimming cycle. Glucose is clipped from the unfolded proteins in this process and the protein binds to the chaperone calnexin. As the calnexin chaperone folds the protein it trims another glucose. Then the protein is released from the calnexin chaperone. If the protein has folded properly it is exported from the ER. However, if the protein has misfolded, glucosyl transferase recognizes the protein and adds a glucose back on. A protein may go through this cycle multiple times in an attempt to refold using calnexin (Alberts, 2002).

ER stress and the Unfolded Protein Response (UPR)

Expression of GRP170 like most ER chaperones is induced by ER stress. Many external factors including disruption of calcium homeostasis, blocking normal glycosylation, nutrient starvation, pathogen infection, and oxidative stress may interfere with protein folding and induce ER stress (Cheon *et al.*, 2011). Additionally, a small initial pool of misfolded proteins can interfere with the normal folding of other proteins ultimately producing highly toxic protein aggregation (Ni & Lee, 2007). When misfolded proteins begin accumulating in the ER, eukaryotic cells induce a conserved stress response known as the unfolded protein response or UPR (Cheon *et al*., 2011). The UPR is an intracellular signaling pathway that upregulates the transcription of the ER resident chaperones. An increase in ER chaperones is vital for cell survival and is used to ameliorate the buildup of misfolded proteins in the lumen of the ER (Cheon *et al*., 2011).

ER Protein Folding Toxins

Many different external factors can induce the UPR. This includes protein folding toxins which result in stress of the ER. In this study, three different ER protein folding toxins were utilized to investigate ER protein folding: tunicamycin, dithiothreitol and thapsigargin. These protein folding toxins interfered with protein folding using different mechanisms.

The toxin most commonly used experimentally to induce the UPR is the antibiotic tunicamycin. This toxin inhibits the enzyme which catalyzes the first committed step of N-linked glycosylation of proteins in the ER. The enzyme which tunicamycin inhibits is known as dolichyl-phosphate (UDP-N-acetylglucosamine) N-acetylglucosaminephosphotransferase 1

(DPAGT1). By blocking protein glycosylation, tunicamycin disrupts protein folding in the ER (Oslowski & Urano, 2011).

A second toxin dithiothreitol is a reducing agent that inhibits disulfide bond formation in the ER. Disulfide bonds help to maintain the shape and stability of secreted proteins. DTT exposure results in a decrease of proper protein folding and an increase in protein misfolding. DTT has the potential to completely inactivate the folding process (Braakman, Helenius, & Helenius, 1992).

The third toxin thapsigargin, is a naturally occurring skin irritant and tumor promoter that is derived from a plant called *Thapsia garganica* (Zwaal *et al.*, 2001). Thapsigargin is an ER calcium pump inhibitor which interrupts protein folding indirectly by hindering the activity of calcium dependent ER chaperones (Zwaal *et al*., 2001).

GRP170, a Member of the DnaK/HSP70 Superfamily of Chaperones

Based on structural similarities, GRP170 is classified as a member of the Dnak/HSP70 superfamily (Easton *et al*., 2000). HSP70 class chaperones assist proteins with proper folding by binding exposed hydrophobic domains of unfolded proteins and consequentially preventing aggregation. Binding is mediated by the beta sandwich domain of HSP70 which binds to the hydrophobic amino acids and the α-helical lid which clamps down. The repetition of the binding and releasing of hydrophobic domains appear to aid in progressive folding into native functional conformations. HSP70 binding is regulated by ATP and ADP. When bound to ATP, HSP70 takes an open confirmation with lower affinity to hydrophobic rejection (Figure 1). Once ATP is hydrolyzed to ADP, the α-helical lid changes confirmation and clamps tightly on hydrophobic domains of unfolded proteins by trapping the peptide against the beta sandwich. In order for the

lid to open, ATP must be exchanged for the ADP in the nucleotide binding domain. A nucleotide exchange factor (NEF) is required to accomplish this exchange. GRP170 has the structural domains required for ATP dependent binding of polypeptides which suggests that it may function in the same way. Similar primary and tertiary structures suggest that GRP170 and HSP70 chaperone proteins using analogous processes. The GRP170 chaperone binds to secreted ER proteins and may be associated with assisting them to fold properly (Easton *et al*., 2000).

Although the members of HSP70 superfamily differ in size and subcellular compartmentalization, they show conservation in their primary amino acid sequences and overall three-dimensional structure (Easton *et al*., 2000). The superfamily includes chaperones structurally similar to the bacterial chaperone dnaK. This includes the cytosolic HSP70 and its ER homologue GRP78, also known as BiP (B cell immunoglobulin-binding protein). The mechanism of chaperoning by HSP70 class proteins is well understood and provided a model for how GRP170 may function.

Other major glucose regulated proteins in mammals includes GRP78 and GRP170. Supporting studies have shown that GRP78 is analogous to cytoplasmic HSP70. These observations lead scientists to propose that GRP170 might be related to the cytosolic HSP110. It was shown that GRP170, HSP70, and HSP110 are in fact related and form a superfamily (Easton *et al*., 2000). HSP70 chaperones are vital for the cellular mechanism that controls the three-dimensional shape of proteins. The functions of the HSP70 chaperone include transporting proteins, regulating signal transduction components, and facilitating protein folding. Further studies could reveal similar functions for GRP170 (Andréasson, Rampelt, Fiaux, Druffel-Augustin, & Bukau, 2010).

The largest of the ER chaperones, GRP170, like other members of the HSP70/dnaK superfamily have a bipartite structure with two major domains, an N-terminal nucleotide binding domain and a C-terminal peptide binding domain (Easton *et al*., 2000). The peptide binding domain is composed of four subdomains: a ß-sandwich domain, a long acidic loop, an α helical domain and a C-terminal random coil domain. Another large member of this superfamily is the 110 kd cytosolic HSP110. These larger family members share the same basic bipartite structure of HSP70. They have a much larger loop/hinge domain between the ß-sandwich domain, α helical lid and an extended C – terminal domain. However, the GRP170 chaperone is localized in the ER and is thought to be just one component of the ER's protein synthesis, folding and modification machinery. HSP110 and hsp70 are localized in the cytoplasm (and nucleus). It is important to consider GRP170 in the context of this ER protein folding process to understand its role in the physiology of *C. elegans*.

Molecular Activity of GRP170

GRP170 has been shown to have functions as a foldase, a nucleotide exchange factor and a holdase (De Keyzer, Steel, Hale, Humphries, & Stirling, 2009; Easton *et al*., 2000). GRP170 binds to unfolded proteins in vitro. There is also evidence that GRP170 binds to a diverse group of partially folded proteins in vivo (Behnke & Hendershot, 2014). GRP170 has been shown to bind to unfolded proteins directly (as a holdase). Another method of binding may be through an ATP dependent binding and releasing cycle (as a foldase) similar to that of HSP70 (Behnke & Hendershot, 2014).

The direct binding of unfolded proteins by GRP170 in vitro uses a mechanism similar to that of HSP70. GRP170 is still capable of binding unfolded proteins in the presence of ATP,

while BiP with ATP releases proteins (Figure 1). This suggests that although the same substrate binds to both chaperones, the ultimate result is different. This strongly supports the research which found GRP170 as an independent chaperone. This leaves scientist speculating about the role ATP plays a role in binding GRP170 to its substrate binding domains. In addition, scientists are left wondering how the substrates are released from the large HSP70s (Behnke & Hendershot, 2014).

In yeast, GRP170 can bind to HSP70 and act as a NEF (Zuo *et al*., 2016). Evidence for this is quite strong. The nucleotide binding domain of GRP170 can serve in place of HSP110's nucleotide binding domain as a NEF for HSP70 (Zuo *et al*., 2016). However, previous studies done in yeast and human cells showed that degradation of nonbinding α-subunits of epithelium sodium channel (ENaC) was reliant on GRP170's chaperoning, but not dependent on its role as an NEF function or the presence of BiP (Behnke *et al*., 2015).

Caenorhabditis elegans GRP170

Studies in *Caenorhabditis elegans* have been used to characterize many components of the metazoan proteostasis networks (Kikis, 2016). Studying the *C. elegans* GRP170s could help with understanding what the protein does in mammals, plants, as well as in yeast (Easton *et al*., 2000). The nematode has two GRP170 loci, the autosomal GRP170A (T24H7.2) and the X-linked GRP170B (T14G8.3) identified as GRP170 homologues (Easton *et al*., 2000). The two loci are believed to have originated from a gene duplication, prior to the divergence of *Caenorhabditis elegans* and *Caenorhabditis briggsae* approximately 100 million years (Stein et al. 2003).

GRP170B protein loci is encoded by a monocistronic gene and is located on the X chromosome. It has been suggested that the monocistronic structure of GRP170B permits it to be regulated independently and UPR induced. GRP170A, on the other hand, is part of a polycistronic operon encoding four unrelated proteins and one snoRNA. The other cistrons on this operon are not ER or stressed related. This polycistronic structure and the association with non-stress gene is consistent with GRP170A having more of a constitutive pattern of expression. This polycistronic structure of GRP170A locus is more consistent with it having a housekeeping role, meaning it is a gene which deals with the normal functions of the cell and is non-inducible by UPR. RNA analysis of the two *C. elegans* loci demonstrated that the GRP170B locus is induced during the UPR but the GRP170A locus is not. This differential expression is consistent with the genomic structure of the two loci (Rockwell, 2016).

Analysis of deletion alleles demonstrate the two loci play different physiological roles. As previously stated, GRP170A is important to the general physiology of the worms (Asrani, 2009; Li, 2016). Deletion of this locus results in delayed development and increased rates of embryonic lethality. Alternatively, loss of GRP170B results in no measurable phenotypes under standard culture conditions.

Loss of both GRP170 loci causes embryonic or early larval arrest (Asrani, 2009). As long as there is one functional allele at either loci, the nematodes can mature into adult hermaphrodites. This suggests the two loci share critical overlapping functions. A functional allele at one GRP170 locus can compensate for loss of alleles at the other locus. Only loss of functional alleles at both loci block early development (Asrani, 2009). The observations that deletion of the GRP170B locus does not have a phenotypic effect under standard culture

conditions along with its stress induction during the UPR suggests that GRP170B role may be in mediating stress physiology (Li, 2016; Rockwell, 2016).

Li (2016) investigated whether *C. elegans* lacking the GRP70B locus were more sensitive to the protein folding toxin tunicamycin. She treated nematodes with a sub-lethal concentration of tunicamycin that slowed maturation. Surprisingly, when she treated GRP170B deficient worms with this concentration of tunicamycin, they were somewhat resistant to the tunicamycin maturing at rates similar to untreated controls. The significance of this observation is unclear. On the surface, it seems to refute the hypothesis that GRP170B mediates ER protein folding stress. It is not known if this stress resistance associated with loss of GRP170B is specifically due to the mechanism of tunicamycin action – blocking ER associated glycosylation. It is possible that stressors affecting other elements of ER protein folding would have a different outcome. Alternatively, the tunicamycin response that Li observed may have been specific to the single concentration she tested and that other concentrations would have different affects (Li, 2016).

To more fully characterize whether GRP170B plays a role in mediating stress, I have tested the response of GRP170B deficient worms to a range of concentrations of tunicamycin. Additionally, I have assayed the response of the GRP170B deficient worms to two toxins which target other elements of ER protein folding, dithiothreitol which targets disulfide bond formation and thapsigargin which disrupts calcium-dependent chaperones (Table 2).

Experimental Objectives

Main objective: To determine the functional significance of GRP170B in mediating the response of *C. elegans* to toxin induced ER protein folding stress, to examine whether loss of GRP170B confers susceptibility to a variety of ER protein folding toxins, and to better understand the role of GRP170B in protein folding capacity of the ER.

Specific Objectives

Analyze how the loss of GRP170B effects maturation of *C. elegans* exposed to varying doses of the three different protein folding toxins.

1.) Analyze response to tunicamycin associated with the loss of GRP170B across a range of tunicamycin concentrations from 0 μg/μl – 640 μg/μl.

2.) Analyze resistance to dithiothreitol associated with the loss of GRP170B strains across a range of dithiothreitol concentrations from 0 μg/μl –1280 μg/μl.

3.) Analyze resistance to thapsigargin associated with the loss of GRP170B strains across a range of thapsigargin concentrations from 0 nM – 5nM.

Materials and Methods

Caenorhabditis Strains

Two nematode strains were used in this study. The standard reference strain N2, the Bristol England isolate and BSC06 (Table 3). BSC06 is isogenic with the N2 strain except that it is homozygous for the ok502 deletion allele at the GRP170B locus (Li, 2016). Nematodes were maintained on nematode growth medium (NGM) plates seeded with OP50 *Escherichia coli* as a food source (Wood, 1988). Cultures were maintained at 15° C unless otherwise noted.

Treatment plates

Standard 30 mm culture dishes were used for toxin treatment of nematodes. A sterile pipette was used to deliver 2.5 ml of NGM media into each dish. The *E. coli* bacterial strain OP50, used as a food source, was grown to saturation in LB broth in a small Erlenmeyer flask. The OP50 was seeded onto the NGM plates using a sterile glass rod. The rod was dipped into the liquid culture, excess bacterium was shaken off the rod by tapping the rod twice against the side of the flask. The rod was then used to spread the OP50 on the plates in a circular motion forming a lawn in the middle. The *E. coli* was grown on the NGM plates by incubating them overnight at 37°C.

Prior to addition of toxins, the *E. coli* lawn was killed with UV radiation. The bacterium was treated with UV light by placing the plates bacteria side down on a UV transilluminator for 5 minutes. Killing the *E. coli* served two purposes. First, it limited potential metabolism of the toxins by living bacterium. Second, killing the bacteria avoided possible complications of toxin killing the bacteria resulting in differences in the viability of the OP50 food source from treatment to treatment. The *E. coli* was killed a day before toxins were applied to the plates.

Toxin Treatment Plate Preparation

Tunicamycin (TM) was dissolved in dimethyl sulfate at a concentration of 10mg/ml. This stock solution was further diluted into DMSO to prepare 10 µg/ml, 20 µg/ml, 40 µg/ml and 80 µg/ml stocks. TM solutions were stored at -20° C until added to NGM treatment plates. Dithiothreitol (DTT) was dissolved in sterile water to a concentration of 154 mg/ml. The DTT was further diluted in sterile water to prepare 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml and 160 µg/ml stocks. DTT solutions were stored at -20° C until added to NGM treatment plates. Thapsigargin (TG) was dissolved in DMSO to prepare a 5mM stock. This stock was further diluted in DMSO to prepare 0.5 µM, 1.25 µM, 2.5 µM and 5 µM stocks. TG solutions were stored at -20° C until added to NGM treatment plates.

Toxins were incorporated into NGM plates by diffusion. After the OP50 was killed by UV light, the diluted toxins were added to the surface of the plates. For TM and DTT 20 µl of each dilution was added to the surface of the 2.5 ml plates resulting in a further 125-fold dilution of the toxins to final concentrations (80-1280 µg/µl). For TG 4 µl was added to the surface of the 4 ml plate resulting in a further 1000 fold dilution of TG resulting in final concentrations between 0.5 nM to 5 nM. Plates were incubated overnight at 4° C to allow diffusion of the toxins through the media. Plates were used immediately for nematode maturation assay.

Nematode Maturation Assay

To obtain young gravid hermaphrodites, L4 stage larva from N2 and BSC06 cultures were transferred to a fresh NGM/OP50 plate and incubated overnight at 20° C allowing them to mature to day 1 adults. Synchronized eggs were obtained by transferring 5 of these adult hermaphrodites to toxin treatment plates. The adult nematodes were then allowed to lay eggs for

4 hours and then were removed. The toxin plates with synchronized eggs were incubated for three days at 20º. The nematodes on each toxin plate were then scored as early larva (L1-L3) or late stage/adult larva (L4-Adult). Typically, 4 independent experiments were conducted for each concentration of toxin tested. However, plates where nematodes moved below the surface of the agar were difficult to score and were not included in the analysis.

Results

The goal of my thesis was to investigate the role of GRP170B in mediating the physiologic response of *Caenorhabditis elegans* to protein folding stress. Protein folding stress was induced by exposure of the nematode to three toxins, tunicamycin, dithiothreitol and thapsigargin. Each toxin disrupts protein folding through a different mechanism. The measure of general physiology monitored to study the effects of protein folding stress was maturation from eggs to late larval (L4)/adult stage. To analyze the role of GRP170B, a nematode strain lacking GRP170B (BSC06) was compared to an isogenic control strain expressing GRP170B (N2). Toxins were applied over a range of doses to monitor the response to both moderate and higher levels of protein folding stress.

Tunicamycin Treatment

Tunicamycin (TM) disrupts normal protein folding by blocking N-linked ER glycosylation (Behnke *et al*., 2015). To investigate the role of GRP170B in the stress response to tunicamycin, eggs of GRP170B deficient worms (BSC06 strain) and control worms (N2) were allowed to develop on media with varying desired doses of TM. After 3 days the number of worms at each stage of larval development was recorded (Figure 2). As the concentration of TM increased, the number of worms failing to mature to the L4/adult stage increased for both BSC06 and N2 worms. This reduction on maturation demonstrating these doses were stressing the worms. The BSC06 GRP170B deficient worms were less sensitive to the TM than the N2 control strain at all tested concentrations (80-640 μg/μl) of TM (Table 4). In the tunicamycin experiments, 100% of the untreated N2 control worms (0 μg/μl tunicamycin) developed into L4/adult stages in 3 days (Figure 3, Table 4). However, the untreated BSC06 worms lacking

GRP170B matured slower with a mean of only 84% developing to these late stages. This is inconsistent with previous studies which found that the N2 and BSC02 strains developed at equal rates with both strains maturing to the adult stage in 3 days (Li, 2016). All the concentrations of tunicamycin tested effected the physiology of the worms as fewer of the worms maturing into adults in both N2 and BSC06 strains. At the lowest concentration of tunicamycin (80 µg/µl) tested, the percentage of N2 worms maturing to L4/adult stages dropped from 100% to 33%. For the BSC06 strain this concentration of tunicamycin decreased the percentage of worms developing to these stages from 84% to 76%. As the concentration of tunicamycin increased, fewer and fewer of the worms of either strain matured to the L4/adult stages. At the highest concentration tested (640 µg/µl) only 2% of the N2 worms and 7% of the BSC06 worms reached the late stages of development.

At all concentrations of tunicamycin tested, the BSC06 strain was more resistant to the tunicamycin protein folding toxin as a greater percentage of BSC06 worms matured to the adult stage compared to N2 worms (Table 4). At 80 µg/µl tunicamycin 2.3 fold more BSC06 worms matured to L4/adults stage compared to N2 worms. At higher concentrations the difference in BSC06 to N2 worms maturing to adult stages was even greater. At 320 µg/µl tunicamycin, 4.3 fold more BSC06 worms reach L4/adult stage compared to N2 worms. Even at the highest concentration of tunicamycin tested, 640 µg/µl, where less than 10% of the worms of either strain reached the late stages of development, 3.5 fold more of the BSC06 worms were maturing to the L4/Adult stage compared to N2 worms (Table 4). These results demonstrate that loss of GRP170B makes the nematode more resistant to tunicamycin at both high and low concentrations.

Dithiothreitol Treatment

Dithiothreitol (DTT) affects protein folding by disrupting disulfide bond formation. To examine the role of dithiothreitol on development of nematodes deficient for GRP170B, eggs of GRP170B deficient worms (BSC06 strain) and eggs of the control strain (N2) were allowed to develop on media with various doses of this dithiothreitol at 20°C. After 3 days the number of worms at each stage was recorded. In contrast to the tunicamycin experiment, in the dithiothreitol experiment more of the untreated BSC06 worms matured to the L4/adult stage (95%) compared to the N2 worms (72%) (Figure 4, Table 5). This was inconsistent with previous developmental studies of these two strains (Li, 2016) and with my results in the tunicamycin experiments described above. This illustrates the type of experimental variation observed throughout my experiments. The lower concentrations of dithiothreitol (80-320 µg/µl) tested had little effect on the maturation of the worms of either strain with means of 80 to 100% of the worms reaching L4/adult stages. The higher concentrations of dithiothreitol tested delayed development in both strains. At 640 µg/µl, the percentage of N2 worms maturing to L4/adult dropped to 74% and the percentage of BSC06 maturing to L4/adult dropped to 66%. At the highest concentration of dithiothreitol tested (1280 µg/µl) no worms of either strain matured to L4/adult stages.

The dithiothreitol experiments provided little evidence that loss of GRP170B increased or decreased sensitivity to the toxin (Table 5). The lower concentrations tested (80-320 µg/µl) showed little physiological effect on the worms of either strain. Although at 640 µg/µl dithiothreitol, both strains were effected by the toxin and the results were similar. Again, at the highest concentration tested 1280 µg/µl, no worms of either strain matured to the L4/adult stages, providing no evidence for an effect of the loss of GRP170B in BSC06.

Thapsigargin Plate Assay

Thapsigargin affects protein folding by inhibiting an ER calcium pump called sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) (Zwaal *et al*., 2001). This disrupts protein folding by interfering with the activity of calcium dependent ER chaperones *(Zwaal et al*., 2001). It was anticipated that the loss of GRP170B would make the worms more sensitive (less resistant) to thapsigargin. To investigate the role of this toxin on development of nematodes, eggs of GRP170B deficient worms (BSC06 strain) and eggs of the control strain (N2) were allowed to develop on media with various doses of thapsigargin at 20°C. After 3 days the percent of worms maturing to L4 stage was recorded (Figure 5). This assay revealed that the worms were slightly less sensitive to the toxin (concentrations used 0.5 nM to 5 nM). A previous study reported the effect of these concentrations on fertility (Zwaal *et al*., 2001). Our study examined the rate of development in response to thapsigargin. The untreated (0nM) N2 strain developed to 98% while the BSC06 worms developed to only 89%. However, previous studies found that the N2 and BSC02 strains developed at equal rates with both strains maturing to the adult stage in 3 days. Therefore, these results were inconsistent with the pervious study (Li, 2016). At all the concentrations tested, there was little physiological effect on the worms of either strain with the worms developing well having only little delays at each dosage (Figure 6, Table 6). At the lowest concentration of thapsigargin (.5nM) tested 99% each strain of worms matured to L4/adult stages. At 1.25 nM, 99% of the BSC06 worms matured fully while 98% of the N2 worms matured to. At 2.5 nM, 100% of the BSC06 worms matured to the advanced developmental stages while 97% of the N2 worms matured fully. Our results revealed that at lower doses of 0.5 to 2.5 µM, most of the worms for both BSC06 and N2 matured to L4 stage with no delay in development observed. This suggested that this level of toxin dose was too low

to affect physiology. At these doses, the BSC06 strain was developing modestly better than the N2 strain deficient for the GRP170B gene, but the results were very similar. At the highest dose of thapsigargin (5 nM), 86% of the BSC06 matured to L4/adult stage while 96% of N2 strain matured to L4/adult stages. At this dose, there is a difference observed in which the N2 worms begin to develop better than the N2 strain of worms (Table 6). The BSC06 strain showed a drop in percent maturity while N2 worms were not affected. This study revealed that thapsigargin had only a modest effect on the rate of development in N2 control nematodes and BSC06 GRP170B deficient nematodes. The worms for both strains were not sensitive to these concentrations of the thapsigargin toxin.

Discussion

The nematode *Caenorhabditis elegans* has two genes encoding the large ER chaperone, GRP170. There is circumstantial evidence that one of the two isoforms encoded by these genes, GRP170B, functions in the nematode's response to protein folding stress. First, deletion of the locus encoding this isoform has little effect on the overall physiology of the nematode under non-stress conditions (Li, 2016). Second, GRP170B mRNA expression is induced during protein folding stress and this induction is dependent on the unfolded protein response (UPR) signal transduction pathway (Rockwell, 2016). To investigate directly the role of GRP170B in protein folding stress, I compared the physiological response of a strain lacking GRP170B (strain BSC06) with an isogenic strain expressing normal GRP170B (strain N2) upon exposure to three different protein folding toxins, tunicamycin (TM), dithiothreitol (DTT) and thapsigargin (TG). As a broad measure of physiological health, the percentage of eggs maturing into L4/Adult stage after three days was measured. Under standard conditions, *C. elegans* matures to the late larva/adult stage in 72 hours. Impaired physiology can slow the rate of development causing a reduction in the number of worms maturing in this time period.

The N2 control strain used in this study seemed to have developed slower than the standard rate reported broadly in the literature. It is well documented that this strain normally develops into adults at 20° C in 72 hours (Wood, 1988). However, in my experiments, a portion of the N2 eggs failed to mature into late stage L4 larva or adults but remained in early larval stages (L1-L3). It is likely that the variation in developmental timing of N2 in our laboratory could be due to accumulations of mutations and genetic drift that can occur from repeated passage of the strains in culture conditions. The N2 strain has been matured in the laboratory by transferred cultures for many generations, allowing accumulations of many random mutations.

Researchers have shown that genetic variants exist in the N2 strain and these variants impact phenotypic differences in the N2 populations (Sterken, Snoek, Kammenga, & Andersen, 2015).

The N2 strain showed the typical rate of development with the use of untreated worm during the tunicamycin and thapsigargin experiments, having100% and 98% of the larva reaching late L4 or adult stage respectively. The BSC06 strain showed a delay in development with 84% and 89% development of the larva to late L4 or adult stage respectively. However, during the dithiothreitol experiment, the untreated BSC06 worms surprisingly showed a more typical developmental role, with 95% of the larva reaching L4 or adult stage and only 72% of N2 nematodes developing to these stages. This complicates the analysis in two ways. First, the two strains were originally designed to be isogenic. However, the possibility of genetic drift suggests they may have genotypic differences. Second, the differences in the base line rate of development further complicate the comparison of the two strains. For simplicity, for each treatment, the relative development of the two strains was compared by dividing the percentage of BSC06 nematodes reaching late stages of development by the percentage of N2 nematodes reaching this stage. In untreated worms this value was typically 1.2, indicating that 20% more BSC06 reached maturity compared to the N2 strain.

Tunicamycin is an antibiotic that acts by inhibiting the enzyme dolichyl-phosphate (UDP-N-acetylglucosamine) N-acetylglucosaminephosphotransferase 1 (DPAGT1) complex which catalyzes the first step of n-glycosylation in the ER. By blocking protein glycosylation, tunicamycin disrupts protein folding in the ER (Heifetz, Keenan, & Elbein, 1979). GRP170B makes worms more sensitive to this antibiotic. It has been extensively used to induce protein folding stress and the UPR (Oslowski & Urano, 2011). Among the protein induced by tunicamycin induced UPR is GRP170B (Rockwell, 2016). This has been previously examined

with respect to GRP170 (Li, 2016). In an earlier study of the physiological effect of tunicamycin on GRP170B deficient nematodes, worms lacking GRP170B surprisingly show a slight resistance to tunicamycin compared to the N2 strain containing GRP170B (Li, 2016).

In this study, BSC06, the GRP170B deficient strain show a developmental delay compared to the N2 worms with approximately 16% more of the N2 worms reaching the L4/adults stages. However, at 80-160 µg/µl TM, an increased percentage of the GRP170B deficient worms reached the later stages of development than the N2 strain of worms (Table 4). BSC06 nematodes were less sensitive (or more resistant) to the toxin and exhibited better development at the lower doses (Figure 3). The reason why GRP170B deficient worms would be resistant to tunicamycin is unclear. However, at these doses of TM, loss of GRP170B clearly does not impair this measure of physiologic health during stress.

At the highest concentration of TM tested (640µg/µl) GRP170B is not essential for advanced development over the tested timeframe. At this high concentration of TM only 7% of the GRP170B deficient nematodes matured to L4/Adult stage while 2% of the control strain developed to L4/Adult stage. For both strains at the highest doses, there was a delay in development observed. This data suggests that the tunicamycin is not allowing the *C. elegans* to develop fully at higher doses and GRP170B shows no role in mitigating protein folding stress at the higher concentrations.

DTT is a reducing agent which affects protein folding by disrupting disulfide bond formation in the ER. Disulfide bonds help maintain the shape and stability of secreted proteins. DTT exposure results in a decrease of proper protein folding and an increase in protein misfolding. The use of DTT could totally inactivate the folding process (Braakman *et al*., 1992). In doing so, DTT also induces UPR in *C. elegans*. Disulfide bond formation has been shown to

be important in stabilizing the folded state of proteins. DTT has also been shown to have a direct role in protein folding (Darby & Creighton, 1997).

In my study, there was no difference in DTT sensitivity between BSC06 worms deficient for GRP170B and N2 control strain. In the absence of the poison, the BSC06 worms deficient for the GRP170B strain developed 32% better than the N2 control strain (Table 5). However, the affect was modest with little difference in sensitivity.

At the lower doses (80-640 µg/µl), there was an increase in the maturation percentage of L4 staged worms for both strain of worms. This suggests that DTT is not affecting the metabolism of the nematodes at the lowest doses. However, at the highest dose (1280 µg/µl) tested neither BSC06 nor N2 strain of worms developed to L4 stage (Table 4 and Figure 4). These data suggest that at high doses, the toxin is delaying the *C. elegans* development.

Thapsigargin is used in animals to induce the UPR (Oslowski & Urano, 2011). It is a conserved sarco-endoplasmic reticulum Ca^{2+} transport ATPase (SERCA) specific suppressor. By inhibiting SERCA, thapsigargin consequently incites apoptosis in virtually all cells. The nematode *C. elegans* possess only one SERCA counterpart which was characterized in the experimental study done by Richard Zwaal *et. al* (2001). This counterpart or homologue is encoded by a gene called sca-1 (Zwaal *et al*., 2001). Thapsigargin treatment results in a reduction of ER calcium levels which affects chaperone activity. When the chaperone activity is affected, unfolded proteins begin to aggregate in the ER (Oslowski & Urano, 2011).

In *Caenorhabditis elegans*, thapsigargin has been shown to affect fertility at doses 1.5 to 5 µM. A previous study revealed that the loss of GRP170B made the worms more sensitive (less resistant) thapsigargin when analyzing fertility (Zwaal *et al*., 2001). In this study I modified the protocol and tested dosages .5 to 5 µM to investigate the effect on development.

With no introduction of the poison, the N2 control strain developed modestly better than the BSC06 worms deficient for the GRP170B. The worms tested with thapsigargin showed no difference between the two strains.

Lower doses of 0.5 to 2.5 µM, had little effect on either strain with most of the nematodes maturing to L4 stage with no delays in development observed. The results illustrate that this level of toxin doses are too low to see an effect on development.

For the highest doses tested 2.5 to 5 µM, BSC06 showed a drop in percent maturity while N2 worms were not affected by the poison. My research has shown that thapsigargin had only a modest effect on the rate of development in N2 control nematodes and BSC06 GRP170B deficient nematodes. The results suggest that thapsigargin is not affecting the physiology of *C. elegan* worms at these concentrations.

Several pilot studies using higher concentrations of thapsigragin (results not shown) was also carried out. This data revealed that N2 wildtype was slightly sensitive, but at the highest dosage the worms were more sensitive to the poison. However, the development was not as delayed as the BSC06 strain. It is worth repeating these experiments at higher doses in order to reveal how thapsigargin is affecting nematodes deficient for GRP170B. The results were unanticipated, but there was no significant affect in the preliminary data.

Experimental variations throughout this study made it difficult to analyze the data statistically and limited our certainty of the conclusion. In an attempt to reduce variations, the lab protocols were followed accordingly, eggs used during the study were all from synchronized adult worms and all experiments were repeated following a strict time schedule. Tunicamycin was successfully tested with the results having some experimental variation making it hard to see a strong affect. Tunicamycin was expected to have less variations since it is not volatile and is

relatively stable. Other researchers have reported similar issues from experimenter to experimenter when working with *C. elegans* (Lithgow, Driscoll, & Phillips, 2017). It was shown that experiments done using the same protocols produced different results between different well-respected laboratories. It was shown that replicability of the experiments relied heavily on the methods utilized and even subtle changes made big impacts on results. These changes include the quality and sterile states of compounds utilized, variances in the microbial environments as well as minute changes to techniques carried out by the experimenter

This study provided insight on what GRP170B physiologic role. Identifying which class of factors is associated with resistance will provide us with evidence to the role in ER protein metabolism associated with GRP170B. In this thesis research high doses of tunicamycin resulted in nematodes that required GRP170B to develop better. For the dithiothreitol, there is not enough evidence to suggest whether or not GRP170B is protected or sensitized to this toxin. For the last toxin thapsigargin, there was also no difference between the N2 control strain and the BSC06 GRP170B deficient strain. The findings of this study are beneficial for future studies and assist with gaining a better understanding for the functions of GRP170B which includes proposed cellular roles acting as a direct chaperone utilizing foldase activity as well as co-chaperone activity for GRP78 using NEF and holdase activity (De Keyzer *et al*., 2009; Easton *et al*., 2000).

Future Research and Recommendations

This thesis has provided insight as to what happens to *C. elegans* at an organismal level when ER stress introduced using tunicamycin, dithiothreitol and thapsigargin. The analysis of GRP170B produced a reasonable dataset and provided information that future studies could benefit from. This research provided data in support how the GRP170B gene functions specifically in *C. elegans* with the use of tunicamycin toxin. The experiments suggest that GRP170B is very important in normal non-stress conditions and stress physiology of the nematode. This is demonstrated well since under normal conditions the worms developed normally, but when the GRP170B gene was knocked out there was some variability when using these toxins. This maybe in response to gene mutation.

Using the toxin tunicamycin revealed that, the GRP170B deficient strain exhibited a slight developmental advantage over N2 control worms at the lowest doses. At low doses of tunicamycin, development of the nematode is not delayed with advanced development in the GRP170B deficient strain. This demonstrates that at low doses of tunicamycin, the loss of GRP170B has an important role in protein folding tolerance. However, at the highest doses there was no difference in development. With treatment of the thapsigargin toxin, there was no difference in development between the two strains. For the DTT, it would be wise to test higher doses in the same range utilized (320-1280 μg/μl) to examine the effects on the nematodes. For the TG, it would be a good idea to test fertility since there is evidence suggesting an effect on fertility using this toxin. UPR induction using SJ4005 would also give insight as to what GRP170B is doing in the nematodes.

In the future, it would be beneficial to compare GRP170 and GRP78 to see if there is a difference with knocking out the small GRP78 versus the large GRP170. Although, it could be a

great experiment the GRP78 strain of worms are already sick which could modify the data. It still would be good to show that this works when a different Grp (BIP) is knocked out. It would be interesting to test other stressors that stress *C. elegans* ER and observe the changes in some stages of development as a result of GRP170B knockout. It would also be beneficial to carry out future experiments using new control strains.

In *C. elegans,* the conserved SERCA (target) of thapsigargin allows for genetic and chemical suppressor analyses to detect therapeutic drug targets. This could result in molecules that are essential for the treatment of the SERCA associated illnesses such as heart disease (Zwaal *et al*., 2001). This type of experimentation is vital and gives researchers insight as to what the GRP170 gene is doing at a molecular level to gain understanding of *C.elegans.*

This study examined the role of GRP170B in mediating the physiologic response of *Caenorhabditis elegans* to protein folding stress. It was hypothesized that loss of GRP170B would make the nematodes more sensitive (or less resistant) to forms of protein folding stressors. Protein folding is a complex area to study. Testing one toxin would not capture the exact role for GRP170B in protein folding stress. It is possible that GRP170B is important for responding to any stress. However, GRP170B may only be important in responding to one or two protein folding toxins. Responding to specific toxins would provide insight on the role in protein folding.

The goal of this study was to see if GRP170B plays a role in stress using the three toxins and to study the function of GRP170B. This is important in understanding the protein folding process. This study showed an effect on nematodes deficient for GRP170B that were treated with low doses of tunicamycin and dithiothreitol. There is a small effect of these toxins on illustrating that the loss of GRP170B confers tolerance to specific agents that interfere with normal ER

protein folding. However, the thapsigargin data showed that loss of GRP170B was not being affected by the toxin at the doses utilized.

Protein metabolism in the ER involves a variety of processes and degradation of proteins through a ERAD. The absence of GRP170B makes the worms less sensitive to tunicamycin. It would be interesting to test some genetic approaches to see if this sensitivity is dependent on ERAD. The loss of ERAD could be examined in the worms to check for this sensitivity. This could be done using the knockout of ERAD under the presence or absence of GRP170B. Another option is to use GRP78 or protein loading dependence under the presence or absence of GRP170B. These experiments could help us in further understanding the sensitivity of the worms in response to the presence or absence GRP170B, and see if ERAD has a major role. This thesis research provides information on nematodes as a good model for studying aspects of human biology. The goal was to figure out where GRP170B is expressed inside the organism. A complete understanding is not yet known and needs to be further examined. This research has provided some important information for future researchers to use when examining the role of GRP170B in *C. elegans* physiology.

Supporting Figures and Tables

Table 1: Wild type and deletion allele conditions

	WT	Deletion Alleles		
Locus	UPR Induction	Rate of Development	Embryo Development	Protein Folding
GRP170A	Non-inducible (Atypical)	Slower	Defective	UPR Induced
GRP170B	Inducible (Normal)	Normal	Normal	Normal

Table 2: Agents that interfere with ER protein folding

Protein Folding Toxin	Target
Tunicamycin	N-Glycosylation
Dithiothreitol	Disulfide bridge formation
Thapsigargin	Calcium homeostasis

Table 3: *Caenorhabditis elegans* strains used in this study

Strain	Type	Allele(s)
N2	Laboratory Standard	Both
BSCO6	Deletion Mutant	T24H7.2 (GRP170A)

Table 4: Mean Percentage of Nematodes Treated with Tunicamycin Maturing to L4/Adult

Tunicamycin concentration (µg/µl)	BSC06 maturing to L4/Adult $\bar{x}$ % ± S.D.[1]	N2 maturing to L4/Adult $\bar{x}$ % ± S.D.	Comparison of BSC06 to N2 strain[2]
0	84 ± 18 (n=2)[3]	100 ± 0	84
80	76 ± 20 (n=4)	33 ± 12 (n=4)	230
160	46 ± 30 (n=4)	12 ± 18 (n=4)	383
320	30 ± 46 (n=4)	7 ± 9 (n=4)	428
640	7 ± 13 (n=4)	2 ± 4 (n=4)	350

[1] Mean ± standard deviation of multiple experiment of the percentage of eggs which matured to at least L4 stage in 3 days.

[2] Relative difference in percentage of BSC06 and N2 eggs maturing to L4/adult stage ($\bar{x}$ of BSC06/$\bar{x}$ of N2). Each experiment analyzed the maturation of eggs laid by 5 young hermaphrodites.

[3] Number of independent experiments analyzed for each treatment.

Table 5: Mean Percentage of Nematodes Treated with Dithiothreitol Maturing to L4/Adult

Dithiothreitol µg/µl	BSC06 Mean % ± S.D.[1]	N2 Mean % ±S.D.	Relative % BSC06 to N2[2]
0	95 ± 95 (n=1)[3]	72 ± 19 (n=3)	132
80	82 ± 28 (n=3)	89 ± 13 (n=4)	92
160	84 ± 24 (n=3)	100 ± 0 (n=4)	84
320	97 ± 4 (n=3)	98 ± 2.6 (n=4)	99
640	66 ± 43 (n=3)	74 ± 50 (n=4)	89
1280	0 ± 0 (n=2)	0 ± 0 (n=4)	N/A[4]

[1] Mean ± standard deviation of multiple experiment of the percentage of eggs which matured to at least L4 stage in 3 days.

[2] Relative difference in percentage of BSC06 and N2 eggs maturing to L4/adult stage ($\bar{x}$ of BSC06/$\bar{x}$ of N2). Each experiment analyzed the maturation of eggs laid by 5 young hermaphrodites.

[3] Number of independent experiments analyzed for each treatment.

[4] Not calculated because no BSC06 matured to L4/adult stage.

Table 6: Mean Percentage of Nematodes Treated with Thapsigargin Maturing to L4/Adult

Thapsigargin nM	BSC06 Mean % ± S.D.[1]	N2 Mean % ±S.D.	Relative % BSC06 to N2[2]
0	89 ± 20 (n=4)[3]	98 ± 1.6 (n=3)	91
.5	99 ± 1.5 (n=4)	99 ± 1.2 (n=3)	100
1.25	99 ± 1.5 (n=4)	98 ± 2.5 (n=3)	101
2.5	100 ± 0 (n=4)	97 ± 1.2 (n=3)	103
5	86 ± 16 (n=4)	96.5 ± 4.9 (n=3)	89

[1] Mean ± standard deviation of multiple experiment of the percentage of eggs which matured to at least L4 stage in 3 days.

[2] Relative difference in percentage of BSC06 and N2 eggs maturing to L4/adult stage ($\bar{x}$ of BSC06/$\bar{x}$ of N2). Each experiment analyzed the maturation of eggs laid by 5 young hermaphrodites.

[3] Number of independent experiments analyzed for each treatment.

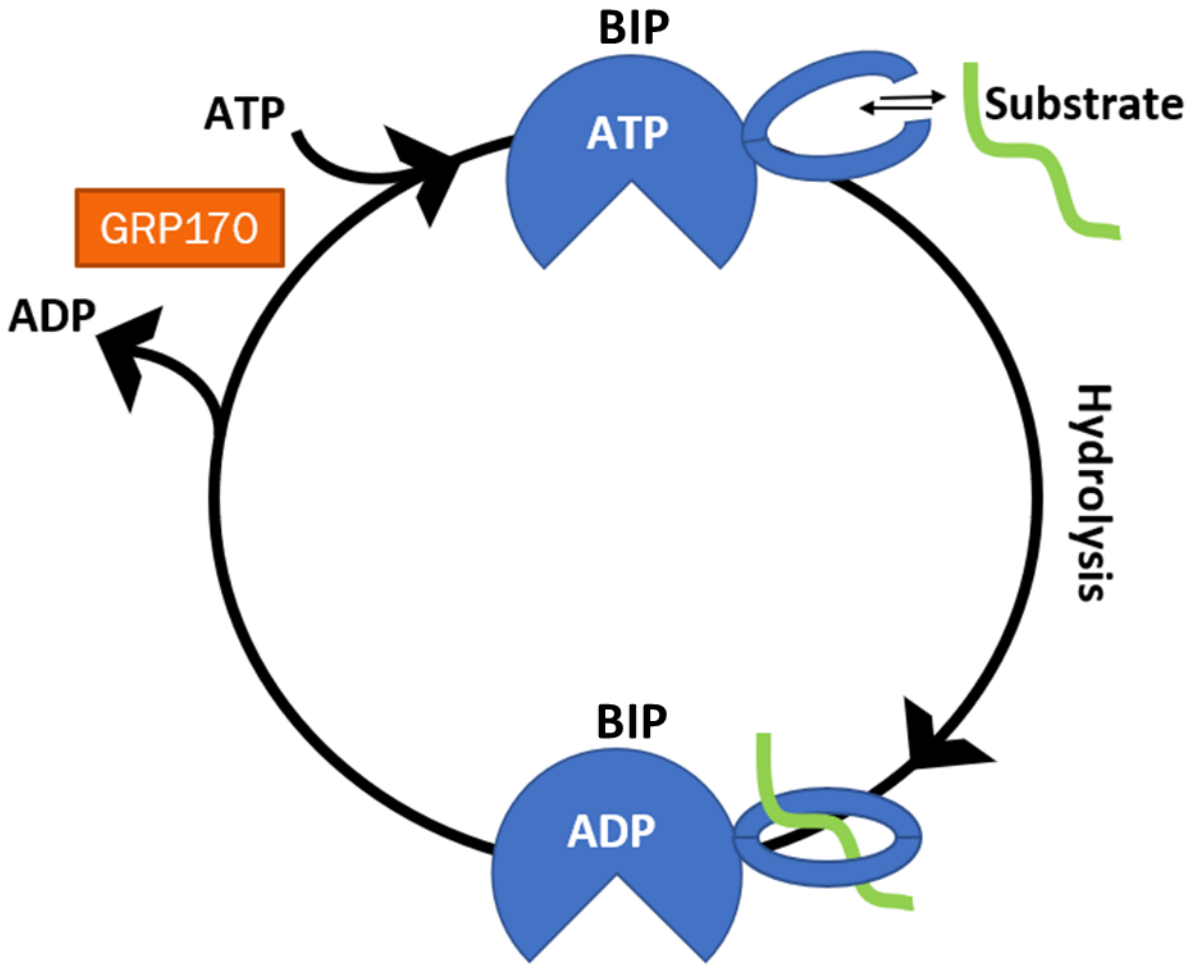

Figure 1: Schematic of BIP (ER HSP70) ATP to ADP cycle with assistance from GRP170 chaperone acting as an NEF.
When ATP is hydrolyzed to ADP the lid clamps down on the peptide preventing aggregation. In order to open and release the peptide GRP170 is required and facilitates a nucleotide exchange factor (Mayer & Bukau, 2005).

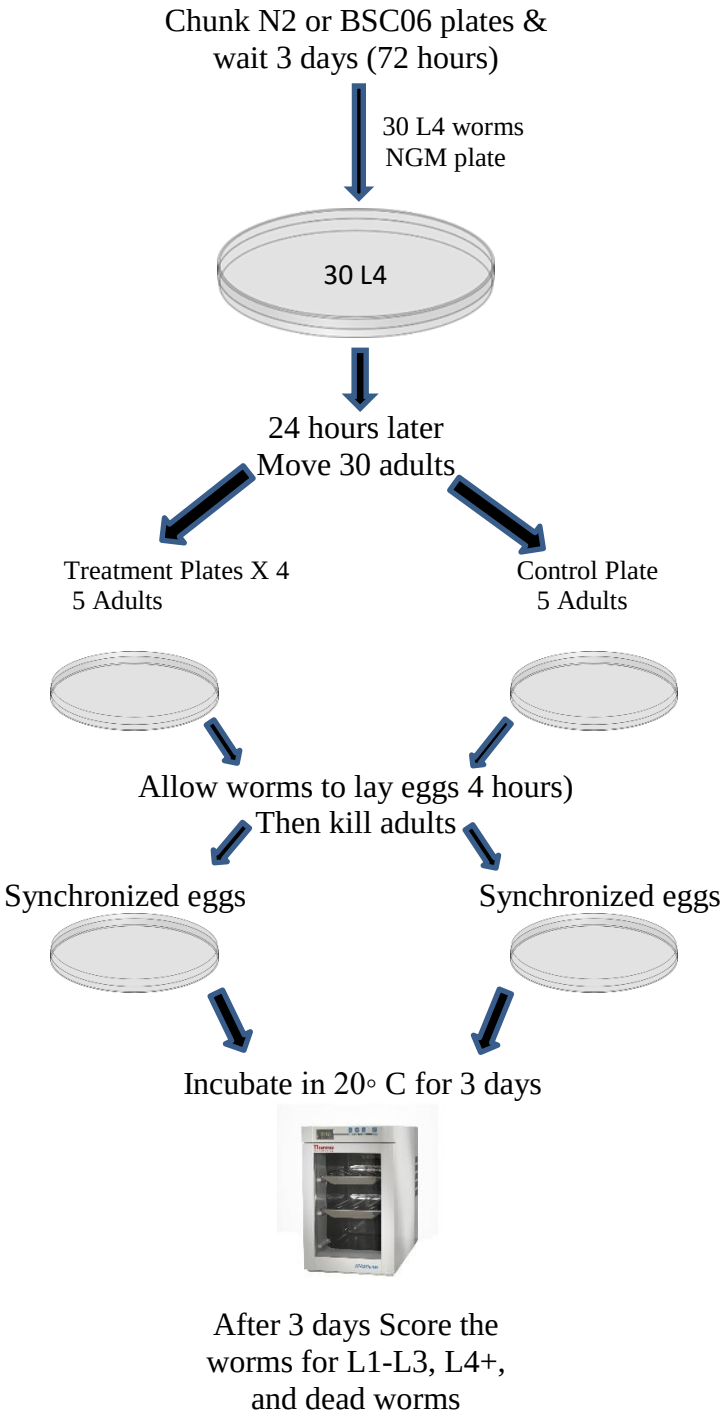

Figure 2: Flow chart of general experimental design
This diagram shows the process the nematodes go through for the treatment plan experiments.

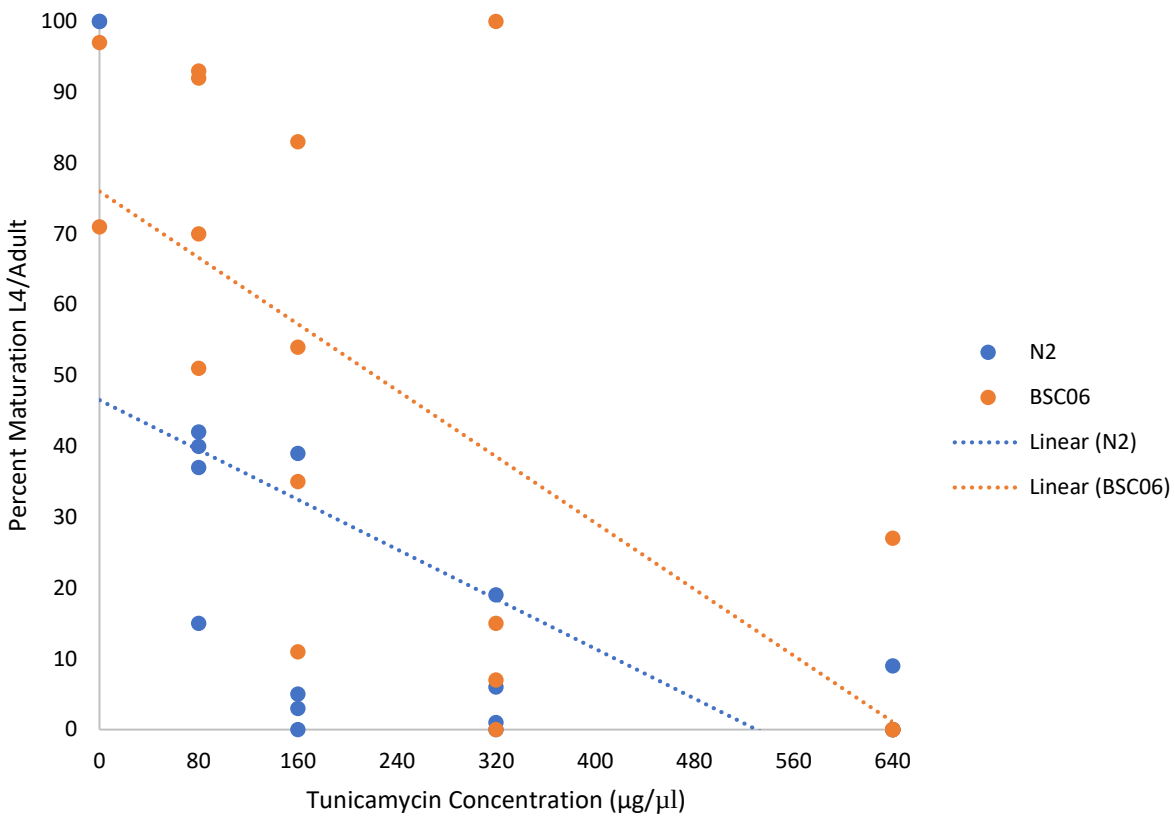

Figure 3: Analysis of Tunicamycin Concentration on Nematode Development
Tunicamycin graphs for N2 and BSC06 worms with double the concentration doses (80-640µg/µl). The orange and blue dots correspond to several different experiments. The graph shows the toxin concentrations and percent maturation to L4 stage nematodes. The BSC06 nematodes are less sensitive to the toxin at all concentrations of tunicamycin. The mutant strain is more sensitive to the toxin at all doses. Both strains show an increase in sensitivity at higher doses from 165 to 640µg/µl.

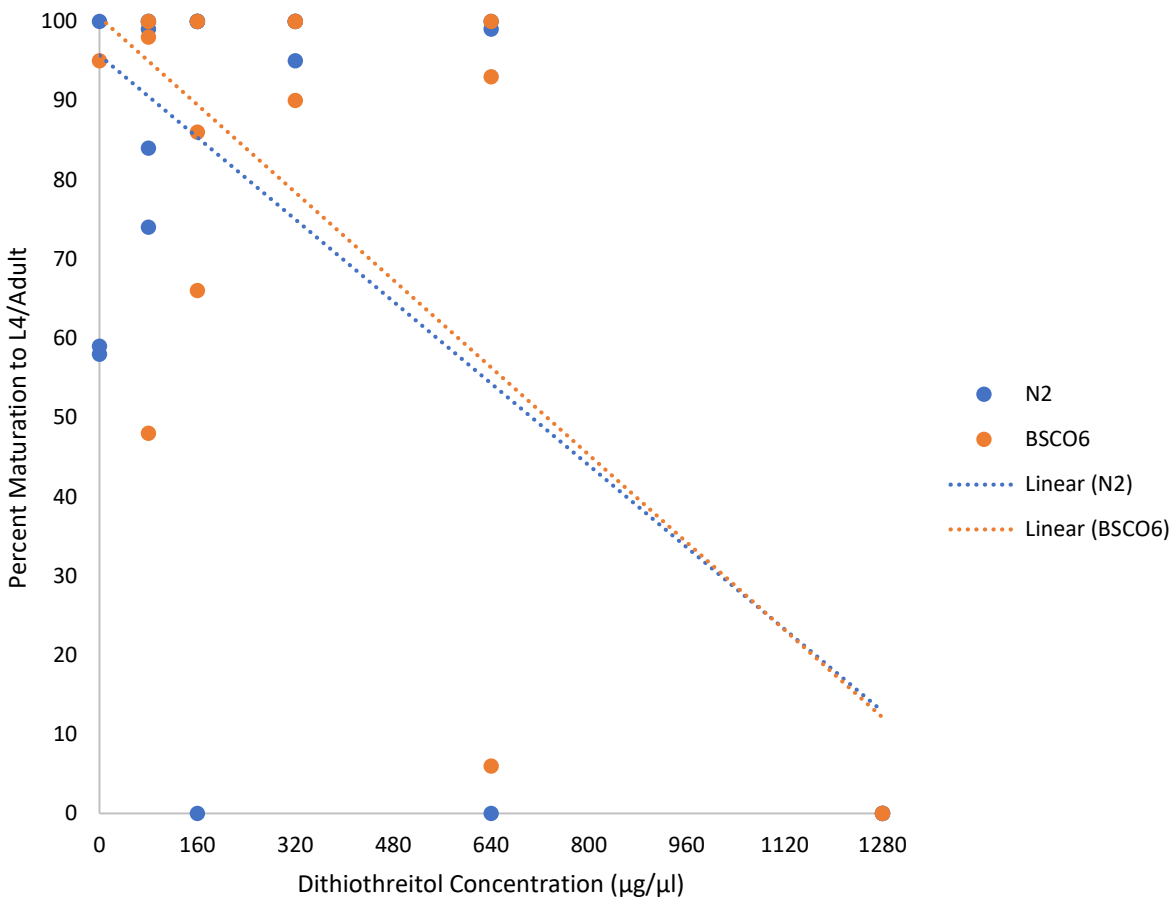

Figure 4: Analysis of Dithiothreitol Concentration on Nematode Development
Dithiothreitol at double the concentration doses (80-1280µg/µl). The orange and blue dots correspond to several different experiments. The graph shows the toxin concentrations and percent maturation to L4 stage nematodes. The N2 nematodes are less sensitive to the toxin at all concentrations of DTT. Both strains exhibit a similar trend with nematode development to L4 stage decreasing as toxin concentration increases.

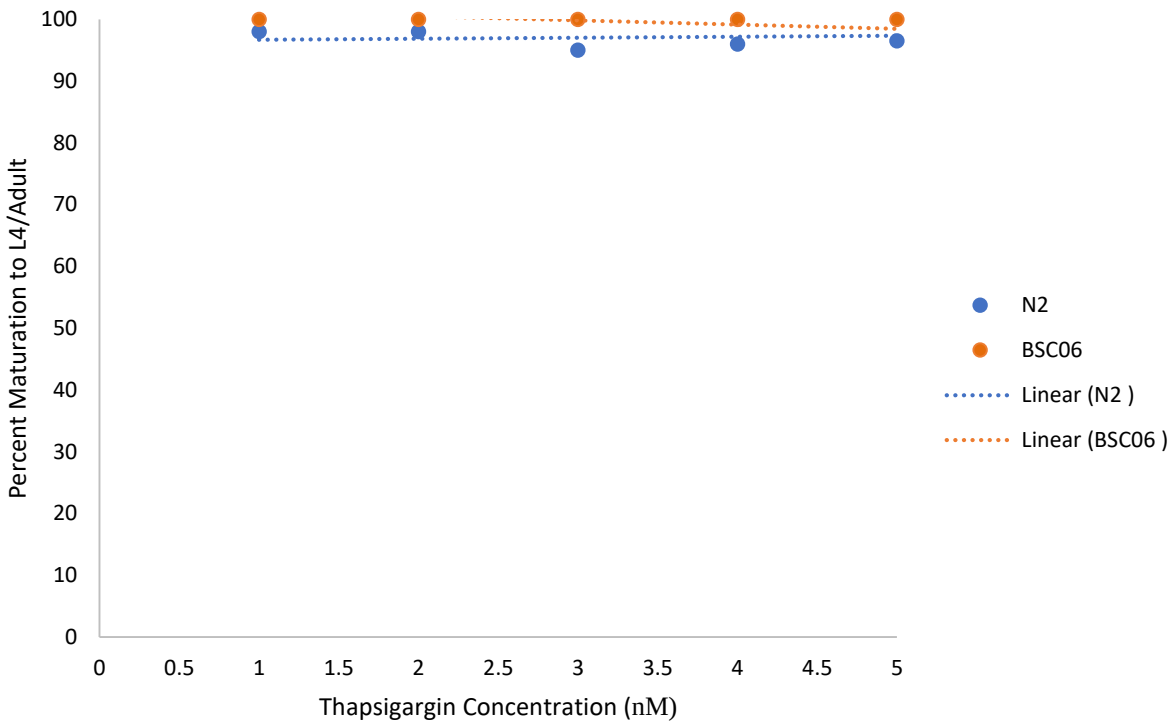

Figure 5: Analysis of Thapsigargin Concentration on Nematode Development
Thapsigargin at examined concentration doses (0.5-5nM). The orange and blue dots correspond to several different experiments. The graph shows the toxin concentrations and percent maturation to L4 stage nematodes. The N2 and the BSC06 nematodes are less sensitive to the toxin at all concentrations of TG. Both strains exhibit a similar trend with nematode development to L4 stage decreasing as toxin concentration increases.

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Appendix

Table A1: Tunicamycin Data for N2 and BSC06 Strain Development

Total Percent Development of Tunicamycin Treated N2 Nematodes							
Toxin Concentration N2 Strain (µg/µl)	L4	L1-L3	Dead	Toxin Concentration BSC06 Strain (µg/µl)	L4	L1-L3	Dead
0	100	0	0	0	71	29	0
0	100	0	0	0	97	3	0
80	42	53	5	80	51	49	0
80	37	41	22	80	92	4	4
80	40	28	32	80	93	5	2
80	15	28	57	80	70	18	12
160	39	50	11	160	35	63	2
160	3	41	56	160	83	8.5	8.5
160	0	54	46	160	54	10	36
160	5	46	49	160	11	83	6
320	6	47	47	320	100	0	0
320	19	34	47	320	7	52	41
320	1	60	39	320	15	45	40
320	0	82	18	320	0	91	9
640	0	16	84	640	0	100	0
640	9	51	40	640	27	39	34
640	0	79	21	640	0	50	50
640	0	79	21	640	0	84	16

Table A2: Dithiothreitol Data for N2 and BSC06 Strain Development

Total Percent Development of Dithiothreitol Treated N2 Nematodes							
Toxin Concentration N2 Strain (µg/µl	L4	L1-L3	Dead	Toxin Concentration BSC06 Strain (µg/µ)	L4	L1-L3	Dead
0	100	0	0	0	95	5	0
0	59	41	0	0	*	*	*
0	58	42	0	0	*	*	*
80	100	0	0	80	48	52	0
80	100	0	0	80	98	2	0
80	84	16	0	80	100	0	0
80	74	26	0	80	*	*	*
160	100	0	0	160	66	34	0
160	100	0	0	160	100	0	0
160	100	0	0	160	86	14	0
160	100	0	0	160	*	*	*
320	100	0	0	320	100	0	0
320	95	5	0	320	90	10	0
320	100	0	0	320	100	0	0
320	100	0	0	320	*	*	*
640	99	1	0	640	100	0	0
640	100	0	0	640	93	7	0
640	99	1	0	640	6	94	0
640	0	100	0	640	*	*	*
1280	0	95	5	1280	0	100	0
1280	0	100	0	1280	0	96	4
1280	0	98	2	1280	*	*	*
1280	0	100	0	1280	*	*	*

* Insufficient data to report.

Table A2: Thapsigargin Data for N2 and BSC06 Strain Development

Total Percent Development of Thapsigargin Treated N2 Nematodes											
Toxin Concentration N2 Strain (nM)	L4	L3	L2	L1	Dead	Toxin Concentration BSC06 Strain (nM)	L4	L3	L2	L1	Dead
0	98	0	0	1	0	0	100	0	0	0	0
0	97	3	0	0	0	0	100	0	0	0	0
0	100	0	0	0	0	0	98	0	2	0	0
0	*	*	*	*	*	0	60	0	0	40	0
0.5	98	2	0	0	0	0.5	100	0	0	0	0
0.5	100	0	0	0	0	0.5	97	0	3	0	0
0.5	100	0	0	0	0	0.5	100	0	0	0	0
0.5	*	*	*	*	*	0.5	98	2	0	0	0
1.25	95	0	5	0	0	1.25	100	0	0	0	0
1.25	100	0	5	0	0	1.25	100	0	0	0	0
1.25	98	0	2	0	0	1.25	98	0	0	2	0
1.25	*	*	*	*	*	1.25	97	2	0	1	0
2.5	96	2	0	2	0	2.5	100	0	0	0	0
2.5	98	2	0	0	0	2.5	100	0	0	0	0
2.5	98	2	0	0	0	2.5	100	0	0	0	0
2.5	*	*	*	*	*	2.5	100	0	0	0	0
5	96.5	0	3.5	0	0	5	100	0	0	0	0
5	93	7	0	0	0	5	69	31	0	0	0
5	100	0	0	0	0	5	100	0	0	0	0
5	*	*	*	*	*	5	77	2	13	8	0

* Insufficient data to report.