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Distinct Physiological Roles for the Two Isoforms of the ER Chaperone GRP170 in Caenorhabditis elegans

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Distinct Physiological Roles for the Two Isoforms of the ER Chaperone GRP170 in *Caenorhabditis elegans*

by

Yuanyuan Li

An Abstract of a Thesis
in Biology

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

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State University of New York
Buffalo State
Department of Biology

ABSTRACT OF THESIS

Distinct Physiological Roles for the Two Isoforms of the
ER Chaperone GRP170 in *Caenorhabditis elegans*

GRP170 is a large molecular chaperone found in the ER of all eukaryotes. The nematode *Caenorhabditis elegans* has two loci encoding GRP170: T24H7.2 (grp170a) and T14G8.3 (grp170b). The phenotypes of nematodes genetically deficient for either grp170a or grp170b were compared to a standard laboratory strain with functional grp170 loci. Worms that were deficient for grp170a developed 32% slower than the control strain. The loss of grp170a had a significant but modest reduction on the life span compared to the control strain. Worms deficient for grp170a also displayed significantly increased embryonic lethality and resulted in 6.9% arrested embryos. The loss of grp170b did not change the rate of development, lifespan, or affect embryonic lethality. These data suggested that grp170a has a more critical role in the physiologic processes associated with completion of development compared to grp170b. To investigate whether either of the two grp170 loci plays a protective role during ER stress, sensitivity of the grp170 deficient worms to the ER toxin tunicamycin was analyzed. A sublethal dose of tunicamycin (3 μg/ml) modestly slowed development of both the control strain and the grp170a deficient strain. Worms deficient for grp170b were unexpectedly resistant to tunicamycin and did not show a developmental delay in the presence of tunicamycin.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Overview

Chaperones are essential proteins that help other polypeptides fold into their functional three dimensional shapes and that prevent unfolded proteins from aggregating. GRP170 is a large chaperone found in the ER of all eukaryotes. It has been proposed to have several molecular activities including functioning as a holdase (De Keyzer et al., 2009), directing chaperone activity by interaction with unfolding protein (Schröder and Kaufman, 2005), and functioning as a co-chaperone by acting as a nucleotide exchange factor for other chaperones (De Keyzer et al., 2009). The relative importance of each of these activities to the cell and overall physiology of animals is not clear. The nematode *Caenorhabditis elegans* has two loci encoding GRP170: T24H7.2 (grp170a) and T14G8.3 (grp170b) (Nikolaidis and Nei, 2004). To better understand the role of GRP170 in the physiology of animals, phenotypes of nematodes genetically deficient for each grp170 locus were compared to a standard laboratory strain with functional grp170 loci. Specifically, three assays were carried out to analyze whether loss of either locus is important to the general physiology of worm: overall rate of development, egg hatching, and lifespan. To investigate the role of the two grp170 isoforms under ER stress conditions, worms were treated with tunicamycin, which creates ER stress by blocking normal protein folding in the ER (Heifetz et al., 1979).

Chaperone and ER protein folding

Protein folding in the Endoplasmic Reticulum (ER) is aided by three classes of chaperones (Ni and Lee, 2007). The first class includes the chaperones homologous to the cytosolic heat shock proteins (HSP's). These chaperones act by binding to exposed hydrophobic residues to prevent aggregation and promote folding. GRP170, the focus of this study, falls into this first class. It shares sequence homology with the cytosolic chaperones Hsp70 and Hsp110 (Easton et al., 2000). The second class is the chaperone lectins, which recognize oligosaccharides on unfolded proteins (Williams, 2006). This class includes the calnexin/calreticulin chaperone system. These chaperones recognize the nascent protein with monoglucosylated N-linked glycans and ensure the proper folding and thus aid in quality control of the newly synthesized glycoproteins. Finally, the third class is the substrate specific chaperones such as HSP47 (Ni and Lee, 2007). HSP47 has been thought to be important to the development of the mouse and functions as a collagen-specific molecular chaperone. The formation of collagen microfibril is impaired in embryos with a lack of HSP47 (Koide et al., 1999).

Many of the ER chaperones, including GRP170, were first identified as proteins induced by glucose starvation in mammalian cell culture. Hence these proteins were named Glucose Regulated Protein or GRP (Lee, 1992; Little et al., 1994). The principal GRP's of mammals are GRP78 (also known as Bip), GRP94, and GRP170 (Olden et al., 1979; Sciandra and Subjeck, 1983; Shen et al., 1987). While GRP's were initially identified by their induction during stress they also play a role in protein folding under non-stress conditions. ER chaperones facilitate

protein folding in the ER in four ways: 1. facilitating co-translational translocation of proteins into the ER lumen, 2. assisting protein folding in the ER lumen, 3. facilitating retro-translocation of terminally misfolded proteins from the ER to the cytoplasm for degradation by proteasomes, ERAD, and 4. contributing to ER luminal calcium storage (Ma and Hendershot, 2004).

Protein folding in the ER starts as soon as the polypeptide chains begin to enter the ER lumen. The process of the co-translational translocation into the ER starts with a signal recognition particle, SRP (Yvonne et al., 2013). The SRP binds the N-terminal signal sequence as it emerges from the ribosome forming the RNC-SRP complex (RNC: Ribosome-Nascent Chain). Then the RNC-SRP complex connects to SRP receptor (SR) on the ER membrane. Afterwards the RNC is transferred from the SR-SRP to the translocon resulting in insertion of signal sequence in the translocon pore and its opening. Then the nascent protein passes through the ER membrane by being threaded into the pore and the SRP also separate with SR. As soon as the protein begins to enter the ER, chaperones will bind and facilitate the protein folding (Yvonne et al., 2013).

It has been proposed that both calnexin and GRP78 aid in the translocation of nascent polypeptide chains into the ER (Ma and Hendershot, 2004). GRP78 has been shown to “plug” the translocon during early stages of protein translocation to maintain the permeability barrier between the ER and cytosol (Hamman et al., 1998). This puts GRP78 in an ideal place to bind nascent chains as they enter the ER (Sanders et al., 1992). GRP78 also assists pulling the polypeptide into the ER lumen by acting as a molecular ratchet (Matlack et al., 1999). Calnexin has been

shown to bind to the N-linked glycan on translocating hemaglutinin polypeptide chains as soon as 111 amino acids have entered the ER (Daniels et al., 2003).

Chaperones also aid in protein folding once the protein is in the lumen of the ER. A newly synthesized polypeptide that has not folded completely or proteins that have folded incorrectly will be retained in the ER via their interactions with the chaperones (Ellgaard and Helenius, 2001) and refolded. This process is believed to involve two major chaperone systems. The first system is comprised of the lectin-like chaperones calnexin and calreticulin. This system is dependent on the presence of both monoglucosylated N-linked glycans and unfolded regions on nascent glycoproteins. If the nascent protein does not fold completely during this time, the resident ER protein UDP glucose glycoprotein glucosyltransferase (UGGT) binds to the unfolded regions and adds a single glucose to the deglucosylated glycan (Trombetta and Parodi, 1992), which provides a binding site for calnexin or calreticulin again (Sousa et al., 1992; Hammond et al., 1994). The second major ER chaperone system involves the ER chaperone GRP78 (Flynn et al., 1991). Grp78 recognizes the unfolded regions on proteins containing hydrophobic residues (Blond-Elguindi et al., 1993). GRP78, in this capacity, like other Hsp70 class proteins**,** associate with unfolded proteins when the chaperones in an "open" ATP-bound state. DnaJ family members (ERdjs) bind to the Hsp70 proteins, and in some cases, to the unfolded substrate, as well, and assist grp78 in catalyzing the rapid hydrolysis of ATP to ADP (Liberek et al., 1991), This ATP hydrolysis "locks" the Hsp70 onto the unfolded protein. DnaJ proteins have been identified in many species and organelles (Cheetham and Caplan, 1998). Yeast ER contains three DnaJ proteins (Cheetham and Caplan,

1998). Recently five mammalian ER DnaJ family members were cloned and one of these, ERdj3, has been shown to interact with unfolded proteins in the ER (Meunier et al., 2002). This implies that ER-resident DnaJ proteins assume similar roles in both the cytosol and the ER.

In the ADP-bound state, the hsp70 chaperones exhibit high affinity to their substrates. The next step in the Hsp70 ATPase cycle is where the ADP in the nucleotide binding site of hsp70 is replaced by ATP. This is followed by a conformational change of hsp70, which "reopens" the Hsp70 chaperone's peptide binding site and releases the unfolded protein substrate. Hsp70 binds both ADP and ATP, which serve to regulate its binding and release from nascent chains (Kassenbrock and Kelly, 1989; Wei and Hendershot, 1995). Thus the ATP hydrolysis and ADP/ATP exchange are key events for substrate binding and Hsp70 release during folding of nascent polypeptides.

The 170 kDa glucose-regulated protein (GRP170), a molecular chaperone resident in the ER, is the focus of my study. A sequence alignment of GRP170 with HSP70 reveals a high degree of similarity between the protein families (Easton et al., 2000). Based on the sequence alignment, Easton et al. (2000) predicted that GRP170 like members of the HSP70 family may be expected to have an ATPase domain, and a C-terminal substrate binding domain. Unlike Hsp70, GRP170 has two domains that may engage in protein-binding (Park et al., 2003). Both domains inhibit the aggregation of denatured proteins *in vitro*. Besides the similar β-sheet region to that of Hsp70. The second protein binding domain is similar to the C-terminal α-helical domains of Hsp70. This helical

domain also binds denatured polypeptides *in vitro* and holds them in a folding-competent state (Park et al., 2003).

ER-Associated Protein Degradation (ERAD) is the cellular process that targets recalcitrant misfolded ER proteins for degradation by the cytosolic proteasome. During conditions of ER stress, a major function of the ER chaperones is to promote protein folding by preventing misfolding or aggregation. If the stress is not resolved rapidly, many unfolded proteins will be targeted for ER associated degradation (ERAD) as one way to decrease the load of malfolded proteins that accumulate in the ER (Nakagawa et al., 2000).This process is conserved in all eukaryotes from yeast to mammals (Nishikawa et al., 2001; Hampton, 2002). The ER chaperones GRP78, calnexin, calreticulin, and calmegin are involved in identifying malfolded glycoproteins for ERAD. (Haas and Wabl, 1983; Munro and Pelham, 1986; Haas and Meo, 1988). Recent data on ERAD in mammalian cells has demonstrated that calnexin and GRP78 play sequential roles in identifying and targeting ERAD substrates for degradation (Molinari et al., 2002).

In addition to the above roles, ER chaperones contribute to ER luminal calcium storage. Sustained ER calcium levels are essential for normal protein folding in this organelle. Lievremont et al. (1997) suggested that levels of ER calcium binding proteins helps regulate levels of calcium in the ER. In the ER, calcium is bound through both high affinity and low affinity interactions to a number of resident ER proteins including calnexin (Wada et al., 1991), calreticulin (Milner et al., 1991), GRP94 (Van et al., 1989), GRP78 (Lievremont et al., 1997), and

CaBP1 (Fullekrug et al., 1994). GRP170 is also a major calcium-binding protein in the ER (Naved et al., 1995).

Molecular activities of GRP170

The protein folding processes discussed above involve a complex network of chaperones. Each chaperone has specific molecular activities important to protein folding in the ER. My thesis focuses on one of these chaperones, GRP170. GRP170 has been proposed to have several molecular activities including: holdase, nucleotide exchange factor and translocation of proteins.

In yeast GRP170, Lhs1p (homologue of GRP170) functions as a holdase, a particular kind of molecular chaperones that assist the non-covalent folding of proteins in an ATP-independent manner (Beissinger and Buchner, 1998). It holds heat denatured proteins in a fold competent state (De Keyzer et al., 2009). Like other members in the Hsp70 family, Lhs1 is comprised of a conserved N-terminal nucleotide binding domain and a C-terminal substrate binding domain. De Keyzer et al. (2009) demonstrated that the Lhs1p holdase activity is a nucleotide-independent process *in vivo* that is not affected by mutations that prevent ATP binding.

Additionally, GRP170 can act as a co-chaperone for GRP78 by acting as a nucleotide exchange factor (NEF). In this role GRP170 removes ADP from the nucleotide binding site of GRP78 and replaces it with ATP (Weitzmann et al., 2006; De Keyzer et al., 2009,). This NEF activity is essential to the binding/release cycling of GRP78 and its chaperoning activity (Brodsky and

Bracher, 2000). In yeast and vertebrates there are two proteins that can function as a NEF for GRP78, GRP170 and SIL1 (Weitzmann et al., 2006; De Keyzer et al., 2009). In these phyla, loss of both GRP170 and SIL1 is lethal. *In vitro* assays also demonstrate that GRP170 can function as a NEF for GRP78 (Weitzmann et al., 2006). The nematode system seems to differ from the yeast and vertebrate systems. While *C. elegans* has GRP170 homologues (Nikolaidis and Nei, 2004), it apparently lacks a homologue for the second class of GRP78 NEF, SIL1 (Wormbase, 2007).

In addition to GRP170's nucleotide exchange activity, Dierks et al. (1996) demonstrated that GRP170 may play a role in translocation of proteins through the ER membrane. They identified two ATP binding proteins with chaperone activity, GRP78 and GRP170. Microsomes preparations of fragmented ER membranes that had been depleted for ATP binding proteins could not import proteins. Dierks et al. (1996) found that the reconstitution of microsomes by adding back their ATP binding proteins restored import of proteins into the microsome lumen. Since GRP78 alone could not restore translocation, they proposed GRP170 also played a role in translocation. GRP170 may promote translocation through its co-chaperone NEF activity. However, Spee et al. (1999) demonstrated GRP170 directly interacts with peptide transport into the ER suggesting it may function through direct interaction with proteins during translocation.

GRP170 in animal physiology

Beyond the important molecular activities, several physiological roles for GRP170 have been identified in animals. First, GRP170 can be induced under physiological stress and have potential protective functions for cells. For example, in radiation induced fibrosarcoma (RIF) murine tumors, GRP170 is induced as ischemia and necrosis develop in the growing tumor (Cai et al., 1993). Similar induction of GRP170 is observed when human breast tumors are compared with normal breast tissue (Yoshitane et al., 1998).

In addition to its protective roles, GRP170 has the ability to facilitate immune responses and can be used for cancer vaccine development (Wang et al., 2003a). Wang et al. (2003b) showed tumor-specific CD8+ T cell responses caused by immunization with tumor-derived GRP170. The immunization with grp170 preparations from autologous tumor significantly delayed progression of the primary cancer and reduced established pulmonary metastases (Wang et al., 2003b). In studies with mice, researchers found that the genetic modification of weakly immunogenic prostate tumor cells (TRAMP-C2) by stable transfection of these cells with a secretable form of GRP170 significantly enhances its immunogenicity *in vivo* (Gao et al., 2008).

GRP170 in *C.elegans*

Most organisms have only one gene encoding GRP170, but *Caenorhabditis elegans* has two similar but distinct genes encoding GRP170: T24H7.2 (grp170a) and T14G8.3 (grp170b) (Nikolaidis and Nei, 2004). The grp170a locus is located on chromosome 2 (Wormbase, 2007). The mRNA transcript for grp170a consists

of seven exons with six intermediate introns. This gene is encoded on a polycistronic operon with three additional genes: T24H7.1, T24H7.3, and T24H7.4. The complete gene is 3533 base pairs and the spliced transcript is 2778 base pairs that encode the ER chaperone GRP170a, a protein consisting of 925 amino acids.

There are two independent deletion alleles for the grp170a locus. The ok2107 allele has a 1337 base pair deletion spanning the 3rd (partially) and 4th exons and introns (Wormbase, 2007). This deletion also results in the loss of the small nucleolar RNA gene, T24H7.6, which lies within the 3rd intron of the mRNA transcript of the gene. Because this deletion affects two loci, it was not used in this study. The tm3109 allele has a smaller deletion resulting in the partial loss of exon 3 of its mRNA transcript (Wormbase, 2007). I chose to analyze this deletion allele for grp170a because it only affected the grp170a ORF and did not directly affect other loci on this polycistronic gene.

The grp170b locus is 8792 base pairs and is located on the X chromosome (Wormbase, 2007). The mRNA transcript of this gene is monocistronic and is made up of 14 exons and 13 introns. The spliced transcript is 2766 base pair and encodes a GRP170b protein of 921 amino acids. A single deletion allele is available for this locus, ok502, with a 2312 base pair deletion resulting in the loss of the 8th to the 13th exon (Wormbase, 2007).

Deletion alleles provide a powerful tool to study the physiological roles of chaperones. Asrani (2009) reported that loss of grp170a slowed development

about 30% but loss of grp170b had no effect on rate of development. Additionally, she demonstrated that worms that had at least one functional allele at either locus could mature into adults. Worms that lacked functional alleles at both loci showed early larval arrest and failed to develop into adults. She proposed that two loci had an overlapping critical function.

To confirm this and further understand the specific physiological roles of the two grp170 loci, I conducted a more comprehensive comparison of the physiological effects associated with loss of either of the two grp170 loci of *C. elegans*.

OBJECTIVES

1. Generate a nematode strain that contains the grp170b deletion allele, ok502, in the genetic background of the standard laboratory strain N2.

2. Investigate the physiologic roles of GRP170a and GRP170b in *C. elegans* by comparing strains deficient in grp170a or grp170b for the following traits:
    a. Rate of development
    b. Life span
    c. Embryonic lethality (including hatching defects).

3. Investigate the potential protective roles of the two grp170 loci during externally induced ER stress by analyzing the sensitivity to the protein folding toxin tunicamycin of worms deficient for grp170a or grp170b.

MATERIALS AND METHODS

1. Materials

Caenorhabditis elegans strains and OP50 *E. coli* were obtained from Caenorhabditis Genetics Center (Caenorabditis Genetics Center, 1999; *C.elegans* Gene Knockout Consortium, 2001) and the Shohei Mitani Laboratory, Tokyo Women's Medical University School of Medicine, Japan. Nucleotides, buffers and Taq Polymerase for PCR reactions were obtained from New England BioLabs (Ipswich, MA). PCR primers were obtained from Integrative DNA Technology (Coralville, IA). Tunicamycin was obtained from Sigma-Aldrich (St. Louis, MO).

2. Strain maintenance

C. elegans were cultured on NGM/OP50 plates at 20°C unless otherwise noted (Stiernagle, 2006). Nematode Growth Medium (NGM) agar was used to maintain *C. elegans* in the laboratory. The OP50 strain of *E. coli* was seeded on the NGM agar plates and served as food for worms. OP50 is a uracil auxotroph that limits its growth on NGM plates. A limited bacterial lawn allows for observation and manipulation of the worms during mating.

3. Generation of males

Male worms were required to conduct genetic crosses. Males were generated using heat shock (Koelle Lab. Protocols, 1998). Six late L4 hermaphrodites were placed on a single NGM/OP50 plate which was then incubated at 30°C for 6 hours to heat shock the worms. The culture was transferred to 20°C and nematodes were allowed to mature and generate progeny for 48 hours.

Plates were examined for male progeny. Male producing cultures were maintained by crossing two L4 stage hermaphrodites of a strain with seven males of the strain.

4. Backcrosses

A standard genetic approach to understanding gene function is to analyze phenotypes associated with loss of functional alleles by comparing the mutant strains to a control strain with functional alleles. To be confident that phenotypic differences are due to the mutant allele, the two strains must be isogenic, which means they have the same genetic background except for the target gene. Therefore, to analyze the phenotypes associated with T14G8.3 (grp170b) deficiencies, the deletion alleles were moved into the genetic background of the standard laboratory strain N2 by repeated genetic backcrosses (Ahringer, 2006)..

The backcross was conducted by mating two L4 stage RB734 hermaphrodites (homozygous for the grp170b deletion allele ok502) with seven N2 males (Figure 1). After the hermaphrodites laid eggs, the adults were removed and the F1 generation eggs were allowed to mature to the L4 stage larva. These F1 larval hermaphrodites were segregated onto individual plates and were allowed to mature and lay F2 eggs for 2-3 days. The F1 hermaphrodites were removed and genotyped (see below). Two types of genotypes were expected. Some of the F1 hermaphrodites were the progeny of RB734 hermaphrodites self-fertilizing and were homozygous for the ok502 deletion allele. These homozygous nematodes did not represent a backcross and were discarded.

Other F1 hermaphrodites were the progeny of N2 males and RB734 hermaphrodites and were heterozygous for the grp170b locus. Only the hybrid offspring of F1 were retained. The F2 L4 stage larva were segregated onto individual plates and allowed to self-fertilize and lay eggs. One quarter of these worms were expected to be homozygous for the grp170b deletion allele ok502 based on standard Mendelian gene segregation. After the F2 worms laid eggs they were removed from the plates and genotyped via duplex PCR. Plates corresponding to ok502 homozygotes were retained. Plates with other genotypes were discarded. This procedure represented a single backcross. It was repeated 5 more times to increase the likelihood to replace the genetic background of Rb734 with N2. The backcrossed strain was named BSC-06. The genetic crosses necessary to move the tm3109 deletion allele of grp170a into the genetic background of N2 generating the strain BSC-07 had been previously accomplished by Gregory J. Wadsworth (unpublished results).

5. Genotyping

Nematodes were genotyped for grp170a and grp170b by using a duplex PCR assay (Asrani, 2009). Template DNA for duplex PCR was isolated using freeze/thaw–protease treatment (adapted from Schedl Lab, 2008). A single worm was suspended in 10µl lysis buffer [1mg/ml Proteinase K in 1X Standard Taq Reaction Buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, pH 8.3)] and then frozen at -80° for 15 minutes to lyse the nematode. Next the worm was incubated at 65° for 60 minutes for protease digestion, and the

Proteinase K was then inactivated by heating the sample at 95°for 15 minutes. The resulting worm lysate was used directly in the PCR reaction.

In duplex PCR, the alleles are distinguished by the size of the PCR products generated by each allele (Asrani, 2009). To distinguish between standard full length and mutant alleles at the grp170b locus, the first forward primer (F1) is complementary to the DNA sequence just before the deletion region (5'-GTAAGGACGGCAAGGACAAG -3'). The second forward primer (F2) is complementary to the DNA sequence within the deletion region (5'-TTAGGCTCGTGACTTGGAAC -3'). The reverse primer (R) is complementary to the DNA sequence immediately after the deletion (5'-CAACATCTCACCTGCTGCTC -3'). The expected size of the standard full length product is 195 bp and is generated by the primer F2 and R. The mutant allele product is identified by the primers F1 and R, and the expected product size is 317 bp.

A similar genotyping assay was used for the T24H7.2 (grp170a) loci. Primer F1: 5'-TGCTCTGAATACGGAATCGAG-3'. Primer F2: 5'-ATGGTCTATGACATGGGAGC-3'. Primer R: 5'-TCGGCATTGGCAGATAGAAC-3' (Asrani, 2009). The expected size of standard full length product is 281 bp. The expected product size of mutant allele is 381 bp.

For a single duplex PCR reaction, 5µl of worm lysate was used as template DNA. DNA was amplified in the 50µl reaction with 1.25 U of Taq DNA Polymerase, 10X Standard Taq reaction buffer, 0.16µM each dNTP, 0.4µM each Primer (F1, F2, R). Cycling parameters were as follows: 1 min at 95°C;

40 cycles of 30 s at 95°C, 30 s at 50°C, 30 s at 72°C, followed by a 7 minute extension at 72°C and a hold at 4°C. The amplified products were analyzed on a 2% agarose gel with ethidium bromide [2% w/v agarose in 1X TBE (89mM Tris base, 89mM Boric acid, 2mM EDTA, pH 8.0), 0.5 µg/ml ethidium bromide]. The gels were photographed under ultraviolet light after electrophoresis (Figure 2). N2 homozygotes showed a single PCR product at approximately 200 bp, ok502 homozygotes exhibited a single PCR product at about 300 bp and both PCR products were observed in heterozygous nematodes.

Phenotypic Assays

1. Rate of development

Rate of development was defined as the number of hours it takes for an adult worm to develop from a freshly laid egg into an egg laying adult (Asrani, 2009). To determine the rate of development, L4 larva were transferred to fresh 60mm NGM/OP50 plates and allowed to mature 24 hours to generate day 1 adult hermaphrodites. The day 1 hermaphrodites were transferred to a fresh NGM/OP50 plate and allowed to lay eggs for 4 hours, after which they were removed from the plates. The eggs were allowed to develop at 20°C. When the worms reached the L4 larva stage, 26 L4 larva of each strain were selected randomly and placed on individual 30mm fresh NGM/OP50 plates. Each larva was examined every hour until it developed into an adult and laid its first egg. The total time taken for an adult worm to develop from an egg

into an egg laying adult was recorded. The rate of development was analyzed by comparing the standard laboratory strain N2 to BSC-07 and BSC-06.

2. Life span

For the life span study, adult nematodes were maintained on NGM media supplemented with ampicillin (100mg/ml) and florodeoxyuridine (FUDR, 150mM). Ampicillin was used to prevent foreign bacterial contamination. FUDR inhibits cell division, reduces eggs production, and prevents eggs from hatching (Sutphin et al., 2009). This eliminates the need to transfer worms every few days in order to separate them from growing larva. Although rare interactions between FUDR and genotypes have been observed, FUDR is still routinely used in lifespan studies (Van and Hekimi, 2011). In this study, all life span assays were conducted on FUDR medium, and no attempt was made to study FUDR genotype interaction. Additionally, the *E. coli* OP50 bacteria seeded on the plates were put into Clean Bench (The Baker Company SG603A-HE 108202) and exposed to a 5 min UV dose which is sufficient to arrest growth of the bacteria. This allowed storage of these plates up to one month (adapted from Sutphin et al., 2009).

To generate a synchronized population of worms, stage L4 larva were transferred to new 60mm fresh NGM/OP50 plates to mature 24 hours to generate day 1 adult hermaphrodites. The day 1 young adult hermaphrodites were placed on standard NGM plates for 4 hours at 20°C to lay eggs. Adults were then removed from those plates. These plates were incubated at 20°C until the eggs had hatched and the worms had developed to the L4 larval

stage. Each L4 larva was transferred to an individual 30mm seeded Amp/FUDR plate. The plates were checked once a day at the same time to determine if the worm had died. Death was determined by noting a lack of movement of the worm and determining if the worm failed to respond when touched by a probe on its head.

3. Embryo lethality and hatching defects

Stage L4 larva were transferred to fresh 60mm NGM/OP50 plates to mature 24 hours to generate day 1 adult hermaphrodites. The day 1 young adult hermaphrodites were placed on individual 30mm NGM/OP50 plates for 4 hours at 20°C to lay eggs. There were eight 30mm NGM/OP50 plates set up for each strain, and then the adults were removed from those plates. Over 300 eggs of each strain were collected. These plates were incubated at 20°C and the number of unhatched eggs of each strain developed after 48 hours were recorded.

4. Tunicamycin sensitivity

The antibiotic tunicamycin blocks normal protein folding in the ER by blocking glycosylation (Heifetz et al., 1979). Over 50 worms of each of the three isogenic strains were treated with tunicamycin (3µg/ml) to allow them to grow in a stress condition. Stage L4 larva were transferred to fresh 60mm NGM/OP50 plates to mature 24 hours to generate young gravid hermaphrodites. The gravid adult hermaphrodites were placed on individual 30mm NGM/OP50 plates with and without tunicamycin for 4 hours at 20°C to lay eggs. Adults were then removed from those plates and the number of

eggs laid on each plate were counted. These plates were incubated at 20°C for 3 days (BSC-07 took 4 days because of the slower rate of development). The number of L1-L3, L4 and older (L4+), and dead worms were recorded for each plate with and without tunicamycin (Richardson et al., 2011).

Statistical Analysis

Data was analyzed by ANOVA using R 3.2.0 (R Development Core Team, 2011). On-line software for the log-rank test was used to analyze survival curve data (Russell, 1999).

RESULTS

Generation of Isogenic Strains

The *C. elegans* strain Rb734, which carries the ok502 deletion allele of the grp170b gene, was generated by the International *C. elegans* Knockout Gene Consortium (2001). The mutagenesis process used by the *C.elegans* Gene Knockout Gene Consortium could have resulted in secondary mutations. To eliminate these secondary mutations and generate a strain isogenic with N2, the Rb734 strain was backcrossed six times with the N2 strain (Figure 1). These six backcrosses resulted in a 98.4% probability of removing any unlinked secondary mutations (Allard, 1999). The strain generated by the six backcrosses was BSC-06, carried the grp170b deletion allele in the N2 genetic background.

Similar backcrosses were conducted for the T24H7.2 (grp170a) locus. The strain carrying the tm3109 deletion allele at the grp170a locus was previously successfully backcrossed six times with the standard laboratory strain N2 to generate BSC-07 (Gregory J. Wadsworth unpublished results).

Physiological role of GRP170 proteins

To distinguish the physiological roles of the GRP170 isoforms encoded by the two grp170 loci in *C.elegans*, three general measures of physiological health were compared in strains BSC-07 (deficient for grp170a), BSC-06 (deficient for grp170b) and N2 (the control strain that has functional alleles at both

grp170 loci). The three general measures were (1) rate of development, (2) life span, and (3) embryonic lethality.

Rate of development

To investigate whether deletion of either grp170 locus causes developmental delay, the time required to complete development was determined for N2, BSC-07 and BSC-06 worm strains (Figure 3). In this egg to egg assay, the rate of development was defined as the time a freshly laid egg took to complete embryonic and larval development, mature into an adult and lay its first egg. Worms that failed to complete embryonic development and hatch from the egg were excluded from this analysis. For N2 and BSC-06, greater than 99% of the eggs hatched while approximately 93% hatched in the BSC-07 strain.

Loss of grp170b did not significantly alter the rate of development. The mean rate of development of BSC-06 worms homozygous for deletion allele (OK502) of the grp170b locus (74 hours) was not significantly different from the mean rate of development of the control strain N2 (72 hours) ($P>0.05$). This suggested that grp170b does not play a role in physiological processes critical for normal development.

In contrast, the loss of grp170a significantly slowed the rate of development. The mean rate of development of BSC-07 worms homozygous for deletion allele (tm3109) of the grp170a locus (95 hours) was significantly slower (32% slower) than the rate of development of the control strain N2 (72 hours) ($P<0.01$). These results suggest that the two grp170 loci play different

physiological roles during development. Specifically, the grp170a locus appears to play a more critical role in the physiologic processes associated with the rate of development than grp170b.

Life span

A second physiological phenotype that was examined was lifespan. The life span of nematodes deficient for either locus were compared to standard laboratory strain N2 at 20°C. Synchronized population of eggs from the 3 population were monitored every day to determine the lifespan of each worm (Figure 4). Worms deficient for grp170b had a mean lifespan of 20 days. This did not significantly differ from the lifespan of the control strain N2, which had a mean lifespan of 22 days ($P>0.05$). However, worms deficient for grp170a had an average lifespan of 19 days, which was significantly shorter than N2 ($P<0.05$).

Another approach for comparing genetic effects on life span is a survival curve analysis (Lionaki and Tavernarakis, 2013). A survival curve was generated showing the percentage of worms of each genotype surviving over the course of the experiment (Figure 5). Consistent with the shorter lifespan, worms deficient for grp170a also had a significantly different survival curve compared to N2 worms ($P<0.01$). Again, loss of grp170b did not significantly affect the survival curve compared to the N2 strain ($P>0.05$).

Embryo lethality and hatching defects

Nematode embryos go through several stages of development before hatching from an egg to generate an L1 larva. To determine if the two grp170 genes play a critical role during embryonic development, eggs of each strain were collected and the number of arrested embryos of each strain developed after 48 hours was recorded (Figure 6).

Loss of grp170b did not significantly affect hatching. The mean percentage of hatching defect (unhatched eggs) for BSC-06 worms homozygous for deletion allele (OK502) of the grp170b locus (0.4%) was not significantly different from the mean percentage for the control strain N2 (0.4%) ($P>0.05$). This suggests that grp170b does not play a critical role in embryonic development.

In contrast, the loss of grp170a significantly increased embryonic lethality, resulting in more arrested embryos. The mean percentage of hatching defect for BSC-07 (6.9%), the strain homozygous for deletion allele (tm3109) of the grp170a locus, was significantly greater than the mean percentage of the control strain N2 (0.4%) ($P<0.01$). The fact that loss of grp170a alleles differentially affects completion of embryogenesis compared to loss of grp170b alleles suggested that the two loci play different physiological roles. Again as with rate of development and lifespan, grp170a plays a more important role in embryo development than grp170b.

Tunicamycin sensitivity

The antibiotic tunicamycin interferes with normal protein folding in the ER by blocking glycosylation (Olden et al, 1979). To test whether worms lacking either grp170 locus were more sensitive to tunicamycin, worms were cultured on media with a sublethal concentration of tunicamycin (3µg/ml) and allowed to develop for 3 days (BSC-07 was allowed to mature 4 days to accommodate its delayed rate of development) (Figure 7). For both tunicamycin treated populations and control populations, the number of worms maturing to late larva/adult stage of development (L4+), the number of larva with delayed development (L1-L3), and the number of dead larva were recorded.

In the standard laboratory strain N2 without tunicamycin, all of the worms matured into L4+ stage by day 3. When cultured with tunicamycin, 30% of the N2 worms showed delayed development (L1-L3) and 11% of N2 worms died. In the control treatment, the grp170a deficient strain (BSC-07, deletion allele: tm3109) showed delayed development, even after 4 days 30% were at L1-L3 stage and 6% dead without tunicamycin. When treated with tunicamycin, worms deficient for grp170a showed an even greater delay in development. At day 4, 80% of the grp170a deficient worms were still in the L1-L3 stage and 16% of worms had died.

Similar to the N2 strain, all grp170b deficient worm (BSC-06, deletion allele: ok502) matured into L4+ stage by day 3 without tunicamycin. However, in contrast to N2 worms, worms deficient for grp170b did not exhibit delayed development when cultured with tunicamycin. The assay was repeated twice

with similar results. This demonstrates that worms with grp170b were more sensitive to tunicamycin than worms without functional grp170b.

DISCUSSION

While most organisms have only one gene encoding GRP170, *Caenorhabditis elegans* has two similar but distinct grp170 loci (Nikolaidis and Nei, 2004). The gene duplication that generated the two grp170 loci occurred prior to the divergence of *C.elegans* and *C. briggsae* estimated to be about 100 million years ago (Stein et al., 2003). Theoretically, duplicated genes should only be maintained if each gene evolves distinct adaptive functions (Nowak et al., 1997). Therefore, the maintenance of these two loci for so many millennia suggest they have evolved separate functions.

Previously, grp170 was shown to be essential for *C.elegans* development (Asrani, 2009). Nematodes with deletion alleles at both the grp170a and grp170b loci showed arrested development at an early larval stage and failed to develop into adults. On the other hand, worms lacking either grp170a or grp170b were viable and matured into adults (Asrani, 2009; Wormbase 2007). This suggests that grp170a and grp170b have a shared critical function in the cells that either locus can perform. However, it does not demonstrate whether they have unique functions to the grp170a or grp170b loci in the nematode.

Recent studies of gene expression have demonstrated that the two grp170 genes are differentially regulated at the mRNA level in *C.elegans* (Rockwell unpublished data). The grp170b gene is highly induced during the Unfolded Protein Response (UPR) stress pathway while grp170a is unaffected by this pathway. These patterns of gene expression are consistent with the chromosomal structure of these two loci (Asrani, 2009). The grp170a locus is part of a polycistronic gene

which includes several non-stress non-UPR inducible loci. The co-regulation of polycistronic loci in *C.elegans* from the same promoter suggests that grp170a should not be inducible. On the other hand, grp170b is in a monocistronic locus and therefore has its own promoter. The UPR inducibility of grp170b and the lack of inducibility of grp170a provides support for a simple model for the different activities of the two loci with grp170a acting as a more housekeeping gene and grp170b acting in a more stress specific role (Asrani, 2009).

To investigate if there are different physiological roles associated with these two loci, three phenotypic were assayed under non-stress condition (Summarized in Table 1). The phenotypic differences between the two grp170 loci suggested they have different physiological roles. With the grp170a locus playing a more important role than grp170b in the general physiology of nematodes. For example, deletion of grp170a alleles delay development 32% compared to the control strain N2. Asrani (2009) obtained similar results with independently generated isogenic lines deficient for grp170a. In addition to slowing the rate of development. Loss of grp170a also significantly reduced lifespan and had a significant effect on the survival curve. Additionally, worms deficient for grp170a exhibited a significantly increasing on the percentages of embryos with hatching defects. All these phenotypes show that grp170a plays an important role in the processes that affect the rate of development, the life span and embryonic development.

Further study of the roles of grp170a during development is warranted. It would be interesting to see whether specific stages of development were impacted by

the loss of grp170a, for instance, the rate of embryogenesis or the rate of larval development. It might give us a further insight into the physiological and developmental role of grp170a.

Based on the results of this study, loss of grp170b did not seem to affect the nematodes physiology under normal non-stressed conditions. For example, deletion of the grp170b did not affect the rate of development and the life span, or the survival curve. During embryonic development, again, loss of grp170b did not significantly alter the percentages of embryos with defects at hatching. They had the same level of embryonic lethality as the control strain. All of these suggest that grp170b locus does not play an important role in the general physiology of nematodes under non-stressed condition.

However, this might be expected if grp170b is a stress related protein as suggested by the mRNA expression data. To investigate whether either grp170 locus plays a special role under stress conditions, the nematodes were exposed to a sublethal dose of the ER toxin tunicamycin. Tunicamycin stresses the ER by inhibiting protein glycosylation which leads to the accumulation of unfolded proteins in the ER (Olden et al, 1979). If grp170b is essential to the physiological response to tunicamycin, we predicted that worms deficient in grp170b exposed to tunicamycin would show increased sensitivity to the drug.

The assay for tunicamycin sensitivity measured a delay in development due to the drug. Analysis of the grp170a deficient strain was complicated by the fact that loss of grp170a itself delays development. Even without tunicamycin, after 4

days about 36% of the grp170a deficient nematodes failed to reach the late larval stage or died. Treatment of grp170a deficient nematodes with tunicamycin further delayed development with 96% of worms failing to reach late larval stages or dying. Because the grp170a deficient worms already have impaired development, it was not possible to conclude that these worms are "more" sensitive to tunicamycin than the control strain.

Further investigation of tunicamycin sensitivity of the grp170a for tunicamycin sensitivity is warranted. In this study, only a single concentration of tunicamycin was tested. It would be worth analyzing a range of concentrations to study the dose response of grp170a deficient worms to tunicamycin. For example it would be interesting to know if grp170 deficient worms lower LD50 (the dose required to kill 50% of the test population) than the control strain. It should also be possible to compare the minimum dose that has a physiologic effect on the worms. On the other hand, to investigate that if grp170 loci are specific to the type of stress induced by tunicamycin. Another toxin is worth trying. For example, Dithiothreitol (DTT). It interrupts normal protein folding in a different stage with tunicamycin.

The grp170b mRNA is induced by UPR stress and so it was reasonable to predict that loss of grp170b would make the nematode more sensitive to UPR stress. However, the opposite effect was observed. While the sublethal dose of tunicamycin slowed development of the control strain, it had no effect on the rate of development of the grp170b deficient strain. This is inconsistent with a model of grp170b playing a protective role against UPR stress. Instead, worms

with grp170b were more sensitive to tunicamycin than worms without functional grp170b.

Although the decreasing sensitivity associated with loss of GRP170b was unexpected, this phenomenon may provide insight into the role of grp170b in nematodes. The HSP70 chaperone, GRP78 and GRP170, have been proposed to play a central role in protein metabolism of the ER. For example, aiding in the translocation of nascent polypeptide chains into the ER (Ma and Hendershot, 2004), identifying and targeting ERAD substrates for degradation (Molinari et al., 2002), etc. Thus there are several possible mechanisms for grp170b enhanced tunicamycin sensitivity. For example, grp170b might allow translocation of excess proteins into the stressed ER; it might promote excessive proteins degradation by ERAD in tunicamycin treated cells; it might be allow export of toxic unfolded proteins; or grp170b might promote excessive ER retention of proteins by folding machinery. Determining how grp170b increases sensitivity to tunicamycin could provide insight into the general role of grp170b in *C. elegans*.

Table 1. Summary of grp170a and grp170b mutant phenotypes

	Mutant Strains[1]	
	grp170a$^{\Delta}$	grp170b$^{\Delta}$
General Physiology[2]		
Rate of Development	Slowed	Unaffected
Life Span	Reduced	Unaffected
Embryonic Defect	Increased	Unaffected
Stress Physiology		
Tunicamycin[3]	Mild Effect	Resistant

[1] The grp170a$^{\Delta}$ strain BSC-07 was homozygous for the grp170a deletion allele, tm3109 and the grp170b$^{\Delta}$ strain BSC-06 was homozygous for the grp170b deletion allele, ok502.

[2] For all measures of physiology the mutant strains were compared to the isogenic standard laboratory strain N2.

[3] Tunicamycin stressed the ER by inhibiting protein glycosylation and caused the accumulation of unfolded protein in the ER.

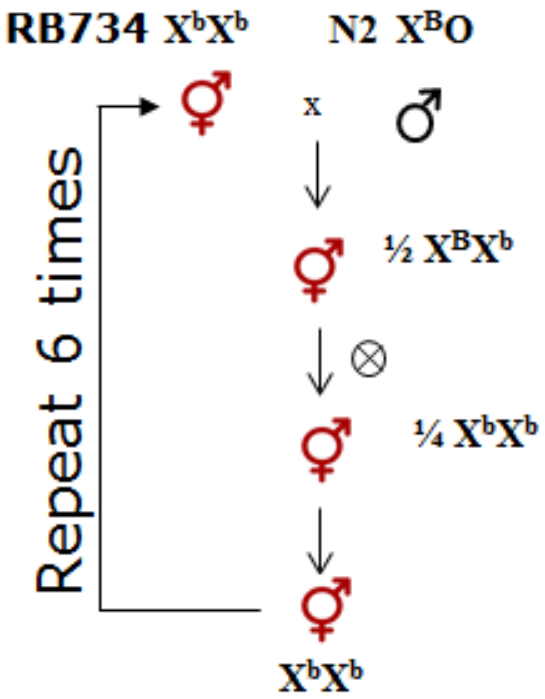

Figure 1. Backcrosses for grp170b deletion allele ok502. To move the T14G8.3 (grp170b) ok502 deletion allele into the N2 genetic background, strain RB734, homozygous for ok502, was mated with N2 males. The F1 heterozygotes were allowed to self-fertilize to generate an F2 population. Genotypes of F1 worms were confirmed with duplex PCR. F2 worms were allowed to lay eggs and they were genotyped to identify ok502 homozygotes. This back cross procedure was repeated a total of six times.

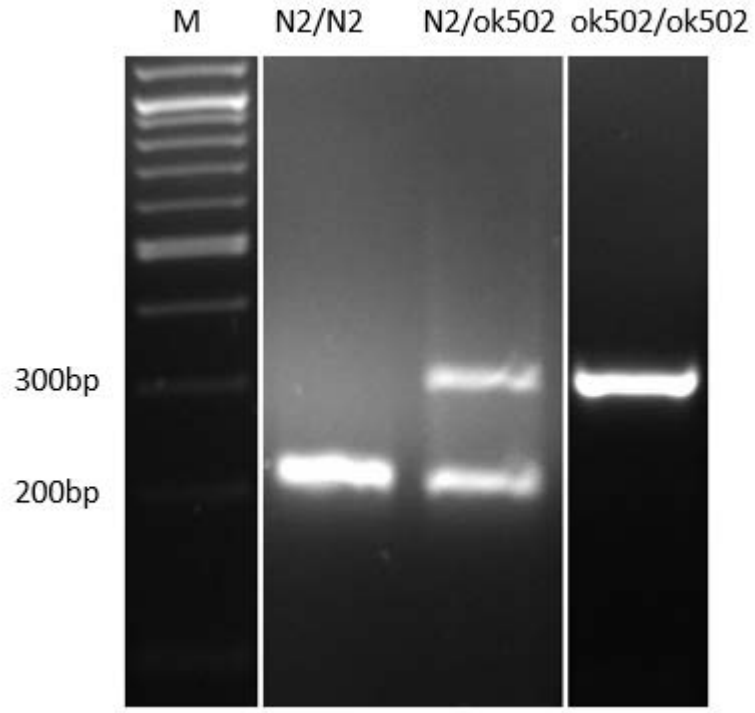

Figure 2. Genotyping T14G8.3 (grp170b) ok502 deletion allele. Worms homozygous for the N2 control allele, homozygous for the ok502 allele and heterozygous were genotyped using duplex PCR. The products were separated by agarose gel electrophoresis. The N2 worm carrying full length alleles at the grp170b locus generated a product of ~200 bp (lane N2/N2), close to the expected product size of 195 bp. A single homozygous mutant worm carrying ok502 deletion alleles at the grp170b locus generated a band at ~300 bp (lane ok502/ok502), close to the expected product size of 317 bp for the mutant allele. A single heterozygote worm carrying ok502 deletion alleles and full length alleles at the grp170b locus generated two bands (lane N2/ok502). DNA standards are shown in the left lane (lane M).

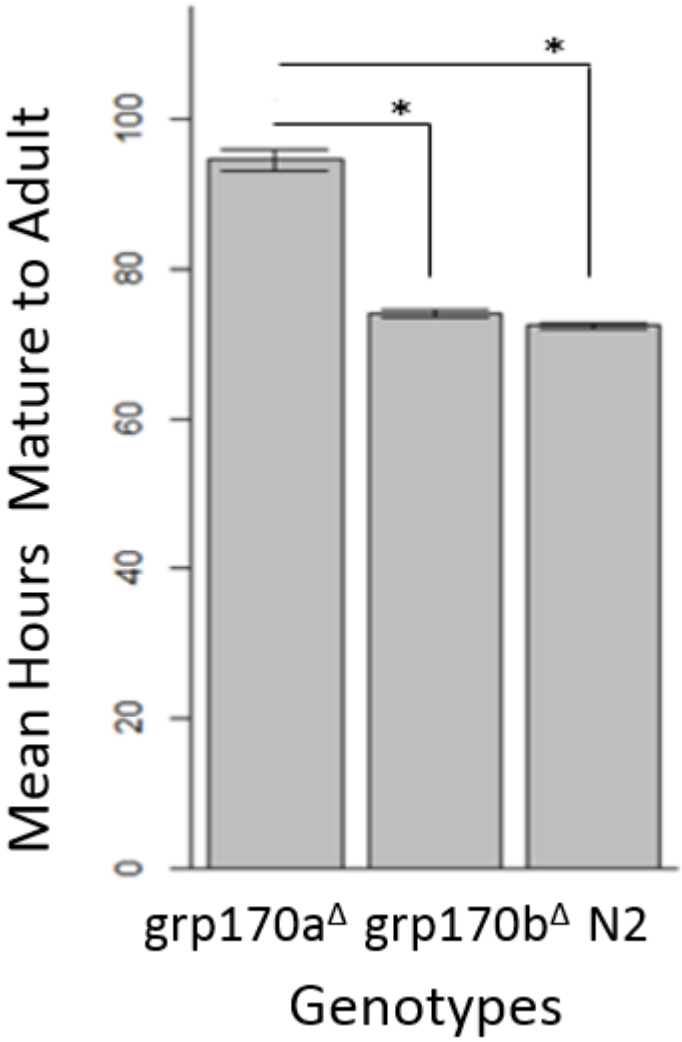

Figure 3. Genetic deficiency for grp170a slowed *C. elegans* development. The rate of development (time for an egg to develop into an egg laying adult) was determined for worms deficient for grp170a (grp170a$^{\Delta}$ strain BSC-07), for grp170b (grp170b$^{\Delta}$ strain BSC-06) and the standard laboratory strain N2. A total of 26 worms were analyzed for each strain. Worms deficient for grp170a developed significantly slower than either N2 ($P<0.01$) or worms deficient for grp170b ($P<0.01$). Development of grp170b deficient worms was not significantly different from N2 ($P>0.4$). [ANOVA test]

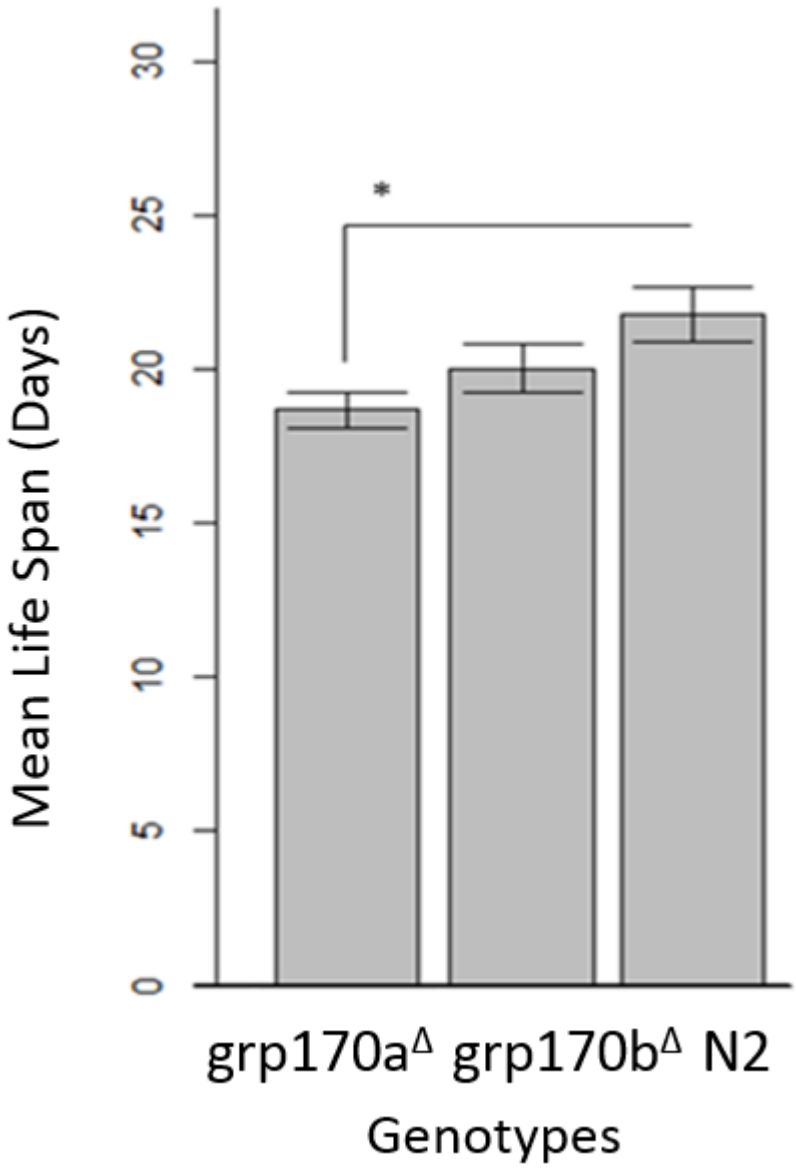

Figure 4. Genetic deficiency for grp170a reduced the life span of *C. elegans*. The life span (days from when an egg was laid until death of the adult) was determined for 34 worms of each of the three strains. Worms deficient for grp170a (grp170a$^{\Delta}$) had a significantly shorter lifespan ($P<0.05$) than N2 worms. The strain deficient for grp170b (grp170b$^{\Delta}$) was not significantly different from N2 strain ($P>0.2$). [ANOVA test]

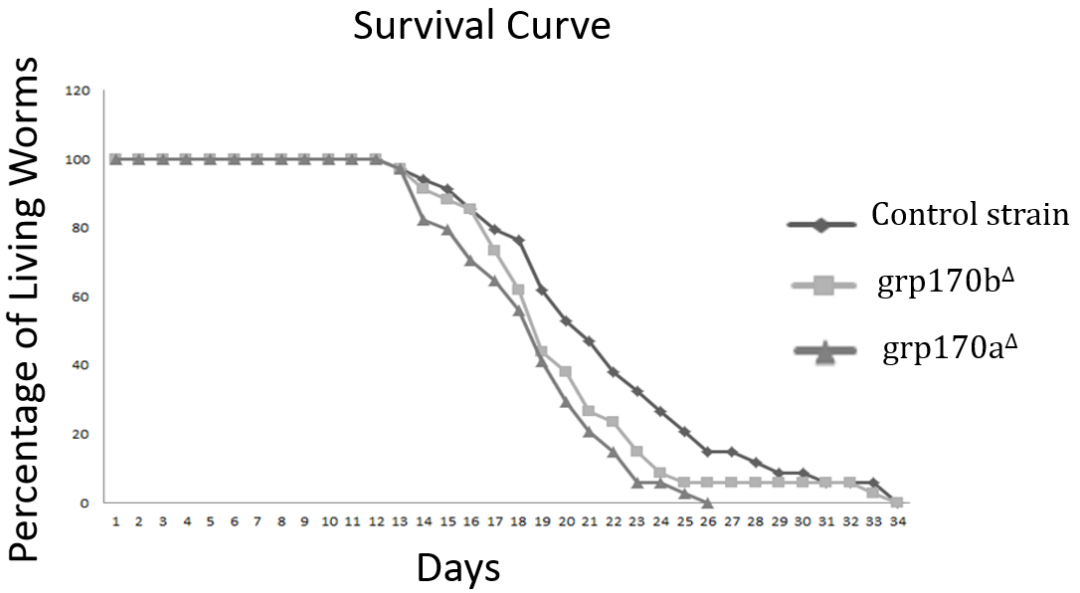

Figure 5. Survival curve of *C. elegans* affected by deletion of grp170a locus. Synchronized population of N2 (control strain,), BSC-06( ) and BSC-07( ) were monitored for 34 days. Each day the surviving worms were counted and the resulting survival curve generated. Worms deficient for grp170a had a significantly different survival curve ($P<0.01$) compared to the control strain N2. The survival curve for grp170b deficient worms was not significantly difference from N2 ($P>0.05$). [Log-rank test]

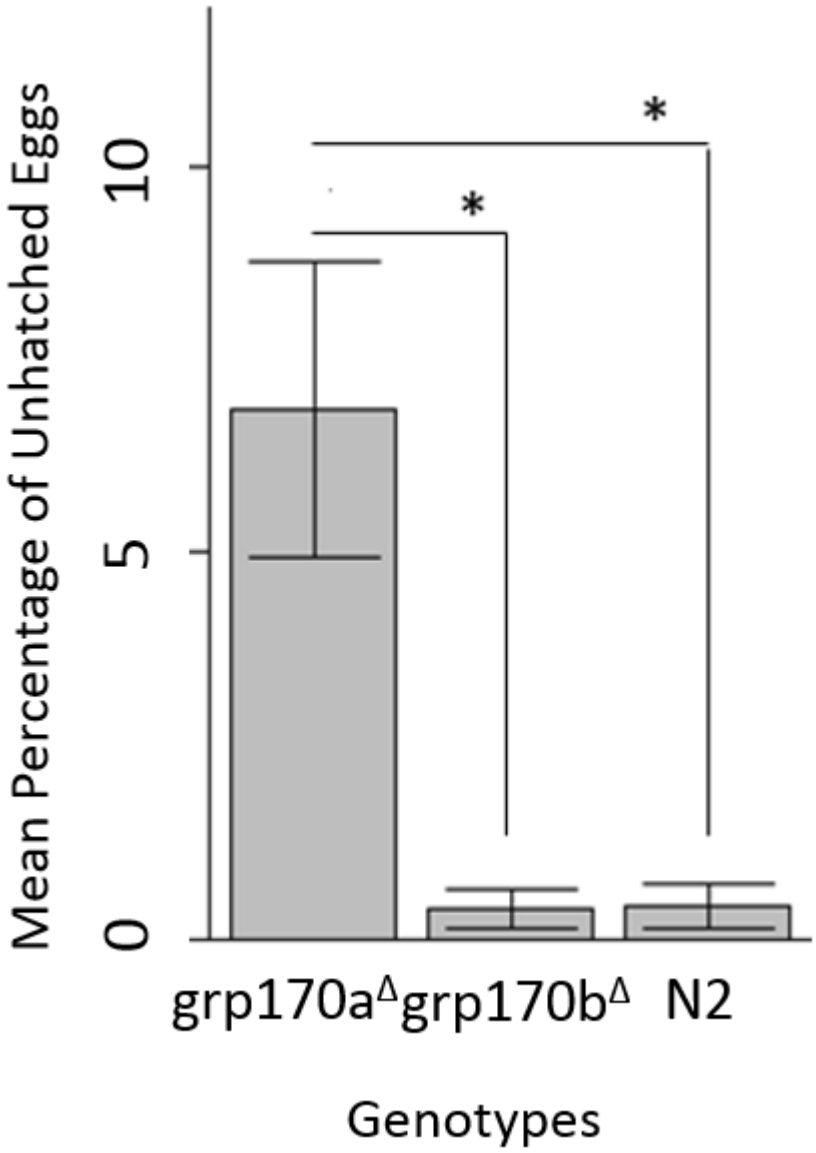

Figure 6. Genetic deficiency for grp170a increased arrested embryos. Over 300 eggs of each strain were monitored to determine the percentages of unhatched eggs after 48 hours (indicative of a hatching defect). Worms deficient for grp170a exhibited a greater hatching defect and was significantly different from either N2 ($P<0.01$) or worms deficient for grp170b ($P<0.01$). The percentage of unhatched eggs for grp170b deficient worms was not significantly difference from N2 ($P>0.9$). [ANOVA test]

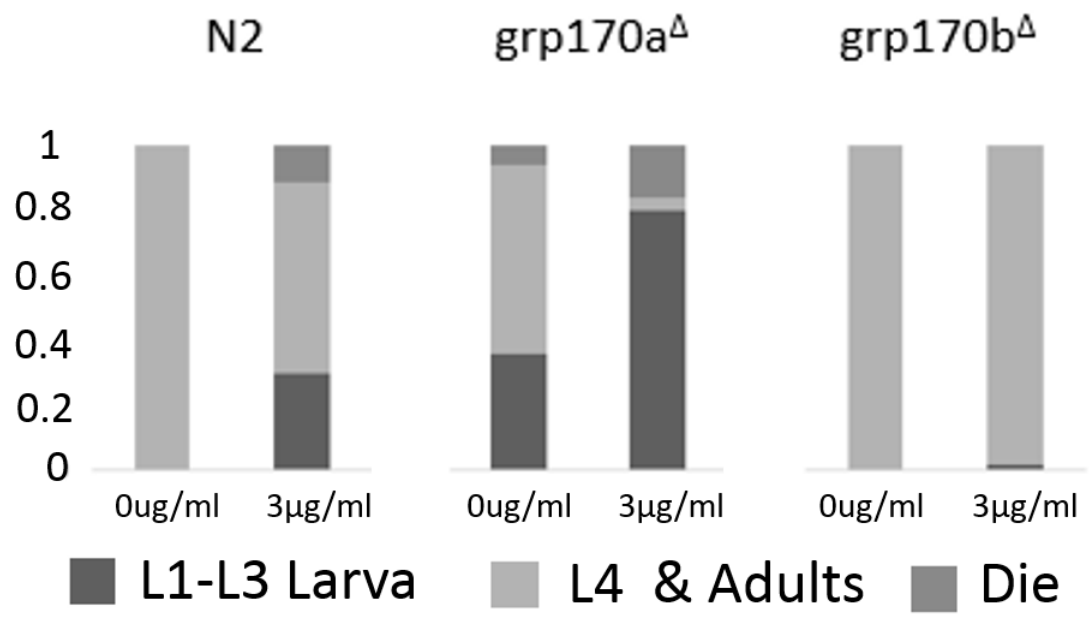

Figure 7. Tunicamycin sensitivity of grp170 deficient strains. Over 50 worms were examined in both 0µg/ml and 3µg/ml tunicamycin treatment for each isogenic strain After 3 days (4 days for grp170a deficient worms) the stage of development of each worm was determined.

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