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Differential Regulation of the Two grp170 Paralogues of Caenorhabditis elegans

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Differential Regulation of the two *grp170* Paralogues of *Caenorhabditis elegans*

By

Antonio Rockwell

An Abstract for a Thesis in
Biology

Master of Arts

August 2015

State University of New York
Buffalo State College
Department of Biology

Abstract of a Thesis

Differential Regulation of the Two *grp*170 Paralogues of *Caenorhabditis elegans*

Caenorhabditis elegans has two loci encoding the large eukaryotic molecular chaperone Grp170, *grp170a* (T24H7.2) and *grp170b* (T14G8.3). To investigate expression of the two *C. elegans grp170* loci during ER stress, the Unfolded Protein Response (UPR) was induced with the glycosylation inhibitor tunicamycin. Levels of *grp170a* mRNA did not significantly change in response to tunicamycin treatment while the levels of *grp170b* mRNA increased 6-fold. ER stress induction of *grp170b* was unaffected in worms defective for the ATF6 and PERK-1 UPR signal transduction pathways. However, worms defective for the IRE-1 dependent UPR pathway failed to induce grp170b. The expression of *grp*170 mRNA was analyzed in nematodes homozygous for the *grp170a* deletion allele tm3109 or for the *grp170b* deletion allele ok502. Expression of *grp170a* mRNA was unaffected by the loss of *grp170b*. In contrast, *grp170b* was induced 83 fold in nematodes deficient for *grp170a*. To further investigate whether loss of either *grp170* loci induces UPR, the expression of another UPR responsive gene, *hsp-4*, was analyzed. In nematodes lacking *grp170b*, expression of *hsp-4* mRNA was not affected. However, in nematodes lacking *grp170a,* the UPR responsive *hsp-4* mRNA was up-regulated 38 fold. These data suggest that while grp170a plays a critical role in ER protein folding, it is not itself inducible by the UPR. On the other hand *grp*170b seems to play a less critical role in protein folding under non-stress conditions, it is the *grp*170 locus that is induced by the UPR.

State University of New York
College at Buffalo
Department of Biology

Differential Regulation of the Two GRP170 aralogues of *Caenorhabditis elegans*

A Thesis in Biology

By

Antonio Rockwell

Submitted in Partial Fulfillment
Of the Requirements
For the Degree of

Masters of Arts
August 2015

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Introduction

Overview

Molecular chaperones are proteins that assist in the proper folding of polypeptides by binding hydrophobic residues of unfolded or newly synthesized polypeptides to prevent these proteins from aggregating (Vabulas et al., 2010, Whitesell and Lindquist, 2005). The endoplasmic reticulum, a major site of protein synthesis in the cell, has a complex chaperoning system (Ron and Walter, 2007). ER chaperones were initially identified as proteins induced by glucose starvation of cultured cells and, therefore, were classified as glucose response proteins (Grp's) (Lee, 1981). In subsequent research, other conditions that interfere in normal ER protein folding were shown to induce these chaperones (Banhegy et al., 2007). These ER stress inducing conditions include treatment with reducing agents that interfere with disulfide bridge formation or agents that block glycosylation. The induction of ER chaperones by ER stress is conserved in all eukaryotes and is part of a larger cellular reaction to unfolded proteins in the ER called the unfolded protein response (UPR) (Ron and Walter, 2007).

The largest ER chaperone is the 170kDa Grp170. It is classified as a member of the Hsp70 superfamily based on structural similarities (Easton et al., 2000; Park et al., 2006). Multiple molecular activities have been identified for Grp170 including acting as a nucleotide exchange factor, a foldase and a holdase (Andréasson et al., 2010; De Keyser et al., 2009; Williams et al., 2015). In most eukaryotic organisms a single locus encodes *grp170* (Easton et al., 2000; Park et al., 2006). However, in the nematode *Caenorhabditis elegans* two loci encode *grp170* (Easton et al., 2000; Nikoladis and Nei, 2004). These loci, formally T24H7.2 and T14G8.3 in *Caenorhabditis elegans,* are also known as

grp170a and *grp170b* respectively. To better understand the roles of *Caenorhabditis elegans grp170* genes I have characterized the regulation of their mRNA expression using RT-qPCR.

ER Chaperones and Protein Folding

The proteins of the ER are synthesized by ribosomes bound to the cytoplasmic face of the ER membrane. As proteins are synthesized they enter the ER lumen by a co-translational translocation process which threads the polypeptide through a pore in the membrane. As a newly synthesized polypeptide enters the lumen it begins to fold into secondary structures (Harding et al., 1999). ER proteins must fold into their proper quaternary structure in order to perform normal biological activities within the cell. Improperly folded proteins within the ER can take several misfolded conformations (Vabulas et al. 2010). These misfolded conformations include partially folded proteins, intermediate structures, oligomers, amorphous aggregates and amyloid fibrils. These conformations tend to aggregate because of interactions between exposed hydrophobic amino acid residues and regions of unstructured polypeptide backbone which are typically buried inside of the native structure. The ER chaperones function to facilitate proper polypeptide folding.

Molecular chaperones play multiple roles in ER polypeptide formation and maintenance (Horwich, 2003). Under normal unstressed conditions, chaperones participate in the following processes: 1) initial protein folding; 2) degradation of misfolded proteins; 3) translocation of proteins across the ER and other membranes; 4)

disassembling oligomeric protein structures; 5) controlling the biological activity of folded regulatory proteins including transcription factors (Bukau and Horwich, 1998). In addition to functioning in normal cell conditions, molecular chaperones also play key roles in stressed conditions. During ER stress, molecular chaperones play a role in preventing protein aggregation, facilitation of ER associated degradation (ERAD) and assisting in refolding improperly formed polypeptides (Bukau and Horwich, 1998).

Hsp70 Chaperone Superfamily

A variety of different chaperones exist within the cell. These chaperones are classified into different categories based on structure, expression patterns and biological functions. One category of chaperones is heat shock proteins (Hsp's). Although these specialized proteins were initially characterized as being induced by heat stress, hsp's can also be induced by a variety of different factors that cause protein aggregation. One of the most well studied groups of Hsp's is the Hsp70 superfamily.

The Hsp70 superfamily represents a major class of molecular chaperones (Easton et al., 2000). Members of this protein family are found in archaea, eukaryotes and prokaryotes, which suggest an evolutionary significant and ancient role in protein folding. Hsp70's can be expressed constitutively within the cell or they can be induced by stress. All members of this chaperone family have a conserved domain structure, which includes: a N-terminal adenosine triphosphatase domain (ATPase; ~400 aa) and a C-terminal substrate-binding domain (SBD; ;180 aa) (Bukau and Horwich, 1998). The substrate-binding domain is divided into three sub domains; the β-sandwich domain, the

α helix domain and a short loop between the β-sandwich domain and the α helix domain. Members of the Hsp70 superfamily function by binding to exposed hydrophobic residues of unfolded proteins and thus prevent the formation of aggregates (Tyson, 1997). The large molecular weight members of the Hsp70 superfamily are considerably larger than the 70 kDa members (in the range from 90 kDa-170 kDa). The large Hsp70s have the same base basic domain structure as the 70 kDa members but have an expanded loop between the ATPase domain and the substrate binding domain and the α helical domain is also expanded (Easton et al., 2000). ER members of the Hsp70 family include *grp170* and *grp78*.

The Unfolded Protein Response (UPR)

Processes that interfere with normal protein structure formation often lead to the accumulation of unfolded or partially folded proteins in the ER (Ron and Walter, 2007). These unfolded proteins can disrupt cellular processes, such as protein transport or ion regulation. The buildup of unfolded proteins in the ER can also trigger apoptosis (Fribley et al., 2009). Additionally, neurodegenerative diseases such as Parkinson's and Alzheimer's, can be caused by an accumulation of unfolded polypeptides (Brehme et al., 2014).

Levels of improperly folded protein are reduced by the unfolded protein response (UPR), which is an adaptive response by the cell to restore homeostasis. The UPR primarily reduces unfolded polypeptides in the ER primarily by using three mechanisms (Liu and Kaufman, 2003). The first mechanism is transcriptional induction of ER

chaperone genes. Induction of molecular chaperone genes increases the capacity of the ER to fold proteins and reduce protein aggregation. A second adaptive mechanism of the UPR is translational attenuation of ER protein synthesis. Translational attenuation reduces further translocation of unfolded proteins into the ER lumen so that newly synthesize proteins are not adding to the pool of unfolded proteins within the ER. The final mechanism is ER-associated degradation (ERAD). ERAD involves the export of misfolded proteins to the cytosol where they are degraded by proteasomes thus reducing levels of unfolded proteins in the ER.

The UPR Signal Transduction

The UPR uses several independent signal transduction pathways that can sense accumulation of unfolded proteins in the ER and induce one or more of the protective mechanism (Figure 1). The primary UPR signal transduction pathway in *C.elegans* is the IRE-1/XBP-1 pathway (Marcella et al., 2001). In *C.elegans* the IRE-1/XBP-1 pathway plays a role in transcriptional induction of ER chaperones. IRE-1 is a transmembrane kinase/endoribonuclease (RNAse) activated by unfolded proteins in lumen of the ER (Tirasophon et al., 1998, Lee 2005). In non-stressed cells, Grp78 keeps IRE-1 in an inactive form by binding to the N-terminal luminal domain. Upon accumulation of unfolded proteins in the ER, Grp78 releases IRE-1 and binds to the unfolded proteins. Freed from its interaction with Grp78, IRE-1 forms homodimers. This homodimer structure promotes autophosphorylation by its kinase domain which activates the endoribonuclease of IRE-1. The active endoribonuclease then can initiate the

nonconventional splicing of a 23 nucleotide intron from the *xbp-1*. The intron of the unspliced *xbp-1* mRNA results in an early stop codon producing a truncated version of XBP-1. Removal of the intron results in a frameshift that allows production of the full length XBP-1 transcription factor (Yoshida et al, 2001, Shen et al., 2001). The spliced mRNA produces the functional XBP-1 protein which is a transcriptional factor that translocates into the nucleus and binds its target sequence in regulatory regions of chaperone genes. This promotes the transcription of chaperones such as *grp170* and *grp78* begins (Lee et al., 2003).

IRE works in parallel with two other signal transduction pathways, the PERK1 and ATF6 pathway. PERK1, also known as PEK1, is a transmembrane kinase that phosphorylates the eukaryotic translation initiation factor 2 subunit α (EIf2α), thereby reducing protein synthesis and counteracting ER protein aggregation (Richardson et al., 2011). Although phosphorylation of eIF2α reduces translation of most mRNAs, it allows for the selective translation of a few mRNAs that contain small open reading frames in their 5' untranslated region. This includes selective translation of the mRNA encoding the transcription factors such as ATF4. ATF4 is a transcriptional activator that increases transcription of some chaperone genes (Wafa, 2013). ATF6 is the critical component of a third UPR signal transduction pathway. ATF6 is an ER-resident transmembrane protein (Lin et al., 2007). Unfolded proteins in the ER trigger proteolysis of the ATF6 cytoplasmic domain also known as ATF6f. ATF6f is released from the ER membrane and becomes a transcription factor that moves into the nucleus and increases transcription of ER chaperones. Strains of *C. elegans* defective in each of the three UPR signal transduction pathways are available (Wormbase 2007).

The *C. elegans grp170* Loci

The two *grp170* loci in *Caenorhabditis elegans* arose from a gene duplication event that took place before the divergence of *C. elegans* and *C. briggssae* approximately 110 million years ago (Nei and Nikolaidis, 2004). The conservation of these two paralogues for such a long period of time suggests that they have important and functionally independent roles in the organism's survival.

The *grp170a* gene, formally designatedT24H7.2 in *C. elegans*, is part of a polycistronic operon on chromosome II (Wormbase, 2007). In *C. elegans* polycistronic operons produce a single precursor RNA containing several open reading frames which are processed into individual mRNA's each containing a single open reading frame. The other loci encoded by this operon include: T24H7.1 which encodes the mitochondrial membrane scaffold protein prohibitin, T24H7.3 which encodes a protein with homology to GSK3-beta interaction protein (a protein which functions as a kinase scaffold in vertebrate cells) and T24H7.4 which encodes blos-4, a protein involved in biogenesis of lysosomes. Additionally, there is a small nucleolar RNA locus, T24H7.6 residing in an intron of the *grp170a* gene. The function of this snoRNA is unknown. Operons in *C. elegans* often encode proteins with related functions and which are co-regulated (Blumenthal and Gleason, 2003). Analysis of the other proteins on the *grp170a* operon suggest *grp170a* may have a housekeeping role (Asrani 2009). The *grp170a* locus consists of 7 exons and 6 introns (Figure 2a). The spliced mRNA has an open reading frame 2778 nucleotides long and encodes a protein predicted to be 925 amino acids long. Two deletion alleles are available for the grp170a locus (Wormbase 2007). The ok2107 allele deletes most of exon 3, all of exon 4 and part of the following intron. In addition to

disrupting *grp170a*, ok2107 also deletes the small nuclear RNA, T24H7.7. The use of ok2107 as a tool to study grp170a function is complicated by its deletion of an unrelated locus. The other deletion allele for grp170a, tm3109, targets just the *grp170a* gene deleting 155 codons and introduces a frameshift mutation in exon 3.

The *grp170b* gene, T14G8.3, is a monocistronic gene on the X chromosome (Wormbase, 2007). Its monocistronic status would allow it to be independently regulated, possibly making it a more suitable locus for induction by the UPR (Asrani 2009). Other UPR inducible chaperones, such as hsp-4 are monocistronic genes (Lui et al., 2003). The *grp170b* gene sequence is 8792 base long, this includes 7 introns and 8 exons (Figure 2b). Its open reading frame is 2766 nucleotides long and it encodes a protein predicted to be 921 amino acids long. One deletion allele is available for the *grp170b* locus, ok502. This is a 2312 base pair deletion spans from exons 8 to 13.

In a genome wide study of gene expression during the UPR using microarray analysis, both *grp170a* and *grp170b* were reported to be induced by the UPR (Shen, 2001). A limitation of microarray analysis is the potential for cross hybridization of related genes. Shen (2001) selected a sample of genes to confirm expression data using the more reliable and gene specific qPCR technique. This sample included *grp170b* which qPCR confirmed was induced by the UPR. However they did not analyze *grp170a* expression by qPCR. It is possible that the positive signal they received for *grp170a* in the microarray analysis was not due to induction of *grp170a* but was an artifact caused when the highly induced *grp170b* probe crossed hybridized with *grp170a*. RT-qPCR analysis of expression of *grp170a* is needed to clarify its expression during the UPR.

Tunicamycin induction of the UPR in *C. elegans*

Tunicamycin is often used experimentally to induce the UPR (Olden et al, 1979*).* Tunicamycin is a mixture of homologous nucleoside compounds produced by several bacteria which blocks N-glycosylation of proteins in the ER in eukaryotes. N-glycosylation is required for proper protein folding in the ER (Elbein, 1987). Therefore tunicamycin inhibits ER protein folding by inhibiting biological glycosylation. During N-glycosylation a glycan sugar is attached to nitrogen group of a specific asparagine residues. Tunicamycin targets UDP-G1cNAc:DolichyLPhosphate GlcNAc-1 –Phosphate transferase which is the last enzyme in the metabolic pathway in the synthesis of N-glycosidically linked oligosaccharides (Heifetz et al, 1979). Although there are a several experimental approaches to induce the UPR in *C.elegans*, tunicamycin offers several advantages. The primary advantage of tunicamycin is its chemical stability. Unlike other chemical inducers such as the unstable dithiothreitol, tunicamycin is not prone to structural changes due to factors such as oxidation.

Reverse Transcriptase Quantitative PCR

Reverse transcriptase quantitative PCR assay (RT-qPCR) is one of the most effective techniques for mRNA detection and quantitation. RT-qPCR is a sensitive technique that can determine if a specific gene is being expressed within a given sample and to what degree the expression is occurring (Dharmaraj, 2015).

Analysis of mRNA by RT-qPCR first requires that cDNA be synthesized from the mRNA template (Figure 3). Reverse transcriptase is the enzyme used to synthesize the

cDNA (Dharmaraj, 2015). This polymerase requires a short double stranded sequence at the 3' end of the mRNA to act as a primer to initiate synthesis. There are several different priming options for converting RNA into cDNA using Reverse Transcriptase (Life Technologies, 2015). One of the most common is Oglio(dt) which anneals to the poly(A) tail of mRNA. There are two big advantages in using Oglio(dt). First, a full length cDNA is generated because synthesis is initiated from the 3' poly(A) tailed end of the mRNA. Second, since oligo(dt) targets the polyA tail, it biases cDNA production to intact mRNA in a pool of total RNA.

There are several advantages to synthesizing cDNA from mRNA in a separate process from the qPCR amplification. (Life Technologies, 2015). The first is that a pool of cDNA is stable and can be stored for long periods of time; and be used for multiple reactions. Second, the control genes can be amplified from the same cDNA pool, which allows consistency for normalization purposes. Finally, optimized reaction buffers and conditions can be chosen for each individual reaction. These advantages make the two step approach (cDNA synthesis followed by PCR amplification) a robust experimental process.

In qPCR, DNA is amplified by a standard PCR reaction with the inclusion of a fluorescent dye specific for double stranded DNA such as sybr-green. The sybr green allows the progress of the PCR reaction to be monitored in real time by measuring the fluorescence associated with increasing quantities of double stranded DNA. Relative mRNA expression levels of target genes are compared as fold changes by normalization using reference genes.

Thesis Objective

To investigate the physiological roles of the two loci encoding GRP170; T24H7.2 (*grp170a*) and T14G8.3 (*grp170b*) in *Caenorhabditis elegans* by analyzing the expression of their mRNA's using RT-qPCR .

Specific Objectives

1) Determine whether the two *C. elegans grp170* genes have diverged in their response to UPR.
2) Determine if loss of either *grp170* loci induces the UPR by examining *hsp4* in nematodes defective for *grp170*a or *grp170b*.
3) Determine what signal transduction pathway is most significant in inducing *grp170* expression during the UPR in *C.elegans*.
4) Investigate possible genetic interactions between the *grp170* loci by characterizing expression of each *grp170* loci in nematodes deficient for its paralogous locus.

I. Methods

A. Primer Design

Primers were designed to specifically target the *grp170b* gene using the Primer-BLAST software (Ye et al., 2012). Standard criteria were used in designing primers (Stratagene, 2004). Criteria included optimizing primer length and amplicon size, limiting the formation of secondary structure, and targeting a GC content between 40-60%. Secondary structure analysis was conducted using OligoAnalyzer Tool (Integrative DNA Technology, 2009). Primer sequences for other analyzed genes were previously described (Table 1).

B. General *C. elegans* Techniques

1. Preparing Worm Cultures

Stocks of *C.elegans* were grown on standard nematode growth media (NGM) (0.25% w/v tryptone, 0.3% w/v NaCl, 1.8% w/v agar, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 µg/ml cholesterol in ethanol, 25 mM KPO_4, pH 6) seeded with OP50 strain of E. coli as a food source (Brenner, 1974). Nematode cultures were grown and maintained between 15°C and 25°C. New cultures were established by transfer of individual adult nematodes or by the chunking method (Steiernagle, 1999).

2. Synchronizing Cultures

Procedure 1

(Tunicamycin Induction, Compensating Expression and UPR Induction experiments)

To generate small synchronized populations of nematodes, thirty mature gravid hermaphrodites were placed in a drop of 40µl of 20% alkaline bleach mixture (8.25mL

ddH2O, 3.75mL 1M NaOH, 3.0mL Bleach) on the OP50/NGM plates. The bleach killed the adults and released the bleach resistant eggs. Eggs were allowed to hatch on the 0P50/NGM media and mature for approximately 2.5 to 3 days into young adults (Steiernagle, 1999).

Procedure 2

(Signal Transduction Pathway experiment)

To generate larger synchronized populations of nematodes, mature cultures with numerous gravid hermaphrodites were washed from plates with sterile H_2O. The washed worms were collected in a 15ml sterile conical centrifuge tube in a total volume of 3.5ml. To lyse approximately 200 adult worms, an alkaline beach solution (8.25mL ddH2O, 3.75mL 1M NaOH, 3.0mL Bleach) was added. The tube was then agitated by vortexing for a few seconds every two minutes for a total of 10 minutes. Samples were centrifuged for 30 seconds at 1300xg to pellet the eggs. The supernatant was removed by aspiration leaving a 100µl volume of pelleted eggs. Next, the eggs were washed by adding 5ml of H_2O. The sample was vortexed briefly and centrifuged for 30 seconds at 1300xg. All but 100µl of supernatant was removed by aspiration. Using a sterile pipet tip, the remaining liquid containing released viable eggs was transferred to edge of a clean NGM plate seeded with OP50 and allowed to mature for 2.5 to 3 days (Stiernagle, 2006).

3. Induction of the UPR by Tunicamycin

For tunicamycin treatments, synchronized populations of worms were washed from culture plates with 1.25ml of M9 Buffer (5.8g Na2HPO4•7H2O, 3.0g KH2PO4, 5.0g NaCl, 0.25g MgSO4•7H2O, ddH2O to 1L) and transferred to a 1.5ml RNase free

microfuge tube. Next, tunicamycin was added to treated samples to a final concentration of 3μg/ml. Control worms had no tunicamycin added to the tubes. Induction of the UPR by these treatment conditions was confirmed using the nematode strain SJ4005. This *C. elegans* strain has a transgene that links the promoter of the *hsp*4 gene with the green fluorescence protein of jellyfish (Shen et al. 2001). Because *hsp4* is highly inducible by the UPR, GFP can be used as a proxy for induction of the UPR. GFP induction was monitored using fluorescence microscopy (Santoro, 2000). Tunicamycin treatment resulted in high levels of GFP induction in the SJ4005 strain, indicating the treatment was effective for induction of the UPR (data not shown).

C. Reverse Transcriptase Quantitative PCR

1. RNA isolation

RNA was isolated using the Trizol Reagent *(*Morimoto Lab Protocols, 2008). Adult *C. elegans* nematodes were washed in triplicate sets (approximately 200 worms) from synchronized plates with 1.5ml of M9 Buffer and transferred with a sterile RNase free pipette tip to RNase free microfuge tubes. The worms were harvested by centrifugation for 1 minute at 4ºC at 14,000 rpm (9,800xg). The supernatant was carefully removed and discarded. The pelleted worms were resuspended in 1ml of Trizol Reagent (Invitrogen, Grand Island, NY) by briefly vortexing and incubated at room temperature for 10 minutes. The sample was then centrifuged for 10 minutes at 4ºC at 12,000 rpm (8,400xg). Supernatant was transferred to fresh RNase free microfuge tube and 200μl of chloroform was added to supernatant. The tube was mixed by vortexing for 15 seconds and then

incubated at room temperature for 5 minutes. The sample was centrifuged at 12,000 rpm (8,400xg) for 14 minutes at 4ºC. The top aqueous layer was removed and transferred to a new RNase free tube. To precipitate the RNA, 500µl of propanol was added and the sample was mixed by inversion. Precipitated RNA was pelleted by centrifuging the sample at 12,000 rpm (8,400xg) for 10 minutes. The supernatant was removed and the pellet was washed by adding 100µl of ethanol followed by centrifugation of the sample at 14,000 rpm (9,800xg) for 5 minutes at 4ºC. The supernatant was removed and the pellet was air dried for 10 minutes. Pellet was dissolved in 25µl of DEPC treated H_2O by incubation in 55ºC water bath for 10 minutes to resuspend pellet. RNA samples were stored at -20ºC or -80ºC until RNA purification.

2. DNase Treatment

Trizol purification of RNA selectively removes DNA. To further limit the possibility of genomic contamination, RNA samples were treated with DNase I using the Ambion DNA-free kit (Invitrogen, Grand Island, NY) following the manufacturer's instructions. For a 20µl reaction, 10µl of purified RNA, 2µl 10x DNase I buffer, 7µl RNase free H20, 1µl DNase were combined in a 1.5ml microfuge tube**.** Tubes were incubated at 37°C for 20-30 minutes. Then 2.5µl of Ambion DNase Inactivation Reagent was added and incubated for 2 minutes at room temperature. Tubes were spun down at 14,000 rpm for 1.5 minutes at 4°C. The inactivation agent traps DNase in the pellet .The supernatant was transferred to an RNase free microfuge tube.

3. RNA Microquantification

Concentrations and purity of the isolated RNA were assessed spectrophotometrically using the Beckman Coulter DU730 spectrophotometer microcuvette. The wavelengths

analyzed were 260nm and 280nm. RNA concentrations were determined using an extinction coefficient at 260nm of 0.025 (µg/ml) cm−1 and initial characterization of RNA purity was determined using the ratio of absorbance at 260nm/280nm (Ausubel et al., 1999).

4. Agilent 2200 Tape Station

To assess the quality of the RNA, samples were analyzed on the Agilent RNA TAPE screen system (Agilent Technologies, Santa Clara, CA). Each sample was prepared by mixing 5µl of Agilent RNA Buffer solution with 1µl of RNA sample. Samples were vortexed using IKA vortexer and adaptor at 2000 rpm for 1 minute followed by brief centrifugation. The sample was denatured (heated to 72ºC for 3 minutes and placed on ice for 2 minutes and then centrifuged) and then loaded into Tape station. The Tape station provides an RNA integrity number which on a scale of 1 to 10 provides an assessment of RNA quality with 1 being heavily degraded RNA and 10 being the highly intact RNA (Mueller et al., 2004)

5. cDNA Synthesis

DNA-free RNA samples were used as a template to synthesize cDNA using Life Technologies iScript kit. Following manufacturer's instructions a 20µl reaction (10µl RNA, 4µl of 5x iScript mix, 1µl Reverse Transcriptase, 5µl RNase free H_2O) was incubated at 25º for 5 min; 42º for 30 min and finally 85º for 5 min. The cDNA was stored at -20º prior to qPCR.

6. qPCR Protocol

The BioRad iScript One-Step RT-PCR Kit with SYBR Green (BioRad, Hercules, CA) was used for qPCR. All qPCR reactions were conducted using a standard assay except for

varying template cDNA source and the primer set. The 28µl standard reactions included 12.5 µl 2x SYBR Green RT-PCR mix, 300nm Forward Primer, 300nm Reverse Primer, 4µl of cDNA (approximately 12µg/ml), and nuclease-free water. Amplification was carried out in the Biorad CFX96 Real-Time System/C1000 Touch Thermal Cycler. The thermal cycling protocol was 50°C for 10 min, 95°C for 5 min, 95°C for 10 sec and then 30 sec, and 55-60°C for 40 cycles at 30 secs per cycle for PCR cycling and data collection. Actin-1(act-1) and peroxisomal membrane protein-3 (pmp-3) were used as control mRNA's (Zhang et al., 2012). The BioRad CFX96 Real-time system software identified the threshold values used in analysis. Specificity of primers was confirmed by electrophoresis on a 1% TBE agarose gel (1x TBE supplemented with 0.5µg/ml ethidium bromide) (Gauley and Heikkila, 2006). Additionally, melt curves were run at the conclusion of amplifications to confirm single PCR products.

7. qPCR Data Analysis

The Pfaffl method was used to analyze relative mRNA levels in samples (Pfaffl, 2004) (Figure 4). The relative difference in mRNA levels between the control and the experimental sample is "R". PCR efficiency, "E", can represent a static efficiency of 2 or the actual efficiency for a given gene as determined by a template dilution experiment. The crossing point, "Cp", is the cycle at which the amplification crosses the threshold value. The change in Cp, "ΔCP", is the difference between the Cp value of the control minus the Cp value of the experimental sample. Target Cp values were normalized to control reference genes. The change of Cp in the target gene is determined by changes in cycle number.

Results

Part I. Development and validation of qPCR assays

Primers development

Efficient and specific primers are necessary for an effective qPCR assay. A pair of qPCR primers were designed and tested for each of the *grp170* loci in C. elegans. The primers were designed to meet criteria based on the established literature for a reliable qPCR assay (Ye et al, 2012). These criteria include primers which generate short amplicons of approximately 300 base pairs; primer length between 17 to 30 base pairs long and primers with melting temperature's (t_m's) within 2° of each other. Primers were also chosen with minimal stable secondary structure [i.e. heter- and homo-dimers with ΔG's not less than -4 kC/mol] and with a G-C content of 40-60% (Stratagene).

The *grp170a* forward and reverse primer are both 20 base pairs long (Table 1). The amplicon length is 289 base pairs. The ΔG values for potential secondary structure are not less than -5 kcal/mol (D'Auria unpublished, 2013). The grp170b forward and reverse primer were also both 20 base pairs long. The amplicon length was 289 base pairs and the ΔG values did not exceed -5kcal/mol. The primer sequence for the control genes and *hsp4* were designed in previous studies (Zhang et al., 2012, Lai et al., 2002).

Analysis of RNA Quality

A. Spectrophotometric Analysis

RT-qPCR requires intact and pure RNA as a template for the reverse transcriptase. RNA was isolated from nematodes using a Trizol extraction method. Initial

analysis of the yield and purity of the RNA was determined spectrophotometrically. The yield and concentration of RNA was determined by measuring absorbance at 260 nm (A_{260}) (Wilfinger et al., 1997). The average concentration of RNA samples were approximately 12 µg/ml and yield was approximately 300ng. The purity of the sample was analyzed by determining the ratio of absorbance at 260nm and 280nm (A_{260}/A_{280}). A ratio between 1.8 and 2.0 is considered satisfactory purity (Wilfinger et al., 1997). 260/280 ration under 1.6 could indicate phenol and/or protein contamination, while a ratio over 2.0 might be indicative of alkaline conditions. All RNA samples used in this study had 260/280 ratios in the acceptable range (1.6 and 2.1) with the average ratio of the RNA samples being 1.76.

B. Agilent Technology's 2200 Tape Machine

The structural integrity of RNA samples is important to the reliability of RT-qPCR (Schroeder et al., 2006). Degraded RNA may not yield full length cDNA for PCR amplification. Traditionally, RNA integrity was determined by analyzing the ratio of 28S:18S ribosomal RNA. However, the traditional method has been criticized because results are considered subjective which can lead to inconsistent findings (Schroeder et al., 2006). However, the recent development of automated quality control instrumentation has made assessing RNA integrity more reliable. The integrity of all RNA samples used for cDNA synthesis were analyzed using the Agilent Technology's 2200 Tape Machine. The tape station examines the entire electrophoretic trace of the RNA and uses an algorithm to report on the integrity of each sample (Mueller et al., 2004). The algorithm allows for the classification of eukaryotic RNA based on a scale of 1 to 10, 1 being an

extremely degraded sample and 10 being a sample perfectly intact. Samples used in this study had values ranging between 5.9-7.6 RIN. Samples above 5 are considered acceptable for RT-qPCR assays (Agilent Technologies 2015, Muller et al., 2004). All RNA used in cDNA synthesis had acceptable structural integrity.

The qPCR Amplification

Quantitative PCR (qPCR) uses real time measurements of the dsDNA produced during PCR amplification. The double stranded DNA is measured using a fluorescence dye such as Sybr green. Sybr green has minimal fluorescence in the absence of double stranded DNA but is highly fluorescent when bound to dsDNA. During cDNA amplification, the Sybr green binds the dsDNA as it is synthesized; resulting in an increase in Sybr green fluorescence. Amplification of the grp170a and grp170b sequences resulted in the expected sigmoidal curve of increasing fluorescence as a function of the number of amplification cycles (Figure 5). Sybr green florescence level is measured in relative fluorescence units (RFU) (see y axis of amplification plot). A threshold value within the exponential phase of amplification was identified by the BioRad CFX96 Real-Time instrument. The point at which the amplification curve crosses the threshold is known as the threshold cycle (Cp). The Cp is used to determine the amount of initial template DNA. Amplification curves shapes are a good indicator of product reliability (Stratagene, 2004). Amplification curves that appear linear or that do not cross the threshold are often unreliable. Amplification curves that have a sigmoidal shape and are well above the threshold (approximately 3000 RFU) usually yield dependable results. Only qPCR reactions generating sigmoidal amplification curves were analyzed.

Analysis PCR Product

A. Melting Curves

RT-qPCR should amplify only the single target amplicon and no other non-target sequences. Melt curves of all amplified products were analyzed at the end of each qPCR reaction to confirm specificity of amplification. Fluorescence of Sybr green was monitored as samples were slowly heated until they fully denatured. The BioRad CFX96 Real-Time generated a melt curve as the rate of change in absorbance's plotted against temperature. A single peak in a melting curve is expected for reactions generating a single amplification product. Multiple peaks or a significant shoulder would indication non-specific amplification. All primers used in this study routinely resulted in products that had single peaks on melting curves (Figure 6).

B. Gel Electrophoresis

To confirm that PCR reactions were specific and yielded a single product, final products were also analyzed by agarose gel electrophoresis (Figures 7). Only PCR reactions that yielded a single product of the expected sized were included in gene expression analysis.

Efficiency of Amplification over a Range of Template cDNA Concentrations

To evaluate significance of differences in amplification curves, the efficiency of qPCR for each primer set must be determined by comparing amplification curves of known dilution of template cDNA. A template dilution experiment was conducted using cDNA generated from RNA of the N2 strain induced for PCR with tunicamycin. The Ct

values for undiluted cDNA and 3, 4 and 5 fold dilutions were analyzed (Figure 8). Amplification of *grp170a, grp170b* and control mRNA's showed efficiencies of PCR between 1.90 and 1.98 which was very close to the maximum theoretical efficiency of 2.0 (Table 2). The efficiency was calculated using log of 3 (3^ (-1/slope)) (Life Technologies Applied Biosystems, 2004; Joshua et al., 2006)

Part II. Analysis of g*rp*170 mRNA expression

C. elegans *grp170* mRNA Expression During the Unfolded Protein Response (UPR)

Synchronized cultures of nematodes of the standard laboratory strain N2 treated with tunicamycin to induce the UPR were compared to untreated controls. Relative levels of *grp170a* and *grp170b* mRNA were determined. Two control genes, *act-1* and *pmp-3* were used to normalized expression levels and allow comparisons between treatments (Figure 9). The *grp170a* locus was not induced by the UPR. The slight increase (55%) in grp170a mRNA during UPR was not significant and far below induction levels observed for other UPR inducible chaperones (Shen et al., 2005). On the other hand, the *grp170b* was highly induced by the UPR. Compared with the untreated control, *grp170b* mRNA levels significantly increased, almost 580%.

Compensating Expression of *C. elegans grp*170 Loci

Deletion alleles for either *grp170a* (tm3109) or *grp170b* (ok502) are associated with only minor phenotypic changes (Wormbase, 2007). This might be explained if loss of one *grp*170 locus as associated with increased expression of the remaining locus. This

type of phenomena has been observed for the *grp78* loci, *hsp-3* and *hsp-4* (Link et.al 2005).

To investigate potential genetic interactions between the two *grp170* loci, expression of each *grp170* locus was analyzed in a strain deficient for the paralogous *grp170* locus. Expression of *grp170a* was not significantly different in nematodes homozygous for the deletion allele ok502 of *grp170b* (strain BSC06) compared to the isogenic N2 strain with function *grp170b* alleles (Figure 10a). Therefore, there is no evidence that *grp170a* alters its expression to compensate for loss of *grp170b*.

On the other hand, the expression of *grp170b* was significantly up-regulated in nematodes homozygous for deletions the deletion allele tm3109 of *grp170a* (strain BSC07) compared to the N2 control strain (Figure 10b). The increase was approximately 8300%.

Analysis of the UPR in *grp*170 Deficient Nematodes.

The *grp*170 chaperones are believed to be part of the protein folding machinery of the ER (Liu and Kaufman, 2003). If the protein encoded by either *C. elegans grp170* locus plays an important role in protein folding, then loss of that locus would interfere in normal protein folding and result in the UPR. Induction of the UPR was assayed by measuring the expression of the UPR inducible *hsp-4* gene (Link, 2005). The expression of *hsp-4* was not induced in *grp170b* deficient worms (strain BSC 06) compared to the control N2 strain (Figure 11). Therefore, loss of *grp170b* does not induce the UPR suggesting that it is not important for general ER protein folding under the conditions analyzed. In contrast expression of *hsp*-4 was highly induced in nematodes

deficient for *grp*170a (strain BSC 07) compared to the control strain. This demonstrates that loss of *grp*170a does induce the UPR and suggests that *grp*170a plays an important role in ER protein folding.

Signal Transduction Pathways

Eukaryotes have several conserved signal transduction pathways that can sense unfolded proteins in the ER and induce the UPR. In *C.elegans* three pathways have been characterized to play a role in the UPR induction, the IRE-1, PEK-1 and ATF6 pathways. These different pathways are responsible for the induction of different genes in the UPR response (Shen et al., 2005).

To test which pathway is responsible for *grp170b* induction by the UPR, *C.elegans* strains defective for elements of the IRE-1, PEK-1 and ATF6 were analyzed. The strains used were RB545 (PEK-1 mutant), RB772 (ATF6 mutant) and SJ17 (XBP-1 mutant). UPR induction of *grp*170b was unaffected by deletion in the PEK-1 and ATF6 signal transduction pathways (Figure 12). There was no significant difference in grp170b mRNA expression between mutant strains defective for these pathways and the standard laboratory strain when treated with tunicamycin. Expression of *grp*170b was induced 420% by tunicamycin induced UPR in the standard N2 control strain, 490% in the PEK-1 mutant and 400% in the ATF-6 mutant. These differences were not significantly different (ANOVA and tukey post hoc test p value for N2/AFT6 = 0.018 and for N2/PEK-1 p= 0.001.) This indicates PEK-1 and the ATF6 pathway are not involved in the UPR induction of *grp170b*. In contrast, the strain blocked for the IRE-1-pathway (caused by a non-functional allele of *xbp-1*) failed to induce *grp170b* during the UPR

(Figure 12). The *grp170b* mRNA levels in worms blocked for the IRE-1 mutant decreased 50% relative to the N2 control but the difference was insignificant (ANOVA and tukey post hoc test p value for N2/xbp-1 = 0.006). This shows that the IRE-1 pathway is essential for induction of *grp170b* mRNA by the UPR. It also confirms grp170b induction by tunicamycin is part of the UPR response.

Discussion

RT-qPCR Analysis Validated through use of Established Quality Standards (MIQE)

RT-qPCR was used to study the expression of the two *grp170* loci of C. elegans. To minimize the potential variables introduced in sample processing and to increase reliability and repeatability in qPCR, every attempt was made to follow the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines for this study (Taylor et al., 2010).

MIQE recommends standard measures for quality of RNA template used in qPCR assays. Purity, quantification and intactness of RNA was determined both with standard spectrophotometric analysis and with the Aligent RNA Screen TAPE system. DNA contamination was minimized with use of a Trizol extraction procedure followed by DNase treatment. Only RNA samples meeting minimum standards outlined in MIQE were used in this analysis.

As part of this study, primers for the *grp170b* locus were designed to meet MIQE standards. Primer sequences for other genes, (*grp170a, act-1 and pmp-3, hsp-4*) were adapted from previous studies (D'auria unpublished, 2013, Zhang et al., 2012, Marcella et al., 2002) Melt curve and electrophoresis analysis of amplification products was used to confirm specificity of all primers for their gene targets. The PCR efficiency and linear dynamic range for analysis was also determined by amplification of a template dilution series.

Differential expression of *C. elegans grp170* loci during UPR

Vertebrate *grp170* genes were first characterized based on their induction during ER stress by the Unfolded Protein Response (UPR). To better understand the physiological roles of *grp170* in *C. elegans*, I studied the expression of the two *grp170* loci at the mRNA level during the UPR using RT-qPCR.

C. elegans grp170a mRNA maintained a consistent level of expression in the UPR stressed and unstressed nematodes. The apparent constitutive expression of this locus suggests it may play more of a housekeeping role in ER protein folding in *C. elegans.* Conversely, *grp170b* mRNA levels increased significantly during the UPR stress compared with unstressed nematodes, which suggest *grp170b* may be the stress inducible form of the *grp170* in *C.elegans*.

The differential expression of the two nematode *grp*170 loci observed in my study was consistent with the molecular structure of the loci (Asrani, 2009). The *grp170b* gene is a monocistronic locus and has its own unique promoter. Its unique promoter would facilitate the independent regulation of this gene during the UPR. In contrast, *grp170a* is part of a polycistronic operon in which multiple genes share a promoter. The three other genes on the operon (T24H7.1, T24H7.3, and T24H7.4) are not stress related and appear to have housekeeping functions. The operon structure of this locus is consistent with the constitutive expression of *grp*170a observed in my study.

A previous high-throughput microarray analysis suggested that both *grp*170a and *grp*170b were induced by the UPR (Shen et al., 2001) which was inconsistent with my data that demonstrates *grp170a* was not induced during UPR. Microarray analysis is an

extremely good tool for transcriptome analysis but it is prone to false positives caused by cross hybridization of related gene sequences (Draghici et al., 2005). Shen et al. (2001) confirmed the UPR inducibility of *grp170b* by RT-qPCR but did not analyze *grp170a* expression by this more reliable assay. The simplest explanation for the discrepancy between the two studies is that the microarray hybridization data for *grp170a* was a false positive that resulted from an artifactual cross hybridization with the highly inducible *grp170b*. Shen et al. (2001) discussed the possibility of cross hybridization in their analysis. An alternative explanation for the discrepancy might involve technical differences in the experiments. However, this seems unlikely as both experiments induced the UPR using tunicamycin and both confirmed induction of UPR through induction of the UPR inducible gene *hsp-4*.

IRE-1/XBP-1 Pathway Necessary for Induction of *grp170b*

The UPR involves three signal transduction pathways that can sense accumulation of unfolded proteins in the ER. When unfolded protein accumulate in the ER, the three response mechanisms are activated and work in parallel to induce cellular responses that restore homeostasis. These pathways are named for the transmembrane signally molecule that is activated by unfolded proteins in each pathway, IRE1, ATF6 and PERK1.

In vertebrates the ATF6 pathway is primarily responsible for the induction of GRP proteins (Lee et al., 2003, Ishikowa et al, 2011) while in nematodes the IRE-1 pathway is essential to the induction of ER Chaperones (Shen et al., 2001). In order to further assess the relative roles of IRE1 and the other two pathways in induction of

grp170b, the ability of tunicamycin to induce expression of *grp170b* was analyzed in mutant nematodes strains deficient for an early component of each of the UPR signal transduction pathways.

Mutant worms deficient for the XBP-1 of the IRE/XBP-1 pathway failed to induce *grp170b* when treated with tunicamycin. This lack of induction demonstrates the IRE1/XBP-1 pathway is required for induction of *grp*170b. This evidence also confirms that *grp170b* induction is dependent on a well characterized the UPR signal transduction pathway. However, *C.elegans* nematode strains deficient for elements of the PERK1 or ATF6 pathways showed significant induction of *grp*170b; the induction levels being similar to N2 nematodes. This evidence suggest the ATF6 and PERK1 pathways do not play a critical role in *grp*170b induction. These results for *grp*170b are consistent with the microarray results reported by Shen et al (2001).

Loss of *grp*170a Results in Induction of the UPR

Certain chaperones play a pivotal role in protein folding in the ER. A recent study done on a suite of ER chaperones in mammalian cells showed that loss of *grp*78 and *grp*94 had different effects on the ER (Eletto et al, 2012). Loss of *grp*78 activated the UPR and a suite of other chaperones, while loss of *grp*94 activated a stress response separate from UPR. These results indicate chaperones vary in how they contribute to general protein folding and ER homeostasis.

In *C. elegans*, genetic mutations in ER chaperones such as *hsp-4* induces the UPR (Link et al., 2005). This demonstrates these chaperones play an important role in ER

protein folding. Further, the induction of the UPR is necessary for nematode survival in the absence of these critical chaperones.

The induction of *hsp-4* is a commonly used indicator of the UPR (Link et al., 2005) and was measured in this study to investigate whether genetic deficiencies for either *grp170a* or *grp170b* induced the UPR. The expression of *hsp-4* mRNA was compared in nematodes deficient for *grp170a* or *grp170b* to the standard lab strain N2 to investigate whether loss of either *grp*170 locus induced the UPR. The nematode strain deficient *grp170b* gene showed no evidence of inducing the UPR as it had *hsp-4* expression levels similar to N2. These data suggest *grp170b* does not play a critical role in protein folding. Conversely, loss of *grp170a* did trigger the UPR as indicated by the induction of *hsp-4*. This suggests *grp170a* plays a crucial role in protein folding and is sufficient in maintain homeostasis in the absence of grp170b. Previous studies have demonstrated that nematodes deficient for both grp170 have an early larval arrest phenotype indicating that at least one grp170 loci must be present for development and maturation (Asrani, 2009).

Loss of grp170a leads to induction grp170b

I have demonstrated that the loss of *grp170a* induces UPR and that UPR induces grp170b. Therefore, I investigated whether loss of *grp170a* would induce *grp170b*. Expression of the *grp170* mRNA's were compared in *C. elegans* strains deficient for *grp170a* or *grp170b* with the standard N2 strain. *C. elegans* that lack grp170b but had a functional *grp170a* gene expressed *grp170a* mRNA at levels similar to N2. These results

showed that *grp170a* maintains constitutive levels of expressions even when the paralogous locus is deleted. On the other hand, *C. elegans* deficient for *grp170a* showed significant induction of of *grp170b* mRNA. This induction was consistent with earlier results that showed loss of grp*170a* induced UPR and UPR induced *grp170b*. Asrani (2009) showed that *grp170b* is essential in nematodes deficient for *grp170a*. However, it is unclear if the up regulation of *grp170b* in *grp170a* deficient nematodes is important to survival in these worms.

Conclusion

The analysis of expression of *grp170a* and *grp170b* mRNA provided insight into diverged physiological roles of these loci. The *grp170a* gene appears to play a greater role in protein folding and maintaining homeostasis within the cell. This is supported by the fact that loss of *grp170a* triggers the UPR as evidenced by induction of *hsp-4* and *grp170b*. Additionally, expression of *grp170a* is not altered by UPR or loss of *grp170b*, which further supports a model where grp170a plays a consistent maintenance or housekeeping role in cells. The *grp*170b locus on the other hand may not be as important for protein folding in the ER, instead it seems to be the stress inducible locus. The *grp*170b gene was induced by the tunicamycin treatment as well as loss of *grp*170a. Conversely, loss of *grp*170b did not result in the induction of *hsp*4. However, the fact that worms lacking *grp*170a require *grp*170b (Asrani, 2009) suggests that *grp*170b can compensate for *grp*170a's role in protein folding.

The differential pattern of expression documented in this study also provides insight into the phenotypic differences observed in nematodes deficient at each *grp170* loci (Li unpublished, 2015). The observation that loss of *grp170a* significantly slows nematode development is consistent with our model of *grp170a* having an important housekeeping function. Further, the loss of *grp170b* was associated with no significant phenotypic effect, consistent with it being less important to a housekeeping/maintenance role in protein folding.

Future research

While this study demonstrated at the whole worm level that *grp*170a functions like a housekeeping constitutive chaperone and *grp*170b is the stress inducible isoform, it didn't provide any insight to where the genes were functioning in the nematode. It is possible that both isoforms function in a basic cellular process that is the same in every cell type. Alternatively, it is possible that the two isoforms have cell type specific functions that were not apparent analyzing whole worm RNA. Experiments analyzing *grp*170a promoter function were consistent with the second alternative (Krone, 2009). A transgene linking the *gfp*170a promoter linked to a GFP reporter open reading frame, indicated that the *grp*170a promoter was highly active in only a few cell types. To fully understand where *grp*170a and *grp*170b are expressed in the nematode, in situ hybridization with gene specific probes could localize the mRNA in the nematode. Results from these experiments could help us understand whether *grp*170a is a true housekeeping gene and whether *grp*170b is a general stress inducible loci or whether they play some cell type specific function.

Table 1. RT-qPCR Primer Characteristics

	Transcript Target[1]				
Primer Attribute	*act-1* (T04C12.6)	*pmp-3* (C54G10.3)	*grp170a* (T24h7.2)	*grp170b* (T14H7.2)	*hsp-4* (F43E2.8)
For Primer (5'-3')	gctggacgtgat cttactgattac	gttcccgtgttca tcactcat	tgttgacgagg agaaggcac	gagccatagc gtggaaggtc	agttgaaatca tcgccaacg
Rev Primer (5'-3')	gtagcagagcttctctcct tgatgtc	Acaccgtcgagaa gctgtaga	ccgtctttggc aagatcgtc	gttggaagac ggtgaggagt	gcccaatcag acgcttgg
Length (F/R)[2]	25nt/24nt	21nt/21nt	20nt/20nt	20nt/20nt	20nt/19nt
Tm[3] (F/R)	61.2°/60.1°	58.3°/60.9°	60.0°/59.7°	56.7°/57.9°	53.7°/56.8°
ΔG Homodimer[4] (F/R) (kcal/mol)	-0.96	-1.34	-1.96	-3.07	-1.47
ΔG Heterodimer (kcal/mol)	4.74	4.64	3.9	-2.12	-0.85
Amplicon Length	114bp	115bp	289bp	289bp	249bp

[1] Transcript targets identified by gene name (in italics) and genetic locus (in parentheses).

[2] F/R indicates an attribute of the forward and reverse primers for a gene separated by a slash.

[3] Tm (DNA melting temperature) was calculated using Primer Blast (Ye et al., 2012).

[4] Thermostability (ΔG) of homodimer formation for each primer and heterodimers formation between the forward and reverse primers were calculated using OligoAnalyzer (Intergrated DNA Technologies, 2009)

Table 2. Primer Efficiency for RT-qPCR

Gene	Efficiency[1]
grp170a	1.90
grp170b	1.93
act-1	1.98
pmp-3	1.93
hsp-4	nd[2]

[1] Efficiency of DNA amplification for each cycle of PCR as determined from template dilution experiment. Perfect efficiency (100%) would correspond to a doubling of the DNA each cycle and have a value of 2. The four tested primers all have an efficiency greater that 90% (1.90-1.98).

[2] Efficiency for *hsp-4* was not determined. A value of 2.0 was used for Pfaffl calculations.

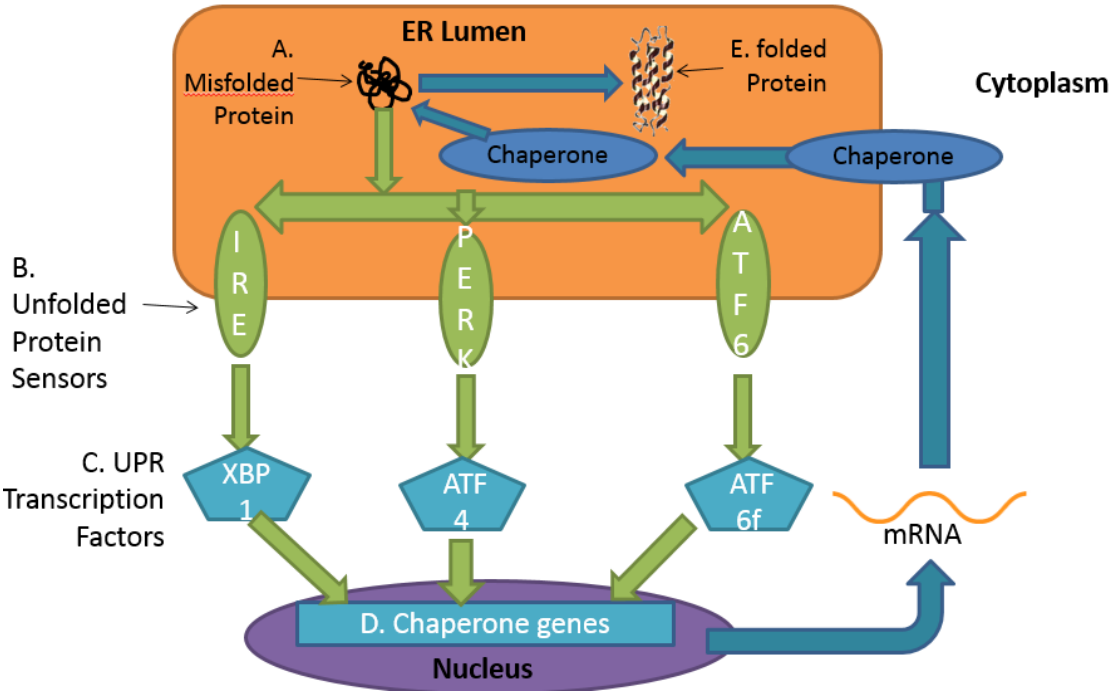

Figure 1: Model of unfolded protein response (UPR) in *C.elegans*.

Three signal transduction pathways (IRE-1, PERK-1 and ATF6) responsible for UPR in *C. elegans* are shown. Misfolded proteins in the ER is sensed by one of three transmembrane proteins. Each sensors activates its own specialized transcription factor (IRE-1 signals XBP-1, PERK-1 signals ATF 4 and ATF6 signals ATF6f. Transcription factors then promote transcription of chaperone genes in nucleus. The mRNA is the translated into a chaperone protein via a co-translation translocation process. This UPR mediated increase in chaperone proteins helps refold misfolded or unfolded polypeptides and restores ER homeostasis.

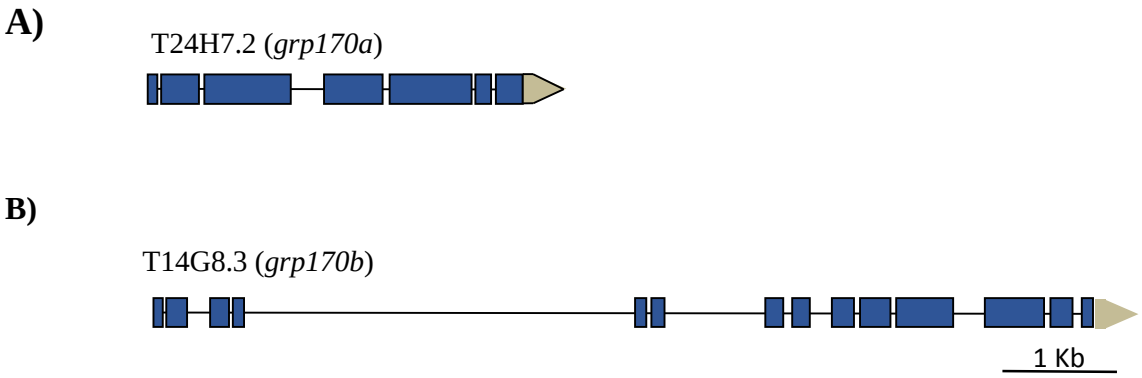

Figure 2: Gene structure of *grp170a* and *grp170b* in *C.elegans.*

A) The *grp170a* locus consists of 7 exons (purple boxes) and 6 introns (black lines). The spliced mRNA has an open reading frame 2778 nucleotides long and encodes a protein predicted to be 925 amino acids long. The *grp170a* locus is part of a polycistronic operons that encodes three other proteins and a small nuclear RNA (T24H7.4, T24H7.3, T24H7.6 and T24H7.1). B) The grp170b locus in monocistronic. It consist of 14 exons and 13 intron. Its open reading frame is 2766 nucleotides long and it encodes a protein predicted to be 921 amino acids long. Figure adapted from gene model in Wormbase (2007).

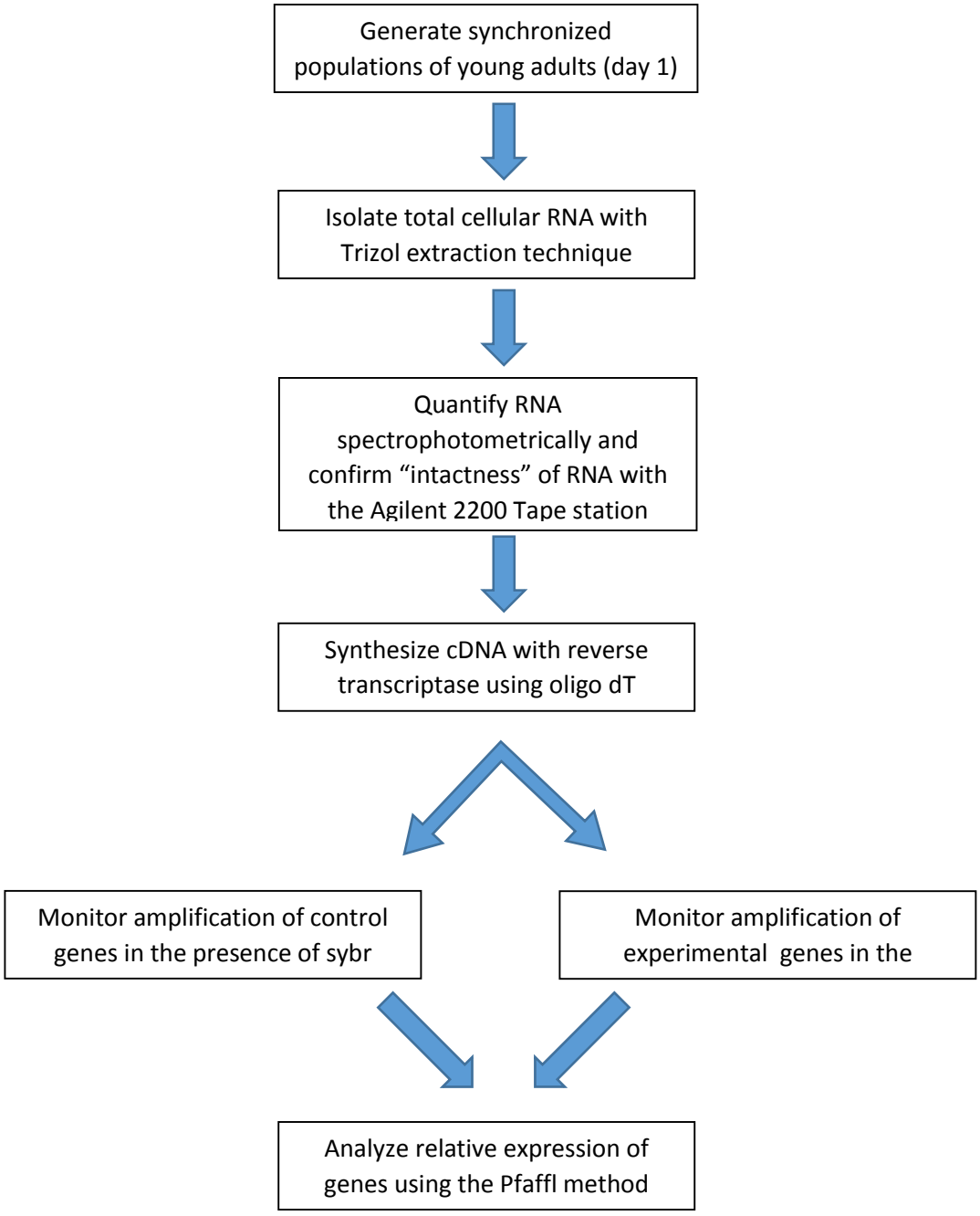

Figure 3. Flow chart of step in qPCR analysis of relative mRNA levels.

Synchronized *C.elegans* populations RNA was isolated. Quality and quantity of RNA was determined. RNA was converted into cDNA and expression levels were determined using RT-qPCR. Expression levels were analyzed using Pfaffl method.

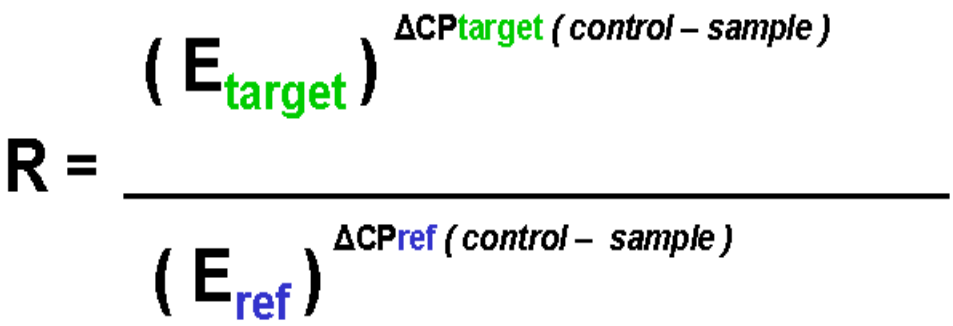

Figure 4. Pfaffl equation.

Method used to determine relative mRNA expression levels represented by R. E target and E ref represent real time PCR efficiencies. ΔCP of target and reference is the difference in CP between control and experimental samples.

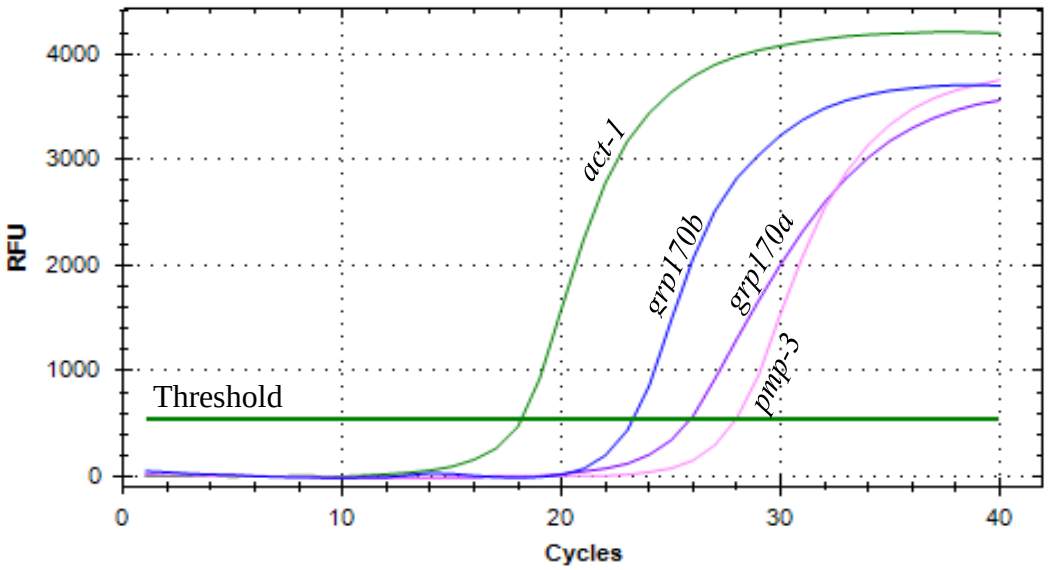

Figure 5 Amplification curve generation of qPCR products.

Sigmoidal amplification curves above the threshold were observed for all four analyzed genes. The relative fluorescence units (RFU) of the sybr green is indicated on the y axis and cycle number for PCR reaction is indicated on the x axis. The threshold (represented by the solid green horizontal line) is approximately 800 relative fluorescence units (RFU). The amplification curves of *act-1* (green curve), *grp170b* (blue curve), *grp170a* (purple curve) and *pmp-3* (pink curve) are labeled on the graph. The Ct values for these samples ranged from approximately 16 and 28 cycles.

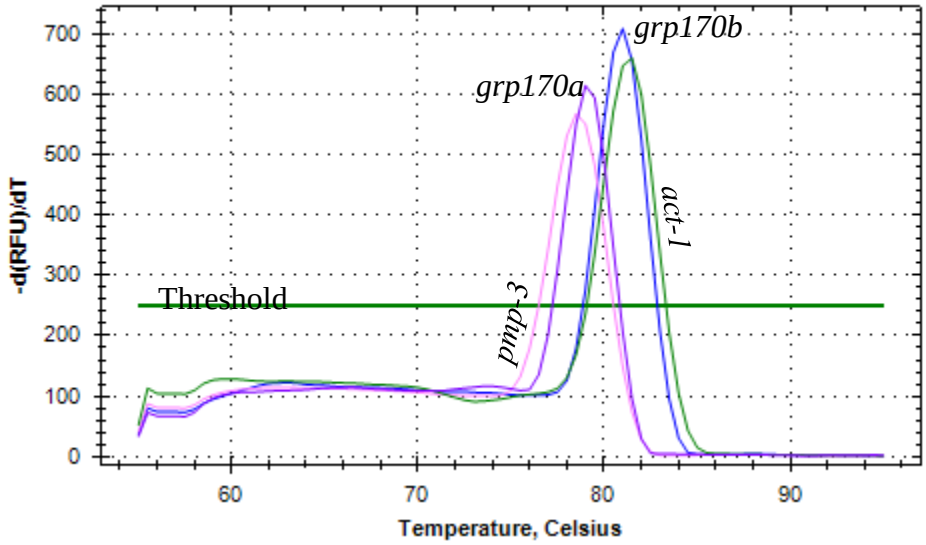

Figure 6. Melt curve quality analysis.

Single peak melt curves were observed for all 4 genes analyzed consistent with the amplification of a single target molecule. The threshold is represented by the solid green line that runs horizontal across the chart at approximately 250 negative relative fluorescence units (-d(RFU)/dt) is indicated on the y axis and temperature (° Celcius) is indicated on the X axis. A single melt peak was observed for the qPCR products of *grp170a* (purple curve), *grp170b* (blue curve), *act-1* (green curve) and *pmp-3* (pink curve).

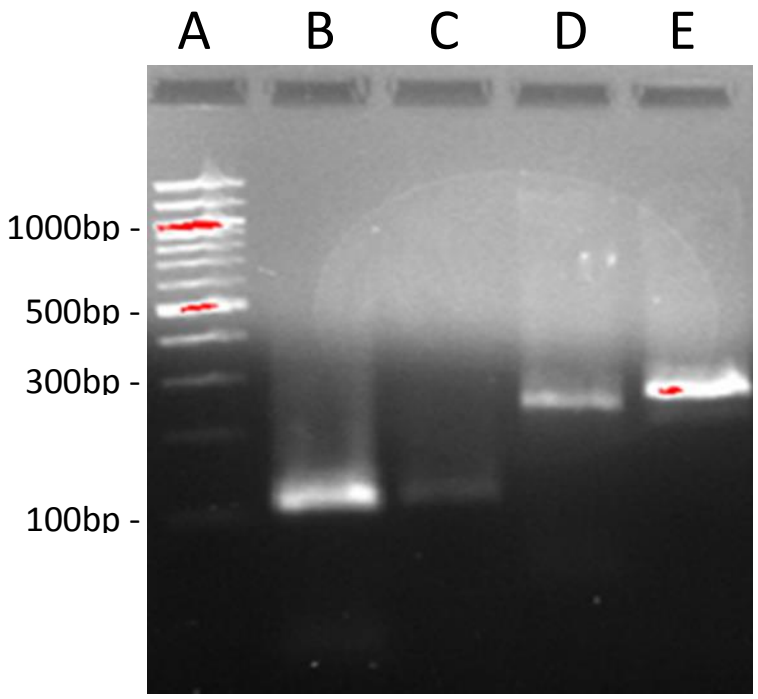

Figure 7. Agarose gel electrophoresis analysis of RT-qPCR products.

A) DNA products of RT-qPCR reactions were analyzed by agarose gel electrophoresis to confirm specificity of amplification. Representative results are shown: lane A: DNA standards (100 bp ladder, New England Biolabs, Ipswich, MA), lanes B-D RT-qPCR products (B: *act-1* ,C: *pmp-3* , D: *grp170a* and E: *grp170b*). Sizes of selected DNA standards are indicated on the left. All RT-qPCR reactions contained a single product as indicated by a single band of DNA. Product sizes were in the range expected for the targeted amplicon (*act-1:* 114bp, *pmp-3*: 115bp *grp170a* 289bp and *grp170b*: 289bp).

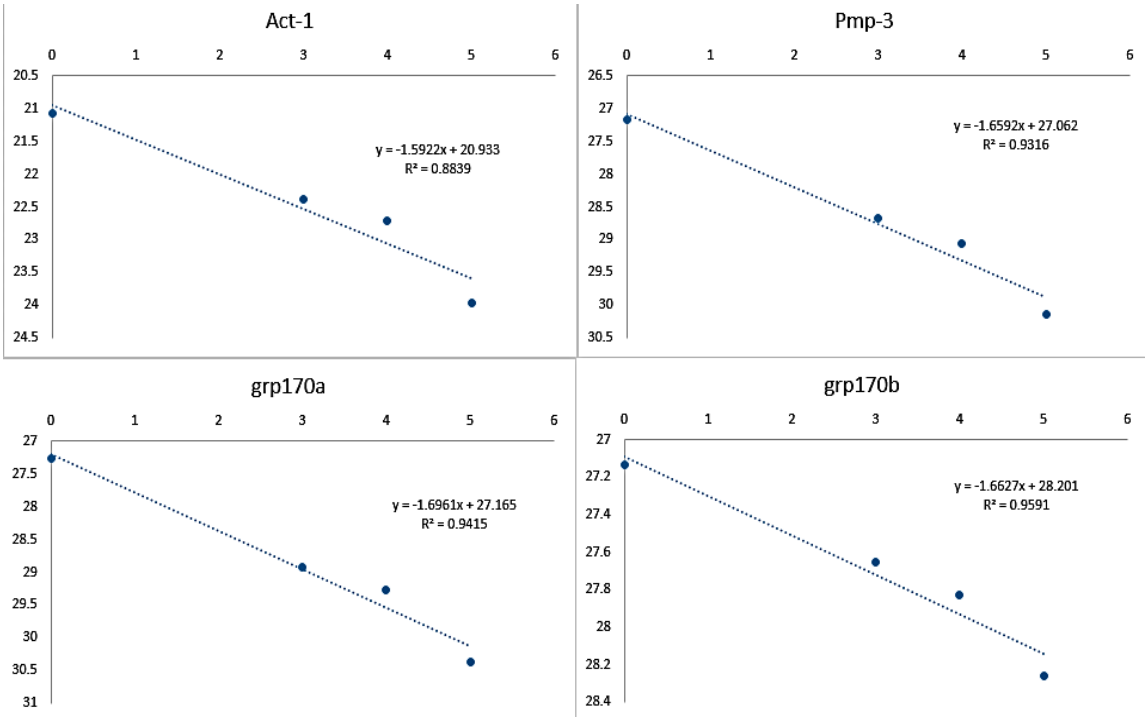

Figure 8. Sample dilutions to test RT-qPCR efficiency.

RT-qPCR was run on diluted samples to test experimental efficiency. The genes diluted were act-1,pmp-3,grp170a and grp170b. The dilutions were a 3,4,5 fold dilution series. Slope (Y value) and goodness of fit (R2) are listed on each graph. Y axis shows cycle of amplification and X axis shows fold change. Consistent linear pattern in each experiment along with high R2 value indicate efficient experimental set-ups.

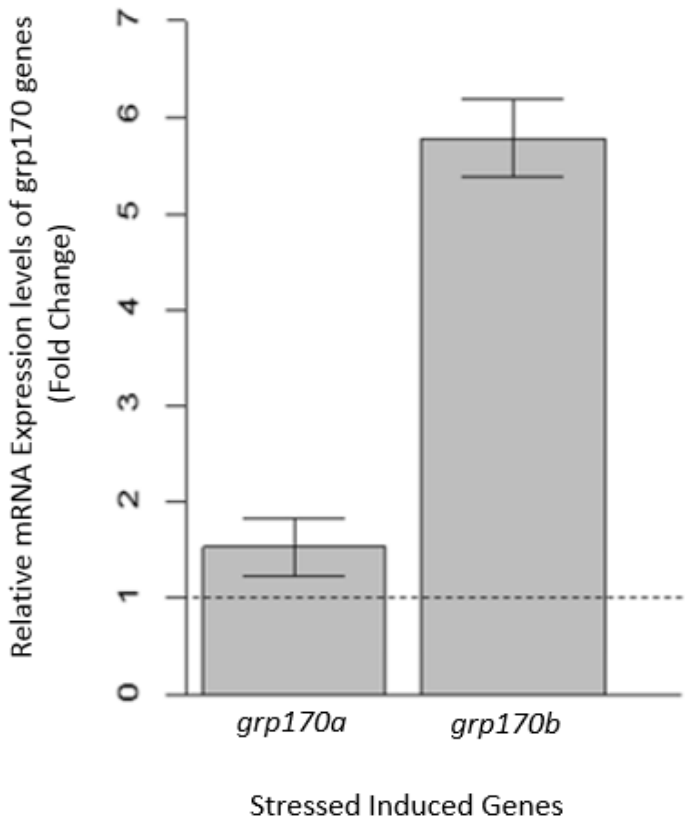

Figure 9. ER stress induction of two grp170 loci.

Relative levels of *grp170a* and *grp170b* mRNA were compared between tunicamycin stressed worms and non-stress control worms in the standard laboratory strain N2 using a qPCR assay. *Act-1* and *pmp-3* were used to normalize expression of target grp170 genes. Dotted line represents the control N2 level of the worms. The *grp170a* mRNA levels were not significantly different in tunicamycin stressed worms compared to non-stressed controls ($p = 0.067$). The *grp170b* locus did show significant induction by tunicamycin when compared to the unstressed control ($p = 0.0007$) (Anova with Tukey post hoc test)

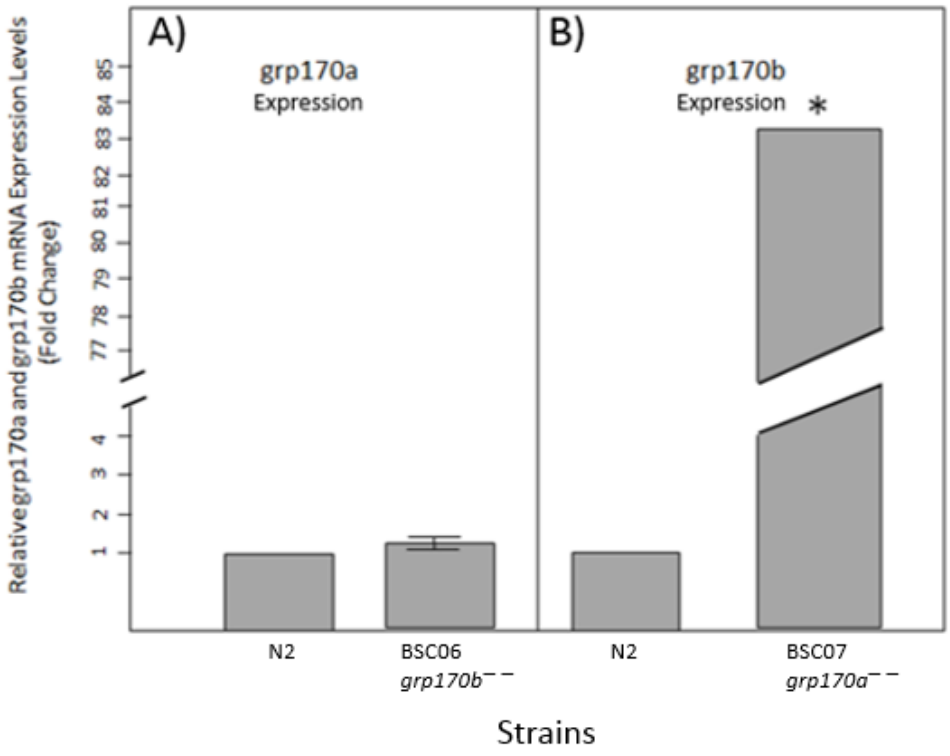

Figure 10. Expression of each *grp*170 locus for the loss of other loci.

A) Relative expression of *grp*170a mRNA was compared in the standard laboratory N2 nematode strain and the isogenic BSC06 strain (homozygous for a deletion allele of *grp170b*) using qPCR. The *grp170a* locus was not significantly different in the two strains (P Value of 0.99) demonstrating that loss of *grp*170b did not influence expression of *grp*170a. **B)** Relative expression of *grp*170b was compared in the N2 strain and the isogenic BSC07 strain (homozygous for a deletion allele of *grp170a).* Expression of *grp170b* mRNA is significantly induced in the absence of a functional *grp170a locus (p = 0.000013).* Asterisk above BSC07 bar represents a large variance of 54.56. (Anova with Tukey post hoc test)

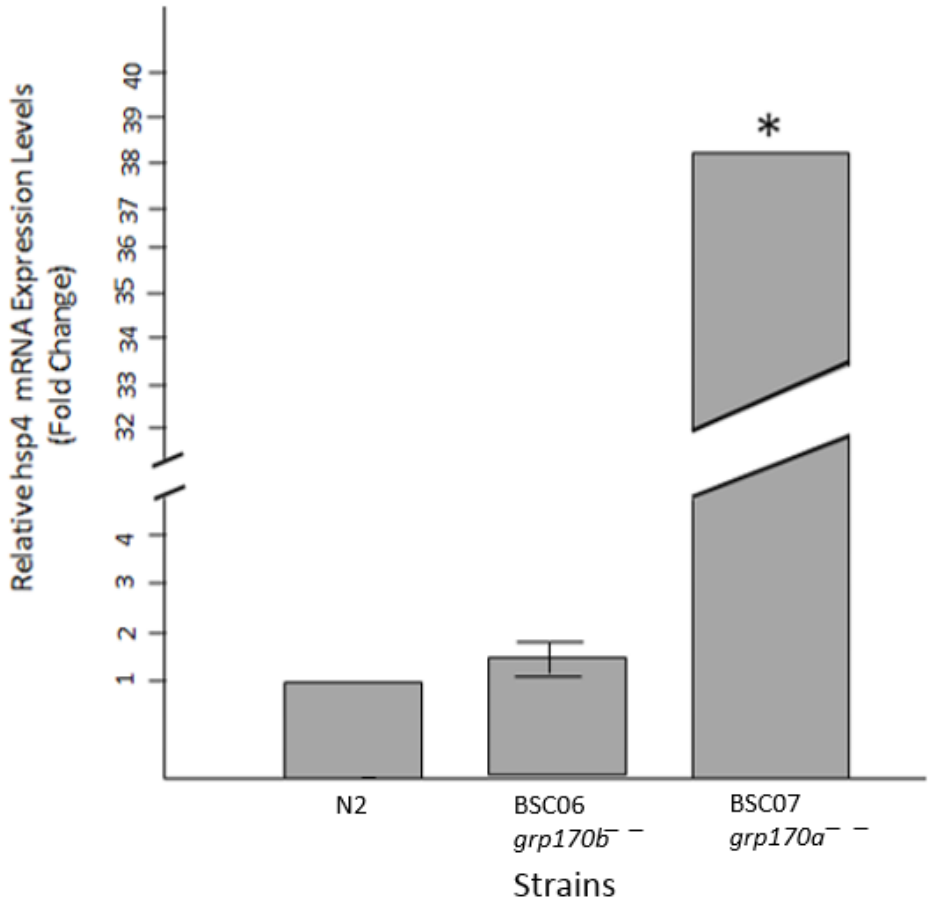

Figure 11: Analysis of induction *hsp*4 mRNA in response to loss of functional alleles at either *grp170* loci.

Expression levels of *hsp*4 mRNA were compared in the standard N2 laboratory strain was compared to the isogenic strains BSC06 (homozygous for a *grp*170b deletion allele) and BSC07 (homozygous for a *grp*170a deletion allele) using qPCR. Deletion alleles for *grp*170b (strain BSC06) did not significantly affect expression of *hsp*4 mRNA (P value of 0.064). Deletion alleles for *grp170a* did significantly increase expression of *hsp4* (P value of 0.0002). Asterisk above BSC07 bar represents a large variance of 71.02. (Anova and a tukey post hoc)

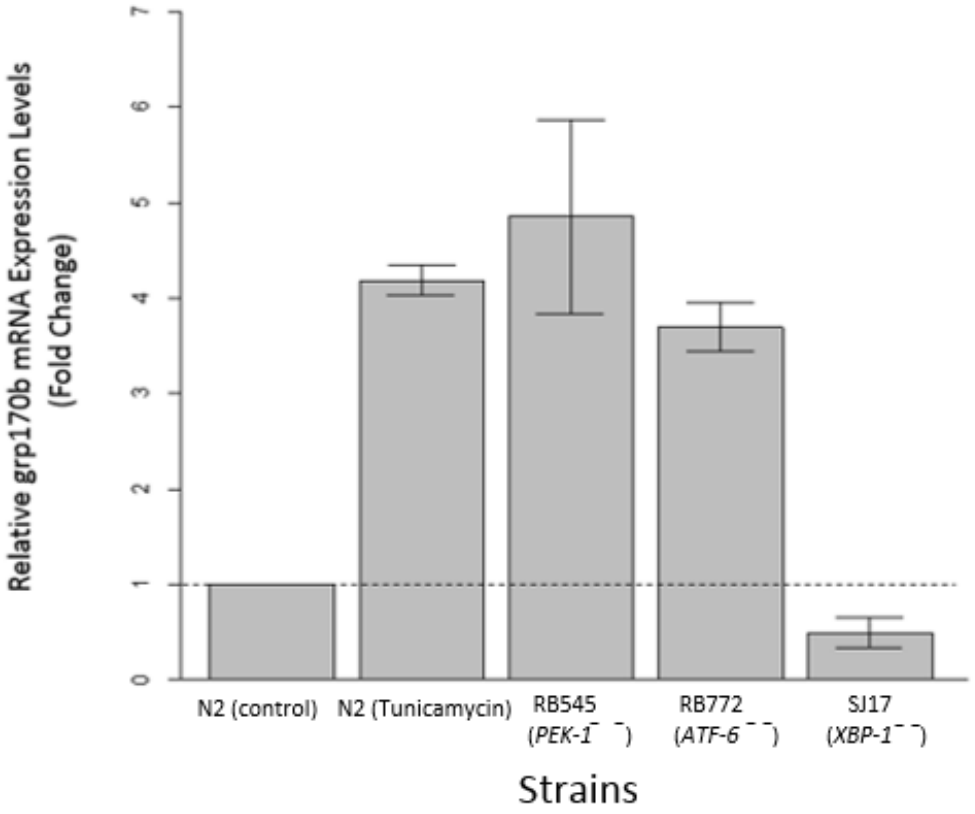

Figure 12 Analysis of UPR signal transduction pathway responsible for stress induction of *grp170b*.

A qPCR assay was used to compare relative mRNA levels in tunicamycin versus control N2, RB545, RB772 and SJ17 worms. Solid bars with the exception of the N2 control represents populations treated with tunicamycin. RB545 mutants are deficient for PEK-1. RB772 mutants are deficient for ATF6. SJ17 mutants are deficient for XBP-1. Dotted line represents control mRNA expression levels. An anova and a tukey post hoc test was used to compare each of the mutant strains to the N2 tunicamycin treated worms. The p values are as follows N2 (tunicamycin) to RB545 was 0.85, N2 (tunicamycin) to RB772 was 0.94 and N2 (tunicamycin) to SJ17 0.002. The p value are as followed between N2 control and the following SJ17 0.93, N2 treated 0.006, RB545 0.001 and RB772 0.018.

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