Determining the Enzymatic Pathway of the Stress Reaction in *Caenorhabditis elegans*

by

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Determining the Enzymatic Pathway of the Stress Reaction in *Caenorhabditis elegans*

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Abstract

Stress pathways are ubiquitous among organisms due to their immense importance in responding to environmental changes. Stress response pathway failure has been implicated in issues ranging from spontaneous fetal abortion to myriad cancers. This experiment attempts to determine the components comprising the ubiquitin stress response of *Caenorhabditis elegans* due to its homologous nature to the human pathway. Gene knockdowns were done for each enzymatic step of the pathway as well as a lifespan study to determine other related functions of these enzymes. The E2 was determined to be the *ubc-18* enzyme, the E3 is still unknown, and the lack of *ubc-18* did not have a significant effect on the lifespans of the worms. This experiment yielded one more component of the stress pathway and, while the lack of the enzyme does not influence life after hatching, the loss of the enzyme may cause problems during development that could become evident later.

Table of Contents

List of Tables

List of Figures

Definition of Terms[1]

Antibody Staining—A technique used to determine whether a specific protein is present in a sample by introducing antibodies into the sample that bind to the antigen (protein) and exploiting this to determine if and where the protein is present within the sample.

Degradation—The process by which multiple ubiquitin molecules are attached to a targeted substrate in order to be broken down by a proteasome.

E1—The first enzyme in the three step pathway of degradation by ubiquitination. This enzyme is responsible for recruiting and activating ubiquitin as well as determining the protein in need of degradation.

E2—The second enzyme in the three step pathway of degradation by ubiquitination. This enzyme is known as the ubiquitin conjugating enzyme (UBC), as it attaches ubiquitin molecules to targeted substrates so that they can be degraded.

E3—The third enzyme in the three step degradation process by ubiquitination. These are known as ubiquitin ligases; they are responsible for recruiting a ubiquitin rich E2 and recognizing a protein substrate so that ubiquitin can be attached to the substrate and the substrate can be degraded by a proteasome.

L4440—A strain of *Escherichia coli* that *Caenorhabditis elegans* consumes when conducting RNAi experiments that alter the physiology of the worms. This acts as a control as it has undergone the same treatment as other RNAi bacteria but is made to have no effect upon the functioning of the worm itself.

LN130—A *C. elegans* worm strain that has a green fluorescent protein (GFP) tag on its ubiquitin and an mCherry marker on its histones so that DNA will fluoresce under a fluorescent microscope.

N2—A wild-type *C. elegans* strain that is used as a control in lifespan experiments.

OP50—A strain of *Escherichia coli* that *Caenorhabditis elegans* consumes normally, under no experimental conditions.

Proteasome—A protein complex functioning to break down unneeded or damaged proteins, it is responsible for the dismantling of the ubiquitinated substrates at the conclusion of the three enzyme stress pathway.

RNAi—RNA interference is the experimental procedure that blocks the production of a specific enzyme in a *C. elegans* nematode by introducing double stranded RNA into the organism that subsequently attaches to a specific portion of the DNA and does not allow translation to take place, which stops that specific enzyme from ever being synthesized. This is accomplished by introducing a vector into the food source of the worms: *Escherichia coli* (*E. coli*). A vector is a strand of DNA that is incorporated into the DNA of the host, *E. coli* in this case. When the worms eat the bacteria, the DNA gets into their cells and prevents the production of the specific enzyme that was being targeted by the RNA molecule.

Ring-Between-Ring (RBR) E3s—An RBR E3 is a class of E3-ligases which contain a highly conserved catalytic component comprising a RING1, an in-between RING, and a RING2 domain. The RING domain specifies which E2 and E3 can interact with.

HECT E3s—These E3s get there name because they have the same protein domain, or specific protein sequence, as another molecule; the name is an acronym for '**H**omologous to the **E**6-**A**P **C**arboxyl **T**erminus'. E6-AP is the ubiquitin ligase present in humans.

Introduction

Stress is an integral part of life for all organisms; this fact has led to the development of many stress responses within the cells of these organisms so that homeostasis can be maintained even under the most strenuous conditions. This allows for the mitigation or circumvention of tissue damage, sometimes even allowing for the evasion of death. For example, some bacteria such as *Escherichia coli* are able to recover from near death due to ultraviolet light by activating antioxidant genes to combat reactive oxygen species present after UV exposure[2]. Heat shock protein synthesis is another form of stress response that is highly conserved among species; heat shock proteins function to prevent the misfolding of proteins when an organism is under stress, so that their cells continue to function properly[3]. Osmotic stress is yet another form of stress that organisms have to cope with, particularly those organisms that are directly exposed to varying levels of salt in the environment, such as fish, aquatic plants, or bacteria. However, all organisms have the ability to respond to changes in osmolarity[4]. This stress response induced by changes in environmental osmolarity levels, as well as other types of stressors, is present in *Caenorhabditis elegans*; by determining this response in these organisms, an equivalent response will be easier to find in more complex organisms like humans, as this stress pathway is very conserved among organisms and has a high homology to the human counterpart, which includes the multiple enzyme steps, the occurrence of polyubiquitination, degradation by a proteasome, *etc.*[5].

The aforementioned osmolarity response by *C. elegans* is a stress response involving a molecule known as ubiquitin. Ubiquitin is responsible for attaching to a protein and signaling a protein complex called a proteasome to degrade that protein[6]. Ubiquitin does

not simply attach to a protein in need of degradation; however, it must go through a pathway in order to attach to the substrate in need of degradation[7] (Figure 1). This ubiquitination stress pathway is composed of three stages, involving at least one enzyme at each stage[8]. The three enzymes at each of these stages are known as the E1, E2, or E3, which is nomenclature used to denote the sequence of events; E1 meaning the first enzyme in the pathway, E2, second, and so forth. The first enzyme (E1) is responsible for recruitment and activation of the ubiquitin molecule, as well as targeting the molecule that needs to be degraded. The second enzyme is a ubiquitin conjugating enzyme, or UBC. It is responsible for removing ubiquitin molecules from E1s, recruiting an E3 molecule, and then transferring the ubiquitin molecules either to that E3 molecule or the targeted substrate, until enough ubiquitin molecules are attached to the substrate that it can be degraded by a proteasome. The third enzyme, or E3, is known as the ubiquitin ligase. It is responsible for attaching to the substrate that is being targeted for degradation and may aid in the transfer of ubiquitin molecules to the substrate itself. Once a polyubiquitin chain is attached to a substrate, it is degraded by a proteasome[6]. This process occurs on the cellular level; the model organism *C. elegans* is very useful in this regard, as the effects of blocking specific genes can be easily accomplished.

The only E1 present in the *C. elegans* ubiquitin pathway is known as UBA-1[9], so the identity of this did not need to be confirmed. There are many more possibilities with the E2: 22 UBCs, as well as 3 additional ubiquitin E2 variants that lack a specific cysteine residue[7], and an additional 3 E2-like enzymes[10]. By utilizing a process of gene blocking called RNAi, the production of specific enzymes are blocked by inhibiting gene expression in order to elucidate the exact components of the ubiquitin pathway. If the

enzymes are unable to be produced, the spheres should cease to be present if the enzymes are responsible for the creation of those spheres. Through the process of gene expression inhibition, the exact enzymes responsible for the formation of the spheres can be determined; this information can be used to further illustrate the exact functions of these enzymes and whether they significantly influence any other cellular processes within the worms. The same process can be used to determine a possible E3. There are 182 E3s in total[7], but the identity of the E2 can be used in order to determine any possible interactions between E2 and E3 and possibly reduce the number of candidates for the E3[11].

Fluorescent microscopy is one technique used to determine whether ubiquitin is the protein responsible for degrading proteins when a cell becomes stressed. By tagging ubiquitin with green fluorescent protein (GFP), the localization of ubiquitin can be witnessed so that it can be determined whether the ubiquitin molecules are responsible for the spheres in question. When a cell is stressed, it will experience an increase in fluorescence, indicating an increase in ubiquitination, which would mean that there is more protein degradation occurring. Therefore, the intensity of fluorescence will indicate whether a stressed worm is experiencing more or less ubiquitination, as well as the amount of protein degradation due to that stressor. This is accomplished in *C. elegans* with the LN130 worm strain. When stressed, gonadal cells exhibit unknown spheres within their nuclei (Figure 2); previous research in Dr. Lynn Boyd's lab points to ubiquitin as a major component of these spheres, leading to the hypothesis that these are areas of major protein degradation and can therefore be manipulated by restricting the enzymes necessary for the creation of the spheres. Many nuclear foci are known to occur

in other worm cells already, such as the *HSF-1* nuclear spheres that form after heat shock[12] or the Cajal bodies that contain myriad protein types found in very active cells[13], but these experiments will focus on determining the identities of the components of the unknown foci within the gonadal cell nuclei of stressed *C. elegans* nematodes (Figure 3).

For determining the identities of the enzymes responsible for sphere formation, initial fluorescent microscopy consists of using a less powerful microscope utilizing RNAi techniques and GFP tags. Once a suspected enzyme is found, a ubiquitin antibody stain needs to be done in order to reduce the likelihood of the spheres being artifacts of GFP or due to something other than the normal processes that occur inside the worms. The antibody stain will elucidate whether the genes are truly responsible for the creation of foci. With fluorescent screening, the foci are influenced according to the amounts of GFP present in the worm and the localization of that GFP. The antibody stain uses a ubiquitin antibody to specifically target and attach to ubiquitin proteins, so that the GFP system is circumvented and it is possible to see whether the differences in foci are actually due to a difference in ubiquitin and not merely the fluorescent capability of the GFP. N2 worms will be used so that it is clear that the foci are forming due to ubiquitin; since N2 worms do not contain GFP, any witnessed foci will be due to the ubiquitin-antibody complex, providing more evidence for the identity of the enzyme in question. Once the identity of the enzymes have been confirmed with some degree of certainty, behavioral and developmental studies can be conducted.

Not only are these enzymes involved in cellular responses, but certain mutations of these enzymes can alter the lifespans[14], behaviors[15], and other innate aspects of *C. elegans*. The enzymes responsible for sphere formation could give insight into whether

the degradation of proteins during cellular stress is necessary to keep an animal functioning and, if so, how removing the destruction process will affect the organism overall. If this affects the lifespans of certain cells, this type of knowledge could lead to the prevention of some cancers, as preventing the stress process from deteriorating could slow the multiplication of high-risk cells. For example, studies using *Saccharomyces cerevisiae*, or yeast, have found that osmotic stress that occurs during specific stages of the cell cycle can cause problems such as DNA rereplication[16], a process which has been implicated in the onset of cancer in some cases[17]. This problem is highly conserved in all eukaryotes and is therefore germane to humans as a possible cause of cancer treatment and prevention in the future. In regards to overall lifespan of an organism, research with oxidative stress on *C. elegans* has shown that the removal of the expression of some genes that protect against this type of damage can lead to a significant decrease in lifespan[18]; if this is true for this stress pathway as well, it could lead to more intense study of this pathway in humans in order to prevent the onset of cancer by ensuring that the stress pathway remains fully functional.

In order to accrue information about the impacts these enzymes have on a worm's lifespan, a lifespan experiment for each suspected enzyme was undertaken. The lifespan experiment will follow a set of worms from birth until death so that an average lifespan can be determined and a comparison between stressed and unstressed worms can be made. This will elucidate whether the different treatment groups have considerably different lifespans or whether blocking the stress pathway enzymes has no effect on the lifespan. Information on brood size will determine whether a significant difference in egg laying and hatching exists between the treatment groups as well. A brood study

determines whether stressing the worms and inducing spheres in the gonadal nuclei has an effect on their progeny and whether it will affect egg laying rates. Number of progeny, development of those individuals, and the amount of eggs laid will determine whether a difference exists between all of the treatment groups.

Because the stress process in worms is homologous to that of humans, determining what enzymes are involved in worm responses may shed light on how humans react under similar circumstances[5]. As mentioned in the preceding paragraphs, osmotic stress has been implicated in the onset of some cells during specific phases of the cell cycle, prevention of which may lead to the decrease in cases of some types of cancer. This study deals mainly with stress to the gonadal tissue and developing embryos, so it may therefore be more appropriate to look at the effects osmotic stress can have upon development and gonadal tissue specifically. Gonad cells and the embryonic stages are the early periods in the worms' development that are very sensitive to external influence and can lead to numerous problems if something like osmotic homeostasis is not maintained. Mammal embryos are sensitive in much the same ways and as such they must be kept in stable environments as well, due to the fact that vast changes in osmolarity can have detrimental effects on many fetal processes. Examples of processes which can be irreparably damaged if osmolarity is not kept in check include the development of the blood brain barrier[19], or the maintenance of the fetal heartbeat[20], problems which, if not corrected immediately, can lead to developmental problems or spontaneous abortion[21]. Understanding stress may be very advantageous for humanity, and this research provides another clue to the human reaction to stress.

Thesis Statement

The purpose of this experiment is to elucidate the second and third enzymes in the ubiquitination process that gonadal cells within *C. elegans* undergo when immersed in a stressful environment. Also, this study will attempt to illuminate the effects that these enzymes have on the overall lifespan of worms and to determine whether blocking its production will increase or decrease a worm's lifespan and amount of progeny.

Methodology

a. E2 Assay

A technique called RNA interference (RNAi) is used to alter the production of specific enzymes so that they are no longer able to be synthesized and carry out their function within the ubiquitination pathway in the worm. Worms that are in the L1 stage, meaning that they have just hatched, are selected and placed on Petri plates containing *Escherichia coli* RNAi bacteria. The bacteria present on these plates have been transformed so that they incorporate and synthesize a vector; this vector is composed of genetic material containing a segment that blocks enzymes by introducing double stranded RNA into the worms' cells as well as a segment with antibiotic resistance so that the bacteria containing the vector are the only ones able to survive on the RNAi plates. The vector does not affect the bacteria, but the bacterial cells replicate the vector and produce double stranded RNA (dsRNA). When eaten by a worm, this dsRNA will pervade the worm's body and block the specific sequence that was present within the vector of the bacteria. Specific mRNAs are able to be targeted due to the specificity of the dsRNA sequence, so that only the desired gene products are knocked down and their enzymes are rendered unable to be synthesized[22]. When these enzymes are knocked down, their function within

the worms can be elucidated by comparing these experimental worms to worms fed non-gene-inhibiting bacteria. In order to sustain populations of worms, they are simply grown on nematode growth media plates with OP50 bacteria as a food source. However, when undergoing an RNAi experiment, the worms are grown on smaller plates with media that contains antibiotics so that bacteria that do not contain the vector are not able to survive, ensuring that the worms cannot eat non-enzyme blocking bacteria. About 30 L1 worms are taken from a starved plate and placed an on RNAi plate in order to ensure that they are only taking up the RNAi bacteria. These worms are grown on the RNAi bacterial lawns until they reach the L4 stage, at which time 10 of them are moved to a new plate containing the same type of bacteria; these worms will be the worms stressed and observed for the presence of spheres. Moving them to a new plate ensures that all the experimental worms are young adults, so that age is not a factor in the presence or absence of spheres.

To analyze the effects of the RNAi, a quantifiable method to view the effects must be available; this is accomplished through fluorescent microscopy. LN130 is a worm strain that possesses a green fluorescent protein (GFP) tag on the ubiquitin, allowing live, stressed worms to be viewed and compared so that it is possible to determine the effect that RNAi is having on the worms. The LN130 strain also possesses an mCherry tag on the histones of a cell, allowing for the location of the DNA to be determined as well.

The stress procedure involves placing 10 worms in a solution of a 500 mM NaCl and water solution for 30 minutes, then immediately imaging them on a florescent microscope. When compared to L4440 vector worms, or those consuming bacteria that do not block any sequences of genes, the worms that fail to form spheres in response to

stress should be the worms that have had the enzyme in question blocked. This is used as a control to compare the effects that any non-control vector may have upon the worms in terms of sphere formation. In this way, the enzyme responsible for sphere formation will be elucidated when sphere formation fails to occur.

b. Antibody Staining

An antibody stain was done to determine whether the spheres were actually ceasing to coalesce in the E2 enzyme knockdown worms or if the effect was merely an artifact of the GFP presence. An antibody stain involves breaking open the worm and allowing exposed antibodies to be targeted in order to circumvent the introduction of protein tags in order to observe spheres, or the lack thereof. This type of procedure uses N2, or wild type, worms in order to image worms as they are normally, without human interactions into their physiology; since N2 worms do not contain GFP, any witnessed foci will be due to the ubiquitin-antibody complex, providing more evidence for the identity of the enzyme in question.

In accordance with the antibody procedures normally conducted on *C. elegans*[23], this antibody stain consisted of taking young adult worms, "freeze-cracking" open their cuticles in order to directly interact with their endogenous proteins, fixing them in methanol so that the original locations and conformations of the antigens are preserved within the specimen, and introducing the primary and secondary antibodies into the specimen so that the antigens can be located and observed by way of fluorescent microscopy. The primary antibody used was a ubiquitin antibody from Santa Cruz, P4D1 (sc-8017), used at a block to antibody concentration of 1:200; it was raised in a mouse. The secondary was also used at a 1:200 concentration and it was a goat anti mouse

antibody; this means that the mouse antibody was injected into a goat so that the goat produced antibodies that would specifically bind to the antibodies of the mouse. The secondary also has a fluorophore attached to it. In this way, the secondary antibody will bind to the primary antibody and also emit light at a frequency that can be viewed on a fluorescent microscope, so that ultimately the location of the ubiquitin can be determined.

c. Lifespan

Wild type, or N2, worms can be used in order to determine the normal lifespan of these worms and can then be compared to an experimental group of worms in order to determine if a difference exists between normal lifespan and the lifespans of the experimental worms. In order to carry out the lifespan test, 80 L4 worms per trial, or 320 total worms, were picked as L1s and put on RNAi plates. There were four separate trials: stressed worms fed L4440 bacteria, unstressed worms fed L4440 bacteria, stressed worms fed E2 bacteria, and unstressed worms fed E2 bacteria. The only difference between the L4440 and E2 bacteria is the double stranded RNA present in the E2 bacteria that blocks production of an enzyme in the worms; all else is exactly the same. Worms were taken from each group and placed on either a watch glass containing a 500 mM NaCl and water solution for the stressed worms or M9 for the unstressed ones; the worms were subjected to their treatment for sixty minutes. These 50 were further split into groups of 10, so that there were 10 worms per plate in order to make counting easier. Each day they were moved over to a new plate until they had stopped laying eggs, at which point they were kept on the same plate until food became scarce and they required a new plate. Every day they were counted and the dead were recorded and then discarded. Their living status was determined by whether they moved due to direct or indirect

stimulation with a pick. If worms failed to move after having the agar around them tapped or being directly prodded, then they were classified as dead and removed from the petri plate.

d. E3 Assay

To determine the possible E3 in the pathway, worms were subject to RNAi once again, in the same manner as the E2 assay: worms were placed on RNAi plates that had been seeded with lawns of RNAi bacteria that block production of a specific enzyme, stressed with a 500 mM NaCl and water solution for 30 minutes, then imaged on a fluorescent microscope.

e. E3 Confirmation

To determine whether the E3s tested did indeed code for the enzymes desired to be knocked down, a minipreparation was done according to the instructions in the Qiagen Miniprep Kit[24]. This involves growing up colonies of bacteria, lysing the bacteria in order to obtain the inner components, purifying the mixture to obtain pure vector that had been within them, and running these samples through a polymerase chain reaction (PCR) machine alongside primers in order to amplify the acquired sequence. After these sequences were acquired, a computer program named Edit Seq was used to compare the derived sequences with the database sequences available on Wormbase[25], a website devoted to *C. elegans* genes and other related information. If they did not match up with the expected sequences, they were then run through NCBI's nucleotide blast database[26], a database that compares any entered sequences with others in the database and delivers the gene possibilities in order of identity likelihood.

Results

a. E2 Assay

After subjecting the worms to RNAi and imaging them on a fluorescent microscope, the images were collected and analyzed. Gonadal cells were counted as sphere forming if it was evident that the nuclei of the cells contained spheres (Figure 3 A) and deemed non-sphere forming if they failed to exemplify spheres whatsoever (Figure 3 B). Fifty gonad cells were analyzed for each treatment. Worms that were unable to synthesize the *ubc-18* enzyme did not form spheres when stressed (Figure 3 B), while the vector control worms, or those fed unaltered, L4440 *E. coli,* formed foci of localized ubiquitin in their nuclei (Figure 3 A). Using a one-way ANOVA, the 19 genes out of 22, as well as the aforementioned 3 E2-like genes and the 3 E2 variants for a total of 25 genes, were compared with the vector L4440 *E. coli* frequency of sphere formation (Table 1). The *ubc-18* gene was the only gene found to cause significant reduction of sphere formation when worms were fed the *ubc-18* inhibiting bacteria, forming spheres in approximately 16% of nuclei as opposed to an overwhelming majority, 83 %, of vector nuclei forming foci ($p<.0001$) (Figure 4).

b. Antibody Stain

Since only the *ubc-18* gene seemed to be responsible for the synthesis of the enzyme responsible for sphere production, an antibody test was done to make sure it was not caused by an artifact of the GFP tagging. The statistical analysis for the antibody portions of the experiment consisted of taking pictures and quantifying the number of gonad cells that contained foci. Gonad cells were categorized according to whether they exhibited foci or not, similarly to the E2 assay above. The quantification of the pictures consisted

of doing a one-way ANOVA test. A significant number of cells failed to form spheres in the antibody stains for the *ubc-18* knockdown worms ($p<.0001$) (Figure 5). This is further proof that *ubc-18* is the second enzyme in this pathway.

c. Lifespan

The effects of *ubc-18* gene blockage was done in order to determine if there was any detectable difference in the length of a worm's life without the enzyme that forms spheres in response to stress. There were no significant differences between any of the lifespan curves according to the Log-rank (Mantel-Cox) test that compares lifespan curves (Figure 6). The average lifespan of the *ubc-18* group did not significantly differ from the lifespan of N2 group, regardless of the presence of a stressor. The median survival for each of the treatments were as follows: 13 days survived for both L4440 vector stressed and unstressed and 14 days for both *ubc-18* stressed and unstressed.

d. E3 Assay

Existing research pointing to possible interactions between E2s and E3s suggests that the *ubc-18* enzyme is most often associated with Ring-Between-Ring (RBR) and HECT E3 enzymes[11] and therefore the search for the E3 will begin with these associated enzymes. There are 182 E3s in total[7]; of those, there are 9 HECT[7] and 10 RBR[27] E3s in *C. elegans*. The E3 assay was done on 25 different E3s including all the E3s from the HECT and RBR list, as well as others that appear closely related to the E3s listed (Table 2). The statistical analysis for this experiment consisted of taking pictures and quantifying the number of gonad cells that contained foci. The quantification of the pictures between trials was compared using a one-way ANOVA test. Gonad cells were categorized according to whether they exhibited foci or not. Ten pictures were taken for

each strain of bacteria, with strains exhibiting less than 70% foci formation being re-examined in order to determine if their enzymes were possible candidates for the stress pathway. There were no strains that differed significantly in terms of sphere formation when compared to the L4440 vector.

e. E3 Confirmation

The results of the E3 confirmation are in the E3 Table in the Appendices section (Table 2). Eleven of the E3s were successfully confirmed to be correctly identified originally. Five of the E3s were unable to be identified on matchup but were subsequently identified when compared to sequences in the government BLAST website[26]. Nine of the genes have yet to be sequenced but will be sequenced and catalogued eventually.

Discussion

The goal of this series of experiments was to determine the enzymes responsible for the ubiquitination pathway and also to ascertain the effects these may have on the worms' lifespan and behavior if the worm lacks these enzymes. As stated earlier, the ubiquitin pathway consists of three enzymes numbered according to their position in the pathway. Since there exists only one E1, the identity of the E1 in this pathway was easily determined to be the *UBA-1* enzyme[9]. The *ubc-18* enzyme has been determined to be responsible for acting as the E2 in this ubiquitination pathway according to all the data obtained to date. The *ubc-18* gene has a known role in pharyngeal development[28], but its involvement in foci formation during gonadal cellular stress was previously unknown. According to the results so far, the inability to form these spheres apparently has little effect on overall lifespan for the worms. Other studies have shown that stress can

influence lifespan in surprising ways, such as extending lifespan when mitochondria are under stress[29], but the *ubc-18* gene has not been linked to any lifespan hindrance or augmentation thus far. The lack of this pharyngeal development gene did not seem to impact the worms as they matured from L1 to adult stages, but this may become much more evident when the parent organism ingests the *ubc-18* gene inhibiting bacteria and their progeny are observed from hatching through to adulthood. Therefore, a future study for this gene will be to conduct a brood study to see whether the blockage of this gene and the introduction of stress will have a statistically identifiable effect between unstressed and L4440 vector cohorts.

The confirmation of *ubc-18* has elucidated one more piece of the puzzle, but the E3 gene is still missing from the stress pathway. Although initially it seemed like the promising E3s were running out, the E3 confirmation has led to the idea that it could still be one of these HECT or RBR E3s that has been misattributed or one that has failed to be elucidated through sequencing so far. Therefore, these will be acquired and tested as soon as possible in order to illuminate the E3 in this pathway or eliminate it as a suspect. However, even acquiring the correct E3 sequences and conducting RNAi with the correct bacteria may fail again. Because of this, the scope of the E3 may need to be widened to include other E3s that had not been previously tested, such as ring genes[7] and other genes that are necessary for other stress pathways. Eventually, all 182 E3s[7] may need to be exhausted in order to determine the third step in the process, or something even more drastic such as combinations on a mass scale.

Another aspect that has as of yet gone untested is whether this phenomenon is present in other organisms. Initial tests have been conducted so far on the possibility of

witnessing ubiquitin and proteasome localization utilizing antibodies in the model organism *Danio rerio* (zebrafish). These fish have been used extensively in drug treatments applicable to humans[30] as well as myriad other experiments that show they are valuable indicators of how human cells react under similar conditions. So far only very limited amounts of these antibodies have been able to permeate the cells of the zebrafish, but further testing will be done in order to elucidate this connection and establish whether a cross-species similarity in this response exists. If zebrafish do exhibit these spheres under stress, it will lend even more credence to the idea that humans also have an identical or very similar response under stress and also elucidate the other functions that these may carry out within the human body.

Because the stress process in worms is homologous to that of all eukaryotes, as well as humans, determining the components of the pathway as well as their contribution to other cellular processes will be invaluable in treatments from fetal distress to various forms of cancer. Osmotic imbalances are the cause of many problems for developing humans, as an upset concentration of solutes can mean the difference between a consistent, life-giving heartbeat and the inability to create osmotic gradient to cause even one beat of the heart[20]. The inability to form a blood brain barrier due to an osmotic disparity is also a problem, as it spells certain death for humans exposed to pathogens in their blood that are able to infiltrate the brain so easily[19]. Under normal circumstances, the pathogen would be unable to cross the barrier and be destroyed as the blood circulates in the circulatory system, but is able to cause lethal harm since the brain remains agonizingly open to attack. Not only afflicting the young, this stress pathway is important to understand since osmotic imbalances can also cause DNA replication issues if they occur at specific points

in the cell cycle[16], which could ultimately cause cancer if uncorrected[17]. Any amount of research into this stress response pathway will elucidate the intricacies involved in how cells respond to external osmotic stress, and hopefully will aid future research into treatments for common ailments afflicting those of all ages.

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Appendices

Enzyme	Gene Sequence	Usage
C17D12.5	C17D12.5	T
F52C6.12	F52C6.12	T
ubc-1	C35B1.1	T
ubc-2 (let-70)	M7.1	T
ubc-3	Y71G12B.15	T
ubc-6	D1022.1	T
ubc-7	F58A4.10	T
ubc-8	Y94H6A.6	T
ubc-9	F29B9.6	NT
ubc-12	R09B3.4	NT
ubc-13	Y54G2A.31	T
ubc-14	Y87G2A.9	T
ubc-15	Y110A2AR.2	T
ubc-16	Y54E5B.4	T
ubc-17	B0403.2	T
ubc-18	R01H2.6	T
ubc-19	Y69H2.6	T
ubc-20	F40G9.3	T
ubc-21	C06E2.3	T
ubc-22	C06E2.7	T
ubc-23	C28G1.1	T
ubc-24	F49E12.4	T
ubc-25	F25H2.8	T
ubc-26	Y110A2AM.3	NT
uev-1	F39B2.2	T
uev-2	F56D2.4	T
uev-3	F26H9.7	T
ufc-1	C40H1.6	T

Key
T=Tested
NT=Not Tested

Table 1: E2 List Used for Assay

This list contains all the E2 enzymes present within a *C. elegans* organism, as well as 3 E2-like enzymes and 3 additional E2 variants. The enzyme names are listed on the right, the gene sequences are listed in the middle, and their usage for RNAi in this experiment is indicated on the right. T indicates "tested," meaning that the enzymes with a T were used during the E2 assay, while NT means "not tested." The three enzymes designated NT were not tested because they were unavailable in the lab at the time of the assay.

Enzyme	Genes Sequence	Confirmation Similarity
ari-1	C27A12.8	99%
ARIHH1 ortholog	C27A12.6	99%
ARIHH1 ortholog	C27A12.7	99%
C17H11.6	C17H11.6	NS
eel-1	Y67D8C	NS
F14F9.3	F14F9.3	97%
F14F9.4	F14F9.4	100%
hecd-1	C34D4	95% similar to C42D4.12
hecw-1	F45H7	NS
ima-1	T19B10	98%
ire-1	C41C4	NS
nsy-1	F59A6	NS
oxi-1	Y39A1C.2	100%
pdr-1	K08E3	NS
RNF14 ortholog	F56D2.2	100%
sek-1	R03G5	NS
skn-1	T19E7	NS
T12E12.1	T12E12.1	NS
tag-349	Y73F8A.34	95% similar to Y73F8A.945
UBE3C ortholog	D2085.4	99%
wwp-1	Y65B4BR	99%
Y49F6B.9	Y49F6B.9	98% similar to T10D4
Y58A7A.3	Y58A7A.3	99% similar to Y75B7AL.2
Y58A7A.4	Y58A7A.4	96% similar to Y47D7A.3
Y92H12A.2	Y92H12A.2	98%

Key
NS=Not Sequenced

Table 2: E3 List Used for Assay and Confirmation

This list contains all of the HECT and RBR E3 enzymes present within a *C. elegans* organism that were tested during this assay. This list varies from the above table in that it does not contain an exhaustive list of E3s. The enzyme names are listed on the right, the gene sequences are listed in the middle, and their usage for RNAi in this experiment is indicated on the right alongside the gene sequencing data. NS indicates a gene that was "not sequenced" at the time due to unavailability and a number as a percentage means that the gene shares that percent similarity to the gene indicated. A number and percentage by itself indicates that the gene was correct and encoded for the expected enzyme, while a percentage next to a gene name indicates that the gene failed upon matchup with its expected gene but subsequently matched up upon being entered in to the BLAST database[26].

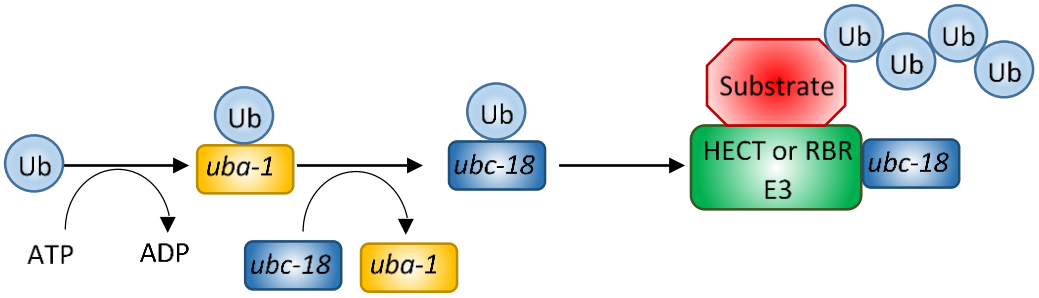


Figure 1: Enzymatic Steps of the Ubiquitination Pathway

A ubiquitin molecule interacts with three distinct enzymes as it proceeds to tag proteins for degradation. The first enzyme (*UBA-1*), or E1, is responsible for recruitment and activation of the ubiquitin molecule, as well as targeting the molecule that needs to be degraded. The second enzyme (*ubc-18*) is a ubiquitin conjugating enzyme, or UBC. It is responsible for removing ubiquitin molecules from E1s and transferring them to an E3 molecule, until enough ubiquitin molecules are attached to the substrate that it can be degraded by a proteasome. The third enzyme, or E3, is known as the ubiquitin ligase. It is responsible for attaching to the substrate that is being targeted for degradation and may aid in the transfer of ubiquitin molecules to the substrate itself. Once a polyubiquitin chain is attached to a substrate, it is degraded by a proteasome. This process occurs on the cellular level; the model organism *C. elegans* is very useful in this regard, as the effects of blocking specific genes can be easily accomplished. This experiment confirmed the E2's identity and has attempted to determine the E3, but this has been unable to be accomplished thus far.

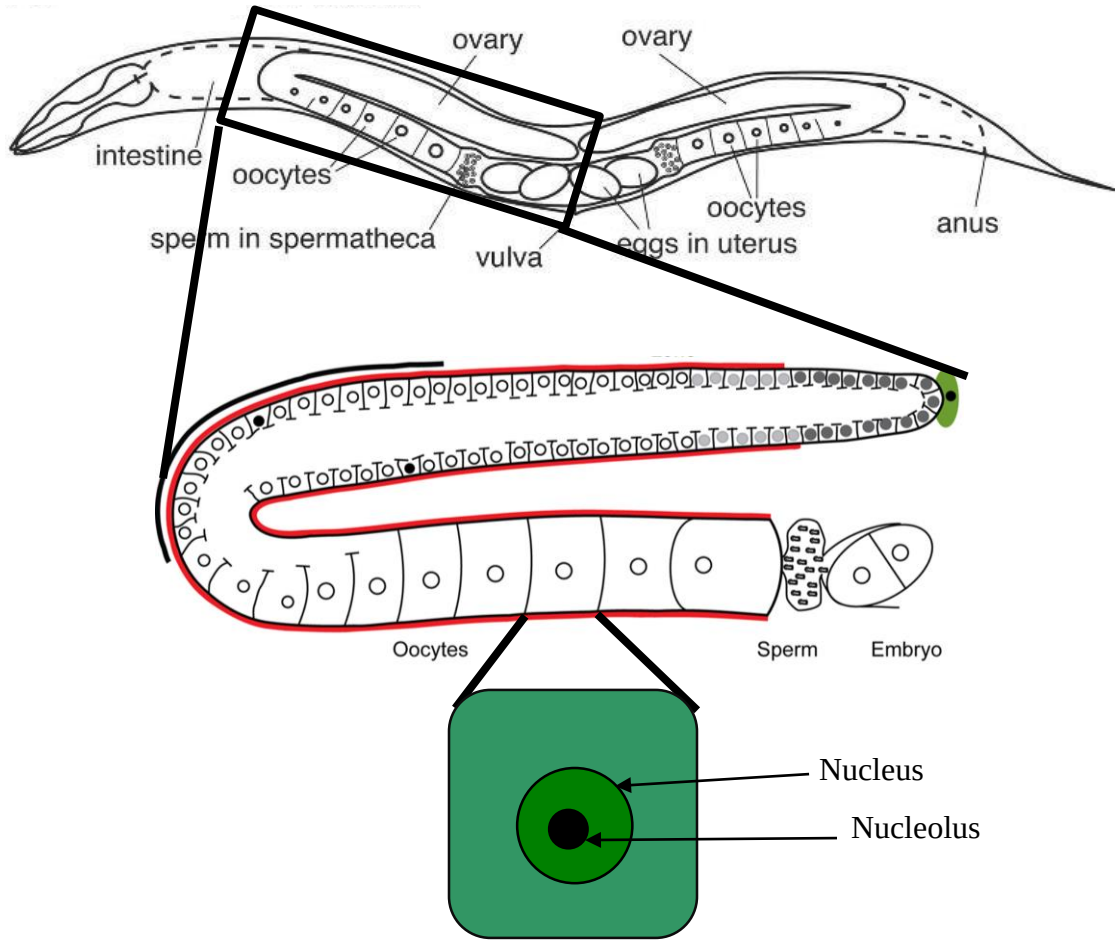


Figure 2: Points of Interest in *C. elegans*

A. Mature *C. elegans*: This is a basic overview of the hermaphroditic worm *C. elegans*, with a box around the ovary, as this was the portion of the worm studied in this experiment. Two ovaries are present in each worm, a distal and proximal gonad.

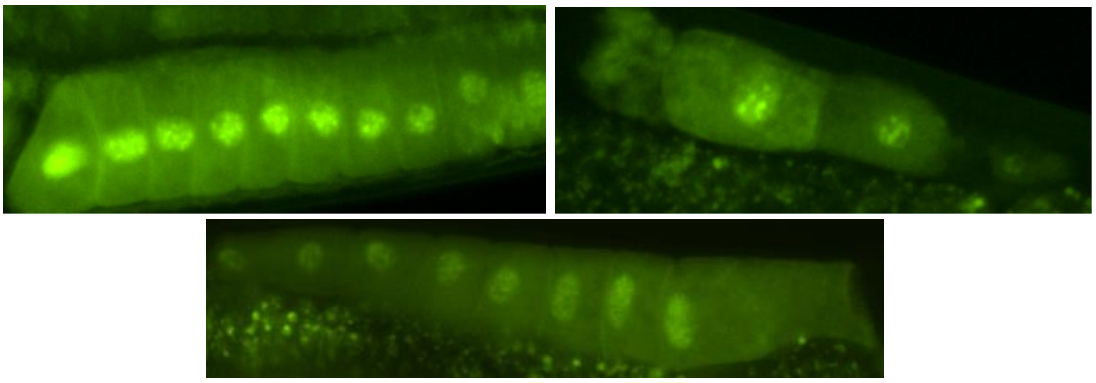
B. *C. elegans* Ovary: This more detailed view of the ovary shows that one end is filled with less mature eggs that slowly mature as they move toward the spermatheca in order to be fertilized. The GFP present in the LN130 strain of these worms allows stress experiments to be done and the effects of that stress to be witnessed.

C. Oocyte in Gonad: This is a basic representation of the more mature oocytes toward the end of the ovary, with an emphasis on the nucleus, as this is where sphere formation occurs when the worms become stressed.

Zarkower, D. 2006. Somatic sex determination. WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.84.1.

Gartner, A, *et al.* 2008. Germline survival and apoptosis. *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.145.1.

A (Vector):



B (*ubc-18*):

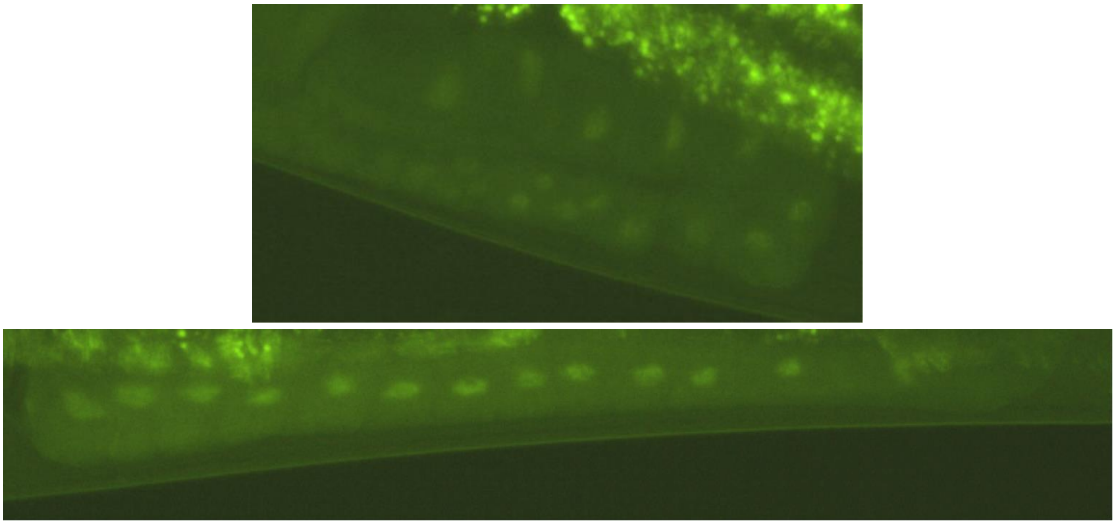


Figure 3: Gonad of NaCl Stressed Worms Fed *ubc-18* and Vector (L4440) Bacteria

Fluorescent microscopy was used to take images of *C. elegans* gonad cells after they had been stressed in a 500 mM NaCl and water solution after 30 minutes. The gonadal cells of the worms that were fed unaltered (L4440) bacteria (A) show distinct foci formation in the nuclei, which are spheres of ubiquitin forming to degrade proteins. A significant number of worms that were unable to synthesize the *ubc-18* enzyme did not form foci (B), as evidenced by the lack of spheres in the nuclei of those worms ($p<.0001$). This means that they were unable to form spheres of ubiquitin when under stress and potentially unable to degrade proteins in their nuclei.

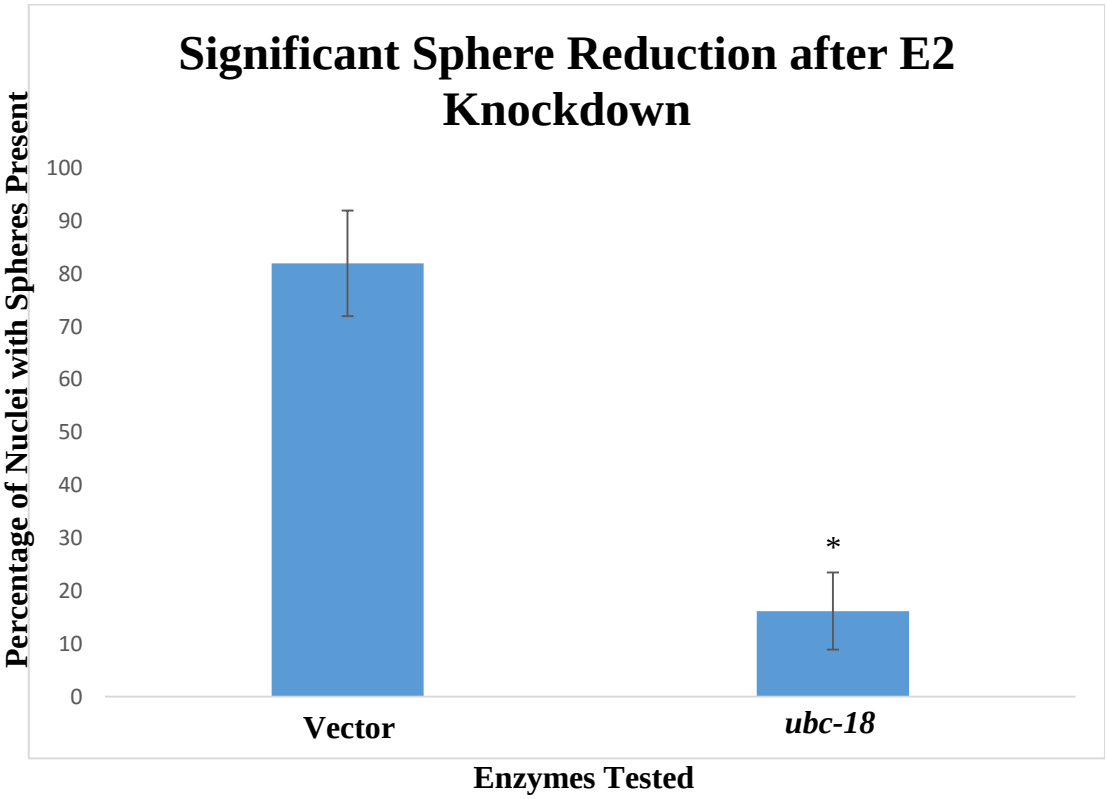


Figure 4: Graphical Comparison of Most Promising E2 Enzymes to the Control

This graph compares the number of gonad cells that contained spheres when their respective worms were stressed for 30 minutes using a 500 mM NaCl and water solution. The *ubc-18* gene is the only gene responsible for significant sphere inhibition, eliminating foci formation in all but 15% of gonad cells (.15 ± .07), while all other enzymes tested in the gene knockdown had > 70% sphere formation within gonad cells. L4440 control (vector) worms exhibited a wide ranging but nonetheless high amount of sphere formation at around 82% (.82 ± .09). The error bars represent the standard error for each treatment.

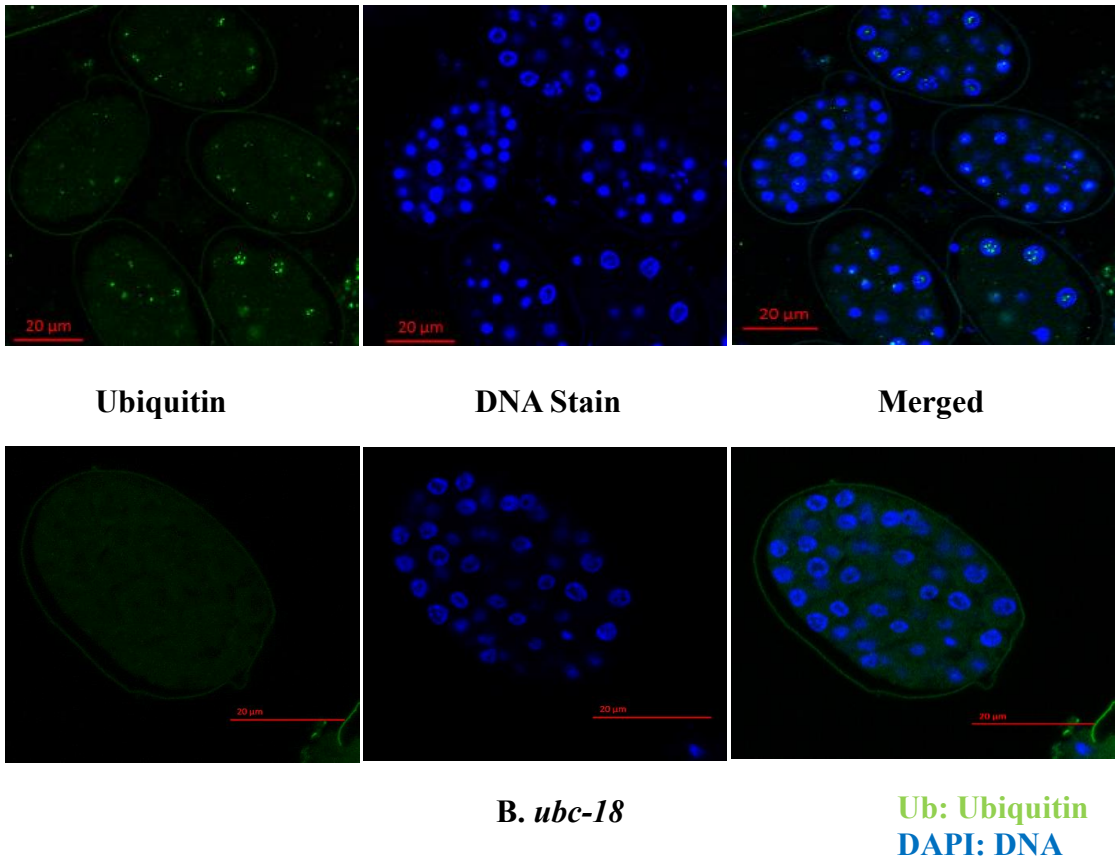


Figure 5: Eggs of NaCl Stressed Worms Fed *ubc-18* and Vector (L4440) Bacteria

Fluorescent microscopy by a confocal was used to take images of *C. elegans* gonad cells after they had been stressed in a 500mM NaCl and water for at least 30 minutes. This antibody stain used DAPI and ubiquitin antibodies to stain distinct parts of the cell: the blue fluorescence is a DNA stain, while the green is a ubiquitin stain. The individual cells of the eggs of the worms that were fed unaltered (L4440) bacteria (A) show distinct foci formation in the nuclei, which are the green foci surrounded by blue stained DNA. These green spheres are spheres of ubiquitin forming to degrade proteins, while the blue shows DNA, which is located within the nucleus. The worms that were unable to synthesize the *ubc-18* enzyme did not form foci (B), as evidenced by the lack of spheres in the nuclei of those worms. This means that they were unable to form spheres of ubiquitin when under stress and potentially unable to degrade proteins in their nuclei.

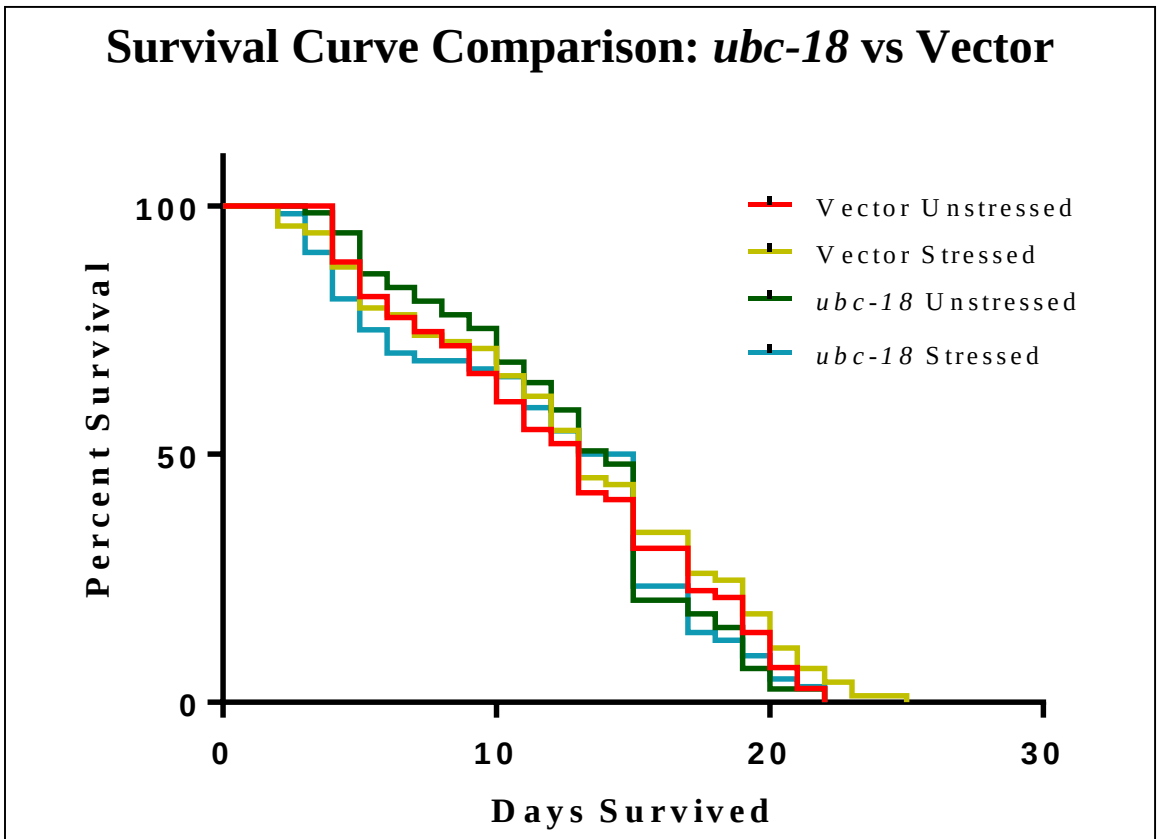


Figure 6: Survival Curve Comparing Stressed and Unstressed Treatment Groups

This survival curve compares *ubc-18* gene knockdown stressed and unstressed worms to L4440 control (vector) stressed and unstressed worms in order to determine whether stress or the inability to form these degradation spheres has a significant impact on lifespan. The data begins with day zero as the first day of adulthood and continues until the deaths of all individual has occurred. According to a Log-rank (Mantel-Cox) test, these curves are insignificantly different ($p = .5$), meaning that none of the groups has a longer or shorter lifespan when compared to the vector unstressed worms. The median lifespans for each treatment are as follows: 13 days survived for both vector stressed and unstressed and 14 days for both *ubc-18* stressed and unstressed.