Examining the Effects of Manipulating Chaperone-Mediated Autophagy on Stress-Induced Nuclear Granules (SINGs) within the Nuclei of Oocytes of *Caenorhabditis elegans*.

by
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A thesis presented to the Honors College of Middle Tennessee State University in partial fulfillment of the requirements for graduation from the University Honors College

Spring 2018

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Acknowledgements

I would like to thank Middle Tennessee State University, the College of Basic and Applied Sciences, the Biology department, and the University Honors College for allowing me the opportunity to participate in this Honors Research Thesis and for providing me the means with which to do so. From laboratory facilities, equipment, and supplies, to world-class faculty mentorship and oversight, so much has been given to me to help me learn and succeed. I am also immensely grateful for the financial provision that has been entrusted to me through scholarships from the Honors College, Biology department, and the Pre-Professional department. The Honors Transfer Fellowship, the Freeman P. Jordan, Jr. Biology Scholarship, and the Roberson-Beauregard Science Scholarship from these departments respectfully have enabled me to maximize my time and energy towards the Honors Thesis.

I would like to deeply thank Dr. Lynn Boyd, chair of the Biology Department. Her years of generous impartation of wisdom and knowledge have been invaluable and instrumental in setting me up for success. Her passion for biological science is infectious and has made an indelible mark upon how I approach science, medicine, and my forthcoming profession. I am thankful for how she adopted me into the heart of the Boyd Lab, introduced me to this project, and provided me the path with which to walk it out successfully. I am also thankful for how she initially encouraged me to become involved in Honors classes in the Honors College. I will be forever grateful for how she has championed my non-traditional career path and given me tremendous opportunities. I would not be where I am today if it were not for her and her mentorship.

I would like to thank the members of the Boyd Lab, namely Dr. Katherine Sampuda, Paola Molina, and Josh Evers for generously teaching and training me with such thoroughness and expertise. I am thankful for how they inspire our entire lab personnel with the research projects that they do and with how hard they work on them. I am also thankful for their kindness and willingness to tirelessly support me. There have been countless hours of training, answering of my questions, and providing me with assistance at the bench, and I am immensely grateful for it. I could not have completed (or even fully understood) this project without them. I would also like to thank Chelsea Campbell, James Hayes, Roseanna Durnell, and Michaela Langley for all of their consistent support and camaraderie during my tenure in the Boyd Lab. I am thankful for their assistance with training me and especially with helping me with all of the maintenance responsibilities. Everyone has been so willing to give me a helping hand, and I am extremely grateful. Also, I am thankful for all of the laughs along the way.

I would like to thank Ms. Judy Albakry, MTSU Honors College Advisor. I am extremely grateful for how she has supported me and gone out of her way to help me and advocate for me. The landscape of an undergraduate's academic career can often be as disorienting as a dark wilderness, but she has truly provided light for my path. I am thankful for all of her help with the Honors Thesis process and for how she has been such a strong source of encouragement and support along the way. She has truly helped me to go farther and faster than I could have gone on my own.

I would like to thank Dean Vile, Dr. Philip Phillips, and the University Honors College staff for allowing me to participate in the Honors Thesis and for overseeing the multiple steps of the process. I am thankful for the constructive feedback along the way.

I especially want to thank Dr. Phillips for including me in the Honors in Thailand study abroad course. One feature of that course that is pertinent to this thesis is that I was able to see my writing skills greatly improve under his instruction and mentorship. Dr. Phillips also opened my world to a much deeper level of objectivity and respect towards other cultures through the study abroad course and it has truly made an impact on how I respect and approach my career in science and medicine.

I would like to thank Dr. Jason Jessen for agreeing to be the second reader of this thesis and for contributing to its success. Additionally, my comprehension of this project has greatly increased as a result of sitting under his teaching in the Cell and Molecular Biology course this semester. I am extremely grateful for his tireless willingness to answer the myriad of questions that I have had about the material. I am very thankful to have had him as my professor.

I would like to thank Dr. John Dubois for introducing me to the Honors Thesis and for taking the time to encourage me to participate in it. I am thankful for all of the hours he has spent advising me along the way. I am thankful for how he has inspired me with his passion for research.

Abstract

Proteostasis is the cyclical protein quality control system of biological organisms. The cycle begins with protein synthesis, followed by specific regulated function within the cell and tissues, and then degradation unto the cycle repeating. Degradation allows for obsolete proteins (e.g. no longer used, damaged, or misfolded proteins) to be broken down into reusable amino acid and polypeptide subunits. This degradation process occurs through two main systems: 1. The ubiquitin-proteasome system; and 2. Lysosomes that utilize autophagy. One of the types of autophagy that the cell uses is called chaperone-mediated autophagy. The purpose of this study was to examine the effects of manipulating chaperone-mediated autophagy on stress-induced nuclear granules (SINGs) within the nuclei of the oocytes of *Caenorhabditis elegans.* Stress-Induced Nuclear Granules (SINGs) form within the nuclei of oocytes of *C. elegans* as a result of the accumulation of misfolded proteins in a suspected area of protein quality control. Ribonucleic acid interference (RNAi) was used to separately knock down the activity of two genes, *lmp-1* and *lmp-2* of lysosomes, which code for the receptor proteins LMP-1 and LMP-2 respectively in *C. elegans* (LAMP-1 and LAMP-2 in mammals and *Homo sapiens*). These proteins are responsible for binding to the chaperone-substrate misfolded protein complex and translocating it across the lysosomal membrane for subsequent degradation. With the translational activity reduced of either the *lmp-1* gene or the *lmp-2* gene, the process of lysosomal chaperone-mediated autophagy was potentially halted, thus causing a buildup of misfolded proteins in the cytosol. An increase of SING formation was then observed during salt stress conditions in the RNAi knockdown stressed models compared to the control models. Two possibilities for this

increase include: 1. Cytosolic misfolded proteins are potentially included in SING formation; or 2. An increase in cytosolic misfolded proteins potentially triggers a chaperone sink in the cytosol, which partially hinders normal chaperone function in the nucleus.

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Introduction

Proteostasis is maintained through a cyclical network of pathways and includes synthesis, regulation, degradation, and recycling of proteins. This organismal capability for inherent protein quality control is a vital area of biological research. While some aspects of proteostasis have been previously characterized, many of its finer details and anomalies remain unexplained. There is a wide array of implications that stem from a better understanding of proteostasis ranging from general biological science advancements to potential prevention and therapeutic methods for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease.

An individual organism's entire complement of proteins is referred to as its proteome. Proteostasis is the cyclical system that keeps the proteome regulated. Proteins undergo a cycle that begins with synthesis, followed by specific function within the cell and tissues, and then degradation unto the cycle repeating. Degradation allows for obsolete proteins (e.g. no longer used, damaged, or misfolded proteins) to be broken down into reusable polypeptide chain and amino acid subunits. The subunits of the degraded proteins can then be reused by the organism as component parts for entirely new proteins.

Environmental chemical stressors are among the many ways that proteins become misfolded. When proteins become misfolded, they become dysfunctional and potentially dangerous and toxic to the organism. This problem can escalate when hydrophobic regions of the amino acid sequences of misfolded proteins become inappropriately exposed and thus attract like hydrophobic regions of other misfolded proteins. This electrostatic attraction (non-covalent interaction) can often progress into large aggregates

of misfolded proteins. Cell damage and death can occur when the cell becomes unable to gainfully manage and repair its toxic concentration of misfolded proteins. Neuronal cell death due to protein aggregation is considered to be one of the best understood causes of neurodegenerative diseases such as Alzheimer's disease (Irvine *et al.* 2008). The proteostatic network of pathways serves as a system of quality control that aids an organism's cells and overall health, particularly when unexpected environmental changes occur with the organism that increase the prevalence of misfolded proteins.

Two of the main systems that help to identify and degrade these misfolded proteins are the ubiquitin-proteasome system and autophagy via lysosomes. Regarding the first system, ubiquitin is a post-translational protein tag that is applied to misfolded proteins causing them to then be targeted by proteasome. Proteasome, which is located all throughout the cell in both the nucleus and the cytoplasm, is a large, two-particle complex containing protease enzymes that degrade these misfolded proteins by a peptide bond-breaking chemical reaction known as proteolysis. Regarding the second system, lysosomes are cytoplasmic membrane-bound organelles that are full of a wide variety of catabolic enzymes responsible for the autophagy of many molecule types including proteins. Lysosomes employ three different forms of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. The specific systems that this study included in its examination were the ubiquitin-proteasome system and chaperone-mediated autophagy.

Chaperone-mediated autophagy is a process that occurs in the cytoplasm of the cell and involves several steps. First, a misfolded cytosolic protein (substrate) is recognized by and bound to a protein called a chaperone (Figure 1). The cell attempts to

recognize these misfolded proteins before the misfolded proteins aggregate and form large non-functional structures. Next, the chaperone-substrate complex targets the lysosome and delivers the misfolded protein (Swatek 2016). The lysosome has receptor proteins called LMP-1 and LMP-2 that bind to the misfolded protein and translocate it across the lysosomal membrane. Finally, once inside the lysosomal lumen, the enzymes of the lysosome degrade the misfolded protein into reusable amino acid and polypeptide chain subunits (Cuervo 2013).

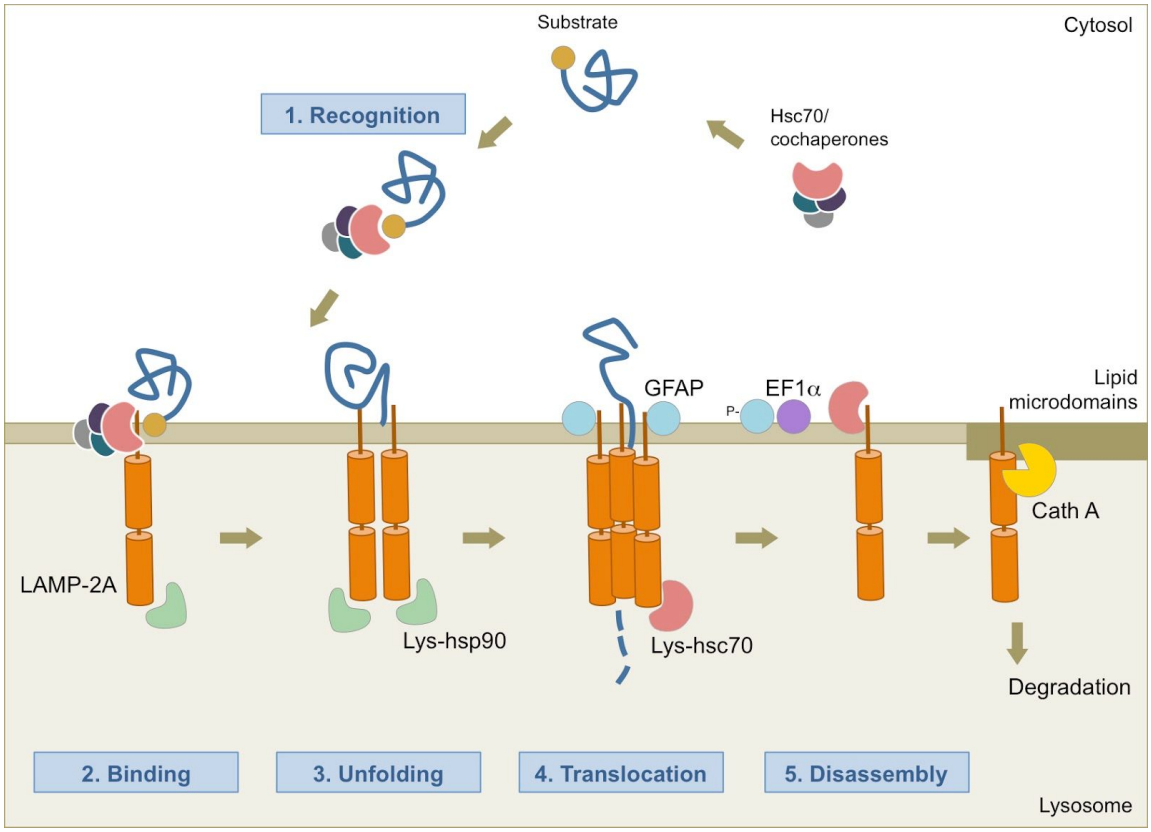


Figure 1 | Chaperone-Mediated Autophagy Process in Lysosome. 1. Recognition of cytosolic misfolded proteins (substrate) by chaperones. Binding of misfolded proteins to chaperones and trafficking to lysosome. 2. Binding of substrate-chaperone complex to lysosomal associated membrane proteins (LMP-1 and LMP-2 in *Caenorhabditis elegans*). 3. Substrate unfolding by membrane proteins. 4. Translocation of misfolded protein and degradation into subunits. 5. Disassembly of LMP-1 and LMP-2 (Cuervolab 2018).

In a recent discovery in April of 2017, researcher Katherine Sampuda, Ph.D., from the Boyd Lab, experimentally discovered Stress-Induced Nuclear Granules (SINGs) forming within the nuclei of oocytes of *Caenorhabditis elegans* as a result of the accumulation of misfolded proteins in a suspected area of protein quality control. The study showed how certain environmental stress scenarios consistently produced SINGs. When nematodes were subjected to environmental stressors such as a high salt (NaCl) concentration, sucrose, and an oxidative (H_2O_2) condition, the oocytes of the organisms presented visible (via microscopy) subnuclear bodies comprised of ubiquitin, proteasome, and the TIAR-2 protein. SINGs also form in early embryos and correlate with embryos not performing cell division and hatching. It is not yet fully known whether SINGs are comprised wholly of exclusively nuclear misfolded proteins, or if they also include contributions from misfolded proteins from the cytoplasm (Sampuda 2017).

This study utilized the organism *Caenorhabditis elegans* (Figure 2) for its control and experimental models. *C. elegans* is a free-living nematode roundworm (worm) and considered to be a model metazoan organism for the biological study of Eukaryotes because of its transparency, fixed number of cells, consistent cell development, ideally fast life cycle (3 days from egg to adult), and ability to reduce the activity of genes by use of ribonucleic acid interference (RNAi) (Corsi *et al.* 2015). Hatched larvae are 0.25mm in length and the adult organism is 1mm in size. The adult form is primarily hermaphroditic. In this study, data were collected from 3,470 distal oocytes across 117 worms.

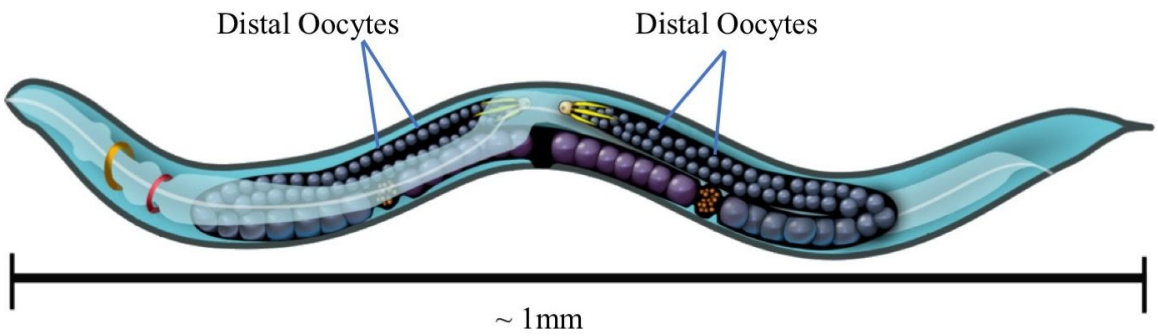


Figure 2 | Anatomical reference of *Caenorhabditis elegans.* Arrows showing distal oocytes in the gonad. Oocytes of the distal gonad were used in this study. Image reproduced with modifications (Schroeder 2015).

The growth, population maintenance, and experimentation of the LN151 (RPT-1::mCherry) strain of *Caenorhabditis elegans* was involved for this study. This transgenic strain has had its RPT-1 protein of proteasome genetically altered to include the mCherry fluorophore marker, which allows it to be visible under the fluorescent confocal microscope. RPT-1 is a regulatory base protein of proteasome, and since SINGs are partly comprised of proteasome, the marker allows for SING formation to be visualized and used as a factor when comparing experimental and control models.

RNAi is genetic technique that was used to reduce the activity of lysosome. RNAi consists of double-stranded RNA molecules that target and neutralize specific messenger RNA (mRNA) in order to inhibit translation or gene expression. The LN151 *Caenorhabditis elegans* strain was fed RNAi bacteria specific for the *lmp-1* and *lmp-2* genes, underwent a salt stress condition for 1 hour, and then was imaged on the fluorescent confocal microscope to quantitatively compare the number of nuclear oocyte SING formations with that of the control models.

The purpose of this study was to examine the effects of manipulating chaperone-mediated autophagy on stress-induced nuclear granules (SINGs) within the nuclei of the oocytes of *Caenorhabditis elegans*. RNAi (RNA interference) was used to

knock down the activity of the *lmp-1* and *lmp-2* genes that code for the LMP-1 and LMP-2 proteins respectively in *C. elegans*, a receptor protein of the lysosome responsible for binding to the chaperone-substrate misfolded protein complex and translocating it across the lysosomal membrane for subsequent degradation. With lysosomal gene activity reduced, the process of chaperone-mediated autophagy was then potentially halted and caused a potential buildup of misfolded proteins in the cytosol. The goal of this project was to observe if increasing the amount of cytosolic misfolded proteins would then increase SING formation.

Materials and Methods

The LN151 (RPT-1::mCherry) transgenic strain of *Caenorhabditis elegans* was acquired from the Boyd Lab and populated at 20ºC in Petri dishes. The Petri dishes were filled with Nematode Growth Medium agar (NGM) and seeded with the uracil auxotroph *E. coli* strain OP50 as a food source. Using a platinum wire pick, the worms were manually transferred to freshly seeded dishes every 2-3 days throughout the entire study to prevent starvation and to promote reproduction. The growth of the worms was multiplied across a series of dishes with their growth stages staggered in order to readily identify the proper growth stage (L4 stage) required for RNAi manipulation.

Control and experimental models utilized worms from the population and treated those worms with RNAi. RNAi was administered to the worms via plasmids transformed into HT115 bacteria. The RNAi bacteria were acquired from the -80ºC storage freezer in the Boyd Lab and transferred to tryptic soy agar dishes containing tetracycline and ampicillin (for quality control) for 24 hours until newly grown single colonies could be obtained. The single colonies were then populated into a sufficient worm food source by

transferring the colonies into tubes containing tryptic soy broth (with tetracycline and ampicillin) and aerating them in an orbital shaker at 37ºC for 15-16 hours to obtain the proper bacterial cell count density. The bacterial broths were then used to seed feeding dishes that contain NGM, ampicillin, and lactose. Control and experimental worms were then moved to their respective dishes to feed on the RNAi bacteria for 24 hours in order to sufficiently induce gene silencing. The control worms were fed RNAi bacteria containing the empty RNAi plasmid (PL4440 vector). The experimental worms were fed RNAi bacteria containing the *lmp-1* and *lmp-2* RNAi plasmids.

The environmental stressor of high salt concentration was used by submerging worms in 1 mL of 500mM NaCl aqueous solution for 1 hour. The worms were removed from the RNAi bacteria and were placed in watch glasses containing the salt solution. After one hour, the worms were placed on microscope slides for imaging.

Vector Unstressed, Vector Stressed, and RNAi treated nematodes were imaged with fluorescent laser scanning microscopy. Fluorescent microscopy images were captured using the ZEISS AxioObserver confocal microscope that is equipped with a 63X/1.4 Plan-Apochromat oil DIC M27 objective and an LSM (Laser Scanning Microscopy) 700 confocal module. The 555nm laser was used for the RPT-1::mCherry fluorescent marker. Microscope imaging settings were held constant for each trial. ZEN software (2009) was used to control the microscope. Captured image dimensions were 512 × 512 pixels with a bit depth of 8 bits. Images were used to qualitatively observe and quantify SING formation. Images were processed with Powerpoint (Sampuda 2017).

The experiment was run across three trials of ~1200 oocytes per trial (~300 oocytes per parameter per trial). To reduce observer bias, trials #2 and #3 were blinded

and randomized by a second party when collecting data. For a positive control, UBC-2, which has a known lethal embryonic phenotype, was used to verify RNAi conditions.

Results

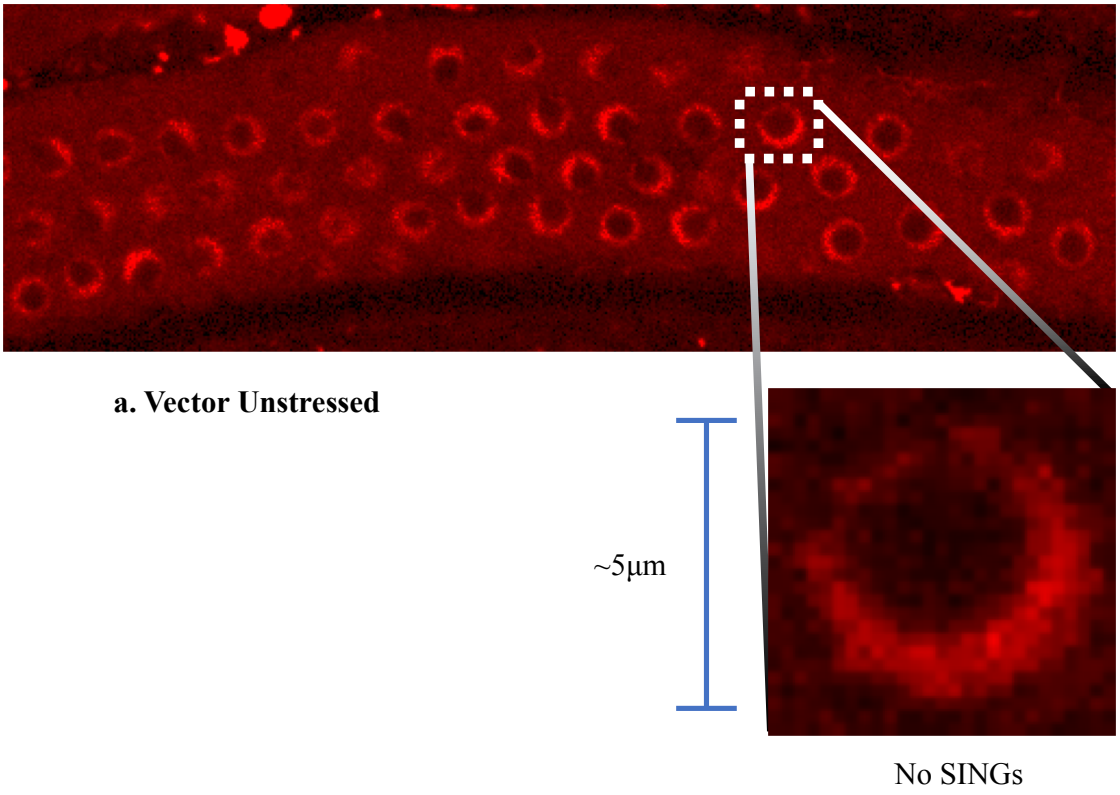
In order to reduce chaperone-mediated autophagy and thus increase the number of cytosolic misfolded proteins, the experimental nematodes were fed the *lmp-1* and *lmp-2* RNAi plasmids. As a negative control, nematodes were treated with bacteria containing the empty RNAi plasmid (PL4440 vector). The environmental stressor of high salt concentration (1mL 500mM aqueous sodium chloride solution for 1 hour) was used with the experimental worms. The negative control was split into two control parameters by treating one set of negative control worms with salt stress while leaving one set unstressed. Having two control parameters provided the ability to observe a negative control for the increase of SINGs between vector unstressed and vector stressed while also allowing for negative control comparison between vector stressed and experimental stressed. All worms were then imaged on the confocal microscope to observe the number of SING formations.

There was an observed correlation between the buildup of cytosolic misfolded proteins (due to the interruption of chaperone-mediated autophagy) and an increase in SING formation. There was an observed increase in SING formation in the nuclei of distal oocytes (Figure 2) in stressed *lmp-1* and *lmp-2* knockdowns when compared to the vector stressed control model. In the first negative control model, the vector unstressed parameter (Figure 3a), 138 SINGs were observed out of 900 oocytes for a total of 15.33% of oocytes (Table 1). In the second negative control model, the vector stressed parameter (Figure 3b), 369 SINGs were observed out of 900 oocytes for a total of 41.00% of

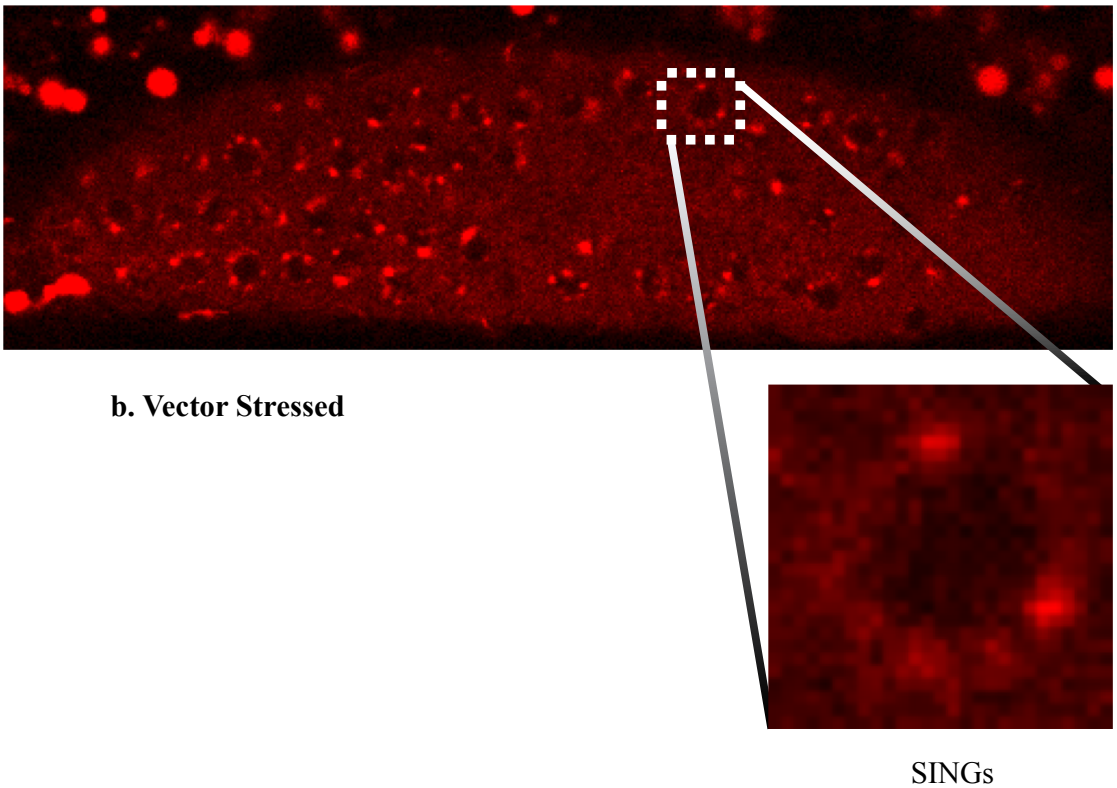
oocytes (Table 1). These results demonstrate that salt stress caused an increase in SING formation between control models.

In the first experimental model, the *lmp-1* knockdown stressed parameter (Figure 3c), there were 450 SINGs observed out of 850 oocytes for a total of 52.94% of oocytes and an 11.94% increase in SINGs from the vector stressed control parameter (Table 1). In the second experimental model, the *lmp-2* knockdown stressed parameter (Figure 3d), there were 416 SINGs observed out of 820 oocytes for a total of 50.73% of oocytes and a 9.73% increase in SING formation compared to the vector stressed control parameter (Table 1). These data were collected from a grand total of 3,470 distal oocytes from 117 worms across three trials of ~1200 oocytes per trial (~300 oocytes per parameter per trial). Exactly 1200 Oocytes were attempted for each of the three trials, but there were rare instances during trial #2 when a small and statistically insignificant number of worms were damaged and became unviewable by the microscope.

All of these results are statistically relevant with a one-tailed *z*-test for variance in percent change. *Z*-values were each less than .0001 between both knockdown parameters and vector stressed (Table 2). This can be interpreted to mean that the differences between both knockdown parameters and vector stressed (two independent proportions) were greater than a 99.99% statistically significant difference (n = ~900) (VassarStats 2018). Furthermore, both knockdown parameters were successfully compared to vector stressed using 99% confidence intervals (Figure 4) and were computed using the Wald Method (GraphPad Software 2018). Error bars representing the confidence interval regions did not overlap and thus confirmed a 99% confidence in the proportional difference between intervals.



a. Vector Unstressed



b. Vector Stressed

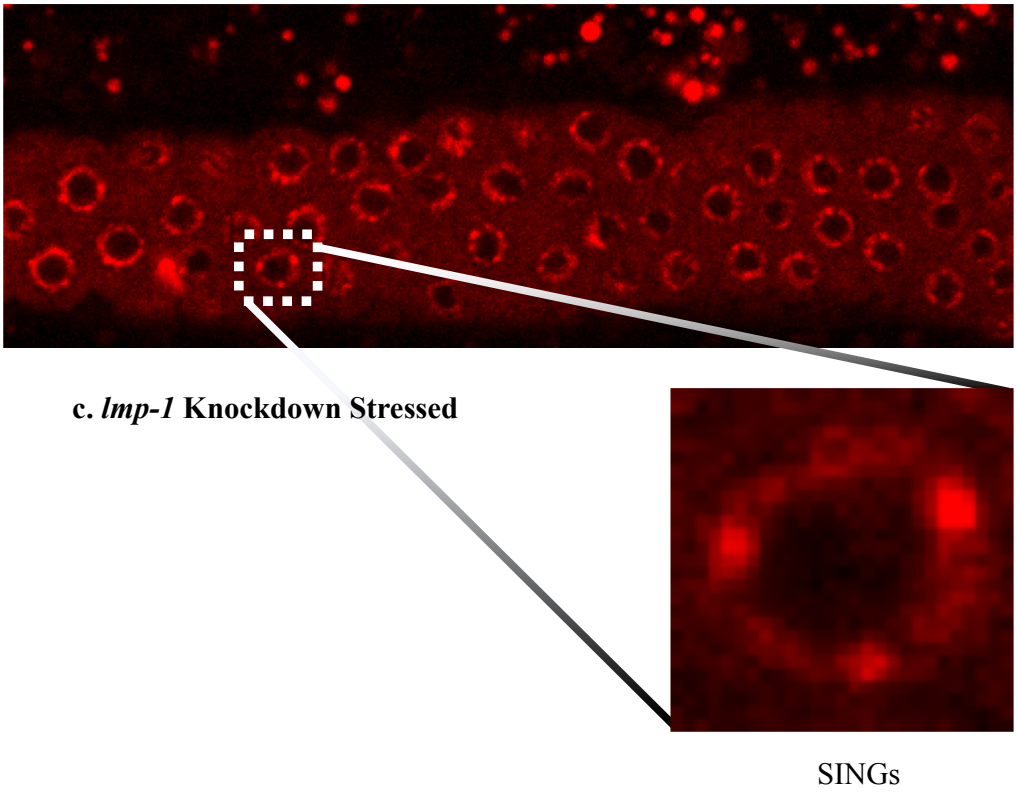


Figure 3 | Confocal imaging of representative distal oocytes with RPT-1::mCherry fluorescent marker. a. Vector unstressed: Little to no apparent SING formation using RNAi vector and no NaCl stress. **b.** Vector Stressed: Some SING formation using RNAi vector and NaCl stress. **c.** *lmp-1* knockdown Stressed: Increased SING formation with NaCl stress using RNAi to knockdown *lmp-1*. **d.** *lmp-2* knockdown Stressed: Increased SING formation with NaCl stress using RNAi to knockdown *lmp-2*.

			Percent Oocytes with SINGs	Percent Δ in SINGs vs. Vector Stressed
Vector Unstressed	**SINGs:**	138	15.33%	
	Oocytes:	900		
Vector Stressed	**SINGs:**	369	41.00%	
	Oocytes:	900		
***lmp-1* Knockdown Stressed**	**SINGs:**	450	52.94%	Δ +11.94%
	Oocytes:	850		
***lmp-2* Knockdown Stressed**	**SINGs:**	416	50.73%	Δ +9.73%
	Oocytes:	820		

Table 1 | Data and Statistical Analysis: Data Collection Totals, Percent Oocytes with SINGs, and Percent Δ in SINGs vs. Vector Stressed. There was an observed relative increase in SING formation between the vector unstressed and vector unstressed control models. There was an observed increase in SING formation in the stressed *lmp-1* and *lmp-2* knockdowns when compared to the vector stressed control model. n = ~900 oocytes for each parameter.

	***z*-Values**
Vector Unstressed to Vector Stressed	< .0001
Vector Stressed to *lmp-1*	< .0001
Vector Stressed to *lmp-2*	< .0001

Table 2 | Data and Statistical Analysis: One-tailed *z*-test for variance in percent change. The results are statistically relevant with an unpaired, one-tailed *z*-test for variance in percent change. The *z*-values were < .0001 between both knockdown parameters and vector stressed (VassarStats 2018).

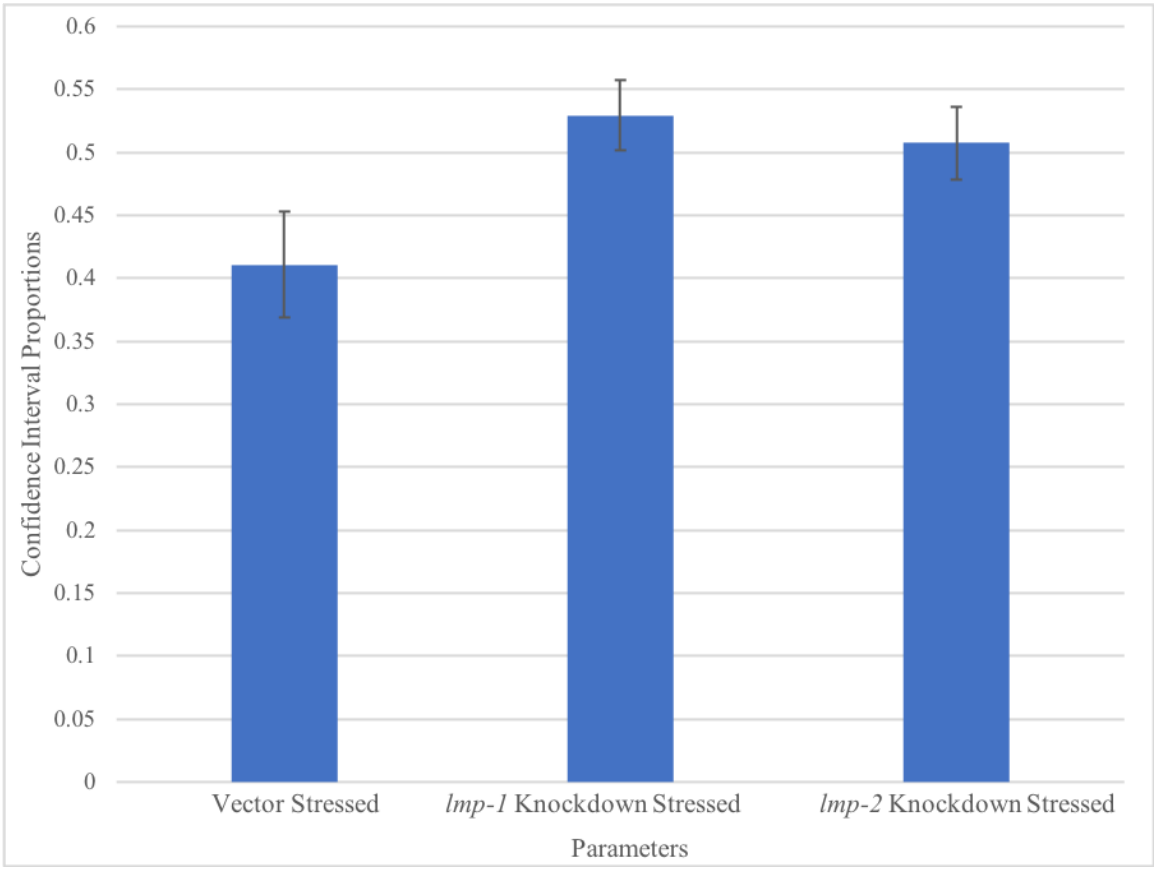


Figure 4 | Data and Statistical Analysis: Confidence Intervals. Both knockdown parameters were successfully compared to vector stressed using a 99% confidence interval and were computed using the Wald Method (GraphPad Software 2018). Error bars representing the confidence interval regions did not overlap and thus confirmed a 99% confidence in the proportional difference between intervals.

Conclusion

SING formation statistically increased in the nuclei of oocytes of worms exposed to environmental stress when *lmp-1* or *lmp-2* genes were knocked down.

Discussion

It has been accepted that cytosolic misfolded proteins are kept and managed in the cytosol. Recently published data has suggested that cytoplasmic misfolded proteins can be translocated and imported into the nucleus for degradation (Prasad 2010). The concept of protein degradation in the nucleus is a relatively newly proposed mechanism.

There have been several studies that have propelled this idea forward and have established that, in some cell types, proteasome is actually 400% more present in the nucleus than in the cytoplasm (Russell *et al.*, 1999; Laporte *et al.*, 2008; Wojcik and DeMartino 2003). In the cytoplasm, proteostasis is managed both through ubiquitin-proteasomal proteolysis and chaperone-mediated autophagy, but since there are no lysosomes in the nucleus, nuclear protein degradation is presumably driven by the the ubiquitin-proteasome system. Considering that in some cell types the abundance of proteasome in the nucleus is greater than in the cytoplasm, it could be hypothesized that the cell can translocate misfolded proteins between the cytosol and nucleus, particularly when cells are exposed to environmental stress (such as high salt stress). If cells have abundant misfolded proteins in the cytoplasm, then cells could respond by importing some of these proteins into the nucleus to relieve the burden of proteolysis on the cytoplasm.

The results of this study support the idea of misfolded protein translocation to the nucleus. The formation of SINGs in stressed oocytes was used as an indicator for proteasomal nuclear localization. Previous work has established that SINGs are a cellular response to stress and protein misfolding. It is possible that cytosolic misfolded proteins are possibly included in SING formation due to a cellular response to an overload of protein misfolding. With chaperone-mediated autophagy interrupted in the cytosol, interaction between the cytosol and the nucleus was observable with the increase of the formation of SINGs in nuclei.

Another possible explanation for an increase in SING formation could be that cytosolic misfolded proteins may trigger a chaperone sink in the cytosol, which partially

hinders normal chaperone function in the nucleus. Chaperone sinks are areas of relatively high concentrations of misfolded proteins that require an increased amount of chaperone involvement. In order to meet the demand of the number of chaperones that are needed for these chaperone sinks, the cell may sometimes recruit chaperones from other areas of the cell (such as from the nucleus).

An important area of study related to proteostasis is the pathology of diseases such as Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis (ALS), Creutzfeldt-Jakob, and Huntington's disease. All of these diseases are caused by the aggregation of misfolded proteins. Although the full pathological pathways of these diseases are not yet fully understood, it has been hypothesized that these protein aggregates could possibly interfere with normal cell pathways and become toxic to the cell's overall health (Gallagher, 2013). Gaining a better understanding of the mechanisms and pathways of proteostasis could one day reveal therapeutic methods to aid the cell when proteostasis fails. As this experiment has suggested, perhaps finding a way to leverage the proteolytic power of the nucleus to assist when the cytosol is overloaded with misfolded proteins could help the cell improve recovery from an environmental stressor and/or a misfolded protein-related disease.

Future Directions

After completion of this thesis, a plausible next direction would be to compare these results with the result of knocking down both *lmp-1* and *lmp-2* simultaneously. In mouse models, some LAMP-1 deficient cells have been shown to up-regulate expression of LAMP-2 to compensate (Andrejewski *et al.,* 1999; Eskelinen *et al. 2006*). Also in mouse models, double deficiency of both LAMP-1 and LAMP-2 show a lethal embryonic

phenotype (Eskelinen *et al. 2006*). Thus, reducing the expression of both proteins could result in a much larger observation of SING formation.

Another small elaboration of this project would be to expand the control models by comparing unstressed *lmp-1* and *lmp-2* knockdowns to stressed *lmp-1* and *lmp-2* knockdowns. The unstressed *lmp-1* and *lmp-2* knockdowns would serve as third and fourth controls for this experiment. This would provide increased data and support for these results.

There is an alternative method to observing misfolded protein traffic in the cell via SING formation, and that would be to inject the cytosol of experimental cells with fluorescent-tagged photoswitchable proteins called Dendra2. Once the Dendra2 protein is present within the cytosol of the experimental cell, the cytosol will be treated with light at both ~400nm and ~490nm to convert the green-colored fluorophore to red-colored (Chudakov *et al.,* 2007). These Dendra2-injected cells then have the ability to show whether misfolded proteins translocate to the nucleus under environmental stress. Once the color conversion has completed, the worm will be treated with environmental salt-stress and then imaged to see if the red-fluorophore is observable in the nucleus due to stress-induced nuclear import.

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Appendix A: Methods Documentation

Trial #1: First Set of 1200 Oocytes (for 20ºC worm population)

Experiment Start Date: 02/08/2018
Strain: LN151

Day #1: Streaking **(Need: 57-65 eggs to begin incubation)**
Date: 2/8/2018 Thursday

1. 1:00pm-2:00pm Suggested Start Time — Actual Time: 5:15pm

    a. Streak RNAi bacteria (from -80ºC freezer) using pipette tips onto large TSA+Tet+Amp plates for single colonies (2 bacterias per plate). Incubate at 37ºC for ~24 hours. After 24hrs., sit out on bench until time to start overnights (for Day #2).
        i. Large plate #1 (2 halves):
            1. Vector
            2. Ubc-2 (Technique control for embryonic lethality)
        ii. Large plate #2 (2 halves):
            1. Target RNAi gene: *lmp-1*
            2. Target RNAi gene: *lmp-2*

    b. (Optional: Hypochlorite Day-2 adult worms for eggs to have L4 worms by Day #3)

Day #2: RNAi Growth
Date: 2/9/2018 Friday

1. 1:00pm-2:00pm Suggested — Scheduled: 5:15pm
   Actual: 4:50pm

    a. Make bacteria overnight growth tubes from Day #1. Use 15 ml plastic tubes. Add a single colony from each bacteria plate to its own 15ml plastic tube containing 2ml of TSB+Amp+Tet.
        i. UBC-2
        ii. Vector
        iii. Target RNAi gene: *lmp-1*
        iv. Target RNAi gene: *lmp-2*

    b. Incubate overnight growth tubes using orbital shaker incubator at 36ºC @ 200 rpms for 15-16 hours (preferably 16 hours)

2. Pull out RNAi (NGM+Amp+Lac) tiny plates from 4ºC refrigerator and sit them out on the bench overnight to reach room temperature for next day.

Day #3: L4 Seeding & Plating **(Need: 57-65 L4 worms)**
Date: 2/10/2018 Saturday

1. 5:00am Suggested Scheduled: 7:50am-8:50am
   Actual: 9:15am

    a. Pull out overnight tubes from orbital shaker incubator and sit out at room temperature.

2. Immediately/relatively soon Actual: 9:25am
   Scheduled: 9:25am

    a. Seed two plates (one extra) for each RNAi bacteria on RNAi plates (NGM+Amp+Lac) with 125 ml of overnight broth and let dry at 37ºC for at least 4 hours to 8 hours.

3. 9:00am-1:00pm Suggested Scheduled: 1:25pm-5:25pm
   Actual: 3:39pm

    a. Plate ~57 L4 worms onto the following plates:
        i. UBC-2 = **5** L4 worms
        ii. Vector = **26-30** L4 worms
            1. Vector unstressed = **13-15** L4 worms
            2. Vector stressed = **13-15** L4 worms
        iii. Target RNAi gene: *lmp-1* = **13-15** L4 worms
        iv. Target RNAi gene: *lmp-2* = **13-15** L4 worms

    b. Incubate worms on RNAi plates for ~24 hrs at 20ºC

Day #4: Stressing & Imaging
Date: 2/11/2018 Sunday

1. 9:00am-1:00pm Suggested Scheduled: 3:45pm
   Actual: 4:30pm

    a. Vector unstressed
        i. Immobilize worms using 18µL Tetramisole
        ii. Prepare 12 unstressed worms from vector plate onto a slide with agar pad.
    b. Vector stressed and Target RNAi stressed

i. Salt-stress Day-1 adults from each RNAi plate:
    1. 1ml of 500mM NaCl in watch glass for 60 mins.
ii. Immobilize worms using 18μL Tetramisole
iii. Prepare 12 unstressed worms per plate onto each slide.

c. Place slides within a humid dark container for temporary storage while imaging.
d. Label slides and hide each label with tape on top and beneath slide.
e. Ask another person to randomize the order of slides for unbiased qualitative data collection.
f. View worms on confocal microscope and take pictures of and collect data from the first 30 distal oocytes encountered of each of the first 10 encountered worms from each RNAi parameter.

Day #5: UBC-2 RNAi Verification
Date: 2/12/2018 Monday

1. 9:00am Suggested (~ ≥ 48hrs. after plating) Scheduled: 4:30pm Actual: 4:40pm

    a. UBC-2 Positive Control:
        i. Verify that the worms have not reproduced exponentially.
            1. Phenotype is embryonic lethality.
            2. This confirms RNAi conditions.

Trial #2: Second set of ~1200 Oocytes **Experiment Start Date:** 02/17/2018
(for 20ºC worm population) **Strain:** LN151

Day #1: Streaking **(Need: 57-65 eggs to begin incubation)**
Date: 2/17/2018 Saturday

1. 1:00pm-2:00pm Suggested Start Time — Actual Time: 4:45pm

    a. Streak RNAi bacteria (from -80ºC freezer) using pipette tips onto large TSA+Tet+Amp plates for single colonies (2 bacterias per plate). Incubate at 37ºC for ~24 hours. After 24hrs., sit out on bench until time to start overnights (for Day #2).
        i. Large plate #1 (2 halves):
            1. Vector
            2. Ubc-2 (Technique control for embryonic lethality)
        ii. Large plate #2 (2 halves):
            1. Target RNAi gene: *lmp-1*
            2. Target RNAi gene: *lmp-2*

    b. (Optional: Hypochlorite Day-2 adult worms for eggs to have L4 worms by Day #3)

Day #2: RNAi Growth
Date: 2/18/2018 Sunday

1. 1:00pm-2:00pm Suggested — Scheduled: 7:40pm
   Actual: 7:52pm

    a. Make bacteria overnight growth tubes from Day #1. Use 15 ml plastic tubes. Add a single colony from each bacteria plate to its own 15ml plastic tube containing 2ml of TSB+Amp+Tet.
        i. UBC-2
        ii. Vector
        iii. Target RNAi gene: *lmp-1*
        iv. Target RNAi gene: *lmp-2*

    b. Incubate overnight growth tubes using orbital shaker incubator at 36ºC @ 200 rpms for 15-16 hours (preferably 16 hours)

2. Pull out RNAi (NGM+Amp+Lac) tiny plates from 4ºC refrigerator and sit them out on the bench overnight to reach room temperature for next day.

Day #3: L4 Seeding & Plating **(Need: 57-65 L4 worms)**
Date: 2/19/2018 Monday

1. 5:00am Suggested Scheduled: 11:00am-12:00pm
   Actual: 12:10pm

    a. Pull out overnight tubes from orbital shaker incubator and sit out at room temperature.

2. Immediately/relatively soon Actual: 12:10pm
   Scheduled: 12:10pm

    a. Seed two plates (one extra) for each RNAi bacteria on RNAi plates (NGM+Amp+Lac) with 125 ml of overnight broth and let dry at 37ºC for at least 4 hours to 8 hours.

3. 9:00am-1:00pm Suggested Scheduled: 4:10pm-8:10pm
   Actual: 5:30pm

    a. Plate ~57 L4 worms onto the following plates:
        i. UBC-2 = **5** L4 worms
        ii. Vector = **26-30** L4 worms
            1. Vector unstressed = **13-15** L4 worms
            2. Vector stressed = **13-15** L4 worms
        iii. Target RNAi gene: *lmp-1* = **13-15** L4 worms
        iv. Target RNAi gene: *lmp-2* = **13-15** L4 worms

    b. Incubate worms on RNAi plates for ~24 hrs at 20ºC

Day #4: Stressing & Imaging
Date: 2/20/2018 Tuesday

2. 9:00am-1:00pm Suggested Scheduled: 5:30pm
   Actual: 5:30pm

    a. Vector unstressed
        i. Immobilize worms using 18µL Tetramisole
        ii. Prepare 12 unstressed worms from vector plate onto a slide with agar pad.
    b. Vector stressed and Target RNAi stressed
        i. Salt-stress Day-1 adults from each RNAi plate:
            1. 1ml of 500mM NaCl in watch glass for 60 mins.

ii. Immobilize worms using 18µL Tetramisole
iii. Prepare 12 unstressed worms per plate onto each slide.

c. Place slides within a humid dark container for temporary storage while imaging.
d. Label slides and hide each label with tape on top and beneath slide.
e. Ask another person to randomize the order of slides for unbiased qualitative data collection.
f. View worms on confocal microscope and take pictures of and collect data from the first 30 distal oocytes encountered of each of the first 10 encountered worms from each RNAi parameter.

Day #5: UBC-2 RNAi Verification
Date: 2/21/2018 Wednesday

2. 9:00am Suggested (~ ≥ 48hrs. after plating) Scheduled: 5:30pm Actual: 5:40pm

a. UBC-2 Positive Control:
i. Verify that the worms have not reproduced exponentially.
1. Phenotype is embryonic lethality.
2. This confirms RNAi conditions.

Trial #3: Third set of 1200 Oocytes **Experiment Start Date:** 02/24/2018
(for 20ºC worm population) **Strain:** LN151

Day #1: Streaking **(Need: 57-65 eggs to begin incubation)**
Date: 2/24/2018 Saturday

2. 1:00pm-2:00pm Suggested Start Time — Actual Time: 7:30pm

    a. Streak RNAi bacteria (from -80ºC freezer) using pipette tips onto large TSA+Tet+Amp plates for single colonies (2 bacterias per plate). Incubate at 37ºC for ~24 hours. After 24hrs., sit out on bench until time to start overnights (for Day #2).
        i. Large plate #1 (2 halves):
            1. Vector
            2. Ubc-2 (Technique control for embryonic lethality)
        ii. Large plate #2 (2 halves):
            1. Target RNAi gene: *lmp-1*
            2. Target RNAi gene: *lmp-2*

    b. (Optional: Hypochlorite Day-2 adult worms for eggs to have L4 worms by Day #3)

Day #2: RNAi Growth
Date: 2/25/2018 Sunday

3. 1:00pm-2:00pm Suggested — Scheduled: 7:30pm
Actual: 5:00pm

    a. Make bacteria overnight growth tubes from Day #1. Use 15 ml plastic tubes. Add a single colony from each bacteria plate to its own 15ml plastic tube containing 2ml of TSB+Amp+Tet.
        i. UBC-2
        ii. Vector
        iii. Target RNAi gene: *lmp-1*
        iv. Target RNAi gene: *lmp-2*

    b. Incubate overnight growth tubes using orbital shaker incubator at 36ºC @ 200 rpms for 15-16 hours (preferably 16 hours)

4. Pull out RNAi (NGM+Amp+Lac) tiny plates from 4ºC refrigerator and sit them out on the bench overnight to reach room temperature for next day.

Day #3: L4 Seeding & Plating **(Need: 57-65 L4 worms)**
Date: 2/26/2018 Monday

4. 5:00am Suggested Scheduled: 8:00am-9:00am
   Actual: 9:00am

    a. Pull out overnight tubes from orbital shaker incubator and sit out at room temperature.

5. Immediately/relatively soon Actual: 11:49am
   Scheduled: 9:00am

    a. Seed two plates (one extra) for each RNAi bacteria on RNAi plates (NGM+Amp+Lac) with 125 ml of overnight broth and let dry at 37ºC for at least 4 hours to 8 hours.

6. 9:00am-1:00pm Suggested Scheduled: 3:49pm-7:49pm
   Actual: 4:54pm
    a. Plate ~57 L4 worms onto the following plates:
        i. UBC-2 = **5** L4 worms
        ii. Vector = **26-30** L4 worms
            1. Vector unstressed = **13-15** L4 worms
            2. Vector stressed = **13-15** L4 worms
        iii. Target RNAi gene: *lmp-1* = **13-15** L4 worms
        iv. Target RNAi gene: *lmp-2* = **13-15** L4 worms

    b. Incubate worms on RNAi plates for ~24 hrs at 20ºC

Day #4: Stressing & Imaging
Date: 2/27/2018 Tuesday

3. 9:00am-1:00pm Suggested Scheduled: 4:55pm
   Actual: 4:45pm
    a. Vector unstressed
        i. Immobilize worms using 18µL Tetramisole
        ii. Prepare 12 unstressed worms from vector plate onto a slide with agar pad.
    b. Vector stressed and Target RNAi stressed
        i. Salt-stress Day-1 adults from each RNAi plate:
            1. 1ml of 500mM NaCl in watch glass for 60 mins.

ii. Immobilize worms using 18µL Tetramisole
iii. Prepare 12 unstressed worms per plate onto each slide.

c. Place slides within a humid dark container for temporary storage while imaging.
d. Label slides and hide each label with tape on top and beneath slide.
e. Ask another person to randomize the order of slides for unbiased qualitative data collection.
f. View worms on confocal microscope and take pictures of and collect data from the first 30 distal oocytes encountered of each of the first 10 encountered worms from each RNAi parameter.

Day #5: UBC-2 RNAi Verification
Date: 2/28/2018 Monday

3. 9:00am Suggested (~ ≥ 48hrs. after plating) Scheduled: 4:45pm
Actual: 3:10pm

a. UBC-2 Positive Control:
i. Verify that the worms have not reproduced exponentially.
1. Phenotype is embryonic lethality.
2. This confirms RNAi conditions.

Appendix B: Data Collection

Trial #1: First set of 1200 Oocytes

Experiment Start Date: 02/08/2018
Confocal Imaging Date: 02/11/2018

Parameter: Vector Unstressed

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
0	30	1	30	1	30	5	30	1	30

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
3	30	5	30	15	30	4	30	2	30

Total # of SINGs:	37
Total # of Oocytes:	300
Percent SING Formation:	12.33%

Parameter: Vector Stressed

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
15	30	2	30	17	30	14	30	9	30

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
2	30	10	30	15	30	20	30	20	30

Total # of SINGs:	124
Total # of Oocytes:	300
Percent SING Formation:	41.33%

Parameter: *lmp-1* Knockdown Stressed

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
17	30	12	30	30	30	26	30	30	30

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
17	30	18	30	15	30	6	30	7	30

Total # of SINGs:	178
Total # of Oocytes:	300
Percent SING Formation:	59.33%

Parameter: *lmp-2* Knockdown Stressed

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
8	30	4	30	30	30	6	30	23	30

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
6	30	10	30	8	30	30	30	30	30

Total # of SINGs:	155
Total # of Oocytes:	300
Percent SING Formation:	%

Trial #2: Second set of ~1200 Oocytes

Experiment Start Date: 02/17/2018
Confocal Imaging Date: 02/20/2018

Parameter: Unknown A (Vector Stressed)

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
5	30	14	30	6	30	9	30	25	30

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
9	30	30	30	5	30	0	30	0	30

Total # of SINGs:	103
Total # of Oocytes:	300
Percent SING Formation:	34.33%

Parameter: Unknown B (*lmp-2* Knockdown Stressed)

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
25	30	3	30	3	30	5	30	0	10

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
15	30	30	30	0	0	0	0	27	30

Total # of SINGs:	108
Total # of Oocytes:	220
Percent SING Formation:	49.09%

Parameter: Unknown C (*lmp-1* Knockdown Stressed)

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
8	30	7	30	13	30	6	30	0	0

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
20	30	5	10	9	30	30	30	30	30

Total # of SINGs:	128
Total # of Oocytes:	250
Percent SING Formation:	51.20%

Parameter: Unknown D (Vector Unstressed)

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
10	30	6	30	6	30	0	30	6	30

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
0	30	5	30	0	30	20	30	10	30

Total # of SINGs:	63
Total # of Oocytes:	300
Percent SING Formation:	21.00%

Trial #3: Third set of 1200 Oocytes **Experiment Start Date:** 02/24/2018
Confocal Imaging Date: 02/27/2018

Parameter: Unknown A (Vector Stressed)

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
22	30	20	30	28	30	11	30	13	30

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
28	30	2	30	5	30	3	30	10	30

Total # of SINGs:	142
Total # of Oocytes:	300
Percent SING Formation:	47.33%

Parameter: Unknown B (Vector Unstressed)

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
3	30	1	30	5	30	4	30	2	30

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
0	30	2	30	5	30	12	30	4	30

Total # of SINGs:	38
Total # of Oocytes:	300
Percent SING Formation:	12.67%

Parameter: Unknown C (*Imp-1* Knockdown Stressed)

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
3	30	25	30	5	30	8	30	5	30

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
30	30	4	30	30	30	30	30	4	30

Total # of SINGs:	144
Total # of Oocytes:	300
Percent SING Formation:	48.00%

Parameter: Unknown D (*lmp-2* Knockdown Stressed)

1st Worm		2nd Worm		3rd Worm		4th Worm		5th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
25	30	13	30	21	30	2	30	6	30

6th Worm		7th Worm		8th Worm		9th Worm		10th Worm	
# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes	# of SINGs	# of Oocytes
13	30	19	30	21	30	3	30	30	30

Total # of SINGs:	153
Total # of Oocytes:	300
Percent SING Formation:	51.00%