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Transgenerational epigenetic inheritance via environmental stress in *Caenorhabditis elegans*

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Transgenerational epigenetic inheritance via environmental stress in

Caenorhabditis elegans

By

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A culminating thesis submitted to the faculty of Dominican University of California and
Buck Institute Research on Aging in partial fulfillment of the requirements for the degree

Master of Science in Biology

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CERTIFICATION OF APPROVAL

This thesis, written under the direction of the candidate's thesis advisor and approved by the department chair, has been presented and accepted by the Department of Natural Sciences and Mathematics in partial fulfillment of the requirements for the degree Master of Science in Biology. The content and research methodologies presented in this work represent the work of the candidate alone.

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LIST OF ABBREVIATIONS

<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
DMSO	Dimethyl sulfoxide
F1, F2, F3	Generations 1, 2, and 3
GFP	Green fluorescent protein
HSPs	Heat shock proteins
L1	Larval stage 1
L4	Larval stage 4
N2	Wild type <i>C. elegans</i>
NGM	Nematode growth media
P ₀	Parental generation
s.e.m.	Standard error of the mean
ThioT	Thioflavin T
UPR ^{mt}	Mitochondrial unfolded protein response
WT	Wild-type

Abstract

Epigenetics is defined as heritable changes in gene expression/function that do not affect DNA sequence. These can include post-translational modifications, chromatin remodeling, and DNA methylation. The majority of epigenetic marks are erased during development in mammals. However, sometimes these marks can remain intact and pass on the traits from parents to offspring. These circumstances are known as transgenerational epigenetics, where information that is passed down from one generation to the next affects traits of the offspring without changing the DNA sequences. These effects are seen in generations that were not initially exposed to the signal or environment that triggered a change in the parental generation. It is unknown how environmental stresses in the parental generation cause epigenetic changes that are then transmitted to their progeny.

Here, *C. elegans* was used in different environmental stresses to measure transgenerational epigenetics. We showed that descendants of N2s coming from 25°C are more thermotolerant than worms from 15° and 20°C. This phenotype was passed down to three generations of N2s not initially exposed to different temperatures. In *C. elegans*, *hsp-6* and *hsp-16.2* expression levels are higher in descendants coming from 25°C than those from 15° and 20°C. We also exposed worms to a chemical compound, Thioflavin T, and saw that the worms had a higher sensitivity to stress and therefore lived shorter than control N2s. Although the mechanism for transmitting traits and phenotypes epigenetically remains unknown, this study provides insight into some possible genes involved in transgenerational epigenetic inheritance.

INTRODUCTION

Transgenerational epigenetics

The epigenome is comprised of a number of chemical compounds and proteins that tell the genome how to function. These compounds and proteins attach themselves to the DNA and turn the gene on or off (“Epigenomics,” 2015). This phenomenon is known as epigenetics, or to be “above” or “on top of” genetics. In other words, epigenetics means changes in gene expression/function that are not caused by changes in the DNA sequence (Rettner, 2013). Epigenetics can include post-translational modification, chromatin remodeling, and DNA methylation. Much of the genome is reset when passed from parent to offspring, but under some circumstances, these chemical tags remain for the next generation.

The term epigenetics was first coined by Conrad Hall Waddington in the 1940s when he heat shocked *Drosophila melanogaster* flies and saw different wing patterns inherited in response to the initial heat shock (Lim & Brunet, 2013). Flies were heat shocked for 4 hours at 40° C, 17 to 23 hours after the pupa formation. Several flies exposed to this heat shock developed crossveinless wings, but the phenotype was not seen in flies raised in normal conditions (Waddington, 1953). Waddington then saw crossveinless wings in progeny of parents who were initially heat shocked, but did not undergo treatment themselves even after 16 generations. This suggested that crossveinless wings were an acquired characteristic from the flies exposed to heat shock and then inherited by their progeny (Waddington, 1953). This example became known as the transgenerational epigenetic theory of inheritance.

Transgenerational epigenetics is when information is passed down from one generation to the next that affects the traits of offspring without changing DNA sequence (Heard & Martienssen, 2014). These effects are found in generations that are not exposed to the initial signal or environment that triggered a change in parental generation. This concept can be seen as a form of Lamarckism, which is the idea that an organism can pass on characteristics acquired through its lifetime (Burkhardt, 2013). Epigenetics has come to be defined as molecular factors and processes around DNA that regulate the genome independent of the DNA sequence (Skinner, Manikkam, & Guerrero-Bosagna, 2010). As the field progressed, the term epigenetics was modified to not only be the molecular aspects, like histone modifications, but also about gene-environment interactions.

Environmental factors affecting epigenetics

Many environmental factors have been known to alter the epigenome. For example, nutritional factors (Heijmans et al., 2008), chemicals including pesticides (Andersen et al., 2008), and maternal care during nursing (Champagne et al., 2015) have all shown the different ways our epigenome can be altered without any changes to our DNA sequences. These environmental factors have been known to affect a multitude of organisms and promote epigenetic inheritance of diseases (Skinner, 2011). Exposure to these environmental factors during critical times in development can change the differentiation programming of cells that can alter the genome (Skinner et al., 2010). The germline is essential in transmitting information from generation to generation. Therefore, if alterations are made during germline development, the offspring can be

affected. This may suggest that ancestral stress conditions can be a significant factor in what is passed down to our children and grandchildren (Skinner et al., 2013).

An example would be the winter of 1944 when Netherlands ran out of food stock because waterways and canals were frozen. Germans banned food from entering the Netherlands because the Netherlands helped the Allied forces in World War II (Roseboom, Painter, Abeelen, Veenendaal, & Rooij, 2011). After the war ended, doctors were able to gain information on how maternal malnutrition affected pregnancy and the development of the baby before birth. It was shown that babies who were born during the famine, and therefore exposed to famine in late pregnancy, weighed lighter than babies not exposed. These babies were also more prone to become schizophrenic and develop anti-social personality disorder (Hoek et al., 1996). Maternal malnutrition later played a part in occurrences of mental illnesses that were seen in children born from the famine (Roseboom et al., 2011). Therefore, the Dutch famine studies have shown that maternal nutrition during a pregnancy may cause negative effects that are then transmitted to the baby epigenetically. But how epigenetic information is passed down between the generations remains unknown (Greer et al., 2014). And if information is passed down, how many generations inherit this information?

C. elegans as a model for transgenerational epigenetic inheritance

Recently, *Caenorhabditis elegans* (*C. elegans*), a transparent microscopic nematode, has become a model for transgenerational epigenetics. *C. elegans* are non-hazardous, non-pathogenic, non-parasitic organisms (Eisenmann, 2005). *C. elegans* is a model organism used to study aging and diseases because of its short lifespan (2-3

weeks). It is also useful because a single worm can produce, 300 to 350 offspring, which allows for duplicates of experiments to occur with ease. *C. elegans* is a good species for epigenetic studies due to the ability to easily control variables and environmental settings, as well as availability of *C. elegans* strains from the *Caenorhabditis* Genetics Center (Saint Paul, Mn).

Previous studies already have shown the utility of *C. elegans* as a model for transgenerational epigenetic inheritance. One example is the inheritance of longevity. Deficiencies in the histone H3 lysine 4 trimethylation (H3K4me3) complex chromatin modifiers ASH-2, WDR-5, and SET-2 extend lifespan in *C. elegans*' progeny even after three generations (Greer et al., 2012). WDR-5, a component of the complex, was knocked down and that lead to the decrease in H3K4me3 levels and an extension of lifespan. They generated genetically wild-type (WT) descendants from the *wdr-5* mutant worm and were still able to see lifespan extension even after three generations (Figure 1).

Figure 1: Lifespan of F3 progeny of wild type (WT) *C. elegans* from *wdr-5* (*ok1417*) mutant parents (Greer et al., 2012).

Another example of transgenerational epigenetics in *C. elegans* is the inheritance of fertility. Mutants of *spr-5*, an ortholog of human H3K4me2, subsequently decreased broodsize until sterility began to occur in the 20th generation (Greer et al., 2014). These results are interesting because both studies involve the H3K4 complex, suggesting that this histone mark may play an important role in epigenetic inheritance (Lim & Brunet, 2013). Another example in *C. elegans* showed that starvation induced developmental arrest lead to the generation of small RNAs that were inherited through at least three consecutive generations not exposed to the initial starvation trigger (Rechavi et al., 2014).

C. elegans have also been used as a model to understand how animals respond to heat stress and how that can induce changes in mRNA levels which remain for two generations after exposure (Schott, Yanai, & Hunter, 2014). Here we proposed to look at the long term effects of environmental stresses, like heat, and see if generations not initially exposed to the trigger pass down thermotolerance epigenetically. *C. elegans* will also be exposed to small chemical compounds that alter sensitivity to stress to see if changes are being passed down epigenetically. This will give us an insight to possible mechanisms and key players that are playing a role in transgenerational epigenetic inheritance.

MATERIALS AND METHODS

C. elegans strains

The *Caenorhabditis* Genetics Center (CGC), located at the University of Minnesota, contains a repository of deletion mutants and point mutation encompassing more than 80% of the *C. elegans* genome. *hsp-6::GFP* [SJ4100] was provided by the CGC. Strains N2 [GL], and *hsp-16.2::GFP* [GL311] were provided by the Lithgow lab.

Preparation of nematode growth media (NGM) plates

C. elegans were grown on NGM plates at either 15, 20, or 25°C. For 1L of NGM, 17g of bacto agar, 3g NaCl, 2.5g bacto peptone, and 975mL of water were added together and then autoclaved for sterilization. After autoclaving, 25mL of KH₂PO₄, 1mL MgSO₄, 1mL CaCl₂, and 1mL cholesterol were added to the beaker containing the agar. After 2 days, medium petri plates (60mm diameter) were seeded with 200μl concentrated *E. coli* OP50, while smaller plates (35mm diameter) were seeded with 80μL of OP50. *E. coli* OP50 was grown and stored as described in “*C. elegans*: A practical approach” (Hope, 1999).

Maintenance of C. elegans

In order to prevent contamination and starvation, *C. elegans* were maintained regularly by being transferred onto petri plates. Plates of worms were maintained differently depending on the temperature the worms were growing at. For worms reared at 15°C, eggs were picked every 7 days, for worms in 20°C, eggs were picked every 3-3.5 days, and at 25°C, eggs were picked every 2-3 days (Hope, 1999)

Preparation of compound plates

After pouring NGM plates and seeding them with OP50, some small NGM plates were spotted with Thioflavin T (ThioT) and DMSO,. Both compounds were dissolved in distilled water. Their respective concentrations were 50 μ M ThioT and .5% DMSO per NGM small plate. Compounds were added after plates were seeded with OP50.

Egg lay

To have an age-synchronized cohort of worms, an egg lay was performed. About 30 to 50 adult hermaphrodite worms were placed onto small NGM agar plates, and left at room temperature for about 1.5 to 2 hours. After eggs were laid, all adult worms were removed to maintain the eggs synchronicity. The plates of eggs were then placed in the incubator at 20°C for further experiments.

Hermaphrodite self-fertility

To measure if progeny size is affected as a result of specific treatment, a fertility assay was performed. Two and a half days after the egg lay, 12 stage L4 worms were transferred to a separate NGM plate for each condition. They were then placed in the 20°C incubator and transferred each day for 5 days or until the worms ceased laying eggs. Egg plates were then placed in the 25°C incubator after each transfer and scored the following day at the larva stage.

Thermotolerance

In order to see if environmental signals change a worm's ability to withstand heat, a thermotolerance assay was performed. Worms coming from their respective temperatures, 15, 20, and 25°C were moved to 20°C for three more generations. After the third generation an egg lay was performed at room temperature in order to synchronize the population. After the egg lay, adult worms were removed so only eggs remained. Plates were placed back into the 20°C incubator until they became stage L4 larval worms. When they reached the L4 stage, 50 hermaphrodite worms in duplicate were placed on their respective plates. Then, plates were placed in a shifting incubator. 5 hours prior to scoring, the incubator's temperature was shifted to 34°C. After 5 hours at 34°C, worms were scored every 1-2hrs until all worms were dead. Dead worms were removed from the plate with the worm pick after they no longer respond to prodding.

Recovery Assay

A recovery assay was done in order to measure the amount of worms alive at an end point after being exposed to heat. Like the thermotolerance assay, worms came from different respective temperatures and were placed in 20°C for three more generations. An egg lay at 20°C was performed for descendants of worms coming from 15, 20, or 25°C. When the eggs reached adulthood, duplicates of 50 adult worms from each condition were placed onto NGM agar plates. Those duplicates were placed into Ziploc bags and submerged in a 34°C water bath for 8hrs. Plates were placed back into the 20°C incubator for a 14hr recovery. Plates were scored once for the amount of worms dead/total number of worms.

Heat Stress Assay

This assay was used in order to properly visualize *hsp-16.2::GFP* transgenic worms.

Stage L4 larval worms were placed in small NGM plates and into the shifting incubator overnight. Prior to collection, worms were heat stressed at 35°C for an hour and then left at room temperature for 3hrs. After the worms were left at room temperature for three hours, they were ready to be visualized under the microscope or collected for future western blot analysis.

Quantification of GFP

In order to view our protein of interest after the environmental assay, we visualized the reporter transgenic worms under a Leica microscope at 40x and quantified the intensity of GFP. After being treated with stress, 15 day one adult hermaphrodite worms descendant from each condition were paralyzed in 1mM levamisole. They were mounted on 1% agarose and covered with a glass coverslip. Quantification of integrated density of GFP was analyzed with ImageJ™ (Madison, WI).

RESULTS

N2 C. elegans grown at 25°C have a lower % of death in a heat bath

The Lithgow Lab has previously published data that wild-type (N2) *C. elegans* that were subjected to a mild thermal stress, 25°C, significantly induced thermotolerance compared to N2s that stayed in 20°C prior to thermotolerance for one generation (Lithgow, White, Melov, & Johnson, 1995). In order to test transgenerational epigenetic inheritance, N2 *C. elegans* were placed into three separate temperatures, 15°C, 20°C, and 25°C. N2 worms were kept in these respective temperatures for three generations prior to experiments in order for them to acclimate. N2s from different temperatures were then placed back into the 20°C incubator. The first, second, and third generation of these worms were then each tested with a stress (Figure 2).

Figure 2. Schematic of experimental design

A recovery assay was done in order to measure the fraction of worms alive at an end point after being exposed to heat. The three separate groups were tested for thermotolerance by being placed in a 35°C water bath for 8 hours and then worms were assayed after a 14-hour recovery. In figure 3, progeny of N2 coming from 15°C (green bar) had a higher percentage of death, between 20-25%, in all three generations. N2s coming from 25°C (purple bar) had the lowest percentage of death, between 3-10%. Generations two and three were statistically significant, but generation one was not. This

could be due to the fact that some water from the bath had leaked into the plates during the first time the experiment was run. In order to make sure that it was the water leakage, two more experiments were run and showed similar data to generations two and three. All three experiments were included in the data pool.

Figure 3. Recovery Assay of *C. elegans* at different temperatures at F1, F2, and F3 progeny. N2s were heat stressed in a water bath for 8hrs at 34°C and then placed in the 20°C incubator for a 14hr recovery. Error bars represent mean plus standard error of the mean (s.e.m.) of three biological replicates. **P < 0.005 versus 15°C (Student's test)

Worm with ancestors grown at 25°C are more thermotolerant than those with ancestors grown at 15°C and 20°C

In order to test thermotolerance in a different way but with more time points, a thermotolerance assay was done. When the progeny of N2 worms raised at different temperatures reached the larval 4 (L4) stage, they were placed into a shifting incubator. 5 hours prior to scoring, the incubator shifted from 20°C to 34°C. Worms were scored every 1-2 hours until all worms were dead 5 hours after the heat shift. Results show that progeny with ancestors grown at 15°C (green line) and 20°C (blue line) died around the same time, 600 to 650 minutes, and had a lower survival rate than those coming from ancestors grown at 25°C (purple line). Interestingly enough, N2s grown at 25°C were able to withstand heat stress for a longer period of time by dying around 700 minutes (Figure 4). These results show that descendants of N2s from 25°C were able to withstand heat stress in two different kinds of assays showing that there is some mechanism that has been induced in the parent generation and passed down transgenerationally to their progeny. These results were also able to replicate a similar pattern that was reported in the Lithgow paper, showing that N2s coming from an original mild heat 25°C would be able to withstand a larger heat stress than those worms not exposed to the stress (Lithgow et al., 1995), but we were able to show this same phenotypic trait for three generations after initial exposure.

Figure 4. Representative graphs of F1, F2, and F3 generation thermotolerances.
C. elegans were heat stressed for 5 hours at 34°C and then scored until all worms were dead. All three generations show statistical significance between the three groups of worms. These pictures are representative graphs for four replicates for each generation. Each replicate contained 100 worms per condition (Log-Rank Test).

Parental generation (P_0) also shows that 25°C N2s have a higher thermotolerance than worms grown at other temperatures

N2 worms taken from 15°C, 20°C, and 25°C directly were also tested for thermotolerance. This is to ensure that the trend of 25°C N2s surviving heat stress longer is inherited from their parents. Figure 5 (below) shows that the N2s from 25°C survived heat stress longer than the worms from the other two temperatures, by surviving up to 650 minutes. Interestingly enough, worms from 20°C lived shorter than 15°C. This could be because 15°C is considered a mild stress in itself compared to normal room temperature that could enable a stress resistance mechanism for the parental generation. N2s grown at 20°C is considered in the lab to be the optimal temperature for fertility and that's why it was chosen to be the control temperature.

Figure 5. Representative graph of parental generation (P_0) thermotolerance. *C. elegans* were heat stressed for about 3 hours at 34°C and then scored until all worms were dead. All three temperatures were statistically significant to each other. This graph is a representative of four replicates. Each replicated contained 100 worms per condition.

hsp-16.2::GFP transgenic worms grown in 25°C have higher levels of heat shock chaperone

Heat shock proteins (HSPs) are proteins, many of which are up-regulated in high temperature environments (Zhou, Pincus, & Slack, 2011). These HSPs function mostly as chaperones correcting misfolded proteins caused by a wide variety of stresses such as heat and oxidative stress. Upregulation of HSPs is a common cellular response to thermal stress. Walker et al. also showed that overexpression of *hsp16.2* induced thermotolerance (Walker & Lithgow, 2003). Therefore, we predict that enhanced organismal resistance to stress could correlate with the modification of HSPs to increase expression levels, and be a mechanism by which the transgenerational effect of thermotolerance is inherited.

hsp-16.2 encodes a 15-kD HSP protein and a heat shock chaperone currently being studied in our lab. Expression of *hsp-16.2* is strongest in the intestine and pharynx (Link, Cypser, Johnson, & Johnson, 1999). L4s of the transgenic worm *hsp-16.2::GFP* (green fluorescent protein) were also placed into three different temperatures 15°C, 20°C, and 25°C and left for three generations. Progeny of worms were then moved to 20°C and then tested for levels of *hsp-16.2* via GFP fluorescence intensity. *hsp-16.2::GFP* worms were heat stressed for an hour at 35°C, then placed in the 20°C incubator for 3 hours. After 3 hours, worms were mounted onto slides and GFP intensity was measured via ImageJ. Figure 6 shows the visualization of GFP in the *hsp-16.2* transgenic worms. Progeny of worms coming from 25°C show a higher level of *hsp-16.2* expression around the intestine and pharynx than worms coming from 15°C or 20°C.

Quantification of GFP intensity was then measured via ImageJ. For each generation, there were higher levels of *hsp-16.2* in progeny of worms coming from 25°C. In the first generation of *hsp-16.2::GFP* worm, there was a statistical significance between worms from 15° and 25°C. Generations two and three were close to being statistically significant, as they still trended towards high levels of *hsp-16.2* expression in the 25°C worms (Figure 7). These findings tell us that worms originally from 25°C are able to promote an increase in *hsp-16.2* expression, as seen with GFP intensity, as opposed to worms grown in 15°C and 20°C for three generations.

15°C

20°C

25°C

Figure 6. GFP visualization of *hsp-16.2* on progeny of worm from 15°C, 20°C, and 25°C. *hsp16.2::GFP* [GL311] were heat stressed for 1 hour at 34°C and then placed in the 20°C incubator for 3 hours. GFP intensity was measured via fluorescent microscopy at 40x.

A

B

C

Figure 7. Heat shock induction of *hsp-16.2::GFP* reporter transgene worms. *hsp16.2::GFP* [GL311] were heat stress at 35°C for an hour and then placed at room temperature for three hours before viewing. (A) First generation *hsp-16.2* worms showed a significant difference in GFP expression between 15°C and 25°C, *P < 0.02 (Student's test). (B & C) Second and third generation *hsp-16.2* worms showed no significant difference between temperatures. Error bars represent mean plus standard error of the mean (s.e.m.) of three biological replicates. Each replicate contained 100 worms per condition (Log-Rank Test).

hsp-6 expression levels are increased in worms grown at 25°C

hsp-6 is another HSP that encodes a mitochondrion-specific chaperone involved in the mitochondrial unfolded protein response (UPR^{mt}) (Yoneda et al., 2004). Its induction is increased when mitochondrial protein folding is disrupted and can be detected from the larval 1 (L1) stage to adulthood. Just like *hsp-16.2* and N2, *hsp-6* worms were placed into three separate temperatures where they remained for three generations. After three generations, *hsp-6* worms were moved to 20°C, where the progeny was tested for *hsp-6* expression levels.

Basal levels of *hsp-6* were visualized and quantified to see if there was a difference in expression from the progeny of ancestors from 25°C, a mild stress. *hsp-6::GFP* transgenic worms, like *hsp-16.2::GFP* were mounted onto a slide where their fluorescence was then measured using ImageJ. There was an increased level of *hsp-6* expression in the progeny of ancestors coming from 25°C (Figure 8). Aside from visualization, GFP intensity was also quantified like *hsp-16.2* worms. Just like *hsp-16.2*, *hsp-6* expression was increased in progeny of worms that came from 25°C than those coming from 15°C and 20°C. In the first generation, there was a statistical significance in GFP levels between 15°C and 20°C compared to 25°C. There was also statistical significance between 15°C and 25°C in the third generation. Although there was no statistically significant effect in the second generation, the trend remained consistent by having increased levels of *hsp-6* in the progeny of ancestors coming from 25°C (Figure 9).

15°C

20°C

25°C

Figure 8. GFP visualization of *hsp-6::GFP* on progeny of worm from 15°C, 20°C, and 25°C. *hsp-6::GFP* [SJ4100] were visualized for *hsp-6* as young adults on normal OP50 plates. GFP intensity of *hsp-6* was measured via fluorescent microscopy at 40x.

A

B

C

Figure 9. Measurement of basal levels of *hsp-6::GFP* reporter transgene worms.

All three generations consist of four biological replicates. (A) First generation *hsp-6* worms showed a significant difference in GFP expression between 15°C and 25°C, * $P < 0.01$ as well as 20°C and 25°C, * $P < 0.01$ (Student's Test). (B) Second generation showed no statistical difference between temperatures. (C) Third generation showed a significant difference in GFP expression between 15°C and 25°C, * $P < 0.01$ (Student's test). Error bars represent mean plus standard error of the mean (s.e.m.) of three biological replicates. Each replicate contained 100 worms per condition (Log-Rank Test).

Chemically induced epigenetic change

We then began to shift our focus onto epigenetic changes caused by chemicals and how they are transgenerationally inherited. This is because humans are exposed every day to chemicals and pesticides that can have harmful effects on the body. Sometimes, these chemicals can alter our sensitivity to stress and chemicals.

Thioflavin T (ThioT) is a benzothiazole, amyloid-binding dye used to visualize misfolded proteins aggregates, amyloids (Figure 10). It binds to protein fibrils and slows aggregation *in vitro* (Biancalana & Koide, 2010). The Lithgow Lab has been testing ThioT on *C. elegans* and it has shown extension of lifespan and slowed aging (Alavez, Vantipalli, Zucker, Klang, & Lithgow, 2011). ThioT is also known to make *C. elegans* thermosensitive (personal communication, Daniel Edgar, Lithgow Lab).

Figure 10. Structure of Thioflavin T (ThioT)

N2s exposed to ThioT have no statistical significance in difference of brood size compared to N2s exposed to DMSO

N2s coming from DMSO and ThioT were checked for a difference in brood size to determine if the compounds were affecting fertility. If fertility is affected, a different mechanism could make the worms die faster rather than the sensitivity to stress caused by ThioT itself. Parental N2s were kept on DMSO and ThioT plates until they started to lay eggs. Once they were adults, worms coming ThioT and DMSO were places on regular NGM plates. An egglay was performed and progeny of worms coming from ThioT or DMSO were tested for fertility. There is no significant difference in broodsize from worms coming from either ThioT or DMSO. This indicated that the fertility mechanism is not manipulated by ThioT or DMSO (Figure 11).

Figure 11. F1 Broodsize (DMSO vs ThioT). Progeny of worms coming from DMSO and ThioT exposure was tested for fertility. One adult worm was placed per plate and transferred everyday for 5 days. Every day the number of eggs laid by a single worm were scored. There is no statistical difference between brood sizes. Error bars represent mean plus standard error of the mean (s.e.m.) of two biological replicated. Each replicate contained 10 adult worms per condition (Log-Rank Test).

N2s exposed to ThioT are more sensitive to heat stress

N2s were used to test sensitivity to heat stress by being exposed to ThioT or DMSO control. N2s were placed onto ThioT and DMSO plates at the L4 stage. Once the N2s became adults, egg lays were performed. Progeny of worms exposed to ThioT were tested to see if sensitivity to heat stress had been passed down epigenetically.

Just like previous experiments, parental generations were tested as well progeny. Just like the N2 thermotolerance from different temperatures, N2s were placed in an overnight incubator. Five hours prior to scoring, the incubator was shifted from room temperature to 34°C. N2s directly placed and exposed to ThioT and DMSO plates were considered parental generation. The prior observation was confirmed; worms exposed to ThioT during the L4 to adult stage died faster than worms exposed to our DMSO vehicle

control. ThioT worms on average lived up to 300 minutes while 90% of DMSO worms were still alive. Worms exposed DMSO lived up to 590 minutes, indicating that ThioT causes a sensitivity to heat stress (Figure 12).

Figure 12. Representative graph of parental generation (P₀) thermotolerance.

N₂s were heat stressed for about 3 hours at 34°C and then scored until all worms were dead. The difference between the deaths of ThioT versus DMSO were statistically significant ****P < 0.0001 (Log-Rank Test). Error bars represent mean plus standard error of the mean (s.e.m.) of three biological replicates. Each replicate contained 100 worms per condition.

In order to test if this characteristic could be passed down epigenetically, progeny of worms exposed to ThioT and DMSO were then tested without being exposed to those compounds. In the first and second generations, progeny of worms initially exposed to ThioT had a lower survival rate than progeny of worms coming from DMSO. The difference in the survival of worms was statistically significant in both generations (Figure 13).

A

B

Figure 13. Representative graphs of F1 and F2 generation thermotolerances. N2s were heat stressed for 5 hours at 34°C and then scored until all worms were dead. Both generations show statistical significance between the two groups of worms. Error bars represent mean plus standard error of the mean (s.e.m.) of four biological replicates. Each replicate contained 100 worms per condition (Log-Rank Test).

DISCUSSION

Transgenerational epigenetics of inheritance can be defined as information passed down from the parental generation initially exposed to some trigger to the next generation without affecting the DNA sequence. How exactly traits and characteristics are being transmitted is unknown. Here we have taken the model organism of *C. elegans* and tested transgenerational inheritance over several phenotypes and heat stress markers.

In this study, we have shown that descendants of N2s coming from 25°C are more thermotolerant than worms from 15° and 20°C. This finding replicates data previously published by the Lithgow Lab where worms pretreated from a mild heat stress induces thermotolerance compared to worms kept at 20°C. These results could implicate that 25°C acts as a mild stressor for N2s and activates a mechanism that helps them survive higher heat stress. This phenotype was passed down to future generations indicating that this unknown mechanism helps the progeny survive heat stress longer without secondary exposure to the mild heat stress again. We have also shown that in *C. elegans*, *hsp-16.2* and *hsp-6* are expressed at higher levels, shown with GFP, in descendants of worms coming from 25°C than those from 15° and 20°C. Since these two HSPs play a role in *C. elegans* when they undergo heat stress, it could be part of a bigger mechanism where more than two HSPs play a role in transmitting information epigenetically of thermotolerance.

We also conducted brief experiments indicating that *C. elegans* exposed to compounds can have an effect epigenetically. Thioflavin T is a compound that has been shown to make *C. elegans* thermosensitive. When the N2s were exposed to ThioT, they

died rapidly compared to N2s exposed to our control DMSO. Progeny of N2s exposed to ThioT were also thermosensitive, and that was seen across two generations. This could indicate that ThioT may be activating a mechanism that is being passed down epigenetically as well. Interestingly enough, ThioT was also shown to extend lifespan, so it would be interesting to investigate what mechanism is making these worms more thermosensitive. This study could indicate that the exposure of different drugs and environmental effects could have opposite effects on *C. elegans* and humans.

C. elegans is a great model to conduct transgenerational epigenetic inheritance due to its short lifespan. There is currently no evidence that indicates heat shock causes mutations in *C. elegans*. We do believe the changes are epigenetic; however, it is possible that some mutations could have arisen that are causing the thermotolerance. One way to address this concern would be to perform whole genome sequencing of the thermotolerant *C. elegans* and compare to the wildtype worm. If DNA sequences are both the same, we could then conclude that this effect is purely epigenetic.

We do understand that when *C. elegans* were moved back to 20°C, the F1 generation as embryos could have been affected since they were exposed to the initial heat stress as early embryos. However, the F2 and F3 generations were never exposed to the initial stressor, and therefore the conclusions we made are based on the F2 and F3 generations. Because the F2 and F3 generations still show the same trend as F1 and P0 generations, it is very likely that it could be because of transgenerational epigenetics and not from descendants experiencing the same initial stressor.

There are several areas in this study that are worth pursuing in the future. Further evaluation is needed to see which mechanisms are being altered allowing worms to become thermotolerant. It is also important to find out how exactly this thermotolerance phenotype is being passed down epigenetically. One way we can look at this is to check for histone modification and methylation/acetylation marks via chromatin immunoprecipitation (ChIP). Using antibodies that attach to histones will give us an insight into which histones are being modified and playing a role in transgenerational epigenetic inheritance of thermotolerance. We can also test if oxidative or ER stress is mediating a response allowing characteristics to be passed down epigenetically. RNA sequencing can be used to examine expression of RNA levels across the genome at a given time. Measuring mRNA abundance can be an important tool in determining how an organism like *C. elegans* is being affected under certain conditions, like thermotolerance and what mechanism are at play during that time.

A gene candidate screen could also be run in order to see what specific genes are playing a role in epigenetics of environmental stress in order to get a better understanding of which pathways are upregulated or downregulated. We have started a list of candidate genes from previous *C. elegans* transgenerational epigenetic studies to give us a foundation of different pathways and possibly mechanism that can be playing important roles. For example, looking into levels of *wdr-5*, a member of the H3K4me3 complex, can show us if histone modifications are playing a role in transmitting thermotolerance (Greer et al., 2012). Another example of a candidate gene would be *rde-4*, which functions in recognizing and cleaving long-trigger dsRNA molecules into siRNAs. *rde-4* is known to play a role in response to different stresses (Rechavi et al., 2014). If factor,

like genes and histones, can be identified with their modifications, then it can help determine how information, like thermotolerance, is being passed down generation to generation.

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