The effects of tumor growth on stress response, body size, and fat content in Caenorhabditis elegans

Reem Yassine
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Abstract
Tumorous growth affects nearby healthy tissues, as seen in wasting disease experienced by cancer patients, but the underlying mechanisms are not well understood. We studied tumor growth in *C. elegans*, a microscopic worm that shares many genes with humans and has the ability to grow tumors in its reproductive system. To test whether tumors cause stress in nearby tissues, we used genetic crosses to create strains containing tumors and fluorescent-based stress reporters for the following physiological stresses: endoplasmic reticulum unfolded protein response (*hsp-4p::GFP*), heat shock (*hsp-6.2p::GFP*), and infection (*irg-1p::GFP*). We found that expression of the *hsp-4p::GFP* reporter was inhibited by the presence of a tumor at baseline conditions. The *hsp-6.2p::GFP* reporter and the *irg-1p::GFP* reporter demonstrated no significant difference in expression between tumorous and non-tumorous worms at baseline. Stress reporter expression following acute heat shock was also examined. There was no significant difference in *hsp-4p::GFP* expression 24 hours following acute heat shock. In comparison to non-tumorous *C. elegans*, tumorous *C. elegans* demonstrated higher *hsp-16.2p::GFP* expression 4 hours following acute heat shock. We also found that tumorous *C. elegans* worms containing a tumor in their reproductive system have larger body size and greater fat content than do wildtype *C. elegans*.

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Department
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First Advisor
Hannah Seidel

Second Advisor
Aaron Liepman

Third Advisor
Marianne Laporte

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THE EFFECTS OF TUMOR GROWTH ON STRESS RESPONSE, BODY SIZE, AND FAT CONTENT IN *CAENORHABDITIS ELEGANS*

By

Reem Yassine

A Senior Thesis Submitted to the

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in Partial Fulfillment of the Requirement for Graduation

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ABSTRACT

Tumorous growth affects nearby healthy tissues, as seen in wasting disease experienced by cancer patients, but the underlying mechanisms are not well understood. We studied tumor growth in *C. elegans*, a microscopic worm that shares many genes with humans and has the ability to grow tumors in its reproductive system. To test whether tumors cause stress in nearby tissues, we used genetic crosses to create strains containing tumors and fluorescent-based stress reporters for the following physiological stresses: endoplasmic reticulum unfolded protein response (*hsp-4p::GFP*), heat shock (*hsp-16.2p::GFP*), and infection (*irg-1p::GFP*). We found that expression of the *hsp-4p::GFP* reporter was inhibited by the presence of a tumor at baseline conditions. The *hsp-16.2p::GFP* reporter and the *irg-1p::GFP* reporter demonstrated no significant difference in expression between tumorous and non-tumorous worms at baseline. Stress reporter expression following acute heat shock was also examined. There was no significant difference in *hsp-4p::GFP* expression 24 hours following acute heat shock. In comparison to non-tumorous *C. elegans*, tumorous *C. elegans* demonstrated higher *hsp-16.2p::GFP* expression 4 hours following acute heat shock. We also found that tumorous *C. elegans* worms containing a tumor in their reproductive system have larger body size and greater fat content than do wildtype *C. elegans*. 
INTRODUCTION

During normal cell growth, cell division is tightly regulated. Cells that are damaged, no longer necessary to the organism, or aging will undergo programmed cell death. In contrast, during abnormal cell growth, cell division is not regulated and results in cells evading programmed cell death leading to uncontrolled cell proliferation. While decreased cell death is one way that cells may over proliferate, increased division is another possible contributor to over proliferation. The abnormal cell mass that develops as a result of the unregulated division of cells is called a tumor. Certain types of cancer such as metastatic breast cancer arise when tumor cells metastasize, migrating to regions in the body other than their original location.

Many cancer patients experience wasting disease, in which they lose excessive amounts of fat and muscle tissue; clinically, this disease is known as cancer cachexia (Aversa 2017). The specific mechanisms by which tumors affect surrounding healthy tissue and contribute to wasting disease are not well understood. Recent studies have reported that the absence of fatty acids, which are the monomers of lipids, plays a vital role in cancer cachexia development (Silverio et al. 2011). A possible mechanism for cancer cachexia development is that a tumor acts as a ‘sink’ and drains surrounding healthy cells of their lipid and peptide building block components.

In addition to depleting lipid building blocks, it has also been found that tumors promote metastasis by expressing cell communication signals known as cytokines that promote tumor growth (McAllister and Weinberg 2010). Cytokines and various growth factors are utilized by the tumorous tissue to promote tumor expansion within the body.
(McAllister and Weinberg 2010). For example, inflammatory cytokines dependent on the unfolded protein response (UPR) promote an intracellular environment that enhances tumor proliferation (van Oosten-Hawl and Morimoto 2017).

Understanding the specific mechanisms underlying wasting disease, such as changes in cytokine communication and lipid monomer production, may allow scientists to develop more precise and improved treatments to combat wasting disease.

*Caenorhabditis elegans* is a Useful Model for Studying Tumors

*C. elegans* is a non-parasitic microscopic worm that shares many genes and tissue types with humans. The *C. elegans* genome contains homologs for 60–80% of human genes. This set includes genes regulating apoptosis, aging, cell signaling, cell cycle, and metabolism (Kyriakakis et al. 2015). *C. elegans* is also useful because certain mutant lines have the ability to grow a tumor within the reproductive system (Francis et al. 1995; Eckmann et al. 2004). *C. elegans* transparency facilitates viewing inner tissues at all life stages allowing for changes in body morphology to be visualized using a microscope (Figure 1) (Kyriakakis et al. 2015).
Figure 1: C. elegans Body Plan

(A) Cartoon representation of C. elegans hermaphrodite morphology when viewed with a light microscope. Adapted from (Zarkower 2006). (B) Light micrograph of a wild-type hermaphrodite showing the same structures.
Anatomy of the Hermaphrodite Gonad

*C. elegans* has two sexes: a XO male and a self-fertilizing XX hermaphrodite.

The hermaphrodite *C. elegans* reproductive system has two symmetrical U-shaped arms. At the tip of each gonad arm, there is a single large somatic cell known at the distal tip cell (DTC). The DTC promotes germ cell mitosis while inhibiting germ cell meiosis (Lints and Hall, 2009). The DTC forms a cap around 6-10 distalmost germ cells (Lints and Hall, 2009). In Figure 2, this cap is found within a region known as the ‘DTC niche’. Cells enter meiosis in the transition zone and then enter pachytene (Lints and Hall, 2009). Most stem cells undergo apoptosis in the loop region and any remaining cells develop into oocytes located in the proximal arm (Lints and Hall, 2009).

![Figure 2: Hermaphrodite C. elegans Distal and Proximal Parts of Single Gonad Arm.](image)

Figure based on (Narbonne et al., 2016).
Regulators of Germline Development in *C. elegans*

The GLP-1/Notch Signaling pathway is important for regulating cell fate during early *C. elegans* development. The GLP-1/Notch Signaling pathway inhibits the activity of the GLD-1 and GLD-2 pathways through the activity of two pumillo and FBF family proteins (PUF), FBF-1 and FBF-2 (Racher and Hansen 2012). The proteins encoded by *gld-3* and *nos-3* genes control important steps in germline development, including entry into meiosis (Eckmann et al., 2004). NOS-3 encodes a protein responsible for repressing the translation repressor of *gld-1*, a gene with a vital role in *C. elegans* meiosis (Eckmann et al., 2004). Both GLD-1 and GLD-2 pathways inhibit cell proliferation and promote entry into meiosis. A contiguous tumor in the gonad results when both *gld-3* and *nos-3* genes are mutated. For our experiment, the JK3182 double mutant strain was used, containing mutations in the *gld-3* and *nos-3* genes.

Figure 3: Meiotic Decision Entry in *C. elegans* Germline. Adapted from (Racher and Hansen 2012).
Phenotypic Effects of Tumor Growth in *C. elegans* Reproductive System

Tumors change the *C. elegans* reproductive system in a number of ways. Germline tumor cells, which are found strictly in the gonads, are mitotically active but fail to differentiate into gametes because meiosis does not occur (Kirienko et al., 2010). Because they do not produce gametes, tumorous animals are sterile. Tumorous animals have swollen gonads due to containing a large number of mitotic nuclei (Kirienko et al., 2010). The germ cells remain in mitosis, and the entire gonad fills with abnormally proliferating cells.

![Figure 4: Comparison of Non-tumorous (N2 strain) and Tumorous (JK3182 strain) Worm Phenotypes.](image)

**Figure 4:** Comparison of Non-tumorous (N2 strain) and Tumorous (JK3182 strain) Worm Phenotypes.
The Significance of Studying Stress Response in Nearby Healthy Tissues

One way tumors may affect healthy tissues is by causing stress response. A stress response is activated to help animals respond appropriately to and survive in a changing or hostile environment. Many organisms, including *C. elegans*, have developed stress responsive pathways that are regulated by exposure to specific environmental stressors (Dues et al. 2016). These pathways are designed to protect cells against a variety of stressors such as heat shock, oxidative stress, infection, and hypoxia.

Heat Stress Results in Activation of Heat Shock Response

Temperatures between 15°C to 25°C are considered within the normal physiological range for the *C. elegans* life cycle (Gómez-Orte et al., 2017). Temperatures above this range qualify as heat stress, resulting in activation of the heat shock response. The heat shock response serves the purpose of preventing cellular degeneration and increases thermal tolerance during heat shock (Rodriguez et al. 2013). Heat shock factor-1 (HSF-1) is a transcription factor that is upregulated in response to heat shock stress. HSF-1 regulates the expression of heat shock proteins (HSPs). HSPs are molecular chaperones responsible for preventing cellular damage during heat shock exposure. HSPs also control protein homeostasis, the process that regulates production, folding, transport, and degradation of proteins within and outside the cell (Rodriguez et al. 2013).

How Are Stress Responses Monitored?

Stress responses can be monitored using stress reporters. A stress reporter is a transgene that is attached to the regulatory sequence of a stress inducible gene of
interest. Stress reporters act as markers for activation of different stress responses. Reporter systems are often fluorescence based using fluorescent proteins to measure gene expression. We used a green fluorescent protein (GFP)-based reporter system to measure specific stress gene expression. GFP is commonly used for experimental purposes due to its characteristic fluorescence when activated by light of a specific wavelength. The GFP reporter developed in the early 1990’s is a derivative of a fluorescent protein from jellyfish (Mishin et al. 2008).

![Diagram of GFP-based Stress Reporter System](image)

**Figure 5:** GFP-based Stress Reporter System. (A) GFP gene insertion used to monitor stress gene expression. The GFP gene is inserted in a transgenic animal’s genome downstream of the promoter for the specific stress-induced gene of focus. (B) Stress responsive gene regulation. Transcription of a stress response gene is regulated by the presence or absence of specific stressor.
We constructed a transgene stress reporter for the heat shock protein 16.2 gene, *hsp-16.2*, (Link et al., 1999). *hsp-16.2* has been shown in previous studies to be induced not only by exposure to heat shock, but a variety of other physiological stresses such as superoxide-generating redox-cycling quinones and expression of human beta amyloid peptide (Link et al., 1999). The second stress reporter we examined is for the heat shock protein 4, *hsp-4* gene, that has also been found to be induced by heat shock (Dues et al. 2016). We hypothesized that both *hsp-16.2* and *hsp-4* gene, expression will be greatly upregulated in non-tumorous worms in response to acute heat shock exposure.

In addition to activation by heat shock, the *hsp-4* gene regulates a stress response pathway that is activated upon increased misfolded or unfolded proteins in the endoplasmic reticulum (ER). The ER is a cellular organelle responsible for protein secretion in the cell. Previous studies have demonstrated that increased ER stress response promotes life span longevity and improves immunity in *C. elegans* (Ron et al., 2007). It has been shown that bacterial infection upregulates the unfolded protein response (UPR) (Bischof et al., 2008). UPR maintains ER homeostasis through regulation of protein degradation and translation, mRNA degradation and chaperone protein production (Ron et al., 2007).

The third stress reporter we examined is for infection stress response. This stress reporter measured expression of the infection response gene-1 (*irg-1*), a gene that is induced by exposure to pathogens such as *Pseudomonas aeruginosa* (Dunbar et al., 2012). Upregulation of *irg-1* is accomplished using the *zip-2* bZIP transcription factor (Dunbar et al., 2012). In response to infection, the *zip-2/irg-1* pathway is upregulated and culminates in the inhibition of mRNA translation in the intestines (Dunbar et al., 2012).
Translational inhibition is a common pathogenic strategy because it may activate immune surveillance pathways that provide host protection. For our project, we examined infection stress reporter expression at baseline.

<table>
<thead>
<tr>
<th></th>
<th>Stress Type</th>
<th>Corresponding Stressor</th>
<th>Strain Code</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp-4</td>
<td>heat shock protein 4</td>
<td>ER unfolded stress, heat shock</td>
<td>SJ4005</td>
<td>(Dues et al. 2016)</td>
</tr>
<tr>
<td>hsp-16.2</td>
<td>heat shock protein 16.2</td>
<td>oxidative, heat shock</td>
<td>CL2070</td>
<td>(Link et al. 1999)</td>
</tr>
<tr>
<td>irg-1</td>
<td>infection response protein</td>
<td>infection, translation inhibition</td>
<td>AU133</td>
<td>(Dunbar et al. 2012)</td>
</tr>
</tbody>
</table>

Table 1: Strain Nomenclature for Each Reporter Gene and Corresponding Stressors.
Another way of monitoring how tumors affect nearby tissues is by examining body size. While collecting preliminary data, we observed that many tumorous animals seemed to have an elongated body shape, and the tumor had a dark and grainy appearance in the worm’s gonads. Previous research reasons that growth factors such as IGF-1 and TGF directly impact cell size by stimulating transcription and translation (Hirose et al. 2003). The phenomenon of a mutation causing increased organ length in *C. elegans* has been previously documented. For example, a mutation in the egg-laying defective 4 (*egl-4*) gene has been identified as the cause of increased cell volume and length in intestine, hypodermis and muscle cells of *C. elegans* (Hirose et al. 2003). We were curious to study if the presence of a tumor in the reproductive system caused by *gld-3(-) nos-3(-)* mutations would impact the overall body size of the worms. This is relevant because

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype Description</th>
<th>Tumor present?</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>wildtype</td>
<td>-</td>
</tr>
<tr>
<td>JK3182</td>
<td><em>gld-3(q730) nos-3(q650)/mln1 [mle14 dpy-10(e128)] II</em></td>
<td>yes</td>
</tr>
<tr>
<td>SJ4005</td>
<td>zcls4 [hsp-4::GFP] V</td>
<td>-</td>
</tr>
<tr>
<td>XHH5</td>
<td>JK3182 derivative containing zcls4 [hsp-4::GFP] V</td>
<td>yes</td>
</tr>
<tr>
<td>CI2070</td>
<td>dvl70 [hsp-16.2p::GFP + rol-6(su1006)]</td>
<td>-</td>
</tr>
<tr>
<td>XHH3</td>
<td>JK3182 derivative containing dvl70 [hsp-16.2p::GFP + rol-6(su1006)]</td>
<td>yes</td>
</tr>
<tr>
<td>AU133</td>
<td>agls17 [myo-2p::mCherry + irg-1p::GFP] IV</td>
<td>-</td>
</tr>
<tr>
<td>XHH1</td>
<td>JK3182 derivative containing agls17 [myo-2p::mCherry + irg-1p::GFP]</td>
<td>yes</td>
</tr>
</tbody>
</table>

Table 2: Descriptions of Strains Used

**Effect of Tumor Growth on Body Size**

Another way of monitoring how tumors affect nearby tissues is by examining body size. While collecting preliminary data, we observed that many tumorous animals seemed to have an elongated body shape, and the tumor had a dark and grainy appearance in the worm’s gonads. Previous research reasons that growth factors such as IGF-1 and TGF directly impact cell size by stimulating transcription and translation (Hirose et al. 2003). The phenomenon of a mutation causing increased organ length in *C. elegans* has been previously documented. For example, a mutation in the egg-laying defective 4 (*egl-4*) gene has been identified as the cause of increased cell volume and length in intestine, hypodermis and muscle cells of *C. elegans* (Hirose et al. 2003). We were curious to study if the presence of a tumor in the reproductive system caused by *gld-3(-) nos-3(-)* mutations would impact the overall body size of the worms. This is relevant because
abnormal cell proliferation in tumorous *C. elegans* may contribute to a significant
difference in body size compared to wildtype. Programmed cell death during normal cell
proliferation limits the number of cells competing for nutrients in the gonad (Kyriakakis
2015). If cells have more nutrients available, their growth may be enhanced in
comparison to tumorous cells, contributing to an overall larger body size for the wildtype.
However, it is also possible that tumor cell proliferation is so extensive that it results in
greater body size as compared to wildtype *C. elegans*.

**Effect of Tumor Growth on Lipid Content**

Another way of monitoring how tumors affect neighboring healthy tissues is by
measuring lipid content. Lipids serve many important functions in the cell, acting as
energy storage molecules, signaling molecules and contributing to the hydrophobic
component of cellular membranes. Lipid droplet formation in the ER requires fat storage-
inducing transmembrane protein-related (FITM-2) protein (Watts and Ristow et al.,
2017). Mutations in the *fitm-2* gene are lethal, indicating that lipid droplet formation is
essential to survival (Watts and Ristow et al., 2017). Due to the significant impact of lipid
content on cell survival, we hoped to gain an understanding of differences in lipid content
amount between non-tumorous and tumorous *C. elegans*.

**Overall Goal of Thesis Project**

As an attempt to better understand the mechanisms underlying wasting disease,
we studied the effects of tumor growth on stress response, body size and fat content in
neighboring healthy tissues of *C. elegans*. First, we examined the relationship between
tumor growth and stress experienced in nearby tissues at baseline. We constructed three worm strains that differ based on the specific reporter gene they contain. We used reporters specific for three types of cellular stress: unfolded proteins in the endoplasmic reticulum; infection; and heat. We wanted to learn if tumors cause nearby tissues to be stressed. We also wanted to learn if tumors inhibit stress response in nearby tissues. We hypothesized that the presence of a tumor will increase the stress response in nearby tissues at baseline. We then measured how tumor growth in one tissue causes changes in overall body size. Our hypothesis was that the body size of tumorous animals will be greater than wildtype animals. Thirdly, we measured the effect of tumor growth on altering stress response in animals exposed to heat stress. We hypothesized that tumorous animals exposed to heat stress will have a greater stress response than wild type animals exposed to the same stressor. Lastly, using Oil Red O, we examined changes in lipid content between non-tumorous and tumorous C. elegans. We hypothesized that the lipid content in nearby tissue would be depleted in tumorous animals compared to wild type animals.

Materials and Methods
Worm Maintenance
Worms were grown at 20°C on Nematode Growth Media (NGM) plates with Escherichia coli OP50 (Brenner, 1974) as the food source. NGM contained 25 ml/L 1 M potassium phosphate buffer (1 M K₂HPO₄ mixed with 1 M KH₂PO₄ to reach a pH of 6.0), 1 mM MgSO₄, 3 g/L NaCl, 2.5 g/L peptone, 20 g/L agar, 1 mM CaCl₂, and 5 μg/ml cholesterol (Seidel et al. 2018).
Desired Genotype Construction and Strain Confirmation

Strains

gld-3(q730) nos-3(q650)/mIn1[mls14 dpy-10(e128)] II (Eckmann et al., 2004), N2

Genetic crosses were used to create three strains of *C. elegans* containing tumors and different fluorescent-based stress reporters. Tumorous strains constructed included XHX5, XHX3, and XHX1 (Table 1). Non-tumorous strains included SJ4005, CL2070, and AU133 (Table 2).

The desired genotype was *gld-3(-) nos-3(-)* with the green balancer. JK3376 adult males were crossed with larval stage 4 (L4) stress reporter positive hermaphrodites. This cross produced the F1 generation. Green F1 males were crossed with non-green F1 L4 hermaphrodites. F2s of the desired genotype were identified by examining the phenotypes of their F3 progeny. When screening for this genotype, we looked for worms that were tumorous, non-green, and non-dumpy. Next, worms homozygous for the stress reporter were identified. Collected F2 animals were either homozygous for the stress reporter, heterozygous for the stress reporter, or did not carry the stress reporter. The F2s that were homozygous for the stress reporter were kept. We selected for these worms by looking in the F3 broods. If an F2 was homozygous for the stress reporter, then all of the F3 animals carried the stress reporter. F3s were classified as carrying or not carrying a particular stress reporter by looking for certain phenotypes unique to each stress reporter. For example, worms homozygous for a *hsp-16.2p::GFP* stress reporter were selected for based on their Roller phenotype. This stress reporter has a linked marker gene that causes
the worms to have a distinct rolling movement rather than sinusoidal undulation. Plates that had both rollers and non-rollers indicated that the strain was heterozygous for the \textit{hsp-16.2p::GFP} stress reporter.

\begin{center}
\begin{tikzpicture}
  \node at (0,0) {green adult JK3376};
  \node at (1.5,-1) {L4 stress reporter (+) hermaphrodite};
  \node at (0,-2) {F1 progeny};
  \node at (1.5,-3) {green F1 males};
  \node at (0,-4) {non-green F1 L4 hermaphrodite};
  \node at (0,-5) {F2 progeny};
  \node at (1.5,-6) {single out green F2 L4 hermaphrodites each onto separate plates};
  \node at (0,-7) {F3 progeny};
  \node at (1.5,-8) {screen F2 progeny for stress reporter homozygosity by examing F3 broods};
  \node at (1.5,-9) {look for phenotype specific to stress reporter in F3};
\end{tikzpicture}
\end{center}
Figure 6: Diagram of Genotype Construction and Strain Confirmation

GFP Balancer

Due to the tumorous strain being sterile, we used a genetic balancer to maintain the strain. The green fluorescent protein component of the desired genotype was maintained throughout multiple generations using a genetic construct known as a balancer. A balancer is a chromosomal rearrangement that allows lethal or sterile mutations (i.e. a tumor) to be maintained and passed down in heterozygotes (Edgley 2006). The desired genotype for our project was the following:

<table>
<thead>
<tr>
<th>Chr. II</th>
<th>Chr. III</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gld-3(-) nos-3(-)</em></td>
<td>+ stress reporter + stress reporter</td>
</tr>
<tr>
<td>Green (GFP balancer)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>gld-3(-) nos-3(-)</th>
<th>gld - 3(-)nos - 3(-)&lt;br&gt;gld - 3(-)nos - 3(-)&lt;br&gt;tumorous, non-green,&lt;br&gt;non-dumpy, sterile&lt;br&gt;Green Balancer&lt;br&gt;non-tumorous, green,&lt;br&gt;non-dumpy, fertile&lt;br&gt;Green Balancer&lt;br&gt;non-tumorous, green,&lt;br&gt;dumpy, fertile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green Balancer</td>
<td>Green Balancer</td>
</tr>
<tr>
<td>gld - 3(-)nos - 3(-)</td>
<td>gld - 3(-)nos - 3(-)</td>
</tr>
</tbody>
</table>
Table 3: Chromosome II Punnett Square Presenting Possible Genotypes and Phenotypes

In the specific strain of *C. elegans* we employed, chromosome II contains the GFP balancer. Balancer chromosomes are composed of many chromosome inversions; these inversions disrupt synapsis between homologs and stops crossing over from occurring (Edgley 2006). One of the chromosome II homologs is a balancer chromosome that results in GFP expression and also contains functional copies of *gld-3* and *nos-3*. *C. elegans* hermaphrodites have the ability to reproduce without male fertilization because their reproductive system contains both oocytes and sperm; this process is referred to as self-fertilization. Worms that are heterozygous for the balancer (green, non-dumpy) were permitted to self-fertilize and the resulting *gld-3(-) nos-3(-)* homozygotes among the progeny were used for experimentation. Worms that were homozygous for the GFP balancer resulted in a dumpy (dpy) phenotype that may have negatively impacted fitness. A homozygous *gld-3(-) nos-3(-)* animal is non-green, tumorous and sterile. A worm with a heterozygous balancer will be green and maintain its fertility so that the *gld-3(-) nos-3(-)* genotype may be passed on to the progeny.

When maintaining the tumorous strain, we picked the green, non-dumpy and fertile worms that have a heterozygous GFP balancer genotype. These worms were then given time to “self”, producing their progeny. For the purpose of our experiments, we used the sterile, non-green and non-dumpy progeny because these are the tumorous worms.
When worms with this genotype self-fertilize, the following genotypes and phenotypes are possible:

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Phenotypes</th>
</tr>
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<tbody>
<tr>
<td><strong>Chromosome II</strong></td>
<td><strong>Chromosome III</strong></td>
</tr>
<tr>
<td>$\text{gld-3(-)}$ $\text{nas-3(-)}$</td>
<td>$\text{gld-3(-)}$ $\text{nas-3(-)}$</td>
</tr>
<tr>
<td>$\text{gld-3(-)}$ $\text{nas-3(-)}$</td>
<td>$\text{gld-3(-)}$ $\text{nas-3(-)}$</td>
</tr>
<tr>
<td>$\text{Green balancer}$ $\text{stress reporter}$</td>
<td>$\text{Green balancer}$ $\text{stress reporter}$</td>
</tr>
<tr>
<td>$\text{Green balancer}$ $\text{stress reporter}$</td>
<td>$\text{Green balancer}$ $\text{stress reporter}$</td>
</tr>
</tbody>
</table>

Table 4: Genetic Crosses and Possible Genotypes.
Baseline vs. Stress Conditions

During baseline conditions, tumorous and non-tumorous *C. elegans* containing the same stress reporter were grown on NGM plates spotted with *Escherichia coli* OP50 (Brenner, 1974) at 20°C and were not exposed to any physiological stressors. During heat stress conditions, *C. elegans* that were initially grown on NGM plates spotted with *Escherichia coli* OP50 (Brenner, 1974) at 20°C were transferred to a 30°C incubator for a duration of 2 hours. Following acute heat shock exposure, the samples were returned to 20°C for recovery. Tumorous and non-tumorous samples containing a specific stress reporter were imaged at two different recovery times, 24 hours or 4 hours following heat shock.

Measurement of Stress Response Using Fluorescence Microscopy

In order to test whether a tumor causes a change in stress gene expression at baseline or following stress conditions such as acute heat shock, staged worms were imaged for GFP expression using a Nikon Epifluorescence microscope set to 10x magnification and an exposure time that did not yield saturation. The fluorescence level of each worm imaged was quantified using the software application ImageJ.

Lipid Content Assay Using Oil Red O

Tumorous and wild type worms were stained using Oil Red O (ORO) to quantify lipids in the intestines. ORO is a fat-soluble red colored dye that stains neutral lipids and
triglycerides. Worms were washed off plates into a 15 ml tube, using 3 ml 0.1%
PBS/Tween. Volume was then brought up to 10 ml with 0.1% PBS/Tween and tubes
were inverted to mix. Tubes were spun at 300 g in a swinging bucket rotor and the
supernatant was aspirated down to 0.5 ml. The washing and spinning at 300 g and
aspirating sequence was repeated twice. Worms were then transferred to a 1.5 ml tube
that was spun at 300 g for 30 seconds. The supernatant was removed. Worms were
incubated in 1 ml 1 X MRWB/2%PF containing 160mM KCl, 40mM NaCl, 30 mM
PIPES at pH of 7.4, and 50% NaOH for thirty minutes with rocking. Worms were spun at
300 g for 30 seconds and then washed two times with 1 ml 0.1% PBS/Tween. Worms
were then equilibrated with 1 ml 60% isopropanol in nanopure water by incubation with
rocking for 15 minutes. Following the equilibration step, worms were settled to bottom of
tube by gravity and the supernatant was aspirated. An 1 mL solution of ORO containing
600 µL of 0.5% Oil Red O in isopropanol and 400 µL of nanopure water was incubated
for 15-60 minutes. Following incubation, ORO solution was filtered through a 0.22 µm
pore filter and then added immediately to worm tubes. Using a Nikon DS-Fi1 camera,
bright field images were taken of N2 and JK3182 worms. To compare staining of lipids
between worm strains, we took red-green-blue images of each worm and then compared
the ratio of red channel to green and blue channels combined.

Body Size Assay

Body sizes of tumorous and wild type animals were compared over the course of
four days. Worms were initially picked as L4s and grown at 20°C on NGM plates spotted
with Escherichia coli OP50 (Brenner, 1974). Body size was measured each of four
consecutive days by using a 2MP USB eyepiece digital camera (#MD200L AmScope) and ImageJ to quantify images.

Results

1. Body Size Comparison

We conducted the body size experiment to analyze if there is a difference in body size between tumorous and non-tumorous *C. elegans* over the course of four days. Results demonstrate that tumorous *C. elegans* on average have a larger body size than wildtype *C. elegans* during days 2 through 4 of adulthood (Fig. 7).

![Body Size of Non-tumorous (N2) and Tumorous (JK3182) Worms During Adulthood](image.png)

**Figure 7.** Body Size of Non-tumorous (N2) and Tumorous (JK3182) Worms During Adulthood
2. Stress Reporter Expression During Baseline Conditions

We were also interested in comparing baseline stress reporter exposure between tumorous and non-tumorous *C. elegans*. We first compared baseline ER unfolded protein response stress reporter expression for non-tumorous and tumorous worms. The purpose of this experiment was to understand if the presence of a tumor causes nearby healthy tissue to express the ER unfolded protein response gene, leading to increased expression of HSP-4 protein. Using epifluorescence microscopy, we compared whole-body green fluorescence intensity in non-tumorous and tumorous worms containing the *hsp-4p::GFP* stress reporter. We also noted local fluorescence intensity differences between non-tumorous and tumorous worms; non-tumorous worms demonstrated higher fluorescence intensity in the pharynx, mid-body and rectum regions (Fig. 8). The data suggest that there may be a significant difference in gene expression related to the ER unfolded protein response between non-tumorous and tumorous worms (Fig. 8). Specifically, it appears that the presence of a tumor inhibits the ER unfolded protein response.
We then compared baseline infection stress reporter expression for non-tumorous and tumorous worms. The purpose of this experiment was to understand if the presence of a tumor causes nearby healthy tissues to express the infection response gene, leading to increased activation of immune protective pathways. Using epifluorescence microscopy, we imaged non-tumorous and tumorous worms that both contained the *irg-1p::GFP* reporter gene and compared whole body fluorescence between the two strains. No local intensity differences were observed between the two strains. The data
demonstrate that the presence of a tumor does not increase or inhibit infection stress gene expression (Fig.9). There is no significant difference between infection stress reporter gene expression in tumorous and non-tumorous worms.

Figure 9: Baseline irg-1p::GFP Stress Reporter Gene Expression. (A) Baseline stress reporter expression for non-tumorous (AU133) and tumorous (XHX1) worms containing the infection and translation inhibition stress reporter. Both strains contain the irg-1p::GFP reporter gene and were imaged using an epifluorescence microscope at 10X magnification and 200 ms exposure time. * p = 0.061 by t-test.

(B) Comparison of non-tumorous (AU133) and tumorous (XHX1) images taken at 200 ms exposure time and 10X magnification.

Next, we examined baseline heat shock stress reporter expression for non-tumorous and tumorous worms (Fig. 10). Both strains contained the hsp-16.2p::GFP reporter gene. We imaged the strains using epifluorescence microscopy and then
compared GFP expression using ImageJ. Similar to the infection stress reporter results, there was no significant difference in heat shock stress reporter expression between tumorous and non-tumorous strains. This result demonstrates that the presence of a tumor does not increase or inhibit HSP-16.2 expression.

Figure 10: Baseline hsp-16.2p::GFP Stress Reporter Gene Expression. (A) Baseline stress reporter expression for non-tumorous (CL2070) and tumorous (XHX3) worms containing the heat shock stress reporter. Both strains contain the hsp-16.2p::GFP reporter gene and were imaged using an epifluorescence microscope at 10X magnification and 450 ms exposure time. *p = 0.806 by t-test. (B) Comparison of non-tumorous (CL2070) and tumorous (XHX3) images taken at 450 ms exposure time and 10X magnification.
3. Stress Reporter Expression Following Heat Shock

After assessing baseline reporter expression, we decided to test stress reporter expression following acute heat shock exposure. We examined hsp-16.2p::GFP reporter expression four hours following acute heat shock exposure. We exposed tumorous and non-tumorous worms, both containing the heat shock stress reporter to a heat shock treatment at 30°C for 2 hours. Worms were then allowed to recover at 20°C for 4 hours. Following the recovery period, GFP fluorescence of worms was imaged using epifluorescence microscopy. The data (Fig. 11) demonstrate that there is a significant difference between tumorous and non-tumorous worms HSP-16.2 protein expression at 4 hours following acute heat shock. The tumorous strain demonstrated higher hsp-16.2p::GFP reporter expression than the non-tumorous strain. We also studied how exposure to acute heat shock impacts ER unfolded protein stress reporter, hsp-4p::GFP, expression 24 hours following acute heat shock exposure. We exposed both strains to a 30°C heat shock condition for 2 hours. Worms were then allowed to recover at 20°C for 24 hours. After 24 hours, GFP fluorescence of worms was imaged using epifluorescence microscopy. The data (Fig. 12) demonstrate there is no significant difference between tumorous and non-tumorous worms HSP-4 protein expression at 24 hours following acute heat shock.
Figure 11: *hsp-16.2p::GFP* Reporter Expression 4 hours Following Heat Shock. *hsp-16.2p::GFP* reporter expression for non-tumorous (CL2070) and tumorous (XHX3) worms 4 hours following heat shock. *p < 0.001* for two sample t-test. *p < 0.006* for control groups by t-test.

Figure 12: *hsp-4p::GFP* Reporter Expression 24 Hours Following Heat Shock. *hsp-4p::GFP* expression for non-tumorous (SJ4005) and tumorous (XHX5) worms 24 hours following heat shock. *p = 0.08* for experimental groups by t-test. *p = 0.43* for control groups by t-test.
4. Using ORO to Assess Lipid Content

Lastly, to compare lipid content between tumorous and non-tumorous *C. elegans*, we stained both strains with a red neutral lipid dye known as ORO and took red-green-blue images of each worm. Fat content was quantified as the ratio of red channel to signal of green and blue channels combined (Fig. 13). The data demonstrates a significant difference between tumorous and non-tumorous *C. elegans* lipid content. Tumorous *C. elegans* have a higher ratio of red channel to the signals of green and blue channels combined, indicating greater lipid content in tumorous worms.

**Figure 13:** Oil Red O Stain of Non-tumorous and Tumorous Worms. A) Oil Red O lipid content ratio for non-tumorous (N2) and tumorous (JK3182) worms. Ratio of red channel to signal of green and blue channel was measured. All red-green-blue images were taken at 10X magnification using a Nikon DS-Fi1 camera. *p = 0.009 by t-test. B) Comparison of non-tumorous (N2) and tumorous (JK3182) worms stained with ORO and imaged at 10X magnification.
Discussion

Our results demonstrated that the presence of a tumor caused by \textit{gld-3(-)} \textit{nos-3(-)} mutations increases overall body size of \textit{C. elegans} (Fig. 7). These findings supported our hypothesis that tumorous worms have larger body size compared to wildtype worms. The significant difference in body size suggests that over proliferation of cells in the reproductive system contributes to an increased body size. While no previous studies have specifically examined the impact of double mutations in \textit{gld-3 nos-3} genes on overall body size, a recent study demonstrates that increased uterus size results from the presence of uterine tumor mass development in aging wildtype \textit{C. elegans} (Wang et al., 2017). This specific study brings attention to a limitation in our study in that specific differences in individual organ size between tumorous and non-tumorous \textit{C. elegans} were not measured. An idea for a future experiment would be to specifically measure and compare sizes of intestines and gonads between double mutant \textit{gld-3 nos-3} worms and wildtype worms. It would be interesting to measure if neighboring tissues, such as the intestine, are impacted by the presence of a tumor in the reproductive system. A study of that nature would help us better understand the impact of tumor growth on neighboring healthy tissues.

We found that there is a significant difference in baseline ER unfolded protein response expression between tumorous and non-tumorous \textit{C. elegans}. The presence of a tumor appears to be inhibiting the ER unfolded protein response (Fig 8.) at baseline conditions. This finding differs from the results for the infection gene stress response and the heat shock stress response at baseline. Data for the infection gene stress response and heat shock stress response indicated no significant difference in stress reporter expression
between tumorous and non-tumorous *C. elegans*. Previous studies have found that the inhibition of the ER unfolded protein response causes misfolding of important proteins and contributes to the development of disease (Carrell and Lomas, 1997). The inhibited hsp-4p::GFP reporter expression in tumorous *C. elegans* suggests inhibited unfolded protein response creates a molecular environment that is favorable to tumor growth and disease development.

We also examined hsp-4p::GFP stress reporter expression twenty-four hours following acute heat shock exposure. We chose a 24 hour recovery time because we were interested in examining the long term impact of heat shock on hsp-4 gene expression. Our results indicated no significant difference between tumorous and non-tumorous worm HSP-4 protein expression at twenty-four hours following acute heat shock. Current research demonstrates the importance of HSP-4 as an ER chaperone responsible for regulation of fasting-induced lipases that break down fat granules in response to starving conditions in *C. elegans* (Hyunsun et al., 2009). *C. elegans* containing a mutation in the hsp-4 gene experience impaired mobility due to decreased energy supply (Hyunsun et al., 2009). These findings suggest that the hsp-4 gene plays a large role in *C. elegans* energy homeostasis. We hypothesized that exposure to heat shock of 30°C for 2 hours would be sufficient to upregulate heat shock response in non-tumorous worms more in than tumorous worms. However, for the hsp-4p::GFP stress reporter, we did not observe any significant difference in expression between tumorous and non-tumorous animals following acute heat shock. A possible limitation to our experimental design is that our heat shock temperature conditions were not sufficient to upregulate heat shock response in either of the strains. Current literature suggests that 35°C rather than 30°C is a more
commonly used temperature for acute heat stress in *C. elegans* (Zevian and Yanowitz, 2015). An idea for future experiments is to reassess *hsp-4* and *hsp-16.2* stress reporter expression at higher acute heat shock experimental conditions such as 35°C and 37°C.

For the *hsp-16.2p::GFP* stress reporter, our results indicated that the tumorous strain had significantly higher reporter expression 4 hours following heat shock than the non-tumorous strain. These results prompt us to question whether the heat shock conditions were successfully inducing *hsp-16.2p::GFP* expression. In further read of the literature, we have learned that heat shock protein levels peak 4 hours into heat shock as opposed to 4 hours after heat shock (Jovic et al., 2017). Due to the discrepancies in heat shock temperature and duration, it is possible that our heat shock conditions were insufficient for inducing proper heat shock response. An idea for a future research experiment is to repeat our heat shock study for strains containing *hsp-16.2p::GFP* and *hsp-4p::GFP* stress reporters using a higher temperature and a longer exposure duration.

Our final experiment examined the difference between tumorous and non-tumorous *C. elegans* neutral lipid content using ORO dye. The results for the ORO stain indicate that *C. elegans* containing the double mutation in *gld-3* and *nos-3* genes have higher lipid content than do wildtype *C. elegans*. Interestingly, this finding rejects our original hypothesis that the presence of a tumor reduces lipid storage in nearby healthy tissues. Current literature indicates that obesity causes fat cells to malfunction and this may contribute to the development of cancer and metabolic disorders (Kyriakakis et al., 2015). Current literature also suggests that adipose tissue does not only serve the role of energy storage for organisms but also works as a metabolically active organ (Paz-Filho et al., 2011). In fact, lipid cells have been found to secrete chemical signals known as
adipocytokines that play a large role in cancer development (Paz-Filho et al., 2011). The two most common adipocytokines are leptin and adiponectin (Paz-Filho et al., 2011). Our finding that tumorous C. elegans have a greater amount of lipid content supports the idea that high lipid content in surrounding healthy tissue could contribute to the success of tumor development and tumor nourishment.
References


