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Shanti Nicole Gunda Bernstein

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CHARACTERIZATION OF REPRODUCTIVE TUMORS IN *CAENORHABDITIS ELEGANS*

By

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Abstract

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Introduction

Tumor characteristics

Organs are composed of specific types of cells and an extracellular matrix (ECM) that helps the organs to carry out their unique functions. Tumors arise in tissues as a result of uncontrolled cell division and are comprised of multiple cell types and an ECM, which come together to form something similar to an abnormal organ. Components of the tumor microenvironment, the surrounding cellular environment in which the tumor resides, can include fibroblasts, blood networks, ECM, and other relevant factors that could encourage tumor growth (Wang et al., 2017). Tumorous cells can also exploit the functions of non-tumorous cells that surround them to promote their growth (Egebald et al., 2010). For instance, fibroblasts that are stimulated by the tumor microenvironment can cause changes in the tumor-associated ECM and instigate tumor

progression via architectural and signaling interactions. Due to changes in the ECM that prompt the overproduction of fibrous cytoskeletal elements, such as intermediate filaments, tumors tend to become stiffer than the normal surrounding tissue, exerting physical pressure on surrounding healthy tissues as they expand. Pressure usually causes injury to the surrounding healthy tissue, leading to discomfort to the organism (Egebald et al., 2010).

Tumor cells at the edge of a tumor can also exploit the surrounding stromal architecture and prompt adjacent tissue stroma to cooperate and support the continued growth of the tumor (Tarin, 2012). Tumorous cells can lose their ability to respond to contact inhibition and continue to grow and proliferate in the absence of space needed to adequately support them. Some tumors may grow to capacity and seem to cease growth, but this varies amongst different types of tumors. Recent studies have proposed that tumors cause adjacent healthy tissue to enter a state that is intermediate between tumorous and healthy tissue through aberrant cell-cell interactions (Aran et al., 2017). Other studies suggest that intermediate filaments — fibrous support elements that help cells maintain their shape — are produced in greater numbers in cells in contact with tumors in order to counter physical stress exerted by tumors (Pirentis et al., 2015). Understanding how tumors create these effects in neighboring tissues is a critical step in understanding how tumors grow and spread within the body.

Caenorhabditis elegans as a tumor model

An excellent model for studying tumor-host interactions is the free-living roundworm *Caenorhabditis elegans. C. elegans* is a small (about 1 mm in length) free-living eukaryotic roundworm that is non-parasitic, has a short lifecycle of about one

week, and shares many genes and tissue types with humans (Kimble and Crittenden, 2005). *C. elegans* is also naturally transparent, and internal functions such as digestion and reproduction, as well as individual cells, can be observed in living specimens. *C. elegans* has been heavily studied for the past five decades and was the first multicellular organism to have its genome completely sequenced (as cited by Corsi et al., 2015). *C. elegans* serves as a choice genetic model for human diseases because many human genes that are associated with the onset of disease, such as those that are responsible for cell cycle regulation, have orthologs in *C. elegans* (Corsi et al., 2015).

In *C. elegans*, the number of sex chromosomes determines the sex. XO animals are males, and XX animals are hermaphrodites (Figure 1). Hermaphrodites can produce oocytes and sperm, and males produce only sperm (Hirsh et al., 1976). Hermaphrodites are able to fertilize their own oocytes with their own sperm, a process called self-fertilization. Hermaphrodites can also be fertilized by the sperm of a male through mating. Within the species, hermaphrodites are more numerous than males because they often reproduce alone more efficiently than by mating with males (Corsi et al., 2015). Entire populations of animals can be maintained solely by hermaphrodites due to their ability to self-fertilize. This feature is useful for geneticists because there is no introduction of genetic variation through mating.

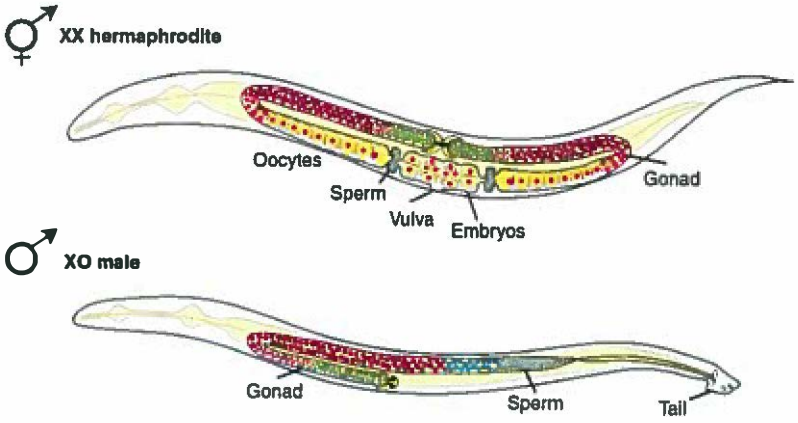

Figure 1: Comparison between hermaphrodite (XX) and male (XO) worm anatomy.
The hermaphrodite gonads are in the shape of two mirroring U-shaped tubes and contain both male and female reproductive structures that allows for self-fertilization. The males have only one U-shaped gonad that is capable only of producing sperm cells, making them capable of sexual reproduction with hermaphrodites. (Reprinted from Epigenetics, Second Edition, 2015).

Basic anatomy of C. elegans

The basic anatomy of *C. elegans* includes the internal organs covered by dermal layers (Figure 2). Anchored to the cuticle are striated, mononucleated body wall muscles and muscles involved in eating, egg laying, mating, and defecation (Moerman and Fire, 1997). The reproductive systems differ between the two sexes in that the gonad in hermaphrodites consists of two mirrored U-shaped tubes, whereas the gonad in the male consists of a single U-shaped tube. During development, hermaphrodites first form an ovotestis and later produce haploid sperm, which is stored in the spermatheca, and oocytes are produced later in adulthood (Corsi et al., 2015). In either sex, the cells undergoing development in the germline do so in a gradient, and observation of germline cells reveals all stages of meiosis and the transition from mitosis to meiosis (Figure 3) (Hubbard and Greenstein, 2005).

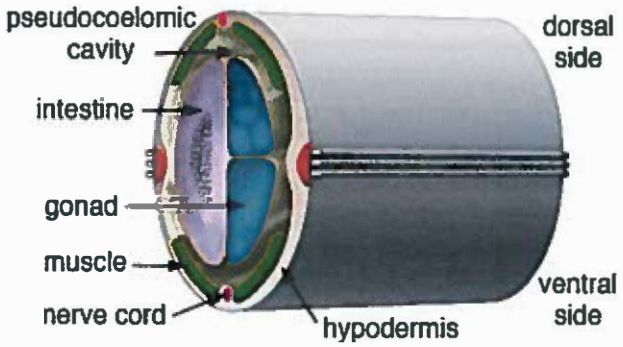

Figure 2: Cross section of *C. elegans*. (Reprinted from Altun and Hall, 2009).

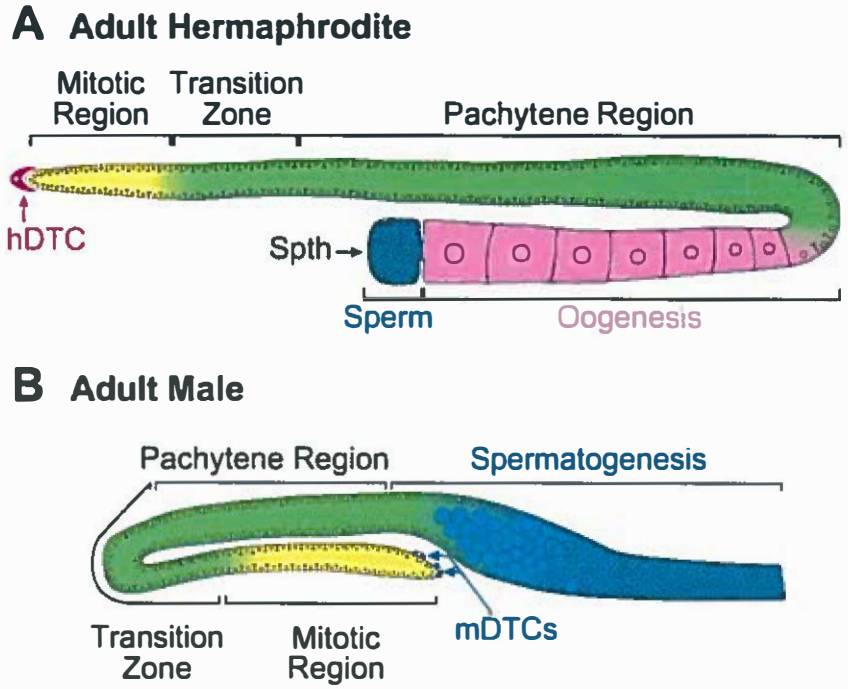

Figure 3: Spatial organization of stem cell differentiation from mitosis to meiosis (Reprinted from Morgan et al., 2010). (hDTC) hermaphrodite Distal Tip Cell; (mDTC) male Distal Tip Cell; (Spth) spematheca.

The C. elegans reproductive system and cell proliferation

The reproductive system in *C. elegans* males and hermaphrodites is organized spatially, which each section of the gonad contributing to a different stage of germ cell development (Figure 3). The mitotically active stem cells of the system are concentrated at the distal end of the gonad, where a distal tip cell functions to nurture them as they grow and divide. As the stem cells grow, they are pushed along the gonad toward the proximal end. As they travel along the organ, the cells exit mitosis and enter meiosis to differentiate into germ cells (Kirienko et al., 2014).

Germ cell development is controlled by a network of genes. The genes of interest in our study are germline proliferation protein 1 (*glp-1*), germline development proteins 1, 2, and 3 (*gld-1, gld-2, gld-3*), and nanos 3 (*nos-3*) (Pepper et al., 2003; Eckmann et al., 2004). In wildtype animals, *glp-1* functions to promote stem cell fate by signaling for controlled division of these cells. The gene combinations of *gld-2 gld-1* and *gld-3 nos-3* in wildtype animals function in tandem in to inhibit stem cell production and prevent their overgrowth (Kraemer et al., 1999; Lee and Schedl, 2010; UniProtKB). The effects of the stem cell promoter and the inhibitors in wildtype animals function coordinately in the Notch signaling pathway to control stem cell growth and prevent over proliferation (Figure 4).

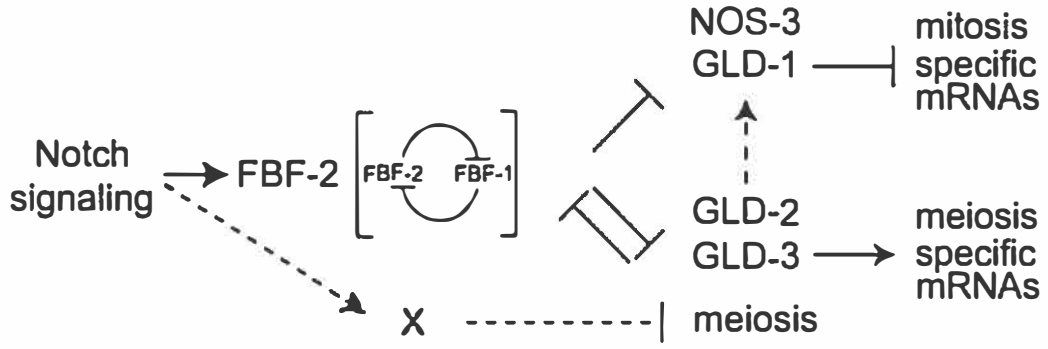

Figure 4: GLD-1, GLD-2, GLD-3 and NOS-3 all have roles in the Notch signaling pathway and dictate the transition from mitosis to meiosis in germline cells (Reprinted from Kimble and Crittenden, 2005).

Within the *C. elegans* germline, tumors form when select genes encoding regulators of germ cell development are mutated. For example, a gain of function mutation in *glp-1*, results in stem cells receiving strong and consistent signals to grow and divide. These signals contribute to over proliferation and stem cell-like cells fill the animal's gonad. The gene combinations *gld-2 gld-1* and *gld-3 nos-3* can experience a loss-of-function mutation (lf), which causes the genes lose their ability to inhibit stem cell growth. Without inhibition, the stem cells are able to proliferate into the gonad and fill the system with stem cell-like cells (Eckmann et al., 2004; Hansen et al., 2004).

The result of either types of mutation in these genes results in a germline tumor (Kimble and Crittenden, 2005). The germline tumors that arise as a result of these sorts of mutations are considered contiguous tumors (Figure 5B), which are tumors that are comprised completely of dividing stem cell-like cells (Kirienko et al., 2014).

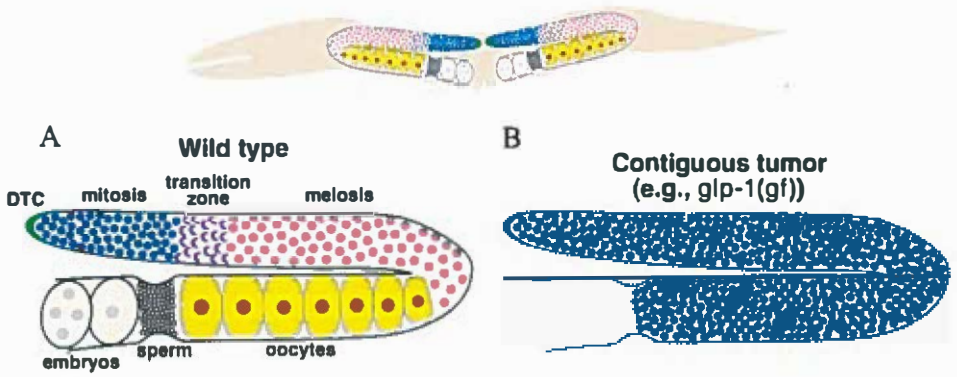

Figure 5: Germline tumor progression from proximal to contiguous.
This figure presents the wild type worm, which has germline cells that transition from mitosis to meiosis. However, in the tumorous germlines, specifically those that harbor contiguous tumors, there is no transition zone and no cells entering meiosis, yielding a nonfunctional gonad. (Reprinted from Kirienko et a. 2014).

Research hypotheses and predictions

Although tumors in *C. elegans* have been well characterized with respect to their formation, little is known about the effects of rupture of these tumors or the pressure of

tumors on other tissues, such as the intestine. This knowledge is important to understand how tumors affect adjacent healthy tissue in an organism. To build a better understanding of reproductive tumors in *C. elegans*, we carried out two sets of experiments focusing first in intestinal intermediate filament proteins and second on tumor rupture.

In our first set of experiments, we studied the arrangement of intermediate filament proteins located in the endotube of the intestine of wildtype and *gld-3 nos-3* tumorous hermaphrodites. Recent research suggests that when tumors expand, they harden at the edges and exert force onto the surrounding host tissue (Voutouri et al., 2014). This feature lead us to hypothesize that intermediate filaments in the intestine adjacent to a germline tumor will congregate at the site of tumor-intestine contact to counter the force of the tumor on intestinal cells.

In a second of set of experiments, we tracked tumor development in *glp-1, gld-2 gld-1,* and *gld-3 nos-3* tumorous animals over the course of their normal reproductive period. The goal of this experiment was to understand if reproductive tumors in *C. elegans* rupture. Rupture is defined as disruption gonad basal lamina, which is the structure that surrounds the gonad and separates the germline cells from the somatic cells (Killian and Hubbard, 2005). If these tumors do rupture, we next wanted to understand how factors like age, sex, and genotype affect tumor rupture. We hypothesized that germline tumors will rupture and that age, sex, and genotype will affect the rate and likelihood of rupture.

Materials and Methods

Animal maintenance

Animals were grown at 20 °C on nematode growth media (NGM) seeded with *Escherichia coli* OP50. NGM contained 3 g/L NaCl, 2.5 g/L peptone, 20 g/L agar, 25 ml/L 1 M potassium phosphate buffer (1 M K_2HPO_4 mixed with 1 M KH_2PO_4 to reach a pH of 6.0), 1 mM CaCl2, 1 mM MgSO4, and 5 µg/ml cholesterol (Seidel et al., 2018).

Strains

Strain name	Genotype	Reference
N2	Wildtype	Byrd et al., 2014
JK3182	*gld-3(q730) nos-3(q650)*	Eckmann et al., 2004
JK4299	*gld-2(q497) gld-1(q361)*	Hansen et al., 2004
JK3520	*unc-32(e189) glp-1(oz112 gf)*	Kershner et al., 2014

Animal synchronization

Gravid hermaphrodites and males of each strain, wildtype, *gld-3 nos-3*, *gld-2 gld-1*, and *gld-1*, were transferred to a sterile tube in M9 (3 g/L KH_2PO_4, 6 g/L $NaHPO_4$, 5 g/L NaCl, 1 mM $MgSO_4$). M9 was added to the sample in excess. A 2:1 solution of bleach and 5M NaOH was added to the sample and vortexed. The sample was incubated at room temperature for 6 minutes and vortexed every minute. The embryos were sedimented at 300 x g for 60 seconds, and the supernatant was aspirated down to 0.5 mL. The embryos were washed twice with excess M9 and the supernatant was aspirated after the second wash. The embryos that were left in the sample were placed onto NGM plates seeded with *E. coli*. The embryo plates were incubated at 20°C for 4 days.

Intermediate filament fluorescent staining

Hermaphrodite wildtype and *gld-3(-) nos-3(-)* animals were picked at larval stage 4 (L4) and placed on NGM seeded with *E. coli* OP50 at 20°C for 1-4 days. Animals were transferred to a dissection dish containing 1X PBS/0.1% Tween-20 0.25mM Levamisole and dissected with a scalpel by cutting at the head between the pharynx and the intestine. The samples were sedimented at 300 x g for 60 seconds. The supernatant was discarded and the samples were fixed in methanol for 10 minutes at -20°C. Samples were washed twice with excess 1X PBS/0.1% Tween-20 and blocked in 3% BSA in 1X PBS/0.1% Tween-20 on a rocker at room temperature for 30 minutes. Block was removed, and samples were incubated in monoclonal antibody MH33 (DSHB Hybridoma Product MH33; Karabinos et al., 2001), and diluted in 1:200 blocking buffer at 4°C overnight. The samples were sedimented, the primary antibody solution was removed, and the samples were washed three times with 1X PBS/0.1% Tween-20 with the supernatant removed after the third wash. Samples were incubated in anti-mouse Alexa 488 (Invitrogen, #A12379) diluted 1:100 in blocking buffer in the dark for 2 hours at room temperature. The samples were then pelleted at 300 x g for 60 seconds, the supernatant was removed, and the samples were washed three times with 1X PBS/0.1% Tween-20, with the supernatant removed after the third wash. DNA was stained by mounting sample in Vectashield containing DAPI (Vector Labs, #H-1200; Seidel et al., 2018) overnight at 4°C.

Tumor time course

Hermaphrodite N2, *gld-3(-) nos-3(-)*, *gld-2(-) gld-1(-)*, *glp-1(gf)* and male *gld-3(-) nos-3(-)* and *gld-2(-) gld-1(-)* animals were picked at larval 4 (L4) stage, placed onto

NGM or NGM with 2% DMSO, and incubated at 20°C. Animals were collected in 1X PBS/0.1% Tween-20 at 0-4 days post-L4 and fixed with 3% paraformaldehyde in 1X PBS/0.1% Tween-20 at room temperature for 30 minutes. The animals were washed once with 1X PBS/0.1% Tween-20 and then fixed with methanol for 15 minutes at -20°C. The animals were washed three times with 1X PBS/0.1% Tween-20, and the supernatant was discarded after the third wash. The samples were mounted and their DNA was stained using Vectashield with DAPI (Vector Labs, #H-1200; Seidel et al., 2018).

Fluorescence imaging

The DNA of samples was visualized using an epifluorescence microscope equipped with a high-powered light source camera (Nikon Eclipse E400 Hg 100W; Photometrics CoolSNAP MYO). Tumor time course samples were defined as rupture if any tumorous cells were found outside of the gonad and loose within the body cavity.

Statistical analysis

Chi-square tests were used to determine statistical significance of tumor rupture rates between hermaphrodite genotypes and between sexes of the same genotype.

Results

IFB-2 in the endotube of the intestine does not change due to a germline tumor

Germline tumors may be able to inflict pressure onto adjacent tissue, like the cells of the intestine. Such pressure might cause intermediate filament proteins to rearrange themselves to counter physical stresses. We therefore stained the intermediate filament protein IFB-2 located in the endotube of the intestine of dissected tumorous hermaphrodites using immunohistochemistry methods to see if the presence of a germline

tumor affected the arrangement of IFB-2. We visualized the intestines with an epifluorescence microscope and did not observe any significant difference in IFB-2 distribution or intestinal morphology (Figure 5). Any aberrations in intestinal morphology were observed in both the wildtype and tumorous animals and were therefore not due to the germline tumor. These results indicate that the presence of a germline tumor does not affect the distribution of IFB-2 in the endotube of the intestine.

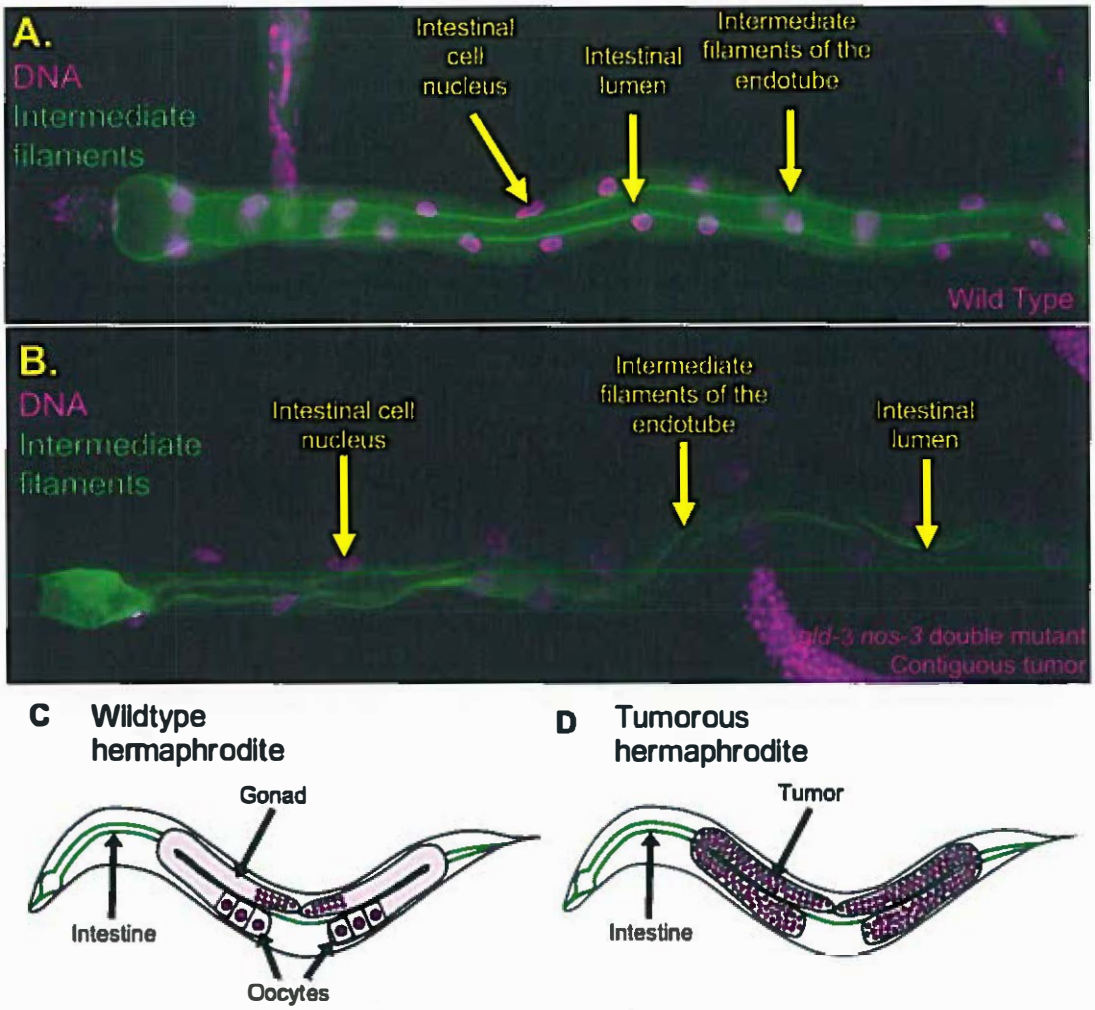

Figure 5: MH33 anti-IFB-2 stained intestine. (A) Wildtype animal. (B) *gld-3 nos-3* tumorous animal. The differences in intestinal morphology shown here were seen in both wildtype and *gld-3 nos-3* tumorous animals. Animals in panels A and B were dissected prior to tissue fix and the gonad is not shown. (C) Normal spatial arrangement of gonad and intestine in wildtype animals. (D) Normal spatial arrangement of tumor and intestine in tumorous animals.

Tumors in hermaphrodites rupture at different rates among tumorous animals

C. elegans hermaphrodites are capable of forming reproductive tumors when certain genes that function to control germline development are mutated. To understand if these tumors rupture, we tracked tumor development over the normal reproductive period of 5 days. On each day, we collected whole animals, fixed their tissues, stained their DNA, and visualized their cells with epifluorescence microscopy. We observed that rate of tumor rupture differed between the three tumorous genotypes tested and rupture generally increased with age (Figure 6). We also observed the *glp-1* and *gld-2 gld-1* tumors to have clusters of DNA that were not observed in the *gld-3 nos-3* tumors (Figure 7A and 7B). The *gld-2 gld-1* tumors also had abnormal nuclear morphology of tumorous cells that was not observed in the hermaphrodites of the other two tumorous genotypes (Figure 7C). These results demonstrate that the *gld-2 gld-1* and *glp-1* tumors rupture more often than the *gld-3 nos-3* tumors and that rupture increases with age. Other characteristics, such as tumor cell nuclear morphology and clusters of DNA, may also be dependent on genotype,.

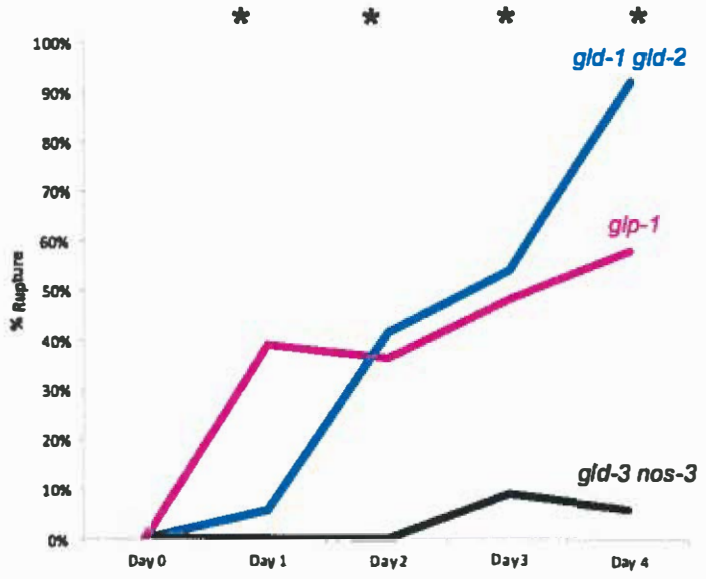

Figure 6: Percentage of ruptured hermaphrodite tumors of each genotype over normal reproductive period. N=33 to 508.
* $p<0.05$, Chi square test indicates statistical significance between each of the three genotypes at each time point.

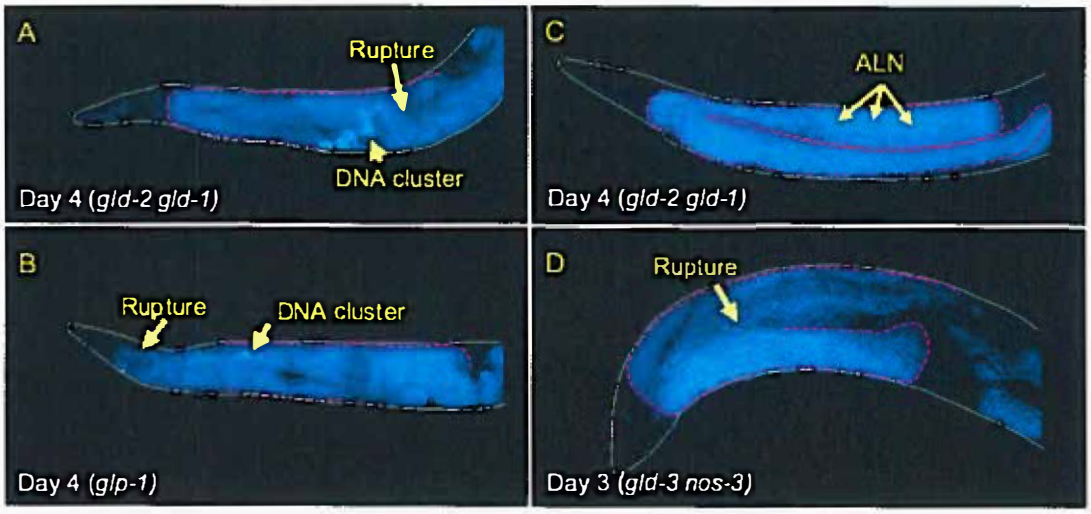

Figure 7: Rupture was identified by the presence of tumor cells within the body cavity. (A and B) *gld-2 gld-1* and *glp-1* tumors presented with clusters of DNA that were not seen in the *gld-3 nos-3* tumors. (C) *gld-2 gld-1* tumors had abnormally large nuclei of tumorous cells that was not seen in either of the other two genotypes of tumors. (D) Rupture was often identified by looking through different optical sections of the tumors at various depths.

Tumors in males seldom rupture

Rupture trends in the hermaphrodites of the three tumorous strains tested were found to be dependent on age and genotype, but it is unknown how sex affects rate of tumor rupture. We used the *gld-2 gld-1* and *gld-3 nos-3* tumorous males in a time course and tracked tumor development using the same methods used for the hermaphrodites. We found that the male tumors were less likely to rupture in comparison to the hermaphrodites (Figure 8). We also observed large clusters of DNA in the *gld-2 gld-1* males that appeared to promote large gaps in the tumor (Figure 9C) and were not observed in the *gld-2 gld-1* hermaphrodites. These clusters were not seen in the *gld-3 nos-3* male tumors, but these males did have abnormal nuclear morphology of the tumorous cells that were not seen in the *gld-3 nos-3* hermaphrodites (Figure 9A). These results indicate that the rate of tumor rupture in the three strains of tumorous *C. elegans*

tested is dependent on sex, as are other characteristics such as tumor cell nuclear morphology and clusters of DNA.

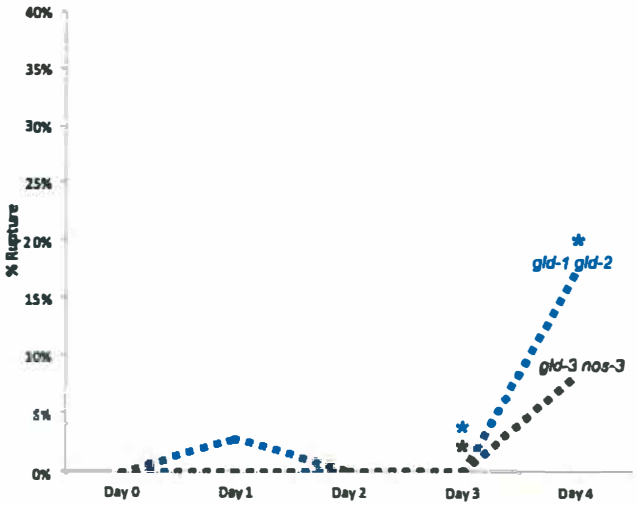

Figure 8: *gld-2 gld-1* and *gld-3 nos-3* tumors in males were much less likely to rupture than those of their hermaphrodite counterparts. N=21 to 116. * p<0.05, Chi square test of rate of rupture between males and rupture of hermaphrodites, represented in Figure 6. Differences were significant on Day 3 between *gld-2 gld-1* males and hermaphrodites and *gld-3 nos-3* males and hermaphrodites., Rupture rates were also statistically different on Day 4 between *gld-2 gld-1* males and hermaphrodites.

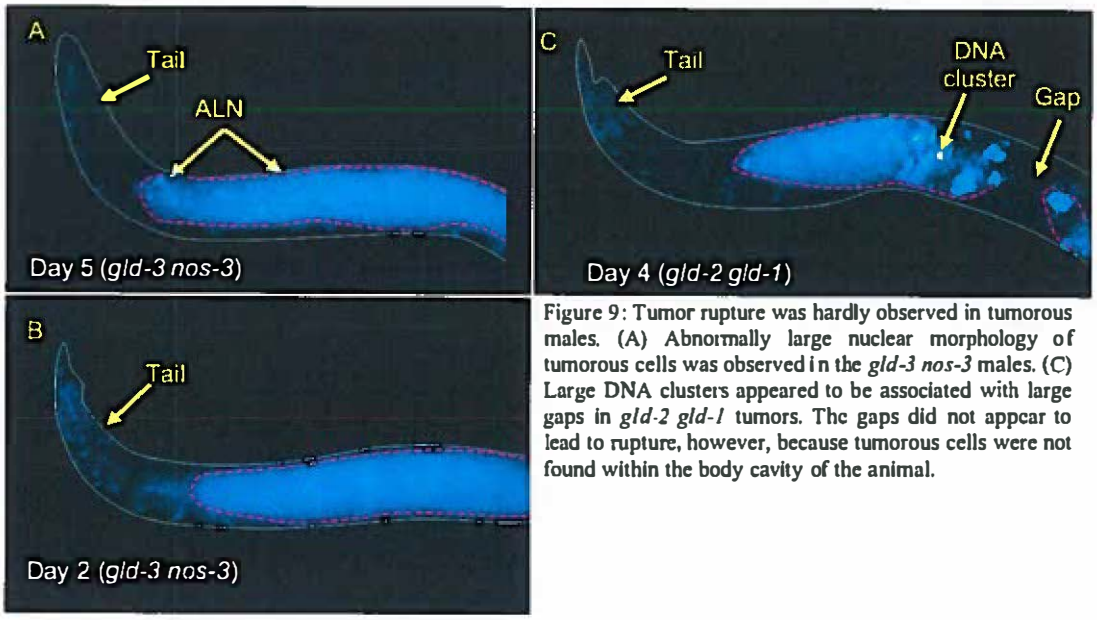

Figure 9: Tumor rupture was hardly observed in tumorous males. (A) Abnormally large nuclear morphology of tumorous cells was observed in the *gld-3 nos-3* males. (C) Large DNA clusters appeared to be associated with large gaps in *gld-2 gld-1* tumors. The gaps did not appear to lead to rupture, however, because tumorous cells were not found within the body cavity of the animal.

Discussion

The purpose of this study was to establish an understanding of the effects of germline tumors on an adjacent tissue and the tumor rupture trends using three tumorous strains of *C. elegans*. We performed two sets of experiments and the first examined the distribution of intermediate filament protein IFB-2 in the endotube of the intestine in the presence of a germline tumor. Through immunohistochemistry and the use of epifluorescence microscopy, we found that the presence of a germline tumor does not affect the distribution of IFB-2. In *C. elegans*, the endotube is part of the apical portion of the intestine that faces the lumen of the intestine. The basolateral portion of the intestine faces the cells of the germline and would most likely experience pressure by the stiff and growing germline tumor in tumorous animals (Huang et al., 2003). From our data, IFB-2 is likely not affected by the germline tumor because the apical surface of intestinal cells is not in direct contact with the tumor. However, other intermediate filament proteins, such as IFB2, IFD2, and IFC1, may be affected by the presence of a germline tumor (Karbinos et al., 2004). Immunohistochemistry methods on laminins found in the basement membrane surrounding the basolateral surface of the intestine could also show aberrant distribution of laminins in response to pressure inflicted by a germline tumor (Huang et al., 2003).

In a second set of experiments, we focused on understanding rate of tumor rupture in three tumorous genotypes of *C. elegans* and some factors that may influence rupture. We examined tumors for signs of rupture using a time course to track tumor development over the normal reproductive period of hermaphrodite and male tumorous animals. We visualized the cells of tumors with epifluorescence microscopy and found that germline

tumors in *C. elegans* are capable of rupture. Our data suggest that tumor rupture is dependent on age, genotype, and sex. Like humans, *C. elegans* experiences morphological changes as it ages. Tumor rupture is likely increased in older animals due to weakening interactions between the cells the cover the tumor (Herndon et al., 2018).

Understanding how sex affects tumor rupture is more complex. In males, the single U-shaped gonad is the site of germ cell development, which occurs in a gradient from the stem cells in the distal end to spermatocytes at the proximal end. The proximal end of the gonad is joined to the somatic gonad, which is comprised of the seminal vesicle and vas deferens (Figure 10). The structures of the somatic gonad are composed of thick cells, whereas a thinner layer of cells called the basal lamina covers the hermaphrodite gonad. These morphological differences may explain why the male gonad doesn't rupture when a germline tumor forms (Lints and Hall, 2009). Our data suggest that tumor cells enter the somatic gonad, but we are uncertain as to whether these cells continue to proliferate or exit the animal through the cloacal opening at the end of the vas deferens. Observation of tumorous males with a fluorescent transgene expressed in tumorous cells may allow us to identify escaped tumor cells from live animals.

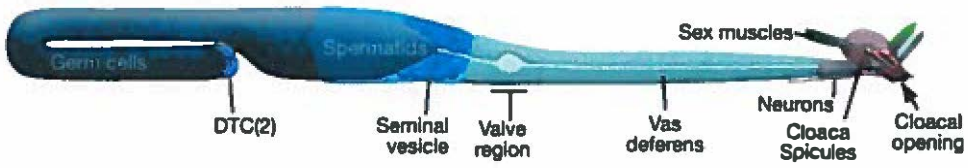

Figure 10: Anatomical structures associated with the male gonad. The seminal vesicle and vas deferens are part of the somatic gonad (reprinted from Lints and Hall, 2009).

The genes of interest in this study work in conjunction with other genes to maintain the germline and promote production of germ cells. *gld-1*, *gld-2*, and *nos-3* are

each involved in germline progression and regulate the transition of developing cells from mitosis to meiosis in both males and hermaphrodites (Kraemer et al., 1999; Lee and Schedl 2010; UniProtKB). *gld-3* is required for survival of the germline and completion of meiosis in both sexes (Eckmann et al., 2014). There are data that suggest that some sexual dimorphism exists between the male and hermaphrodite distal gonad, the area where the stem cells are housed, and that in males the mitotic cell cycle of these cells is shorter than that seen in hermaphrodites (Morgan et al., 2010). These differences in sexes could result in differences in cell signaling by germline genes, providing a possible explanation for the differences we observed in tumor rupture between males and hermaphrodites.

In the future, we want to continue to learn more about the tumor characteristics we observed in the three tumorous strains studied, such as abnormal nuclear morphology of tumor cells, large DNA clusters, and gaps in the tumors. We also want to understand how tumor rupture is affected when the animal is exposed to stressors, like starvation or chemical compounds like dimethyl sulfoxide (DMSO). Starvation is a stressor that causes stem cells in the hermaphrodite germline to stop dividing and we want to understand if there are similar effects on the stem cell-like cells of germline tumors (Salinas et al., 2006).

DMSO is a compound commonly used to enhance drug administration because the compound makes membranes more permeable to the drug of interest (Capriotti and Capriotti, 2012). Data on Chinese hamster cells indicates that DMSO is able to increase membrane permeability by weakening the calcium-dependent cadherins that form barriers between cells, allowing substances to pass (Fiore et al., 2002). Cells exposed to

DMSO have also been shown to arrest in phase G_1, which is a point in the cell cycle associated with cell growth, but also a point where cells can enter G_0, if necessary (Fiore et al., 2002). Due to this effect on the cell cycle, dysfunctional growth inhibition features, like contact inhibition, become restored when exposed to DMSO. For this reason, we believe that growing tumorous *C. elegans* on media infused with DMSO will inhibit tumor cell division and decrease tumor rupture rates.

We would also like to study the effects ruptured tumor cells have on surrounding healthy tissues. We are uncertain as to whether or not tumor cells are able to continue to grow and divide once they've left the gonad and entered the pseudocoelomic cavity. Recent data indicate that germ cells that enter the body cavity in *C. elegans* can prompt muscle cells to enwrap them, similar to that of the distal tip cell in the germline (Gordon et al., 2019). These germ cells are causing the muscle cells to function in a way that is completely different than that of its normal function and we would like to understand if cells of ruptured germline tumors are capable of inducing similar changes. A better understanding of these cellular interactions could establish *C. elegans* a good model for tumor metastasis.

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