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Understanding the role of sog-4 and sog-6 in the GLP-1/ NOTCH Signaling Pathway

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Understanding the role of *sog-4* and *sog-6 in* the GLP-1/ NOTCH Signaling Pathway

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Abstract

Notch signaling is a highly conserved signaling mechanism that is important for many developmental processes in animals. In the roundworm, *Caenorhabditis elegans (C. elegans)*, GLP-1 signaling, a form of Notch signaling, is necessary for mitotic proliferation of the germline. *glp-1(ts)* mutants display a sterile phenotype at 20 °C. Previously, 14 extragenic suppressors were found that rescued the embryonic and germline temperature sensitive defects caused by improper functioning of GLP in a *glp-1(ts)* mutant. These mutations were mapped to six genes. These genes are referred to as suppressors of g*lp-1* or *sog* mutants. The current study serves to determine the identities of two of these genes, *sog-4* and *sog-6*, at the molecular level using whole genome sequence analyses and RNA interference experiments. Whole genome sequence data support the possibility that *sog-4* may correspond *oac-49*, while RNAi results suggest that *sog-4* is not *oac-49*, a gene whose function is to regulate protein turnover. Both Whole genome sequence data and RNAi data support the possibility that *sog-6* may correspond to F28D1.2. Understanding how *sog-4* and *sog-6* function to regulate the GLP-1/ Notch pathway can give meaningful insight as to how they can be used to regulate diseases that result from improper Notch signaling.

Executive Summary

Cell signaling is the mechanism by which cells communicate with one another. In order to make sense of and respond to changes in their environment, cells need to be able to receive signals and process these signals correctly. Notch signaling is an evolutionary conserved mechanism that is important in the decision of cell proliferation versus differentiation. In mammals, Notch signaling is important for proper development of a variety of organs. Notch gene mutations can result in improper functioning of cells, often leading to a variety of diseases, which include T-cell acute Lymphoblastic Leukemia/ Lymphoma, Multiple Sclerosis, and Alagille Syndrome.

Notch signaling is common to all metazoan organisms; therefore, the model organism *C. elegans* can be used to elucidate the components of the pathway and their functions. What has been determined previously is that Notch signaling involves a ligand or signal present on the outside of one cell that binds with a Notch receptor present on an adjacent cell. Binding of the signal to the receptor results in removal of the intracellular portion of the receptor. This inner portion goes to the nucleus where it binds to DNA and activates the expression of target genes.

There are two Notch genes in *C. elegans, lin-12* and *glp-1*. *glp-1* controls the process by which germline stem cells switch from mitotic division to meiotic division. A loss of function mutation, *glp-1(0),* prevents *glp-1* signaling from occurring, so the germ line stem cells are unable to proliferate. They are still able to undergo meiosis, but they only make sperm. As a result of this, the hermaphrodites are sterile.

Previous experiments discovered genetic suppressors of a *glp-1(ts)* temperature sensitive mutant that partially rescued the *glp-1* sterile phenotype and embryonic defect found in these worms. These mutations were mapped to 6 gene regions. These genes are referred to as

suppressors of *glp-1* or *sog*. However, the genetic identities of most of these *sog* mutants are unknown. Therefore, the goal of this project is to determine the identities of two of these suppressor genes, *sog-4* and *sog-6*, by whole genome sequencing (WGS) and RNA interference (RNAi). By sequencing the genome of two alleles of *sog-4, sog-4(q304)* and *sog-4(q301),* the whole genome sequences of *sog-4(q304)* and *sog-4(q301)* can be compared to each other to identify the gene in which both alleles are mutated. This gene would be the candidate for *sog-4*. The same procedure is also performed with *sog-6* alleles.

In addition to sequencing, RNA interference was also carried out. RNAi prevents a particular gene from functioning by degrading the mRNA sequence it encodes, and therefore preventing proteins from being made. In this study, RNAi is used to knock-down the products of the candidate genes discovered by WGS. This procedure is conducted on either *glp-1(ts)* mutants or *glp-1(gf)* gain of function mutants. The knockdown phenotypes of each candidate gene are analyzed to determine their effects on *glp-1* sterility. The loss of *sog* gene function should suppress a *glp-1(ts)* sterile phenotype and enhance a *glp-1(gf)* sterile phenotype.

If similar results are found from both sequencing data and RNAi experiments, it can then be hypothesized that *sog-4* or *sog-6* corresponds to the particular gene being tested. Moreover, once each *sog* gene has been identified, additional experiments can then be carried out to elucidate each gene's function within the Notch pathway.

In short, these experiments are both important and relevant because, as mentioned previously, Notch is common to both *C. elegans* and human. Whereas improper signaling in *C. elegans* can result in sterile worms, improper signaling in humans can result in a variety of diseases. However, although the basic components of the Notch pathway have been identified, there are still components within the pathway that function as regulators, and are not necessary

for Notch signaling to occur. With that said, these experiments allow for the identification of two regulators of Notch in *C. elegans.* If these regulators are found to be novel to Notch signaling, then this can potentially allow for their discovery in human systems, where they can be used as potential treatments that regulate the severity of diseases caused by improper Notch signaling.

Table of Contents

Acknowledgments…….7

Advice to Future Honors Students…….8

Introduction……9

Methods…….25

Results……...28

Discussion……..…37

Works Cited……...……...40

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Advice to Future Honors Students

To members of the next graduating class, I have three pieces of advice for you. First, start working on the basic parts of your capstone by the end of junior year or by the beginning of senior year. This is extremely helpful because, the final semester can get extremely hectic or even unpredictable. For me, I was still working on my results during my final semester, and getting this part done early meant I could dedicate more of my time to getting results. My second piece of advice, reach out for help if you need to. I constantly meet with my Advisor every week, and during these meetings I am able to discuss the progress of my thesis and ask for help and suggestions. She was able to offer a lot of support in terms of where to get information or how to present the information I had, so doing this was very helpful. Third and finally, back up everything. There have been several occasions when I have lost parts of my thesis draft. However, I have saved a copy for every day I make corrections or added new data, so when I lost my most recent copy, I could turn to the copy from the day before. Though I had to re-add all the changes that were made, this was definitely more ideal that rewriting everything.

Introduction

The development of cells to tissues, organs, organ systems, and later to a multicellular organism requires intricate short and long-range communication between cells. Developing cells must be competent, or able to respond to incoming signals they receive from their environment. Through the process of induction, cells can secrete signals, causing nearby cells to differentiate into new cell types (http://www.nature.com/scitable/topicpage/cell-differentiation-and-tissue-14046412). With that stated, Notch is a highly conserved signaling mechanism involved in many developmental and differentiation processes. Notch signaling permits neighboring cells to communicate with each other, which can produce many downstream responses, including cell-fate specification, progenitor cell maintenance, boundary formation, cell proliferation and apoptosis (Brou 2016). However, improper regulation of Notch signaling can occur, as a result of mutations in the Notch receptor, its ligand, or other genes known to function in the pathway (Brou 2016). These mutations can lead to a variety of cancers and neurodegenerative diseases (Louvi *et al.*, 2012). Therefore, a proper understanding of the components of this pathway and their function to regulate the pathway is necessary to understand how they are improperly regulated in these diseases. This knowledge can help to create proper therapies to treat these diseases.

Humans have four types of Notch receptors, NOTCH-1, NOTCH-2, NOTCH-3, and NOTCH-4 (Zhou 2010), whereas worms have two Notch receptors, LIN-12 and GLP-1 (Greenwald 2005). In order to understand how these receptors function in human development, we must first understand their regulation in simpler systems. With that said, *C. elegans* has been very useful model to study Notch signaling, as it has allowed for a better understanding of the structural components of this pathway and their biochemical functions.

C. elegans as a Model Organism

C. elegans is a free-living roundworm that is about 1mm in size as an adult (Worm Classroom). It is usually found in soil environments where it feeds on microbes that eat dead or decaying plant material. It has a fully sequenced genome and approximately 35% of its genome encodes proteins similar to human proteins (Worm Classroom). Additionally, *C. elegans* shares many processes in common with humans, such as embryonic development, morphogenesis, aging, and nerve function (Alton and Hall 2009). Therefore, it is a useful model to study these processes.

Coupled with this, the roundworm has several features that allow for its study in lab settings. Due to its small size, it can be easily viewed under the microscope. Its transparency allows researchers to view processes such as embryogenesis and organogenesis in the developing worm. With the use of fluorescent-tagged proteins, researchers can specifically view the tissues and organs of study. Furthermore, researchers can view enlarged images of the developing cells and organs by use of Differential Interference Contrast (DIC) Microscopy. This type of optics uses polarized light to provide an enlarged and highly contrasted image of cells (Abramowitz and Davidson; 2016)

Another reason that *C. elegans* is a useful model organism to study in the lab is because it can be easily grown and maintained. Worms can be grown on petri dishes that have a bacterial lawn of *Escherichia coli* (*E. coli*), which serves as a food supply for the worms as they move around the plate. Also, *C. elegans* have a very short life cycle. It takes approximately 3 days for an egg to develop into an adult, and its life span is about 2-3 weeks at 20°C (Stiernagle 2006). This short life cycle is useful because it allows researchers to carry out more experiments in a shorter period of time, without being constrained by the life cycle of the worm.

C. elegans Life Cycle

C. elegans develops through an embryonic stage, and then four larval stages (L1, L2, L3, and L4), before reaching adulthood (Figure 1). When conditions are unfavorable due to overcrowding (high pheromone conditions), starvation, or extreme changes in temperature, the late L1 larva may take an alternative pathway known as dauer development (Hu 2007). During dauer development, worms develop from the late L1 stage to a pre-dauer L2 stage (L2d) before entering the dauer stage. When conditions become more favorable, worms leave dauer stage, and enter into the L4 stage before reaching adulthood (Hu 2007). Phenotypically, dauer worms are thin as a result of a reduced hypodermis (Riddle *et al*., 1997). They also have a closed cuticle with an internal plug at the opening of the pharynx (Riddle *et al*., 1997). This prevents pharyngeal pumping, which is the method worms use to intake food. Therefore, dauer worms do not feed (Riddle *et al*.,1997). Additionally, dauer worms tend to remain immobile on agar plates, which might help them to conserve energy.

As previously mentioned, *C. elegans* has a very short life cycle. However, their developmental timeline can be manipulated by growing the worms at different temperatures. At 20ºC, development from embryo to adulthood takes about 2.5 days (Worm Classroom). However, at a lower temperature of 15ºC degrees, development usually takes up to 6 days (Worm Classroom). Thus, temperature can be manipulated to speed up or slow down development, as needed for experimental use. In the experiments to follow, temperature sensitive worms were used. These worms exhibit wild type phenotype at the permissive temperature, and the mutant phenotype at the restrictive temperature.

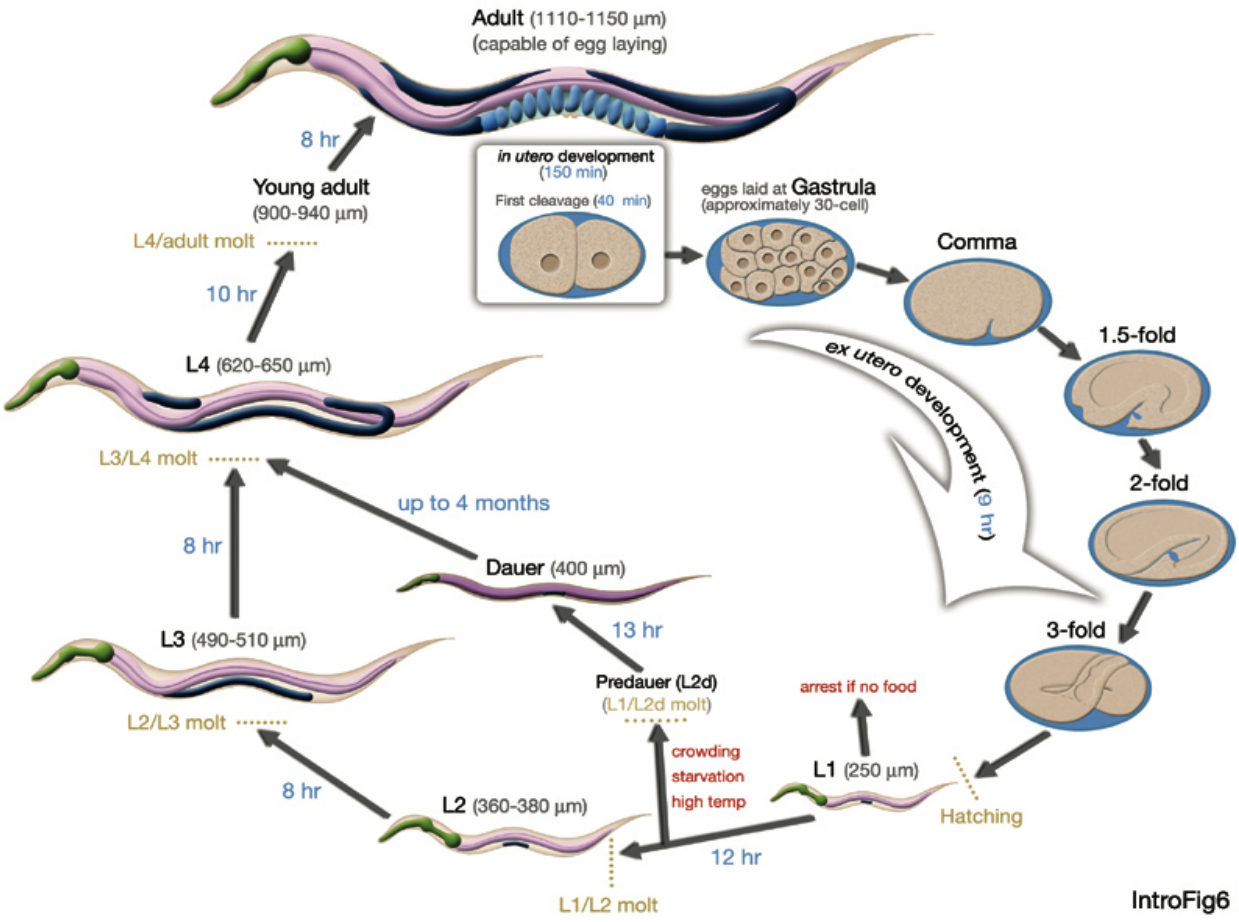

Figure 1. ***C. elegans*** **Life Cycle.** The figure shows the normal developmental cycle of *C. elegans* at 22 °C as the worm moves from embryonic to larval then to adult stages. The arrows depict the relative length of time spent at each stage. Also included in the figure are the times it takes to move from one stage to the next. Dauer development is also depicted. Figure from (Alton and Hall 2006).

C. elegans Reproduction

C. elegans have two sexes, males (XO) and hermaphrodites (XX). Hermaphrodites are very common in nature (Figure 2). They produce both sperm and oocytes, and can self-fertilize to produce offspring. Male worms occur at a much lower rate (0.1%) due to spontaneous meiotic non-disjunction in hermaphrodites (Hodgkin 1997), and they cannot self-fertilize. However, when a hermaphrodite is mated with male, the frequency of males can increase to 50% of the cross progeny (Altun 2009).

About 50 hours after hatching at 22ºC, a hermaphrodite worm begins to lay its first eggs (Lewis and Fleming, 1995; Byerly *et al.*, 1976). The worm makes eggs for 4 days then continues to live without laying eggs for an additional 10-15 days. Normally, hermaphrodites make approximately 300 offspring through self-fertilization (http://wormclassroom.org/short-history-c-elegans-research). However, the number of offspring can increase to 1200-1400 if males are mated to hermaphrodites (Worm Classroom).

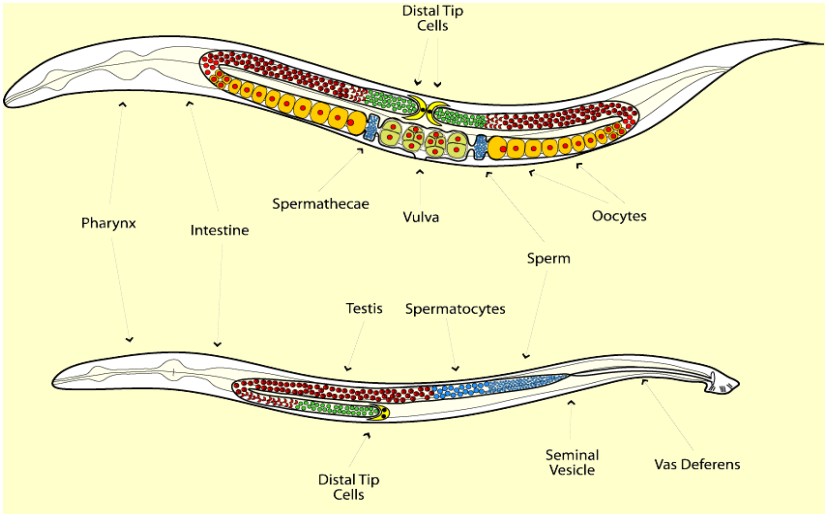

Figure 2. Male and Hermaphrodite germline. The top part of the image shows a hermaphrodite worm. The bottom image shows a male worm. Hermaphrodites produce both sperm and oocytes, and can self-fertilize. Males only produce sperm and can be mated to hermaphrodites. Figure from (University of Calgary: The Hansen Lab 2016).

C. elegans Gonad Development

During embryogenesis, the P_o blastomere undergoes two asymmetric divisions, which culminate in the formation of P_4, the primordial germ cell (Hubbard and Greenstein 2005). P_4 forms all germ cells. Approximately after the 100-cell stage, P_4 divides into two primordial germ cells, Z2 and Z3 (Seydoux and Strome 1999) (Figure 3A). These cells join the somatic gonad precursor cells, Z1 and Z4, and all four cells stop proliferation until the L1 stage (Seydoux and Strome 1999). Z1 and Z4 further divide to form 12 cells during the L1 stage. Ten cells form the somatic gonad primordium, while the other two form Distal Tip Cells (DTCs), which are important for germline proliferation (Hubbard and Greenstein 2005). By the L3 stage the somatic and germ cells rearrange to form the gonad (Figure 3B). The DTCs move to the anterior and posterior ends of the gonad arm, while the other ten cells move to the center (Lints and Hal 2009). As the gonad arms lengthen, germ cells begin to proliferate Figure 2 (Hubbard and Greenstein 2005). This proliferation is controlled by signaling from the DTCs to the germ cells.

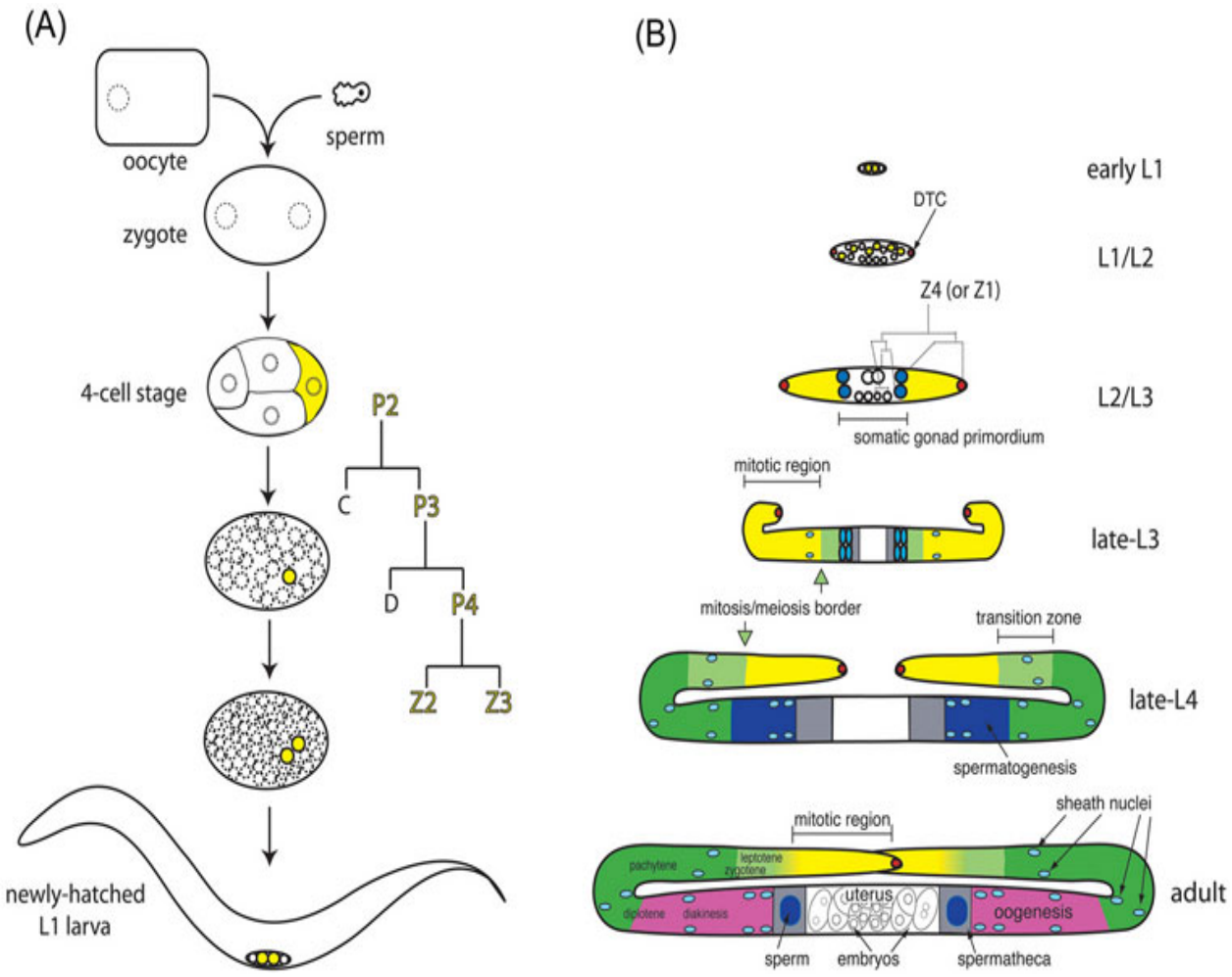

Figure 3. Fertilization and Gonad development in *C. elegans*. (A) The schematic depicts the process of fertilization and embryonic development of the germline. The germline lineage is colored yellow. (B) This schematic depicts the formation of the post-embryonic gonad in a hermaphrodite worm. Figure from (Hubbard and Greenstein 2005).

Notch Signaling

Notch signaling is a highly conserved process found in animals that regulates cell proliferation, differentiation and apoptosis. Notch signaling utilizes a juxtacrine mechanism, a contact dependent form of signaling that consists of three main elements, a ligand, receptor, and transcription factors (Figure 5). Essentially, Notch is a membrane bound transcription factor, and its release is controlled by binding a DSL (Delta, Serrate, LAG-2) type ligand. The ligand on an adjacent cell binds to the extracellular portion of the Notch receptor. This binding results in release of the Notch Intracellular Domain (NICD) after two proteolytic cleavage events. The first cleavage occurs after the ligand binds to the receptor and exposes a cleavage site known as Site 2, which is then cleaved by an ADAM family protease (Greenwald 2005). The second cleavage takes place within the lipid bilayer at Site 3, and is performed by γ-secretase. S3 cleavage releases the NCID, which then translocates to the nucleus where it where it associates with transcription factors of the CSL (CBF1/Suppressor of Hairless/LAG-1) family to regulate the expression of target genes (Greenwald 2005).

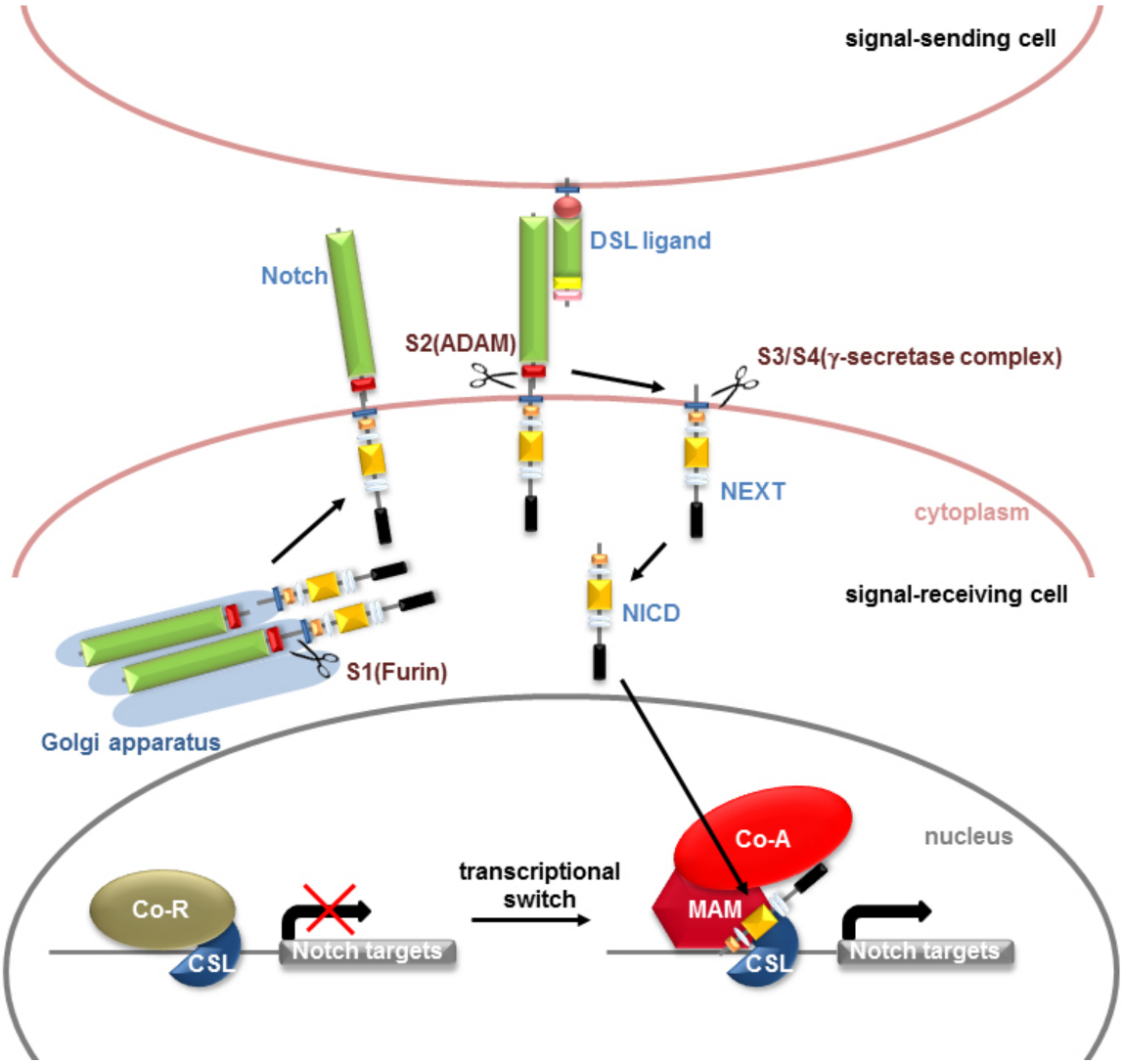

Figure 5. Notch Signaling requires the binding of a signal molecule (ligand), followed by two cleavage events, S2 and S3 cleavage, to release the Notch Intracellular Domain (NICD), a part of the receptor that can regulate gene expression. Figure from (Alhiyari 2014).

GLP-1 Signaling

GLP-1/Notch signaling is mediated by interaction of a ligand and receptor to produce downstream effects. The DSL (Delta, Serrate, and LAG-2) family of proteins are the ligands for Notch. LAG-2 is a Notch ligand expressed in *C. elegans.* Within the gonad of the worm, GLP-1 signaling occurs when LAG-2 produced by the somatic gonad binds to the GLP-1 receptor in the germline. The NICD (Notch Intracellular Domain) is then released and transported to the germ cell nuclei. There, the GLP-1 NICD interacts with the CSL protein, LAG-1 (Figure 4).

Early in larval development, germline proliferation is controlled by DTCs (Distal Tip Cells) as well as the AC (Anchor Cells)/VU (Ventral Uterine) precursor cells, Z1.ppp and Z4.aaa (Pepper *et al*., 2003). These AC/VU precursor cells make LAG-2, which promotes germline proliferation via Notch signaling. However, when worms transition from the L2 to L3 stage, the somatic gonadal cells are rearranged to form the somatic gonadal primordium, and one of the two AC/VU precursors adopts the anchor cell (AC) fate (Kimble and Hirsh, 1979). The AC continues to make LAG-2, but it no longer contacts the germ cells or causes them to proliferate (Kimble and Hirsh, 1979; Seydoux *et al.,* 1990). However, experiments have shown that removal of the obstructing somatic gonadal cells allows the AC to promote germline proliferation (Seydoux *et al*., 1990).

As mentioned previously, *glp-1* signaling in the adult gonad occurs when the DTCs signal the nearby germline stem cells to mitotically divide. In hermaphrodites, there is one DTC at the end of each of the two gonad arms (Figure 2). The DTC produces LAG-2, which binds to GLP-1 on the germline stem cell membrane (Figure 3b). Germ cells that receive the signal from the DTC undergo mitotic division. Cells that do not receive the signal undergo meiosis (Figure 3a). These data were obtained from experiments where either of the two DTCs in hermaphrodite

worms was laser ablated. This procedure caused all germline stem cells (GSCs) near the ablated DTC to end proliferation (mitosis) and start differentiation (meiosis) (Kimble and White, 1980). Therefore, the result was a worm with normal GLP-1 signaling in the gonad arm with a DTC, and loss of GLP-1 signaling in the other gonad arm where the DTC was removed.

Unlike hermaphrodite worms, males have one gonad arm, with two distal tip cells (Figure 2). Similar experiments were done on male worms where one DTC was moved from its normal location. This caused all cells adjacent to the new location to undergo mitosis (Kimble and White, 1980). Therefore, signaling from the DTC controls cell fate within the germline.

The *glp-1* gene was found in two different forward genetic screens identifying mutations essential for proper development of *C. elegans*. The first was a screen for sterile mutants. It was later determined that a loss of zygotic *glp-1* decreases the germline stem-cell (GSC) population and causes the GSCs to prematurely enter into meiosis (Austin and Kimble, 1987). It was also determined that a gain-of-function mutation of *glp-1* results in a germline tumor due to constant proliferation of the germ cells. This results in a decrease in the number of germ cells that enter meiosis and produce sperm and oocytes (Berry *et al.*, 1997; Pepper *et al*., 2003). The second screen was for maternal-effect embryonic lethal mutations; it was discovered that a loss of maternal *glp-1* prevents induction of the anterior pharynx at the 12-cell stage of embryogenesis (Priess *et al*., 1987). *glp-1* has also been found to function in the development of the 4-cell stage embryo. Other forms of GLP-1 signaling can be found in the formation of a bilaterally symmetrical head (Priess 2005). Therefore, *glp-1* is important in several developmental and inductive events in *C. elegans*.

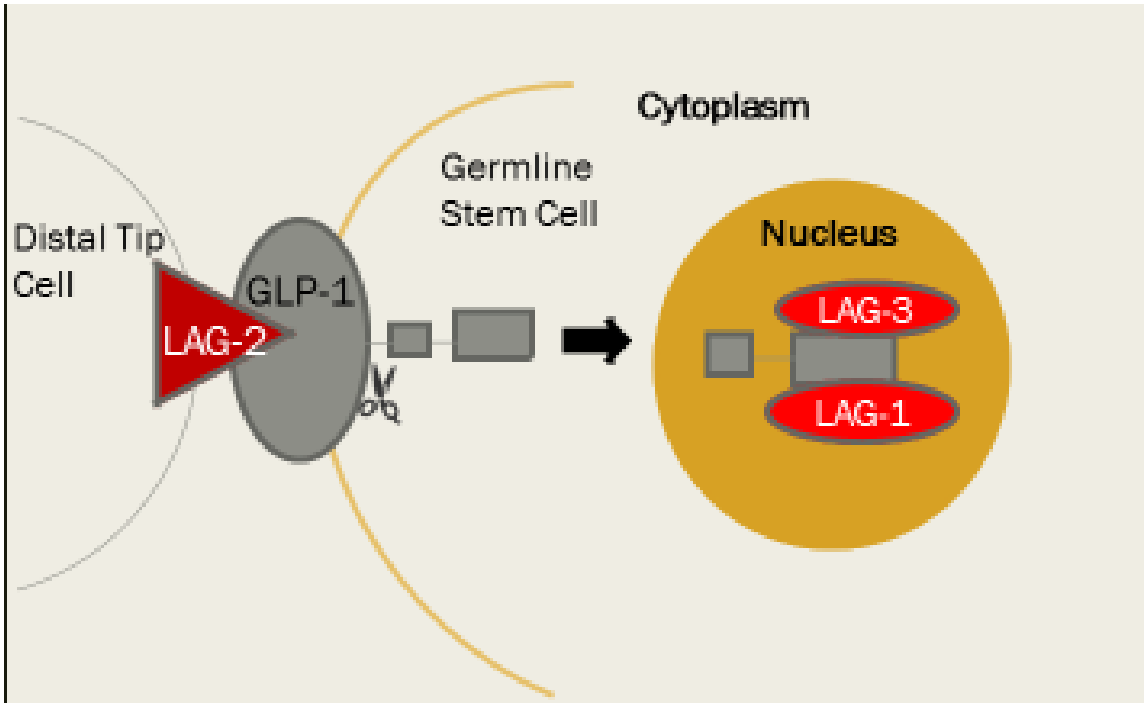

Figure 4. GLP-1 Signaling. This figure shows components of GLP-1 signaling pathway which includes a ligand (LAG-2), a receptor (GLP-1), the cleaved NCID, as well as transcription factors (LAG-3 and LAG-1). The NICD is part of the GLP-1 receptor that can regulate gene expression.

LIN-12 Signaling

The *lin-12* gene is important in a number cell fate decisions. One of the most understood of these events is the anchor cell vs. ventral uterine precursor cell decision. During early gonadogenesis in hermaphrodites, *lin-12* mediates lateral specification in two somatic gonadal cells to form the anchor cell (AC) and a ventral uterine precursor cell (VU). This process is known as the "AC-VU decision." Initially, two equivalent cells, Z1.ppp and Z4.aaa, mentioned above, have an equal chance of becoming either the AC or the VU. Additionally, both Z1.ppp and Z4.aaa express LIN-12 receptor and its LAG-2 ligand. However, by chance *lin-12* expression becomes upregulated through positive feedback and restricted to one cell, and *lag-2* expression becomes upregulated and restricted to the other cell. The result is that the cell with *lin-12* expression becomes VU and the other with *lag-2* expression becomes the AC (Figure 6). The AC is a terminally differentiated cell that undergoes no further division. In contrast, the VU precursor cell further divides to form descendants that contribute to the ventral uterus.

Experimental studies from laser ablation of these cells have provided support for the cell-cell interactions that determine the fates of these cells. When either Z1.ppp or Z4.aaa was destroyed with a laser, the remaining cell always became the anchor cell (Kimble et. al. 1981). These observations suggested that signaling was occurring between these cells. Additional experiments determined that, if all somatic gonadal cells except Z1.ppp and Z4.aaa were laser ablated, then the "AC-VU" decision could still occur. This suggested that Z1.ppp and Z4.aaa were necessary for this decision, and that no other cells were necessary.

Other experimental evidence suggested that *lin-12* was important for the VU cell fate in Z1.ppp and Z4.aaa. This is because a loss of function of *lin-12* resulted in both cells becoming anchor cells. In other words, the VU precursor cells could not form when there was no *lin-12*. In

contrast, a gain of function of *lin-12* resulted in both cells becoming VU cells (Greenwald *et al*., 1983). Thus, *lin-12* is important in formation of the VU cell.

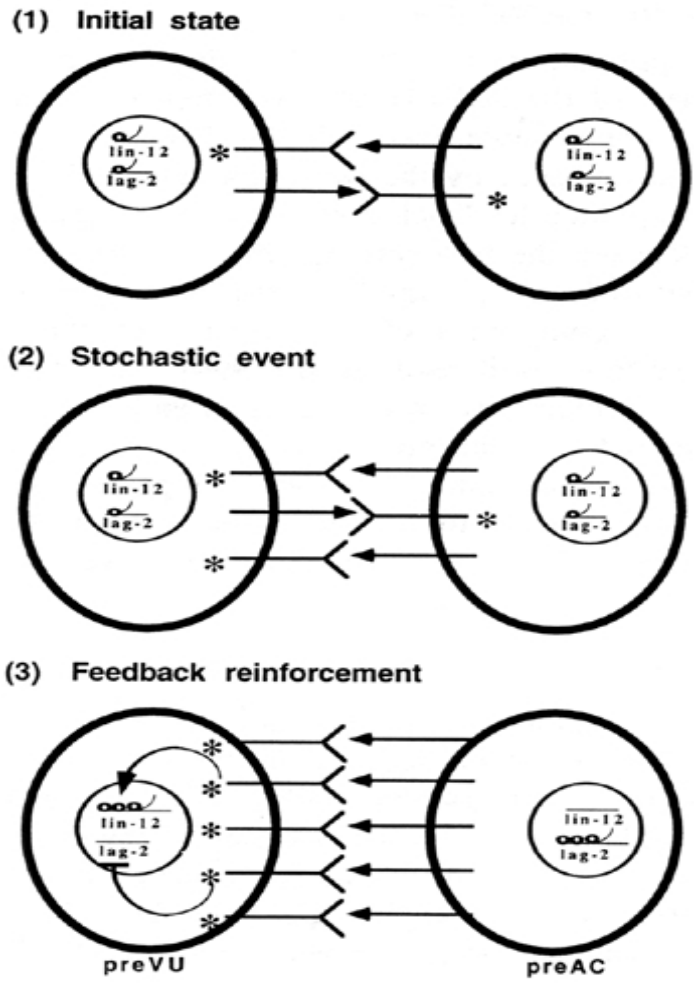

Figure 6. Anchor Cell vs. Ventral Uterine Precursor Cell Decision. (1) Two cells Z1.ppp and Z4.aaa shown here both have equal expression of *lin-12* and its ligand *lag-2.* (2) Due to a chance event, *lin-12* increases in one cell, which means its ligand, *lag-2* increases in the other cell. (3) The increase of these genes undergoes a feedback mechanism that leads to further increase of these genes. The cell with *lin-12* becomes the VU, and the cell without *lin-12* or the cell with *lag-2* becomes the Anchor Cell. Figure from (Riddle *et al*., 1997).

Regulation of Notch Signaling

Though not essential for Notch signaling, many genes have been found to influence Notch activity by either acting as a suppressor or enhancer through of the receptor and other components of the pathway. By interacting with Notch, these gene products may have serious impacts on the development and induction processes that take place in *C. elegans*. In a screen for suppressors of *glp-1* temperature sensitive mutants, 14 extragenic *suppressors of glp-1* (*sog*) were found that could partially rescue the embryonic and germline defects of a temperature sensitive loss of function *glp-1* mutant (Maine and Kimble 1993). This was determined by measuring the average brood size and percent fertility of *glp-1(ts);sog* mutants (Maine and Kimble 1993). Results showed that *glp-1(ts);sog* mutants had larger brood sizes and more viable offspring compared to *glp-1(ts)* mutants. Additionally, complementation tests and mapping were conducted, and the 14 suppressors were mapped to six genes, designated as *sog-1* to *sog-6*. However, actual genetic identities of many these s*og* mutations are still unknown. Identifying these genes would allow for an understanding of their function within the GLP-1 signaling pathway, and may provide a better understanding of the pathway itself.

Previously, the gene corresponding to *sog-1* was identified as *ubr-5* (ubiquitin protein ligase E3 component n-recognin 5 (Safdar *et al*., in press). UBR-5 is a HECT-type E3 ubiquitin ligase that is important for the ubiquitination and protein turnover (Safdar *et al*., in press). *ubr-5(om2)* also suppresses a loss of function mutation in *lin-12* (Safdar *et al.*, in press). The remaining five *sog* genes have yet to be identified, and the following experiments are aimed at identifying *sog-4* and *sog-6*.

Summary

In this experiment, I set out to determine the identities of *sog-4* and *sog-6* by Whole Genome Ssquencing (WGS) comparisons of their alleles and RNAi. From WGS data, one candidate gene was found for *sog-4*, whereas 6 candidate genes were found for *sog-6*. RNAi then was carried out to knockdown the candidate gene product(s) in *glp-1(ar202*) worms; F1 offspring was counted to determine if the sterile phenotype was enhanced after knockdown. The percent of sterile worms resulting from the gene knockdown was compared to either a non-RNAi or an RNAi control. The data from each comparison were put into a graph using excel and Z tests were conducted using Minitab Statistical Software. Fold change comparisons were calculated. The results suggest that F28D1.2 could be *sog-6* since it had the highest fold change when compared to both the non-RNAi and RNAi controls. However, results also suggest that the only candidate gene for *sog-4*, *oac-49,* may not be *sog-4.*

Methods and Materials

Strains and Culture

Worms were maintained on agar plates seeded with *E. coli* as described (Brenner 1974). Nomenclature follows guidelines of Horvitz *et al.,* (1979). Genes are named by a three letter abbreviation that includes lowercase italicized letters, a hyphen, and a number (e.g. *dpy-5* for the *dumpy-5* gene). Mutations used in this study include *glp-1(q231)* (germline proliferation defective), *unc-32(e189), sog-4* (suppressor of *glp-1),* and *sog-6(q306).* The *sog-4* alleles used were *sog-4(q304)* and *sog-4(q301).* All were recovered by mutagenesis with Ethylmethanesulfonate (EMS) (Maine and Kimble 1993). The *glp-1(q231)* allele used in this experiment is temperature sensitive. Mutants produce viable offspring at 15ºC; however, when raised to 20ºC, approximately 98% produce inviable progeny and 2% produce no progeny (Maine and Kimble 1993).

Whole Genome Sequencing

DNA prepared from *sog-4(q304), sog-4(q301),* and sog-*6(q306)* mutants was sent to the Beijing Genomics Institute (BGI) for whole genome sequencing. Previous genetic assays mapped *sog-4* and *sog-6* to chromosomes V and IV, respectively. With that said, the sequencing information revealed a number of mutations on these individual chromosomes that may correspond to the *sog-4* gene and *sog-6* gene, respectively. However, some of these mutations have also been detected in other *unc-32(e189)glp-1(q231)* strains, so these mutations are hypothesized to come from the original background strain.

Polymerase Chain Reaction Sequencing

Interestingly, one of the mutations detected by whole genome sequencing of *sog-4(q304)* was in a gene region corresponding to *apx-1*, a gene known to function in the GLP-1/Pathway. APX-1 is a DSL-type ligand that activates GLP-1 or LIN-12 in a number of tissues. Maternally supplied *apx-1* in the 4-cell stage embryo is a ligand for GLP-1 that specifies the fate of the ABp Blastomere (Mello *et., al* 1994). Later, in larval development APX-1 functions alongside LAG-2 and DSL-1 as ligands in the LIN-12 mediated lateral signaling that forms the primary and secondary vulva lineages (Mango *et al.*, 1994).

To further investigate whether or not *apx-1* was mutated in *sog-4*, PCR and DNA sequencing were carried out. Forward and reverse primers corresponding to the gene region of *apx-1*were purchased (Invitrogen). DNA from *sog-4(q304)* worms were isolated and amplified using Standard PCR protocol (Chin-Sang 2014). The DNA corresponding to *apx-1* was isolated by gel electrophoresis and spin column extraction, and the product was sent out for sequencing (Genewiz). The results from the sequencing showed that there was a nucleotide base change in *apx-1*, however the codon remained the same. This is a silent mutation, so it should have no effect on the protein function. Therefore, *apx-1* was ruled out as a possibility for *sog-4.*

RNA Interference

RNA interference is a form of reverse genetic screening that knocks down a known mRNA and therefore protein, and allows for screening of the loss of function phenotypes associated with that gene. In this study, RNAi was carried out by feeding using the protocol and feeding constructs from the Ahringer Lab (Ahringer 2006). A region of the gene of interest was cloned into a bacterial feeding vector (L4440), which was then transformed into a dsRNase

deficient *E. coli* strain, HT115(DE3). The bacterial cultures were seeded on plates that contained IPTG (Isopropyl-D-thiogalactopyranoside), a chemical that triggers the T7 promoters of the plasmid to begin transcription (Ahringer 2006). The two ssRNA hybridize to form duplex dsRNA. Once the worms consume the transformed bacteria, the dsRNA is processed into siRNAs that associate with other proteins and form a complex that identifies mRNA corresponding to the gene inserted. This complex would bind to the mRNA and degrade it, preventing translation from occurring.

RNAi was conducted in two sets of experiments. The first set of experiments, RNAi was done on 5 *glp-1(ts)* gravid hermaphrodite worms. The genes knocked down were the candidate *sog-4* and *sog-6* genes based on the whole genome sequencing results. With that said, 5 *glp-1(ts)* worms were placed on RNAi plates or OP50 (no RNAi) control plates and allowed to lay eggs at the nonrestrictive temperature of 15ºC for approximately 24 hours. After this time, these worms were moved to a new plate and kept at 15 ºC, while the original plate was shifted to the restrictive temperature of 20 ºC. RNAi plates kept at 20 ºC were then screened for fertile F2 progeny and compared to control plates to determine if *glp-1(ts)* was suppressed.

In the second set of experiments, RNAi was conducted on 2 *glp-1(ar202)* L4 hermaphrodite worms. The genes knocked down were the same *sog-4* and *sog-6* candidates. Two *glp-1(ar202)* worms were placed on RNAi or OP50 plates and allowed to lay eggs at 20 ºC for two days. After this time, the worms were moved to a new plate and kept at 20 ºC. The worms were monitored as they aged, and when they reached adulthood, they were counted to determine the number of fertile and sterility progeny resulting from each treatment. These numbers were compared to OP50. Additionally, the RNAi procedure was done with *E. coli* that contained an "empty" feeding vector to control for any effects the RNAi produced on worm development.

Results

Candidate sog-4 genes identified by WGS

After comparing the sequencing data of the two *sog-4* alleles, *sog-4(q304)* and *sog-4(301),* to each other, to sequencing data for a *sog-6* allele, and to the two *sog-1* alleles, one candidate *sog-4* gene was found. More explicitly, this gene was the only gene on chromosome V that was mutated differently in both *sog-4* alleles, but was not mutated in *sog-1* or *sog-6.* The gene was found in the predicted region of *sog-4*, according to previous map data. This gene, *oac-49*, encodes an o-acyl transferase. O-acyl transferases are enzymes functions by transferring an acyl group from one protein to another (Mulder *et al.,* 2005). Since this enzyme was not previously identified in regulating the Notch signaling, it could essentially function anywhere within the GLP-1/Notch pathway.

Candidate sog-6 genes identified by WGS

Sequencing data revealed six genes located on chromosome IV that could potentially be *sog-6*. These include F28D1.2, Y45F10A.6, K09B11.10, Y64G10A.1, Y40H7A.10, and Y105C5A.15. K09B11.10 corresponds to the *mam-3* gene, while Y64G10A.1 corresponds to the *tbc-9* gene. *tbc-9* encodes a protein with a tre-2/bub2/cdc16 domain. TBC-9 is similar to human TBC1D8 (TBC1 domain 8) and members of the EF-hand domain containing family (McKay *et al.,* 2003). TBC1D8 (TBC1 Domain 8) and members of the EF-Hand Domain family are expressed in the pharynx and the intestine (McKay *et al.,* 2003). *tbc-9* has not been previously found to be important in Notch signaling.

Test for suppression of glp-1(q231)

RNA interference was used to knockdown three of six potential *sog-6* genes (F28D1.2, Y45F10A.6, K09B11.10) as well as the candidate *sog-4* gene, *oac-49.* Suppression of *glp-1(ts)* mutant 20º C was tested for by quantifying the number of viable F2 progeny. None of the possible *sog* genes showed signs of suppression as the number of F2 offspring in each gene tested, ranged from 0-5 (Table 1), which was very similar to the non RNAi treated OP50 control. Also, this number is much lower than 300, which is the amount of offspring produced by a wild-type worm. In addition, of 0-5 offspring, all died before reaching adulthood, therefore none were viable. This suggested that the genes tested might not be *sog-4* or *sog-6* because knockdown of these genes were unable to suppress a *glp-1(ts*) mutant at the restrictive temperature. Also, these results could mean that the *sog-6* phenotype could be due to a combination of the candidate genes working together. With that said, previous knockdown of *ubr-5* was also unable to suppress a *glp-1(ts).* Therefore, RNAi was further conducted on these genes to determine whether or not they could enhance a *glp-1* temperature sensitive phenotype.

Tests for enhancement of glp-1(ar202)

Another RNA interference assay was conducted using the same candidate *sog* genes previously mentioned. RNAi was done using *glp-1(gof)* (gain of function) mutants. Knockdown of *sog* should result in an enhancement of the *glp-1(gof)* mutant phenotype, which is a sterile worm. After RNAi was carried out, the viable offspring was counted to determine the percentage fertile and sterile progeny. The data are summarized in the table below (Table 2), which includes the percentage of the total offspring that were fertile, sterile, and fertile in one gonad arm, but sterile in the other.

Table 1. **Test for suppression of *glp-1(ts).*** This table depicts the results from RNAi of 3 candidate *sog-6* genes and the candidate *sog-4* gene. *glp-1(ts)* worms were used in this experiment. The number viable progeny at 20°C was counted on each RNAi plate and compared to an OP50 control.

Gene	# of offspring (F2)	# of viable offspring (F2)
T26H2.7	3	0
KO9B11.10	3	0
Y45F10A.6	5	0
F28D1.2	1	0
OP50 (Control)	0	0

Table 2. Test for enhancement of *glp-1(ar202)*. Table summarizing the results from RNAi on the 3 candidate *sog-6* genes and the candidate *sog-4* gene. Empty vector RNAi as well as OP50 plates were used as controls. Two *glp-1(ar202)* worms were used in this experiment. The numbers of sterile and fertile progeny were counted, and the percentages of sterile and fertile offspring were calculated. Each gene knockdown was tested alongside an OP50 control. Experiments done together are labeled with the same letter (A-E).

Gene Knocked Down		#Fertile (%fertile)	#Sterile (%sterile)
A.	T26H2.7	195 (70%)	82 (30%)
A.	OP50	181(71%)	74 (29%)
B.	KO9B11.10	387(78%)	110(22%)
B.	OP50	502(87%)	73 (13%)
C.	Y45F10A.6	182(59%)	128(41%)
C.	OP50	363(83%)	76(17%)
D.	F28D1.2	131(47%)	147 (53%)
D.	OP50	182(83%)	37(17%)
E.	Y45F10A.6	115(50%)	115(50%)
E.	OP50	176(69%)	79(31%)
E.	Empty vector	177(57%)	135(43%)

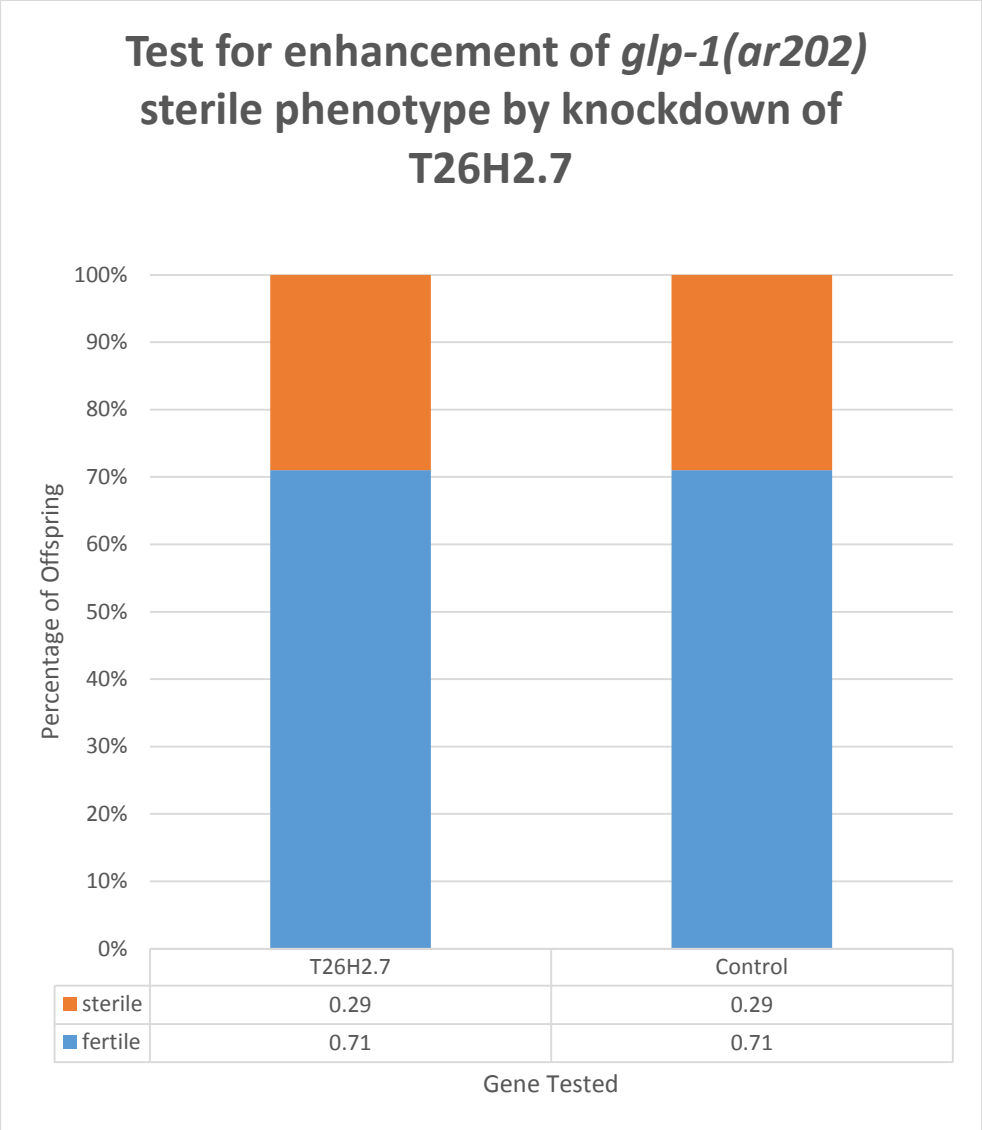

Figure 7. This graph depicts the results of RNAi on T26H2.7. The percent fertile and sterile offspring of 2 *glp-1(ar202)* were compared between T26H2.7 and OP50 (Control) plates. These percentages can be found below the graph. The data contains information in the summarized graph (Table 2). 277 worms were tested in the T26H2.7 group and 255 worms were tested in the control group. These results reflect one trial.

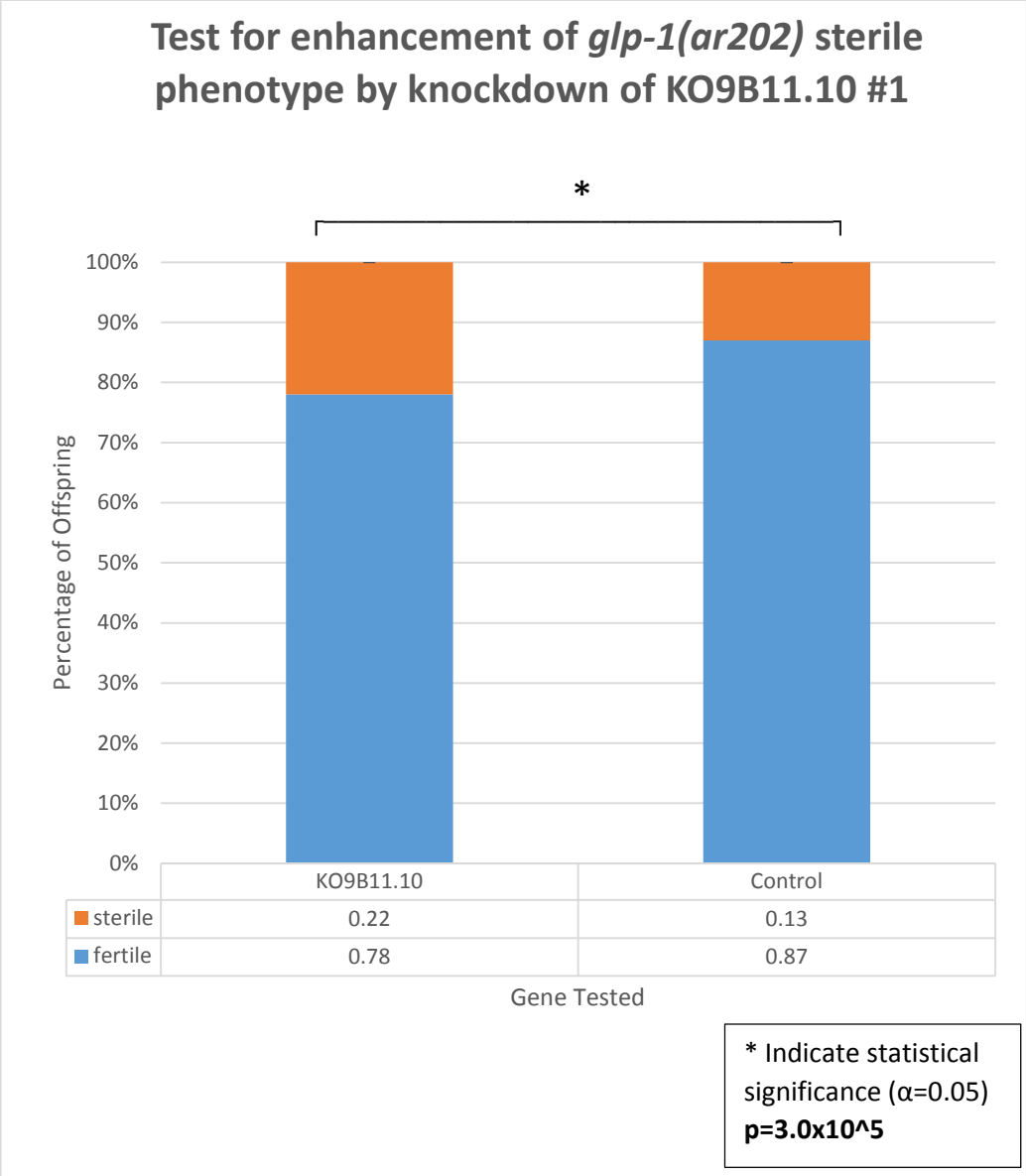

Figure 8. This graph depicts the results of RNAi on K09B11.10. The percent fertile and sterile offspring of 2 *glp-1(ar202)* were compared between K09B11.10 and control plates. These percentages can be found below the graph. The data contains information in the summarized graph (Table 2). 497 worms were tested in the K09B11.10 RNAi group and 575 worms were tested in the control group. These results reflect one trial.

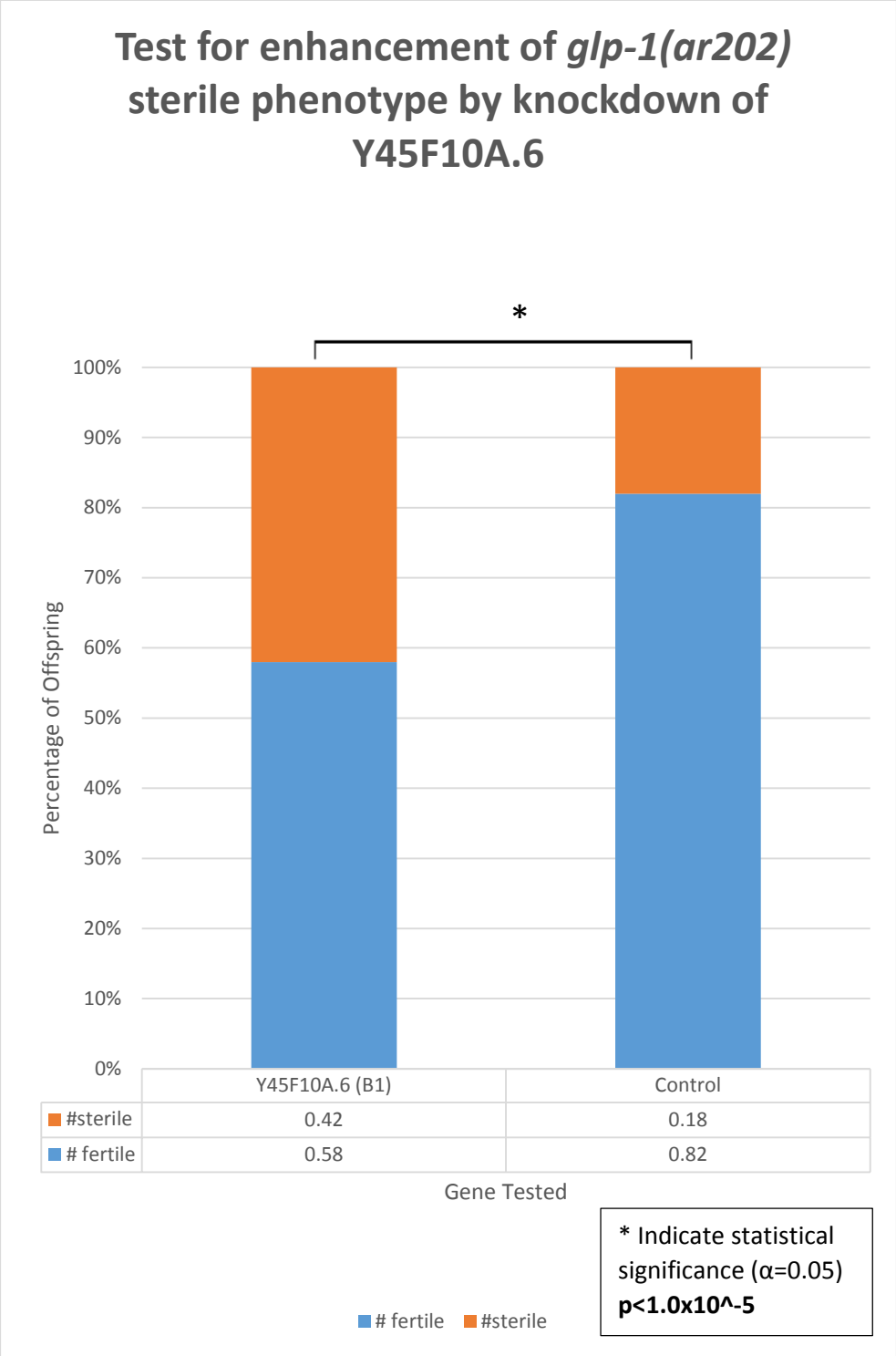

Figure 9. This graph depicts the results of RNAi on Y45F10A.6. The percent fertile and sterile offspring of 2 *glp-1(ar202)* were compared between Y45F10A.6 and control plates. These percentages can be found below the graph. The data contains information in the summarized graph (Table 2). 310 worms were tested in the Y45F10A.6 RNAi group, and 439 worms were tested in the control group. Results reflect one trial.

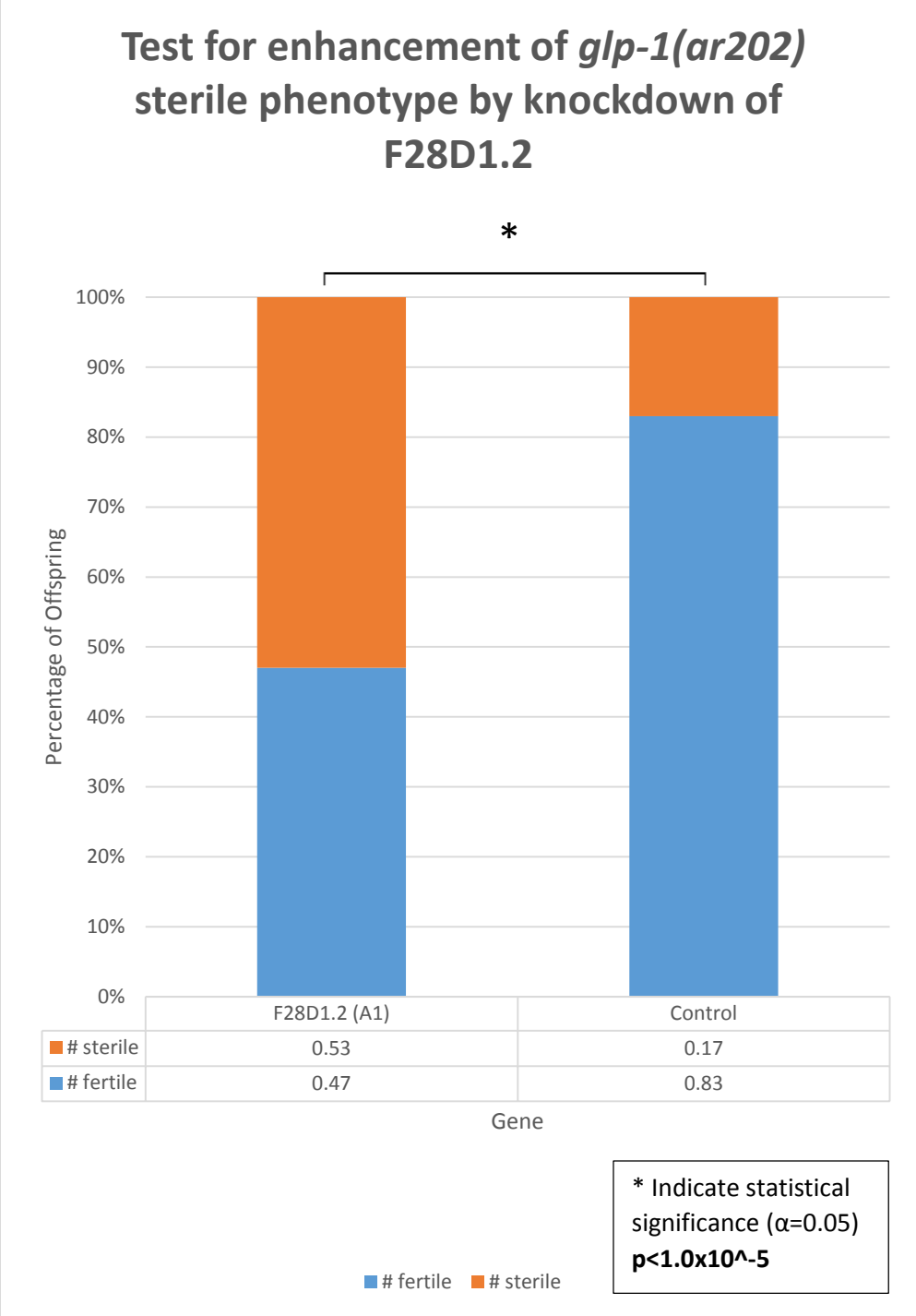

Figure 10. This graph depicts the results of RNAi on F28D1.2. The percent fertile and sterile offspring of 2 *glp-1(ar202)* were compared between F28D1.2 and control plates. These percentages can be found below the graph. The data contains information in the summarized graph (Table 2). 278 worms were tested in the F28D1.2 group, and 219 worms were tested in the control group.

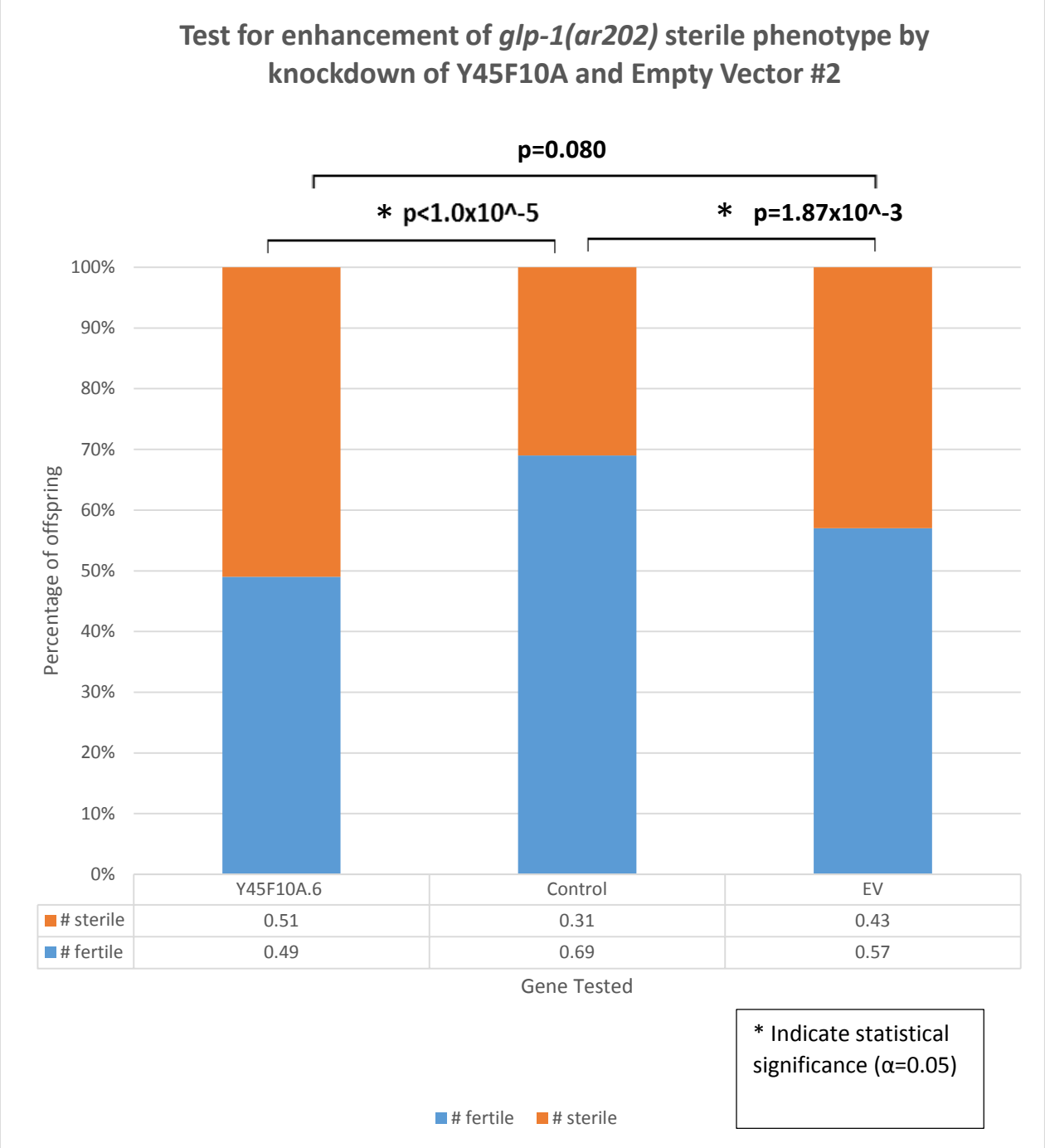

Figure 11. This graph depicts the results of RNAi on Y45F10A.6 and an empty vector, and an OP50 control. The percent fertile and sterile offspring of 2 *glp-1(ar202)* were compared among, Y45F10A.6, empty vector and control plates. These percentages can be found below the graph. The data contains information in the summarized graph (Table 2). 228 worms were tested in the Y45F10A.6 group, 255 worms were tested in the OP50 group, and 312 worms were tested in the empty vector group. These results reflect one trial.

Table 3. Comparisons of knockdown phenotype of sterility in *glp-1(ar202)* worms using candidate *sog-4/sog-6* genes, RNAi and non-RNAi controls. OP50 are non RNAi controls, while Empty Vectors (EV) are RNAi controls. The fold change shows how much the sterile phenotype in the first gene listed is different from the second gene listed. Numbers greater than one show an increase in sterility, while numbers less than one show a decrease in sterility.

Gene comparisons	**Fold change in sterility**	**Z Test Results**
T26H2.7A (*oac-49)* to OP50	1.02	--
K09B11.10 to OP50	1.71	**p=3.0x10^5**
Y45F10A.6 (#1) to OP50	2.38	**p<1.0x10^-5**
F28D1.2 to OP50	**3.15**	**p<1.0x10^-5**
EV to OP50	1.38	**P=1.87x10^-3**
Y45F10A.6 (#2) to OP50	1.62	**p<1.0x10^-5**
Y45F10A.6 (#2) to EV	1.18	**p=0.080**

Fold change is calculated by using the equation below.

Equation 1: % sterile of gene knocked down ÷ %sterile of control

Discussion

As previously mentioned, the goal of this study is to determine the genetic identities of *sog-4* and *sog-6* by WGS and RNAi. The first gene tested was the only candidate *sog-4* gene, *oac-49*. Knockdown of this gene was tested alongside a "non-RNAi", OP50 control. Comparison of the *oac-49* to the control results suggests that knockdown of *oac-49* did not enhance a *glp-1(ar202)* phenotype (Figure 7). The data shown in Table 2 show similar percentages of sterile offspring in the both RNAi and control experiment. With that said, WGS comparison and the RNAi experiments showed conflicting results. WGS suggests the *oac-49* is *sog-4*, while RNAi suggest *oac-49* is not *sog-4*. Therefore, these contradictory results could suggest that *sog-4* might not be a protein coding gene.

After testing the *oac-49* gene, three of the candidate *sog-6* genes were tested using similar methods. Results from knockdown of the genes (F28D1.2, Y45F10A.6, and K09B11.10) all showed an increase in *glp-1*(*ar202*) phenotype compared to non-RNAi controls (Figures 8-11). The percent of sterile *glp-1(ar202)* offspring were higher in the RNAi experiments than in the controls. Z test analysis determined that these differences were statistically significant ($p < 0.05$) compared non-RNAi controls. These results could mean two things. Firstly, enhancement of the sterile phenotype after knockdown of each of three genes suggests that all three genes could be working together to produce the *sog-6* phenotype. Additionally, these results could also suggest that the methods and chemicals used to conduct RNAi could be causing enhancement of the *glp-1(ar202)* phenotype, rather than genes knocked down.

To determine whether or not RNAi was contributing to enhancement of the *glp-1(ar202*) phenotype, results from Empty Vector RNAi and non-RNAi controls were compared using a Z-test. Empty vector was used as a negative control in this experiment. There is no gene inserted

into the bacterial feeding plasmid. Therefore, there is no mRNA targeted for degradation. In the best case scenario, worms should have near identical numbers of sterile and fertile offspring compared to OP50 (non-RNAi control). However, the results in Figure 11 suggested that this was not the case. *glp-1(ar202)* worms on empty vector RNAi plates had an increase in the number of sterile offspring compared to non-RNAi control worms. This difference was statistically significant ($p=1.87x10^{-3}$, $\alpha =0.05$). Therefore, engaging the RNAi machinery may have altered germline physiology and led to increased proliferation.

Furthermore, since the all the genes knocked down for *sog-6* were significantly different from OP50 controls, and the Empty Vector knockdown was also different from OP50, the next step was to determine whether or not RNAi of any of the candidate *sog-6* genes were different from the Empty Vector RNAi. Y45F10A.6 was chosen because it was the first gene tested that showed enhancement of the sterile phenotype. Comparing Y45F10A.6 to Empty Vector (Figure 11), the results showed that the difference in both groups was most likely due to chance ($p=0.08$). This result could mean that the procedure of RNAi rather that the genes being knocked down could be acting to produce this phenotype.

Only one candidate *sog-6* gene was tested alongside the EV. Due to the variability seen in the non-RNAi controls tested in each experiment, it is difficult to compare F28D1.2 and K09B11.10 to the EV control since these experiments were not done together. Therefore, to further understand these results, the other candidate *sog-6* genes should also be tested alongside the EV control, and any increase in sterility found in the EV should be used as a baseline to determine the amount of increase in sterility in the gene tested.

The reason for the variability in experiments conducted at different times might be due to the fact that *glp-1(ar202)* are extremely sensitive to changes in temperature. Therefore, since the

experiments were carried out at different times, the conditions of the room in which the worms were picked or the incubator in which they were stored in could have been different for the worms being tested, and this could have produced the variability in genes being tested.

With that said, we then went on to analyze how different the amount of sterile offspring in each of the candidate genes were to the non-RNAi control, by measuring the fold change in amount sterile offspring. Results show that F28D1.2 had the largest fold increase in sterility compared to the control than any of the other *sog-6* candidate genes (Table 3). These results also showed that similarities in the fold changes between the other candidate the *sog-6* genes and the empty vector. Y45F10A.6 also had a higher fold change than its non-RNAi control, but the results suggest that it is not *sog-6*. Therefore, these results suggest F28D1.2 could be *sog-6*. However, further experiments should be conducted in which F28D1.2 is directly tested alongside and EV control and a non-RNAi control.

In short, future experiments should be conducted to test all six candidate *sog-6* genes alongside an empty vector control, and non-RNAi control. This would allow for better comparisons between the candidate gene and the EV. Additionally, these experiments should be conducted multiple times to determine whether or not the results are consistent. If variability is found in the results, then a different method, such as CRISPR/*cas-9* should be used to knockout the candidate *sog-4* and *sog-6* genes and determine whether *glp-1(ts)* phenotype is suppressed or whether a *glp-1(ar202)* phenotype is enhanced.

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