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Genetic Analysis of Germline Development in the Model Organism *C. elegans*

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May/2007

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Abstract

My research investigated the relationships among several transcriptional and/or post-transcriptional regulators in the *C. elegans* germ line. I examined the relationship between the *rha-1* gene and three other regulatory genes, *ego-1*, *glp-1* and *csr-1*. These genes function in germline proliferation and differentiation and are required for fertility. Because these genes regulate similar processes, we have investigated the relationships among them.

The *rha-1* gene encodes an RNA helicase that is required for germline development, chromatin regulation, and RNA interference (RNAi). The *ego-1* gene encodes an RNA-directed RNA polymerase that is also required for these processes. Although many of the *rha-1* and *ego-1* defects are similar, the *rha-1* null phenotype is temperature-sensitive (*ts*) whereas the *ego-1* null phenotype is not. The *glp-1* gene encodes a Notch-type receptor that receives an inductive signal from distal tip cell (DTC), which maintains germline proliferation. In the absence of GLP-1 signaling, germ cells that are normally mitotic instead enter meiosis and undergo gametogenesis. The *csr-1* gene encodes an Argonaute-type RNA binding protein that is required for germline development and chromatin regulation. Mutations in *ego-1* and *csr-1* enhance the phenotype of a weak *glp-1* mutation.

We constructed double mutant strains carrying the *rha-1* null allele and either a null allele of *ego-1* or *csr-1*, or a *ts* allele of *glp-1*. Our data suggest that EGO-1 and RHA-1 proteins work together to regulate some aspects of development and in parallel to regulate others. Preliminary genetic data also suggest that CSR-1 and RHA-1 work together to regulate development, but act antagonistically to regulate oogenesis onset or a very early step in oogenesis. Additionally, our research suggests that RHA-1 does not regulate germline proliferation by promoting GLP-1 signaling. These findings have improved our understanding of how germline development is regulated in this model organism.

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Preface:

As a freshman entering Syracuse University in the fall of 2003 my heart was set on one achievement; acceptance into medical school upon graduating from Syracuse University. Since my childhood I have been fascinated by the human body, its capabilities, intricacies, and most remarkably its limitations. This life long interest in the human body made a career in medicine and a major in biology an easy decision. It wasn't until I began fulfilling my biology major requirements with courses far more in-depth than any of my previous biology course work in high school, that I began to realize there was in fact much more to biology and medicine than I had ever realized. It was at this point that I found myself not only learning about in-depth biological processes but also about how research today is continually uncovering more about them. It was at this point I realized that every diagnosis and procedure a doctor performs is the result of scientific research which allows the medical world to understand and ultimately treat the illnesses that patients present with.

With this eye-opening realization I began to question my career aspirations. I thought perhaps a career in medical research would better fulfill my curiosity of the human body and medicine. With this I spent some time learning about the different biology labs at Syracuse University and their research focuses. It was during this research I learned about the Maine lab, which focuses on genetic regulation of development, cell-signaling, germline

development and RNA silencing. It was the genetic aspect of this lab's research I was attracted to, as genetics was an area of biology in which I excelled, and I enjoyed and could easily see impacting medicine.

During the end of my fall semester as a sophomore I spent some time learning more about the research done in the Maine lab. Dr. Maine explained her research and the model organism, *C. elegans*, which the Maine Lab uses to study their research questions. I also spent time reading past publications by Dr. Maine and members of the lab. Their terminology was difficult and overwhelming at first. The genetics was far more detailed than anything I had learned in class and trying to see the difference between a male and a hermaphrodite *C. elegans* under a microscope seemed impossible. However, with persistence and practice, I was soon easily able to distinguish males from hermaphrodites as well as features of worms in each of the developmental stages. It was at this point that Dr. Maine discussed with me the option of the honors thesis program, which I knew right away was something I wanted to do. It would give me the perfect chance to explore research and determine if perhaps this was the career path I wanted to pursue.

Around this time it had been discovered that the EGO-1 and GLP-1 proteins in *C. elegans* interact at the genetic level (Qiao *et al.* 2005). Previous research had also shown that the *rha-1* gene plays a similar role to *ego-1* gene in *C. elegans* germline development, specifically germ cell proliferation and meiosis. The realization that both of these genes are important for similar processes is what led to my investigation of the relationship between several

transcriptional and/or post-transcriptional regulators in the germ line of *C. elegans*. Specifically I have looked the following genes: *ego-1*, *csr-1*, *glp-1* and *rha-1*.

The observation that *rha-1* mutant have a meiotic silencing defect only occurs at high temperatures is one reason that we suspected there are other genes involved with mitotic silencing at lower temperatures. However, it is this temperature sensitive nature of the *rha-1* gene as well as other genes in the *C. elegans* that added great challenges to my experiment. Even the slightest variation in temperature can cause significant effects on the development of the germ line. Thus it was essential that all incubators remained constant. Unfortunately, during both fall and spring semesters of my junior year, our lab was experiencing difficulties with our 25°C incubator. Due to spikes in the temperature at times, there were several experiments which I had to repeat. Additionally, I was looking at the relationship between the *rha-1* and *csr-1* genes using RNAi feeding. Experiments were done twice, producing similar results during the fall semester of 2005. After December break of 2005 this experiment was again repeated and different results were found. After several attempts we were unable to repeat the initial results, and suspect this could have been the result of problems with our 25°C incubator. A second challenge of my research was the importance of time intervals. While characterizing the phenotypes of different mutants we looked at the development of the germ line at different stages. At times this meant going into lab at late or very early hours. While this was exciting it often presented

a challenge for me as I worked to balance this schedule with my academics, extra circulars and personal life.

It didn't take long for me to realize that genetic analysis and research is far from easy. I often found that my predictions were incorrect and my results were often negative. However, with time, I began to realize that proving hypotheses wrong was in fact progress. I was able to rule out possible genetic interactions and make new predictions. I also found that while finding negative results was often disappointing, it is the uncertainty and suspense that makes research so exciting and intellectually challenging.

Over the past five semesters I have sharpened my skills in both developmental and genetic biology. I have built a solid foundation for basic research techniques. I have learned everything from PCR, DNA extraction, basic genetic crosses, germline characterization of *C. elegans* as well as how to produce a research poster and write a thesis. As I reflect, however, I think the most valuable lesson I have learned from my experience is the importance of basic biological research.

With research funding at a low it seems people are often eager to spend funding on medical research because they feel it more directly impacts people. I must admit that as an enthusiastic pre-medical student, I felt the same way at the start of my research project. From my experience, however, I have learned that many major discoveries in biology have later led to medical advancements. Basic research provides knowledge about the intricate mechanisms that sustain life and how alteration of these mechanisms affects

living systems. And as I have learned, basic research is often not glamorous, and likely won't appear as a news headline. It does, however, provide vital insight for future headlines. Thus it is my hope that my research aimed at understanding the relationships among transcriptional and/or post-transcriptional regulators will improve our understanding of how these processes are regulated in this model organism. While this is just a small piece of a much larger puzzle, it has been exciting, educational, and rewarding to have been part of it.

As I graduate, my goal remains to attend medical school; however my career aspirations have changed as a result of my research experience at Syracuse University. While I wish to work in a clinical setting I also aspire to supplement my practice with research. Not only do I think my interactions with patients will provide me with invaluable insight and motivation with respect to my research, but also a combination of research and clinical work will allow me to expand my scientific knowledge while at the same time interact with people during pivotal and personal moments in their lives.

Acknowledgements:

First and foremost I would like to thank my research advisor, Dr. Eleanor Maine, for her time, patience, support and willingness to teach. The knowledge I have gained from working in her lab far surpasses anything I learned from my classes. I would also like to thank the other members of the Maine lab for their help and willingness to answer questions. I also appreciate the time Dr. Larry Wolf and Dr. John Belote have spent planning and providing feedback during thesis seminars. Lastly, I would like to thank my friends and family for their enormous amounts of support and continued confidence in my potential.

Advice to Future Honors Students

There is so much to explore in the world of biology and research that it very easily may seem like a field without an end. Becoming a successful research scientist is a life long process that involves continual learning as technology advances and problems facing the field change. Thus, as an honors student my most important piece of advice is to have specific goals in mind before you begin. Pick a very definite area of this overwhelming field that you will focus your time on and work on becoming an expert in it. Once you have done this, realistically figure out the amount of time, resources, and effort you will need to apply towards achieving your goals. There will never be a definite end to your area of research and there will always be more questions you could ask, thus setting goals that allow you to track your progress is important.

Secondly, time management and organization is of the utmost importance when writing your thesis. For the biology thesis it is difficult to get an early start with the writing component because most students work on obtaining their results right up until graduation. However, there are many parts of the thesis that can be done without the results and it is vital that you start these early. Writing, revising and adding to the thesis is extremely time consuming and thus the sooner you can have a draft prepared the higher quality project you will accomplish in the end. Also, as you go through the honors seminar course, each presentation you do covers one of the sections in the thesis. Knowing this, I advice you to do quality and detailed presentations

from which you can essentially write your thesis. Without very clear and organized documentation of your work, it can be quite difficult to remember what you did several semesters past.

Finally, take advantage of the Honors Thesis Program and Undergraduate Research that Syracuse University has to offer. As students of this university we are fortunate to have these programs available to us. There are many other universities where very few undergraduates are able to take part in an independent research study. However, such an experience is far more educational than any textbook could ever be. The opportunity is what you make of it, and thus I encourage you to not only join the program, but dedicate yourself to your project and thesis. Use the vast resources this campus has to help you, including your advisor, graduate students, the honors program, the writing center, and even the librarians. Lastly, and of utmost importance, enjoy yourself throughout the process!

Introduction

Eukaryotic organisms contain thousands of genes and gene products. The cellular control of both the amount of these gene products and the timing of their appearance is vital to the survival of an organism. Part of this process involves cellular differentiation in which a cell becomes a specific type with a specialized function. During cell differentiation the morphology of the cell may change dramatically, however the genetic material is not altered. Thus, the specialization of cells is accomplished by the regulation of gene expression. Regulation can occur at any step of gene expression, starting with chemical and structural modification of DNA or chromatin through post-translational modifications of a protein. Regulation of genes is at the core of biological systems and consequently errors in regulation can have devastating effects. One such devastating effect is the uncontrolled cell growth responsible for the development of cancers. Thus, understanding the role and process of gene regulation at a basic level may lead to improved understandings of diseases and therapeutic methods to treat and prevent them.

The Maine Lab focuses on the regulation of cell fate during development in the model organism, *Caenorhabditis elegans* (*C. elegans*). The lab examines how cells choose between cell proliferation and differentiation and how these choices are coordinated with other developmental events. The purpose of my research was to investigate the relationships among several transcriptional and/or post-transcriptional

regulators in the germ line. This included the following genes: *ego-1*, *csr-1*, *glp-1* and *rha-1*.

Caenorhabditis elegans as a Model Organism for Development

C. elegans is a free-living nematode about 1.5 millimeters in length as a fully developed adult. The phylum Nematoda consists of smooth-skinned worms with long cylindrical bodies, tapered at the ends, and is often referred to as roundworms. *C. elegans* live in soil in many parts of the world and survives by feeding on microbes naturally found in the soil. The laboratory strain, however, is usually fed a weakened strain of *E. coli* bacteria (Brenner, 1974).

C. elegans is an ideal model system because it is a multicellular eukaryotic organism which is simple enough to be studied in great detail, yet similar enough to humans that scientists are able to use their findings to improve our understanding of human biology. Similar biological characteristics between humans and *C. elegans* include complex development and growth, presence of a nervous system, and demonstration of behavior and aging. In addition, many of the genes and cellular mechanisms in this model organism have been conserved and are found in the human genome. Conserved cellular mechanisms include the cell cycle, apoptosis and RNA interference.

This model organism is cheap and easily maintained in a laboratory setting. The body is transparent, making observation of every cell possible

under the microscope. Additionally, *C. elegans* was the first eukaryotic organism to have its genome completely sequenced, and today many mutants are available for biological research. The availability of these mutant strains makes the organism advantageous for genetic study.

Individual *C. elegans* are one of two sexes, hermaphrodite or male.

Figure 1 depicts the anatomy of the hermaphrodite with various structures labeled. The male, unlike the hermaphrodite, has a fanned tail used in mating. The anatomy of the male, with a detailed view of the fanned tail, can be seen in Figure 2. Hermaphrodites can self-fertilize or mate with males, but cannot fertilize each other. The ability to create new strains by crossing males with hermaphrodites, combined with natural inbreeding by self-fertilization, is advantageous for the study of developmental genetics.

An additional advantage of this model organism is its short life cycle. The life cycle (egg to egg-laying parent) is about five and a half days at 15°C, three and a half days at 20°C, and 2.5 days at 25°C (Brenner, 1974). The short life cycle of *C. elegans* make it possible to study numerous generations in a short amount of time.

C. elegans Germline Development and Anatomy

Cells of an animal are classified as one of two types: somatic cells or germ cells. Somatic cells form the body and compose organs and tissues that maintain the live of an individual. The germ line is the cell lineage from which reproductive cells are derived. Sperm and oocytes differentiate from

germ cells and transmit genetic information to the next generation. It is the production of these gametes, and their union, that results in the formation of new individuals. Germ cells are unique because of their ability undergo meiosis and form haploid gametes as well as their totipotent property. The stages of mitosis and meiosis in the *C. elegans* germ line are depicted in Figure 3. Germ cells share a common cytoplasm and are considered syncytial.

Development in *C. elegans* includes embryogenesis, four distinct larval stages, and an adult stage. Gonad development occurs during the four larval stages (L1-L4) each of which ends in a molt. Molting is when a new, stage-specific cuticle is synthesized and the old cuticle is shed by the organism. Animals are sexually mature as soon as they complete the L4-to-adult molt. An adult hermaphrodite gonad has two symmetrical U-shaped arms joined by a uterus and vulva. Each of the arms contains a somatic cell, called the distal tip cell (DTC), which maintains proliferation of the distal germline as seen in Figure 4. During the L1 and L2 stages, all germ cells proliferate and produce daughter cells with a diploid number of chromosomes. However, during the later portion of L3 stage, proximal cells, which are those closest to the vulva, enter meiosis and undergo gametogenesis. The first 40 diploid daughter cells in each gonad arm produce sperm, which are visible at the proximal end of the gonad by the end of L4 stage. All succeeding germ cells make oocytes. Male gonad development is similar to that of the hermaphrodite; however, all germ cells develop into sperm, and there is only one gonadal arm, which contains both of the DTCs.

RNA Interference

The introduction of double stranded RNA into cells can be used as a method to interfere with the function of genes. Specifically, this method has been used in *C. elegans* as a way to reduce gene expression. In RNA interference (RNAi), double stranded RNA (dsRNA) is recognized by an endonuclease, Dicer, which processes the double stranded RNAs into small interfering RNAs (siRNAs) approximately 21-26 nucleotides in length (Matzke and Birchler, 2005). These siRNAs, along with proteins, form what is called an RNA-induced silencing complex (RISC). The siRNAs in RISC bind to cellular mRNA complementary in sequence. RISC has an endonuclease activity that cuts the mRNA and, in doing so, inactivates it. The mechanism of RNAi is outlined in Figure 5.

RNA interference is widely found in eukaryotic species, and recent research has indicated its involvement in many distinct biological phenomena (Tuschl *et al.*, 1999). It is believed to serve as a host defense mechanism against viral infection and to silence transposons in the germ line of eukaryotic genomes. Components of the RNAi machinery are also involved in transcriptional silencing via histone and DNA modifications. Research on this natural phenomenon has led to its use as a research tool. RNA interference is widely used as a way to reduce the expression of specific genes in experimental organisms like the *C. elegans*. Additionally, research today focuses on the uses of this mechanism as a therapeutic strategy in which the

expression of problematic genes could be regulated (Mello and Conte, 2004).

The ego-1 Gene

The *ego-1* gene encodes an RNA-directed RNA polymerase (RdRP) (Smardon *et al.*, 2000). Cellular RdRPs function in RNA-mediated silencing and development in many species (Aniquist, 2002; Mantzke and Birchler, 2005; Tijsteman *et al.*, 2002). *ego-1* mutants have developmental defects, which include reduced germline mitosis, premature entry into meiosis, slow progression through early meiosis, and abnormal oogenesis (Qiao *et al.*, 1995; Smardon *et al.*, 2000). Additionally, chromatin structure is not regulated correctly, and RNA interference (RNAi) is defective in the *ego-1* mutant (Maine *et al.*, 2005; Smardon *et al.*, 2000). In *C. elegans*, unpaired (non-synapsed) chromatin is silenced (i.e., not expressed) during meiosis. This process, called meiotic silencing of unpaired DNA (MSUC), is a wide spread phenomenon that has been observed in many organisms (Hynes and Todd, 2003; Lee, 2005; Matzke and Birchler, 2005). The mechanisms of meiotic silencing are poorly understood, however it is known to occur at the transcriptional level in some species (e.g., *C. elegans*, mouse) and posttranscriptional level in other species (e.g., *N. crassa*) (Hynes and Todd, 2003; Lee, 2005; Matzke and Birchler, 2005). During meiosis in *C. elegans*, unsynapsed DNA normally receives a high density of the “silencing” modification, dimethylation of the lysine 9 residue of histone H3 (H3K9me2). In *ego-1* mutants, H3K9me2 does not accumulate on unpaired DNA. We are

trying to understand whether the *ego-1* developmental phenotype is caused by the defects in chromatin regulation and/or RNA-mediated silencing.

The rha-1 Gene

The *rha-1* gene encodes the *C. elegans* RNA helicase A (RHA) ortholog. RNA helicase activity catalyzes the unwinding of an RNA helix. Research has shown that RHA has a variety of functions in the regulation of transcription. In *Drosophila*, RHA is part of the dosage compensation complex (Kelly and Kuroda, 2000). Dosage compensation is a regulatory mechanism which equalizes the activity of gene expression between the homogametic and heterogametic sexes of an organism. In *Drosophila* dosage compensation is achieved by increasing the transcription of the single male X chromosome. In humans, RHA interacts with proteins to help export RNA from the nucleus (Tang and Wong-Staal, 2000). This protein is conserved in nematodes and mammals, however little research with respect to this protein has been done on nematodes. Previous research has shown that *rha-1*, like *ego-1*, functions in germline development, regulation of germline chromatin, and RNAi (Walstrom *et al.*, 2005; Robert *et al.*, 2004). RHA-1 activity is also important for germ cell proliferation and meiosis. The *rha-1* null [*rha-1(0)*] chromatin and developmental defects are temperature sensitive while the RNAi defects are not temperature sensitive. One chromatin defect is loss of H3K9me2 on unpaired chromatin during meiosis. This histone modification defect may cause the sterile phenotype.

The glp-1 Gene

The *glp-1* gene encodes a Notch-type receptor protein. Germline proliferation depends on a signal from the distal tip cells (DTCs) that is mediated by GLP-1 protein in the germ line (Kimble and Simpson, 1997; Westlund *et al.*, 1997). When the DTCs are removed, germs cells that are normally mitotic instead enter meiosis and undergo gametogenesis (Kimble and White, 1981). *ego-1* was first identified as on the basis of genetic interactions with the GLP-1/Notch pathway (Qiao *et al.* 1995). Recent data indicate that EGO-1 is not a component, regulator, or target of the GLP-1/Notch pathway, but instead acts in parallel with it to promote germline proliferation (Vought *et al.* 2005).

The csr-1 Gene

Argonaute proteins are a class of RNA-binding proteins. It is known that Argonaute proteins are involved in: RNAi; regulation of gene expression by miRNA (microRNA) and siRNA (small-interfering RNA); meiotic silencing of unpaired DNA in *N. crassa*; and regulation of heterochromatin formation in *S. pombe* (Grishok *et al.*, 2001; Lee *et al.*, 2003; Yigit *et al.*, 2006). CSR-1/AGO is one of 26 *C. elegans* Argonaute genes. The *csr-1* gene was recently discovered to be an *ego* gene with a developmental phenotype similar to *ego-1* (X. She and E. Maine, unpublished data). *csr-1* male animals have a altered H3K9me2 accumulation pattern (X. She and E. Maine,

unpublished data). Thus, research suggests that *csr-1*, like *ego-1*, functions in germline developmental, RNAi, and meiotic silencing of unpaired chromatin (X. She and E. Maine, unpublished data). It is these phenotypic similarities between *csr-1* and *ego-1* that makes this gene of particular interest to our research.

We designed experiments to further explore the relationships among the *rha-1*, *ego-1*, *glp-1* and *csr-1* genes. These genes all function in germline proliferation and differentiation and are required for fertility. They are also all involved in transcriptional and/or post-transcriptional regulation in the germ line. Because these genes regulate similar processes, we developed and characterized the following double mutants: *ego-1(0); rha-1(0)*, *csr-1(0); rha-1(0)* and *rha-1(0); glp-1(ts)*. By characterizing the phenotypes of these double mutants we have improved our understanding of the relationships between these genes and how they work both together and independently to regulate similar processes in the germ line of *C. elegans*.

Materials and Methods

Strains

Nematodes were maintained on agar plates seeded with OP50, an *Escherichia coli* strain with limited growth, as described by Brenner (Brenner, 1974). All strains were cultivated at 20°C except for *ts* alleles, which were used in experiments at 25°C. Standard maintenance conditions, as described by Lewis and Fleming, were used for all strains (Lewis and Fleming, 1995). The wild-type strain was *C. elegans* variant Bristol (N2) (Chen *et al.*, 2003; <http://wormbase.org>). Mutant nomenclature follows standard guidelines (Horvitz *et al.*, 1979).

Animals were passaged in one of two ways. Overgrown plates were passaged by using a sterilized spatula to move a chunk of agar, containing hundreds of animals, from an old plate to a fresh plate. Alternatively, single animals were moved using a worm picker made of platinum wire mounted on the tip of a Pasteur pipette. The platinum wire was flamed between transfers to avoid contamination of worm stocks.

Strains containing the following mutations and chromosomal rearrangements were used in this study: LG I: *ego-1 (om71)*, *ego-1(om84)*, *unc-15(e73)*, *unc-15[ccIs4251]*; LG II: *rha-1(tm329)*, *unc-4(e266)*; LG III: *glp-1(bn18)*; LG IV: *him-8(el489)*, *csr-1(tm892)*, *nT1 [IV; IV]*.

Construction of Mutant Strains

ego-1(om84) homozygous animals are sterile and thus can not be maintained as a homozygous stock. In order to generate an *ego-1(om84); rha-1(tm329)* double mutant, we constructed a strain where *ego-1(om84)* was balanced by a double mutant *ccIs4251 unc-15* chromosome. This chromosome has an *ego-1(+)* allele. Homozygous *unc-15* animals have a paralyzed phenotype which is easily viewed under the microscope. These animals are slow moving compared to wildtype adults and tend to curl their bodies. Heterozygous animals were identified by using the chromosomally integrated transgene insertion *ccIs4251* expressing green fluorescent protein (GFP). GFP is a protein from jellyfish which fluoresces green when exposed to blue light. *ego-1* heterozygous animals could be isolated by looking for the presence of GFP and wildtype body movement. Homozygous wildtype animals lacked both the paralyzed phenotype and GFP.

Genetic crosses were carried out using *ego-1(0)/ccIs4251* and *rha-1(0)* in order to generate an *ego-1(0)/ccIs4251; rha-1(tm329)* strain. Here, "0" is used to designate the null allele of each gene. The genetic crosses performed to obtain this strain are outlined in Figure 6. *rha-1(0)* males were mated with *ego-1(0);ccIs4251unc-15* hermaphrodites, and *ego-1(0)/+;rha-1(0)/+* F1 males were identified as non-GFP expressing. These males were mated with *ego-1(0);ccIs4251unc-15* hermaphrodites. Green hermaphrodite progeny were placed on individual plates and allowed to produce self-progeny, which were screened for the presence of sterile *ego-1(0)/ccIs4251;*

rha-1(0)/+ hermaphrodites. Fertile siblings were chosen from those plates, placed onto individual plates, and allowed to produce progeny, some of which will be genotype *ego-1(0)/ccIs4251; rha-1(0)/rha-1(0)*. Two sets of progeny from each of these lines were then selected. One set was placed at 25°C and one set at 20°C. Animals grown at 25°C were screened for sterility, which was expected to be approximately 50% if the line was *rha-1(-/-)*. Two plates were found to contain a high % of sterile hermaphrodites. Homozygosity for *rha-1(tm329)* was confirmed by PCR.

To isolate genomic DNA for PCR, worms were rinsed with M9 buffer, and pelleted by centrifugation at 1,500 rpm for 1-2 minutes. M9 supernatant was removed, and worms were washed with 10 volumes of DNA Disruption Buffer (200mM NaCl, 50mM EDTA, 100mM Tris (pH 8.5), 0.5% SDS, dH₂O) two times, pelleting worms in between each wash. 1/100 volume of 20 mg/ml Proteinase K was added and worms were incubated for 1-2 hours at 65°C with periodic mixing. Solution was extracted with 1:1 solution of phenol: chloroform and spun at high speed for 2 minutes. The aqueous layer was moved to a fresh tube and the extraction was repeated. The aqueous layer was extracted with chloroform; nucleic acid was precipitated by adding 2 volumes of ethanol, mixing and leaving at -20°C for 1 hour. The solution was centrifuged at 4°C for 30 minutes at high speed to pellet the nucleic acid; supernatant was removed and the pellet was rinsed with 70% ethanol two times and left to dry. The dry pellet was resuspended in TE (10mM Tris (pH

7.5), 1mM EDTA). The quality of the DNA was confirmed by running 2µl of DNA with 1µl of dye and 4µl of water on a 2% agarose gel.

The extracted DNA product was then amplified, as follows. The PCR mixture included: 1µl DNA oligonucleotide 3784, 1µl DNA oligonucleotide 2221, 1µl DNA oligonucleotide 3430, 2.5µl 10X PCR buffer, 0.5µl dNTPs, 1.5µl 22mm MgCl₂, 0.25µl Taq polymerase, 16.75µl double distilled water, and 0.5µl DNA sample. PCR mixture was placed under oil to allow the drop of liquid containing the lysed worms to mix with it under the oil. The following PCR program was used: 94°CX30", (92°CX30", 58°CX30, 72°CX30) X40, 72°CX5, 4° hold. A 1.2% agarose gel was used to determine the genotype of the animals. The wildtype amplification product is 1586 base pairs while the deletion amplification product is 527 base pairs as a result of the 1059 base pair deletion as seen in Figure 7 (Walstrom *et al.*, 2005). There is also a smaller, 377 base pair, wildtype amplification product that is generated using an oligonucleotide primer within the deleted region; hence, no corresponding product is generated from the deletion allele.

Phenotypic Characterization of Double Mutants

The phenotypes of the *ego-1(0); rha-1(0)* and the *csr-1(0);rha-1(0)* double mutants were characterized at specific developmental times points. Developmental phenotypes of double mutants were compared with individual single mutants. Synchronized *ego-1(0); rha-1(0)* animals and controls were recovered as follows: embryos were picked to seeded plates, and L1 larvae

that hatched within a 4 hour time period were picked to a fresh plate and allowed to grow at 25°C. Synchronized *csr-1(0);rha-1(0)* animals and controls were recovered as follows: adults were placed on plates for 4 hours and allowed to lay eggs; after 4 hours, adults were removed from plates and embryos were left at 25°C to hatch and grow for 60 hours. Single mutant control strains were maintained together with the double mutants at 25°C. After the appropriate time periods, animals were washed with M9 buffer to remove bacteria, fixed in -20°C methanol for 10-15 minutes, stained with DAPI (1 mg/ml) for 10-15 minutes, and mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) for viewing under fluorescent light. The percent of gonad arms with oocytes, mature sperm, and primary spermatocytes was determined. In addition, the organization of the germ line was evaluated.

The phenotype of the *rha-1(0);glp-1(ts)* double mutant was characterized at the semi-permissive temperature of 20°C. Double mutant fertility was assayed in the dissecting microscope. The presence of mitotic germ cells was determined by picking L4 larvae, letting them age for 24 hours, and then DAPI-staining and examining them in the compound microscope.

Mutagenesis

rha-1;him-8 animals were washed off of OP50 plates with M9 buffer and placed in eppendorf tube using a glass pipette. This step was performed

twice to ensure that all animals were transferred. The eppendorf tube was centrifuged for 2-3 minutes at 1,000-2,000 rpm. The supernatant (OP50 bacteria and M9 solution) was removed using a pipette. If the supernatant was cloudy, additional M9 was added to the tube, animals were rewashed, and the tube was spun again.

Separately, an EMS (Methanesulfonic acid Ethyl Ester) solution was made. These steps were carefully performed under the hood because EMS is a mutagen that tends to cause single nucleotide changes in DNA (cytosine is converted to thymine or guanine is converted to adenine). At a much lower rate, EMS has also been shown to induce nucleotide deletions (Sulston and Hodgkin, 1988). EMS solution was prepared by centrifuging a mixture of 1 μ l EMS to 0.5 ml M9. The solution was then added to the washed nematode pellet, and the tube was gently inverted and wrapped with parafilm. The animals were left in EMS for 5 hours at 20°C with periodic re-suspension by inversion of the tube.

After the 5 hour time period, animals were pelleted as done previously. EMS solution was removed and placed in beaker with 4N NaOH to inactivate the EMS. M9 was added to the tube containing the mutagenized animals and gently mixed to wash. Tube was again centrifuged. Animals were removed from the bottom of the tube with a glass capillary pipette and placed onto an unseeded Nematode Growth Medium plate. Plate was left uncovered in the fume hood until solution evaporated.

Mutagenized young adults were individually picked onto plates and stored at 20°C. F1 progeny were picked onto plates and stored at 20°C. F2 progeny were screened twice for sterile mutants. Any sterile F2 progeny were characterized by DAPI staining at various different time points. DAPI staining was used to determine if the new mutation enhanced the *rha-1* chromatin defect at 20°C. Mutants that appeared to enhance the *rha-1* phenotype were further tested by DAPI staining at several developmental time points and their germline morphology was analyzed under the microscope. Those which had similar germline morphology to *rha-1* were further tested with antibody staining specific for H3K9me2. This antibody staining was used to determine if proper silencing via the dimethylation of H3K9me2 was taking place.

Results

Characterization of the ego-1(om84); rha-1(tm329) double mutant phenotype

The *ego-1* and *rha-1* genes both play a role in germline development and are involved in germ cell proliferation, meiosis, chromatin structure and RNAi. Studies have shown that many of the *rha-1* and *ego-1* defects are similar and thus we speculated these genes may be acting in the same pathway. We generated and characterized the phenotype of an *ego-1(om84);rha-1(tm329)* double mutant to investigate this hypothesis. Animals were examined at 25°C because the *rha-1* allele, although a molecular null, has a temperature sensitive phenotype.

The overall germline organization of the double mutant was evaluated. In general, single mutants had a well-organized germline with well-defined mitotic, leptotene, zygotene, pachytene, diplotene and diakinesis regions. Refer to Figure 3 for an overview of mitosis and meiosis in the germ line of nematodes. Double mutant germ lines, however, had a “disheveled” look. Nuclei with different morphologies were scattered, nuclei looked ragged and abnormal, and the distal end of the arm was often bulbous (Figure 8).

Detailed characterization of gamete formation at 25°C revealed a severe defect in the double mutant. The percent of germ lines with oocytes was drastically reduced in the double mutant as compared with either single mutant. For animals 44-48 hours post-hatch, 69% of *rha-1(0)* animals and 75% of *ego-1(0)* animals had oocytes, while only 9% of *ego-1(0); rha-1(0)* double mutants had oocytes (Table 1). If the effect of the two single mutants

were additive, then we would expect approximately 52% of the double mutant animals to have oocytes. For animals 48-52 hours post-hatch, 68% of *rha-1(0)* and 100% of *ego-1(0)* animals had oocytes, while only 31% of *ego-1(0); rha-1(0)* double mutants animals had oocytes (Table 1). If the effect of the two single mutants were additive, we would expect approximately 68% of the double mutant animals to have oocytes.

In addition to the oogenesis defect, there seemed to be a weak detrimental effect on spermatogenesis in the double mutant. In particular, there was a delayed onset or reduced rate of spermatogenesis. At 44-48 hours post-hatch, 91% of *rha-1(0)* and 100% of *ego-1(0)* animals had primary spermatocytes, while only 82% of double mutants had them. Interestingly, we also observed that the double mutant occasionally had oocytes but no sperm, a phenotype which was not observed in either single mutant.

Characterizing of rha-1;csr-1 double mutant

The *csr-1* gene was recently discovered to be an *ego* gene with a developmental phenotype similar to *ego-1* (X. She and E. Maine, unpublished data). Research suggests that *csr-1*, like *ego-1*, functions in germline developmental, RNAi, and meiotic silencing of unpaired chromatin (X. She and E. Maine, unpublished data). It is these phenotypic similarities between *csr-1* and *ego-1* that makes this gene of particular interest to our research. We hypothesized that *csr-1* might interact with *rha-1* in the same way that *ego-1*

interacts with *rha-1*. Thus, we have characterized the phenotype of an *rha-1(0);csr-1(0)* double mutant at 25°C.

The overall germline organization of the double mutant was evaluated. In general, *csr-1* single mutants had a smaller and less organized germline than *rha-1* single mutants. Overall, *rha-1* single mutants had a well-organized germline, as described above. The *rha-1(0);csr-1(0)* germline closely resembled that of the *rha-1(0)* single mutant. In general, it was larger and better organized than the *csr-1(0)* single mutant germline (Figure 9)

At 25°C, initial characterization revealed an enhancement of gamete formation in the double mutant. The percent of germ lines with oocytes was greater in the double mutant compared with either single mutant. For animals 60 hours post-egg-lay, 65% of *rha-1(0)* animals and 36% of *csr-1(0)* had oocytes, while 80% of *rha-1(0);csr-1(0)* double mutants had oocytes (Table 2). If the effect of the two single mutants was additive, we would expect approximately 23% of the double mutant animals to have oocytes.

In addition to the oogenesis enhancement, there seemed to be a weak detrimental effect on spermatogenesis in the double. Only 90% of double mutants had sperm, while 96% of the *rha-1(0)* single mutants and 100% of the *csr-1(0)* animals had sperm.

Characterization of rha-1(0);glp-1(ts)

Previous research has identified several genes whose loss-of-function mutations enhance *glp-1* (Qiao et al., 1995). These genes include *ego-1*, *ego-*

2, *ego-3*, *ego-4*, *ego-5* (Qiao *et al.*, 1995), *lag-1* (originally identified by Lambie and Kimble, 1991), and *glp-4* (originally identified by Beanan and Strome, 1992). Since the *rha-1(0)* mutant resembles *ego-1* mutants in several (although not all) respects, we tested whether *rha-1(0)* also enhanced *glp-1*.

At 25°C, the *glp-1(bn18ts)* phenotype is severe; germ cells prematurely enter meiosis and undergo gametogenesis (Kodoyianni *et al.*, 1992). At 20°C, however, 99% of *glp-1(bn18ts)* animals maintain a mitotic population until adulthood (Qiao *et al.*, 1995). Thus, we screened *rha-1(0);glp-1(ts)* at 20°C, a temperature at which *glp-1* function is only slightly reduced. Evaluating at this temperature minimized the defects caused by the *glp-1* mutation alone.

Strong enhancers of the of the *glp-1(ts)* 20°C phenotype produce a severe Glp-1 phenotype, in which germs cells prematurely enter meiosis and undergo gametogenesis (e.g., Qiao *et al.* 1995). However, we observed no enhancement of *glp-1* by *rha-1*. Our data showed that 100% of both single mutant and double mutant germ lines had a mitotic zone. These data are summarized in Table 3.

Genetic screen for enhancers of rha-1

Chromatin defects of *rha-1* mutants are *ts* (Walstrom *et al.* 2005). Therefore, we suspected that other genes function redundantly with *rha-1* at lower temperatures to regulate the chromatin structure. We mutagenized *rha-1(0)* animals and screened for mutations that enhanced the *rha-1* chromatin

defect at 20°C (see Methods). In our pilot study, we screened 4932 haploid
genomes and found no mutations that enhanced this defect.

Discussion

ego-1 and rha-1 work both together and in parallel

If *ego-1* and *rha-1* were acting in the same pathway, we would expect the double mutant null phenotype to resemble either the *ego-1(0)* or *rha-1(0)* single mutant, although the interpretation is complicated by the fact that the *rha-1(0)* phenotype is *ts*. With respect to most aspects of the phenotype, the double mutant resembles *rha-1*. This suggests that the two proteins work together to regulate germline proliferation and organization. The oogenesis defect did not, however, resemble either of the single mutants. This defect is significantly worse in the double mutant than in *rha-1* alone. This suggests that these proteins work in parallel, rather than together, to regulate either the early stages or entry into oogenesis. Overall, the results suggest that *ego-1* and *rha-1* work together to regulate some processes and independently to regulate others.

The csr-1 and rha-1 work together and antagonistically

If *csr-1* and *rha-1* were acting in the same pathway, we would expect the double mutant null phenotype to resemble either the *csr-1(0)* or *rha-1(0)* single mutant, although the interpretation is again complicated by the fact that the *rha-1(0)* phenotype is *ts*. With respect to the germ line size and organization, the double mutant resembles *rha-1*. However, the oogenesis defect is significantly less severe in the double mutant than in *rha-1* or *csr-1* alone. This interaction is the opposite of what we observed between *ego-1*

and *rha-1*. This is a surprising due to the previously observed similarities between *ego-1* and *csr-1*. We expected *ego-1* and *csr-1* to interact similarly with *rha-1*. These results, however, are preliminary and only a small number of animals have been looked at in detail. I plan to examine a larger number of animals in order to confirm these initial findings. However, if the preliminary result are confirmed, they would suggest that *csr-1* and *rha-1* act antagonistically (in a genetic sense) to regulate oogenesis onset or a very early step in oogenesis. Furthermore, these findings would imply that EGO-1 and CSR-1 proteins function differently to regulate oogenesis. Given this possibility, it might be informative to examine oogenesis in the *ego-1;csr-1* double mutant. Overall, at this point, we suspect that the CSR-1 and RHA-1 proteins may work together to regulate germline proliferation and organization and independently to regulate either entry or early stages of oogenesis.

rha-1 does not enhance *glp-1(ts)*

If *rha-1* enhanced *glp-1*, we would have seen germs cells prematurely enter meiosis and undergo gametogenesis. Our results, however, showed no such enhancement. Thus, from our results, we suspect that RHA-1 does not regulate germline proliferation by promoting GLP-1 signaling. However, because our experiment was carried out at 20°C, it is possible that there was too much RHA-1 activity to see an effect. While looking at animals at 25°C would eliminate this difficulty with respect to RHA-1 activity, the GLP-1

phenotype is too severe at this temperature. Due to this difficulty, we can not conclusively say that there is no genetic interaction between these genes.

Conclusion

Although it is not possible to make definitive conclusion about the relationships between RHA-1 and these other germline regulators, my research has continued to improve our understanding of how different aspects of germline development are regulated in this model organism. The regulation of gene expression by the *rha-1*, *ego-1*, *glp-1* and *csr-1* gene products helps to accomplish development in *C. elegans*. This process of regulated gene expression is essential to biological systems and consequently errors in regulation can have devastating effects. Thus, any advancement in our understanding of the role and process of gene regulation at a basic level may lead to new or improved understanding of diseases and therapeutic methods to treat and prevent them.

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FIGURE 1: *C. elegans* Hermaphrodite Anatomy

Taken from Altun, Z. F. and Hall, D. H. 2005. Handbook of *C. elegans* Anatomy. In *WormAtlas*. <http://www.wormatlas.org/handbook/contents.htm>.
A. Differential interference contrast image of an adult hermaphrodite. Lateral side is to the left. **B.** Diagram of anatomical structures of adult hermaphrodite. Dotted lines and numbers mark the level of each section.

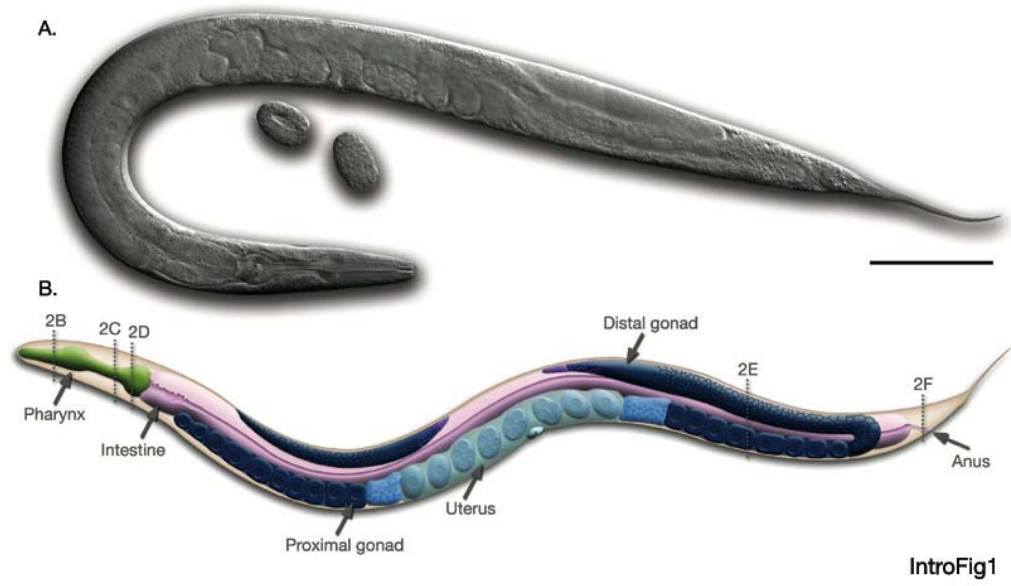
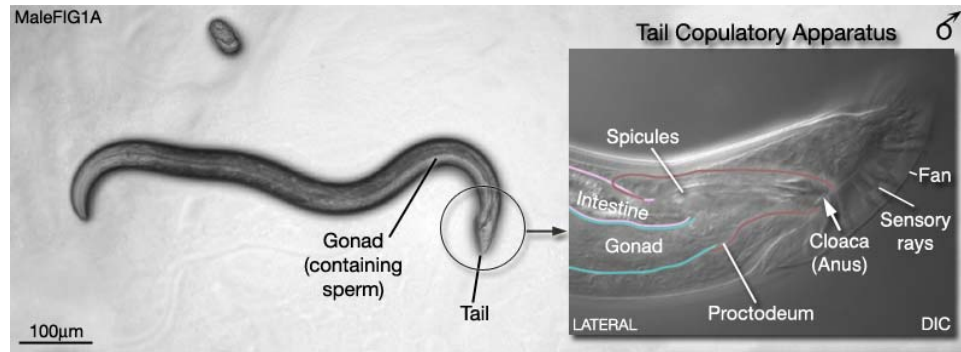


FIGURE 2: *C. elegans* Male Anatomy

Taken from Lints, R. and Hall, D. H. 2005. Handbook of *C. elegans* Male Anatomy. In *WormAtlas*.
<http://www.wormatlas.org/handbook/contents.htm>. Under the microscope, an adult male can be distinguished from a hermaphrodite by its slim body and distinctive tale, which has a copulatory apparatus.



**FIGURE 3: DAPI Stained Adult Hermaphrodite Germ Cell
Nuclei Undergoing Mitosis and Meiosis as Seen Under the Microscope**

Taken from Taken from Altun, Z. F. and Hall, D. H. 2005. Handbook of *C. elegans* Anatomy. In *WormAtlas*.<http://www.wormatlas.org/handbook/contents.htm>. Mitotic germ cells are uniform in appearance. In the transition zone, germ cells enter the early phase of meiotic prophase (leptotene and zygotene). Nuclei then enter the pachytene stage. As nuclei reach the loop of the arm they enter diplotene stage and become organized into a single line. Oocytes continue to diakinesis, where they arrest until maturation.

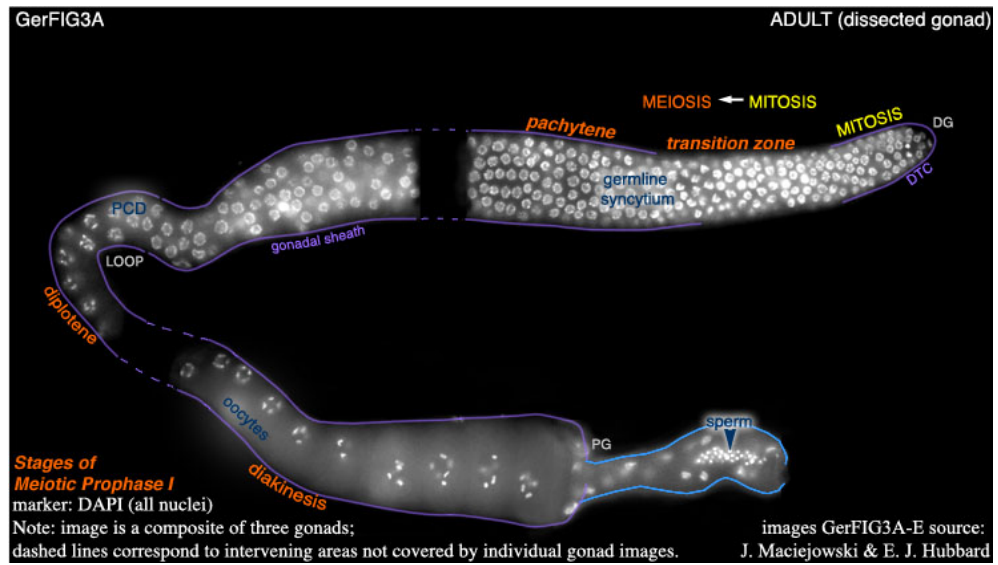


FIGURE 4: Germline Development in *C. elegans*

Taken from Altun, Z. F. and Hall, D. H. 2005. Handbook of *C. elegans* Anatomy. In *WormAtlas*.

<http://www.wormatlas.org/handbook/contents.htm>. Germline development begins at L1 and continues through early adulthood. At hatching the L1 larva contain the germ line precursors Z2 and Z3. During L2 somatic gonad blast cells divide and separate into anterior and posterior populations. These cells continue to divide during L3 and L4, forming the sheath, spermatheca, and uterus. During L3 stage most proximal germ cells enter meiotic prophase. Proximal germ cells continue to enter meiosis and as development progresses a larger number of cells are in meiotic prophase. At adulthood only the distal mitotic stem-cell population continues to proliferate. In hermaphrodites the first 40 germ cells enter meiotic prophase in each arm and produce approximately 160 sperm. After the first 40 germ cells all other germ cells differentiate into oocytes.

GerFIG6

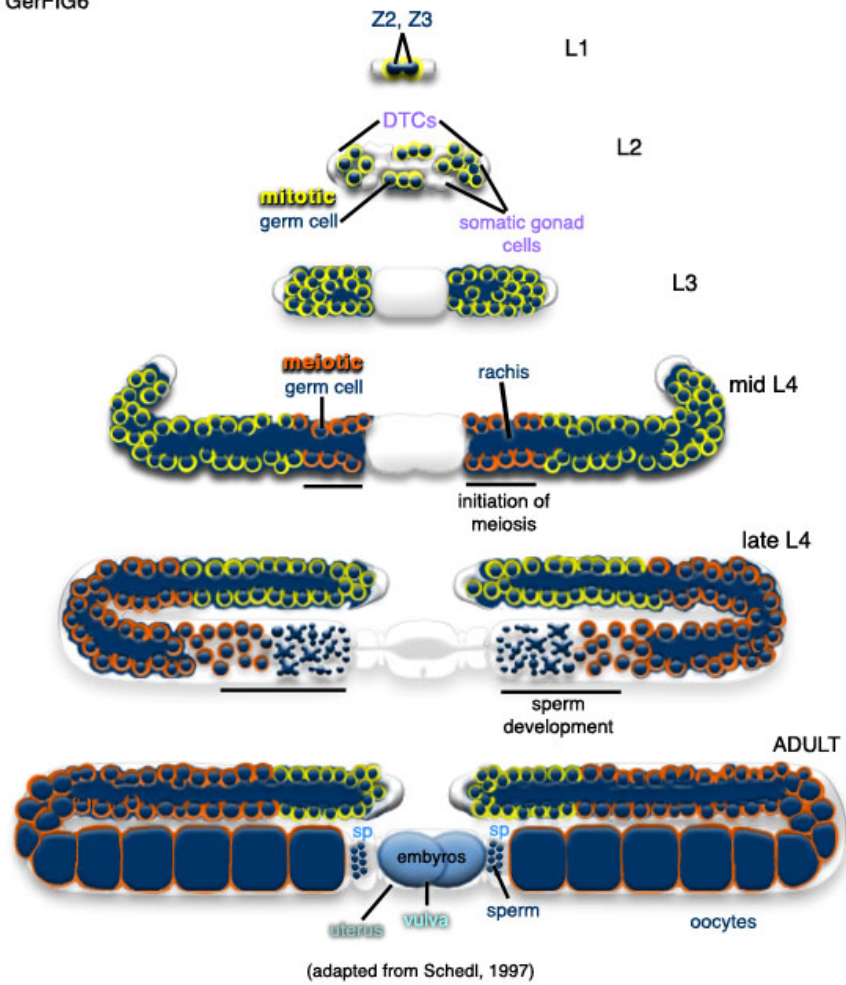


FIGURE 5: Mechanism of RNA Interference (RNAi)

This schematic drawing is adapted from Ambion, Inc., Foster City, CA. <http://www.ambion.com>. When dsRNA enters the RNAi pathway it is processed into 20-25 nucleotide siRNAs by dicer endonuclease. siRNAs assemble with the protein components to form RNA-induced silencing complexes (RISC). siRNA is unwound and the complex is activated. The siRNA strand guides the complex to the cognate mRNA. The mRNA is cleaved, resulting in its deactivation.

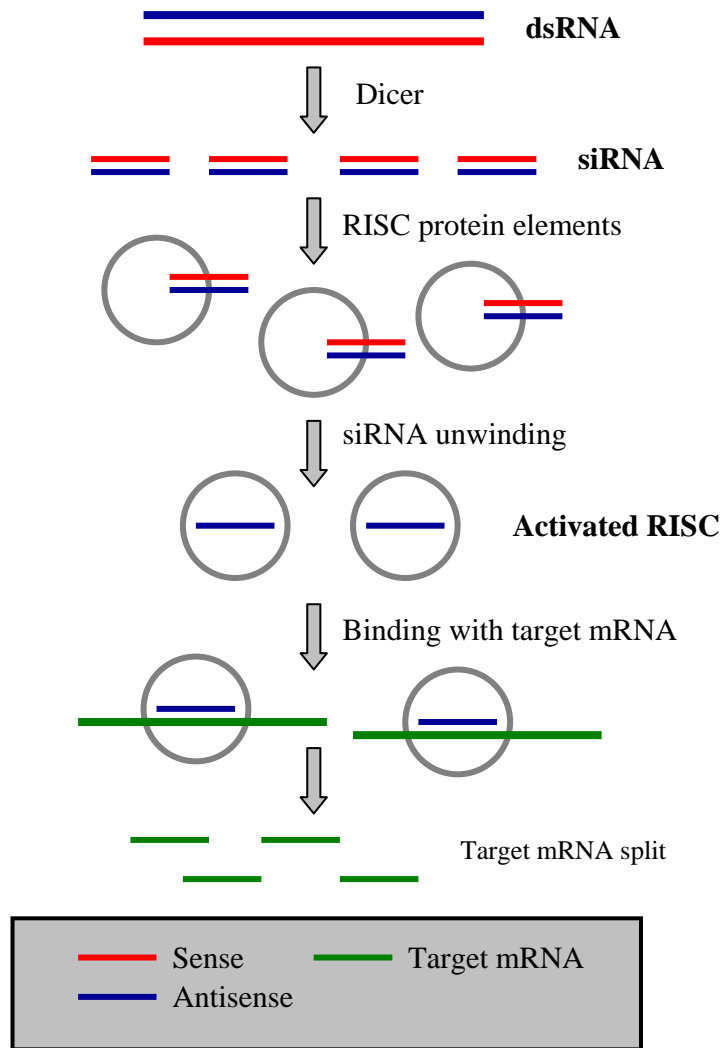


FIGURE 6: Creating an *ego-1(om84); rha-1(tm329)* Double Mutant

The *ego-1(0)* strain used in this experiment was *ego-1(om84)/ccIs4251unc-15(e73)*. The *rha-1(0)* strain used in this experiment was *rha-1(tm329)*. *rha-1(0)* males were mated with *ego-1(0)/ccIs4251unc-15* hermaphrodites. *om84/+;rha-1/+* F1 progeny were identified as GFP-expressing. These males were mated with *ego-1(0);ccIs4251unc-15* hermaphrodites. Green hermaphrodite progeny were placed on individual plates and allowed to produce self-progeny, which were screened for the presence of sterile GFP-expressing hermaphrodites, which would be the *ego-1(om84)/ccIs4251unc-15;rha-1(0/0)* animals. Fertile siblings were chosen from these plates, placed onto individual plates, some of which will be *ego-1(0)/ccIs4251;rha-1(0/0)*. Two sets of progeny from each of these lines were then selected. One set was placed at 25°C and one set at 20°C. Animals grown at 25°C were screened for a high percentage of sterility, which was expected to be ~50% if the line was *rha-1(0/0)*. Two plates contained a high percentage of sterile hermaphrodites. The corresponding plates of these strains maintained at 20°C were tested for the presence of *rha-1(0/0)* genotype by PCR.

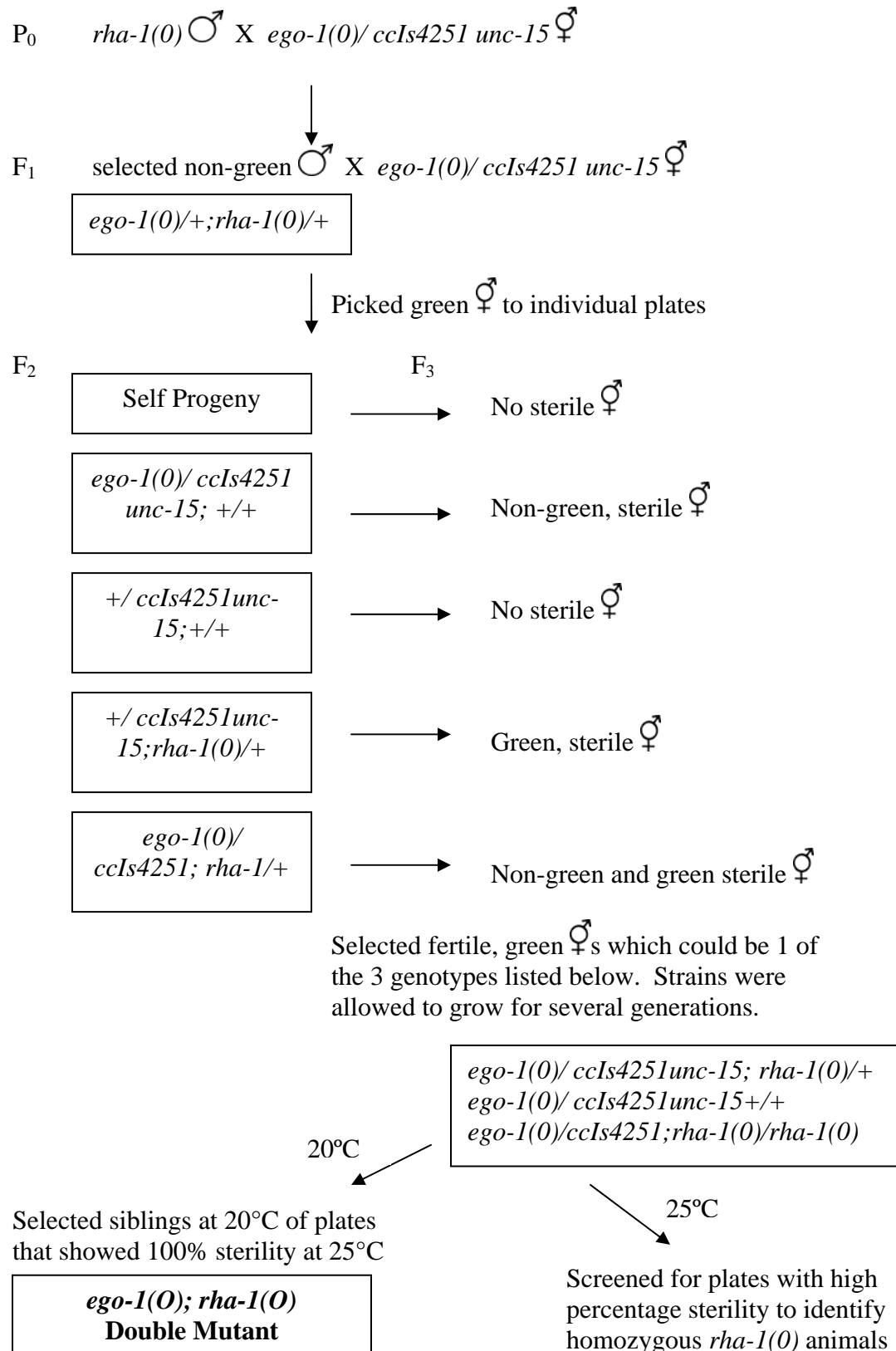
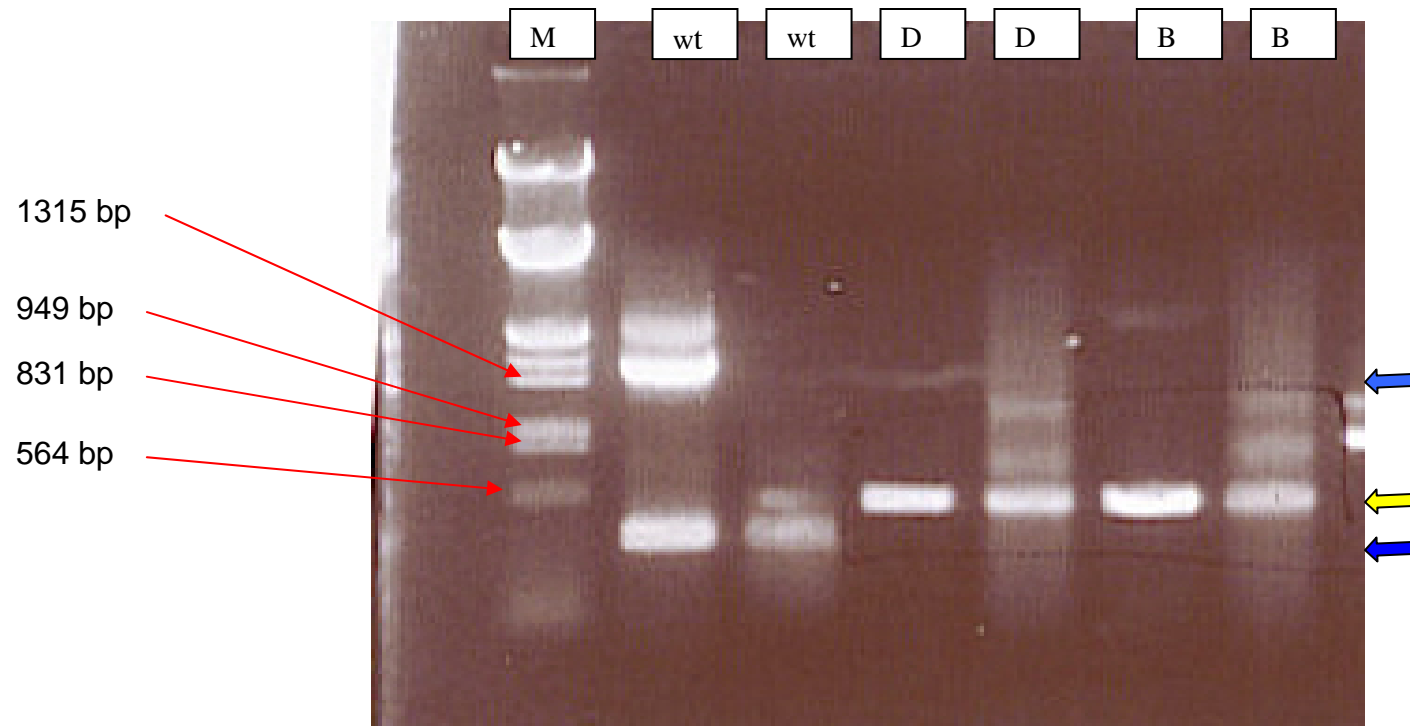


FIGURE 7: PCR Confirmation of *ego-1(om84); rha-1(tm329)* Double Mutant

After building the *ego-1(om84); rha-1(tm329)* strain, genomic DNA was extracted from the animals and PCR was used to confirm the presence of the *rha-1(tm329)* deletion. Three primers were used in the PCR reaction, 2221, 3430 and 3784. The size of the deletion allele product is 527bp while the size of the larger wildtype product is 1586bp. The marker is labeled M on the PCR gel. Wildtype PCR product is labeled wt. Two bands are seen in the wild type product (labeled with blue arrows); the larger band corresponds to product generated by primers 2221 and 3784 and the smaller band corresponds to product generated by primers 3430 and 3784. The second wildtype sample had a very small amount of product and thus the reaction did not work as well. Smaller products usually amplify better, explaining why only the smaller band appears in this wildtype control. In potential mutant strains, labeled B and D, only one band (labeled in yellow) is present because the complementary sequence of primer 3430 has been deleted. Other faint bands are the result of nonspecific product.



Primers:

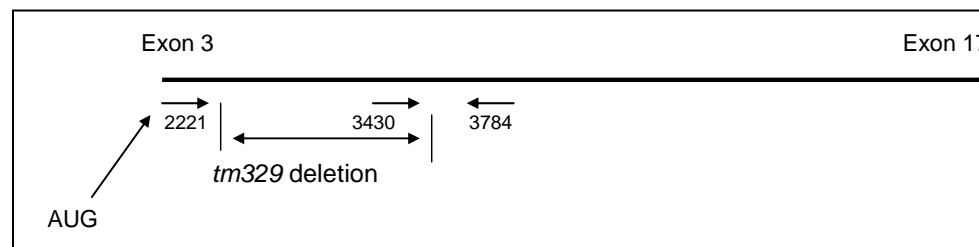
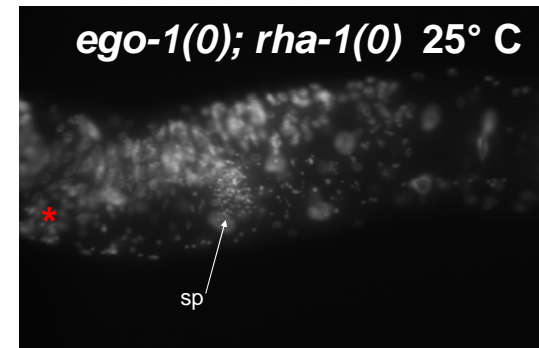
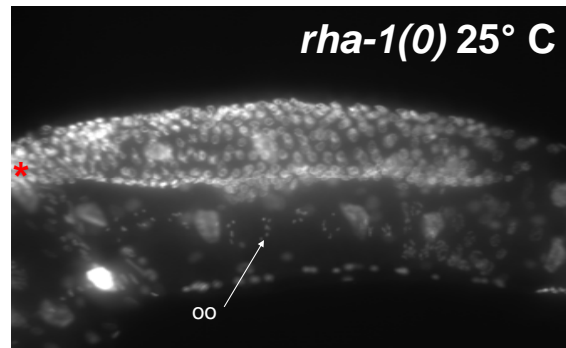
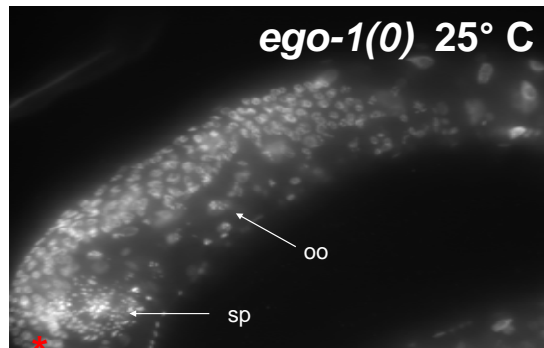


FIGURE 8: Characterization of the *ego-1(om84); rha-1(tm329)* Double Mutant Phenotype

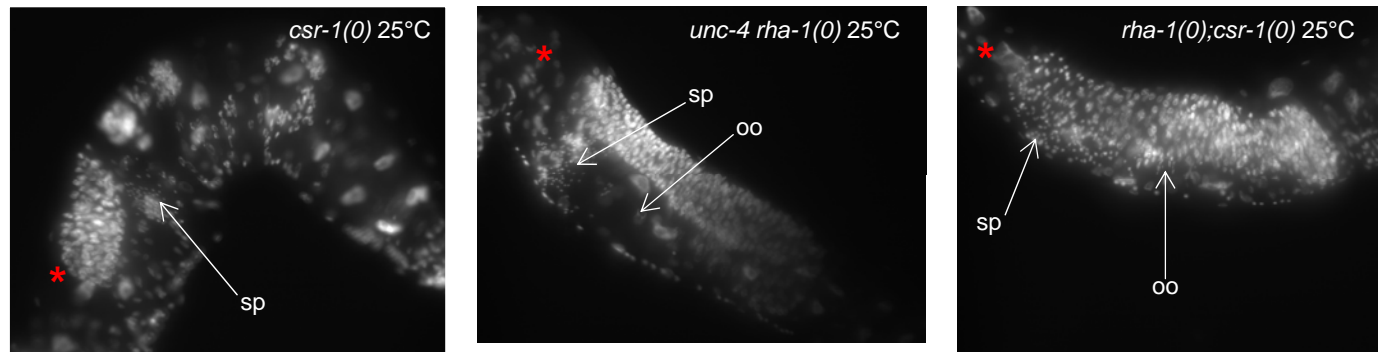
The phenotypes of the *ego-1(om84); rha-1(tm329)* double mutant was characterized at specific developmental times points. Developmental phenotypes of *ego-1(om84); rha-1(tm329)* were compared with single mutants *ego-1(om84)* and *rha-1(tm329)*. Single mutant control strains were maintained together with the double mutants at 25°C. After the appropriate time periods, animals were stained with DAPI and viewed under fluorescent light. The percent of gonad arms with oocytes, mature sperm, and primary spermatocytes was determined. In addition the organization of the germ line was evaluated. In general, single mutants had a well-organized germline with well-defined regions of mitosis, leptotene, zygotene, pachytene, diplotene and diakinesis. Double mutant germlines, however, had a “disheveled” look. Nuclei with different morphologies were scattered, nuclei looked ragged and abnormal, and the distal end of the arm was often bulbous. The percent of germlines with oocytes is drastically reduced in the double mutant as compared with either single mutant. In addition to the oogenesis defect, there seems to be a weak detrimental effect on spermatogenesis in the double mutant. In particular, there is a delayed onset or reduced rate of spermatogenesis.



*****, distal end of the germ line; sp, sperm; oo, oocytes

FIGURE 9: Characterization of the *csr-1(0);rha-1(0)* Double Mutant Phenotype

The phenotype of the *rha-1(0); csr-1(0)* double mutant was characterized at a specific developmental times point and compared with the phenotype of *csr-1(0)* and *unc-4 rha-1(0)* mutants. (Full genotype of the double mutant strain was *unc-4 rha-1(0); csr-1(0)*.) *csr-1* and *rha-1* control strains were maintained together with the *rha-1;csr-1* double mutant at 25°C. After the appropriate time period, animals were stained with DAPI and viewed under fluorescent light. The percent of gonad arms with oocytes and mature sperm was determined. In addition, the organization of the germ line was evaluated. In general, *csr-1(0)* single mutants had a smaller and less organized germline than *rha-1(0)* single mutants. Overall, *rha-1(0)* single mutants had a well-organized germline. In general, the *rha-1(0);csr-1(0)* germline closely resembled that of the *rha-1(0)* single mutant: it was larger and better organized than the *csr-1(0)* single mutant germline. The percent of germlines with oocytes was enhanced in the double mutant as compared with either single mutant.



*, distal end of the germ line; sp, sperm; oo, oocytes

**TABLE 1: Observed Germline Morphology in age 44-48 and 48-52
Hour Post-Hatch of *rha-1(0)*, *ego-1(om84)*, and *om84;rha-1(0)* Animals at 25°C**

Age (post-hatch)	Genotype	% w/ oocytes	% w/ sperm	% w/ gametes
44-48 hr	<i>rha-1(0)</i> (n=84)	69	81	89
	<i>ego-1(0)</i> (n=24)	75	100	100
	<i>ego-1(0);rha-1(0)</i> (n=34)	9	68	88
48-52 hr	<i>rha-1(0)</i> (n=44)	68	93	93
	<i>ego-1(0)</i> (n=28)	100	100	100
	<i>ego-1(0);rha-1(0)</i> (n=28)	31	91	91

Embryos were picked to a lightly seeded plate, and L1 that hatched within a 4-hr window were picked to a fresh plate. Plates were maintained together at 25°C. After the given time frame, animals were fixed and stained with DAPI. Germline morphology was examined under the dissecting microscope. n is the number of gonad arms scored.

**TABLE 2: Observed Germline Morphology of
rha-1(tm329), *csr-1(tm892)*, and *rha-1(tm329);csr-1(tm892)*
 Animals 60 hours post-egg-lay at 25°C**

Genotype (# germlines)	% w/ oocytes	% w/ sperm
<i>rha-1(0)</i> (n=26)	65	96
<i>csr-1(0)</i> (n=14)	36	100
<i>rha-1(0);csr-1(0)</i> (n=10)	80	90

Adults were placed on seeded plate, allowed to lay eggs for 4 hr, and removed. Embryos were left to hatch and grow for 60 hr. Plates were maintained together at 25°C. After 60 hr, animals were fixed and stained with DAPI. Germline morphology was examined under the dissecting microscope. n, the number of gonad arms scored.

TABLE 3: Observed Germline Morphology in *rha-1(ts);glp-1(ts)* at 20°C

Genotype	# Germlines screened	% Germ lines w/ mitotic germ cells
<i>glp-1(ts)</i>	17	100
<i>rha-1(0)</i>	17	100
<i>rha-1(0);glp-1(ts)</i>	19	100

The presence of mitotic germ cells was determined by analyzing DAPI stained L4+24 hour germlines with the compound microscope.