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Reverse Genetics: Downregulating chk-1 and fasn-1 in the Gonads of C. elegans

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Reverse Genetics: Downregulating *chk-1* and *fasn-1* in the Gonads of *C. elegans*

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TABLE OF CONTENTS

INTRODUCTION AND BACKGROUND INFORMATION

The Motivation for this Research

In the world of biological scientific research, there is perhaps no more important first step in any experiment than finding the ideal model organism for the intended work. When testing how a medicine may be used to treat certain human conditions, mice are often the model organism of choice (Bryda, 2013). When looking for an example of inheritable genotypic mutations, fruit flies can be used to visualize the difference in eye color and determine the type of inheritance that trait displays (Bellen et al., 2010). When easily visualizing the phenotypic changes that occur due to genetic mutations, the nematode *Caenorhabditis elegans* is a practical choice (Culetto and Sattelle., 2000; Chiu et al., 2011). *C. elegans* live for 2 weeks, lay approximately 300-1000 eggs in their lifetime, are hermaphroditic over 99% of the time which allows for self-fertilization (Hodgkin, 1983), and are easily stored on plates seeded with the bacteria they will use for food (Corsi, 2006). While these traits make them easy to use, several others make them interesting to study. They have similar molecular signals for regulating growth as those seen in humans (Chiu, 2011), have orthologs for approximately 42% of the genes associated with human diseases (Culetto and Sattelle, 2000), and have readily visible cells and tissues due to the organism being transparent throughout its life (Corsi, 2006). Altogether, these traits make *C. elegans* an amazing model organism for humanity.

Yet another known characteristic of these animals is their extremely specific cell lineage. This means that all *C. elegans* individuals of the same sex developed from zygote to adult in the same way (Sulston, 1983). There is no somatic cellular variation in the development process. All of this allows for the mapping of where each individual cell in an adult came from in the embryo (Sulston, 1983) which makes for an extremely convenient model organism as there is an

invariant and standardized process by which development occurs in wild-type organisms. This makes it easy to identify the effects any mutation or experiment would have on *C. elegans*.

C. elegans individuals are either males or hermaphrodites. The males produce only sperm during their adult life via their testes, while the hermaphrodites, which are phenotypically female, will produce a limited quantity of sperm before switching to produce oocytes for the rest of their lives via their ovotestes (Corsi, 2006). Thus, hermaphrodites can self-fertilize and reproduce clonally. Of note, males are significantly rarer than hermaphrodites, comprising only 0.1 – 0.01% of any given population (Maures *et al*., 2014) due to *C. elegans* typically self-fertilizing as opposed to mating. For this reason, male *C. elegans* received significantly less attention from the scientific community than hermaphroditic *C. elegans*, especially with regards to the regulatory genes of gonadal differentiation. This research intends to focus on the male *C. elegans* to broaden this field of study.

C. elegans General Information and Gonadogenesis

The gonadal region of *C. elegans* is of particular importance because it is crucial in the reproductive process and serves as a commonly studied area of the animal. *C. elegans* develop their gonads from a 4-cell primordium (Kimble and Hirsch, 1979) in a process that is further described in the following section. This process is highly regulated, has well defined stages of development that are readily identifiable under a microscope, and there is currently a great deal of knowledge about what genes are highly expressed during these developmental and differentiation stages (Kroetz *et al*., 2015). The exact function of each gene in these developmental processes is largely unexplored, and this research aims to explore two such genes that are potentially involved in regulating male somatic gonadogenesis.

During embryogenesis, a four-celled primordium present during the first larval stage (L1), will differentiate into a mature gonad. The somatic gonads, which refers to the organs and structures in the gonads not associated with the nematode's germ line, will differentiate from cells termed Z1 and Z4 of this primordium as shown in Figure 1 (Kimble and Hirsch, 1979; Kroetz et al., 2015). By the end of the second larval stage (L2) of the embryo's development, Z1 and Z4 will have differentiated into 10 or 12 total cells depending on whether the animal will become a male or hermaphrodite, respectively. If the animal will be a hermaphrodite, two of these cells will be distal tip cells (DTC) responsible for regulating germline patterning and gonad elongation, one of the cells will be an anchor cell (AC) responsible for patterning the cells of the vulva, and the remaining nine cells will be blast cells responsible for dividing into the remaining portions of the

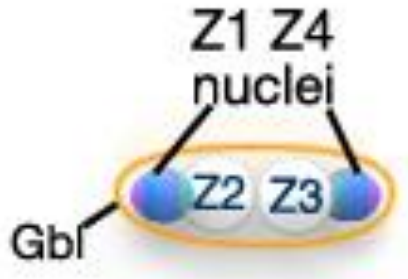

Figure 1: Diagram showing the gonadal primordium at the L1 stage of *C. elegans.* Originally from Wormatlas.org: https://www.wormatlas.org/male/somaticgonad/Somaticgonadframeset.html

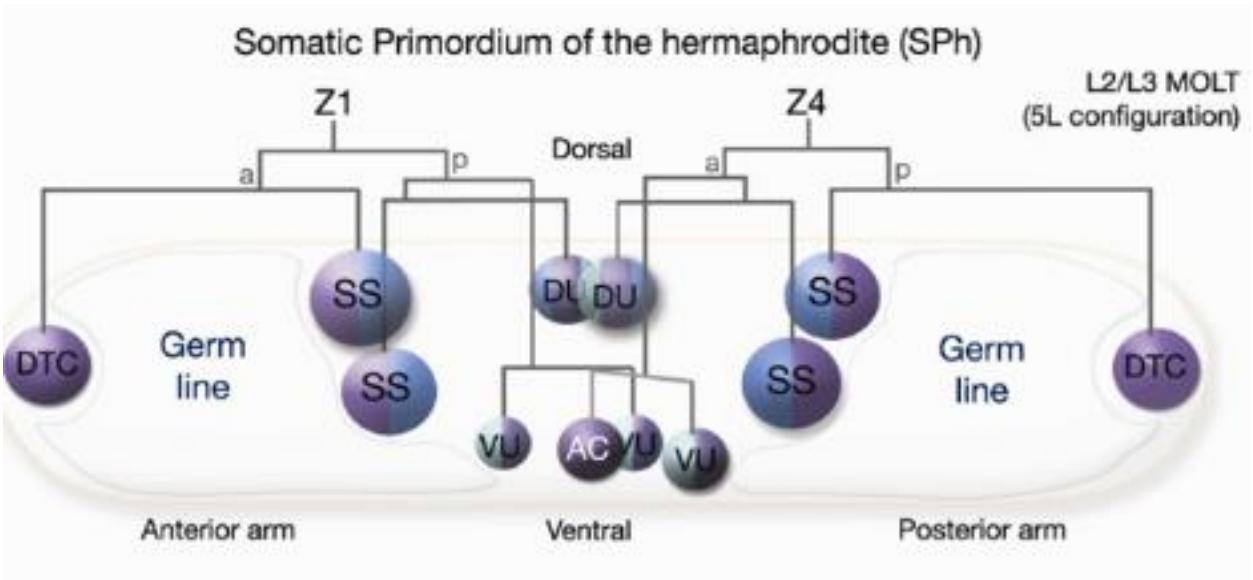

Figure 2: Diagram of the somatic gonadal primordium at the late L2 / early L3 stage of hermaphroditic *C. elegans.* Originally from Wormatlas.org: https://www.wormatlas.org/hermaphrodite/somatic%20gonad/Somframeset.html

somatic gonad (Kimble and Hirsch, 1979), as shown in Figure 2. In males, the 10 total cells will contain two DTCs that only regulate germline patterning (how the germ cells switch from mitosis to meiosis; Killian and Hubbard, 2005), one linker cell (LC) that regulates gonad elongation, and the remaining 7 cells are blast cell precursors for the vas deferens (3 cells) or the seminal vesicles (4 cells) (Kimble and Hirsch, 1979), shown in Figure 3. The blast cells and blast cell precursors in both hermaphrodites and males will continue to divide by mitosis as the worm develops, eventually developing into either the 56-celled asymmetrical testes or the 143-celled

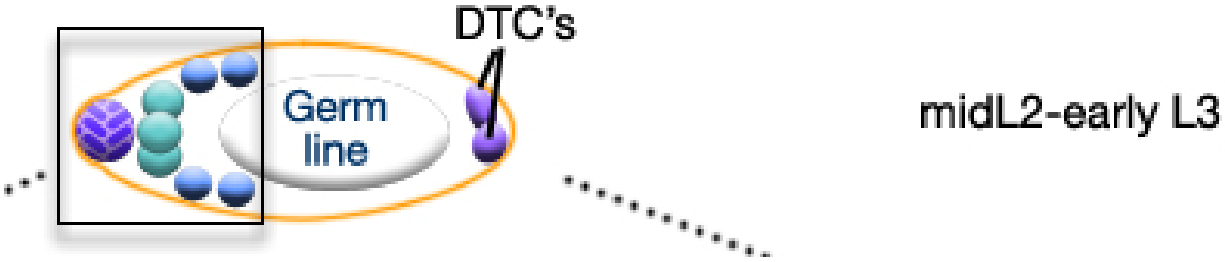

Figure 3: Diagram showing the asymmetrically separating gonadal primordium at the midL2 to early L3 stage of male *C. elegans.* Originally from Wormatlas.org

symmetrical ovotestes (Kimble and Hirsch, 1979).

The him-8 Gene

Because this research intends to focus on male gonadal differentiation, having only 0.1% of the *C. elegans* population be males presents a logistical challenge that must be overcome. There are three potential ways to overcome this challenge and increase the frequency of males in a self-fertilizing hermaphrodite's offspring. First, there is heat shocking the hermaphrodite while it is an L4 which would increase the incidence of male progeny in the F_1 generation to approximately 3-5% (Corsi, 2006). Despite its simplicity, this method presents several issues such as the F_1 generation males potentially having undesirable stress-induced epigenetic changes that persist for subsequent generations (Walsh *et al*., 2020). For the second option, there is crossing males with hermaphrodites which increases male frequency in the progeny to 50% but

which would require the repeated selection of males from nearly every generation of progeny to prevent the population from reverting to being almost exclusively hermaphrodites within a few generations. (Walsh *et al*., 2020). This is impractical. Finally, mutating the hermaphrodite's genome in one of several *him* genes can variably increase the frequency of males in self-fertilizing hermaphrodite's offspring (Hodgkin *et al.*, 1979; Walsh *et al*., 2020). This is the best option for this research, as it is the least variable in terms of success and provides the largest increase in the frequency of male offspring. In the case of the *him-8* mutation that this research will induce, it increases the incidence of males to approximately 40% and has no practical effect on brood size or the general health of both the male progeny and hermaphroditic parent (Broverman and Meneely, 1994). Thus, for any genes of interest in this research that are associated with male gonadal differentiation, the *him-8* gene mutation will need to be simultaneously induced.

Genes of Interest

While there is a substantial difference in morphology between the males and hermaphrodites, and the genes directly regulating this process have been extensively investigated in both sexes, little has been found regarding which genes are important for forming the somatic gonad (Hodgkin *et al*., 1987; Zarkower and Hodgkin, 1992; Chang *et al*., 2004; Kalis *et al*., 2010). Recent transcriptome analysis of the developing somatic gonads in males and hermaphrodites has revealed a suite of genes that are upregulated in the somatic gonads during their development (Kroetz *et al*., 2015), which can now be further explored in the context of gonadal formation. These genes' mRNA transcripts were highly expressed in the somatic gonads when compared to the whole animal or when comparing developing male somatic gonads to

developing hermaphroditic somatic gonads. Thus, it is reasonable to assume that they play some role in regulating somatic differentiation.

Of the genes examined by Kroetz et al (2015), *chk-1* and *fasn-1* were chosen to be the

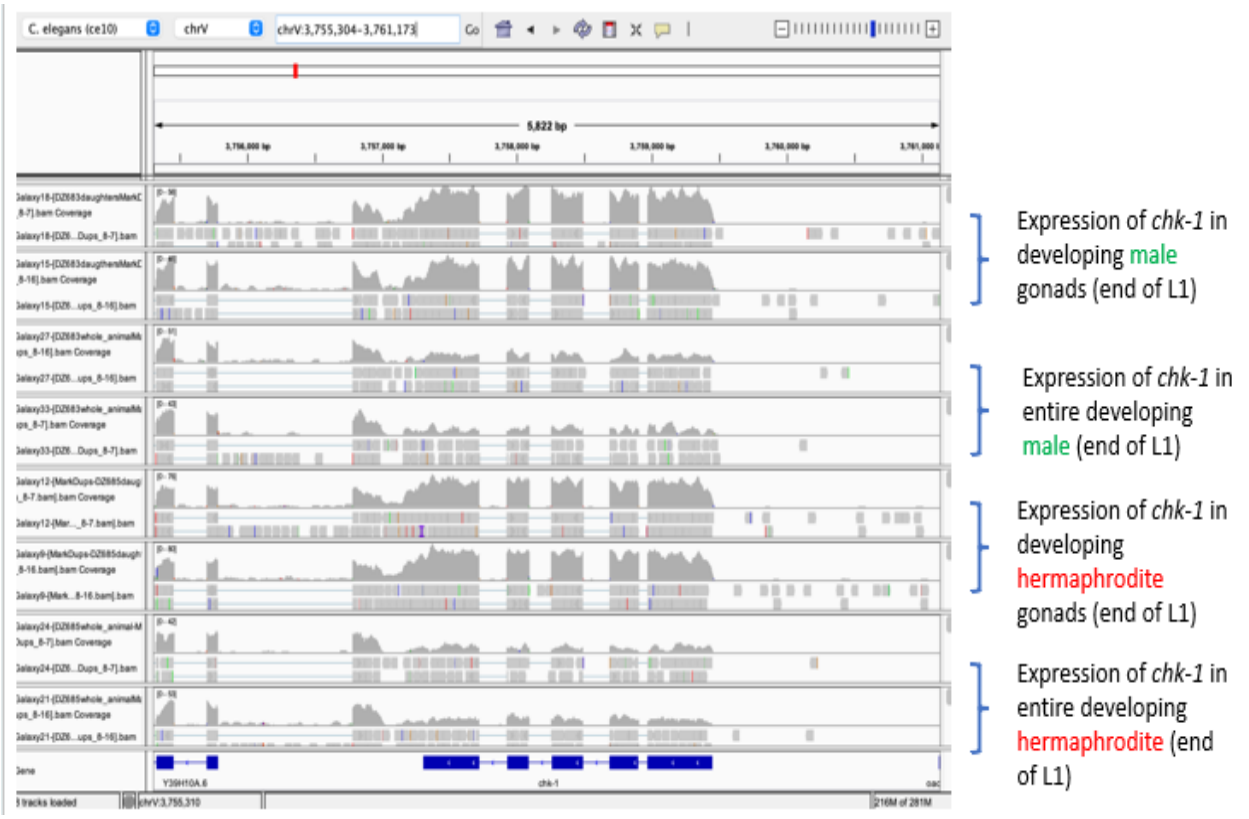

Figure 4: Image showing the transcriptome analysis of *chk-1* in developing hermaphrodites and males at the end of the L1 stage just before the four-celled somatic gonad primordium divides.

focus of this research. The gene *chk-1* was shown to be 5.97 times more highly expressed in developing male somatic gonad cells as compared to the rest of the male's developing cells at the L1 stage of development (Kroetz *et al*., 2015). The same is true in the hermaphrodites, as *chk-1* was found to be 5.27 times more highly expressed in the developing somatic gonad cells as compared to the rest of the animal (Kroetz *et al*., 2015). This is summarized in Figure 4.

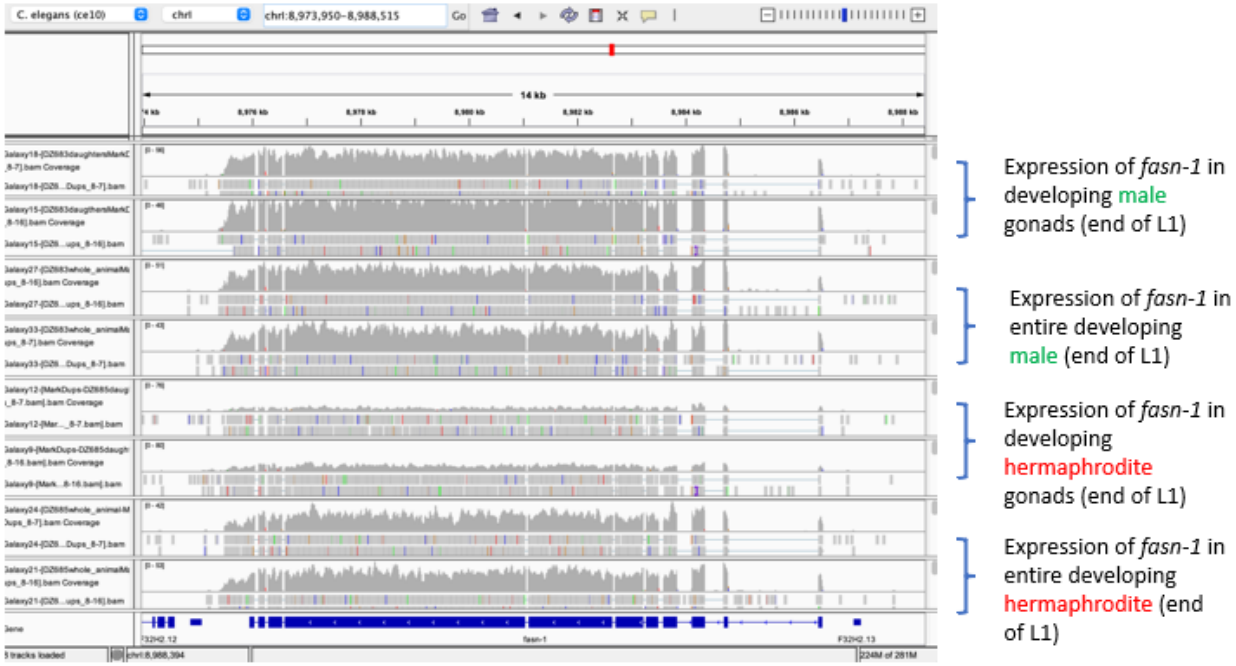

Figure 5: Image showing the transcriptome analysis of *fasn-1* in developing hermaphrodites and males at the end of the L1 stage just before the four-celled somatic gonad primordium divides.

The gene *fasn-1* had a different but interesting pattern of mRNA enrichment. The *fasn*-1 gene was shown to be expressed 8.77 times more in the developing male somatic gonad primordium cells than in developing hermaphroditic somatic gonad primordium cells at this stage (Kroetz *et al*., 2015) as shown in Figure 5. Because each of these genes were upregulated in somatic gonad primordium cells, they are likely to be regulators of gonadal differentiation, making them prime candidates for further study. The *fasn-1* gene is of particular interest as it being more prevalent in the transcriptome of developing male gonads as opposed to developing hermaphrodite gonads makes it a potentially important regulator of male gonadal differentiation which, as stated, is a relatively uninvestigated area.

The gene *chk-1* has been shown to have two functions in developing *C. elegans*. It serves as a regulatory gene that is activated in response to cell damage and prevents the damaged cell

from entering mitosis before the cell can either be repaired or destroyed. Specifically, it is a G2-M checkpoint gene responsible for arresting the cell cycle in the G2 phase should the cell's DNA have been damaged (Kalogeropoulos *et al*., 2004). If the cell is unable to repair the damaged DNA, *chk*-1 is also responsible for beginning apoptosis of the cell (Kalogeropoulos *et al*., 2004). The gene *chk-1* has also been shown to regulate the development of germ line cells in embryonic *C. elegans* (Kalogeropoulos *et al*., 2004).

In other studies, it was found that silencing *chk-1* via RNAi , which leads to downregulating the expression of *chk-1* at the mRNA-level organism-wide, has resulted in two possible phenotypes. The adult hermaphrodite's cells were consistently unable to repair DNA damage caused by ionizing radiation after *chk-1* had been inhibited by RNAi (Kalogeropoulos *et al*., 2004). Additionally, these cells were also unable to enter DNA damage-induced apoptosis (Kalogeropoulos *et al*., 2004). This is consistent with the above claims regarding the role of *chk-1* in mitosis and apoptosis. In the embryonic progeny of this adult, the most prominent of the two observed phenotypes was embryo lethality in approximately 90% of offspring (Kalogeropoulos *et al*., 2004; Olsen *et al*., 2006). Of the 10% of the embryos that did not die, they developed into sterile adults which were found to have abnormally shaped germ cells in the distal regions of the gonad and no germ cells in the rest of the gonad (Kalogeropoulos *et al*., 2004). Interestingly, a separate study found that *C. elegans* which survived the *chk*-1 RNAi treatment had 15-25% longer lifespans than wildtype (WT) worms and were more resistant to thermal stress (Olsen *et al*., 2006). The 10% that survived are theorized to have developed under extremely low concentrations of *chk-1*, as researchers concluded that *chk-1* expression may have not been entirely disrupted (Kalogeropoulos *et al*., 2004). This lack of germ cells is consistent with *chk-1* being an important regulatory gene in germ line development.

Interestingly, the research of Kalogeropoulos *et al*. comments that *chk-1* "probably" was not necessary for somatic cell cycles as the germline defect was the only postembryonic phenotype detected in their research (Kalogeropoulos *et al*., 2004). However, this ignores the possibility that the *chk-1* expression they theorize as having been not entirely disrupted in the 10% of progeny that survived may have been significant enough for somatic cell development in the gonads. This research aims to specifically downregulate the expression of *chk-1* in the somatic gonads to conclusively determine whether it plays a significant role in somatic gonadogenesis.

The gene *fasn-1* codes for a fatty acid synthase, an enzyme responsible for synthesizing fatty acids to store energy for later use by the organism (Lee K. *et al*., 2010). Specifically, it performs the elongation of malonyl-CoA in a repeated process designed to create palmitic acid (most often) and other unsaturated fatty acids (Lee K. *et al*., 2010). Silencing *fasn-1* via RNAi has also been reported to cause embryonic and larval lethality because these fatty acids are necessary for providing the energy needed during embryonic development (Lee K. *et al*., 2010; Fraser *et al*., 2000). For reference, removing the two complex fatty acids C15ISO and C17ISO from a worm embryo has been shown to be lethal (Kniazeva *et al*., 2004). It thus makes sense how in any way silencing *fasn-1*, which is responsible for creating the precursor molecules for these complex fatty acids, would be similarly lethal.

The *fasn-1* gene, like many genes observed in *C. elegans*, is an ortholog of a gene observed in vertebrate animals called Fatty Acid Synthase (*FASN*). Research comparing *FASN* to *fasn-1* has shown that both are targets for tumor-suppressing genes such as p53 in vertebrates and the p53 homolog in invertebrates, *cep-1* (D'Erchia *et al*., 2006). As a p53 homolog, *cep-1* plays an important role in regulating apoptosis within developing cells and is thus capable of

targeting and inhibiting genes that are extremely active during mitosis such as *fasn-1* (D'Erchia *et al*., 2006). Because *fasn-1* is a target of an apoptosis-inducing gene such as *cep-1*, it both shows the importance of *fasn-1* and provides another pathway through which the degradation of the proteins produced by this gene in the somatic gonads might affect the animal. More than just a gene responsible for creating membrane fatty acids, *fasn-1* is also involved in a similar apoptosis-inducing pathway as *chk-1* (D'Erchia *et al*., 2006).

While these two genes may have vastly different primary functions, both have been shown to be upregulated in the developing gonads of embryonic *C. elegans*, hinting at their significance in this process (Kroetz *et al*., 2015). This research aims to use CRISPR (clustered regularly interspaced short palindromic repeats) to genetically modify these two genes so that any proteins they produce are tagged with GFP. This allows future researchers to selectively degrade these proteins from the proteome and subsequently examine the phenotypic effects this would have in the worm.

CRISPR and Cas9

The CRISPR technique in this research is used to cut double stranded DNA (dsDNA) at specific locations (Ran *et al*., 2013). The two components of this CRISPR system are Cas9 endonuclease, which will cut the dsDNA, and guide-RNAs (gRNAs) made from gRNA oligonucleotides which will 'guide' Cas9 to the correct location(s) to cut at a precise sequence of dsDNA (Ran *et al*. 2013). A third component, a homologous DNA sequence used as a template, will be provided to direct the repair of this cut. While Cas9 endonuclease will be provided, the gRNAs and the homologous template will be generated by this researcher.

CRISPR examines dsDNA for the protospacer adjacent motif (PAM) section (shown in purple in Figure 6) and then scans upstream of that motif for a sequence that is complementary to the section of gRNA called the spacer (Ran *et al*., 2013). The PAM sequence is, from 5' to 3', NGG, which denotes any nucleotide followed by two guanines. An additional section of gRNA, which is not directly interacting with the target DNA, is intended to bind the gRNA to the Cas9 nuclease so that the two can move together as one unit (Ran *et al*., 2013). This is what is called the scaffold in Figure 6. Once the spacer is bound to the target DNA, Cas9 will introduce a double-stranded break (DSB) approximately 3-4 base pairs (bps) upstream of the PAM (Ran *et al*., 2013). For this research, the designed gRNA spacers will be homologous to sequences of DNA upstream and downstream of the STOP codon in the last exon of the target genes *chk-1* and *fasn-1*, respectively. This will introduce two DSBs on each gene, resulting in the removal of the sequence between these DSBs.

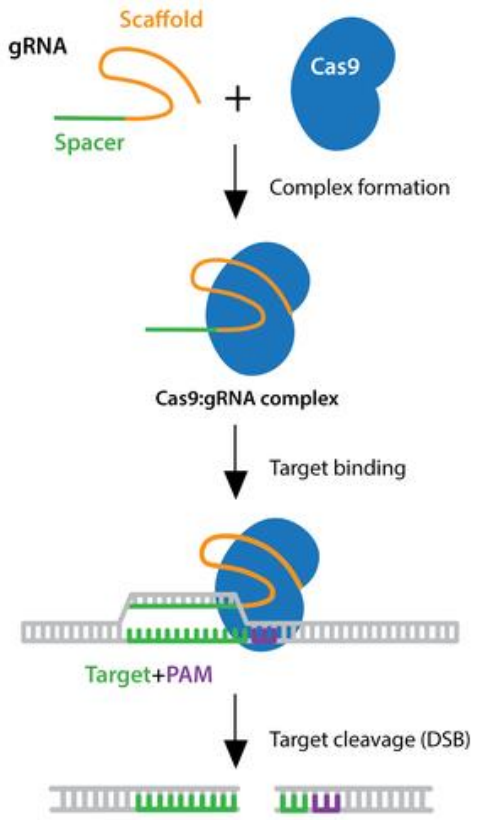

Figure 6: Diagram showing the general structure, requirements, and end-result of the CRISPR technique. From: https://www.addgene.org/guides/crispr/#web-references

While an organism can and often does use the faster but error-prone non-homologous end joining (NHEJ) process to rejoin any blunt dsDNA cuts (Xu and Li, 2020), it can also use the slower but more correct homology directed repair process shown in Figure 7. In HDR, the cell repairs the cut in its DNA by referencing a template section of DNA that is homologous to the area that was cut (Zhang *et al*., 2014). This can be accomplished in two general ways. First, by referencing a homologous chromosome, or second, by using an artificially inserted homologous DNA template. This homologous DNA template allows geneticists to repair the DSB with precise insertions, deletions, or mutations, which is useful when performing research that requires careful gene editing (Ran *et al.*, 2013). These artificial homology templates are homologous to the DNA surrounding the cuts Cas9 introduced but can also contain inserts not

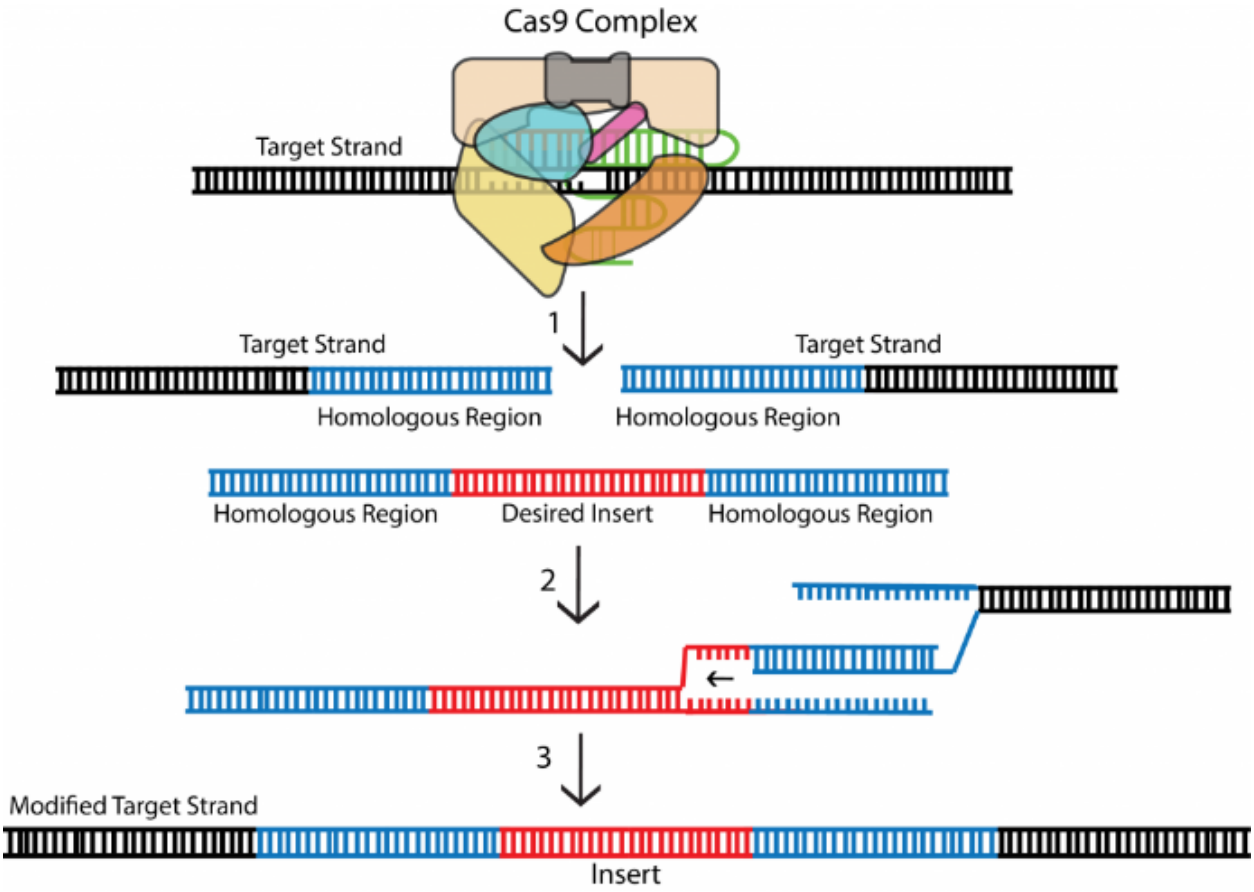

Figure 7: Diagram showing HDR and how it can be used to insert a desired sequence of DNA into a genome at a DSB. From https://sites.tufts.edu/crispr/genome-editing/homology-directed-repair/

originally present in the organism's genome that are to be studied (Ran *et al*., 2013). For the purposes of this experiment, the homologous template will 'seal' the cuts the Cas9 enzyme created while simultaneously inserting GFP and the SEC into the *chk-1* and *fasn-1* genes.

In the case of *fasn-1*, as it is a gene with potential involvement in developing male somatic gonads, the *him-8* gene will be simultaneously mutated using CRISPR and HDR at that locus as well. This will cause the hermaphrodites containing the *fasn-1* mutation to produce a higher percentage of male progeny. So, CRISPR will be happening simultaneously at two different loci for the *fasn-1* individuals.

These *chk-1* and *fasn-1* genes will be fused to the DNA sequence of GFP causing a GFP-fusion protein to be expressed. Thus, everywhere in the organism where these genes' products are present will glow green when examined under a microscope with epifluorescences, allowing us to determine the time and the place where the tagged proteins are expressed. More importantly, tagging these proteins with GFP provides a useful target for degradation systems to differentiate modified *chk-1* and *fasn-1* proteins from the rest of the organism's proteins. In addition to being tagged with GFP, these new *chk-1* and *fasn-1* genes will contain a region of DNA useful in determining whether CRISPR has been successful in these *C. elegans* individuals after they are injected.

This region of DNA is called a self-excising cassette (SEC) and it is inserted 3' to the GFP sequence (Dickinson *et al.*, 2015). It is a sequence of DNA that contains several useful tools for selecting *C. elegans* individuals that have successfully undergone CRISPR. First, the SEC contains a drug resistance gene for hygromycin (*hygR*) which allows for the selection of individuals containing the SEC in general. Second, the SEC contains a dominant phenotypic marker for the gene *sqt-1* that, when expressed, causes worms to demonstrate the rolling

phenotype wherein they move in a circular pattern from the L3 stage until the end of their lives. Third, the SEC is flanked on either end by 34bp sequences of DNA called *loxP* sites. Finally, the SEC contains a Cre recombinase that, when heat-shocked, will catalyze the cleavage of DNA at the *loxP* sites and subsequently re-ligate the *C. elegans* genome back to itself (Dickinson *et al.*, 2015). Once the SEC removes itself from the genome of *C. elegans*, the *loxP* site that will remain in the genome will be within a synthetic intron and thus will not affect the proteome of the animal. This process of self-excision, as well as a general picture of the entire SEC, is in Figure 8.

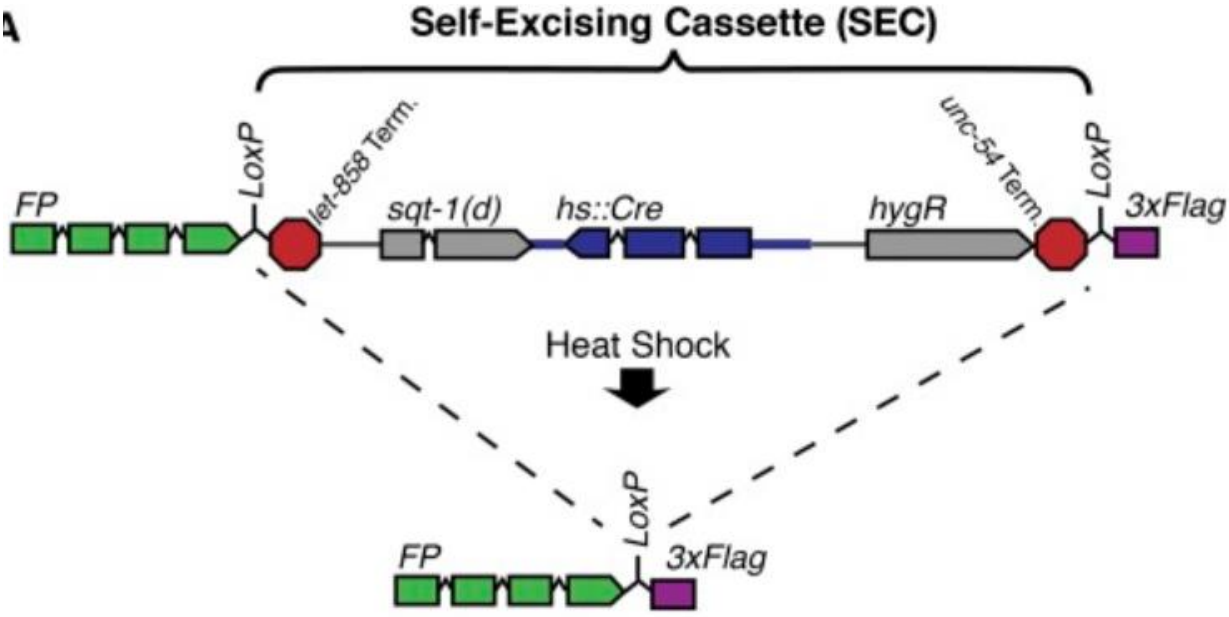

Figure 8: Overview of the SEC, its component genes, and the process by which it will remove itself from the genome of *C. elegans*. FP represents the fluorescent protein of choice, which will be GFP. From Dickinson *et al.* (2015)

Gibson Assembly

Generating the homology repair template that HDR will use involves combining several fragments of DNA together in one plasmid which will be accomplished using Gibson Assembly.

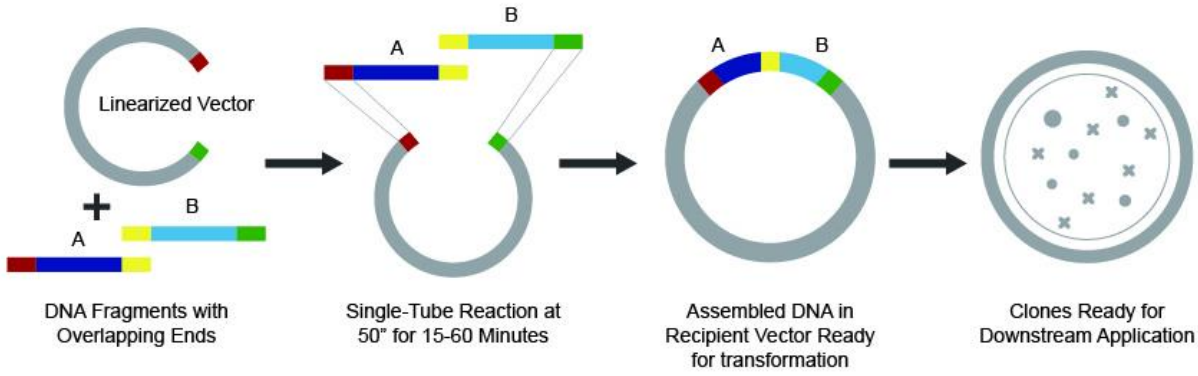

Figure 9: Overview of Gibson Assembly in a plasmid using 2 separate DNA strands. From: https://www.moleculARcloud.org/what-is-Gibson-assembly.html

Gibson Assembly (Gibson) refers to a method by which multiple fragments of DNA can be joined together in a single isothermal reaction. For the purposes of this experiment, Gibson Assembly was used to join three fragments of DNA per gene of interest, the two homology arms and the plasmid vector pDD282. These sections were generated separately and are only now being joined together because each section of HA needed to flank the GFP gene being inserted, so one had to be created upstream of the GFP gene and one had to be created downstream of it. This final plasmid, which includes the GFP sequence required to produce the 'marked' proteins of interest and the SEC for determining if CRISPR worked, acts as the homologous template from which the cell's repair machinery will attempt to 'fix' the cut introduced by Cas9 during CRISPR. The full homology template will need to be formed before it is inserted into the plasmid, as all fragments are joined together simultaneously as shown in Figure 9.

To accomplish this, the homology arms (HA) and pDD282 vector are combined with DNA ligase, DNA polymerase, and an exonuclease which are collectively responsible for

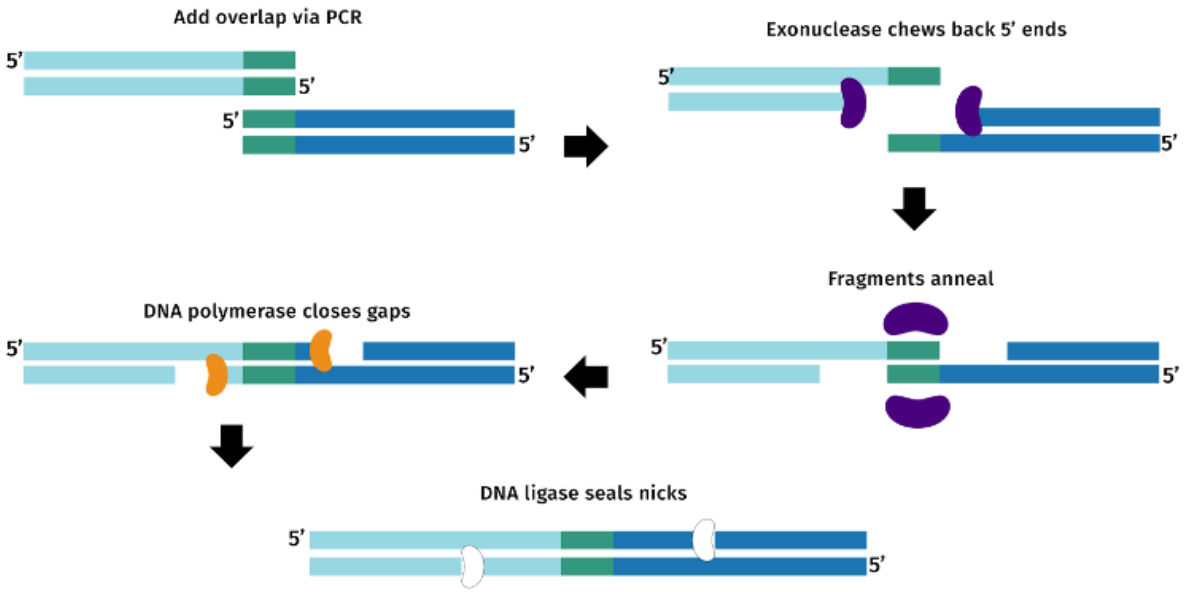

Figure 10: Gibson Assembly as it happens linearly. Originally from: https://www.snapgene.com/guides/gibson-assembly

conjoining the DNA fragments. First, the exonuclease will remove between 20 - 40 of the nucleotides on the 5' ends of each individual DNA strand such that the remaining overlapping regions of the respective 3' ends can bind to one another as shown in the second and third steps of Figure 10 (Gibson et al., 2009). The DNA polymerase will then insert the missing nucleotides in the remaining 5' to 3' gaps in the fourth step before, in the final step, the DNA ligase will seal the 'nick' or final gap in between the DNA strands. This process happens at every site where joining should occur to create a single, conjoined, circular pDD282 plasmid that contains the desired HAs (Gibson et al., 2009).

An Experimental Overview

The goal of this research is to mutate the *chk-1* and *fasn-1* genes in *C. elegans* so that any proteins these genes produce are tagged with GFP and hopefully also functional. This will be accomplished using the CRISPR/Cas9 technique to engineer a GFP knock-in for each gene. Following this, the injected worms will be allowed to self-fertilize and reproduce before being treated with hygromycin to select for those individuals containing the SEC which conveys hygromycin resistance. This will allow for easy visual identification of which *C. elegans* individuals had their genomes modified by CRISPR.

This research will allow future researchers to downregulate the proteins produced by the genes *chk-1* and *fasn-1* specifically in the gonads of developing male *C. elegans* by using degradation systems that specifically target proteins tagged with GFP. This method of studying the effects of *chk-1* and *fasn-1* is used because both genes of interest are essential for the organism to live; they cannot simply be removed from the genome and studied that way because the organism would die. Downregulating the proteins these genes produce prevents the organism from dying in this way while still allowing for the exploration of what role each gene may play in gonadogenesis. These genes' role in gonadogenesis could then be explored by examining the phenotype of nematodes whose gonads are developing without the expression of one of these two genes.

In summary, the goal of this experiment is to create the gRNA and HA constructs for *chk-1* and *fasn-1* to be used in CRISPR and attempt to edit the genes via CRISPR. This would allow future researchers to understand how each gene drives the process of gonadal differentiation by selectively degrading those proteins tagged with GFP such as by using a ZIF-1

degron system. This would allow those researchers to explore these genes' roles in the somatic gonad.

METHODS

Transformation Procedure

For all transformation procedures conducted in this research, 1.7 mL tubes of frozen *E. coli* were removed from a -80ºC freezer at the same time the DNA to be transformed was removed from the -20 ºC freezer. Once thawed, a variable µL volume of the DNA to be transformed was added to the *E. coli* tube and incubated on ice for 5 minutes. The tube was then heat shocked at 42ºC for 45 seconds and then immediately placed back on ice before 1.0 mL of LB was added. This mixture incubated on ice for 2 minutes before being moved to an incubator set to 37ºC for 50 minutes. After this final incubation, the tube was removed from the incubator and spun in a centrifuge at 16.1 rcf for 1 minute which resulted in a small pellet forming in the bottom of the tube. Approximately 900.0 µL of the supernatant was pipetted out of the tube before the pellet was gently resuspended in the remaining liquid (approximately 100 µL). Mixtures were then either plated on LB+KAN or LB+AMP plates as specified.

APEX PCR Protocol

For all APEX PCR procedures conducted in this research, a master mix was first made containing solutions that would be required for all reactions ran using this protocol. To account for pipetting error, an extra reaction's worth of master mix was typically made at this time. Also, to account for mutations during PCR, two reactions were typically done for every pair of HA primers used in this step. For each reaction that would be run, 12.5 µL of APEX 2X Hot Start Master Mix BLUE was added to the 1.7 mL tube which will contain the master mix along with

1.0 µL of N2 genomic DNA and 9 µL of de-ionized and autoclaved water for a total of 22.5 µL per reaction. Now, in separate 0.2 mL PCR tubes, 22.5 µL of this mix would be added to each tube before adding 1.25 µL of each desired 10 µM HA primer to bring the total volume to 25 µL. These PCR tubes were then placed in the BioRad MJ Mini PCR machine and run using the following protocol outlined in Table 1. After the reaction was finished, these PCR products were stored at -20°C until needed again.

Table 1: APEX PCR Protocol

Step Number	Machine Instructions
1	95°C FOR 15:00 minutes
2	95°C for 0:30 seconds
3	55°C for 0:30 seconds
4	72°C for 1:00 minute
5	GOTO Step 2, 34 times
6	72°C for 5:00 minutes
7	10°C for FOREVER
8	END

Sequencing, Analysis, and Dehydrated Primers

All genetic sequencing was performed by Eurofins Genomics LLC (Eurofins) which used the Sanger sequencing method. Analysis of sequenced products was conducted by aligning the sequence returned by Eurofins with the published sequence modified to contain any desired mutations. Additionally, primers produced by Eurofins and mailed in a dehydrated state after being designed.

Creating the pRB1017 Plasmid

The gRNA plasmid pRB1017 conferring kanamycin (KAN) resistance was obtained. (Addgene plasmid # 59936 ; http://n2t.net/addgene:59936 ; RRID:Addgene_59936).

The received pRB1017 plasmid was transformed according to the *Transformation Procedure* outlined above using 5 µL of pRB1017 plasmid. The resulting 100 µL were then plated in agar petri dishes that contained kanamycin (called LB+KAN plates) to select for bacteria that successfully took up the plasmid. These plates were allowed to grow overnight at 37 ºC after which colonies were individually selected to grow overnight in a test tube containing a 5.0 mL culture of LB and 5.0 µL 1000X KAN shaking at 37ºC overnight to further replicate the plasmid. These bacteria were then miniprepped according to the ZymoPURE Plasmid Miniprep kit protocol to successfully extract the pRB1017 plasmid. A notable change this research made to this protocol is that the final elution step was conducted twice and 35 µL of Elution Buffer was used as opposed to the recommended 25 µL.

The circularized plasmid was then digested with *BsaI* enzyme to linearize it. Doing this allows for the insertion of the designed gRNA primers in a later step. After miniprepping, 18 µL of each miniprep product was added to a 1.7 mL tube along with 4 µL of New England BioLabs (NEB) 10X rCutSmart buffer, 3 µL of NEB BsaI-HF v2 enzyme, and 15 µL water for a total of 40 µL per 1.7 mL tube. This reaction was incubated overnight at 37ºC and stored at -20ºC. Once digestion was complete, each reaction was run on a 1% agarose gel. Each gel in this research contained an NEB 1kb plus DNA ladder in the first column for reference. Before pipetting each solution into the gel, 6.67 µL of NEB 6X Purple Gel Loading Dye was added to each 40 µL tube so that the BioRad ChemiDoc would be able to detect the migration of the DNA. The resulting solution was pipetted into two consecutive wells of the gel (because each well could only hold 25 µL) and was run on a 1.0% agarose gel submerged in 1X TAE buffer at 120 V for 30 minutes.

The gel was imaged in a BioRad ChemiDoc imaging system on a Blue Sample Tray under the SYBR Gold imaging protocol. The brightest, top-most band at approximately 2507 bp was excised using a razor blade and stored in a 1.7 mL tube at -20ºC. This band contained the cut plasmid while the plasmids that did not cut were separated in the gel.

These excised gel slices were eluted according to the Zymoclean Gel DNA Recovery Kit protocol with the only change being the same as mentioned before regarding the final step. These eluted products were then used in a ligation reaction which would cause the plasmids that were only cut once to re-ligate to themselves. Running this ligation product on a gel would separate the desirable twice-cut plasmids from the re-ligated single-cut plasmids as the re-ligated plasmids would not migrate as far down the gel as the twice-cut plasmids would. Removing these incompatible single-cut plasmids is important because they could re-ligate to themselves after the initial treatment with *BsaI* which would generate several transformed bacterial colonies that would not contain the desired gRNA insert. This reaction contained the entire 35 µL elution product, 4 µL NEB 10X T4 DNA ligase buffer, and 2 µL NEB T4 DNA ligase for a total of 41 µL. This reaction was allowed to incubate overnight at 15ºC and was immediately ran on a 1% agarose gel the following morning under the same conditions as the previous gel.

From this gel, the brightest band that was approximately the length of the linearized plasmid, 2507 bp, was excised (Fire *et al*, 2014) and eluted according to the Zymoclean Gel DNA Recovery Kit protocol with the same modification to the last step as previously mentioned. This 35 µL eluted product contained pRB1017 BsaI digest plasmid which would be capable of accepting the gRNA primers we previously designed. It was stored at -20ºC.

Designing gRNA

Primer sequences for gRNA in *C. elegans* were selected using the CRISPOR tool (https://crispor.tefor.net) developed by Tefor Infrastructure and modified so that they would insert into the vector plasmid pRB1017 at the sticky ends that remained following the *BsaI* digest. These modifications were specifically the 5'-TCTT-3' sequence that is at the beginning of each forward gRNA primer and the 5'-AAAC-3' sequence that is at the beginning of each reverse gRNA primer. The approximately 700 base pair (bp) long sections of DNA immediately 5' and 3' of the STOP codon for each gene were copied into the tool and the search parameters were set to identify primers containing a PAM sequence with the nucleotides NGG. Gene sequences were found and copied from the website wormbase (https://wormbase.org/#012-34-5).

Table 2: gRNA Primers

Sequence name	Sequence
Forward *chk-1* gRNA (Before STOP)	TCTTGTCGGCGTCCTCGATCAATG
Reverse *chk-1* gRNA (Before STOP)	AAACCATTGATCGAGGACGCCGAC
Forward *chk-1* gRNA (After STOP)	TCTTGGAGGAATGATGAGGCAGTG
Reverse *chk-1* gRNA (After STOP)	AAACCACTGCCTCATCATTCCTCC
Forward *fasn-1* gRNA (Before STOP)	TCTTGGCCGTGTCAGAAGATTGCG
Reverse *fasn-1* gRNA (Before STOP)	AAACCGCAATCTTCTGACACGGCC
Forward *fasn-1* gRNA (After STOP)	TCTTGTTCAATTGAAATCAAGGGT
Reverse *fasn-1* gRNA (After STOP)	AAACACCCTTGATTTCAATTGAAC

This led to one 5' and one 3' gRNA primer being chosen per sequence. Requirements for these primers were that the predicted efficiency according to Doench (2016) be above approximately 60 and have as few off-target mismatches as possible. The primers chosen for this research are shown in Table 2.

Designing HA Primers

Homology template primer sequences were identified by examining the unspliced gene of interest and copying approximately 700 bps immediately 5' and immediately 3'of the STOP codon of the gene into a DNA analysis program. Of note, the 700 bp sequence immediately 3' of the STOP codon contained the STOP codon as the first 3 bps of its sequence (TAA). This was done to ensure that the GFP sequence was immediately upstream of the STOP codon of each gene and would thus be attached to the end of the protein *chk-1* or *fasn-1* produces. Each 700 bp sequence required two HA primers (so 4 total primers per gene). For the 700 bp sequence upstream of the STOP codon, the last ~25 bps had to be one of these primers while for the 700 bp sequence downstream of the STOP codon, the first ~25 bps had to be one of these primers. The other upstream primer had to be any sequence of ~25 bps towards the beginning of the 700 bp sequence while the other downstream primer had to be any ~25 bp sequence towards the end of the 700 bp sequence. The reverse complement was taken for the primer at the 3' end of each 700 bp sequence before the following calculations. Requirements for all primers were that the

Table 3: Homologous Template Primers

Sequence Name	Sequence
56: 5' Forward *chk-1* HA	acgttgtaaaacgacggccagtcgccggcaTGCTTCGTTCTCCCAGCCGAC
57: 5' Reverse *chk-1* HA	catcgatgctcctgaggctcccgatgctccGACATCtACATTGATCGAGGACG
58: 3' Forward *chk-1* HA	cgtgattacaaggatgacgatgacaagagaTAATTGTTTTTTTTTGTTTGTTCGTTTTTTG
61: 3' Forward gRNA mutation chk-1	GATGAGGCAGTGGtGACAAACTCTAG
59: 3' Reverse *chk-1* HA	ggaaacagctatgaccatgttatcgatttcTACTCTTTAAAGGCGCACACACAC
60: 3' Reverse gRNA mutation *chk-1*	CTAGAGTTTGTCaCCACTGCCTCATC
66: 5' Forward *fasn-1* HA	acgttgtaaaacgacggccagtcgccggcaGATTCTTCTCGATGGATCCCATC
69: 5' Forward gRNA mutation *fasn-1*	gtaaatcttacCTCaCAgTCcTCgGACACGGCCG
67: 5' Reverse *fasn-1* HA	catcgatgctcctgaggctcccgatgctccTTGAAGAATAATCCTGTTGATGTGTTC
68: 5' Reverse gRNA mutation *fasn-1*	CGGCCGTGTCcGAgGAcTGtGAGgtaagatttac
70: 3' Forward *fasn-1* HA	cgtgattacaaggatgacgatgacaagagaTAAATCAACAATCACATATCCATAATC
73: 3' Forward gRNA mutation *fasn-1*	GAAATCAAGGGTGGaCAGTCCTAG
71: 3' Reverse *fasn-1* HA	ggaaacagctatgaccatgttatcgatttcAATAGGACATCTTCATCGTCATCC
72: 3' Reverse gRNA mutation *fasn-1*	CTAGGACTGtCCACCCTTGATTTC

melting temperature (calculated by assigning the value of 2 to A and T nucleotides and assigning 4 to G and C nucleotides and adding up the number of individual nucleotides) be at least 68 and that all primer sequences end with G or C. Primers were allowed to deviate from the 25 bp length to achieve these goals but did not deviate more than a few bps. The primers chosen for this research are shown in Table 3.

The long leading sequence of lowercase nucleotides in Table 3 that are in the sequences containing HA (for homology arm) coded overlap areas for Gibson Assembly into pDD282 and were added onto each primer after they were designed. The lowercase letters highlighted in light blue are the silent mutations that were induced in each sequence of the HA to prevent the Cas9 endonuclease from re-cutting the homology template once it was successfully inserted into *C. elegans* genome. These silent mutations were inserted into the PAM site, if possible, as only a single nucleotide would need to be mutated for the gRNA to not recognize the cleavage site. But if a silent mutation could not be induced in the PAM site (because all potential mutations were not silent), such as with the 5' forward and reverse HA for *fasn-1*, then four separate silent mutations would need to be inserted into the HA itself. The light blue highlighted letters represent what the nucleotide was changed to, and not what the original sequence was.

Annealing and Ligation of gRNA to pRB1017

Now, the gRNA and HA primers will undergo separate processes to create the plasmids required for CRISPR. First, the gRNA. The designed gRNA oligonucleotides were generated by Eurofins Genomics as single-stranded oligonucleotide sequences that needed to be annealed to one another before they could be ligated into the pRB1017 BsaI digest plasmid.

The gRNA oligonucleotides received from Eurofins Genomics were first diluted to 100 µM. Once diluted, the primer oligos were added to PCR tubes containing 25 µL water, 10 µL of the 5' oligo, 10 µL of the 3' oligo, and 5 µL of NEB restriction enzyme buffer 3 for a total of 50 µL per PCR tube. These tubes were gently mixed by pipetting and then placed in a thermocycler under a protocol that would heat each tube to 95ºC before incrementally dropping the temperature to 20ºC over approximately 20 minutes. This process allows for the single-stranded 5' and 3' primers to properly anneal to one another (as they are mostly complementary to one another), creating a double stranded primer that is ideally devoid of any incorrect bindings.

These annealed oligos were ligated to pRB1017 in a 20 µL reaction within a PCR tube. The following were added to this PCR tube in the order they are mentioned: 14 µL water, 2 µL 10X T4 DNA ligase buffer, 2 µL of pRB1017 BsaI digest plasmid, 1 µL of the annealed oligos, and 1 µL of T4 DNA ligase. This reaction tube was allowed to incubate overnight at 15ºC and stored at -20ºC until they are transformed into *E. coli*.

Transforming the Plasmid into Bacteria and Plating

Once the ligation procedure was complete, these gRNA-containing plasmids were transformed into *E. coli* according to the *Transformation Procedure* section above and plated onto LB+KAN plates where the bacterial colonies were allowed to grow overnight at 37ºC. After 12-16 hours, at least two colonies were selected from the plate and miniprepped according to the ZymoPURE Plasmid Miniprep kit protocol but with 35 µL of elution buffer used in the last step as opposed to the recommended 25 µL. This miniprepped product was sequenced according to the Eurofins Genomics sequencing protocol and subsequently checked for the correct insertion of the gRNA with no unwanted mutations. Once confirmed, these products were stored at -20ºC until they are needed to make the injection mix.

Homology Arm Creation and One-step PCR

Simultaneous to the steps involving the gRNA primers, the HA primers were also being processed. The dehydrated HA primers received from Eurofins were hydrated to a concentration of 100 µM and stored at -20ºC until used. A separate tube was made for each primer at a concentration of 10 µM and the *APEX PCR Protocol* was followed. The resulting PCR products were then run on a 1% agarose gel at 120V for 30 minutes to see if the desired product had formed. Each PCR product would generate a different sized band based on what primers were used. These bands were excised from the gel using a razor and then eluted according to the Zymoclean Gel DNA Recovery Kit protocol and subsequently divided based on whether they required two-step PCR to fully form the homology arms. One notable change to this protocol

5’ *chk-1* HA (56 + 57):

TGCTTCGTTCTCCCAGCCGACaaagactgaggatcttctgcttactcagcatattgatatgtcgcagaccaactcggttag
gaaaaaaccgtcagaaattggcggatttttagctaaaatctccaatttttcggtcgtaaattggcatttttgataatttaggatgaaaattcgctaa
tgacacggaaatggttgcaaaattagaggtttttgctaaatttacttttttttttgattaaaaaacccagaatttagtttaaatccccctaaaatttca
gaatctcctccaacgaatggtgtgccgtatgacacgtttctgcgtcgtcaccgacatccgttccacgtaccaaaaagtggctcgagccagtg
agcacgccggcttcggcgttcgtgagactgacgactaccgtctactagtcacgtggcgcgaggtttcgatgatggtcagtctgtacacgatg
ggcgatattcccgacaagccacgtgtcatggtagatttccgcaggtctcgcggtgacggaatccagtttaagaagatgttcatggacgttaga
aaccgtatgcatgagtggatatgtaccgacggaaacaactggctcgccaatcttggatatgtgccaagaaatccgcagatagttaatggtgg
tggagtcaatgtggagcactcggCGTCCTCGATCAATGTaGATGTC

Figure 11: The intended full 5’ HA for *chk-1* using primers 56 and 57. The capital letters represent the homology arm primers while the yellow highlighted region represents the gRNA primer. The light blue highlighted and lowercase nucleotide within the uppercase region represents the mutation that was inserted to prevent re-cutting during CRISPR

was to the final step where 35 µL of DNA Elution Buffer was used. Only the 5’ *chk-1* HA would

be generated using one-step PCR as all other HA required two-step PCR. The final HA for the original 5' *chk-1* HA is in Figure 11.

Two-step PCR

Two-step PCR involves running another round of PCR with homology arm primers that could not have induced a mutation in the gRNA-specific PAM due to there being no overlap between the PAM and the HA. The first step of PCR was to create two sections of each homology arm that contained the mutated PAM on either the 5' or 3' end. That was what the mutations in the gRNA primers were for. Then, these two sections of the homology arm would be placed in another PCR reaction with the forward and reverse HA primers for that specific HA. To do this, the *APEX PCR Protocol* was run again with several modifications.

First, the 9 µL of de-ionized water was no longer a fixed amount and would instead be added last to bring the total volume to 25 µL. This is because the 1.0 µL of N2 genomic DNA was replaced with an appropriate ratio of the previous step's PCR products that was determined by running said PCR products on a 1.0% agarose gel for 30 minutes at 120V and then visually comparing brightness of each band shown on a BioRad ChemiDoc. If one band was approximately 5X brighter than the other, for example, then 5 times the volume of the dimmer primer was used compared to the brighter primer in this step. The volume of each PCR product used here ranged from 0.4 µL to 4 µL depending on the brightness of the bands. Finally, the 1.25 µL of primers used are the outermost primers for each homology arm. In Table 3, they are noted by an HA in their sequence name. The PCR products made from this reaction are the full

homology arms shown in Figure 12 and are stored at -20ºC until they are ready to be used in Gibson Assembly.

3’ *chk-1* HA:

TAATTGTTTTTTTTTGTTTGTTCGTTTTTtgttccatcagactattgagatttaaagcacaatatttatcatcatcatcat
cactttaaacatctccctatcccattaatttttaaatttaacgtgaattaacgccgaattacaagcctgtgaatgaatgtttgtttttatctctgcgtct
agagtttgtcaccactgcctcatcattcctcctctttctctttccctctctcacttttgcacaatatctcgctgcccctcgcctccgttcgatagtcgt
cagctgagagagtgtctgcagcagacaagacaattcttactaaataatacgcacgaatatattcttatgaatttttctacccccctactgtcattag
gtctcaactggaaaatctggaattcgtttttgtagttttcaaaatatcagtggcgcgtcgttcccacttaacaactcaaagtggttcggaaaccca
tcggagaagaggctgacgagccagccgttgtcccggccaagcgacagaggtttgcaatttaaaaaaagctaaatatgacgattttgcgata
ctgtaccaggtctcaacacgacaaatttctataaaattaatgcaaaaGTGTGTGTGCGCCTTTAAAGAGTA

5’ *fasn-1* HA:

GATTCTTCTCGATGGATCCCATCtctacatgcaaacttacagaaacgtaagttgagctagtttatggctttcgaaattaaat
ttgaaacttctcaggtataccgtatggcctttggagtaacgggagattccctggtcaacaatccactcttcgaatctgagatcatgtgtgccatg
actcttcgtttcgccaatgtggattacaagaagttccgatttgaactccttcaacaaccaggattcaaggcccgtgtgcaaaaggtggtggatc
aagtcatgctaactggtctcttcaagtcaccagagaccgttgcatttgcctgtgaggcaatgcattccaagttcctcatggctgacaagtgagtt
tcagaaacctaaaaaatttatttaaatttaaacattacaggtacaagccaagaagaaacttcggtggacacatcacactgatcagagctgag
caaggagctgctcgtgaggaggacgttggagaagattatggagttgcggccgtgtccgaggactgtggaggtaagatttacaagttgtagta
gtgaattaaagctttctttaatctaaatcaaaacttaaaattaattgtttacaggtgctcaaagttaaaggagatcacgataccttcgtgcaagg
aaagtcatcttcagtaacagttGAACACATCAACAGGATTATTCTTCAA

3’ *fasn-1* HA:

TAAATCAACAATCACATATCCATAATCtcatccaatctctcccctcaatgttttaatattttggctttatattttgactttca
aactgtttatttctttttcggttgcttctttttctcaaacctttgattttcaaacgtgtcacccaatattgctctttgctcaagactcatgagtgtgtgta
ttagttttcaatggattagttagttttctaatgttttctaggactgtccaccttgatttcaattgaattctaatcattttccatatgtttagtaccatgcttt
cttctctctttgctcgatctgtctgtacttgacccccttttccccctcttcacaggtctctctctcccccagtgccatgccgttacggttacgtag
gttactgattctttcccattccttcttctcgaaacgtttctttttgttttaaattttctttataactcaattttcacgcggaaccaattgtttttaatgtgtttc
attttgtaacttttcatttttttttttcttgtttgttctcaatgtttttgcttctagatttctctattgagctgaataaattctttttgaaaaataacacatttcttct
ttatctgtgataatacaagcatcaatagttaggattgtaatgttttcaggaaacttcagcggtttaGAAATTGGGATGACGATG
AAGATGTCCTATT

Figure 12: The full 3’ HA for *chk-1* and both the 5’ and 3’ HA for *fasn-1*. The capital letters represent the homology arm primers while the yellow highlighted region represents the gRNA primer. The light blue highlighted and lowercase nucleotides within the uppercase regions represent the mutations that were inserted to prevent re-cutting during CRISPR.

Performing Gibson Assembly

For this experiment, Gibson Assembly was performed in a PCR tube using 0.5 µL of a pDD282 plasmid that confers ampicillin resistance, 3.0 µL of 2X HIFI DNA Assembly mix, and

a mixture of the two PCR products that generate the complete HA in a ratio that added up to a total of 6.0 µL. The ratio of HA was determined by running each HA on a 1.0% Agar gel in a gel electrophoresis machine for 30 minutes at 120V and then visually comparing the brightness of each PCR product once the gel was imaged in a BioRad ChemiDoc. The completed 6.0 µL mixture was then added to a thermocycler and run at 50ºC for 3 hours before being removed and stored in a freezer at -20ºC overnight.

These mixtures were transformed according to the transformation procedure outlined in *Transformation Procedures* with the notable exception that the 50-minute incubation occurred at 30 ºC and not 37 ºC. This change was implemented because the plasmid being used for Gibson Assembly, pDD282, will mutate when grown at temperatures above 30ºC whereas the pRB1017 plasmid the gRNA was inserted into did not generate mutations above 30ºC. The resulting 100 µL solution was spread onto an LB+AMP plate to select for bacteria with the Ampicillin resistance that pDD282 confers and stored overnight in an incubator set to 30ºC.

The next morning the plates were checked for colonies which, if present, were selected with a 200 µL pipette tip grown overnight in LB + AMP broth at 30ºC, and miniprepped according to the ZymoPURE plasmid miniprep kit protocol with the same modification to the final elution step as mentioned above. These miniprep products were sequenced according to the Eurofins protocol using the primers USA20 and USA21 which are outlined in Table 4 below. This was the first of two sequencing checks to determine if the Gibson Assembly had been

Table 4: Sequencing Primers

Sequence name	Sequence
M13 Forward Primer (USA20)	tgtaaaacgacggccagt
M13 Reverse Primer (USA21)	caggaaacagctatgaccatg

successfully conducted and it was to confirm that there were no unwanted mutations in the homology arm portion of the template. The second check was to determine that there were no mutations in the GFP region of the template.

Injection Mix Creation

Injecting the previously created homology template and two gRNA products alongside the provided Cas9 and *him-8* (only needed for *fasn-1*) at the same time necessitated the creation of an injection mix. This mix had a final volume of 20 µL and various concentrations of the ingredients as outlined in Table 5 below. If the gene to be modified was not shown to be exclusively upregulated in male developing gonads in previous research (Kroetz *et al*., 2015), remove both rows that mention *him-8* and keep all other concentrations the same. The water mentioned at the bottom of the table was nuclease-free water and was used last to bring the final volume of the mixture to 20 µL. All volumes were accurate to the thousandth place when pipetting for this mixture, and it was stored at -20ºC once complete.

Table 5: Injection Mix Concentrations

Plasmid / Item to be Added	Final Concentration in the Mix (ng / µL)
pDD162 (Cas9)	50
pRB1017 – gRNA 5' *chk-1 or fasn-1*	25
pRB1017 – gRNA 3' *chk-1 or fasn-1*	25
pDD282 – HA (Gibson Product) *chk-1 or fasn-1*	10
pRB1017 – gRNA *him-8* for *fasn-1 only*	25
Him-8 ssOND	500 nM
Str-1::gfp	25
water	-

To determine the concentration of each item created in previous steps, a nanodrop machine was used. The concentration that this machine read was used as the C1 variable in the equation C1V1 = C2V2 to help solve for what volume of every item in the left column would be needed in this mix. The C2 variable in this equation was the concentration listed in the right column of Table 5, while the V2 variable was always 20 µL.

When ready to inject, the injection mix was removed from the freezer and thawed before being pipetted onto a fine filter and spun at 16.1 rcf for 1 minute to remove any lasting impurities. Then, the injection mix was taken up into a small, thin tube using capillary action before being transferred to an injection needle using mouth pipetting. Once the mix had been transferred, the needle was placed under a microscope and observed until all air bubbles had risen to the top of the solution. Once all air bubbles were out of the mix, the needle was placed into an injection apparatus.

Injection and Hygromycin Treatment

The worms that would be injected were adult hermaphroditic *C. elegans* that were immobilized in heavy mineral oil on a microscope slide prior to injection. The injection mix was injected into each *C. elegans* in the distal arm of the gonad where the most immature germ cells were. Doing this meant that these germ cells might take up the injection mix and, should the injection have been successful, and CRISPR activate, undergo CRISPR. Once injected, two such worms were plated onto their own plates and incubated between 22 and 23 ºC so that the uninjected animals could lay eggs and develop without any negative selection over 4 days. In total, approximately 60 worms were injected and placed onto thirty total plates.

After the four-day incubation period, these plates were treated with 500 µL of 5 mg/mL hygromycin-B. The 500 µL volume of hygromycin-B was pipetted directly onto each plate and then swirled around so that the entire surface of the plate was covered in the antibiotic. These plates were allowed to dry out uncovered for approximately 10 minutes or until there was no more liquid hygromycin observed on the plate. At the same time this is occurring, the worms were also observed to see if they had eaten through too much of their bacteria (food) by looking to see if there were still tracks from where the worms had moved through the bacteria. If there weren't any visible tracks, those worms would need to be transferred to new plates that had food. Once this had been decided, two new plates that had bacteria but no worms on them were also treated with hygromycin-B and allowed to dry out in preparation. This is two new plates per plate that had injected and treated worms but no bacteria. Once these new plates had dried, the agar-plate that contained the worms was split in two using a heat-sterilized instrument and one half was flipped onto the new, bacteria-laden plate so that the worms would be sandwiched between the two pieces of agar. The same was done with the other half of the original plate. Now, the worms that potentially contained the injection mix would be treated with hygromycin-B and be able to survive due to the new supply of bacteria. In the first round of injections, 6 plates were chunked according to this method. In the second round, another 6 were chunked.

RESULTS

Designed gRNA and HA

The designed DNA products this research created were successfully inserted into the plasmids pRB1017 and pDD282 as confirmed by DNA alignment analysis for *fasn-1*. Figure 13

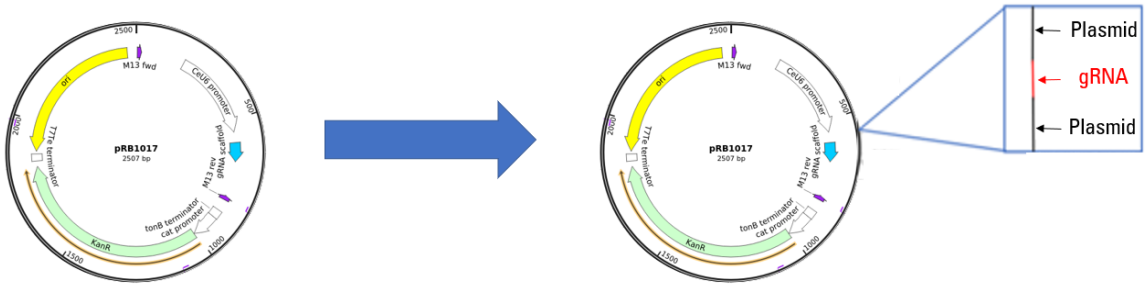

Figure 13: Images adapted from Addgene for plasmid pRB1017. A summary depiction of the gRNA primers being inserted into the plasmid has been added.

shows pRB1017 as well as a summary depiction of the gRNA primers inserted into pRB1017. Figure 14 shows pDD282 and another summary depiction of the HA template inserted into pDD282. The *chk-1* gene's gRNA was successfully inserted into pRB1017, but the homology template was not successfully inserted into pDD282 as one of the two homology arms was not generated by PCR. This lack of PCR product is shown in Figure 15 where the red 5' *chk-1* HA was not present. Every other column had bands that were the correct length and excisable (the column immediately to the right of the ladder was for another researcher). Replacement primers

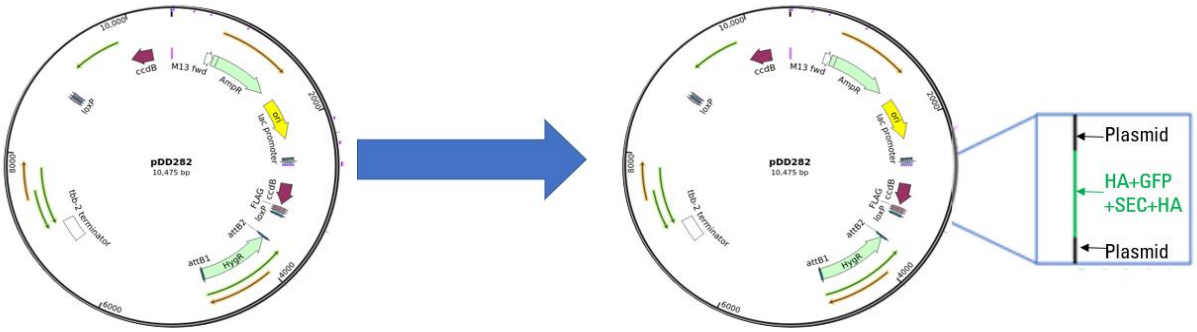

Figure 14: Images adapted from Addgene for plasmid pDD282. A summary depiction of the HA template being inserted into the plasmid has been added.

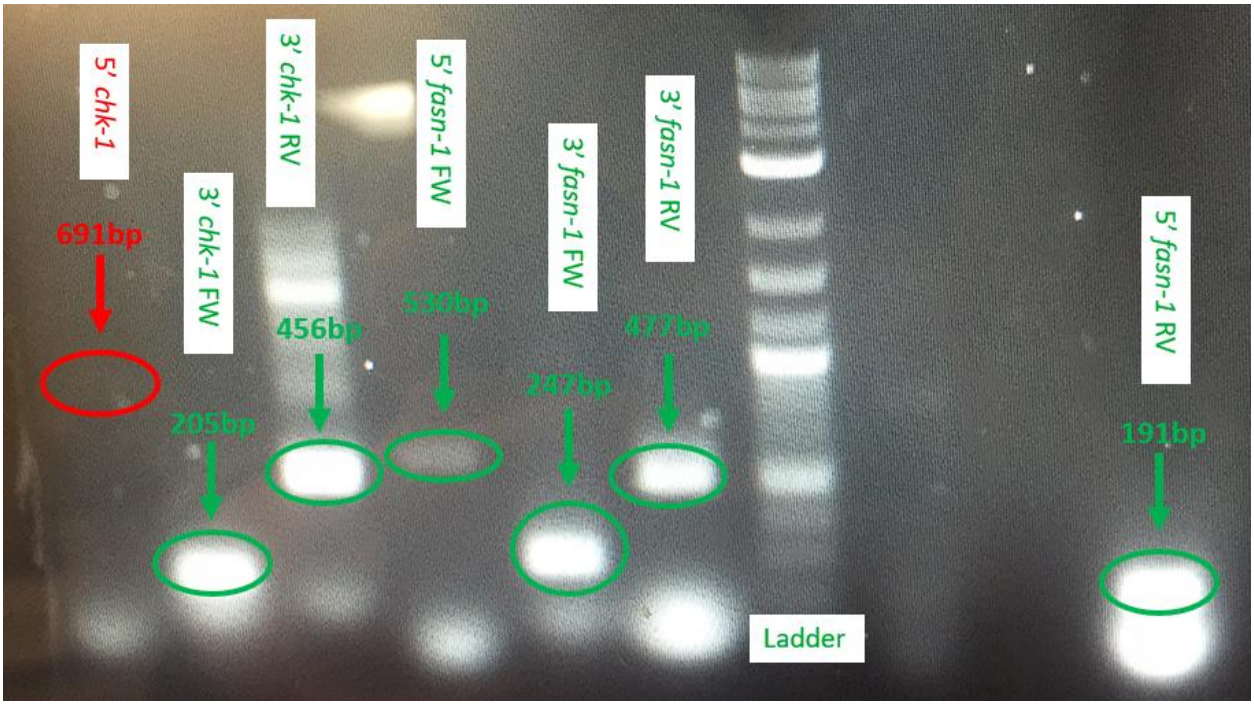

Figure 15: A picture of a gel containing the PCR products from the first round of PCR which was imaged using a BioRad ChemiDoc system. Each well is labelled with the HA fragment it contained while the base pair (bp) size of each fragment is also given.

were ordered for the unworking 5' *chk-1* HA, but these were not successfully inserted into pDD282 by the deadline for this research.

Once the *fasn-1* injection mix had been injected into 30 individual *C. elegans* and these individuals were subsequently treated with hygromycin, no rolling phenotypes were observed over the next five days. Following this, another 30 individuals were injected, grown for 5 days, treated with hygromycin (chunked if needed), and observed for the rolling phenotype over the next 4 days. No rollers were found on this examination either.

This research also had an unintended side benefit, in that students in Dr. Kroetz's Genetics class next year will continue the research by injecting the products created for *fasn-1* again.

Research Challenges: chk-1 gRNA

Several challenges arose during this research process. First, there was approximately a year of issues regarding the gRNA for *chk-1*. As described above, the gRNA oligonucleotide

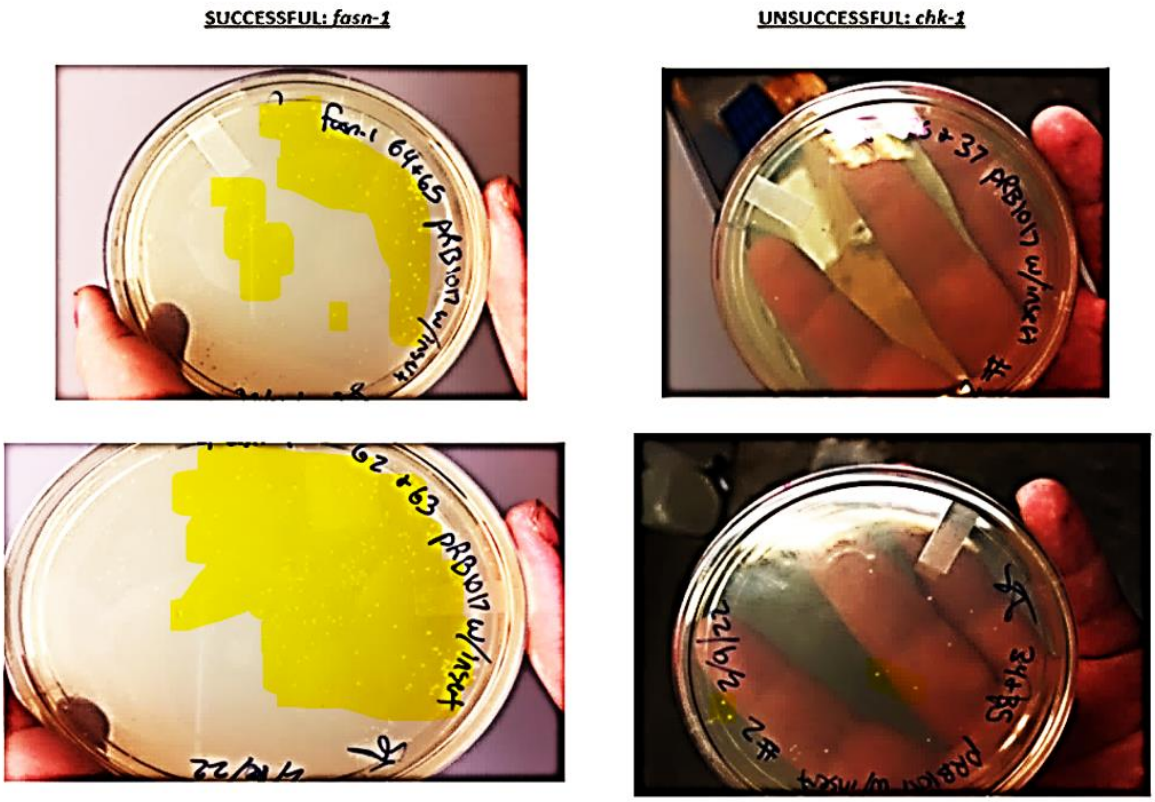

Figure 16: An image comparing the successful *fasn-1* gRNA plates to two of many unsuccessful *chk-1* plates. The *fasn-1* plates had significantly more colonies (white dots highlighted in yellow) than the *chk-1* plates which would often have no colonies as seen in the top right.

sequence for *chk-1* had several processes that it needed to undergo to be inserted into pRB1017 for use in CRISPR. It needed to be re-annealed, ligated into pRB1017, then was heat shocked to transform the plasmid into competent *E. coli* bacteria, and finally the bacteria would be plated on LB+KAN plates. Those bacteria with pRB1017 have resistance to Kanamycin and would thus survive this treatment. Approximately 6-7 times over the course of a year, each of the above steps would be completed according to the written procedures and then when the plated bacteria would be checked for colonies there would either be no colonies or very few colonies.

While having very few colonies is not necessarily a sign of the bacteria failing to take up these few colonies were consistently determined by DNA sequencing to have either have failed to take up a pRB1017 plasmid that contained the *chk-1* gRNA sequence or to have successfully taken up the pRB1017 plasmid, but not a copy that contained the *chk-1* gRNA sequence. An example of the successful *fasn-1* gRNA plates and the unsuccessful *chk-1* gRNA plates is shown in Figure 16.

Several theories as to why the *chk-1* gRNA was not being taken up were proposed and tested. Initially it was considered that perhaps pipetting error was the cause so the procedures were conducted again without any changes but with new 2 µL pipette tips. This failed to solve the issue. Another possibility was that the heat shock transformation was not being conducted correctly. So, the procedures were repeated with extra attention paid to heat shocking the tubes at *exactly* 42 ºC for *exactly* 45 seconds. This also did not work. Then it was considered whether the pRB1017 plasmid was nonfunctional over the time spent re-testing so two new pRB1017 plasmids were made. The gRNA insertion procedures were repeated this time with both *fasn-1* and *chk-1* gRNA. The *fasn-1* gRNA plates produced colonies while the *chk-1* gRNA plates did not produce colonies which ruled out several potential issues that were being considered. The issue had, at this point, been isolated to the *chk-1* gRNA oligonucleotides themselves.

At this point, two potential sources of error remained. Either the lab had sent oligonucleotide sequences that were not correct (this is very unlikely), or the gRNA sequences had been hydrated to the wrong concentration when they were originally received from Eurofins (this is much more likely the case). Thankfully, both sources of error had the same solution: re-ordering the *chk-1* gRNA sequences. Once re-ordered, the procedure for inserting the gRNA sequences into the pRB1017 plasmid was repeated and, thankfully, several dozen bacterial

Table 6: Graded APEX PCR Protocol	
Step Number	**Machine Instructions**
1	95°C FOR 15:00 minutes
2	95°C for 0:30 seconds
3	52/68°C for 0:30 seconds
4	72°C for 1:00 minute
5	GOTO Step 2, 29 times
6	72°C for 5:00 minutes
7	10°C for FOREVER
8	END

colonies were present on the LB+KAN plates after the transformation. The selected colonies were sequenced and contained the desired gRNA sequence.

Research Challenges: The 5' Homology Arm of chk-1

Additionally, the 5' *chk-1* HA also was not generating a PCR product. As mentioned above regarding Figure 11, the columns containing 56-57 were showing no bands where the homology arm was supposed to be. Several theories as to why the PCR product was not being generated were tested. First, to test for pipetting error or other one-off mistakes, this HA was run separately on another gel at a lower voltage (80V) for a longer period (40 minutes) and again showed no bands. Next, it was considered that the temperature at which the PCR reaction ran could be denaturing the DNA. To test for this, several PCR reactions were run with the procedure listed in Table 6 in which step 3 has been modified from the original PCR protocol so that the PCR reactions ran on a gradient of temperature from 52 - 68 ºC. These PCR products were run on a gel but also produced no bands as shown in Figure 17.

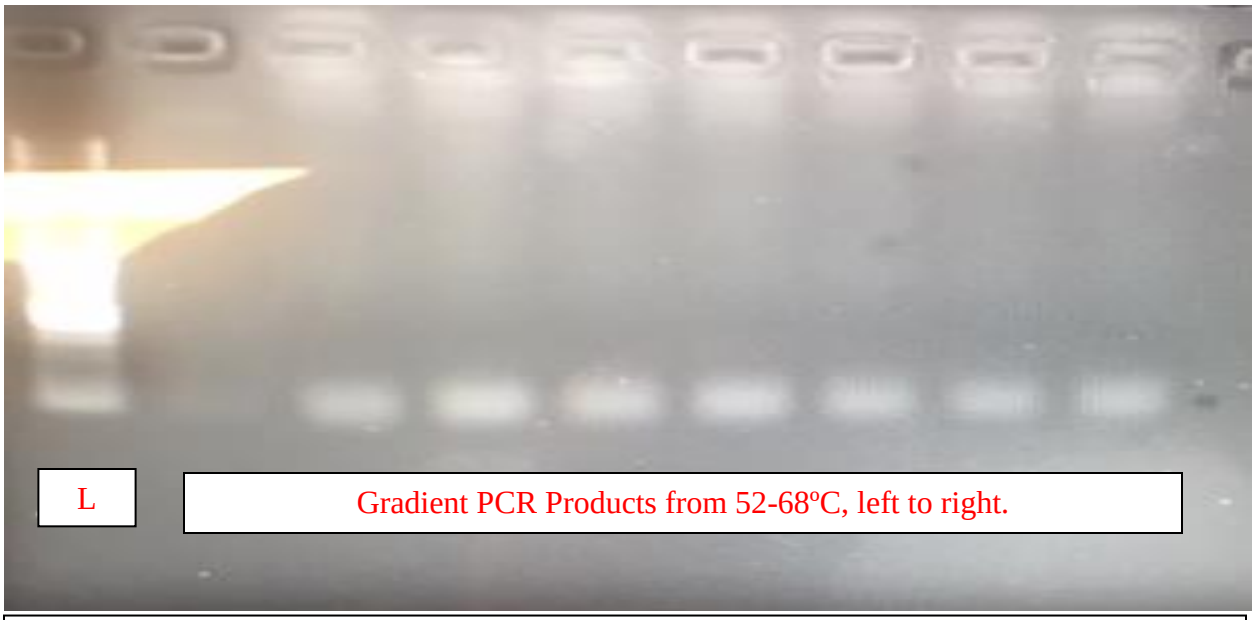

Figure 17: Graded PCR products for the 5' HA of *chk-1*

At this stage, it was determined that examining the originally designed HA primer would be the best next step to determine if there were any problems when originally designing these primers. Upon re-examination, it had been determined that primer 56 had the potential to create very strong secondary structures, which means that it was much more likely to fold on itself and create unusable hairpins as opposed to linear DNA. To remedy this, another primer was ordered from Eurofins that was less likely to produce these strong, unusable secondary structures. This primer is listed in Table 7, and it would still be paired with primer 57 for the purposes of 1-step

5' *chk-1* HA (141 + 57):

CTGAGGATCTTCTGCTTACTCAGCatattgatatgtcgcagaccaactcggttaggaaaaaaccgtcagaaattggcg
gatttttagctaaaatctccaatttttcggtcgtaaattggcatttttgataatttttaggatgaaaattcgctaatgacacggaaatggttgcaaaatt
agaggtttttgctaaatttacttttttttttgattaaaaaacccagaatttagtttaaatccccctaaaatttttcagaatctcctccaacgaatggtgtg
ccgtatgacacgtttctgcgtcgtcaccgacatccgttccacgtaccaaaaagtggctcgagccagtgagcacgccggcttcggcgttcgtg
agactgacgactaccgtctactagtcacgtggcgcgaggtttcgatgatggtcagtctgtacacgatgggcgatattcccgacaagccacgt
gtcatggtagatttccgcaggtctcgcggtgacggaatccagtttaagaagatgttcatggacgttagaaaccgtatgcatgagtggatatgta
ccgacggaaacaactggctcgccaatcttggatatgtgccaagaaatccgcagatagttaatggtggtggagtcaatgtggagcactcggC
GTCCTCGATCAATGTaGATGTC

Figure 18: The full 5' HA for *chk-1* with the redesigned primer 141. The capital letters represent the homology arm primers while the highlighted region represents the gRNA primer. The light blue highlighted and lowercase nucleotide within the uppercase region represents the PAM mutation.

Table 7: New 5' HA Primer for *chk-1*	
Sequence name	**Sequence**
141: 5' Forward *chk-1* HA	acgttgtaaaacgacggccagtcgccggcaCTGAGGATCTTCTGCTTACTCAGC

PCR. The final HA product for using primers 141 and 57 is shown below in Figure 18. The gel for this new HA was not run by the research deadline and thus this problem is unclear with regards to whether the final solution used was correct.

Unsuccessful vs. Successful Markers for CRISPR

The SEC portion of the mutation that was inserted into both *chk-1* and *fasn-1* provides several identifiable markers that can be used to determine if CRISPR was successful or not. First, there was the marker for hygromycin resistance, which could be tested for by treating each plate containing injected worms and their progeny with hygromycin-B. It is reasonable to assume that

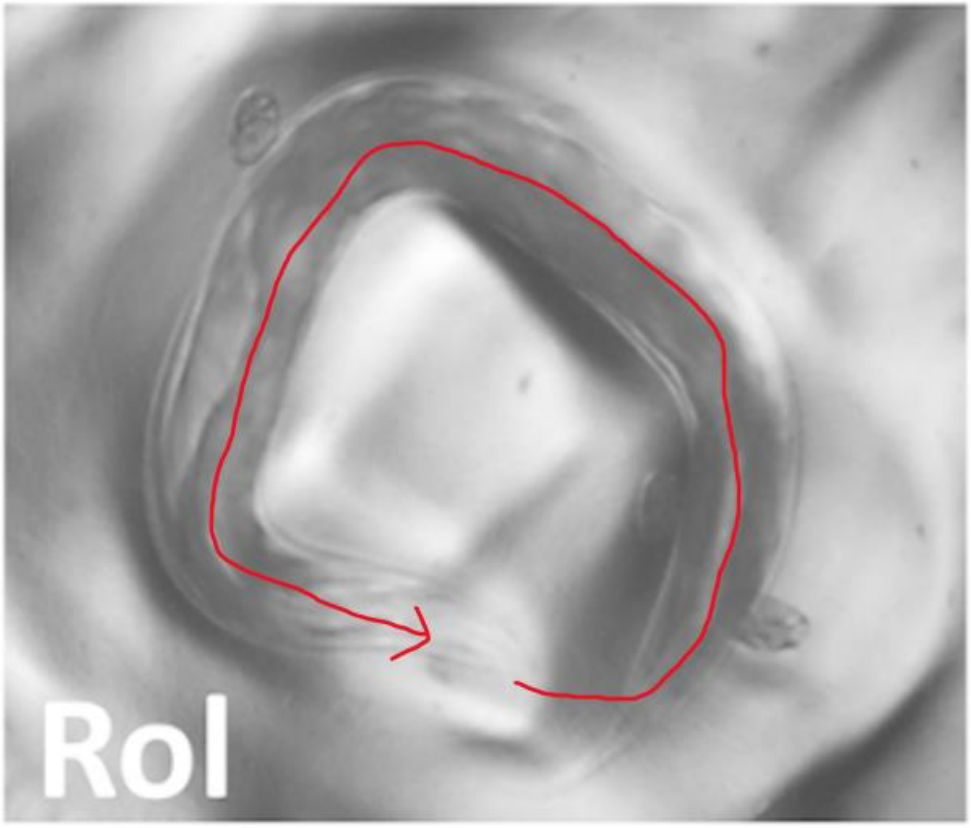

Figure 19: Image showing the roller phenotype in a *C. elegans* individual. The worm will continue to move in a circular pattern roughly along the red line. Adapted from Corsi *et al.*, 2015

any individuals that are still alive and reproducing on the treated plates 4-5 days after treatment were potentially modified by CRISPR. Another indicative marker is observing the rolling phenotype in said survivors that is conferred by the *sqt-1* gene within the SEC. This is when any individual worm exclusively moves in the pattern shown in Figure 19 after they have reached the L3 stage of their life cycle. Selection for these worms is done using a heat-sterilized pick to individually transfer the worms that are rolling to another, smaller plate where they will be allowed to self-fertilize and reproduce.

The final two markers for whether CRISPR occurred involve observing the worms under a powerful microscope with epifluorescences to determine which regions of the animal are expressing GFP-bound protein. Two very bright glowing regions that are observed near the head of the animal, as shown in Figure 20, are indicative of CRISPR not having worked. These two regions are neurons in the head that produce GFP-tagged proteins of the *str-1* gene if the injected

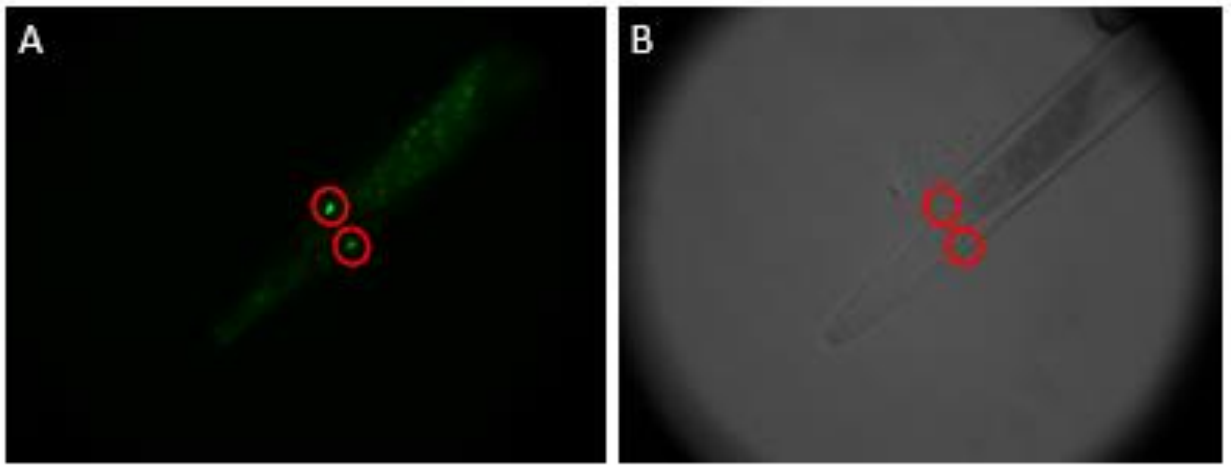

Figure 20: Images taken by Dr. Mary Kroetz of the worms produced from the research of Peyton Young.

A: The head region of an individual *C. elegans* under epifluorescence with the two brightly glowing neurons that are indicative of unsuccessful CRISPR.

B: The same image as 'A' but not under epifluorescence with the locations of the two neurons circled in red. These are not visible under these conditions.

DNA remained as an extra chromosomal array as opposed to having been inserted into the

worm's genome via CRISPR. While the DNA within these extra chromosomal arrays is transcribed (hence why the worms would have demonstrated the rolling phenotype in the first place), it is not maintained by the cells normal DNA repair machinery, can have variable expression levels even from cell to cell within an individual, and is not reliably inherited by the affected individual's progeny. Thus, while an extrachromosomal array does confer hygromycin resistance and the rolling phenotype to the worm, it is not the desired result of CRISPR.

The second of the two markers is indicative of CRISPR having worked, and it is when the two brightly glowing regions from Figure 20 are not observed while the region that the gene

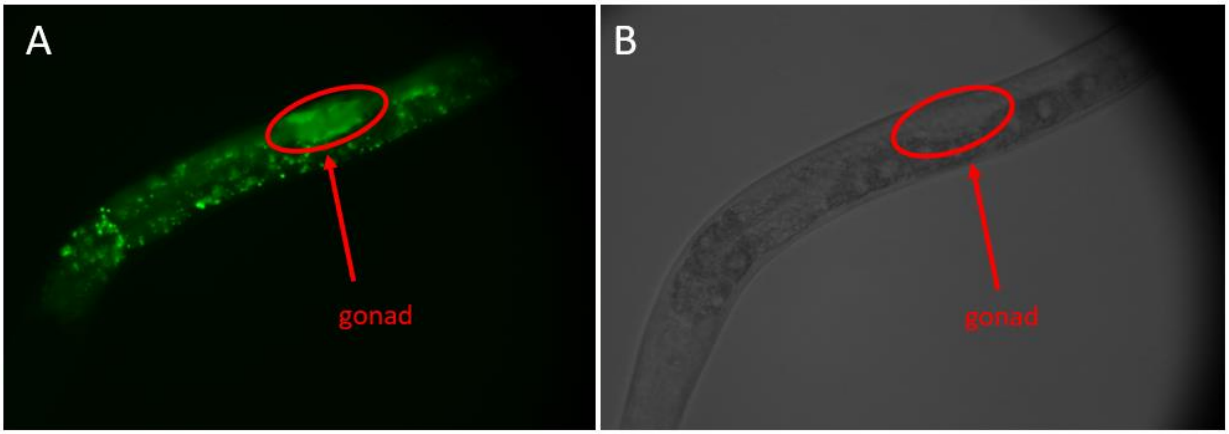

Figure 21: Images taken by Dr. Mary Kroetz of the worms produced from the research of Peyton Young.

A: The central region of an individual *C. elegans* under epifluorescence showing the brightly glowing body of the worm with the green-glowing gonad identified.

B: The central region of an individual *C. elegans* not under epifluorescence where the gonad bulges into the intestinal tract slightly.

of interest is expressed in is glowing. For Figure 21, the green region labelled gonad is highlighted because that is the region where the gene of interest in these pictures, *pig-1*, is expressed. Marked in Figure 21, this region appears to glow as a green smudge and is conclusive evidence that CRISPR was successful. This is the final step in concluding that CRISPR was

successful and that the observed individual should be plated separately and allowed to self-fertilize to make many more progeny with this exact genotype.

DISCUSSION

Interpreting Results

The roles that *chk-1* and *fasn-1* have in various *C. elegans* cellular pathways have been previously explored but never in the context of somatic gonadogenesis. This research intended to create the tools necessary for future researchers to investigate how these two genes are involved in gonadogenesis. This was to be accomplished by creating the gRNA and HA constructs for *chk-1* and *fasn-1* to be used in CRISPR and to subsequently attempt to edit these genes in *C. elegans* via CRISPR.

The gRNA constructs were successfully created for both genes and the HA were successfully created for *fasn-1*. Two attempts were made at conducting CRISPR with the *fasn-1* gene but neither of these attempts were successful at producing worms demonstrating the roller phenotype.

Future Study: The Degron System

To fully realize what roles *fasn-1* and *chk-1* play in the gonadogenesis process, future research must include a method to degrade the GFP-tagged proteins these genes produce translated in the gonads. Following this, the somatic gonads developing in the protein's absence will be observed for phenotypic abnormalities. This will be accomplished using the ZIF-1 nanobody degron system (Z1D).

The Z1D system is a method of depleting specific proteins from specific cell tissues in a variety of organisms, including *C. elegans*. For future research, the Z1D system would direct proteins fused to a GFP to the proteasome for degradation via ubiquitylation, which is the binding of ubiquitin to specific amino acid residues in a protein of interest (POI). Lysine is the target amino acid for this research and is the most commonly ubiquitylated amino acid via this method. This system will be responsible for degrading the proteins produced by *chk-1* and *fasn-1* in the gonads. The rest of the organism will also produce GFP-tagged *chk-1* and *fasn*-1 proteins, but these will not be degraded in the proteasome because the degron system is not present organism-wide. It is exclusively expressed in the gonads, which will avoid the embryo lethality caused by RNAi of these genes (Kalogeropoulos et al., 2004; Vrabilik and Watts, 2012; Kniaveza *et al*. 2004)

Specifically, this system works by first binding an epiDEG molecule to GFP of the target protein (via the anti-GFP nanobody as shown in Figure 22). The ZIF-1 protein will then recruit

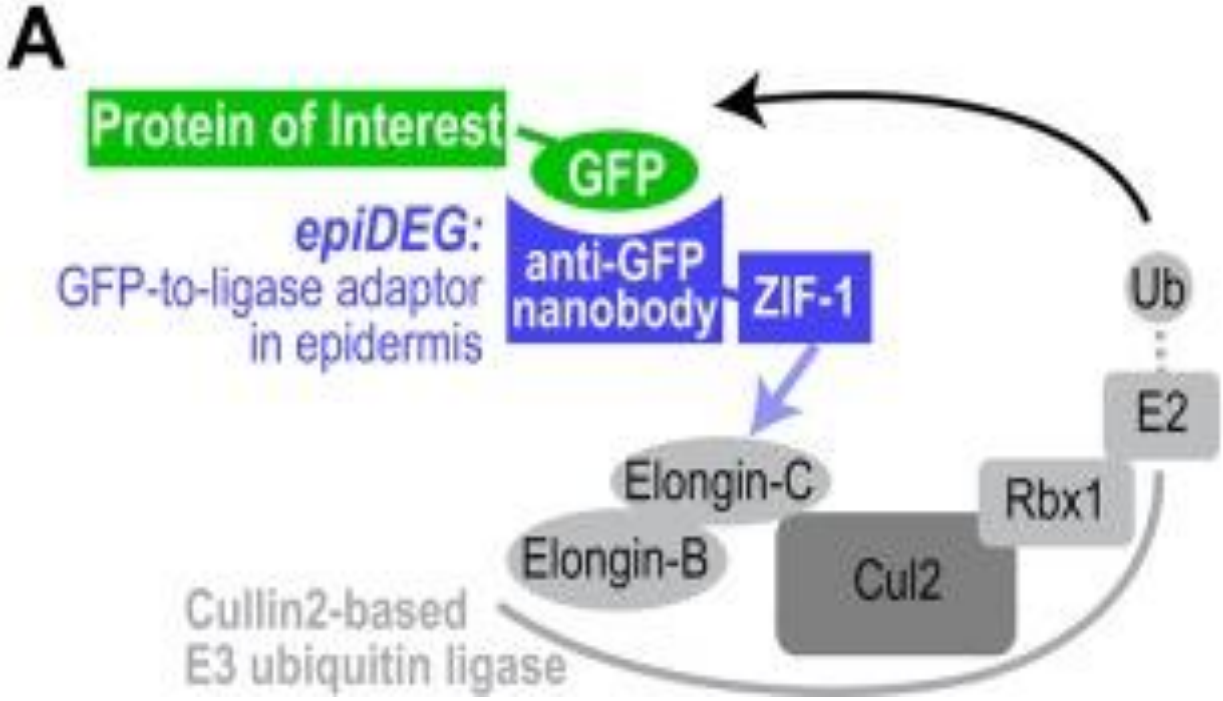

Figure 22: Diagram showing how the ZIF-1 degron system ubiquitylates proteins of interest (POI) for degradation. From Wang et al., 2017.

the Cullin2-based E3 ubiquitin ligase complex via Elongin-C as shown. This complex is responsible for inducing ubiquitin to bind to lysine residues throughout the GFP–protein fusion which will 'mark' them for transport to proteasomes via normal cellular processes where they will be degraded (Wang, 2017).

To create an individual that contains both the degron system and the GFP-tagged gene of interest, several rounds of crossbreeding will need to occur. Individuals that contain the mutations which tag *chk-1* and *fasn-1* proteins with GFP will be cross-bred with individuals who contain the degron system so that their progeny will be heterozygous for both traits. These heterozygotes will reproduce via self-fertilization. Doing this will result in approximately 1 out of every 16 progeny being homozygous recessive for both the GFP-tagged genes and the degron system. This system will be responsible for removing the expression of the target genes from the gonad in the doubly homozygous individual that future researchers might be studying.

The products of this research will be used in future Genetics classes where the overall goal of determining what roles *chk-1* and *fasn-1* play in gonadogenesis will continue to be pursued. The techniques used and the products made by this research are an example of how in-depth performing CRISPR modifications really is and are an excellent guide to future Genetics researchers in Dr. Kroetz's lab. Each gene has the potential to be a major regulator of somatic gonadogenesis in *C. elegans* which, because of how similar their genome is to humans, can have major implications for how our own developmental processes are regulated. Ultimately, this research achieved its goal of designing the precursor molecules needed for future researchers to perform CRISPR on and study the effects of *chk-1* and *fasn-1*.

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