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Using CRISPR to Genetically Engineer Two Genes Involved in Gonadogenesis in the Model Organism C. elegans

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Using CRISPR to Genetically Engineer Two Genes Involved in Gonadogenesis in the Model Organism *C. elegans*

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Introduction

Background Information

C. elegans are a microscopic nematode that are often used as model organisms, particularly because of their similarities to humans. It shares many features with humans, particularly seen through the comparison of the human proteome to *C. elegans* proteome. Of the known protein sequences of *C. elegans*, 40%-80% have human homologous genes, making *C. elegans* an ideal organism for study of human proteins and their functions (Lai *et al.* 2000). Additionally, like humans, *C. elegans* has a nervous system, develops through differentiation and specialization of cells in the embryo, and reproduces via sperm and egg. These similarities, coupled with their small size and quick life cycle, make them an excellent model organism to study in the lab.

There are two sexes of *C. elegans*, a self-fertilizing hermaphrodite and a male, and most exist as hermaphrodites, with only occurring about 0.2-0.5% due to non-disjunction (Hodgkin, *et. al*, 1979). This scenario, where there exists an XO male (in which the male has only one X chromosome) and an XX hermaphrodite, rather than females, differs from the more traditionally seen male and female model (Hodgkin, 1987). The males and hermaphrodites arise from morphologically identical primordia, as seen in *Figure 1*.

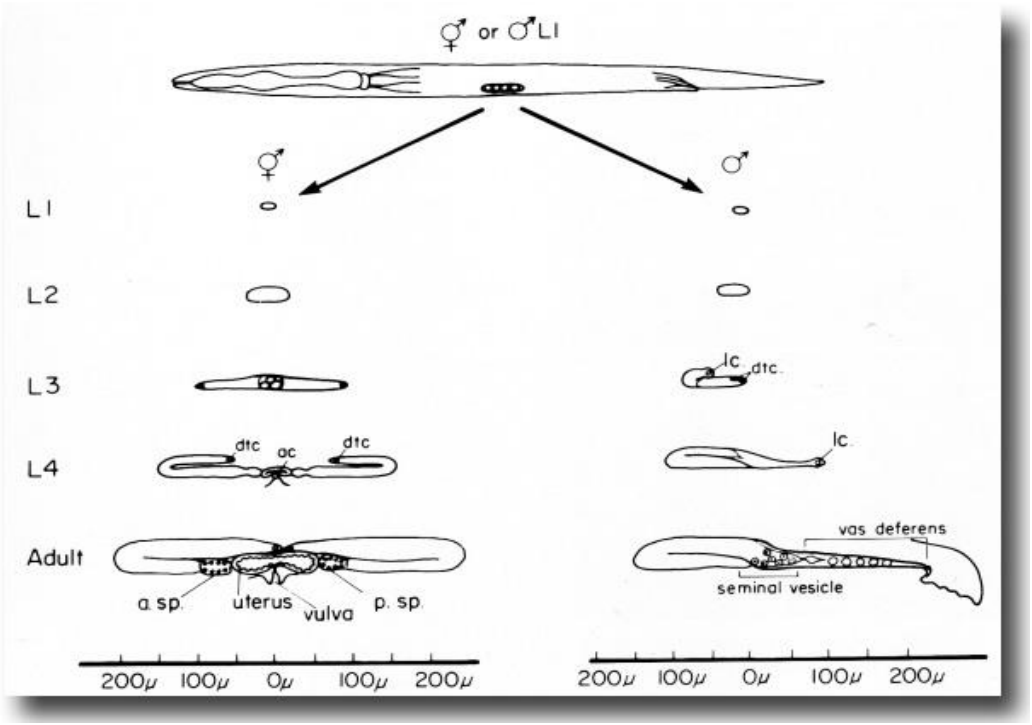


Figure 1. Hermaphrodite and Male Gonad Formation (Kimble and Hirsch, 1979). Above the formation of the gonad in both the male and hermaphrodite sexes can be observed, as well as the differing structures at the adult stage of *C. elegans.*

In hermaphrodites, the primordium develops to a two-armed gonad that has mirror symmetry, with structures including a uterus, vulva, and a spermatheca, which allows for storage of sperm. In the males, however, the primordium develops into an asymmetrical structure with several key structures, including a testis, a loop region, a seminal vesicle, and a vas deferens (Klass *et al.* 1976). The differences in the symmetrical anatomy of the hermaphrodites compared to the asymmetrical anatomy of the males be seen in *Figure* 1 and below, in more detail, in *Figure 2.*

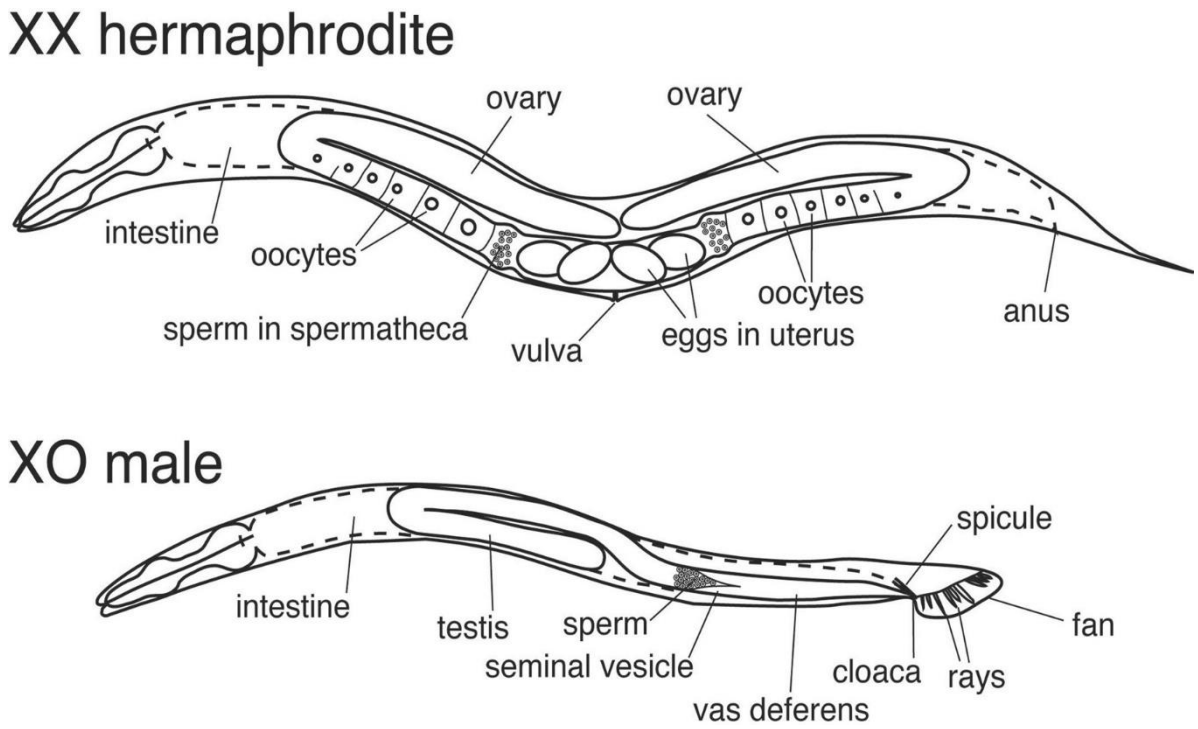


Figure 2. Internal Anatomy of the gonad of C. elegans (Zarkower, 2006). Above the symmetrical anatomy can be observed in the hermaphrodites. Additionally, the asymmetrical anatomy can also be observed in the males, as well as other important structures used for identification.

Due to the asexual nature of the *C. elegans* reproductive process, males arise from the spontaneous nondisjunction (failure of chromosomes to properly separate) of an X chromosome during meiosis, as could be inferred by the XO chromosome pattern in the males. Alternatively, they can also arise from a hermaphrodite cross with an existing male (Loxterkamp, et. al, 2021). The males and hermaphrodites differ greatly, and not only in the structure of their gonads. Males are generally small compared to the hermaphrodites and have a blunt fan-shaped tail, rather than the long-pointed tail of the hermaphrodites, as seen in *Figure 1.* Additionally, males are unable to produce eggs, and therefore must rely on the hermaphrodites to reproduce. Neurologically, hermaphrodites possess only 302 neurons, while males have 385. These extra neurons found in the male are tied to mating and generally function within the tail region (Loxterkamp, et. al, 2021).

Of the two sexes discussed, the focus of this research is on the males. Due to the low numbers of naturally occurring male species within *C. elegans*, they are studied significantly less than their hermaphrodite counterparts. As such, much less is known about the development of specific organs.

Life Cycles

This research especially focuses on the development of the gonad and the genes involved in this development. To understand this process, the analysis of the life cycle stages of *C. elegans* is essential. These stages, which both sexes undergo, can be seen below in *Figure 3.*

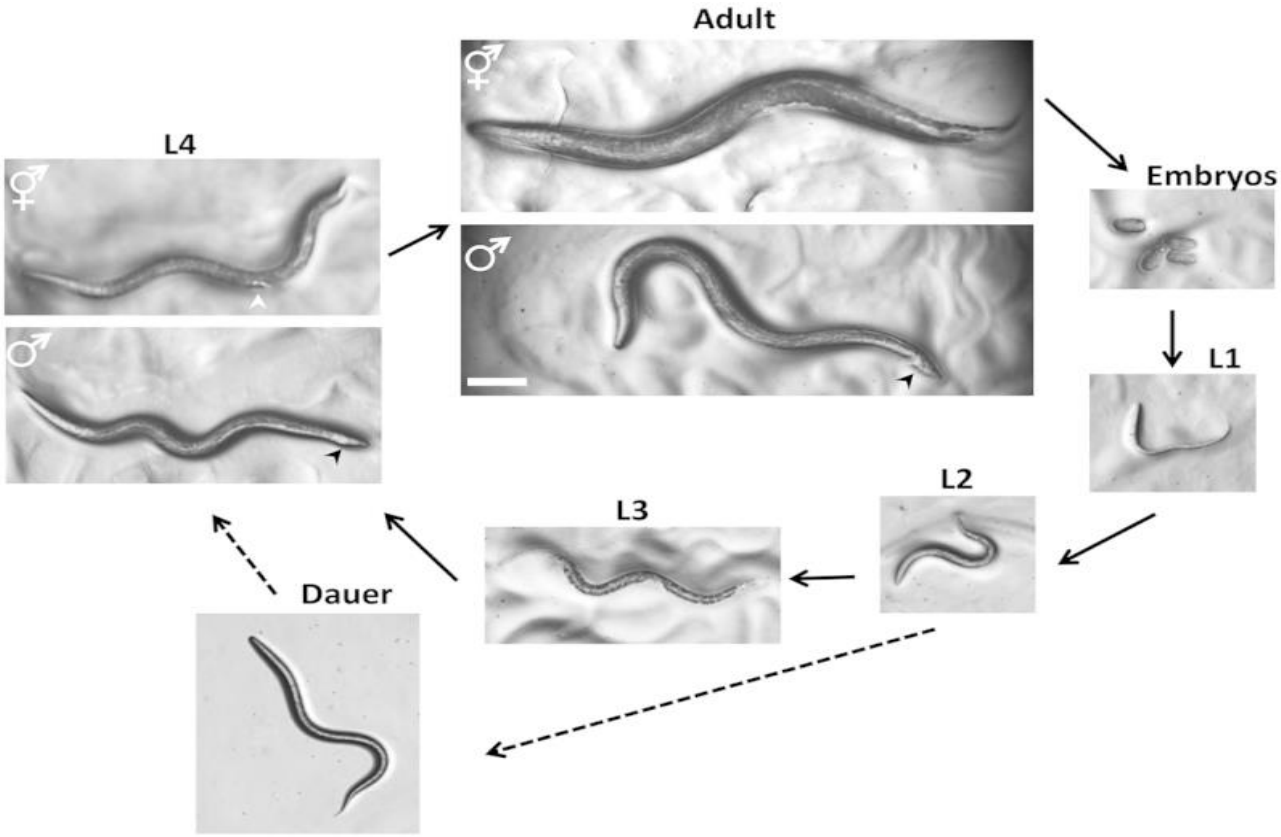


Figure 3. Developmental Stages of C. elegans (Corsi, et. al 2015). Pictured above are the developmental stages of both the hermaphrodite and the male sexes of *C. elegans.*

As seen above, the two sexes begin as embryos and then typically follow a progression from L1-L4 (larval 1 – larval 4) stage, until the final transition into adulthood. It is important to note that the sexes are not easily distinguished until the L4 stage under a stereomicroscope used for worm maintenance. After this stage, structures that are unique to the males (such as the fan on the tail seen in *Figure* 2) are more easily identified. The Dauer stage seen in *Figure 3*, which can occur in place of the L3 stage, typically arises from poor conditions, such as low food or overcrowding with other organisms (Corsi, et. al, 2015). These stages are critical to this research, as the gene products must be targeted in the earliest stages of development, in order to prevent any of the effects from occurring in the gonad. They were also relevant in the determination of which genes to study, as the gene products of the two genes of interest were observed at high levels in the early developmental stages, specifically mid to late the L1 stage.

Genes of Interest

Two genes were chosen for study based on two main criteria: high levels of mRNA expression in the male gonad in the early developmental stages compared to mRNA expression levels in the entire organism and the genes necessity to the survival of the organism. If a gene is necessary for an organism's survival, it is termed as an essential gene. This means that the gene plays an important role in some other part of the *C. elegans* that is integral to the survival of the worm. Therefore, complete removal of the gene would lead to the death of the organism and an inability to study the effects of the gene in gonadal development.

The two genes that were chosen were C10E2.6 and *pig-1*. These genes were found to fit the aforementioned criteria. For C10E2.6, the evidence of this can be seen below in *Figure 4.*

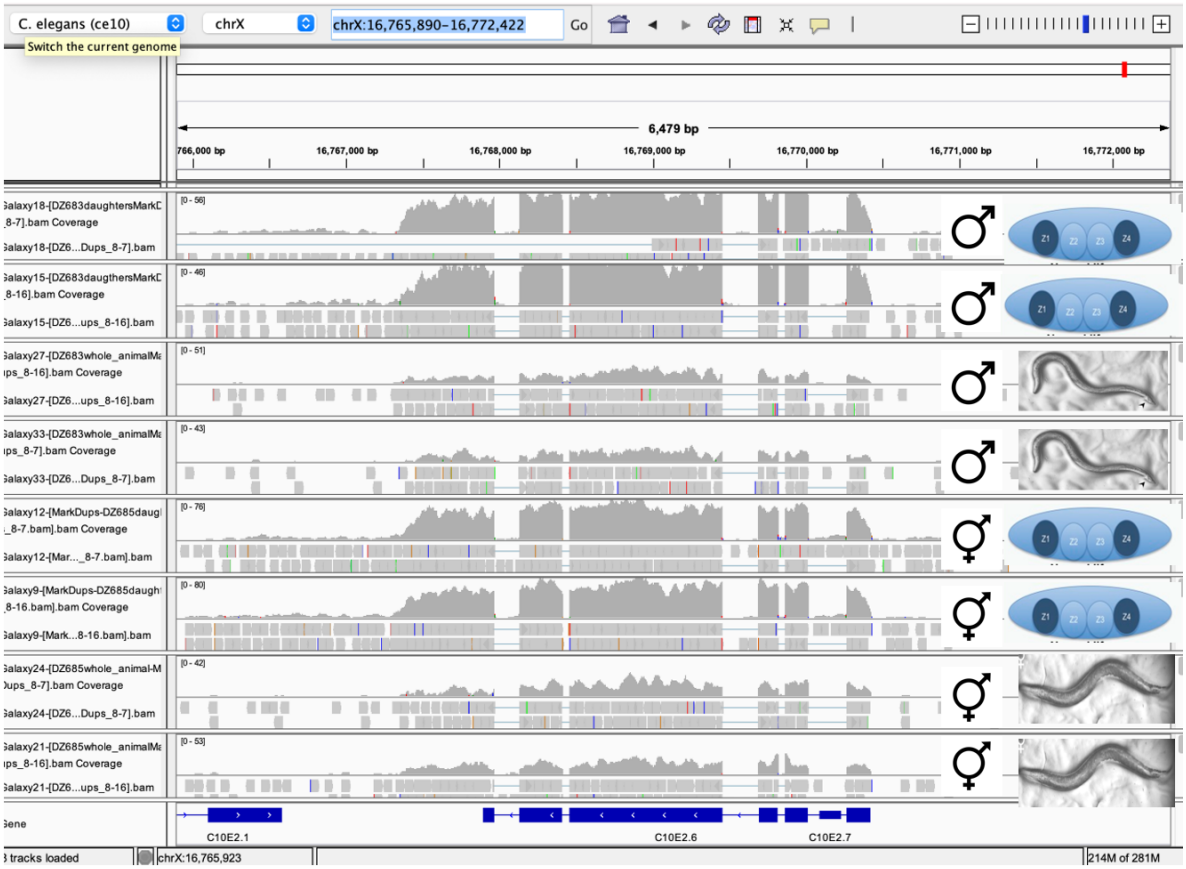


Figure 4. Expression Levels of C10E2.6. This figure shows the enrichment of C10E2.6 in the gonad and whole animal. Each expression observation was done in duplicate. The males (♂) and hermaphrodites (⚥) are represented by their appropriate symbols. The gonads are represented by their four primordium cells in blue. The whole animal is represented by an image of the appropriate sex. From top to bottom, the rows are expression in male gonad (in duplicate), male whole animal (in duplicate), hermaphrodite gonad (in duplicate), and hermaphrodite whole animal (in duplicate). The expression levels for each base pair can be seen by the height of the grey on each line.

C10E2.6 was observed in the male gonad in order to determine the level of expression when compared to the whole male animal. This likely indicates that this gene is important for the formation of the gonad. It was found that expression of C10E2.6 was 7.15 times higher in the male gonad than in the rest of the organism. This same observation occurred in the hermaphrodite gonad as well. Expression was found to be 2.98 times higher than the whole animal, though this was determined to be an insignificant difference (Kroetz and Zarkower, 2015).

This high expression of the genes in the gonad leads to the conclusion that the gene plays a role in gonadal development. The scenario is similar in *pig-1*, as seen below in *Figure 5*.

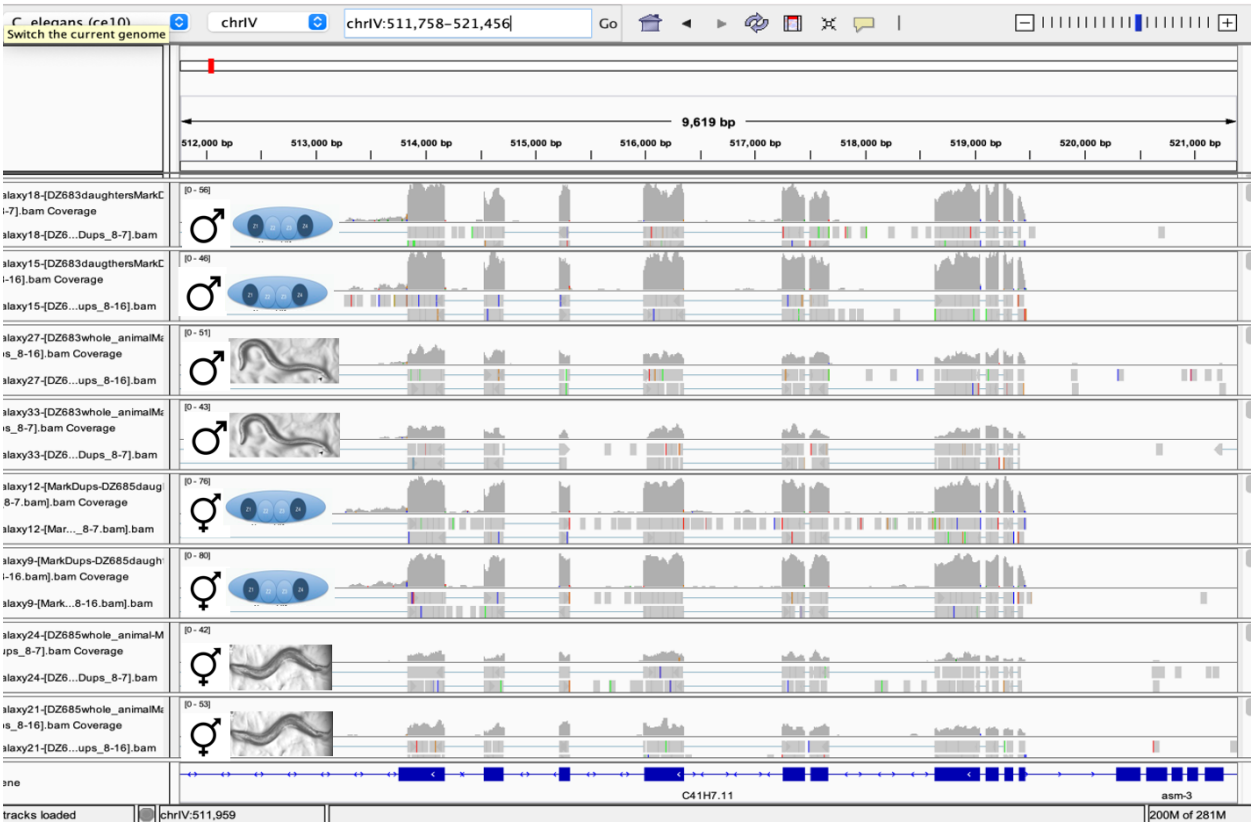


Figure 5. Expression Levels of pig-1 (Kroetz and Zarkower, 2015). This figure shows the enrichment of *pig-1* in the gonad and the whole animal. Each expression observation was done in duplicate. The males (♂) and hermaphrodites (⚥) are represented by their appropriate symbols. The gonads are represented by their four primordium cells in blue. The whole animal is represented by an image of the appropriate sex. From top to bottom, the rows are expression in male gonad (in duplicate), male whole animal (in duplicate), hermaphrodite gonad (in duplicate), and hermaphrodite whole animal (in duplicate).

As seen in *Figure 5*, similarly to C10E2.6, the expression of the gene product of *pig-1* is observed at higher levels in the male gonad. For *pig-1*, the expression in the gonad was 4.92 times higher in the males. As with C10E2.6, the same observation occurred with the hermaphrodites. For *pig-1*, however, the number was found to be significant at 4.35 times higher. Again, this leads to the conclusion that the gene plays a role in gonadal development.

The effects of the two genes will be studied individually, but the same method will be used for analysis. Both of these genes were observed to be highly expressed during development of the gonad in the early stages of development. Additionally, it has been reported that when these genes were not expressed, the organism did not survive (illustrating the essentiality of the genes to life). Aside from their possible roles in gonadal development, C10E2.6 and *pig-1* also have other interesting characteristics. C10E2.6 is expressed in the tails of *C. elegans* and is predicted to play a role as a transmembrane protein. It also has orthologs in the human genomes that are related to the cause of exercise induced hyperinsulinemic hypoglycemia. This disease is characterized by a drop in blood sugar and a spike in insulin after exercise. *pig-1* is involved with serine/threonine kinase activity, cell differentiation, and apoptosis. It also has a human ortholog of MELK, which is an oncogenic kinase that plays a role in metastasis of lung cancers (Tang *et. al,* 2020). While these other roles in the worm could be indicative of the genes' roles in gonadal development, it is possible that the roles are unique from the other gene functions. Therefore, the goal of this research is to alter the genome so that the genes of interest are fused to GFP. Similar to *Figure 6*, allowing for observation of areas of high gene expression. In the future, researchers can study the gene function within the gonad using the GFP tagged worms. The two genes are essential to the organism's survival, so complete removal of the gene would result in death. To avoid this, C10E2.6 and *pig-1* will be tagged with GFP which will eventually allow degradation of these genes from gonadal cells in future research.

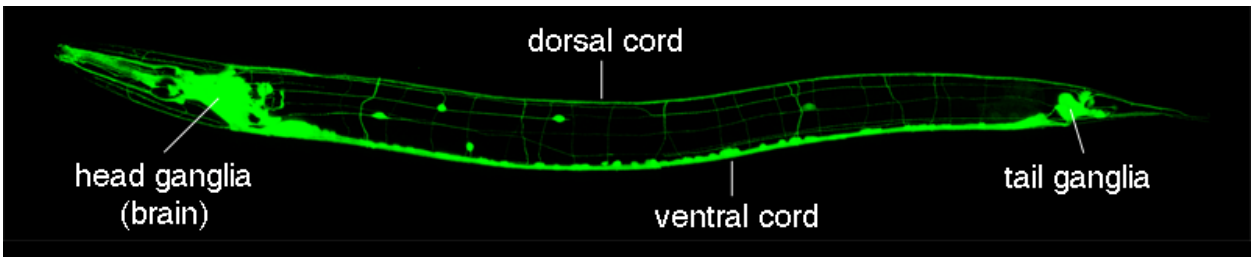


Figure 6. GFP in C. elegans (Hutter, 2008). Nervous system genes were tagged and visualized with GFP.

This study will include several methods such as CRISPR (clustered regularly interspaced short palindromic repeats), *him-8* mutations to increase the incidence of males in the population, and Homology Directed Repair.

CRISPR/Cas9

For this research, a plasmid which contains the Cas9 sequence will be used. CRISPR/Cas9's structure, as well as its interactions with target DNA are seen in *Figure 7.*

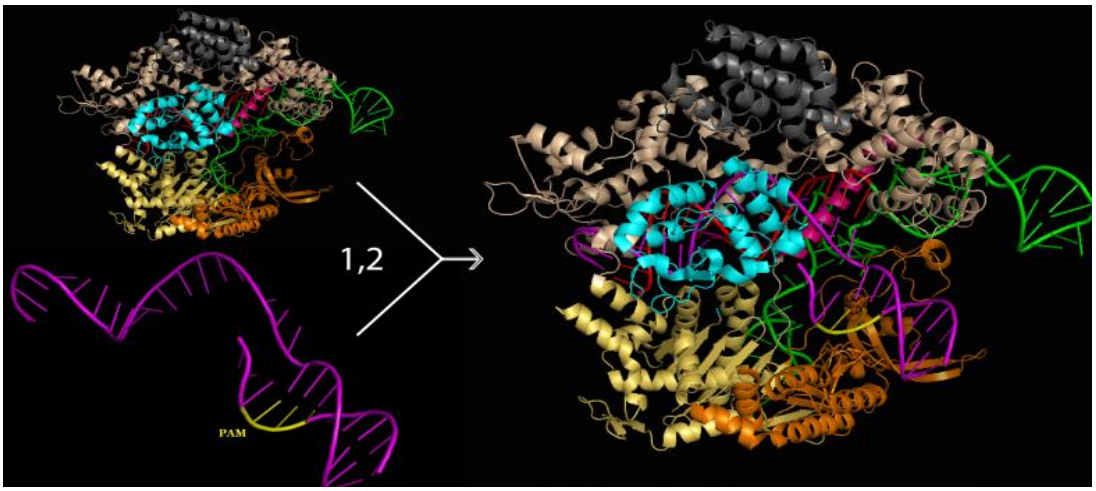


Figure 7. CRISPR/Cas9 Structure (Anders *et al.* 2014). In the image, 1 is CRISPR/Cas9 complex, with the guide RNA (gDNA) seen in stick configuration in green. 2 is the target DNA in purple stick configuration. The combination on the right is the CRISPR/Cas9 complex bound to the target DNA.

The complex used contains a Cas9 endonuclease, gRNA, and homology arms. Cas9 acts as a pair of molecular scissors, able to cut at specific points in the DNA (Harrison, et. al, 2014). The cutting of the DNA is directed by guide RNA (gRNA), which was designed specifically for the gene of interest (Saey, 2017). In this research, that is *pig*-1 and C10E2.6. The gRNAs anneal to a sequence in the genome that is 5'-N_{20}-3' that are directly followed by 5'-NGG-3' PAM site, where N is any nucleotide. The 5'-NGG-3' is not part of the gRNA but is

instead found in the target DNA. The PAM site is used for identification of the cutting location by the Cas9. Following this identification, the Cas9 uses the gRNA to confirm the correct region for cutting by comparing the gRNA sequence to the sequence upstream of the PAM site in the genome (SYNTHEGO, 2019). If the sequences match, the Cas9 will cut. *Figure* 8 illustrates the mechanism by which CRISPR functions.

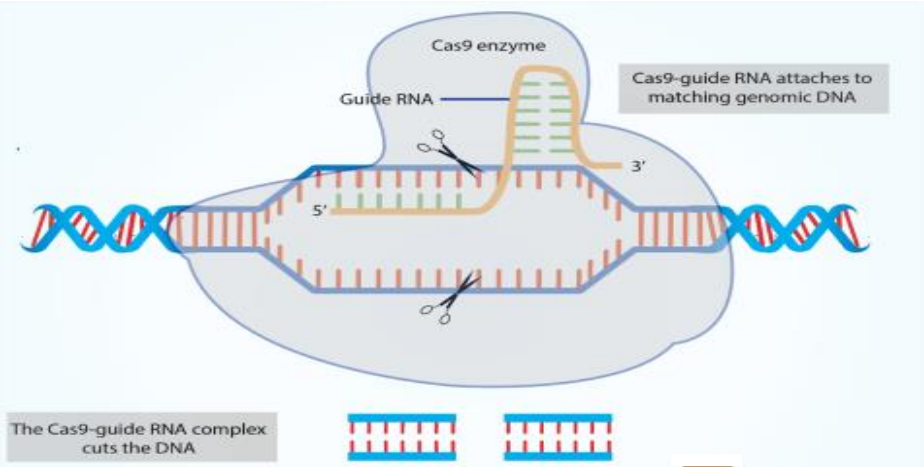


Figure 8. CRISPR and how it functions (Sampath, 2018). This figure illustrates how CRISPR works to edit specific areas of interest within the genome. The gRNA can be seen in orange and the Cas9 in gray.

Homology Directed Repair

After the DNA is cut at a specific point by the gRNA led Cas9, a repair will need to be made. There are two methods that can be used: non-homologous end joining (NHEJ) and homology directed repair (HDR). NHEJ utilizes single base pair overhangs at the cut regions and does not require a template for repair. HDR relies on homologous arms for a repair template. For this experiment, HDR was the method used, as it was necessary to insert the GFP into the worm's genome. The homology arms, also designed and inserted into a plasmid in this experiment, were used as a template to repair the cut DNA. Homology arms are long segments of DNA that are homologous to the genome The homology arms used as the repair template consist

of long segments of DNA that are exact copies of DNA surrounding the cut made by Cas9. Using the homology arms as a template, the DNA will repair itself. This can be seen in *Figure 9.*

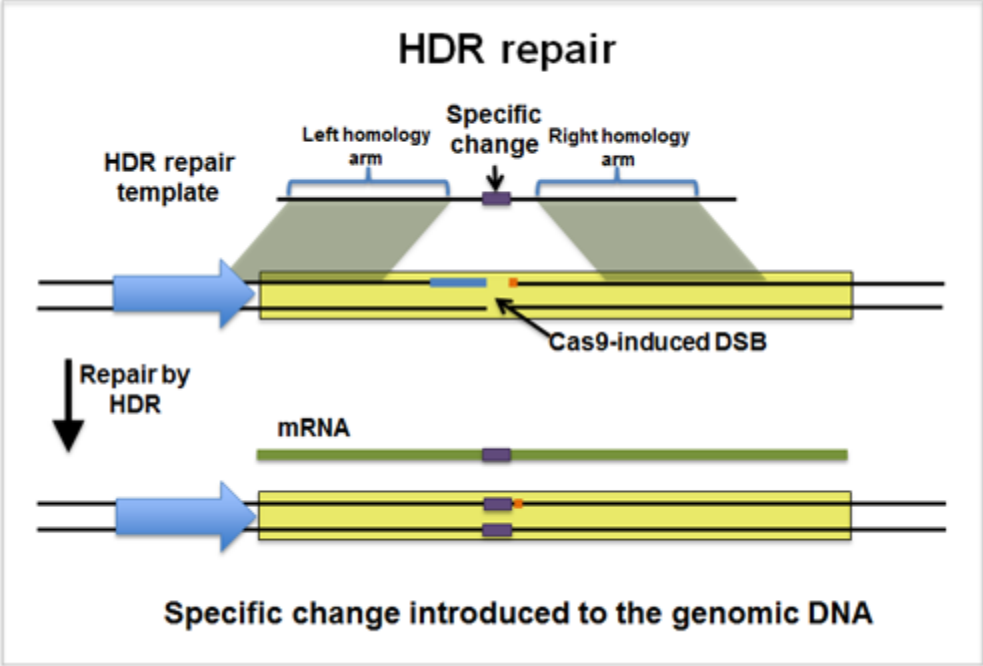


Figure 9. Homology Directed Repair. The graphic illustrates the method by which the DNA will repair itself using the homology arms (Cortez, 2015). The yellow represents the worm genome where the cut was made.

Figure 9 represents the repair of the DNA by the homology arms. As stated earlier, the two genes in our study are essential to the organism's survival, so complete removal of the gene would result in death. Therefore, the gene products must be downregulated in specifically the gonadal region but allowed to function normally in the rest of the organism for future studies.

To achieve this result in *C. elegans*, homology arms were designed with the aim to insert a construct, consisting of green fluorescent protein (GFP) and a self-excising cassette (SEC), into the genome. The specific change, referenced in purple in *Figure 9*, will include the GFP and SEC. Specifically, the GFP sequence will be fused to the two genes of interest, which will allow for visualization of the genes of interest. Additionally, within the SEC there is a

sequence which codes for the *sqt-1* gene. When expressed, this gene will cause the worms to exhibit irregular movement, which will be referred to as rolling. The worms will often crawl in circle or roll side-over-side. This will be another indicator that the insertion has occurred. *sqt-1* is a dominant gene, so both homozygotes and heterozygotes will exhibit this behavior.

Gibson Assembly Background

Gibson Assembly allows for the assembly of fragments of DNA into a circular plasmid *in vitro* (Gibson, et. al, 2010). For the purposes of this research, Gibson Assembly was used for site-directed mutagenesis, more specifically, the insertion of the homology arms into the vector pDD282 so that it can be used as a repair template. Gibson Assembly is the process through which these homology arms are assembled and then subsequently inserted into the plasmid, pDD282. The insertion of the homology arms, if successful, occurs on either side of GFP within the plasmid. To complete Gibson Assembly, the homology arms are combined with DNA ligase, DNA polymerase, and an exonuclease (Gibson, et. al, 2010). The exonuclease degrades the 5' end DNA of the homology arms and the cut plasmid so that there are overlapping single-stranded DNA fragments, which then bind. The DNA polymerase repairs the gaps in the DNA and the DNA ligase repairs the nicks left in the DNA, leading to the formation of the desired product (Birla, 2015).

him-8

As stated previously, males are found in low numbers in normal worm populations. This presents a challenge for this research, which focuses primarily on the male sex. In a typical population of hermaphrodites, males only occur about 0.2-0.5% due to non-disjunction (Hodgkin, *et. al,* 1979). Because this research is interested in males, *him-8* (high incidence of

males) was mutated in order to increase the likelihood of male occurrence within a population. By mutating *him-8* there is an increase in male occurrence from ~0.5% to 35% (Walsh, *et. al*, 2020). The *him-8* gene is involved in meiotic segregation of X chromosomes, so mutation to this gene will allow for an increase in the number of non-disjunctions (Phillips, *et. al,* 2005). Through this dramatic increase in non-disjunction events, the male population will be studied much more efficiently. These edits will take place simultaneously to the edits on the genes of interest. This means that multiple Cas9 edits will take place in a single worm, in order to achieve a more population that is easier to study. These edits will take place at the *him-8* locus on Chromosome IV, which are not involved with the genes of interest. Therefore, these edits will have no effect on the study of *pig-1* and C10E2.6, other than to increase the incidence of males.

Methods

Choosing Cas9 Target Site

For each gene, a site for mutation must be chosen. This site must be 100-200 base pairs long and centered on the N-terminus or C-terminus (Dickinson, *et. al*, 2015). A 3' section was chosen for C10E2.6 and a 5' section was chosen for *pig-1*. A 5' section was used for *pig-1* because this gene had a number of isoforms, and the enrichment of mRNA transcripts was found to more clearly enriched in the gonad at the 5' end of the gene. Otherwise, it is often more simple to fuse the GFP sequence to the 3' end of the gene. These sections were submitted to CRISPOR (https://crispor.tefor.net), which aids in the evaluation and design of guide sequences for CRISPR/Cas9. The sequences for insertion were chosen based on the efficiency and specificity score. Specificity scores are a prediction of how likely an RNA sequence will lead to a cut at a location other than the target sequence. Efficiency scores predict how likely CRISPR will cut at the chosen location. The scores for the chosen sequences can be seen in *Table 1*.

Designing gRNA

For CRISPR to make specific cuts in the desired location, a guide RNA is required. This guide RNA (or gRNA) must be designed specifically for the genes of interest. After the target site was chosen, it was evaluated to find the best sequences for each gene. These sequences can be seen in *Table 1*.

Table 1: gRNA

Primer Name	Primer Sequence	Primer Information
46. FW gRNA *pig-1*	TCTTGGCAAAACGCGAGACGCAGA	to anneal with oligo 47, ligate into pRB1017, cuts 149 bp upstream of pig-1 start, scored 60 efficiency by Doensch 2016 on CRISPOR.tefor.net
47. RV gRNA *pig-1*	AAACTCTGCGTCTCGCGTTTTGCC	to anneal with oligo 46, ligate into pRB1017, cuts 149 bp upstream of pig-1 start, scored 60 efficiency by Doensch 2016 on CRISPOR.tefor.net
48. FW gRNA *pig-1*	TCTTGCAAGTATGAAGTGCTCCAG	to anneal with oligo 49, ligate into pRB1017, cuts 24 bp downstream of pig-1 start, scored 68 efficiency by Doensch 2016 on CRISPOR.tefor.net
49. RV gRNA *pig-1*	AAACCTGGAGCACTTCATACTTGC	to anneal with oligo 48, ligate into pRB1017, cuts 24 bp downstream of pig-1 start, scored 68 efficiency by Doensch 2016 on CRISPOR.tefor.net
115. FW gRNA C10E2.6	TCTTGCAGAAAAAAGCCAAGGCCA	to anneal with oligo 116, ligate into pRB1017, cuts 20 bp upstream of C10E2.6 stop, scored 59 efficiency by Doensch 2016 on CRISPOR.tefor.net
116. RV gRNA C10E2.6	AAACTGGCCTTGGCTTTTTTCTGC	to anneal with oligo 115, ligate into pRB1017, cuts 20 bp upstream of C10E2.6 stop, scored 59 efficiency by Doensch 2016 on CRISPOR.tefor.net

117. FW gRNA C10E2.6	TCTTGAAATCATGGCAGATCATCA	to anneal with oligo 118, ligate into pRB1017, cuts 13 bp downstream of C10E2.6 stop, scored 70 efficiency by Doensch 2016 on CRISPOR.tefor.net
118. RV gRNA C10E2.6	AAACTGATGATCTGCCATGATTTC	to anneal with oligo 118, ligate into pRB1017, cuts 13 bp downstream of C10E2.6 stop, scored 70 efficiency by Doensch 2016 on CRISPOR.tefor.net

The evaluation through CRISPOR returns target sites that are 5'-N_{20}-3' and are directly followed by 5'-NGG-3', where the 5'-NGG-3' is not part of the gRNA but is instead found in the target DNA. Additionally, either TCTT for the forward or AAAC for the reverse was added to each sequence to allow for sticky end overhands of the cut plasmid. The plasmid was cut so that sequences complementary to the ends was exposed, allowing for insertion of the desired gRNA sequences. The chosen gene's sequences were then ordered through Eurofins. Upon arrival, they were rehydrated to 100uM. The sequences were then ready to begin insertion into the pRB1017 plasmid.

Designing the Plasmid pRB1017

The first step in inserting the gRNA into a plasmid was the designing of the pRB1017 plasmid, which will house the gRNA after insertion. In its original state, the plasmid has kanamycin (KAN) resistance, which was used as a selective property to determine uptake by the bacteria, *E. coli. E. coli* was transformed to insert the pRB1017 plasmid through the following protocol.

Transformation Protocol

5 µL of pRB1017 was added to the *E. coli.* After an incubation of 5 minutes on ice, it was heat-shocked at 42ºC for 45 seconds. The tubes were then placed back on ice and 1mL of LB was added. After a period of two minutes on ice, the tubes were moved to an incubator at 37 ºC for 50 minutes. Following incubation, the tubes were spun for 1 minute at 16.1 RCF (relative centrifugal force) in a centrifuge. Approximately 900 µL of the supernatant was decanted and the pellets were resuspended in the remaining supernatant. The resuspended solution was pipetted onto LB plates treated with KAN to ensure that only bacteria with the plasmid would survive. The plates were incubated at 37 ºC overnight to allow for growth. *E. coli* was then picked from the plates and grown up in a shaker set to 37 ºC in 5mL of LB and 5 µL of KAN to ensure the plasmid was taken up.

Miniprep Protocol

This solution was then miniprepped according to ZymoPURE Plasmid miniprep kit protocol to extract the pRB1017 plasmid. Two changes were made to this protocol: in the final elution step, 35 µL of buffer was used, rather than 25 µL and the 35 µL was run through the filter twice to increase the DNA in the final elution product.

BSA1 Cutting and Separation

Following the miniprep, the plasmid was cut with the *BSA1* enzyme. 18 µL of the miniprep, 4 µL of New England BioLabs (NEB) 10X rCutSmart buffer, 3 µL of NEB BsaI-HF v2 enzyme, and 15 µL water were incubated in a water bath at 37ºC overnight. To separate the cut plasmid from the uncut plasmid, the incubated solution was run on a 1% agarose gel, along with a NEB 1kb plus DNA ladder (this ladder was used in all other gels as a reference). The bands for the cut

plasmid were excised and purified according to ZymoClean Gel Purification kit protocol. One change was made to this protocol: 35 µL of elution buffer was used in the final step. Following purification, the vector was ligated with all of the purified plasmid (~35 µL), 4 µL NEB 10X T4 DNA ligase buffer, and 2 µL NEB T4 DNA ligase overnight at 15 ºC in an incubator. This allows for the separation of plasmid that was cut only once from plasmid that was cut twice. Plasmids that are cut only once can ligate to itself, giving a false positive for gRNA insertions after transformation. Therefore, the ligation was again run on a 1% agarose gel. The bands for the twice-cut plasmid were excised and purified. The ligated once-cut plasmids were discarded. At this point, the plasmid is ready for the insertion of designed gRNA.

Insertion of gRNA

Following the preparation of the pRB1017 plasmid and the gRNA, the insertion is ready to be performed. The first step in the insertion is annealing, followed by a ligation, and finally a transformation.

Annealing

For each reaction, 25 µL of water, 10 µL of oligo 1 (forward gRNA), 10 µL of oligo 2 (complimentary reverse gRNA), and 5 µL of NEB restriction enzyme buffer 3 were added to PCR tubes. The solution was pipetted up and down to mix, and then added to the thermocycler. The thermocycler began at 95 ºC for 2 minutes before dropping the temperature slowly to 20 ºC over the course of 20 minutes.

Ligation

The ligation reaction consisted of 14 µL of water, 2 µL of NEB 10X T4 DNA ligase buffer, 2 µL of pRB1017 *BSA1* digest plasmid, 1 µL of annealed oligos (diluted 1:10), and 1 µL

of NEB T4 DNA ligase. A control was also made by replacing the 1 µL of annealed oligos with 1 µL of water. The solutions were mixed by pipetting up and down and then incubated at 15 ºC overnight.

Transformation

The transformation follows the same protocol as seen in *Transformation Protocol* section, with the exchange of 5 µL of the pRB1017 plasmid being replaced with 5 µL of the annealing product. Following the transformation, the colonies were picked and grown in a shaker at 37 ºC overnight and miniprepped.

Sequencing

4 µL of miniprep and 6 µL of primer USA20 were placed in a tube and sent Eurofins for Sanger sequencing. The returned sequence was analyzed via multiple alignment on CLUSTLW, comparing the pRB1017 plasmid sequence to the transformation product to ensure the insertion of the gRNA.

Designing Homology Arms

Homology arms were designed for each gene of interest. For C10E2.6, 500-700 base pairs before and after the stop codon were used to create the four homology arm primers. For *pig-1* 500-700 base pairs before the start codon were used to create the four homology arm primers. The sequences can be seen below in *Table 2.*

Table 2: Homology Arm Primers		
Homology Arm Primer Name	Homology Arm Primer Sequence	Homology Arm Primer Information
42. 5’ FW C10E2.6 HA	acgttgtaaaacgacggccagtcgccggcaGTTTTACAGATCTGCGGACTTCTC	Forward primer for generating 5' Homology arm 527 bp upstream of C10E2.6 stop codon, to be used with primer 43 to insert gRNA mutation, Tm = 70
43. 5’ RV C10E2.6 HA	catcgatgctcctgaggctcccgatgctccCTTTTTGATAGCCAGGGCtTTGGCCTTG	Reverse primer for generating 5' Homology arm 527 bp upstream of C10E2.6 stop codon, to be used with primer 42 to insert gRNA mutation, Tm = 80
44. 3’ FW C10E2.6 HA	cgtgattacaaggatgacgatgacaagagaTAGATGCTCCTGTCtCTGATGATC	Forward primer for generating 5' Homology arm 675 bp downstream of C10E2.6 stop codon, to be used with primer 45 to insert gRNA mutation, Tm = 72
45. 3’ RV C10E2.6 HA	ggaaacagctatgaccatgttatcgatttcCGTTTTGCTTTTGTGGTACTTCAG	Reverse primer for generating 5' Homology arm 675 bp downstream of C10E2.6 stop codon, to be used with primer 44 to insert gRNA mutation, Tm = 68
50. 5’ FW *pig-1* HA	acgttgtaaaacgacggccagtcgccggcaGTTGCGTGTCGCGTCGCGGC	Forward primer for generating 5' Homology arm 596 bp upstream of pig-1 d start codon, to be used with primer 53 to insert gRNA mutation, Tm = 68

51. 5’ RV *pig-1* HA	tccagtgaacaattcttctcctttactcatGCTGAAATTAAAAAA AAAATTTTACATGA	Reverse primer for generating 5' Homology arm 596 bp upstream of pig-1 d start codon, to be used with primer 52 to insert gRNA mutation, Tm = 68
54. 3’ FW *pig-1* HA	cgtgattacaaggatgacgatgacaagagaATGAGCAAGTATG AAGTGCTCCAGGGcTTTTACG	Reverse primer for generating 5' Homology arm 609 bp downstream of pig-1 d start codon, to be used with primer 55 to insert gRNA mutation, Tm = 70
114. 3’ RV *pig-1* HA	tccagtgaacaattcttctcctttactcatGCTGAAATTAAAAAA AAAATTTTACATGAAAAATCTGG	Reverse primer for generating 5' Homology arm 596 bp upstream of pig-1 d start codon, to be used with primer 52 to insert gRNA mutation, 9 nt were added because the original primer 51 was mis-priming Tm = 92

In *Table 2*, the lower-case letters represent the sequence that overlaps with the enzyme digested pDD282 plasmid, the uppercase is the designed primer to amplify the homology arm, and the highlighted is a single base mutation to prevent repeat cutting by the Cas9. The 5’ reverse primer and the 3’ forward primer were restricted in choice. For C10E2.6, the 5’ reverse primer had to be the last 20-25 base pairs before the stop codon, while the 3’ forward had to be first 20-25 following the stop. For *pig-1*, the 5’ reverse primer had to be the last 20-25 base pairs before the start codon, while the 3’ forward primer had to be the first 20-25 base pairs following the start. These restrictions are because of the insertion site for the GFP. For C10E2.6, the desired location for the GFP to insert is before the stop codon. For *pig-1*, the desired location for the insertion is after the start codon. The other two primers (5’ forward and 3’ reverse) were chosen through GC%. A score was calculated for each using T_m = (2*#AT)+(4*#GC). This score had to be in the 68-74 range for the primer to be chosen. The scores can be seen in *Table 2*. To prevent repeat cutting by Cas9, a single base silent mutation was introduced into the homology

arms of C10E2.6 in segment of overlap with the gRNA, seen in highlighted, lower case in *Table 2.* This mutation was made at the PAM site of the gRNA, so that Cas9 would no longer be able to recognize this region and cut after the genome is repaired. However, in the *pig-1* 5' homology arm, the homology primers did not overlap with the gRNA, so two additional primers were designed to insert the mutation to prevent repeat cutting. All these primers were ordered through Eurofins and rehydrated to 100uM. After rehydration, the primers were diluted to 10uM in a separate tube and a PCR reaction was performed.

PCR (Polymerase Chain Reaction)

Table 3: APEX Protocol	
Step Number	Machine Instructions
1	95°C for 15 minutes
2	95°C for 30 seconds
3	55°C for 30 seconds
4	72°C for 1 minute
5	Return to Step 2 (x34)
6	72°C for 5 minutes
7	10°C for forever
8	End

One-step PCR

For the 5’ C10E2.6, 3’ C10E2.6, 3’ *pig-1* homology arms, traditional PCR (polymerase chain reaction) was used to amplify the product. The APEX PCR protocol was used (see *Table 3)*. A master mix of 12.5 µL APEX 2X Hot Start Master Mix BLUE, 1 µL of DNA template (N2 genomic DNA), and 9 µL of water per reaction was made (i.e. two reactions would double the master mix quantities). 22.5 µL of the master mix was then added to a PCR tube, followed by 1.25 µL of primer 1 and 1.25 µL of primer 2 (in this experiment, this is the forward and reverse primers for each homology arm – 5’ forward and 5’ reverse). To account for mutations during PCR, two separate reactions were run in the PCR machine for each reaction. After PCR, the reaction was run through a 1% agarose gel, excised, and purified. The final product should be the completed homology arm, seen below in *Figure 10.*

Figure 10. Designed Homology Arms. This image shows the entirety of the 5’ and 3’ C10E2.6, as well as the 3’ *pig-*

1 homology arm. The capital letters represent the homology arm primers. The yellow highlight represents the gRNA sequence. The pink highlight represents the single base mutation in the -NGG region to prevent repeat cutting.

Two-step PCR

The 5' *pig-1* homology arm did not have overlap with the gRNA region, so an additional round of PCR was performed. The first round followed the APEX protocol seen in *One-Step* section, with the exception of what primers were used. For the first round of two step PCR, the 5' forward *pig-1* homology arm was used with gRNA mutation #53 (see *Table* 2) as the two primers and the 5' reverse *pig-1* homology arm were used with gRNA mutation #52 (see *Table* 2) as the two primers. These PCR products were run through a 1% agarose gel, excised, and purified. In the second round of two step PCR, two main changes were made. The first was the purified product from the first round was used as the template in place of the genomic DNA. To account for concentration differences, the two products were run on a 1% agarose gel side-by-side. Based on band brightness, the products were added in ratio to ensure the concentrations of the two products would be similar. The second was the primers were the outermost primers of the homology arm (5' forward and 5' reverse primers). The second round PCR product was run on a 1% agarose gel, excised, and purified. This led to a final product of the 5' *pig-1* homology arm with the desired mutations in the gRNA region, as seen in *Figure 11*.

5’ *pig-1* –

GTTGCGTGTCGCGTCGCGGCtcgattttaaatagttgtaatactaaatgtatttgtccgtgtggagtacacgactttgtccggcgggcgactgtcaatgaagcgcg
aaaaattcaatgaggaagacacgcgcaacactattatattgtggtttgaaaatttctataactatgccttttcaaaataaatattttcattcgcgtcctgacacgagctaaaa
tttgcagctacagcactcggcgagtacggtagtctctatttttttgcattccctgtttttcgcttacgacgccttttttctttattgaaaaaaaaaacgaagtttcatgaacaatttt
accatttaaatatttaaatatgttttgtctttaaagaattgtatttcttcccaatccgaaaaagcgatctaacgtgattgcgagagactgtggcaaaacgcGAGACGCAG
ACaGATGCAAGCGCGCcctactgctaattgcgcgaaataacctttattcaaaatctgaacgcatttaaattcgtctcccaaacttttttcgctttgattttatcgatatt
ttccagattttTCATGTAAAATTTTTTTTTTAATTTCAGC

Figure 11. Designed Homology Arms with Two Step PCR. This image shows the entirety of the 5’ *pig-1* homology arm. The capital letters represent the homology arm primers. The yellow highlight represents the gRNA primer. The pink highlight represents the single base mutation to prevent repeat cutting. The bold, underlined, and capitalized represents the gRNA mutation primers that were used in two step PCR.

Gibson Assembly

Gibson Assembly is the insertion of the homology arms into the vector pDD282. To achieve this, 0.5 µL of pDD282, 3 µL of 2X Hifi DNA assembly mix which contains the enzymes, and 2.5 µL total of the two homology arms in ratio so that concentration is equal. To determine the ratio, complementary homology arms were run side-by-side in a 1% agarose gel. This mix was run in a PCR machine at 50 ºC for a minimum of three hours to allow for four linear pieces of DNA to make a singular circular plasmid. The Gibson assembly mix was then transformed on a plate with ampicillin resistance according to previous transformation protocol, with one exception. The plates were incubated at 30 ºC instead of 37 ºC. This is because the pDD282 plasmid grows at 30 ºC rather than 37 ºC because the pDD282 plasmid has a sequence that is sensitive to higher temperatures.

Gibson PCR

Table 4. USA Primers	
Primer Name	Primer Sequence
USA20 (binds in vector only)	tgtaaaacgacggccagt
USA21 (binds in vector only)	caggaaacagctatgaccatg
USA22 (binds in GFP)	cgctcttggacgtatccctctg
USA51	tccaaagttttatcaaggccata
USA54	caaaaattcggaaactaaacttcaa

Colony PCR

After colonies were observed, they were picked and placed in a PCR tube along with 50 µL of fresh water to test for insertion of homology arms. 5 µL was then run through a PCR reaction consisting of 1.25 µL of each primer (see *Table 5*), 12.5 µL of APEX 2X Hot Start Master Mix BLUE, 5 µL of water, and 5 µL of the aforementioned colony + water solution. Each colony had two reaction tubes – a 5' and a 3' to verify both homology arms.

Table 5: Gibson PCR Primers	
Gene Name	Primers Used
5' C10E2.6	43 (see *Table 2*) + USA20 (see *Table 4)*
3' C10E2.6	44 (see *Table 2*) + USA21 (see *Table 4*)
5' *pig-1*	114 (see *Table 2*) + USA20 (see *Table 4*)
3' *pig-1*	54 (see *Table 2*) + USA21 (see *Table 4*)

For each reaction tube, one primer is in the vector (the USA primers) and the other is in the homology arms (the *Table 2* primers). This ensures that a product will only be made if there is a correct insertion of the homology arm into the plasmid. The reactions were run in PCR machine under the APEX protocol (see *Table 3*). After PCR protocol was complete, the product was run on a 1% agarose gel. The desired result is a band for the 5' homology arm and a band for the 3' homology arm from a single colony. This ensures that the chosen colonies have both the 5' and the 3' homology arms inserted into the plasmid. If the colony produced a band for both the 5' and the 3' ends, 40uL of the water + colony solution was added to 5mL of LB and 5 µL of ampicillin, as this indicates that Gibson Assembly was successful. This was grown up in a shaker overnight at 30°C.

Gibson Analysis

The colonies with a band for both the 5' and 3' were sent for sequencing to Eurofins in two rounds. The first round was sent with primer USA20 for the 5' and USA21 for 3' to check for mutations in the homology arms and to determine if they were correctly inserted. The second round was sent with different primers for each gene. For *pig-1*, primer USA51 was used for the 5' and USA54 was used for the 3'. For C10E2.6, primer USA22 was used for the 5' and 44 (see *Table 2*) for 3'. The second round was an extra check to ensure the homology arms had either no mutations, only silent mutations, or mutations in non-coding regions that are not highly conserved.

Injection Mix

To create the injection mix, the concentrations of each plasmid were determined by placing 2 µL of each plasmid seen in *Table 6* and *Table 7* in a spectrophotometer, in this case, a

Nanovue. $C_1V_1=C_2V_2$ was then used to calculate the amount of each plasmid (and water) that would be needed for the final volume to equal 20 µL. The results of these calculations can be seen in *Table 6* and *Table 7.*

Table 6: Volumes of Plasmid for Injection Mix (no *him-8* [*pig-1])*			
Plasmid	Concentration of plasmid stock (ng/µL)	Concentration of plasmid needed in injection mix (ng/µL)	Volume for injection mix (µL)
pDD162 (Cas9)	545.5	50	1.833
pRB1017 – gRNA – 5’	163	25	3.067
pRB1017 – gRNA – 3’	217.5	25	2.299
pDD282 - HA	99	10	2.020
str-1 : GFP	232	25	2.155
Water	-	-	6.606

Table 6. Volumes of Plasmids for Injection Mix (no him-8). In the above table, the pDD162 plasmid provides the sequence for the Cas9 protein to allow for cutting of the genome. The pRB1017 gRNA for 5’ and 3’ provides the guide sequences to indicate where the Cas9 should cut. The pDD282 plasmid provides the repair sequence with the homology arms. *str-1* is a reporter gene that indicates the presence of an amosomal array.

For *pig-1*, we did not use the *him-8* protocol, as the gene product was significantly upregulated in gonad compared the whole animal for both the hermaphrodite and the male, and the *him-8* protocol had not yet been tested. The injection mix was made according to *Table 6* and placed in a 1.7mL tube for storage. *str-1,* which has not been previously mentioned, is a reporter

gene that indicates the presence of an extrachromosomal array by causing two neurons in the head to glow. Extrachromosomal arrays are primers of DNA in the nucleus of a worm that consist of many copies of the DNA that can spontaneously form during injection (Yochem & Herman, 2003). The extrachromosomal arrays are not incorporated into the genome, so the presence of an extrachromosomal array after multiple generations of *C. elegans* means that CRISPR edits did not occur. If the injection was performed successfully, but the edits were not made in the genome by CRISPR, the worms will roll but *str-1* will be expressed. If *str-1* is not expressed, but the worms are rolling, that indicates that the CRISPR edits took place and the gene for rolling is incorporated in the genome.

Table 7: Volumes of Plasmid for Injection Mix (*him-8* [C10E2.6])

Plasmid	Concentration of plasmid stock (ng/µL)	Concentration of plasmid needed in injection mix (ng/ µL)	Volume for injection mix (µL)
pDD162 (Cas9)	545.5	50	1.833
pRB1017 – gRNA 5’	341	25	1.466
pRB1017 – gRNA -3’	340	25	1.471
pDD282-HA	78	10	2.564
pRB1017-gRNA *him-8*	206	25	2.427
him-8 ssOND	100 µM	500 nM	0.1 (1.0 with 1:10 dilution)
str-1::gfp	232	25	2.155

water	-	-	6.784

For C10E2.6, *him-8* protocol was used for the injection mix. This is because the gene is upregulated specifically in the male gonad compared to the whole animal, and not in the hermaphrodite. The mix was made according to the volumes in *Table 7* and placed in a 1.7mL tube for storage.

Injection

Needle Preparation

To prepare for injection, the injection mix was run through an additional filter and spun at 16.1 RCF in a centrifuge for one minute. This was to remove any remaining solid impurities that may clog the needle. The injection mix was then taken up in small tube by capillary action. The mix was then transferred via mouth pipette to a needle for injection. All air bubbles were allowed to filter out. The needle with the injection mix was loaded to the injection apparatus.

Worm Preparation

Hermaphrodite worms were allowed to grow to adulthood prior to injection. The worms were then immobilized in heavy mineral oil on a cover slip on an inverted compound microscope and placed under a scope to allow for visualization during the injection.

Injection of Worms

The hermaphrodite worms were injected with the injection mix in the immature germ cells of the distal arm of the gonad. These cells are referred to as a syncytium, which means the germ cell nuclei share a common cytoplasm, allowing for the uptake of DNA in multiple

progenies with a single injection. This allows for the progeny to take up the injection mix and transcribe and translate the DNA in the worms to express the gRNA, Cas9, and homologous repair template, and, ultimately, be edited by CRISPR. After injection, the worms were transferred to new plates, each with two animals. The plates were incubated at 22-23°C for four days to allow for the worms to lay eggs without selection.

Observation of Worms

Treatment with Hygromycin

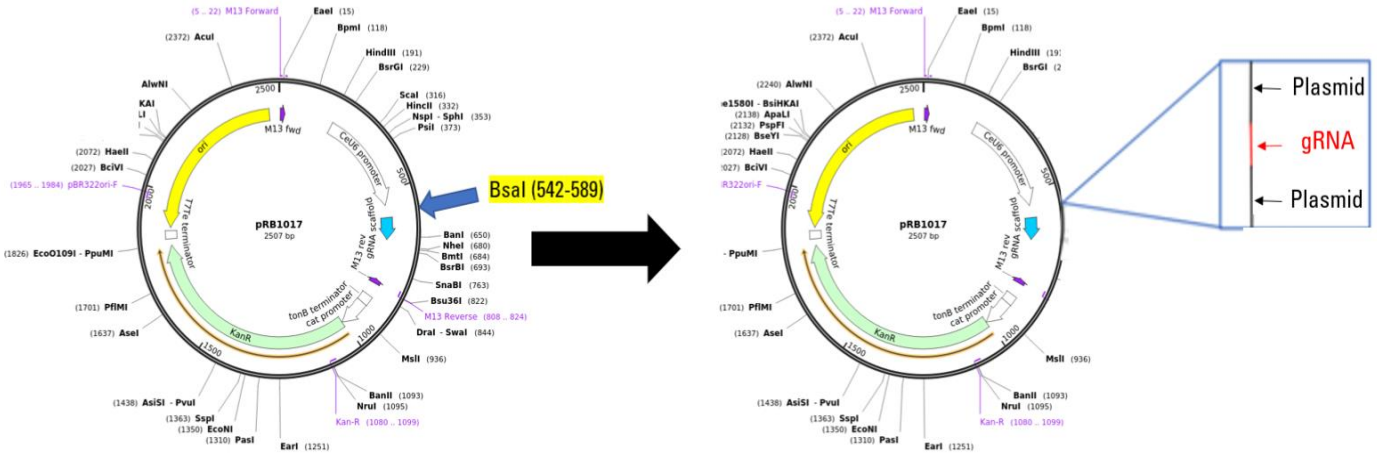
50mg/mL hygromycin was diluted to 5 mg/mL in water. 500 µL of the diluted hygromycin was then added to each worm plate and swirled to ensure the plate was coated with the drug. The liquid was allowed to dry before placing the plates back at 22-23°C. This allows for selection of worms that have the injected DNA, as the SEC confers a resistance to hygromycin. The worms were then observed for the presence of rolling worms.

Results

gRNA and Homology Arm Insertion

The designed DNA products this research created were successfully inserted into the pRB1017 plasmid and the pDD282 plasmid as confirmed by DNA alignment analysis through CLUSTLW. All designed gRNAs were inserted into pRB1017 between the *BSA1* restriction sites. The C-terminus HDR templates for *pig-1* were inserted into pDD282 between the ClaI and SpeI restriction sites. The N-terminus HDR template for C10E2.6 was inserted between the AvrII and SpeI restriction sites. *Figure 11* shows the insertion of these products within the plasmid, as well as the location of the restriction sites.

A.



B.

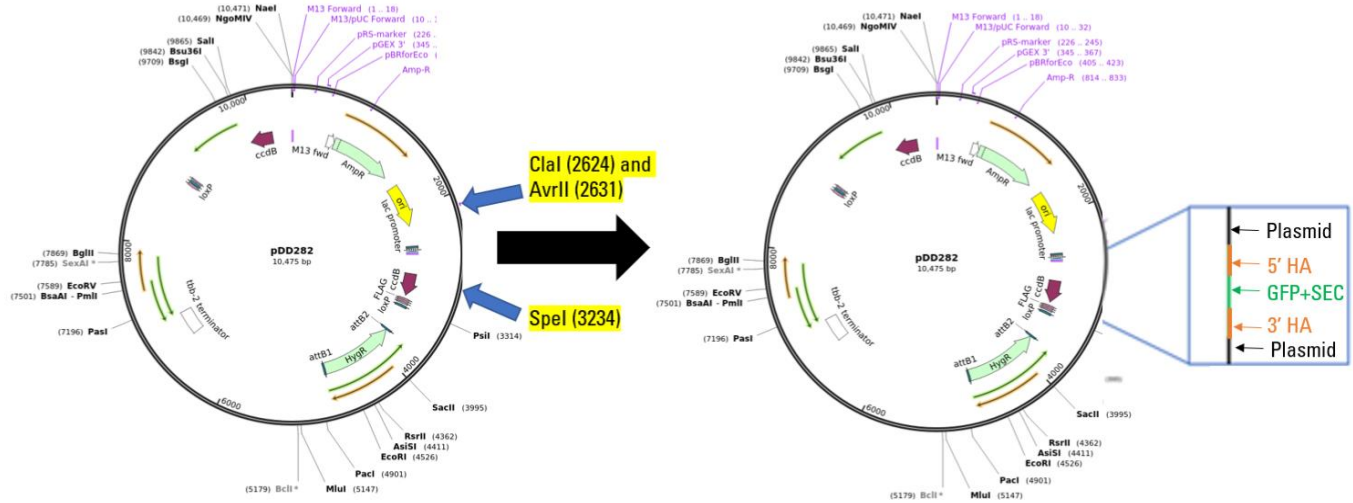


Figure 11: Images adapted from Addgene for plasmids pRB1017 and pDD282. Restriction sites used for this research were added to the images as well as a summary depiction of the gRNA and HDR. templates inserted into their respective plasmids. Image A represents the gRNA insertion plasmid. Image B represents the HA insertion plasmid

These successfully designed constructs were used in the creation of an injection mix, which was successfully injected into *C. elegans.*

Injected Worm Analysis

For C10E2.6, 30 worms were successfully injected with the injection mix and treated with hygromycin. After the injected worms were allowed to grow and reproduce, rollers (rolling worms) were observed on one plate. Twelve of these rolling worms were isolated and grown on individual plates to ensure that the progeny would be from the same parent. The worms were then analyzed and categorized into four categories: homozygous, heterozygous, extrachromosomal arrays, or no rollers. These categories were determined based on the percentages of progeny that were rolling. If approximately 100% of the progeny are rolling, the parent is likely homozygous for the CRISPR edits. If approximately 75% of the progeny are rolling, the parent is likely heterozygous for the CRISPR edits. See *Figure 12* for a Punnett Square representation of possible progeny.

Homozygous Parent

	X	X
X	XX	XX
X	XX	XX

Heterozygous Parent

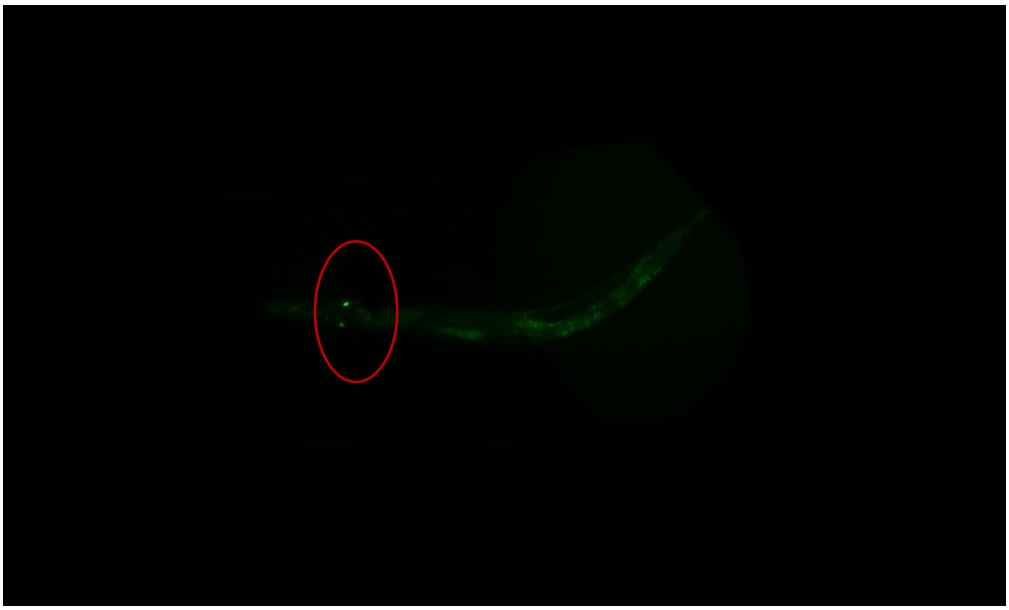
	X	x
X	XX	Xx
x	Xx	xx

Figure 12. Punnett Square of Possible Progeny Percentages. In the above Punnett Squares, X represents the *sqt-1* gene to express rolling behavior. x represents non-rolling expression. Rolling is dominant, so any organism is at least one X will roll.

If approximately 30% or less were rolling, the parent worm was likely rolling as a result of the extrachromosomal array and the CRISPR edit did not occur. Finally, if the progeny had no rollers, a non-rolling worm was likely picked as a mistake. For C10E2.6, there were no homozygous, heterozygous, or non-rolling plates. All plates appeared to have about 30% or fewer rolling progeny. Additionally, plates generated multiple males. This indicates that *him-8*

was successfully mutated via CRISPR. However, not all males were rollers. To confirm that none of the worms had CRISPR edits, several of the rolling worms were checked for *str-1*. All the worms expressed *str-1*, as seen in *Figure 13.*

A.



B.

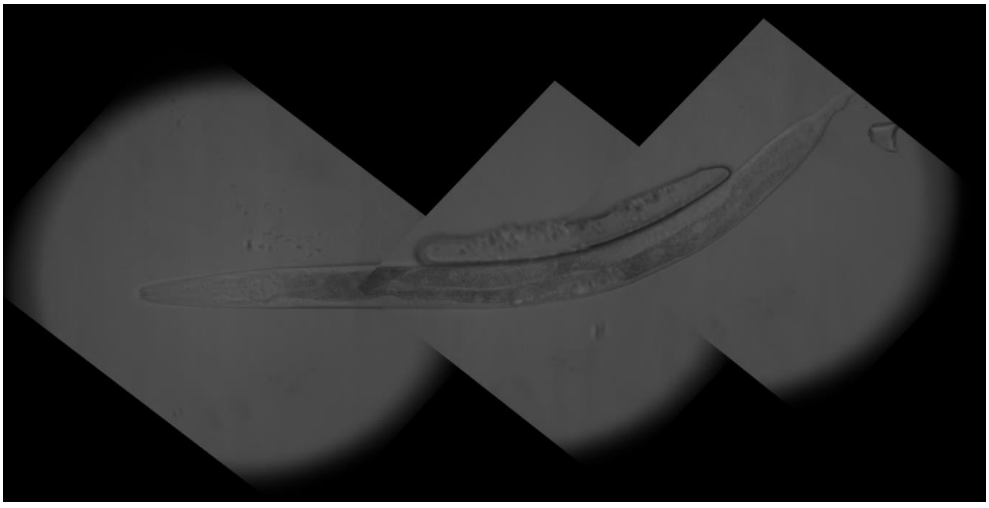
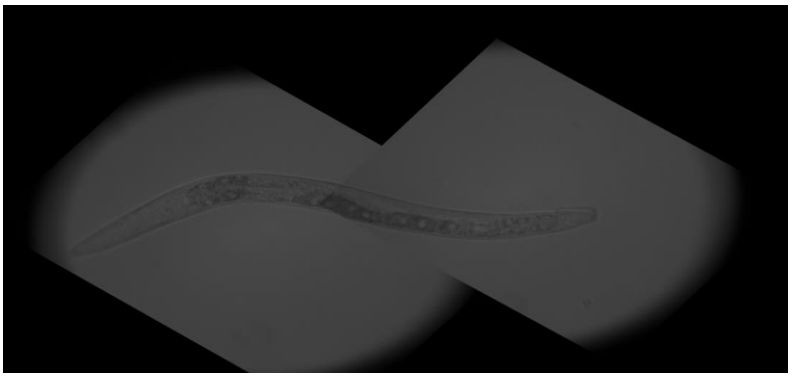


Figure 13. str-1 worm. Image A. is a compilation of three images to construct the entire worm. Circled in red are the two *str-1* glowing neurons. Image B. is also a compilation of three images to construct the same worm seen in image A, without the light to excite the GFP. At the top of the worm, there is an air bubble which can be disregarded.

For *pig-1*, 60 worms were successfully injected with the injection mix, in two batches of 30. For the first batch, one plate was found to have rollers. Twelve rollers were again picked,

sorted, and analyzed for *str-1*. All picked worms were found to have the *str-1* GFP expression, as seen in C10E2.6. For the second batch, three plates were found to have rollers, so were labelled A, B, and C. Twelve worms were picked from each plate. For the B and C worms, all were sorted into the extrachromosomal array category. They were analyzed and confirmed to have the *str-1* GFP expression. For the A plates, four were found to have 100% rolling progeny. Several worms from the 100% rolling progeny plates were then analyzed for GFP expression. None of the worms were found to express the *str-1* GFP. More importantly, all of the worms in the larval stages were found to have GFP expression in the gonad, as seen in *Figure 14.*

A.



B.

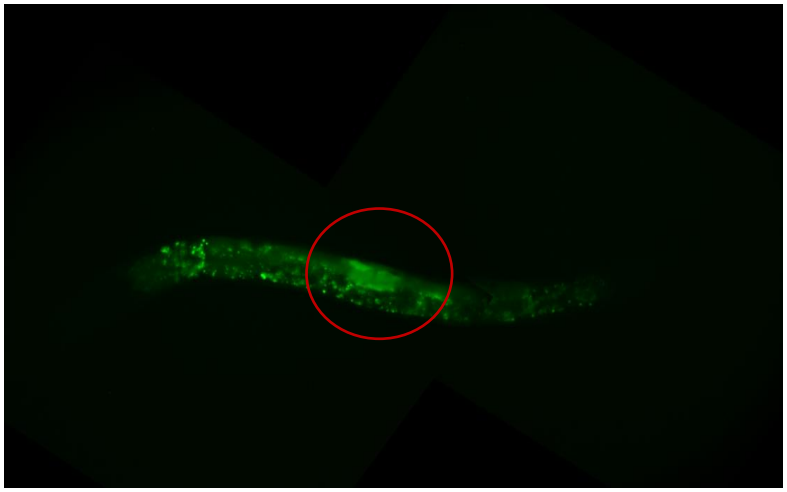


Figure 14. CRISPR worm. Image A. is a compilation of two images to construct the entire worm. Circled in red is the gonad, where GFP can be seen, indicating successful CRISPR edits. Image B. is also a compilation of two images to construct the same worm seen in image A, without the light to excite the GFP.

In *Figure 14*, *str-1* is not seen in the head of the worm. Interestingly, GFP was only found to be expressed in the gonad in the larval stages of the *pig-1* worms. In the adult worms that were analyzed, GFP was no longer expressed in the gonad.

Discussion

Results Interpretation

This research's goal was to fuse GFP to the two genes of interest for visualization. The success of achieving that goal for the two genes of interest can be determined by the analysis of the worm progeny for the *pig-1* and C10E2.6.

C10E2.6

Out of the 30 C10E2.6 injected worms, the single plate with rollers was found to have GFP expression in *str-1*. This indicates the presence of an extrachromosomal array, meaning the rollers were false rollers. The worms are able to roll as a result of the *sqt-1* gene being present in the extrachromosomal array, but the expression of *str-1* indicates that the DNA was not taken up into the genome. Although C10E2.6 was not successful for the CRISPR edits for the gene of interest, the presence of multiple males indicates that CRISPR did occur for the *him-8* gene. This is interesting because the two CRISPR edits (for the GFP fusing to the gene of interest and the *him-8* mutation) were both in the same injection mix and could occur simultaneously. However, because only one plate was found with rollers, *him-8* was perhaps easier to edit than C10E2.6 likely occurred due to chance.

With the success of the *him-8* mutation in this research, future injections in the Kroetz lab, as well as the Genetics class (BIOL-240) at Bellarmine University will use the *him-8* CRISPR method to increase the incidence of males. This will allow for much more efficient

studies and experiments with the male sex of *C. elegans* as a direct result of this research. Additionally, all the necessary constructs for CRISPR have been designed and generated in this research. These constructs will be used by future BIOL-240 students to attempt CRISPR edits. While this research did not yield results for C10E2.6 edits in the genome, it is very likely that results will be found in the near future, as a result of this study.

pig-1

Out of the 60 injected *pig-1* worms, the C plate with rollers was found to be the only one with 100% rolling progeny, no *str-1*, and GFP in the gonad. This is highly indicative of a successful CRISPR edit for the genes of interest. This means that GFP was likely fused to the gene within the worm's genome. The absence of GFP expression in the adult worms gonad is also notable, as the GFP was seen in the earlier larval stages. This could mean that *pig-1* is no longer used in the gonad after the worm becomes fully developed. However, more research must be done to confirm this, along with the exact function of the gene within the larval stages of the gonad.

Research Challenges

Throughout the research process, two main challenges were encountered. The first being insertion of the C10E2.6 gRNA into the pRB1017 plasmid. The initial attempts at inserting the gRNA into the plasmid were unsuccessful, so several aspects of the process were tested to problem-shoot. The same plasmid was retested to ensure that the issue was not pipetting or other human error. A plasmid made by a different researcher was tested to determine if the plasmid was the problem. The ratios of DNA in the ligation were also tested, including a 1:3, 1:10, and 1:20 dilution, but it was found that the original 1:10 dilution produced the most colonies when

transformed. The pRB1017 plasmid was also remade once more, to ensure the plasmid was not too old to use, which was also unsuccessful. The remade plasmid was tested with the *pig-1* gRNAs, which were successful on the first round of insertion. They were again successful, indicating that the issue likely lied with the primers. After reordering the primers from Eurofins, the insertion was successful. This indicates that there was likely some impurity or error in the initial dilution of the primers that was causing the issues with insertion.

The other significant issue arose with the *pig-1* 3' RV homology arm. When it was run on gels, it initially gave bands of the incorrect size. It was rerun on a 1% gel several times to ensure that the issue was not human error. After the repetition of this failed to yield different results, the primer was run through a BLAST search to determine if the issue lied with primer. It was found that the primer also primed a different region which gives bands the same size as the incorrect bands that were seen in this experiment. To remedy this, a new primer was ordered with nine additional bases added to the primer to make a more specific match. This new primer yielded the correct band size on the first run.

Next Steps

CRISPR edits were successfully observed in the *pig-1* worms, but not the C10E2.6 worms. However, as only one round of C10E2.6 was injected into 30 worms, while *pig-1* had double that number, it is possible that C10E2.6 would have successful results if more worms were injected. This result may be achieved by future BIOL-240 students. After the successful observation of GFP in the gonad, the CRISPR edits could also be confirmed through PCR and sequencing. The worms could be lysed and run in a PCR machine with primers in the 5' region of the gene and in the GFP. If a product is produced, that indicates that the gene is correctly fused to GFP. Following this confirmation, the PCR product could be sent for sequencing to

ensure the edits occurred correctly. Despite these confirmations not being performed for this research, the presence of GFP in the gonad of multiple worms, as well as the progeny being 100% rollers, is a very strong indication that CRISPR was successful in the *pig-1* worms, ultimately achieving the goal of the research for one of the two genes of interest.

Future Research

Degron System

As previously stated, *pig-1* was successfully edited by CRISPR and fused with GFP. C10E2.6 was not edited in this research, but likely will be by the BIOL-240 students using the designed constructs from this research. The genes of interest were (or will be) tagged with GFP, not only for visualization, but also for degradation in future research. When fused to a protein, GFP allows the visualization of the protein. Now that worms have been successfully edited by CRISPR and GFP is fused to the sequence, after further confirmation, the worms can be crossed with degron worms, which will destroy GFP and the proteins attached to it (the destruction is isolated to the gonad), allowing for study of the function of the genes of interest within the gonad. The degron system allows for the rapid depletion of the gene product, or protein, within specific tissues without removal of the gene itself from the genome. This is necessary, as complete removal, as stated earlier, would lead to the death of the organism. Previously, the degron system has been used to study other developmental events, such as molting, meiosis, spermatogenesis, and organogenesis (Ashley, et. al, 2021) The system could be used to study the organogenesis of the gonads in future research. The specific degron system used will be a GFP-mediated-tissue-specific protein degradation. This system involves the fusion of a GFP nanobody and a ubiquitin ligase. The degron worms, which will be crossed with worms with GFP-fusion protein, contain the degron system, which may be passed on to the progeny.

The progeny is where the effects will be seen. The GFP will act as a tag on each protein produced by the two genes of interest and GFP nanobody will bind to this tag. Therefore, any protein with GFP attached to it, will be targeted by the nanobody and subsequently ubiquitinated by the ubiquitin ligase. Because the degron is only expressed in the gonad, this will allow for the removal of the products within the gonad (but not the entire organism) for the study of the gene function (Wang, et. al, 2017).

Real World Applications

Due to the similarity seen in the proteome of *C. elegans* and humans, there are many applications of this research to more applicable daily life scenarios. As mentioned in the introduction, both C10E2.6 and *pig-1* both have human orthologs which play a role in human diseases. The disease linked to C10E2.6, exercise induced hyperinsulinemic hypoglycemia, is characterized by a spike in insulin and a drop in blood sugar levels. This is particularly worrying, as insulin is a hormone that decreases blood sugar levels. The spike in insulin will further decrease the already rapidly dropping blood sugar levels, leading to fainting, shakiness, dizziness, and more (Alexander, 2022). Because *C. elegans* is an ideal model organism, especially considering the proteome similarity, further study of the function of C10E2.6 could help to better the understanding of this disease. Similarly, *pig-1* also has a human ortholog related to MELK, which is an oncogenic kinase that plays a role in metastasis of lung cancers (Tang *et. al*, 2020). As with C10E2.6, further study of *pig-1* in an organism that closely models humans may lead to developments in the treatment or exact causes of this disease. By better understanding the functions of these genes, the body of knowledge about these orthologs, and their diseases, may also increase.

Additionally, by studying the function of these genes within the gonad, this research and future related projects contribute information about reproductive systems in general. As they are a common site of problems, this is a relevant research project. Infertility affects 50-70 million couples worldwide (Szamatowicz and Szamatowicz, 2020), endometriosis affects 10-15% of women in their reproductive years (Mehedintu, *et al.,* 2014), and testicular cancer is the most common solid tumor in young men (Baird *et al.,* 2018). While there are certainly many factors that play a role in these issues, such as environment or lifestyle, genetics is a key player as well. By studying the process of organogenesis and sexually dimorphic development in *C. elegans* as it is related to genetics, this research may lead to a better understanding of the process of organ formation in different species, possibly leading to developments in human disease and pathology that affect such a significant number of the population.

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