

Characterization of *spe-43* homologs: *K05F1.1* and *F57A8.6*

by

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ABSTRACT

Sexual reproduction relies on the presence of a viable sperm cell, which fuses with an egg cell in the process of fertilization. To increase understanding of the molecular processes involved in sexual reproduction, a reverse genetics method can be utilized to learn the role of specific proteins in fertility. *C. elegans* are an excellent organism to model these processes with, as they reproduce through sexual reproduction. *C. elegans* exhibits two sexes: hermaphrodites, which can self-fertilize, as well as males, which produce sperm to fertilize eggs from the hermaphrodites. Sperm activation is the process through which post-meiotic spermatids are altered to become sperm suitable for fertilization. In *C. elegans*, the process is triggered through two different pathways: TRY-5 and SPE-8. The TRY-5 pathway and the SPE-8 pathway are active in the male reproductive tract, while only the SPE-8 pathway is active in hermaphrodites. Hermaphrodites with mutations in genes required for the SPE-8 pathway experience self-sterility. In contrast, males with non-functional TRY-5 pathway components show no fertility defect unless the SPE-8 pathway is also disabled. *spe-43* is a protein-encoding gene required for sperm activation through the SPE-8 pathway. *spe-43* has three different paralogs: *K05F1.1*, *F57A8.6*, and *T10E9.4*. All three paralogs are protein-encoding genes hypothesized to be involved in sperm activation. To study the role of *K05F1.1* and *F57A8.6* in *C. elegans* fertility, deletion mutants were made to knock out the genes' function. Quantitative experiments, including hermaphrodite self-fertility assays and male fertility assays, display no significant difference in progeny produced by *K05F1.1*(*syb4161*) deletion mutants compared to wild type organisms. Similarly, imaging such as DAPI staining and sperm dissections have shown that loss of *K05F1.1* has no visible impact on sperm production, localization, and sperm morphology. When knocking out *K05F1.1* and *spe-8* together, males were able to produce viable progeny. The result implies that the *K05F1.1* protein is neither part of the TRY-5 pathway, nor the SPE-8 pathway. In contrast, *F57A8.6*(*syb4189*) deletion mutants had significantly fewer progeny produced compared to wild type organisms in quantitative experiments.

Chapter 1

Introduction

1.1 Sexual Reproduction

Sexual reproduction is the process utilized by most animals and many plants to produce offspring. Successful sexual reproduction requires a competent sperm cell to fertilize an egg cell. The fertilized egg cell develops into an embryo. While the basic processes required for sexual reproduction are generally understood, many specifics are still unknown, especially on a molecular level. Having a more developed understanding of the molecular interactions and processes involved in fertility would enable the compilation of a more comprehensive model of sexual reproduction. In researching the molecular processes required for fertility, it is helpful to model the process in an organism which reproduces sexually.

1.2 *C. elegans* as a model organism

Caenorhabditis elegans are small, transparent nematodes often used as models in genetic and developmental biology studies. Adult *C. elegans* are approximately 1 millimeter long and can be maintained on petri dishes seeded with *E. coli*, making maintenance and storage very simple. Moreover, the transparent bodies of *C. elegans* offer advantages, such as allowing for the imaging of internal structures of intact organisms without the need for dissection (Cori et al., 2015).

C. elegans reproduce through sexual reproduction. They exhibit two sexes: hermaphrodites and males. Hermaphrodites produce both sperm and eggs, and can

self-fertilize. In contrast, males produce sperm that outcompetes hermaphrodite sperm (Singson, 2001). The ability to maintain hermaphrodites through self-fertilization is helpful because it allows for maintenance of mutant strains with little extra effort—the progeny produced by a hermaphrodite that is homozygous for a chosen mutation will also be homozygous for said mutation. When dealing with mutations that cause hermaphrodite self-sterility due to sperm defects, the ability to maintain *C. elegans* by crossing hermaphrodites to males overcomes the issue of sterility. Moreover, the ability of male sperm to outcompete hermaphrodite sperm allows for genetic manipulation by enabling the introduction of new mutations into existing strains (Corsi et al., 2015). *C. elegans* is also known to produce a large number of progeny, meaning a large population can arise from the active maintenance of a few worms.

The life cycle of *C. elegans* is very short, with development from egg to adulthood taking approximately 4 days at 20°C. It is divided into six distinct stages: an embryonic stage, four larval stages (1-4), and adulthood. In the L4 stage, hermaphrodites produce sperm, switching to oocytes in adulthood. As such, *C. elegans* hermaphrodites have limited sperm for self-fertilization. In comparison, males continue to produce sperm into adulthood (Altun and Hall, 2009).

1.3 Spermiogenesis

As previously stated, sexual reproduction requires an activated spermatozoa to bind to an oocyte in the process of fertilization. Once fertilized, the egg is then activated, becoming an embryo. Spermiogenesis is the process through which post-meiotic spermatids are altered to become spermatozoa suitable for fertilization (Ma et al., 2012). In *C. elegans* specifically, inactivated spermatids develop a pseudopod for locomotion (Smith, 2014). During spermiogenesis, membranous organelles also fuse

to the plasma membrane of spermatids, allowing for cell signaling and binding to egg cells (Ward, Hogan, and Nelson, 1981).

In *C. elegans*, two pathways trigger spermiogenesis: the TRY-5 and SPE-8 pathways. The TRY-5 pathway and the SPE-8 pathway are active in the male reproductive tract, while only the SPE-8 pathway is active in hermaphrodites. The TRY-5 pathway relies on the male-specific TRY-5 protease, which is hypothesized to be an extracellular activator of spermiogenesis (Smith and Stanfield, 2011; Shakes, 2011). The SPE-8 pathway comprises multiple proteins that facilitate spermiogenesis in hermaphrodites and males (Geldziler et al., 2005; Minniti et al., 1996; Nance et al., 2000; Nane et al., 1999; Muhlrads et al., 2014; Krauchunas et al., 2018).

In hermaphrodite self-fertilization, only the SPE-8 pathway triggers spermiogenesis. Therefore, mutations in genes required for the SPE-8 pathway result in hermaphrodite self-sterility. In contrast, males with non-functional TRY-5 pathway components show no fertility defect unless the SPE-8 pathway is also disabled, due to males' ability to trigger spermiogenesis through either pathway (Smith and Stanfield, 2011). In imaging of the *C. elegans* germline, defects in spermiogenesis are evident, as indicated by the decreased quantity of spermatozoa in the spermatheca.

1.4 *spe-43*

spe-43 is a protein-encoding gene required for spermiogenesis that functions in the SPE-8 pathway. The SPE-43 protein is a transmembrane protein with a DX domain of unknown function. The phenotypes associated with *spe-43* loss of function align with the expectations for organisms that do not undergo spermiogenesis. Knocking out *spe-43* results in sterile hermaphrodite *C. elegans*, but no change in male fertility. Imaging of *spe-43(eb63)* mutant germlines shows that over time,

spermatids are pushed from the spermatheca but are unable to crawl back due to the lack of a functional pseudopod. Inactive *spe-43* spermatids are identical to wild type, but when exposed to an in vitro activator, they do not undergo spermatogenesis and instead form spikes (Krauchunas et al., 2018).

1.4.1 *spe-43* Paralogs

The *spe-43* gene has three predicted paralogs in the *C. elegans* genome: *T10E9.4*, *F57A8.6*, and *K05F1.1*. The paralogs were identified through FlyRNAI classification (FlyRNAI). There is no published data on the function of any of the paralogs. *K05F1.1* and *F57A8.6* are both predicted to encode proteins with DX domains of unknown function, like *spe-43*. Expression of *K05F1.1* measured in fragments per kilobase of transcript per million mapped reads (FPKM) found the highest expression level in the late embryonic stage of *C. elegans* lifecycle. After that, male L4s had the highest expression levels of *K05F1.1*. In contrast, *F57A8.6* showed no expression in the embryonic stage and had much higher expression than *K05F1.1* in the male L4 stage (Wormbase).

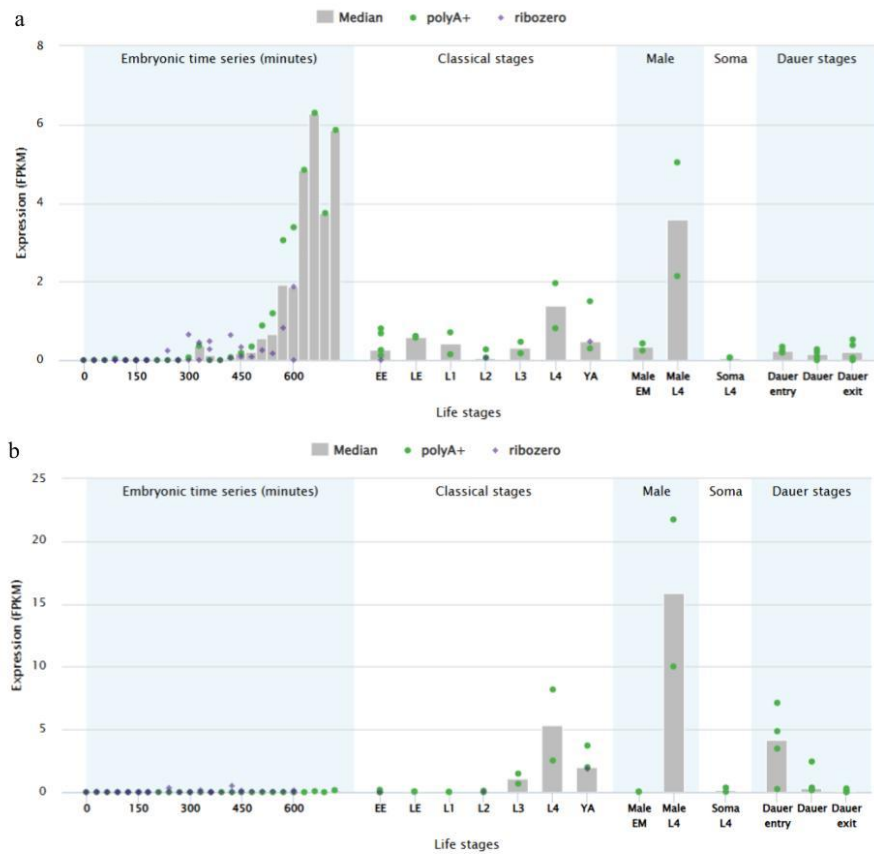


Figure 1.4.1 Expression of *K05F1.1* (a) and *F57A8.6* (b) throughout the *C. elegans* life cycle. Expression is measured in fragments per kilobase of transcript per million mapped reads (FPKM). Taken from Wormbase.

A 2014 study identified mRNAs expressed in spermatogenesis and oogenesis using RNA-Seq. The study identified the expression of *K05F1.1*, *F57A8.6*, and *T10E9.4* during spermatogenesis (Ortiz et al., 2014). A reverse transcription polymerase chain reaction (RT-PCR) performed in our lab delivered similar results. The RT-PCR utilized cDNA from sperm-producing *him-5* mutants and non-sperm-producing *fem-1* mutants. For all three predicted paralogs, the *him-5* mutant showed

expression, while the *fem-1* mutant did not. The result indicates that all three genes are sperm-expressed. The focus of this thesis is *K05F1.1* and *F57A8.6*.

Since *K05F1.1* and *F57A8.6* are expressed in sperm and are predicted paralogs of *spe-43*, which are known to play a role in sperm activation, we hypothesized that *K05F1.1* and *F57A8.6* likewise play a role in the development or function of sperm. Characterizing deletion mutants of both genes enables us to understand if the predicted paralogs play a similar role to *spe-43* in spermiogenesis. Characterization of *K05F1.1* returned mostly negative data. Results imply that deletion of *K05F1.1* does not impact sperm activation in *C. elegans*.

Various *F57A8.6* fertility assays indicate that deletion of *F57A8.6* does impact *C. elegans* fertilization, although its mechanisms remain unclear.

Chapter 2

Materials and Methods

2.1 Strain List

Table 1. Strain list of all *C. elegans* strains used

Strain Name	Genotype
PHX4161	<i>K05F1.1(syb4161)</i>
PHX4189	<i>F57A8.6(syb4189)</i>
PHX4144	<i>T10E9.4(syb4144)</i>
CB1489	<i>him-8(e1489)</i>
BA786	<i>spe-8(hc53)</i>
FX30179	<i>tmC20 [unc-14(tmIs1219) dpy-5(tm9715)]</i>
DG4915	<i>fog-2(oz40); his-72(uge30)[gfp::his-72]</i>
BS553	<i>fog-2(oz40)</i>

2.2 Genetic Crosses

Five *C. elegans* strains were produced using genetic crosses. Parental crosses were set up with 3-5 plates consisting of 4 L4 hermaphrodites and 12-15 young adult males per plate. 3-5 plates consisting of 5 F₁ L4 hermaphrodite offspring were picked from the parent plates to self-fertilize. An exception is the *K05F1.1(syb4161); spe-8(hc53)* cross, which involved picking 5 plates of 4 F₁ L4 hermaphrodites and 12-15 young adult sibling males from the parent plates to produce the F₂ generation. The

second exception was the *K05F1.1(syb4161); spe-8(hc53)/tmC20* cross, in which offspring produced at the parental generation were crossed to *spe-8(hc52)* mutant males in the F₁ generation. Individual L4 hermaphrodites from the F₂ generation were picked to separate wells in 12-well plates. Again, the *K05F1.1(syb4161); spe-8(hc53)* cross involved an altered procedure where a sibling hermaphrodite and male were crossed 1:1 in the 12-well plates.

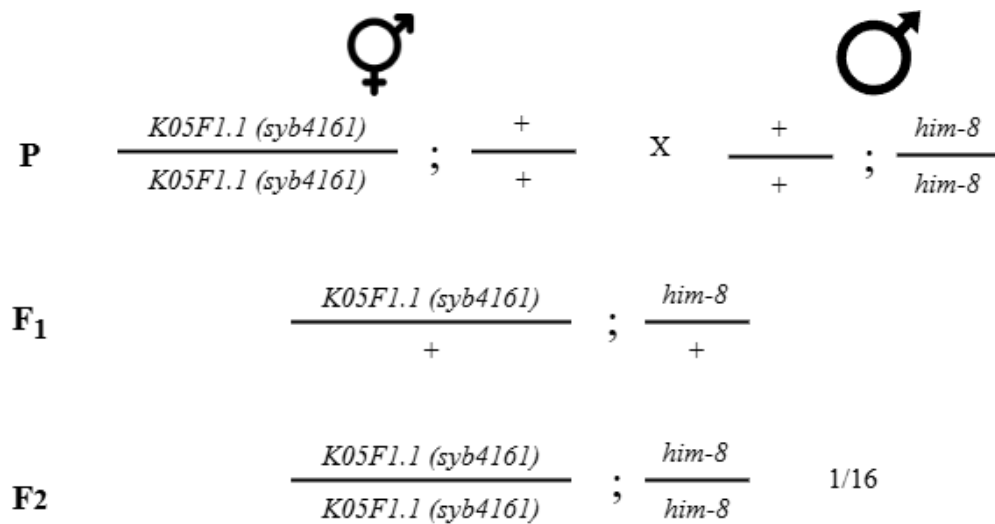


Figure 2.2.1 *K05F1.1(syb4161); him-8(e1489)* Genetic Cross *K05F1.1(syb4161)* hermaphrodites were crossed to *him-8(e1489)* males to produce a homozygous *K05F1.1(syb4161); him-8(e1489)* double mutant. The F₂ generation was a product of F₁ hermaphrodite self-fertilization.

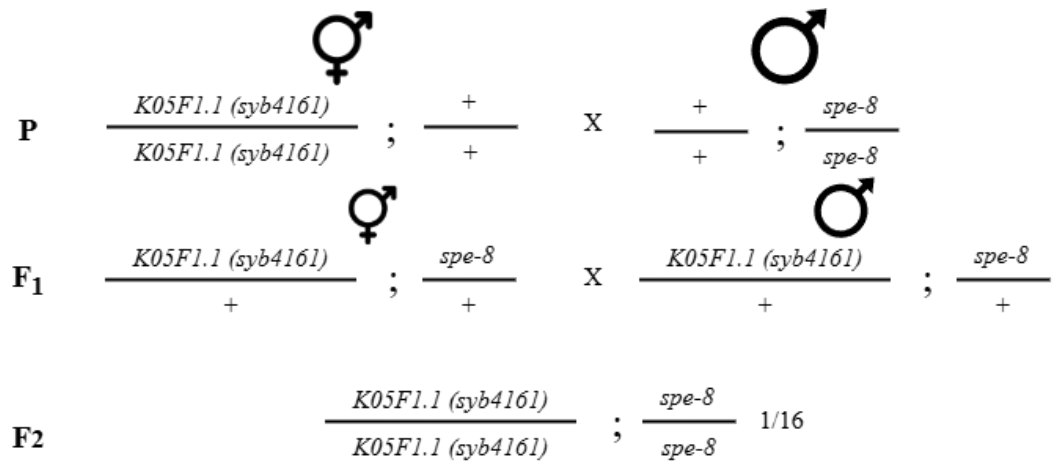


Figure 2.2.4 *K05F1.1(syb4161); spe-8(hc53)* Genetic Cross *K05F1.1(syb4161)* hermaphrodites were crossed to *spe-8(hc53)* mutant males. In the F₁ generation, heterozygous males were crossed to hermaphrodites. In the F₂ generation, males and hermaphrodites from the same plates were crossed in 1:1 crosses.

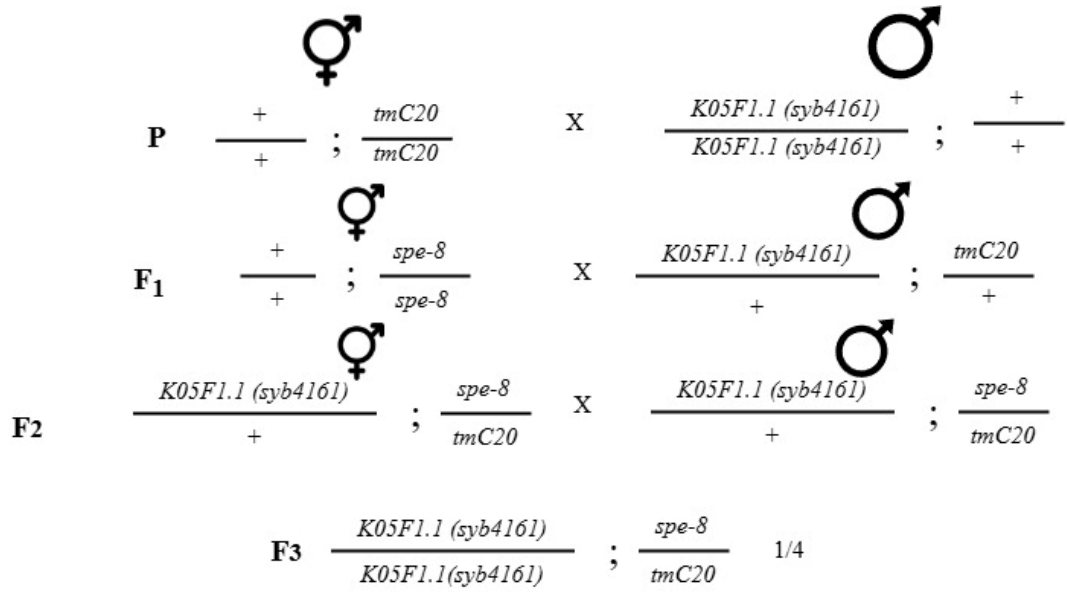


Figure 2.2.5 *K05F1.1(syb4161); spe-8(hc53)/tmC20* genetic cross. In the P generation, *tmC20* hermaphrodites were crossed to *K05F1.1(syb4161)* males. In the F₁ generation, fluorescent offspring were crossed to *spe-8(hc53)* males. In the F₂ generation, sibling organisms were crossed in one-to-one crosses. F₃ organisms were singled out, and L4 hermaphrodites were left to self-fertilize.

Table 2: Primer sequences used

Primer	Annealing Temperature (°C)	Primer Sequence
K05F1.1 Forward	51 (LongAmp)	5' ATG ACC AGC AAG ATG GAA TA 3'
K05F1.1 Reverse	51(LongAmp)	5' CCT ATC ACA CAT ACA TCT CC 3'
T10E9.4 Forward	57(LongAmp)	5' CTGCTAACTGCCTGCGTTAT 3'
T10E9.4 Reverse	57(LongAmp)	5' GGCTTGAGGTCTGGTTCTGA 3'
F57A8.6 Forward	57(LongAmp)	5' TCCGTTTGCGTGGTTTCA 3'
F57A8.6 Reverse	57(LongAmp)	5' GGT GTC TTA CAC TTT CCA CTC C 3'
spe-8 Common Forward	50 (GoTaq)	5' CGCCTTCAAATCCATCTGTG 3'
spe-8 wild type reverse	50 (GoTaq)	5' GCTGCAAATACAGATGTCTTCAGC GTAGGAATGTGCAC 3'
spe-8 mutant reverse	50 (GoTaq)	5' GCTGCAAATACAGATGTCTTCAGC GTAGGAATGTGCAT 3'

To identify *him-8(e1489)* homozygous organisms in the *K05F1.1(syb4161)*; *him-8(e1489)* genetic cross, wells were scored for the presence of F₃ males. Only wells containing males were genotyped for the *K05F1.1(syb4161)* mutation. In crosses with the *F57A8.6(syb4189)* mutation, F₂ wells were screened for the presence of oocytes. Only wells with oocytes were genotyped for *F57A8.6(syb4189)* mutation.

Lysis was completed on organisms of the F₂ generation. Organisms were frozen at -80°C and then incubated in a mixture of worm lysis buffer and proteinase K.

After incubation, the samples were diluted with distilled water and stored at -80°C until required for PCR.

K05F1.1(syb4161), *F57A8.6(syb4189)*, and *T10E9.4(syb4144)* genotyping was completed using LongAmp polymerase. *spe-8(hc53)* genotyping was completed using GoTaq polymerase.

Electrophoresis was performed using 1% agar stained with ethidium bromide. Samples were loaded with 7 µL of a solution consisting of 7 µL of the sample and 1 µL of loading dye. Each gel was run for 45 minutes.

Five vials of each strain were frozen. Before freezing, the plates were chunked, and samples of *C. elegans* strains were incubated at 20 °C until the plates were almost starved. A freezer stock solution was prepared using 3 mL of 1x M9 buffer and 3 mL of freezing solution. 1 mL was pipetted onto each plate, then the entire batch was pipetted into the vial of solution once the worms were detached from the plate. 1 mL of the freezer solution containing worm samples was placed into each cryovial, which was then stored in the -80°C freezer.

To determine the viability of the frozen samples, a vial of the frozen sample was thawed and placed on an agar plate. Once the plate dried, the worms were observed for movement (any visible motion or signs of tracks in *E. coli* lawns).

2.3 Hermaphrodite Self-Fertility Assays

Individual hermaphrodites in the L4 stage were singled out onto seeded agar plates and placed in temperature-controlled incubators. Every 24 hours the hermaphrodite was moved to a new plate and returned to the incubator. After the hermaphrodite was removed, plates were left in the incubators for varying quantities of time depending on the assay temperature: at 16°C, plates were left for 4 days, at

20°C, plates were left for 3 days, and at 25°C, plates were left for 2 days. After incubating for the specified number of days, offspring were counted on each plate. The process continued until the parent hermaphrodite stopped laying viable embryos, bagged, meaning eggs prematurely hatched within the hermaphrodite's germline, or died. During data analysis, all hermaphrodites that died or bagged were omitted from the final dataset.

2.4 Hermaphrodite Cross-Fertility Assays

Individual L4 stage hermaphrodites were singled out onto seeded agar plates and left to grow for 48 hours at 20°C. After 48 hours, organisms were transferred to new seeded plates that contained 4 young adult N2 males. The plates were kept at 20°C for 24 hours to allow mating, after which the males were removed and the hermaphrodites were transferred to new plates. The hermaphrodites were transferred every 24 hours until the end of the assay, when the parent hermaphrodite stopped laying viable embryos. As the experiment was completed at 20°C, progeny was counted three days after the hermaphrodite was picked to a new plate. Organisms that died prematurely or crawled off the plate were omitted from the dataset. However, organisms that bagged were included in the dataset.

2.5 Male Fertility Assays

Individual *fog-2(oz40)* hermaphrodites in the L4 stage were singled out onto seeded agar plates with 4 young adult males and left to mate at 20°C for 24 hours. After those 24 hours, the males were removed from the plate, and the hermaphrodites were transferred to new, seeded plates. This process continued every 24 hours until the end of the assay. As all male fertility assays were completed at 20°C, progeny were

counted three days after the hermaphrodite was removed from the plate. The process continued until the parent hermaphrodite stopped laying viable embryos. During data analysis, worms that died were omitted from the final dataset. However, organisms that bagged were included in the dataset.

2.6 DAPI Staining

Twenty-five to thirty L4 hermaphrodites were picked onto a seeded agar plate and incubated for 24 hours at 20°C. After 24 hours, organisms were fixed using cold methanol and stained with DAPI in Vectashield before being picked onto agar pads on microscope slides. About 8 organisms were picked to each agar pad before a cover slip was added. Slides were stored out of light until imaging. Imaging was completed using a Zeiss Axio Observer microscope with a 20x objective.

2.7 Sperm Imaging

Twenty-five to thirty young adult males were picked to a seeded agar plate with one L4 hermaphrodite and left to grow for 24 hours at 20°C. Males were dissected on Histabond microscope slides with 20 μ L of sperm media or sperm media containing 200 μ g/mL Pronase. Slides treated with Pronase were left in a humidity chamber for about 10 minutes before applying a coverslip and imaging. DIC imaging was completed using a Zeiss Axio Observer microscope with a 40x objective.

Chapter 3

Results

3.1 Loss of *K05F1.1* has no Impact on Fertility

K05F1.1 impact on fertility was measured using brood size assays. To begin characterizing *K05F1.1*, hermaphrodite self-fertility was measured at 16°C, 20°C, and 25°C.

At 16°C and 20°C, there was no significant difference between the number of progeny produced by the N2 controls and the *K05F1.1(syb4161)* mutants. One self-fertility assay, completed at 25°C, resulted in significantly more progeny being produced by the *K05F1.1(syb4161)* deletion mutants. When completing a second replicate at 25°C, there was no significant difference in the number of progeny produced (not pictured). The results imply that deletion of *K05F1.1* does not directly impact hermaphrodite self-fertility.

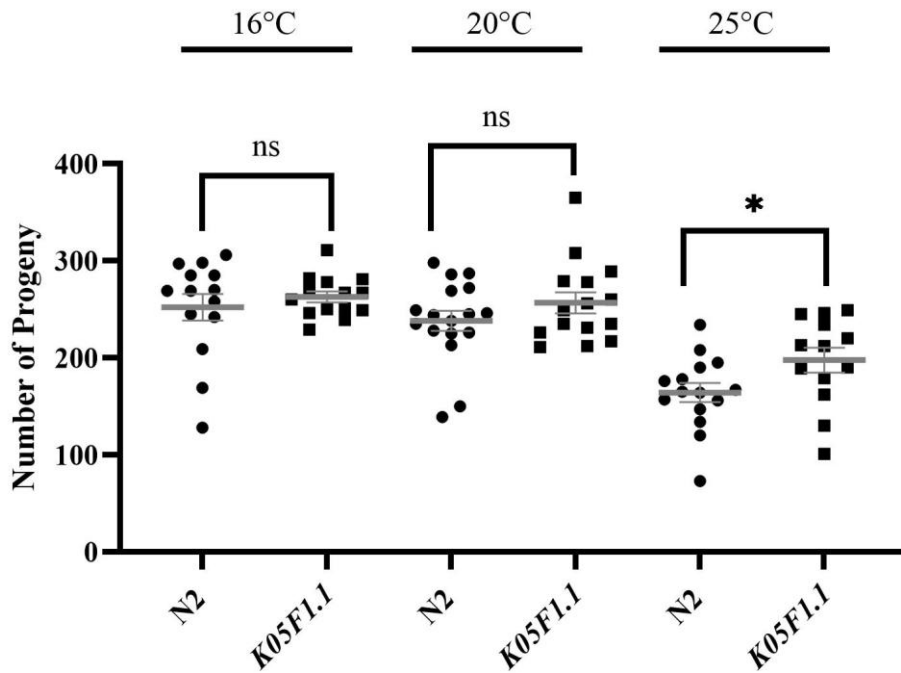


Figure 3.1.1 Hermaphrodite self-fertility assays comparing wild-type and *K05F1.1(syb4161)* mutant *C. elegans* at 16°C, 20°C, and 25°C. Each point indicates the number of progeny produced by a single hermaphrodite. $N \geq 15$

To continue characterization of *K05F1.1(syb4161)* deletion mutants, a *K05F1.1(syb4161); him-8(e1489)* mutant strain was created to test male fertility. The *him-8(e1489)* mutation results in a higher incidence of males in *C. elegans*, enabling the production of male progeny in each generation. Homozygous *him-8(e1489)* mutants can be identified by their ability to produce male offspring without mating with males. Plates were phenotypically screened for the presence of *him-8(e1489)* by searching for males in each well. Only the worms that produced males were genotyped for *K05F1.1*.

The wild-type allele of *K05F1.1* is expected to produce a 1765 base pair PCR product, while the mutant allele would produce a 698 base pair product. Of the samples, samples 5, 9, and 22 all had two visible bands, one corresponding with the wild-type band length and one corresponding with the mutant band length, meaning those samples were heterozygous for *K05F1.1*. Samples 19 and 41 both had a singular band corresponding with the wild-type band length, meaning samples 19 and 41 were both homozygous wild types. Samples 10 and 32 were both homozygous for *K05F1.1(syb4161)*, because there was only one band, which was associated with the mutant band length. Therefore, the wells used to maintain the *K05F1.1 (syb4161); him-8 (e1489)* strain were wells 10 and 32. The N2 sample was expected to have a singular 1763 base pair band, but the sample smeared. The smear was due to overloading the gel. The third band in the heterozygous sample columns is not currently understood.

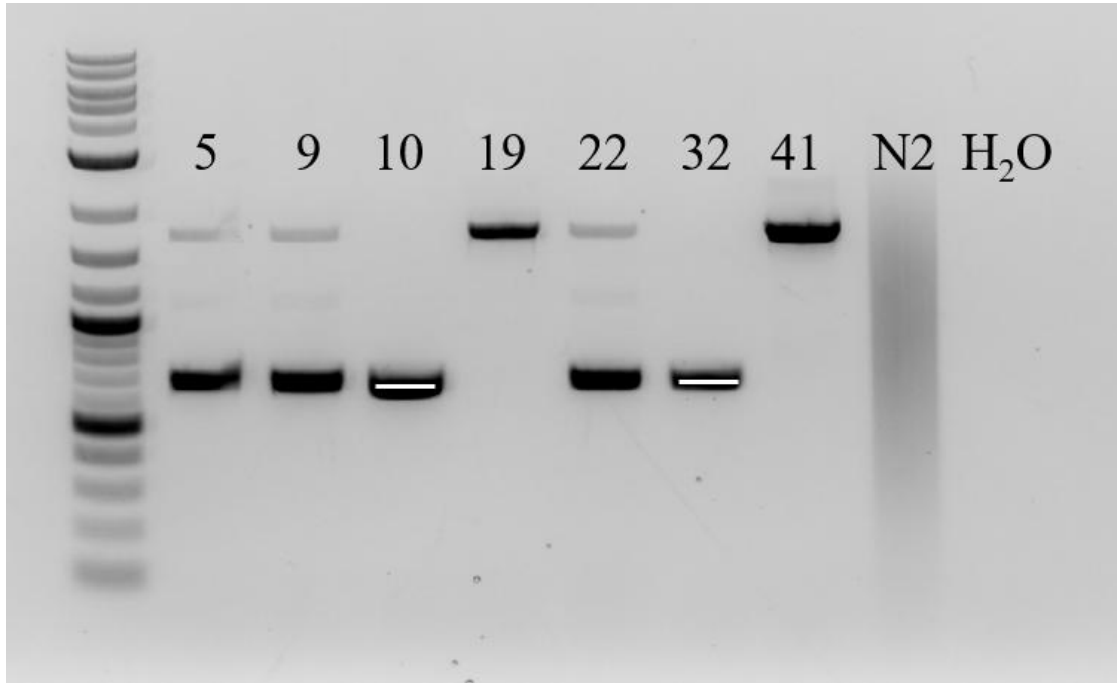


Figure 3.1.2 PCR products run on a 1% agarose gel identify worms homozygous for *K05F1.1(syb4161)* in the *K05F1.1(syb4161); him-8(e1489)* genetic cross. Each lane represents a single F₂ hermaphrodite.

Male fertility assays were completed using the *K05F1.1(syb4161); him-8(e1489)* strain. The male fertility assays yielded inconsistent results. The first assay resulted in no significant difference in the number of progeny produced. However, there were many *him-8(e1489)* and *K05F1.1(syb4161); him-8(e1489)* hermaphrodites which bagged, meaning eggs prematurely hatched within the hermaphrodite's germline, resulting in premature death and stunted production of progeny. The *K05F1.1(syb4161); him-8(e1489)* organisms which bagged did so later in the assay, after all other organisms had stopped laying. The quantity of offspring produced by those organisms were generally aligned with the organisms that had stopped laying naturally. However, the *him-8(e1489)* organisms that bagged did so within the first

few days of the assay, leading to noticeably fewer progeny produced. There were also many hermaphrodites that did not mate with the males on the plates. The unexpectedly low number of progeny for the control males in the first male fertility assay encouraged us to outcross the *him-8(e1489)* line. The second and third assays were both completed with twice-outcrossed *him-8(e1489)* *C. elegans*. As the bagging in the *K05F1.1(syb4161); him-8(e1489)* dataset did not lead to as many noticeable outliers, the strain was not outcrossed. The two male fertility assays following the *him-8(e1489)* outcrossing produced inconclusive results. The first resulted in significant differences in the number of progeny produced, with a P-value of 0.0048. The second resulted in no significant difference in number of progeny produced.

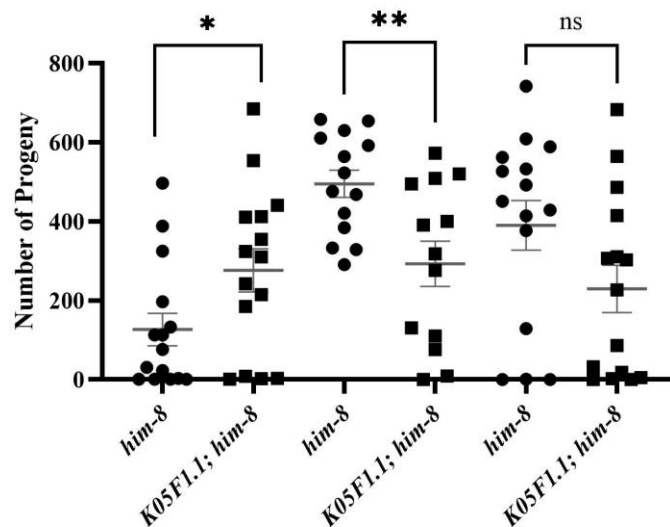


Figure 3.1.3 Male fertility assays comparing *him-8(e1489)* and *K05F1.1(syb4161)* males crossed with *fog-2(oz40)* hermaphrodites in three separate trials. All three replicates were done at 20°C. Each point indicates the number of progeny produced by a single hermaphrodite. N≥15.

DIC and DAPI imaging were completed on N2 and *K05F1.1(syb4161)* mutant organisms to allow for visualization of sperm localization within the germline of mutant organisms. If *K05F1.1* is necessary for sperm activation, we would likely see spermatozoa spread throughout the uterus, rather than being localized to the spermatheca, as in wild-type organisms. In *C. elegans*, sperm are pushed out of the spermatheca by the oocytes during fertilization; however, due to the pseudopod they develop during sperm activation, they can crawl back into the spermatheca. In contrast, spermatids that do not undergo sperm activation would be pushed through the spermatheca but would be unable to return due to the lack of a viable pseudopod.

Sperm are localized to the spermatheca in both N2 and *K05F1.1(syb4161)* hermaphrodites. There is no visible difference between the reproductive tracts of the mutant compared to control. Quantities of sperm in the spermatheca appear similar, although no specific count was completed. Embryos had visible eggshells observed by DIC imaging. The results imply that *K05F1.1(syb4161)* deletion does not impact the success of sperm activation in *C. elegans*.

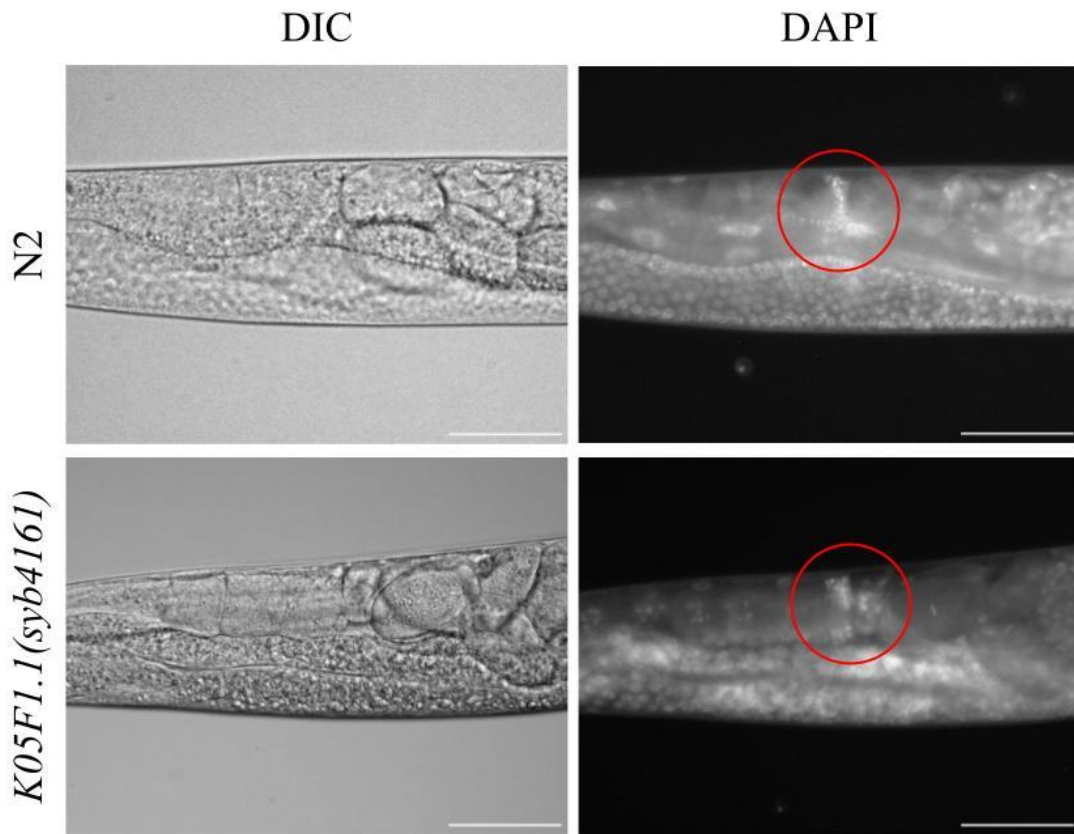


Figure 3.1.4 Representative DIC and DAPI imaging of one day adult N2 and *K05F1.1(syb4161)* spermatheca depicting sperm localization in the spermatheca. Scale bar equals 50 microns. A red circle outlines the spermatheca.

To follow up the DAPI imaging, DIC imaging of male inactivated spermatids was completed to identify any morphological differences between N2 spermatids and *K05F1.1(syb4161)* mutant spermatids. Based on the previous negative data, we did not expect to see a difference between mutant spermatids and controls.

N2 and *K05F1.1(syb4161)* mutant spermatids are not visibly different. Both are round with a visible nucleus in the center. Three separate imaging sessions

produced similar results, where spermatids could not be distinguished between control and mutant based off appearance. The results imply that *K05F1.1(syb4161)* deletion does not impact production or morphology of inactivated spermatids.

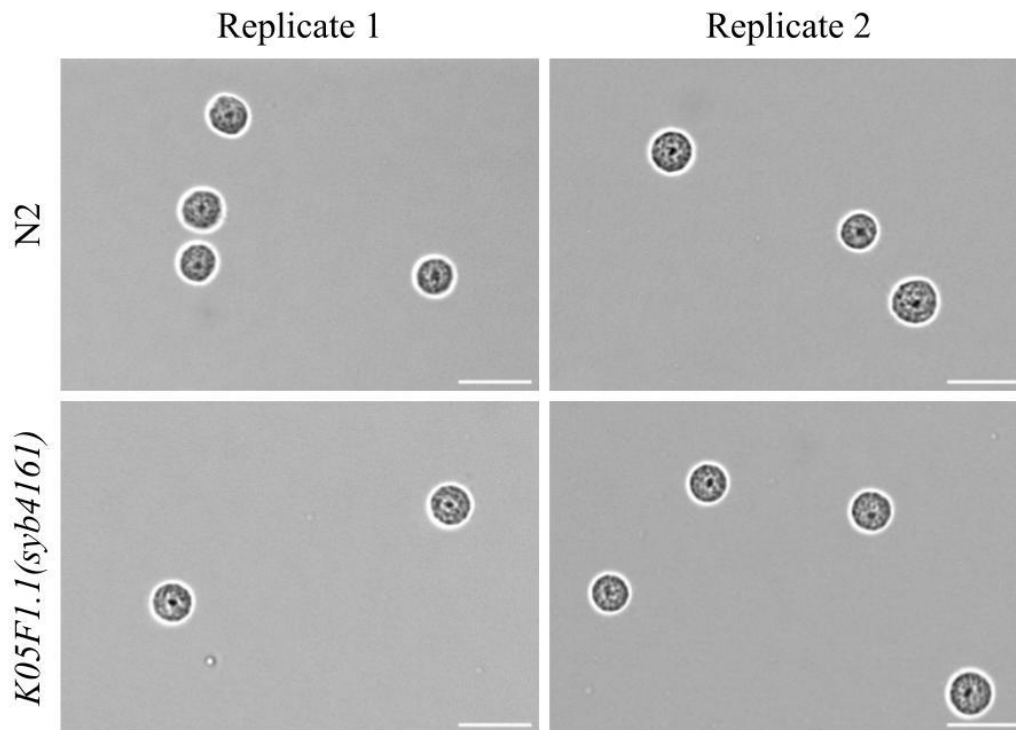


Figure 3.1.5 Representative DIC Images of Inactivated N2 and *K05F1.1(syb4161)* *C. elegans* Spermatids. Spermatids were imaged with a 40X objective. Scale bar equals 10 microns.

DIC imaging of activated male spermatozoa was also completed to determine whether *K05F1.1(syb4161)* had an impact on sperm activation that resulted in different appearances of spermatozoa. Based on the previous negative data and the DAPI imaging results, the expectation was that N2 and *K05F1.1(syb4161)* deletion mutants would produce identical spermatozoa.

Activated spermatozoa could not be distinguished between N2 and *K05F1.1(syb4161)* organisms. All spermatozoa developed pseudopods and had a visible nucleus in the cell. Three different trials yielded similar results, with representative images shown below. The results indicate that *K05F1.1(syb4161)* deletion does not independently impact activation of male spermatozoa.

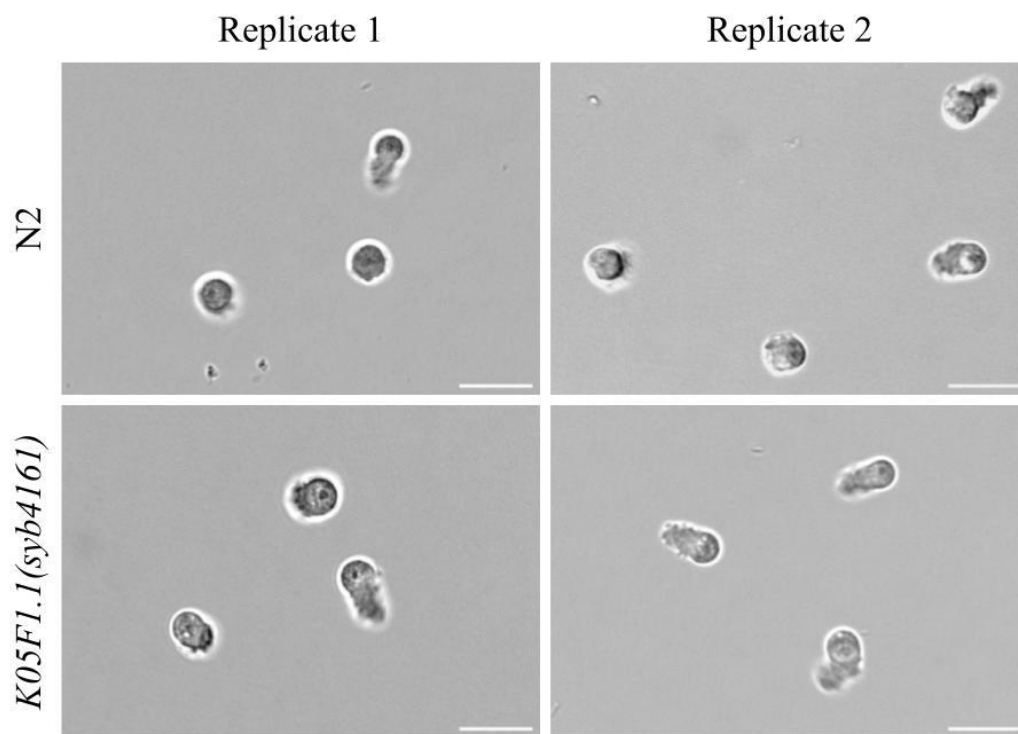


Figure 3.1.6 Representative DIC images of N2 and *K05F1.1(syb4161)* *C. elegans* spermatozoa activated with Pronase. The spermatozoa were imaged with a 40X objective. Scale bar equals 10 microns.

Due to the negative data produced in the previous experiments, we hypothesized that *K05F1.1* may be a part of the TRY-5 pathway. To determine

whether *K05F1.1* is a part of the TRY-5 pathway, a *K05F1.1(syb4161); spe-8(hc53)* double mutant was created. If involved in the TRY-5 pathway, deletion of *K05F1.1* alone would not impact fertility. Deletion of *K05F1.1* and a *spe-8* group gene would, however, result in impaired fertility.

Parental hermaphrodites and males from the F₂ generation were lysed together and genotyped for *K05F1.1* and *spe-8*. Wells where neither parent could be found were fully omitted from the dataset. The wild-type allele of *K05F1.1* was expected to produce a 1765 base pair PCR product, while the mutant allele would produce a 698 base pair product. Samples 3, 4, 9, 11, 12, 13, 14, 15, 16, 20, 23, 29, 30, 34, 36, 38, 41, and 47 (34 through 47 not depicted in representative gel image) had two bands: one corresponding with the expected wild-type band length and one corresponding with the expected mutant band length. The presence of both bands indicates that the worms were heterozygous for the *K05F1.1* mutation. Samples 21, 22, 27, 33, 37, 39, 46, and 48 each had a singular band corresponding with the mutant band length, indicating that those samples were homozygous for the *K05F1.1(syb4161)* mutant allele.

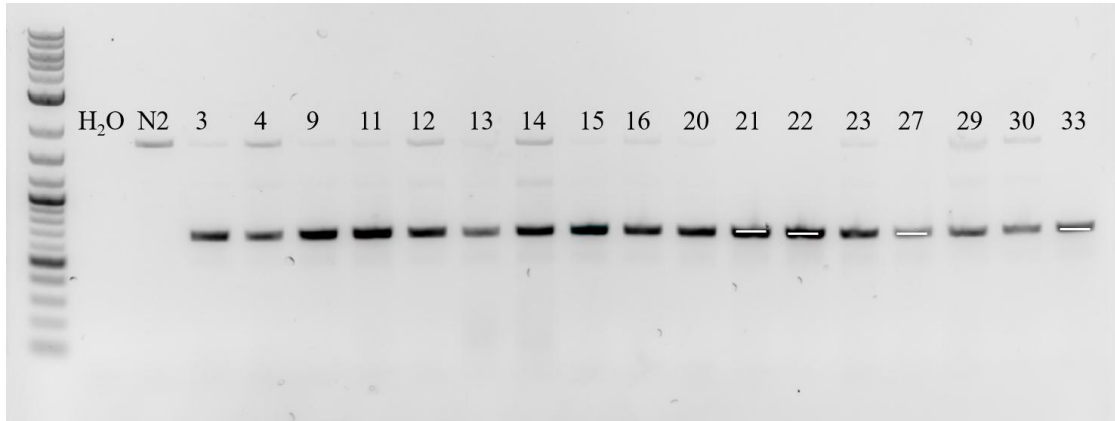


Figure 3.1.7 PCR products run on a 1% agarose gel identify worms homozygous for *K05F1.1(syb4161)* in the *K05F1.1(syb4161); spe-8(hc53)* genetic cross. Each lane represents the parental hermaphrodite and male. Additional samples were run on a separate gel.

None of the F₂ worms were homozygous *spe-8(hc53)* mutants (gel not pictured), so heterozygous *spe-8* worms were mated in one-to-one crosses for an additional generation. The F₃ worms were genotyped using super-selective *spe-8* primers. Super-selective primers only amplify the allele of interest, meaning the PCR products will only produce a band if the allele is present in the sample. In the representative gel image, lanes 2 through 5 were the PCR products made with primers that specifically amplify the wild-type allele of *spe-8*. Lanes 6 through 9 contained PCR products made with primers that specifically amplify the *hc53* allele of *spe-8*. Both the N2 and the *spe-8* controls contained a single hermaphrodite, while sample 44 consisted of the hermaphrodite and male F₃ organism. The N2 sample only produced a band using the wild-type primer set. Both the *spe-8* sample and sample 44 only produced a band using the mutant primer set. As sample 44 produced a band utilizing the mutant primer set, but not using the wild type primer set, we can conclude that the parents were homozygous for the *spe-8(hc53)* mutation. Sample 44 was the only F₃

sample to produce a band with the primers that detect the mutant allele, but not with the primers that detect the wild-type allele. However, these worms did not produce any progeny, so the strain could not be maintained. The sterility of the *K05F1.1(syb4161); spe-8(hc53)* crossed organisms implies that presence of both mutant alleles together may result in sterility. However, due to the extremely small sample size, a conclusion cannot be drawn from only one well.

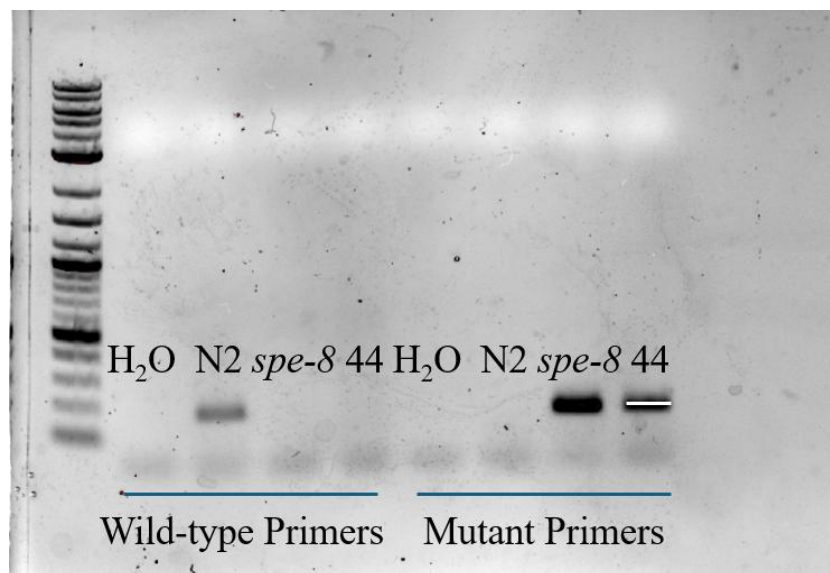


Figure 3.1.8 PCR products run on a 1% agarose gel identify worms homozygous for *spe-8(hc53)* in the *K05F1.1(syb4161); spe-8(hc53)* genetic cross.

To enable the maintenance of a *K05F1.1(syb4161); spe-8(hc53)* mutant, an additional genetic cross was created using *tmC20* as a balancer to balance *spe-8(hc53)*. Balancers allow for maintenance of mutant organisms by simplifying maintenance of heterozygotes. *tmC20* specifically produces fluorescence when the allele is present in an organism, and dumpy worms when both copies of the allele contain the balancer.

Throughout the cross, *tmC20* heterozygotes were identified by looking for fluorescent worms that were not dumpy.

The F₂ generation was genotyped to find *K05F1.1* heterozygotes. During this process, worms were individually lysed, and only wells that contained *K05F1.1* heterozygotes had the second parent genotyped. Siblings from the F₂ wells were crossed in one-to-one crosses for two additional generations in hopes of producing an F₃ generation with hermaphrodites and males (gels not depicted). However, each generation would result in no homozygous *K05F1.1(syb4161)* hermaphrodites being crossed to homozygous *K05F1.1(syb4161)* males, due to the low probability of both instances. To increase the probability of singling out a homozygous organism, the successful F₃ generation was completed using only hermaphrodite self-fertilization.

The wild type allele of *K05F1.1* was expected to produce a 1765 base pair PCR product, while the mutant allele would produce a 698 base pair product. Samples 8, and 11 had one band associated with the wild-type band length, meaning those samples were homozygous for the wild type allele. Samples 3, 4, 5, 13, 14, 19, and 20 had two bands: one corresponding with the expected wild-type band length and one corresponding with the expected mutant band length. The presence of both bands indicates that the worms were heterozygous for *K05F1.1*. Samples 2, 6, 9, 10, and 15 each had a singular band corresponding with the mutant band length, indicating that those samples were homozygous for the *K05F1.1(syb4161)* mutant allele.

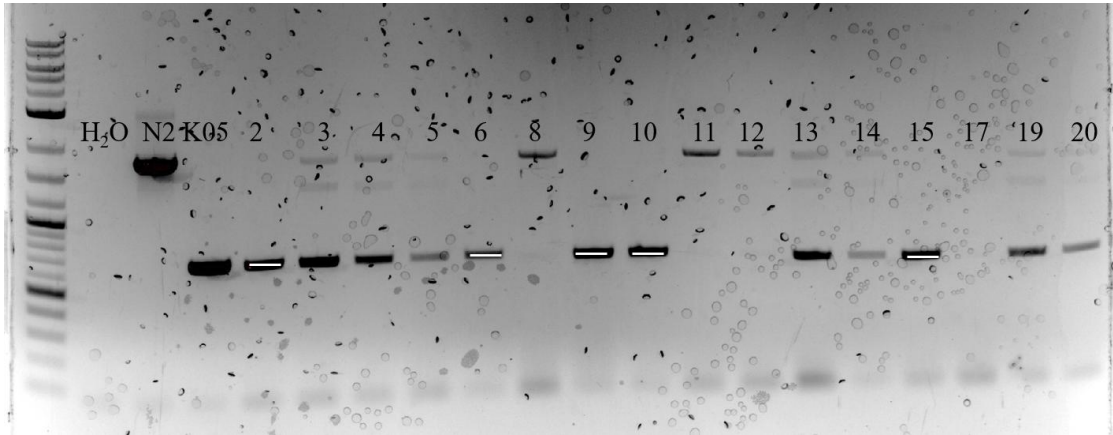


Figure 3.1.9 PCR products run on a 1% agarose gel identify worms homozygous for *spe-8(hc53)* in the *K05F1.1(syb4161); spe-8(hc53)/tmC20* balanced genetic cross.

The samples that were homozygous for *K05F1.1(syb4161)* deletion were then phenotypically screened for *spe-8(hc53)*. Homozygous *spe-8(hc53)* mutation is characterized by hermaphrodites which are unable to produce self-progeny. Non-fluorescent hermaphrodites, which should be *spe-8(hc53)* homozygotes, were singled out and plated with four per plate to different seeded agar plates labelled by each well number. As none of the worms produced any progeny, the *spe-8(hc53)* genotype was confirmed. Organisms were maintained using a fluorescent scope to identify fluorescent, non-dumpy worms.

To determine whether the previous result of *K05F1.1(syb4161); spe-8(hc53)* males being sterile was replicable, two plates of *K05F1.1(syb4161); spe-8(hc53)* homozygous males were crossed with *fog-2(oz40)* hermaphrodites to determine whether progeny were produced. After four days, the plate had visible progeny, indicating that *K05F1.1(syb4161); spe-8(hc53)* males are not sterile.

To confirm these results, a shortened male fertility assay was completed by crossing N2 males with *fog-2(oz40)* hermaphrodites and *K05F1.1(syb4161); spe-8(hc53)* males to *fog-2(oz40)* hermaphrodites. The assay found that within the first two days of progeny production, there was no significant difference in progeny produced.

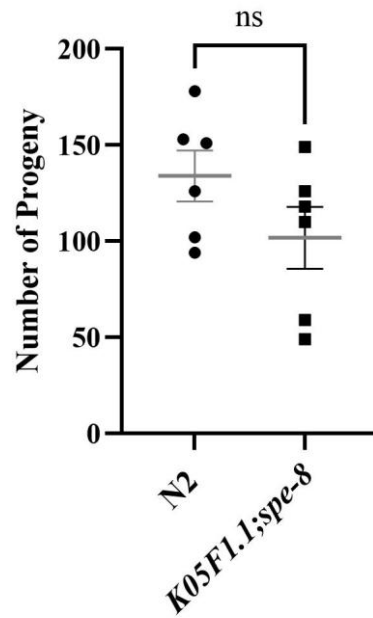


Figure 3.1.10 Male fertility assays comparing wild-type and *K05F1.1(syb4161); spe-8(hc53)* males crossed with *fog-2(oz40)* hermaphrodites. Experiment was completed at 20°C, and was modified to only include progeny from the first 48 hours following mating to hermaphrodites. Each point indicates the number of progeny produced by a single hermaphrodite. N=6.

3.2 Loss of *F57A8.6* Impacts Hermaphrodite Fertility

Experiments done by other members of our lab have found that *F57A8.6* does play a role in fertility. Specifically, hermaphrodite self-fertility assays have found *F57A8.6(syb4189)* mutants produce significantly fewer progeny than N2s at multiple different temperatures. To confirm *F57A8.6* has an impact on fertility, two additional

experiments were completed: a 16°C hermaphrodite self-fertility assay and a 25°C hermaphrodite self-fertility assay.

At 16°C mutant hermaphrodites were significantly subfertile in comparison to N2 control organisms. The P value was <0.0001. The 25°C repeat produced similar results. *F57A8.6(syb4189)* mutant hermaphrodites were significantly sub-fertile in comparison to N2 organisms. The P value was <0.0001. The hermaphrodites kept at 25°C had fewer progeny produced in comparison to the 16°C assay. My results are consistent with the lab's previous findings and our expectations.

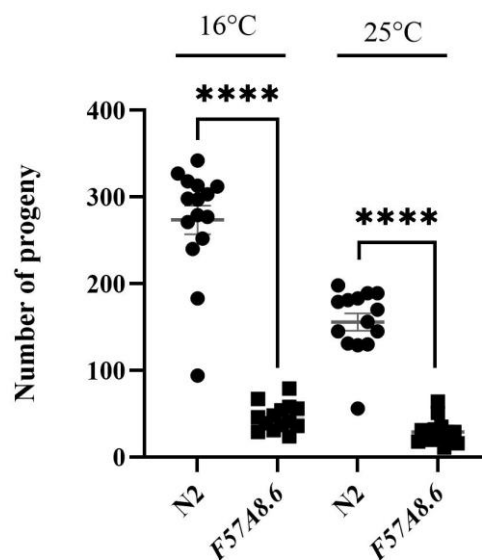


Figure 3.2.1 15°C and 25°C self-fertility assays comparing *F57A8.6(syb4189)* and N2 organisms depicted significantly lower fertility in the mutant organism. Each point indicates the total number of progeny produced by a single hermaphrodite. N=15

To determine whether the *F57A8.6(syb4189)* mutation impacted sperm-specific processes, a cross-fertility assay was completed by crossing mutant and control hermaphrodites to N2 males. The experiment had already been completed in the lab, and the purpose of the repeat was primarily to ensure that the previous results were replicable.

At 20°C, there was no significant difference in the number of progeny produced by *F57A8.6(syb4189)* mutant hermaphrodites crossed to N2 males when compared to *fog-2(oz40)* mutants crossed to N2 males. The *fog-2(oz40)* samples had a larger distribution of values than *F57A8.6*, which in general had much more similar values. The result means that loss of *F57A8.6* does not impact hermaphrodite-specific fertility processes, indicating the mutation as playing a role in sperm-specific processes.

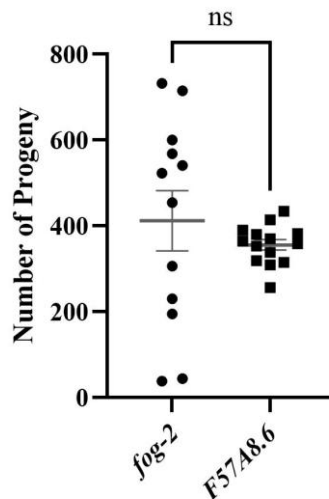


Figure 3.2.2 A hermaphrodite cross-fertility assay at 20°C comparing *fog-2(oz40)* and *F57A8.6(syb4189)* hermaphrodites crossed to wild-type N2 males showed no significant difference in the progeny produced. Each point indicates progeny produced by a single hermaphrodite. $N \geq 12$

3.3 Creation of Additional Double Mutant Strains to Characterize *spe-43* Paralogs

To characterize the impacts of removing multiple *spe-43* paralogs at once, two additional mutant strains were created. One mutant strain was *T10E9.4(syb4144)*; *F57A8.6(syb4189)*; *him-8(e1489)*.

To minimize the number of samples required for genotyping, wells were first scanned for oocytes. A high amount of oocyte production is a phenotype associated with *F57A8.6(syb4189)* homozygosity. Parent organisms from wells with oocytes were first genotyped for *T10E9.4(syb4144)*. The wild type allele of *T10E9.4* produced products with 1710 base pairs, while a mutant *T10E9.4(syb4144)* PCR product had 819 base pairs. The N2 control, sample 7, sample 24, and sample 44 all showed a singular band corresponding with the expected wild type band length. The samples were homozygous wild types. Samples 18, 21, 22, and 27 all had two bands, one corresponding to the mutant band length and one corresponding to the wild type band length, indicating that they were heterozygous for the *T10E9.4(syb4144)* allele. Samples 4, 11, 19, and 45 all had a singular band corresponding with the mutant band length, indicating that the samples were homozygous for the *T10E9.4(syb4144)* allele.

Worms that were homozygous for *T10E9.4(syb4144)* were further genotyped to identify homozygous *F57A8.6(syb4189)* organisms. Therefore, since the parent worms from wells 4, 11, 19, and 45 were homozygous for *T10E9.4(syb4144)* their DNA was used for the *F57A8.6* PCR.

The wild type *F57A8.6* allele produced a 1610 base pair PCR product, while the mutant allele produced a PCR product that was 635 base pairs. The N2 sample showed a singular band corresponding with wild type band length. Since samples 4, 11, and 45 all had only one band at the mutant band length, they are homozygous for

the *F57A8.6* mutation. Sample 19 was heterozygous, having both a band indicating wild type genotype and a band indicating *F57A8.6* genotype. Finally, the N2 sample was the only one wild type homozygote. The parental hermaphrodite from wells 4, 11, and 45 were homozygous for both *F57A8.6* deletion and *T10E9.4* deletion. Adolescents from those wells were placed on agar plates to maintain the *T10E9.4(syb4144); F57A8.6(syb4189); him-8(e1489)* strain.

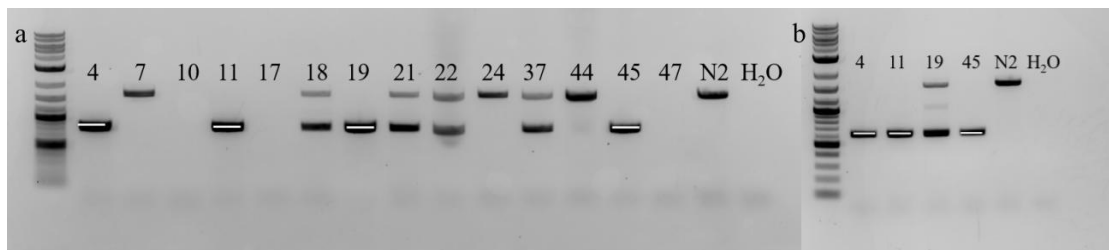


Figure 3.3.1 PCR products run on a 1% agarose gel identify worms homozygous for *T10E9.4(syb4144)* (a) and *F57A8.6(syb4189)* (b) in the *T10E9.4(syb4144); F57A8.6(syb4189); him-8(e1489)* genetic cross. Each lane represents a single F2 hermaphrodite.

Triple mutant organisms exhibit noticeably decreased fertility compared to wild-type organisms at 20°C. When attempting to maintain the strain with the usual number of organisms (five L4 hermaphrodites), the plate would have very few L4s available when transferring organisms for the next generation. Instead, maintenance needed to be completed using 8-10 L4 hermaphrodites to produce similar quantities of offspring to those found on an N2 plate. At 16°C, it was impossible to maintain the triple mutant. Viable embryos and young worms were visible on the plate, as were a few fully grown worms, but there were no L4 hermaphrodites to pick, even after waiting the appropriate number of days for the new generation.

The second double mutant produced to characterize the impacts of removing two *spe-43* paralogs at once was a *K05F1.1(syb4161); F57A8.6(syb4189); him-8(e1489)* triple mutant. Since *F57A8.6(syb4189)* homozygosity is associated with a phenotype of hermaphrodites laying oocytes, wells were first screened for the presence of oocytes. Only worms that produced oocytes were PCR genotyped for *K05F1.1* and *F57A8.6*.

The wild-type allele of *K05F1.1* was expected to produce a 1765 base pair PCR product, while the mutant allele would produce a 698 base pair product. Samples 8, 27, and 47 had a single band at the wild-type band length, indicating that the worm was homozygous for the wild-type *K05F1.1* allele. Samples 2 and 14 exhibited two bands, one corresponding to the expected wild-type band length and the other corresponding to the mutant band length. The result indicates that 2 and 14 were heterozygous. Samples 4, 9, 13, 15, 16, 18, 24, 34, and 48 each had a single band corresponding with the mutant band length, which indicates that each of the samples was homozygous for the *K05F1.1(syb4161)* deletion allele.

Samples used for *F57A8.6* genotyping were determined based on the results of the *K05F1.1* gel. Samples that were homozygous for the *K05F1.1(syb4161)* deletion mutant were genotyped for *F57A8.6*.

The wild-type *F57A8.6* allele produced a 1610 base pair PCR product, while the mutant allele produced a PCR product that was 635 base pairs. Sample 9 had a single band corresponding to the wild-type band length, indicating that the sample was homozygous for the wild-type allele. Sample 34 had two bands, one corresponding with the wild-type band length while the other corresponded with the mutant band length, indicating the sample was heterozygous. Samples 4, 13, 15, 16, 18, and 23 all

had one band at the expected mutant band length, indicating that those samples were homozygous for the *F57A8.6(syb4189)* deletion allele. L4 hermaphrodites from the wells which were homozygous for both *K05F1.1(syb4161)* and *F57A8.6(syb4189)* were picked to maintain the *K05F1.1(syb4161); F57A8.6(syb4189); him-8(e1489)* triple mutant. Mutants were subfertile, but not to the same extent as the *T10E9.4(syb4144); F57A8.6(syb4189); him-8(e1489)* strain.

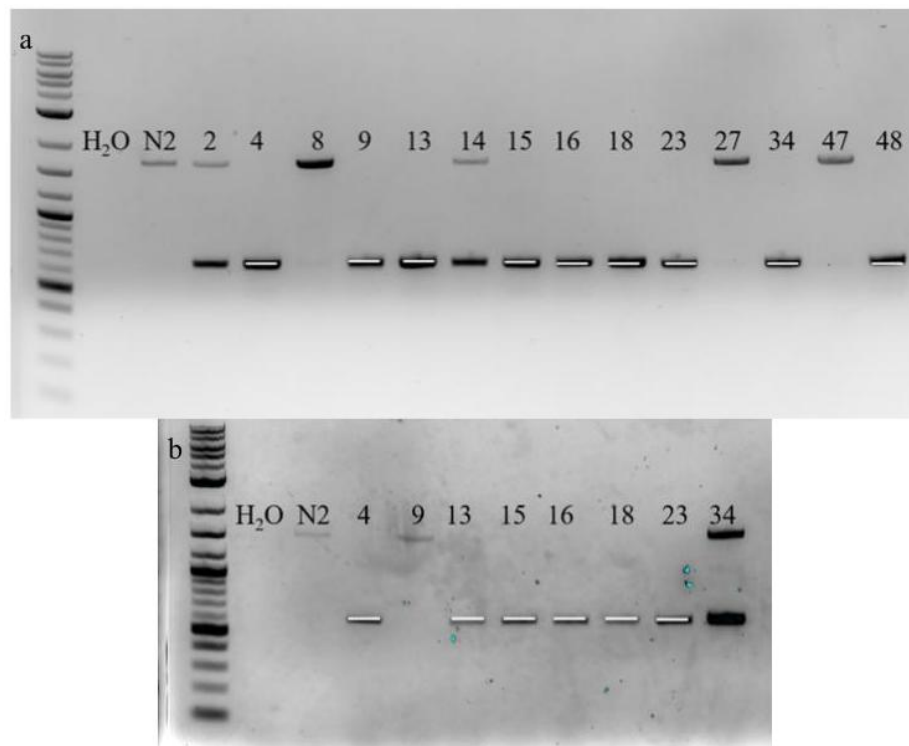


Figure 3.3.2 PCR products run on a 1% agarose gel identify worms homozygous for *K05F1.1(syb4161)* (a) and *F57A8.6(syb4189)* (b) in the *K05F1.1(syb4161); F57A8.6(syb4189); him-8(e1489)* genetic cross. Each lane represents a single F2 hermaphrodite

Chapter 4

Discussion

4.1 *K05F1.1(syb4161)* Assays

Overall, *K05F1.1(syb4161)* fertility assays suggest that *K05F1.1* does not play a role in sperm activation. Most assays showed no significant difference between wild-type organisms and *K05F1.1(syb4161)* deletion mutants. The results do not align with expectations for SPE-8 group proteins.

DAPI imaging revealed similar quantities of sperm in the spermatheca of *K05F1.1(syb4161)* mutants and wild-type N2s. The result suggests that *K05F1.1* is not required for sperm activation, as sperm need to activate and form a pseudopod to travel back to the spermatheca once pushed out. Moreover, oocytes can be observed in the germline of both wild-type and mutant organisms, and shelled embryos are visible in the uteri, indicating that progeny are still produced despite the deletion of *K05F1.1*. The result corroborates the data produced by the brood size assays. Moreover, *K05F1.1(syb4161)* mutant spermatids and spermatozoa are both identical to N2. Therefore, based on these data, I conclude that the absence of the K05F1.1 protein does not cause sperm defects.

The results collected from assaying *K05F1.1(syb4161)* deletion mutants all suggest that the K05F1.1 protein is not involved in the SPE-8 pathway. Results of the initial *K05F1.1(syb4161); spe-8(hc53)* cross indicated that the K05F1.1 protein may be involved in the TRY-5 pathway instead, as it appeared that knocking out *K05F1.1* and *spe-8* together resulted in sterility. This phenotype is associated with TRY-5 pathway components, because unless both pathways are disabled, the SPE-8 pathway will still allow for sperm activation in both hermaphrodites and males. Once the SPE-8

pathway is knocked out, males with mutations in the TRY-5 pathway are sterile, as there is no viable pathway to enable sperm activation. The results, however, were based on a single cross consisting of one hermaphrodite and one male. The results after making the balancer show that *K05F1.1(syb4161); spe-8(hc53)* mutant males were able to produce viable spermatozoa, as they were able to produce progeny when crossed to *fog-2(oz40)* worms, which do not produce sperm. Ultimately, the fertility of the *K05F1.1(syb4161); spe-8(hc53)* males indicates that *K05F1.1* does not play a mandatory role in either pathway.

4.2 *F57A8.6(syb4189)* Mutant Fertility Assays

The results of the 16°C and 25°C hermaphrodite self-fertility assays indicate that *F57A8.6* plays a role in *C. elegans* fertility. The cross-fertility assay showed that the progeny count matches wild-type quantities when crossed with N2 males. The three results together imply that the role *F57A8.6* plays in *C. elegans* fertility is related to production of hermaphrodite sperm. When wild-type sperm is provided to organisms with deletion of *F57A8.6*, the mutants are still able to produce progeny, so the role of *F57A8.6* is likely sperm-specific. If *F57A8.6* played a role in egg-specific processes, the presence of wild-type male sperm would not be enough to recover progeny count. The results of *F57A8.6(syb4189)* fertility assays I performed align with SPE-8 group proteins; however, with all current data we do not know which pathway the gene is involved in.

4.3 Genetic Crosses

The successful completion of the double mutant strains will enable future characterization to see if deletion of multiple paralogs results in a stronger phenotype.

Already, observations from general maintenance imply that double mutants are sub-fertile. The observations of the *T10E9.4(syb4144); F57A8.6(syb4189); him-8(e1489)* strain when incubated at 16°C are still not understood. The observed lack of progeny may be due to decreased fertility or high mortality. Based on the hypothesis that *F57A8.6* and *T10E9.4* both encode for proteins involved in fertilization, my primary hypothesis is that the strain has decreased fertility at 16°C. Further studies are needed to determine the significance of these observations and whether they are quantifiable.

4.4 Future Directions

To complete the characterization of *K05F1.1*, the final step would be to identify the protein's localization. Tagging *K05F1.1* protein with a fluorescent protein and completing additional imaging would enable us to confirm where *K05F1.1* localizes and could contribute to a better understanding of the protein's function.

The *T10E9.4* deletion mutant and each of the double mutant strains I created will need to be characterized to determine how they impact *C. elegans* fertility. Characterizing the double mutants could note whether any of the proteins have overlapping functions, or how they interact with each other to impact fertility overall. Experiments similar to those completed in this thesis would likely be the first steps in determining the extent of sub-fertility in the mutants, since general maintenance has already indicated that *T10E9.4* deletion mutants and each of the double mutants are sub-fertile.

Understanding the functionality of *spe-43* paralogs in *C. elegans* fertility provides insight into the molecular processes that are required for sperm activation in other organisms. Although *spe-43* and its paralogs do not have direct human homologs, biological processes are often analogous between different species.

Developing stronger understanding of the interactions required for sperm activation in a model organism will enable a better understanding of the underlying mechanisms used for sperm activation in all sexually reproducing organisms. Ultimately, more concise knowledge of sperm activation and fertility processes will allow for the development of pharmaceuticals such as contraceptives. It also will enable more precise understanding of fertility defects and infertility.

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