PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF *SPE-43*, A Gene REQUIRED FOR *CAENORHABDITIS ELEGANS* SPERMIOGENESIS

By

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ABSTRACT OF THE THESIS

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The reproduction of a genetically distinct new organism is a delicate and tightly regulated process. It requires a lot of molecular interactions and time-coordinated events. Before the fertilization process takes place, both male and female gametes need to properly develop and be fully capable to find one another, fuse, and promote the transition into a developing embryo. Different types of research performed in *Caenorhabtidis elegans* and *Ascaris suum* have helped provide a fairly clear picture of the cellular components and processes necessary for sperm development and differentiation. Still, some of the molecular mechanisms for these processes collectively known as spermatogenesis and spermiogenesis, are not fully understood. For those purposes, this thesis elucidates a new gene member required for *C. elegans* spermiogenesis: *spe-43*(*eb63*).

The first chapter of this thesis provides an introduction to the importance of *C. elegans* fertility research and its impact on modern society reproductive technologies. Also in this chapter we will review some basic knowledge about the *C. elegans* reproductive world such as gender, reproductive tract, gamete development, nematode

sperm, spermatogenesis, spermiogenesis and fertilization.

The second chapter of the present work outlines the phenotypic and partial molecular characterization of the *spe-43* gene. This new gene belongs to a family of genes called the *spe-8* group, which they all interact in a specific spermiogenesis pathway of the *C. elegans*. This spermiogenesis process is not only critical for sperm development and fertilization in *C. elegans*, but represents a unique and important model both for the study of differentiation without *de novo* protein synthesis, cell maturation, cell mobility and for the evolutionary development of an androdioecious species from a gonochoristic one.

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That would not have been possible without people such as Dr. Michael J. Leibowitz and Dr. Jerome Langer, external advisors and directors for The Bridge to Doctorate Program Fellowship at the University of Medical and Dentistry of New Jersey and Dr. Richard Padgett, Cell Develoment Biology director, external advisor and committee member for The Waksman Institute Pre-doctoral Fellowship at Rutgers University. I would also like to thank my committee faculty members, as well as other faculty that have helped me at many times: Dr. Monica Driscoll, Dr. Kim McKim, Dr. Chris Rongo and Dr. Richard Padgett. They gave me important suggestions for my projects, provided me deeper scientific insights and lastly, took part of their time to evaluate my dissertation.

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Table of Contents

List of Tables

List of Figures

Chapter 1

I.1 The importance of the fertilization process: Research and its applications for the modern reproductive technologies

Throughout the course of human history, different societies have made dedicated efforts to understand the fertilization process. The study of fertilization has been highly influential for developing better methods for improving fertility and for contraceptive techniques. During the fertilization process two gametes, an egg and a sperm, most find each other and fuse to generate a diploid zygote that will become a new organism (WASSARMAN *et al.* 2001). Reproductive success requires a series of exact and well-coordinated events that include gamete activation, recognition, signaling, adhesion and fusion (GELDZILER *et al.* 2011). Understanding of fertilization has important implications for the medical, economic and social cultural fields. Research using genetic and molecular approaches has been key for the undertanding of the different and complicated biological events of this process.

The applications of reproductive research are numerous and have boomed during the past decades. Approximately, 3.5 million births since 1978 have been accounted for Assisted Reproductive Technologies (ART) (CHAMBERS *et al.* 2009). Newer and improved fertility techniques have now been develop and used. These include ovarian stimulation, intracytoplasmic injection of sperm and in vitro maturation of oocytes. The costs of these processes are high; the average cost in the US of the commonly used In Vitro Fertilization Technique is approximately $12,513 per cycle (CHAMBERS *et al.* 2009).

Conversely, developing nations needs for newer, cost-efficient and safe male and female contraceptive technologies remain in high demand, mainly because of the high impact of unwanted pregnancy in terms of mortality and financial burden (AITKEN *et al.* 2008). While an estimated 42 million abortions occur every year world wide, nearly half of those were performed unsafely, making it one of the leading causes of maternal mortality (HADDAD and NOUR 2009; SHAH and AHMAN 2009). Around 68,000 women die of unsafe abortion annually (HADDAD and NOUR 2009). Of those women who survive unsafe abortion, 5 million will suffer long-term health complications (HADDAD and NOUR 2009). Both of the primary methods for preventing unsafe abortion—less restrictive abortion laws and greater contraceptive use—are highly needed particularly in developing nations, where most unsafe abortions (97%) occur (HADDAD and NOUR 2009). According to the World Health Organization (WHO), every 8 minutes a woman in a developing nation will die of complications arising from an unsafe abortion (HADDAD and NOUR 2009).

More research is critically needed for understanding the fertilization and reproduction processes. Many studies in this field have focused on a limited number of experimental systems: marine invertebrates and mammals. Yet, many genetic and biochemical aspects of this processes are still controversial and poorly understood (CHATTERJEE *et al.* 2005). As a different approach, this thesis focuses on the importance of *Caenorhabditis elegans* fertilization process and its contribution to sperm and oocyte development research.

I.2 A tiny worm with a big scientific role:

Caenorhabditis elegans as an experimental model organism

The *C. elegans* model has been an excellent organism to study and understand many biological processes. Molecular and genetic techniques have been used in the *C. elegans* model system to examine different cellular and developmental pathways such as neurological and fertilization cellular pathways. Research with *C. elegans* has contributed significantly to novel knowledge and understanding of multicellular organism development. In the context of fertilization, this model has been emerging as a model system for studying gamete formation, spermiogenesis, fertilization and embryonic development. These processes have all been very challenging to study in many other multicellular organisms, including humans.

C. elegans is a free-living, transparent nematode of about 1 mm in length that feeds on bacteria and lives in temperate soil environments worldwide (Figure 1.1). In 1963, Sydney Brenner proposed the use of this nematode for researching animal development, particular for neural development (BRENNER 1974). Since then, the *C. elegans* organism has been used extensively for molecular and genetic research in many different developmental biological fields (BRENNER 1974). This nematode was chose by Brenner because it was a simple multicellular eukaryotic organism, inexpensive to grow in mass population on standard bacterial plates or in broth, and because they were easy to handle and manipulate under a low power binocular microscope (CORSI 2006).

The *C. elegans* worm has many advantages for scientific research. These include efficient storage methods, mutant generation and isolation methods, short reproductive

cycles, and high progeny generation. These worms can be stored for many years by freezing them at -80^{o}C and when subsequently thawed, worms remain viable. This freezing method is a unique advantage of this worm over many other model organism (BRENNER 1974) .

C. elegans presents an excellent model organism for describing the molecular underpinnings of the processes of cell divisions, differentiation and development (GREENSTEIN 2005; KIMBLE and CRITTENDEN 2005; L'HERNAULT 2006). Both light and fluorescent microscopy techniques can be used successfully in the *C. elegans* model thanks to their transparent cuticle that allows live imaging of their internal processes (Figure 1.2). Every developmental stage of the worm, from undifferentiated stem cell, to fertilization competent egg and finally the developed embryo, it can all be viewed progressively when studied *in vivo* (Figure 1.2)

As stated above, *C. elegans* is a great model organism for scientific research. Mutants can be easily found. A major way to produce and isolate new mutants is by inducing mutations in the *C. elegans* genome using chemical or radiation mutagens such as ethylmethanesulfonate (EMS), diethyl sulfate (DES), *N*-nitroso-*N*-ethylurea (ENU), or formaldehyde; (2) irradiation with X-rays, γ-rays, UV light, or ionizing particles. These techniques enable rapid and successful forward genetic analysis. *C. elegans* are highly sensitive to temperature and can be grown at lower temperatures to slow their growth or higher temperatures to speed their growth. Temperature sensitive (ts) mutations may also be isolated, at which a defect is present only at certain temperature, allowing for simple isolation and propagation of mutations that might otherwise be lethal or sterile.

The *C. elegans* life cycle, from hatching to mature reproductive age adult is 3.5

days at 20^{o}C, enabling rapid generation of individuals for analysis. *C. elegans* can be males or hermaphrodites, but mostly are self-fertilizing hermaphrodites. The ability to cross males with hermaphrodites makes *C. elegans* ideal for isolation and mutant characterization. Homozygous recessive individuals are easily produced. Even mutants with mating defects can be proliferated through selfing. Conversely, crossing a self-sterile homozygous mutant hermaphrodite to a wild-type male can propagate a mutation that disrupts sperm function in a hermaphrodite. The availability of crossing also allows rapid genetic mapping and strain construction.

In addition, *C. elegans* represent the first multicellular organism to have a completely sequenced genome, publish in 1998 (CONSORTIUM 1998). The *C. elegans* genome sequence is ~100 million base pairs long and consist of six chromosomes (named **I**, **II**, **III**, **IV**, **V** and **X**) and a mitochondrial genome. Many of their genes are arranged in operons, classifying them among the few eukaryotes currently known to have operons (BLUMENTHAL 2004). The worm genome contains approximately 22,227 protein-coding genes (CONSORTIUM 1998; SPIETH and LAWSON 2006). The number of known RNA genes has approximately 16,000 RNA genes (CONSORTIUM 1998; SPIETH and LAWSON 2006). Recently, genomes of several other species of *Caenorhabditis* and other nematodes have been sequenced and published, making identification and study of orthologous easy (COGHLAN *et al.* 2006; SULSTON and HORVITZ 1977).

Transgenic worms can be produced faster and easier than other model organisms by injection of DNA into the germ line, by use of the MOS transposon recombination system such as *mut-2* or through microparticle to generate successful arrays (BESSEREAU *et al.* 2001; GRANGER *et al.* 2004; KADANDALE *et al.* 2009; PRAITIS 2006; WILLIAMS *et*

al. 2005). The most commonly used method is the injection of DNA into the germ line. This method consist in introducing a DNA construct tagged with a fluorescence marker protein (gfp and rfp), expressed under the direction of either their own, or a tissue specific promoter (KADANDALE *et al.* 2009; MELLO and FIRE 1995).

Also, the complete linage history of every adult worm somatic cell has been catalogued and many mutants affecting the linage pattern have been characterized (KIMBLE and HIRSH 1979; SULSTON and HORVITZ 1977; SULSTON *et al.* 1983). The adult hermaphrodites have exactly 959 somatic nuclei and adult males have 1031 (SULSTON and HORVITZ 1977; SULSTON *et al.* 1983). The nervous system of a hermaphrodite worm consists of 302 neurons and of 381 in males (KOSINSKI and ZAREMBA 2007).

Other groundbreaking discoveries in apoptosis, cell death mechanism, RNAi (RNA mediated interference), and fluorescent protein tagging (GFP) (CHALFIE *et al.* 1994; FIRE *et al.* 1998; HEDGECOCK *et al.* 1983) have been successfully accomplished in *C. elegans* and have led to an number of Nobel Prizes. Understanding the RNAi pathway in *C. elegans* has uncovered a whole new researching field. In *C. elegans*, RNAi knockdown can be achieved by different successful methods (soaking, feeding and microinjection of nucleic acids) (JULIE AHRINGER 2006). RNAi and its ability of knocking down the maternal complementation of the protein has been widely used to examine the germline function of all adult stages in the *C. elegans* worm. This allows for the investigation of many genes that would otherwise be embryonic lethal. Many fluorescent and antigenic markers are available for cellular compartments and components, including nuclear, plasma membrance, golgi, and endosomal (HADWIGER *et al.* 2010; SATO *et al.* 2008). Genetic deletion mutants are available for study. This capability, combined with whole

genome-sequencing and gene expression studies, enables rapid and global reverse genetic analysis strategies (JIANG *et al.* 2001; REINKE *et al.* 2000).

C. elegans have been key for providing major scientific insight and bringing solid foundation to understanding the genetically and molecular signaling processes for fertilization. Every stage of the *C. elegans* life cycle can be viewed progressively *in vivo* under light or fluorescence microscopy. Similar to mammals and other higher eukaryotic organism, *C. elegans* undergoes a progression of steps for fertilization to occur. These steps include: resumption of meiosis, cytoskeletal rearrangement, ovulation, fertilization, the block to polyspermy, completion of meiosis, extrusion of a chitinous, trilamellar eggshell, formation of a female and male pronucleus, fusion of the two pronuclei, and the initiation of the mitotic divisions (MCNALLY and MCNALLY 2005b). In addition, this model organism also carries out the same basic developmental processes as mammals and other higher eukaryotic organisms during the oocyte-to-embryo transition.

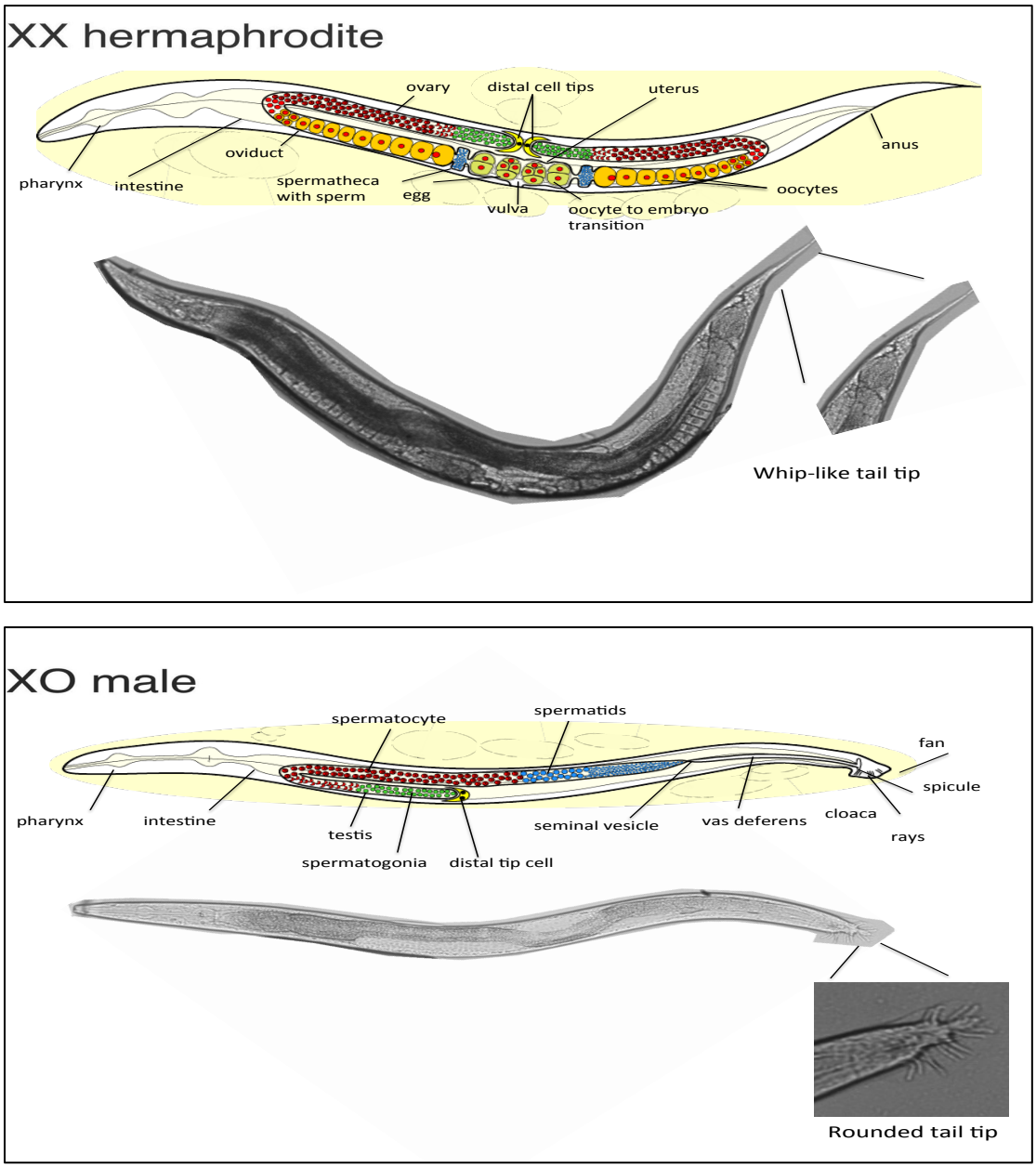


Figure 1.1, *C. elegans* adult hermaphrodite and male.

Adult hermaphrodite and male; differential interference contrast (DIC) image at 20X magnification. Reproductive and other prominent anatomical features are diagrammed below the DIC micrographs. Cartoon images provide from http://homepages.ucalgary.ca/~dhansen/worms.gif

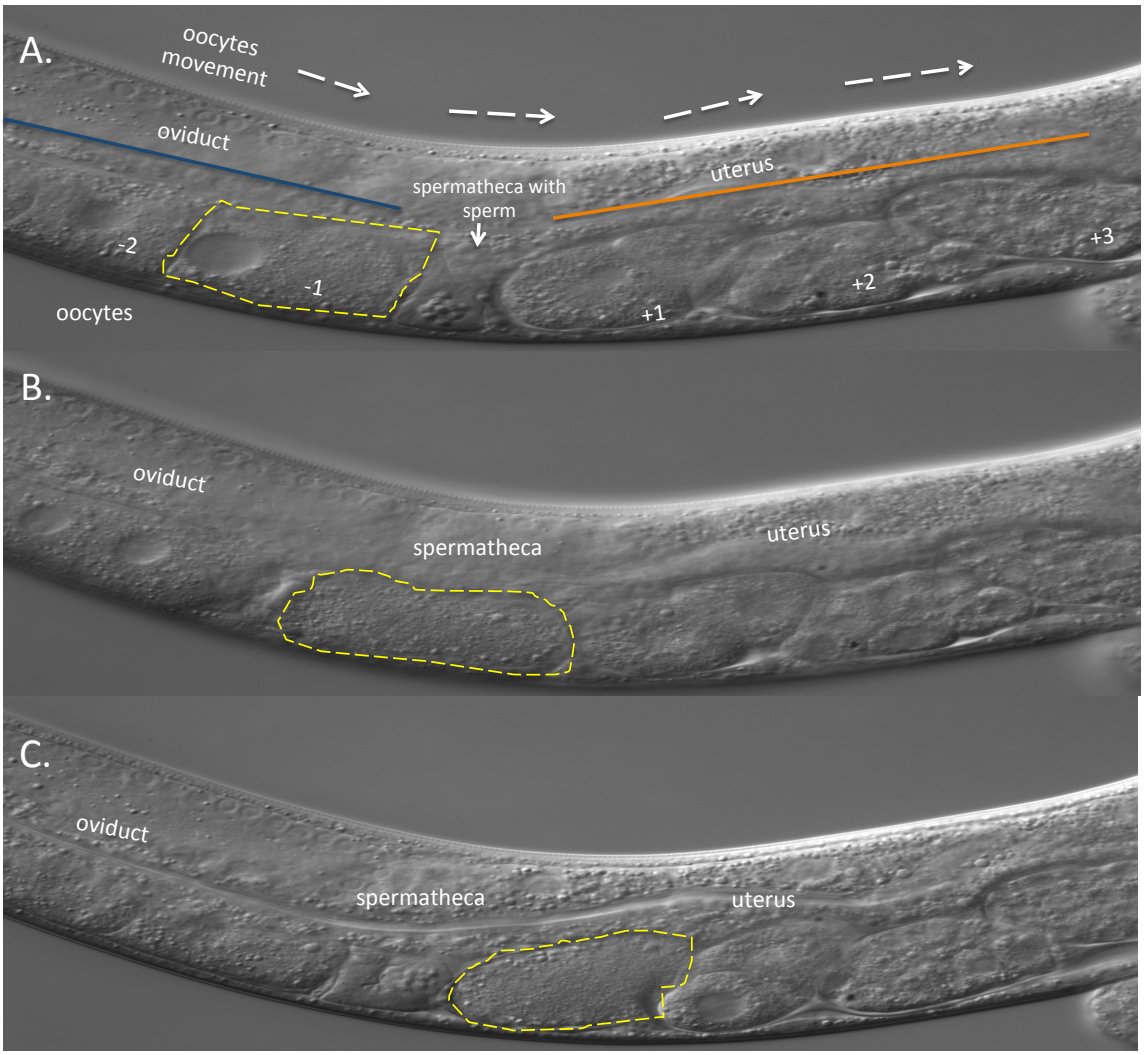


Figure 1.2, N2 *C. elegans* wild type adult hermaphrodite reproductive tract: ovulation and fertilization process.

Wild type adult hermaphrodite germline centered on spermatheca at 40X magnification. (A) Dashed lines surround proximal oocyte (-1 position). (B) Dashed lines surround an oocyte resume of meiosis and ovulated into the spermatheca. (C) Dashed lines surround newly fertilized embryo.

I.3 An Introduction on the *C. elegans* reproductive world: Gender, Reproductive Tract, Gamete development and Fertilization

In the *C. elegans* world their two type of worms, males or hermaphrodite, in which both of them have four larvae development stages before adulthood (Figure 1.1 and Figure 1.3). Self-fertilizing hermaphrodite are common while males are rare (~ 1 in 1000). In the L4 development stage (last larvae stage), the *C. elegans* hermaphrodites produce sperm and store them in the spermatheca (site of fertilization). In their adult stage they will switch to oocyte production. By having this unique self-fertilizing mechanism, they can produce approximately 300 progeny. Males produce only sperm and are capable of fertilizing hermaphrodites to produce outcross progeny (WARD and CARREL 1979). When mated, hermaphrodites can increase their reproductive potential to produce approximately 1,000 offspring.

As a species, *C. elegans* gender is determined by the ratio of autosome pairs to X-chromosomes. Hermaphrodites contain two X chromosomes (X) per diploid somatic nucleus. About 1 in every 1000 progeny, non-mated hermaphrodites produce a male (HODGKIN *et al.* 1979). Male worms contain only one X chromosome (XO), in each somatic nucleus. This effect occurs by aberrant non-disjunction of the X chromosome pair during meiosis, resulting in a nullo-X and diplo-X gametes (HODGKIN *et al.* 1979). Because males are somatically XO, half of their sperm bear an X chromosome and half do not. Therefore, when male are mated with an hermaphrodite (XX), whose oocytes almost all contain a single X chromosome, about half of the resulting outcross progeny will be males and half will be hermaphrodites. A number of genes that regulate germline

sex determination have been characterized. Some of these (e.g., her-1, fem-1, fem-2, fem-3, tra-1, tra-2 and tra-3) are also involved in somatic sex determination, while some genes (e.g., the fog and mog genes and gld-1), are specific to germlines sex determination (KUWABARA 1999).

Homozygous *C. elegans* males can also be obtained in higher progeny ratio by promoting non-disjunction of the X chromosome, without the need for crossing. Different and successful methods for promoting non-disjunction of the chromosome X have been develop, such as: heat shock (SULSTON and HODGKIN 1988), exposure to ethanol (LYONS and HECHT 1997), *him-14* dsRNA feeding (KILLIAN and HUBBARD 2004) (KILLIAN and HUBBARD 2001) and crossing it to *him-5* or *him-8* male strains. These techniques for obtaining homozygous male lines enable us to recover male progeny in subsequent generations without the need for constant crossing.

The *C. elegans* organism is a reproductive dynamo and its reproductive tract plays a mayor role for it success. Is very important to understand their reproductive tract and germline cell cycle, because of the different development events before and after the fertilization process. Between males and hermaphrodite *C. elegans* highly noticeable anatomical difference exists. Hermaphrodites are bigger in size and they contain a much complex and larger reproductive tract than males.

The hermaphrodite reproductive tract is U shape bi-lobed gonad called ovotestis (Figure 1.1). This U shape gonad is located inside middle cavity of the worm and contain different compartment, such as: oviduct, two spermatheca, a central uterus and vulva. These compartments are surrounded by different muscles and tissues needed for *C. elegans* reproduction. The end of each lobe nearest the uterus is designated the proximal

end. Between the uterus and each proximal end is a spermatheca (site of sperm storage and site of fertilization in hermaphrodite). On the other hand males have J-shape single-lobed gonad arm (testi), which forms a hairpin shape extending from a single distal tip cell to the seminal vesicle and vas deferens, near the cloaca (Figure 1.1) (KLASS *et al.* 1976). They also contain an elaborate tail that has a spicule structure and copulatory apparatus that they use for locating, courting and mating with hermaphrodites.

Not only males and hermaphrodites *C. elegans* have anatomic differences but also there are differences in sperm size, sperm activation mechanism and the site of activation. The hermaphrodite sperm activates in the spermatheca and are smaller than male's sperm. Male sperm are bigger in size and their activation initiate after their introduction through the hermaphrodite vulva. Once inside the hermaphrodite male sperm quickly out-compete hermaphroditic self-sperm for fertilization of the oocytes (GELDZILER *et al.* 2005b; SINGSON 2001; SINGSON *et al.* 1999; WARD and CARREL 1979).

Precision is highly important for the regulation of the cell cycle, especially in the germ cells. In *C. elegans* this process can be clearly observed by DNA staining in fixed specimens, or by live imaging of fluorescent-tagged histones. In hermaphrodites the production of sperm and oocytes are specifically coordinated, reflecting its existence as a primarily androdioecious species. Gamete production in both males and hermaphrodites begin at the L4 larval development stage, when nuclei in the gonad primordium begin dividing.

Under the influences of the two somatic cells at the distal end of each gonad lobe call distal tip cells (DTC's) (one at the distal tip of each ovotesti hermaphrodite lobe, two at the distal tip of a male testi) the germline begins to divide mitotically (AUSTIN and

KIMBLE 1987). The DTC's are a niche for the germ cells, which proliferate from dedicated progenitor stem cell (KIMBLE and CRITTENDEN 2005). These germ cells are maintained at mitosis by the binding of LAG-2 (protein ligand secreted by the DTC) to GLP-1 (corresponding membrane protein receptor on germ cells). This ligand-receptor interaction activates a signaling cascade, which affects mRNA stability in the early germ cells, and prevents the transition from mitosis to meiosis (HUBBARD and GREENSTEIN 2000; KIMBLE and CRITTENDEN 2005).

Throughout all of these nuclear divisions, germ cells are only partially compartmentalized within a plasma membrane. A membrane opening joins every nucleus to a common central core of cytoplasm, called rachis (HIRSH *et al.* 1976; L'HERNAULT 2006; RIDDLE *et al.* 1997). As germ nuclei accumulate, the oldest ones become displaced farther from the distal tip cell they start to differentiate and begin to enter prophase of meiosis I in the transition zone (Figure 1.3) (HUBBARD and GREENSTEIN 2000; KIMBLE and CRITTENDEN 2005). Well-defined regions of the rachis correspond sequentially to pachytene, diplotene, and diakinesis of prophase I (Figure 1.3, Figure 1.4) (HUBBARD and GREENSTEIN 2000).

At the L4 stage in hermaphrodites, the first 35 or so cells in each lobe that enter meiosis to produce primary spermatocytes, ultimately producing a total of around 300 sperm. The spermatocytes are stored undifferentiated in each of two spermathecas, one corresponding to the anterior and posterior gonad arms (Figure 1.1) (L'HERNAULT 2006). Thereafter L4 stage, they switch permanently to oocyte production. The arrest of *C. elegans* germ cells in prophase corresponds to the arrest of mammalian oocytes at metaphase I. As oocytes approach the bend of the gonad arm they are individualized from

the rachis and begin to accumulate yolk. As oocytes progress sequentially closer to the spermatheca, they are often referred to numerically by position with the oocyte closest to the spermatheca referred to as -1 (Figure 1.1, Figure 1.2). This -1 oocyte ovulation event occurs approximately every 23 minutes when sperm are abundant (Figure 1.2) (McCarter *et al.* 1999a; McNally and McNally 2005a). The abundance in sperm is highly important for the fertilization process since they are a limited resource in unmated hermaphrodites.

A nematode sperm specific small protein of 126 amino acid call Major Sperm Protein (MSP) stimulates the -1 oocytes. MSP is a core component of the sperm cytoskeleton and signaling molecule (hormone acting molecule), in which are necessary for *C. elegans* reproduction. MSP stimulated -1 oocyte from both arm of the gonad, undergoes nuclear-envelope breakdown (NEBD) and cytoskeletal rearrangement in preparation for ovulation (Harris *et al.* 2006; Kuwabara 2003). When the -1 oocyte undergoes NEBD simultaneously reenters the meiotic cell cycle at metaphase I of meiosis I approximately five minutes before ovulation will occur (Figure 1) (McCarter *et al.* 1999a; McNally and McNally 2005a). Furthermore, MSP signal promotes the overlying of somatic sheath cells (a type of smooth muscle covering the oviduct) to dilate the spermathecal valve and force the spermatheca to engulf the -1 oocyte (Figure 1.5)(Miller *et al.* 2001).

When the first ovulation of the -1 oocyte event pushes the developed spermatids (immature sperm cell) into the spermatheca, it stimulates sperm activation to a spermatozoan (mature sperm cell) (Figure 1.2) (Chu and Shakes 2013; L'Hernault 2006). When the mature oocyte enters the spermatheca it will be fertilized by a single

sperm, were it will exit through the uterus and vulva to be laid (MCCARTER *et al.* 1999a).

Other sperm and oocyte specific molecules of the mature gametes are highly important for the fertilization process, such as spe-9 (surface spermatozoa protein ligand) and oocyte specific molecules such as egg-1 and egg-2 (surface protein receptors on the egg) are necessary for sperm/egg interaction and sperm entry (KADANDALE *et al.* 2005; PUTIRI *et al.* 2004; SINGSON *et al.* 1998). After the entry of the sperm, an increase in intracellular calcium will correspond to the release of the cortical granules to the cell surface (SINGARAVELU and SINGSON 2013). A chitin-containing, trilammelar eggshell will be secreted to provide support to the developing embryo (JOHNSTON *et al.* 2006).

The six pairs of sister chromatids, corresponding to the *C. elegans* wild type hermaphrodites, will complete meiosis I and extrude a polar body containing the excess genetic material (MCNALLY and MCNALLY 2005a). Then six pairs of chromatids undergo the meiosis II, I they will divide and extrude a second polar body. Finally the formation of a female pronucleus will form by the decondensation of the female chromosome at the anterior pole of the embryo. The male pronucleus at the posterior pole will decondense of the male chromosomes. Both pronuclei will fuse to form a single diploid nucleus, and the mitotic divisions of embryotic development will begin, the oocyte-to-embryo transition is now completed.

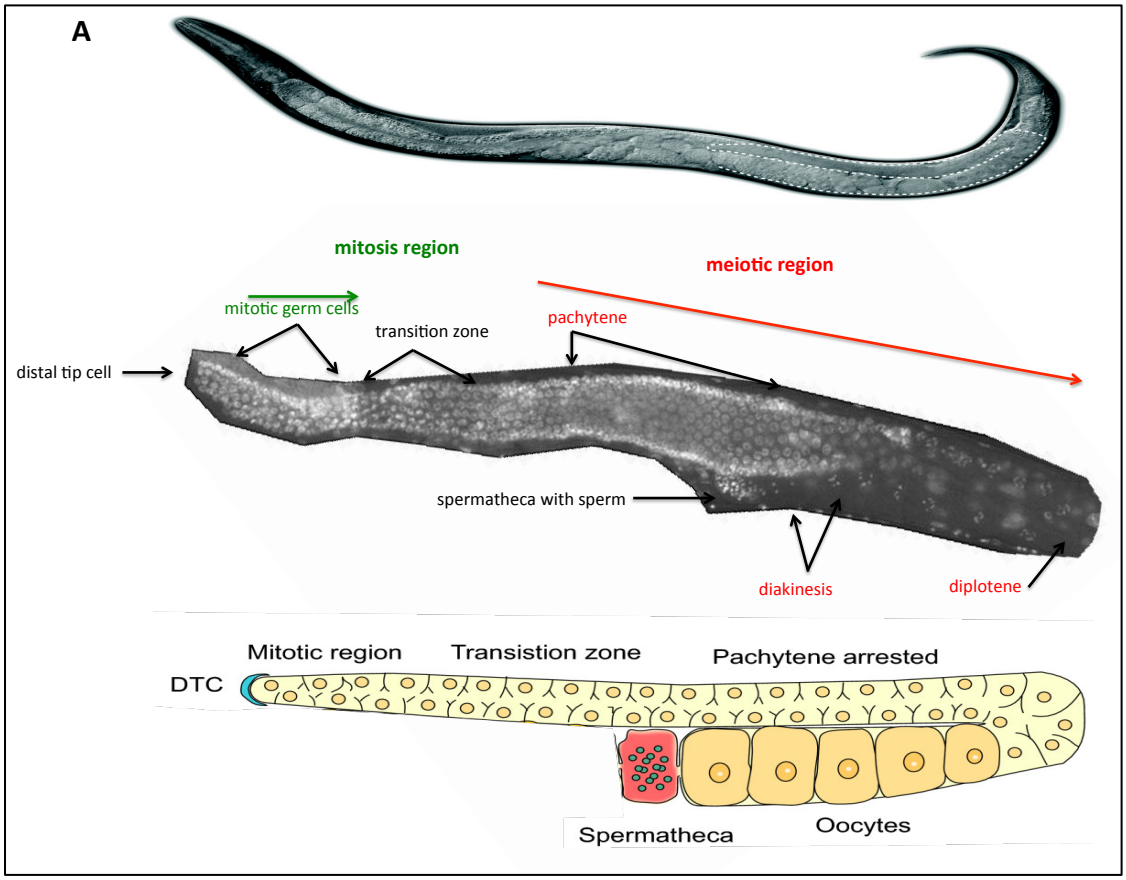


Figure 1.3, One arm of the hermaphrodite U shape gonad with its germline inside

The germline of an adult hermaphrodite fixed and stained with DAPI. The germline extends from distal-tip cell to spermatheca. Regions corresponding to stages of mitosis, and meiotic development are marked in green or red. The oocytes individuate from the rachis and begin to accumulate yolk proteins as the round the bend of the germline. An illustration diagramed of the DAPI image above. Cartoon scheme of the *C. elegans* gonad arm provide from (BOXEM 2006).

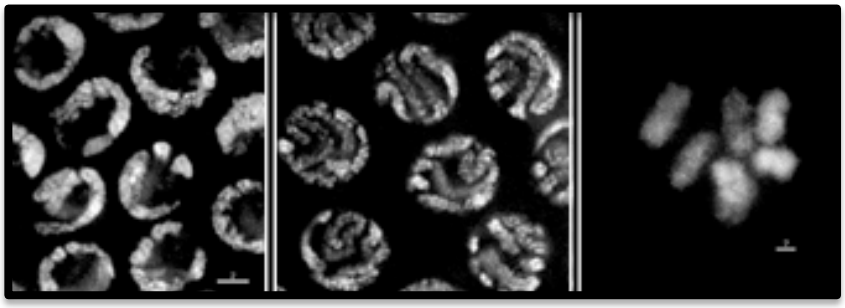


Figure 1.4, *C. elegans* adult hermaphrodite oocyte meiotic stages

Different germline maturation stages from the transition zone to the diakinesis stage. Germline is stain with DAPI, image has been crop and amplified.

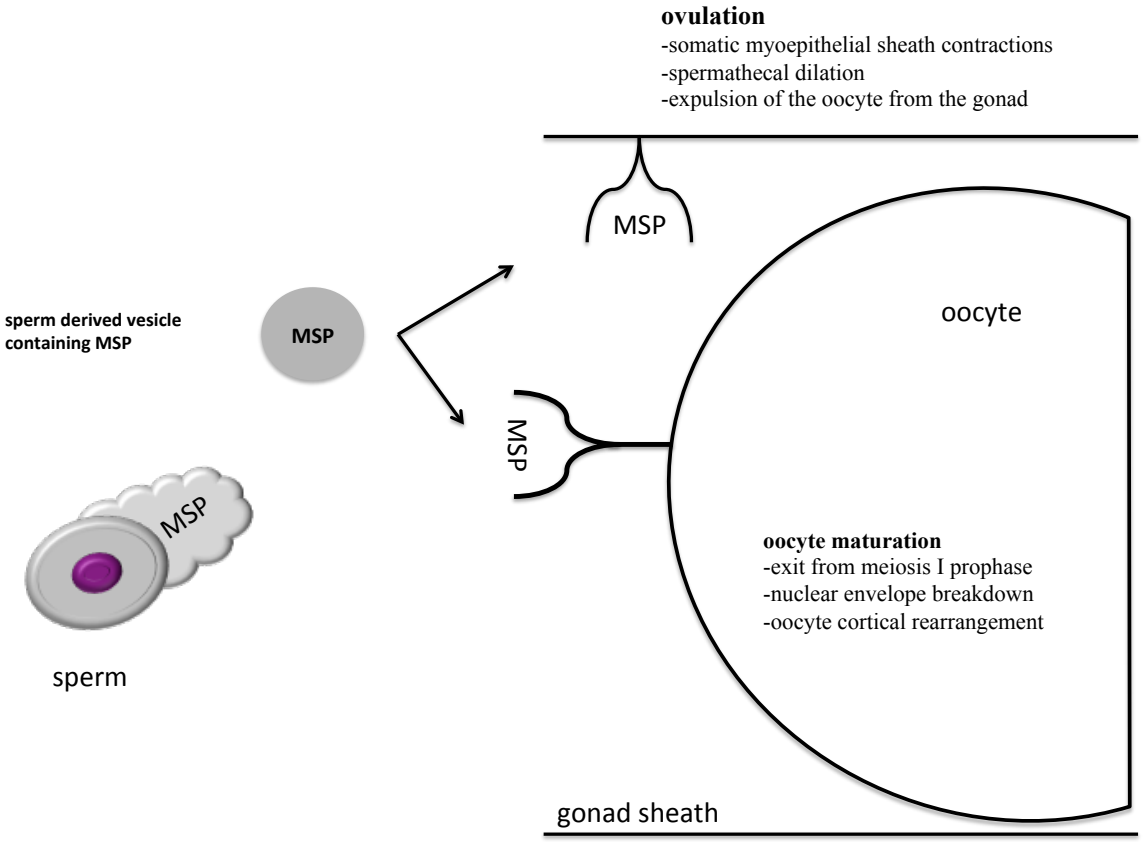


Figure 1.5, Nematode sperm specific small protein, Major Sperm Protein (MSP)

The 126 amino acid MSP protein is a core component of the sperm cytoskeleton and signaling molecule (hormone acting molecule), in which stimulates the -1 oocytes to be ovulated. MSP signal promotes oocye maturation, overlying of somatic sheath cells to dilate the spermathecal valve and force the spermatheca to engulf the -1 oocyte.

I.4 A mature sperm is a motile sperm: Spermatogenesis and Spermiogenesis as the two events that make this possible

The sperm, in sexually reproductive organisms, are mostly tiny cell but with a major role for existence. A sperm's role in the fertilization process is indispensible; it activates the oocyte, increases the ovulation rate, contributes the haploid paternal genome and triggers the developmental program that results in a new individual (PATRIZIO *et al.* 2003; SINGSON 2001). This cell contains nucleus in which it contains the paternal genetic material, mitochondria for energy generation, and cellular structures devoted to motility and interaction with the oocyte. They lack the machinery for RNA and Protein synthesis, as well as other component, such as endoplasmic reticulum and the golgi apparatus.

Sperm morphology will depend on the environment in which they function (i.e., sea water or soil) and will vary considerably, but despite the variability in their morphology, all sperm must accomplish similar tasks. First, they need to be motile and locate the egg. Second, they must interact and bind to the egg, often in a specie-specific manner. Third, they must fuse with or enter the egg and trigger the developmental program that results in a new individual (SINGSON 2001).

The *C. elegans* spermatozoon is a bizarre cell, but they are highly efficient at fertilizing; essentially every sperm fertilizes an oocyte (LAMUNYON and WARD 1995; SINGSON 2001; WARD and CARREL 1979). Like any other sperm cell from other organisms, their prime function is to fertilize oocyte. In *C. elegans*, the abundance of sperm in the spermatheca is highly important for fertilizing the oocyte, but also for oocyte maturation and ovulation (Figure 1.5) (KOSINSKI *et al.* 2005; KUWABARA 2003; MCCARTER *et al.* 1999a; MCNALLY and MCNALLY 2005a; MILLER *et al.* 1999).

Between a non-activated and activated sperm cell, the cell morphology will change drastically (Figure 1.6). In non-activated sperm it will contain a spherical cellular structure with no pseudopod formation. But in activated or mature spermatozoan, it will contain two main structures: cell body (which contains mainly the nucleus and mitochondria) and the pseudopod (Figure 1.6). The pseudopod is made-up of a dynamic network of protein polymers, which are needed mostly for sperm movement and interaction with the oocyte. Despite lacking a flagellum to help them move by swimming (like in mammalian sperm), the nematode spermatozoa crawl using the pseudopod (ROBERTS and STEWART 1995; ROBERTS and STEWART 2000; SEPSENWOL *et al.* 1989; SEPSENWOL and TAFT 1990; THERIOT 1996; WOLGEMUTH *et al.* 2005).

Even among other types of crawling cells nematode spermatozoa are distinctive. Crawling cells typically achieve their motility through actin and myosin based cytoskeleton, but nematode sperm have little of these proteins (NELSON *et al.* 1982). In spite of having centriole, they also lack of tubulin another major cytoskeletal component of most motile cell types. Rather, their pseudopod cytoskeleton is composed mainly of MSP, which compromises almost 15 % of their protein.

Ascaris sum as been essential for most of the biochemical and microscopic studies leading to our understanding of the MSP cytoskeleton on sperm cells, because of their quantities and large size (~25µm long) compared to *C. elegans* sperm (~6µm long). The MSP from *Ascaris* is 86% identical to *Caenorhabditis* MSP in sequence (SCOTT *et al.* 1989). The *Ascaris* sperm pseudopod contains a complex filamentous projection composed of a tube-shaped fiber meshwork of polymerized MSP made up of about 15-20 branched fiber complex, each about 200 nm wide, called villipodia. The proximal end of

the pseudopod membrane structure disappears near the cell body. The spaces between fiber complexes are filled with more diffuse fiber networks.

This MSP cytoskeleton structure is very dynamic, in which the fiber complex assembly and disassemble in a rapid fashion. The fiber complex begins assembling at the pseudopod leading edge occurs simultaneously with the network's disassembly at the pseudopod base, where it joins the cell body. This assembly and disassembly process proceed at the same rate, so that the entire cytoskeleton undergoes a treadmilling. The length of this protein fiber network remains constant; as the sperm cell crawls forward, individual landmarks on the cytoskeleton remain stationary with respect to the substrate. Developed *in vitro* assay system from *Ascaris* sperm extract recapitulates MSP cytoskeleton formation (ITALIANO *et al.* 1996). When ATP is added to the extract, formation of the tube-shaped fiber meshworks of MSP filaments is observed, similar to those seen in live sperm. Each of these fiber tubes is attached to a membrane vesicle at the end. These vesicles are derived from inside-out fragments of the pseudopod plasma membrane, and contain a phosphotyrosylated protein. The forward movement of the pseudopod leading edge can occur directly from MSP polymerization, by the growth of the meshworks of MSP assembly onto the vesicle-attached end. This motility process is similar to the proposed mechanism for actin-based motility in more conventional cells (THERIOT 1994; THERIOT 1996; THERIOT and MITCHISON 1992)

A mayor contributor for sperm development is the pH in the environment and in the cell. The Moerland lab, found strong correlation evidence between the assembly state of MSP and intracellular pH in *Ascaris* sperm were observer by using Fluorescence imaging microscopy cell loaded with pH sensitive dye, 2',7'-bis-(2-carboxyethyl_-5-(and 6)-

carboxyfluorescein acetoxymethyl ester (BCECF-AM) (KING *et al.* 1994a). In spermatid in which the MSP is not polymerize, had a more acidic environment pHi of 6.2, whereas in primary spermatocytes, in which all MSP is assembled in to filaments in fibrous bodies, had a pHi of 6.8.These researches further found that sperm activation prevented this rise. Interestingly, actively motile spermatozoa consistently exhibited a pH gradient of 0.15 units along the length of the pseudopod, from the most acidic region at the base to the most alkaline region at the tip. Abolishing the gradient by adding weak acids resulted in complete disassembly of the MSP cytoskeleton and retraction of the pseudopod. Upon washing the acid out, a pHi gradient was reestablish and the MSP cytoskeleton reformed.

Roberts and colleagues were able to uncouple *Ascaris* sperm cytoskeleton polymerization and depolymerization while monitoring cell movement by video microscopy by manipulating intracellular pH (ITALIANO *et al.* 1999; ITALIANO *et al.* 2001). They used conditions that prevented the MSP polymerization, but left depolymerization at the cell body unaffected causing the leading edge and the bulk of the pseudopod to cease movement, allowing the cell body to continue its forward motion. These experiments suggested a "push-pull" model for spermatozoan motility. In the sperm motility model the MSP polymerization propels the front of the pseudopod forward, while depolymerization at the pseudopod base pulls the cell body forward.

In similar experiments done by this group of researchers, successful blocking cytoskeleton treadmilling (and crawling) was accomplish by treating the sperm with phenylarsine oxide (PAO), in which is a protein tyrosine phosphatase inhibitor. By treating the cells with dimercaptopropanol, a PAO atagonist, the effect was completely reversed. These experimental results, plus the previous finding of a phosphotyrosine

protein in vesicle in the *in vitro* motility reconstitution assay, implicate protein-tyrosine phosphorylation (or dephosphorylation) in the pseudopod cytoskeleton dynamics.

There are enough reason to justify an interest in learning the development and function of this unique and very efficient cell. Beyond this, the workings of nematode sperm may contribute to newer understanding of important processes like cellular development, cell-to-cell interaction, assembly and dynamics of cytoskeletal filaments, cellular motility and behavior of disease in higher organism, such as our own specie. Indeed, recent studies on nematode sperm are indicating significant clues on the basis, Alzheimer' s disease (HARRISON 1995), deafness (YASUNAGA *et al.* 2000), heart disease, muscular dystrophies (BASHIR *et al.* 1998; DAVIS *et al.* 2000) and cellular movement (ROBERTS and STEWART 2000; THERIOT 1996).

Through research done in *C. elegans* and those on the parasitic nematode, *Ascaris suum*, have provided a fairly clear emerging picture of the cellular components and processes of spermatogenesis and spermiogenesis, the molecular mechanism for these processes are still not understood fully (L'HERNAULT and ROBERTS 1995; ROBERTS and STEWART 1995; THERIOT 1996). Similar to other sperm, such as mammalian sperm, *C. elegans* sperm differentiation produces unique morphological cell structure. These morphological cell structures are required for species-specific fertilization such as acrosome formation in mammals and membranous organelle formation in *C. elegans.* These unique cell structures are needed for finding interacting and fusing with the female gamete to form a new organism. In the *C. elegans* model organism, the spermatogenesis pathway as been highly study, many mutants have been identified during each step of this

process, including a number that act during the acquisition of sperm maturation and motility, referred to as spermiogenesis (L'HERNAULT 2006).

Research focused on spermatogenesis and spermiogenesis in *C. elegans* has also offered highly informative knowledge for evolutionary divergence. Some species of *Caenorhabditis* such as *elegans* or *briggsae* are androdioecious, having hermaphrodites and males. While other species of *Caenorhabditis* such as *remanei* are gonochoristic having female and male sexes. Thus the divergence of the reproductive systems in androdioecious and gonochoristic species, which are closely related, offers an excellent tool for studying evolution in development (BALDI *et al.* 2009). Recent work on the pathway, which regulates whether germ cells form sperm or oocytes, has given insight into the evolution of hermaphroditism in *Caenorhabditis* (BALDI *et al.* 2009). This work made use of the inherent differences in the regulation of germ cell differentiation between hermaphrodites, which must produce eggs and sperm, and males, which produce only sperm. A similar dichotomy in differentiation also exists during spermiogenesis in hermaphrodites and males (GELDZILER *et al.* 2005b; MINNITI *et al.* 1996; NANCE *et al.* 2000; NANCE *et al.* 1999; SHAKES and WARD 1989). As previously discussed, hermaphrodites produce first sperm and then oocytes.

In hermaphrodites the first ovulation pushes the sessile spermatids into the spermatheca and stimulates spermiogenesis (Figure 1.2) (HUBBARD and GREENSTEIN 2000). Males the spermiogenesis process diverges, as the sperm must be transferred with the seminal fluid during mating in the sessile, undifferentiated form. Mutant studies in *C. elegans*, have shown that premature activation of the male sperm prevents proper transfer to the hermaphrodite reproductive tract (STANFIELD and VILLENEUVE 2006). Research on

the spermiogenesis pathway also has demonstrated that different molecules, which act in the spermiogenesis pathway between males and hermaphrodite in *C. elegans*, appear also to diverge (SHAKES 2011; SMITH and STANFIELD 2011). Therefore, the differences between activation methods in hermaphrodites, females, and males could be easily exploited to study the evolution of fertilization.

The process of spermiogenesis occurs in a brief series of well-defined and coordinated events. Before each gamete (sperm and egg) is properly formed and fully capable of fertilization, both male and hermaphrodite derived spermatozoa (male gamete); meiosis must occur and produce a haploid germ cell. Then differentiation events must follow for the sperm to gain motility and capacity for fertilization. This differentiation process in which an immature sperm cell becomes a mature sperm cell occurs in the absence of *de novo* protein synthesis. During the development and formation of the haploid gamete, the ribosomes, actin and tubulin are packaged into the residual body, in which then must be extruded from immature spermatocyte (L'HERNAULT 2006). Thus, the maturation of sperm must occur with a limited body of resources.

Within seconds after receiving the signal to differentiate, the first of a series of profound changes in the spermatid plasma membrane are apparent. Thin dynamic cellular membrane protrusions called microvilli, that form a spike like formation in which first appear over the surface of the spherical immature sperm cell called a spermatid (Figure 1.7). These cellular extensions congregate at one pole of the cell, where they coalesce and expand to form a single pseudopod covered with numerous knobby projections, termed villopodia (Figure 1.7).

This fully mature sperm (spermatozoon) instantly starts crawling in the direction of the pseudopod (NELSON and WARD 1980). This sperm structure called the pseudopod joins the cell body of the mature sperm. In transmission electron micrographs the mature sperm appears to form a boundary between the pseudopod and cell body. This boundary seems to form of a thick layer of laminar membranes or vesicles (KING *et al.* 1994b; NELSON and WARD 1980), which may function to maintain the recycling of pseudopod membrane.

Another membrane transformation occurs at the level of the plasma membrane surrounding the cell body. This time it is involves the membranous organelle (MO), a membranous structure unique and essential to nematode sperm development. This organelle, changes in shape and function throughout spermatogenesis. During early sperm development serves as a transport vehicle to move necessary molecules from spermatocytes into the developing spermatids (ROBERTS *et al.* 1986; SHAKES and WARD 1989). At the initiation of spermiogenesis, before the formation of the pseudopod, most of the membranous organelle fuses with the plasma membrane of the cell body. This fusion forms stable fusion pores in which expel their glycoproteinaceous content to the outside of the cell. Is still unclear the regulators and cellular components that controls this process, but it is known by previous research that it's essential for proper sperm function. Some mutants with defective MOs functions, failed to fuse and these spermatozoa form shortened pseudopodia and are non-motile.

Equally dramatic and crucial to the membrane changes accompanying spermiogenesis are the transformations of the cytoskeleton. Nematode spermatozoa are

distinctive among amoeboid cell in that they contain almost no actin, tubulin or myosin (NELSON *et al.* 1982), these molecules typically are accountable for motility functions in eukaryotic cells (CRAMER *et al.* 1994; THERIOT 1994; THERIOT 1996). In contrast, the interior of the *C. elegans* spermatozoon pseudopod is largely composed of a small, basic protein call major sperm protein, or MSP (WARD *et al.* 1988; WARD and KLASS 1982). This critical cytoskeletal protein comprises about 15% of the total protein and 40% of the soluble protein of a *C. elegans* sperm cell. MSP is synthesized in spermatocytes, where it is assembled into paracrystalline arrays of filaments, called fibrous bodies (FBs). It's needed for pseudopod formation and as a sperm signal molecule to both the soma and oocyte indicating the presence of sperm (CHENG *et al.* 2008; MCGOVERN *et al.* 2007). Each FB forms associate intimately with a membranous organelle, to form the FB-MO complexes that segregate to the budding spermatids. Rapidly after a spermatid detaches from its residual body its MOs release their FBs and MSP depolymerizes distributes freely throughout the spermatid's cytosol, where it remains dispersed until the beginning of spermatogenesis.

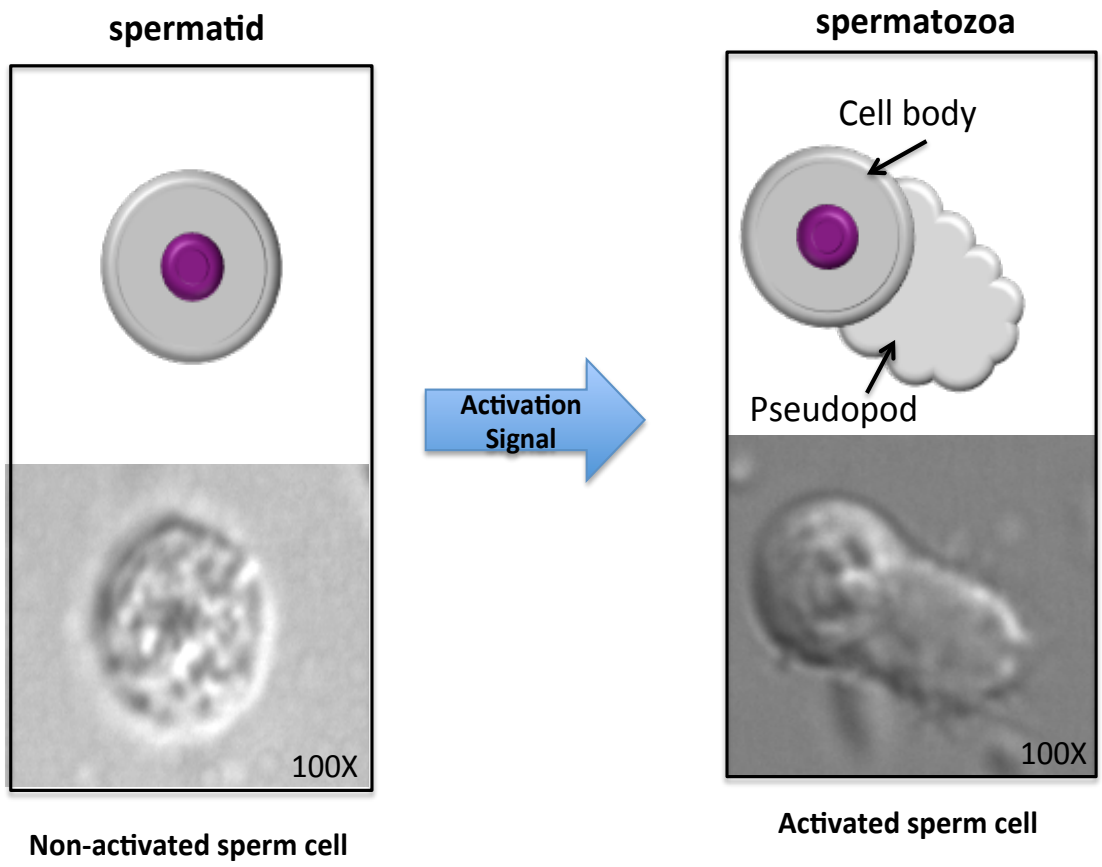


Figure 1.6, Non-activated spermatid and activated spermatozoa cell

Non-activated sperm are spherical cellular structure with no pseudopod formation. But in activated or mature spermatozoon, it will contain two main structures: cell body and the pseudopod. The pseudopod is made-up of a dynamic network of protein polymers of MSP, needed mostly for sperm movement and interaction with the oocyte.

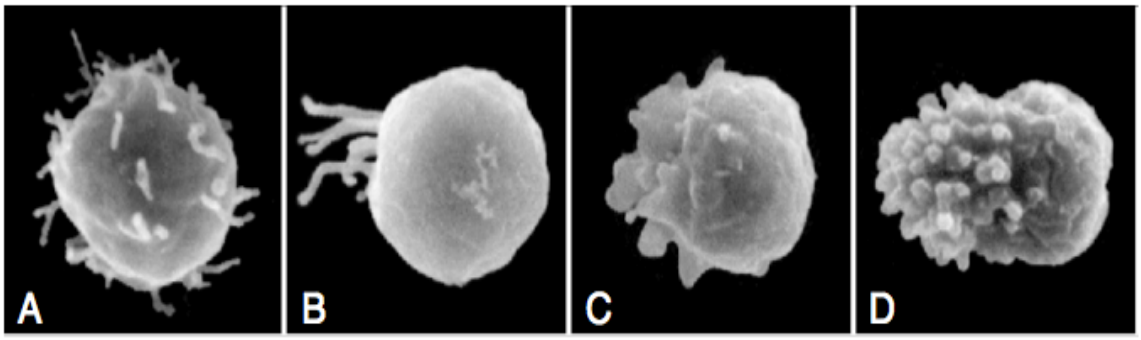


Figure 1.7, Scanning electron micrograph of the spermiogeneis process in *C. elegans* sperm

A spermatid prior to spermiogenesis showing microvilli distribute around the cell surface (A). Microvilli aggregation at one pole of the cell (B). Microvilli fused, forming a pseudopod (C). The end result of spermiogenesis, a mature spermatozoon form (D). Scanning electron micrographs adapted from (MUHLRAD 2001; NELSON and WARD 1980).

I.5 What are the molecules and the molecular events that trigger the spermiogenesis pathway?

During the process of spermiogenesis an immature sperm (spermatid) develops into a motile mature sperm called spermatozoon. Different cellular pathways tightly regulate this process. Both male and hermaphrodite sperm require "activation" by external signals. In males, the signal is probably present in the seminal fluid, and supplied during copulation. Hermaphrodites presumably have the activator in their spermathecae. The nature of the activator signal is unknown in *C. elegans*, but in *Ascaris suum*, a partially purified glycoprotein extracted from the vas deferens can induce spermiogenesis in vitro (SEPSENWOL and TAFT 1990).

C. elegans is useful model organism for studying spermatogenesis, by using genetic techniques. Spermatogenesis-defective mutants are given the "spe" designation indicating their defect in the larger process of spermatogenesis. These mutants can be identified as self-sterile hermaphrodites whose sterility can be rescued by mating with wild-type males. By using different phenotypic and genetic approaches over 50 different genes in the spermatogenesis pathway have been identified (L'HERNAULT and ROBERTS 1995).

Five of these genes *spe-8, spe-12, spe-19, spe-20* and *spe-27*, have been previously elucidate as members of the spermiogenesis pathway, but do not appear to be require for earlier steps in sperm development in *C. elegans* (GELDZILER *et al.* 2005b; MINNITI *et al.* 1996; NANCE *et al.* 2000; NANCE *et al.* 1999; SHAKES and WARD 1989). Genes of this class are referred to as *spe-8* class genes, based on the canonical phenotypes of the spe-8 gene, the first elucidated in the pathway. Worms with mutations in these genes are

characterized by self-sterile hermaphrodites, and fertile males capable of siring progeny, the ability to trans-activate hermaphrodite spermatids, and the ability to be suppressed by the *spe-6* mutation (MUHLRAD 2001; MUHLRAD and WARD 2002; NISHIMURA and L'HERNAULT 2010).

The *spe-6* gene encodes a protein serine/threonine casein type kinase, necessary to inhibit spermiogenesis until the proper seminal vesicle fluid signal is received (MUHLRAD 2001; MUHLRAD and WARD 2002). After the spermatogenesis-promoting signal is receive it would be carried through the *spe-8* class genes and lift the inhibition by *spe-6* (MUHLRAD 2001; MUHLRAD and WARD 2002).

Microscopy techniques such as light magnification and electron magnification have been done on mutant *spe-8* class animal's spermatids, they appear to be wild-type. Unmated mutant hermaphrodite produce normal numbers of spermatids but are self-sterile, because spermatids fail to undergo spermiogenesis to form functional spermatozoa. When mated to sterile males the *spe-8* class hermaphrodites become self-fertile (Transactivated). This demonstrate that in *spe-8*, *spe-12*, *spe-19*, *spe-27*, *spe-29* mutants, the hermaphrodite's sperm have the required machinery to effect spermiogenesis by the male seminal fluid pathway. It also suggests that the mutant hermaphrodite either lacks the activators signal or that her spermatids do not respond to her own signal but do respond to the male signal. Additional result indicates that the fault in fertilization does not lie in one of the steps of sperm production.

Artificial insemination experiments have shown that when only spermatids (but not seminal fluid) from *spe-27* mutant males were introduce into a mutant hermaphrodite, neither they nor the hermaphrodite's own spermatid undergo spermiogenesis. This result

is different when wild type spermatids artificially inseminated into a mutant hermaphrodite can undergo spermiogenesis, but do not cause the hermaphrodite's spermatids to do so. These experiments demonstrate that transfer of sperm form the males is not sufficient to initiate spermiogenesis of a mutant hermaphrodite' self-sperm. Conversely, if a *spe-27* mutant hermaphrodite is mated to a male harboring a mutation that prevents that male form producing sperm (e.g., *fog-1* mutants, which have feminized germlines) its sperm will still undergo spermiogenesis, indicating that the male's sperm are not necessary to cause activation of the hermaphrodite's sperm (ELLIS and KIMBLE 1994; LAMUNYON and WARD 1994; LAMUNYON and WARD 1995; MINNITI *et al.* 1996).

It has been previously hypothesized that these *spe-8* class molecules function in the same pathway with one another, due to their similar phenotypes and additive effects, such as reduced brood sizes, when examined in double mutants (NANCE *et al.* 2000). The activation signal and the molecular regulation for this pathway are unclear, although the activation of the pathway by proteases may provide a possible clue.

It is understood that an unidentified activation signal would bind to a receptor on the surface of the spermatid beginning sperm activation (MUHLRAD 2001; MUHLRAD and WARD 2002). Because this phenotypic event affects males and hermaphrodites differently, male worms with mutations in any of the *spe-8* class of genes are fertile, while their hermaphrodite counterparts are sterile, it is believed that separate male and hermaphrodite sperm activators exist (GELDZILER *et al.* 2005b; MUHLRAD 2001; MUHLRAD and WARD 2002; SHAKES 2011; SMITH and STANFIELD 2011). It also suggests that the mutant hermaphrodite either lacks the activators signal or that her spermatids do not respond to her own signal but do respond to the male signal.

By using this model, the male *C. elegans* would function through a pathway, which does not include the spe-8 molecules, or which bypasses, partially or fully, the *spe-8* class of molecules (GELDZILER *et al.* 2005b; MUHLRAD 2001; MUHLRAD and WARD 2002; SHAKES 2011; SMITH and STANFIELD 2011). There are also notion that the defected spermiogenesis mutants genes is in their sperm, and not in the production of an extracellular signal, is further supported by an in vitro phenotype exhibited by spermatids form both males and hermaphrodites from all of these mutants.

Experiments have shown that mutants in the *spe-8* class of genes do have a negative impact on male fertility, such as reduced competitiveness and reduced ability to undergo *in vitro* activation in the presence of certain chemicals such as protease (e.g. pronase) and chemicals that raise intracellular pH (e.g. triethanolamine (TEA) (GELDZILER *et al.* 2005b; MINNITI *et al.* 1996; NANCE *et al.* 2000; NANCE *et al.* 1999; SHAKES and WARD 1989). Spermatids from wild-type worms can be activated to undergo spermiogenesis in vitro by adding these classes of activator molecules. Spermatids from the spermiogenesis mutants can activate normally in vitro when exposed to TEA, and the spermatozoa so formed are viable a assayed through artificial insemination experiments (LAMUNYON and WARD 1994). However, when exposed to Pronase these mutant spermatids seen to begin to undergo spermiogenesis, but instead of forming a motile pseudopod, they form long rigid spikes radiating outward from the cell periphery, after which further morphogenesis ceases (MINNITI *et al.* 1996; NANCE *et al.* 2000; NANCE *et al.* 1999; SHAKES and WARD 1989).

Already some characterized molecules in the *spe-8* class of mutants have provided the strongest clues to the process of spermiogenesis in *Caenorhabditis elegans*. Is not yet

clear but it's hypothesized that one of the already discovered *spe-8* genes might act as a transmembrane receptor in the spermatid plasma membrane and that this membrane protein receptor will receive the sperm activation signal in hermaphrodites. The candidates for the transmembrane receptors molecule are SPE-12, SPE-19, or SPE-29. However between these three candidates membrane protein SPE-19 is the more likely candidate for the receptor molecule for the sperm activation signal. Because SPE-12 is believed to sit on top of the plasma membrane rather than being embedded directly into it, and SPE-29 is a really small protein. It is also thought that if SPE- 19 is the transmembrane protein receptor in the plasma membrane of the spermatid, it will be changed after sperm activation, either by cleavage or modifications such as phosphorylation.

The spermiogenesisi activation pathway of spe-8 class of proteins has been hypothesized as follow: SWM-1 or another protease inhibitor is stimulated to release its protease upon release of the activation signal (STANFIELD and VILLENEUVE 2006). The protease then cleaves SPE-19 and SPE-8 or another kinase is stimulated to phosphorylate its cytoplasmic tail. The cleaved or phosphorylated tail of SPE-19 is released and interacts downstream with other members of the *spe-8* class of molecules eventually leading to an intracellular increase in pH. It is very clear in this hypothesis that not all members of the activation pathway have been discovered yet. Therefore, further members of the sperm activation pathway must be discovered and elucidated, before the full processes of sperm activation pathway are known in *Caenorhabditis elegans*.

The following chapter of this dissertation describes a new member of the *spe-8* gene class, *spe-43*, which may serve a central role in the spermatogenesis process in *C.*

elegans spermatids. This new study can enlighten us about important cellular processes in higher organism, including humans. The effect of *spe-43* mutations on spermiogenesis suggest that the gene may have a vital role in the decision making of the spermatid to complete its differentiation process to a mature spermatozoon. Such a decision in the regulation of differentiation is one of the most important developmental problems faced by metazoan cells. Defects in differentiation can lead to medical consequence such as tissue and organ deformity and cancer. Nematode spermiogenesis is a particular useful model for examining cellular differentiation. Because it involves entirely post-translational control, it occurs rapidly, is triggered by extrinsic signals, results in a dramatic morphological transformation, can be observed in vitro, and is amenable to genetic analysis.

Chapter 2

II. 1 An introduction to the *spe-43* gene and its role in *C. elegans* spermatogenesis

Developing sperm in the nematode *Caenorhabditis elegans* accumulate as immotile spherical spermatids, which are incompetent for fertilization. In response to extracellular signals, the cell undergoes a differentiation process called spermiogenesis. After the activation signal has been received by a receptor(s), a rapid and dramatic morphological transformation to mature amoeboid spermatozoa, with the ability to crawl occurs. Because this maturation happens without any new mRNA or protein synthesis, spermiogenesis initiation affords the opportunity to study a signaling pathway that acts post-translationally to regulate cellular morphogenesis in a genetically tractable organism. Furthermore, nematode sperm afford a unique system for studying the acquisition of cellular motility because they derive their crawling motility from an unconventional cytoskeleton, containing neither actin or myosin filaments (ITALIANO *et al.* 2001).

Many sperm development defective mutants (*spe*) have been isolated as self-sterile hermaphrodites that can be rescued by crossing with wild-type males (KIMBLE and WARD 1988; L'HERNAULT 2006). Several genes involved in transducing the signal that initiate spermatogenesis have been characterized trough the years such as: *spe-8*, *spe-12*, *spe-19*, *spe-27* and *spe-29* (GELDZILER *et al.* 2005b; LHERNAULT *et al.* 1988; MINNITI *et al.* 1996; NANCE *et al.* 2000; NANCE *et al.* 1999; SHAKES and WARD 1989). In this chapter, I describe the isolation of a new gene called *spe-43* that is required for the spermatogenesis pathway in hermaphrodites.

The *spe-43* gene was identified in an Ethyl methanesulfonate (EMS) mutagenesis screen for mutant genes with fertility defects. It was immediately classified at a *spe-8* class gene, based on its unique characteristic mutant phenotypes. Mutant hermaphrodites were self-sterile hermaphrodites, but the males were fertile. When compared with wild-type hermaphrodites, the *spe-43* uterus is filled with unshelled, non-developing oocytes with endomitotic nuclei (Figures 2.1). These self-sterile hermaphrodites are capable of producing outcross progeny with fertile males, either wild type or *spe-43,* indicating that both the soma and oocytes are phenotypically normal. Conversely, the mutant males are predominantly phenotypically normal, and capable of siring outcross progeny with hermaphrodites. Furthermore, germ cell production in the hermaphrodite appears phenotypically normal, and spermatids appear normal under light microscopy. However, when exposed to Pronase to activate the sperm in vitro, these mutant spermatids seem to begin to undergo spermiogenesis, but instead of forming a motile pseudopod, they form long rigid spikes radiating outward from the cell periphery, after which further morphogenesis ceases.

II.2 Materials and Methods

Strains and general nematode methods

Maintenance and general genetic crosses of *C. elegans* strain were performed as described in (BRENNER 1974) and (LEWIS and FLEMING 1995; WOOD 1988). Genetic nomenclature follows that describe by (HODGKIN 1995; HORVITZ *et al.* 1979). For temperature-sensitive strains the permissive temperature was 16ºC and the restrictive temperature was 25ºC. For some experiments nematodes were reared at 20ºC. Strains used in experiments included: wild-type variety Bristol, strain N2; *spe-43* (*eb63*); *spe-13*(*hc137*); *spe-45 (as8); him-5* (*e1490*); *spe-19(eb52)*; *spe-8(hc53)dpy-5(e61)*; *dpy-5*(*e61*); *spe-6*(*hc163*); *spe-8*(*hc40*); *spe-9*(eb19); *fer-1* (hc13ts) *him-5* (e1490) ; *fem-1* (e1965); MT4150 (*unc-17*; *dpy-4*);The Hawaiian strain CB4856 was used for SNP mapping. The *spe-43* strain was identified in an Ethyl methanesulfonate (EMS) mutagenesis screen for genes with fertility defects by Dr. Andrew Singson while in the lab of Dr. Steve L'Hernault at Emory University.

spe-43 (eb63) stable male strain generation

Wild type N2 male worms were cross to homozygous *spe-43* hermaphrodite at 20ºC in a 4:1 mating ratio to generate *spe-43*/+. After mating for 2 days, the parental worm were removed, to prevent parental and progeny mating. The F1 heterozygous *spe-43*/+ male progeny were pick and cross to *spe-43* homozygous hermaphrodites at 20ºC for 48 hrs in 4:1 mating ratio. Male F2 worm progeny were isolated and single mated to homozygous *spe-43* hermaphrodite. F3 *spe-43* homozygous hermaphrodite were isolated

in to individual 24-well plates and score for infertility, 24/24 *spe-43* homozygous hermaphrodite were sterile.

Statistical analyses

Appropriate statistical tests for significance (t test, ANOVA) were performed using Minitab (Minitab Software, Inc. State College, PA).

Progeny counts

To determine brood sizes, virgin L4 hermaphrodites were picked onto individual OP50-seeded Petri plates at 16ºC, 20ºC and 25ºC and transferred to fresh plates daily until they stopped laying eggs. They were allowed to self-fertilize for at least 24 hours, parents were removed to allow all viable eggs to hatch. Oocytes/progeny were counted daily, live progeny and dead embryos were counted daily as they were aspirated from the plate with a pipette and vacuum. Hermaphrodites that died or missing from the plate, were excluded from the analysis. All errors reported are given as standard error of the mean.

Light microscopy

Differential interference contrast microscopy (DIC) images of live worms or dissected worms were observe on a Zeiss Universal microscope and captured with a Optronics microscope camera using Magnafire imagine software (Karl Storz Industrial – America, Inc. El Segundo, CA).

Diamidino-2-phenylindole (DAPI) staning

To examine sperm cell nuclei, adult hermaphrodite worms were washed once in M9, then fixed in cold methanol for 2-4 minutes then stained with DNA-binding dye 4, 6-diamidino-2-phenylindole (DAPI) (Vectorshield, Burlingame, CA) and mounted on 2% agarose slides for viewing using both fluorescence and Nomarski imaging.

Male fertility assay

To prove the fertility of male *spe-43* worms, two experiments were performed. Young adult males were cross to a L4 *dpy-5* hermaphrodite at 16ºC, 20ºC and 25ºC in a 4:1 ratio for 48 hrs. After the 2 days, the male worms were removed from the plates and only the *dpy-5* hermaphrodites were left on the plates. Only outcross wild type broods were counted daily until the *dpy-5* hermaphrodite had stopped producing outcross progeny. After each day the *dpy-5* hermaphrodites were transfered into new OP-50 seeded plates. Hermaphrodites that died, missing or not laying outcross progeny on the plate, were excluded from the analysis. As a positive control, N2 wild-type worms and *him-5* male worms were also mated to *dpy-5* hermaphrodites.

To further confirm male fertility, male worms were cross to L4 fem-1 hermaphrodite in a 4:1 mating ratio at 25ºC for 48 hrs. After the 2 days, the male worms were removed from the plates and only the fem-1 hermaphrodites were left on the plates. Outcross progeny were counted daily until the fem-1 hermaphrodite had stopped producing outcross progeny. After each day, the fem-1 hermaphrodites were transfers into new OP-50 seeded plate. Hermaphrodites that died, missing or not laying outcross progeny on the plate, were excluded from the analysis. As a positive control, N2 wild-

type worms and *him-5* male worm were to a fem-1 hermaphrodite background. Has a negative control, *spe-13 (hc-137ts)* was cross to fem-1 background.

Transactivation assay

spe-43 L4 hermaphrodites were crossed 1:4 ratio to *fer-1; him-5* homozygous males at 25ºC for 2 days, a temperature at which *fer-1* worm animals are sterile due to a sperm motility defect (Achanzar and Ward, 1997; Shakes and Ward, 1989b). The progeny for each *spe-43* hermaphrodite cross were counted. For control purpose we mated double mutant males *fer-1(hc13ts);him-5(e1490)* to *spe-9(eb19)* hermaphrodite and double mutant males *fer-1(hc13ts);him-5(e1490)* to *fem-1(e1965)* hermaphrodite worm at 25ºC for 2 days in a 4:1 mating ratio.

***In vitro* sperm activation**

L4 young males were isolated for 24 hrs in plate with OP-50 spotted on them. After 24 hrs the male worms reproductive tract was dissected in pH 7.8 sperm medium (SM) and dextrose solution both with and without the known in vitro activators Pronase (Garner et al., 1974) (200 Ag/ml) or triethanolamine (120 mM at pH 7.8).

***In vivo* sperm activation**

L4 males were crossed to *fem-1* hermaphrodites for 24-36 hours in a 4:1 mating ratio at 25ºC, a temperature at which *fem-1* mutant animals are sterile due to a feminization effect. After the time of mating *fem-1* hermaphrodite spermatheca was dissected in pH 7.8 sperm medium (SM) and dextrose solution.

Sperm measurements experiments

Sperm from the different worms were activated *in vivo* and observed in DIC microscopy (Zeizz Universal Microscope, Thornwood, NY) images were captured with a Optronics microscope camera using Magnafire imagine software (Karl Storz Industrial – America, Inc. El Segundo, CA). Sperm images were then analyze and measure by ImageJ computer software using a Mac OS X snow leopard.

Sperm motility assay

Sperm from the different worms were activated *in vivo* and observed in DIC microscopy (Zeizz Universal Microscope, Thornwood, NY) images were captured with a Optronics microscope camera using Magnafire imagine software (Karl Storz Industrial – America, Inc. El Segundo, CA). Sperm images were automerged by Adobe Photoshop software program and measure by ImageJ software using a Mac OS X snow leopard.

Sperm Competition assay

Twenty-four L4 dpy-5 hermaphrodites were picked and individual crosses to male's worms in a 1:4 ration mating, for 48 hrs. Adult worms were then removed from the plates, and progeny were scored after 2 days when the Dpy phenotype could be scored unambiguously. The number of both Dpy (self) and Non-Dpy (outcrossed) progeny were then scored as previously described (Singson et al., 1999).

Epistasis experiments with *spe-6*

Epistasis analysis with *spe-6* mutants was conducted to further link the *spe-43* gene to part of the *spe-8* class pathway involved in the spermiogenesis process. *spe-43* epistasis experiments were performed as described by (GELDZILER *et al.* 2005b; MUHLRAD 2001; MUHLRAD and WARD 2002) using the weak hypomorphic allele *spe-6*(*hc163*). Homozygous double mutants *spe-6*, *dpy-18* hermaphrodites were mated to either homozygous *spe-43* or *fog-2* (a non-spermiogenesis hermaphrodite- specific sterile control) males. F1 heterozygotes were plated and allowed to self-fertilize. Dpy F2s were then plated and scored for sterility. Approximately one fourth of all Dpy progeny would be expected to be sterile unless this phenotype was suppressed by *spe-6*(*hc163*). 0/210 Dpys were sterile when crossed to *spe-43* animals, while 42/143 Dpys were sterile using the *fog-2* control. To ensure that the homozygous *spe-43* class of animals was present, individual Dpy hermaphrodites were then crossed to homozygous *spe-43* males, and progeny were scored for sterility. Animals for whom all progeny were sterile (2 of 19) indicated that the *spe-43* homozygous class of animal was present.

Genetic mapping and Recombination mapping

A combination of different mapping techniques were utilize for mapping the *spe-43* genetic region at the right arm of chromosome IV. These different mapping techniques take advantage in the complementation and recombination events in *C. elegans* model. To assign the *spe-43* genetic region to a linkage group we perform a standard linkage mapping. Male *spe-43*(*eb63*) were crossed in a 4:1 ration to different double mutant markers for the five autosomal chromosomes in *C. elegans* has describe in (HOPE 1999).

These different mating crosses were carried out at 20ºC for 32 hours. The frequency of fertile recombinants, ***R,*** was calculated from the fraction of plates showing no offspring, f = (1- ***R)5.*** Plates that showed offspring were screened for the presence or absence of Dpy (Unc) animals.

Two and three factor mapping was conducted for determining the localization of spe-43 on chromosome IV. For two factor mapping, *spe-43* males were cross to single mutant markers *dpy-13* and to *unc-26* strain at 20ºC for 36 hrs at 4:1 ratio, the F1 recombination broods from the different crosses were isolated and the percent of recombinants was determine has describe (HOPE 1999). To perform the three-factor mapping, *spe-43* males were cross to a double mutant strain MT4150 (*unc-17*; *dpy-4*) at 20ºC for 36 hours at 4:1 ratio. The recombinants from the F1 progeny of the mating cross were isolated and the percent of recombinant was determined has describe (HOPE 1999).

Using deficiencies mapping, we also narrowed down the *spe-43* genetic region and to have a clearer boundary of this genetic region. Male *spe-43* worms were mated with two different deficiency worm lines (*sDf21, sDf22*) (HE/BAILLIE 1983a; HE/BAILLIE 1983b) at 20ºC for 36 hours at 4:1 ratio, has describe (HOPE 1999). Heterozygous broods from the different crosses were isolated and score for 100 % infertility. *sDf21*/*eb63* and *sDf22*/*eb63* were *spe* and therefor these deficiencies fail to complement *spe-43*, consistent with other mapping data.

To even narrow more the physical location of the *spe-43* genetic area to ~ 512,330bp region, SNP mapping was performed. Approximately, 12 cosmids and 10 sperm enriched genes were found in this genetic region. *unc-17*; *spe-43* and *spe-43*; *dpy-4* double mutants were created and used for SNP mapping. These worms were first crossed

into Hawaiian strain CB4856, and their progeny allowed to self-fertilizing. Recombinants were then picked and assayed for the presence of single nucleotide polymorphisms as describe in (FAY and BENDER 2006; HOPE 1999). SNP mapping using these *unc-17* non-*spe-43* (n = 10) and *dpy-4* non-*spe-43* (n = 50) recombinants enabled us to map *spe-43* to the 512,330 kbp interval between cosmids C25G4 (map position 6.17 cM, 12,744,715 – 12,743,016 bp) to F55G11 (map position 6.74 cM, 12,969,265 – 12,967,424 bp) an interval predicted to contain approximately 5 sperm-enriched genes and 17 cosmid on the right arm of chromosome IV (Reinke et al., 2004).

Complementation tests between *spe-43* and *spe-14*, *spe-45*, *spe-36*, *spe-17*

We performed a complementation test to determine if *spe-43* complemented to *spe-14, spe-17, spe-36* and *spe45*. We tested the ability of *spe-43* to complement the sterile phenotype of these other genes. To test this, we crossed to *spe-43* males to hermaphrodite of the different genes at 25ºC for 36 hrs. F1 hermaphrodites (*spe-14/eb63, spe-17/eb63, spe-36/eb63* and *spe-45/eb63*) were picked to individual plates and score for self-fertility at 25ºC. All the F1 progeny from the different crosses were fertile, meaning, that they complemented *spe-43*; they were not mutations in the same gene.

Preparation of *spe-43* Genomic DNA

For the Genomic DNA extraction we used a modified protocol from the original Andrew Fires Genomic DNA preparation protocol. Fifth teen small OP50 seeded 60mm plates full of mutant *spe-43* worms were use for making the DNA library. To remove the worms form the plate and from the OP50 bacteria they were wash off with M9 buffer

with 10% sucrose and collected in a falcon tube. To clean the worms and get rid of the OP50 bacteria from the worms, they were constantly washed and moved in a Revolver Rotator 360° Sample Mixer, rotisserie for 3 hours; vortexed and spun in a centrifuge at a top speed of 13,000 RPM every twenty minutes for 3 hours. After the washes were performed, the pellet was re-suspend by adding 225µl of worm lysis buffer with protease K to the ~25µl of worm pellet (Protease K; 10µl of 20mg/ml in TE pH 8.0, store at -20ºC)(Worm Lysis Buffer; 0.1M Tris-Cl pH 8.5, 0.1M NaCl, 50 mM EDTA, pH 8.0, 1% SDS. Store at RT) and moved to a 1.5ml eppendorf tube and incubated at 62ºC for 60 minutes. Samples were vortexed 4-5 times during the incubation and the solution should clear as the worms disintegrate. 15 µl RNase A was added and mixed in the sample by inverting ~25 times. Samples were then incubated at 37°C for 15–60 min. Then incubated for 3 min on ice to quickly cool the samples. 50µl of 4M NaCl was then added and mixed thoroughly by inversion. 40 µl CTAB solution was added to the solution and incubated 10 minutes at 37ºC. (CTAB/NaCl solution; 10 % CTAB in 0.7M NaCl; Dissolve 4.1 g NaCl in 80 ml distilled water and slowly add 10 g CTAB (Sigma M-7635) while heating and stirring. If necessary, heat to 65ºC to dissolve. Adjust final volume to 100 ml) After CTAB solution is added and incubated for 10 minutes at 37ºC, a phenol/chloroform DNA extraction and DNA ethanol precipitation were performed followed by drying the DNA and re-suspension in 20µl of TE. When nanodrop the DNA spectrophotometer, the final concentration should be100-700ng/µl.

II. 3 Results

The *spe-43* mutant phenotype: a sterile mutant

Until now five genes of the *spe-8* class spermiogenesis pathway have be characterize: *spe-8*, *spe-12*, *spe-19*, *spe-20* and *spe-27* (GELDZILER *et al.* 2005b; MINNITI *et al.* 1996; NANCE *et al.* 2000; NANCE *et al.* 1999; SHAKES and WARD 1989). Theses genes are involved in transducing a signal(s) that initiate spermiogenesis in *C. elegans* (GELDZILER *et al.* 2005b; MINNITI *et al.* 1996; NANCE *et al.* 2000; NANCE *et al.* 1999; SHAKES and WARD 1989). Virgin hermaphrodites mutated in any of these genes are self-sterile because their spermatids fail to activate to develop to maturity (spermatozoa). In contrast, mutant males have nearly wild-type fertility. Moreover mutant's hermaphrodites from this group that have mated (either to wild-type or males carrying the same mutations) are both cross and self-fertile. This odd phenotype suggest that spermatids respond differently to hermaphrodite and male-supplied spermiogenesis initiation signals "activators", and that mutants in these five genes are most deficient in their response to the hermaphrodite-supplied activator.

spe-43 was immediately classified as a *spe-8* class gene based on its unique mutant phenotypic characteristic. Microscopy techniques such as light magnification show that *spe-43* hermaphrodite worms have normal gonad morphology and their pre-fertilization events are the same as wild-type hermaphrodites (Table 2.1). But different from wild-type hermaphrodites, the *spe-43* uterus is filled with unshelled, non-developing oocytes with endomitotic nuclei. Another characteristic that distinguish *spe-43* hermaphrodite from wild type hermaphrodite is that on the OP50 culture plate you can observe shelled eggs in wild type worms and mesh of round opaque cell (unfertilized oocytes) in *spe-43*.

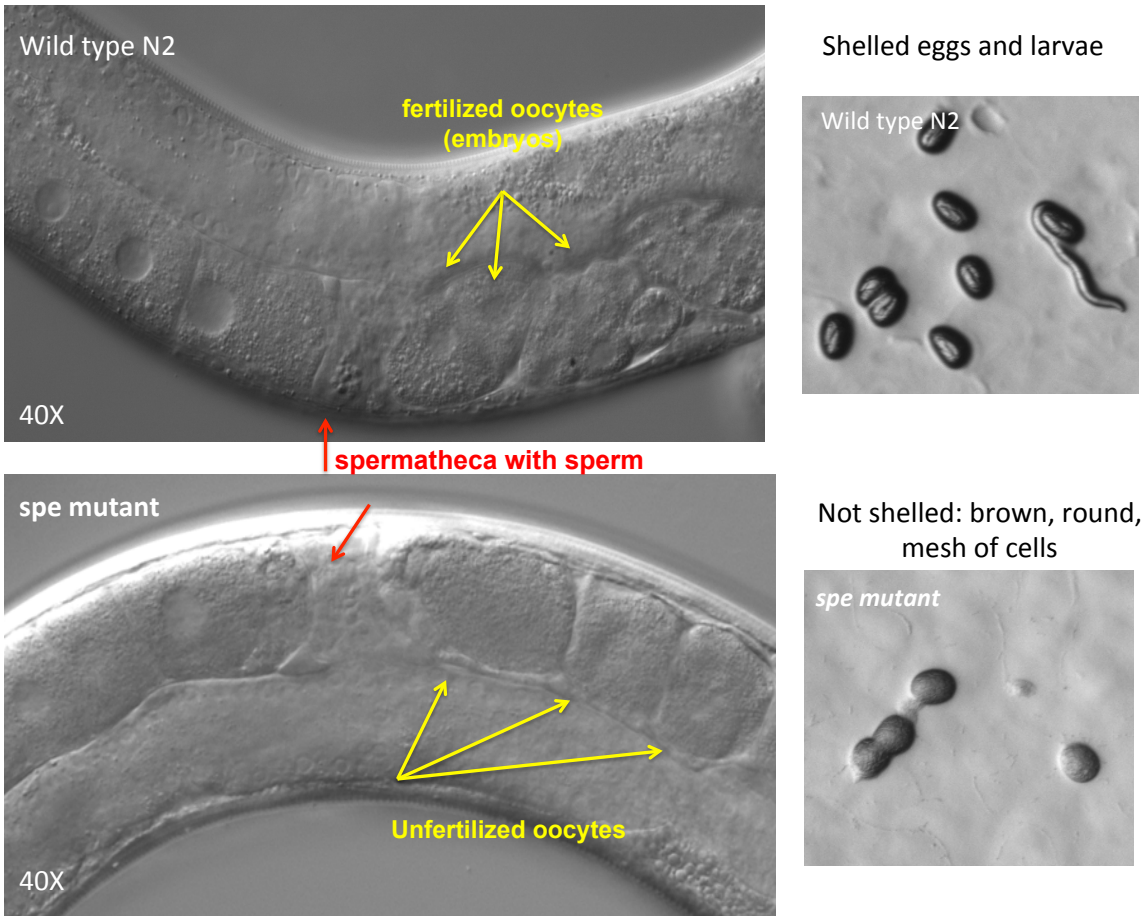


Figure 2.1, Example a mutant with *spe* defective fertilization phenotype identical to *spe-43* mutants

Wild-type hermaphrodites, contains embryos (fertilize oocyte in their uterus). The mutant uterus is filled with unshelled, non-developing oocytes with endomitotic nuclei. In OP50 culture plate you can observe shelled eggs for wild-type hermaphrodite worms and mesh of round opaque cells (unfertilized oocytes) for *spe* hermaphrodites.

Pre-fertilization events	spe-43 (eb63)
Gonad Morphology	Normal
Increase in Size of oocyte	+
Nucleolus Disappearance	+
Nuclear Migration	+
Nuclear Envelope Break Down (NEBD)	+
Cortical Rearrangement	+
Ovulation	+

Table 2.1, Normal pre-fertilization events in *spe-43*

Considering that *spe-43* mutant hermaphrodite is sterile, they have a normal gonad development, mutant worms have normal looking gonad morphology and their pre-fertilization events are identical to wild type hermaphrodites (Table 2.1).

Generation of *spe-43* (*eb63*) mutant stable male strain

The *spe-43* gene was identified in an Ethyl methanesulfonate (EMS) mutagenesis screen for genes defective in sperm function. It was immediately classified at a *spe-8* class gene, based on its unique characteristic mutant phenotypes. Taking advantage of its phenotypic *spe-8* class unique characteristics, I generated a stable homozygous *spe-43* mutant male strain. This approach was helpful because it was needed for further experiments and for better maintenance of the mutant strain.

Wild-type males were mated to L4 *spe-43* hermaphrodite in a 4:1 mating ratio, to generate *spe-43*/+. After mating 48 hours, the parental worms were remove, to prevent parental and progeny from mating. The F1 heterozygous *spe-43*/+ males progeny were picked and crossed to isolated *spe-43* homozygous L4 hermaphrodites at 20ºC for 48 hrs in 4:1 mating ratio. Male F2 worm progeny were isolated and single mated to homozygous *spe-43* L4 hermaphrodite, for getting the *spe-43* homozygote strain. F3 *spe-43* homozygous hermaphrodites were isolated in to individual 24-well plates and score for infertility, 24/24 *spe-43* homozygous hermaphrodite were sterile.

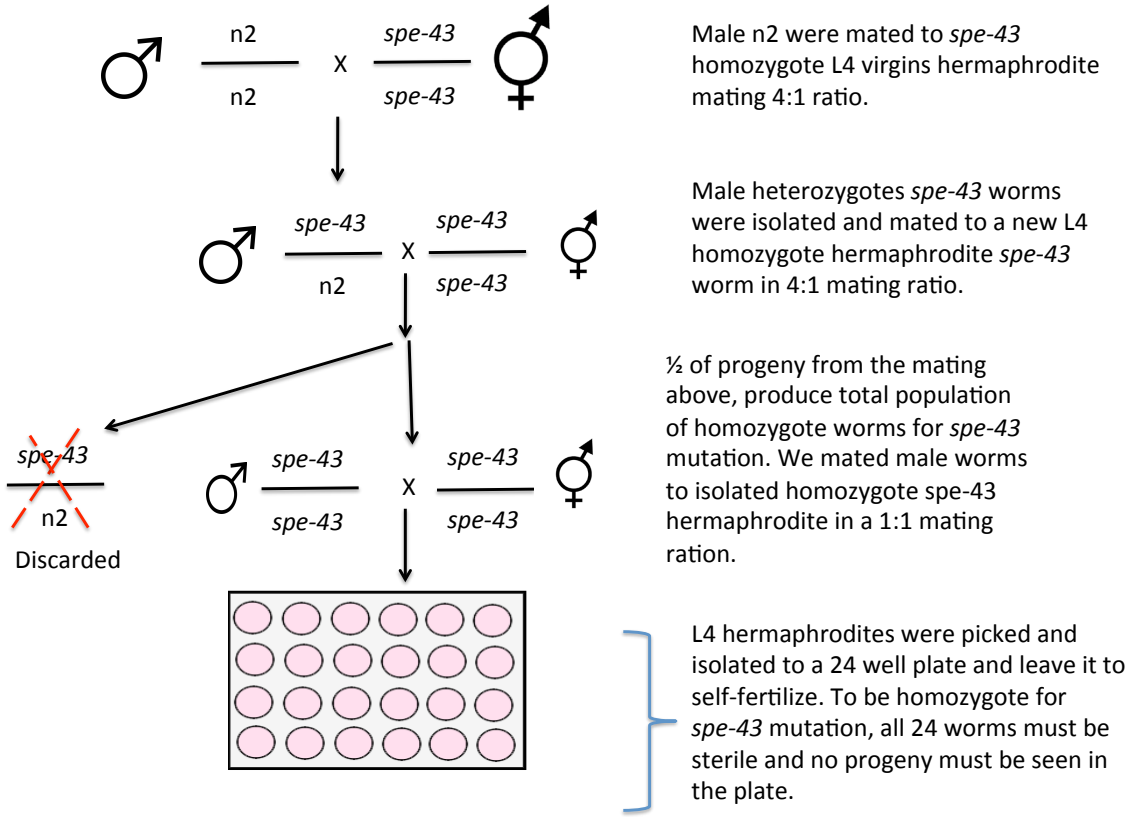


Figure 2.2, Generation of *spe-43*(*eb63*) stable mutant male strain

Male homozygote *spe-43* mutants were generated and mated to *spe-43* hermaphrodites.

spe-43 hermaphrodites are sterile, but ovulate at high levels; _spe-43_ males are efficient maters

One strain of *spe-43* has been isolated thus far, *eb63*. When grown at 16ºC, 20ºC, or 25ºC the strain is completely sterile, producing no viable progeny (Figure 2.3 and 2.4). This result shows that *spe-43* mutants are not temperature sensitive (*ts*) and they are a recessive mutation. Brood counts were performed using N2 worms as a positive control. While *spe-43* hermaphrodites produced no progeny, ovulations were counted as the production of unshelled, undeveloped oocytes (Figures 2.3 A and 2.4). When comparing the numbers of ovulations by wild-type N2 hermaphrodites, counted as production of both eggs and unfertilized oocytes, and *spe-43* hermaphrodites, counted as production of unfertilized oocytes, it was determined that *spe-43* hermaphrodites ovulate at near wild-type levels at both temperatures (Figures 2.3A and 2.4). Two important implication we can get from this experiment, first that *spe-43* hermaphrodites are capable of producing mature oocytes and undergoing ovulation, and second that the *spe-43* hermaphrodite derived sperm are capable of producing the sperm-derived, MSP signal necessary for wild-type levels of ovulation.

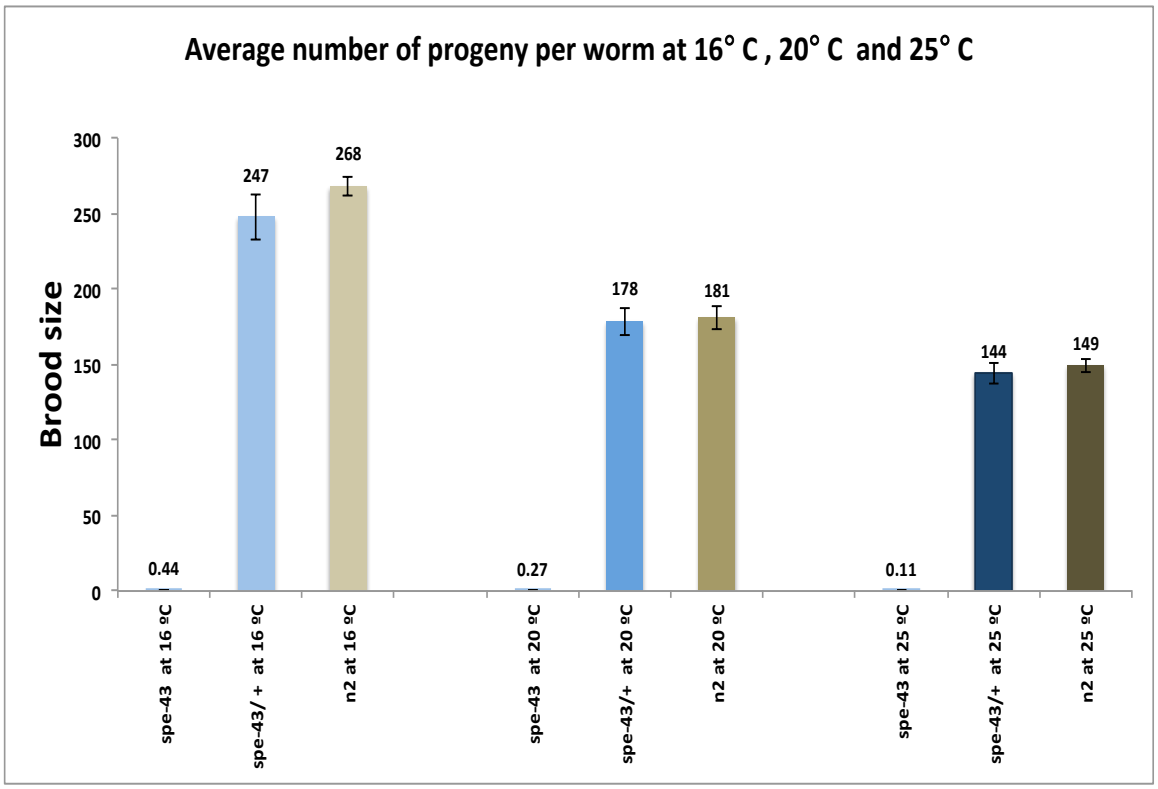


Figure 2.3, Progeny Counts of *spe-43* at 16ºC, 20ºC, or 25ºC

Brood analysis of *spe-43* hermaphrodites at 16ºC, 20ºC, or 25ºC showed that they are sterile at different culture temperatures, meaning that they are not temperature sensitive and that the *spe-43* mutation is recessive. Color intensity indicated the different temperature. Error bars are calculated as standard error. P-value between wild type and *spe-43* at 16ºC: 1.36E-20. P-value between wild type and *spe-43* at 20ºC: 1.97E-14. P-value between wild type and *spe-43* at 25ºC: 2.63E-27.

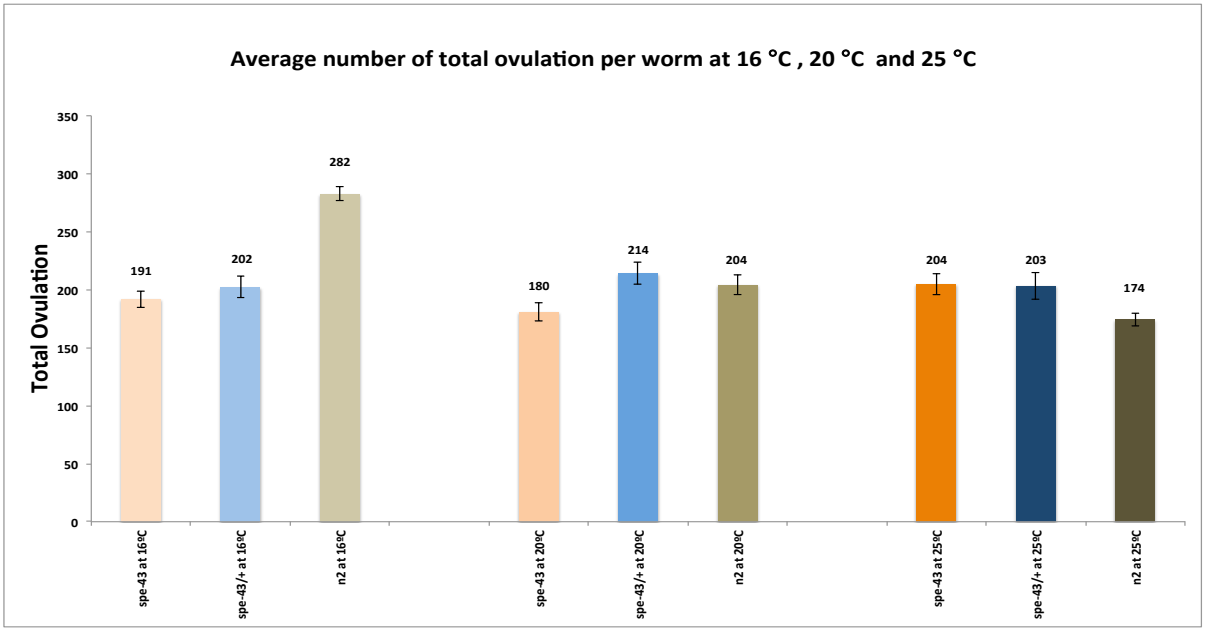


Figure 2.4, Ovulation count of *spe-43* at 16ºC, 20ºC, or 25ºC

The total ovulation is the measurement of progeny plus oocyte laid by a single hermaphrodite worm. For the most part, *spe-43* mutant hermaphrodites ovulate at wild-type levels, but are sterile as compared to N2 wild type. *spe-43* contains enough sperm in the spermatheca for sending the MSP signal for normal oocyte maturation and ovulation. Error bars are calculated as standard error. P-value between wild type and *spe-43* at 16ºC: 3.43E-11. P-value between wild type and *spe-43* at 20ºC: 0.049. P-value between wild type and *spe-43* at 25ºC: 0.006.

spe-43* sterility is suppressed by mutations in *spe-6

The a *spe-6* gene encodes a Casein Kinase 1 like protein and that is required for major sperm protein (MSP) assembly for both early and late spermiogenesis in *C. elegans* (MUHLRAD and WARD 2002). It has previously been shown that the weak hypomorphic allele *spe-6*(*hc163*), can also suppress sterility in the four hermaphrodite-specific activation genes of *spe-8* class (MUHLRAD and WARD 2002).

Homozygous double mutant *spe-6*, *dpy-18* hermaphrodites were mated to either homozygous *spe-19* or *fog-2* (a non-spermiogenesis hermaphrodite- specific sterile control) males. F1 heterozygotes were plated and allowed to self-fertilize. Dpy F2s were then plated and scored for sterility. Approximately one fourth of all Dpy progeny would be expected to be sterile unless this phenotype was suppressed by *spe-6(hc163)*. 0/210 Dpys were sterile when crossed to *spe-43* animals, while 20/ 112 Dpys were sterile using the *fog-2* control. To ensure that the homozygous *spe-43* class of animals was present, individual Dpy hermaphrodites were then crossed to homozygous *spe-43* males, and progeny were scored for sterility. Animals for whom all progeny were sterile (4 of 11) indicated that the *spe-43*(*eb63*) homozygous class of animal was present. The fertility of *spe-6*(*hc163*); *spe-43*(*eb63*) double mutants, suggest that *spe-43* functions in the same genetic pathway as *spe-8*, *spe-12*, *spe-19*, *spe-27* and *spe-29* and that *spe-43* functions upstream of *spe-6*.

spe-43 hermaphrodite sperm is localized in the spermatheca, but has faster turn over than wild-type hermaphrodite sperm

In wild-type hermaphrodites, sperm or spermatid derived MSP promotes ovulation to occur once approximately every 23 minutes (McCarter *et al.* 1999b). When this process occurs, the spermatheca expands to engulf the oocyte, and after a time, then contracts to expel the newly fertilized embryo. In fertilization defective mutants of the *spe-8* class, the process of ovulation still occurs at near wild-type levels, although no viable progeny are produced. Again, in wild-type animals the movements of oocytes through the spermatheca can push sperm into the uterus, when this occurs the sperm must crawl upstream to reenter the spermatheca. Thus in the *spe-8* class mutant *spe-43* sperm are lost from the spermatheca at a much higher rate than in wild-type, as the sperm do not activate to form the pseudopod, and are therefore incapable of tracking back to the spermatheca (Geldziler *et al.* 2005a).

In order to determine whether the same process occurs in *spe-43* mutant hermaphrodites, age matched N2 wild type and *spe-43* hermaphrodites were grown at 20ºC for four days. On each day subsequent to L4 stage, hermaphrodites were fixed and stained with the DNA DAPI dye and imaged under fluorescence microscopy. On the first day of the adult stage, a comparable number of sperm appear in the spermatheca of N2 and *spe-43* animals (Figures 2.5 A and B). By two days of the adult stage, *spe-43* sperm were depleted faster and substantially fewer sperm appear in the spermatheca of *spe-43* animals in comparison to N2 animals (Figures 2.5 C and D). On the third and forth day of the adult stage, almost no sperm were seen in the spermatheca of *spe-43* hermaphrodites, while a number of sperm still remained in the N2 hermaphrodite

spermatheca (Figure 2.5 E-H). This indicates that *spe-43* sperm have a faster loss rate from the reproductive tract than wild type-sperm. In addition, sperm nuclei were often seen in aberrantly high concentrations in locations throughout the *spe- 43* hermaphrodite uterus, indicating that sperm were not retracting to the spermatheca (Figure 2.5 D, F, H).

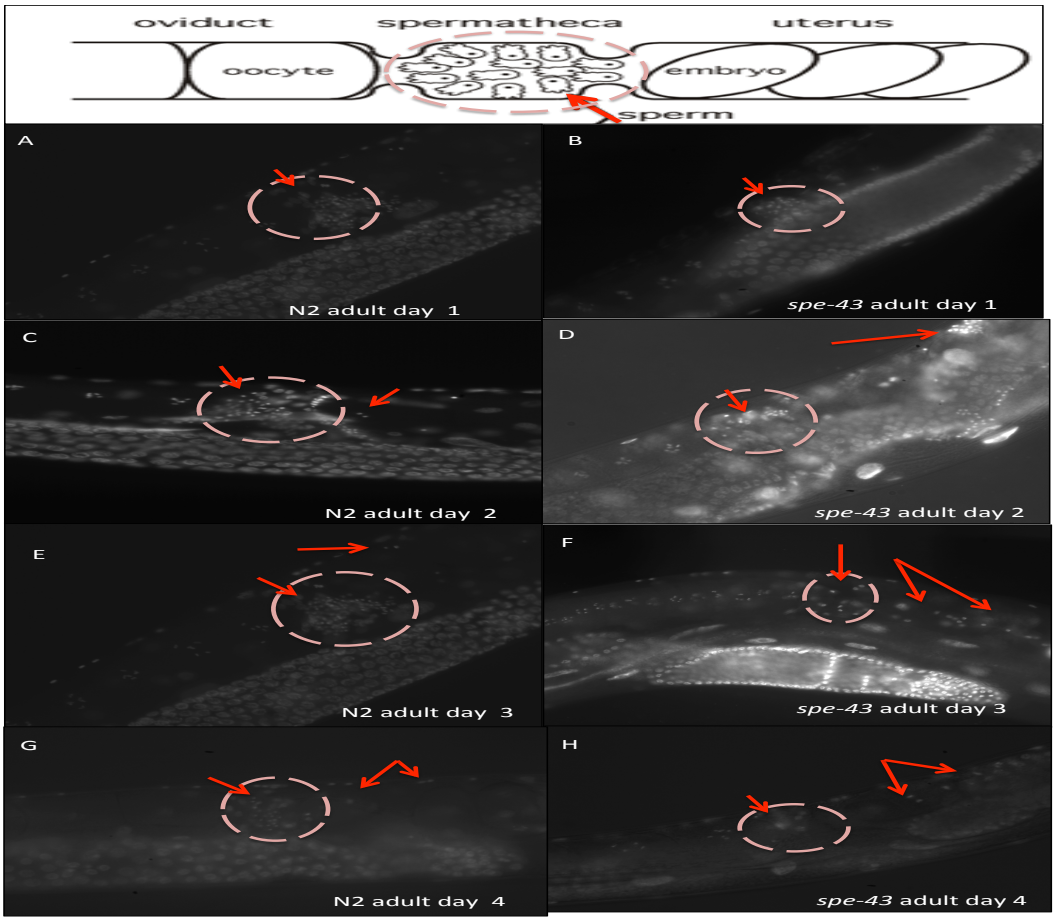


Figure 2.5, DAPI staining of *spe-43* and wild type

Age matched N2 wild type and *spe-43* hermaphrodites were grown at 20ºC for four days. On each day hermaphrodite was fixed and stained with the DNA dye DAPI and imaged with fluorescence microscopy. On day one of the adult stage, a comparable number of sperm appear in the spermatheca of both N2 and *spe-43* animals (A and B). By the subsequent days of the adult stage, *spe-43* sperm were depleted faster and substantially fewer sperm appear in the spermatheca of *spe-43* animals in comparison to N2 animals (C and D). Indicates that *spe-43* sperm have a faster turn over rate from the reproductive tract than wild type sperm and that sperm were not migrating to the spermatheca (D, F, H).

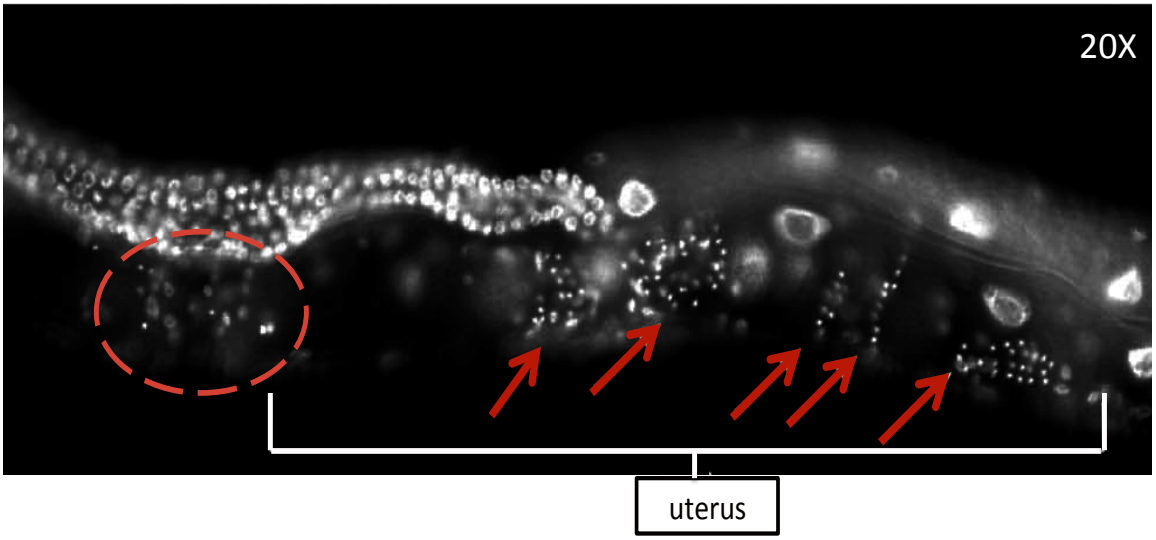


Figure 2.6, Sperm DAPI staining indicating *spe-43* mutant sperm are lost from the reproductive tract

spe-43 hermaphrodite sperm are unable to migrate back to the spermatheca after displacement by passing oocytes. Spermatids can be found in the uterus and near the vulva of *spe-43* hermaphrodites. Indicating that these mutant sperm do have proper motility or are not fully developed.

***spe-43*, sperm *in vitro* activation defects and transactivation**

As with other *spe-8* class mutants, *spe-43* produces normal number of spermatids, but these fail to undergo spermiogenesis to form mature spermatozoa. It's still unclear what is the molecular activator(s) and what is the molecular mechanism that triggers spermiogenesis. It is known that the defect in all *spe-8* class mutant genes is in sperm, and not in the production of an extracellular signal. This is supported by an *in vitro* phenotype exhibited by spermatids from both males and hermaphrodite from all *spe-8* mutants. Spermatids from wild-type worms can be activated to undergo spermiogenesis *in vitro* by adding one of several classes of molecules, such as protease (Pronase) or chemicals that raise intracellular pH (e.g Triethanolamine (TEA)) (Figure 2.6 and 2.7). Spermatid from the spermiogenesis mutant can activate normally *in vitro* when exposed to TEA, forming viable sepermatozoa (GELDZILER *et al.* 2005b; LAMUNYON and WARD 1994). However when exposed to Pronase these mutants spermatids seem to begin to undergo spermiogenesis, but instead of forming a motile pseudopod, they form long rigid spikes radiating outward for the cell periphery, after which further morphogenesis cease (Figure 2.6).

This spike like formation phenotype is due of MSP bundles that project but fail to coalesce into a pseudopod. *spe-43* animals produce morphologically normal spermatids, but these fail to efficiently undergo activation *in vitro. T*hese results suggest that *spe-43* is a classic *spe-8* class sperm activation mutant. Virgin *spe-43* hermaphrodites were also dissected and their sperm examined using Nomarski imaging. Although they produced morphologically normal spermatids, no activated spermatozoa were noted (Figure 2.7B) suggesting that *spe-43* hermaphrodite sperm are unable to undergo activation in vivo.

Upon treatment with Pronase, *spe-43* hermaphrodite-derived spermatids behaved similarly to their male-derived counterparts, with many arresting at the spiked intermediate stage (Figure 2.7 D). These results suggest the sperm-loss phenotype described above is due to motility defects resulting from defective activation.

Each of the *spe-8* class mutants exhibit a phenomenon termed transactivation whereby mutant hermaphrodite spermatids can form functional spermatozoa after exposure to male seminal fluid (LAMUNYON and WARD 1995; SINGSON *et al.* 1999). Transactivation ability varies among alleles; the greater the allele's severity, the lesser the transactivation (GELDZILER *et al.* 2005b). To determine whether *spe-43* hermaphrodites exhibit this behavior, we performed transactivation *spe-43* using a temperature-sensitive allele of *fer-1*, whose male seminal fluid is known to be a potent trans-activator for hermaphrodite spermatids (Figure 2.8) (SHAKES and WARD 1989). Homozygous *spe-43* L4 hermaphrodites were crossed to *fer-1*; *him-5* homozygous males at 25ºC, and their progeny were counted. Has control we crossed *fer-1*(*hc13ts*); *him-5* to mutants are defective in fertilization events: *spe-9*(*eb19*) and *fem-1*(*e1965*).

At this restrictive temperature, *fer-1* sperm are non-motile, and therefore no progeny would be expected unless seminal fluid is capable of activating hermaphrodite spermatids. A significant difference was observed between *spe-43* trans-activated hermaphrodites and controls hermaphrodites. An average progeny of 63 worms per plate was observed compared to 0 of the controls. These results are consistent with previously published transactivation results in other severe loss-of-function activation mutants and suggest *spe-43* alleles is also a loss-of-function as well as a key component for *C. elegans* sperm activation.

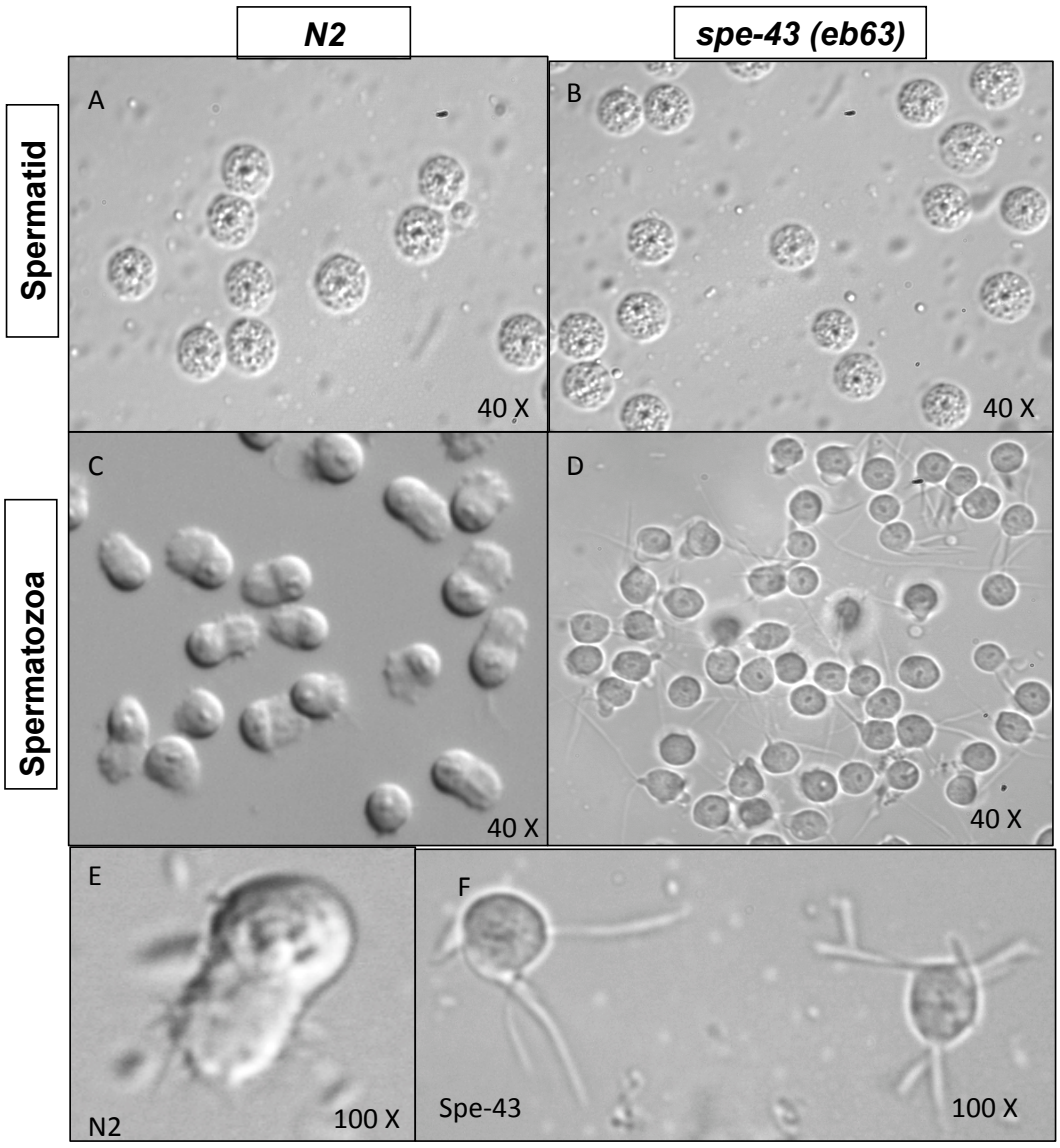


Figure 2.7, *spe-43* and N2 in vitro sperm activation phenotypes

Dissected wild type and spe-43 spermatids from young adult males, 40X resolution (A and B). Spermatozoa dissected from a wild type, young adult male, and activated with pronase. 40X resolution. (C) Spermatozoa dissected from a *spe-43 (eb63*) young adults males, and activated with pronase. MSP does not coalesce to form pseudopod 40X resolution (D). Spermatozoa dissected from a wild type and *spe-43*, young adult male, and activated with pronase, 100X resolution (E and F).

***spe-43* transactivation**					
Genotypes/crosses (n > 15 for all groups)	**Average numbers of eggs**	**SEM**	**Average number of oocyte**	**SEM**	**Eggs as % of ovulation events**
Unmated *spe-9(eb19)*	0.18	0.10	77.7	4.86	0.24
fer-1(hc1) male x spe-9(eb19) hermaphrodite	0.26	0.11	70.9	4.18	0.37
Unmated *spe-19(eb52)* hermaphrodite	0.29	0.14	89.4	5.84	0.34
fer-1(hc1) male x *spe-19(eb52)* hermaphrodite	1.4	0.25	99.2	4.05	1.39
Unmated *spe-8(hc53);dpy-5(e61)* hermaphrodite	0.22	0.12	79.5	4.23	0.28
fer-1(hc1) male x *spe-8(hc53);dpy-5(e61)* hermaphrodite	47.4	2.68	39.7	2.22	54.5
Unmated *spe-43(eb63)*	0.23	0.13	88	6.14	0.25
fer-1(hc1) male x *spe-43* hermaphrodite	49	2.85	41.9	2.63	53.9

Table 2.2, *spe-43* hermaphrodite sperm can be trans-activated by *fer-1* (*hc13ts*) mutant seminal fluid

When *spe-43* L4 hermaphrodite were cross to *fer-1* mutant males at 25ºC self-progeny were observe form the *spe-43* hermaphrodites similar to other *spe-8* class mutants.

Mutations in *spe-43* do not affect male fertility in vivo and their sperm compete with hermaphrodite derived sperm

Of the sperm activation genes known in *C. elegans*, the majority exhibit a hermaphrodite-specific phenotype (GELDZILER *et al.* 2005b). In contrasts to *spe-43* self-sterile hermaphrodites, male *spe-43* worms are efficient maters. After being mated to the *spe-43* males, mutant *spe-43* mutant hermaphrodite will successfully sire outcross progeny. This indicates that *spe-43* male sperm activation is successful to produce a motile mature sperm with appropriate MSP to move to the spermatheca and fertilize the maturing oocytes. To further address this issue in spe-43, we generated male lines, to measure the *spe-43* male fertilization efficiency and to verify *spe-43* males were not temperature sensitive mutants. *spe-43* males were cross to a *dpy-5*(*e61*) and *fem-1* hermaphrodite in a 4:1 mating ratio at 16ºC, 20ºC and 25ºC. This experiment shows that *spe-43* males are efficient maters and have wild type like fertilization potency.

Previous studies showed that males that inseminate fertilization-defective sperm that sperm competition within a hermaphrodite does not require successful fertilization. However, sperm competition does require normal sperm motility. Additionally, sperm competition is not an absolute process. Because oocytes not fertilized by male-derived sperm can sometimes fertilize hermaphrodite-derived sperm. These results indicate that outcrossed progeny result from a wild-type cross is because male-derived sperm are competitively superior and hermaphrodite-derived sperm become unavailable to oocytes.

When male's *spe-43* nematodes were cross to *dpy-5* L4 hermaphrodite also showed that *spe-43* male sperm also competes to fertilize the mature oocyte. After 48 hours of mating, the male-derived sperm fertilizes nearly all of the oocytes, showing that in the

brood count analysis. This result indicates that male *spe-43* sperm undergo activation *in vivo*, are motile and can migrate to the recipient hermaphrodite's spermatheca. Also indicates that *spe-43* male-derived sperm have a competitive superiority over the hermaphrodite-derived sperm, resulting in a functional suppression of hermaphrodite self-fertility.

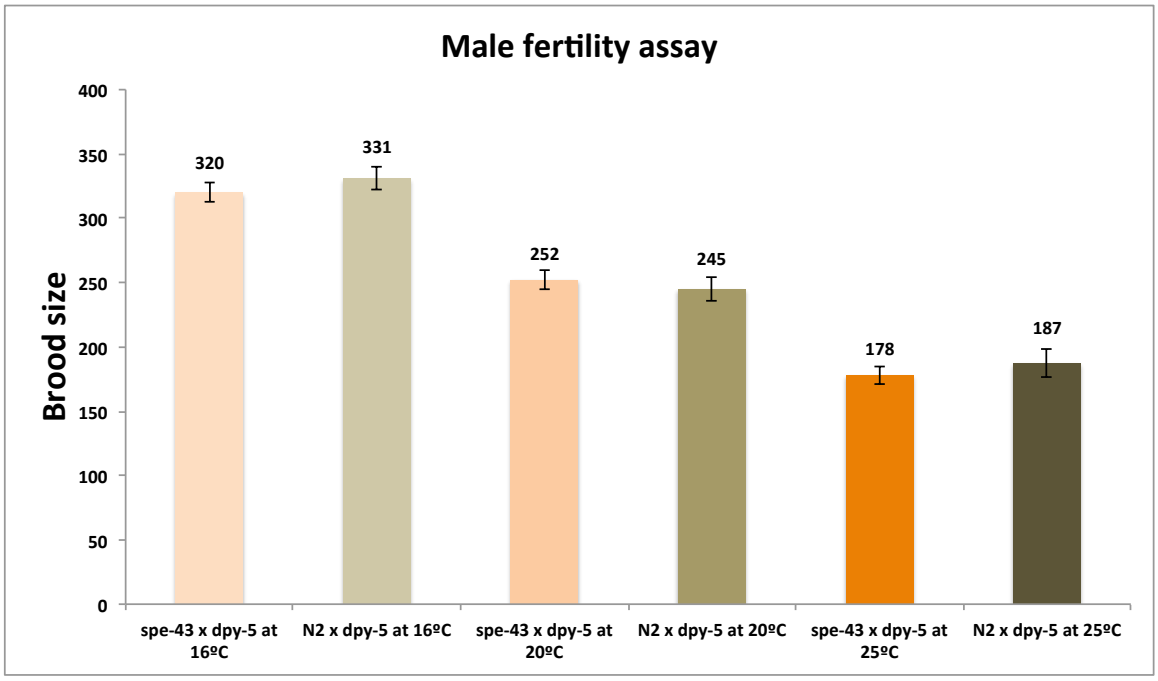


Figure 2.8, *spe-43* mutant males are effective maters

spe-43 mutant males shows wild type mating efficiency after 48 hours of matting with *dpy-5* hermaphrodite at 16ºC, 20ºC and 25ºC. P value at 16ºC: 0.359. P value at 20ºC: 0.539. P value at 25ºC: 0.501.

Genotype	Self progeny	Outcross progeny	Total progeny	n	Ability to compete
n2					
dpy-5 (*e61*) hermaphrodites	41 ± 8	0	32	37	yes
N2 X dpy-5 (*e61*) hermaphrodites	2 ± 3	45 ± 5	40	35	
spe-43					
dpy-5 (*e61*) hermaphrodites	41 ± 8	0	32	37	yes
spe-43 (*eb63*) X dpy-5 (*e61*) hermaphrodites	13 ± 7	33 ± 9	40	58	
spe-13					
dpy-5 (*e61*) hermaphrodites	28 ± 2	0	28	18	yes
him-5 (*e1490*) x dpy-5 (*e61*)	18 ± 3	21 ± 3	39	20	
spe-13 (hc137ts) ; *him-5* (*e1490*) x dpy-5 (*e61*)	15 ± 2	0	15	18	
fer-1					
dpy-5 (*e61*) hermaphrodites	35 ± 2	0	41	24	no
him-5 (*e1490*) x dpy-5 (*e61*)	23 ± 2	29 ± 4	52	23	
fer-1 (*hc13ts*); *him-5* (*e1490*) x dpy-5 (*e61*)	39 ± 4	0	39	21	

Table 2.3, male sperm competition assay

spe-43 male-derived sperm compete to fertilize the mature oocytes compared to other mutant male-derived sperm with fertilization-defective sperm, but it lack of full competitive superiority.

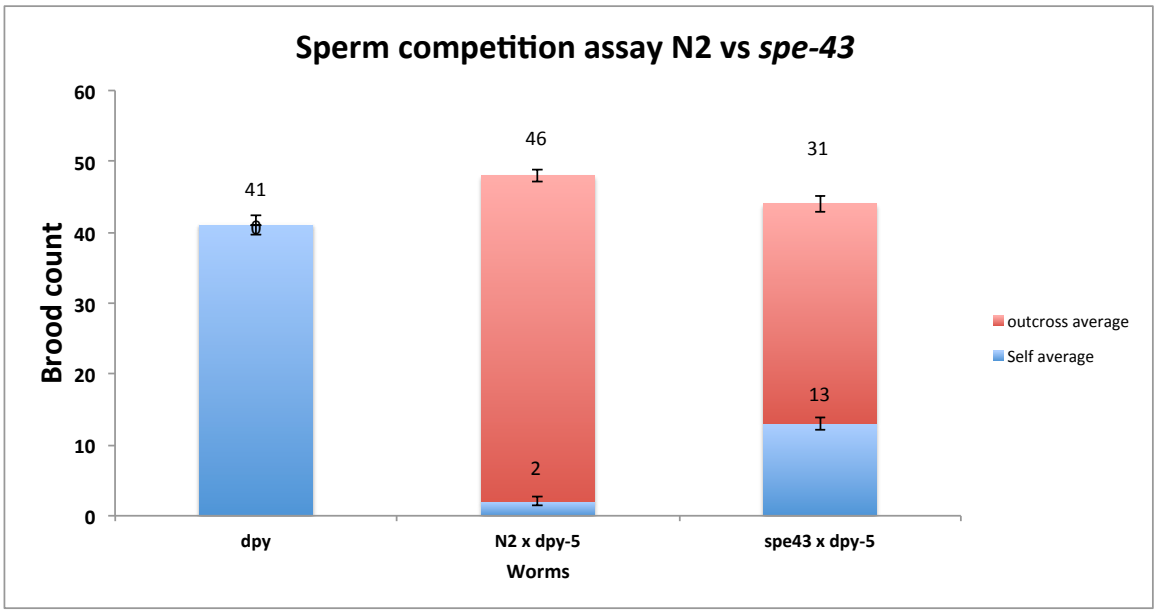


Figure 2.9, *spe-43* sperm competition vs wild type competition

spe-43 male sperm is competitive but is not fully competitive has wild type. P-value 1.34E-16.

In the *C. elegans* sperm world size maters for influencing outcomes of sperm competition

In most of the sexually reproductive organism the size, quantity, strength and motility of the sperm are some of the important factors that make the difference between an organism that reproduces and a one that doesn't reproduce. As a result, sperm competition is now recognized as an almost universal feature of reproductive ecology across animal phyla (LaMunyon and Ward 1999). Selective pressure on males to succeed at fertilization has resulted in the evolution of myriad traits including behaviors (e.g. frequent copulations, mate guarding), morphologies (e.g. penile accessories for sperm removal), and secretions (e.g. mating plugs and anti-aphrodisiacs) (LaMunyon and Ward 1999). Less clear is the role of sperm competition in the evolution of sperm themselves, including morphology, motility, and numbers. Recent, theoretical work has suggested that sperm size may instead increase in response to sperm competition when larger sperm experience greater survivorship and/or when larger sperm are better competitors (Parker 1993). Finally, experimental studies have shown that the larger, amoeboid sperm in *C. elegans* take precedence over smaller sperm (Radwan 1996) and that larger sperm are preferentially stored in the spermatheca (LaMunyon and Ward 1999)

In the hermaphroditic nematode *C. elegans*, sperm from hermaphrodites are always outcompeted by much bigger sperm from males. Larger sperm crawl faster than smaller sperm and are able to physically displace small hermaphrodite-derived sperm from the spermatheca, taking precedence in fertilization (LaMunyon and Ward 1999). However, there is a cost to larger sperm: they are produced at a slower rate. Male sperm compete

equally with other male sperm, which are similar in size (LAMUNYON and WARD 1999). Using new technique called *in vivo* sperm activation our laboratory activated sperm from *spe-43* males were isolated from a recipient *fem-1*(*e1965*) L4 hermaphrodite (Figure 2.10).

Our measurements of sperm size and sperm motility were taken from mature amoeboid spermatozoa that were dissected sperm medium after a 48 hour mating between *spe-43* males and L4 *fem-1* hermaphrodites at 25ºC in a 4:1 mating ratio (Figure 2.10). As control we did the same experimental procedure but with N2 males mated with *fem-1* hermaphrodites (Figure 2.10). After dissection, sperm were observed under Nomarski DIC optics, the sperm images were video captured and analyzed using Image J computer software (Figure 2.10). Sperm cell body diameter, total length and pseudopod length were measure and compared (Figure 2.11). My result shows that wild-type N2 male sperm have bigger cell body diameter and pseudopod length. N2 sperm are bigger and wider by almost two-fold compared to *spe-43* sperm, giving them a competitive advantage to fertilize (Figure 2.10 and 2.11).

In *C. elegans*, the smaller sperm are always outcompeted by much bigger sperm. Larger sperm crawl faster and cover move distance in less time than smaller sperm. The larger and wider N2 male sperm are able to physically displace hermaphrodite sperm from the spermatheca, taking superiority in fertilization (Figure 2.12). The *spe-43* male sperm is smaller and covers less traveling distance than wild type N2 male sperm, but it is a sperm that competes successfully and outcompetes hermaphrodite sperm. When *spe-43* males were mated to *dpy-5* hermaphrodites there sperm showed competitive superiority to fertilize the mature oocyte. *spe-43* male-derived sperm fertilizes nearly all

of the oocytes, showing that in the brood count analysis. This result indicates that the *spe-43* male sperm has some competitive superiority over the hermaphrodite sperm, resulting in a functional suppression of hermaphrodite self-fertility. This size difference between the different sperm makes a big difference in sperm competitive nature but it may not be the only factor that drives sperm competition. Other molecular factors maybe involve in the competitive nature of the sperm.

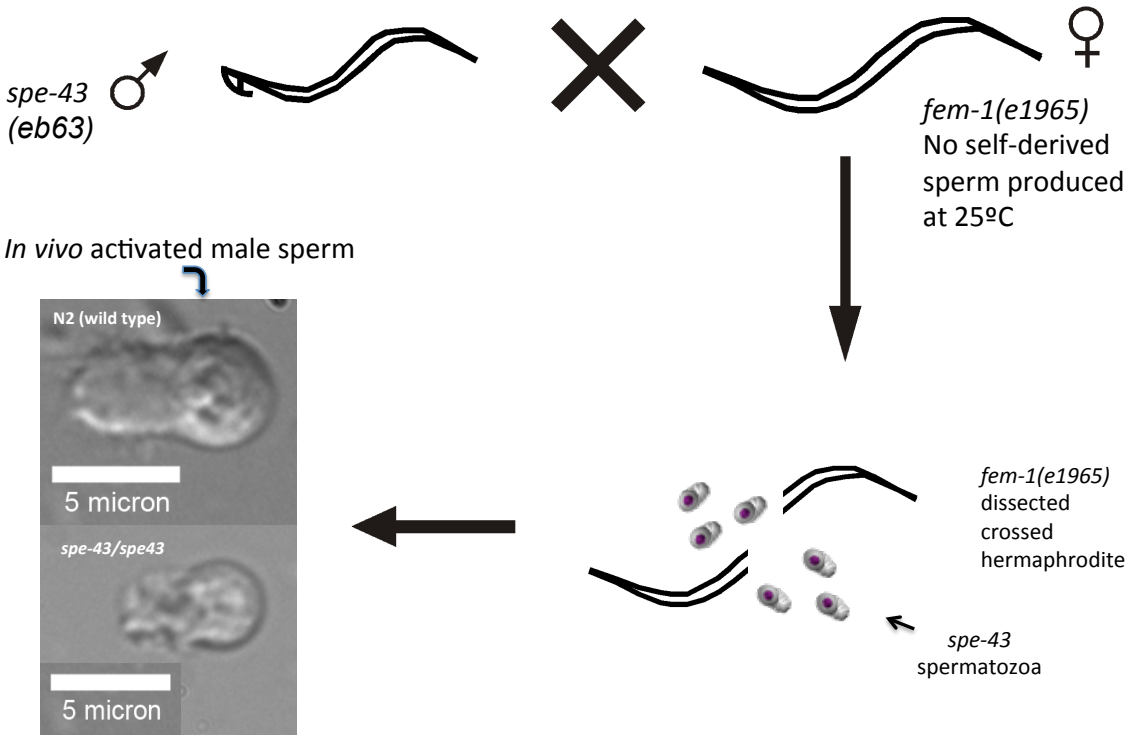


Figure 2.10, *in vivo* activation of N2 and *spe-43* male sperm

The larger and wider N2 male sperm are able to physically displace *spe-43* male sperm from the spermatheca, taking superiority in fertilization. Results show that wild type N2 male sperm have bigger cell body diameter and pseudopod length. Been bigger and wider sperm it provides a competitive advantage to fertilize.

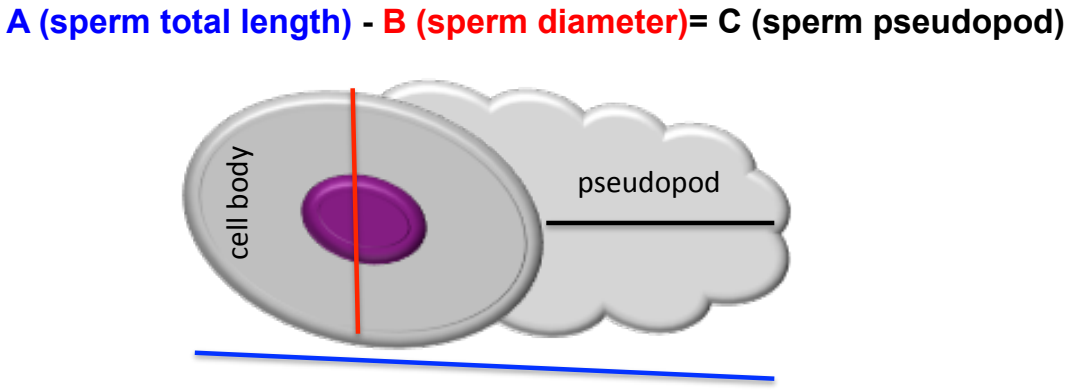


Figure 2.11, Sperm measurements specification parameters

To determine the pseudopod length, the sperm cell body diameter and total length were measure and subtracted.

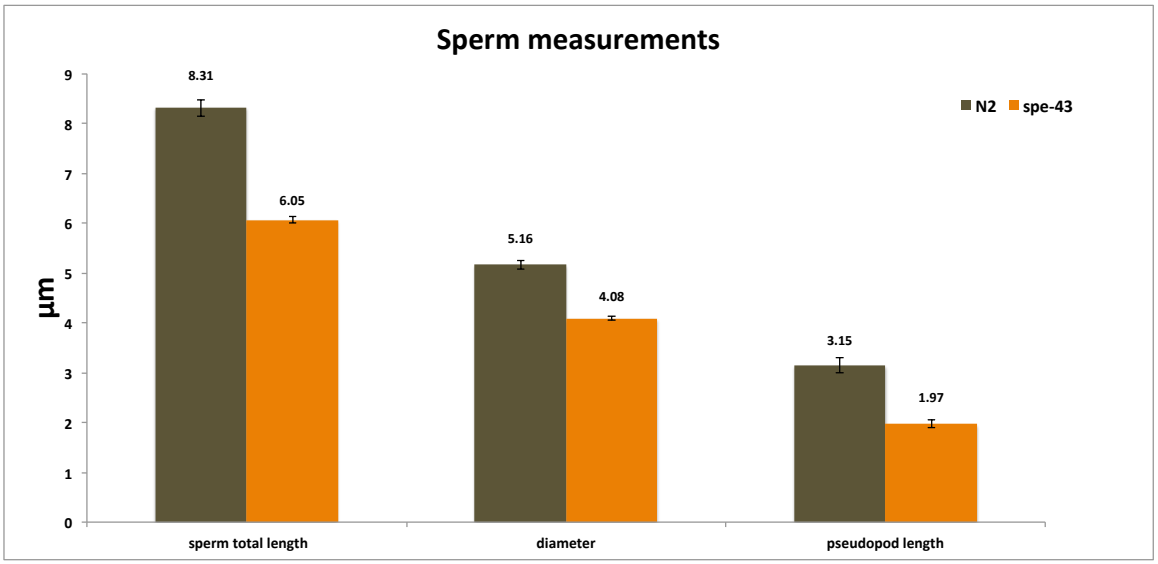


Figure 2.12, Sperm measurements data analysis

N2 male sperm are larger than *spe-43* male sperm, providing them a better competitive advantage to fertilize. But male *spe-43* sperm are efficient and competes with the hermaphrodite sperm to fertilize the oocyte, indicating that there may be other factors involve in sperm competition.

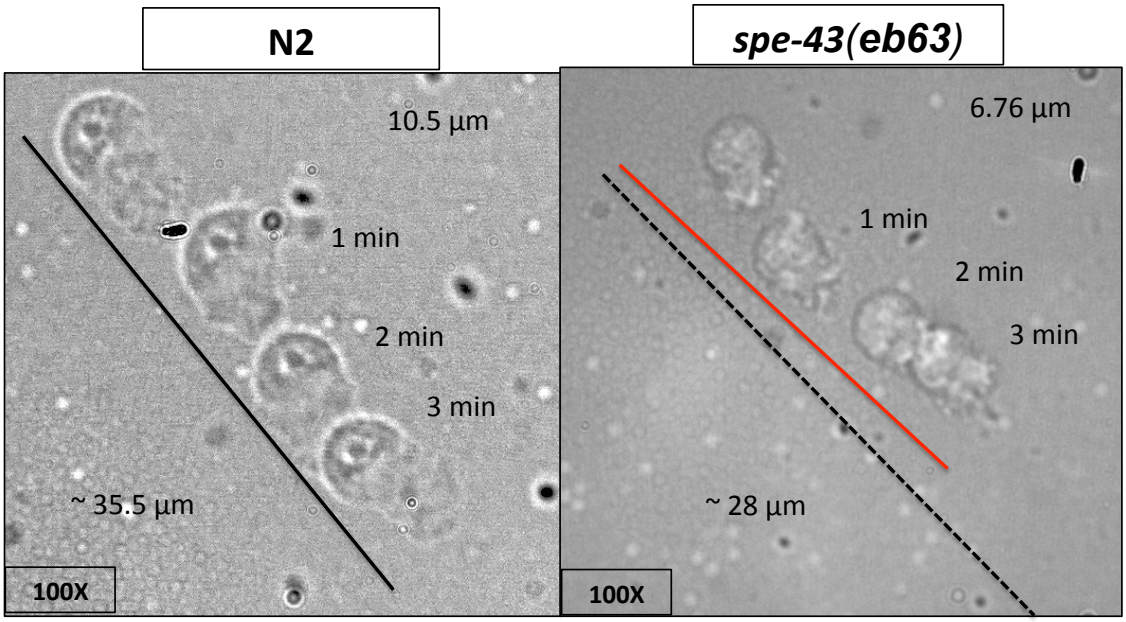


Figure 2.13, *spe-43* male sperm are smaller and slower than wild type sperm

The larger N2 male sperm crawl faster and cover move distance in less time than smaller *spe-43* sperm. By having these characteristics N2 male are more efficient with regards to fertilizing oocytes.

Identification of *spe-43* region on chromosome IV

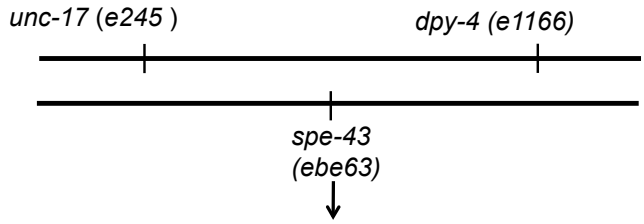
To determine the genetic region of the *spe-43* gene product, we use a series of genetic mapping technics to narrow down the physical location. A combination of two-point, three-point, deficiency and SNP mapping were utilized to limit the region approximately 750,000 bp on the far right arm of chromosome IV.

Strain MT4150, which includes markers *unc-17* (-3.11 cM), and *dpy-4* (12.74 cM), was selected for use in three-point mapping. The three-point mapping and deletion complementation mapping placed *spe-43* between the *unc-22* and *unc-30* markers (11,972,708 bp - 13,119,096 bp) (Figure 2.12), recombinant strains Unc non Dpy and Dpy non Unc were saved for SNP mapping. Recombinants were crossed to the CB4863 Hawaiian strain to generate SNP mapping strains, twenty strains were isolated for the *dpy-4* non *unc-17* worms, and nine strains were isolated for the *unc-17* non *dpy-4* strains. Two SNP's were informative with regards to narrowing down *spe-43* genomic region between C25G4: 2292 and F55G11: 20691 (12,455,094 bp - 12,967,424 bp). This result indicated that the genetic region of interest of *spe-43* is approximately 512,330 bp (Figure 2.13).

Between this genetic region of interest, we have found some sperm enriched gene candidates that could be the *spe-43* gene (**Sam Ward sperm enrich candidate genes table)** (Table 2.3). There were no further informative SNP's in this region, therefore microinjection of fosmids or cosmids must be used to narrow the region and get transgenic rescue of *spe-43* mutation. Among this region of *spe-43* there are 27 fosmids and 20 cosmid candidates. Recently a whole genome sequencing approach for determining the genetic region of *spe-43* is been used. Until right now we have extracted

the genomic DNA of the *spe-43* worms and we are in the phase of making the DNA libraries needed before our samples are sent out for sequencing.

A



Recombinants	Sterile	Total	% recombination
Unc non Dpy	9	35	5.43
Dpy non Unc	20	43	2

B

Genotype	Deletion genomic region on chromosome IV	Complementation
sDf21/ebe63	5.69855 – 10.0502 cM	_
sDf22/ebe63	5.92212 - 8.38345 cM	_

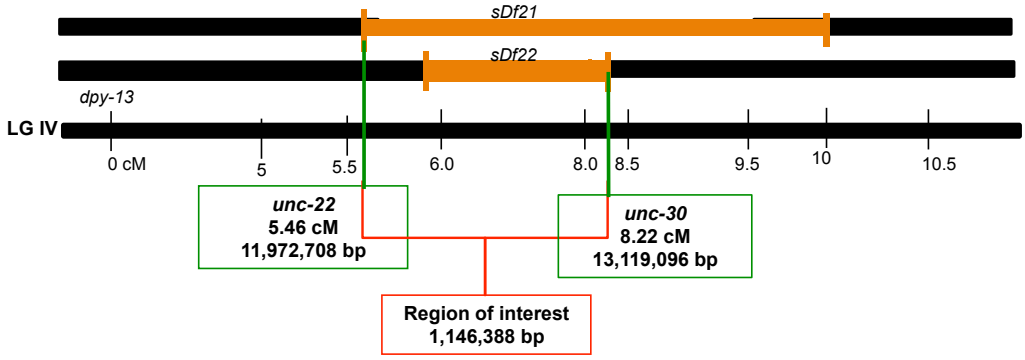


Figure 2.14, Three-point and deletion mapping data

A; three-point mapping data, *spe-43* is closer to *dpy-4* gene.

B; Deletion complementation mapping, *spe-43* region is under *sDf 21* and *sDf22* region.

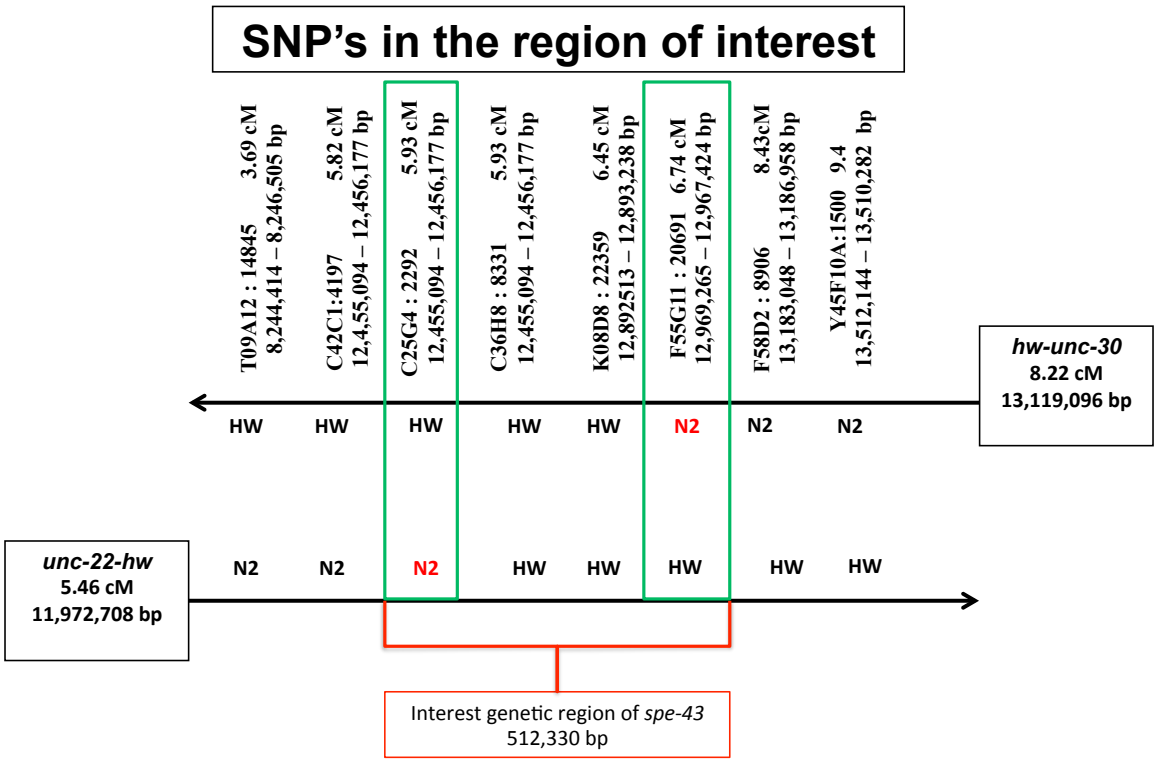


Figure 2.15, Significant SNP's use to narrow down the genetic region of *spe-43*.

The interest genetic region is 512,330 bp between the SNP's C25G4: 2292 and F55G11: 20691.

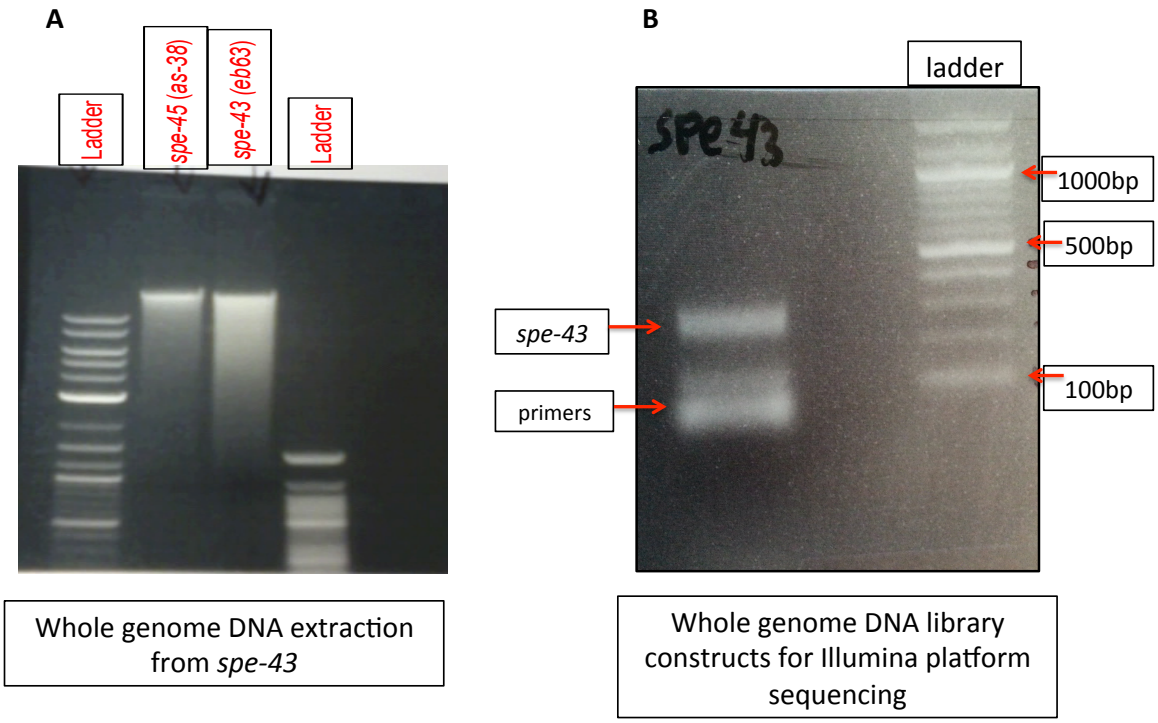


Figure 2.16, Genomic DNA extraction of *spe-43*

spe-43 Genomic DNA extraction was performed using a modified protocol of Dr. Andrew Fire lab (A). Whole Genome DNA library construct for Illumina platform sequencing were perform at Dr. Sam GU laboratory at Rutgers University (B).

11.4 Discussion, Conclusion and Perspective

Spermiogenesis is the rapid maturation of a spherical immotile spermatid to an asymmetrical, crawling spermatozoon. Mutant *C. elegans* hermaphrodites defective in this process have been studied and organized into a specific group, called the *spe-8* group. These defective genes identified have been organized into the same group because of their identical phenotypic traits: hermaphrodite are self-sterile, mated hermaphrodite are self and outcross-fertile, males are fertile and spermatid form males and hermaphrodites have in vitro activation defects. This led to the hypothesis that these genes encode part of the machinery that receives or processes the spermiogenesis initiation signal(s) in spermatids. The fact that all of these mutants have practically identical phenotypes confounded efforts to order the genes in a formal pathway, although genetic analysis have confirmed that at least some of these genes functionally interact with each other, revealing a common signaling pathway by which spermatids initiate spermiogenesis in response to an extracellular stimulus (LHERNAULT *et al.* 1988; MINNITI *et al.* 1996; NANCE *et al.* 2000; NANCE *et al.* 1999; SHAKES and WARD 1989). Of these five genes: *spe-12*, *spe-19*, *spe-27* and *spe-29*, appear to be unrelated to any other genes currently in the gene databases, therefore their genetic sequences alone shed little light on the nature of their activity. Genetic interaction and epistasis studies between these genes suggest their products may act together in a complex (NANCE *et al.* 2000; NANCE *et al.* 1999) (Figure 2.15).

The basic understanding of the hermaphroditic *C. elegans* spermiogenesis pathway, the *spe-8* class pathway, is *spe-6* encodes a protein kinase in spermatids that restrain the

spermiogenesis machinery, preventing it from engaging until the *spe-8, spe-12, spe-19, spe-27, spe-29* gene products have relayed the signal to commence spermiogenesis (Figure 2.15). In this signaling model, SPE-6 maintains a phosphorylation of its target protein substrate(s), which in turn serves as a break on spermiogenesis. When stimulated by an extracellualar signal, the SPE-8, -12, -19, -27, -29 gene products antagonize the SPE-6 kinase activity, relieving the break so that spermiogenesis proceeds, forming crawling spermatozoon (Figure2.15). In this dissertation I provide new phenotypic and genetic evidence of a new gene, called *spe*-43, is also a component of this pathway.

Recessive loss-of-function mutation in *spe-43* lead to the inability of spermatids to undergo spermiogenesis in response to stimulation by an external activation signal. Therefore, the wild type SPE-43 protein may be positive initiator of the spermiogenesis-signaling pathway. While we do not have yet the genetic sequence of the *spe-43* gene, by phenotypic analysis we are confident that the *spe-43* gene is highly involve in the maturation of the spermatid. The SPE-43 protein could fit any of a number of roles in spermiogenesis initiation pathway, such as regulator or a structural role. As mention previously, *spe-43* hermaphrodite have a faster depletion of sperm in the spermatheca than in wild type, meaning that hermaphrodite *spe-43* sperm lack to mature to gain motility. This result indicates that is greatly needed for the pseudopod formation upon activation by an external activation signal. SPE-43 is required for assembling MSP into the filaments that form from fibrous bodies to create a function pseudopod. Extending this hypothesis to the newfound role of *spe-43* in spermiogenesis initiation, *spe-43* may be a nexus between the signal transduction machinery and the MSP polymerization machinery in activating spermatids.

As is often the case, the studies in this dissertation elicit many more question than answers. What is the exact genetic region of the *spe-43* gene? What regulates *spe-43*? What is the encoded protein for the *spe-43* gene? How exactly does this pathway effect the major cellular changes that accompany spermiogenesis? How does SPE-43 interacts with the other SPE-8 class proteins? Some long-standing questions also remain unanswered: What is the activator? Why do the spermiogenesis mutations affect hermaphrodites and males so differently? Where in the cell do the spermiogenesis signaling protein reside.

The genetic approach has so far been our most successful strategy for unraveling the spermiogenesis-signaling pathway. But other biochemical and molecular techniques will be necessary for studying spermiogenesis signaling in greater mechanistic detail. The studies of *C. elegans* sperm develop and maturation is relevant beyond the nematode world. It could be important for the studies of other higher eukaryotes. Until recently, MSP was thought to be unique to nematode sperm. We know now that, not only can the mechanism of MSP-based motility teach us about more conventional motility system (without the complications of the myriad cellular roles of actin), but that there are actually MSP homolog in other organism, including humans. These studies give us important clues about genes, molecular mechanism that can be understood better and impact our treatment and prevention of human disease.

Having gained experienced in identifying and trying to characterize a gene has been a gratifying and special experience. It has been giving me a clear perspective of all the great possibilities and applications of scientific research. Learning about *spe-43* gene and it characteristic and molecular traits blaze many trails for pursuing spermiogenesis

initiation and hopefully its molecular identification. The cloning of this gene its still novel and important. What we learn from here will surely aide in our understanding of how this gene work in worms and maybe even how genes like it function in ourselves.

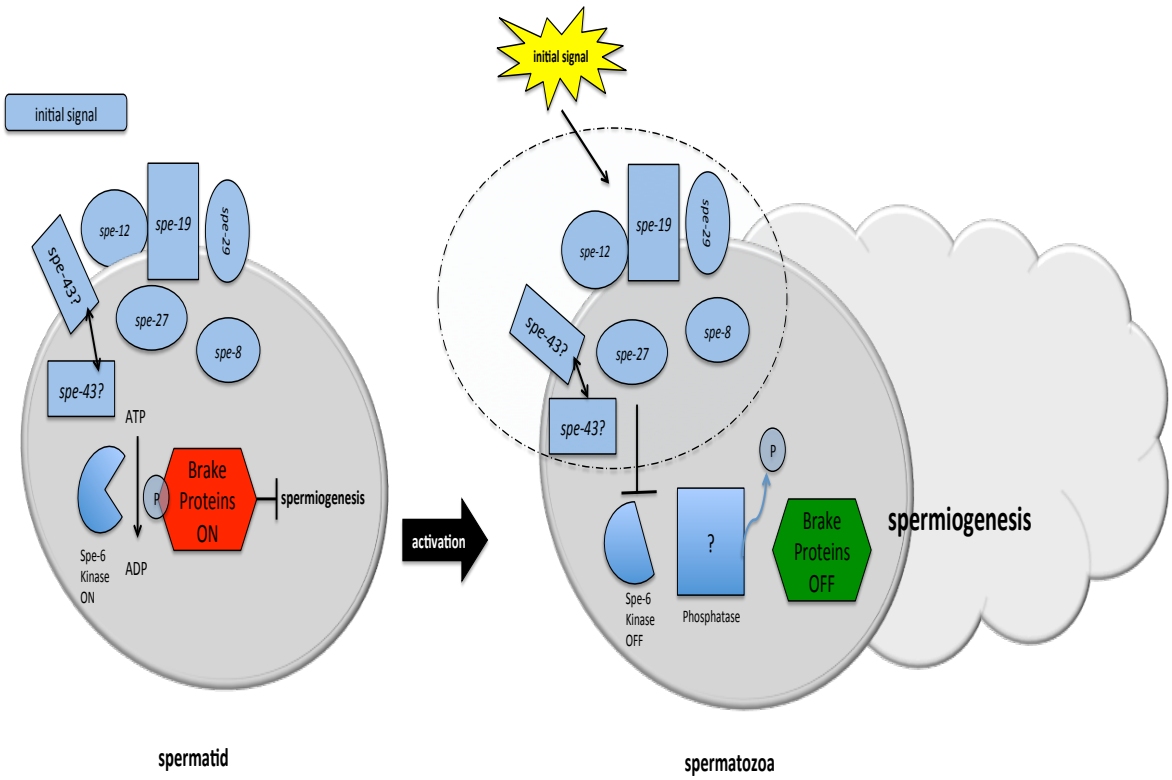


Figure 2.17, A model for the *spe-8* hermaphroditic spermiogenesis signaling initiation pathway.

In a spermatid that has not been exposed to the spermiogenesis initiation signal, the activate SPE-6 protein kinase phosphorylates, and thereby activates, a spermiogenesis "brake protein". Upon exposure of the spermatid to the initiation signal, the SPE-8, -12, -19, -27, -29 and -43 proteins inhibit SPE-6. A hypothetical phosphatase activity can then irreversibly dephosphorylate the spermiogenesis brake protein, rendering it inactive, ensuring that spermiogenesis proceeds completely.

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