5-2011

The deletion of a sperm-specific transcription factor causes cell cycle and sperm differentiation defects in Caenorhabditis elegans

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A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honors in Biology at the College of William and Mary.

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Williamsburg, Virginia
Spring 2011

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Abstract

Germ cells are highly dynamic in that they undergo several dramatic transitions throughout their lives. They begin as highly proliferative stem cells. Eventually, they become mitotically proliferating germ cell precursors. In *Caenorhabditis elegans*, these precursor cells do not even have a specified cell fate. Only after the cells move past the mitotic zone do the precursor cells enter meiosis and initiate the sexual specification process. As the gametes move through meiosis, sex-specific proteins are transcribed and translated. These proteins contribute to the differentiation pathway that creates a functional, haploid gamete. The regulation of these sperm-specific genes and proteins is not well understood. To further understand the process of how gametes progress through the early stages of meiosis, we examined a deletion mutation in the gene for a sperm-specific transcription factor, SPE-44. This deletion causes catastrophic failure of both the meiotic and differentiation pathways of spermatogenesis. *spe-44* mutants fail to create functional sperm, as their cells arrest in M phase. Instead of sperm, *spe-44* gonads contain terminal cells of varying size and composition. Smaller, older cells contain supernumerary microtubule asters and a variable amount of DNA. Phospho-histone H3, an M-phase marker, persists in the terminal, small cells of the mutant. As for sperm differentiation, *spe-44* mutants contain the Major Sperm Protein (MSP) but fail to assemble it into paracrystals after synthesis. The membranous organelles (MOs), Golgi-derived and nematode sperm-specific organelles which associate with the assembled MSP paracrystals, mislocalize within the small cells of the mutant and fail to dock on the cell cortex. During wildtype spermatogenesis, the SPE-44 protein itself localizes to the chromatin of the developing spermatocytes during pachytene and disappears in the

karyosome stage. Microarray data from Harold Smith of the NIH show that *spe-44* mutants do not express many of the sperm-enriched genes that are necessary for sperm production in *C. elegans*. SPE-7, a nematode sperm-specific protein, is among the gene products not present in the *spe-44* mutant. SPE-44 appears to function as the link between sperm specification and sperm production; it is the first sperm-specific transcriptional regulator in the *C. elegans* germ line to be described.

Introduction

The visible, physical identities of all living things- from fruit flies to elephants- are a direct result of the activities inside of the cells of these organisms. Thus, understanding the structures and dynamics of cells can give us tremendous insight into the nature of life itself. In this regard, there is no better place to start than germ cells. They are the metaphorical seeds that give rise to all animals, and the ability to reproduce is an integral part of the very definition of life. Germ cell precursors proliferate and their descendants differentiate to become either sperm or oocytes, depending on the sex of the organism. The primary focus of this study is the formation of sperm, or spermatogenesis, and its regulation by a sperm-specific transcription factor.

Meiosis

During sexual reproduction, diploid precursor cells undergo meiotic divisions resulting in haploid gametes. The process in which unspecified germ cells become functional oocytes or sperm is called gametogenesis. At a specific point during this process, mitotically-dividing germ cells switch over to meiosis. Unlike mitosis, meiosis results in four genetically variable daughter cells, not two identical ones. The daughter cells of meiosis contain half the chromosome number of the parent cell. During meiosis, a crossing over event occurs between paired homologs, which does not occur in mitosis. In addition to the transition from mitosis to meiosis, gametogenesis also includes chromatin remodeling.

During the extended process of meiosis, the organization and compaction of chromatin begins long before the nuclear divisions of meiosis. First, homologous

chromosomes must pair and line up next to each other in preparation for crossing over. During this time, the synaptonemal complex (SC) begins to form between the homologs, locking them together (von Wettstein et al., 1984). Pachytene is defined as the stage when the SC is fully formed (Roeder and Bailis 2000). After pairing, crossing over occurs between the paired homologs, which introduces even greater genetic variation into the process by creating unique combinations of alleles on a single chromosome. After crossing over, the chromosomes begin to condense in preparation for division. Diplotene is considered the stage when the SC has disassembled, but homologs are still held together by chiasmata- the points where the chromosomes have crossed over one another (Champion and Hawley, 2002). Next, the nuclear envelope breaks down as the chromosomes further condense in a phase known as diakinesis. During diakinesis, the centrosomes separate and position themselves at opposite poles of the cell. At this point, the cell has entered prometaphase and begins its nuclear divisions.

Caenorhabditis elegans as a model organism

C. elegans is a species of roundworm nematode that has proved to be exceedingly useful in the study of genetics and molecular biology. For classical genetic studies, the small physical size of the organism, large brood, and rapid development contribute to its utility. In addition, *C. elegans* was the first organism to have its genome fully sequenced, which makes it ideal for gene knockdown studies through the production of RNA interference libraries. Finally, a large number of lines containing knock out mutations exist, which can be procured from several consortiums. It is foreseeable that in the near future a mutant will exist for every gene in the *C. elegans* genome. For the study of

spermatogenesis, the large gonad of the worm occupies a considerable portion of its body volume, and the gonad is visible through the transparent body wall (Figure 1A). When the worm is dissected, the gonad and developing spermatids spill out of the body cavity. This allows us to view spermatogenesis *en vivo*, in real time (Wormbook).

Spermatogenesis

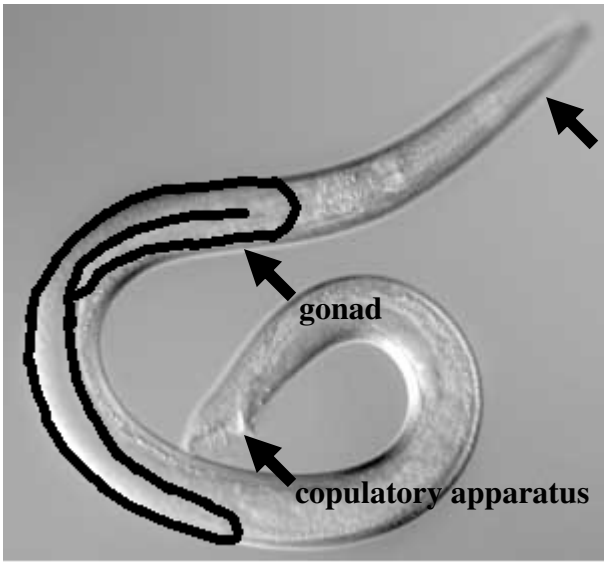
In most organisms, spermatogenesis involves a complex differentiation process in which both the final acculumation of sperm specific products and the final shaping of the sperm nucleus occurs only after the meiotic divisions have been completed. Furthermore the last step of this differentiation process involves sperm-specific products accumulating and the final shaping of the sperm occurring after the completion of the meiotic divisions inside the testis of the animal. After the meiotic divisions are complete, a functional sperm undergoes cytoplasmic shedding and become motile. There are a variety of mechanisms for sperm motility, which differ between species. Although there are outward, visible differences between the spermatogenesis programs of *Caenorhabditis elegans* and mammals, many molecular and intracellular mechanisms are conserved.

In mammalian spermatogenesis, the meiotic divisions take place prior to spermiogenesis. Spermiogenesis is the process of differentiation from a round spermatid into a motile spermatozoa that is capable of fertilizing an egg. During spermiogenesis in mammals, the DNA becomes condensed and the acrosom, a structure necessary for fertilization. The long, tubulin-based flagellum is formed, allowing the spermatozoa to swim. The developing spermatozoa sheds its cytoplasm to become streamlined and thus, faster. The combined processes of spermatogenesis and spermiogenesis in mammals can

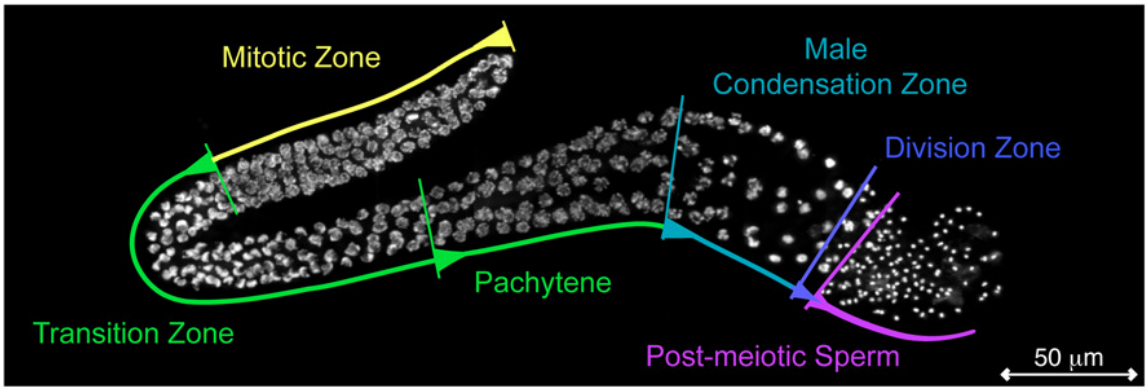
Figure 1

Whole body of a *C. elegans* male (A) and a photograph of its gonad with fluorescent DNA dye (B). A) The *C. elegans* male is built for reproduction. Much of its body mass is dedicated to the cells of the germ line (outlined in black), and its copulatory apparatus is used in mating with hermaphrodites. B) The gonad of a male *C. elegans* is temporally arranged. The proliferating germ line precursor cells are located in the mitotic zone at the distal tip. Near the hairpin turn, the cells switch from mitosis to meiosis and their cell fate (sperm or oocyte) is decided. Cells undergo an extended meiotic prophase until their nuclear envelopes break down at the end of the condensation zone and division occurs, producing hundreds of haploid spermatids (figure taken from Shakes et al., 2009).

A


head
gonad
copulatory apparatus

B


Mitotic Zone
Male
Condensation Zone
Division Zone
Pachytene
Transition Zone
Post-meiotic Sperm
50 μm

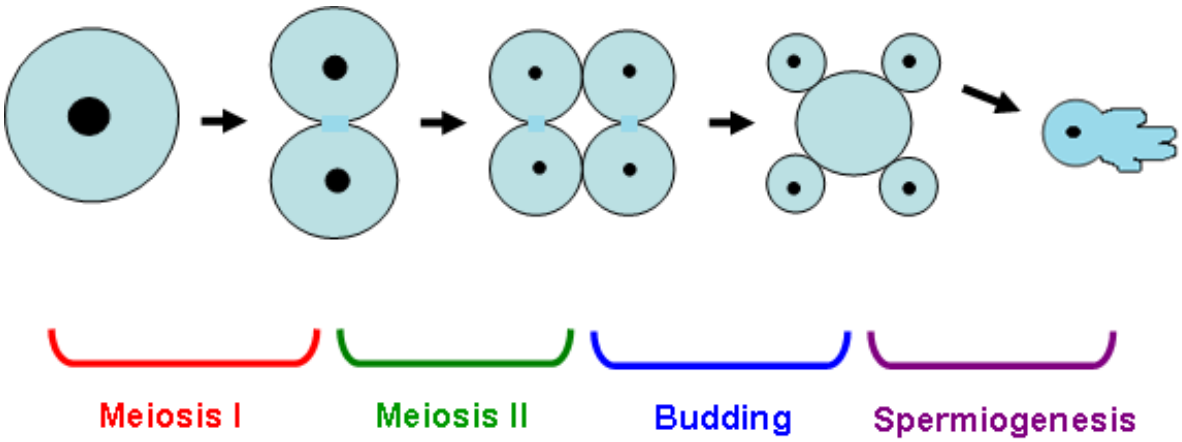
take up to a month from start to finish (for a complete review of mammalian spermatogenesis, see Wistuba et. al., 2007).

Spermatogenesis in *C. elegans*

In *C. elegans*, spermatogenesis occurs in a linear process along the elongated testis; it begins with the formation of primary spermatocytes (Figure 1B). During *C. elegans* spermatogenesis, meiotic prophase includes a final karyosome stage during which chromosomes form a single, highly-condensed mass within the nuclear envelope (Shakes et al., 2009). At this time, most transcription has ceased. Spermatocytes are large, diploid cells which are cytoplasmically continuous until they bud off at the proximal end of the gonad (Figure 2). After budding off, primary spermatocytes begin the meiotic divisions. During the first meiotic division, two secondary spermatocytes form, which may either separate into two distinct cells or remain connected via a small cytoplasmic bridge (Figure 2). During the second meiotic division, cytoplasmic shedding occurs. This is accomplished by the partitioning of the four (or two, in secondaries that have separated) nuclei into small spermatids, which bud off of a large, central residual body (Ward et al., 1981). The residual body contains the majority of the cytoplasm that is unnecessary for sperm function, including ribosomes, endoplasmic reticulum, actin, and microtubules. Once budding has occurred, the spermatids remain quiescent inside the male. During ejaculation, the sperm becomes activated. The spherical spermatids become bipolar and motile as each individual cell extends a single pseudopod (Nelson and Ward 1980; Shakes and Ward 1989; Stanfield and Villeneuve 2006). Nematode sperm are unique in that they crawl using pseudopodia, rather than swim using flagella.

Figure 2

A cartoon representation of spermatogenesis and spermiogenesis. Primary spermatocytes bud off of the syncytial gonad and begin their divisions, ultimately resulting in four haploid spermatids and a residual body. These spermatids are then activated and become spermatozoa during spermiogenesis (adapted from Worm Atlas).


Meiosis I
Meiosis II
Budding
Spermiogenesis

The molecular biology of *C. elegans* spermatogenesis

The process of *C. elegans* spermatogenesis can be broken down into several modules, which may be understood separately but ultimately work in concert to produce the haploid gamete. Thus, the disruption of a single module, such as chromatin dynamics, can secondarily affect the centrosome duplication cycle, ultimately halting meiosis and resulting in cell cycle arrests. For the purposes of this thesis, three modules will be considered: centrosome duplication and tubulin dynamics; meiosis and chromatin remodeling; and fibrous body-membranous organelle formation.

Centrosomes and tubulin: The proper duplication and orientation of centrosomes and their associated tubulin during meiosis is particularly critical in spermatogenesis. In most systems, including *C. elegans*, the sperm supplies the centrosome(s) to the developing embryo (Albertson 1984). In mitosis, the duplication of centrosomes is tightly coupled with DNA synthesis and is regulated by cyclin-dependent kinase and cyclin A/E (O'Connell et al., 2001). Although the exact molecular action of centrosome duplication has not yet been elucidated, the process proceeds in three basic steps. First, the two centrioles within the centrosome must separate, which occurs during G1 phase. Then, in S phase, daughter centrioles form. Finally, the two pairs of centrioles orient themselves and prepare for M phase (Peters et al., 2009). Meiosis varies from this basic pattern in that a second centrosome duplication occurs, which results in haploid nuclei containing two centrioles. For centrosome duplication in mitosis and meiosis in *C. elegans*, the ZYG-1 protein is essential, and the truncation of ZYG-1 in *C. elegans* results in overduplication of centrosomes during meiosis (Peters et al., 2009). The pericentriolar material must contain the right combination of proteins for duplication to occur, and

SPD-2 is a coiled-coil, centrosome-associated protein that recruits the proteins that make up the pericentriolar material (PCM) to the centrosome (Wormbase). In *spd-2(RNAi)* embryos, spindles failed to form properly (Pelletier et al., 2004). Microtubules remain in a network pattern in developing spermatocytes and only begin to nucleate from the centrosomes as the cell enters diakinesis and buds from the syncytial gonad (Shakes et al., 2009).

Nematode sperm contains centrioles that are contributed to the developing embryo during fertilization, but nematode sperm are unique in that they crawl using pseudopodia and do not use tubulin or actin. Thus, during the final budding division of *C. elegans* spermatogenesis, tubulin (except for the centrioles), actin, and myosin are deposited into a residual body (Ward et al., 1981), which is degraded and reabsorbed.

Chromatin dynamics: During meiosis (and mitosis), chromatin must undergo global changes in order to properly separate and segregate. Protein complexes known as condensins have been shown to supercoil DNA *in vitro*, and condensin knockout mutants show incomplete segregation and unequal daughter nuclei (Csankovski et al., 2009). Incomplete separation can result in chromatin bridging and tearing, which leads to daughter cells with incorrect complements of chromosomes. Condensins work alongside other chromatin modifiers to affect how tightly histone proteins bind the DNA, which also affects transcription.

Fibrous body-membranous organelle formation: This is one of the aspects of *C. elegans* cell biology that is not fundamentally conserved. As previously discussed, nematode sperm are models of efficiency, and any unnecessary structures are cast aside. During the budding division after meiosis II, actin and tubulin, two essential cytoskeletal

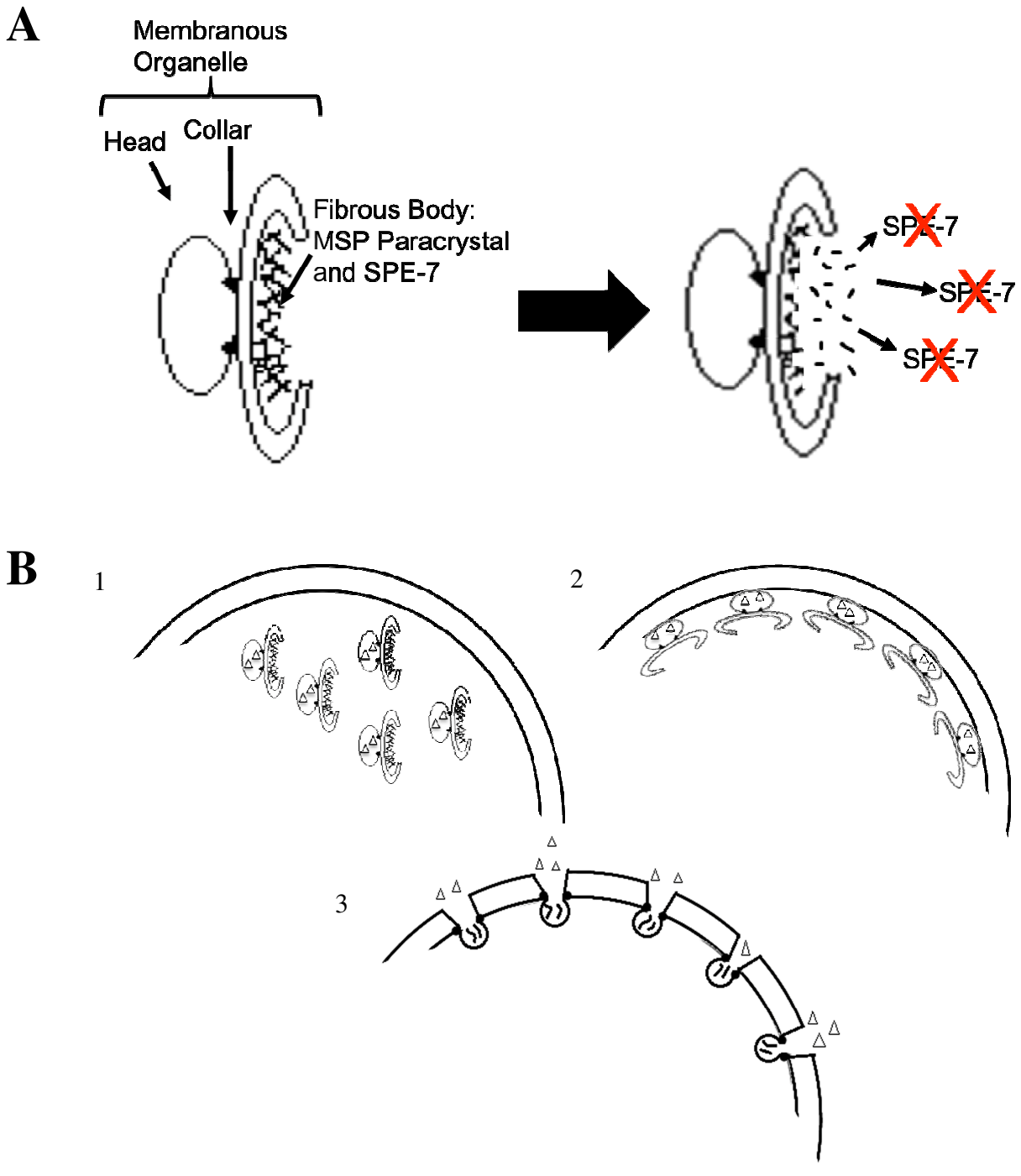
proteins, are essentially thrown away in the residual body along with the motor protein myosin (Ward and Klass, 1982). The functional spermatozoa manage to crawl using the Major Sperm Protein (MSP). The term "MSP" encompasses a family of proteins found in large quantities in nematode sperm. In motile spermatozoa, MSP localizes to the extending pseudopod (Ward and Klass, 1982). Within the pseudopod, MSP forms dynamic filamentous fiber complexes that stretch the membrane in the direction of motion, in a manner similar to actin-based cell motility (Sepsenwol, 1989).

Formation of the Fibrous Body-Membranous Organelle (FB-MO) complex is a sign of both sperm specification and development. As it is initially synthesized, MSP rapidly assembles into fibrous bodies. Then, after the second meiotic division, MSP must be segregated into the developing spermatids. *C. elegans* accomplishes this by sequestering newly synthesized MSP in a paracrystalline structure known as the Fibrous Body (FB) (Wolf et al., 1978; Figure 3A). The FB forms within the outpocketing of the Membranous Organelle (MO). The FB-MO structure is fully formed by the time the primary spermatocytes bud off the syncytial gonad.

MSP first polymerizes in primary spermatocytes, forming fibrous bodies. Then, the FB-MO complexes are partitioned during the meiotic divisions into the haploid spermatids, where MSP begins to depolymerize. As the fibrous body is depolymerizing, the MO docks on the cell membrane (Figure 3B-2). Finally, the MO fuses with the cell membrane, releasing its contents (L'Hernault and Arduengo, 1992).

Figure 3

The Major Sperm Protein (MSP) assembles into a paracrystal known as the Fibrous Body (FB). This structure is associated with the Golgi-derived Membranous Organelle (MO). SPE-7 associates with the Fibrous Body. A) A diagram showing how the nematode-specific SPE-7 protein interacts with the Fibrous Body-Membranous Organelle (FB-MO). The SPE-7 signal disappears in the spermatids of wild type animals, leading us to believe that it dissociates and is degraded and/or deactivated. B) A diagram depicting the localization of the FB-MO in different stages of spermatogenesis and spermiogenesis. In the primary spermatocyte, SPE-7 associates with the FB-MO in the cytosol (1) (Presler 2010). The FB-MOs are packaged into the spermatids during the budding division, at which point SPE-7 comes off of the FB-MO and the MSP paracrystal begins to disassemble. By the time the MO becomes docked at the cortex (2), the paracrystal has dissolved. The proteins at the collar of the MO associate with the cell membrane, and the vesicle fuses with the membrane, releasing the contents of the MO into the extracellular space (3).


A
Membranous Organelle
Head
Collar
Fibrous Body: MSP Paracrystal and SPE-7
SPE-7
SPE-7
SPE-7
B
1
2
3

Gamete-specific gene regulation

A variety of methods for gene regulation can be found in *C. elegans* and nematode lineages in general. In this study, we will specifically focus on the regulation of the germ line, particularly spermatogenesis.

Operons: Despite the presence of operons in the *C. elegans* genome, this mechanism is not widely used for regulation of spermatogenesis. Operons are clusters of genes that are driven by a single promoter and transcribed as a single mRNA. This manner of gene regulation is rarely seen in eukaryotes. In *C. elegans*, the individual genes are then spliced out of the transcript before translation (Spieth et al., 1993). Although 15 percent of all the known *C. elegans* genes and over 30 percent of germline-enriched genes reside in operons, virtually no spermatogenesis-specific genes are found in operons (Reinke and Cutter, 2009).

Endogenous RNA interference: In contrast to operon-mediated gene regulation, endogenous RNA interference (*endo* RNAi) has been shown to play a significant role in the regulation of spermatogenesis. Traditionally, RNAi is thought of as a mechanism for defense against foreign RNA and transposable elements. However, some small interfering RNAs target the organism's own genes, resulting in changes in expression. A group of proteins including ERI-1/3/5 (Enhanced RNA Interference), Dicer, and the RNA-directed RNA polymerase RRF-3 (RNA-dependent RNA polymerase Family) make up the ERI/Dicer complex which mediates this *endo* RNAi (Duchaine et al., 2006). Mutations in some of these genes, including *rrf*-3, can result in temperature-sensitive, sperm-specific sterility (Pavelec et al., 2009). In such an *rrf-3* mutants, there are decreased levels in a subset of spermatogenesis-specific siRNAs (Gent et al., 2009).

Transcription factors: Although the study of these different modes of regulation is relevant to the study of spermatogenesis regulation, the focus of this thesis is on regulation by transcription factors. The decision to become a spermatocyte or oocyte is critical in the *C. elegans* germ line. A single mutation is sufficient to cause a somatic male to produce oocytes instead of sperm. In the hermaphrodites, the germ line progenitor cells have the potential to undergo either spermatogenesis or oogenesis. A series of inhibitory interactions determine the sex of the somatic tissue as well as which type of gamete a germ line will produce. Although the sex-specification pathway has been well characterized (Wormbook), the process by which the "decision" to make oocytes or sperm alters gene regulation and the production of gametes is yet to be elucidated. The regulation of spermatogenesis is a large task with many contributors. In some cases, transcription of sperm genes is regulated by transcription factor proteins which bind specific sequences in the promoter region. In this way, the regulation spermatogenesis and oogenesis can be differentiated. Generally, the regulatory regions of sperm genes are found in the promoter, while the regulatory regions for oocyte genes are located in the 3' UTR (Reinke et al., 2004; Merritt et al., 2008).

One transcription factor, ELT-1 (Erythrocyte-like Transcription Factor), has been shown to regulate genes required for both spermatogenesis and intestinal development (Castillo-Olivares et al., 2009). The *elt-1* gene encodes a GATA transcription factor containing two zinc finger domains. This is unusual in *C. elegans* transcription factors because most contain only one zinc finger domain. The presence of two in ELT-1 suggests that it more closely resembles the regulatory proteins found in vertebrates, and in fact, ELT-1 shows considerable homology to the GATA-1 protein found in mice

(Spieth et al., 1991). Although it undoubtedly plays a role in the regulation of spermatogenesis, it is unlikely that ELT-1 is a master switch for turning on sperm production. GATA binding sites were only discovered in a handful of the 1343 sperm-enriched genes uncovered by Reinke et al. (2004).

Previous study of SPE-44

Recently, Harold Smith of the National Institute of Health has been studying a gene coding for a predicted sperm-specific transcription factor, SPE-44. In microarray studies, over 500 genes were discovered to be transcriptionally down-regulated in spe-44 mutants. As 343 of these genes were already classified as sperm-specific genes (Reinke et al., 2004), these results suggest that SPE-44 lies near or at the top of gene regulatory cascade that regulates spermatogenesis. Preliminary *in situ* hybridization reveals that peak expression of *spe-44* mRNA occurs in the third larval stage (L3) and fades in the fourth stage (L4) (Harold Smith, personal communication). *spe-44* is mutationally represented by the *ok1400* allele which was originally isolated by the Vancouver Gene Knockout Consortium as a clean, 1577 bp deletion of the C25G4.4 gene on chromosome IV mutant (Moerman and Barsted, 2008). It was subsequently characterized as a Spe mutation by Harold Smith (personal communication). Initial analyses revealed a classic Spe phenotype: sperm-specific infertility, which was effectively rescued by outcrosses to wild type males and injection of a transgene (Harold Smith, personal communication). Thus, the oocytes of *spe-44* mutants are unaffected. *spe-44* expression is regulated by the *endo* RNAi pathway; levels of siRNAs complementary to *spe-44* drop in ERI/Dicer mutants and are accompanied by an increase in *spe-44* mRNA levels (Harold Smith,

personal communication). In the *spe-44* mutant, dissected gonads revealed large, early-arrested cells and an absence of spermatids and/or residual bodies, but no further studies of the mutant phenotype were completed.

In this thesis…

We are interested in the exact nature of the Spe phenotype in the mutant, and we aim to determine what role SPE-44 is playing as a regulator of germ line gene expression. In this thesis, the *spe-44* mutant will be inspected using classical genetics, immunocytological and Differential Interference Contrast (DIC) imaging, and proteomics. We will ask the following questions:

- What role does SPE-44 play in the germ line?
- How does the deletion of *spe-44* affect the meiotic cell cycle?
- Does the *spe-44* mutant exhibit sperm differentiation defects?
- Where does SPE-44 localize in the temporal and spatial organization of the germ line?

Materials and Methods

Strains and Genetics

The mutant allele for the *spe-44* gene is known as *ok1400*, which eliminates 80 percent of the coding region and is a putative null. The strain used as the source of mutants was *dpy-20 spe-44/let-92 unc-22*. Such worms are phenotypically wild type, but produce wild type, *dpy-20 spe-44*, and dead embryos (*let-92 unc-22*). The linked recessive *dpy-20* marked the allele of interest on chromosome IV. Dpy-20 encodes a BED zinc-finger protein, and mutation of this gene causes a shorter, wider body shape (Wormbase) that is easily identified during crosses involving *spe-44*. *dpy-20* homozygotes are also *spe-44* homozygotes. The second chromosome contains a lethal mutation (*let-92)* to prevent *let-92 unc-22* homozygotes from surviving as well as a morphological marker (*unc-22*) to identify recombinant individuals in the event of recombination.

To produce male homozygotes, *dpy-20 spe-44* hermaphrodites were crossed to male heterozygotes (+ + */dpy-20 spe-44*). The *him-8* mutation leads to a high incidence of male progeny from hermaphrodite self-crosses.

spe-44 mutant strains and *him-8* (High Incidence of Males) control strains were raised and kept at 20ºC as described by Brenner (1973). All crosses were also performed at this temperature.

Immunofluorescence and DIC

Male worms were dissected in 5µL of sperm media (Shaham, 2006) containing 250 µM levamisole (a paralytic) using a 27.5 gauge needle. For various experiments, three different types of microscope slides were used, and all were Fisher brand. For most DIC experiments, uncharged glass slides were used. For experiments using immunofluorescence or a combination of DIC and immunofluorescence, positively-charged slides (Fisher Scientific # 12-550-19) were used. At times, a 1:1 solution of poly-L-lysine (Sigma # P8920) and deionized water was used to add additional positive charge. In order to create a monolayer of cells, gentle pressure was applied to 18x18mm (DIC) or 24x40mm (immunofluorescence) Fisher brand coverslips that were initially supported by four dots of Dow Corning High Vacuum grease.

Slides destined for immunofluorescence studies were freeze-cracked in liquid N_2 and fixed in -20ºC methanol overnight. After fixation, slides were washed in a 1x phosphate-buffered saline (PBS) solution three times, for five minutes each. Next, slides were transferred into a blocking solution (PBS+0.5% BSA and 0.1% Tween 20) for 20 minutes. Samples were prepared using the various antibody protocols described below. Then, slides were mounted with Biomedia Gel containing fluorescent 4',6-diamidino-2-phenylindole (DAPI) to label DNA.

In DIC preparations, the DNA marker Hoechst 33428 (100x dilution of 1 mg/mL stock) was added to the sperm media and levamisole mixture. Appropriate contrast was achieved by manipulation of a Wollaston prism.

An Olympus BX60 microscope equipped with epifluorescence and a Cooke cooled CCD camera was used for all microscopy and imaging. Images were cropped and optimized using Photoshop CS4. The following antibodies were used in our study:

Anti-α-tubulin

A 1:100 dilution of FITC (fluorescein isothiocyanate) direct labeled mouse anti-α-tubulin antibody (Sigma DM1A) was applied to samples and incubated for two hours at 20ºC. For preparations involving more than one antibody, the direct-label tubulin antibody should be applied with the secondary antibody. After incubation, a dip-wash in 1x PBS is necessary before mounting.

Anti-MSP

A 1:400 dilution of the unlabeled mouse anti-Major Sperm Protein antibody (4D5, a gift from D. Greenstein) was applied to samples and incubated for two hours at 20ºC. The slides were then washed in 1x PBS for two minutes before a 1:50 dilution of an affinity-purified, FITC-conjugated, anti-mouse IgG secondary antibody (Jackson Lab) was added. A second two-hour incubation at 20ºC was followed by a three-minute wash in 1x PBS before mounting.

Anti-Phospho-histone H3 (ser 10)

A 1:150 dilution of the unlabeled primary rabbit anti-Phospho-histone H3 (ser 10) antibody (Upstate Biotechnology) was applied to samples and incubated for two hours at room temperature, which was followed by two five-minute washes in 1x PBS. A 1:100

dilution of an affinity-purified, TRITC-conjugated, anti-mouse IgG secondary antibody (tetramethyl rhodamine isothiocyanate) secondary antibody (Jackson Lab) was used to label the primary antibody. The slides were then washed twice for two minutes each in 1x PBS.

1CB4

Although its antigen is unknown, the 1CB4 antibody effectively labels the Membranous Organelle (MO), a Golgi-derived structure found only in nematode spermatogenesis (Okamoto and Thomson, 1985). A 1:50 dilution of the unlabeled mouse antibody (Jackson Lab) was applied to samples and incubated for two hours at 20ºC. The samples were then washed three times for five minutes each in 1x PBS. A 1:50 dilution of an affinity-purified, FITC-conjugated, anti-mouse IgG secondary antibody (Jackson Lab) was applied and incubated for 1.5 hours at 20ºC, and then the slides were dip-washed in 1x PBS before mounting.

Anti-SPE-7

A 1:1250 dilution of primary rabbit anti-SPE-7 antibody was applied to the samples and incubated for two hours at 20ºC. After washing the slides twice for five minutes each, a 1:100 dilution of an affinity-purified, TRITC-conjugated, anti-mouse IgG secondary antibody was added. The samples were incubated for one hour at 20ºC and then washed for three minutes in 1x PBS before mounting.

This protocol was optimized with the addition of a pre-incubation of the secondary antibody in acetone worm powder to eliminate non-specific binding. The

secondary was incubated with the powder for two hours at 20ºC on a Labquake Shaker. The mixture was spun at maximum speed in an Eppendorf 5415 C centrifuge. The supernatant was then removed from the pellet and applied to the samples.

Anti-SPE-44

A variety of conditions were tested with the affinity-purified SPE-44 antibody (Harold Smith), including dilutions of 1:5, 1:15, 1:45, and 1:50. It was found that all dilutions effectively labeled the protein, with some non-specific binding occurring at all concentrations. The primary antibody was applied to samples and incubated for two hours at room temperature, which was followed by one two-minute wash in 1x PBS. A 1:100 dilution of an affinity-purified, TRITC-conjugated, anti-mouse IgG secondary antibody (Jackson Lab) was used to label the primary antibody. The slides were then washed twice for two minutes each in 1x PBS before mounting.

In the future, we would recommend using acetone powder in conjunction with greater dilutions of the primary antibody to decrease non-specific binding.

Western Blot

To collect a sample, approximately 500 worms were washed from agar plates using M9 and pelleted via centrifugation. The pellet was flash frozen using liquid nitrogen and stored at -80ºC.

First, β-Mercaptoethanol (BME) was added to 4x LDS Sample Buffer (Invitrogen). Then, the BME-Sample Buffer mixture was incubated in a hot water bath (100 ºC) for five minutes. Hot BME-Sample Buffer mixture was added to samples, and

samples were run on pre-cast gels (Invitrogen) in 1x MOPS running buffer at 150V for 55 minutes. The gel was equilibrated in 50 mL of 1x Semi-Dry Transfer Buffer for 10 minutes. The PVDF membrane was soaked in 10 mL of MeOH, followed by 1x Semi-Dry Transfer Buffer. The contents of the gel were transferred to the membrane for 30 minutes at 19V. The membrane was soaked in blocking solution (4% TBST - Tris-Buffered Saline plus Tween - milk) overnight. The anti-SPE-7 primary antibody was diluted in 4% TBST milk, and the membrane was incubated in the antibody solution (anti-SPE-7 1:5000) for two hours at room temperature. Meanwhile, the ECL (enhanced chemiluminescent) goat anti-rabbit secondary antibody (Abcam) was incubated in worm acetone powder. After incubation, the membrane was washed 3x30 minutes in TBST solution. Immobilon, a detection agent, was applied to the membrane and placed in a film cassette to be developed.

Explanation of Methods

Wild type controls: The *him-8* (High Incidence of Males) mutation results in a failure of X chromosome segregation during meiosis. This causes a greater number of XO (male) progeny to be sired by XX (hermaphrodite) self-crossing (Wormbase). For our purposes, *him-8* worms were considered wild type. By outcrossing *dpy-20 spe-44* hermaphrodites with *him-8* males in a 1:2 ratio, we could obtain progeny from self-infertile hermaphrodites, producing a burst of phenotypically wild type progeny, which were also fertile. We used these fertile progeny in a second outcross with *him-8* males to

create *him-8; dpy-20 spe-44* homozygote males, which are referred to as simply *spe-44* males in this thesis for brevity.

DIC optics: Differential Interference Contrast (DIC) imaging uses a specialized Wollaston prism to section white polarized light. The light then passes through transparent, unstained tissue. Light travels more slowly through tissues with a high refractive index, which creates a phase difference by the time the light has reached the objective (LOCI). Since the sample does not need to be fixed or stained, DIC is extremely useful in the examination of live tissue.

Combination DIC/immunofluorescence imaging: Young adult males were cultivated by visually confirming the L4 larval stage and dissecting 12 hours post-L4. To get a better picture of what the actual cell membranes looked like, we used DIC and immunocytology in concert by quickly viewing the samples under DIC, then freeze-cracking and fixing them. Anti-α-tubulin antibodies and DNA dye were applied after fixing.

Results

spe-44 mutants are sterile

To formally confirm the self-sterility of *spe-44* hermaphrodites, we picked twenty homozygous, L4 *spe-44 dpy-20* hermaphrodites to individual plates and monitored these worms for the production of offspring. All twenty failed to produce either viable or dead embryos. Instead they laid unfertilized oocytes, a phenotype that suggested that they failed to produce any functional sperm. Although six of these 20 hermaphrodites died as young adults due to a hernia (gut and gonad extruding through the vulva), the other 14 remained viable throughout the normal period of adult fertility and thus had the opportunity to produce progeny. In contrast, *spe-44* / + heterozygotes are fully fertile, indicating that *ok1400* is a recessive allele. Importantly, *spe-44(ok1400)* hermaphrodites crossed to wild type males proved to be fully fertile. Taken together, these results confirm that the fertility defects in *spe-44(ok1400)* are sperm-specific.

spe-44 mutants lack spermatids

Since the *ok1400* allele exhibited sperm-specific fertility defects, we dissected male gonads to more closely examine the specific nature of the spermatogenesis-defective phenotype. Upon first inspection of isolated *spe-44* gonads under DIC optics, we observed gonads of normal size with maturing spermatocytes that detached from the syncytial gonad. Although the more distal portions of the gonad appeared normal (data not shown), the proximal portion of the mutant gonad contained cells of varying size and structure. We failed to observe any haploid spermatids (Figure 4A). The largest cells,

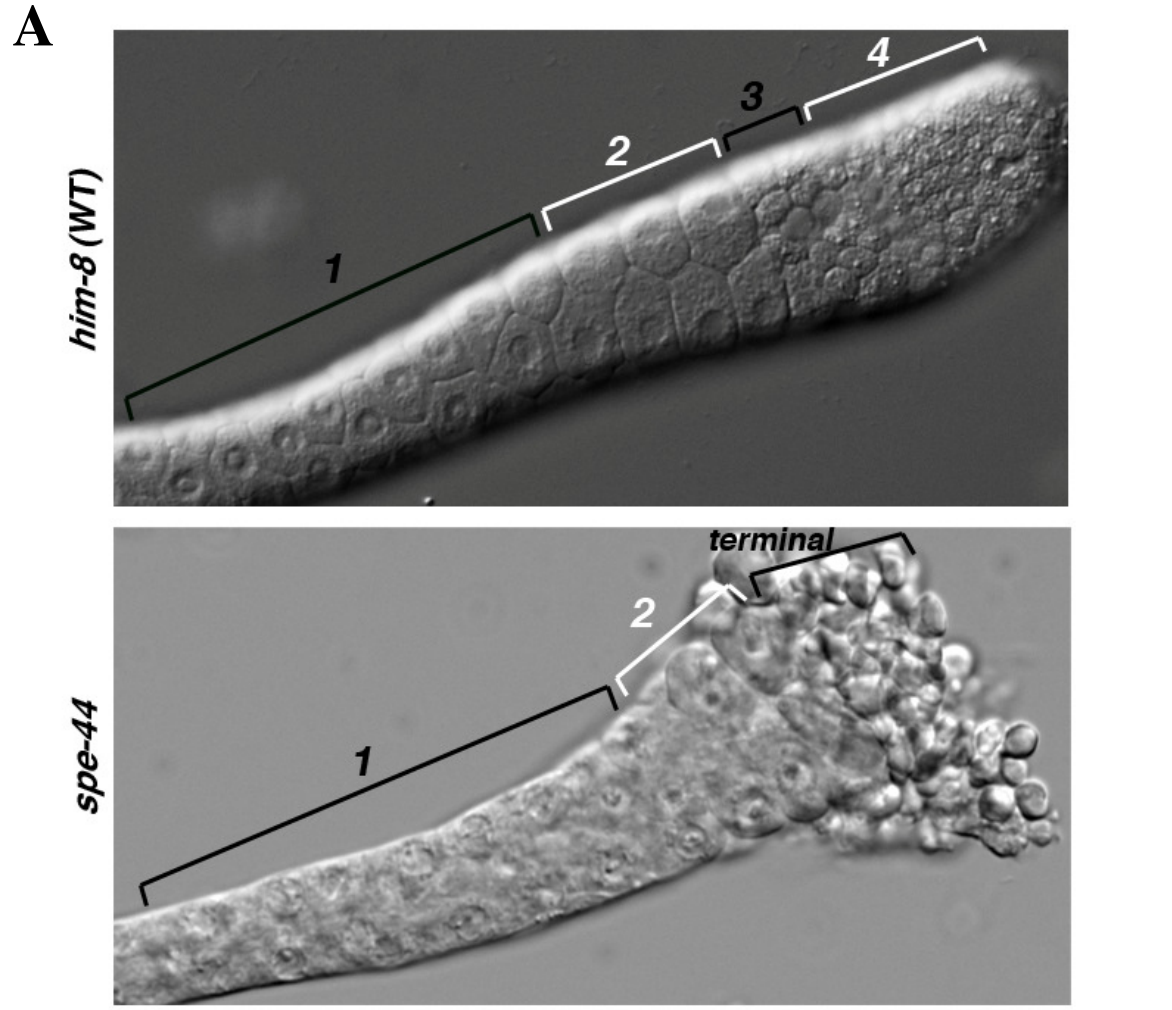
comparable in size to primary spermatocytes, often contained visible vacuoles, a feature that is never observed in wild type spermatocytes (Figure 4B). *Spe-44* mutants lack spermatids, which are seen in wild type as small, round cells containing a visible "button" of highly compact DNA under DIC optics. Instead, the smallest *spe-44* cells appear larger than wild type spermatids, and do not contain the "button" of DNA. Many large and small *spe-44* spermatocytes also lacked the grainy texture that is characteristic of functional sperm. This grainy texture is due to the presence of refractive bodies, presumably organelles, which segregate into spermatids and not into the residual body. All *spe-44* cells appear smooth like wild type residual bodies, even when still attached to the syncytial gonad.

The "oblong" cell in Figure 4B hints at a potential connection between the large spermatocytes that bud off the rachis and the terminal small cells. In wild type, primary spermatocytes undergo a traditional actin/myosin based cytokinesis after meiosis I. However, after the second meiotic division, wild type secondaries undergo an asymmetric budding division. Since no budding divisions were observed in the mutant, we predict that either some type of abnormal cytokinesis is occurring or that the actin/myosin based first cytokinesis is occurring irrespective of the lack of clear nuclear division. These occasional oblong cells may be an intermediate stage of either process.

In comparison to the wild type controls, the male gonads in *spe-44* mutants were very sensitive to excessive squashing, making it difficult to create a monolayer of spermatocytes without destroying the integrity of the cell membranes. This observation suggests potential abnormalities in the cytoskeletal integrity of the mutant sperm.

Figure 4

Differential Interference Contrast images comparing wild type male gonads to the *spe-44* mutant. A) Isolated wild type and mutant male gonads. The *spe-44* mutant consistently lacks spermatids and residual bodies (n = 25 worms). Labels in WT: 1) pachytene 2) condensation zone 3) division zone 4) haploid spermatids and residual bodies. Labels in *spe-44*: 1) pachytene 2) condensation zone and terminal phenotype zone (400x magnification) B) Cut outs of individual cells exhibiting the sequential stages of spermatogenesis in wild type and potentially equivalent stages in the *spe-44* mutant. Large vacuoles are present in various cell types in the mutant (arrowheads). (Pictures taken at 600x magnification)


A
him-8 (WT)
1
2
3
4
spe-44
1
2
terminal
B
him-8 (WT)
primary
secondary
meiosis II
budding
spermatids
spe-44
primary
irregular shape
oblong
vacuoles
small cells

Cell cycle defects in *spe-44* spermatocytes

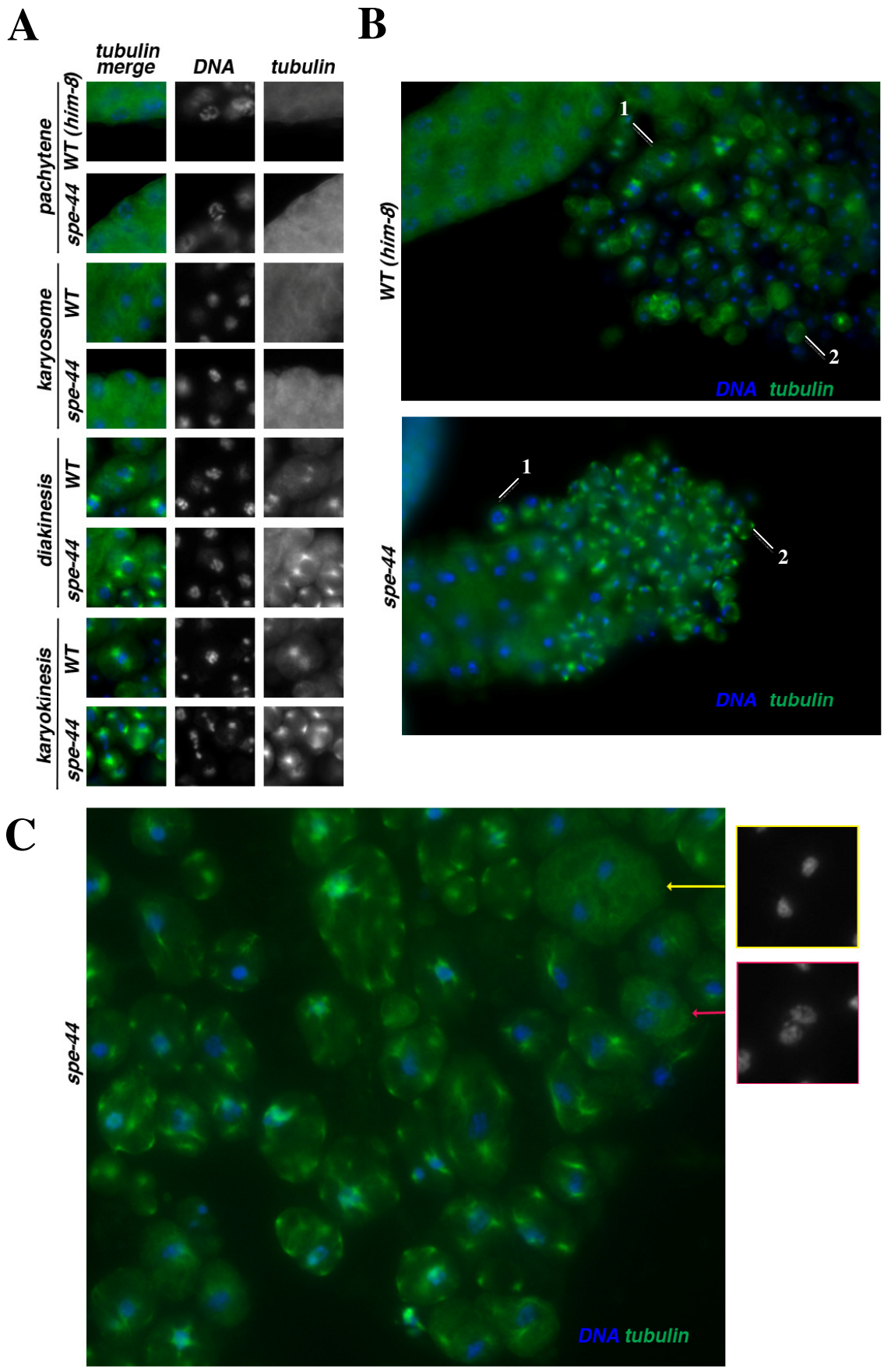
To determine whether the defects in *spe-44* spermatogenesis involved defects in meiotic cell cycle progression, we next analyzed the mutant gonads using DAPI, a fluorescent DNA stain, and an antibody against α-tubulin. In combination, these two markers can be used to stage progression through the meiotic divisions based on the chromatin morphology and the structure of the microtubule spindles. The temporal morphology of the DNA along the syncytial germ line corresponds to the various stages of meiosis (Figure 1B). The mitotic region at the distal tip as well as the transition zone (from mitosis to meiosis) appeared normal (data not shown). Also, pachytene, karyosome, and diakinesis stages showed typical morphology and spatial organization (Figure 4A). As the nuclear divisions commence in wild type, the mature primary spermatocytes bud off the cytoplasmically continuous gonad to become independent cells.

In general, the DAPI-tubulin pattern in the large, primary spermatocytes of the mutant appears normal. In *spe-44* mutants, primary spermatocytes budding off the rachis appear very similar to wild type: the homologs have resolved after crossing over and have become highly condensed. The pair of microtubule asters have separated and nucleated, extending microtubule filaments. However, we observed that some large cells in the mutant may have two nuclei, although these were only observed in older, well-squashed adult worms (more than 40 hours past larval stage 4, see Figure 4C). This phenotype was observed in 10% (4/40) heavily-squashed gonads.

Inspection of the *spe-44* male germ line with an anti-α-tubulin antibody revealed abnormal persistence of supernumerary, stable microtubule asters. Cells still attached to

Figure 5

DAPI (blue) and anti-tubulin (green) staining in wild type and mutant gonads. A) Cut-outs of wild type (*him-8)* and *spe-44* spermatocytes show typical progression through pachytene and karyosome stages. However, during the meiotic division phase, the DNA in *spe-44* cells exhibits chromosome segregation defects. In both wild type and *spe-44* spermatocytes, distinct microtubule asters are first observed in diakinesis. Small, spermatid-like cells contain active, supernumerary asters and varying amounts of DNA, ranging from none to four DNA dots (n = 62 worms). (400x magnification) B) Fields showing spermatocytes budding off of the rachis in wild type and the mutant. Labels in WT: 1) primary spermatocyte budding off rachis 2) residual body (green) surrounded by haploid spermatids (blue). Labels in *spe-44*: 1) primary spermatocyte budding off rachis 2) terminal cell with multiple microtubule asters (green) and DNA (blue) (400x magnification) C) In older, heavily-squashed specimens, some spermatocytes have double-nuclei (arrows and DAPI-only inserts). (600x magnification)


A
tubulin merge
DNA
tubulin
pachytene
WT (him-8)
spe-44
karyosome
WT
spe-44
diakinesis
WT
spe-44
karyokinesis
WT
spe-44
B
WT (him-8)
1
2
DNA tubulin
spe-44
1
2
DNA tubulin
C
spe-44
DNA tubulin

the syncytial germ line exhibited a normal network pattern (Figure 5B). After the primary spermatocytes detach from the rachis, the tubulin formed two asters which began to migrate to the opposite ends of the cell. From this point, it is unclear exactly what is happening, but chromosome segregation is definitely abnormal (Figure 5A). Coordinated meiotic chromosome segregation does not occur. In addition, some single cells contain up to eight microtubule asters. The smallest and presumably the oldest cells are positioned the farthest from the rachis. Multiple fragments of DNA are observed in some small cells, while others contain none. Spindles with overlapping microtubules were rare. Instead, the supernumerary asters observed in the *spe-44* mutant were found in one of two places: associated with either the chromatin or the cell cortex.

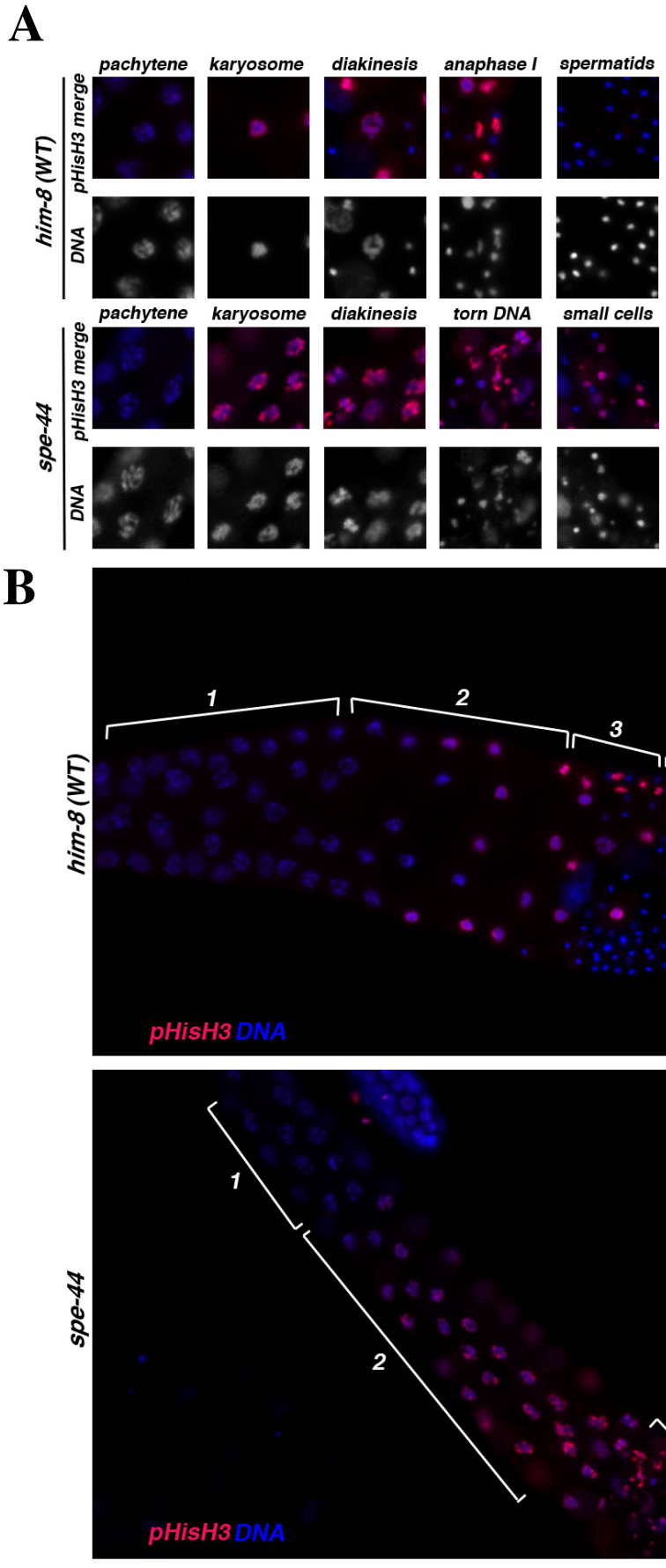
Phospho-histone H3 (ser10) staining persists in the mutant

The striking stability of the microtubule spindles in *spe-44* spermatocytes, suggests that the cells may be arresting in M-phase of the cell cycle. To critically test that hypothesis, we analyzed the pattern of anti-phospho-histone H3 (ser10) labeling in the mutant spermatocytes, as this particular post-translational modification of the H3 histone tail serves as a highly conserved marker of an M-phase state in organisms ranging from mammals to plants and protozoa (Crusio et al., 2002).

In wild type male gonads of *C. elegans*, anti-pHisH3 (ser10) staining is first detected as the synaptonemal complex begins to break down. It then appears most intense during the karyosome and division phases before fading in the spermatids (Shakes et al., 2009, Figure 6). In the *spe-44* mutant, pHisH3 staining first appears in pachytene in the mutant, as in wild type. However, pHisH3 staining persists in the later, smaller

Figure 6

DAPI and anti-phospho-histone H3 (ser 10) staining in wild type and the *spe-44* mutant. A) Cut-outs of various stages of spermatocytes in wild type and the *spe-44* mutant. During both wild type and *spe-44* spermatogenesis, pHisH3 can be first detected in karyosome spermatocytes. In wild type, anti-pHisH3 labels chromatin throughout the meiotic divisions but does not label the chromatin of spermatids. In *spe-44*, anti-pHisH3 labeling persists through the terminal arrest stages (n = 17 worms). B) Full gonad pictures show persistent pHisH3 labeling in the smaller, terminally-arrested, sperm-like *spe-44* spermatocytes, while pHisH3 labeling is completely absent in wild type spermatids. Labels in WT: 1) pachytene 2) condensation zone 3) division zone 4) haploid spermatids. Labels in *spe-44*: 1) pachytene 2) condensation zone and terminal phenotype zone.


A
pachytene
karyosome
diakinesis
anaphase I
spermatids
him-8 (WT)
pHisH3 merge
DNA
pachytene
karyosome
diakinesis
torn DNA
small cells
spe-44
pHisH3 merge
DNA
B
him-8 (WT)
1
2
3
4
pHisH3 DNA
spe-44
1
2
terminal
pHisH3 DNA

terminally-arrested cells of the mutant (Figure 6A). This indicates that the chromatin is remaining in an M phase state. This pattern was previously described by Golden et al., (2000) in *mat* (Metaphase to Anaphase Transition) mutants, which were shown to not exit M phase. Here, we also see the chromosome missegregation phenotype that was observed in the samples treated with anti-tubulin (Figure 5).

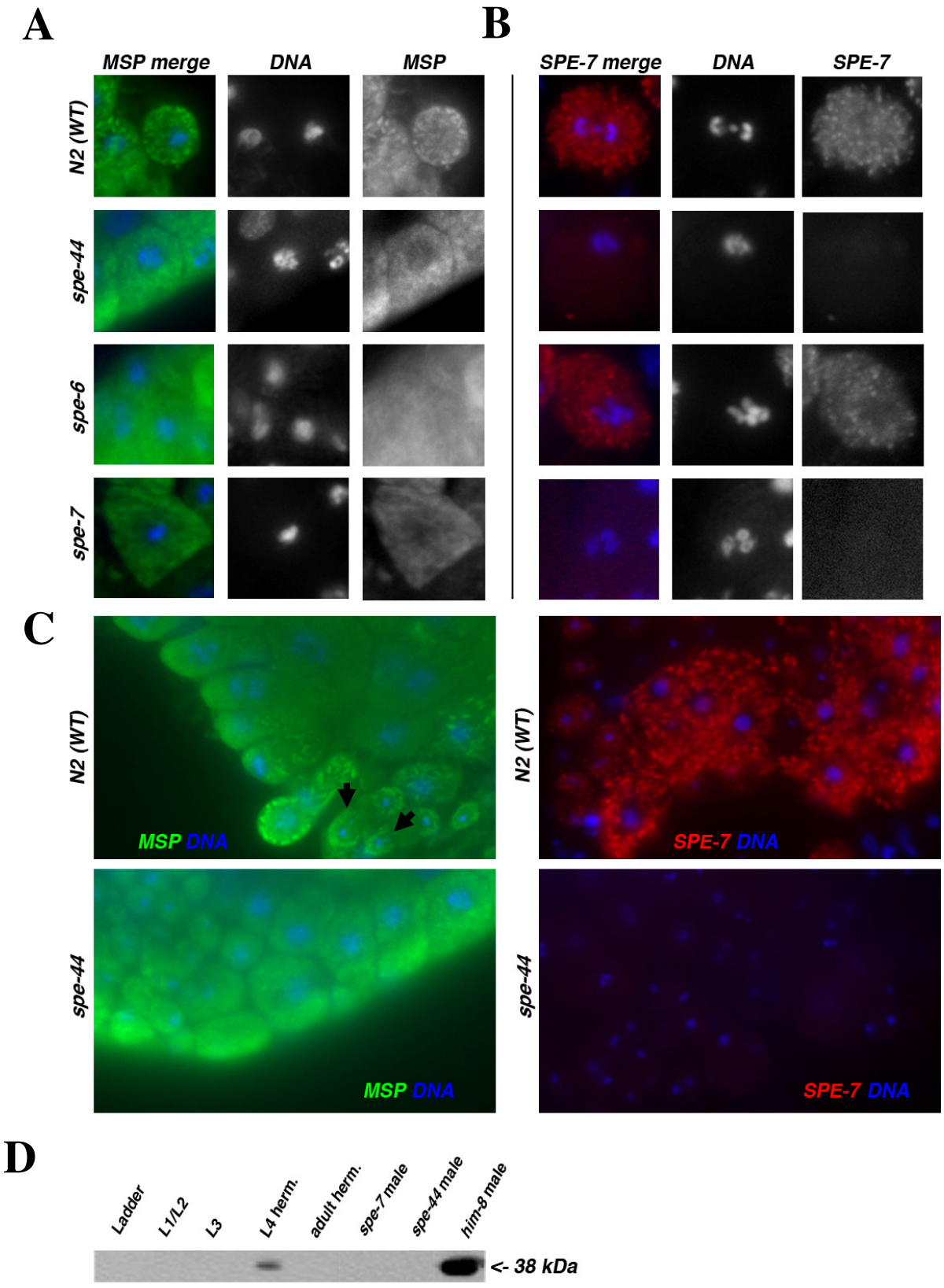
Mutants have defects in the sperm differentiation program

Given the irregular state of the cell cycle, we began to question the differentiation pathways. Cell cycle and sperm differentiation pathways were previously linked in *C. elegans* in Marc Presler's honor thesis (2010). One excellent marker for progression through the sperm differentiation is the major sperm protein (MSP) that ultimately serves as the primary motility protein in motile spermatozoa. The monoclonal MSP antibody shows a dynamic pattern in wild type (Figure 7A).

In the *spe-44* mutant male, MSP is produced at the right time and place, yet it fails to assemble into fibrous bodies (FBs) (Figure 7A). This phenotype was also observed in other known spermatogenesis (Spe) mutants, including *spe-6* (Varkey et al., 1993) and *spe-7* (Presler, 2010); Figure 7A). *spe-6* encodes a casein kinase that is essential for both the formation of the FB (Varkey et al., 1993) and for the subsequent process of spermatid activation (Muhlrad and Ward 2002). *spe-7* encodes an unknown protein product which has been shown to colocalize with the FBs (Presler, 2010) (Figure 3A). Not surprisingly, the antibody signal for SPE-7 antibody is undetectable in *spe-7* mutants (Presler, 2010 and Figure 7B). Interestingly, SPE-7 is also undetectable in *spe-44* mutants (Figure 7B).

Figure 7

Major Sperm Protein (MSP) and SPE-7 in wild type and variety of sperm-specific mutants, including *spe*-44 A) MSP is present, but mislocalizes in the *spe-44* (n = 12 worms)*, spe-6* (n = 9 worms)*, and spe-7* (n = 19 worms) mutants. Arrowheads: MSP localizing to the budding spermatids in wild type. B) SPE-7 localization appears similar to wild type in the *spe-6* mutant (n = 8 worms), but the protein appears to be absent in the *spe-44* mutant (n = 11) worms. C) Larger fields show the dynamic localization of MSP and SPE-7 in wild type and their abnormal patterns in a *spe-44* background. D) A western blot with an anti-SPE-7 antibody in various wild type and mutant samples.


A
B
MSP merge
DNA
MSP
SPE-7 merge
DNA
SPE-7
N2 (WT)
spe-44
spe-6
spe-7
C
N2 (WT)
MSP DNA
N2 (WT)
SPE-7 DNA
spe-44
MSP DNA
spe-44
SPE-7 DNA
D
Ladder
L1/L2
L3
L4 herm.
adult herm.
spe-7 male
spe-44 male
him-8 male
<- 38 kDa

To confirm this result, we performed a Western Blot using the same anti-SPE-7 antibody. We found that the 38 kDa SPE-7 protein was present only in gonads that were actively undergoing spermatogenesis (*him-8* males and L4 hermaphrodites). Hermaphrodites undergo a burst of spermatogenesis during the fourth larval (L4) stage before switching to oogenesis as they reach adulthood, so we would expect SPE-7 protein to be present in L4 hermaphrodites but not in adult hermaphrodites. Harold Smith (personal communication) has subsequently confirmed via microarrays that *spe-7* mRNA levels are greatly decreased in *spe-44* mutants.

To investigate the formation and dynamics of the membranous organelle, we observed the immunostaining pattern of the monoclonal 1CB4 antibody. Although its antigen is unknown, the antibody has been shown to be an effective MO marker. In wild type, staining first appears in the pachytene portion of the syncytial gonad. Staining becomes more concentrated and brighter as primary spermatocytes bud off the gonad. Staining is visible in the spermatids, and localizes to the cell cortex (Figure 3; Figure 8). In the *spe-44* mutant, MO localization appears to be normal until the small cell stage. At this point, staining does not localize to the cortex as in the wild type spermatids (Figure 8). Defects in MO morphology and localization have been shown to cause sperm-specific sterility in *C. elegans* (Shakes and Ward, 1989; Machaca and L'Hernault, 1997; Zhu and L'Hernault, 2003).

Anti-SPE-44 antibody localizes to the nucleus

Having completed a brief characterization of the *spe-44* mutant, we acquired a SPE-44 affinity-purified antibody from Harold Smith. We studied its pattern in mutant

and wild type males and wild type hermaphrodites at a variety of stages of germ line development. The life cycle of *C. elegans* can be divided into five larval stages (L1, L2, L3, L4, and adulthood)**.** Although germ line precursor cells are continually undergoing mitosis, it is not until L3 that the gonad begins meiosis and reaches sufficient size for dissection. We staged populations of wild type (*him-8*) male worms and dissected them at several time points: L3, L4, and adulthood. When we applied the anti-SPE-44 antibody, we saw that the antibody appeared to be in the meiotic nuclei of L3 males and hermaphrodites. SPE-44 first appears in early to mid-pachytene and persists until the karyosome stage, when it fades. This is consistent with the findings of Shakes et al. (2009), that reveal that global transcription is strongly down-regulated by DNA condensation events of karysome formation As expected, the SPE-44 signal within the syncytial gonad of either sperm-producing *spe-44* mutants or the oocyte-producing adult hermaphrodites is not above background levels (Figure 9B). However, the antibody does label the maturing oocytes and other somatic tissues. The staining observed around the spermatids (large arrowheads) can be dismissed as background, as it is also present in the *spe-44* small cell debris (Figure 9B). The staining seen in the oocyte (Figure 9B) of the hermaphrodite is also seen in fertilized embryos (data not shown). It is unclear why the antibody is labeling these structures, although we anticipate that adjustment of the antibody protocol will reduce non-specific staining.

Figure 8

1CB4 staining reveals mislocalization of the membranous organelle in the small cells of *spe-44* mutant gonads. Staining first appears in pachytene in wild type and the mutant. Throughout meiotic prophase, the MOs appear to localize in the cytoplasm. After the meiotic divisions, MOs are segregated into the budding spermatids, and individual MOs can be seen docked at the cortex in wild type spermatids. However, 1CB4 does not label discrete organelles in the cortex in the terminal small cells of the *spe-44* mutant (n = 22 worms). The membranes also appear to aggregate in these cells. Although it appears as though 1CB4 is labeling the DNA in these cells, this is most likely an artifact of long exposures creating a bleed-through effect of the DAPI stain. (600x magnification)

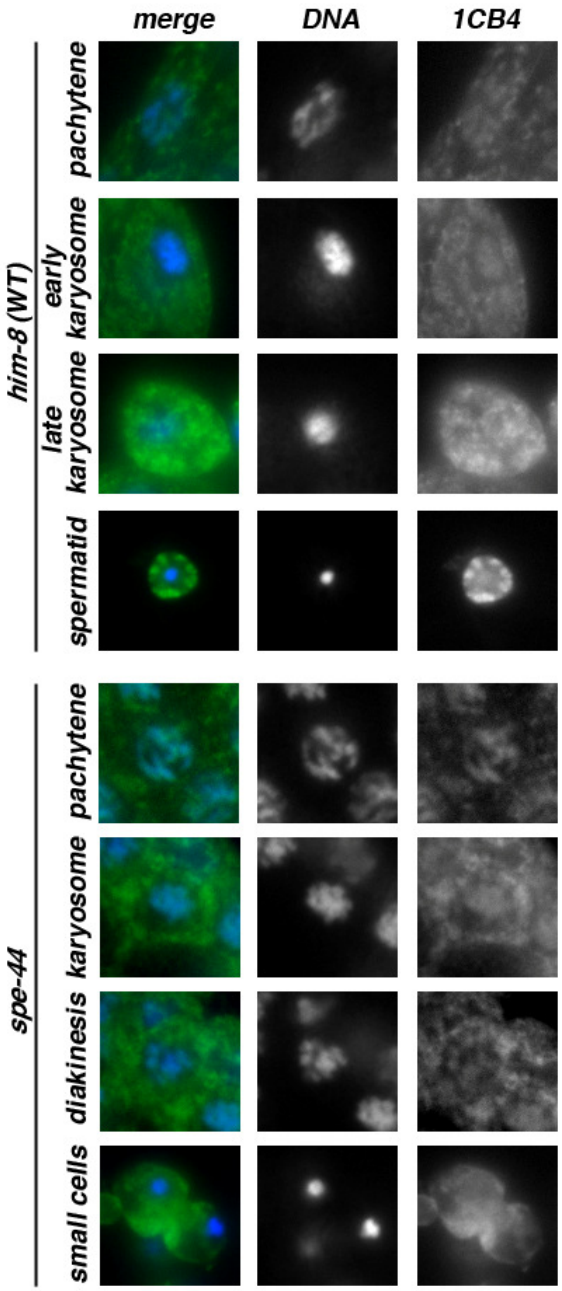
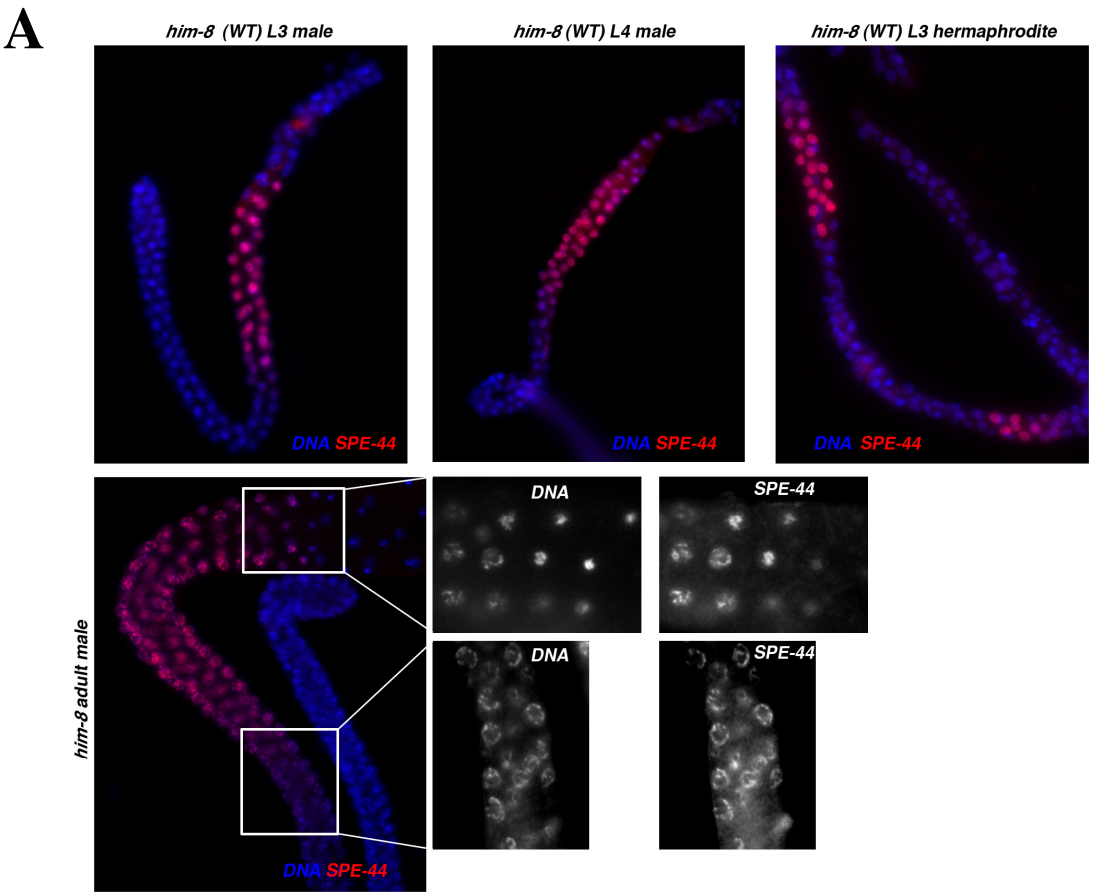

merge
DNA
1CB4
him-8 (WT)
pachytene
early karyosome
late karyosome
spermatid
spe-44
pachytene
karyosome
diakinesis
small cells

Figure 9

The SPE-44 antibody localizes to the nucleus. A) Whole gonads showing SPE-44 localization in various stage of wild type males and hermaphrodites (400x magnification) with cut outs showing the loss of SPE-44 signal during the diakinesis to karyosome transition (top) and initial "turn-on" of SPE-44 signal in early pachytene (n = 36 worms) (600x magnification). B) SPE-44 staining consistently fades in the *spe-44* mutant (n = 14 worms) and *him-8* adult hermaphrodite (n = 7 worms), although it does label the cortex of unfertilized oocyte as well as the developing embryo. (Left to right) Arrows show the nuclei of two maturing oocytes and a collection of haploid sperm nuclei (400x magnification)


A
him-8 (WT) L3 male
him-8 (WT) L4 male
him-8 (WT) L3 hermaphrodite
DNA SPE-44
him-8 adult male
DNA
SPE-44
B
him-8 (WT) adult male
him-8 (WT) adult hermaphrodite
spe-44 adult male
merge
SPE-44
DNA

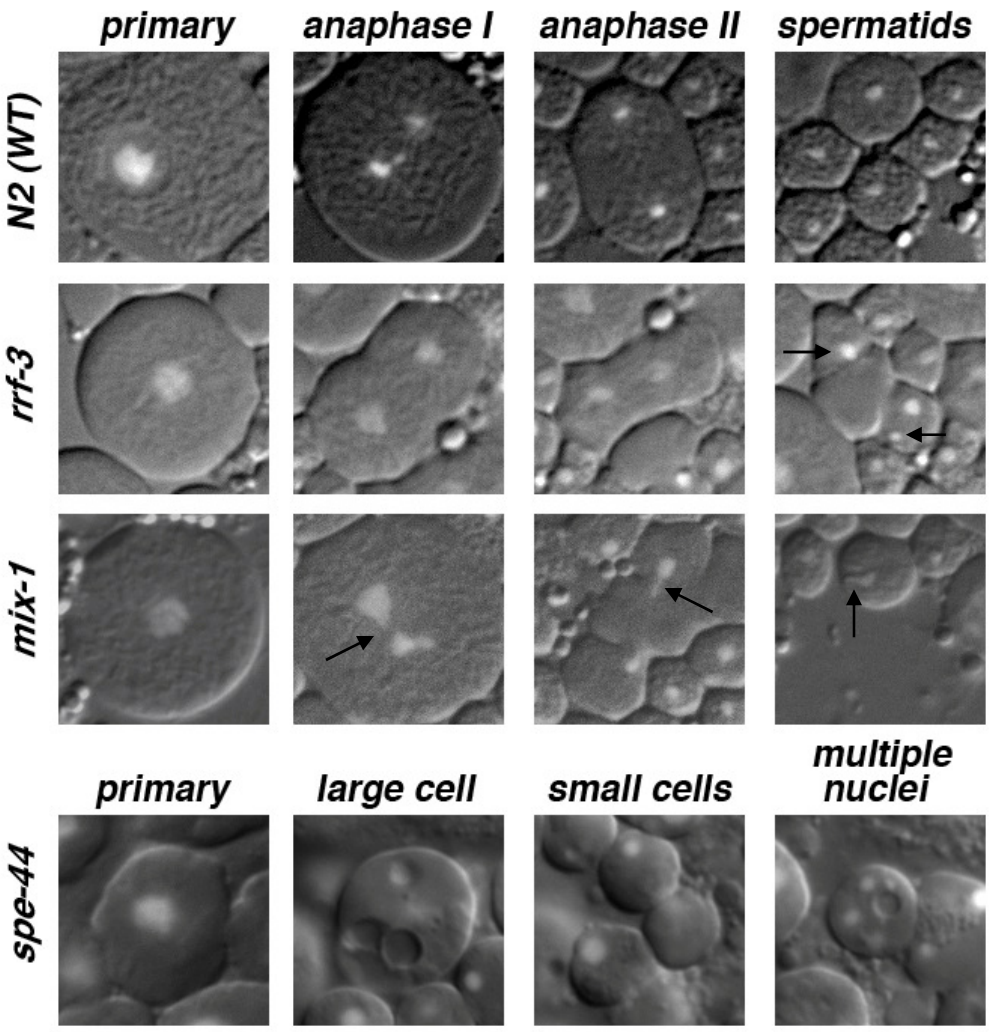
Spe-44 is severe in comparison to other spermatogenesis mutants

To better understand the *spe-44* defects, we compared these defects to those observed in other spermatogenesis mutants, *mix-1(tx29) and rrf-3(mg373),* using DIC microscopy and imaging. We specifically selected mutants based on their roles in gene regulation and chromosome segregation. *mix-1* encodes an SMC2 homolog that is a fundamental component of the condensin complex. When it is absent or mutated, DNA segregation fails in mitosis and meiosis, and it may play a role in gene silencing via chromatin remodeling (Csankovzski et al., 2009). In order to prevent the embryonic death that would result from a lack of MIX-1, a temperature-sensitive strain, *tx29*, was used. Worms were allowed to grow at the permissive temperature (16ºC) until males reached the L4 larval stage, at which point they were upshifted to the non-permissive temperature (25 ºC). After a 24-hour incubation period, adult males were dissected. The same protocol was utilized for the temperature-sensitive strain of *rrf-3*, mg373. *rrf-3* encodes an RNA-directed RNA polymerase, which is a necessary part of the RNAi pathway of the worm. RRF-3 is necessary for inhibition of exogenous and endogenous siRNA. RRF-3 down-regulates genes that are not needed or counterproductive to spermatogenesis (Gent et al., 2009).

Due to the fact that the mutants were not upshifted until the L4 larval stage, some viable spermatids were produced. However, we observed that upshifted *mix-1* mutant gonads showed a metaphase arrest in some cases (data not shown) as well as missegregated DNA (Figure 12). *Rrf-3* mutant spermatocytes always reached the budding stage in a wild type manner, at which point some spermatids had too many

Figure 10

A comparison of spermatogenesis defects in *spe-44* with those in *mix-1* (condensin) and *rrf-3* (RNA-dependent RNA polymerase) mutants. Cells are simultaneously imaged with DIC optics and fluorescence (Hoechst labeled DNA). Arrows show "lagging" haploid nuclei in *rrf-3* (n = 21 worms) and chromatin bridging at all levels of division in *mix-1* (n = 28 worms). (600x magnification)


primary
anaphase I
anaphase II
spermatids
N2 (WT)
rrf-3
mix-1
primary
large cell
small cells
multiple nuclei
spe-44

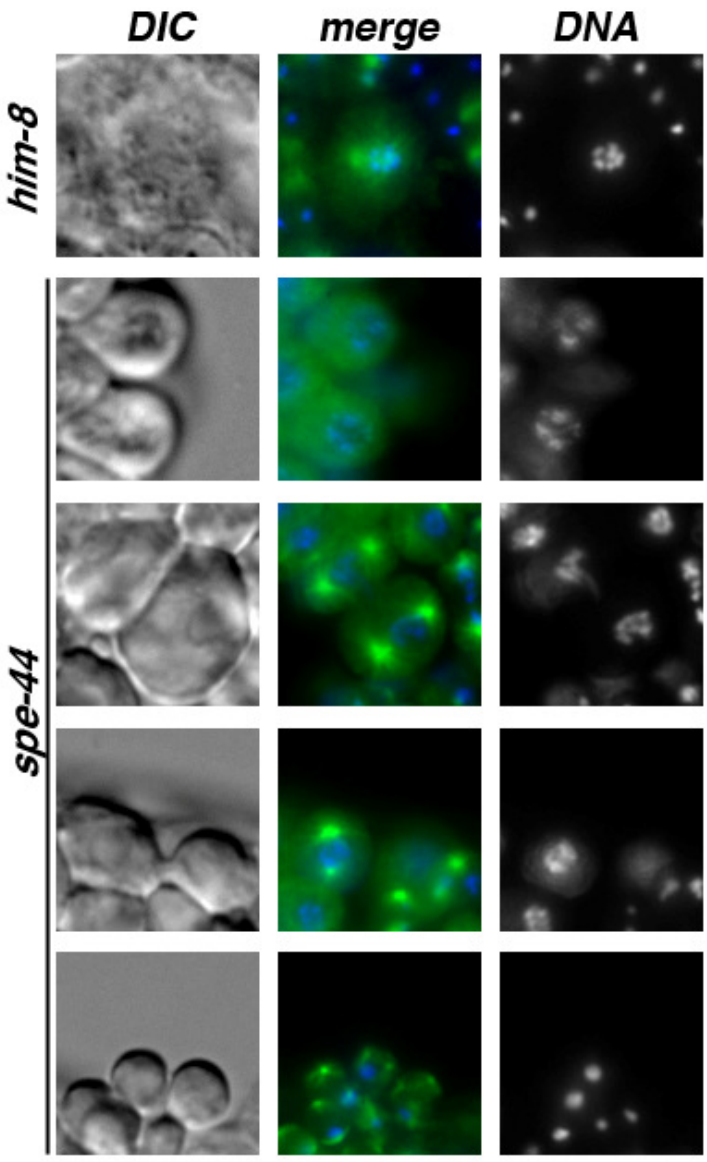
nuclei (Figure 10) or none at all (data not shown). We also confirmed the findings of Gent et al. (2009): tubulin protein persists inside *rrf-3* spermatids (data not shown). Neither *rrf-3* nor *mix-1* mutants exhibited the severe phenotype observed in *spe-44* (Figure 12). This comparison highlights the potential targets of SPE-44, as the mutant does exhibit chromosome segregation problems as well as persistent tubulin patterns.

Double nuclei and the presence of smaller cells are only observed in older *spe-44* males

We noticed an occasional "double nuclei" pattern in what appeared to be primary spermatocytes (Figure 4C). To further investigate this phenomenon, we carefully squashed younger adult males to contrast the older, heavily-squashed specimens. In the younger mutant male gonads (n = 16 worms), cells preparing to bud off the syncytial gonad appeared to have only one nucleus (Figure 11). Also, the microtubules nucleating from the meiotic spindles did not appear to be touching the DNA, indicating the possible presence of a nuclear envelope as late as diakinesis. We observed a cytoplasmic bridge between two cells (Figure 11), but the two cells did not appear to be secondary spermatocytes, which often show cytoplasmic bridging. Small, individual cells contained DNA and microtubule asters, but not as many as seen in older cells (Figure 4C). Overall, it was determined that younger males did not exhibit the same double nuclei phenotype as the older males.

Figure 11

DIC and immunocytology of young, lightly-squashed wild type (*him-8*) and *spe*-44 mutant gonads.


DIC
merge
DNA
him-8
spe-44

Discussion

Summary

The *spe-44* mutant exhibits sperm-specific sterility. *spe-44* males and hermaphrodites fail to produce viable sperm. Further investigation revealed that the resulting cells of the mutant contain supernumerary asters and a variable amount of DNA. The persistent microtubule asters and phospho-histone H3 (ser10) staining suggest that primary spermatocytes arrest in M phase and proceed to break down via abnormal cytokinesis. From the standpoint of sperm differentiation, the Major Sperm Protein fails to polymerize into fibrous bodies, and the membranous organelles fail to dock on the cell membrane. SPE-7, a sperm-specific cytoplasmic protein that associates with the fibrous body, is absent in *spe-44* mutants. The *spe-44* protein itself localizes to the nucleus and pachytene chromosomes of sperm-producing germlines. The protein is first detected in pachytene, and the antibody signal fades by the karyosome stage. Harold Smith reported that SPE-44 contained a DNA binding domain common in transcription factors and that SPE-44 positively regulates 343 of the 1343 sperm-enriched genes categorized by Reinke et al. (2004). These data lead us to conclude that *spe-44* encodes a sperm-specific transcription factor. SPE-44 is the first cell-type-specific transcriptional regulator to be described in the *C. elegans* germ line.

The aberrant cell cycle of *spe-44*

In addition to the stable microtubule aster phenotype, the persistent phospho-histone H3 (ser10) staining seen in *spe-44* mutants supports the hypothesis that these

spermatocytes enter M phase normally but then fail to exit. pHisH3 staining appears in the wild type and mutant in late pachytene and intensifies in the condensation zone. Although pHisH3 staining is undetectable in wild type spermatids, chromatin staining remains bright in the small nuclei of the terminal stage *spe-44* cells. In the mutant, the last point in meiosis where the DNA appears normal is diakinesis. In wild type, diakinesis is characterized by homolog pairing and migration of the centrosomes to opposite poles of the cell in preparation for division. Normally, at the end of diakinesis, the nuclear envelope breaks down. In the *spe-44* mutant, it appears that the microtubules do not touch the DNA of the large spermatocytes, suggesting the presence of a nuclear envelope. The meiotic program begins to malfunction after this point.

As we have yet to confirm the presence or absence of the nuclear envelope in *spe-44* spermatocytes, we consider the use of anti-Nuclear Pore or anti-Lamin antibodies an important experiment for the future. The cell cycle regulator Polo-Like Kinase-1 (PLK-1) can also be an informative cell cycle marker. Polo-like kinases are serine/threonine kinases with diverse cell cycle targets including cellular checkpoints (van Vugt and Medema, 2005). In *C. elegans* spermatogenesis, anti-PLK-1 labels the centrosomes during diakinesis in addition to the chromosomes during the meiotic divisions (Shakes et al., 2009), and PLK-1 eventually segregates into particulate structures in the residual bodies during the budding division. This antibody in a *spe-44* background could also help further delineate how the mutant cells progress through the proper meiotic and sperm development programs.

Finally, the ultimate cell cycle marker, cyclin, should be investigated in the *spe-44* mutant. Cyclin proteins do just what their name suggests: their levels change in

relation to the position of a cell within the cell cycle. Cyclin B has a role in both spermatogenesis (Shakes et al., 2009) and oogenesis (Lévesque and Sirard, 1996). Cyclin B levels increase during prophase, peak between metaphase I and metaphase II, and drop precipitously during anaphase II of oogenesis (Abrieu et al., 2001). However, in *C. elegans* spermatogenesis, cyclin B is segregated to the residual body instead of being degraded (Diane Shakes, unpublished data). Based on our assumption that the *spe-44* spermatocyte arrests in early M phase, cyclin should still be present in the terminal small cells of the mutant. Immunocytology using an anti-cyclin B antibody would indicate whether defects in SPE-44 lead to abnormalities within the meiotic program.

Centrosome Duplication

The multiple-aster phenotype in the *spe-44* mutant is striking, but it is not without precedent. The *zyg-1* mutant described by Peters et al. (2009) also featured as many as 10 spindle poles in meiosis II. These extra spindles resulted in the formation of greater than four spermatids per residual body, and although these sperm were capable of fertilization, the resulting embryos did not survive. ZYG-1 also plays a role in centrosome duplication during mitosis, but the same *zyg-1* mutant that produces extra spindles in meiotic cells halts centrosome duplication in mitotic cells. It is possible that the extra round of centrosome duplication that must occur during meiosis is a result of the interaction of sperm-specific proteins with ZYG-1. SPD-2, another centrosome-associated protein, works in concert with ZYG-1 to recruit pericentriolar material (PCM) to the centrosome (Wormbase), and in *Drosophila melanogaster*, SPD-2 is necessary for recruitment of PCM to the centrioles of the fertilizing sperm (Dix and Raff, 2007). A

centrosome marker such as anti-SPD-2 or γ-tubulin would be useful for detecting the exact number of centrioles present and the timeline of centriole duplication in the *spe-44* mutant. This could help determine whether the cause of the supernumerary aster phenotype is due to overduplication or premature centriole splitting.

spe-44 and apoptosis

We have observed that the terminal phenotype of the *spe-44* mutant is small cells with large vacuoles. We propose two alternative explanations for the origin of the small cells: cells may shrink through autophagy or split through cell division. Autophagy most likely evolved in response to starvation conditions, and it occurs when double-membrane vesicles form, encompassing proteins, organelles, and cytoplasm (Kourtis and Tavernarakis, 2009). Autophagy in *C. elegans* is inversely related to apoptosis, and defective autophagy triggers apoptotic pathways (Takacs-Vellai et al., 2005). It is also possible that although *spe-44* spermatocytes fail to undergo clear nuclear divisions, they may still be completing cytokinesis.

Deterioration of germ cells due to a defective transcription factor is not unprecedented. Apoptotic male germ lines have been documented in mice with mutations in a transcription factor. In 2001, Martianov et al. described the *TLF* (TATA binding protein-Like Factor) null mutant mice phenotype. *TLF* is regulates transcription globally, just like *spe-44*. These mutant mice reached adulthood, but failed to produce viable sperm. The arrested spermatids contained large vacuoles and underwent rapid apoptosis. In *C. elegans*, knockdown of TLF via RNAi results in embryonic lethality rather than sperm-specific sterility (Dantonel et al., 2000; Kaltenbach et al., 2000), which

indicates the amount of diversity in gene regulation among organisms. Nevertheless, this is an instance of a mutation in a transcriptional regulator causing errors in sperm-specific processes and resulting in apoptosis.

To determine whether apoptosis or autophagy are at work in the *spe-44* mutant, we propose staining with acridine orange, a known apoptotic marker, which labels acidic lysosomes and engulfed apoptotic vesicles (Darzynkiewicz, 1990).

Targets of SPE-44

SPE-44 contains a SAND domain (Harold Smith, personal communication), an 80-amino acid DNA-binding sequence. It is found in nuclear proteins and shows dissociation constants similar to those of monomeric DNA binding domains (Bottomley et al., 2001). More specifically, the KDWK motif of the SAND domain binds DNA, interacting with chromatin and allowing RNA polymerase II to bypass the nucleosome (Carles and Fletcher, 2010). Thus, it appears that SPE-44 functions by affecting DNA-histone interactions to chromatin to allow transcription to occur. This is consistent with the nuclear localization of the antibody in individual spermatocytes and the temporal localization in pachytene cells within the gonad. Transcription has ceased by the karyosome stage (Shakes et al., 2009), so transcriptional regulators would be turned off as the DNA condenses and prepares for division.

SPE-44 regulates the mRNA levels of 535 genes (Harold Smith, personal communication). Of these, 343 are sperm-enriched, including *spe-7* and *spe-4*. We have three different confirmations that *spe-7* is regulated by SPE-44: microarray (Harold Smith, personal communication), immunocytology, and Western Blot. In the future,

these genes should be analyzed for conserved domains as potential binding sites for SPE-44 by using either chromatin immunoprecipitation with sequencing or computational analyses. These experiments will elucidate which genes are directly influenced by SPE-44 and which are regulated indirectly by other target genes.

spe-44 in the context of other spermatogenesis mutants

We have examined the *spe-44* mutant gonad next to a number of other spermatogenesis mutants. It is our hope to be able to define some hierarchy or working model for the interactions between these genes. We know from Harold Smith (personal communication), that *spe-44* mRNA levels are increased in ERI/DICER mutants, which led us to investigate the *rrf-3(mg373)*. This mutant contains a defective gene that codes for an RNA-directed RNA polymerase, which plays a role in the RNAi pathway of *C. elegans* (Gent et al., 2009). We suspect that RRF-3 has some relationship with SPE-44, but it is yet unclear who is regulating whom. Small RNAs have been shown to greatly affect germ line development and spermatogenesis (Wang and Reinke, 2008). However, our analysis suggests that the *spe-44* phenotype is more severe than the *rrf-3* phenotype. Future experiments include analyzing the anti-SPE-44 antibody pattern in the *rrf-3* mutant background as well as the phenotype of double mutants. If the phenotype of the *rrf-3;spe-44* double mutant is more severe than that of *spe-44*, we can assume that SPE-44 does not regulate RRF-3. However, if the phenotype appears similar to that of *spe-44* mutants, we would predict that the two proteins are functioning in parallel in germ line regulation.

A second mutant of interest is *mix-1(tx29)*, which exhibits chromosome segregation defects at non-permissive temperatures. MIX-1 plays a variety of roles in chromatin modification. It is an SMC2 homolog, and plays a role in the condensin complex that condenses chromosomes prior to division (Hagstrom et al., 2002). MIX-1 also plays a role in the Dosage Compensation Complex (DCC) of *C. elegans*, which downregulates genes found on the "extra" X-chromosome of XX hermaphrodites (Csankovzski et al., 2009). This opens the possibility for a more general role for MIX-1 in gene regulation. The *mix-1(tx29)* mutant spermatocytes arrest in metaphase I, an arrest point further into the cell cycle than the proposed diakinesis arrest of *spe-44*. Clearly, chromatin dynamics play a role in all cell divisions, not only meiosis, so it would be difficult to analyze a non-temperature sensitive mutant of mix-1. However, the fact that *spe-44* and *mix-1* mutants share both an arrest and DNA missegregation phenotype may suggest that SPE-44 potentially regulates other chromatin modifiers, although it is improbable that *spe-44* affects *mix-1* expression directly because MIX-1 is involved in all cellular divisions. It is more likely that SPE-44 positively regulates a different protein that could potentially interact with the condensin complex. It is important to note that although both *spe-44* and *mix-1* exhibit chromosome segregation defects, the *mix-1* mutant does not exhibit the sperm differentiation defects seen in *spe-44*. Chromosome segregation defects do not automatically create problems for the sperm differentiation pathway.

Lastly, we consider the mutants that lacked fibrous bodies (FB) like *spe-44*: *spe-6* and *spe-7*. *spe-7* encodes a novel, nematode-specific and sperm-specific protein involved in FB formation (Presler, 2010). s*pe-7* mutant gonads show a bizarre tubulin pattern, in

which the spindles appear normal until anaphase I, where the cells arrest. After the arrest, the tubulin resumes a diffuse pattern as seen in the spermatocytes that have not yet entered M phase. This phenotype is quite different from the one observed in the *spe-44* mutant, in which stable spindles persist past the arrest point. We also know that SPE-44 positively regulates the expression of *spe-7*, among many other genes. *spe-7* is one of the 343 sperm-enriched genes that are upregulated by SPE-44, and thus we can assume that some other, earlier actor is also being regulated by SPE-44 and causing the more severe phenotype. If we were to examine the SPE-44 localization pattern within *spe-7* mutant gonads, we would anticipate finding a wild type pattern.

Unlike *spe-7*, *spe-6* expression is not regulated by SPE-44 (Harold Smith, personal communication). However, *spe-6* mutant gonads also exhibit a lack of fibrous bodies. This indicates that there are multiple factors involved in the polymerization of MSP and its segregation into the spermatids. The *spe-6* mutant phenotype was first described by Varkey et al. (1993) as a spermatogenesis defective mutant, with an arrest point around diakinesis. Although *spe-6* encodes a casein kinase, its exact phosphorylation targets are yet unknown (Muhlrad and Ward, 2002).

Conclusions

Before now, one might have assumed that the differentiation of a highly specialized cell such as a spermatocyte or spermatozoa required a hierarchy of cell-type specific transcription factors. However, our collaborative study of *spe-44* provides the first direct evidence of a wide-acting, sperm-specific transcription factor functioning downstream of the sperm/oocyte decision. SPE-44 is specifically expressed during

spermatogenesis, and the majority the genes which it regulates are sperm-enriched. The identification of a transcription factor that regulates such a large number of sperm-specific genes provides an important link between the sex determination and sperm differentiation pathways. Although many more questions remain regarding the function and regulation of SPE-44, we have established a foothold, which we and others may use to further scale the metaphorical mountain that is germ line regulation.

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Acknowledgments

We are grateful to the Jeffress Memorial Trust and Howard Hughes Medical Institute Science Education and Research Program at William and Mary for funding our project. On a personal note, this thesis would not have been possible without the diligent tutelage of Dr. Diane Shakes who encouraged me as a woman in science. I am also indebted to Dr. Harold Smith, who collaborated on the project and provided invaluable insight. I would like to thank Kari Messina for her great advice and friendship, along with the rest of the Shakes Lab. Finally, I would like to thank my parents, Jan and Pat Guevel, for allowing me the opportunity to study at William and Mary and daring me to follow my dreams.