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A novel *C. elegans* gene interfaces cell cycle and development in spermatogenesis

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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May 2010
The last thing one discovers in composing a work is what to put first.
-TS Eliot

Abstract

The spermatogenesis pathway in the model organism *Caenorhabditis elegans* offers an excellent opportunity to study the interplay between the meiotic cell cycle and the developmental program of cellular differentiation. While these two parallel processes cooperate to produce highly specialized gametes, the intricate mechanisms enabling that coordination are not yet fully understood. To further investigate how the cell cycle can integrate with the cellular differentiation program in *C. elegans*, we characterized the sterile, spermatogenesis-defective mutant *spe7*, whose primary spermatocytes arrest at Anaphase I while failing to either divide into secondary spermatocytes or bud to form individual spermatids. Here we show that the spindles regress to an interphase/G2-like pattern following the Anaphase I arrest by reorganizing into a network pattern. PLK-1 (polo-like kinase), a protein required for the G2/M-phase transition, ceases to localize the centrosomes after the arrest point, but properly localizes to the Metaphase I plate and jumps off the chromosomes at Anaphase 1. Despite the interphase/G2-like tubulin and PLK-1 configuration in the arrested primary spermatocytes, cyclin B levels remain elevated and the nuclear envelope stays vesiculated. Although the events of pre-division chromatin modifications progress normally, aberrant chromosome configurations are seen following Metaphase I. The molecular identity of *spe7* is F32A11.3, which is listed as a spermatogenesis-enriched protein in whole genome microarray studies. In homology searches, F32A11.3 is most similar to a second *C. elegans* gene and to single genes in other *Caenorhabditis* species; it also exhibits weak homology to the vertebrate sperm protein, zonadhesion. SPE-7 antibody staining shows a dynamic localization pattern, with
the active form likely functioning at Metaphase I. These findings suggest potential
necessity of an intact spindle for the budding division, implying cell cycle components
are required not just for chromosome segregation, but for early spermiogenesis as well.
Furthermore, the mutant phenotype and dynamics patterns of SPE-7 reflect not only the
modular and dynamic nature of the spermatogenesis, but SPE-7’s likely role as a key
linker between the cell cycle and developmental pathways as well. The possible gene
duplication event points to an evolutionary pressure for innovation in the germline.
Introduction

All life hinges on successful cell division. Sexually reproducing organisms rely on a special type of cell division called meiosis to produce the gametes required to engender the next generation. These gametes, sperm in particular, are a marvel of biological evolution. Sperm production requires not only the meiotic divisions that halve the amount of genetic information in each daughter cell, but the complex developmental program that transforms a bulky, sessile, unspecialized germ cell into a streamlined, motile, highly specialized spermatozoon ready to fertilize a waiting oocyte. The fact that two such highly specialized cells with half the normal genetic information can join to form a totipotent embryo capable of originating an entire organism is a true wonder of cellular biology. The details of this transformation inform important questions not only on how the meiotic divisions or developmental program occur, but how these two coordinate at the molecular level to produce working gametes.

The nematode *Caenorhabditis elegans* provides an excellent system to study questions of cell cycle and development interaction. *C. elegans* is a model system with a fully sequenced genome, mapped cell-lineages, transparent body wall, and a gonad that takes up a substantial part of the worm’s mass (Figure 1). These features assist study of genetics and development, but the gonad in particular provides an excellent tool to study spermatogenesis, or the process of making sperm.

The progression down the spermatogenesis pathway occurs in a linear fashion in *C. elegans*, allowing for a convenient visualization of all the different stages of both the cell cycle and development pathways (Figure 2). The process begins with the distal tip,
Figure 1) A beautiful *C. elegans* hermaphrodite, showing the transparent body wall that allows study of cell morphology with light microscopy. The substantial gonad is seen on the right, unfertilized oocytes in the middle, with two freshly laid embryos at the bottom of the frame. *C. elegans* hermaphrodites are more accurately described as females that produce a short burst of sperm before switching to oocyte production, allowing for some self-fertility (Brenner, 2009).

Figure 2) Wildtype male, fixed gonad stained with DAPI to mark the DNA. The process starts at the distal tip and proceeds in a linear fashion until the haploid spermatids.
supporting the mitotic proliferation of the germline. After this, the diploid cells initiate the meiotic stage as their homologous chromosomes pair and undergo meiotic recombination in preparation for the meiotic divisions. Primary spermatocytes then bud off the rachis, a shared cytoplasmic core in the gonad, and undergo the meiotic divisions (Phillips et al, 2009). The meiotic divisions produce a haploid daughter cell called the spermatid, which will go through spermiogenesis to become a fully functioning, motile spermatozoon.

*C. elegans* present several striking modifications to spermatogenesis as typified by the mammalian pathways. In *C. elegans*, the developing sperm cells do not require adjunct support cells comparable to the Sertoli cells in mammalian testes. Once budded from the central cytoplasmic core of the rachis, the spermatocytes are largely independent. Meiosis requires two divisions to half the number of chromosomes in the cell, and thus requires extensive chromatin modification; *C. elegans* spermatogenesis adds a stage called the karyosome in the late G2 stage just before pro-metaphase I (Figure 6). First molecularly characterized in *Drosophila malanogaster* oocytes, the karyosome stage exhibits condensed chromatin within an intact nuclear envelope (Barbosa et al, 2007). Recently discovered in *C. elegans* spermatogenesis, the karyosome is transcriptionally repressed and may serve as a pre-sorting mechanism for the ensuing chromatin divisions (Shakes et al, 2009).

One of the more apparent deviations from the mammalian program is the formation of a structure called the residual body (Kimble and Ward, 1981). Instead of two cytokinesis events driven by an actin-myosin cleavage furrow, *C. elegans* conclude
the meiotic divisions with a single asymmetric cytokinesis event, where each spermatid simultaneously buds off from a central, membrane bound structure. Called the residual body because it is left behind after the haploid spermatids bud, it contains excess cytoplasm and cellular components not required for functioning spermatozoa (Figure 6, see “metaphase II” and “residual body” to visualize formation). Mammalian spermatogenesis includes a similar step called cytoplasmic shedding (Wu et al, 2010), where excess cytoplasm is dumped during the final steps of an extended post-meiotic differentiation program (spermiogenesis), an event that serves as part of the streamlining process for small, motile spermatozoa. *C. elegans* combines this step with the meiotic divisions.

The contents of the residual body constitute another remarkable shift from the textbook conception of spermatogenesis. Spermatids throw away all cellular machinery not needed for a functioning spermatozoon into the residual body. Surprisingly, this includes ribosomes, voltage-gated ion-channels, actin, myosin, nuclear envelope and tubulin (Machaca et al, 1996; Ward,1986). Spindles are discarded into the residual body after proper chromatin segregation, along with the other cytoskeletal components. A nuclear envelope never reassembles around the haploid chromatin. The absence of ribosomes means that, follow the meiotic divisions, the spermatid is translationally inactive and already contains the needed components for transforming into a working spermatozoon. A translationally inactive spermatid deviates widely from the standard spermiogenesis program, which can take weeks translating sperm-specific proteins required to activate the cell. *C. elegans* spermatids contain only a highly condensed, haploid chromatin mass, a centriole which detaches from the spindle, mitochondria, and a
unique structure called the Fibrous Body/Membranous Organelle (FBMO). The Membranous Organelle (MO) is a golgi-derived structure that helps to shuttle sperm-specific proteins through the meiotic divisions into the spermatid (L'Hernault, 2006). One of these is the Fibrous Body, a paracrystalline configuration of the novel Major Sperm Protein (MSP), which acts as the motility protein in activated *C. elegans* spermatozoa. *C. elegans* spermatozoa are amoeboid, and therefore do not require a complex swimming flagella; instead, they crawl to their oocytes (Singson 2000). A relatively simple motor protein like MSP can then replaces actin, myosin, and tubulin for motility function, allowing the spermatid to discard them in the residual body.

Another surprising resident of the residual body are cyclins. These proteins play a key role in governing the meiotic divisions and cueing the overlapping development program. The cell cycle consists of the G1, S, G2, M-phases. Different types of cyclin (A-E) fluctuate to allow progression through stage of the cell cycle. For example, a spike in Cyclin B concentration allows progression through M-phase, and the subsequent crash is required for cell cycle exit and return to G1, also known as interphase. Cyclins perform this regulatory role by binding to specific Cyclin-dependent Kinases, which subsequently phosphorylate substrates to modulate cell-cycle function (e.g., phosphorylation of lamin facilitates nuclear envelope breakdown). Most meiotic divisions show a slight drop in Cyclin B after Meiosis I, followed by a dramatic degradation of Cyclin B to allow a return to interphase. In unpublished results from the Shakes’ Lab, *C. elegans* do not degrade Cyclin B. Instead, Cyclin B remains at high levels after segregation to the residual body. Spermatids appear to skip the step of returning to M-phase after meiosis II.
Are these alterations to the cell cycle and developmental program adaptive? *C. elegans* spermatogenesis occurs impressively fast. While other species take weeks after the meiotic divisions to churn through the developmental program, *C. elegans* takes merely minutes. The alternative molecular methods detailed above may empower a rapid progression through spermatogenesis. This speed may be achieved through an overlap of the two parallel pathways of cell cycle and development. Managing two overlapping pathways requires a complex set of molecular cooperation and regulation, and we can exploit the evolutionary pressures for speed and molecular teamwork in *C. elegans* to study just how dynamically proteins of seemingly specific function can interact.

Since the cell cycle and the developmental program occur separately for the most part in other organisms, the intuitive assumption is the overlapping nature of these two programs makes them functionally interlocked, where each program cannot proceed without the other. Studies of Anaphase Promoting Complex mutants in *C. elegans* challenge this assumption (Golden et al., 2000; Sadler and Shakes, 2000, Furuta et al., 2000). The Anaphase Promoting Complex with Cdc20 (APC/C) is an E3 ubiquitin ligase activated by M-phase CdK that promotes the transition into Anaphase by degrading the inhibitory of securin, and enzyme that can degrade the cohesins that constitute the synaptonemal complex holding bivalents together at the metaphase I plate (Simpson et al, 2010). An APC mutant causes an arrest in metaphase I. The loss of APC/C function leaves seperase inhibited, allowing the cohesins to remain intact. The inability of the chromosomes to separate triggers the metaphase I checkpoint and causes a cell cycle arrest. Unexpectedly, the developmental program is able to continue despite the cell cycle arrest. Though the microtubules and chromatin remain frozen in a metaphase I
configuration, the budding division continues to produce anucleated spermatids that mature to crawling spermatozoa capable of fertilization, still containing a single centrosome, mitochondria, and the FBMO complex (Sadler and Shakes, 2000). The APC study reveals an abject decoupling of the cell cycle and developmental programs, where the latter continues nearly unaffected by the cell cycle arrest to produce crawling sperm with no DNA. These results present a more nuanced model of the precise relationship between the two overlapping pathways in *C. elegans*. However, if points exists in spermatogenesis where each may operate independently, surely points exist where each converge onto one or two key proteins.

Recently, a new tool box for studying early meiosis in *C. elegans* was published by Shakes et al (2009) that detailed various cytological landmarks to screen for spermatogenesis defects through immunofluorescence. In addition to detailing the dynamics of tubulin, and the study characterized the localization pattern of the important cell cycle protein called PLK-1. With a role in the G2/M transition and spindle polarization (Li and Li, 2006), PLK-1 stains the nuclear envelope during G2 but marks the centrosomes at the G2/M transition. At metaphase, PLK-1 co-stains both the centrosomes and the chromosomes, but localizes in-between the dividing bivalents during anaphase I. The dynamic localization pattern corresponding with important spots in the cell cycle makes PLK-1 an ideal marker progression through the cell cycle.

Given the new tools to allow more precise investigation of early arrest mutations, we turned out attention to *spe-7*. While the APC mutation allows the developmental progression during a cell cycle arrest, *spe7* causes a simultaneous arrest of the cell cycle and the developmental program. The *spe-7* mutant arrests at anaphase I while failing to
perform a successful budding division. The proximity of the arrest point to the APC mutant, but subsequent arrest of both the cell cycle and development is a foothold for understanding the components required for both parallel pathways. Here we show that the spe-7 mutant disassembles its spindles after Anaphase I, while showing an interphase-like PLK-1 pattern. Despite these two data points, Cyclin B and Nuclear Envelope markers reveal that the arrested primaries are not dropping out of the cell cycle. Further, we identify the molecular identity of spe-7 as the spermatogenesis-enriched gene F32A11.3, with a close homologue in C. elegans ZK873.6. Finally, we identify the cellular localization of the SPE-7 protein and suggest potential models, recognizing future experiments for further understanding of the phenotype and protein function.

The early meiotic of activity in oocytes is well studied in the C. elegans literature, and more broadly in Xenopus and mammal systems. The early meiotic system of sperm, however, remains poorly understood by the field. The immense modification and variation on cell division function in C. elegans spermatogenesis offers unmined potential to explore previously unknown links between cellular components while showing how evolutionary pressures can drive dynamic function and interactions at the cellular level.
Results

**spe-7 exclusively affects spermatogenesis**

*C. elegans* hermaphrodites produce a burst of sperm before the germline switches to oocyte production during the L4 larval stage which immediately precedes adulthood. Young adult hermaphrodites are therefore capable of self-fertilization, allowing them to produce around 200 progeny until the initial stockpile of sperm dwindles, after which point they lay a few unfertilized oocytes. Screening for hermaphrodites, that lay only unfertilized oocytes but can produce fertilized embryos when crossed to healthy males, is a convenient method for detecting spermatogenesis defect mutants (Ward and Miwa, 1977). The initial inability of such hermaphrodites to produce fertilized oocytes indicates defective or absent sperm, while the restoration of fertility in the presence of healthy sperm suggests the defects are limited to spermatogenesis, rather than a broader fertility malfunctions or general meiotic flaws.

In wildtype worms, fertilization occurs sequentially, with sperm competing for access to individual oocytes as they enter the spermatheca one by one. *spe-7(mn252)* mutants lay unfertilized oocytes (Table 1), with no mature spermatozoa observed in the spermatheca of hermaphrodite worms (Figure 1). However, mutant hermaphrodites produce large broods of healthy embryos when mated with wildtype males (*n > 100*). The lack of sperm in the mutants and the subsequent restoration of fertility in the presence of functioning spermatozoa suggest that oogenesis proceeds normally while spermatogenesis is specifically affected by the *spe-7* mutation.
**Analysis of Sterile Phenotype**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Growth Temp</th>
<th>n</th>
<th>Unfertilized Oocytes (SE)</th>
<th>Embryos (SE)</th>
<th>%Hatch</th>
<th>Progeny (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>spe-7</em></td>
<td>16°</td>
<td>22</td>
<td>9.55 ± 2.04</td>
<td>0</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20°</td>
<td>20</td>
<td>17.05 ± 1.44</td>
<td>0.7 ± 0.19</td>
<td>100%</td>
<td>0.7 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>25°</td>
<td>17</td>
<td>47.59 ± 4.25</td>
<td>2.41 ± 0.50</td>
<td>100%</td>
<td>2.41 ± 0.50</td>
</tr>
<tr>
<td><em>unc-4;him-8</em> (control)</td>
<td>16°</td>
<td>8</td>
<td>18.25 ± 5.87</td>
<td>183.75 ± 2.88</td>
<td>87%</td>
<td>160 ± 2.57</td>
</tr>
<tr>
<td></td>
<td>20°</td>
<td>9</td>
<td>27.22 ± 4.82</td>
<td>187.89 ± 9.9</td>
<td>90%</td>
<td>170 ± 9.8</td>
</tr>
<tr>
<td></td>
<td>25°</td>
<td>7</td>
<td>12.86 ± 2.6</td>
<td>135.14 ± 6.01</td>
<td>73%</td>
<td>99.57 ± 3.29</td>
</tr>
</tbody>
</table>

Table 1) *spe-7* fertility compared with a wildtype control. Data are given as an average with Standard Error at the mean.

![Image](image1.png)

Figure 3) An unmated, *spe7* hermaphrodite showing unfertilized oocytes with no sperm in the spermatheca (center frame).
The *spe7* mutation is recessive; *spe-7* homozygotes exhibit the sterile phenotype while heterozygous *spe7/+* animals exhibit wildtype fertility, allowing propagation of the mutation through backcrossing. While a dominant sterility mutation would likely result in the extinction of the mutant line, a recessive gene allows the “storage” of the mutation in heterozygotes, where a simple backcross between a homozygous/heterozygous hermaphrodite and a fertile heterozygote male can sire mutants for study.

**Mutant arrests cell cycle at anaphase I**

To investigate the cause of the sterile phenotype, we dissected *spe7* males, which exclusively produce sperm. After examining samples under differential interference contrast (DIC) optics and the lipid-soluble DNA dye, Hoechst 33342, we determined that the mutants arrest spermatogenesis at the diploid, primary spermatocytes stage with their chromosomes frozen in an Anaphase I configuration (Figure 4). The Anaphase I arrest point is an unusual arrest point during the meiotic divisions since the cells have cleared the important metaphase I checkpoint, and by current understanding, are expected to have all the components in place for proper division. This Anaphase I point in *spe7* mutants suggests an additional level of complexity regulates meiotic progression during *C. elegans* spermatogenesis.

The cells also exhibit cytokinesis defects, failing to either divide into secondary spermatocytes via an actin/myosin based process or complete the non-actin/myosin based budding division associated with the formation of individual spermatids (Figure 4). While an arrest of the cell cycle may intuitively result in the corresponding arrest of the developmental program, previous studies of the APC mutants
Figure 4) Anaphase I arrest. A) Wildtype sperm spread. B) spe7 at 20°C, arrows showing anaphase I division. C) spe7 at 16°C, arrows show anaphase I and metal-anaI intermediate. D) spe7 at 25°C, arrows mark anaI and anaII-like separation. E) spe7 at 16°C DAPI field showing with arrows showing all chromatin aberrations present in one field including anaI, metal-anaII, and separation. F) Two common classifications of chromatin aberrations close up.
reveal that primary spermatocytes with chromosomes arrested in a Metaphase I configuration can continue with the budding division to produce functioning spermatozoa that lack DNA (Sadler and Shakes, 2000; Golden et al., 2000). This finding shows that early meiotic arrests do not automatically inhibit the progression of the developmental program of spermatogenesis. The simultaneous cessation of meiotic and developmental activity in spe7 mutants suggests the mutation is required for each pathway to progress normally, and may not function specifically as either a cell cycle or developmental protein.

Though spe-7 spermatocytes most commonly arrest in Anaphase I, the DIC/Hoechst microscopy reveals additional chromatin defects that differ according to temperature (Figure 4B-E). At 25°C, the chromosomes progress past an Anaphase I configuration into a sloppy anaphase II-like partial separation, but in the absence of an associated budding division (Figure 4D). At 20°C, the anaphase I arrest point is much more consistent, with fewer instances of partial sister chromatid separation (Figure 4B). Males grown at 16°C show similarities to the unusual separation phenotype past Anaphase I, but with far fewer spermatocytes per field classified as post-anaphase I (Figure 4E). Interestingly, many of these arrested primary spermatocytes more closely resemble an intermediate between Metaphase I and Anaphase I (Figure F). The large number of mutant spermatocytes with their chromosomes in an intermediate phase between a clean Metaphase I and Anaphase I implies either a slowed separation phenotype or an earlier, albeit variable, arrest point at 16°C. The study could mean that while the arrest point most consistently occurs at Anaphase I, a decrease in temperature nudge the arrest point closer to metaphase, while higher temperatures allow some
aberrant progression past Anaphase I. Our current data does not distinguish between the two models of shifting arrest points or slowed progression.

No budding divisions were observed at any temperature, although a few spermatids (<10) were sporadically observed in 25°C males indicated that such divisions did, on occasion, occur.

Eliminating Phenotypic Variation: Aging, Maternal, and Temperature Effects

Given the variation of chromatin and cell morphologies observed in our early DIC studies, we sought to reduce this variation by controlling for age, maternal source, and temperature. These steps allowed us to more clearly discern between abnormalities caused by our protocol rather than the spe-7 mutation.

To assess the affect of aging, we performed an age study on a synced population of L4s with individual males dissected at precise time intervals (12, 18, 24, 36, and 48 hours past L4). We found that as the mutant males age, they accumulate arrested primary spermatocytes in their gonads. This observation suggests that primaries continue to bud from the shared cytoplasmic core of the rachis successfully throughout the lifetime of the worm. However, as males aged, their gonads accumulated cellular debris in the form of cytoplasm unbound by a membrane. This pattern suggested that, over time, the older primary spermatocytes begin to lyse, assuming the source of the unbound cytoplasm is ruptured primaries. The amount of this debris increased as the worms aged, beginning 36 hours after the L4 larval stage (Figure 5). The cause of this phenomenon remains unclear. The cells may have lysed in the worm before dissection, or were more fragile than the
Figure 5) Age Study of *spe7* males taken with DIC optics. A) 48 hours post-L4: the gonad accumulates arrested primaries. Arrows indicate cellular debris. B) 24 hours post-L4: arrow shows intact cells. C) 12 hours post-L4: arrow shows intact cells, but too few in number for convenient microscopy work.
younger primaries and lysed when the cover slip was placed on the dissected worm. Whole worm mounts (not dissected) were inconclusive in determining whether the older primaries are intact within the gonad. The lysed primaries do not stain with the apoptosis marker Acridine Orange, so programmed cell death is an unlikely culprit. We speculate that arrested primaries become frail after prolonged detachment from the rachis because of the immense cytoskeletal modifications that occur during meiosis.

The presence of lysed cells within older gonads poses challenges to the immunocytological analysis dissected gonad. For example, is a drop in a particular antibody signal due to a regulated change in protein levels or to cell lysis? Because of this, we carefully aged all our samples before dissection to 24 hours after the L4 larval stage. Since our work required studies at different temperatures, we compensated for varying metabolic rate at each temperature (see Methods and Materials). These times ensured that we only studied gonads with intact cells. Importantly, we observed the same range of chromatin aberrations in both the 48 and 24 hour samples, thus 24 hours provided the ideal time frame for consistency in phenotype.

Our initial studies of the sterile mutant phenotype showed some variability in the severity of infertility. To rule out variability due to different maternal contributions, we compared sperm spreads of homozygous spe-7 males derived from backcrosses to either heterozygous or homozygous hermaphrodites. Though we have previously shown that spe7 is spermatogenesis-specific, a heterozygous “mother” could load a protein product into the oocyte that would partially remediate the spe-7 defects in her homozygous sons (Gupta et al, 2007). However, we found that the phenotype of males born from heterozygous mothers were indistinguishable from those born from homozygous mothers.
(data not shown). We therefore ruled out maternal contribution or a heterozygous effect on the spe7 phenotype.

Though the spe7 mutant was initially classified as non-conditional mutant, we performed a fertility study at the conventional growth temperatures of 16°, 20°, and 25°C (Table 1). Surprisingly, we found that while spe7 mutants are largely infertile at all three temperatures, the fertility defect is most severe at low temperatures. Wildtype worms, as our data shows, become slightly less fertile as the temperature increases, a phenomenon that is known to be associated with impaired sperm function (Gupta et al, 2007). Thus the cold-sensitivity of spe7 mutants is unusual, as conventional temperature-sensitive mutations are permissive at lower temperatures and the protein defects worsen with increases in thermal energy. Mutant worms at 25°C produce an average of 2.4±0.5 embryos with a range of 1-6; significantly higher than the zero progeny produced at 16°C. The observation that spe-7 hermaphrodites continue to lay oocytes over a number of days (up to 4-5 days at 25°C compared to 1-2 in WT at the same temp) suggests a lingering signal from non-functional spermatocytes, as sperm produce an MSP (Major Sperm Protein) signal that triggers laying behavior (McGovern et al, 2007) (Appendix Data). Most importantly, the 100% hatch rates for the small number of 25°C and 20°C embryos that are produced by spe-7 hermaphrodites suggests that these rare spe-7 spermatozoa are completely functional if they manage to bypass the meiotic chromosome segregation and cell division defects. Furthermore, with an n=70 hermaphrodites, progeny are never produced at 16°C. These fertility differences led us to closely control for temperature in all our experiments, informing the previously mentioned DIC studies as well.
Microtubules morph to network pattern in arrested primaries

Because abnormal chromosome segregation can arise from either chromatin-based defects or microtubule-based defects, we further investigated the nature of the Anaphase I arrest by examining the chromatin and microtubule patterns in isolated and fixed spe7 male gonads using the DNA dye, DAPI and FITC-conjugated monoclonal antibodies against $\alpha$-tubulin. This experiment could also inform the nature of the cytokinesis failure, since the spindle poles help guide cell polarity during typical cytokinesis. Any defects in spindle morphology that could affect cytokinesis would be visible under an anti-\(\alpha\)-tubulin preparation.

During wildtype spermatogenesis, the microtubules are organized into a network pattern throughout meiotic prophase (Shakes et al, 2009). Immediately prior to the meiotic divisions, the tubulin reorganizes into two microtubule asters centered on the centrosomes, which migrate around the nuclear envelope immediately prior to nuclear envelope breakdown. With two microtubule asters now in place at opposite sides of the nucleus, the resulting meiotic spindle is now ready to perform to initial reductive division that separates the homologous chromosome pairs during meiosis I. After the second round of meiotic divisions and a dramatic polarization event, the intact spindles are discarded into a central residual body as the spermatids bud off from this central structure. The spindles always stay in an organized pattern until they are segregated into the residual body (Figure 6). The initial steps of microtubule organization proceed normally in spe7. Some mutant patterns are seen as early as metaphase I, but these defects will be addressed a following section. Globally, intact spindles are present until Anaphase I. After the arrest point, however, the microtubule arrangement morphs from a
Figure 6) Wildtype images featuring immunocytological shot of tubulin (green), DNA (blue), PLK-1 (red), and a false-color merge of all three.
highly organized spindle structure into a network-like tubulin configuration (Figure 7). The pattern resembles the pre-division configuration seen in late G2 before the asters form around the centrosomes. Whether the disassembly of the spindle is responsible for the arrest point or is a consequence of a larger cell cycle phenomenon remains unclear. However, additional data suggests the morphing of the microtubule arrangement from a functional spindle into a network pattern is one component of a broader cell cycle defect associated with the spe-7 mutation.

To further analyze potential defects in the dynamics of microtubule nucleation within spe-7 mutants, we carried out immunohistological studies using an antibody against PLK-1 (polo-like kinase), a regulatory enzyme that associates with the centrosomes during interphase and helps the spindles establish polarity of the spindles as cells enter the meiotic divisions (Chase et al., 2000). According to Shakes et al., 2009, the PLK-1 antibody localizes dually to the nuclear envelope and cytosol in diplotene and early karyosome cells. The protein then marks the centrosomes to indicate the transition from prophase to the meiotic divisions. During Metaphase I, PLK-1 associates both the centrosome and the metaphase chromosomes, but jumps off the chromosomes during anaphase I, remaining in the space between the separating bivalents (Figure 6). PLK-1 stains the centrosomes continuously until it eventually detaches from the centrosomes and segregates to the residual body within which it assumes punctuate pattern (Shakes, unpublished observations). The dynamic localization patterns of PLK-1 serve not only as insight into tubulin dynamics, but as landmarks for proper progression of spermatocytes through the meiotic cell cycle.
Figure 7) Whole gonad images of spe7 and wildtype with merge, tubulin and DAPI. The tubulin configuration dramatically disassembles from organized spindles to a network pattern, seen most strikingly between spe7 at 20°C and wildtype, though the pattern is analogous at each temperature.
PLK-1 shows a normal localization in *spe7* mutants through Anaphase I. PLK-1 signals the healthy entry into the meiotic divisions with successful localization to the centrosomes; PLK-1 strongly stains the metaphase plate and, importantly, jumps off the chromosomes as the cell progresses to Anaphase I. One possible explanation for the Anaphase I arrest was an illicit progression of the cell cycle past an activated Metaphase I checkpoint because of scrambled molecular communications caused by the *spe7* loss of function. The proper decoupling of PLK-1 from the chromosomes in Anaphase I, however, suggests the arrested cells progressed past the metaphase checkpoint properly.

The PLK-1 staining in the arrest primaries, however, shows an aberrant pattern (Figure 8). Instead of remaining clearly between the chromosomes as characterized in Shakes et al, PLK-1 begins to localize in a small punctuate pattern, presumably in the cytosol (though a relocalization to the vesiculated nuclear envelope remains possible). At 16°C, PLK-1 stains the aberrant chromatin dot formation detailed previously in Figure 4F, suggesting that though most cells progress to anaphase I, some show abnormal post-metaphase separation (data not shown). Intriguingly, PLK-1 no longer marks the centrosomes after the disassembly of the spindle. These observations denote distinctly abnormal localization patterns for a cell in M-phase in parallel with the aberrant tubulin patterns. The dual aberrations of tubulin and polo-like kinase suggest that disassembling spindles are not the cause of the arrest, but a consequence of a larger coordination failure sparked by the loss of SPE-7 function.
Figure 8) *spe7* with tubulin (green) and PLK-1 (red). The tubulin reassembles into a network pattern visibly similar to the G2 stage karyosome. The DAPI (blue) shows the Anaphase I configuration. The PLK-1 stains normally until the anaphase I arrest. For context, refer to Figure 6 to see the wildtype equivalent.
**Arrested primaries do not drop out of M-phase**

Two distinct models could explain the observed network microtubule patterns of terminally arrested spe-7 spermatocytes. In one model, spe-7 spermatocytes could be aberrantly exiting M-phase and entering into a G1 state. In a second model, spe-7 spermatocytes could be regressing and assuming a late prophase state. To distinguish between these two possibilities, we examined the patterns of cyclin B since M-phase exit should be associated with the proteolytic destruction of cyclin B whereas a return to meiotic prophase should be associated with high, albeit potentially inhibited, cyclin B levels. In the textbook version of the cell cycle, cyclin B is targeted for destruction in the proteosome by the E3 ubiquitin ligase called the Anaphase Promoting Complex (APC), causing cyclin B concentrations to crash and subsequently allowing exit from the cell cycle after Anaphase I. In most organisms, haploid spermatids return to interphase after completing Meiosis II and complete a final burst of sperm-specific transcription prior to undergoing a complete turn-off of transcription. However during *C. elegans* spermatogenesis, the haploid spermatids do not return to interphase following the meiotic divisions (Shakes, Sadler, Aument, and Giliard unpublished). A normal feature of all meiotic programs is to maintain some level of cyclin B between the meiotic divisions; in Xenopus, a drop in cyclin BMI levels that facilitates progression through meiosis I is accompanied by the translation of cyclin BMII to levels that support the assembly of a metaphase II spindle (Hochegger et al., 2001). One potential explanation of the spe-7 phenotype would be a complete loss of cyclin B following meiosis I that does not support the assembly of metaphase II spindles.
Cyclin B levels, however, do not fall in arrested spermatocytes (Figure 10). In *C. elegans*, the sustained levels of Cyclin B indicate that the cell remains in the cell cycle. As an additional marker of a cell cycle drop out, we stained the Nuclear Pore Complex (NPC) to see if the Nuclear Envelope, which normally vesiculates during Anaphase I, showed any signs of reforming into a membrane. Though the immense chromatin modifications undergone during M-phase may inhibit the complete reassembly of the nuclear envelope, any aggregations of the Nuclear Pore Complex in the arrested primaries could signify an attempt at nuclear envelope reassembly cued by a cell cycle exit. However, our studies show that the membrane remains vesiculated in the arrested primaries (Figure 10). Despite the interphase-like pattern of tubulin, the localization patterns of the NPC protein and Cyclin B indicate that the arrested primary spermatocytes are not dropping out of the cell cycle. However, high cyclin concentrations do not necessarily mean high levels of cyclin activity. Cyclin B, even when paired with cdk, can exist at high concentrations in the cell with an inhibitory phosphatase attached by the cell cycle regulator wee-1 kinase (Lamitina et al, 2002). Since high levels of inhibited Cyclin B are normally associated with the G2 stage of the cell cycle, *spe-7* primaries may regress from M-phase back into a G2-like state.

The complication to the model of G2 regression is the unique feature of *C. elegans* spermatogenesis that segregates Cyclin B away from the budding spermatids into the residual body without degradation, so residual bodies contain high levels of Cyclin B (Figure 9). Therefore, high levels of Cyclin B in arrested primaries do not preclude an arrest in the cell cycle with a continuation down the developmental pathway similar to the APC mutants. A decoupling of the cell cycle and developmental pathways suggests a
Figure 9) Cyclin B (green) costained with DAPI (blue) in wildtype worms. The levels do no fade and stay high when segregated to the residual body (Shakes, unpublished).

Figure 10) spe7 staining of Cyclin B and Nuclear Pore Complex. A) Arrows show levels of Cyclin B in both metaphase I cells and anaphase I arrested cells. B) Arrows mark breakdown of nuclear envelope with no apparent reformation.
third model which our cyclin results cannot distinguish between, but predicts that cellular components involved in the developmental pathway will take on residual body formations in the arrested primary. Currently, the scope of our PLK-1 and tubulin data does not conclusively differentiate between a G2 regression and a developmental continuation. We will outline the evidence and critical experiments for these two competing models in the discussion.

**Predicted centrosomes defects**

In addition to the network pattern, the earliest signs of defective tubulin configurations come before metaphase I (Figure 11). Additional aberrant tubulin patterns besides the disassembly into a network pattern occur after the anaphase I arrest as well (Figure 11), and may indicate partial separation of the centrosomes to an attempted metaphase II plate. Whether this pattern occurs before or instead of the disassembly into a network pattern remains unclear. Intriguingly, some of the three spindle phenotypes still stain with PLK-1 on the centrosomes, while others do not. More experiments are required to test whether these were caused by improper centrosome migration, which can be determined through gamma tubulin studies and the centrosome marker SPD-2.
Figure 11) Aberrant spindle formation. Though rare, metaphase I defects are observed with abnormal centrosome separation. These patterns are indicative of other seen in field. The PLK-1 anaphase I arrest does NOT stain the centrosomes here, but data not shown documents this phenomenon.
**APC Double Mutant**

In APC mutant spermatocytes, the developmental program continues despite the cell cycle arrest in Metaphase I; the budding division generates spermatid in the continued presence of an arrested metaphase I spindle. In contrast, spe-7 spermatocytes proceeds through metaphase I to arrest in anaphase I, yet this later arrest point does not support the budding division or the formation of spermatids. If the key requirement for a budding division is a stable, bipolar spindle, we wondered whether APC; spe-7 double mutants would exhibit the “best of both” and occasionally produce spermatids, or a “worst of both” to determine what aspects of spe-7 are required for the continuation of the developmental program. We found that double mutants show the “worst of both” phenotype, with both Metaphase and Anaphase I arrests. Strikingly, the doubles still show the network tubulin pattern in arrested cells, along with the failure to complete any budding divisions.

![Image](image-url)

**Figure 12** APC/spe7 double mutant. A) merge picture of tubulin and PLK-1 and DAPI. B) Tubulin showing metaphase I spindles, but with arrow marking the characteristic spe7 network pattern. The same place is marked in A. C) PLK-1, interphase-like pattern. D) DAPI, shows primarily metaphase I arrests.
**SNP-mapping**

The initial mapping of *spe-7* to Chromosome two was performed by Bob Herman, and was given the allele designation *mn252*. Alana Noritake from Diane Shakes’ lab narrowed the position to the right of *rol-1* (6.65) and mostly likely between 10-25 map units.

To further address the role of *spe7* in the spermatogenesis pathway, we performed SNP (single nucleotide polymorphism)-mapping to help determine its molecular identity. SNP-mapping employs similar methods of using recombination ratios between two points on a chromosome to map the position of a gene. However, instead of using morphological markers, SNP-mapping exploits the silent, single nucleotide changes that occur between two isolated populations of the same species. These occur much more frequently in the genome than functional morphological markers, thus allowing a higher degree of resolution in the genetic mapping. Restriction digests or sequencing is required to confirm the particular SNP. We crossed worms from the standard lab strain isolated in Bristol, England and crossed them with the evolutionarily isolated strain from Hawaii (CB4856). Both strains have fully sequenced genomes, with the Hawaiian strain containing noted points where silent mutations have occurred to diverge from the Bristol strain. Multiple homozygous recombinants were generated to perform the molecular mapping steps.

Our data from the SNP-mapping experiments narrowed the potential range for *spe-7* between 14.49 map units to 16.99 map units, generating a candidate list of 43 genes.
Microarray Data and predicted inter-actors

To trim the candidate list produced by SNP-mapping, we looked to the vast microarray data and predicted inter-actors available on the online bioinformatics site Wormbase. Out of the 43 candidate genes, only a handful were classified as “spermatogenesis enriched” from microarray data performed by Valarie Reinke’s lab (Reinke, 2000). One of these was the gene F32A11.3, which also showed a predicated interactor list of 40 genes, many of them also spermatogenesis enriched. Given the seemingly integrative nature of spe7 protein, we decided a spermatogenesis-enriched protein with a large number of interactors was the most likely candidate for the molecular identity of spe7.

Sequencing

We PCR amplified and sequenced F32A11.3 from a genomic preparation of spe7(mn252) DNA to locate any potential mutations that could account for the loss of function seen in the spe7 mutants. The gene is about 2kb, so we PCR amplified the gene using three overlapping sections, with additional primers to obtain clear bidirectional reads all gene fragments from multiple, independent PCR amplifications (Figure 13). We found a C/T point mutation at the 1681st nucleotide, confirmed by a bidirectional read of three independent PCR products (Figure 14). The mutation results in a pre-mature stop codon at the beginning of the fourth and final exon. The nonsense mutation would leave the last 40 amino acids untranslated (Figure 15). F32A11.3 shows the closest homology to other nematode species, but is novel to C. elegans. The most homologous gene is another C. elegans gene called ZK783.6, but shows a weak homology to the mucin
domain of zonadhesion, a mammalian sperm protein. ZK783.6, whose function remains unknown, appears to be a sister gene that arose from a duplication event. Microarray studies suggest the sister gene is oogenesis enriched.

Figure 13) Diagram of F32A11.3 with the gene fragments used to PCR an amplicon for sequencing as the labeled lines. Inset primers for better reads toward the middle of the fragment are represented by arrows. The gene runs from right to left across the page, with exons represented by the blue boxes.

Figure 14) Raw data showing the bidirectional sequencing read confirming a C/T substitution from the F32A11.3 gene isolated from spe7 mutants (top two) and the wildtype sequence (bottom one).
Figure 15) Sequencing Summary: The C/T point mutation changes the translated amino acid from a Glutamine to a Stop. The arrows mark the position on the gene as well as the precise amino acid where the truncation occurs on the protein sequence. The protein sequence above is color coded to show each exon in alternating colors. The exon on the far right of the gene diagram corresponds to the top red sequence of amino acids. The region colored white is the sequence used to generate the SPE-7 antibody. Importantly, the antibody will still bind even after the truncation because of the location before the nonsense mutation.
RNAi

RNAi allows researchers to silence the expression of a particular gene in a living organism, essentially turning off the function of a specific protein product. Turning off a gene in a living worm should produce a recognizable phenocopy of the corresponding mutant phenotype in a previously healthy worm. We performed RNAi to determine if we could reproduce the spe7 phenotype by knocking down the F32A11.3 phenotype, thus providing an *in vivo* confirmation of our candidate gene. We observed signs characteristic of a spe7 loss of function phenotype in wildtype worms treated with the F32A11.3 double stranded RNA complex (Figure 16). Through DIC/Hoechst microscopy, we saw the characteristic Anaphase I arrest, including some of the tell-tale aberrant chromatin morphology (marked with arrows) detailed in Figure 4. The RNAi experiment provided a confirmation of the sequencing data that F32A11.3 is the molecular identity of *spe-7*. In addition, since RNAi knockdowns invariably represent loss-of-function phenotypes, this result indicates that the truncation of *spe-7* in the *mn252* allele represents a loss-of-function rather than a neomorphic function.

![Figure 16](image16.png)

Figure 16) RNAi. A) F32A11.3 RNAi, and B) is *spe7*. The two are nearly identical.
Transgenics

Another way to confirm the identity of gene is to perform a rescue experiment through transgenically introducing a healthy copy of a gene into a mutant worm and seeing if the gene rescues the mutant phenotype. In collaboration with the Singson lab, who did the transgenic injections, we attempted to rescue spe-7 with genomic (cosmid) clones containing F32A11.3, but did not find any successful rescue, despite analyzing several independent lines.

Localization of SPE7

Towards the goal of analyzing the cellular localization pattern of SPE-7, we ordered production of polyclonal anti-peptide antibodies from Yenzyme Custom Antibody Service. After pre-screening eight rabbits for non-specific nematode antigens, two rabbits were selected for immunization against a peptide fragment (Figure 15) that was predicted to be both antigenic and on the protein surface. Yenzyme ultimately sent us both sera and affinity-purified antibody. We examined methanol-fixed male him8 gonads initially incubated with the polyclonal SPE-7 antibody followed by incubation with a FITC-conjugated secondary antibody and counterstained with the DNA dye DAPI to characterize the expression pattern of SPE-7 in wildtype spermatogenesis.

SPE-7 exhibits a dynamic localization pattern. SPE-7 could first be detected in late pachytene spermatocytes, consistent with the expression pattern of other spermatogenesis-specific proteins (Figure 17, A-C). The SPE-7 labeling becomes more pronounced in diplotene and karyosome stage spermatocytes, localizing in speckled, punctate formations outside the nucleus. The punctate formations reach peak intensity at
Figure 17) anti-SPE-7 localization in wildtype. A) anti-SPE-7. B) DAPI. C) Merge. Individual cut outs show the change in localization at metaphase I and the lack of signal in the spermatid.
the karyosome stage. Intriguingly, the localization pattern exhibits a dramatic shift from the punctate formations to a diffuse localization during Metaphase I (Figure 17). SPE7 returns to a punctate pattern in Anaphase I, but the signal is less intense than in the karyosome spermatocytes. The antibody continues to label punctuate structures through Metaphase II, Anaphase II, the budding division, and stains spermatids immediately after the budding division. The SPE-7 signal was then rapidly lost after the budding division such that most spermatids have no detectable SPE-7 labeling. Though we suspect signal fades in the late spermatid because of proteolysis, various post-translational modifications could also explain the loss of signal. A phosphorylation/glycosylation event near the antigenic site could inhibit binding of the antibody, or cause a conformational change that excludes the antibody all-together. However, the peptide region used to generate the SPE-7 antigen contains no predicted phosphorylation or methylation sites and it lacks either an N-terminal signal sequence or a transmembrane domain that would expose it to glycosylation enzymes (data not shown).

*SPE7 dynamically localizes with the Membranous Organelle*

Previous ultrastructural studies identify only four structures that are not partitioned to the residual body and remain within the spermatid after the budding division: the condensed, haploid chromatin mass, the associated centriole, mitochondria, and a unique structure called the Membranous Organelle (Chatterjee et al, 2005). The Membranous Organelle (MO) is a Golgi-derived organelle that, among other functions, carries proteins vital for the transformation of a spermatid into a functioning spermatozoon through the various cytokinesis events in spermatogenesis. The
membranous organelle therefore functions as one of the few organelles present from early spermatogenesis all the way through to the mature spermatozoa.

Proteins associating with organelles often exhibit a similar type of punctate pattern as seen with the SPE7 staining under immunofluorescence. SPE7’s presence in the spermatid narrows the likely associated organelle to the mitochondria or the MO. To determine the specific cellular localization of SPE7, we co-stained the anti-SPE7 antibody with the monoclonal 1CB4, which, within nematode sperm marks the Membranous Organelle (Figure 18). We show a convincing co-localization between the two antibodies, implying an association of SPE7 with the Membranous Organelle. However, the diffuse SPE7 pattern observed in Metaphase I primaries specifically does not co-localize with the 1CB4 pattern (Figure 18), suggesting that during Metaphase I the SPE7 protein disassociates from the MO and diffuses throughout the cytosol before reassociating with the MO in Anaphase I. Given the close proximity of the SPE7 trafficking to the arrest point in the loss-of-function mutant, we propose that SPE7 becomes active once released into the cytosol, while otherwise remaining sequestered in a nonfunctional form on the MO.
Figure 18) Strong co-localization between the MO and SPE-7. The pattern is most striking when observing that the spaces with no staining are identical in each panel, except in Metaphase I, where the SPE-7 covers the entire cell, unlike the 1CB4, which remains more punctate. This result supports the co-localization with 1CB4, except for in metaphase I.
**SPE-7 signal fades in mutant background**

To further understand the loss of function seen in spe7 mutants, we stained male gonads with the anti-SPE-7 antibody to determine how the pattern differs in mutants from the previously characterized wildtype pattern. The nonsense mutation produces a truncated protein in the mutant spermatocytes. Missing domains of proteins can interfere with general function or localization, as well as targeting the existing fragment for degradation. Antibody patterns in mutant gonads can look similar to wildtype, implying a loss of general function without degradation, localized to the wrong spot in the cell, implying a mislocalization, or show a lack of signal, implying degradation. Because the piece of SPE-7 that is untranslated due to the premature stop codon is highly conserved, we expected that the mutant pattern would not result in a general loss of function, but instead are mis-localized within the cell or degraded entirely.

We found that anti-SPE7 signal fades almost entirely in a mutant background (Figure 19). Though some samples exhibited weak antibody binding around the chromatin, the general brightness does not score above the general glow produced by the background of extraneous secondary antibodies. Though this result could suggest the absence of the highly conserved 3’ prime region of SPE-7 results in misfolding and subsequent degradation of the protein, the mutant protein could fold in such a manner as to exclude the antibody. A western blot is needed to confirm whether the decrease in antibody binding corresponds to a decrease in protein levels.
Figure 19) SPE-7 signal fades in mutant background. All shots were taken at 2000mmsecs exposure, with maximal intensity (MI) measured through IPlab (scale of 0-4095) A) Wildtype staining, MI=3740. B) Secondary antibody only (control), MI=424. C) spe-7, MI=642. *spe-7* stains about as brightly as the expected secondary antibody background.
Discussion

Summary

We have shown that spe-7 is a recessive, sperm-specific mutation that causes an Anaphase I arrest in diploid spermatocytes. The defect responds to temperature, with a slight increase in fertility at higher temperatures, indicating a cold sensitivity. The arrest point is variable at different temperatures as well, and though no spermatids were observed, mutants do produce a small number of functioning spermatozoa at 20°C and 25°C. Otherwise, mutants fail to successfully perform either the actin-myosin based division that gives rise to secondary spermatocytes or the specialized budding division that gives rise to sperm; especially at 16°C, under which conditions spermatids are never seen.

Primary spermatocytes continue to bud off the rachis for the entire lifetime of the mutant worm. As mutant males age, their gonads accumulate cellular debris unbound by a membrane, which we presume originates from older arrested primaries that lysed. Arrested primaries do not undergo apoptosis.

Microtubule organization proceeds normally through the initial stages of organization, though some defects are detected as early as metaphase I. After the anaphase I arrest, the spindles disassemble and reorganize into network pattern similar to that of G2 spermatocytes. The cell cycle regulator Polo-like Kinase-1 (PLK-1) also exhibits normal localization through the anaphase I arrest, after which it improperly jumps off the centrosomes and assumes a punctate pattern. We are currently unsure whether this pattern more closely resembles that in residual bodies or in G2 spermatocytes.
Despite the interphase-like patterns observed by microtubule and polo-like kinase-1, the arrested primaries are not dropping out of the cell cycle. Cyclin B levels remain high in the arrested spermatocytes indicating that they are not exiting the cell cycle. On the other hand, cells with high concentrations of Cyclin B can be inhibited for CDK activity as in G2 spermatocytes. Since the *C. elegans* spermatogenesis program includes the unusual feature of never returning to interphase after anaphase II, elevated Cyclin B would also be consistent with a continuation of the normal developmental pathway despite the anaphase I arrest. Importantly, the nuclear envelope also shows no signs of reformation in the arrested primaries.

The APC/spe-7 double mutant exhibits the “worst of both” phenotype. Primary spermatocytes arrested at metaphase I but subsequently disassembled their spindle and failed to complete any budding division.

SNP-mapping narrowed the potential genetic position of *spe-7* between 14.49 map units to 16.99. Microarray data and lists of potential interactors listed on the bioinformatics website Wormbase allowed us to select the most likely candidate for sequencing from the candidate list produced from our SNP-mapping. Sequencing of the gene F32A11.3 confirmed a point mutation at the start of the final of four exons. The nucleotide substitution caused nonsense mutation, which would prematurely halt translation to produce a protein without the C-terminal domain that is highly conserved across the *Caenorhabditis* clade. Further study of the F32A11.3 sequence revealed the closest homology was another *C. elegans* gene, which appears to have arisen from a gene duplication event within *C. elegans*. 
Though we were unable to show a viable rescue with transgenics, we produced a function pheno-copy of the spe-7 phenotype using RNAi of F32A11.3. The RNAi experiment revealed the full spectrum of previously observed spe-7 phenotypes including arrest at anaphase I, between metaphase I and anaphase I, post anaphase I, and cell lysing. Elucidating the molecular identity of spe-7 allowed us to produce a polyclonal antibody, and thus study of the in vivo localization of the protein. We found that SPE-7 is first detectable in late pachytene spermatocytes and co-localizes with the Membranous Organelle (MO) until metaphase I, when SPE-7 appears to detach from the MO before reattaching in anaphase I. SPE-7 levels intensify until peaking in karyosome spermatocytes, then taper off in metaphase II, anaphase II, and the budding division until the signal finally disappears in early spermatids. We cannot conclusively claim whether or not SPE-7 levels fade during metaphase I, as the differential patterns make comparison difficult, but when the punctate pattern reemerges in anaphase I, the levels are noticeably dimmer. The antigenic region contains no likely phosphorylation, glycosylation, or predicated methylation site. Thus, we hypothesize that the fading signal is due to degradation. In metaphase arrested cells, SPE-7 locks into metaphase I configuration. However, in these APC mutants a subset of primaries with metaphase I chromatin configurations contained SPE-7 localization more similar to a karyosome cell. These results suggest that cytoplasmic SPE-7 may not be required for the process of getting to metaphase I and that the relocalization of SPE-7 is facilitating a function for SPE-7 at the onset of the metaphase to anaphase transition. Despite this, SPE-7 is not targeted for destruction by the Anaphase Promoting Complex, and fades in metaphase arrested APC spermatocytes. Though the metaphase arrest obstructs SPE-7 trafficking back to the MO,
the components responsible for the fading SPE-7 pathway still function despite the cell cycle arrest.

In a \textit{spe-7} background, SPE-7 signal is nearly undetectable. The truncated protein product likely is likely targeted to the proteosome, indicating a total loss of function for the \textit{mn252} allele of \textit{spe-7}. However, it remains possible that the truncation event causes abnormal folding that excludes the antibody.

\textit{Shifting arrest points or slowed progression?}

\textit{spe-7}’s precise arrest point remains one of the more puzzling components of the mutant phenotype. Though the anaphase I arrest is present at all three temperatures we studied, many of the observed arrested primaries lacked lagging X chromosome characteristic of a classic anaphase I configuration. For simplicity, we classified these cells as anaphase I arrested, but the lagging X “catching up” with the rest of the chromatid block may mean \textit{spe7} primaries progress as far as possible toward metaphase II without actually forming a metaphase II plate. The anti-\(\alpha\)-tubulin experiment supports the former claim; though we noted spindle formations that may indicate partial centrosome separation after anaphase I (Figure 7 at 25\(^\circ\)C, Figure 11), we never observed proper metaphase II spindle assembly (Figure 11, Figure 8). More importantly, PLK-I never relocalized the chromatin after anaphase I to mark a metaphase II plate. The consensus arrest point may require a more subtle classification as between a true anaphase I and metaphase II to reconcile these data points, perhaps as a “late anaphase I” arrest.
Though we never saw a true metaphase II plate, our data shows aberrant chromatin configurations that likely denote partial separation of the sister chromatids after the anaphase I arrest (Figure 4F). Because no metaphase II plate forms, the separation is likely not tubulin-driven movement and more likely a passive drifting. Though this observation is most striking at 25\(^\circ\) and 20\(^\circ\)C, the partial separation phenotype remains present at 16\(^\circ\)C, just with fewer cells per field exhibiting the separation. Mutant males grown at 16\(^\circ\)C show an additional phenotype with close chromatin masses that resembles an intermediate between metaphase I and anaphase I (Figure 4C). Our fertility data shows 16\(^\circ\)C as the most severe phenotype, yet it shows the greatest range of arrest points before and after late anaphase I (Figure 4E). Our RNAi data supports the more severe phenotype should show just this, since a knockdown of SPE-7 produce all the observed chromatin morphologies (Figure 16).

Additionally, one of the aberrant chromatin separation phenotypes with the small chromatin dots (Figure 4F) frequently seen at 25\(^\circ\) and 20\(^\circ\)C, stained with PLK-1 at 16\(^\circ\)C. The small chromatin dot phenotype is potentially an arrested metaphase I “pentagonal” array that continued to separate abnormally. Though this is a relatively rare morphology, the staining with PLK-1 is evidence for shifted arrest point more toward metaphase I at 16\(^\circ\)C, and is likely a distinct form of aberrant chromatin separation that results from an earlier arrest than the sister chromatid drifting. However, at the higher temperature, the chromatin dots were invariably PLK-minus, indicative of an anaphase state whether they appears to arrest in a metaphase I-like configuration or, more commonly, separate subsequent to anaphase I. This result may suggest that the most severe phenotype may trigger an incomplete metaphase checkpoint, were the cell cycle largely stops, but with
some centrosome separation and continued spindle forces. At higher temperatures, the increased stability of microtubules could potentially further aberrant separation of homologues past a weak or incomplete checkpoint. The data together could suggest a variable arrest point highly dependent on temperature.

The alternative to the variable arrest model is one of slowed chromosome segregation. We would expect the chromosome segregation to proceed as normal, but at a pace slow enough to reveal chromatin intermediates rarely captured through microscopy. Spermatogenesis may end with a whimper rather than a precise arrest point, with most cells only reaching anaphase I before stopping. Because anaphase I is normally a rapid step in the cell cycle, the act of quickly dividing chromatin would present a substantial obstacle to a slow progression phenotype. If this model were true, the spe-7 mutant would offer novel insight into the details of chromosome movement during the meiotic divisions. Currently, our data does not distinguish between the two. A future experiment is to examine the meiotic progression through time in spermatocytes expressing GFP-tubulin and mCherry histone. Though possible, this experiment is challenging due to the problem of expressing GFP constructs within the male gonad and the sensitivity of spermatogenesis to heat.

**Competing models for cell cycle dynamics in spe-7**

The morphing of microtubule organization from a functional spindle to an interphase-like network pattern, along with the lack of centrosome staining by PLK-1 and subsequent interphase-like punctate pattern, suggest a cell cycle drop out. Our data suggests three competing models for cell cycle progression in spe-7 mutants: forward jump to G1, regression back to G2, or developmental progression to a residual body state.
An exit from the cell cycle by jumping from anaphase I to G1 predicts a drop of Cyclin B in the arrested primaries, as Cyclin B levels are low at the G1 stage. Cyclin B levels, however, remain high in the arrested spermatocytes. One possibility remains for the G1 jump: a recent paper shows that a knockdown specifically in Cyclin B3 in *C. elegans* result in chromatin defects around anaphase I transition (van der Voet, et al 2009). Because our Cyclin B antibody was generated with the *Drosophila* ortholog, our data may not show a potential drop specifically in Cyclin B3 that could explain the phenotype.

Two cell stages contain elevated Cyclin B levels while exhibiting network tubulin patterns: G2 spermatocytes (high but inhibited) and residual bodies (high and active). The latter suggests a decoupling of the developmental program from the meiotic cell cycle, where aspects of the developmental program are progressing to their residual body formations (Figure 9). We know this is possible given the APC mutation, though a key difference remains that *spe-7* rarely shows successful budding division. To distinguish between these two models, we must consider future experiments as well as our current results:

A continuation of the developmental pathway despite a meiotic cell arrest means that certain molecular players would eventually assume a residual body pattern while the arrested cell cycle components remain frozen. Network microtubule patterns are classically a hallmark of an interphase, including G2, cell. However, microtubules do reassemble into a distinctive network pattern once segregated to the residual body as well (Figures 6). The key difference between the network pattern in G2 and that seen in a residual body is that tubulin remains in an end-gathered state, marking a different level of organization. After the centrioles detach from the spindle and follow the chromatin into
the spermatid, the tubulin remains centrosome nucleated by gamma tubulin. An organized spindle may segregate more efficiently than a disassembled one, thus the selection pressure for gamma tubulin to continue nucleating the microtubules in the residual body after the disassociation with the centrioles. The network pattern recorded in spe-7 arrested primaries is visually distinct from either the expected G2 or residual body formations, but images at high magnification of tubulin in arrested spe-7 primaries reveal the network pattern as more closely resembling a G2 pattern (Figure 8). We will need to stain cells with a gamma tubulin marker to see whether or not the microtubules remain end-gathered to distinguish between the two models, though we predict that the gamma tubulin staining will reveal tubulin is not end-gathered in arrested primaries and therefore more closely resembles a G2 pattern.

PLK-1 may further diagnose the cell cycle point present in arrested primaries. If the developmental program progressed, we would expect PLK-1 to assume a residual body formation in the arrested primaries. Again, the patterns of a residual body and G2 pattern are similar for PLK-1 localization. PLK-1 assumes a speckled pattern with different size punctates in the residual body, while it localizes around the nuclear envelope and more uniform speckles in the cytosol during a typical G2 pattern. The PLK-1 pattern in spe7 arrested primaries more closely resembles the G2 pattern visually, but we cannot make this claim conclusively (Figure 8, 6). A future experiment to confirm a G2 could co-stain PLK-1 with a nuclear envelope marker to see if PLK-1 gathers around the vesiculated nuclear envelope in arrested primaries. Though the nuclear envelope appears to not reform, thus affecting expected PLK-1 pattern, it is unlikely that a nuclear envelope could reform completely around the immense chromatin modifications.
occurring after the initial breakdown. If we stained nuclear lamin instead of nuclear pore, we might increase our sensitivity to detect small, partial assembly events; we would expect PLK-1 to group around these partial assembly events. Both these results would provide dual support for a regression to G2.

Another method is to more closely study the residual body pattern in wildtype. Though we cannot confidently claim whether the arrested pattern is visually closer to a G2 pattern or residual body, further study of the residual body pattern could make this possible. Given our current observations however, we predict to see a pattern conclusively distinct from the residual body pattern.

Thus far, we have outlined experiments to discern between a cell cycle regression and a developmental progression using tubulin, nuclear envelope and PLK-1. While PLK-1’s dynamic role in the cell cycle is useful to mark the stage of a cell within the larger pathway, PLK-1, tubulin, and nuclear envelope markers are all distinctly cell cycle proteins and do not directly mark the developmental program of spermatogenesis. An alternative approach is to mark a protein whose function is strictly developmental and see if the pattern continues past the configurations typically seen in anaphase I. MSP would work wonderfully, as it is sequestered in a paracrystal throughout the meiotic divisions before it releases into the cytosol in the spermatid to form a pseudopod. We could expect that if the developmental program continues despite the cell cycle arrest and lack of budding division, MSP might release into the cytosol in the arrested spermatids. This would support a continuation of the developmental progression, thought it is possible MSP release is somehow dependant on segregation to the spermatid for release. We predict, however, that MSP will remain sequestered.
Given our current information, we predict that arrested primaries regress to a G2-like stage, with anucleated tubulin networks, small reformations of the nuclear envelope with PLK-1 closely grouped, and MSP in paracrystals. Because the G2 hypothesis may depend on some form of Cyclin B inhibition, we could expect wee-1 activity, though the presence of wee-1/myt-1 in sperm is debated in the literature (Lamitina and L’Hernault, 2002). The developmental progression remains a distinct possibility, or a novel combination of the two. Either way, the phenotype frames SPE-7 as an integrating protein that takes cues from both cell cycle and developmental players.

Spermatogenesis may require an intact spindle for budding division

spe-7 was initially considered an interesting phenotype in the context of the APC mutants. Since the arrest points of each mutant were so close, we wondered why the APC mutation allowed a successful budding division while spe-7 did not. Although the failure to produce spermatids may seem intuitive for a cell cycle arrest, the APC mutants show that an intact metaphase I plate can support successful budding divisions for the cytokinesis steps and segregation of the MO, mitochondria, and centriole. The striking difference between APC and spe7 is that spe-7 mutants disassemble their spindles after metaphase I.

Wildtype budding division occurs with elevated levels of cyclin B and with spindles remaining in an M-phase like structure. We performed an anti-α-tubulin/anti-PLK-1 prep on an APC/spe-7 double mutant, hypothesizing that since the APC arrest occurred slightly earlier, it could stabilize the spindles of the double mutants and possibly support spermatid formation. Instead, we found that the double mutants still arrested in
metaphase I AND developed spindle defects. Strikingly, with its nonfunctional spindle, the double mutant no longer produced any anucleated spermatids, normally a hallmark of the APC phenotype. Additionally, our fertility data supports that even a partially functioning spindle may support a budding division. Wildtype worms are typically more fertile at lower temperatures, with brood counts dropping as the temperature increases. Intriguingly, our fertility data shows that though spe7 hermaphrodites are mostly self-sterile, worms grown at 25°C are more fertile than those grown at 16°C (Table 1). A higher permissive temperature usually denotes flawed microtubule processes, as tubulin polymerizes better at higher temperatures. Our immunocytoology confirms the tubulin defect, and interestingly, shows that some formations resembling a flawed metaphase II do organize, especially at higher temperatures. Taken together, the increased polymerization efficiency of tubulin at higher temperatures may counteract the effects of spe-7 just long enough to keep a spindle intact enough to complete a few (<6) successful budding divisions.

Since microtubules are involved mainly in chromatin separation in the meiotic divisions, a potential model requiring spindles for the budding division may seem counterintuitive. However, the budding division in *C. elegans* occurs without an actin myosin ring, so the role of cytokinesis must occur by another mechanism. Instead of a standard cleavage furrow, the spermatids are hypothesized to construct a lipid wall between themselves and the residual body using vesiculated membrane packets that coalesce to complete cytokinesis in a process similar to plant cell division (Ward, 2000). These vesiculated membrane packets would need to travel up the intact spindle to successfully bud off from the primary. Additionally, segregation of the MO and
mitochondria likely requires these organelles to travel up an intact spindle. With closer examination of the nucleated tubulin pattern in wildtype budding figure, one can imagine the spindle structure not only as a machine for chromosome separation, but a highway for organelle segregation and cytokinesis (Figure 6). Though we typically think of microtubules in the context of chromosome division during the cell cycle, their main function within an interphase cell is a cytoskeletal protein with vital roles in vesicular transport. This model proposes that *C. elegans* cleverly combines both features of microtubule function for a superlatively efficient set of meiotic divisions and sperm individualization. Combining the cell cycle and transport function of microtubules allows the necessary components for sperm to crawl away from a centralized region, leaving items behind that are not needed for sperm function. Since MSP allows sperm motility without requiring the tedious reorganizing tubulin into a complex flagellum, a dually functional spindle can stay behind in the forming residual body to scaffold rapid chromatin/organelle segregation and subsequent cytokinesis. Performing division from a centralized furrow would make this strategy difficult, as segregation, chromosome separation and cytokinesis would have to occur in a stepwise fashion.

Actin patterns in *C. elegans* suggest that current spindle function is an adaptive trick which bypasses the normal actin/myosin ring based divisions. An actin ring still forms to mark the middle of the cell; this could help polarize the cell or is just residual from basal function. If actin and microtubules are still closely linked in staging the cytoskeletal function of the spindles, the *spe-7* defect could affect the normal localization of actin throughout the meiotic divisions. Microtubule reassembly interfering with actin function may explain the lysing primaries seen in older *spe-7* mutants.
RNAi as a tool for exploring early spermatogenesis mutants

RNAi is a remarkable tool resource for determining gene function and confirming mapping results. Many genes that function in spermatogenesis, however, remain poorly classified in part because of the difficulties experienced by researchers in getting RNAi to work in the male C. elegans gonad. Many of the well-studied sperm defects occur well downstream of the events discussed in this study, with mutations affecting motility or fertilization. RNAi achieves poor knockdown at these points in the spermatogenesis pathway. Our success with RNAi in the male C. elegans gonad shows exciting potential for RNAi as a window into the complex function of early meiotic sperm-specific proteins. We propose that because mutations affecting fertilization or motility occur long after the primaries bud off the rachis and spermatid individualization, the time may mitigate the effects the RNAi knockdown. In early meiosis however, RNAi seems much more effective at administering a knockdown to show a loss of function phenotype. We propose that future researchers not shy from RNAi in male gonads, especially if the gene of study may work in early spermatogenesis.

Lingering questions for SPE-7, model for function

The mutant characterization and anti-SPE-7 antibody localization patterns partially intimate the molecular function of SPE-7, though a complete understanding of the SPE-7 will require a catalogue of its interactors, some of which are expected to link SPE-7 to better understood molecular pathways. A yeast-two hybrid screen will provide vital insight to SPE-7’s molecular binding partners and elucidate important clues for function. Until we complete this next experiment, important questions remain that hint at the cellular function of SPE-7.
**SPE-7 trafficking**

Our data convincingly shows a change in confirmation from SPE-7’s punctate pattern within karyosome spermatocytes to a diffuse metaphase I configuration. In some pictures, however, the signal appears to fade slightly in metaphase I cells; the drop in brightness could be an illusion created by the concentrated points spreading out into the cell, so though the overall brightness remains the same, the cell appears dimmer. We will use the program IP Lab to measure the pixel density in whole-cell karyosome and metaphase primaries to see if the overall brightness changes. A drop in signal during metaphase I could complicate the hypothesis of diffuse pattern as the active form.

Our current assumption is that SPE-7 remains sequestered on the Membranous Organelle, but why would a protein follow the MO all the way to the spermatid only to be degraded shortly after the budding division? *C. elegans* spermatogenesis is built for efficiency and speed. The MO evolved to cooperate with sperm specific proteins, and remains one of the only organelles present throughout spermatogenesis. Our studies in the APC mutant reveal that the trafficking between the MO and the cytosol occurs rapidly. The function must require quick action, but importantly, quick re-tethering to the MO after the job is performed. If the tethering to the MO sequesters SPE-7 function, the cell may find re-tethering more efficient or faster than outright degradation. Once attached to the MO again after metaphase I, the cell is free to leisurely degrade the protein off the membrane before MO docking with the spermatid membrane. Additionally, SPE-7 may in fact hold additional jobs while attached to the MO, possibly as a recruitment center for other cytosolic proteins. The choice of the MO makes more
sense in this light, allowing SPE-7 function all the way into the early spermatid. However, the multiple MOs may serve as a convenient membrane with a collectively large surface area in primary spermatocytes from which to base a rapid journey to the cytoplasm and back, whose distribution around the cell may assist even diffusion through the cytosol after SPE-7 releases from the membrane.

Evidence for diffuse pattern as SPE-7’s active form

The primary evidence for the diffuse pattern as the active form of SPE-7 is the proximity of the shift in pattern to the arrest point. Moreover, the evidence from the APC mutants show that cells progress to metaphase I before SPE-7 fully detaches from the Membranous Organelle, implying that the protein is not required for the transition into metaphase I, and given the mutant phenotype, acts during the metaphase to anaphase transition. The breakdown of the nuclear envelope may serve as a cue for the release of SPE-7 into the cytosol.

Furthermore, APC mutants show no spindle defects, possibly because the metaphase I arrest allows SPE-7 to enter its active form and stabilize the spindle to permit budding figures despite the arrest in cell cycle. Moreover, minimal defects are seen in spe-7 before the differential metaphase pattern. However, some strange tubulin formations are observed at metaphase I may justify that SPE-7 is required in late in the stage, or perhaps for proper centrosome regulation.

Isolating and analyzing additional spe-7 alleles would prove useful in exploring the neomorphic effects of a partial function mutant. Particularly, a mutant that fails to detach from the MO at metaphase I would help pinpoint the active form of SPE-7 in spermatogenesis. Conversely, a mutant that failed to reattach after the release of SPE-7
into the cytosol would prove informative as well. Though the release of SPE-7 into the cytosol at metaphase I in APC mutants appears sufficient to stabilize the spindle, cytosolic SPE-7 may interfere with processes further down the pathway that the cell cycle arrest masks. SPE-7 trafficking likely functions to regulate function, but the exact reason for this remains unclear.

Fully stated, SPE-7 is a protein that likely functions with a large number of interactors to integrate, interface, and coordinate between the cell cycle and developmental components of spermatogenesis. Because we predict SPE-7 to activate so briefly, it may serve as a regulator that sets off a cascade of various events that must begin as the cell enters anaphase I. Along with organizing tubulin and directing cell cycle progression, the strange chromosome separation phenotypes suggest some upstream role in chromatin segregation. SPE-7 may help modulate motor proteins like the chromokinesin klp-19, involved in microtubule pulling forces, which could interfere with tubulin/chromosome interaction and result in abnormal chromatin segregation. In fact, klp-19/KIF4 was recently shown to play an essential role in segregating the meiotic chromosome of *C. elegans* oocytes (Wignal et al., 2009), and knockdowns of KIF4 in Xenopus oocytes results in an inability to form metaphase II spindles (Perez et al, 2002).

*Sister Gene*

*spe-7* has a sister gene that likely arose from a gene duplication in *C. elegans*. Microarray data predicts that this paralog may function in oogenesis or early embryos. The dynamics of these two proteins are a wonderful example of how gene duplication events and subsequent mutations can lead to an increased complexity of molecular interactions in related tissue. The role of the sister gene serves as a type of ectopic
experiment to shed light on the *spe-7* function. Early experiments show that ZK783.6 may use the same regulatory trick of jumping on and off a membrane in mitotically dividing embryos (Leah Towarnicky, unpublished observations). The function may be highly related, or perhaps the sister gene is required for another aspect of chromosome segregation all together, but uses the same ability to sequester on a membrane as part of its function. The persistence of ZK783.6 points to a broader pressure for innovation in the germline, with an evolutionary selection toward speed and efficiency played out at a molecular level.

*In conclusion* . . .

The original characterization of the APC phenotype in *C. elegans* spermatocytes called into question the depth of the connection between the cell cycle, microtubules formation, chromatin modification, and cellular development. The *spe-7* mutant presents not only a stunning illustration of the intricately connected nature of these features, but the subtlety of their cooperation. Comparing the two mutation shows how some events in the cell cycle can converge on one key protein, while others allow some portions of the gametogenesis program to continue. Successful cell division powers the perpetuation of all living creatures, and the elegant union between the cell cycle and the developmental programs conceives the specialized cells that seed the persistence of multi-cellular life.
Methods and Materials

**Strains and Genetics**

We used the strain *unc-4 spe-7/mnc1;him-8* as a source of mutants to study. The *unc-4* mutants contain a neuronal defect that causes the inability to crawl backwards, therefore serving as a convenient morphological marker. *unc-4* is a recessive gene located on chromosome II, and segregates with *spe-7* during crosses. We selected homozygous *spe-7* worms by picking *unc-4* homozygotes.

To breed mutant males for study, we crossed heterozygous males to homozygous hermaphrodites. Previously, we crossed *him-8* into the lab strain to increase the amount of males produced by the strain. The balancer *mnc1* prevented recombination between *unc-4* and *spe-7*, so picking *unc-4* homozygotes almost always meant we picked *spe-7* mutants.

We used a standard *him-8* line for our wildtype males. However, to perform our controls for *spe-7* studies we used the line *unc-4;him-8*. We noticed that *unc-4* mutants show slowed aging compared with wildtype worms, so an *unc-4* line without *spe-7* was used for wildtype controls to assure comparable development.

Strain stocks were grown at 16°C, but individual crosses were performed at 20°C and 25°C when required for experiments.
**Aging Worms**

We used 24 hours at 20°C as our baseline for aging worms. Since we performed experiments at multiple temperatures, we compensated for metabolic differences at different temperatures. We dissected worms at 48 hours for 16°C worms and 12 hours for 25°C to give us roughly the same metabolic age at each temperature.

**Immunofluorescence and DIC**

We dissected males with 27.5 gauge needles in 5µL of sperm media with a 1:100 levamisole dilution on Fisher brand Superfrost® slides. We squashed the tissue with 24x60mm cover slips to allow observation of additional cellular detail. The samples were freeze cracked in liquid nitrogen and fixed in cold methanol overnight. Once fixed, the tissue was washed for five-ten minutes in 1X PBS then soaked in blocking solution for at least twenty minutes. After treatment with various antibody protocols, samples were then mounted in Biomedia Gel/Mount mixed with the fluorescent stain DAPI to mark DNA.

For DIC samples, we skipped the fixation steps and immediately studied the live tissue, using the lipid soluble DNA dye Hoescht 33428 to stain the chromatin. The Wollastrom Prism settings should yield a pearl grey background for these preps.

All samples were examined with an Olympus BX60 fluorescence microscope with a Cooke cooled CCD camera attached.

**anti-SPE-7:**

Using a 1:1250 dilution of primary antibody, samples were allowed 3-6 hours to incubate at room temperature, followed by a five minutes wash in PBS. The TRITC-
conjugated antibody at 1:100 dilution served as the secondary antibody. The prep concluded with a 3 minutes wash in PBS, followed by mounting with DAPI. The antibody is generous with both concentration and incubation time, but the procedure outlined here is optimal.

**Anti-PLK-1:**

In a 1:500 dilution, samples should incubate at 4°C overnight. The samples require a 2 x 5 minute wash in PBS after the overnight incubation. We marked the primary PLK-1 polyclonal antibody with the goat-anti-rabbit IgG TRITC secondary antibody and incubated for one to two hours at room temperature. Conclude the prep with a two-three minute wash in PBS.

**Anti-tubulin:**

Using a 1:100 dilution of the primary mouse anti-α-tubulin FITC conjugated primary antibody, we incubated samples for one to two hours at room temperatures. If staining with other antibodies, add your anti-α-tubulin at the secondary antibody incubation. The anti-α-tubulin antibody requires only dip-washing, but will still sustain more extended PBS washes.

**Anti-nuclear pore:**

In a 1:800 dilution, we incubated dissected tissue for two hours at room temperature. We marked the anti-nuclear pore primary antibody with an anti-mouse FITC
conjugated secondary antibody (Jackson Lab). The primary antibody is washed for 2 x 5 minutes, while the secondary is washed for 5 minutes before mounting with DAPI.

**Anti-Cyclin B:** anti-Cyclin B was generated from *Drosophila*, but cross-reacts with the *C. elegans* Cyclin B because of the high homology. Because the match is not perfect, however, the prep requires a higher concentration of primary antibody. Using a 1:3 dilution of primary antibody, we incubated the initial antibody for (check this) 2-3 hours at room temperature and washed lightly. The secondary FITC conjugated antibody was left on for 1-2 hours are room temperature at 1:50 dilution and washed for 2 x 3 minutes.

**1CB4-**

The antibody 1CB4 marks the Membranous Organelle structure unique to nematode spermatogenesis. We used a 1:50 dilution of the 1CB4 primary antibody for two to three hours at room temperature and washed the samples for 3 x 5 minutes. In a 1:50 dilution of anti-rabbit TRITC conjugated secondary antibody, we incubated the samples for 1.5 hours at room temperature and dip washed.

*Sequencing*

The wildtype sequence of F32A11.3 was obtained from the online bioinformatics site Wormbase. The sequence allowed us to generate primer sets to sequence the gene and search for mutations in F32A11.3 in the *spe-7* line. We amplified sections of the gene
through PCR reaction for the sequencing reaction. F32A11.3 is 1kb, and because sequencing reads on PCR fragments often only yields accurate reads of 500 base pairs, we divided the gene into three 500bp overlapping sections to amplify by PCR reaction independently. Amplifying shorter sections allowed for accurate reads, with the overlapping allowing us to check regions of the gene on independent amplicons. We also generated primer sets slightly inset from the main primers to allow a more accurate sequencing read toward the middle of each amplicon if needed.

Since we were hunting for a likely point mutation, we required highly accurate readings. We used a high fidelity *taq* polymerase kit with proofreading function to ensure the amplified product accurately reflected the gene sequence in *spe-7* mutant. Bands with high enough concentrations for sequencing were produced using a PCR reaction with 2 μM Mg+ and 5 μliters of BSA. Amplicons were obtained for sequencing by gel purification with the Qiagen® kit. 20-100 nanograms of PCR product are required for a successful sequencing reaction using DNA from a PCR reaction. The most accurate and convenient way to determine the PCR product concentration is with the NanoDrop®. Because a limited amount of volume is allowed for the PCR template during the PCR reaction, more concentrated samples give more freedom to control the final mass of DNA for the sequencing reaction. If the PCR reaction is giving low yields, resuspend the DNA in the least amount of Elution Buffer possible to increase concentration in the final solution. If the concentration is still insufficient, a speed vac can help raise the concentration of a sample if the PCR yield is low or drops during the gel purification step. Before using this technique, resuspend the DNA sample in clean water rather then Elution Buffer, as EB contains ETDA to help preserve samples. The ETDA concentration
will increase along with the DNA if suspended in EB during the speed vac. Elevated
ETDA levels can damage the DNA, thus clouding the sequencing result. Additionally,
the process of speed vacing can denature DNA molecules, which would also interfere
with clean results. Use these methods gingerly, and only as a last resort.

The sequencing reaction was performed by The Molecular Core Facility at the
College of William and Mary.

All primers were diluted to 100 μM:

**MARCP1:**
Left: aatctgacaagaagggggtcc
Right: ggagcaaccttgggtttttgc (forward)

gcaaaagccaaagtggctcc (reverse complimentary)

amplicon length: 864
annealing temperature: 53°C
elelongation: 55 seconds

**MARCPA:**
Left: atctggttgaccggcatga

**MARCPB:**
Right: caatgtactcggactggctgg (forward)

**MARCP2:**
Left: gcaggagagaagctgggaat
Right: ttcaccttcggacatccaccact

agttgcgtggatgtggaagagaa (reverse complimentary)

amplicon length: 992
annealing temperature: 51°C
el elongation: 55 seconds

**MARCPB:**
Left: cttatacaattttggccgg (forward version of MarcP3R)

**MARCPF:**
RNAi

The F32A11.3 RNAi construct was provided generously by Dr. Aurora Esquela-Kerscher’s lab at Eastern Virginia Medical School. We streaked out a sample of the *E. coli* on ampicillin plates and picked the several colonies to build frozen stocks. The colonies containing the plasmid vector enabling F32A11.3 production transfers ampicillin resistance, so picking colonies that can grow on amp plates should select for cultures that contain the required vector. To prepare for the RNAi experiment, we soaked tiny agar plates with 22µL of the transcription activator IPTG solution overnight. We then seeded the plates with 125µL of fresh overnight culture grown from frozen stocks. The IPTG “activates” the RNAi construct production, encouraging the synthesis of double stranded
mRNA of the F32A11.3 transcript. We placed L4 hermaphrodites on the bacterial bed the next morning and allowed them to lay embryos for 24 hours. The parental generation was killed and the F1 allowed to mature to the L4 larval stage, where we separated males and hermaphrodites by transferring to plates with freshly RNAi activated E. Coli beds. After 24-48 hours at room temperature, the worms were dissected for study. The double stranded RNA constructs become absorbed into the C. elegans tissue as they feed on the bacteria. The double stranded RNA triggers the degradation of endogenous mRNA transcripts, effectively silencing the protein product through translational knockdown.
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