SPE-7, a Novel Regulator of MSP Assembly in C elegans Spermatocytes

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SPE-7, A Novel Regulator of MSP Assembly in *C. elegans* Spermatocytes

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of the College of William and Mary in Candidacy for the Degree of
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Through the process of spermatogenesis, bulky, undifferentiated spermatocytes transform into fully functional, differentiated haploid spermatozoa. In C. elegans, the transcriptional and translational constraints that accompany spermatogenesis require that any protein required either for spermatogenesis or the subsequent functioning of the mature spermatozoon must be synthesized in developing spermatocytes. As a result, many proteins required for later functions are sequestered in a specialized organelle complex known as the fibrous body-membranous organelle. Mutation of spe-7 results in sperm-specific sterility as a consequence of cell cycle progression and fibrous body defects and is associated with a spermatocyte arrest phenotype. Here we report the identification of spe-7 as a novel regulator of fibrous body assembly. Expression of spe-7 occurs specifically during spermatogenesis. The SPE-7 protein initially localizes to fibrous bodies in developing spermatocytes, but rapidly disappears from spermatids following budding. Analysis of SPE-7 dynamics in mutants with defects in the developmental program of spermatogenesis reveals that SPE-7 disappearance requires the physical separation of spermatid and residual body as well as the intracellular partitioning of fibrous bodies and other cellular components to spermatids. SPE-7 disappearance does not require disassociation of the fibrous body-membranous organelle complex or progression of the meiotic cell cycle beyond metaphase I. Our findings indicate that SPE-7 acts as a scaffolding protein in the initial assembly of the fibrous body and suggest a model in which SPE-7 must be actively degraded in spermatids to prevent defects in fibrous body disassembly and subsequent pseudopod formation.
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Introduction

Both sperm and oocytes are essential to reconstitute a diploid genome; yet the two are morphologically and functionally different from one another. In contrast to large, nutrient-rich, sessile oocytes; sperm are small, highly differentiated, motile cells that must travel to waiting oocytes in order to contribute genetically to the next generation. The formation of functional sperm requires a drastic transformation of undifferentiated germ cells into specialized, motile spermatozoa. This transformation is achieved through the distinct yet presumably coordinated programs of meiotic cell cycle progression and the cellular differentiation program of spermatogenesis.

The fundamental processes of spermatogenesis are conserved across multiple phyla with high levels of gene conservation even amongst very different species (Bonilla and Xu, 2008). The development of sperm can be divided into two parts: spermatogenesis, or the production of haploid spermatids, and spermiogenesis, the transformation of spermatids into mature spermatozoa. Sperm production is maintained throughout adulthood via a germline stem cell population that resides within a stem cell microenvironment called the stem cell niche (for review see White-Cooper and Bausek, 2010). Asymmetric divisions of spermatogonial stem cells result in uncommitted germ cells that undergo limited mitotic proliferation before commitment to the spermatogenic differentiation pathway to become a spermatocyte. The transition from uncommitted germ cell to spermatocyte involves coordination of two key processes: 1) exit from the mitotic cell cycle and simultaneous entry into the meiotic cell cycle and 2) commitment to the differentiation pathway of spermatogenesis. Upon meiotic entry, a final round of
DNA replication is followed by an extended meiotic prophase and the accompanying molecular events of chromosome pairing, synapsis, recombination, and transcription of sperm-specific genes. Following the meiotic divisions, haploid spermatids undergo the dramatic morphological changes of spermiogenesis before transforming into mature spermatozoa.

The nematode *C. elegans* is an ideal model for the study of spermatogenesis. Both sexes, male and hermaphrodite, have large gonads relative to their adult body mass and employ spermatogenesis at some stage during development. The gonad is temporally and spatially arranged with gamete formation occurring in a linear fashion along the length of the gonad (Seydoux and Schedl, 2001; Figure 1). As such, germ cells can be accurately staged based solely on chromatin morphology and location within distinct zones of the gonad.
Figure 1. The progression of germ cell nuclei during gamete formation in *C. elegans*. DAPI-stained nuclei in isolated, methanol-fixed wildtype male gonads. Regions of the gonad are labeled: mitotic (green), transition (yellow), pachytene (red), condensation (blue), meiotic division zone (orange), and post-meiotic haploid spermatids (white). Scale bar=20 μm.

**Comparison of spermatogenesis in *C. elegans* and mammals**

Through comparison and contrast of nematode spermatogenesis and the typified mammalian pathway of spermatogenesis, we attempt to illustrate that the most basic processes of spermatogenesis are maintained with nematodes having evolved a much more efficient method of sperm production (Figure 2).

In all spermatogenesis programs, extensive chromatin modification is required both before and after the two meiotic divisions. However while fully differentiated sperm in both mammals and nematodes share a common characteristic of having hypercondensed, highly modified, and transcriptionally quiescent chromatin, the spermatogenesis program of mammals and nematodes differ in the point at which transcription ceases relative to the meiotic divisions. In mammals, transcriptional down-regulation occurs post-meiotically and requires the incorporation of sperm-specific nuclear basic proteins (SNBPs) (Sassone-Corsi, 2002; Tanaka and Baba, 2005). Following meiosis, somatic histones are replaced first by sperm-specific histone variants, then by transition proteins, and ultimately by protamines (Braun, 2001; Govin et al., 2004; Kimmins and Sassone-Corsi, 2005). As chromatin-remodeling factors are incorporated in a stepwise fashion, chromatin becomes increasingly compacted. Conversely in *C. elegans*, transcriptional down-regulation occurs prior to the meiotic divisions and requires incorporation of SNBPs (Chu et al., 2006). In *C. elegans*, there is
an additional level of chromatin remodeling, the karyosome stage, that occurs immediately prior to the meiotic divisions (Shakes et al., 2009). During this karyosome stage chromosomes aggregate into a compact mass within a membrane bound nucleus and transcription is globally down regulated. Karyosome formation was first described in *Drosophila melanogaster* and is more typically a typical feature of oogenesis (Gruzova and Parfenov, 1993). In contrast, karyosome formation in *C. elegans* is a sperm-specific feature (Shakes et al., 2009). In short, because transcription halts before the karyosome stage and post-meiotic transcription is absent, proteins required throughout spermatogenesis and spermiogenesis must be transcribed before the meiotic divisions.

As spermatids engage in spermiogenesis, excess cytoplasm and extracellular components are discarded into cellular “waste bins” called residual bodies that facilitate the morphological changes required to become a spermatozoon (Roosen-Runge, 1977; Figure 2). This jettisoning of cytoplasm and non-essential organelles is a shared feature of both mammalian and nematode spermatogenesis, yet the two systems differ in relative timing of the event and the components discarded. In mammals, post-meiotic transcription and translation of proteins required for spermiogenesis and sperm function influence the timing of residual body formation with protein translation ceasing prior to activation of spermatozoa. Upon the completion of spermiogenesis, streamlined spermatozoa contain the bare minimum (a nucleus, centriole, and mitochondria) required to find and fertilize an oocyte. In *C. elegans*, not only is transcription downregulated before the meiotic divisions, but the translational machinery is also discarded into residual bodies immediately after anaphase II (Ward, 1981) eliminating any post-meiotic
translation that may facilitate spermiogenesis. As a result, translation of proteins occurs prior to residual body formation. Additionally the typical cytoskeletal components, actin and tubulin, are discarded into residual bodies (Ward, 1981).

Vertebrate spermatozoa are flagellated and, following the meiotic divisions, spermatids undergo extensive cytoskeletal changes to remodel tubulin into basal bodies that later develop into the inner core of the flagella. In contrast, *C. elegans* spermatozoa acquire motility via a pseudopod. While polymerization-based movement is typically a result of actin-based processes, *C. elegans* spermatozoa utilize another motility protein, the major sperm protein (MSP), for cell motility.
Figure 2. Comparison of vertebrate and C. elegans spermatogenesis. In both vertebrate and C. elegans spermatogenesis, the first and second meiotic divisions are typically accompanied by incomplete cytokinesis events resulting in syncytial secondary spermatocytes and haploid spermatids. Spermiogenesis in vertebrates initiates as spermatids bud from the syncytium. Post-meiotic transcription and translation facilitates the extensive cytoskeletal and cytoplasmic remodeling required for the transformation from spermatid to spermatozoon. Excess cytoplasm resulting from this transformation is discarded into residual bodies immediately prior to activation. Following spermiogenesis, spermatozoa mature and achieve motility via a flagellum. In C. elegans spermatogenesis, spermiogenesis begins immediately following anaphase II as cellular materials including ribosomes that are unneeded in the spermatozoa are partitioned to the residual body.

The details of C. elegans spermatogenesis

Spermatogenesis begins in the immediate vicinity of the distal tip cell, a somatic cell that serves as the stem cell niche. In males, the distal tip cell actually refers to two cells positioned at the distal end of a single armed gonad (Kimble and Crittenden, 2007; Byrd and Kimble, 2009). The distal tip cell proximally maintains the undifferentiated state of uncommitted germ cells by promoting mitotic cell division and inhibiting the meiotic program. As uncommitted germ cells move away from the distal tip cell, they transition into meiosis.

The cytological changes that accompany sperm development in C. elegans have been described in detail (Ward et al., 1981; Wolf et al., 1981; Ward, 1986; Kimble and Ward, 1988; Figure 3). The 4N primary spermatocyte initially develops in syncytium with a cytoplasmic core called the rachis. With entry into the division zone, the primary spermatocyte detaches from the rachis and develops independently for the remainder of spermatogenesis. During the first meiotic division, cytokinesis is often incomplete resulting in spermatocytes connected by cytoplasmic bridges. Following meiosis II, C. elegans spermatids undergo one asymmetric partitioning event during which spermatids
bud from a central anucleate residual body. The residual body contains cytoplasm and typical cellular components not required in mature spermatids including most voltage-gated ion channels, ribosomes, lysosomes, the Golgi apparatus, and the endoplasmic reticulum as well as components not typically discarded including tubulin and actin (Ward et al., 1986; Machacha et al., 1996). Spermatids retain centrioles, mitochondria, and sperm-specific organelles unique to nematode spermatogenesis.

Figure 3. Development of *C. elegans* spermatocytes. Spermatocytes begin to detach from the rachis by the karyosome stage (k) and are fully detached by diakinesis (d). The division zone is represented both schematically and with DIC-Hoechst as follows: ml (metaphase I), al (anaphase I), mII (metaphase II), all (anaphase II), budding figure, and spermatozoa. Scale bar=10μm.

As translation stops with formation of the karyosome and translation ends immediately after anaphase II, proteins required for spermiogenesis and in mature spermatozoa are transcribed before the meiotic divisions, and translated before residual
body formation. During the meiotic divisions, such proteins are sequestered in a non-functional state within the fibrous-body membranous organelle complex or FB-MO. The FB-MO is a transient organelle that develops before the first meiotic division (Wolf et al., 1978; Ward et al., 1981; Figure 4). Reviews suggest that the FB is a paracrystal comprised solely of major sperm protein (MSP) filaments (L'Hernault, 2006; L'Hernault, 2009; Nishimura and L'Hernault, 2010). However our results suggest that FBs contain additional proteins. The MO is a Golgi-derived organelle with three distinct regions: a vesicular head enriched with glycoproteins, a body made of membranous arms that enfold the FB, and an electron-dense collar which separates the head from the body (Roberts et al., 1986). In developing spermatocytes, the FB and MO are intimately associated; however as spermatids separate from their respective residual bodies, FB-MO complexes disassociate after having delivered the sperm-specific proteins to spermatids. Individual MOs then dock but do not yet fuse with the plasma membrane. During sperm activation, MOs fuse with the plasma membrane releasing the glycoproteins contained within the MO head into the extracellular space.
Figure 4. FB-MO morphology during spermatogenesis. Represented by 1° insert, the FB-MO is composed of a head region (A), an electron-dense collar (B), and an MSP-enriched FB paracrystal. MSP assembly into FBs occurs in association with the MO in primary spermatocytes. The FB expands in size through the meiotic divisions where it is largest in secondary spermatocytes. FB-MOs are partitioned into spermatids where the complex disassociates as spermatids mature. During spermatid maturation, MOs dock at the plasma membrane, FBs are completely disassembled, and MSP distributes throughout the cytosol. During sperm activation, MOs fuse with the plasma membrane and cytosolic MSP polarizes to the pseudopod whereupon polymerization factors regulate its assembly/disassembly into filaments.

The FB-MO complex is critical to successful spermatogenesis as it 1) sequesters MSP during the period of sperm development when MSP has no functional role and 2) ensures that sperm-specific proteins required for spermiogenesis and/or sperm function are properly partitioned into spermatids. In spe-6 mutant spermatocytes that fail to properly sequester MSP into FBs, spermatocytes arrest early with defects in chromosome segregation and residual body formation (Varkey et al., 1993) suggesting that, when unassembled into FBs, cytosolic MSP can interfere with normal cell cycle dynamics.

Proper FB assembly requires at least three regulatory factors: SPE-6, SPE-44, and SPE-7. SPE-6 is a member of the casein kinase I superfamily that, in addition to other roles in meiotic cell cycle progression and spermiogenesis, is required for assembly of MSP into FBs (Varkey et al., 1993; Mulhrad and Ward, 2002). SPE-44 is a transcription factor necessary for the expression of hundreds of genes roughly equating to one-third of all sperm-specific genes (Kulkarni et al., 2012). One of its direct downstream targets is the novel gene spe-7, which is the focus of this thesis.
Ensuring that MSP arrives in spermatids is the role of the FB-MO complex; but once partitioned into spermatids, individual FBs must be promptly disassembled releasing MSP allowing for its reassembly into filaments within the pseudopod of activated spermatozoa. While complete disassembly is not essential to motility, mutants with defects in FB disassembly have not only small, aberrant pseudopods but also defects in motility and/or fertilization (Ward et al., 1981). To date, two PP1 phosphatases are known to be required for FB disassembly, GSP-3 and GSP-4 (Wu et al., 2012).

The multi-faceted MSP protein

MSP is a small (14 kDa) but highly abundant protein that comprises 15% of total and 40% of soluble protein in *C. elegans* spermatids (Klass and Hirsh, 1982; Ward and Klass, 1982; Roberts et al., 1986). MSP is highly conserved among nematodes and is encoded by a multigene family (Burke and Ward, 1983; Scott et al., 1989; Smith, 2006). While MSP functions similarly to actin as the motive force in amoeboid spermatozoa, it bears no sequence homology or structural similarity to actin (King et al., 1992; Bullock et al., 1996) and is specifically expressed only during spermatogenesis (Klass and Hirsh, 1981). MSP can self-assemble into filaments with distinct structural and polymerization properties throughout spermatogenesis (Roberts and Stewart, 2000). Monomeric MSP readily associates into dimers that form an Ig-fold (King et al., 1992; Bullock et al., 1996). These dimers assemble into two-stranded polymers whose subunits are constructed from two loosely connected helical subfilaments (Stewart et al., 1994). With regards to motility, these MSP subfilaments assemble into macrofibers that when linked
to other macrofibers perform as a single unit to advance the leading edge (Seppesenwol et al., 1989). In contrast to actin, MSP does not associate with nucleotides or motor proteins and MSP is incapable of forming polar filaments in the absence of external factors (Haaf et al., 1996; Italiano et al., 1996).

pH plays a significant role in the assembly of various MSP structures. The assembly of MSP into FBs, disassembly of FBs into individual MSP dimers, and reassembly of MSP into filaments that occurs during spermatogenesis is accompanied by changes in intracellular pH (King et al., 1994). The assembly of MSP into FBs within spermatocytes occurs in the relatively high pH environment of 6.8. The disassembly of FBs into MSP dimers occurs in spermatids coincident with a decrease in pH to 6.2. A rapid increase to pH 6.4 in spermatozoa results in the reassembly of MSP dimers into MSP filaments.

In contrast to what is known about the assembly of MSP within the pseudopod, the structure and assembly of MSP within FBs is not well understood. Early ultrastructural studies of C. elegans spermatocytes via TEM depict the fibrous body as a ‘dark and striated aggregate of microfilaments’ contained within a double-layered membrane (the MO) (Wolf et al., 1978). Ward et al. (1981) later described ‘crystalline inclusions’ in a subset of fertilization-defective mutants and suggested MSP as the structural component. Ward and Klass (1982) went on to show the FB as a crystalline inclusion comprised of MSP. Further analysis of MSP revealed that when assembled into FBs, MSP is organized as an array of parallel fibers 4-5 nm in diameter that is partially enclosed by the MO (Kimble and Ward, 1988). The terms crystalline inclusion and
paracrystal suggest high local concentrations of one protein and it has generally been presumed that the FB is comprised solely of MSP. As mutants with defects in FB assembly are identified, we are beginning to better understand the assembly of MSP into FBs.

MSP is not only a structural protein required only for locomotion, but it also acts as an extracellular signaling molecule for oocyte maturation and egg-laying rate (Miller et al., 2001). Through activation of the MAP kinase cascade, MSP signaling activates both resumption of the meiotic cell cycle in oocytes as well as contraction of sheath cells, the smooth muscle-like cells of the somatic gonad that regulate ovulation.

The acquisition of motility via MSP

Intracellular pH is a major regulatory component of fiber assembly in the pseudopod of spermatozoa; however, a change in pH alone is insufficient to account for the precise control of MSP filament formation throughout development. *In vivo* and *in vitro* filament assembly occurs in association with membranes/membrane proteins, either with the MOs in spermatocytes or at the plasma membrane in activated spermatids and spermatozoa. This observation suggests that association with membrane proteins adds an additional level of complexity to the regulation of MSP filament assembly.

Extensive biochemical studies in the nematode *Ascaris suum* have elucidated many of the external factors required to modulate the assembly and disassembly of MSP fibers at the leading edge. Key to the assembly process is the phosphorylated membrane protein MPOP. MPOP recruits MPAK (MSP polymerization-activating kinase), a ser/thr
kinase, to the vesicle surface generating the localized polymerization required for advancement of the plasma membrane (LeClaire et al., 2003; Yi et al., 2007). MPAK in turn phosphorylates MFP2 (MSP fiber protein 2). Phosphorylated MFP2 can then bind MSP accelerating the assembly of MSP filaments (Buttery et al., 2003; Grant et al., 2005). In contrast, MFP1 decreases the fiber growth rate by reducing the number of filaments formed at the vesicle surface (Buttery et al., 2003). A third factor, MFP3, binds to assembled MSP filaments. In its phosphorylated state, MFP3 stabilizes newly formed filaments, whereas dephosphorylation of MFP3 by a PP2A phosphatase localized near the cell body results in localized filament disassembly and pseudopod contraction (Yi et al., 2009).

The novel gene spe-7

spe-7(mn252) was originally isolated by Bob Herman (unpublished) and mapped to the right arm of chromosome II by Diane Shakes and Steve L’Hernault (Shakes PhD thesis, 1989). spe-7 encodes a protein required for successful spermatogenesis. spe-7 (mn252) mutants exhibit simultaneous arrests of the cell cycle and developmental programs of spermatogenesis (M. Presler Honors Thesis, 2010). Presler showed that spe-7 mutants exhibit different phenotypes depending on the temperature at which they are grown (Figure 5). spe-7 mutants raised at 16°C exhibit the most severe phenotype; mutant spermatocytes arrest prior to anaphase I with both chromatin and cytological defects. At 20°C, spe-7 spermatocytes arrest with an anaphase I-like separation. At 25°C, mutant spermatocytes arrest with an anaphase II-like separation but most fail to undergo either cytokinesis or the post-meiotic budding division and thus arrest as terminal
spermatocytes with 3-4 haploid (potentially aneuploid) chromatin masses. On occasion, self-fertilizing spe-7 hermaphrodites that have been raised at 25°C, produce as many as eight progeny revealing that the few sperm produced are normal.

Figure 5. DIC-Hoechst of wildtype and spe-7 mutant spermatocytes. As compared to wildtype spermatocytes, spe-7 mutants at different temperatures exhibit distinct cell cycle arrests. Representative cells of normal and aberrant stages are identified as follows: (k) karyosome, (ml) metaphase I, (al) anaphase I, (all) anaphase II, (rb) residual body, (dots) haploid chromatin masses, and (s) spermatids. While 20°C mutants typically arrest at anaphase I, few spermatocytes progress beyond this stage and are represented by M2* for
aberrant meiosis II. 25°C spermatocytes arrest with multiple chromatin dots. Scale bar=20 μm.

Using SNP (single nucleotide polymorphism)-mapping, the spe-7 locus was narrowed to a 2.5 map unit interval between +14.49 and +16.99, an interval containing 43 genes (Presler Honors Thesis, 2010). Of these 43 genes, a smaller subset was identified as spermatogenesis-specific by microarray (Reinke et al., 2000). Of these, F32A11.3 was the most promising candidate based on its genetic map position. F32A11.3 was PCR amplified and sequenced from spe-7(mn252) genomic DNA and it was determined that in spe-7(mn252) mutants, F32A11.3 has a C/T point mutation at nucleotide 1681 resulting in a pre-mature stop codon (Figure 6). F32A11.3 is a novel C. elegans gene, but homologs are present in other nematode species. In follow-up studies, RNAi knockdown of F32A11.3 resulted in the same phenotype as spe-7 mutants confirming the molecular identity of spe-7. This RNAi result also suggested that the truncation of spe-7 results in a loss-of-function rather than a neomorphic phenotype.
Figure 6. spe-7(mn252) encodes a premature stop codon. Wildtype spe-7 encodes CAA which corresponds to a glutamine residue at position 301 as represented by red ‘Q’. However, in spe-7 mutants, a C/T point mutation results in TAA or stop codon, effectively removing the c-terminus. The full length SPE-7 protein has a predicted molecular weight of 37 kDa compared to the truncated SPE-7 protein which, if stable, has a predicted molecular weight of 33 kDa.

An affinity purified, polyclonal antibody generated against a predicted antigenic SPE-7 peptide was used to determine where SPE-7 functions within the cell (Figure 6). Initial immunolocalization studies by Presler suggested that SPE-7 is detected during late pachytene consistent with other spermatogenesis-specific proteins (2010). SPE-7 localization was shown to be very dynamic, moving from a punctate to diffuse pattern during metaphase I and returning to the same punctate pattern during anaphase I. In addition, SPE-7 was transiently observed in budding spermatids before becoming undetectable.
Specific Aims of Thesis

The objective of this thesis is to further characterize the SPE-7 protein, its direct role in proper fibrous body assembly and its indirect role in proper cell cycle progression. Through immunocytological studies, we address the subcellular localization of SPE-7 to fibrous body complexes throughout spermatogenesis, the relationship of SPE-7 dynamics and proper cell cycle progression, and factors that contribute to wildtype SPE-7 dynamics. With a biochemical approach we address the disappearance of SPE-7 and potential modifications to the SPE-7 protein that may facilitate its dynamic localization.

This study adds new details to our understanding of the wildtype pathway of nematode spermatogenesis. First, we show that not only is the fibrous body not exclusively composed of MSP as previously assumed, but that in addition to localizing to fibrous bodies the novel protein SPE-7 is required for its assembly. Second, the role of SPE-7 in fibrous body assembly appears to be regulated by the kinase SPE-6 thus identifying another potential target and role of SPE-6 in spermatogenesis. Lastly, we report for the first time the presence of proteasomes in *C. elegans* spermatids providing a possible mechanism for SPE-7 disappearance.
Materials and Methods

Strains and Genetics

With the exception of spe-7(mn252), gsp-3(tm1647) gsp-4(y418), and spe-44(ok1400), C. elegans strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota) and are derived from the wild-type isolate N2 (Bristol). Strains used include: CB1489 him-8(e1489), CB4108 fog-2(q71), JK816 fem-3(q20gf), RV110 uba-1(it129), SL754 spe-39(eb9)/nT1 [unc-?(n754) let-?], BA782 spe-10(hc104) him-5(e1490), BA606 spe-6(hc49) unc-25(e156); eDp6, DS175 unc-4(e120)spe-7(mn252);him-8(e1489)/mnc1, SL48 dpy-5(e61) spe-4(q347)/sDf5, XC26 gsp-3(tm1647) gsp-4(y418)/hT2[bli-4(e937) let-?(q782) qIs48]; him-8(e1489) from Diana Chu, RV120 spe-44(ok1400) dpy-20(e1282)/let-92(s677) unc-22(s7) from Harold Smith.

Nematode strains were maintained on MYOB plates (Church et al., 1995) seeded with E. coli strain OP50 or NA22 and cultured at 20°C unless otherwise noted. Genetic manipulations were carried out as described by Brenner (1974).

Worm Maintenance

Worms were isolated one of two ways: either through bleaching or daily transfers. For bleaching, gravid adult hermaphrodites were washed from plates with M9 buffer and subjected to 5 minute bleaching to isolate embryos (4 parts 0.625 M potassium hydroxide: 1 part bleach). Washed embryos hatched on unseeded plates and were then transferred to seeded plates as L1 larvae. For isolation of males through daily transfers, 8-10 hermaphrodites were allowed to lay embryos for 24 hours after which point they were
moved to a new seeded plate. The resulting embryos were then incubated at the appropriate temperature for the specific strain and experiment.

For all wildtype controls, *him-8* (*e1489*) males were used. For mutant strains, males were obtained from *him-8* double mutants or heterozygote *him-8* males backcrossed to homozygous mutant hermaphrodites.

For temperature sensitive alleles, embryos were isolated through bleaching or daily transfers. Isolated embryos were then shifted to the restrictive temperature, usually 25°C, and worms were collected as adults.

To ensure an abundance of intact residual bodies rather than cellular debris in studies with *spe-10* (*hc104*) mutant males, L4 males were isolated and collected 12-24 hours later. Collection after 48 hours yielded predominately cytoplasts with very few residual bodies.

**Immunostaining and Microscopy**

Male gonads were dissected in 5-10 microliters of sperm media (50 mM HEPES, 25 mM KCl, 1 mM MgSO₄, 45 mM NaCl, and 5 mM CaCl₂, pH 7.8) on ColorFrost Plus slides (Fisher Scientific) with 1:100 levamisole dilution using established antibody staining protocols (Miller and Shakes, 1995). A 24X60 coverslip with four corner dots of silicone grease was placed over isolated gonads. The prepared slides were then freeze cracked in liquid nitrogen. Following freezing, coverslips were removed and slides were placed in cold methanol overnight at -20°C. Slides were washed with three consecutive five-minute washes in 1X PBS followed by a thirty-minute room temperature incubation
in blocking solution (PBS+0.5% BSA, 0.1% Tween 20, and 0.04% Azide). Primary and secondary incubations were conducted at room temperature in a humid chamber. After incubation in various antibodies, slides were prepared with either GelMount (Biomedia corp.) or Fluoro-Gel II mounting media (Electron Microscopy Sciences), both with DAPI, as a combined mounting and anti-fade media. Following application of mounting media, slides were allowed to settle overnight at 4°C before sealing with nail polish.

For DIC/Hoechst preparations, we studied live cells using the lipid soluble Hoechst 33342 (Sigma-Aldrich) at 100 mg/ml to stain chromatin.

Images were acquired with an Olympus BX60 fluorescence microscope equipped with either a Cooke Sensicam cooled CCD camera or EXi Aqua camera (QImaging). When required, images were analyzed with iVision or ImageJ (www.nih.gov) and minimally enhanced with Photoshop.

**Antibodies**

The affinity purified anti-SPE-7 polyclonal antibody was raised in rabbits against amino acids 266-279 of SPE-7 (Yenzyme; Figure 6). Antibody specificity was confirmed by Western blot of spe-7 mutant animals. Samples were incubated for two hours at room temperature with a 1:1250 dilution of primary antibody. Following primary, samples were washed for four minutes in PBS. Slides were then incubated at room temperature for two hours in TRITC-conjugated goat-anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories) at either 1:50 or 1:100 dilution.
The monoclonal anti-MSP (clone 4D5) antibody was provided by David Greenstein. Samples were incubated with dilutions ranging from 1:600-1:800 for two hours at room temperature. Slides were washed for three minutes before addition of secondary antibody. FITC- or DyLight-conjugated goat-anti-mouse secondary antibody (Jackson Immunoresearch Laboratories) was used at either 1:50 or 1:100 dilutions. Samples were washed for three minutes following secondary incubation.

The monoclonal antibody 1CB4 (Okamoto and Thomson, 1985), provided by Steve L’Hernault, was used to label the MO in nematode spermatocytes. Samples were incubated with a 1:50 dilution and incubated for two hours at room temperature followed by a 1-minute wash in 1X PBS. Samples were incubated in 1:50-1:100 dilution of goat-anti-mouse secondary antibody for two hours at room temperature. Before mounting, slides were dip washed in 1X PBS.

The monoclonal antibody PAS-7 (Hadwiger et al., 2010; Developmental Hybridoma Center, University of Iowa) was used to label 20S proteasomal subunits. Samples were incubated in a 1:20 dilution overnight at 4°C followed by a 3-minute wash in 1X PBS. Samples were then incubated in 1:50-1:100 dilution of Dylight-conjugated goat-anti-mouse secondary antibody for two hours at room temperature. Following secondary, slides were washed for 3-minutes in 1X PBS.

**Worm Collection for Western Blot**

For Western blot analysis, 50-100 worms were collected in 15-25 µl of M9 buffer contained within the cap of a 1.5 µl Eppendorf tube. Tubes were centrifuged for 1 minute
at 15,000 relative centrifugal force (rcf), immediately frozen in liquid nitrogen, and
stored at -80°C.

**Large Scale Worm Growth for Immunoprecipitations**

To obtain age-synchronized populations, *fem-3(q20gf)* were allowed to grow on
MYOB plates enriched with peptone seeded with *E. coli* (NA22) for four days at the
permissive temperature of 16°C and collected as gravid adults. Plates were washed with
M9 buffer and bleached to isolate embryos. Embryos were allowed to hatch overnight on
unseeded plates at 16°C and starved L1 larvae were shifted to the non-permissive
temperature of 25°C and collected as adults. Adults were washed off plates with M9
buffer, pelleted, and dropped via Pasteur pipette into liquid nitrogen. Samples were stored
at -80°C. This method typically yielded 1-2 mls of pelleted worms.

**Western blotting and Immunoprecipitations**

Western blot analysis was performed on worm lysates obtained from one freeze-
thaw cycle, homogenized with 4:100 beta-mercaptoethanol (MP Biomedicals) to sample
buffer (NuPAGE® LDS 4X Sample Buffer, Invitrogen) heated to 100°C, boiled for 5
minutes, and centrifuged for 8 minutes at 15,000 rcf. Lysates from 50-100 worms were
loaded per lane, proteins were resolved at 150V via SDS-PAGE (NuPAGE® Novex 4-
12% Bis-Tris; Invitrogen), and transferred to a polyvinylidene difluoride membrane (GE
Healthcare). After blocking overnight with Tris-buffered saline with Tween 20 (TBST;
1M Tris HCL pH 8.0, 5M NaCl, 0.1 % Tween 20) containing either 4% non-fat dry milk
(Carnation) or 5% bovine serum albumin (Sigma-Aldrich), membranes were incubated with appropriate primary antibody diluted in blocking buffer (4% milk or 5% BSA in 1X TBST) for two hours at room temperature, incubated with 1:20000 peroxidase-conjugated secondary antibody (Abcam) for two hours at room temperature and developed by enhanced chemiluminescence (Immobilon™ Western Chemiluminescent HRP substrate, Millipore). SPE-7 protein was detected by a 1:5000 dilution of rabbit anti-SPE-7 polyclonal antibody (1°; Yenzyme) followed by a 1:20000 dilution HRP-conjugated goat-anti-rabbit IgG polyclonal antibody (2°; Abcam product # ab6721). MSP was detected by a 1:10000 dilution of mouse anti-MSP monoclonal antibody 4A5 (1°; Kosinski et al., 2005) followed by a 1:20000 dilution of HRP-conjugated goat-anti-mouse IgG polyclonal antibody (2°; Abcam product # ab6789). In some cases, membranes were stripped and reprobed with another antibody.

For immunoprecipitations, 1-2 ml fem-3(gf) worm pellets were homogenized by grinding in liquid nitrogen with mortar and pestle. Following homogenization, samples were incubated in 40-50 μl lysis buffer B70 (Jedamzik and Eckmann, 2009; 0.1% Triton X-100, 5 mM MgOAc, 1 mM NaF, 70 mM KAc, 50 mM Hapes, 10% glycerol, 2 mM Na3VO4). The lysates were pre-cleared using 40 μl protein A-coupled sepharose beads (nProtein A Sepharose™ 4 Fast Flow; GE Healthcare) on ice for 30 minutes and then incubated with 5 mg of anti-SPE-7 antibody on ice for 2 hours. The beads were harvested by centrifugation (4°C) at 15,000 rcf for 10 minutes and washed three times with buffer B70. Proteins were then eluted with 40-50 μl NuPAGE® LDS Sample Buffer, pH 8.4 (Invitrogen).
Experimental Replicates

All conclusions were based on experimental replicates of 3 or more. Experimental conclusions were based on numbers of gonads scored represented in the following table:

<table>
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<th>Antibody</th>
<th>Genetic Background</th>
<th>Gonads Scored (n)</th>
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<td>1CB4 &amp; anti-SPE-7</td>
<td>him-8</td>
<td>&gt;80</td>
</tr>
<tr>
<td></td>
<td>spe-4</td>
<td>&gt;60</td>
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<tr>
<td></td>
<td>spe-39</td>
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<td>30-40</td>
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Table 1. Experimental gonads scored.
Results

*spe-7(mn252)* represents a null allele.

To confirm specificity of the anti-SPE-7 antibody, we used indirect immunofluorescence and Western blots to compare SPE-7 localization patterns in wildtype and mutant backgrounds (Figure 7A). As the antigenic site of the SPE-7 antibody is located before the truncation, anti-SPE-7 should recognize both full-length and truncated forms of the SPE-7 protein. Comparison of immunostained wildtype and *spe-7(mn252)* mutant male gonads reveals that the full-length SPE-7 protein is present in wildtype gonads only. From this immunocytological study we know that SPE-7 does not localize to the chromatin but in a punctate, cytosolic pattern and that SPE-7 is noticeably absent from spermatids.

To show that SPE-7 is present in sperm-producing gonads only, we collected wildtype males and *fog-2(1f)* hermaphrodites for Western blot analysis. FOG-2 is required for hermaphrodite spermatogenesis and loss of function mutations in *fog-2* transform XX hermaphrodites into females (Schedl and Kimble, 1988). As *fog-2* hermaphrodites do not undergo spermatogenesis, we would expect no SPE-7 signal in this mutant. We probed whole worm lysates from both wildtype and mutant backgrounds with anti-SPE-7 via Western blot and found no SPE-7 signal in *fog-2* hermaphrodites (Figure 7B).

To confirm that our antibody specifically targets the SPE-7 protein, we needed to show the lack of a SPE-7 band in the *spe-7* mutant. As previously mentioned, Presler was able to phenocopy the *spe-7(mn252)* phenotype with RNAi suggesting that this allele is null. Therefore, we expect to see neither full-length or truncated SPE-7 in a *spe-7(mn252)*
background. Additionally, from previous immunocytological analyses we know that full-length SPE-7 is present in wildtype males but not spe-7(mn252) mutants. We chose to investigate the presence of SPE-7 not only in spe-7 mutants, but in spe-44(ok1400) mutants also (Figure 7). SPE-44 is a sperm-specific transcription factor required for spe-7 expression (Kulkarni et al., 2012). Western blot analysis of wildtype, spe-7, and spe-44 mutant males with anti-SPE-7 revealed a band of higher than predicted molecular weight (~42 kDa) in our wildtype sample but not spe-7 or spe-44 mutants (Figure 7C). In addition to the 42 kDa band, we observed non-specific binding at both higher and lower molecular weights. For the most part, this non-specific binding was observed across all samples not indicative of higher/lower molecular weight or truncated SPE-7 products. Therefore, although the band recognized by anti-SPE-7 is of a higher molecular weight than expected, we concluded that this 42 kDa band is SPE-7 and that no truncated SPE-7 products are stable in the mutant.
Figure 7. SPE-7 antibody is specific to SPE-7 protein. (A) Anti-SPE-7 antibody recognizes the SPE-7 peptide in wildtype (top, DAPI (blue); SPE-7 red) but not spe-7 mutant males (bottom, DAPI (left), SPE-7 (right)). (B) Anti-SPE-7 antibody identified a 42-kDa band on Western blots (WB) in lysates of male whole worms, but not hermaphrodites that fail to undergo spermatogenesis (fog-2 (lf)). (C) Anti-SPE-7 antibody detected SPE-7 in the wildtype (WT) male lysate, but not in lysates from either spe-7(mn252) or spe-44(ok1400) mutants. The same number of worms was loaded per lane for all samples. Non-specific binding at ~50 kDa is observed in all samples although the intensity varied across the samples.
SPE-7 is required for proper FB assembly.

spe-7 represents one of a limited number of early arrest spermatogenesis-defective (spe) mutants that have been previously described in C. elegans (Nishimura and L’Hernault, 2010). Presler showed that spe-7 mutants arrest as primary spermatocytes with defects in chromatin segregation, residual body formation, and cytokinesis. A similar phenotype was described for spe-6 mutants by Varkey et al. (1993) who attributed the observed cell cycle and developmental defects to a failure in sequestering MSP into FBs. MSP is required in spermatozoa where it serves as the motility protein and a signal for oocyte maturation; yet it has no function in developing spermatocytes. As transcription ceases prior to the meiotic divisions and translation after, MSP is synthesized early and sequestered into FBs. Therefore the formation of FBs is critical to spermatogenesis as to prevent interference of key regulatory processes from abundant, cytosolic MSP.

To determine if spe-7 spermatocytes also fail to sequester MSP, we examined the distribution of MSP in the context of fibrous body formation (Figure 8). In wildtype spermatocytes, the fibrous body appears as an oblong inclusion early in meiosis. However in spe-7 spermatocytes, MSP fails to assemble into fibrous bodies and instead distributes diffusely throughout the cytoplasm. This phenotype is identical to that of spe-6 and spe-44 mutants. SPE-6 is a kinase with multiple roles throughout spermatogenesis, one of which is in FB assembly (Varkey et al., 1993).
SPE-7 is expressed specifically during spermatogenesis.

Previous microarray analyses by Reinke et al., (2000) identified clusters of genes whose expression was specifically enriched during either spermatogenesis or oogenesis. In hermaphrodite only populations, the spermatogenesis-enriched genes were expressed almost exclusively during the L4 larval (sperm-producing) stage whereas oogenesis-enriched genes were expressed exclusively in adults consistent with their switch to exclusively producing oocytes. In these initial studies, spe-7 (F32A11.3) was identified as one of these spermatogenesis-enriched genes. While microarray analyses are informative at the level of gene expression, we wanted to analyze the SPE-7 protein in terms of its initial synthesis and subsequent turnover. To confirm and extend these microarray
findings, we analyzed the levels of SPE-7 protein in wildtype (N2) hermaphrodites at several developmental time points (Figure 9). Hermaphrodite samples were staged as L1/L2, L3, L4, and adult. Following separation via SDS-PAGE and Western blot analysis, we found that SPE-7 protein is present during the L4 larval stage of hermaphrodites. In wildtype young adult males, which continually produce sperm, SPE-7 protein levels are higher than in hermaphrodites at the same stage. While spermatozoa are present in the spermatheca of adult hermaphrodites, no SPE-7 signal is observed. This result is consistent with previous immunocytological studies in which SPE-7 signal disappears in young spermatids (Figure 7A). As several bands of similar molecular weight are present across all samples, we disregarded these bands as non-specific to the anti-SPE-7 antibody. In addition, we observed two sets of non-specific bands at ~35 and 150 kDa that were present in only adult hermaphrodites and wildtype males. While unsure of the identity of these proteins, the molecular weight is different from that of SPE-7.
Figure 9. SPE-7 is expressed during spermatogenesis. SDS-PAGE and Western blot analysis of developmentally staged hermaphrodites immunoblotted with anti-SPE-7. Hermaphrodites produce fewer sperm during spermatogenesis as compared to males as evidenced by lower amounts of SPE-7 protein. Staged, hermaphrodite samples were washed off synchronous plates while wildtype males were picked as L4 larvae and collected as young adult males. Arrow corresponds to SPE-7 band.

**SPE-7 structures are assembled prior to FBs.**

With Western blot analyses, we described the overall presence/absence of SPE-7 at different developmental stages. As SPE-7 is expressed only during spermatogenesis, we wanted to use the temporal-linear arrangement of the gonad to analyze the relative timing of SPE-7 synthesis with regards to both the stages of spermatogenesis and assembly of sperm-specific organelles, specifically the FB. During wildtype
spermatogenesis, MSP is expressed early in meiotic prophase, and the FB assembles during diplotene (Figure 10). However, comparative immunostaining of SPE-7 and MSP relative to chromatin morphology in wildtype males revealed that SPE-7 structures are assembled during pachytene, before the assembly of MSP into FBs. Conversely, in newly formed spermatids, SPE-7 structures disappear prior to FB disassembly whereas MSP disperses to the cytoplasm but remains stable and abundant.

Figure 10. Immunolocalization analysis of SPE-7 (red) and MSP (green) in isolated and methanol fixed male gonads. The various stages of meiotic progression can be distinguished by the morphology of the DAPI-stained nuclei (blue). Zones within the meiotic region are highlighted as follows: pachytene (orange), condensation zone (diplotene/karyosome) (red), division zone (purple) and post-meiotic spermatids (blue). SPE-7 structures assemble during pachytene prior to assembly of FBs. SPE-7, but not MSP, is first detected in pachytene stage cells (arrow). Conversely, MSP but not SPE-7 is present in spermatids (characterized by the bright, punctate chromatin masses in the DAPI and merge images). Scale bar=15μm.
SPE-7 initially associates with MOs before dynamically localizing to FBs.

Until now, the FB has been assumed to be primarily and/or exclusively composed of MSP, and few additional FB components have yet to be described in the published literature. Furthermore, while work by Roberts and others have elucidated key steps in the assembly and disassembly of MSP fibers within the pseudopod (Buttery et al., 2003; Grant et al., 2005; Yi et al., 2007; Yi et al., 2009), little is known regarding either the assembly of MSP into FBs within developing spermatocytes or the subsequent disassembly of FBs within maturing spermatids. As judged by MSP localization patterns (Figure 10), FB formation begins at diplotene, and the FBs continue to grow in size until the FBs are partitioned into spermatids and the ribosomes are partitioned into the residual bodies. Therefore, FBs are largest in secondary spermatocytes. From their initial formation and through the meiotic divisions, the FBs remain cradled within the membranous arms of the MO but are released from the MO immediately after the budding division (Ward and Klass, 1982; Roberts et al., 1986; Figure 4). Within the newly formed spermatids, the fibrous bodies do not immediately disassemble but remain intact during a recently described period of spermatid maturation (Chu and Shakes, 2012).

Since spe-7 (mn252) mutants lack fibrous bodies, we wanted to determine if SPE-7 localizes to wildtype fibrous bodies where it may participate in proper assembly. Although the observed SPE-7 localization pattern suggested that it was colocalizing with MSP in the fibrous bodies, the small size and close association of the fibrous body and MO within the FB-MO complex makes it challenging to distinguish between these two
cellular compartments by light microscopy alone. Therefore to more definitively
determine whether SPE-7 preferentially localizes to the fibrous body rather than to the
MO, we examined the co-localization patterns of SPE-7 and the MO marker, 1CB4,
within spe-4 spermatocytes. spe-4 encodes a presenilin required for proper timing of FB-
MO disassociation (Arduengo et al., 1998). Whereas wildtype FB-MO complexes first
disassociate in post-meiotic maturing spermatids, those in spe-4 spermatocytes
disassociate prematurely as the spermatocytes enter the meiotic division stage (Arduengo
et al., 1998). spe-4 mutant spermatocytes ultimately arrest as undivided terminally
arrested spermatocytes with distinct FBs and MOs (Arduengo et al., 1998; Figure 11). If
SPE-7 localizes to MOs, SPE-7 should co-localize with the 1CB4 antigen throughout the
meiotic period. However if SPE-7 localizes to FBs, SPE-7 should separate from the
1CB4 antigen as the mutant spermatocytes enter the meiotic division phase. In terminal
spe-4 spermatocytes, we observed the latter (Figure 11) indicating that SPE-7 localizes to
the FB rather than the MO.
Figure 11. SPE-7 preferentially localizes to FBs and persists in spe-4(q347) terminal spermatocytes. Immunolocalization analysis of methanol fixed spermatocytes that were double labeled with antibodies to SPE-7 (red) and the MO-marker 1CB4 (green) and stained with DAPI (blue). (A, A') Sequence of karyosome to budding figure spermatocytes from flattened male gonad preparations; white arrow represents spermatids enlarged in adjacent panels. (B-E) Wildtype spermatid from A, enlarged 2.5X. (B'-E') Terminal spe-4 spermatocyte from A', enlarged 2.5X. (B, B') 1CB4. (C, C') DAPI. (D, D') SPE-7. (E, E') Merge. Scale bar=15μm.
We show that SPE-7 localizes to FBs and that SPE-7 assembles into smaller “pre-FB” structures prior to the addition of MSP. However, we did not know whether SPE-7 requires its own assembly factors. Given the close association of FBs and MOs, we hypothesized that association with the MO may be required for SPE-7 assembly. In wildtype spermatocytes, the assembly of SPE-7 into “pre-FB” complexes, consisting of SPE-7 and other potential proteins, occurs during late pachytene with the addition of MSP occurring quickly thereafter during the transition to diplotene. By examining the progression of events in developing wildtype spermatocytes as they transition through pachytene and the subsequent diplotene and karyosome stages, we can determine the site of SPE-7 localization in these pre-FB complexes and during the subsequent addition of MSP. From direct comparisons of MSP/MO (via 1CB4) and SPE-7/MO co-localization patterns in wildtype and spe-4 mutants, we have concluded that the assembly of SPE-7 structures prior to FB assembly occurs adjacently to the MO (Figure 12). These results suggest that SPE-7 may require association with the MO membrane or membrane proteins to properly assemble into its pre-FB conformation.
Our studies of SPE-7 localization in spe-4 mutants confirmed that SPE-7 localizes to the FB rather than the MO but provided little additional information as to its precise subcellular localization within the FB itself. To further understand SPE-7 localization patterns and dynamics, we immunostained fixed wildtype male gonads with SPE-7 and MSP and focused our analysis on the division zone where SPE-7 expression was previously shown (Figure 13). Our results showed that SPE-7 structures assemble during pachytene, prior to FB assembly. In diplotene–stage primary spermatocytes, SPE-7 precisely colocalizes with MSP (Figure 13). However, as spermatocytes initiate the meiotic divisions SPE-7 remains associated with the FB but becomes enriched into focal points at multiple edges (typically four). As the FBs reach their maximal size during metaphase II, SPE-7 accumulates at the two ends of the fully elongated FB. This pattern
of localization persists through the post anaphase II budding division. SPE-7 segregates to the spermatids and quickly becomes undetectable. In contrast, MSP remains assembled in rod shaped FBs until the completion of the spermatid maturation process, at which point the FBs disassemble and MSP disperses throughout the cytoplasm.
Figure 13. SPE-7 localizes to FBs in a dynamic pattern during the meiotic divisions and then disappears quickly in spermatids. (A) Immunolocalization of SPE-7 (red), MSP (green), and DAPI-stained DNA (blue) during spermatogenesis. Yellow coloration indicates colocalization. (B) Schematic representation of SPE-7 localization. SPE-7 (pink), MSP (yellow), colocalization (orange). Scale bar=15μm.

The secondary antibodies that recognize anti-MSP and anti-SPE-7 are FITC-conjugated goat-anti-mouse and TRITC-conjugated goat-anti-rabbit, respectively. Due to potential overlap of emission spectra of both fluorophores, we wanted to confirm the observed patterns of fluorescence with single labeled immunopreparations. To distinguish actual antibody signal from bleedthrough, we prepared isolated gonads with either anti-SPE-7 or anti-MSP. Using ImageJ, we measured the lengths of individual FB (as represented by MSP) and SPE-7 structures (Figure 14). During the karyosome stage, SPE-7 and FB lengths are comparable consistent with observed colocalization during this stage. When quantitated, we discovered that SPE-7 structures are, on average, longer than MSP structures consistent with our previous observation of enriched SPE-7 localization to the ends of FBs.
Figure 14. Quantitation of the average length of FB versus SPE-7 structures during different stages of spermatogenesis. Abbreviations as follows: karyosome (karyo), meta I (metaphase I), ana I (anaphase I), metaphase II (meta II), budding (budding figure), sperm (newly formed spermatid before the disappearance of SPE-7) Each unit of length corresponds to 50 pixels. Error bars represents standard deviation. Averages of statistical significance as determined by the student’s t-test are represented with asterisks: p<0.5 (*), p<0.01 (**), p<0.0001 (****). All measurements were acquired from 600X images.

**SPE-7 is presumably degraded in spermatids, but not via an ubiquitin-mediated program.**

In immunolocalization studies of SPE-7 in wildtype spermatocytes, SPE-7 became rapidly undetectable in newly formed spermatids immediately after budding. However, the specific nature of SPE-7’s “disappearance” remained unclear. We were uncertain whether SPE-7 was being proteolytically degraded or if the antigenic site was merely being masked by either a post-translational modification or overall change in the protein shape. If the SPE-7 antigen is inaccessible to anti-SPE-7 due to overall changes in
protein conformation, proteins should unfold when heated in the presence of SDS and beta-mercaptoethanol making the antigenic region readily accessible. We attempted to address this question using a biochemical approach.

In a first set of experiments, we investigated the levels of SPE-7 over time in aged, celibate males (Figure 15A-C). Left unmated, C. elegans males can accumulate ~3000 fully mature spermatids without expanding the meiotic zone. Therefore, we can determine relative levels of SPE-7 to increasing levels of MSP as an indication of spermatid accumulation. If SPE-7 were not degraded, we would expect to see levels of SPE-7 increase in parallel with increasing levels of MSP. If SPE-7 were degraded, we would expect to see MSP levels increase as SPE-7 levels remain the same or even decrease over time. We collected celibate males at 24, 48, and 72 hours post-L4 and analyzed SPE-7 and MSP protein levels via SDS-PAGE and Western blot. SPE-7 levels are highest at +24 hours. Over a period of 72 hours, SPE-7 levels gradually decrease while MSP levels increase, reaching a peak at 48 hours, before leveling off.

As an additional level of confirmation, we investigated the relative levels of SPE-7 and MSP via immunocytology (Figure 15D). As with our Western blot analysis, we isolated L4 males and subsequently collected them over the course of three days. We observed a similar pattern in fixed and isolated gonads as with the Western blots: as spermatids accumulate over time, the zone of SPE-7 positive cells diminishes gradually.

Individual findings from two separate experimental approaches support a model of SPE-7 degradation rather than epitope masking due to folding constraints. Although these experiments do not directly address the possibility of the antigenic site being
masked by a post-translational modification, our bioinformatics analyses predicts that the peptide against which the anti-SPE-7 antibody was generated is not a direct substrate for post-translational modifications.

Figure 15. Relative levels of SPE-7 and MSP in aged, celibate males collected at 24, 48, and 72 hours. (A, B, C) Immunolocalization of SPE-7 (red), MSP (green), and DAPI-stained DNA (blue). White arrows in A, B, and C mark the zone of SPE-7 expression. (D) Western blot showing levels of SPE-7 and MSP in aged, celibate males.
In most cells, rapid degradation of a specific protein, particularly of cytosolic proteins is achieved by ubiquitin-dependent proteolysis. Ubiquitin is a small, highly conserved, and ubiquitous protein that can be covalently attached to lysine residues of target proteins by a cohort of three enzymes: an E1-ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase (Hershko et al., 1983; Figure 16). Attachment of single ubiquitins (monoubiquitination) to a protein targets the protein for endocytosis and trafficking of cell surface proteins whereas attachment of poly-Ub chains (polyubiquitination) targets proteins for destruction within the proteasome (Schnell and Hicke, 2003; Haglund and Dikic, 2005; Mukhopadhyay and Riezman, 2007). The *C. elegans* genome encodes a single E1 ubiquitin-activating enzyme (Kulkarni and Smith, 2008), twenty putative E2 ubiquitin-conjugating enzymes (Jones et al., 2001), and hundreds of E3 ubiquitin ligases that provide substrate specificity (wormbook.org). In *C. elegans*, UBA-1 is the only E1 enzyme (Kulkarni and Smith, 2008).
Figure 16. The ubiquitin enzymatic cascade. Ubiquitin is attached to a protein by a series of enzyme-mediated reactions. Repeated cycles of the ubiquitin cascade result in a chain of ubiquitin molecules that target the substrate for proteolysis by the 26S proteasome. Following degradation of targeted proteins, ubiquitin is recycled to target additional proteins for subcellular relocalization or degradation via the proteasome. Figure adapted from Petsko and Ringe, 2004.

The single E1 enzyme in *C. elegans* is genetically represented by a single temperature-sensitive mutant, *uba-1(it129)* (Kulkarni and Smith, 2008). Shifting *uba-1(it129)* animals to restrictive temperatures at various points in development results in several different phenotypes (Kulkarni and Smith, 2008). While upshifting as embryos or adults results in larval or maternal effect (embryonic) lethality, respectively, a sperm-specific phenotype is observed when mutants are upshifted as L2/L3 larvae. The sperm produced by L2/L3 upshifted males are at best incompatible of fertilization and at worst compromised in terms of its motility.
If SPE-7 is being specifically targeted for degradation by an E3 ubiquitin ligase, then we predicted that SPE-7 might be stabilized in *uba-1 (it129ts)* worms, and the stabilization of SPE-7 might even have a role in the previously described *uba-1* sperm phenotype (Kulkarni and Smith, 2008). To test this hypothesis, we investigated the SPE-7 localization pattern in *uba-1 (it129)* mutant males (Figure 17). *uba-1 (it129)* mutants were shifted to the restrictive temperature as L2 larvae and as worms reached adulthood, we confirmed the *uba-1* phenotype by the presence of unfertilized oocytes on plates and male tail defects as described by Kulkarni and Smith.

To our surprise, SPE-7 was not stabilized in *uba-1* spermatids; it disappeared as quickly during spermatid maturation as in as our wildtype controls. This result suggested that SPE-7 is not a target of ubiquitin-mediated degradation. Unfortunately, our attempts to independently verify this result by ascertaining whether immunoprecipitated SPE-7 is poly-ubiquitinated when probed with anti-ubiquitin antibodies via Western blot analysis were inconclusive.
Figure 17. SPE-7 does not persist in *uba-1* mutant spermatids. Wildtype and *uba-1* mutant spermatocytes were methanol fixed and immunostained with anti-SPE-7 (red) and DAPI to label DNA (blue). (A) Wildtype monolayer spermatocytes represent sequence from pachytene to budding figure spermatocytes. Spermatids can be distinguished by tiny punctate chromatin masses. (B) *uba-1* monolayer spermatocytes represent sequence from pachytene to budding figure spermatocytes. Scale bar=15 μm.

Our results indicate that the rapid “disappearance” of SPE-7 is unaffected in *uba-1* mutants; however, ubiquitin-dependent degradation is only one pathway of protein destruction. Certain proteins undergo proteosome-mediated degradation via an alternate program that acts independently of ubiquitin (Asher et al., 2006). To date, nothing had been reported regarding whether or not, proteosome-mediated protein degradation occurs in transcriptionally and translationally inactive *C. elegans* spermatids. Thus, to determine whether or not this explanation was even plausible, we decided to test whether or not proteasomes were present in spermatids using a monoclonal antibody against an alpha subunit of the 20S subcomplex of the 26S proteasome called PAS-7 (Hadwiger et al., 2010). The published localization pattern of PAS-7 describes a punctate pattern in the cytoplasm in addition to nuclear staining (Hadwiger et al., 2010). Immunocytology with
anti-PAS-7 showed that PAS-7 subunits, observed as punctate structures, are indeed present in spermatids as well as residual bodies (Figure 18). We also observed the previously described nuclear staining. Taken together, these results suggest that while SPE-7 is not degraded via an ubiquitin-dependent pathway, proteasomal degradation via an ubiquitin-independent pathway remains a plausible explanation for its disappearance as proteasomes are present within spermatids.

Figure 18. Proteasomes are present in wildtype spermatids. Wildtype spermatocytes were methanol fixed and immunostained with anti-PAS-7 (green) and DAPI to label DNA (blue). (A) Sequence of karyosome to budding figure spermatocytes from flattened male gonad preparations. (B-E) Spermatocytes from A, enlarged 2X. (B) Anaphase II. (C) Budding figure. (D) Residual body. (E) Spermatids. Scale bar=15µm.

SPE-7 disappearance requires spermatid-residual body separation and proper FB-MO partitioning into spermatids.

Coincident with the timing of SPE-7 disappearance in wildtype spermatocytes are the developmental events of residual body formation/spermatid budding, the partitioning of FB-MO complexes to spermatids, and FB-MO disassociation. To determine the
dependence/independence of SPE-7 disappearance relative to these cellular events, we investigated SPE-7 localization in spermatogenesis mutants that either arrest prior to the formation of spermatids or produce aberrant spermatids.

As SPE-7 disappears quickly follows the budding division, we hypothesized that processes that occur immediately prior to, or coinciding with, spermatid-residual body separation might influence SPE-7 disappearance. We chose to first address the dependence of SPE-7 disappearance on residual body formation. To determine the relationship between these two events, we investigated SPE-7 dynamics via immunohistochemistry in spe-4 (q347) mutants (Figure 11). Previously, we used spe-4 mutants to show that SPE-7 assembles near MOs but clearly co-localizes with the FBs when FB-MO complexes prematurely disassemble in terminal spermatocytes. In spe-4 spermatocytes, meiotic chromosome segregation occurs normally and produces four, distinct haploid chromatin masses yet both the cytokinesis event following meiosis I and the asymmetric budding division following meiosis II fail to occur (Arduengo et al., 1998). In the absence of this normally MO-associated member of the presenillin protein family, spe-4 spermatocytes not only arrest as single large spermatocytes but they also fail to differentially partition their cellular organelles into separate residual body and spermatid compartments. In the absence of these events, FBs persist within the terminally arrested spe-4 spermatocytes and they contain SPE-7 (Figure 11). This result suggests that SPE-7 disappearance requires either the partitioning or physical separation events associated with the post-anaphase II budding division.
To distinguish between a requirement for the physical act of spermatid budding \textit{per se} and the differential partitioning of FB-MO to the intracellular distinct environment of the spermatid compartment, we next analyzed SPE-7 localization patterns in \textit{spe-10} (\textit{hc104}) mutants. SPE-10, a transmembrane palmitoyl transferase, is required for proper partitioning of FB-MO complexes into spermatids (Shakes and Ward, 1989; Gleason et al., 2006). In \textit{spe-10} (\textit{hc104}) mutants, FB-MO complexes break down prior to spermatid-residual body separation, and a subset of these “naked” fibrous bodies lacking associated MO membranes persist within the residual bodies for a short time (Shakes and Ward, 1989; Gleason et al., 2006). Many of these naked fibrous bodies also bud from the residual bodies as cytoplasts (Figure 19A). Analysis of \textit{spe-10} mutants revealed that, following the budding division, when FBs ended up within residual bodies rather than in the spermatids, many of them continued to label with anti-SPE-7 antibodies (Table 2; Figure 20). Likewise when FBs budded from the mutant residual bodies as independent cytoplasts, SPE-7 remained particularly stable within these structures (Figure 19E).

<table>
<thead>
<tr>
<th>Analysis of \textit{spe-10} residual bodies</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>No FBs</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>SPE-7 negative FBs</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>SPE-7 positive FBs</td>
<td>40</td>
<td>68</td>
</tr>
</tbody>
</table>

Table 2. FB analysis of SPE-7 in residual bodies.
Figure 19. Cytoplasts present in spe-10 (hc104) mutants. (A) DIC image of arrested spe-10 spermatocytes and cytoplasts. White arrow represents a cytoplast that has recently budded from residual body. Black arrow represents off-center nuclei, a distinguishing phenotype of spe-10 mutant spermatids. Scale bar=10 μm. (B) 3X enlargement of a cell cluster containing a cytoplast. (C) 3X enlargement of off-center nucleus represented with an asterisk. (D) Anti-SPE-7 in isolated wildtype gonads. (E) Anti-SPE-7 in isolated spe-10 gonads with numerous SPE-7 foci in spermatid zone. Inset represents 2X enlargement of SPE-7 foci in (E). Scale bar=20 μm.
Figure 20. SPE-7 is present in FBs that missegregate to the residual bodies during spe-10 (hc104) spermatogenesis. Immunolocalization analysis of methanol fixed spermatocytes double labeled with antibodies to SPE-7 (red) and MSP (green) and stained with DAPI (blue). (A, A’) Sequence of karyosome to budding figure spermatocytes from flattened male gonad preparations. White arrows denote residual bodies; white arrowheads distinguish spermatids. (B-E) Wildtype budding figure represented by white arrow in A, enlarged 2.5X. (B’-E’) spe-10 budding figure represented by white arrow in A’, enlarged 2.5X. (B, B’) MSP. (C, C’) SPE-7. (D, D’) DAPI. (E, E’) Merge. Scale bar=10μm.

Lastly, we investigated the role of FB-MO disassociation on SPE-7 disappearance using yet again spe-4 (hc49) mutants. FB-MO disassociation presumably occurs at the time of SPE-7 disappearance (Figure 11). In wildtype spermatids, FB-MO disassembly
normally occurs concomitantly with other developmental events making it hard to discern the effect of FB-MO disassembly on SPE-7 disappearance. However, as described above, the primary defect in spe-4 spermatocyte is precocious FB-MO disassembly. In these mutants, SPE-7 persists despite premature disassembly of FB-MO complexes suggesting that FB-MO disassembly itself does not serve as the trigger for SPE-7 disappearance.

SPE-7 is necessary, but not sufficient, for fibrous body assembly.

As previously characterized by Varkey et al. (1993), the casein kinase homolog spe-6 is required for both proper FB assembly and cell cycle progression through the meiotic divisions. As SPE-6 has many functions during spermatogenesis and SPE-6 and SPE-7 are both required for FB assembly, we hypothesized that SPE-6 may regulate SPE-7 resulting in proper FB assembly. If SPE-6 regulates SPE-7, then in spe-6 mutants we would expect aberrant SPE-7 localization patterns. If SPE-6 does not regulate SPE-7, we would expect a normal SPE-7 localization pattern in spe-6 mutants. Upon examination of SPE-7 in spe-6 mutants, we discovered that while SPE-7 assembles into pre-FB structures, these structures assemble later than in wildtype. In wildtype gonads, pre-FB complexes initially assemble during pachytene in contrast to spe-6 mutants where we first observe pre-FB complexes during the karyosome stage. Pre-FB complexes also appear larger than their wildtype counterparts and remain morphologically similar to those of the late pachytene/karyosome stage observed in wildtype spermatocytes (Figure 21). This result suggests that in the absence of SPE-6 and MSP assembly into FBs, SPE-7
assembles as normal but does not undergo the additional structural changes that accompany growing FBs.

Figure 21. Altered SPE-7 localization dynamics in spe-6(hc49) mutants. Immunolocalization analysis of SPE-7 in wildtype and spe-6 backgrounds. (A, A’) DAPI. (B, B’) SPE-7. (C) Sequence of wildtype SPE-7 localization from pachytene to anaphase I, enlarged 2X. (C’) Sequence of SPE-7 localization from pachytene to karyosome, enlarged 2X. Scale bar=20μm.

**Delays in FB disassembly can occur independently of SPE-7 stabilization.**

Critical to the acquisition of motility in spermatozoa is FB disassembly. FBs disassemble releasing MSP so that, in crawling spermatozoa, MSP can reassemble into the filaments and, ultimately, macrofibers that treadmill to advance the leading edge of the pseudopod. In mutants that fail to disassemble FBs, pseudopod formation is aberrant and motility is impaired (Ward et al., 1981). Unpublished data from both our lab and that
of Diana Chu suggests that in a population of spermatids, there are many more spermatids with intact FBs than would be implied by review articles in the literature (Roberts et al., 1986; L’Hernault, 2006). As a result, our new model highlights the transition of recently budded spermatids into mature spermatids as an important maturation process (Wu et al., 2010; Chu and Shakes, 2012).

In gonad preparations from wildtype males, SPE-7 is observed in only a small population of maturing spermatids with intact FBs. This observation suggests a model in which the disappearance of SPE-7 may be a prerequisite and potentially even a causative factor in the ultimate disassembly of FBs. Furthermore, as an additional corollary, we wondered whether we might observe a stabilization of SPE-7 in a subset of mutants whose spermatids fail to disassemble their FBs.

We first chose to examine SPE-7 in the FB disassembly double mutant gsp-3(tm1647) gsp-4(y418). gsp-3 and gsp-4 encode two homologs of PP1-gamma (Chu et al., 2000), a PP1-phosphatase required for mouse spermatogenesis (Varmuza et al., 1999). In C. elegans, these nearly identical proteins are required for meiotic chromosome segregation, efficient FB/MSP disassembly in maturing spermatids, and proper formation and treadmilling of the pseudopod (Wu et al., 2012). To address whether SPE-7 disappearance is required for FB disassembly and if SPE-7 disappearance is regulated by GSP-3/4, we investigated SPE-7 localization in gsp-3/4 mutants (Figure 22). If SPE-7 disappearance is required for ultimate FB disassembly and a target of GSP-3/4, we would expect that SPE-7 would persist in gsp-3/4 mutants. If SPE-7 is not required for FB disassembly and is not a target of GSP-3/4, we would expect the timing of SPE-7 loss to
be unaltered in $gsp-3/4$ mutants. In $gsp-3(tm1647)$ $gsp-4(y418)$ spermatids, the
disappearance of SPE-7 does not coincide with FB disassembly; MSP remained locked in
FB-like structures while the timely disappearance of SPE-7 remained unaffected. This
result suggests that SPE-7 loss does not directly trigger FB disassembly nor is SPE-7 a

Figure 22. SPE-7 disappears despite delayed FB disassembly. Immunolocalization
analysis of SPE-7 (red), MSP (green), and DAPI to label DNA (blue) in isolated and
methanol fixed gonads. (A) Sequence of pachytene to budding figure spermatocytes from
flattened male gonad preparations. (B-E) Spermatocytes corresponding to red, blue,
yellow, and orange arrows respectively in A, enlarged 2X. (B) Pachytene. (C)
Karyosome. (D) Metaphase I. (E) Spermatids with intact FBs. Scale bar= 15μm.

Another mutant in which spermatids fail to rapidly disassemble FBs is the $fem-3$
gain-of-function mutant, $fem-3(q20gf)$ (Chu and Shakes labs, unpublished data). $fem-3$
encodes a novel protein that functions as a component of a CUL-2/ElonginB/C-based
ubiquitin ligase and whose expression directs spermatogenesis in $C.\ elegans$
hermaphrodites (Barton et al., 1987; Starostina et al., 2007). In wildtype hermaphrodites, fem-3 must be negatively regulated to allow the switch to oogenesis. Gain-of-function alleles masculinize the germline, but do not affect the soma resulting in genetically XX animals that are somatically female (hermaphrodite) animals but produce only sperm (Barton et al., 1987). To independently confirm the role of SPE-7 in FB disassembly, we investigated SPE-7 localization in fem-3(q20gf) mutants. Analysis of spermatids in restrictively grown fem-3(gf) mutants revealed that, as in gsp-3/4 mutants, the timing of SPE-7 disappearance is similar to that of wildtype males (Figure 23); only a few newly formed spermatids label with anti-SPE-7 antibody even though a much larger population of spermatids are delayed in disassembling their FBs.

Figure 23. Unchanged SPE-7 dynamics in fem-3(q20) gain-of-function mutants. Immunolocalization analysis of SPE-7 (red), MSP (green), and DAPI-stained DNA (blue). (A) Sequence of pachytene to budding figure spermatocytes from flattened male
gonad preparations. Arrows represent enlargements. (B-E) Spermatocytes from (A) enlarged 2X. (B) Pachytene. (C) Karyosome. (D) Anaphase I. (E) Budding spermatids. (F) Late spermatids with intact FBs. Scale bar=20 μm.

**SPE-7 disappearance does not require progression of the cell cycle beyond metaphase I.**

Taken together, our results are consistent with a model in which the disappearance of SPE-7 is not linked to either GSP-3/4 or an unknown factor related to FEM-3, which function later to promote the disassembly of the FBs. Instead, the disappearance of SPE-7 requires the completion of the post-meiotic budding division during which the FB moves from the cellular environment of an arrested primary spermatocyte/residual body or residual body-derived cytoplast into the environment of the maturing spermatid. However beyond this developmental event, another factor that could be required for the disappearance of SPE-7 is progression through the meiotic cell cycle. To determine if the disappearance of SPE-7 requires or is linked to progression through the cell cycle, we examined SPE-7 localization in a mutant lacking a subunit of the anaphase-promoting complex, *emb-27*, an ortholog of *cdc-16* (Cassada et al., 1981; Golden et al., 2000). In restrictively grown *emb-27(g48ts)* mutants, the cell cycle of affected spermatocytes arrests at metaphase I while the developmental program of spermatogenesis proceeds resulting in the formation of haploid, anucleate spermatids capable of activation into crawling and fertilization-competent spermatozoa (Golden et al., 2000; Sadler and Shakes, 2000). All spermatids label with anti-MSP, but most are anucleate as documented by the absence of DAPI-stained chromatin. If SPE-7 disappearance is linked to meiotic cell cycle progression, we would predict that SPE-7
would be stabilized in *emb-27* mutants as the cell cycle arrests before spermatids are formed. If SPE-7 disappearance is independent of cell cycle progression, we would predict that SPE-7 disappearance would occur as normal despite the metaphase arrest.

Immunocytological analyses of SPE-7 in these mutants showed that SPE-7 is absent from anucleate spermatids (Figure 24). Therefore, SPE-7 disappearance is not linked to cell cycle progression beyond metaphase I. However we cannot rule out whether normal SPE-7 dynamics may depend on cell cycle events prior to metaphase I.

Figure 24. SPE-7 disappearance is independent of cell cycle progression beyond metaphase I. Immunolocalization analysis of methanol fixed gonads double labeled with antibodies to SPE-7 (red) and MSP (green) and stained with DAPI (blue). (A-D) Wildtype. White arrowhead represents karyosome stage spermatocytes; white arrow corresponds to spermatids. (A'-D') restrictively grown *emb-27(g48ts)*. White arrowhead represents karyosome stage spermatocytes; white arrow corresponds to anucleate spermatids. (A, A’) MSP. (B, B’) DAPI. (C, C’) SPE-7. (D, D’) Merge. Scale bar=15um.
Bioinformatics

With bioinformatics analyses, we attempted to further understand the nature of SPE-7. To predict possible protein structures, we utilized the bioinformatics program I-TASSER. Starting from the amino acid sequence, I-TASSER generates three-dimensional models from multiple threading alignments (Roy et al., 2010). The protein function is then determined by matching the obtained structure to structures of known proteins with known functions. Each model is provided a confidence score (c-score) or an estimate of the accuracy of the model. C-scores are typically in the range [-5, 2] with higher scores corresponding to higher confidence. Generally, models with c-scores > -1.5 have a correct fold. I-TASSER predictions reveal that SPE-7 lacks a well-defined secondary structure of high probability (Figure 26). The C-scores for SPE-7 are of no greater than -1.8, but the predictions share a common characteristic: large loop regions with little secondary structure in the form of alpha helices/beta pleated sheets. We then analyzed the amino acid sequence for a possible explanation of the lack of structure. Analysis of the sequence revealed that SPE-7 is enriched in proline and glutamine residues (Table 3) as compared to SPE-6 and MSP, two proteins with resolved structure. Investigation into the literature reveals that an abundance of proline and short stretches of glutamine residues is a common characteristic of unstructured proteins/protein regions as proline and glutamine are known disorder-promoting residues (Dunker et al., 2002). We also investigated potential post-translational modifications. SPE-7 contains several predicted phosphorylation and few predicted ubiquitination sites and one region has homology to a mucin domain suggesting potential glycosylation sites (NetPhos 2.0,
Technical University of Denmark). However, as SPE-7 is cytoplasmic we do not expect the protein to be glycosylated. Of the possible kinases, there is an abundance of Casein Kinase 1 (CK1) phosphorylation recognition motifs as well as CK2, ERK, SRC, CDK1, and CDK2 (phosida.com; Table 4). The likelihood of phosphorylation, especially by a CK1, in addition to the different localization patterns of SPE-7 throughout spermatogenesis directed us to investigate the presence of different/modified forms of SPE-7.

<table>
<thead>
<tr>
<th>Amino Acid Frequencies</th>
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<tbody>
<tr>
<td>Residue</td>
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<tr>
<td>---------</td>
</tr>
<tr>
<td>Ala</td>
</tr>
<tr>
<td>Arg</td>
</tr>
<tr>
<td>Asn</td>
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<tr>
<td>Asp</td>
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<tr>
<td>Cys</td>
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<td>Gln</td>
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<tr>
<td>Gly</td>
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<tr>
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<td>Ile</td>
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<tr>
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<td>Lys</td>
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</tr>
<tr>
<td>Trp</td>
</tr>
<tr>
<td>Tyr</td>
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<tr>
<td>Val</td>
</tr>
</tbody>
</table>

Table 3. Amino acid composition of SPE-7 as compared to SPE-6 and four identical MSP proteins, MSP-10/36/56/76. Protein sequences were obtained from wormbase.org and
compared for differences in amino acid composition. Number of amino acid residues/protein are represented first, followed by percentages in parentheses.

Figure 25. I-Tasser protein structure alignment predictions of SPE-7. Images are just one view of a 3-D structural prediction. Few alpha helices are observed and large portions of the protein are exposed as unstructured regions.

<table>
<thead>
<tr>
<th>Potential Kinases</th>
<th>Potential Phosphorylated Residues</th>
</tr>
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<tbody>
<tr>
<td>ALK</td>
<td>Y52, Y225, Y252</td>
</tr>
<tr>
<td>CDK1</td>
<td>S65</td>
</tr>
<tr>
<td>CDK2</td>
<td>S65</td>
</tr>
<tr>
<td>CK1</td>
<td>S6, T26, S57, S61, S64, S220, S245, S336</td>
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<tr>
<td>CK2</td>
<td>S348</td>
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<tr>
<td>DNA damage response kinase</td>
<td>S43, T103, T281</td>
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<tr>
<td>EGFR</td>
<td>Y225</td>
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<tr>
<td>ERK</td>
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<td>GSK3</td>
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<tr>
<td>NEK6</td>
<td>S43, S54, S59, S61</td>
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<td>PLK1</td>
<td>T253</td>
</tr>
<tr>
<td>SRC</td>
<td>Y169</td>
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</table>

Table 4. SPE-7 has multiple potential phosphorylation sites by various kinases. Most predicted sites are serine/threonine residues, however there are several sites of predicted tyrosine phosphorylation.
**Discussion and Future Directions**

This work identifies *spe-7* as a key regulator of FB assembly in nematode spermatocytes. Through a combination of genetic, biochemical, and bioinformatics approaches, we have garnered information regarding the expression, role, and nature of the protein SPE-7.

The *spe-7* gene is expressed specifically during spermatogenesis and mutation of *spe-7* results in sperm-specific sterility as a consequence of defective FB assembly. In wildtype spermatocytes, SPE-7 is expressed prior to the formation of FBs and assembles into pre-FB complexes, localizes to FBs upon incorporation of MSP, undergoes conformational changes during FB growth, and rapidly disappears after FBs are partitioned into spermatids. Initial results suggested that SPE-7 associates and disassociates from FBs (Presler, unpublished); however we show here that SPE-7 remains associated with FBs through the budding division albeit in a very dynamic pattern consistent with its proposed role as a nucleator of proper FB assembly. SPE-7-only foci are observed by late pachytene of meiotic prophase and SPE-7 co-localizes with MSP/developing FBs by diplotene. Upon entry into the meiotic divisions, SPE-7 positions itself on the FB periphery ultimately localizing to the ends of rod-shaped FBs during metaphase I where it persists through the budding divisions until it subsequently becomes undetectable in maturing spermatids. Our results suggest this disappearance is a result of SPE-7 degradation rather than epitope masking as SDS-PAGE and Western blot analysis of celibate males show that as males accumulate spermatids, MSP levels increase whereas SPE-7 levels decrease over time.
However, SPE-7 does not seem to be the target of ubiquitin-dependent degradation. Examining the possibility of ubiquitination of SPE-7, we investigated the localization pattern and dynamics of SPE-7 in *uba-1* mutants. Immunocytological studies with these mutants showed that despite the absence of the only E1 activating enzyme in *C. elegans*, the SPE-7 protein does not persist in spermatids. But we cannot eliminate the possibility of SPE-7 degradation as ubiquitin-independent pathways exist (Asher et al., 2006). Using a transgenic SPE-7:GFP fusion protein, we could better distinguish between degradation of SPE-7 and epitope masking.

SPE-7 regulates FB assembly, but not FB disassembly. In maturing wildtype spermatids, SPE-7 disappears prior to the disassembly of FBs. As a result, we hypothesized that SPE-7 disappearance might be necessary and even directly trigger FB disassembly. However in two examples of delayed FB disassembly, FBs do not disassemble and SPE-7 does not persist. In both the phosphatase mutant, *gsp-3/4*, and *fem-3(gf)* mutants, FB disassembly is delayed yet SPE-7 dynamics are normal and the timing of disappearance is unaffected. While the ultimate disappearance of SPE-7 may be necessary, SPE-7 disappearance is not sufficient to trigger FB disassembly.

We used various genetic mutants to determine cellular correlates of SPE-7 disappearance. We have shown that SPE-7 disappearance occurs independently of cell cycle progression beyond metaphase I (as shown with *emb-27*), after cytokinesis (shown with *spe-4*) and proper residual body formation (*spe-10*). In *emb-27* mutants, SPE-7 does not persist despite a metaphase I arrest suggesting that regardless of an early cell cycle arrest, the developmental cues triggering SPE-7 disappearance are present. However, we
cannot speak to the dependence of SPE-7 on cell cycle progression prior to metaphase I. Our analysis of SPE-7 in spe-4 mutants reveals that SPE-7 disappearance is likewise unlinked to chromatin maturation, as terminal spermatocytes arrest with haploid nuclei and stable SPE-7 aggregates. SPE-7 disappearance does appear to rely on the budding division that follows meiosis II. Additionally, investigation of SPE-7 in spe-10 suggests that proper residual body formation and spermatid budding contribute to SPE-7 disappearance as SPE-7 disappears when partitioned into spermatids, but not when missegregated to residual bodies. This result suggests that the differing environments of residual body versus spermatid play a role in the timing of SPE-7 disappearance.

SPE-7 alone is not sufficient to direct FB assembly. Both SPE-7 and SPE-6 are required for FB assembly as both mutants fail to properly assemble FBs. On the premise that mutants with similar phenotypes may interact, we investigated SPE-7 dynamics in spe-6(hc49). We discovered that in the absence of SPE-6, SPE-7 and pre-FB complexes assemble. However, SPE-7 localization is perturbed as SPE-7 persists in a pre-FB conformation lacking the same localization dynamics as observed in wildtype spermatocytes. As spe-6 encodes a kinase, we hypothesize that SPE-7 requires activity of SPE-6 to incorporate MSP into FBs as SPE-7 is locked in a pre-FB conformation in spe-6 mutants. It is also possible that SPE-6 phosphorylates MSP, resulting in its assembly into FBs. We have not yet determined if SPE-6 directly regulates SPE-7, but bioinformatics data predicts multiple SPE-7 residues to be phosphorylated by members of the Casein Kinase 1 superfamily. SPE-6 is therefore a likely candidate, but there are multiple sperm-enriched CK1s (Manning, 2005).
Taken together, this work has identified SPE-7 as a critical regulator of FB assembly. We show that the FB is not solely composed of MSP as previously assumed, but requires external factors for proper assembly and development. By following wildtype SPE-7 dynamics, we know that SPE-7 disappearance is a critical step in the spermatid maturation process but that SPE-7 disappearance does not directly regulate FB disassembly or its timing.

![Figure 26](image-url)

**Figure 26.** Model of FB assembly. The pre-FB complex, comprised of SPE-7, assembles prior to the meiotic divisions requiring the kinase SPE-6 to incorporate MSP into FBs. This incorporation results in localization changes to SPE-7. SPE-7 localizes to developing FBs as four foci in primary spermatocytes. As the FB develops and elongates, SPE-7 enriches at opposite ends of the rod-shaped FB before ultimately disappearing in maturing spermatids. Following SPE-7 disappearance, the FB disassembles and MSP is released into the cytosol. Several mutants disrupt normal SPE-7 localization dynamics resulting in the stabilization of SPE-7 in terminal cells. *spe-6* mutants arrest with SPE-7 is a pre-FB conformation; *spe-4* mutants arrest with SPE-7 associated with FBs; *spe-10* mutants missegregate SPE-7 into residual bodies where it remains stable in residual bodies and budded cytoplasts.

**SPE-7 may be an intrinsically unstructured protein.**

In recent years, the traditional structure-function paradigm has shifted as to incorporate a burgeoning class of proteins that lack structure but retain function. These
intrinsically unstructured proteins (IUPs) are highly flexible with basic regulatory roles in key cellular processes (Tompa, 2002). IUPs are disordered under physiological conditions but obtain structure upon simultaneous phosphorylation and binding of a target substrate. Most unstructured proteins have an amino acid sequence enriched in proline, glutamic acid, lysine, serine, and glutamine residues as these are disorder-promoting amino acids (Dunker et al., 2002). Conversely, the amino acid sequence is typically depleted in tryptophan, tyrosine, phenylalanine, cysteine, isoleucine, and asparagine residues as these are order-promoting amino acids. One distinct class of IUPs is the assembler class. The assemblers regulate the assembly of large, multiprotein complexes as they recruit and tether individual globular subunits.

From our bioinformatics and experimental data, we hypothesize that SPE-7 falls into the assembler class of intrinsically unstructured proteins. The SPE-7 sequence is abundant in prolines and glutamines and lacks a predicted secondary structure. When analyzed via SDS-PAGE and Western blot, SPE-7 protein is consistently observed at a higher molecular weight than anticipated. This migration shift has been previously documented for other IUPs. Because of their unusual amino acid composition, IUPs bind less SDS than usual and their experimental molecular weight is 1.2 times higher than the molecular weight calculated from sequence data (Tompa, 2002). The experimental molecular weight of SPE-7, amino acid composition, role in FB assembly, and lack of structure suggests SPE-7 as an assembler IUP.

Identification of IUPs has historically been a result of x-ray crystallography of IUPs and accompanying proteins from which IUPs obtain structure in addition to other
structural techniques like NMR. Additional information about the nature of IUPs has been garnered in recent years allowing for less labor-intensive techniques. In addition to molecular weight data, it is known that IUPs remain soluble at higher temperatures (Tompa, 2002). As IUPs do not have a hydrophobic core, they can often be purified by treatment with heat. Preliminary data suggests that when treated with heat, a band of the same molecular weight as SPE-7 is present in the supernatant of whole worm lysates (Figure 27) in addition to bands of higher intensity and varying molecular weights. However when compared to the lysate and pellet lanes, the heat treatment eliminated a majority of the bands.

Further analysis of IUPs by two-dimensional gel electrophoresis has identified novel IUPs (Csizmök et al., 2006). By combining native gel electrophoresis of heat-treated proteins followed by a denaturing gel containing 8M urea, IUPs can be separated from globular proteins. As IUPs are as denatured in urea as they are in native gels, they will migrate the same distance in both dimensions ultimately resolving on the diagonal. Any remaining heat-stable globular proteins will unfold in urea and migrate slowly resolving above the diagonal. Therefore, placement above or on the resulting diagonal allows resolution of possible IUPs.

As a future direction, we could extend our preliminary heat-treatment experiments to include this novel 2D gel electrophoresis approach followed by mass spectrometry to identify SPE-7 as an IUP.
SPE-7 may be degraded via an ubiquitin-independent pathway. Our data suggests that SPE-7 is not degraded via an ubiquitin-mediated pathway. There is however an alternate degradation pathway that does not require ubiquitin (Asher et al., 2006). Proteins that are inherently unstable often undergo “default” degradation by the 20S proteasome providing a new hypothesis for SPE-7 disappearance.

In recent years, extensive unstructured regions have been shown to serve as a signal for ubiquitin-independent degradation (UID) (Asher et al., 2006). The binding of substrates regulates degradation of proteins with highly disordered regions. As proteins interact and bind, unstructured regions are masked thereby inhibiting 20S proteasomal
degradation. A prominent example of this “cooperative stability” is with the tumor suppressor p14ARF and the viral oncogene SV40 LT that together inhibit p53 degradation (Asher et al., 2002). Together these proteins assemble larger protein complexes that protect p53 from degradation from the 20S proteasome. We hypothesize a similar scenario for SPE-7; when bound to MSP (and possibly a suite of other proteins), SPE-7 adopts transient structure making its unstructured regions inaccessible by the 20S proteasome. Once SPE-7 disassociates from MSP in the spermatids, it is again unstructured and a target for UID. We have shown that the 20S proteasomal subunit PAS-7 is present in spermatids making UID a likely possibility.

Several approaches exist for identification of proteins degraded via UID. Using purified SPE-7, we can perform degradation assays by incubating SPE-7 in vitro with purified 20S proteasomes and analyzing the degradation products by SDS-PAGE. In addition, we can perform the same degradation assays but include a known “gatekeeper” of the 20S proteasome, NQO1 (Asher et al., 2005). NQO1 binds the 20S proteasome, protecting IUPs from 20S proteasomal degradation, and is an important regulator of UID (Asher et al., 2006). We can then compare the degradation products from both assays by SDS-PAGE to determine if a “default” 20S proteasomal pathway is responsible for SPE-7 disappearance.

In a more straightforward approach, we can employ RNAi to address the same question. RNAi is a powerful tool used to manipulate expression of target genes in C. elegans and is performed by feeding (Timmons & Fire, 1998), soaking (Tabara et al., 1998), or injection (Fire et al., 1998). To independently determine possible degradation of
SPE-7 via an UID we can perform RNAi against PAS-7 as PAS-7 is present in spermatids and may contribute to UID. Loss of PAS-7 via RNAi knockdown results in embryonic and larval lethality, sterility, and abnormal meiotic progression (Takahashi et al., 2002; Rual et al., 2004; Fernandez et al., 2005; Sonnichsen et al., 2005). Following knockdown of *pas-7* expression, we can examine SPE-7 localization via immunohistochemistry.

**SPE-7 disappearance may be triggered by a change in intracellular pH.**

The assembly-disassembly-reassembly of MSP during spermatogenesis occurs concomitantly with changes in intracellular pH (King et al., 1994). MSP assembly occurs in spermatocytes, pH 6.8; disassembly occurs in spermatids, pH 6.2; and reassembly into filaments occurs in spermatozoa, pH 6.4. While the shift from 6.8 to 6.2 is only a difference of 0.6 units, in logarithmic terms this difference is substantial ($10^6$). The pseudopod itself establishes a pH gradient with pH 0.15 units higher at the leading edge where fibers assemble than at the base where fibers disassemble. We hypothesize that SPE-7 disappearance occurs promptly following separation of spermatid from residual body coinciding with a shift in pH. Consistent with experimental data, we hypothesize that SPE-7 disappearance is dependent on a change in pH within spermatids.

We can test our hypothesis of a pH-mediated trigger of SPE-7 disappearance by first determining if SPE-7 disappearance occurs before or after a change in pH. To this end, we anticipate using a membrane permeable, intracellular pH indicator to precisely determine the timing of pH change with regards to SPE-7 disappearance. The pH
indicator 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxyethyl ester (BCECF, AM; pH range of 6.5-7.5) would be preferred given that throughout spermatogenesis, the intracellular pH remains close to neutral. The range of BCECF, AM is such that in spermatocytes, pH 6.8, there would be a fluorescence signal; however the pH of spermatids would be out of range and there would be no fluorescence signal. By comparing the presence/absence of signal to the timing of SPE-7 disappearance, we can determine if SPE-7 disappearance correlates with a decrease in intracellular pH.

**SPE-7 may act as a molecular “ruler” for FB assembly.**

Purified MSP has been shown to polymerize *in vitro* with the addition of polyethylene glycol (Castillo-Olivares and Smith, 2008). The resulting crystals resemble the subfilaments that hierarchically assemble into macrofibers and are indistinguishable from subfilaments polymerized *in vivo*. We have observed MSP assemblages of similar size and shape with spe-7 mutants grown at 25°C. While these assemblages can occur in the absence of wildtype SPE-7, they are structurally more similar to MSP filaments found within the pseudopod. This data suggests the role of SPE-7 not only as a FB nucleator, but also as a regulator of FB shape and size. By extending the Castillo-Olivares study to include SPE-7 and SPE-6, we can determine if *in vitro* polymerized MSP crystals appear more like FBs with the addition of these known FB assembly factors.

**In conclusion…**

Successful *C. elegans* spermatogenesis relies on the crosstalk between cell cycle progression and cellular differentiation. The novel protein SPE-7 proves to be a critical
regulator of both of these processes; however its primary role is in the nucleation and regulation of sperm specific fibrous bodies. Outside of nematodes, SPE-7 shares no sequence or structure homology and may very well be an intrinsically unstructured protein. However like the typical cytoskeletal components, actin and tubulin, which rely on unstructured assembler proteins/domains like caldesmon (actin) and the MAP2 microtubule-binding domain, respectively, for polymerization and bundling, the highly abundant nematode cytoskeletal protein MSP appears to require an analogous organizer. Therefore, SPE-7 may be functionally homologous to previously described organizational/scaffolding proteins.
References


Appendix: Experiments without definite conclusions

Throughout the work of this thesis, several questions were left without fully developed conclusions. Our preliminary results for such experiments are included here, but require additional replicates or further analysis in order to fully address the experimental question.

We showed that SPE-7 and the pre-FB complex initially associates with the MO prior to formation of the FB. Our results suggested that formation of the pre-FB requires MO membrane proteins to properly assemble. However, it was unclear whether the pre-FB requires only membrane proteins or a fully developed, intact MO for proper assembly. To critically test this question, we investigated SPE-7 dynamics in a mutant with abnormal MOs, spe-39. SPE-39 is required for early FB-MO morphogenesis as it is involved in membrane fusion of MO vesicles and, in spe-39 spermatocytes, normal MOs are replaced with numerous small Golgi-like vesicles throughout the cytoplasm (Zhu and L'Hernault, 2003). FBs still form but are disorganized and often inaccurately partitioned to both the residual body and spermatids during the subsequent budding division. We questioned whether the presence of more MO-like structures would result in more pre-FB/SPE-7 structures. Our results show that in spe-39 spermatocytes, SPE-7 assembles into the pre-FB complex at the same time as observed in wildtype and despite the absence of fully formed MOs, every SPE-7 focus associates with an MO-like structure (Figure 28). This result suggests that, in spe-39 spermatocytes, MO associated lipids or membrane proteins that normally interact with SPE-7 are still present and associated with
these early SPE-7 enriched pre-FB complexes despite defects in normal MO vesicle fusion.

We also noticed very large SPE-7 structures in later spermatocytes. Preliminary quantitation of SPE-7 structures in wildtype and spe-39 spermatocytes reveals that, on average, the pre-FB complex grows larger in spe-39 spermatocytes (Figure 29). As we were interested in the assembly of the pre-FB complex, we limited our measurements to the first five SPE-7 positive cells. By measuring the lengths of SPE-7 structures in the first five spermatocytes, we found that while comparable in cell (+2), in cells (+3), (+4), and (+5), the sizes of SPE-7 foci are significantly larger in spe-39 mutant spermatocytes than in wildtype. This result suggests that in spe-39 spermatocytes, the initial assembly of pre-FB complexes is normal. However, in the absence of properly developed MOs, SPE-7 structures grow in an unregulated fashion. Examination of SPE-7 in (+5) spermatocytes reveals that, in contrast to wildtype, there is greater variability in size as some SPE-7 structures are more than double the size of SPE-7 structures in wildtype (Figure 30). From this preliminary data it appears that MOs are required not only for the initial assembly of the pre-FB complex, but to regulate the size of SPE-7 structures and possibly the developing FB in later spermatocytes.
Figure 28. Pre-FBs complexes appear larger in spe-39(eb9) mutant spermatocytes. Immunolocalization analysis of SPE-7 (red), MO marker 1CB4 (green), and DNA (blue) in isolated and formaldehyde fixed male gonads. The first five SPE-7 positive cells are marked with arrows and numbers with cell (0) being the most proximal SPE-7 negative.
spermatocyte. Spermatocytes marked with numbered arrows are represented below larger image and enlarged 1.5X. Scale bar=30 μm.

Figure 29. Quantitation of lengths of SPE-7 structures in wildtype (n=40) and spe-39 (n=40) spermatocytes. Pre-FB structures were measured in the earliest spe-7 expressing cells with (+1) spermatocytes being the first SPE-7 positive spermatocyte. Each unit of length corresponds to 50 pixels. Error bars represent standard deviation. Averages of statistical significance as determined by the student's t-test are represented with asterisks: p<0.5 (*), p<0.0005 (**), p<0.0001 (****). All measurements were acquired from 600X images.
Multiple SPE-7 bands are present in wildtype spermatocytes.

Bioinformatics predictions of multiple potential phosphorylation sites within SPE-7 suggested that phosphorylation events could play a role into the shifting localization pattern of SPE-7 throughout the meiotic divisions. To address this hypothesis, we investigated whether we could identify multiple, modified forms of SPE-7. To do this, we isolated SPE-7 from whole worm lysates via immunoprecipitation and subsequently analyzed the immunoprecipitated material by Western blot analyses (Figure 31). Our results show two major bands: a 42 kDa band corresponding to the experimentally determined molecular weight of SPE-7 as well as a band at 37 kDa. From previous experiments, we show that the experimental molecular weight of SPE-7 is 42 kDa, therefore it is uncertain whether the 37 kDa band is specific to anti-SPE-7 as it is also present in the SPE-7 IP. Bands of smaller molecular weight are also present in both
samples, but are of greater intensity in the IP suggesting that the same bands are present in both samples, but are proportionally different. While it is also possible that the smaller molecular weight bands are partially degraded SPE-7 products, these results suggest that the observed bands represent different forms of SPE-7 are present throughout spermatogenesis.

Figure 31. Multiple SPE-7 bands of differing molecular weight are present in SPE-7 immunoprecipitations. Immunoprecipitation with anti-SPE-7 in fem-3(q20gf) mutants followed by SDS-PAGE and Western blot analysis with anti-SPE-7. (M) Marker. (IP) SPE-7 immunoprecipitation. (L) Whole worm lysate. The SPE-7 band is distinguished by a black arrow at 42 kDa.

As SPE-7 has predicted sites of tyrosine, serine, and threonine phosphorylation we wanted to test each systematically beginning with potential tyrosine phosphorylation. To specifically test whether SPE-7 is a target of tyrosine phosphorylation, we
immunoprecipitated SPE-7 in the presence and absence of sodium orthovanadate, a tyrosine phosphatase inhibitor, and probed for the presence of phosphorylated tyrosine with anti-phosphotyrosine antibodies. So far we have been unsuccessful in our attempts to determine tyrosine phosphorylation of SPE-7. Therefore, while we presume that multiple bands of SPE-7 is the result of a possible post-translational modification, we must test not only for phosphorylation of tyrosines, but serines and threonines as well with antibodies specific to these modifications.

**MSP self assembles in spe-7 mutants.**

Presler previously showed that spe-7(mn252) mutants were inherently cold-sensitive with the most severe phenotype observed at 16°C and the least severe phenotype at 25°C. However, this conclusion was based on two distinct criteria: chromatin morphology as visualized with DIC-Hoechst and the sporadic production of a few unviable progeny (0-8) by hermaphrodites raised at 25°C. Our continued analysis of spe-7 mutants at each temperature reveals that while proper FBs never form, MSP is not completely cytoplasmic at each temperature as previously described. In contrast to wildtype, spe-7 spermatocytes grown at 16° and 20°C fail to assemble MSP into FBs and MSP remains diffuse and cytosolic. However when males are raised at 25°C, MSP assembles into long filaments that resemble the filaments observed within the pseudopod of mature spermatozoa (Figure 32). This observation suggests that in the absence of SPE-7, MSP can self-assemble at higher temperatures. To test whether the self-assembly of MSP at higher temperatures is specific to spe-7(mn252) mutants only, we investigated the
possibility of MSP self-assembly in spe-6(hc49) mutants. Both SPE-7 and MSP are present in spe-6 mutants, but FBs do not assemble normally. However in these mutants, elevated temperatures do not induce MSP self-assembly as observed in spe-7 mutants; instead MSP remains diffuse throughout the cytoplasm. Therefore it is not elevated temperatures alone that induce self-assembly of MSP in spe-7 mutants, but SPE-6 or another unknown factor that may contribute to FB assembly. To further explore the nature of this unknown factor, we also examined the localization patterns of MSP within the spermatocytes of spe-44 males raised at the same temperatures. The gonads of spe-44 males have both MSP and SPE-6 (Kulkarni et al., 2012; Leah Towarnicky, unpublished data) but lack SPE-7 and hundreds of other spermatogenesis-enriched proteins (Kulkarni et al., 2012). Our results show that in 25°C spe-44 spermatocytes, despite the presence of SPE-6 that may or may not contribute to the assembly of MSP at higher temperatures, MSP self-assembly does not occur. Taken together, this result suggests that genes specifically downstream of spe-44 may result in MSP self-assembly at 25°C. As we observed this assembly in spe-7 but not spe-6 mutants, we would predict that in a spe-6;spe-7 double mutant, no assembly would occur as SPE-6 is absent. However, this experiment has not been performed.
Figure 32. MSP self assembles in spe-7 mutants grown at 25°C, but not spe-6 or spe-44 mutants. Immunolocalization of MSP in karyosome staged wildtype, spe-7, spe-6, and spe-44 mutant spermatocytes. Scale bar=10μm.
Vita

Kari Lynn Messina (née Price) grew up in Callahan, FL and attended West Nassau High School. Following graduation, Kari attended The Florida State University in Tallahassee, FL where she earned a Bachelor of Science degree in Biological Sciences. Hoping to obtain more research experience before tackling a Ph.D. program, Kari entered the Master’s program in Biology at the College of William and Mary. In the Shakes’ lab, Kari was introduced to the world’s most fantastic model organism, Caenorhabditis elegans. Kari likes C. elegans so much that she has chosen to attend the University of California-Davis where there are multiple worm labs to choose from.