Cytological and Bioinformatical Analysis of SPE-26, a C. elegans Kelch-like Protein that Functions during the Karyosome Stage of Spermatogenesis

Stephen A. Gurley
College of William and Mary

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Cytological and Bioinformatical Analysis of SPE-26, a *C. elegans* Kelch-like Protein that Functions during the Karyosome Stage of Spermatogenesis

This thesis is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Biology from the College of William & Mary

Stephen Andrew Gurley

Accepted for: ______________________

__________________________________

Advisor: Dr. Diane Shakes

__________________________________

Dr. Lizabeth Allison

__________________________________

Dr. Shantá Hinton

__________________________________

Dr. Robert Scholnick

Williamsburg, VA

April 27, 2017
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Abstract

Prior to the meiotic divisions, spermatocytes undergo extended developmental processing termed meiotic prophase. As these spermatocytes transition out of meiotic prophase, they must cease transcription and prepare the chromosomes for the meiotic division. Our knowledge of the mechanisms that drive this transition remains incomplete.

In this study, we analyze through cytological and bioinformatics methods a poorly understood Kelch-like protein required for *Caenorhabditis elegans* spermatogenesis, SPE-26. Analysis of *spe-26* mutants reveals precocious meiotic spindle maturation relative to chromatin morphology beginning during the poorly-understood spermatogenesis-specific karyosome stage of late meiotic prophase. *spe-26* spermatocytes also display chromosome alignment and segregation defects during the meiotic divisions. Quantitative and qualitative analysis reveal that karyosome entry occurs normally, while similar analysis reveals that *spe-26* spermatocytes accumulate within the karyosome stage, suggesting SPE-26 is required for progression through and/or out of the karyosome stage. Using a newly generated antibody, immunofluorescence assays show that SPE-26 concentrates in the nucleus of karyosome stage spermatocytes, is shed to the residual body during the budding division, and is thus absent from spermatids. Next, we use various bioinformatics tools to make predictions of SPE-26 structure and function. We present the predicted secondary and tertiary structures of SPE-26, and compare sequence and structural features to its closest homologs, in addition to other well-studied Kelch-like proteins. Lastly, we predict various functional sites along the structure of SPE-26, including potential phosphorylation sites and a potential nuclear localization signal (NLS).
Chapter 1: Introduction

Cell Biology in Context

One of the overarching goals of cell biology is to understand the myriad complex processes that allow cells to function and proliferate. Among the most important of these processes is cellular division, which has often been likened to an intricately choreographed dance. In order for these cell division events to be successful, each "dancer" (i.e. proteins, protein complexes, organelles) must perform each step correctly and at the correct time. One of the most important steps of cellular division is the equitable allocation of genetic material; each daughter cell must receive the appropriate genetic information in order to survive. Errors and missteps in this "dance" can have resounding repercussions throughout the cell; proteins could end up in the wrong locations, different cellular processes could be out of sync with each other, chromosomes could fail to segregate to each daughter cell, or even, in some cases, the cell could die. Many times, errors in cellular division are the basis for certain human diseases, the most infamous being cancer. Thus, many cell biologists are actively investigating the mechanisms that allow cells to successfully execute the tightly coordinated events of cell division.

In sexually reproducing eukaryotes, genetic material is encoded in the form of genes located on chromosomes. Genetic material is passed on from one generation to the next by gametes, sperm and egg cells that are produced through highly specialized cellular processes. The production of gametes requires: 1) a transcriptional switch to the expression and subsequent translation of gamete-specific genes; 2) the assembly of gamete-specific cellular structures; and 3) a specialized form of cellular division and chromosome segregation termed meiosis. Meiosis differs from mitosis, the general form of cell division, in several key ways (Figure 1-1). During
mitosis, one cell divides into two genetically identical and diploid (possessing two copies of each chromosome) daughter cells, whereas during meiosis, a spermatocyte or oocyte divides twice, in two sequential divisions, to produce four daughter gamete cells. During the highly specialized first division, meiosis I, paired homologous chromosomes are separated, such that each daughter cell is haploid (containing only one copy of each chromosome). In the second division, meiosis II, sister chromatids are separated in a manner similar to mitotic division. The reduction division in meiosis I ensures gametes are haploid, so that when a sperm cell fuses with an egg cell (fertilization), the resulting diploid cell has a full set of chromosomes (and therefore genes) from each parent.

Spermatogenesis is the process by which diploid sperm progenitors first differentiate into specialized spermatocytes that then divide via two sequential meiotic divisions to generate four haploid sperm cells. To ensure successful transmission of genes to progeny, spermatogenesis is an extremely complex and highly regulated process (Chu and Shakes, 2013).

Defects in spermatogenesis are thought to cause human reproductive health problems that are rising in incidence. Recent evidence suggests that, globally, as many as 10-15% of couples desiring pregnancy are infertile (Cooke and Saunders, 2002). Some research suggests that male infertility is the single most common cause of infertility in these couples (Irvine, 1998), with chromosomal abnormalities in sperm being the single most common cause amongst male infertility cases, followed by sperm structural defects (Seshagiri, 2001). Infertility caused by defects in sperm structure (e.g. flagellar motility) can be treated with some success using intra-
cytoplasmic sperm injections (ISCI); however, this treatment does not correct chromosomal abnormalities (Irvine, 1998; Seshagiri, 2001).

**Spermatogenesis in other animals**

All animals have a shared evolutionary lineage, which explains why many cellular processes occur similarly throughout the animal kingdom. This also explains why cell biology insights discovered in one animal can often be applied to other animal models, as is the case for spermatogenesis (Kotaja et al., 2004; L’Hernault, 2006). While the overall process of spermatogenesis remains highly conserved, there remain important distinctions between different animals. In mammals, spermatogenesis only occurs with the aid of various accessory somatic cells and hormones that create the appropriate cellular microenvironment through signaling (Griswold, 1998). The location of these cells in the periphery of the tubular basement membrane creates a spatial signaling gradient that contributes to the centripetal spatial organization of mammalian testes. This organization complicates analysis of cellular progression through spermatogenesis, because multiple tubules must be analyzed in order to observe each stage of spermatogenesis. In another commonly studied organism, the fruit fly *Drosophila melanogaster*, spermatogenesis occurs in a tubular testis. In *D. melanogaster*, germline stem cells first differentiate into sperm-fated cells, then mitotically proliferate but maintain intercellular bridges between daughter cells in groupings called cysts (Demarco et al., 2014). Each cyst typically yields 64 spermatozoa. Like mammalian spermatogenesis, *D. melanogaster* spermatogenesis also requires the aid of accessory somatic cells. Unlike mammals, however, *D. melanogaster* spermatocytes progress through spermatogenesis along a linear array, making analysis of
progression through spermatogenesis simpler because all stages of spermatogenesis are observable within a single gonad.

**Caenorhabditis elegans as a model for spermatogenesis**

The nematode roundworm *Caenorhabditis elegans* has emerged as a powerful model organism for basic and applied biomedical research. *C. elegans* is small in size (1mm long), and possesses a relatively short generation time (about 3 days), making it ideal for laboratory manipulation (Brenner, 1974). *C. elegans* is an andro-dioecious (male/hermaphrodite) species, in which hermaphrodites produce both sperm and oocytes and are self-fertile (Brenner, 1974). This makes selfing and outcrossing for genetic crosses in *C. elegans* much simpler compared to dioecious (male/female) species. Further, the entire *C. elegans* genome has been sequenced and annotated, and is publicly available online (Harris et al., 2014), allowing the development of a powerful genetic toolkit that allows for robust analysis of *C. elegans* genes and their functions. Genomic analysis has shown that 60-80% of human genes have an identified homolog in the *C. elegans* genome, meaning that predictions of the function of human genes can be made through study of their *C. elegans* homologs (Kaletta and Hengartner, 2006). Numerous forward and reverse genetics techniques critical to modern molecular biology were originally optimized, and are still used frequently in the *C. elegans* model (Friedland et al., 2014; Frøkjær-Jensen, 2013).

In addition to the many assets of *C. elegans* as a model organism in other subfields, several unique biological features of this species make it a particularly useful model with which to study gametogenesis. In *C. elegans*, the gonad comprises approximately half the total body mass of the worm (Chu and Shakes, 2013). Conveniently for reproductive cell biologists, *C. elegans* germlines are arranged linearly and temporally along the length of the gonad; that is, as
germline stem cells differentiate into spermatocytes and then subsequently undergo meiosis, they move proximally along the length of the gonad (Figure 1-2a and 1-2c) (L’Hernault, 2006). Like goods moving along an assembly line at a factory, cells at the distal end of the germline are beginning gametogenesis, while cells at the proximal end have completed gametogenesis. This means that, upon dissection, the entire cellular progression of gametogenesis can be observed within a single germline (Chu and Shakes, 2013). Furthermore, C. elegans gametes develop without the aid of somatic accessory cells (e.g. cyst cells in D. melanogaster or Sertoli cells in mammals) (Chu and Shakes, 2013). This drastically simplifies cytological analysis of C. elegans gonads.

The unique biological characteristics of C. elegans allow for the design of elegant genetic screens to identify genes required for spermatogenesis specifically. Other model systems, such as mice or D. melanogaster, are male-female organisms, so spermatogenesis-specific defects cannot immediately be differentiated from other male fertility defects (e.g. copulation apparatus, behavior), which requires further genetic analysis. In C. elegans, however, spermatogenesis-specific defects are screened by identifying self-infertile mutant hermaphrodites that lay unfertilized oocytes that regain fertility upon mating with wild-type males (L’Hernault, 2006). This has made C. elegans an extremely powerful system for the screening of spermatogenesis (spe) genes (Chu and Shakes, 2013). Currently, nearly 50 spe genes have thus been identified as required for spermatogenesis in C. elegans, (Harris et al., 2014; L’Hernault, 2006). One such gene, spe-26 will be the subject of investigation in this thesis. Hence, these factors make C. elegans an attractive model for investigating spermatogenesis.
Overview of Spermatogenesis in *C. elegans*

**Glossary of Terms:**

<table>
<thead>
<tr>
<th>Term:</th>
<th>Definition:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermatogenesis</td>
<td>The process by which germline stem cells become spermatozoa, including a differentiation into spermatocytes, the meiotic divisions, and sperm activation of spermatids into spermatozoa.</td>
</tr>
<tr>
<td>Spermatocytes</td>
<td>Differentiated, meiotic cells undergoing spermatogenesis that give rise to four haploid spermatids.</td>
</tr>
<tr>
<td>Spermatids</td>
<td>Products of the meiotic divisions, these haploid cells remain inactive within the seminal vesicle until insemination, when they undergo sperm activation in the spermatheca to become motile spermatozoa.</td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>The final products of spermatogenesis (and the direct products of sperm activation), these fully-motile gametes fertilize oocytes and possess non-actin or -tubulin based motility using a pseudopod.</td>
</tr>
<tr>
<td>Spermatheca</td>
<td>The site of sperm storage and fertilization within the hermaphrodite gonad, spermatids undergo sperm activation in the spermatheca in the case of hermaphrodite-produced sperm. Ovulation moves oocytes from the proximal end of the gonad into the spermatheca.</td>
</tr>
<tr>
<td>Sperm Activation</td>
<td>The last subprocess of spermatogenesis, the process by which sessile spermatids develop into active and motile spermatozoa. In hermaphrodite-produced sperm, this process occurs in the spermatheca. In male-produced sperm, this process occurs upon insemination within the mated hermaphrodite uterus.</td>
</tr>
</tbody>
</table>

Spermatogenesis in the two *C. elegans* sexes is largely similar, with several important differences. Male worms only possess a single-armed gonad, while hermaphrodites possess a two-armed gonad. Males continuously produce sperm from their last larval stage (L4) throughout their adult life (Chu and Shakes, 2013). Hermaphrodites, however, only produce
about 300 sperm, after which they undergo a one-time switch as they transition into adulthood, and their germlines permanently switch to oocyte production. The sex of *C. elegans* progeny is determined by copy number of the X chromosome; unlike humans, there is no Y chromosome. Hermaphrodites are diploid for the X chromosome, while males are haplo-X, meaning they possess an unpaired X chromosome (L’Hernault, 2006). One consequence is that during the separation of homologs in anaphase I, the X chromosome "lags" behind the other chromosomes being pulled apart from the metaphase I plate.

Due to the linear and temporal organization of *C. elegans* germlines, the stage of spermatogenesis can be identified for each spermatocyte. This is achieved by comparing each cell’s relative location along the length of the gonad, its nuclear chromatin morphology, and by using known cell cycle and spermatogenesis markers through immunofluorescence (Chu and Shakes, 2013). Hence, the *C. elegans* germline has been subdivided into distinct zones (Figure 1-2A and 1-2C).

**Mitotic Proliferative Zone:** The distal-most region of the sperm-producing germline (L4 hermaphrodites or L4-adult males) is the mitotic region, which contains germline stem cells that are, at this point, non-fated (Chu and Shakes, 2013). Maintenance of these germline stem cells in mitotic proliferation is achieved through juxtacrine signaling by a single somatic distal tip cell, which extends protrusions along the distal end of the gonad to prevent meiotic entry of the germline stem cells.

**Meiotic Prophase and Early Gametogenesis:** Upon exit from the range of the distal tip cell's juxtacrine signaling, germline stem cells transition not only to meiosis, but also become
sperm-fated. Homologous chromosomes pair and a protein-based scaffold, the synaptonemal complex, forms between them. Once their homologs have paired and fully synapsed, the cells enter one of the longest stages of meiotic prophase in these germlines, pachytene, during which crossing over occurs. Crossing over is a meiosis-specific event, during which genetic material is exchanged between homologs (Figure 1-3). Following pachytene is diplotene, during which the synaptonemal complex dissociates and homologs disjoin. Pachytene and diplotene are the last stages in which spermatocytes are actively transcribing genes. Unlike mammalian (Bettegowda and Wilkinson, 2010) or Drosophila (Fabian and Brill, 2012) sperm, there is no post-meiotic burst of transcription in *C. elegans* sperm, meaning that all products required for sperm function must be transcribed by the end of diplotene (Chu and Shakes, 2013)

**Karyosome Stage:** Following desynapsis, spermatocytes enter a final phase of meiotic prophase, which is a spermatogenesis-specific stage in *C. elegans* called the karyosome stage (Figure 1-2c). During this phase, the chromatin hypercondenses and the chromosomes coalesce into a single mass in the center of the nucleus, detached from the envelope (Shakes et al., 2009). Prior work in the Shakes lab (Shakes et al., 2009) first characterized this stage in *C. elegans*. Cytological suggests that karyosome cells are the first transcriptionally inactive cells of spermatogenesis (Shakes et al., 2009). In karyosome cells, chromosomes appear punctate, and are brightly tagged by a marker for chromatin compaction, phospho-Histone H3 Ser10 (Shakes et al., 2009). Upon karyosome exit and entrance to diakinesis, spermatocytes detach from the shared syncytial rachis, a central core of cytoplasm that interconnects the developing germ cells from the distal mitotic zone through the karyosome zone (Shakes et al., 2009). From these results, our lab has previously proposed that the karyosome stage might be a last holding stage for spermatocytes on the rachis before they achieve "cellular independence" and begin the
meiotic divisions without somatic support as seen in mammals and *Drosophila* (Shakes et al., 2009).

**Into and Through the Meiotic Divisions:** At the end of the karyosome stage, meiotic centriole-based spindles begin to nucleate, although the centrioles remain close to each other and the spindles are not yet full-sized. As cells enter diakinesis, chromosomes dissociate from one another, and meiotic spindles continue to grow and centrioles continue to migrate toward opposite poles of the cell. During the transition from diakinesis to prometaphase, the nuclear envelope breaks down as the chromosomes begin moving towards alignment at the metaphase plate, an event referred to as chromosome congression. Once the chromosomes are fully aligned, during metaphase I, homologs separate and segregate in anaphase I. In XO males, presence of a lagging X helps to distinguish anaphase I from anaphase II. During metaphase II, one of the two resulting secondary spermatocytes possesses an X chromosome, while the other does not. Cytokinesis between these two secondary spermatocytes is often incomplete with these cells remaining connected by an intercellular bridge. During anaphase II, four haploid cells are formed, all of which are initially connected by a shared cytoplasmic space.

**Post-Meiotic Development:** Following anaphase II, a polarization event results in contents unnecessary for sperm motility and fertilization being shed to a central cytoplasmic space, the residual body. The haploid sessile spermatids then bud off from the residual body, at which point they exist as individual cells. In males, these spherical spermatids are metabolically quiescent until they are activated. During sperm activation, cells extend a pseudopod and a vesicular fusion event places new proteins on the cell surface (Chu and Shakes, 2013; L’Hernault, 2006). Activated sperm are then motile and fertilization-competent.
Karyosome Stage in *C. elegans* and Beyond

The gametes of many other organisms, including humans and *D. melanogaster*, also pass through a karyosome stage. In these organisms karyosome formation is oogenesis-specific. In humans, very little is known about the karyosome, also sometimes called the 'karyosphere,' besides that the chromatin is extremely transcriptionally inactive (Parfenov et al., 1989). Studies in *D. melanogaster* have found that karyosome formation is regulated in part by the ratio of F-actin:G-actin present in the oocyte nucleus (Djagaeva et al., 2005). Actin has recently emerged as an important key player in nuclear chromatin dynamics. Work across several different model systems, including *Xenopus laevis, Sacchromyces cerevisiae, Drosophila*, and mammals has demonstrated actin's evolutionarily widespread and complex roles within the nucleus such as chromatin remodeling and positioning (Olave et al., 2002). A key step in karyosome formation is the detachment of chromatin from the nuclear envelope. In *D. melanogaster*, this process is in part regulated by the phosphorylation of a chromatin binding protein (BAF) by the kinase NHK-1 (also known as VRK) (Lancaster et al., 2007, 2010). Little is known about the *C. elegans* homologs of these proteins (BAF-1 and VRK-1 respectively), and preliminary experiments in loss of function mutants have not proven fruitful due to defects in germline proliferation (Shakes et al., 2009).

Protein Structure and Function, and their Implications for Human Disease

Proteins carry out a dizzying array of functions within a cell, performing many of the roles to ensure the "dance" of cell division goes smoothly. Proteins function in part because of their unique structure, as they are built with a specific sequence of different building blocks, amino acids, and also in part because of their location within the cell. Many human diseases are caused by protein malfunction (Walker and LeVine III, 2000). In some cases, the structure of an entire
protein is disrupted, causing aberrant aggregates that are oftentimes toxic. Many times, these toxic aggregates are mislocalized within a cell, causing disruptions in various cellular processes, as is the case in Alzheimer's disease. In other cases, the folding of only one specific domain, or functional piece of a protein, is disrupted, while the remaining aspects of the protein's structure remain intact. Molecular and cell biologists have developed several approaches to elucidate the function of unknown proteins and determine their involvement in various cellular processes. One approach is to study the cellular and developmental repercussions of mutants for the gene that encodes that protein. By determining malfunctions in cellular events in mutants, biologists can infer the normal function of the protein. Another approach is to study the localization of the protein within the cell, and use that information to infer the function of the protein. Proteins located on a cell's plasma membrane are typically involved in signaling or transport, while proteins located within the cell nucleus are typically involved in gene expression, and DNA replication and repair. A third approach, bioinformatics, has developed since the turn of the 21st century. Bioinformatics pools together data about a given protein’s sequence, structure, and function into immense online databases. These databases can then be utilized to develop prediction tools that can predict unknown aspects of protein structure and function. Many scientists, from basic researchers studying fundamental cellular processes, to translational scientists who apply scientific findings to developing cures and treatments for diseases, use these approaches to understand the function of proteins.
**spe-26: Prior knowledge and unanswered questions**

One gene required for spermatogenesis is *spe-26*. Originally characterized in 1995 through analysis of loss of function mutants, *spe-26* is a gene of unknown function (Varkey et al., 1995). These mutants experienced severe meiotic division defects, resulting in large, multinucleate cells that have failed to divide (Varkey et al., 1995). Northern blot and *in situ* hybridization experiments in WT (wild-type) worms showed enriched expression of *spe-26* transcript in spermatocytes throughout the germline (Varkey et al., 1995). Sequence analysis revealed the presence of several known domains within the structure of SPE-26. Most notably, *spe-26* contains several β-propeller-shaped Kelch domains, making it a member of the Kelch-like superfamily of proteins characterized by a windmill tertiary structure composed of many Kelch domains (Stogios and Privé, 2004; Varkey et al., 1995). Kelch, the founding and most well-studied member of this superfamily, is an actin binding protein required for ring canal development during fruit fly oogenesis (Robinson and Cooley, 1997). Kelch is named after the German word for “cup,” because of the cup-like morphology of *kelch* oocytes in *Drosophila* (Robinson and Cooley, 1997). In Kelch and many other Kelch-like proteins such as *Limulus* scruin, the Kelch repeat domains and windmill structure bind actin, making actin-binding the canonical function of Kelch-repeat domains (Adams and Kelso, 2000). Members of this important protein family, however, are now known to perform tremendously diverse cellular functions beyond actin binding from neuronal differentiation, to cytoskeletal rearrangements, to enzymatic activity, and have been found in organisms across the animal kingdom, including humans (Adams and Kelso, 2000). Within this family, there are several subclasses with additional protein domains. Via sequence homology, SPE-26 is most closely related to a large subclass generally characterized by the presence of two additional domains, BTB and BACK
Despite this sequence homology, SPE-26 is unusual because it contains a BACK domain without the typically-associated N-terminal BTB domain, which typically function together to regulate transcription or allow for protein dimerization (Stogios and Privé, 2004). Indeed, SPE-26 is the only known *C. elegans* protein and one of only a handful of known Kelch proteins that contain a BACK domain without a BTB domain (Stogios and Privé, 2004). Interestingly, there are three such proteins in humans, whose functions remain to be elucidated (Stogios and Privé, 2004).

There still remain, however, many unanswered questions about SPE-26. Most importantly, where does SPE-6 localize within cells and what is its function at the molecular level? Knowing where within the spermatocyte SPE-26 localizes, can yield important clues about its function. Varkey et al. (1995) initially speculated that SPE-26 may bind actin, similarly to *Drosophila* Kelch. However, now that we know about the tremendously diverse functions of Kelch-like proteins, further data are required to make more specific predictions. BTB domains can also be associated with E3 ubiquitination and sumoylation, and BACK domains are thought to orient substrates for binding (Stogios and Privé, 2004). Despite their prevalence, little is known about the function of the BTB and BACK domains beyond a few studied proteins. The unusual structure of SPE-26, with a BACK domain lacking the usually accompanying BTB domain, may lend it a unique function, and insights into SPE-26 function may broaden our understanding of these domains.

**Key Aims and Research Questions**
Using our existing knowledge of the mutant phenotype and considering the diversity of function within the Kelch superfamily, we have generated several competing models of SPE-26 function that will guide these cytological and bioinformatic investigations. In one model, SPE-26 protein may be functioning primarily as a cell cycle regulator, and in some way be regulating the events of late meiotic prophase in preparation for the meiotic divisions. Toward this end, we will use various cell cycle markers to compare spe-26 mutant and WT spermatocytes to determine if the entry and exit from various substages of late meiotic prophase are disrupted, or if the localization of these markers is altered. Our second model is that SPE-26 protein functions primarily to modulate the activity of certain cytoskeletal elements involved in meiotic divisions, particularly actin. Indeed, this model is supported by the nuclear actin organizing function of Drosophila Kelch and its association with karyosome chromatin condensation (Djagaeva et al., 2005). Toward this end, we will elaborate on the cytoskeletal morphologies that Varkey et al. (1995) described, and attempt to find the earliest point during spermatogenesis at which the spe-26 mutant phenotype diverges from WT. We will also study the post-meiotic budding division in spe-26 mutants. In parallel studies, we will examine how the localization pattern of SPE-26 protein may be altered in the subset of spermatogenesis-defective mutants with defects transitioning into or through the meiotic divisions.

This thesis is based on two major aims: first, furthering our understanding of C. elegans spermatogenesis, particularly the karyosome stage; and second, drawing insights as to the function of SPE-26 protein. More specifically, I have employed three distinct approaches to
characterize SPE-26 and, in the process, further explore the formation and progression through the karyosome stage of meiotic prophase.

1. Using new markers to characterize the cellular and developmental defects of spe-26 mutants, particularly those that impact entry into, through and beyond the karyosome stage (Chapter 2).

2. Using a newly generated antibody against SPE-26, to characterize its sub-cellular localization pattern during different phases of spermatogenesis (Chapter 2).

3. Using bioinformatics databases and modeling programs, to predict SPE-26 structure and function (Chapter 3).

**Figure 1-1: Comparison of Mitosis and Meiosis**

A. Diploid organisms contain a pair of copies of each chromosome, one inherited from each parent (one grey, one black). These two chromosomes are thus described as a pair of
homologs. After cells have prepared for division and during division itself, chromosomes are comprised of two identical chromatids.

B. During mitosis, sister chromatids are separated into two daughter cells. Each daughter cell is diploid.

C. During meiosis, two sequential divisions result in four haploid daughter cells called gametes, used for sexual reproduction. In meiosis I, the homologs of primary gametocytes are separated, resulting in two secondary gametocytes. In meiosis II, the sister chromatids of each chromosome are separated, resulting in four haploid gametes.

Figure 1-2: Schematics of *C. elegans* Spermatogenesis

A. *C. elegans* germlines are organized in a linear and temporal array. At the distal end of the germline is the distal tip cell, which creates a stem cell niche termed the mitotic zone through juxtacrine signaling. As cells leave this stem cell niche, they enter an extended meiotic prophase. Crossing over occurs in the pachytene stage. Germline cells from the
mitotic zone until the late meiotic prophase substage karyosome share a syncytial space called the rachis, which provides nutrients to cells. Karyosome cells cinch off from the rachis, and progress through diakinesis and the meiotic divisions.

B. A cross-sectional view of the *C. elegans* germline. Germline cells surround a shared syncytial space termed the rachis.

C. Diagram of late meiotic prophase and meiotic divisions in *C. elegans* spermatogenesis.

Pictured are simplified diagrams of cells, highlighting their connections to the rachis (which end after the karyosome stage), relative size and shape, presence/absence of nuclear envelope (which breaks down during diakinesis), and MTOC presence (denoted by *). Further, grey coloring denotes "waste" products that are shed from sperm into the residual body during the budding division.

**Figure 1-3: Overview of Meiotic Recombination**

One key feature of the meiotic program is recombination of homologous chromosomes during prophase. During this process, chromatids on paired homologs are bound together by a structure called the synaptonemal complex, which forms during early meiotic prophase. This complex holds homologs in place as dsDNA cuts allow crossing over and recombination of genetic
material in a complex process during pachytene. The synaptonemal complex breaks down during diplotene, and the absence of a synaptonemal complex marks the completion of diplotene.
Chapter 2: SPE-26, a *C. elegans* Kelch-like protein, concentrates in the nucleus of karyosome-stage spermatocytes and is required for normal meiotic chromosome segregation.

The following chapter is a draft of the results and discussion sections of a manuscript currently in preparation.

**Authors:** Stephen Gurley, Sindhura Kolachana, Amanda Rones, Elena Parcell, and Diane C. Shakes.

**Co-author roles:**

Gurley collected the majority of the data reported herein (with occasional assistance from Kolechana, Parcell, Rones, and Shakes) with the following exceptions: Kolechana, Parcell, and Rones collected and analyzed the Nuclear Pore/PHisH3ser10 data presented in Figure 2-6; Kolechana and Rones collected and analyzed SYP-4 data and performed quantitative analysis on karyosome number presented in Figure 2-5. Gurley wrote the initial draft of the manuscript, and then edited it with thorough input from Dr. Shakes. Kolechana, Parcell, and Rones have also provided helpful suggestions for the manuscript text.
Results:

Late meiotic prophase and division defects of spe-26 mutants

Varkey et al. originally described both "large" meiotic microtubule asters as well as chromosome segregation and cytokinesis defects in spe-26 mutant spermatocytes, suggesting a connection between SPE-26 and cytoskeletal events during meiotic divisions (Varkey et al., 1995). What remained unclear, however, was the point of divergence in spe-26 mutants when microtubules became aberrantly large. In order to determine exactly when during spermatogenesis the spe-26 microtubule defects are first detectable, immunocytology was used to visualize microtubule and chromatin patterns in developing spermatocytes from late meiotic prophase and through the meiotic divisions.

By comparing the entire sequence of spermatogenesis in whole germlines of WT and spe-26 males, qualitative differences in the relative sizes of these zones could be determined. The zone of karyosome stage spermatocytes, which in WT is only a few cells long (representative image shown in Figure 2-1A, yellow zone), is noticeably expanded in spe-26 male germlines (representative image shown in Figure 2-1B, yellow zone). To examine cell-level defects in more detail, this analysis was repeated in flattened germline preparations. In WT germlines, microtubules within meiotic prophase spermatocytes remained in a non-centrosomal, network pattern, without MTOCs and meiotic spindles present, until the very end of the karyosome stage when only 1-2 cells with both networked microtubules and tiny microtubule asters could be observed (Figure 2-1C, late K). In spe-26 germlines, however, many more karyosome cells possessed both microtubule asters and networked tubulin and many of these asters were quite large (Figure 2-1D and 2-1E late K). Furthermore, within these mutant karyosome spermatocytes, the two microtubule asters had already separated and migrated to opposite sides
of the nucleus. When these cells reach the diakinesis stage, the migration state of their asters, along with these asters’ large size, are both indicative of a maturing microtubule spindle (Figure 2-1D and E, Dk). Despite these maturing spindles, these cells contain an intact nuclear envelope, exhibit pre-M phase chromatin morphology, and are still attached to the rachis, as evidenced by the presence of networked microtubules as well as astral microtubules (Figure 2-1D and E, Dk). We believe that these are the "large" spindles that Varkey et al. reported; they are prematurely large relative to other markers for spermatogenesis progression.

In WT spermatocytes, the entry into meiotic M-phase (diakinesis to metaphase I) is accompanied by cellular detachment from the rachis, the disassembly of networked microtubules during diakinesis, (Figure 2-1C, Dk), and nuclear envelope breakdown (NEBD) (Figure 2-1C, Dk and Met I). spe-26 mutants exhibit multiple spermatogenesis defects during M-phase. While spermatocytes in the moderately severe (as described by Varkey et al. in terms of spermatid production defect) hc138 allele formed a normal metaphase plate just over half the time (Figure 2-1D, Met I inserts), those in the more severe hc139 allele rarely formed a normal metaphase plate (Figure 2-1E, Met I inserts, only 8/40 were normal). In both alleles, defective metaphase I plates displayed several defects, including chromosome alignment failure and microtubule-chromosome attachment failure (Figure 2-1D and 2-1E Met I defects, abnormal). Later, during anaphase I, chromosome segregation occurs unequally (Figures 2-1D and 2-1E, Ana I inserts). Despite the chromosome segregation defects in meiosis I, these mutant spermatocytes duplicate their meiotic spindles as they attempt the second meiotic division, with the chromosomes again dividing unequally (Figures 2-1D and 2-1E, Met II and Ana II inserts).

Following anaphase II, WT spermatocytes discard cellular contents unnecessary for nematode sperm function—like microtubules and actin microfilaments—into a central residual
body (Figure 2-1C). The discarding of microtubules in WT is achieved through microtubules redistributing from the centrosome to the residual body as non-centrosomal microtubules (Winter et al. manuscript in preparation). Subsequently, WT spermatids separate from the residual body through a specialized form of cell division termed the budding division. While the microtubules of spe-26 terminal cells do partition away from the haploid sperm nuclei, spermatids fail to separate from the residual body (Figure 2-1D and 2-1E). It remains unclear whether these cells attempt the budding division, then fail, or if they never try. Preliminary DIC microscopy evidence suggests they attempt to bud is made but fail (data not shown). This preliminary experiment, however, requires further corroboration. Importantly, later stage terminal cells in alleles were observed with diffuse tubulin throughout the cell, suggesting eventual loss of microtubule organization (Figure 2-1E and data for other alleles not shown).

**SPE-26 localization**

These microtubule, chromosome segregation, and cytokinesis defects of spe-26 mutants suggest several possible functions for the SPE-26 protein. SPE-26 may function to organize meiotic cytoskeletal machinery necessary for divisions and cytokinesis (a function consistent with many canonical members of the Kelch-like superfamily, see next chapter), or it may be in some way preparing chromosomes for the meiotic division. Further, it was unknown whether or not the more moderate alleles it112 and hc138, expressed a stable but modified version of the spe-26 protein, or no stable spe-26 protein at restrictive temperature. We predicted that severity of spermatid production failure phenotype of these different alleles might correspond with different levels of SPE-26 protein.
In order to test these hypotheses and further analyze cellular distribution of SPE-26, we generated anti-peptide polyclonal antisera against SPE-26 (Figure 2-2A, antibody binding site). The specificity of this polyclonal anti-SPE-26 antibody was tested with a western blot and with immunofluorescence. In the western blot experiment, actin was used as a loading control, and MSP (a major component of nematode sperm) was used as to verify sperm production in the various alleles. The predicted molecular weight of SPE-26 is 65kDa (WormBase, 2004). Adult \textit{him-8} (effectively WT) males have both developing spermatocytes and post-meiotic sperm (MSP positive) whereas \textit{him-8} hermaphrodites have post-meiotic sperm (MSP positive), but lack developing spermatocytes since they have shifted over to producing only oocytes. A band at approximately 65kDa was observed in \textit{him-8} adult males but not \textit{him-8} adult hermaphrodites, suggesting SPE-26 is present in spermatocytes but not oocyte producing germlines (Figure 2-2B and 2C). \textit{fem-3} (gain of function) mutants are somatically female but produce spermatocytes throughout their lives (MSP positive), while \textit{fem-3} (loss of function) mutants never produce spermatocytes and only produce oocytes (MSP negative). A strong 65kDa band was observed in \textit{fem-3} (gain of function) mutants but not \textit{fem-3} (loss of function) mutants, further indicating a spermatocyte-only expression (Figure 2-2B and 2C).

Three \textit{spe-26} mutant alleles were also used for this experiment, all of which were previously shown to be MSP positive at both permissive and restrictive temperatures (unpublished results). \textit{spe-26} (\textit{it112}) and \textit{spe-26} (\textit{hc138}) are temperature sensitive (ts) point mutations, while \textit{spe-26} (\textit{hc139}) is a ts premature stop that stops the SPE-26 protein before the antibody binding site (Figure2-2A). Amongst the \textit{spe-26} mutants analyzed, a band at 65kDa was not observed in \textit{hc139}, as expected, nor was such a band observed in \textit{hc138} (Figure 2-2B). Unexpectedly, a band was observed in \textit{it112} raised at restrictive temperature, which Varkey et al.
(1995) describe as the most moderate allele which does produce some sperm even at restrictive temperatures (Figure 2-2B and 2C). Furthermore, equal levels of SPE-26 were observed in it112 worms raised at both 16°C and 25°C (Figure 2-2C). These results suggest that it112 SPE-26 is actually present in it112 spermatocytes, albeit with at least some aspects of its function disrupted. Puzzlingly, two, closely-sized bands both at a higher molecular weight, approximately 75kDa, were observed in some of the samples. In him-8 males, only the higher of the two bands was observed, while in him-8 adult hermaphrodites, only the lower of the two bands was observed (Figure 2-2B and C). In fem-3 (gain of function) and fem-3 (loss of function), both bands were observed (Figure 2-2B). It is unclear whether this band indicates an alternate form of SPE-26, perhaps with some post-translational modification, or an unrelated protein of similar structure. Expression data on WormBase indicate that SPE-26 may be expressed (albeit perhaps spliced differently) in gut tissue in all adult worms, perhaps justifying the presence of other bands (WormBase, 2004). This model would not, however, explain the differential presence/absence of the higher or lower band dependent upon sex of worm. Importantly, the presumed male-specific SPE-26 band at 65kDa has been experimentally verified in multiple, independent replicates of this western blot experiment.

Given the finding that SPE-26 is present in spermatocytes but not in spermatids, SPE-26 was analyzed using epifluorescence microscopy to determine when specifically in spermatogenesis SPE-26 is expressed, and if and how its localization changes throughout spermatogenesis. Our immunofluorescence results corroborated many of our findings from our western blot: SPE-26 is expressed in germlines undergoing spermatogenesis, such as WT adult males (Figure 2-2D) and WT larval L4 hermaphrodites (Figure 2-2F, left), but not in germlines exclusively undergoing oogenesis, such as WT adult hermaphrodites (Figure 2-2F, right). In WT
male germlines, SPE-26 can be first detected in mid-pachytene stage spermatocytes (figure 2D, P label and insert, represent first pachytene cells that express SPE-26). Further, after initial diffuse (both nuclear and cytoplasmic) localization, high levels of SPE-26 suggests the protein concentrates in the nucleoplasm during the karyosome stage, primarily surrounding rather than colocalizing with the tightly compact chromatin (Figure 2-2D, K label and insert, Figure 2-2E). SPE-26 remains concentrated in the nucleoplasm through diakinesis. Once the nuclear envelope breaks down (NEBD) and the meiotic divisions initiate, SPE-26 again distributes throughout the cell. During post-meiotic polarization and the budding division, SPE-26 localizes to the residual body (Figure 2-2D, Bud.). As a result, spermatids possess no SPE-26, consistent with the lack of the 65kDa SPE-26 band in our western blot analysis of him-8 adult hermaphrodites (Figure 2-2B and C, him-8 hermaphrodite lanes).

In order to determine the effect of various mutations on SPE-26 protein localization, similar immunofluorescence experiments were conducted, this time with various mutant alleles (Figure 2-2E). Mutants lacking SPE-44, a transcription regulator gene required for the expression of many spermatogenesis-specific genes (Kulkarni et al. 2012) did not express SPE-26 protein (Figure 2-2G, spe-44), suggesting that spe-26 expression requires SPE-44 either directly or indirectly. Furthermore, our antibody failed to specifically label anything beyond baseline background staining in non-conditional spe-26 (eb8) and weakly temperature sensitive spe-26 (hc139), which both contain premature stop codons before the epitope of our SPE-26 antibody (Figure 2-2G). This further indicated that the epifluorescence signal observed in WT by our anti-SPE-26 polyclonal antibody is specific, and not simply background. Conversely, a strong signal in spe-26 (it112) male germlines was detected after pachytene (Figure 2-2G), potentially indicating SPE-26 is indeed present, consistent with the 65kD presumed SPE-26 band
observed in our western blot (Figure 2-2B and C, it112 lanes). In these spe-26 (it112) germlines, however, SPE-26 failed to concentrate within the nucleoplasm during the karyosome stage, suggesting a mislocalization phenotype (compare Figures 2-2D and 2-2G).

In the absence of a differentially localized signal, it was difficult to definitively confirm that the signal in it112 was indeed real and not simply background nonspecific staining (Figure 2-3A and B). Thus, to verify our anti-SPE-26 observations in spe-26 (it112) germlines and verify that dim, non-nuclear signal in WT male karyosome spermatocytes was indeed simply background, we prepared samples in a preliminary experiment similarly to our previous experiment, but instead analyzed them using a confocal microscope. Confocal microscopy minimizes out-of-focus light from other z-planes, and facilitates the imaging of several z-planes of a sample nearly simultaneously. With out-of-focus light minimized, the contrast in SPE-26 signal intensity in him-8 spermatocytes (Figure 2-3C and 3F) between karyosome nuclei and cytoplasm is made clearer than in epifluorescence. Next, him-8 SPE-26 (Figure 2-3C and 3F) staining was compared with spe-26 (it112) SPE-26 staining (Figure 2-3D and G) after imaging both with identical laser intensities and exposures (optimized for him-8 staining). Given the results of the previous experiment (Figure 2-2E), spe-26 (hc139) SPE-26 staining was included as a negative control (Figure 3-E, 3H). For analysis, the SPE-26 (red) channel in each image was heat-scaled with the lowest possible intensity (0) colored coldest (purple), and the highest possible intensity (4095) colored warmest (red) (Figures 2-3I, J, and K). Since these images were taken with the exact same laser intensity, intensity of staining in each could be compared to determine if there was a difference in signal intensity between karyosome cell cytoplasm in him-8 (Figure 2-3I) and in spe-26 (it112) (Figure 2-3J). This analysis indicated a noticeable difference in signal intensity, with spe-26 (it112) cells staining brighter than him-8 cytoplasm of
similarly-staged cells (see arrows Figure 2-3D and 2-3E). Hence, these preliminary results suggest that spe-26 (it112) spermatocytes possess an altered version of SPE-26, which fails to concentrate within the nucleus. The WT SPE-26 staining results also support the conclusion that WT SPE-26 exhibits a nucleus-concentrated localization in karyosome stage spermatocytes.

Further Cell Cycle Defects in spe-26 mutants

Given the WT accumulation of SPE-26 inside the nucleus specifically during the karyosome stage, the SPE-26 mislocalization defects observed in spe-26 (it112), and onset of meiotic microtubule defects during the karyosome stage in all studied spe-26 mutants; we sought to investigate any further cell cycle defects during the karyosome stage. To test this, the monoclonal antibody MPM-2 was employed in immunofluorescence. MPM-2 is an M-phase marker that specifically binds to the diverse phosphorylated substrates of cyclin-dependent kinase 1 (CDK1)/Cyclin B. Standard epifluorescence experiments using MPM-2 were inconclusive regarding any localization defects in spe-26 mutants compared to WT due to signal from out of focus light interfering (data not shown). To remedy this, a preliminary experiment using confocal microscopy was used to analyze MPM-2-stained slides. While MPM-2 staining is brightest during M-phase (see red arrows Figure 2-4), specific staining in WT begins during late diplotene as MPF begins phosphorylating targets (Figure 2-4A). At this point, MPM-2 signal is only observable in the cytoplasm, suggesting that MPF's targets are initially entirely cytoplasmic. When WT spermatocytes reach the karyosome stage, however, MPM-2 signal localization becomes more complex. In some WT karyosome stage cells, MPM-2 staining remained present only in the cytoplasm (Figure 2-4A, dark yellow arrow). In other WT karyosome stage cells, MPM-2 was observed everywhere in the cell except for the space
occupied by the compact karyosome, including the nucleoplasm (Figure 2-4A, light yellow arrow). One hypothesis is that the former localization is observable in early karyosome stage cells, while the latter is observable in later karyosome stage cells, although this hypothesis is yet untested. Interestingly, similar analysis of spe-26 mutant spermatocytes revealed a failure of MPM-2 signal to ever enter the nucleoplasm during the karyosome stage (Figure 2-4B, 2-4C, and 2-4D, see dark yellow arrows). Despite this, after nuclear envelope breakdown (NEBD), MPM2 localizes throughout both WT and mutant spermatocytes, as they fully enter M-phase. This preliminary result, taken together with the premature spindle maturation defect (Figure 2-1D and E, Dk), suggests that, in the absence of SPE-26, the onset of M-phase events within the nucleus are delayed relative to those in the cytoplasm.

**Analysis of key karyosome stage features in spe-26 mutants.**

Given these numerous defects in spe-26 mutants beginning around the karyosome stage, we sought to determine if the transition into or out of the karyosome stage was in any way altered. One hypothesis that would explain both these defects and the timing of SPE-26 localization shifts is that SPE-26 is required for normal karyosome entry. Some of the defining features of the karyosome stage, as originally described by Shakes et al. in 2009, include the absence of a synaptonemal complex, coalescence of condensed chromosomes into a single mass, and transcriptional inactivity. To determine if SPE-26 is required for any of these processes, the cytology of karyosome entry was compared in WT and spe-26 (it112) and (hc139) using several cellular markers originally used by Shakes et al. to define the karyosome stage. If SPE-26 is required for specific aspects of karyosome entry, one would expect timing defects in one or more
of these processes and a buildup of cells transitioning from the diplotene stage to the karyosome stage.

Failure to disassemble the synaptonemal complex prior to the karyosome stage might prevent chromosomes from fully coalescing into a tight, compact structure. Using an antibody against SYP-4, a central element in the meiotic synaptonemal complex (Smolikov et al., 2009), synaptonemal complex disassembly in WT and spe-26 spermatocytes was compared. In WT, the synaptonemal complex begins to breakdown during diplotene (Smolikov et al., 2009; Figure 2-5A). Diplotene-karyosome transition cells were defined in WT as cells with intermediate chromatin morphology, and SYP-4 staining revealed these cells are SYP-4 negative, indicating a complete disassembly of the synaptonemal complex (Figure 2-5A). Once these cells transition into the karyosome stage, they remain SYP-4 negative (Figure 2-5A). Analysis of spe-26 alleles revealed no defect in the timing of synaptonemal complex disassembly (figure 2-5B and 2-5C). SYP-4/DAPI images were then quantitatively analyzed to determine the average number of diplotene-karyosome transition cells/germline as well as the average number of karyosome stage cells/germline. The average numbers of transition cells and karyosome cells per germline were then compared between WT and spe-26 (it112) and (hc139) (2-5D). Unexpectedly, there was no significant difference in average number of diplotene-karyosome transition cells / germline between WT (him-5) and spe-26 (it112) and there was actually a slight but significant decrease between WT and spe-26 (hc139) (2-5D). This result suggests that SPE-26 is not required for karyosome entry. Conversely, there was a large and significant increase in the number of karyosome stage cells in spe-26 (hc139) but not in the less severe allele spe-26 (it112) (2-5D).

Given these results, we next sought to determine if any of the other defining characteristics of karyosome were disrupted in spe-26 karyosome cells. To compare relative
chromatin compaction between WT and spe-26, we next used an antibody that binds a phospho-epitope of histone H3 only when phosphorylated at serine 10 (pHisH3-serine10), a configuration that in karyosome cells suggests chromosome hypercompaction (Shakes et al., 2009). When anti-pHisH3-ser10 antibody is used in conjunction with an antibody against the nuclear pore complex, the small size of karyosomes relative to the nucleus, and the chromosomes' detachment from the nuclear envelope, can be better analyzed. In WT, pHisH3-ser10 staining increases in intensity as cells transition from diplotene to karyosome, reaching its brightest intensity during the karyosome stage (Shakes et al., 2009; figure 2-6A). WT chromatin begins detaching from the nuclear envelope during the diplotene stage (Figure 2-6A). This detachment remains incomplete until cells transition from diplotene to the karyosome stage (Figure 2-6A). Analysis of both itl12 and hc139 reveals that neither chromosome compaction nor detachment are noticeably affected by the absence of SPE-26, suggesting SPE-26 is not required for either of these processes (Figure 2-6B, 2-6C).

Given that spe-26 spermatocyte chromosomes successfully coalesce during the karyosome stage, we sought to determine whether, like in WT, this corresponded with transcriptional inactivity. This association is particularly salient given the known involvement of other BTB-BACK-Kelch proteins in transcriptional regulation (Adams and Kelso, 2000). Previous, unpublished data suggest that spe-26 spermatocytes overproduce certain proteins required for spermatogenesis, particularly MSP (Uyehara, 2014). One potential explanation for this could be that SPE-26 functions in transcriptional regulation, either of only spermatogenesis-specific genes or a more global regulation. SPE-44 is an important transcriptional regulator of spermatogenesis genes; and in WT gonads, it localizes along the length of autosomes from the early pachytene stage through the diplotene-karyosome transition (Figure 2-7A). spe-26
spermatocytes exhibit a similar pattern (Figure 2-7B, 2-7C), suggesting that neither the initial binding of SPE-44 to the chromosomes nor its subsequent release from the chromosomes is altered in these mutant spermatocytes.

While our SPE-44 experiments suggested spermatogenesis-specific, SPE-44-regulated expression is not altered in spe-26 mutants, we next sought to determine whether or not global transcription is properly suppressed during the karyosome stage (Shakes et al., 2009) was disrupted in these mutant spermatocytes. This analysis was performed using an antibody against RNA polymerase II with serine 2 of its carboxyl terminal domain (CTD) phosphorylated, a configuration that only occurs during transcriptional elongation. Positive RNA polymerase II pCTD-ser2 staining suggests transcriptional activity, while lack of staining suggests transcriptional inactivity. Based on this RNA polymerase pCTD-ser2 staining, global transcription decreases in WT diplotene-karyosome transition cells, and karyosome cells are completely transcriptionally inactive as described by Shakes et al. (Figure 2-8A). Similar staining in both studied mutant alleles (Figure 2-8B, 2-8C) suggests that spe-26 karyosome spermatocytes are indeed transcriptionally inactive.

**Discussion:**

Here, we have reported a cytological analysis of the protein SPE-26. Using a newly generated polyclonal antibody, we have shown that the SPE-26 protein is present only in spermatocytes and not post-meiotic spermatids. Further, SPE-26 is initially translated in the cytoplasm during mid-pachytene, later concentrates to the nucleoplasm during the karyosome stage, and is ultimately discarded into the residual bodies (see summary figure 2-9A, SPE-26). In addition to elaborating on previously reported spe-26 phenotypes (chromosome segregation,
microtubule, and RB formation defects), we also investigated which aspects of late meiotic prophase and the meiotic divisions were or were not occurring normally. The key events of karyosome formation, namely synaptonemal complex breakdown, chromosome coalescence, and termination of transcription, all occur normally in spe-26 mutants (summary Figure 2-9B, SYP-4, pHisH3, RNA poly, and SPE-44), suggesting SPE-26 is not required for karyosome entry. Post-meiotic cells are also able to successfully redistribute/partition their meiotic spindles. Several processes subsequent to karyosome formation, however, occurred aberrantly. spe-26 germlines possessed more karyosome cells than WT, suggesting a delay in karyosome exit. Further, spe-26 meiotic spindles achieved metaphase size despite intact nuclear envelope, and diakinesis stage chromatin (summary Figure 2-9B, meiotic spindle maturity). Following nuclear envelope breakdown, these spermatocytes often fail to form normal metaphase I plates resulting in aberrant chromosome segregation in both meiotic divisions. Following the meiotic divisions, cytokinesis fails, resulting in large, multinucleate terminal cells.

When taken together with our other findings, the precociously metaphase-sized spindle phenotype is intriguing. The higher number of karyosome cells per germline—but not diplotene-karyosome transition cells—in these mutants suggests that a key signal for transition out of the karyosome stage is delayed. Another interpretation of this finding is that there is some yet undefined cell cycle checkpoint during the karyosome stage, and these cells are temporarily arresting at this checkpoint before progressing out of the karyosome stage. Our preliminary observations suggest that, in spe-26 karyosome stage spermatocytes, phosphorylation of nuclear CDK-1/Cyclin B targets (MPM-2 staining) may be delayed (summary Figure 2-9B, MPM-2). Perhaps this delayed signal reaching the nucleus could, in part, explain the apparent desynchrony
of meiotic spindle size and chromatin morphology. In this way, perhaps spe-26 meiotic spindles are not necessarily growing more quickly, but are progressing at a normal pace through late meiotic prophase, while the nucleus lags behind, out of sync with cytoplasmic events.

Our results also advance our understanding of the spermatogenesis-specific substage of late meiotic prophase, the karyosome stage. Work in other model systems has revealed that the karyosome stage is present in other organisms, most notably D. melanogaster and humans, although in those two species, the karyosome stage is specific to oogenesis rather than spermatogenesis (Lancaster et al., 2007; Parfenov et al., 1989). In D. melanogaster, chromosome detachment from the nuclear envelope in karyosome formation is facilitated by the kinase NHK-1 phosphorylating the chromatin binding protein BAF (Lancaster et al., 2007). Little is known about any potential spermatogenesis-specific function of the C. elegans homologs of BAF and NHK-1, which are BAF-1 and VRK-1 respectively. BAF-1 and VRK-1 function are required for nuclear envelope assembly in embryos and meiotic spindle and chromatin organization in oogenesis meiosis II (Gorjanacz et al., 2007). It is unknown whether these functions are conserved in spermatogenesis. Previous work in the Shakes lab has revealed the timing of histone H3 ser10 phosphorylation during spermatogenesis, which results in chromosome hypercompaction, which suggests that this phosphorylation is unlikely to be carried out by aurora kinase (AIR-2) as it is in C. elegans oogenesis and murine spermatogenesis (Shakes et al., 2009). However, the responsible kinase has yet to be identified. Further, it is unclear what causes the sudden concentration of SPE-26 in the nucleus during the karyosome stage, but one possibility is that phosphorylation of SPE-26 causes a conformational change, which might reveal a previously hidden nuclear localization signal (NLS) (see Chapter 3). Our
results indicate that, in spe-26 mutants, chromosome detachment from the nuclear envelope and hypercompaction occur correctly, and SPE-26 fails to concentrate in the nucleus. In hc138 and hc139, we did not detect any SPE-26 protein. In it112, we did detect SPE-26 protein; however, our results indicate that it112 SPE-26 remains diffuse throughout the cell during the karyosome stage. This may suggest that it112 SPE-26 is altered such that its hypothesized NLS is no longer functional.

When first described by Varkey et al. (1995), the Kelch-like protein SPE-26 was assumed to be actin binding, like D. melanogaster Kelch, which stabilizes cytoplasmic bridges between nurse cells and oocytes during oogenesis. Advances in proteomics have revealed a tremendous diversity in Kelch-like protein function, from transcriptional regulation to enzymatic activity (Adams and Kelso, 2000). This more complete knowledge of the functions of Kelch-like proteins, taken together with our results, complicate any conclusions made about SPE-26 function. The cytokinesis failure observed in spe-26 mutants may suggest SPE-26 is required for this actin-driven process. Work in mammals and D. melanogaster has revealed that nuclear actin functions in complex with BAF to detach chromatin from the nuclear envelope during karyosome formation (Olave et al., 2002). If SPE-26 does indeed bind actin in a cytokinesis context, it is then intriguing that chromatin detachment, another process that involves actin in other organisms, occurs normally in spe-26 mutants. This could suggest that SPE-26 binds actin in one context, cytokinesis, but not in another, chromosome detachment. Cytoplasmic actin dynamics and interactions are known to differ from those of nuclear actin (Olave et al., 2002). While the role of nuclear actin in C. elegans spermatogenesis remains poorly understood, this finding suggests that SPE-26 may be functioning beyond actin binding within the nucleus. It is unlikely that SPE-26 regulates transcription given the localization of the protein to the nucleus.
during a stage characterized by its transcriptional inactivity. SPE-26 could, however, perform a chromatin binding function that in some way prepares the chromosomes for meiotic chromosome segregation. Another possibility is that SPE-26 is performing some enzymatic function within the nucleus during this time. This function could in some way regulate the progression through the karyosome stage. Future studies are needed in order to identify the function(s) of this protein.

Still unclear is the localization and possible function of SPE-26 during the meiotic divisions. While SPE-26 is eventually shed to the residual body in WT (Figure 2-2B), our results remain inconclusive regarding the role of SPE-26 during the meiotic divisions. Preliminary evidence suggests a possible colocalization of SPE-26 with metaphase chromosomes during the meiotic divisions; however, a similar localization pattern was observed in spe-26 (hc139) spermatocytes (See Chapter 4: Future Directions, Figure 4-1), suggesting this pattern might be nonspecific staining. Alternative methods of fixing and treating the samples may reduce chromatin staining if it is indeed nonspecific (for more detail, see Chapter 4: Future Directions).

The localization of SPE-26 protein to the nucleus during the poorly understood karyosome stage highlights our lack of mechanistic understanding of the nuclear and chromosome processing events that occur during this stage. SPE-26 is now one of very few proteins in C. elegans linked specifically to the karyosome stage. It is unknown whether this association is C. elegans specific or if Kelch-like proteins in other organisms are also linked to the karyosome stage. Future studies can use SPE-26, along with other markers previously described by Shakes et al., to further investigate the events that occur during the karyosome stage.
**Figure 2-1: Specific microtubule defects in spe-26 mutants** Immunofluorescence microscopy using a chromatin stain (DAPI) (Blue) and a direct-FITC-labeled antibody against tubulin (Green),

(A-B) Isolated, intact male WT (A) or spe-26 (it112) (B) germline. Scale bars indicate 40 μm. Colored lines indicate stage of spermatogenesis of cells contained in those demarcated regions, with color being carried forward from introduction Figure 2: dark blue = late pachytene; purple = diplotene; yellow = karyosome; red = meiotic divisions; baby blue = post-meiotic products, including spermatids and residual bodies.

(C-E) Flattened spread of individual spermatocytes isolated from a WT (A), spe-26 (hc138) (B), or spe-26 (hc139) (C) male germline. Inserts are enlarged 3x. Scale bar indicates 10μm. Cartoons are reproduced from introduction Figure 2c and adjusted accordingly to reflect phenotype observed. For metaphase I inserts, images shown are representative of the breadth of defect severity observed in each strain. Normal/abnormal
metaphase I counts for \textit{hc138} were obtained from 8 germlines, and for \textit{hc139} 20 germlines.
Figure 2-2: A newly generated SPE-26 antibody reveals nuclear localization of SPE-26 during the karyosome stage.

(A) Structural diagram of SPE-26 protein annotated with locations of mutant alleles and location of antibody binding site of SPE-26.

(B-C) Western blot analysis using anti-actin as a loading control, anti-MSP as a phenotype verification, and anti-SPE-26 antibodies. WT and temperature-sensitive strains raised at the non-permissive temperature of 25ºC for all samples to ensure expression of the mutant phenotypes, and samples consisted of 50 worms homogenized together. Labels on right indicate bands at predicted molecular weights of proteins assayed: S= SPE-26 at MW = 65kDa, A= Actin at MW = 42kDa, and M = MSP at MW = 14kDa. Arrows marked with “?” indicate bands of unknown protein identity.

(D-G) Immunofluorescence microscopy using anti-SPE-26 antibody (Red) on (D) WT male, (E) WT male costained with anti-Nuclear Pore Complex antibody, (F) WT hermaphrodite larval L4 and adults, and (G) several mutant (spe-44, and several alleles of spe-26) germline. Inserts are 2x magnified cells from the whole germline image of pachytene (p), diplotene (d), karyosome (k), diakinesis (dk), and meiotically dividing cells (m), as well as spermatids (s). Budding figure (Bud.) is from another germline imaged during the same experiment as the one presented. Larger image scalebar indicates 40µm, and insert scalebar indicates 15µm.
Figure 2-3: WT SPE-26 concentrates in the nucleus during the karyosome stage, while spe-26(it112) SPE-26 remains diffuse.

A-B: Isolated, intact male WT (A) or spe-26 (it112) (B) germline. Samples were prepared with DAPI (Blue) and anti-SPE-26 antibody (Red) in the same experiment. Inserts labels: P = pachytene (blue), D = diplotene (green), trans. = diplotene-karyosome transition (yellow-green), and K = karyosome (yellow). Inserts are magnified 2x. Scale bars indicate 40 µm and 10 µm for inserts.

C-H: One plane from multi-stack confocal micrographs of samples prepared with DAPI (blue) (C-E) and anti-SPE-26 antibody (red) (F-H). All images were taken using the exact same laser settings and exposures, and are 60x.

I-K: SPE-26 (red) channel (C-H) heat-scaled from lowest possible intensity (purple, 0) to largest possible intensity (red, 4095), to allow a more robust qualitative comparison of him-8 and spe-26 (it112) SPE-26 localization. Arrows indicate karyosome cells. spe-26 (hc139) is included as a negative control, as determined in the previous experiment (see Figure 2D). Scalebars indicated 10µm.
Figure 2-4: M-phase signal is late entering the nucleus spe-26 spermatocytes. One plane from multi-stack confocal micrographs of samples stained with DAPI (blue) and M-phase marker MPM-2 (green). Red arrows indicate metaphase I cells, while yellow arrows indicate similarly-staged karyosome cells. Images are 60x, and scalebar indicates 10µm.

(A) *him-5* (effectively WT) male germline.
(B-D) Male germlines of three *spe-26* alleles including: *spe-26* (*it112*) (B), *spe-26* (*hc138*) (C), and *spe-26* (*hc139*) (D).
Figure 2-5: Synaptonemal complex breakdown occurs normally, while karyosome cells accumulate in spe-26 mutants.

Immunofluorescence microscopy of intact germlines of WT (A), spe-26 (it112) (B), and spe-26 (hc139) (C) using anti-SYP-4 (Green) and DAPI (Red) staining. Inserts labels: P = pachytene (blue), D = diplotene (green), trans. = diplotene-karyosome transition (yellow-green), and K = karyosome (yellow). Inserts are magnified 2x. Scalebar = 40µm.

Using these images, the number of diplotene-karyosome transition cells and the number of karyosome stage cells / germline were quantified for him-5 (n = 28), spe-26 (it112) (n = 20), and spe-26 (hc139) (n = 16). The average number of each type of cell per germline for each strain is shown in (D). Error bars indicate standard deviation. Significance was calculated using a two-tailed t test, and * : p < 0.05; *** p < 0.0001; NS : not significant.
Figure 2-6: Chromosome detachment and compaction occurs normally in spe-26 mutants. Immunofluorescence microscopy of intact germlines of WT (A), spe-26 (it112) (B), and spe-26 (hc139) (C) using anti-pHisH3ser10 (Red), anti-Nuclear Pore Complex (Green), and DAPI (Blue) staining. Arrows and single cells: P = pachytene (blue), D = diplotene (green), trans. = diplotene-karyosome transition (yellow-green), and K = karyosome (yellow). Inserts are magnified 2x. Scalebar = 40µm whole germline, 10µm inserts.

Figure 2-7: The sperm-specific regulator SPE-44 detaches from chromatin normally in spe-26 mutants.

Immunofluorescence microscopy of intact germlines of WT (A), spe-26 (it112) (B), and spe-26 (hc139) (C) using anti-SPE-44 and staining. Arrows and single cells: P = pachytene (blue), D =
diplotene (green), trans. = diplotene-karyosome transition (yellow-green), and K = karyosome (yellow). Inserts are magnified 2x. Scalebar = 40µm whole germline, 10µm inserts.

Figure 2-8: Transcriptional termination occurs normally in spe-26 mutants. Immunofluorescence microscopy of intact germlines of WT (A), spe-26 (it112) (B), and spe-26 (hc139) (C) using antibodies against the elongating form of RNA polymerase II (anti-anti-RNA polymerase pCTDser2) and DAPI staining. Arrows and individual cells: P = pachytene (blue), D = diplotene (green), trans. = diplotene-karyosome transition (yellow-green), and K = karyosome (yellow). Inserts are magnified 2x. Scalebar = 40µm whole germline, 10µm inserts.
Figure 2-9: Summary of Cytological Markers Before and After the Karyosome Stage. Summary cartoon showing the relative amount of signal for various markers in WT (A) and spe-26 (B).
Chapter 3: Investigation of SPE-26 Structure and Function Using Bioinformatics

Prologue:

The advent of large-scale structural databases and various bioinformatics techniques has revolutionized the study of protein structure and function. These databases and techniques allow inferences to be made regarding predicted protein structures and functions that inform bench-lab experimentation in protein biology and biochemistry.

The model organism *C. elegans* is attractive for the study of protein biology due to key achievements of the *C. elegans* research community. These achievements include assembly of a fully sequenced and annotated genome, transcriptome, and proteome, which are available in public online databases. One extremely useful resource is WormBase, an online, collaboratively curated collection of databases. WormBase cross-links information to other databases, including non-nematode databases, thus expediting searches for orthologs in other models (Harris et al. 2013). These resources have contributed to a highly collaborative and connected *C. elegans* research community.

Bioinformatic resources provide insights into SPE-26 structure and function without doing biochemical investigation. This chapter will focus on those insights from modern bioinformatics resources regarding SPE-26 function.

Methods:

Several methods were used to align SPE-26 sequence with known proteins to determine homology and to predict its structure. Protein Basic Local Alignment Tool (Protein BLAST) is a National Center for Biotechnology Information (NCBI)-created tool that finds local regions of similarity between a queried protein sequence and a multitude of proteins in several different
databases, including the NCBI's Protein Databank (PDB) and SwissProt (Altschul et al. 1990; Boratyn et al. 2012). Protein BLAST was used to query SPE-26 sequence to determine highest sequence homology matches. Iterative Threading Assembly Refinement (I-TASSER) uses the threading approach to align query protein with template protein sequences in PBD (Zhang et al. 2008). I-TASSER then predicts a protein's 3D structure using these templates, performing iterations with fragments of each template. I-TASSER was used to predict SPE-26 structure.

The online protein structure prediction software Phyre2 predicts both secondary and tertiary aspects of protein structure using multi-sequence alignments processed through a query hidden Markov model to predict aspects of structure and to calculate relative confidence of predictions (Kelley et al. 2015).

Several databases were used to predict post-translational modifications of SPE-26 using its protein sequence. UbPred predicts potential ubiquitination sites on query protein sequences though forest-based prediction software trained on published sequences known to be ubiquitination sites (Radivojac et al. 2010). The Consensus Approach (COACH) software uses a consensus approach to predict ligand-binding sites in a query protein sequence through templates from the BioLiP database (Yang et al. 2013). COACH works in tandem with I-TASSER to consider predicted structure when predicting ligand binding sites. NetPhosK predicts potential serine, threonine, and tyrosine phosphorylation sites on query protein sequences with reference to both generic and kinases specific phosphorylation sites (Blom et al. 1999).

Other tools were used to predict aspects of SPE-26 functionality. cNLS Mapper predicts potentiality nuclear localization signals (NLSs) through analysis of cumulative contribution of amino acids sequences in the query protein to likelihood of NLS function, as determined experimentally using amino acid replacement analyses in yeast (Kosugi et al. 2009). cNLS
mapper predicts NLSs that interact with either importin alpha or importin beta, the two subunits required for eukaryotic nuclear import. Finally, DisEMBL predicts likelihood of local disorder/lack of structure in a stretch of an amino acid sequence through calculation of local values of several different parameters that estimate likelihood of disorder (Linding et al. 2003).

**Background:**

**Kelch-like Superfamily**

Members of the Kelch-like superfamily all possess 4-7 ß-propeller-shaped Kelch domains that together form a windmill-like structure (a representative structure is shown later in figure 3-3b). Initially, all Kelch-like family members were thought to function as cytoskeletal organizers, like the two most well-known members Kelch and Scruiin. Kelch is the founding and most well-studied member of the Kelch-like superfamily of proteins, and is an actin binding protein required for ring canal development during *D. melanogaster* oogenesis (Robinson et al. 1997). In *D. melanogaster*, Kelch bundles and organizes actin filaments to facilitate ring canal formation (Robinson et al., 1997). Scruiin is an actin filament cross-linker protein discovered in the horseshoe crab *Limulus polyphemus* (Way et al. 1995). Yet another well-known Kelch-like protein is Diablo in *Drosophila*, a substrate-specific adaptor for E3 ubiquitin ligases involved in proteasomal degradation (Strutt et al. 2013). Modern sequencing and alignment techniques have led to the discovery of Kelch-like proteins in organisms as evolutionarily diverse as yeast and humans (Adams et al. 2000). As more members of this evolutionarily widespread family were discovered, knowledge of this family's tremendously diverse cellular functions grew. Kelch-like proteins are now known to function in diverse contexts outside of cytoskeletal rearrangements,
from transcriptional regulation to such enzymatic activities as phosphorylation and galactose oxidation (Adams et al. 2000).

Within this protein superfamily, there are several subclasses defined by the presence of additional protein domains (Figure 3-1, adapted from Stogios et al. 2004). According to sequence homology, SPE-26 is most closely related to a large subclass called the BTB-BACK-Kelch (BBK) proteins. BBK proteins are generally characterized by the presence of two additional domains, BTB and BACK (Stogios et al. 2004), and are known to perform a diverse array of cellular functions. The BTB domain, also sometimes referred to as a POZ domain (for Pox virus and Zinc-finger domain), is a protein-protein interaction domain known to permit di-oligomerization and is often found in transcription factors and interactors with E3 ubiquitin-ligases (Perez-Torrado 2006). Amongst known BTB proteins, there is high sequence variation in the BTB domains, though the structure of the BTB domain is thought to be conserved (Perez-Torrado, 2006). Little is known about the BACK domain, which is named because of its location between an N-terminal BTB and several C-terminal Kelch domains (Stogios et al. 2004; Stogios et al. 2005). For BBK proteins involved in E3 ubiquitin ligase binding, the BACK domain is thought to aid in substrate orientation and function in tandem with the BTB domain; however, it is unclear whether this functions applies to BBK proteins of other functions as well (Stogios et al. 2004). The function of the BACK domain remains poorly understood although most BTB-Kelch proteins also contain BACK domains.

One well-known group of BBK proteins is the human KLHL family of proteins (Dhanoa 2013). Much like other Kelch-like groupings, KLHL proteins carry out a diverse array of cellular function. Misfunction of many of these genes have been implicated in various forms of cancer, including KLHL6, KLHL19 (more commonly known as KEAP1), and KLHL20. Some evidence
has shown that the *C. elegans* KLHL19/KEAP1 homolog is required for cytokinesis and polar body formation in oogenesis (Skop *et al.* 2004). Several others have been implicated in congenital diseases, such as KLHL3, KLHL7, and KLHL16 (also known as GAN). Interestingly, mutations in KLHL16/GAN are implicated in Giant Axonal Neuropathy, a debilitating disease involving aberrant accumulation of intermediate filaments in neurons that drastically decreases lifespan and sensorimotor function (Blomont *et al.* 2000).

Puzzlingly, despite high sequence homology with BBK proteins, SPE-26 is unusual because it does not contain an identifiable BTB domain (Figure 3-1; Figure 3-2a; Stogios *et al* 2004; Wormbase.org). While BTB domains do contain high sequence variability (Stogios *et al.* 2004), the sequence homology alignment tools compiled in WormBase, including hmmpanter (Huaiyu *et al.* 2016), interpro (Finn *et al.* 2017), pfam (Finn *et al.* 2016), SMART (Schultz *et al.* 1998), and SUPERFAMILY (Gough *et al.* 2001), that all recognize BTB domains in other proteins fail to do so with SPE-26 (Wormbase.org). Indeed, SPE-26 is the only known *C. elegans* protein, and one of only a handful of known proteins in any organism (including three predicted proteins of unknown function in humans), that contains a BACK and Kelch domain but does not also include a BTB domain (Stogios *et al* 2004). These rare proteins may challenge the hypothesis that BACK domains always function in tandem with BTB domains. However, it is unknown if the BACK domain in these BTB-less proteins are actually functional. It is also possible that SPE-26 and these human proteins contain BTB domains that have drastically diverged from other BTB proteins, such that they are no longer recognized by sequence alignment databases. It is not currently known if this N-terminal, non-BTB region is structured in SPE-26.
Structural Location of spe-26 Mutations

While Varkey et al. (1995) initially described the location of the spe-26 mutations along the structure of SPE-26 protein in relation to the Kelch domains, we can now also describe the locations of these mutations relative to the BACK domain (Figure 3-2b).

As reported by Varkey et al., the moderately-severe, temperature sensitive it112 mutation S360N is located on one of the Kelch domains (Figure 3-2b), and is hypothesized to disrupt the function of at least that Kelch domain. The temperature sensitivity and mild phenotype of it112 indicates that the amino acid change of serine (with a small hydroxyl side chain) to asparagine (with a larger carboxamide side chain) is less significant compared to the other known point mutations for spe-26. Interestingly, recent findings in other Kelch proteins suggests that while repeat Kelch domains function most efficiently in tandem, they are also known to act individually to some extent (Adams et al. 2000). This might explain the less severe spermatid production and division defect phenotypes observed in it112. SPE-26 protein was detected in it112 male worms using western blot analysis (2-2B and C) and it112 spermatocytes are positive for SPE-26 antibody (2-2F). These data indicate that this altered version of the protein is still present in these cells. However, this altered SPE-26 (it112) protein mislocalizes during the karyosome stage, remaining in the cytoplasm rather than concentrating in the nucleus (Figure 2-2F). This mislocalization phenotype may suggest that SPE-26 requires all of its Kelch domains in order to localize correctly. It remains to be determined whether this SPE-26 (it112) protein is functional at all or if only its localization is disrupted.
hc139 and hc140 are both temperature sensitive mutants with molecular lesions at the C-terminal region of the protein (Figure 3-2B). While hc139 and hc140 have different types of molecular lesions, both exhibit a similarly severe spermatogenesis defects and both are barely fertile even at permissive temperature (hc139 shown in Figure 2-2F; both shown in Varkey et al. 1995). hc139 is a premature stop mutation W460amb that cuts the protein short in the middle of one of its C-terminal Kelch domains. This truncation occurs N-terminal to the epitope recognized by our anti-SPE-26 polyclonal antibody. Accordingly, no signal is detected in either western blot analysis or immunofluorescence using SPE-26 antibody (2-2B and 2-2F). hc139 worms are weakly fertile at 16 degrees (Varkey et al. 1995), despite truncation of the SPE-26 protein in one of its critical domains. hc140 is a point mutation G446E within one of its C-terminal Kelch domains. It is unclear why this point mutation results in a more severe phenotype than the similarly-lesioned it112. One possibility could be that the replacement of glycine 446 (small hydrogen side group) with glutamate (propanoate side group) is more disruptive than the replacement of serine (hydroxyl side group) 360 with asparagine (carboxamine side group).

hc138 is a moderately-severe temperature sensitive point mutation G223A at the at the end of the BACK domain (Figure 3-2b). Varkey et al. describe hc138 as more severe phenotypically than it112 but less severe than hc139. The location of this mutation suggests that the BACK domain of SPE-26 is to some extent required for its function. hc138 SPE-26 is likely degraded because no SPE-26 protein was detected in hc138 using either western blot analysis or immunofluorescence.

eb8 is a non-conditional premature stop mutation Q441och that cuts the protein short in the middle of one of its C-terminal Kelch domains. This truncation occurs N-terminal to the epitope recognized by our anti-SPE-26 polyclonal antibody. Accordingly, no SPE-26 is detected using
immunofluorescence in this mutant allele (Figure 2-2F). This mutation conveys the most severe sterility phenotype, complicating study of this strain, and preventing the acquisition of large samples of this strain for biochemistry experiments.

Results and Discussion:

Structural and Functional Predictions of SPE-26

The absence of a BTB domain in SPE-26 will likely alter the structure and function of this protein. Further, it was unknown how this absence of a BTB domain affected the homology of SPE-26 to other Kelch-like proteins.

In order to determine the closest sequence homologs to SPE-26, the SPE-26 amino acid sequence was submitted to Protein BLAST, and all known proteins in PDB were queried. The top eight closest known sequence homologs of C. elegans SPE-26 are other nematode species' versions of SPE-26 and other nematode Kelch-like proteins of unknown function (Table 3-1; Figure 3-3a) (Altschul et al. 1990; Boratyn et al. 2012). The top four nematode homologs have extremely low E values that are rounded down to 0 (Table 3-1). Because the SPE-26 protein sequence is highly conserved within nematodes, SPE-26 may serve a conserved function in nematodes. Non-nematode BBK proteins, though, may not be informative to understand SPE-26 function. The closest non-Nematoda homologs included the BBK proteins Drosophila diablo and human KLHL18 (Table 3-2). Dbo (DIABLO) is a Drosophila protein known to be a substrate-specific adaptor for E3 ubiquitous ligases involved in polyubiquitin-mediated proteasomal degradation (Strutt et al. 2013). KLHL18 is known to bind Cullin-3, a core component of E3 ubiquitin-ligases in humans, and has been shown to break down promyelocytic protein, a known cancer progression factor (Dhanoa 2013).
Because SPE-26 has sequence homology to other BBK proteins, I-TASSER structure prediction software was used to determine if SPE-26 was predicted to be structurally homologous as well (Yang et al. 2015; Roy et al. 2010; Zhang, 2008). The templates used to predict this structure are shown in Table 3. I-TASSER results predict a windmill Kelch domain superstructure characteristic of the Kelch-like superfamily. Less obviously recognizable structured domains were also predicted in SPE-26 (Figure 3-3b).

The first 130 amino acid non-BTB domain at the N-terminus of SPE-26 did not align with any of the templates. While SPE-26 does not contain a BTB domain, it is unknown whether the first 130 amino acids sequence is actually structured in some unrecognized way or if instead it is truly unstructured. The I-TASSER model did not predict any large unstructured regions, which suggests this region structured in some unrecognized way. To corroborate this prediction, the local disorder probability prediction software Dis.EMBL was used to analyze the protein sequence of SPE-26 (Linding et al. 2003). Dis.EMBL results indicated that small sections of this region (AA 40-60 and 70-80) have local disorder probability greater than 0.5, but the average disorder probability for the entire AA1-130 region is below 0.5. Therefore, this section is likely indeed structured (Figure 3-3c).

The DisEMBL results indicated that this region contains secondary structure. In order to both corroborate this prediction and to determine the nature of this secondary structure, the online prediction tool Phyre2 was used to analyze SPE-26 sequence. Phyre2 performs multiple sequence alignments to predict secondary structure, while considering known aspects of tertiary structure to refine these secondary structure predictions (Kelley et al. 2015). Phyre2 results predicted with high confidence that the first 130AA region forms several large (15-20 AA in length) alpha helices and several shorter (5-10AA in length) beta pleated sheets (Table 3-5).
Interestingly, BTB domains are similarly composed of several large alpha helices that are flanked by short beta pleated sheets (Ahmad et al. 1998), supporting our hypothesis that this region might contain a domain derived but diverged from the BTB domain. It remains unknown whether or not this similarity in structure corresponds to similarity to BTB function or whether this region is functional at all.

Due to the large functional diversity within the BBK family and greater Kelch-like superfamily, it was unclear which possible molecular functions and interactions of SPE-26 would support the localization change from diffuse to nuclear-localized during the karyosome stage. Ubiquitination is one way in which proteins' structure, function, and localization can be modulated. To determine if any ubiquination sites were present along the structure of SPE-26, the online software UbPred was used. UbPred uses a random-forest based approach to predict potential ubiquitination sites along a queried sequence (Radivojac et al. 2010). UbPred did not predict with high confidence the presence of any such sites. Hence, we can conclude that ubiquitination does not explain the SPE-26 localization change.

Next, because of the well-documented role of BBK proteins as transcription factors and the functional association of BTB domains and zinc-finger domains, and because SPE-26 concentrates in the nucleus, we hypothesized that SPE-26 may function as a transcription factor. To test this hypothesis, the online prediction software COACH was used to predict any potential ion binding sites on the structure of SPE-26 (Yang et al. 2013). COACH results failed to identify ion binding sites on SPE-26 (data not shown). This failure to identify binding sites does not support the hypothesis that SPE-26, like other BBK proteins, functions as a transcription factor.

Given the preliminary MPM-2 data suggesting a mislocalization in spe-26 spermatocytes (Figure 2-4), it was unclear whether or not SPE-26 itself was phosphorylated by CDK1/Cyclin B.
Interestingly, both Diablo and KLHL18 are known to have functionally-relevant phosphorylation sites. Most of the functionally-relevant phosphorylation sites of DIABLO are at its C-terminus and its N-terminus, with few in the intervening regions (Mertins et al. 2016). KLHL18 contains predicted phosphorylation sites in its BACK domain and its C-terminal Kelch domain, but not in its BTB domain (Zhou 2011, data shared to PhosphositePlus). In order to determine if SPE-26 had any predicted phosphorylation sites, particularly CDK1 phosphorylation sites, the online prediction tool NetPhosK was used to identify any such sites along the sequence of SPE-26. NetPhosK compares query sequences to highly conserved consensus sequences for well-known kinases (Blom et al. 2004). NetPhosK identified many potential sites for phosphorylation along the length of SPE-26, most of which are predicted to be phosphorylated by protein kinase C and casein kinase II with high phosphorylation potential (>0.7) (Figure 3-3d; Table 3-6). Protein kinase C is a canonical signal transduction kinase (Newton 1995), while casein kinase II performs many cellular functions, including playing a role in gene expression and apoptosis (Gao et al. 2006). Interestingly, these predicted sites are found throughout the length of SPE-26, allowing for comparisons to both DIABLO and KLHL18. There were few predicted phosphorylation sites in the BACK domain of SPE-26, but many were found in the N-terminal non-BTB region as well as its Kelch domains (Figure 3-3d). The NetPhosK results did identify several CDK1/cdc2 phosphorylation sites on SPE-26, primarily located in between the BACK domain and the Kelch domains, as well as inside the Kelch domains. Taken together with findings in the literature, this suggests SPE-26 may have significant phosphorylations, and future investigations might seek to verify these predictions.

One explanation for SPE-26 translocation the nucleus is a nuclear localization signal (NLS) somewhere along the protein structure. To this end, we used cNLS, an NLS prediction
software, to predict potential alpha and beta importin-recognized NLSs (Kosugi et al. 2009). cNLS predicted an alpha NLS at AA538-568 (located at the C-terminus of the protein, containing the antibody binding site). The sequence of the predicted NLS is RNFLERRGTINEQSMEMDDDYEDDAGASYMSI, and had a cutoff score of 2.9. This cutoff score indicates presence in both the cytoplasm and nucleus. While this finding is consistent with SPE-26 presence in both compartments in pachytene and diplotene, this finding fails to explain how SPE-26 later concentrates in the nucleus only during the karyosome stage. This suggests that this movement may be coordinated by some other cellular process that occurs during the karyosome stage.

**Conclusion:**

While the function of SPE-26 remains unknown, bioinformatics analyses allow us to better predict this function. pBLAST and I-TASSER results suggest SPE-26 may share many structural and functional characteristics with known Kelch proteins (particularly BBK proteins). The strong homology of *C. elegans* SPE-26 to other nematode proteins, and its lack of a BTB domain suggest that SPE-26 may function dissimilarly to known BBK proteins. The prediction of secondary structure similar to the BTB domain in the N-terminal non-BTB region of SPE-26, along with this region's homology to other nematode proteins, may suggest a nematode-specific domain that diverged from the BTB domain over evolutionary time. The identification of predicted phosphorylation sites in this region further suggest its functional importance, especially when taken together with the functional significance of phosphorylation sites in the BTB domain of *Drosophila* DIABLO. Interestingly, none of the known spe-26 mutations are located in this region, so we cannot use mutant analysis to directly study this region. Hence, these methods have provided a more thorough understanding of the molecular biology and biochemistry of SPE-26.
Figure 3-1: Venn Diagram Comparison of Number of BTB, BACK, and/or Kelch-containing proteins in several representative organisms. Adapted from Stogios et al. 2004 with minor changes. Number of proteins containing BTB, BACK, and/or Kelch domains in *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens*. Color Legend: Blue= BTB; Red= BACK; Green= Kelch. Asterisk denotes SPE-26, the only protein in *C. elegans* that contains a BACK domain and several Kelch domains, but not a BTB domain.
Figure 3-2: Structural Diagrams of SPE-26 protein.
Diagrams were created in the program Illustrator for Biological Sequences, described in Wenzhong et al. (2015). Labels denote domain location, given in residue number. Dark blue regions represent intervening protein regions between known domains.

A. Comparison of SPE-26 protein to a canonical BBK protein, human KEAP1. KEAP1 (also known as KLHL19) is a well-studied and canonical BBK protein. Despite large sequence homology to BBK proteins, SPE-26 does not contain a BTB domain. It is unknown whether or not the N-terminal Non-BTB region of SPE-26 is structured.

B. Annotated structural diagram of SPE-26 protein. Indicated are location and type of mutations, as well as the location of the antibody binding site on the protein.
Figure 3-3: Bioinformatics results.

A. Structural features of SPE-26. Subset of pBLAST results of SPE-26 query. pBLAST fails to identify a BTB domain in SPE-26 sequence.

B. I-TASSER predicted structural model of SPE-26 using top threaded templates.

C. N-Terminal Non-BTB region of SPE-26 is not predicted to be unstructured. X axis displays residue number, while Y axis displays predicted disorder probability using three prediction software procedures.
D. NetPhosK prediction of phosphorylation sites along the length of SPE-26. X axis displays residue number, while Y axis shows phosphorylation potential (0-1) of various Ser, Thr, Tyr kinases relative to their cutoff threshold (=0.5).

Table 3-1: Top 10 BLAST hits.

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<th>Protein Name</th>
<th>Species</th>
<th>Accession #</th>
<th>Max Score</th>
<th>Total Score</th>
<th>Query Cover</th>
<th>E value</th>
<th>Percent Identity</th>
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<td>Caenorhabditis elegans</td>
<td>NP_501919.1</td>
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<td>1195</td>
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<td>100%</td>
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<td>EGT47747.1</td>
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<td>885</td>
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<td>0</td>
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<td>99%</td>
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<td>66%</td>
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<td>8.00 E-72</td>
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<td>3.00 E-48</td>
<td>31%</td>
</tr>
<tr>
<td>PREDICTED: kelch-like protein 10</td>
<td>cuculus canorus</td>
<td>XP_009553780.1</td>
<td>174</td>
<td>174</td>
<td>77%</td>
<td>7.00 E-44</td>
<td>27%</td>
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</table>
### Table 3-2: Non-nematode BLAST hits.

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<th>Protein Name</th>
<th>Species</th>
<th>Accession #</th>
<th>Max Score</th>
<th>Total Score</th>
<th>Query Cover</th>
<th>E value</th>
<th>Percent Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PREDICTED: kelch-like protein 10</td>
<td><em>Danio rerio</em></td>
<td>XP_017207308.1</td>
<td>157</td>
<td>157</td>
<td>71%</td>
<td>3.00E-40</td>
<td>26%</td>
</tr>
<tr>
<td>PREDICTED: kelch-like protein 18 isoform X7</td>
<td><em>Homo sapiens</em></td>
<td>XP_016861524.1</td>
<td>147</td>
<td>147</td>
<td>73%</td>
<td>2.00E-37</td>
<td>27%</td>
</tr>
<tr>
<td>kelch-like protein 10</td>
<td><em>Mus musculus</em></td>
<td>NP_080003.1</td>
<td>146</td>
<td>146</td>
<td>81%</td>
<td>4.00E-36</td>
<td>25%</td>
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<tr>
<td>diablo, isoform B</td>
<td><em>Drosophila melanogaster</em></td>
<td>NP_001261892.1</td>
<td>115</td>
<td>115</td>
<td>77%</td>
<td>1.00E-25</td>
<td>24%</td>
</tr>
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</table>

### Table 3-3: Top I-TASSER Templates

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<tr>
<th>Top Threading Templates</th>
<th>Known function (if any)</th>
<th>Structure Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Unknown Kelch Protein (<em>Plasmodium falciparum</em>)</td>
<td>Unknown</td>
<td>Jiang, DQ et al 2015</td>
</tr>
<tr>
<td>2 KLHL3-Cul3 complex (<em>H. sapien</em>)</td>
<td>Protein Binding</td>
<td>Ji, AX et al. 2016</td>
</tr>
<tr>
<td>3 Unknown Kelch Protein (<em>Plasmodium falciparum</em>)</td>
<td>Unknown</td>
<td>Jiang, DQ et al 2015</td>
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<tr>
<td>4 KLHL19/KEAP1-Nrf2 complex (<em>H. sapien</em>)</td>
<td>Transcription</td>
<td>Tong, KI et al. 2007</td>
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<tr>
<td>5 KLHL12 (<em>H. sapien</em>)</td>
<td>Protein Binding</td>
<td>Canning, P et al. 2013</td>
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</table>
Table 3-4: UbPred Data: Condensed

Output:

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<th>Residue</th>
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<th>Ubiquitinated</th>
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<td>0.43</td>
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<tr>
<td>30</td>
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<tr>
<td>87</td>
<td>0.51</td>
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</tr>
<tr>
<td>158</td>
<td>0.56</td>
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</tr>
<tr>
<td>173</td>
<td>0.43</td>
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<th>Ubiquitinated</th>
</tr>
</thead>
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<tr>
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<td>0.63</td>
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</tr>
<tr>
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<td>0.59</td>
<td>No</td>
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<tr>
<td>456</td>
<td>0.52</td>
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<td>472</td>
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<th>Specificity</th>
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<td>0.464</td>
<td>0.903</td>
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<td>Medium confidence</td>
<td>0.69 ≤ s ≤ 0.84</td>
<td>0.346</td>
<td>0.950</td>
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<td>High confidence</td>
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<td>0.197</td>
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Table 3-5: Secondary structure and disorder prediction

Phyre2

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<th>Disorder</th>
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</table>

N-terminal non-BTB region

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<th>Disorder</th>
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N-terminal non-BTB region

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<th>Disorder</th>
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<th>Disorder</th>
<th>Disorder</th>
<th>Disorder</th>
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Non-BTB region

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BACK domain

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Kelch Domain

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</table>

Confidence Key

**High(9)  Low (0)**

? Disordered (8%) Alpha helix (26%)

Beta strand (36%)
<table>
<thead>
<tr>
<th>Position</th>
<th>Residue</th>
<th>Score</th>
<th>Kinase</th>
<th>Position</th>
<th>Residue</th>
<th>Score</th>
<th>Kinase</th>
</tr>
</thead>
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<td>0.589</td>
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<td>PKC</td>
<td>479</td>
<td>T</td>
<td>0.706</td>
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<td>S</td>
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Table 3-6: NetPhosK Phosphorylation site predictions
Chapter 4: Future Directions

Here, we report an extension and elaboration of the cytological analysis of SPE-26 begun by Varkey et al. (1995). spe-26 mutants exhibit several defects beginning in the karyosome stage: cells are slow to exit the karyosome stage, meiotic spindle asters reach full size precociously, a key M-phase marker might arrive in the nucleus late, and chromosomes fail to align at the metaphase plate and segregate correctly. While these processes are disrupted, several other processes occur normally, namely those involved in the formation of the karyosome stage. This analysis is complemented by the finding that SPE-26 protein concentrates in the nucleus during the karyosome stage. We also report various predictions of SPE-26 protein function using various bioinformatics tools. We describe the predicted secondary structure of the N-terminal region of the SPE-26 protein and a potential nuclear localization signal (NLS). We also report the results of a phosphorylation site predictor, which predicts several likely phosphorylation sites in the non-BTB region and the Kelch domains of SPE-26.

Investigating the role of SPE-26 during the meiotic divisions

One incomplete line of investigation is the localization and potential direct functioning of SPE-26 during the meiotic divisions. One challenge in this line of investigation is a limitation of the current SPE-26 antibody used. When this antibody is used in immunofluorescence experiments in WT, SPE-26 brightly stains along the metaphase plate of spermatocytes (Figure 4-1A). While these data in isolation suggest an association between SPE-26 and the chromatin beyond karyosome, observations of similar patterns in spe-26 (hc139) (which lacks the antigenic site) suggest this might actually be a cross reactive signal or simply nonspecific background staining (Figure 4-1B). Given the location of this polyclonal antibody's epitope, which is C-terminal to the Kelch domains of SPE-26, if this staining is cross reactive signal, then this result suggests this region may have homology with some other unidentified Kelch-like protein found
within spermatocytes. If this signal is shown to simply be nonspecific background signal, there are several ways in which this signal could be reduced, so that only specific staining is reduced. These include:

• Incubate sample with goat antiserum after blocking solution and prior to incubation with primary antibody.

• Incubate antibody solution on a spe-26 (hc139) sample, any nonspecific or cross reactive antibodies would bind to this sample. Then, pipette up the remaining sample, and incubate it with a WT sample.

• Incubate primary anti-SPE-26 antibody and/or secondary antibody with concentrated worm powder, which is composed of pulverized WT adult hermaphrodite samples to allow nonspecific or cross reactive antibodies to bind these generic C. elegans epitopes. Then, remove remainder solution from powder, and incubate with sample.

In theory, these techniques would leave only specific, non-cross reactive anti-SPE-26 antibodies, so any remaining signal observed should be specific.

The experiments above seek to get around certain limitations of cytological investigation using this newly generated antibody; however, a completely independent investigative method would be the generation of a spe-26::GFP or spe-26::Mcherry construct strain. Such a tool would allow live imaging studies of SPE-26 localization in WT. This could be coupled with the extremely powerful genetic toolkit, such as crossing into another previously identified spe mutant to determine the relationship between SPE-26 and known SPE gene pathways. If such a construct proves to be difficult to design or create, the C. elegans genetic toolkit could be utilized in other ways.
Another possibility is to use RNAi, another commonly used genetic knockout technique, to knockdown SPE-26 levels in WT or *hc139* worms. This would ensure that little or no SPE-26 protein is produced. If the meiotic division chromosome staining is still observed in *spe-26* RNAi treated worms, then we could conclude that the meiotic localization pattern is entirely nonspecific. A western blot on *spe-26* RNAi treated worm samples could determine if the higher molecular weight bands observed in figures 2-2B and 2-2C are indeed SPE-26-specific. Presence of these bands in *spe-26* RNAi treated worm samples would suggest these bands are cross reactive signals. Comparison of mutant chromosome segregation, precocious spindle maturity, and karyosome exit phenotypes between *spe-26* RNAi and analyzed *spe-26* alleles, particularly *it112*, could reveal any aspects of SPE-26 function conserved in these mutant versions of the protein.

After the role of WT SPE-26 during the meiotic divisions is explored, another interesting question that remains is whether or not this hypothetical post-karyosome function of SPE-26 is preserved in *spe-26 (it112)*. While we report here that *it112* SPE-26 fails to localize specifically to the nucleus during the karyosome stage, it is unclear whether any function of SPE-26 after NEBD during the meiotic divisions (if there is any) occurs normally or abnormally. The preservation of some, but not all, of SPE-26 functions could potentially explain the less severe spermatid formation defects observed in *spe-26 (it112)*. The anti-SPE-26 antibody could be used in immunofluorescence experiments with various other *spe* mutants. For example, preliminary evidence suggests that SPE-26 concentrates in the nucleus correctly in diakinesis-arresting *spe-6* mutants.
Investigating the higher molecular weight band observed in western blot experiments

Another remaining question is the identity of the second, larger molecular weight band observed in western blot analysis in WT males and hermaphrodites, as well as in \textit{fem-3 (gain of function)} and \textit{fem-3 (loss of function)}. The presence of this band at the same molecular weight in multiple, independent replications of this western blot experiment suggest this could be a cross reactive signal, rather than nonspecific staining. While our whole-germline analysis of WT hermaphrodite germlines suggested no specific staining, perhaps reanalysis at a higher magnification of flattened hermaphrodite germlines might reveal some staining, cross reactive or otherwise, in oocytes. In order to investigate this hypothesis, a subsequent western blot should be performed using a sample of L3 hermaphrodites, which have proliferating germline stem cells and early spermatocytes in meiotic prophase but no oocytes. Absence of the higher molecular weight band would indicate that the band is an unidentified protein present in oogenesis. Presence of this band would suggest this higher band is specifically not involved in oogenesis, and would indicate expression in another unrelated tissue. Another interesting possibility is a similar localization pattern as the one presented in Figure 5-1A, of colocalization with chromosomes during the meiotic divisions. One speculative interpretation of this hypothetical result is a cross reactive protein with a chromatin binding function specifically during metaphase.

While we have primarily focused on the role of SPE-26 during and after the localization of SPE-26 to the nucleoplasm during the karyosome stage, the possibility of a function before this localization has not yet been ruled out. However, the existence of such a function is not supported our data. Analysis of cell cycle markers associated with progression through pachytene, diplotene, and into the karyosome stage revealed no noticeable difference between \textit{spe-26} mutants and WT until the karyosome stage.
Identifying SPE-26 binding partners

These cytological investigations into SPE-26 function should be complemented by robust molecular biological and biochemical techniques. Bioinformatics can only predict so much about SPE-26 function without corresponding wetlab experimentation. Most importantly, any molecular interactor(s) of SPE-26 should be determined. This is important for several reasons. First, knowledge of binding partners may inform us in attempting to determine the function of SPE-26 in spermatogenesis. Given the apparent importance of SPE-26 for timely exit from the karyosome stage, the interaction between SPE-26 and its interactor(s) could reveal a great deal about the mechanisms that control this poorly understood stage. Second, this interaction will be important from a protein biochemistry point of view, because it would be one of the first studied interactions known for a BACK-Kelch protein. While the canonical hypothesis is that BACK domains function largely as accessory domains for BTB and Kelch domains, this hypothesis could be challenged if the BACK domain of SPE-26 is revealed to be essential for its molecular interactions (in absence of a BTB domain). Hence, the most important next step is to identify any molecular interactor(s) of SPE-26.

One particularly interesting and time-efficient method for doing so is a yeast 2-hybrid screen, which will identify all *C. elegans* genes that can interact with *spe-26*. One limitation of this method is that not all of these predicted interactors may actually interact with SPE-26 *in vivo*. Corroboration of these results with an immunoprecipitation (IP) of sample from *C. elegans* would provide evidence for these interactors' significance *in vivo*. Further experimentation performing an IP of *spe-26* mutant samples may illuminate how these mutant forms of the protein interact (if at all) with an identified interactor. Because of the location and type of
mutations relative to the antibody binding site, this would only be possible with the \textit{it112} and \textit{hc138} alleles. An IP using \textit{it112} sample may inform us of the function of the Kelch domains in SPE-26; while an IP using \textit{hc138} may inform us of the importance of the protein's BACK domain. Another alternative is the creation of a \textit{spe-26::FLAG} construct strain that would allow for the use of anti-FLAG antibody in a western blot or perhaps even coupled with an IP.

\textbf{Cell cycle dynamics in \textit{spe-26} mutants}

The cell stage-specific localization of SPE-26 suggests regulation through some molecular mechanism. One important class of molecular regulators in the cell are kinases, which control the function and localization of their specific targets through phosphorylation. The prediction of CDK1 phosphorylation sites on the structure of SPE-26 complements our preliminary finding that phosphorylation of nuclear CDK1/Cyclin epitopes arises late to enter the nucleus during the karyosome stage of \textit{spe-26} mutants. More experiments are needed to corroborate this preliminary result. A more conclusive result of such experiment along with the simultaneously-occurring SPE-26 localization defect of \textit{spe-26} mutants (diffuse in \textit{it112}, absent in other mutants) would suggest interaction between SPE-26 and CDK1/Cyclin. Further investigation regarding the specific localization of CDK1 and stability of Cyclin B, particularly after the karyosome stage, might reveal further associations between this protein in SPE-26 elsewhere in the cell cycle. Additionally, other important meiotic cell cycle markers may prove to be disrupted in \textit{spe-26} mutants. PLK-1, the \textit{C. elegans} Polo Kinase, is required for NEBD and meiotic chromosome segregation (Chase et al., 2000). Given the NEBD and meiotic chromosome segregation defects described here, it seems likely that PLK-1 function may also be disrupted in its timing, localization, or function in \textit{spe-26} spermatocytes. Yet another important
cell cycle kinase is AIR-2, the *C. elegans* aurora kinase, which is required for meiotic microtubule attachment to centromeres (Bishop et al., 2005). One interpretation of the metaphase I microtubule attachment phenotype described in Figure 2-1 is that the process of kinesin attachment to centromere is disrupted in *spe-26* mutants. Thus, AIR-2 may also prove to be disrupted in its timing, localization, or function as a result of *spe-26* mutation. These various markers help cell biologists pinpoint the exact timing of certain cellular events; for this question, they can be used to pinpoint when exactly the mutant phenotypes are noticeable.

**Terminal Cell Analysis**

One intriguing aspect of the *spe-26* mutant phenotype is the relative success of spermatocytes to successfully segregate unnecessary contents to one side of the terminal cell, in an attempt to bud off from a residual body that proves fruitless due to cytokinesis errors. While our results suggest that the meiotic microtubules are drastically segregating and reorganizing to form networked microtubules, the extent to which this process occurs as normal remains unknown. One remaining question is whether or not these microtubules are releasing from the centrosome in their reorganization. Recent work suggests that WT meiotic microtubules reorganize in the residual body into non-centrosomal microtubules (Winter *et al.* 2017, awaiting publication). Organization of *spe-26* terminal microtubules relative to centrosomes remains unknown. Analysis with such markers for the centrosomal proteins SPD-2 and gamma-tubulin may reveal whether or not this post-meiotic microtubule reorganization proceeds normally.

**Final Conclusion**

Here, we have investigated, using cytological and bioinformatics methods, the *C. elegans* Kelch-like protein SPE-26. We show that SPE-26 concentrates in the nucleus during the poorly-
understood karyosome stage of late meiotic prophase. While we do know more about which specific processes are disrupted when SPE-26 is mutated, we do not yet know specifically the molecular or cellular function of SPE-26. The cytokinesis failures observed in spe-26 mutants suggest SPE-26 may be required for this actin-driven process. Our results also suggest a function in aiding spermatocytes exiting the karyosome stage, however our knowledge of how spermatocytes do this remains incomplete. Before they can perform their function as gametes, spermatocytes must undergo a complex and highly coordinated series of developmental processes, many of which we do not fully understand. Furthering our knowledge of one “dancer,” SPE-26, and one “step,” the karyosome stage, in the intricately choreographed “dance” of spermatogenesis, will give us a more complete understanding of this process that is fundamental to sexually-reproducing life.
Figure 4-1: Chromatin colocalization of SPE-26 during meiotic divisions observed in WT and spe-26 (hc139).
(A-B) Immunofluorescence microscopy using anti-SPE-26 antibody (Red), anti-Tubulin antibody (Green) and DAPI (Blue) on flattened germlines of male WT (him-5) (A) and spe-26 (hc139) (B). Insert labels colors are as follows: K = karyosome; Dk = diakinesis, Met I = metaphase I; Met II = metaphase II; Bud = budding division; Term. = terminal cell. Spread scalebars indicate 25 micrometers, and insert scalebars indicate 10 micrometers.
Chapter 5: Materials and Methods

C. elegans culturing:

After obtaining strains from the Caenorhabditis Genetics Center, worms were grown using standard methods for C. elegans culturing and husbandry (Brenner, 1974). For genetics and immunocytology experiments, small scale amounts of worms were grown on 35mm MYOB agar plates (Church et al., 1995) seeded with OP-50 strain E. coli. For SDS-PAGE/western blot experiments, large scale amounts of worms were grown on 100mm MYOB plates enriched with peptone (10g/L agar) and seeded with NA22 strain of E. coli. Strains obtained from the CGC include: CB1489 him-8 (e1489), RV120 spe-44(ok1400); dpy-20 (e1282)/let-92(s677); unc-22 (s7), BA793 spe-26 (hc138); dpy-13 (e184), BA825 spe-26 (hc140); dpy-20 (e1282), BA837 spe-26 (it112), BA824 spe-26 (hc139); dpy-20 (e1282), SL305 spe-26 (eb8) IV/nT1 [unc-? (n754) let-?]. Additional strains were generated by crossing DR466 him-5 (e1490) into these strains obtain more male worms; these double-mutant strains include: spe-26 (hc138); dpy-13 (e184); him-5 (e1490), spe-26 (it112); him-5 (e1490), spe-26 (hc139); dpy-20 (e1282); him-5 (e1490).

Immunocytology:

Worms were first picked to an un-seeded MYOB agar plate to ensure worms were free of excess bacteria. About 10 worms were then picked into 5µL of Edgars buffer (Edgar, 1995: 60mM NaCl; 32mM KCl; 3 mM Na₂HPO₄; 2 mM MgCl₂; 2 mM CaCl₂; 5 mM Hepes; 0.2% glucose; pH 7.2) with 1mM levamisole on a positively charged Superfrost Plus slide (Fischer Scientific cat # 12-550-15). Worms were then dissected using a 0.3mm x 13mm syringe in order to isolate individual gonads. Next, 24mm x 40mm coverslips with four rectangularly placed dots of silicon grease were applied to the slides. For whole gonad preparations, no pressure was applied to the cover slip; for cell monolayer preparations, however, a small amount of pressure...
was applied to the cover slip. Unless otherwise noted, slides were then immediately placed into a dewar of $\text{N}_2$ for a flash freeze. To fix the samples to the slide, slides were removed from the dewar and coverslips were removed to freeze-crack the samples and immediately placed in a coplin jar filled with methanol pre-chilled to -20°C. Specimens were fixed in -20°C at least overnight or for a maximum of a month. Unless otherwise noted, slides were then rehydrated in 3x5 minutes in 1xPBS and subsequently a blocking solution (PBS with 0.04% NaN$_3$; 0.1% Tween-20; 0.5% BSA) for at least 20 minutes.

For certain immunocytology experiments (anti-SYP-4), an alternative fixation procedure was used. After dissection, flash freeze, and coverslip removal as above, slides were placed into prechilled -20°C methanol for exactly 1 minute, after which they were removed and a fixation solution consisting of 4% aldehyde, 1xPBS, 0.25M HEPES buffer, 0.5M EDTA, and 3.2uL MgSO$_4$, was added for 30 minutes. After this time, slides were washed for 5 minutes in a solution of 1xPBS-Tween (1mL Tween-20/L 1xPBS). Afterwards, slides were submerged in blocking solution (see above) for at least 20 minutes.

Next, slides were incubated in 25µL primary antibody diluted in antibody buffer (PBS with 0.04% NaN$_3$; 3% BSA) in a dark humidity chamber for 1.5-2 hours at room temperature unless otherwise noted. Dilution factor depended on the antibody being used: anti-Tubulin--1:100 (Sigma prod # 2168); anti-SPE-26-- 1:300 (from Yenzyme, see below; from 50% glycerol stock); anti-Nuclear Pore Complex-- 1:200 (Covance, Catlog # MMS-120p); anti-MPM2-- 1:200 (courtesy of Golden lab); anti-SYP-4-- 1:200 (courtesy of Villeneuve Lab); anti-RNA Polymerase II pSer_ CTD--1:800 (Millipore cat #05-598; stored in 50% glycerol stock); anti-
SPE-44--1:200 (courtesy of Harold Smith). Following this incubation, slides were washed in 1x PBS buffer before adding and incubating in diluted secondary antibody (see Table 5-1 for specific antibody conditions). Two types of secondary antibody were used for this thesis: anti-Mouse Dylite 488 FITC, which was diluted 1:100 (Jackson Immunoresearch Laboratories Catalog #: 211-482-171); and anti-Rabbit TRITC, which was diluted 1:400 (Jackson Immunoresearch Laboratories Catalog #: 111-025-144). After this incubation, slides were again washed in 1xPBS. Lastly, coverslips with 5µL of Fluoro-Gel II (Electron Microscopic Science) containing 6-diamidino-2-phenylindole (DAPI) were applied to slides.

Table 5-1: Antibody incubation and wash conditions.

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<th>Primary Incubation Time and Temperature</th>
<th>Length and Number of Post-Primary Wash</th>
<th>Incubation Time and Temperature</th>
<th>Length and Number of Post-Secondary Wash</th>
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Rabbit anti-SPE-26 antibody used in this thesis was generated and affinity purified by Yenzyme against the following epitope (see figure 3-2B Bioinformatics Chapter for relative location details) near the C-terminus of the protein: CLERRGTINEQSMEMDDDY.

All epifluorescence images were acquired using an Olympus BX60 microscope equipped with a QImaging EXi Aqua cooled CCD camera with an Olympus PlanApo 40x or 60x objective lens and IPLab software. All confocal images were acquired using a fully automated Nikon TE2000 inverted microscope equipped with BioRad scanning lasers, and were taken with 40x or 60x objective lenses. In some cases, the levels adjust function in Adobe Photoshop was used to spread the data containing regions of the image across the full range of tonalities.

For the SPE-26 heat scale intensity experiment, we used the intensity scale feature of Nikon NIS elements program for confocal imaging, and the "Rainbow" color setting.

**SDS-PAGE and Western Blotting**

For most experiments, 100 worms were picked to 20µL of M9 buffer with Roche cOmplete EDTA-free protease inhibitors (Sigma Aldrich cat # 11 873 580 001) on a 2mL screwcap tube cap, flash-frozen in N\textsubscript{2} \text{liq}, and then stored -80°C. Next, 20µL of 2x Laemmli SDS sample buffer (Biorad cat #1610737)+ 25µL Beta mercaptoethanol and heated to 95°C for 5 min. After heating, samples were vortexed for 30s and then centrifuged at 15,000rpm at room temperature (Eppendorf Centrifuge 5424) for 3min. For SDS-PAGE, Bio-Rad Mini-Protein TGX pre-cast tris-glycine gels any kD were used (cat # 4569035). 20µL of samples were loaded into each lane. 6µL of protein standards (Biorad Precision Plus Protein Dual Color cat #
1610374) were loaded into ladder lanes. Gels were run at 100V for between 60-100 minutes. 
Next, proteins were transferred to a PVDF membrane (GE # 10600021) using a standard semi-
dry transfer method at 19V for 30 minutes. Following transfer, membranes were then blocked
overnight at 4°C in a solution of 4% weight:volume Carnation instant non-fat, dried milk in
1xTBS-Tween (10x TBS recipe: 10mL 1M Tris-HCl, pH 8.0, 20mL 5MNaCl, and 970mL
dionized water; 1xTBS-T 100mL 10xTBS, 1mL Tween, 900mL ultrapure water; all recipes from
SD Hinton). Membranes were then incubated for 1-2 hours with a primary antibody diluted in
4% milk in 1xTBS-T. Following primary incubation, membranes underwent 6x5min washes in
1xTBS-T. Next, membranes were incubated for 1-2 hours with a secondary antibody diluted in
4% milk in 1xTBS-T and washed 6x5min in 1xTBS-T. For detection, 1.75mL of ECL (GE
Healthcare Amersham ECL Prime RPN2232) was applied to the membrane. After 5 minutes,
membranes were exposed in a dark room to X-ray film and developed using a Konica Minolta
catalog # SRX-101A film processor.
References


Uyehara, C.M. (2014). New Insights into Fibrous Body Protein Complexes Involved in C.
