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Determining SPE-16 interacting proteins during *Caenorhabditis elegans* spermatogenesis

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Abstract

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By Katherine Hays Reiter

The *spe-16* gene affects how sperm develop and move in the nematode *Caenorhabditis elegans*. My lab has cloned *spe-16* and found that it encodes an ubiquitin E3 ligase, homologous to Mind bomb, which is found in arthropods and vertebrates. Protein ubiquitination is used to tag proteins for removal from cells and also to direct their trafficking within the cell. This suggests that SPE-16 affects sperm development and movement by controlling ubiquitination of one or more substrate proteins. The goal of this work was to define proteins that are potential substrates for SPE-16. *spe-16* mutants are temperature sensitive so that hermaphrodites and males produce viable sperm at 20°C, but defective sperm when grown at 25°C. Consequently, large numbers of homozygous mutants can be propagated at 20°C and subsequently shifted to 25°C so that the mutant phenotype can be examined. My overall goal was to discover SPE-16 substrates and two experimental approaches were taken. First, I used a genetic suppressor approach where the goal was to explore whether *spe-16* self-sterile mutants could be restored to self-fertility. Such suppressors could define gene products that interact with SPE-16, however, none were recovered despite extensive screening of three *spe-16* mutants. Next, I took a biochemical approach that included 1D and 2D gel electrophoresis, Western blots, and ubiquitin assays. These approaches suggest that *spe-16* mutants affect the distribution and abundance of several proteins, but further research is needed to fully evaluate these potential substrates.

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This thesis is dedicated to my parents, John and Beth Reiter.

Table of Contents

List of Tables and Figures

Tables.

Figures.

CHAPTER I

Introduction

A. Overview of *Caenorhabditis elegans*

Caenorhabditis elegans is a 1 mm, transparent soil nematode found around the world. It first appeared as a model organism in laboratory studies during the 1960s through the efforts of Sydney Brenner (Brenner, 1974). Since then, it has become a common model for the study of development and behavior due to its short life cycle and sophisticated genetics (Riddle, 1997).

C. elegans can be easily maintained in the laboratory at 16°C, 20°C, or 25°C, mimicking its natural soil habitat temperatures. Laboratory cultures are fed *E. coli* on either NGM agar plates or in liquid culture and can retain viability for years if stored at -80°C (Riddle, 1997). The nematode life cycle starts with a fertilized egg, has four larval stages, and culminates in a sexually mature adult after 3.5 days of growth at 25°C. Adult hermaphrodites produce approximately 200-300 self-progeny per brood (depending on growth temperatures) and can live for up to two weeks.

The *C. elegans* haploid genome consists of 8×10^7 base pairs comprising 6 chromosomes: 5 autosomal and 1 X chromosome (Wood, 1988). *C. elegans* is a self-fertilizing hermaphrodite and also forms spontaneous males, created by X chromosome nondisjunction at meiosis in hermaphrodites at a frequency of 0.1% (Riddle, 1997). Males can be mated with hermaphrodites to produce cross progeny with a 1:1 ratio of males to hermaphrodites.

B. Spermatogenesis

Spermatogenesis is the process by which sperm are created in animals. Germ cells in both hermaphrodites and males undergo spermatogenesis during the L4 larval stage, and then the hermaphrodite switches to oogenesis while males continue to make sperm during the adult stage.

C. elegans germ cells begin development in a gonadal syncytium with a central cytoplasmic core, named the rachis. As meiosis begins, 4N primary spermatocytes bud from the rachis and undergo meiosis I, developing into 2N secondary spermatocytes (Figure 1A). As secondary spermatocytes bud from the residual body they form spermatids and leave unneeded cellular structures behind (L'Hernault, 2006). The budded spermatid contains its nucleus, mitochondria and fibrous body-membraneous organelles (FB-MOs), but lacks ribosomes and actin, and therefore does not undergo further transcription or translation (Ward et al., 1983; Ward, 1986). FB-MOs contain the cytoskeletal component, which in nematodes is the major sperm protein (MSP) (Roberts et al., 1986). During budding the FB double membrane retracts, releasing MSP (Figure 1B). The MSP filaments depolymerize and disperse throughout the cytoplasm, localizing to the pseudopod during later sperm development stages (Ward and Klass, 1982).

C. Spermiogenesis

Spermiogenesis is the final stage of spermatogenesis in which spermatids develop into mature spermatozoa. Nematodes produce nonflagellated amoeboid sperm that, like other crawling cells, use pseudopodia for motility. During spermiogenesis, several cellular processes occur which lead to pseudopod extension. Membranous organelles

fuse with the cell plasma membrane and deposit glycoproteins onto the surface of the cell body (Figure 1B). The cell body houses the remaining cellular components of the sperm, while MSP localizes to the forming pseudopod (Ward and Klass, 1982).

In hermaphrodites, sessile spermatids are produced during the L4 larval stage and are pushed into the spermatheca by oocytes, where they mature into motile spermatozoa. Male-derived spermatids do not undergo spermiogenesis until they are deposited in the hermaphrodite during copulation. The *in vivo* molecular signals that promote spermiogenesis are unknown. Spermatids can be activated in vitro, however, by using several initiators, including weak bases such as TEA, the ionophore monensin, and proteases (Ward et al., 1983). As the fertilized egg moves to the uterus, spermatozoa must crawl back to the spermatheca for future fertilization attempts. Male-derived spermatids, stored in the seminal vesicle, are larger than hermaphrodite-derived sperm (LaMunyon and Ward, 1998) and can out-compete hermaphrodite self-sperm to produce cross-progeny (Ward and Carrel, 1979; LaMunyon and Ward, 1995).

C. elegans pseudopods have unique cytoskeletal properties, since they lack the common actin and myosin based membrane flow mechanisms. A larger nematode sperm, *Ascaris suum*, has been used extensively to study the biochemistry and cell biology of amoeboid crawling (Sepsenwol et al., 1989). *A. suum* membrane flow is thought to be a recycling process in which there is evidence of new materials incorporated at the leading edge of the pseudopod, which track toward the cell body and disappear at the cell body/pseudopodial junction (Sepsenwol et al., 1989). Membrane flow has been tested in *C. elegans* using labeled beads, lectins and phospholipids, with similar results (Roberts and Ward, 1982). The presence of vesicles at the cell body/pseudopodial junction

suggests that endocytosis may mediate the internalization and recycling of cellular components from the pseudopod (Sepsenwol, 1989). It is assumed that incorporation and internalization must occur at the same rate in order for motility to occur.

D. Mutations affecting spermatogenesis

Over 44 genes are known to be required for proper spermatogenesis development (L'Hernault, 2006). Many mutants that cause self-sterility have been identified, the majority affecting spermatogenesis; such mutants are called SPErmatogenesis-defective mutants, or SPE mutants. Certain mutants affect the structure and/or function of pseudopodia, including *spe-16*.

E. Introduction to spe-16

spe-16 is a gene located on chromosome III that is required for normal sperm development in *C. elegans*. All *spe-16* mutants are temperature sensitive, so hermaphrodites and males can produce viable sperm at 20°C, but are self-sterile at 25°C. This allows homozygous *spe-16* mutants to be maintained at 20°C, but the mutant phenotype can be turned on and off with ease. Since *spe-16* mutants can maintain self-fertility at 20°C, it is possible that another component is acting in conjunction with *spe-16* at low temperatures. *spe-16* mutants also have abnormal pseudopod morphology, and extend and retract the pseudopod rather than maintaining the normal treadmilling motion (Figure 2) (Ratliff et al., personal communication).

The *spe-16* gene product is a 765 amino acid protein and it has homology to Mind bomb. Mind bomb is an E3 ubiquitin ligase that acts in intracellular signaling via the

Notch pathway in many animals, as described below (Itoh et al., 2003). The presence of 8 ankryin repeats and 2 RING (Really Interesting New Gene) finger domains in SPE-16 also identify it as a possible E3 ubiquitin ligase protein with high specificity for its substrate (Figure 3) (Ratliff et al., personal communication). Ankyrin repeats are known to be involved in protein-protein interactions, while RING fingers mediate ubiquitin transfer (Itoh et al., 2003). This suggests that SPE-16 facilitates ubiquitination of one or more substrate proteins involved in sperm differentiation.

Mind bomb is expressed in mammalian testes, but an embryonic lethal phenotype kills animals before its effects can be studied in this tissue (Krebs et al., 2004). The use of *C. elegans* to study Mind bomb is advantageous since a mutant phenotype is not lethal. The prior work of others has not linked Mind bomb to spermatogenesis, so perhaps SPE-16 may be participating in some other, as yet to be discovered, way. Several *spe-16* missense mutations have been isolated (Figure 4). Their corresponding single amino-acid changes presumably cause conformational changes in SPE-16 that affect its function, perhaps by disrupting its interaction with substrate proteins.

F. Introduction to Notch signaling

Notch is the receptor in a conserved signaling pathway involved in cellular development and differentiation. Mind bomb is a key component of the Notch pathway, and mutations in Mind bomb have been found to cause developmental defects in *Drosophila,* including arrested appendage development (Bray, 2006). *C. elegans* has two Notch receptors, LIN-12 and GLP-1, which interact with the DSL-1 ligands, LAG-2 and APX-1 (Bray, 2006). Notch ligands are membrane bound, limiting their signaling to

nearby cells. The signaling pathway begins when a DSL-1 ligand binds the Notch receptor (Figure 5). Two cleavage events occur, the first activating the receptor, while the second cleaves an intracellular domain that localizes to the nucleus. This free domain interacts with DNA-binding proteins within the nucleus to regulate gene transcription (LeBorgne et al., 2005).

Endocytosis of DSL ligands plays a key part in receptor activation. It is postulated that the internalization of ligands allows for post-translational modifications that activate the ligand and allow it to be recycled back to the membrane (LeBorgne et al., 2005). Mind bomb catalyzed ubiquitylation may play a role in ligand internalization, as well. It is hypothesized that the ubiquitylation of the ligand, by Mind bomb, activates the ligand in a way that allows it to interact with the Notch receptor (Figure 6). Prior to ligand activation, ligands are vulnerable to degradation (Bray, 2006). The exact interaction between Mind bomb and DSL ligands is unknown, and knowing the type of ubiquitin conjugation would help to elucidate the Notch pathway.

Notch signaling is known to play a role in *C. elegans* hermaphroditic gonad differentiation (Greenwald, 1998). Vulval development begins with two equivalent cells that can differentiate into anchor cells (AC) or ventral uterine precursor cells (VU). Differentiation occurs after lateral signaling, in which the fate of one cell inhibits other cells from having that same fate. LIN-12 and LAG-2 interactions mediate the fate of these cells, in which LIN-12 activated cells have a VU fate, whereas non-activated cells turn into anchor cells (Greenwald, 1998).

G. Introduction to ubiquitin pathways

Ubiquitin is a conserved 76 amino acid protein encoded by eukaryotic genomes (Kipreos, 2005). Used in cell signaling pathways, ubiquitin can be covalently linked to proteins to target their degradation or to modify their function. The number and type of ubiquitin attachment defines the result of the conjugation event. Mono-ubiquitination targets the protein for endocytosis, membrane trafficking, or protein sorting, whereas multi-ubiquitination is only known to target for endocytosis and membrane trafficking (LeBorgne et al., 2005). Poly-ubiquitination is a degradation signal that sends proteins to the 26S proteosome to be recycled.

Several steps are involved in ubiquitin conjugation, beginning with the ATP dependent E1 ubiquitin-activating enzyme, which binds ubiquitin. The one E1 in *C. elegans* transfers ubiquitin to one of 22 E2 ubiquitin-conjugating enzymes. The E2, bound with ubiquitin, then binds one of hundreds of E3 ubiquitin-protein ligases. Ubiquitin can either be directly transferred to the substrate, or is transferred to the substrate via the E3. There are four types of E3 ubiquitin ligases: HECT domain, U-box, monomeric RING finger and multisubunit RING finger complexes. Complexed E3 ligases do not bind substrates directly, rather, other components within the complex perform this duty (Kipreos, 2005). The SPE-16 protein is one of 152 RING finger proteins encoded by the *C. elegans* genome (Kipreos, 2005). How SPE-16 participates in ubiquitination during *C. elegans* spermatogenesis and which of the more than 500 sperm proteins are its substrate(s) (Ward, 1987) is the focus of this thesis.

CHAPTER II

Genetic Suppression of *spe-16* mutants

A. Introduction

spe-16 is a gene required for sperm development in the nematode *Caenorhabditis elegans*. *spe-16* mutants are self-sterile because they have defects in spermiogenesis, the final step of sperm differentiation. Our lab has discovered that *spe-16* encodes a homolog of Mind bomb, a conserved E3 ubiquitin ligase (Figure 3). This suggests that SPE-16 facilitates ubiquitylation of substrate proteins involved in sperm differentiation. Nine alleles of *spe-16* have been recovered and each is a unique point mutation that causes the same phenotypic defects (Figure 4). Three mutations *(hc54, eb35, eb42)* are missense and the affected residues reside in the ankyrin repeat region of SPE-16. Their corresponding single amino-acid changes presumably cause conformational changes in SPE-16 that affect its function, perhaps by disrupting its interaction with substrate proteins. I hypothesize that extragenic mutations will partially restore self-fertility to *spe-16* mutants and that such mutations will reveal SPE-16 substrates. Genetic crosses can determine whether verified suppressor candidates are intragenic revertants of *spe-16* or extragenic mutations in new genes.

B. Materials and Methods

Worm Strains: The following strains were used in the EMS mutagenesis screen: *spe-16(hc54) dpy-18(e364), spe-16(eb42) dpy-18(e364), spe-16(eb35) dpy-18(e364)*. All strains were mutants of the Bristol (N2) strain. *dpy-18(e364)* was used as a genetic marker to aid in subsequent analysis following mutagenesis. *dpy-18* is located on chromosome III, position 8.62, and is characterized by worms being shorter than wild type. *spe-16* is located nearby on chromosome III at position 8.20 and therefore its

linkage with *dpy-18* can be used to evaluate intra and extra-chromosomal suppressions. Each of the utilized *spe-16* alleles is a result of a missense mutation within an ankyrin repeat, with *hc54* changing amino acid 247 from a leucine to phenylalanine in repeat 1, *eb42* changing amino acid 292 from a leucine to glutamine and *eb35* changing amino acid 291 from a methionine to isoleucine. Both *eb42* and *eb35* are within ankyrin repeat 2 (Figure 4).

Leakiness at high growth temperatures: All *spe-16* mutants are temperature sensitive at 25°C. *spe-16* worms produce <1 progeny at 25°C, on average, compared to half wild-type N2 brood size at 16°C. In order to evaluate the effects of *dpy-18(e364)* on brood size, L4 worms from each strain were plated onto NGM plates spotted with *E. coli*, and their progeny were counted at 25°C over 6 days. Hatched progeny were removed to reduce counting error.

Induction of mutation: Each of the three strains was mutagenized with ethyl methanesulfonate (EMS) under standard conditions. EMS is a potent mutagen causing mostly G/C – A/T transitions (Riddle, 1997). Strains were raised at 20°C in liquid culture and young adults, prior to self-fertilization, were mutagenized. A total of 6.6 million *spe-16(eb35) dpy-18 (e364),* 3.3 million *spe-16(hc54) dpy-18(e364),* and 1.6 million *spe-16(eb42) dpy-18(e364)* haploid genomes were mutagenized. Worms were washed with M-9 buffer and concentrated by centrifugation. In a 6 cm Petri dish, 1 ml of EMS in M-9 buffer was added to 3 ml of packed worms to bring the final concentration to 50 mM

EMS. After 4 hours, the worms were washed in 12 ml of M-9 six times. Worms were then put back into liquid culture at 20°C.

Synchronization of worms: In order to synchronize generation times during the screen, fertilized eggs were harvested from young adults of each generation using a standard Clorox treatment. Young adult worms were settled out of liquid culture and separated from contamination and dauers by floating on Renografin, which is a density gradient solution (L'Hernault and Roberts, 1995). After washing with M-9 buffer to remove excess Renografin, a 20% solution of Clorox/ 0.5 M NaOH was added to worms in a ratio of 10:1 of packed worms. The worms were vortexed for 3.5 minutes to dissolve everything except the eggs and then subjected to centrifugation for 2 minutes. Pelleted eggs were washed in M-9 buffer and put back into liquid culture at 20°C.

Suppression screen: Mutagenized worms were allowed to grow in synchrony for two generations at 20°C to allow for potential suppressors to become homozygous. F2 larvae were shifted to 25°C and were Cloroxed 1 day after they began laying eggs to reduce false positive results from the intrinsic leakiness of *spe-16* mutants (Tables 1A-C). The Clorox cycle was performed twice more and F4 eggs were placed on high density egg plates to reduce loss (Lewis and Fleming, 1995). Plates with larger numbers of progeny were picked to test for potential suppressors (Figure 6).

C. Results

Leakiness at high growth temperatures: Following 6 days of counting progeny, *spe-16*(*hc54) dpy-18(e364)* had an average of 0.3 progeny at 25°C, with a maximum of two progeny from a single worm. *spe-16(eb35) dpy-18(e364)* had an average of 0.3 progeny at 25°C with a maximum of 4 progeny from a single worm. *spe-16(eb42) dpy-18(e364)* had an average of 2.3 progeny at 25°C with a maximum of 8 progeny from a single worm. These data helped to define the number of false positives that might be seen in liquid culture. The highest number of progeny from each of the mutants occurred on day 2 of egg laying (Tables 1A-C).

Suppression screen: During inspection of L4 prior to cloroxing, I found that generation times were longer than wild type, therefore reducing anticipated yield. Mutagenesis could have affected development, thereby decreasing synchrony. After Clorox treatment to collect F4 progeny, L1 worms were present in mutagenized cultures from each mutant strain. After one month, none of the mutants had exponentially increased in the number of progeny.

D. Discussion

Although no candidate suppressors were isolated, this potentially reflects certain characteristics of the SPE-16 *in vivo* role that could provide insight into future experiments. EMS produces G/C to A/T transitions efficiently, as well as deletions and chromosomal arrangements less frequently (Anderson, 1995), but perhaps in order to achieve the required compensatory change we needed to have a higher frequency of A/T

to G/C transition. This type of transition could be accomplished by using another mutagen, N-nitroso-N-ethylurea (ENU), which has a similar efficiency to EMS (Riddle, 1997). Other mutagens, such as formaldehyde or UV-irradiation, yield kilobase sized deletions (Riddle, 1997), which are too large for the goal of this experiment. Since EMS produces random mutations, there could have been many locations in the *C. elegans* genome, or even in the target protein, that were hit, however, none of them affect its interaction with SPE-16. Perhaps an amino acid change in the substrate will not alter its structure to restore its binding to SPE-16. Monomeric RING finger E3s bind both the substrate and E2, which is indicated by SPE-16s RING fingers mediating ubiquitin transfer, as well as 8 ankyrin repeats, which show high specificity in protein-protein interactions. SPE-16 may also ubiquitinate multiple substrates at different stages of sperm development. In this case, a suppressor mutation would be needed in each substrate to restore partial fertility. The probability of the needed suppressors occurring in one animal is quite low. For instance, in *C. elegans*, the average forward mutation rate using EMS is 1:2000 (Brenner, 1974). Therefore, if two substrates, corresponding to two genes, needed to be mutagenized, the probability would be 1 in 4 million. Presumably we need a very specific mutation, if not multiple changes in the same substrate, to restore partial function. In this case, the frequency drops dramatically.

CHAPTER III

Biochemical Analyses

A. Introduction

To understand how the ubiquitin conjugation pathway is involved with SPE-16, biochemical analyses were pursued as a direct visualization method. Perhaps SPE-16 is involved in regulating a protein that is essential for cell polarity. In mutants, this pathway may cease when a substrate is not ubiquitinated, resulting in downregulation of the downstream product (Figure 8). On the other hand, perhaps SPE-16 is involved in protein degradation. In mutants, the potential build-up of excess protein could be detrimental to pseudopodium function. If this is the case, we might expect a build-up of ubiquitin that either cannot be transferred to the substrate or remains attached to the E2 (Figure 8). Through direct visualization methods, each of these hypotheses can be refined, giving further insight into SPE-16's activity in ubiquitination.

B. Materials and Methods

Sperm isolation: In order to analyze protein modifications between wild type and *spe-16* sperm, spermatids were isolated from *him-5(e1490)* and *spe-16(eb37) him-5(e1490)* males using a standard sperm isolation protocol (L'Hernault and Roberts, 1995). Worms were grown in liquid culture at 20°C and males were prepped for sperm isolation when hermaphrodite siblings were loaded with eggs. Contamination and debris was removed with Renografin and worms were filtered to separate hermaphrodites, males, and larvae. A 35 μm Nitex cloth filter will separate hermaphrodites from males, and a 20 μm filter separates males from larvae. Males were washed in sperm medium containing PMSF and drops were placed between two plexiglass squash plates. The spermatids were isolated by placing the plates under 13,000 lbs of pressure in a hydraulic

press. Carcasses were filtered from sperm by using a 10 μm filter and sperm were centrifuged in a 10% Percoll gradient to reduce debris. Sperm number was manually counted using a hemocytometer. Sperm were rinsed in SM salts and stored as pellets at -80°C for future use.

2D electrophoresis: To visualize protein differences between *him-5(e1490)* and *spe-16(eb37) him-5(e1490)* spermatids, each strain was analyzed by 2D polyacrylamide gel electrophoresis (PAGE). Proteins were solubilized using a urea/CHAPS buffer (Bio-Rad) and aliquotted to 1.56 x 10^6, 3.12 x 10^6, and 4.68 x 10^6 spermatid concentrations, which should correspond to 25 μg, 50 μg and 75 μg of protein, respectively. Forty μl 2.5M DTT and 5 μl BioLytes 3-10 range were added to 1 ml of urea/CHAPS buffer. Each aliquot received 185 μl of the solubilizing solution and was run on 11 cm immobilized pH gradient (IPG) strips with a range of 3-10. Gels were overlaid with mineral oil and run linearly at 50,000 V/hrs at 20°C for 12 hours in a Bio-Rad Protean Isoelectric Focusing (IEF) cell.

Focused strips were washed in 4 ml equilibrium buffer with 0.2 g 2.5% DTT each for 20 minutes. Strips were rewashed in 4 ml equilibrium buffer with 0.24 g 3% iodoacetamide for 20 minutes and unused strips were frozen at -20°C. IPG strips that had 50 μg spermatid protein from either *him-5(e1490)* and *spe-16(eb37) him-5(e1490)* were run on a second dimension that was a 1.5 mm 10-20% gradient SDS-PAGE with a 4% stacker. Gels were run nonlinearly at 200V for 3.25 hours. Both gels were silver stained to visualize proteins (Figures 9-10).

2D Western blot analysis: IPG strips containing 75 µg were run on a 15% SDS-PAGE gel with a 4% stacker at 200V for 3.25 hours. Gels were transferred onto PVDF membranes at 100 V for 50 minutes at room temperature. Membranes were probed with primary antibody MCA Ubi-1 (Encor, Lot 120103) at a 1:1000 dilution overnight at 4°C. Secondary antibody probes were performed with GAM-HRP (Bio-Rad, Cat 170-6516) at a 1:5000 dilution for 1 hour at room temperature. Membranes were developed using ECL kit and exposed for 1 and 2 minutes (Figures 11-12).

1D SDS-PAGE analysis: *him-5(e1490)* and *spe-16(eb37) him-5(e1490)* spermatids were run on a 15% SDS-PAGE gel with 5% stacker. 1.0 x 10^6 spermatids were run per lane against a control ubiquitylated protein lysate (Biomol; Cat UW0130) using a pre-stained SDS-PAGE standard broad range marker (Bio-Rad; Cat 161-0318). The gel contained two lanes of each strain so that strips could be cut up to allow Western blots against MSP and ubiquitin. The gel was run at 150 V for 90 minutes.

1D Western blot analysis: The SDS-PAGE gel comparing *him-5(e1490)* and *spe-16(eb37) him-5(e1490)* was transferred to PVDF at 100 V for 1 hour at 4°C. Half of the membrane was probed with primary antibody MCA Ubi-1 (Encor, Lot 120103) at a 1:1000 dilution, and the other half was probed with MSP antibody (TR-20; (Roberts et al., 1986) at a 1:100 dilution for 1.5 hours at room temperature. Both membranes were probed with ImmunoPure secondary antibody GAM-HRP (Lot JG126199; Thermo Scientific) at a 1:16000 dilution for 30 minutes. Membranes were developed with high

sensitivity super signal West Dura Extended Duration Substrate (Thermo Scientific; Lot JD120018A) and exposed for 2 and 20 minutes (Figure 13).

As an indirect method of testing loading concentrations, membranes were re-probed with SP56 antibody (Roberts et al., 1986) at a 1: 500 dilution overnight at 4°C. SP56 was anticipated to not be affected by either *him-5(e1490)* or *spe-16(eb37) him-5(e1490)* and should serve as a gel loading control. Both membranes were probed with ImmunoPure secondary antibody GAM-HRP (Thermo Scientific; Lot JG126199) at a 1:16000 dilution for 30 minutes. Membranes were developed with high sensitivity super signal West Dura Extended Duration Substrate (Thermo Scientific; Lot JD120018A) and exposed for 0.5 and 2 minutes (Figure 14).

Ubiquitin binding assay: To clarify samples for further analysis, 25 µg spermatid protein lysates were run over a ubiquitin binding matrix. RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Roche protease inhibitor) was used to lyse cells. Wild type and mutant lysate as well as control ubiquitinylated protein lysate (Biomol; Cat UW0130) were mixed with an ubiquitin binding resin (Biomol UW0125) overnight at 4°C. The resin was put in a column and proteins were eluted using 0.1M Gly-HCl pH 2.5 and 2M Tris-HCl pH 9.0. Proteins were concentrated by adding 160 µl chloroform, 480 µl methanol, and bringing the volume to 1.3 ml with distilled water. Samples were centrifuged, leaving a protein layer between the aqueous and organic phase. Five hundred µl methanol was added to the organic phase and pelleted. Concentrated protein was dissolved in sample buffer and frozen at -80°C for future use.

1D assay SDS-PAGE gel: Concentrated protein samples of eluted fractions, unbound fractions, and lysates of *him-5(e1490), spe-16(eb37) him-5(e1490),* and control ubiquitinylated lysate were run on an SDS-PAGE gel, as described above.

1D assay Western blot analysis: The gel was transferred to PVDF at 100V for 1 hour at 4°C. Membranes were probed with primary antibody MCA Ubi-1 (Encor, Lot 120103) at a 1:1000 dilution overnight at 4°C. Membranes were probed with ImmunoPure secondary antibody GAM-HRP (Thermo Scientific; Lot JG126199) at a 1:16000 dilution for 30 minutes. Membranes were developed with high sensitivity super signal West Dura Extended Duration Substrate (Thermo Scientific; Lot JD120018A) and exposed for 0.5, 2 and 20 minutes (data not shown).

C. Results

2D electrophoresis: The silver stained 2D SDS-PAGE showed a lot of similarities and differences between *him-5(e1490)* and *spe-16(eb37) him-5(e1490)*. Specifically, proteins at approximately 15-17 kD were at a lower concentration in *spe-16(eb37) him-5(e1490).* The *spe-16(eb37) him-5(e1490)* gel had lower protein concentrations overall, except for an increased signal at 50kD. Because the gel was run nonlinearly, the exact pI of proteins can not be estimated on the gel (Figures 9-10).

2D western blot analysis: A western blot was performed only on the lower portion of the gel (<25 kD). A spot appeared in the *him-5(e1490)* blot at approximately

10 kD, which was absent in the *spe-16(eb37) him-5(e1490)* blot. The spot does not seem to be present in the 2D SDS-PAGE silver stained gel (Figures 11-12).

1D western blot analysis: The initial blotting of the membrane with Ubi-1 and MSP showed differences in concentrations between wild type and mutant, but not total shifts toward one or the other. For example, at approximately 27 and 31 kD, the ubiquitin signal seems similar between wild type and mutant, but at approximately 6, 35, and 47 kD, *spe-16*(*eb37) him-5(e1490)* has a much higher concentration of protein (Figure 13). In contrast, the *him-5* signal is higher at 28 and 116 kD as compared to *spe-16 him-5*. A clear MSP signal appears at 15 kD, and both MSP (Figure 13, left panel) and SP56 (Figure 14) were both at a much higher concentration in wild type, as compared to *spe-16(eb37).*

Ubiquitin binding assay: A 20 minute exposure of the assay Western blot indicated a faint band at approximately 7kD in the control lysate and control unbound fraction. A light band could also be seen in the *him-5(e1490)* lysate and *spe-16* eluted fraction (data not shown).

D. Discussion

I performed 2D electrophoresis to discover if *spe-16* mutants produce sperm that have differences in protein composition and/or abundance as compared to wild-type. Several differences in protein composition/abundance were observed on 2D gels of *spe-16* versus wild-type. The type of 2D gels I ran used a first dimension (isoelectric

focusing) that was not run to equilibrium. While this approach does not allow determination of precise pI's, it has the advantage of maximizing the number of proteins observed on the gel. I found that *spe-16* mutant sperm appeared to have diminished abundance of proteins in the 15-17 kD region. Published *C. elegans* sperm 2D gels, where isoelectric focusing was performed until equilibrium was reached, suggest the protein spots I observed correspond to MSP isoforms, which run at approximately 15 kD at pI's of 7.1, 8.7, and 8.8 (Burke and Ward, 1983; Ward, 1987). This suggests that the *spe-16* associated psuedopodial defects could be related to changes in MSP abundance. In *C. elegans,* actin constitutes less than 0.02% of total spermatid protein (Nelson et al., 1982), whereas MSP is highly concentrated in the pseudopod. No detectable modification of MSP during localization has been identified, but perhaps SPE-16 regulates MSP synthesis or function during earlier developmental stages.

I loaded my gels based on equivalent numbers of spermatids, which were determined by manually counting aliquots. In theory, this should be the best way to compare mutant and wild type spermatid samples. However, when wild type was compared to *spe-16* mutants on gels, the protein concentrations were visibly different between the two samples. While a counting error is a possible explanation, it is also possible that *spe-16* worms have sperm with an overall lower protein concentration. The downstream effect of Notch signaling is to regulate gene transcription, therefore, perhaps a malfunctioning Mind bomb component results in inadequately activated ligands, and thus, reduces gene transcription (Figures 5-6). If this is the case, this might also affect spermatozoa, since transcription or translation do not occur after spermatids have completed budding (Figure 1).

My 2D SDS-PAGE analysis and Western blots were run on different gels (with different protein concentrations) and are not directly comparable, but, some conclusions can still be drawn. No ubiquitin signal was observed at 15 kD, which suggests that MSP is unlikely to undergo post-translational ubiquitinylation in spermatids, consistent with prior published results (Burke and Ward 1983). However, the clear differences between 2D gels of *spe-16* and *him-5* spermatids suggest a possible regulatory connection between SPE-16 function and MSP abundance. The lower portion of the 2D gel was blotted because of the readily apparent differences in wild type vs. mutant gels around 15-17 kD (Figures 9-10). The lack of a corresponding band at 10 kD in the 2D SDS-PAGE gel could be due to the protein running off the gel or low abundance of the protein that was detectable on a Western blot.

Since MSP concentration differences could be a result of *spe-16(eb37) him-5(e1490)* regulation, the membrane was probed with SP56, which is a protein not known to be affected by either *spe-16* or *him-5*, and therefore might serve as a loading control. SP56 signal was higher in wild type, suggesting either that wild type loading concentrations were higher than *spe-16,* or that SP56 is in some way affected by SPE-16 (Figure 14). SP56 is a monoclonal antibody that is directed toward several minor sperm proteins, found exclusively in sperm, which share a common antigenic determinant (Ward et al., 1986). SP56 has been shown to bind membrane-bound vesicles during spermatid budding from the residual body (Roberts et al., 1986), and therefore its connection with vesicle proteins may link it in some way to *spe-16*. Even if *him-5* gel lanes contained more protein than the *spe-16 him-5* lanes, this does not explain all of the differences in concentrations between wild type and *spe-16* probed with the ubiquitin

antibody Ubi-1. For instance, *spe-16* has a higher concentration of protein at approximately 47, 35 and 6 kD (Figure 13).

I also attempted to perform ubiquitin affinity chromatography on *spe-16* and wild type spermatids. All identified bands from the ubiquitin assay ran at approximately 6 kD, however, ubiquitin is 8.5 kD and the antibody used recognizes mono and poly ubiquitination, rather than free ubiquitin. No signal was seen in the control elution fraction, indicating that there may have been a problem during the binding procedure, and therefore the assay needs to be repeated. There are faint bands appearing in *him-5(e1490)* and *spe-16(eb37) him-5(e1490)* lysates that are lacking in the eluted fraction when one would expect that the signal should have appeared in both the lysate and eluted fraction. This result is most likely due to inadequate resin binding. Consequently, new samples were lysed in RIPA buffer including ubiquitin aldehyde and Ada-(Ahx)3-(Leu)3-vinyl sulfone as de-ubiquitinylation and proteosome inhibitors, respectively, and run on SDS-PAGE. The blot was probed with ubiquitin conjugate specific HRP-linked antibody (Biomol; Cat KW0150), both with and without a GAM-HRP secondary antibody (Thermo Scientific; Lot JG126199), both resulting in no signal (data not shown). In conclusion, the ubiquitin binding assay needs to be optimized before further progress will be possible.

CHAPTER IV

Future experiments

The optimization of recent experiments and several new experiments is needed in order to further understand the role played by SPE-16 during spermatogenesis. To account for protein concentration differences between the two samples, methods, such as a Bradford Assay (vanKley and Hale, 1977), should be performed and correlated with hemocytometer based cell counts. If *spe-16* sperm do indeed have decreased protein levels, this should be accounted for in any future biochemical analyses. All of my data used spermatids purified from male worms; however, the role of SPE-16 may not be confined to the spermatid development stage. Activated spermatozoa should be compared in the event that SPE-16 is involved in post-translational modification during spermiogenesis. One way to indicate the timing of SPE-16 activity would be to use an E3 ligase inhibitor. By comparing sperm phenotypes after using the inhibitor on wild-type sperm during spermatogenesis, followed by sperm activation, as well as using the inhibitor during spermiogenesis, would help to reveal SPE-16 timing. Ubiquitin affinity bead purification would correlate directly with ubiquitin changes in sperm, and should be repeated under optimum conditions. Large protein samples should be used in the event that SPE-16 substrates are in low concentration. Once conditions are optimized, sperm protein should be analyzed using Difference Gel Electrophoresis (DIGE) to quantify changes in protein distribution, which could be a result of SPE-16 activity. The goal would be to eventually characterize potential candidates using mass-spectrometry.

CHAPTER V

Conclusions

Fertility research is currently a major field in reproductive biology, and *C. elegans* has proven to be an excellent model organism for understanding its protein and genetic counterparts in humans. A homolog of SPE-16, Mind bomb, is expressed in mammalian testes, but a mutant phenotype kills animals before its effect can be studied in this tissue (Krebs et al., 2004). The use of *C. elegans* to study Mind bomb is advantageous since a mutant phenotype does not kill the nematode, but does produce a corresponding sterile phenotype at high temperatures. Current results suggest that SPE-16 may play a role in ubiquitinylating multiple substrates that may act in regulating cell polarity (Figures 9-10, 13). By increasing our understanding of the role played by SPE-16 in this pathway, we might be able to elucidate what role Mind bomb plays in mammalian testes. Spermatogenesis research in *C. elegans* is also beneficial for researchers interested in controlling pathogenic species of nematodes for agricultural and health purposes.

Tables and Figures

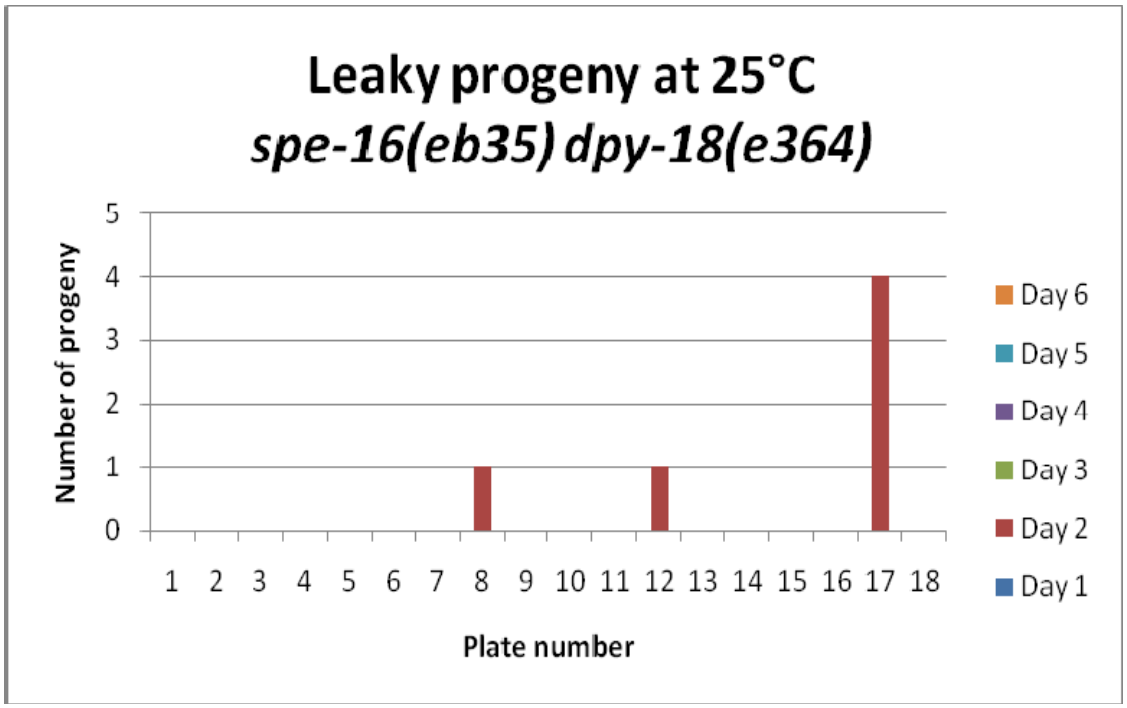

Table 1A. *spe-16(eb35) dpy-18(e364)* progeny counts at 25°C growth temperature.

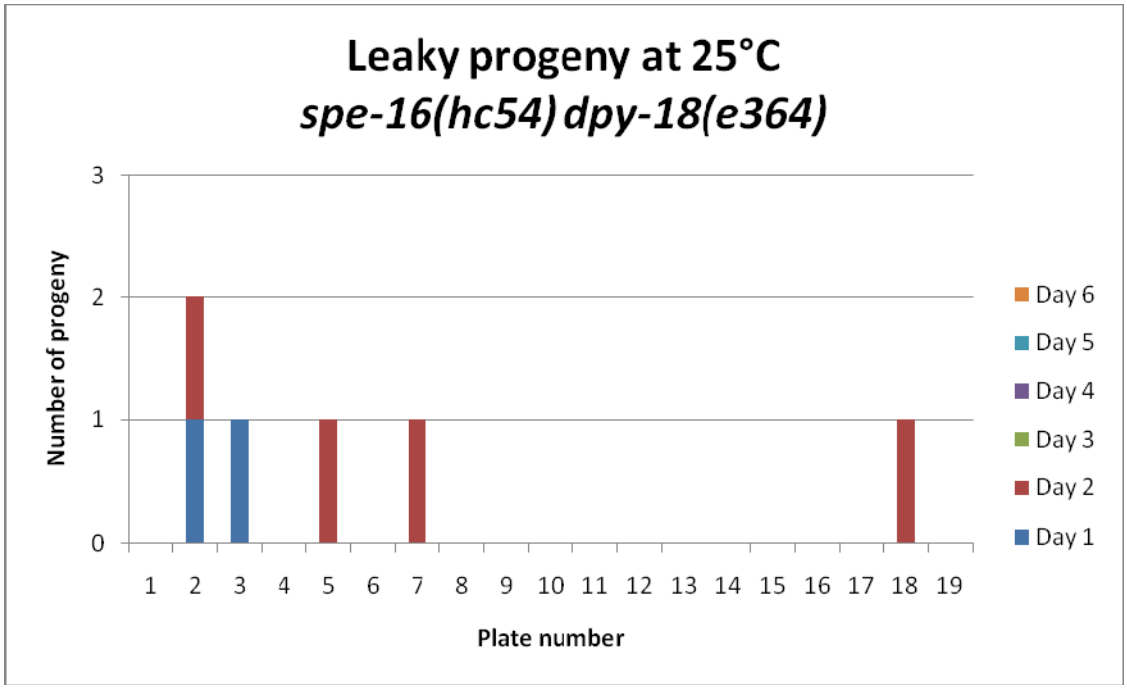

Table 1B. *spe-16(hc54) him-5(e364)* progeny counts at 25°C growth temperature.

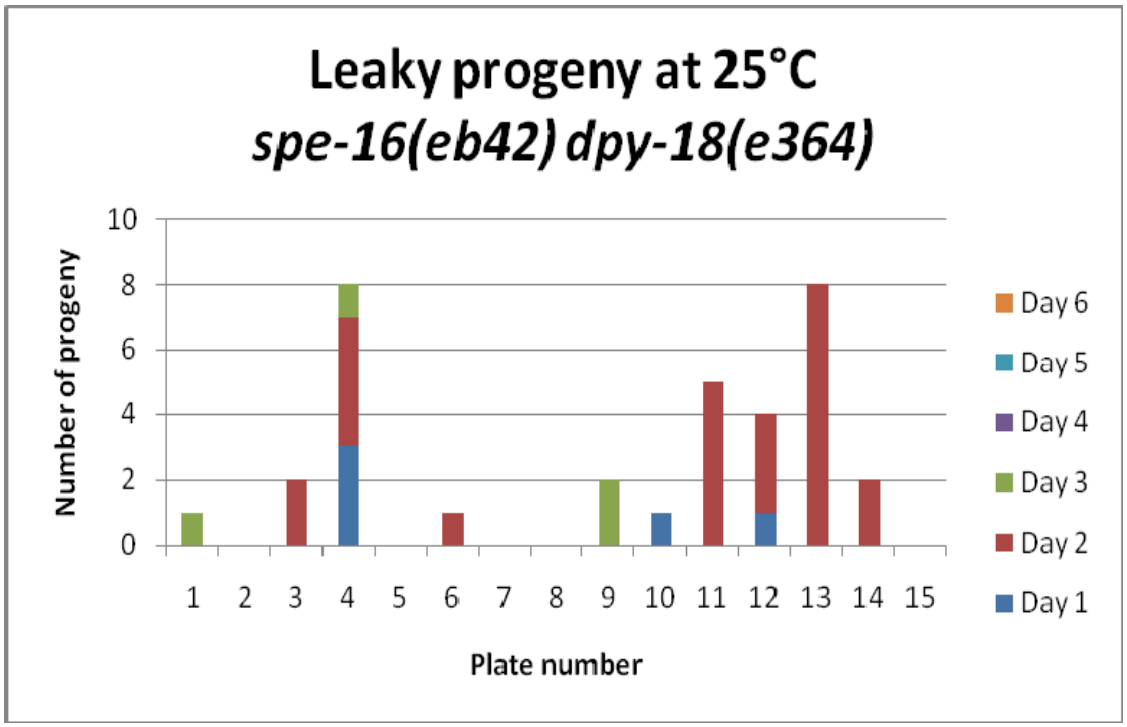

Table 1C. *spe-16(eb42) dpy-18(e364)* progeny counts at 25°C growth temperature.

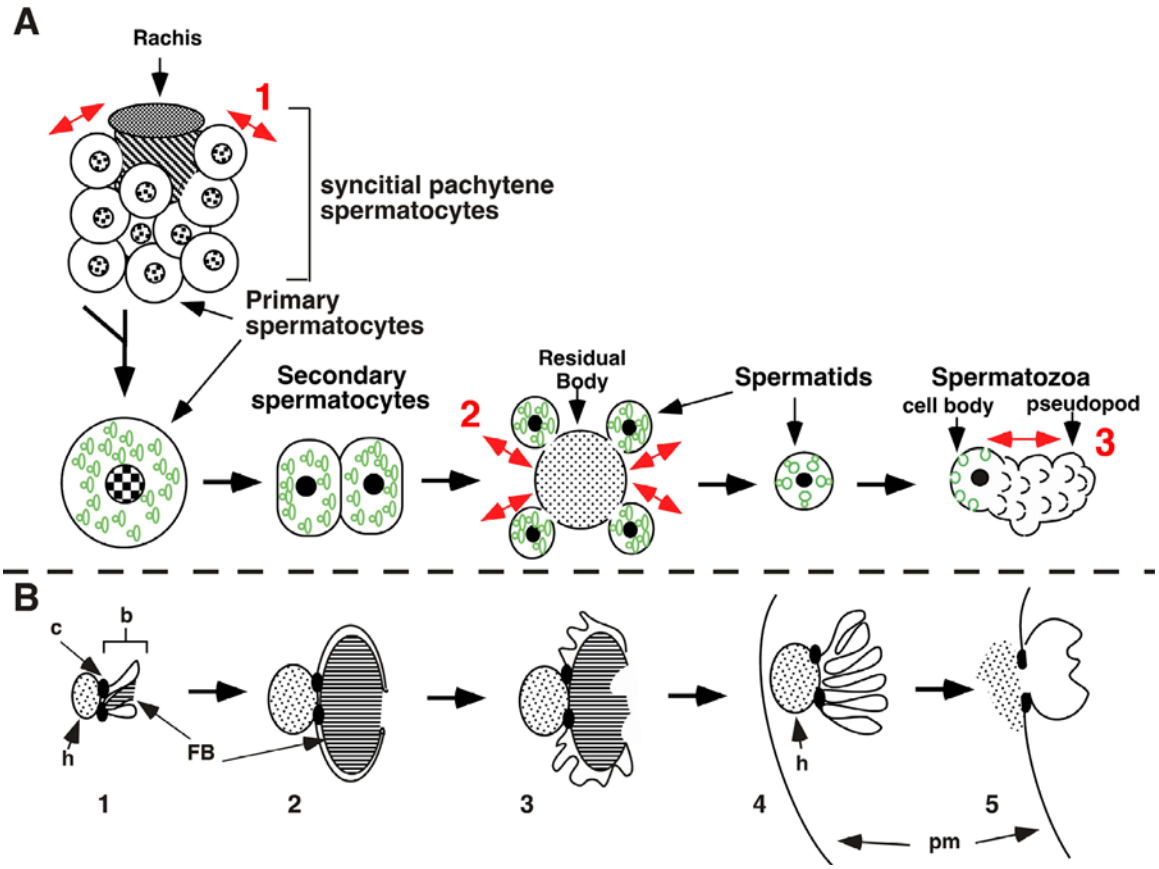

Figure 1. A. Spermatogenesis. (1) Germ cells begin development in syncitium with the rachis. Primary spermatocytes bud from the rachis and undergo meiosis. (2) As secondary spermatocytes bud from the residual body they form spermatids, which later undergo activation to form mature, motile spermatozoa (3).
B. FB-MO fusion during spermiogenesis. Fibrous body-membranous organelles contain the cytoskeletal component, MSP. While secondary spermatocytes bud from the residual body, the FB double membrane retracts, releasing MSP into the cytoplasm (3*). MO heads fuse with the sperm plasma membrane and release its contents to the extracellular region (5*). (From L'Hernault, 2006).

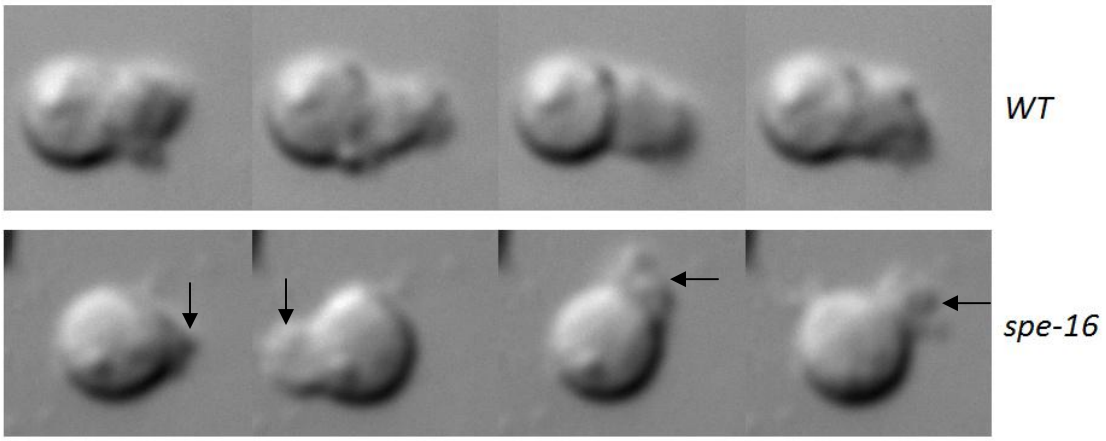

Figure 2. Time lapse image comparing wild-type and sperm pseudopodia defects of *spe-16* (From Ratliff et al., personal communication).

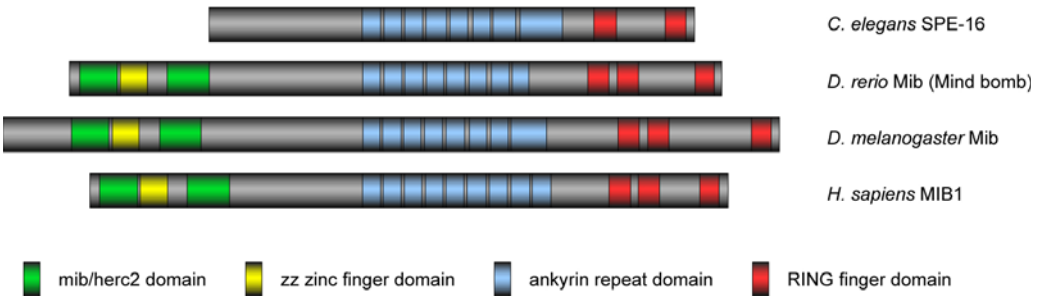

Figure 3. SPE-16 homology to Mind bomb (From Ratliff et al., personal communication).

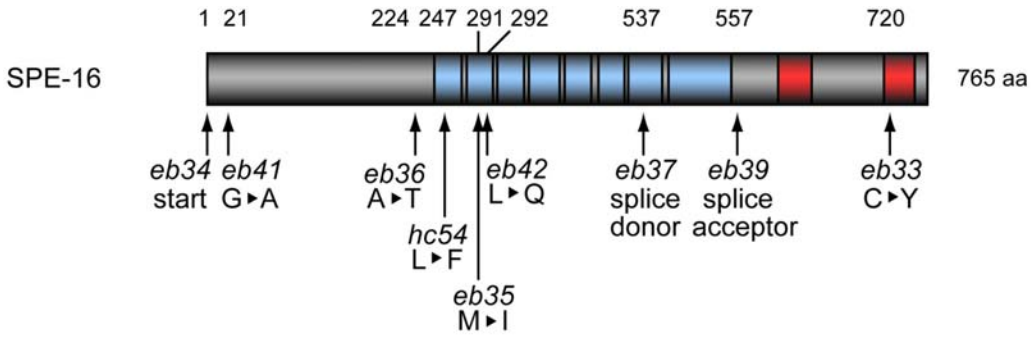

Figure 4. *spe-16* allele distribution. Red domains indicate RING fingers, which mediate ubiquitin transfer to a substrate. Blue domains indicate ankyrin repeats, which are involved in protein-protein interactions (From Ratliff et al., personal communication).

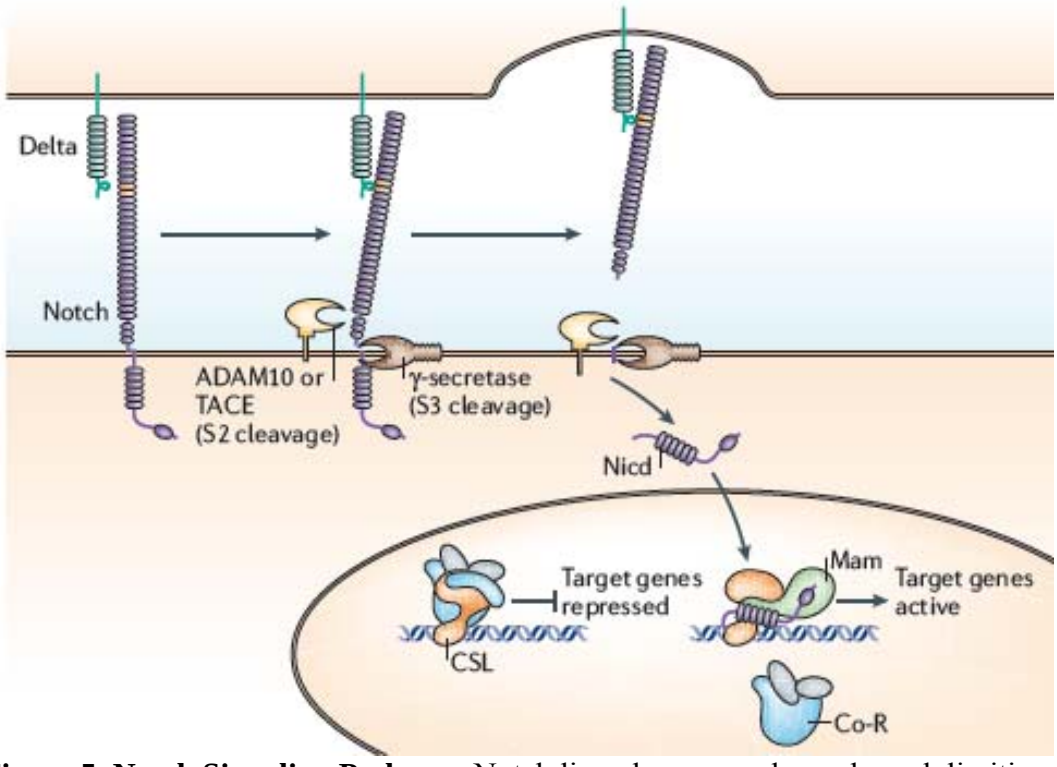

Figure 5. Notch Signaling Pathway. Notch ligands are membrane bound, limiting their signaling to nearby cells. The signaling pathway begins when a DSL-1 ligand binds the Notch receptor. Two cleavage events occur, the first activating the receptor, while the second cleaves an intracellular domain that localizes to the nucleus. This free domain interacts with DNA-binding proteins within the nucleus to regulate gene transcription (From Bray, 2006).

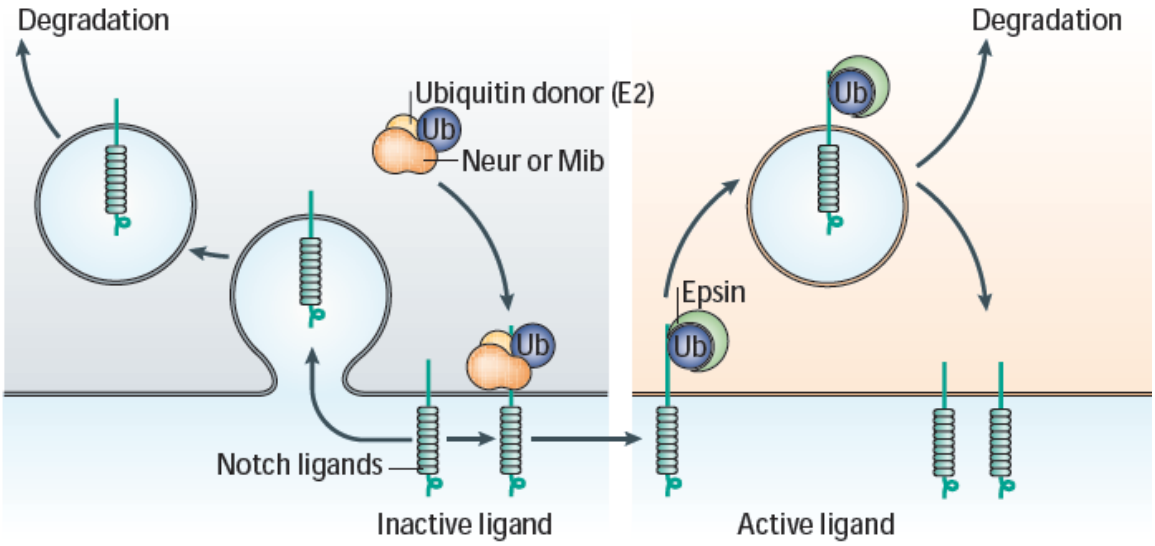

Figure 6. Hypothesized ubiquitination roles in Notch signaling. Membrane bound Notch ligands may be ubiquitinated by Mind bomb, activating the ligand in a way that allows it to interact with the Notch receptor (From Bray, 2006).

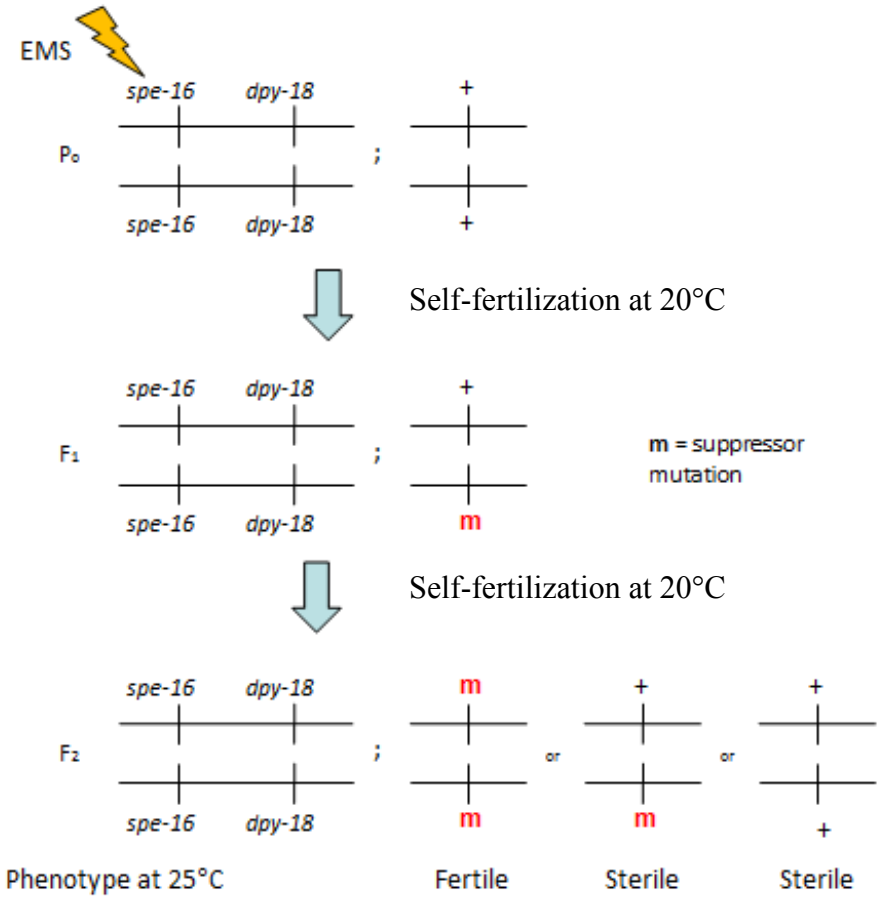

Figure 7. EMS mutagenesis scheme. Each of the three *spe-16* mutant strains was mutagenized with ethyl methane sulfonate (EMS) under standard conditions. Mutagenized worms were allowed to grow in synchrony for two generations at 20°C to allow potential suppressors to become homozygous. F2 larvae were shifted to 25°C and the progeny were assessed for potential suppressors.

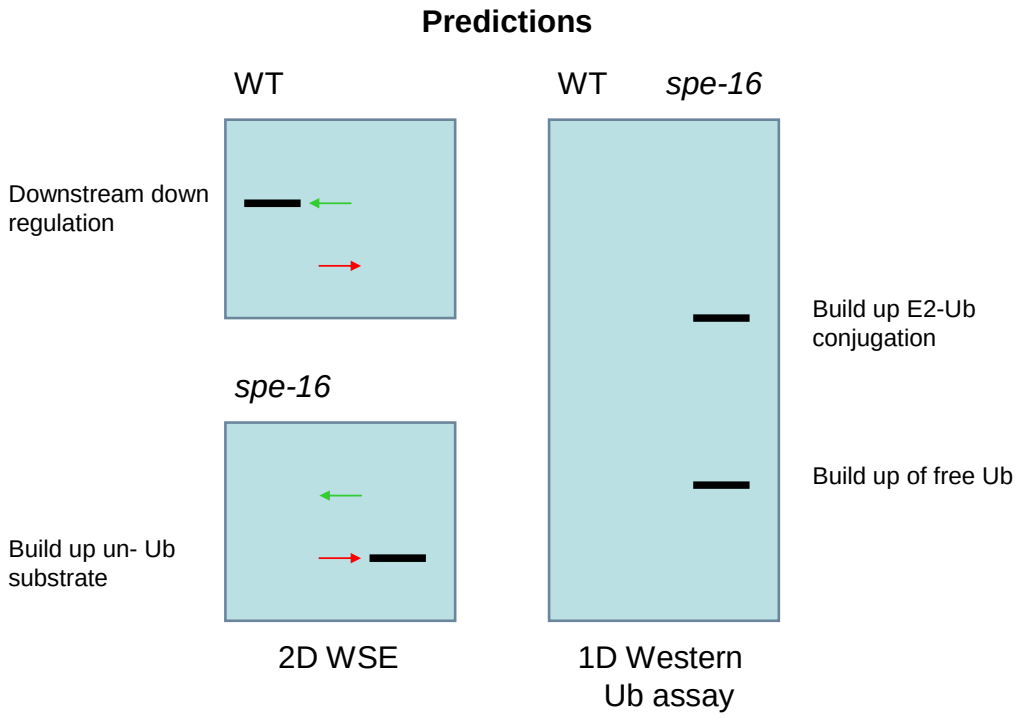

Figure 8. Predictions of biochemical analyses. Perhaps SPE-16 is involved in regulating a protein that is essential for cell polarity. In mutants, this pathway may cease when a substrate is not ubiquitinated, resulting in downregulation of the downstream product, as indicated on the left by the predicted 2D electrophoresis results from whole sperm extract. On the other hand, perhaps SPE-16 is involved in protein degradation. In mutants, the potential build-up of excess protein could be detrimental to pseudopodium function. If this is the case, we might expect a build-up of ubiquitin that either cannot be transferred to the substrate or remains attached to the E2, as indicated in the predicted ubiquitin Western on the right.

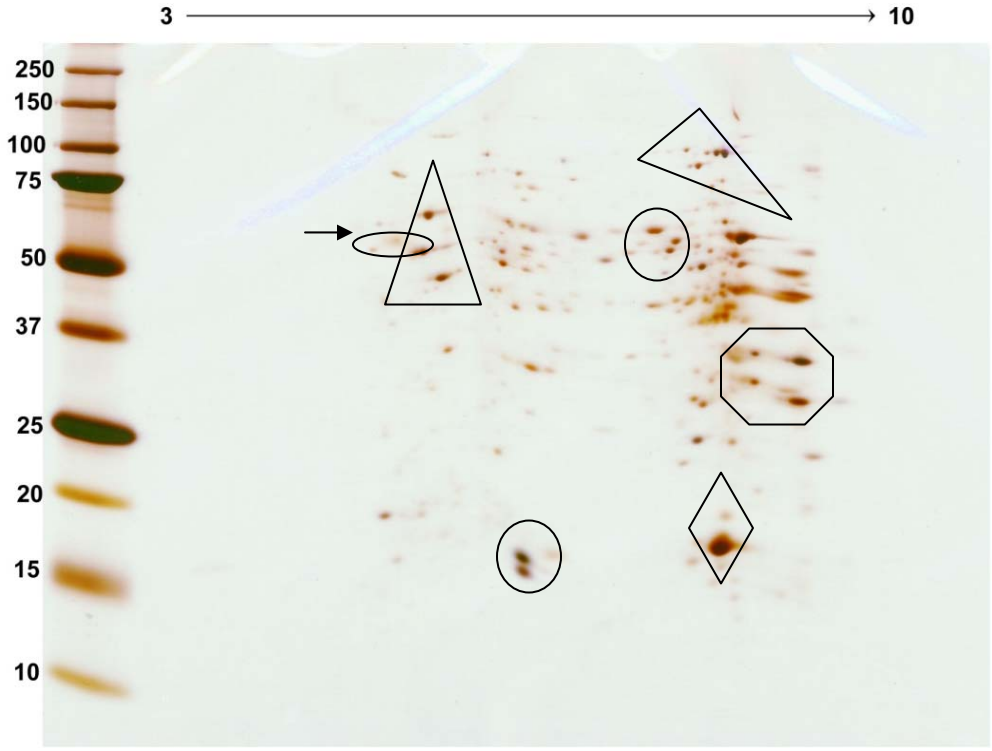

Figure 9. *him-5(e1490)* 2D SDS-PAGE silver stained. Signals at 15-17 kD could correspond to known MSP proteins. A lower signal was seen at approximately 50 kD as compared to *spe-16*.

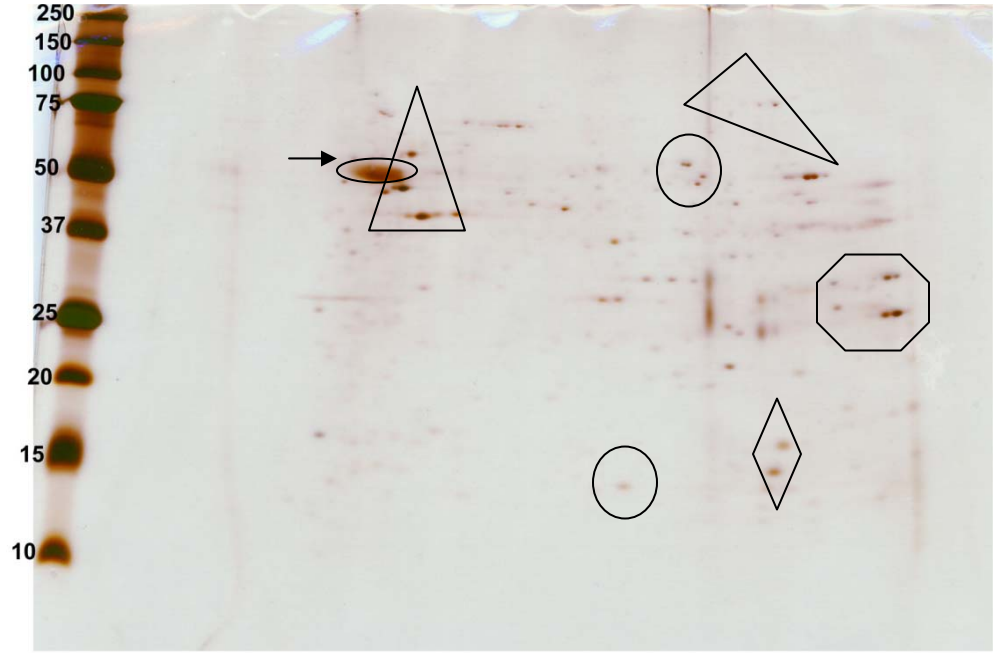

Figure 10. *spe-16(eb37) him-5(e1490)* 2D SDS-PAGE silver stained. Signals at 15-17 kD were lower than corresponding *him-5* signals. A higher signal is seen at approximately 50 kD compared to *him-5*.

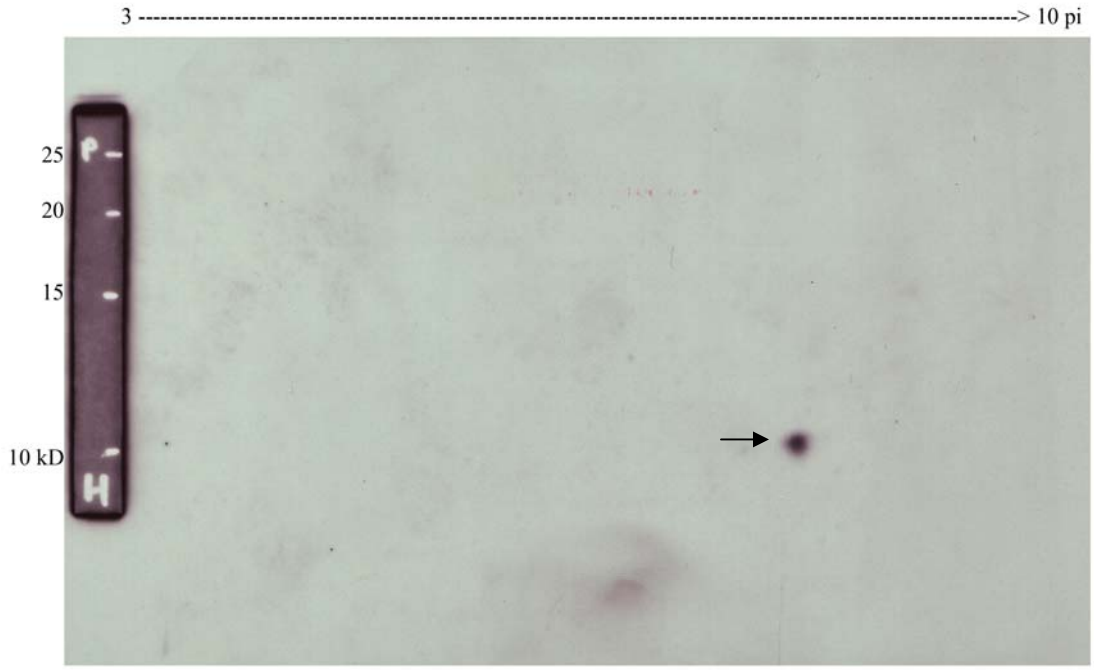

Figure 11. A 2D Western blot of whole sperm extract from *him-5(e1490)* males, probed with a 1:1000 dilution of anti-ubiquitin primary antibody. A clear signal appears at 10 kD.

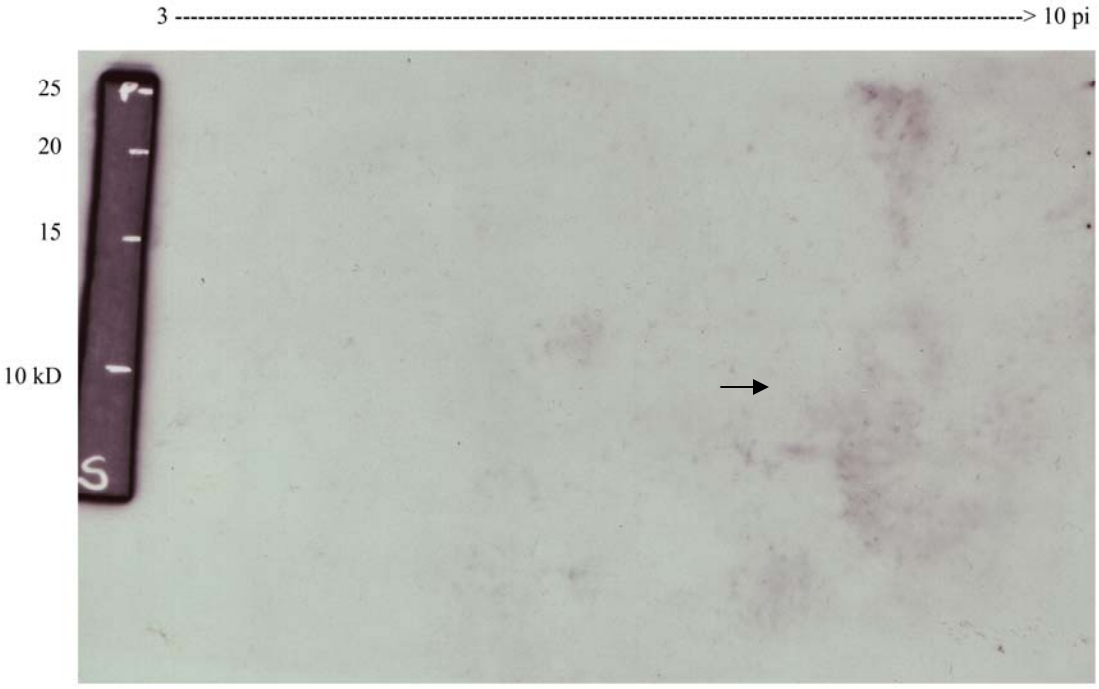

Figure 12. A 2D Western blot of whole sperm extract from *spe-16(eb37) him-5(e1490)* males, probed with a 1:1000 dilution of anti-ubiquitin primary antibody. No signals appear.

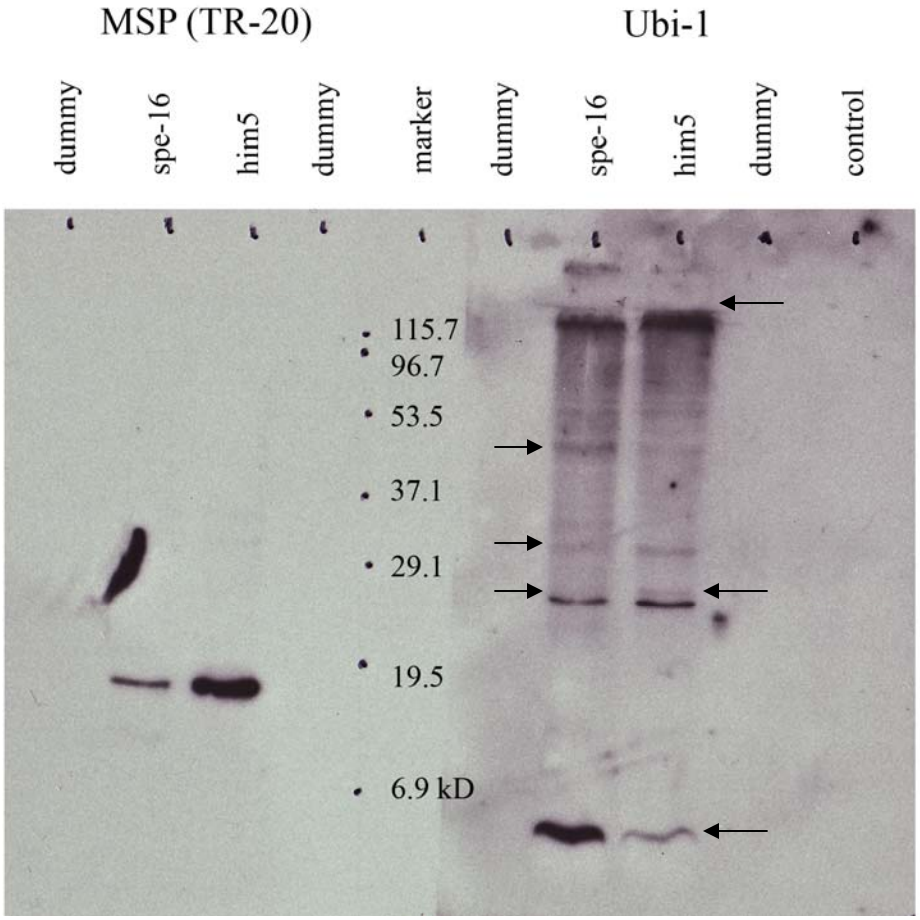

Figure 13. A Western blot of whole spermatid extract from *spe-16(eb37) him-5(e1490)* males, probed with 1:100 MSP and 1:1000 anti-ubiquitin antibodies. A clear anti-MSP signal appears at 15 kD. There are several anti-ubiquitin bands occuring at approximately 27, 31, 35, 47, and 116 kD.

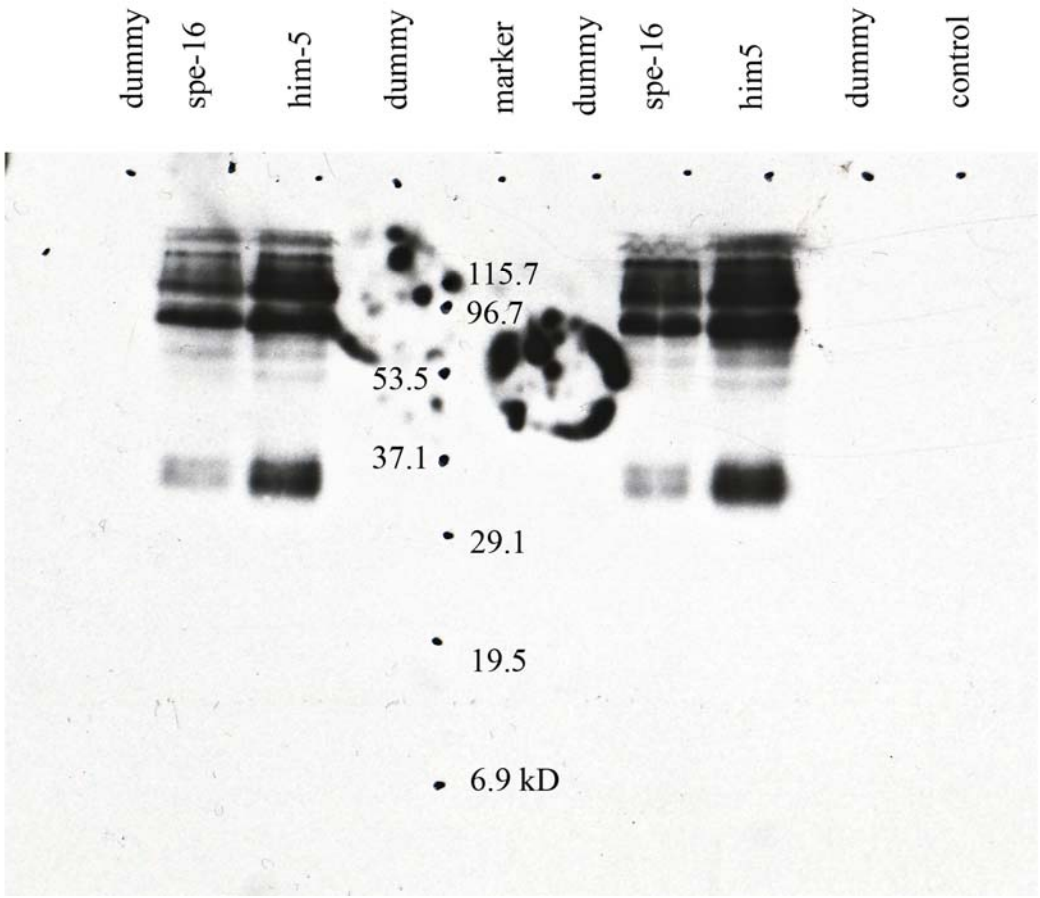

Figure 14. A Western blot of whole spermatid extract from *spe-16(eb37) him-5(e1490)* males, probed with SP56 anti-body at a 1:500 dilution. *him-5(e1490)* lanes show higher protein concentrations compared to *spe-16*.

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