IDENTIFICATION OF A VESICLE BUDDING MECHANISM FOR

THE RELEASE OF A MEIOTIC MATURATION HORMONE FROM

CAENORHABDITIS ELEGANS SPERM

MARY E. KOSINSKI

Dissertation under the direction of Professor David I. Greenstein

Fertilization is a complex process involving several steps, including sperm activation, oocyte maturation, chemotaxis, gamete recognition, and cell fusion. Many of these essential steps are controlled and regulated by intercellular communication between gametes. This thesis work examines the communication events that occur between sperm and oocytes cells that facilitate fertilization.

Oocyte meiotic maturation is one example of a step in fertilization in which intercellular signaling between gametes is required. In many species, sperm prepare the oocyte for fertilization by providing signals for meiotic maturation. Oocyte meiotic maturation is defined by the transition between diakinesis and metaphase I and is accompanied by MAP kinase activation, nuclear envelope breakdown, and meiotic spindle assembly. *C. elegans* sperm signal oocyte meiotic maturation using the major sperm protein (MSP) as a hormone. Interestingly, the MSP also functions as the central cytoskeletal protein required for the amoeboid motility of nematode sperm. The

discovery of MSP's signaling role raised the question of how sperm export MSP to signal oocytes at a distance. MSP lacks a hydrophobic leader sequence and *C. elegans* sperm lack many standard secretory components, such as ribosomes, ER, or Golgi.

Using light and electron microscopy we analyzed the mechanism of MSP release from sperm. We demonstrate that sperm bud novel MSP vesicles to signal distant oocytes. These 150-300 nm MSP vesicles contain both an inner and an outer membrane, with MSP sandwiched in between. Budding protrusions from the cell body contain MSP, but not the MSD proteins, which counteract MSP filament assembly, suggesting that MSP may generate the protrusive force for its own vesicular export. MSP vesicles are labile structures that generate long-range MSP gradients for signaling at oocyte and sheath cell surfaces. Both spermatozoa and non-motile spermatids bud MSP vesicles, but their stability and signaling properties differ. Spermatozoa generate a long-range, short-acting signal, whereas spermatids generate a long-acting signal. EM results suggest that differential vesicle stability affects the physical and temporal range of signaling. We hypothesize that the MSP vesicle release mechanism is in itself signal dependent and signals derived from the female animal initiate MSP vesicle release.

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Mary E. Kosinski

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Approved:

David Greenstein

David Miller

Christopher Wright

Gary Olsen

Bruce Appel

To my amazing family;

my daughter Macy,

my beloved husband Joe,

and our expectant baby

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CHAPTER I

INTRODUCTION

The propagation of a species is essential for its persistence. Several strategies

have evolved to achieve this task; however, the unique strategy of sexual reproduction

has fascinated and intrigued biologist for many years. Sexual reproduction is the union

of two parental sex cells (gametes), resulting in a genetically distinct individual. The

process of sexual reproduction is both critical and complex. Several steps regulate this

process such as the formation of gamete cells, release and recognition, fusion of the

gametes, and the programmed development that must follow creating an individual.

Despite the complexity of this process, sexual reproduction is accomplished by many

species. I too am intrigued and fascinated by this process and have chosen to study one

aspect of this complicated process. In my thesis work, I have focused on the

communication events that occur between sperm cells and oocyte cells and how these

signals are released to initiating gamete fusion.

Gametic Interactions

Communication between gametes is essential for sexual reproduction. The fusion

of two gametes is a complex process involving several steps that are mediated by

intercellular communication. The use of intercellular communication is shared in animals

with highly divergent reproductive strategies to control a medley of essential

reproductive processes, such as oocyte meiotic maturation, sperm activation, chemotaxis,

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gamete recognition, cell fusion, and egg activation (for reviews see Ward and Kopf, 1993; Vacquier, 1998; Runft et al., 2002; Wasserman, 2002; Kuwabara, 2003). Gamete communication and cellular interaction are essential for regulating many of these steps in fertilization (Hardy, 2002).

Meiosis and Oocyte Meiotic Maturation

The meiotic division is a critical step that is required for sexual reproduction. Meiosis is derived from the Greek word "diminution" meaning a reduction. As this definition suggests, meiosis is a nuclear division that only germ cells undergo in which the chromosome complement is precisely halved. Haploid gametes must be generated to create a diploid zygote when maternal and paternal pronuclei fuse. To accomplish this reduction, gametes undergo one round of DNA replication followed by two consecutive rounds of division (meiosis I and meiosis II) (Fig. 1). As in mitosis, (normal cell division) meiosis I, and meiosis II are divided into four stages prophase, metaphase, anaphase, and telophase (representing condensing, aligning, attaching to the spindle, and separating respectively). During S phase before mitosis diploid cells (2N) undergo chromosome duplication and become tetraploid (4N). Mitosis begins with prophase in which duplicated chromosomes condense. Chromosomes then align along the cell equator during metaphase and attach to microtubule spindles. Separation occurs during anaphase and division is completed during telophase resulting in newly formed daughter cells. Each daughter cell is genetically identical and the diploid complement is restored (Fig. 1).
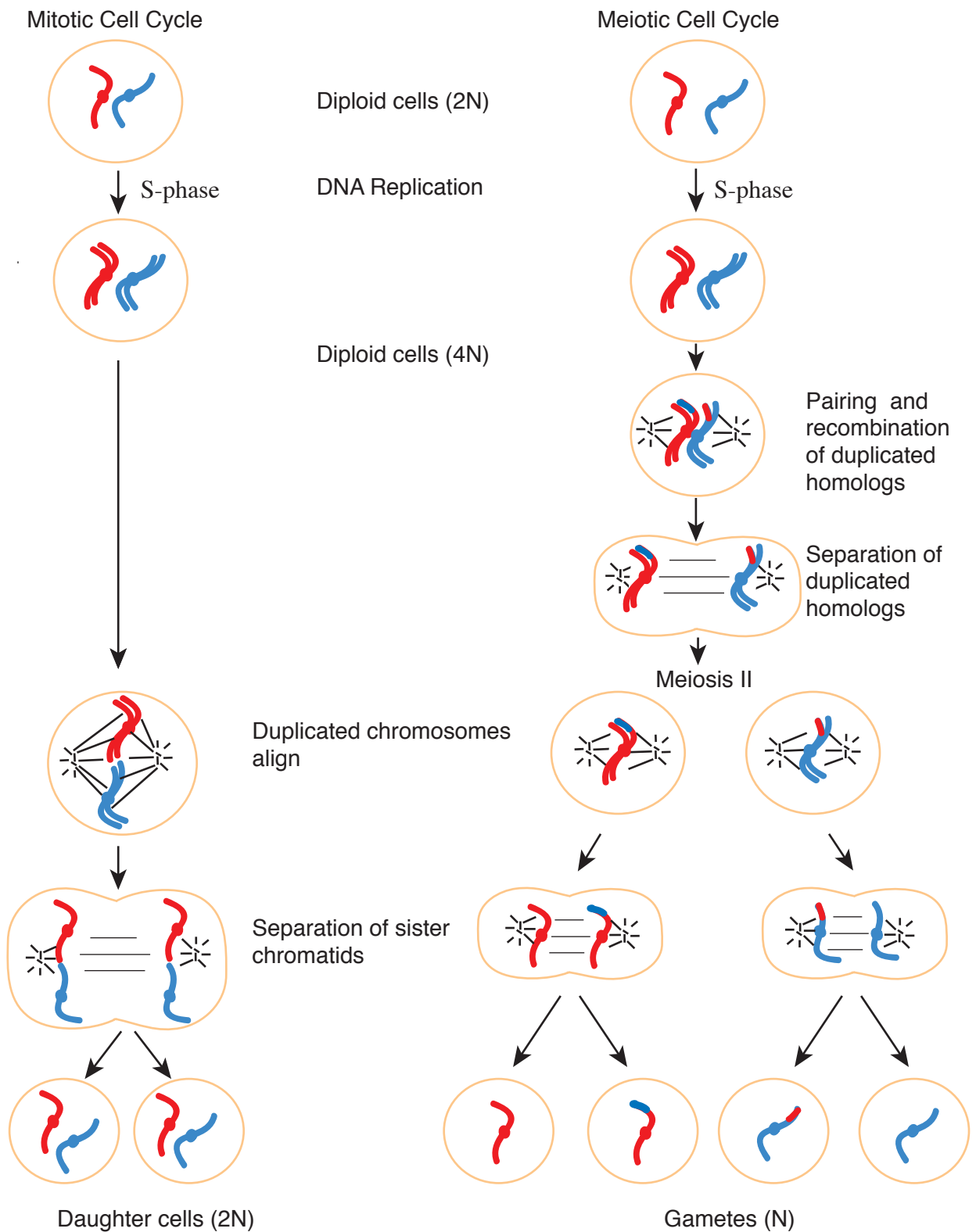
Figure 1. Comparison of mitosis and meiosis. During mitosis duplicated chromosome align and sister chromatids are separated generating two diploid genetically identical cells. In contrast during meiosis I duplicated homologous chromosomes align and homologs are separated. Meiosis II is similar to mitosis in that sister chromatids are separated however four haploid gametes are generated. Figure adapted from Alberts et al., 2002

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Meiosis differs from mitosis in two major ways. First, prophase I is prolonged and thus is further divided into five stages, leptotene, zygotene, pachytene, diplotene, and diakinesis. In prophase of meiosis I, duplicated chromosomes pair with their homologs forming bivalents (homologous chromosome pairs and their sister chromatids). During prophase I chromosomes condense, pair with homologs and synapse. Pairing of homologous chromosomes allows for the formation of synapsis and cross-over reactions thus facilitating exchange of genetic material from one homolog to another (Fig. 1). Following meiotic recombination, homologs become physically linked through the chiasmata structure. This physical linkage remains until cohesion between sister chromatids is lost at arms during anaphase I. Thus the meiotic recombination process provides two important functions, one of genetic exchange and also a structural role required in chromosome alignment. Often oocytes arrest or pause at one or even two places during prophase I, awaiting signals before completing meiosis. The second major difference occurs during anaphase I when the bivalents align along the spindle and homologous chromosomes are separated rather than the separation of chromatids as in mitosis. Separation of the homologous chromosome generates two diploid cells (2N) one with the maternal homolog and one with the paternal homolog (Fig. 1). The second division of meiosis (meiosis II) is similar to the mitotic division in that chromosome, previously duplicated before meiosis I (2N), align at the spindle and sister chromatids are separated. The division results in four haploid (N) gametes that are genetically different (Fig. 1).

Although both sperm and oocytes depend upon meiosis for the generation of haploid gamete nuclei, they each regulate meiosis differently. Sperm proceed through the meiotic

divisions uninterrupted, while oocytes often arrest during one, and sometimes two stages following DNA replication depending on the species. This unique arrest and resumption in the cell cycle exhibited by oocytes during meiosis, was recognized early by developmental biologist (Wilson, 1925). These physiological changes that occur within the oocytes prior to fertilization, cell cycle arrest, cell cycle resumption, nuclear envelope breakdown, and meiotic spindle assembly, have been termed meiotic maturation.

The underlying principle of oocyte meiotic maturation is to prepare the oocyte for successful fertilization. This arrest in the oocyte meiotic cycle allows the oocyte time to grow and accumulate nutrients. Thus, the timing of oocyte meiotic maturation must be tightly coordinated with oocyte development and fertilization. This coordination is achieved through signals that regulate oocyte meiotic progression (Ferrell, 1999, Masui, 2001). Signals regulating the meiotic divisions, and the timing of the meiotic divisions vary greatly among species. For example in some species such as nematodes, sponges and mollusks, signals released from sperm regulate oocyte meiotic maturation (Miller et al., 2001). Despite these differences, studies also reveal striking similarities and conservation in the molecular pathways regulating oocyte meiotic maturation among diverse animals. For example, the Maturation Promoting Factor (MPF) was discovered from amphibian oocytes and later revealed to be the universal meiotic cell cycle progression (Masui and Market, 1971; reviewed by Masui, 2001; reviewed by Morgan, 1995).

Additional Examples of Gametic Communication

Long-range signaling mechanisms are often utilized for gamete communication to control such processes as chemotaxis. For example, many marine invertebrate eggs secrete a long-range chemoattractant to direct sperm after they are released into the sea. L-tryptophan in red abalone (Riffell et al., 2002) and Resact and Speract in sea urchins (Garbers, 1989; Kaupp et al, 2003) are utilized as chemoattractants. Interestingly, sperm chemotaxis is also thought to occur in the mammalian female reproductive tract (Eisenback and Tur-Kaspa, 1999). Recently the hOR17-4 olfactory receptor was shown to function in sperm chemotaxis in humans (Spehr et al., 2003).

Many species also utilize short-range signaling during the interaction of sperm and egg, as components of the egg surface function at multiple steps in the fertilization process (Evans and Florman, 2002, Primakoff and Myles, 2002). In many species oocyte surface proteins, such as ZP3 in mammals, mediate contact-dependent signaling that induces the acrosome reaction, a specialized exocytotic event during which the acrosomal contents are released, thereby facilitating zona penetration and gamete fusion (Bleil and Wassarman, 1980; Wassarman. 2002; Talbot et al., 2003). Extensive studies in diverse organisms are just beginning to elucidate the basic mechanisms underlying gametic interactions. It is becoming clear that similarities between the multiple strategies occur. For example, members of the conserved transient receptor potential (Trp) class of cation channels are required for the ZP3-induced acrosome reaction (Jungnickel et al., 2001) in mouse sperm and also fertilization in the nematode *Caenorhabditis elegans* (Xu and Sternberg, 2003). By contrast, the lysins required for vitelline envelope penetration in abalone, are rapidly evolving under positive selection and thus differ greatly from other

species (Kresge et al., 2001). Although many advances have been made in understanding how gametes communicate during fertilization, much about gametic communication remains unknown.

In this document I will focus on the communication occurring between sperm cells and oocytes in *C. elegans*. Communication between sperm and oocytes is highly regulated and complex in *C. elegans*. Our results suggest that nutritional cues may trigger sperm to release a hormone signaling oocytes to undergo oocytes meiotic maturation. These studies are beginning to dissect pathways and mechanisms regulating gametic communication during fertilization in *C. elegans*.

C. elegans as a Genetic Model For Studying Gametic Interactions

C. elegans is an excellent model for studying gametic signaling because of the wealth of genomic, genetic, and cell biological resources available. *C. elegans* is one of the only genetic model organisms in which the events of oocyte development, meiotic maturation, ovulation, and fertilization can be directly observed in the intact animal (reviewed by Hubbard and Greenstein, 2000). Further the signal from sperm that initiates oocyte meiotic maturation, major sperm protein (MSP), has been identified (Miller et al., 2001 and Miller et al., 2003). The hermaphrodite sex of *C.elegans* allows signaling pathways to be studied in the presence of both sperm and oocytes within the same animal. In addition, mutants are readily available that contain only oocytes or only sperm to uncouple the two cell types. Further, DNA microarrays have recently profiled germline gene expression in diverse of backgrounds (Reinke et al., 2000). In addition to whole animal analysis, large quantities of sperm and oocytes can be isolated (Klass and Hirsh,

1981; Arroian et al., 1997). The characteristics of *C. elegans* coupled with the available tools and techniques enable a multidisciplinary analysis of gametic interaction to be performed.

C. elegans Reproductive Development

C. elegans has two natural sexes, hermaphrodites and males. Hermaphrodites are self-fertile and are essentially modified females that produce a limited number of sperm early in gametogenesis before switching exclusively to producing oocytes as adults (Meyer, 1997). The X to autosome chromosome ratio determines gender, such that hermaphrodites have two X chromosomes and two sets of autosomes (2X:2A) and males have one X chromosome and two sets of autosomes (1X:2A).

Oogenesis

The reproductive system of the adult hermaphrodite contains two U-shaped gonad arms each terminating proximally at a spermatheca, a specialized sperm storage compartment (Fig. 2A). The two spermathecae share a centrally located uterus that collects the newly fertilized embryos. Embryos are laid via the vulva, an opening located in the middle of the uterus. Hermaphrodite gonadal development begins upon hatching when the precursor gonad cells (Z1, Z2, Z3, and Z4) divide and reorganize generating a gonad primordiaum consisting of somatic and germ line cells. During the L3 and L4 stage the somatic precursor cells, Z1 and Z4 divide giving rise to the spermatheca, uterus, and sheath cells that surround germ cells. The germ cells of the two gonadal arms are generated from the Z2 and Z3 presursor cells. Each U shaped gonad arm is extended by a

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distal tip cell (DTC), establishing the most distal arm of the gonad. The DTC sends processes, which encompass approximately 19 germ cells, promoting mitosis and inhibiting meiosis within these distal cells (Hall et al., 1999). The DTCs maintain the mitotic proliferation of the germ cells by expressing the membrane bound LAG-2 ligand homologous to *Drosophila* Delta (the ligand for the Notch receptor). LAG-2 interacts with GLP-1, a Notch-family receptor, promoting mitosis and preventing the entry into meiosis. GLP-1 regulates the decision between mitosis and meiosis and is expressed in proliferating distal cells (Austin and Kimble, 1989; Lambrie and Kimble, 1991; Crittenden et al., 1994; Henderson et al., 1994; Tax et al., 1994). Thus these distal cells that respond to the LAG-2 ligand and activate GLD-1, are thought to constitute the germline stem cell population.

As germ cells flow proximally they exit the mitotic cell cycle and progress through the meiotic cell cycle beginning with meiotic prophase. Entry into the meiotic pathway requires GLD-1 and GLD-2 function. Hermaphrodites with *gld-1* null mutations develop germline tumors. These tumors are caused by the premature exit from meiosis and reentry into mitosis, causing cells to proliferate (Francis et al., 1995). GLD-1 encodes a KH/STAR domain RNA binding protein that likely sequesters mRNA in translational repression (Lee and Schedl 2001; Lee and Schedl, 2004). GLD-2 is a mRNA polyA polymerase which suggests that meiotic progression involves translational regulation. Once oocytes are formed, GLD-1 expression is lost suggesting proteins required during oocytes growth may be inhibited by GLD-1 (Jones et al., 1996). In addition to GLD-1, the proteins comprising the RAS/MAPK kinase signaling pathway
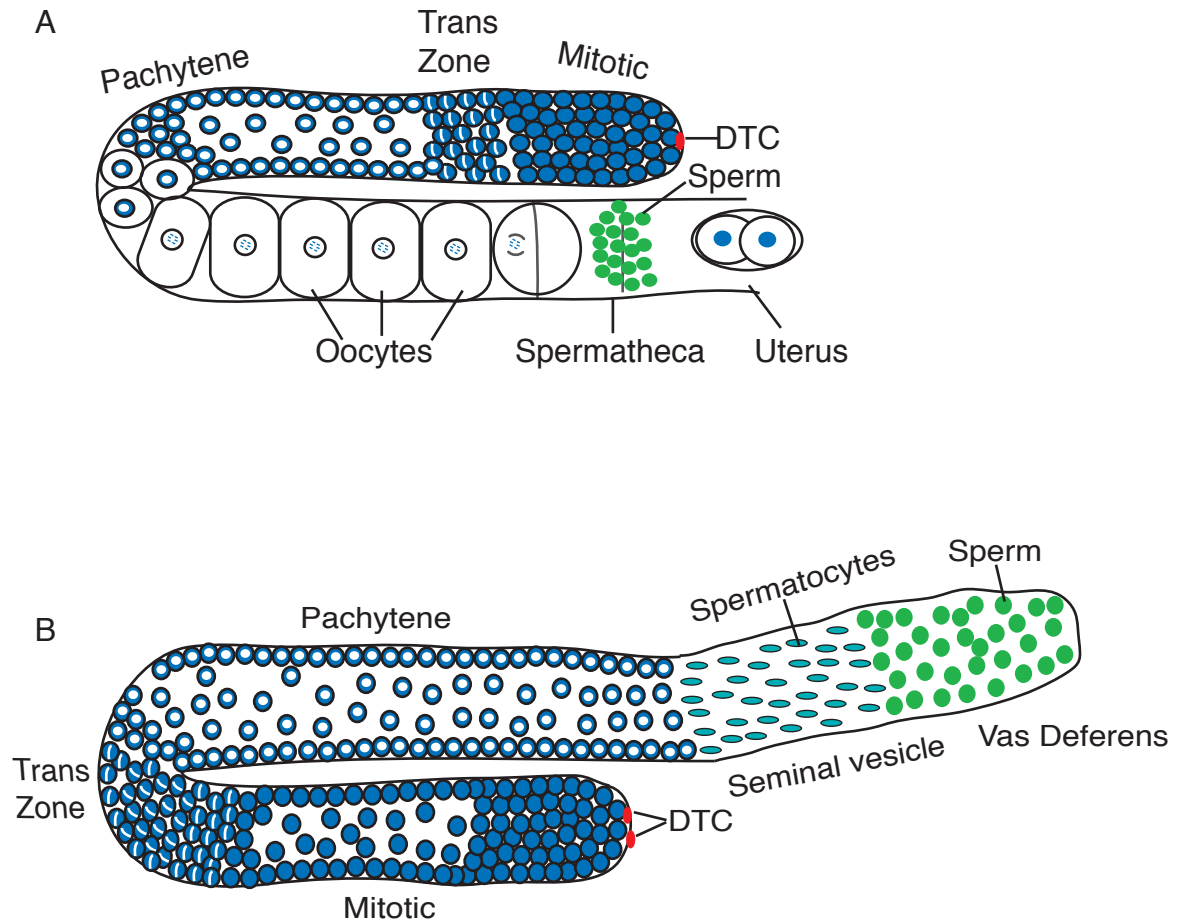
Figure 2. Cartoon of hermaphrodite and male gonad. (A) One arm of the hermaphrodite gonad. The DTC defines the distal region of the arm while sperm define the proximal region of the arm. (B) The male gonad arm. The two DTCs also define the distal arm while sperm define the proximal region.

are required for meiotic progression (Church et al., 1995; Hsu et al., 2002). Mutations in these proteins cause a pachytene arrest phenotype.

As distal nuclei transit the loop region of the gonad and enter the diplotene stage their chromosomes begin to condense and individual oocytes begin to grow and form. During the diakinesis stage of meiotic prophase six discrete highly condensed bivalents can be visualized. Oocytes begin the process of cellularization at the loop region such that the germline nuclei become fully enclosed by membrane. Growing oocytes begin to accumulate yolk lipoprotein particles that are synthesized in the intestine and secreted into the pseudocoelom (Kimble and Sharrock, 1983). Pores in gonadal sheath cells allow the passage of yolk lipoprotein particles from the body cavity onto oocytes cell surfaces (Hall et al., 1999). Once at the oocytes cell surface yolk lipoproteins bind the RME-2 yolk receptor, a member of the low-density lipoprotein receptor superfamily, and are endocytosed within the oocyte (Grant and Hirsh, 1999; Grant et al., 2001; Zhang et al., 2001). Interestingly, the uptake of yolk is not essential, as *rme-2 (bl008)* null mutant strains are viable but have reduced fertility (Grant and Hirsh, 1999). Cholesterol bound to vitellogenins is also mediated through RME-2 receptor-mediated endocytosis (Matyash et al., 2001).

Oocyte Meiotic Maturation

C. elegans oocytes arrest in diakinesis of meiotic prophase until signaled by sperm to complete meiosis. In the presence of sperm, oocytes develop in the proximal arm of the gonad in an assembly line manner, such that the most proximal oocyte matures

and is ovulated into the spermatheca where it will be fertilized. The second oocyte then assumes the most proximal position and will mature next and so forth (McCarter et al., 1999). The landmark events of oocyte maturation, ovulation and fertilization have been carefully recorded (McCarter et al., 1999). The oocyte nucleolus disappears 70 minutes prior to fertilization. Nuclear envelope breakdown occurs 6 minutes prior to fertilization followed by cortical rearrangement (3 minutes) and ovulation (0.7 minutes). The oocyte is fertilized within the spermatheca and pushed into the uterus approximately 3 min after fertilization. This process occurs approximately every 23 minutes for each gonad arm when sperm are abundant. When sperm are absent this rate is lowered dramatically to approximately $1/40^{th}$ of the wild type peak rate (0.1 vs. 2.5 maturations per gonad arm per hour) (McCarter et al., 1999).

Developing oocytes in the proximal gonad arm are surrounded by the gonadal sheath cells, which play roles in both structure and integrity of the gonad (McCarter et al., 1997; Rose et al., 1997). The myoepithelial sheath cells surrounding the oocytes contract to drive ovulation such that the most proximal oocyte is pushed/pulled into the spermatheca allowing fertilization to occur (Rose et al., 1997; Hall et al., 1999). In addition to their contractile role, the sheath cells have recently been shown to inhibit oocyte meiotic maturation when sperm are absent (Miller et al., 2003). This inhibitory pathway has been defined by the POU-homeobox gene *ceh-18* (Greenstein et al., 1994).

In *C. elegans*, sperm utilize the major sperm protein (MSP) as a hormone to promote oocyte meiotic maturation and gonadal sheath cell contraction at a distance (Miller et al., 2001). MSP promotes oocyte meiotic maturation, in part by binding the VAB-1 Eph receptor protein-tyrosine kinase on oocytes and by antagonizing an

inhibitory somatic gonadal sheath cell pathway defined by *ceh-18* (Miller et al., 2003). Since hermaphrodites produce a fixed number of sperm, oocyte meiotic maturation occurs constitutively until sperm become limiting. In mutant female animals, where sperm are absent, oocytes arrest in meiotic prophase until insemination. Thus, the MSP hormone functions as the linchpin of a sperm-sensing mechanism linking meiotic maturation and sperm availability, thereby ensuring efficient fertilization.

Males and Spermatogenesis

In contrast to hermaphrodites, males only produce sperm and must mate with hermaphrodites to produce progeny. The adult male animal consists of a single U-shaped gonad arm that terminates proximally at the seminal vesicle and vas deferens (Fig. 2B). Males also have two DTCs, however they both remain at the distal end of the single gonad and only one has the gonadal extension function. The organization of the male gonad is similar to the adult hermaphrodite gonad in that spermatogenesis proceeds in a linear fashion both temporally and spatially, such that cells located more distally are at earlier stages of spermatogenesis than those found proximally (Fig. 2B). In males spermatogenesis begins in the L4 stage and continues throughout the adult stage. Although the anatomy of the hermaphrodite and male gonad differ, the process of spermatogenesis is similar in both. However, sperm derived from males are approximately 50% larger and have an increased motility compared to hermaphrodite sperm. Male sperm can out compete hermaphrodite sperm from the premium fertilization positions, in the distal most region of the spermatheca (Laymunyon and Ward, 1998; Singson et al., 1999).

As in hermaphrodites, the DTCs maintain the mitotic population of germ cells at the distal region of the male gonad. Unlike oogenesis, spermatogenesis does not pause in meiosis, both meiotic divisions precede uninterrupted, resulting in four haploid spermatids at the proximal end of the gonad. As syncytial spermatocytes enter meiosis at the loop region of the gonad arm they begin to separate and bud from the rachis (a central cytoplasmic core) becoming primary spermatocytes. This budding is the first partitioning event of spermatogenesis (Fig. 3). Nuclei, mitochondria, endoplasmic reticulum (ER), ribosomes, and the nematode sperm specific organelle called the fibrous body-membranous organelle (FBMO) are segregated to the budding spermatocyte and the rachis retain only cytoplasm, ER and ribosomes (Wolfe et al, 1978). The budded primary spermatocyte then undergoes two divisions giving rise to four haploid spermatids. In contrast to *Drosophila melenogaster* and mammalian spermatogenesis these divisions require no input from other cells and can proceed *in vitro* once initiated (Ward et al., 1981; Nelson and Ward, 1980; Machaca et al., 1996).

The first spermatogenic division is symmetrical such that both daughter cells (secondary spermatocytes) receive equal amounts of cytoplasm, organelles, and nuclear material. While the mitotic division of the second division of spermatogenesis is symmetrical, in that it generates four equivalent meiotic products, the actual division is asymmetrical, in that the residual body receives all organelles and products not required for the fertilization process. The resultant spermatids are extremely streamlined. During anaphase of the second meiosis the cell becomes elongated and segregation of

most proximal germline — 1° — budding — rachis — spermatocytes 2° — or — residual body — spermatid — spermatozoon — pseudopod — budding

Asymetrical division

Spermatids form a pseudopod

Meiosis I   Meiosis II
Spermatogenesis
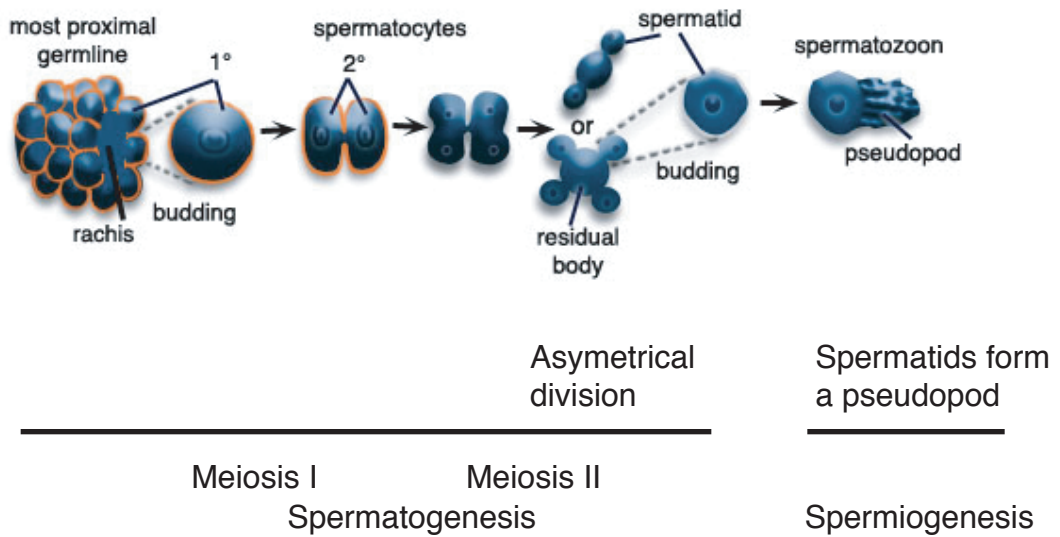
Spermiogenesis

Figure 3. Wild-type spermatogenesis. Spermatocytes bud from the rachis and undergo meiosis II as individual cells. Primary spermatocytes divide to generate secondary spermatocytes which then undergo an asymetrical division generating spermatids. Spermatids bud from the residual body and undergo spermiogenesis when mated into the hermaphrodite uterus, or pushed into the spermatheca in a hemaphrodite. Adapted from Worm Atlas and L'Hernault.

chromosomes, mitochondria, and FBMOs are partitioned to the extreme ends of the cell, which will later become spermatids (Wolf et al., 1978; Ward et al., 1981). Once elongated the middle portion of the cell begins to round up forming the residual body (Fig. 3). Separation of the residual body from the spermatids appears to occur by gradual accumulation of membrane vesicles at the point of division such that new membrane is formed and severs the cytoplasmic connection between the residual body and the spermatid (Ward et al., 1981). Cellular components not required for fertilization, such as ER, ribosomes, actin and most of the tubulin (except that associated with the centrioles), are discarded into the residual body (Nelson et al., 1982). The resultant non-motile spermatids are stored within the seminal vesicle until ejaculation into the hermaphrodite, at which time they become activated and motile (Fig. 3).

Spermiogenesis

Non-motile spermatids activate to form pseudopods and become polarized, motile spermatozoa capable of fertilizing oocytes during the process of spermiogenesis. Spermiogenesis commences when spermatids enter the spermatheca during ovulation in the hermaphrodite or in the uterus following insemination by mating. Spermiogensis can also be simulated *in vitro* by treatment with a number of drugs and proteases, such as monesin and Pronase (Nelson and Ward, 1980; Ward et al., 1983; A. Singson personal communication). Spermatids that are unable to undergo spermiogenesis cannot fertilize oocytes and are rapidly cleared from the reproductive tract due to their inability to crawl back to the spermatheca (Argon and Ward 1980). *spe-8*, *spe-12* (Shakes and Ward, 1989), *spe-27* (Minniti et al., 1996), and *spe-29* (Nance and Ward, 2000) mutants affect

hermaphrodite sperm activation. In these mutants, hermaphrodites are self-sterile but

males are fertile because spermatids are activated normally during mating. These

mutations suggest that hermaphrodites and males activate spermatids differently and thus

two different pathways of spermatid activation exist. The evolution of two different

pseudopod activation pathways fits the evolution of the nematode. It is likely the

ancestral species was that of male/female. Thus, when hermaphrodism evolved it is

likely a new method of spermatid activation also had to evolve, since spermatids are

located within the gonad arm.

Nematode Sperm

Nematode sperm is unique in that they crawl by amoeboid locomotion. *Ascaris*

suum, a parasitic nematode that dwells in pig intestines, is very similar to *C. elegans*

except they are much larger in size, approximately 400 times larger. Accordingly,

Ascaris sperm are approximately (20-25 μm in diameter) are also proportionally larger

than *C. elegans* sperm (4-5 μm in diameter). This larger size and abundant quantity of

sperm has made *Ascaris* very amenable for studying sperm locomotion (reviewed by

Roberts and Stewart, 1995). *C. elegans* and *Ascaris* spermatozoa differ greatly from

other spermatozoa, as do the spermatozoa of other nematodes, in that they lack a

flagellum and acrosome (reviewed by Roberts and Stewart, 1995). Motility exhibited by

C. elegans and *Ascaris* is also unusual. These polarized cells have a single pseudopod at

one end of the cell. In addition these spermatozoa lack appreciable amounts of common

cytoskeletal proteins. Little actin is present (less than 0.2% of sperm protein) and neither

microfilaments nor myosin can be detected (reviewed by Roberts et al., 1989). The only

microtubules present in spermatozoa are those that form the centrioles. Further,

treatment of spermatozoa with cytochalasin B, D, E, colchicine or oncobendazole have

no affect on spermatozoan motility, reiterating that actin and tubulin are not required

(Wolfe et al., 1978; Ward et al., 1981; Ward 1986). Despite this lack of requirement for

actin and tubulin the pseudopods of nematode sperm cells exhibit surface movements and

membrane flow. The rate of membrane flow closely approximates the rate of forward

movement (reviewed by Roberts and Stewart, 1995). Thus the mechanism of nematode

motility is novel.

Major Sperm Protein and Motility

One of the most abundant proteins in nematode sperm is the major sperm protein

(MSP), accounting for about 15% of the total sperm protein (Klass and Hirsh, 1981;

Nelson and Ward, 1981). *C. elegans* MSP is not homogeneous but rather a family of

closely related 12-14 kDa basic polypeptides encoded by 40 genes. The MSP

polypeptides are so closely related that they only differ by 1 to 4 amino acids (Ward et

al., 1988). By contrast *Ascaris* contains only 2 MSP genes suggesting perhaps gene

duplication occurred in the hermaphrodite speices. *Ascaris* MSP is 82% identical to

C.elegans MSP suggesting that MSP function is also highly conserved (Bennett and

Ward, 1986; King et al., 1982; Ward et al., 1988).

MSP has been studied for many years, and its role in nematode locomotion has

been well characterized. In *C. elegans,* MSP has been shown to localize to the fibrous

body of spermatocytes and spermatids and in the psedopods of spermatozoa (Ward and

Klass, 1982). Likewise, *C. elegans* anti-MSP antibodies detect are filament bundles in

Ascaris pseudopods (Sepsenwol et al., 1989). These filament bundles span the length of the pseudopod and drive protrusion.

MSP monomers are assembled into symmetrical dimers that polymerize into MSP filaments. These filaments then bundle forming thick, branched, meshworks called MSP fibers. The leading edge each MSP fiber contains a vesicle. MSP filaments polymerize at the vesicle surface allowing fibers to push their vesicle forward as they elongate similar to the way a column of cross linked actin filaments pushes forward (Italiano et al., 1996). At the opposite end of the fiber, near the base of the cell body, MSP filaments disassemble causing filaments to shrink in length and diameter and ultimately pulling the cell body forward. The rates of assembly and disassembly are balanced such that the pseudopodial leading edge and the cell body advance at the same speed (Roberts and Stewart, 2000). This is in contrast to actin where the rate of assembly occurs faster then the rate of disassembly. The polymerization and retraction of the MSP cytoskeletal system have been reconstituted *in vitro* in cell free extracts of sperm (Italiano et al., 1996; Miao et al., 2003). MSP assembly requires a pH sensitive soluble factor and a membrane protein (Italiano et al., 1996). MSP assembly also requires the addition of ATP, however the role of ATP remains unclear since MSP does not bind or hydrolyze ATP. Recently in *Ascaris*, two cytosolic soluble proteins, MPF1 and MPF2 were identified and shown to affect MSP fiber growth rates. MPF1 decreases the rate of fiber growth while MPF2 increases the rate of fiber growth (Buttery et al., 2003). These proteins have homologs in *C. elegans*, termed the MSD proteins. Disassembly of MSP fibers requires a phosphate and occurs at a more acidic pH (Miao et al., 2003). Thus assembly and disassembly can

be completely uncoupled with each mechanism requiring a different set of molecules and different pH triggers (Miao et al., 2003).

Motility of *Ascaris* sperm occurs through a push-pull mechanism of locomotion. The polymerizing filaments create a protrusive force along the leading edge that pushes against the membrane. Traction force, created by the disassembly of MSP filaments at the base of the lamellipodium, pulls the cell body forward. To accomplish directional movement the area between the leading edge and the base of the cell body is attached to the substrate (Roberts and Stewart, 2000). Sperm maintain a pH gradient that spans the pseudopod such that the leading edge has more alkaline conditions allowing MSP filaments to assemble and bundle driving protrusion. At the rear of the pseudopod near the base of the cell body more acidic conditions are prevalent resulting in an unbundling and disassembly of MSP filaments (Roberts and King, 1991; King et al., 1994).

Major Sperm Protein and Cell Signaling

Despite the well-characterized role of MSP in nematode sperm motility, its role in cell signaling was not evident until recently. Previously it was shown that a sperm-associated signal promotes oocyte meiotic maturation independent of fertilization (McCarter et al., 1999). Genetically altered XX female animals that do not produce sperm undergo oocyte maturation and ovulation at very low rates, approximately <0.1 maturations per gonad per hour. Mating to wild-type males or fertilization incompetent sperm defective (*spe*) mutants restores the normal rate of oocyte maturation to that of wild type hermaphrodites where sperm are plentiful, a rate of approximately 2.5 maturations per gonad, per hour.

Recently MSP was discovered to be the sperm derived signal triggering oocytes meiotic maturation and gonadal sheath cell contraction (Miller et al., 2001). An *in vitro* bioassay, in which supernatant from media incubated with sperm was injected into *fog-2(q71)* female animals and injected animals were assayed for oocyte meiotic maturation. Mass spectrometry analysis revealed that MSP was the active factor promoting oocyte meiotic maturation. In addition, recombinant bacterial produced MSP was sufficient to promote oocyte maturation and sheath contraction in the nanomolar concentration range Further, injection of MSP antibodies into the uterus of hermaphrodites results in a reduction in ovulation rates. *C. elegans* MSP can signal in *C. remanei* (Miller et al., 2001) and *Ascaris* MSP can signal in *C. elegans* (M. Kosinski and D. Greenstein., unpublished results), consistent with observations of heterospecific matings in the genus *Caenorhabditis* and the high protein identity of *Ascaris* and *C. elegans* MSP (Hill and L'Hernault, 2001; Ward et al., 1988).

MAPK activation plays critical roles in regulating meiotic progression in animal oocytes (Ferrell, 1999), including *C. elegans* oocytes. Proximal oocytes exhibit MAPK activation in the presence of sperm (Miller et al., 2001; Page et al., 2001) and MSP was shown to be sufficient to activate MAPK in oocytes (Miller et al., 2001). Additional pathways and proteins activated by MSP within the oocyte and the gonadal sheath cells remain to be identified.

One MSP oocyte receptor, the VAB-1 Eph receptor protein-tyrosine kinase, has been identified (Miller et al., 2003; reviewed by Kuwabara, 2003). *In situ* MSP binding showed specific and saturable binding of labeled MSP to oocyte and proximal sheath cell membranes at low nanomolar concentrations. Further, VAB-1 was shown to be sufficient

to confer MSP binding activity to living COS-7 cells (Miller et al., 2003). More recently, MSP was shown to directly bind the VAB-1 ectodomain *in vitro* (H. Cheng and D. G., unpublished results). *vab-1* functions in parallel to a somatic gonadal pathway, defined by the POU-class homeobox gene *ceh-18* (Greenstein et al., 1994; Rose et al., 1997; Miller et al., 2003). The *ceh-18* and *vab-1* parallel pathways negatively regulate oocyte meiotic maturation, MAPK activation, and gonadal sheath cell contraction in hermaphrodite and female gonads (Fig. 4). MSP disrupts this negative regulation in part by binding by VAB-1 Eph receptor protein-tyrosine kinase on the oocyte cell surface thereby promoting meiotic maturation, MAPK activation and gonadal sheath cell contraction (Fig. 4)(Miller et al., 2003). Together these parallel pathways constitute a sperm-sensing checkpoint to conserve metabolically costly oocytes when sperm are unavailable for fertilization. Additional MSP receptors likely exist since animals in which *vab-1* and *ceh-18* are eliminated show oocyte meiotic maturation rates above female levels and respond to MSP.

Once oocytes receive the MSP signal one or more signal transduction pathway are likely activated within the oocyte triggering MAPK activation and oocytes maturation. Some components of this pathway have been identified, however several remain to be determined. For example, *oma-1* and *oma-2* are required for maturation and ovulation and are thought to function in the regulating of translation or the stability of proteins required for maturation and ovulation (Detwiler et al., 2001). Acting either in an upstream or a parallel pathway to *oma-1* and *oma-2* is the *cgh-1*, a DEAD-box helicase. Mutations in *cgh-1* lead to ectopic MAPK activation, suggesting *cgh-1* functions to inhibit MAPK (I. Yammamoto and D. Greenstein unpublished results). Although the
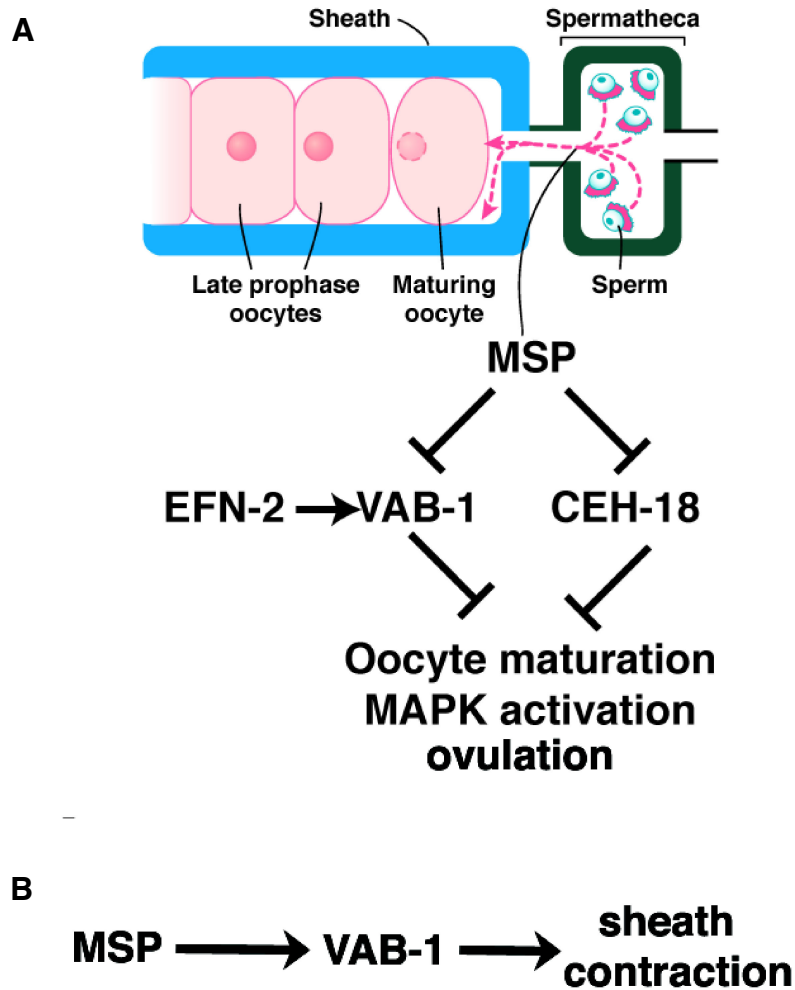
Figure 4. Model for MSP signaling. Two parallel pathways, defined by VAB-1 and CEH-18, act to inhibit (A) oocyte meiotic maturation and (B) gonadal sheath cell contraction. In the presence of sperm MSP removes this negative regulation resulting in maturation and sheath cell contraction. MSP has been shown to directly bind the VAB-1 Eph receptor on oocyte surfaces. Receptors regulating the CEH-18 pathway remain to be identified.

signal transduction pathway MSP activates within the oocytes has been identified several components of it remains to be determined.

The MSP signal defines a new class of signaling molecules. MSP's unique characteristics coupled with the cell type it is localized within suggest a novel release mechanism may be at play. For example, MSP is a cytoskeletal protein, most of which are not typically secreted from cells. In addition, MSP does not possess a recognizable signal sequence and translational processing of MSP does not occur, further suggesting a typical signal sequence is not present (Miller et al., 2001). Finally nematode sperm are devoid of secretory organelles, suggesting MSP release does not occur through the conventional secretory pathway. These observations suggest the classical protein secretion pathway does not act in the release of MSP from spermatozoa and such the mechanism regulating the release of MSP remained unknown until this work identified the novel release mechanism.

MSP domains are widespread and found in yeast, plants and animals. Proteins containing MSP domains have been associated with vesicle trafficing. Thus, similar signaling functions for MSP domain proteins may exist in other organisms. MSP is known to be released and signal oocyte meiotic maturation at a distance due to the anatomy of *C. elegans* (a constriction of the distal spermatheca prevents spermatozoa from physically reaching). However, when this work commenced, little was known about the release mechanism of MSP signaling. Thus, this work aimed to determine the secretory mechanism nematode spermatozoa use to release MSP and trigger oocytes meiotic maturation and ovulation. To build a foundation upon which to further compare

and contrast MSP release from spermatozoa with standard protein release, I would like to first review the classic protein secretory pathway.

Protein Secretion

Protein secretion is a fundamental process of living cells. Eukaryotic protein secretion is typically carried out through the classical or ER/Golgi protein secretion pathway. In this pathway, newly synthesized proteins pass in a sequential manner through a series of membrane bound compartments, mainly the ER and the Golgi complex (Palade, 1975; reviewed by Duden, 2003). Each compartment provides a specialized environment that facilitates the various stages in protein biogenesis, modifications and sorting (reviewed by Lee et al., 2004). This pathway of protein export from eukaryotic cells has been well characterized and can be blocked with drugs such as brefeldin A, a classical inhibitor of ER/ Golgi dependent protein secretion (Misumi et al., 1986 and Oric et al., 1991). The majority of proteins are exported from cells by this ER/Golgi pathway, and until fairly recently this was believed to be the only pathway of protein export. Although *C. elegans* spermatozoa do not release MSP by the classical ER/Golgi pathway, as these organelles are removed during meiosis, the release of MSP may have similarities or hallmarks of this classical pathway and thus a brief review is provided to compare and contrast these release mechanisms.

The Classical Secretory Pathway

Targeting of proteins through the secretory pathway is facilitated through signal sequences, also called sorting sequences. Proteins targeted to the ER commonly contain

a continuous stretch of amino acids located at the amino terminus of the polypeptides. These sequences are usually 15-60 residues long and contain a central hydrophobic region (reviewed by Sakaguchi, 1997). Once protein sorting has been completed this type of signal sequence is usually cleaved by the signal peptidase complex, located with in the lumen of the ER, and degraded (Sakaguchi, 1997).

Transport of proteins from the ER to the Golgi complex and finally to the cell surface usually occurs via small vesicles. Distinct sets of coated vesicles function at different steps in the secretory pathway. Transport between the ER and Golgi is facilitated by COPII and retrograde transport, from the Golgi to the ER, occurs via COPI. Clathrin coated vesicles function in a number of transport steps such as movement from the plasma membrane to the Golgi (Lee et al., 2004). In addition to the role of vesicle formation coat proteins also facilitate the docking of vesicles at the target surface.

Coated Vesicles

Most secretory vesicles bud at specific coated areas of the membrane to form coated vesicles. Coated vesicles contain a characteristic cage of proteins covering their surface that acts by forming a meshwork and deforming the membrane. Thus vesicles containing the same type of coat proteins generate the same type of appearance (reviewed by Lee et al., 2004). The coat matrix functions are two fold, to shield the hydrophobic membrane from the aqueous environment and provide stored energy needed to bend the membrane to allow vesicle formation.

Coated vesicle formation follows a mechanism common to most vesicle budding processes. Coated vesicle budding occurs at specific sites within the ER membrane

designated ER exit sites (ERES). Both fluorescent and immunoelectron microscopy have

demonstrated the localization of COPII proteins to specific sites on the ER membrane

that give rise to COPII vesicles (Kuge et al., 1994; Oric et al., 1991; Pagano et al., 1999).

Precisely how these sites are maintained is unclear and the full repertoire of proteins

marking these sites has not been characterized (reviewed by Lee, 2004). Coat

components must be specifically recruited to these sites of the membrane. In some cases

small GTP-binding proteins recruit coat proteins and drive coat assembly. For example

in COPII vesicle formation, Sar-1GTP recruits two cytosolic complexes; Sec23-Sec24

and Sec13-Sec31. These five components are sufficient to deform the membrane and

generate COPII vesicle in add back experiments (Matsuoka et al., 1998). Sar1 and Arf1,

the COPI GTP binding protein, are unique exchange factors in that the exchange reaction

can only proceed when these proteins are bound at the membrane surface (Antonny et al.,

2001; Paris et al., 1997). In clathrin coated vesicles, coat proteins are not recruited

through GTP binding proteins but rather the heterotetrameric adaptor proteins recruit

clathrin from the cytosol and the β subunits of the adaptor proteins trigger assembly of

clathrin into a lattice meshwork at the membrane (Brodsky et al., 2001; Gallusser &

Kirchhausen, 1993).

Once recruited, coat proteins interact with specific cargo proteins, weakly binding

these proteins to help facilitate capture upon vesicle formation. In COPII vesicles the

Sec24 subunit interacts with the majority of cargo proteins and in clathrin coated vesicles

the β and μ subunits of the adaptor proteins bind cargo (Ohno et al., 1995 and Owen &

Evans 1998). Once coat proteins are recruited and cargo proteins are selected coat

polymerization must occur. In COPII vesicle formation the GTP binding proteins initiate

coat polymerization when activated. Specifically, the Sec23 subunit is the GTPase

activating protein (GAP) for Sar1. The released energy and polymerization of coat

proteins allows membrane curvature and vesicle release. Sec13-31 forms the outer layer

of the coat in COPII vesicles and likely functions as a structural scaffold to crosslink

adjacent 23-24 complexes forming a coat lattice that propagates membrane curvature

(Matsuoka et al., 2001). The facilitation of bending the lipid bilayer is the most energetic

and difficult steps in the vesicle formation. Thus the process is eased at regions where

membranes are already curved. There is some evidence that this priming step occurs in

clathrin-coated vesicles at the trans-Golgi, however this priming step has yet to be

identified at the plasma membrane (Brodsky et al., 2001). During formation at the

plasma membrane, clathrin subunits, consisting of 3 large and 3 small polypeptide chains,

are nucleated onto the membrane by adaptor proteins, AP1 and AP2. Adaptors

incorporate transmembrane molecules by interacting with the cytoplasm and then trigger

clathrin subunits drawing them into a lattice meshwork (Brodsky et al., 2001). This

polyhedral lattice then distorts the membrane allowing vesicle formation. Scission of the

assembled vesicle from the plasma membrane relies on the function of the GTPase

dynamin (Hinshaw et al., 2000 and Brodsky et al., 2001). While it is known that

dynamin is required in vesicle scisson its exact role during the scisson process remains

under debate (Sever et al., 2000). Dynamin may function as a regulatory GTPase, simply

recruiting other proteins that actually mediate vesicle scission. The function of dynamin

self-assembly would be to stimulate the GTPase activity, which in turn may function as a

sensor of vesicle closure (Brodsky et al., 2001). Alternatively, the dynamin self-

assembly may be the mechanical force behind vesicle scission, either through membrane

constriction or through membrane rupture, due to the spring-like action caused by a conformational change (Brodsky et al., 2001).

Released vesicles must be correctly delivered to their target surface. In mammals as well as in many animals the cytoskeleton mediates trafficking, such that microtubules facilitate vesicle movement from ER to Golgi while actin directs retrograde vesicular flow (Fucini et al., 2002; Stamnes 2002).

Proteins destined for secretion to the extracellular space are released via exocytosis. Many signaling molecules such as hormones, neurotransmitters and digestive enzymes must be secreted as well as proteins and lipids, which become incorporated into the plasma membrane. Generally, secreted proteins leave the trans Golgi apparatus in secretory vesicles. Once vesicles reach their target surface, they must dock, fuse with the plasma membrane and release their contents via exocytosis. Docking and fusion are distinct and separable. In the case of synaptic vesicles, fusion with the plasma membrane is not immediate, but rather docked vesicles are primed and await signals, typically intracellular calcium, before membrane fusion and release of neurotransmitters (Sudhof, 2004). This allows nerve cells to rapidly release neurotransmitter upon receiving a signal.

Intercellular membrane docking and fusion are typically mediated through SNARE proteins, which are present on both the target membrane (t-SNAREs) and on the vesicle (v-SNAREs). SNARE proteins have been well characterized in nerve cells where they mediate the docking and fusion of synaptic vesicles at the plasma membrane of nerve terminals. Synaptic vesicles dock at particular sites called, active zones. Once docked at these zones they are primed in preparation for fusion in response to a signal, typically

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calcium (Richmond and Broadie, 2002). Synaptic vesicles require three proteins to mediate synaptic vesicle docking and fusion: synaptobrevin (V-SNARE), located on synaptic vesicles, syntaxin (t-SNARE), and SNAP-25, both located on the presynaptic plasma membrane (Sollner et al., 1993; Sudhof, 2004). Synaptobrevin and syntaxin mutations result in an accumulation of docked vesicles that do not undergo fusion (Broadie et al., 1995). Membrane fusion requires very close proximity, within 1.5 nm, of the two membranes. In addition water must be displaced from the membrane, a charged surface and thus highly energetically unfavorable. SNAREs may facilitate membrane fusion by using energy, released from the helices of SNARE proteins wrapping around one another, to push out water molecules (Sudhof, 2004).

In addition to secretion, cells can signal to adjacent cells via gap junctions. Gap junctions are narrow channels connecting neighboring cells. Only small signaling molecules, such as calcium or cAMP, can pass through these junctions, whereas, macromolecules like proteins cannot fit. Thus the majority of proteins destined to leave the cell do so through this classical ER/Golgi secretion pathway.

Many differences are readily observed between the MSP release from spermatozoa and this classical secretory pathway. First, MSP lacks a recognizable signal sequence and thus it is unclear how MSP is targeted for release from sperm. *C. elegans* spermatozoa have discarded the ER and Golgi during meiosis, thus their role is not necessary for MSP release. Second, it is unclear if upon release MSP vesicles must dock at target sites. It is possible that MSP vesicles simply degrade at specific sites and release their cargo, MSP. Despite these differences, a number of similarities emerge between MSP release and this classical protein secretion pathway. For example, MSP may be

released at specific sites along the plasma membrane, similar to the ERES sites that from

which coated vesicles bud. Finally, MSP may be recruited by membrane proteins located

at these sites, comparable to the recruitment of cargo proteins in coated vesicles. Proteins

involved in MSP vesicle scission must also be required but remain to be identified.

Dynamin is present within *C. elegans* sperm however does not play a role in signaling,

since animals lacking dynmin appear to signal at wild-type rates (M. Kosinski and D.

Greenstein, unpublished results). Together these observations suggest that MSP release

does not occur through the classic ER/Golgi pathway and might occur through some type

of novel release mechanism. However, perhaps remnants or components of this typical

secretion pathway exist.

Non-Classical Protein Secretion

Until fairly recently, it was thought that the classical or ER/Golgi secretory

pathway was the only pathway cells utilize to secrete proteins. Researchers are now

discovering a growing number of proteins being released from cells by unconventional

pathways, termed non-canonical or leaderless secretory pathways (Fig. 5) (reviewed by

Nickel, 2003). Non-canonical or leaderless secretory pathways are widespread, and in

general, poorly understood. Non-canonical secretory pathways are being discovered in a

vast variety of mechanisms including reproduction (Kuchler et al., 1997; Groos et al.,

1999). Generally, proteins in this class of secretion are released either by translocation,

multivesicular lysozymes, or through vesicles pinching off from the plasma membrane

(Fig. 5) (Rubartelli and Sitia, 1997; reviewed by Nickel, 2003).   The most direct pathway

for secretion is translocation through the plasma membrane mediated by transporters

(Fig. 5A). Mechanisms for the translocation of proteins across a membrane often require

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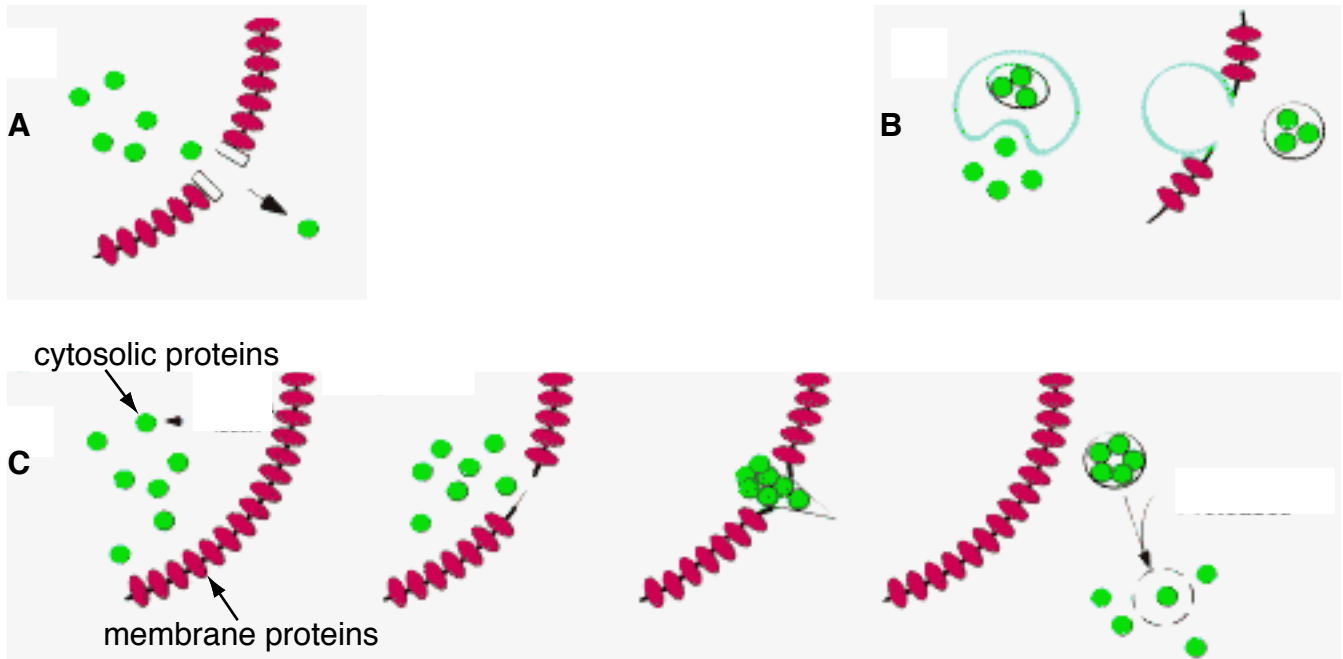
Figure 5. Various non-canonial cytosolic protein secretion mechanisms. (A) Translocation mediated by transmembrane transports. (B) Fusion of multivesicular vesicles releases vesicles into the extracellular space. (C) Ectocytosis. Cytosolic proteins concentrate at the plasma membrane and aggregate. These aggregates become included within protrusions that pinch off creating free vesicles. Adapted from Hughes, 1999.

interactions between chaperone proteins with unfolded or partially unfolded proteins (Hughes, 1999). *Saccharomyces cerevisiae* utilize a leaderless secretory pathway to release the mating pheromone, a-factor. This small peptide is transported directly across the plasma membrane via an ATP-driven pump that belongs to the family of ATP-binding cassette (ABC) transporters.

In addition to protein translocation via transporters, proteins such as Interleukin 1B (IL1B) is shown to be exported from cells through exocytosis in the absence of a functional ER/Golgi system (Rubartelli et al., 1990 and Cooper et al., 1990). A fraction of cytosolic IL-1B is localized within structures that display ultrastructural features and markers typical of the organelles such as late endosomes and early lysosomes suggesting a portion of IL1B is secreted out of the cells via these vesicles (Fig. 5B) (Andrei et al., 1999). Similar to exocytosis the mechanism of membrane shedding called ectocytosis has been implicated in protein secretion (Fig. 5C). During ectocytosis cytosolic proteins concentrate at the plasma membrane forming aggregates, these aggregates become included in blebs that push out of the membrane. These evaginating protrusions then pinch off into the extracellular space (Hughes et al., 1999). Annexin I is secreted by this mechanism from rat colon epithelium, and human lung adenocarcinoma cells (Hughes et al., 1999). Galectin 1 and galectin 3 are thought to be released by ectocytosis from undifferentiated myoblasts, and BHK cells. Galectin 3 can be detected in vesicles blebbing from the surface of BHK cells (Sato et al., 1993). Electron microscopy of mouse macrophages revealed labile, morphologically heterogeneous vesicle about 0.5 um in size (Mehul et al., 1997; Hughes, 1999). Under cultured conditions analysis has shown these vesicles are released quite quickly and have a half-life of about one hour.

Similar to ectocytosis, another method of secretion, termed apocrine secretion has been proposed. Apocrine secretion involves a protrusion of the cytoplasm into the lumen, creating apical blebs that then pinch off (Hermo and Jacks, 2002). These protrusions have been seen in a variety of male reproductive tissues from various species (Aumuller et al., 1997; 1999; Hermo and Robaire, 2002; Hermo et al., 2002). This type of secretion has also been reported in other sex related glands such as sweat glands (Schaumburg-Lever and Lever 1975; Kurosumi et al., 1984), the mammary gland (Kurosumi et al., 1968), epididymis (Manin et al., 1995) and the coagulating gland (Groos et al., 1999). Despite the growing number of examples of proteins released via apocrine secretion, the mechanisms and molecular pathways remain unclear and complex (reviewed by Gesase and Satoh, 2003).

As highlighted above, several different pathways of non-canonical protein secretion exist. A growing number of proteins released via non-canonical secretion mechanisms are emerging, however just as many key questions about these mechanisms are emerging as well. As highlighted above and further examined in this work, several similarities exist between MSP release and the various models of the non-canonical mechanisms. Further illumination of the molecular mechanisms of nonclassical protein export from eukaryotic cells as well as the identification of additional nonconical pathways will greatly further our current understanding of protein secretion. My thesis work has identified another non-canonical release mechanism, the release of MSP from sperm in *C. elegans*. Proteins containing MSP domains are widespread and thus the signaling function and the novel release mechanism may exist in other cells.

The newly defined signaling role for MSP raises the intriguing question as to how

MSP is released from spermatozoa to signal oocytes. As with other proteins released by

non-conventional secretory mechanisms, MSP lacks a signal sequence and spermatozoa

do not have an ER or Gogi apparatus. These properties indicate that MSP release most

likely occurs through a novel mechanism.

CHAPTER II

ANALYSIS OF MSP RELEASE

Introduction

Intercellular communication between sperm and oocyte is fundamental for sexual reproduction (Hardy, 2002). Long- and short-range signaling mechanisms control a medley of essential reproductive processes, including: sperm chemotaxis, oocyte meiotic maturation, gamete recognition, cell fusion, and egg activation. Studies of diverse organisms reveal striking cell biological parallels in the molecular underpinnings of gametic interactions. Many marine invertebrates broadcast sperm into the sea, which then depend on long-range chemotactic cues to locate and fertilize eggs (Ward et al., 1985). Sperm chemotaxis also occurs in the mammalian female reproductive tract (Eisenbach and Tur-Kaspa, 1999) and may involve the function of conserved olfactory receptors (Spehr et al., 2003). Egg surface components, such as the ZP3 glycoprotein in mammals and fucose sulfate polymer in sea urchins, mediate short-range signaling that induces the acrosome reaction, a highly specialized exocytic event needed for zona penetration and gamete fusion (Wassarman et al., 2001; Neill and Vacquier, 2004).

In many animals, including many species of sponges, annelids, mollusks, and nematodes, sperm promote the resumption of meiosis in arrested oocytes (Masui, 1985; McCarter et al., 1999). In *C. elegans*, sperm utilize the major sperm protein (MSP) as a hormone to promote oocyte meiotic maturation and gonadal sheath cell contraction at a

distance (Miller et al., 2001). MSP is also the key cytoskeletal element required for

amoeboid locomotion of nematode sperm (Italiano et al., 1996). MSP promotes oocyte

meiotic maturation, in part by binding the VAB-1 Eph receptor protein-tyrosine kinase on

oocytes, and by antagonizing an inhibitory somatic gonadal sheath cell pathway (Miller

et al., 2003). *C. elegans* hermaphrodites reproduce by selfing or mating with males

(Hubbard and Greenstein, 2000). Since a hermaphrodite produces only a fixed number of

sperm, oocyte meiotic maturation occurs constitutively until sperm become limiting. In

females lacking sperm, oocytes arrest in meiotic prophase until insemination. Thus, the

MSP hormone functions as the linchpin of a sperm-sensing mechanism linking meiotic

maturation and sperm availability. Proteins with MSP domains are widespread and five

human genes encode proteins containing this domain. Recently, a mutation in the MSP

domain of VAPB was shown to cause spinal muscular atrophy and amyotrophic lateral

sclerosis type 8 (Nishimura et al., 2004). Studies of MSP signaling, motility, or release in

C. elegans may thus provide information about the functions of this conserved domain.

MSP release likely occurs through an unconventional mechanism because sperm

lack cellular components required in standard models of protein secretion, such as

ribosomes, ER and Golgi. Moreover, MSP was defined as a cytoplasmic protein lacking

an N-terminal leader sequence, and there is no evidence for proteolytic processing (Klass

and Hirsh, 1981; Miller et al., 2001). Here we address the question of how sperm release

MSP to signal oocytes and sheath cells at a distance in a complex reproductive tract (Fig.

6). We demonstrate that spermatids and spermatozoa release MSP by a novel vesicle

budding mechanism. Spermatids and spermatozoa differ in their signaling potencies:

spermatozoa produce a long-range signal that is temporally labile; whereas spermatids
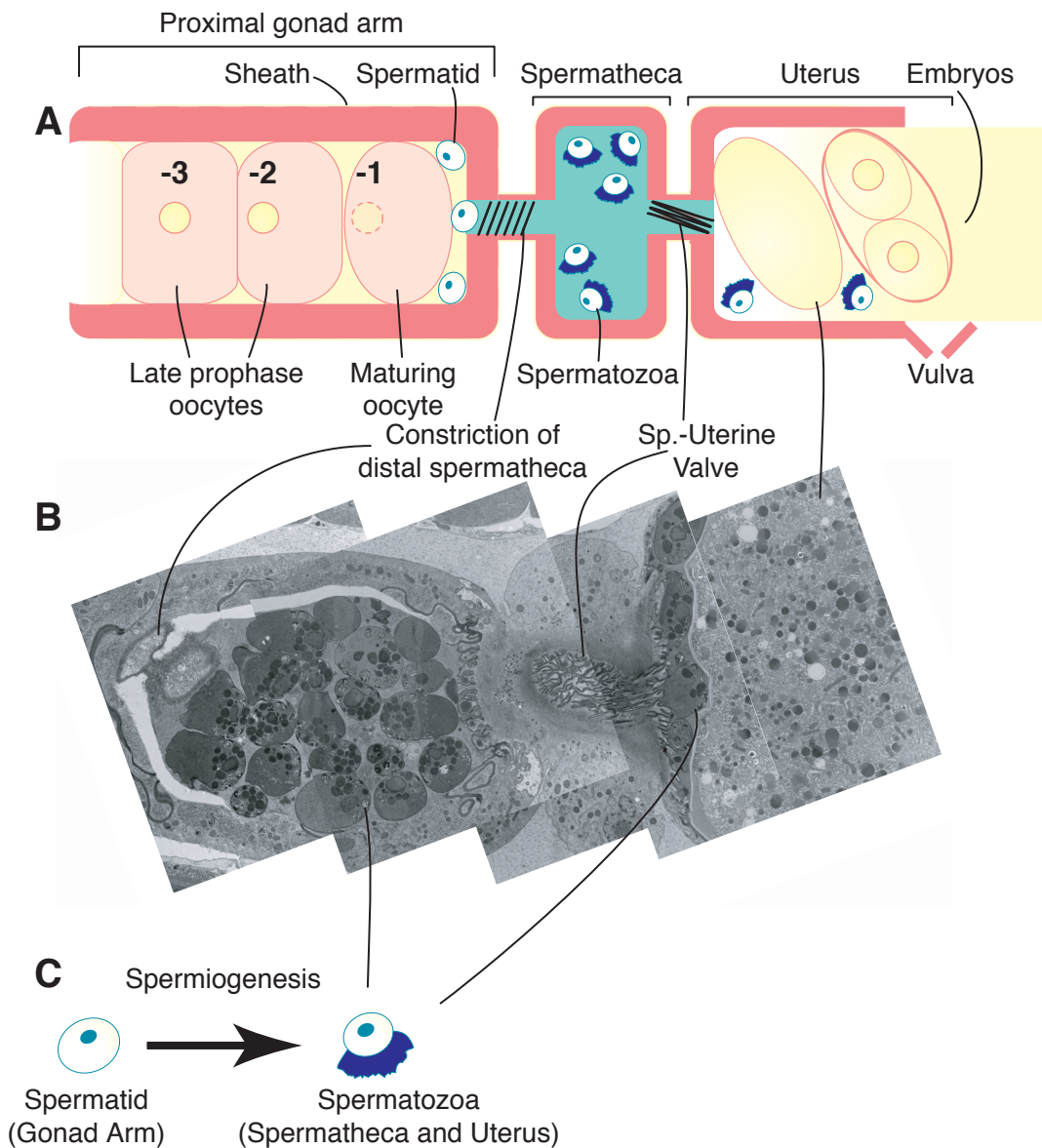
Figure 6. Anatomy of MSP signaling. (A) Diagram of the hermaphrodite reproductive tract. Oocytes undergo meiotic maturation in an assembly line fashion in response to MSP signaling. At ovulation, the distal constriction of the spermatheca dilates, the oocyte enters, and is fertilized. (B) Electron micrograph of the spermatheca. Spermatozoa are unable to enter the proximal gonad because the constriction of the distal spermatheca provides a barrier. Some spermatozoa enter the uterus with embryos, and must then crawl back. (C) Spermiogenesis is the process during which non-motile spermatids become fertilization-competent motile spermatozoa with a pseudopod. Spermiogenesis occurs when spermatids enter the spermatheca during the first ovulations in hermaphrodites, or as they enter the uterus during mating.

provide a long acting more local signal. We propose that differential vesicle stability

determines the physical and temporal range of signaling.

Materials and methods

Nematode strains and phenotypic analysis

Standard techniques were used for nematode culture at 20°C. Wild-type nematode

strains were: *C. elegans* N2, *C. briggsae* AF16, *C. remanei* PB206, *Poikilolaimus*

regenfussi SB199, *Acrobeloides maximus* DF5048 (Thorne, 1925), and *Zeldia puncta*

PDL0003 (De Ley et al., 1990). Mutations and rearrangements were (Riddle et al.,

1997): LGI: *spe-8(hc50)*LGIV: *spe-27(it110), unc-24(e138), fem-3(e1996), nT1(IV,*

*V)*LGV: *emo-1(oz1), fog-2(q71)*.

Oocyte meiotic maturations rates and MAPK activation were analyzed as

described (Miller et al., 2001). Spermatozoa were labeled using 75 µM MitoTracker Red

CMXRos (Molecular Probes) by modifying the method of Hill and L'Hernault (2001).

spe-8(hc50) hermaphrodites were feminized using RNAi feeding of L1 larvae (Kamath et

al., 2001).

Antibodies, western blotting, and immunocytochemistry

Standard methods were used to raise, purify and characterize antibodies (Harlow

and Lane, 1988). Peptides were purchased from Open Biosystems and purified by

HPLC. Three fixation methods were used: dissected gonads with 3% paraformaldehyde

(method 1; Rose et al., 1997) or methanol (method 2), or whole-mounts with Bouin's

reagent (method 3; Nonet et al., 1997). 14 different antibody preparations were used to

examine MSP localization. The only differences observed were the sensitivity of detection and the fixation methods required. Polyclonal antibodies were affinity-purified using peptides coupled to CNBr-activated sepharose (Amersham Biosciences) or SulfoLink resin (Pierce). For purification of monoclonal antibodies, hybridomas were grown in serum-free medium and purified on protein A/G columns (Amersham Biosciences). The N-terminal-specific antibodies were raised to MSP(1-22) AQSVPPGDIQTQPGTKIVFNAP (2 rabbits, method 1). C-terminal-specific antibodies were raised to: MSP(107-126) EWFQGDMVRRKNLPIEYNP (2 rabbits, methods 1 and 2); and CGG-MSP(106-126) CGGREWFQGDMVRRKNLPIEYNP (2 rabbits, method 1; 5 mice, methods 1 and 3; 2 monoclonal hybridomas, method 1 and immunoEM). We also used mAbTR-20 raised to MSP (Ward et al., 1986, method 1 and 3 and immunoEM). Antibodies to MSD proteins were raised to CGG-MSD(53-73) CGGDPSGSKDITITRTAGAPKEDK (2 rabbits, method 1 and 3). Other antibodies used were: RME-2 (Grant and Hirsh, 1999), and Cy2-, Cy3-, or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories).

For western blotting, protein lysates were prepared from 10 staged adults, and analyzed by electrophoresis on 4-12% NuPage gels (Invitrogen). The signal was detected with SuperSignal West Femto reagent (Pierce). Blots were quantitated using a VersaDoc imager with QuantityOne software (Bio-Rad).

Fluorescence microscopy

Wide-field fluorescence microscopy employed Zeiss Axioskop or Axioplan microscopes using 63x and 100x (NA1.4) objective lenses. Images were acquired with

an ORCA ER (Hamamatsu) charge-coupled device camera using OpenLab (Improvision) or MetaMorph (Universal Imaging) acquisition software. Pixel intensities were measured in arbitrary fluorescent units. All exposures were within the dynamic range of the detector. Measurements at 10 different points within areas of interest were averaged, and background levels subtracted as described (Miller et al., 2003). DNA was detected with DAPI.

Confocal images were acquired on a Zeiss LSM510 microscope using a pinhole of 1.42 Airy units and 63x and 100x (NA1.4) objective lenses. Gain and offset were set so that all data was within the dynamic range of the PMT. Band pass filters were used to optically isolate the Cy2, Cy3, and Cy5 fluorophores, and no cross talk was observed. For the reconstructions shown in Fig. 9, B and E, serial images were smoothed with a Gaussian filter and an isosurface and voltex were constructed with Amira (Amiravis). The images were transferred to QuickTime format using VR Worx (VR Toolbox). DNA in some samples was detected with propidium iodide (Molecular Probes).

Electron microscopy

Samples were prepared for TEM by high-pressure freezing and freeze substitution (Howe et al., 2001; Müller-Reichert et al., 2003). Wild-type (n=2), *fog-2(q71)* (n=3), and *spe-8(hc50)* (n=3) animals were viewed in serial longitudinal sections. For immunoEM, wild-type (n=2) and *spe-8(hc50)* (n=2) samples were prepared according to Lonsdale et al. (1999), using 0.25% glutaraldehyde as fixative. Thin layer embedding in LR White (Ted Pella) was used so tissue preservation could be assessed by light microscopy and the sample could be oriented for sectioning (Lonsdale et al., 2001). Longitudinal thin

sections (70 nm) were placed on formvar-coated grids and stained with 5-10 μg/ml mAb4D5 anti-MSP.   Secondary antibodies conjugated with 10 nm gold particles (Amersham Biosciences) were used for detection.   Grids were examined using a Philips CM-12, 120keV electron microscope at 80 kV.   Mated (n=1) and unmated (n=1) *fog-2(q71)* female samples were also prepared for TEM and immunoEM by conventional methods (Hall et al., 1999).   Immunolabeling of spermatozoa within mated females was comparable to that obtained by the HPF method, but extracellular spaces in the spermatheca were not well preserved, and MSP vesicles were not seen.   No labeling was observed in unmated females.

Results

Release of MSP from spermatozoa

Previous studies reported the intracellular localization of MSP during spermatogenesis (Klass and Hirsh, 1981; Ward and Klass, 1982).   To examine MSP release from spermatozoa, we raised a battery of polyclonal and monoclonal antibodies to N- and C-terminal MSP peptides.   The antibodies detect an abundant ~15 kilodalton polypeptide, co-migrating with purified MSP, in western blots of total protein extracts from mated *fog-2(q71)* females and males, but not unmated females or *E. coli* extracts (Fig. 7 A, and unpublished data).

Figure 7. Evidence that spermatozoa release MSP. (A) Western blot. Males and mated females contain MSP, but unmated females do not. Minor N- and C-terminal fragments (*N and *C) result from scission of MSP during the boiling step of lysate preparation (data not shown). The male lysate was over-exposed to visualize *N (center lane, 15 s exposure time). (B and C) Detection of MSP (red) in the proximal gonad arm of mated females (left panels in B). MSP extends beyond the distal constriction (arrowhead) of the spermatheca (sp). A sharp boundary in staining intensity is observed between the –1 and –2 oocytes (arrow). DNA (blue) is shown in the merged images (lower panels in B). No MSP staining is seen in unmated females (right panels in B). The unmated control was over-exposed to visualize the outline of the gonad. The relative fluorescence intensity of the MSP signal is shown (C). Bars, 10 μm. (D) The distance that the MSP signal extends from spermatozoa in mated females. Asterisk, $P < 0.001$, error bars represent SD. (E) The relative intensity of the MSP signal (fold above background) in the proximal gonad. Asterisk, $P<0.02$, when compared to all the other measurements shown. Dagger, $P>0.15$, when compared with the other *emo-1(oz1)* mated female values, but $P<0.05$, when compared with the unmated female controls. (F) Punctate distribution of extracellular MSP. Projections of confocal 3D data stacks from mated females prepared by gonad dissection (left panel, MSP is red) or whole-mount fixation (right panel, MSP is pink and DNA is red). Large MSP puncta (arrows) are outside spermatozoa (s) in both the spermatheca (left panel, sp) and the uterus (u). More diffuse MSP fills the spermatheca (left panel) and extracellular spaces surrounding embryos (e, right panel). No MSP is observed in the distal gonad (dg). Bars, 5 μm. (G) MSP puncta (arrows) in close proximity to spermatozoa (s) in the uterus, detected by wide-field microscopy. Note the extended pseudopod (ps, bottom panels) and the sperm DNA (blue). Bars, 10 μm.
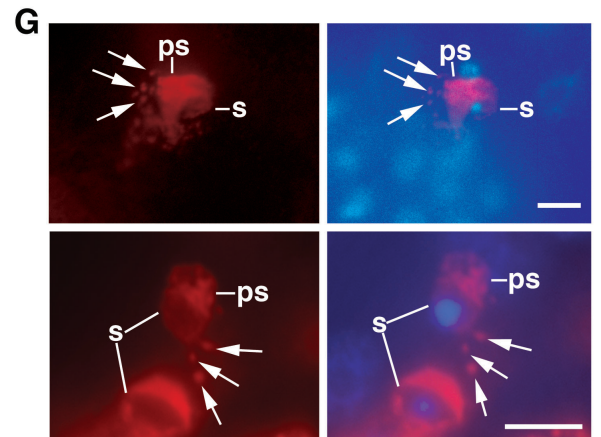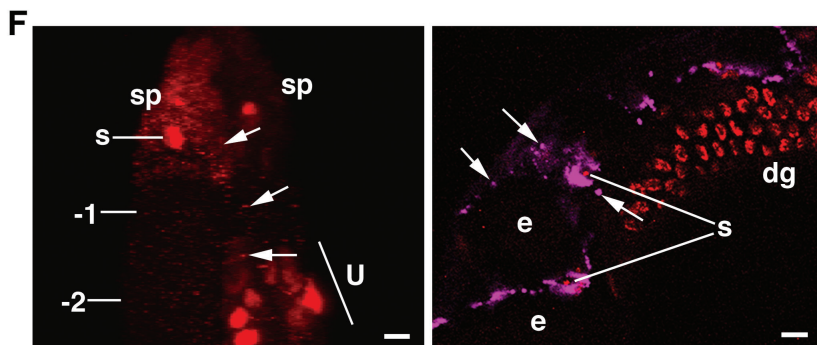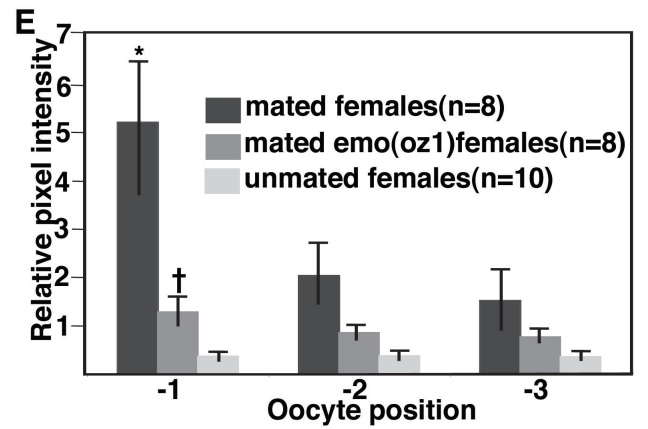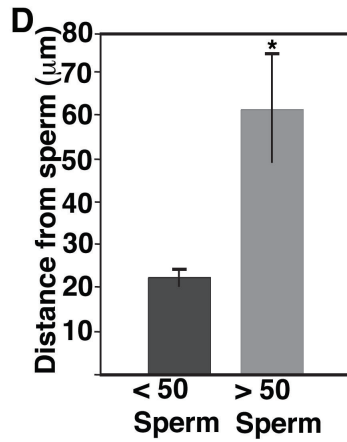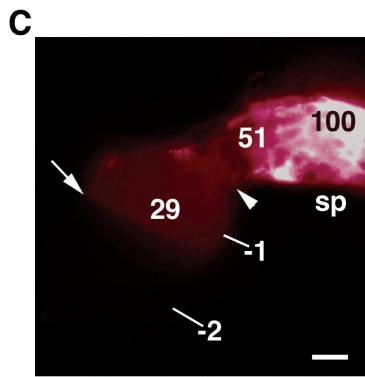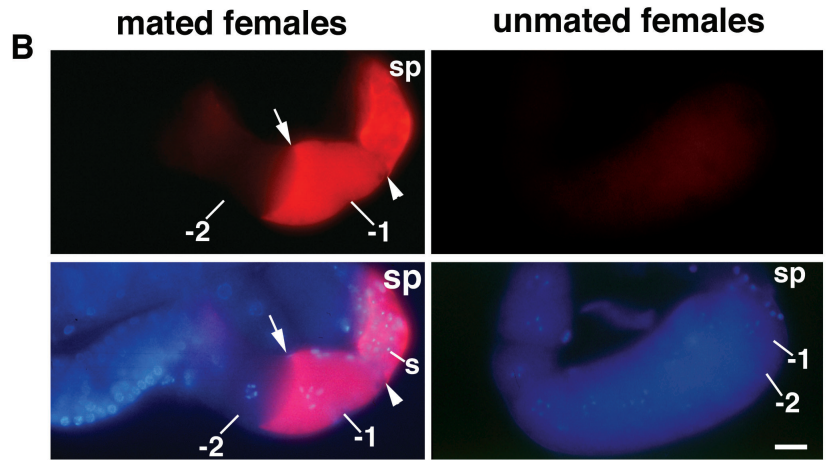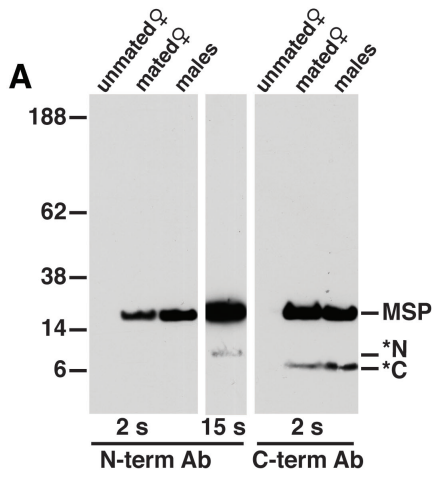
To analyze MSP release from spermatozoa *in vivo*, we examined gonads of mated *fog-2(q71)* female animals using immunofluorescence (Fig. 7, B and C). In mated females, spermatozoa are only observed in the spermatheca and uterus, however, we observed MSP extending past the distal constriction into the proximal gonad arm. This staining represents MSP that is extracellular to spermatozoa. By contrast, all unmated females showed no staining (Fig. 7 B, right panel; n=51). Using anti-MSP mAbTR-20 and visual inspection, 91% of mated female gonad arms exhibited extracellular MSP localization (n=36), with 39% showing extracellular MSP as far as the most proximal (–1) oocyte, the rest exhibiting extracellular MSP only within the spermatheca. Within the spermatheca, MSP staining was judged to be outside of spermatozoa if staining extended at least 5 μm beyond the pseudopod or cell body. MSP staining extended on average 33.6 ±19 μm (maximal range=90 μm; n=12) from spermatozoa, which are approximately 5 μm in size. When the spermatheca contained more than 50 spermatozoa, MSP extended to an average maximal distance of approximately 60 μm from spermatozoa (Fig. 7 D). By contrast, when the spermatheca contained less than 50 spermatozoa, MSP extended to an average maximal distance of approximately 22 μm from spermatozoa (Fig. 7 D). In adult hermaphrodites, we detected MSP outside of spermatozoa during days 1-3 of adulthood (Fig. 8, A-C), but not at day 5, when no spermatozoa remain (Fig. 8 E). Thus, the distribution of extracellular MSP in the gonad correlates with sperm availability. Extracellular MSP was seen in mated *C. remanei* females, as well as in *C. briggsae*, and *Poikilolaimus regenfussi* hermaphrodites (unpublished data).
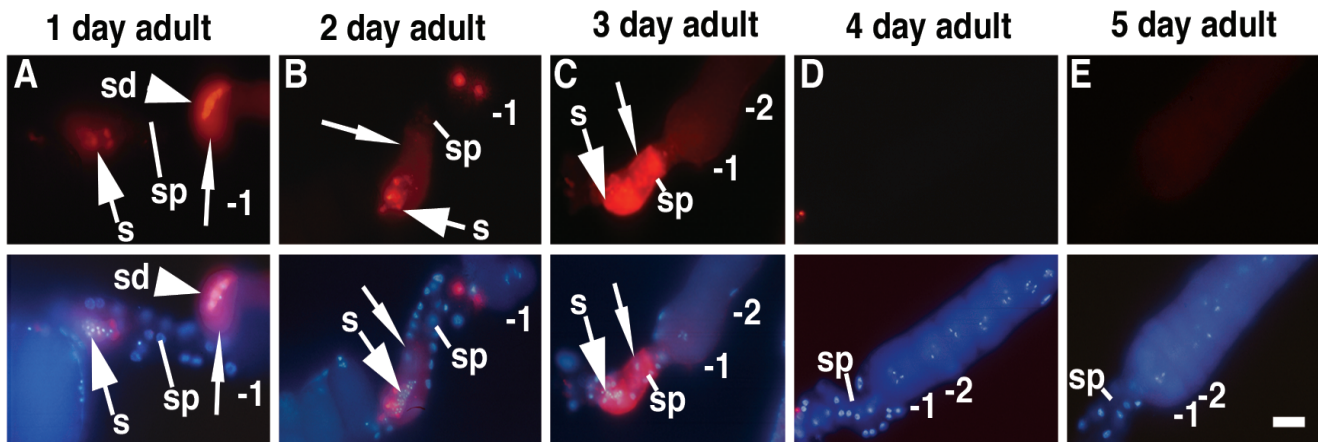
Figure 8. Time course of MSP release in wild-type hermaphrodites. MSP (red) and DNA (blue) detected on the indicated days of adulthood. (A) Day 1, MSP labeling is partitioned between spermatids in the gonad arm (arrowhead) and spermatozoa (arrows) in the spermatheca (sp). Extracellular MSP is most apparent in the gonad arm (thin arrow). (B) Day 2, few spermatids remain in the gonad arm (arrowhead), and most extracllular MSP is present in the spermatheca (thin arrows). (C) Day 3, Extracellular MSP is largely confined to the spermatheca (thin arrows), with only a slight haze over the –1 oocyte. (D, E) Day 4 and 5, MSP and spermatozoa are barely detectable, and oocytes stack in the gonad arm. Bar, 20 mm.

In mated females, extracellular MSP exhibits a graded distribution, with a sharp

boundary between the –1 and –2 oocytes (Fig. 7, B and C). Fluorescence intensity

measurements indicate that MSP is localized in a graded manner from the spermatheca to

the –1 oocyte (Fig. 7 C, n=10). Fluorescence intensity measurements also indicate that

there is significant MSP staining over the –2 and –3 oocytes (Fig. 7 E). Proximal

oocytes bind MSP and express the VAB-1 MSP/Eph receptor and unidentified MSP

receptors (Miller et al., 2003). One explanation for the sharp boundary in staining

intensity between the –1 and –2 oocytes is that MSP receptors may act as a sink for MSP

vectorially presented from the spermatheca. To test this hypothesis, we examined

extracellular MSP localization in mated emo-1/sec-61g(oz1) females, which are defective

for secretion in the germ line (Iwasaki et al., 1996) and MSP binding to oocytes (Miller et

al., 2003). Mated emo-1(oz1) females animals did not exhibit a sharp boundary between

the most proximal two oocytes and quantitative analysis showed no significant difference

in staining intensity of the –1 to –3 oocytes (Fig. 7 E). Instead, MSP extended further

distally in mated emo-1(oz1) females, compared with unmated controls, frequently

reaching more than 100 mm away (unpublished data). These results suggest that

receptors may influence boundary formation by restricting diffusion.

Extracellular MSP is punctate and diffuse and localizes to the oocyte cell surface

With confocal microscopy, extracellular MSP appeared both punctate and diffuse

in the spermatheca, the gonad arm, and the uterus (Fig. 7 F). Analysis of 3D data stacks

indicated that punctate extracellular MSP was enriched near spermatozoa on the

spermathecal walls (Fig. 7 F). The largest puncta were at the diffraction limit of our

microscope (≈0.5 μm) and found nearby spermatozoa. In the proximal gonad arm, MSP was more diffuse and localized in focal plane slices near the oocyte surface (Fig. 7 F, Video 1; see below for further confirmation). In the uterus, we observed large MSP puncta close to spermatozoa (Fig. 7F , right panel). We also observed diffuse MSP in extracellular spaces surrounding embryos in the uterus (Fig. 7 F). We were able to visualize MSP puncta near spermatozoa in the uterus and spermatheca using wide-field microscopy, when these regions were less crowded with spermatozoa (Fig. 7 G). These results are consistent with the possibility that the large MSP puncta arise from spermatozoa and generate a diffuse MSP signal in the proximal gonad.

To pinpoint the localization of MSP at the oocyte cell surface, we conducted a 3D confocal analysis of MSP localization in mated females using the RME-2 yolk receptor to mark the oocyte plasma membrane and early endosomal compartments (Grant and Hirsh, 1999). 3D image reconstructions of the data indicate that MSP localizes in three regions: 1) in superficial focal planes at the oocyte cell surface with RME-2 just beneath; 2) in the same plane as the RME-2 signal; and 3) within the oocyte beneath the plasma membrane (Fig. 9). These results are consistent with data showing that MSP is an extracellular signal that binds receptors on the oocyte surface, and suggests the MSP signal is endocytosed.

Specificity of MSP release and apparent budding from spermatozoa

Retrospective sperm counting experiments indicate that every spermatozoa fertilizes an oocyte (Ward and Carrel, 1979). Nonetheless, we used vital dye labeling

Figure 9. Localization of exported MSP and specificity of release. (A) Localization of MSP at the surface of the –1 oocyte. Single confocal sections at the indicated level of a 3D data stack through a mated female gonad stained for MSP (red) and RME-2 yolk receptor (green). No spermatozoa were seen in the indicated region (line) of the spermatheca (sp), thus the staining observed is extracellular to spermatozoa. Bars, 20 μm. (B) Single angle views of a 3D reconstruction of the data stack represented in (A). The image is cut to show surface and interior views of the –1 oocyte, at the indicated angles. Overlap between the MSP (red) and RME-2 (green) signals is yellow. Note the oocyte surface is slightly compressed where it abuts the spermatheca. The entire reconstruction is presented as Movie 4. (C) Intact and viable spermatozoa release MSP. A mated female stained for DNA (blue), MitoTracker (red), and MSP (green). Note, the MitoTracker staining is limited to the spermatozoa (s), but the MSP staining extends at least 50 μm from the most distal spermatozoa (arrowhead). (D) MSP localizes to extracellular puncta and apparent buds at the spermatozoa surface. Projections of confocal 3D data stacks from mated females stained in whole-mount for MSP (red) and MSD proteins (green), with overlap in yellow. Images are superimposed on the DIC channel, showing spermatozoa (s) in the uterus. Puncta (arrows) and surface blebs (arrowheads) contain MSP, but not MSD proteins. (E) Budding generates MSP puncta. Single angle views of a 3D reconstruction of MSP (red) and MSD (green) staining, with overlap in yellow. The image is cut to show interior and surface views of the spermatozoa. Apparent sites of budding contain MSP, but not MSD.

A

1μm 4μm 9μm

sp
-1
-2

B

0° 135° 210°

sp—

C

DNA Mito MSP Merge

—sp
s

D

s
s

E

0° 45° 120°
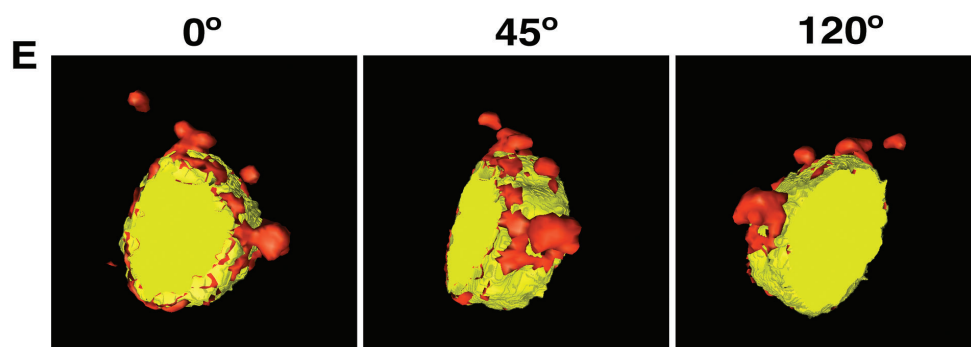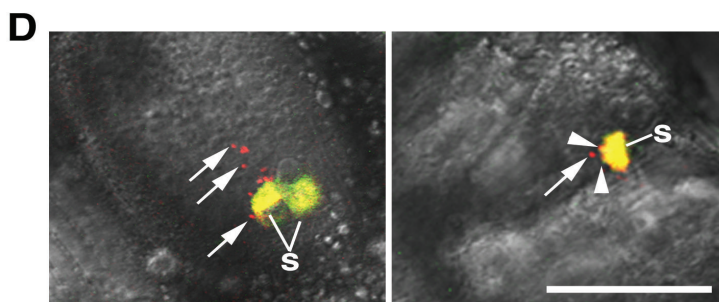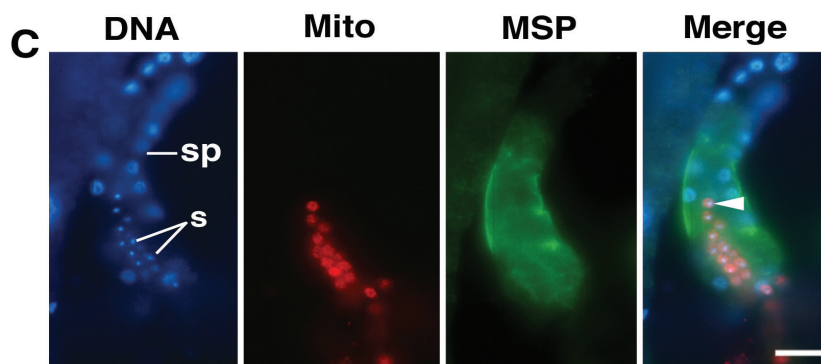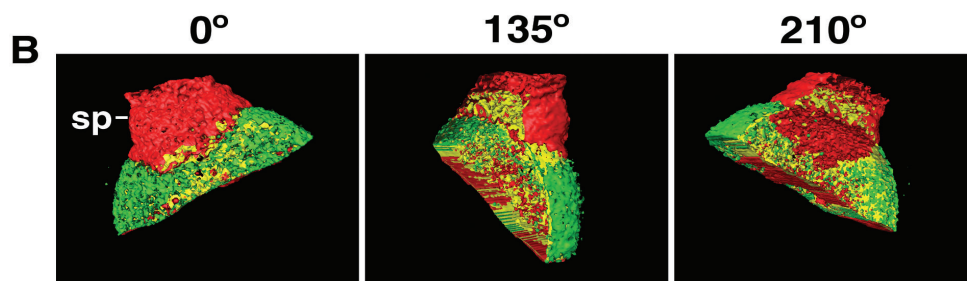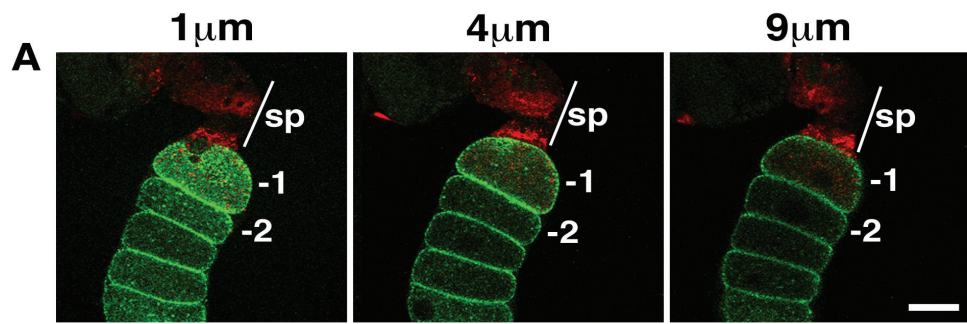
with MitoTracker Red to address whether MSP release results from lysis, the expectation being that lysis would disrupt the structure and integrity of spermatozoa dispersing the label. To label spermatozoa, males were soaked in MitoTracker Red and mated to unlabeled females. Labeled spermatozoa were able to crawl to the spermatheca of unlabeled females and produce viable progeny. The labeled mitochondria were located in a tight cluster in the cell body surrounding the spermatozoa chromatin (Fig. 9 C). By contrast, we observed MSP release from the labeled spermatozoa in all cases, (Fig. 9 C, n=12). By these criteria, the labeled spermatozoa were intact and functional, excluding lysis.

In addition, we generated antibodies to MSD-1(F44D12.3), MSD-2(F44D12.5), MSD-3(F44D12.7), and MSD-4(C35D10.11), identical members of a family of sperm-specific 11 kD proteins containing an MSP domain distinct from that of MSP (referred to here collectively as MSD, for Major Sperm Domain Proteins). The *Ascaris* ortholog, MFP1, is a component of the MSP cytoskeleton that decreases the rate of MSP fiber assembly *in vitro* (Buttery et al., 2003). We examined the localization of MSP and MSD in mated females by confocal microscopy and generated 3D image reconstructions of the data. While MSP and MSD exhibit extensive co-localization within the pseudopod and cell body of spermatozoa, only MSP localizes to extracellular puncta (Fig. 9, D and E). At the margins of the spermatozoa, we observed protrusions containing MSP but not MSD. These results suggest MSP protrusions may give rise to free MSP puncta by a specific budding process, a possibility confirmed by electron microscopy (see below).

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Release of MSP by vesicle budding

To address the mechanism of MSP release at an ultrastructural level, we used

transmission electron microscopy (TEM) of adult hermaphrodites. In order to minimize

processing artifacts and give the best possible preservation of cell ultrastructure, we used

high pressure freezing and freeze substitution (HPF) techniques to prepare samples for

TEM (McDonald, 1999: Mueller-Reichert et al., 2003). The HPF method provided

excellent morphology of germline and somatic tissues, including extracellular spaces in

the spermatheca and uterus (Fig. 10). Using this approach, we detect novel 150-300 nm

vesicles in extracellular spaces of the spermatheca (apical luminal regions) in close

proximity to spermatozoa (Fig. 10 A, left panel). These vesicles have not been observed

in previous EM studies, which relied on conventional fixation techniques (L'Hernault,

1997, our unpublished results). We analyzed vesicles in serial sections (n=30), and

confirmed that they are free structures unattached to spermatozoa or somatic cells (Fig.

10 B, and unpublished results). These vesicles have both an inner and an outer

membrane. In serial sections, the central core narrows, and thus may be encapsulated by

the inner membrane. By tilting the vesicles within the beam of the electron microscope,

we were able to visualize inner and outer leaflets of both membranes (Fig. 10 C). Tilting

of the vesicles also indicates that they have a scalloped appearance formed by multiple

bends of the outer membrane (Fig. 10 D), and that the inner membrane is more regularly

shaped (Fig. 10 C). The annulus between the inner and outer membranes displays an

electron density similar to the cytoplasm of spermatozoa. The inner core varies in

appearance, containing irregular electron dense material (Fig. 10, B-D). In addition to

the spermatheca, we detect these vesicles in the uterine lumen, in extracellular spaces

Figure 10. Detection of a new class of vesicle by electron microscopy. (A) Low power views of vesicles (arrows) in the extracellular space near spermatozoa (s) in the spermatheca (left panel), in the spermathecal-uterine junction region (middle panel), and in an extracellular space of the uterus formed by close packing of embryos (e). Pseudopods (ps) and an apical junction between spermathecal cells (arrowhead) are indicated. Bars, 500 nm. (B) Serial section analysis of two vesicles in the spermatheca. Inset is a magnified view. Bars, 125 nm. Sections are 75 nm thick. (C) The vesicles possess two concentric lipid bilayers. Vesicles were tilted through the indicated angles in the EM beam to visualize the individual leaflets of the inner and outer membranes. Bar, 100 nm. (D) Tilting of a vesicle to visualize its scalloped appearance. Bar, 100 nm. (E) No MSP vesicles are observed in the spermathecal lumen (sl) of a *fog-2(q71)* female, instead the lumen is filled with material that resembles yolk lipoprotein particles, bar,
500 nm.
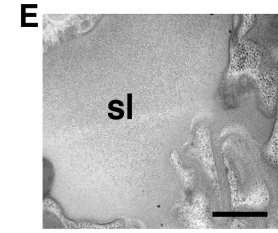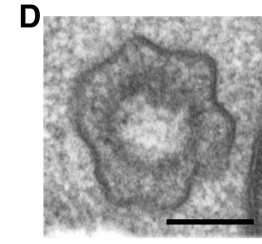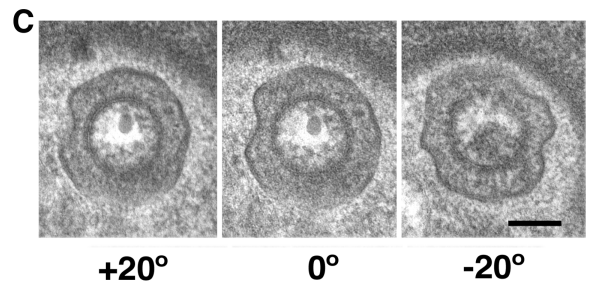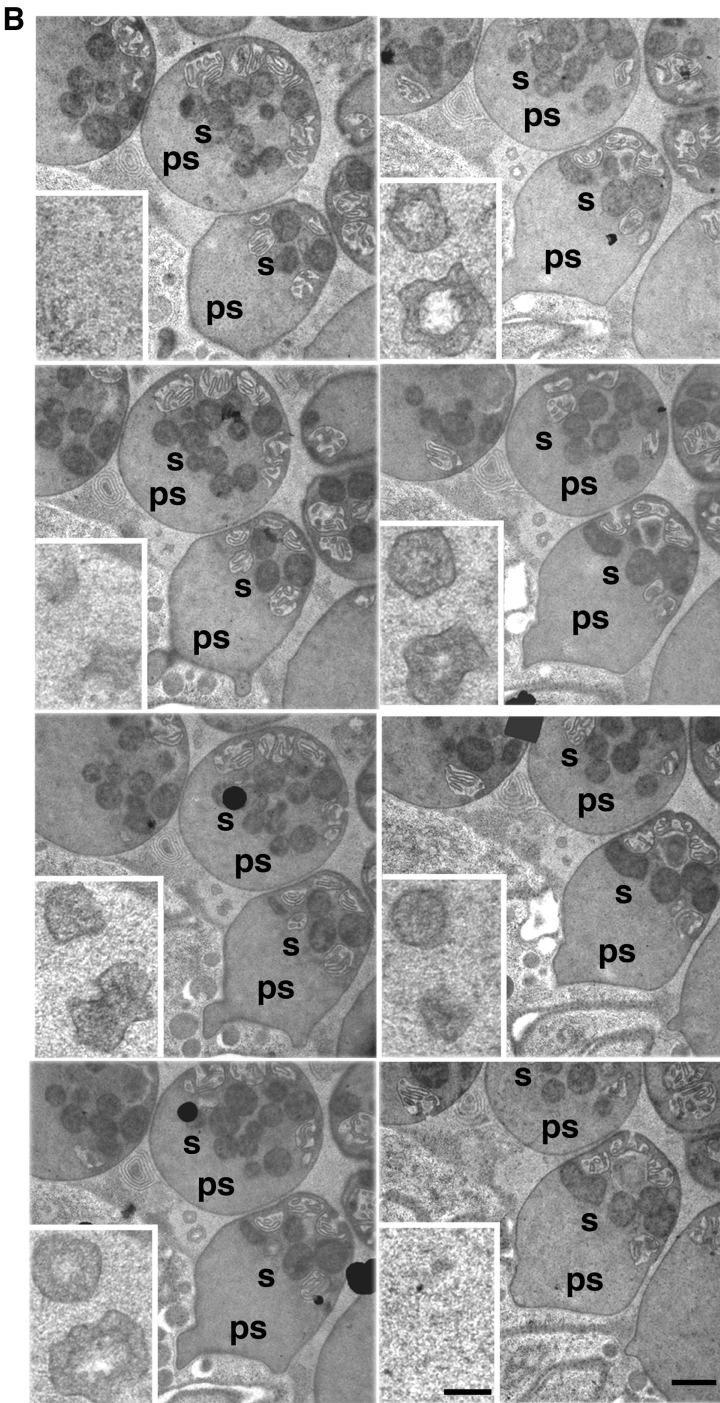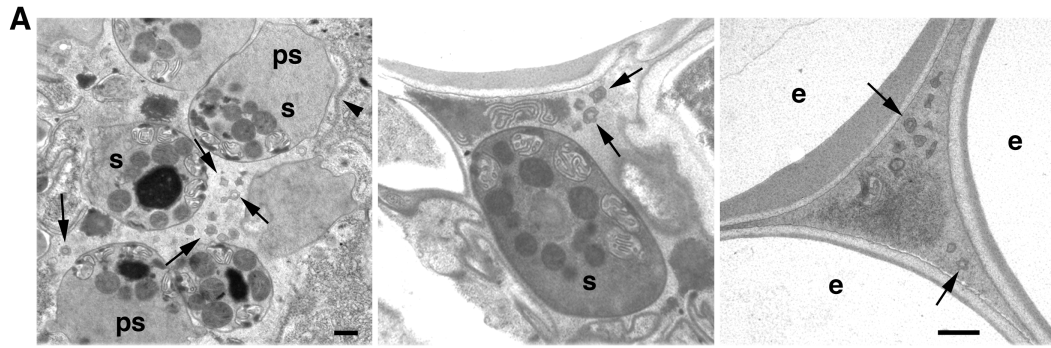
near spermatozoa (Fig. 10 A, middle panel), and within extracellular crevices formed by close packing of embryos (Fig. 10 A, right panel). Vesicles with this characteristic shape and morphology were not observed within the reproductive tract (gonad arm, spermatheca and uterus) of unmated females (Fig. 10 E, and unpublished results). Instead, the spermathecal and uterine lumens were filled with material similar to yolk lipoprotein particles as described by Hall et al., (1999; Fig. 10 E, and unpublished results.). These vesicles were not observed in extracellular (or cellular) spaces of other tissues (unpublished results). Thus, EM analysis defines a novel class of extracellular vesicle associated with spermatozoa, and provides an attractive candidate at the ultrastructural level for the MSP puncta described by fluorescence microscopy above.

To determine whether these novel vesicles contain MSP, we performed TEM on hermaphrodite samples prepared by HPF and post-embedding immunohistochemistry (immunoEM; Fig. 11). We observed strong labeling within the pseudopod and cell body of spermatozoa (Fig. 11, A and B, left and middle panels). Within the cell body, MSP was enriched in close association with the plasma membrane (Fig. 11 B, left panel and inset). We also detected small protrusions of the plasma membrane of the cell body containing MSP (Fig. 11 B, middle panel and inset). By contrast, MSP labeling was largely excluded from the major cellular organelles of the spermatozoa, such as mitochondria, membranous organelles (MOs), and the nucleus (spermatozoa do not possess a nuclear envelope) (Fig. 11, A and B). No appreciable MSP labeling was observed in the distal germ line, the intestine, body-wall muscle, spermathecal cells,

Figure 11. Vesicles contain MSP and form by budding. (A) Detection of MSP by immunoEM. Low power view of two spermatozoa in the spermathecal-uterine junction region of an adult hermaphrodite. Intense labeling in pseudopods (ps), bar, 500 nm. (B) Detection of MSP at the plasma membrane of the spermatozoa cell body. Left panel, MSP labeling excluded from cellular organelles (mitochondria (m), membranous organelles (mo), and the nucleus (n)). Inset, magnified view showing MSP associated with the plasma membrane. Middle panel, MSP associated with the plasma membrane and a protrusion (arrow), magnified in the inset. Note free labeling in the extracellular space (brackets). Right panel, MSP is not detected in distal germ cells. Bars, 500 nm and 125 nm (inset). (C) MSP is contained within the vesicles. A gallery of 7 vesicles (the lower right two panels are views of the same vesicle in non-adjacent sections). MSP is located in the annulus between the inner and outer membranes. Bars, 100 nm. (D) Vesicle budding from spermatids. Non-adjacent sections of two different spermatids in the hermaphrodite gonad. Views 1 and 1' and 2 and 2' are corresponding pairs of non-adjacent sections, respectively. The budding vesicles contain MSP in both views. Vesicles connect to the cell body by a stalk (thin arrows) and the plasma membrane at the budding site appears intact (thick arrow). MSP is enriched in a cross-sectional view at the base of the budding projection (arrowheads in lower panels). Bar, 100 nm. (E) MSP associated with an apical junction of spermathecal cells (arrow). Free MSP in the extracellular space is indicated (brackets). Bars, 500 nm. (F) MSP associated with a lipid whorl structure in the extracellular space of the spermatheca. Bar, 500 nm.
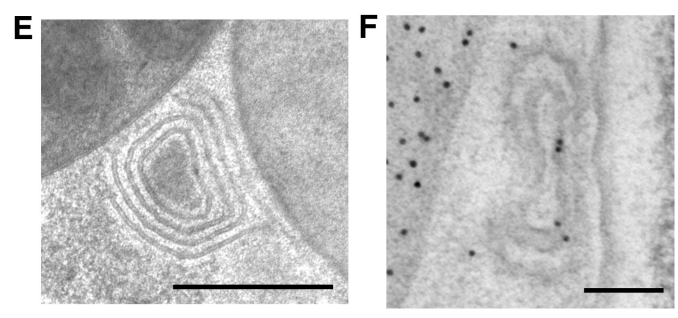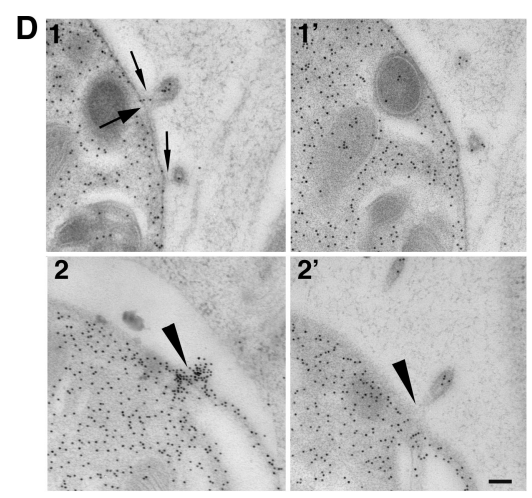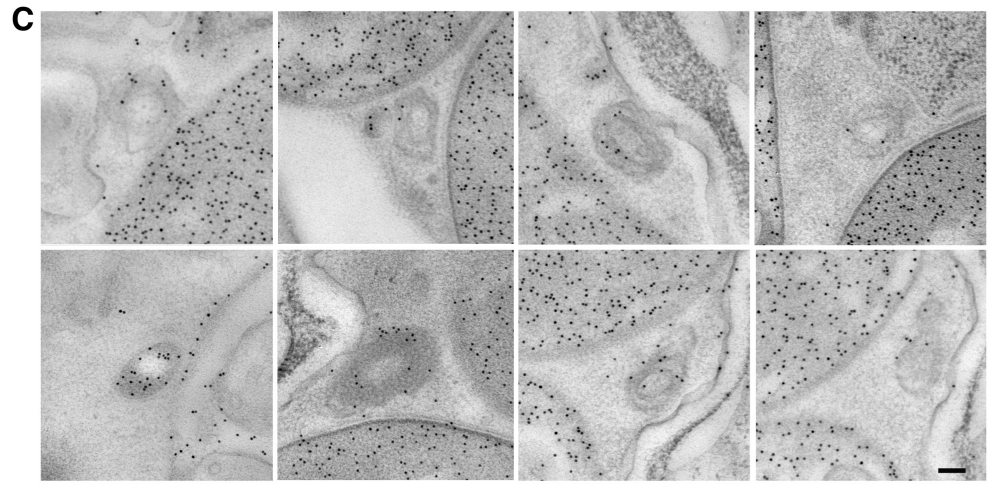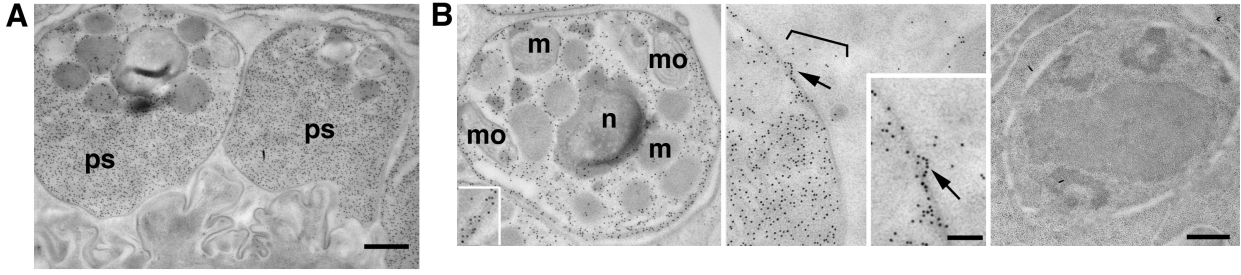
uterine cells, or in *E. coli* surrounding the animal (Fig. 11 B, right panel, and unpublished results). Thus, detection of MSP by immunoEM was highly specific.

Using HPF followed by immunoEM, we detected MSP labeling of the novel vesicles in extracellular spaces of the spermatheca (Fig. 11 C; 7 out of 9 vesicles labeled). Labeling was chiefly found within the annular ring between the inner and outer membranes. Since the only cells in the spermatheca and uterus observed to contain MSP are spermatozoa, these vesicles likely correspond to the MSP puncta, which apparently bud from them (see above). Consistent with this idea, we observed three cases of budding from spermatids in the gonad arm (Fig. 11 D). Two buds were viewed in non-adjacent sections (Fig. 11 D, top panels, views 1 and 1'). The buds exhibit MSP labeling in both views and are connected to the cell body by a thin stalk (thin arrows), with the plasma membrane beneath the budding site apparently intact (thick arrow). A third example of vesicle budding from a spermatid in the gonad arm was found (Fig. 11 D, bottom panels, views 2 and 2') in which MSP intensely labeled the site of budding at the base of the projection in a circular pattern, possibly representing a cross-sectional view of a cylindrical network of MSP filaments (Fig. 11 D, bottom left panel). Free MSP labeling in extracellular spaces could also be observed by immunoEM of adult hermaphrodites (Fig. 11 B, middle panel), but was generally less prevalent then when detected by immunofluoresence in mated females (see above). In adult hermaphrodites, we also observed unique lipid whorl deposits in the extracellular spaces of the spermatheca and uterus (Fig. 11 E). These electron-dense whorls were not observed in the spermatheca or uterus of unmated females (Fig. 10 E, and unpublished results). In several cases, we observed MSP vesicles apparently fusing, possibly contributing the

formation of the whorls (unpublished results). Residual MSP labeling was associated

with the lipid whorl deposits and thus they may represent an end fate of the MSP vesicles

(Fig. 11 F).

Spermatids and spermatozoa differ in temporal and spatial signaling properties

The development of both male and female gametes in the hermaphrodite gonad

provides two contexts for MSP signaling: spermatids signal nearby oocytes within the

proximal gonad, whereas spermatozoa signal remotely from the spermatheca (Fig. 6). To

compare the temporal and spatial signaling activities of spermatids and spermatozoa, we

analyzed *spe-8(hc50)* and *spe-27(it110)* mutants, which are defective in hermaphrodite

spermiogenesis. *spe-8* and *spe-27* mutants produce morphologically normal spermatids

that can be activated for spermiogenesis and fertilization by male seminal fluid

(L'Hernault, 1997).   In the wild type, meiotic maturation rates progressively decline

toward unmated female levels as spermatozoa run out (Table 1). By contrast, *spe-8* and

spe-27 mutants exhibit maturation rates that are more constant over time (Table 1). This

observation is surprising because the mutant spermatids are rapidly cleared from the

reproductive tract because they cannot crawl. To compare further the signaling potencies

of spermatids and spermatozoa, we conducted a time-course analysis of MAPK activation

Table 1. Time Course Analysis of MSP Signaling

Genotype[a]	Meiotic maturation rates (maturations per gonad arm per hr)				
	1-day adult	2-day adult	3-day adult	4-day adult	5-day adult
fog-2(q71)	0.07 ± 0.07 (n=18)	0.17 ± 0.13 (n=18)	0.22 ± 0.20 (n=18)	0.20 ± 0.20 (n=18)	0.21 ± 0.16 (n=19)
Wild type	2.23 ± 0.67[b] (n=30)	2.87 ± 0.57[b] (n=30)	1.38 ± 0.83[b] (n=30)	0.82 ± 0.54[b] (n=28)	0.30 ± 0.30[c] (n=26)
Wild type mated on day 3	N. A.	N. A.	N. A.	2.32 ± 0.82[b, d] (n=37)	1.68 ± 0.73[b, d] (n=42)
fog-2(q71) mated	1.80 ± 0.41[b, e] (n=32)	2.63 ± 0.58[b, f] (n=30)	2.71 ± 0.73[b, d] (n=30)	2.47 ± 0.95[b, d] (n=29)	1.51 ± 0.87[b, d] (n=26)
tra-3(e2333)	1.77 ± 0.38[b, e] (n=34)	2.40 ± 0.54[b, e] (n=25)	2.30 ± 0.63[b, d] (n=28)	1.22 ± 0.40[b, d] (n=24)	0.56 ± 0.43[d, g] (n=19)
spe-9(eb19)	1.17 ± 0.73[b, d] (n=18)	1.98 ± 0.69[b, f] (n=18)	2.17 ± 0.50[b, d] (n=14)	2.35 ± 0.53[b, d] (n=12)	1.73 ± 0.64[b, d] (n=16)
spe-8(hc50)	1.73 ± 0.67[b, h] (n=20)	1.35 ± 0.82[b, d] (n=20)	0.99 ± 0.60[b, f] (n=30)	0.64 ± 0.55[f, g] (n=28)	0.94 ± 0.66[b, d] (n=21)
spe-8(hc50) mated	N. D.	N. D.	N. D.	N. D.	2.27 ± 0.90[b, d] (n=10)
spe-27(it110)	1.54 ± 0.84[b, e] (n=20)	1.01 ± 0.43[b, h] (n=20)	1.27 ± 0.68[b, i] (n=30)	1.05 ± 0.58[b, f] (n=30)	0.90 ± 0.62[b, d] (n=32)

Maturation rates were measured in 5-hour intervals at various times after mid-L4 stage. Standard deviations are shown. Statistical significance was assessed using Student's t-test; N. A., not applicable; N. D., not done. [a]Genotypes analyzed: *fog-2(q71)* produce no sperm; *tra-3(e2333)* produce approximately 50% more sperm; *spe-9(eb19)* are defective in fertilization; *spe-8(hc50)* and *spe-27(it110)* produce non-motile spermatids lacking a pseudopod. [b]$P < 0.001$, [g]$P < 0.01$, and [c]$P > 0.5$, compared to *fog-2(q71)* female values at the same time point. [d]$P < 0.001$, [e]$P < 0.01$, [h]$P < 0.05$, [f]$P > 0.2$, and [i]$P > 0.5$ compared to wild-type hermaphrodite values at the same time point.

(Fig. 12 A). In the wild type, the percentage of gonad arms that exhibit MAPK activation in oocytes declines as sperm are used for fertilization, paralleling the decline in total MSP levels and sperm numbers (Fig. 12 B). By contrast, in *spe-8* mutants, MAPK activation remains high at times (days 3 and 4) when sperm are depleted or no longer present. Consistent with this observation, residual MSP was faintly detected in *spe-8* mutants at these late times (Fig. 12 B). As a control, we feminized *spe-8* (n=20) and *spe-27* (n=19) mutants using *fem-1(RNAi)*, which resulted in low meiotic maturation rates and a stacked oocyte phenotype comparable to *fog-2(q71)* females.

We next examined MSP release from spermatids. Wild-type, *spe-8*, and *spe-27* spermatids release MSP primarily in punctate form within the gonad arm (Fig. 7, C and 12D, and unpublished results). MSP puncta appear widely distributed in the proximal gonad arm (Fig. 12, C and D), whereas extracellular MSP produced by *spe-8* spermatids in the spermatheca appears more diffuse (Fig. 12 C, top panels). At late times, we observed diffuse extracellular MSP in the spermatheca in *spe-8* mutants, despite the absence spermatids (Fig. 12 E). By contrast, when spermatozoa are depleted in the wild type, no extracellular MSP is observed (Fig. 7, D and E). This perdurance of extracellular MSP provides an explanation for the signaling observed at late times in *spe-8* and *spe-27* mutants. Taken together, these results suggest that spermatids provide a long-acting form of the MSP-signal, whereas spermatozoa provide a long-range, temporally labile signal

To investigate the potential basis for the increased stability of MSP signaling in *spe-8* mutants, we conducted HPF and TEM experiments. In *spe-8(hc50)* adult

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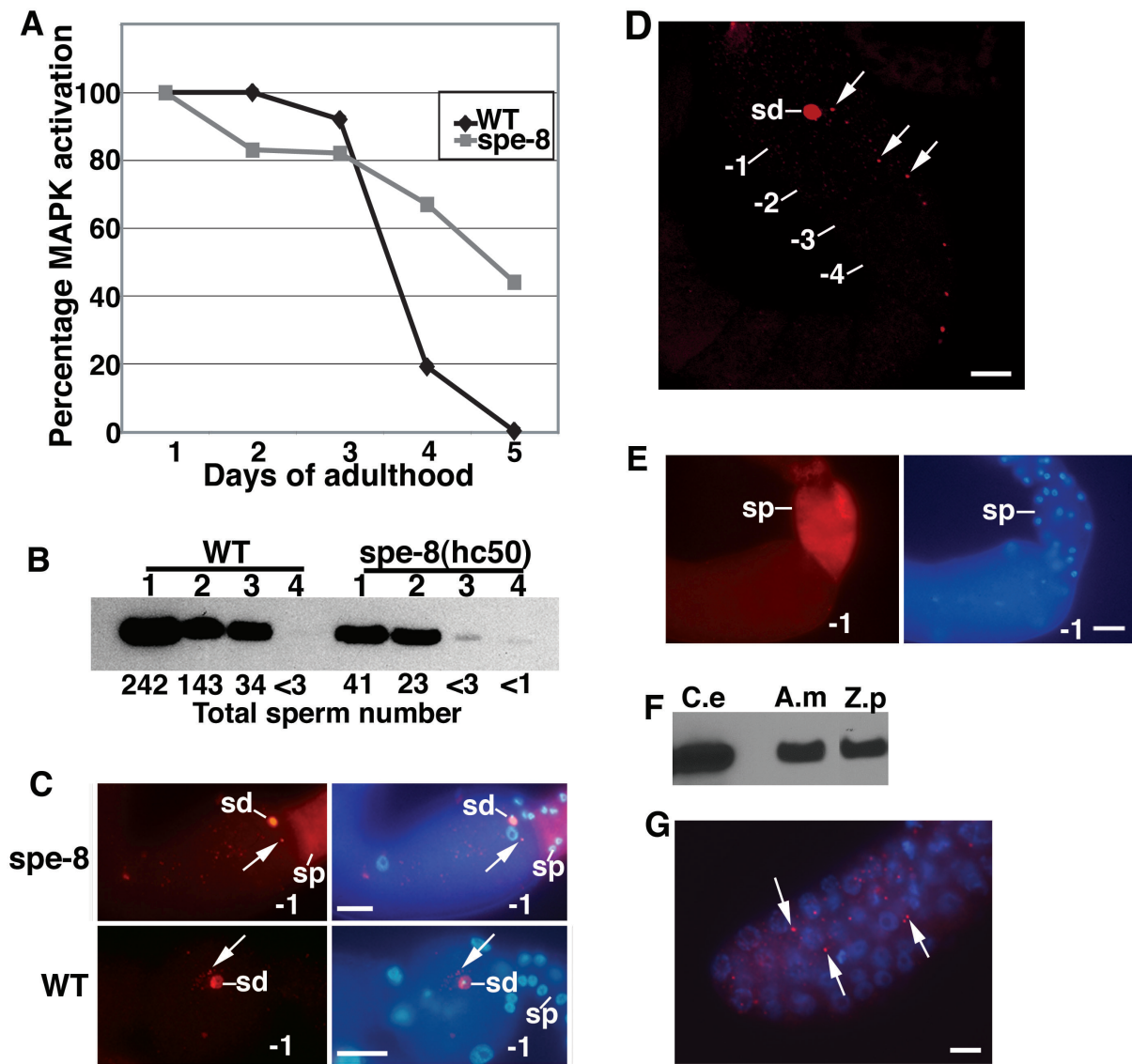
Figure 12. Spermatids provide a long-acting MSP signal. (A) Time course analysis of MAPK activation in the wild type and spe-8(hc50) mutants. The percentage of gonad arms with activated MAPK was measured by staining dissected gonads at the indicated times of adulthood. (B) Time course of total MSP levels. Western blots of MSP in spe-8(hc50) and the wild type (10 animals/lane). The number of spermatids and spermatozoa were counted at each time. The data represents the average of three trials. (C) Detection of MSP puncta (arrows) located near spermatids (sd) in the proximal gonad arm of spe-8(hc50) and wild-type hermaphrodites. MSP (red) and DNA (blue) were detected. MSP is detected in the spermatheca (sp) of spe-8(hc50) hermaphrodites, but no spermatids are observed. Bars, 20 mm thoughout. (D) Projection of a confocal 3D data stack showing MSP puncta (arrow) distributed widely in the proximal gonad far from the single spe-8(hc50) spermatid (sd) seen. Bar, 10 mm. (E) MSP perdures in spe-8(hc50) mutants. MSP (red) staining is observed, but spermatids are not, confirmed by viewing the DNA (blue) signal in multiple focal planes. (F) Western blot of MSP in C. elegans (C. e) and the Cephalobid nematodes Acrobeloides maximus (A. m) and Zeldia punctata (Z. p). (G) Detection of MSP puncta (arrows) in the A. m. gonad. Only the distal arm is shown.

hermaphrodites, we observed MSP vesicles in the gonad arm, spermatheca and uterus (Fig. 13, and unpublished data). The MSP vesicles were particularly abundant in the spermathecal-uterine junction region (Fig. 13 A). In one respect, the MSP vesicles in *spe-8* mutants differed from those of the wild type (Fig. 10), the outer electron-dense layer of the *spe-8* MSP vesicles did not exhibit clearly distinguishable inner and outer leaflets (Fig. 13, B and C, and unpublished results). It is not clear whether this difference is a consequence of their increased stability, or whether it represents some fundamental difference in their assembly. One observation in *spe-8* mutants, however, may shed some additional light on how the MSP vesicles may form. In fertilization-defective mutants, such as *spe-8(hc50)*, the unfertilized oocytes sometimes lyse in the spermatheca or uterus because they do not form egg shells. In these cases, oocyte cytoplasm and organelles filled the spermathecal lumen (Fig. 13 C), and we observed that the interior of the MSP vesicles contained material markedly similar to that found in the extracellular space (Fig. 13 C). This observation suggests that the internal ring of the MSP vesicle may derive from the extracellular space, a possibility that will require further experiments to address fully.

Since *spe-8* mutants do not form pseudopods, we examined the morphology of the cell body in detail to uncover additional information related to the formation of the MSP vesicles. As seen for wild-type spermatids (Fig. 11 D), we observed protrusions of the plasma membrane (Fig. 13, D-I). These varied in length from 100 nm to 2 μm in size (Fig. 13, D-I, and unpublished results), and often had a bent appearance (Fig. 13 E). These protrusions contained MSP labeling (Fig. 13, F and I). Often, pairs of closely

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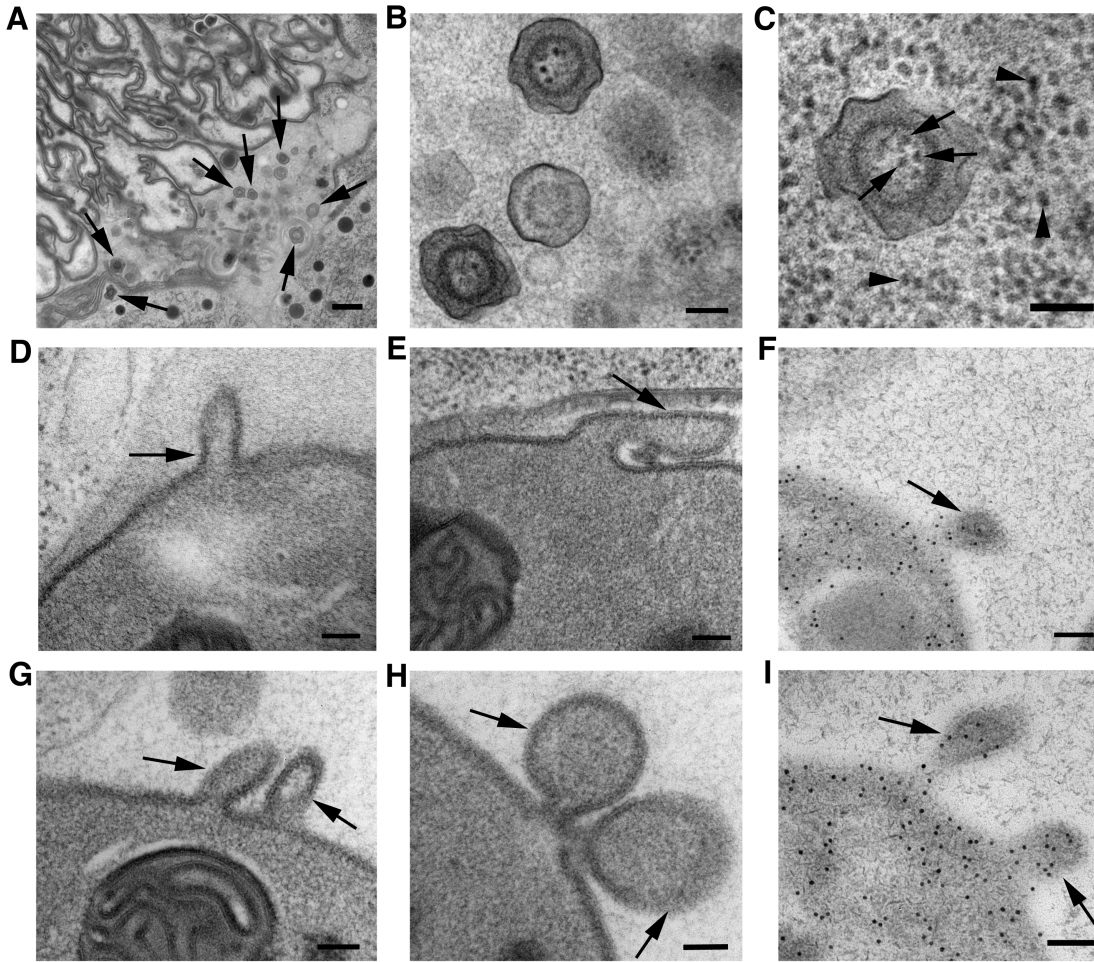
Figure 13. Production of MSP vesicles in the spermiogenesis-defective *spe-8(hc50)* mutant.
(A) Low power view of the spermathecal-uterine junction region. MSP vesicles (arrows) are abundant in extracellular spaces of this region. (B) High magnfication view of MSP vesicles located in the region shown in (A) from an adjacent section. (C) MSP vesicle in the spermathecal lumen surrounded by cytoplasmic debris from a lysed oocyte (arrowheads). Note, the inner ring of the MSP vesicle contains material (arrows) similar to the oocyte cytoplasmic contents.
(D-I) Protrusions (arrows) from the cell body of spermatids located in the gonad arm.
(F and I) Detection of MSP in protrusions. Bars, 500 nm for (A) and 100 nm for (B-I).

spaced protrusions formed in close proximity to one another (Fig. 13, G-I). These observations provide additional evidence for a vesicle budding process at the cell body of spermatids and spermatozoa and raise the question of whether closely spaced buds may contribute to the formation of MSP vesicles.

Since spermatids release MSP, motility and a pseudopod are dispensable. This result prompted us to examine whether parthenogenetic nematodes, which reproduce without sperm, have MSP. We analyzed the highly divergent Cephalobid parthenogens, *Acrobeloides maximus* and *Zeldia punctata* using monoclonal antibodies raised to the highly conserved MSP C-terminus. We detected MSP by western blot of both *A. maximus* and *Z. punctata* (Fig. 12 F). Immunostaining of *A. maximus* indicated that punctate immunoreactivity was widely distributed in the female germline (Fig. 12 G and unpublished results). While the functional roles of MSP in *A. maximus* and *Z. punctata* will require additional study, these results indicate that MSP can be conserved in evolution for functions unrelated to motility of spermatozoa.

Discussion

A vesicle budding mechanism for MSP release

Here we show that *C. elegans* sperm use a novel vesicle budding mechanism to deliver the MSP signal to oocytes and sheath cells. We used an array of microscopic modalities and multiple specific antibodies to examine MSP localization in mated females and hermaphrodites. In particular, mated females provide an ideal format for analyzing MSP release because spermatozoa, and thus MSP, are supplied entirely by mating. Since the distal constriction of the spermatheca constitutes a barrier to sperm

entry, staining in the proximal gonad is due to MSP that is extracellular to spermatozoa. Using fluorescence microscopy, we observed two forms of extracellular MSP: a punctate form, and a diffuse form. Three observations suggest that MSP puncta may represent the precursor to the diffuse form. First, confocal microscopy provides evidence for budding of MSP puncta from spermatozoa, identifying their origin. Second, the punctate form is absent from the proximal gonad arm of mated females, whereas the diffuse form can reach responding oocytes. Finally, when spermatids have been cleared from the reproductive tract in spermiogenesis defective mutants, only the diffuse form is observed.

Several lines of evidence rule out alternative explanations for these observations, such as lysis or leaching of proteins from spermatids or spermatozoa during fixation. First, MSP release is highly specific, as abundant and soluble sperm-specific components of the MSP cytoskeleton, the MSD proteins are not observed in MSP puncta or in buds. Second, vital dye labeling experiments indicate that spermatozoa remain intact and functional, despite releasing MSP. Third, in spermiogenesis-defective hermaphrodites, after spermatids clear from the reproductive tract, extracellular MSP staining is still detected, and thus must originate prior to fixation. Fourth, multiple MSP antibodies and fixation conditions yield consistent results. Finally, electron and light microscopy paint congruent pictures of MSP release.

Using TEM, we detected a new class of vesicle, the MSP vesicle, in the spermatheca and uterus of adult hermaphrodites. ImmunoEM demonstrates that these vesicles contain MSP and likely correspond to the MSP puncta observed by confocal microscopy. The observation that MSP vesicles are more abundant in *spe-8* mutants, which produce a long-acting MSP signal, provides correlative data that MSP vesicles

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may represent signaling intermediates. While the precise steps by which the MSP

vesicles form remain to be determined, our observations are consistent with the

possibility that protrusions from the cell body may bend back upon themselves and pinch

off, thereby encapsulating lumenal material within a double-membraned vesicle. MSP

vesicles are likely to be labile structures because they are not detectable by conventional

electron microscopy and they appear to fuse to generate lipid whorls. Thus, instability of

the MSP vesicles may liberate MSP in a free form able to bind oocytes and sheath cells

via the VAB-1 Eph receptor protein-tyrosine kinase and unidentified receptors (Miller et

al., 2003). Taken together then, these results suggest that MSP release from spermatozoa

and spermatids occurs in two stages: first budding of MSP vesicles, and then vesicle

disintegration. Since both spermatids and spermatozoa release MSP via vesicle budding,

neither a pseudopod nor motility is required.

Vesicle budding, a nexus for the motility and signaling functions of MSP

Does vesicle budding utilize activities of MSP that are also required for amoeboid

locomotion of nematode spermatozoa? Several features of MSP vesicle budding suggest

this is indeed the case. ImmunoEM shows that MSP is enriched at, and associated with,

the plasma membrane of the cell body of spermatids and spermatozoa. Localized MSP

filament assembly may generate the protrusive force driving vesicle budding at the

plasma membrane of the cell body, analogous to the leading edge protrusion that drives

pseudopodial extension (Italiano et al., 1996, Bottino et al., 2002). Consistent with this

idea, we observed MSP in protrusions of the plasma membrane of the cell body by

immunoEM (Fig. 11, and Fig. 13). Confocal microscopy and 3D-image reconstructions

identify MSP-containing protrusions, which are likely to correspond to the sites of budding. Whereas, MSP is concentrated at these budding sites, the MSD proteins are absent. *In vitro* studies of MSP-based motility in *Ascaris* identified MFD1, the ortholog of the MSD proteins, as an activity that decreases the rate of MSP fiber growth (Buttery et al., 2003). Thus, the absence of the MSD proteins at the vesicle budding sites, may favor MSP filament assembly and membrane protrusion.

In vesicle budding processes, bending of the lipid bilayer is energetically costly due to a strong hydrophobic effect (Chernomordik and Kozlov, 2003). Likely TEM views of the vesicle budding process demonstrate the involvement of bent and looped intermediates (Fig. 11, and Fig. 13). Several observations provide initial indications of how membrane bending may be achieved during vesicle budding. Localized polymerization of MSP filaments may provide the protrusive force that drives membrane bending. MSP filaments are flexible and have a short persistence length, and are thus conducive to bending (Italiano et al., 1996; Bottino et al., 2002). TEM views of MSP vesicles indicate that they have a regular, highly bent scalloped appearance, which suggests the involvement of vesicle coating proteins. Thus, MSP polymerization may provide the energy driving membrane protrusion and bending, while uncharacterized coat proteins may store this energy and stabilize the bent configuration. Since MSP cytoskeletal dynamics powers retraction in amoeboid motility (Miao et al., 2003), an attractive idea is that disassembly of MSP filaments at the base of the projection may play a role in scission. While MSP itself may play a prominent role in vesicle budding, other proteins are also likely to be involved. Since SNARE proteins play critical roles in vesicle fusion processes (Bonifacino and Glick, 2004), members of this family may

function in sealing off the MSP vesicle during budding. In this regard, it is interesting to note that VAP-33, a protein with an MSP domain, interacts with synaptobrevin and has been implicated in vesicle trafficking (Skehel et al., 1995; Soussan et al., 1999).

How is MSP vesicle budding regulated? Vesicle budding results in loss of MSP and plasma membrane from spermatids and spermatozoa, so there must be a trade-off between MSP signaling and motility. The best evidence that MSP vesicle budding is regulated comes from two sets of related observations: first, MSP release does not occur from spermatids within or dissected from males; and second, extracts of female animals promote vesicle budding from spermatids *in vitro* (unpublished results). The possibility that MSP release may depend on extracellular cues from the hermaphrodite reproductive tract may have precedents in MSP-based motility because spermatozoa are likely to sense directional cues as they navigate from the uterus to the spermatheca. In this view then, MSP cytoskeletal dynamics would drive pseudopodial extension and crawling in response to one set of cues, and vesicle budding in response to another. Identification of the putative cues will provide the most direct test of this hypothesis.

Vesicle budding provides a basis for long and short range MSP signaling

Our results suggest two modes of MSP signaling: spermatids appear to provide a long-acting form of the MSP-signal, whereas spermatozoa provide a long-range, temporally labile signal. This plasticity is well adapted for the developmental stages of MSP signaling: spermatids signal neighboring oocytes from within the gonad, and spermatozoa must signal from far-flung regions including the spermatheca and the uterus. For the sperm-sensing mechanism (Miller et al., 2003) to generate a biologically

meaningful output, extracellular MSP levels must be valid and reliable indicators of sperm availability. A block to spermiogenesis short-circuits the control mechanism.

Our results suggest that differential MSP vesicle stability may provide a mechanistic basis for the distinct signaling activities of spermatids and spermatozoa. In *spe-8* mutants, MSP vesicles are more stable, and signaling persists after the spermatids are swept from the reproductive tract by ovulated oocytes. While it is not possible to completely exclude the possibility that *spe-8* mutant spermatids release the MSP signal in a way this qualitatively or quantitatively different from wild-type spermatids in the gonad, the isolation of a large class of sperm-defective mutants on the basis that they lay unfertilized oocytes in high quantity suggests that many mutants that disrupt spermiogenesis may have this property (L'Hernault, 1997). If wild-type spermatids do indeed produce a long acting signal within the gonad arm, then some mechanism must exist to eliminate this form of the MSP signal after ovulations have commenced and the spermatids have entered the spermatheca and undergone spermiogenesis. Otherwise, meiotic maturation might continue at a brisk pace after sperm are depleted and thus oocytes would be wasted. Possibly, the presence of spermatozoa may destabilize MSP vesicles from spermatids in trans. While the actual determinants of MSP vesicle stability are unclear, both intrinsic and extrinsic factors may contribute. During spermiogenesis, ER/Golgi derived organelles, the membranous organelles (MOs), fuse with the plasma membrane to transfer their contents to the cell surface and the extracellular environment (L'Hernault, 1997; Xu and Sternberg, 2003). MO fusion is not required for the budding process because spermatids, which have unfused MOs bud vesicles, moreover, *fer-1* mutants, which are defective in MO fusion (Achanzar and Ward, 1997), are able to signal

(McCarter et al., 1999). Nonetheless, MO fusion generates a difference between the plasma membrane protein composition of spermatids and spermatozoa that might affect the stability of their respective MSP vesicles.

MSP vesicle budding and unconventional secretory mechanisms

How general is the MSP vesicle budding mechanism? MSPs are highly conserved in nematodes where they play both cytoskeletal and signaling roles. Proteins with MSP domains are also found in fungi, plants, and animals. Genetic studies demonstrate that an MSP domain protein, DVAP-33A, functions as an instructive signal during bouton formation at the neuromuscular junction in *Drosophila* (Pennetta et al., 2002). A mutation in the MSP domain of VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis type 8 in humans (Nishimura et al., 2004). Our observation that MSP localizes to membranes and apparently drives vesicle budding may define a general activity for the MSP domain in other proteins.

As a molecular mechanism, vesicle budding provides a general means for releasing cytoplasmic proteins from cells. It is now becoming clear, that diverse intracellular proteins can be secreted from cells by novel means independent of a signal peptide or the ER/Golgi system (Nickel, 2003). Proteins released by non-classical secretory pathways fit into two broad groups: those that are located within vesicular compartments within the cell and those that are cytoplasmic. For example, IL-1β is associated with secretory lysozymes and is released by an unconventional mechanism (Stinchcombe et al., 2004). By contrast, galectin 1 and 3 (Cooper and Barondes, 1990), fibroblast growth factors 1 and 2 (Mignatti et al., 1992), and HIV-Tat (Chang et al., 1997)

are likely cytoplasmic, yet exported from cells. For some members of both groups (e.g., IL-1β, galectin 1, FGF-2), there is evidence for release within vesicles (Cooper and Barondes, 1990; MacKenzie et al., 2001).

With classical ER/Golgi-dependent protein secretion mechanisms so robust, it is reasonable to ask why cells should bother with unconventional export pathways? In the case of MSP, nematode spermatids and spermatozoa simply do not have any other option, having jettisoned their ribosomes, ER/Golgi, and actin during meiosis II. A similar argument explains why spermatozoa from many vertebrate and invertebrate species rely on the acrosome reaction for zona penetration. A variety of highly specialized cells (e.g., melanocytes, platelets, cytotoxic T-lymphocytes, mammary gland cells, and sweat gland cells) rely on unconventional protein export pathways (Nickel, 2003; Stinchcombe et al., 2004). Possibly, non-canonical secretion mechanisms provide highly specialized cells with greater flexibility in dynamic environments in which cell positions or developmental status are changeable, as for MSP vesicle budding.

CHAPTER III

A FEMALE SIGNAL MAY INITIATE MSP RELEASE FROM SPERM

Introduction

Multiple signaling events are crucial to ensure successful completion of fertilization. The union of haploid cells generate a diploid zygote requires multiple intercellular signaling between the gametes. Although sperm proceed directly through meiosis uninterrupted to generate haploid gametes, oocytes often pause in meiotic prophase and await signals before they resume meiosis. This resumption of meiosis, meiotic maturation, is defined by the transition between diakinesis and metaphase of meiosis I. The hallmarks of meiotic maturation are nuclear envelope breakdown, cortical cytoskeletal rearrangement, and meiotic spindle assembly (Masui and Clark 1979). To ensure successful reproduction, the completion of oocyte meiosis must be coordinated with oocyte development and fertilization. This pause in oocyte meiosis allows oocytes to achieve this coordination by integrating many signals. For example in starfish 1-methyladenine triggers meiotic maturation (Kanatani et al., 1969), while in amphibians progesterone triggers meiotic maturation (reviewed by Ferrell, 1999). In other animals such as, sponges, mollusks and nematodes sperm promote the resumption of meiosis (Masui, 1985; McCarter et al., 1999). In mammals, signals that trigger meiotic maturation remain to be identified, however follicle cells negatively regulate meiotic maturation (Pincus and Enzmann, 1935).

In addition to intercellular signals regulating fertilization, external signals also can control fertilization. Many animals must assess their environmental conditions and adjust their reproductive cycle accordingly. Favorable environmental conditions, such as adequate nutrition, help to ensure the success of offspring. In fact many bovine females become sterile due to inadequate nutritional intake, either by stopping egg production all together or by inhibiting egg growth (Mognet et al, 1997). In *Drosophila* the insulin pathway is the link between nutrition and proliferation responses in oocytes (Drummond-Barbosa and Spradling, 2003).

To enhance our understanding of signals that regulate reproduction we are using the model organism *C. elegans*. Recently we have established that *C. elegans* sperm signal distant oocytes to undergo oocyte meiotic maturation and gonadal sheath cell contraction utilizing the cytoskeletal protein MSP as a hormone (Miller et al., 2001; Miller et al., 2003). Further we have shown that MSP is exported from sperm by a vesicle budding mechanism (Kosinski et al., 2005). MSP vesicles are proposed to represent labile signaling intermediates such that decomposition of the MSP vesicles releases MSP in a free form able to bind oocyte and sheath cell receptors. MSP has a dual role, functioning as a hormone to signal oocytes meiotic maturation and as the central cytoskeletal protein required for amoeboid locomotion of nematode sperm (Italiano et al., 1996). The trade-off between MSP signaling and motility suggests that MSP export must be highly regulated.

Here we hypothesize that MSP vesicular export is initiated by a signal derived from the female animal. According to our model, this female signal is in part the yolk protein or components of the yolk complex encoded by the vitellogenin genes, suggesting

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that female nutrition may be tied to fertility. We have developed an *in vitro* assay in which we suggest the vesicle budding process can be induced when initiated by female extracts. Extracts generated from males do not result in vesicle formation. Our partial purification reveals that our activity co-migrates with the yolk complex on a sucrose gradient.

Materials and methods

Nematode strains

Standard techniques were used to culture *C. elegans* at 20oC. Strains used were C. elegans N2, *fog-2(q71)V*(Schedl and Kimble, 1988), and *rme-2(bl008)IV* (Grant and Hirsh, 1999). To generate virgin males, wild-type nematode males were picked as young L4 animals and then grown overnight at 20ºC in the absence of females or hermaphrodites. For extract preparation males were separated from female animals with 35μm screens.

In vitro assay and immunocytochemistry

Virgin males were picked and washed in M9 buffer. Males were dissected in SM buffer (50mM NaCl, 25mM Kcl, 5mMCaCl$_2$, 1mM MgSo$_4$, 5mM Hepes, pH 7.8, osmolarity of 220 (Nelson and Ward, 1980) on slides coated with peanut lectin 0.5mg/ml (Sigma). Dissected spermatids were then analyzed or fixed with paraformeldehyde in a humid chamber for 1-2 hours. Spermatids were then stained with MSP antibodies according to Kosinski et al., 2005. Images were acquired on a Zeiss Axioskop or Axioplan microscopes with an ORCA ER (Hamamatsu) charge-coupled device camera.

Activity purification

Approximately 20 100mm plates containing young adult (day 1 of adulthood) *fog-2 (q71)* animals were washed and males were separated using 35mm screens. The removal of males was ascertained by visualization under the microscope. Female animals were diluted in 3 mls of M9 buffer and passed through a French press 3 times at 12,000 psi. Supernant was centrifuged at 10,000x g for 30 minutes followed by a heat step of 65°C for 10 minutes. Ultracentrifugation at 135,000x g was then performed to remove insoluble material, which was then run on a 10 to 30 % sucrose gradient. 1 ml fractions were assayed for activity, and analyzed by electrophoresis on 4-12% NuPage gels (Invitrogen) for protein abundance. Fractions that showed activity in the *in vitro* assay were analyzed by Western analysis for yolk antibodies (YP88, YP115, YP170, Sharrock, 1983). Positive signal was detected with SuperSignal West Femto reagent (Pierce).

Results

Female extracts signal spermatids

Previously we have shown that both spermatids and spermatozoa release vesicles containing MSP *in vivo*. We reasoned that spermatids isolated *in vitro* should also release MSP vesicles. To test this idea we developed an *in vitro* assay in which spermatids from virgin male animals were dissected and adhered to slides. In our assay spermatids were quiescent with a uniformly circular shape (Fig. 14A).
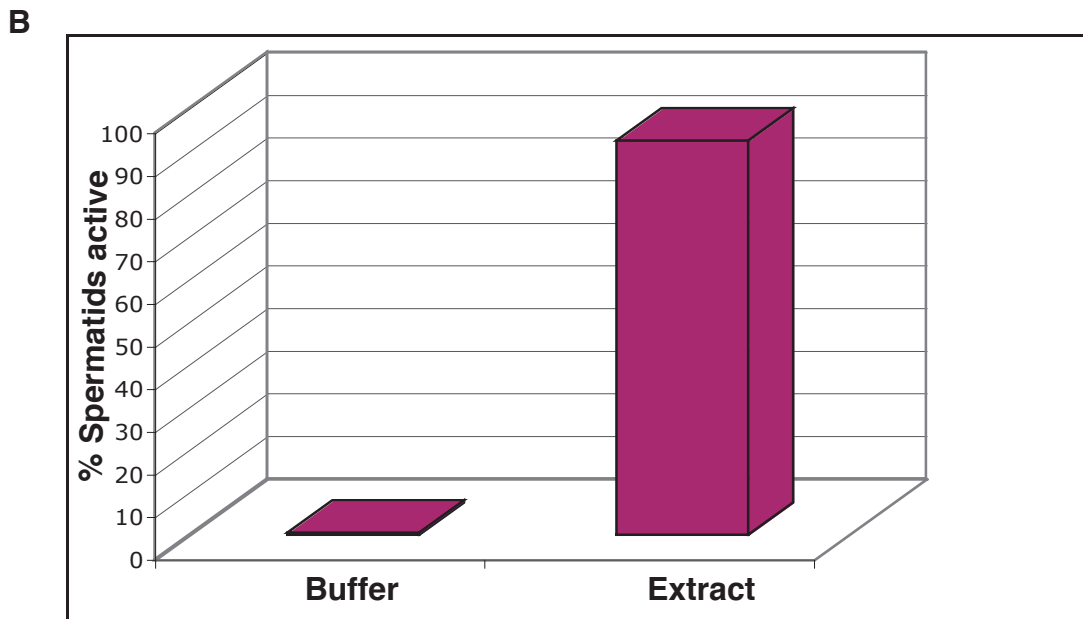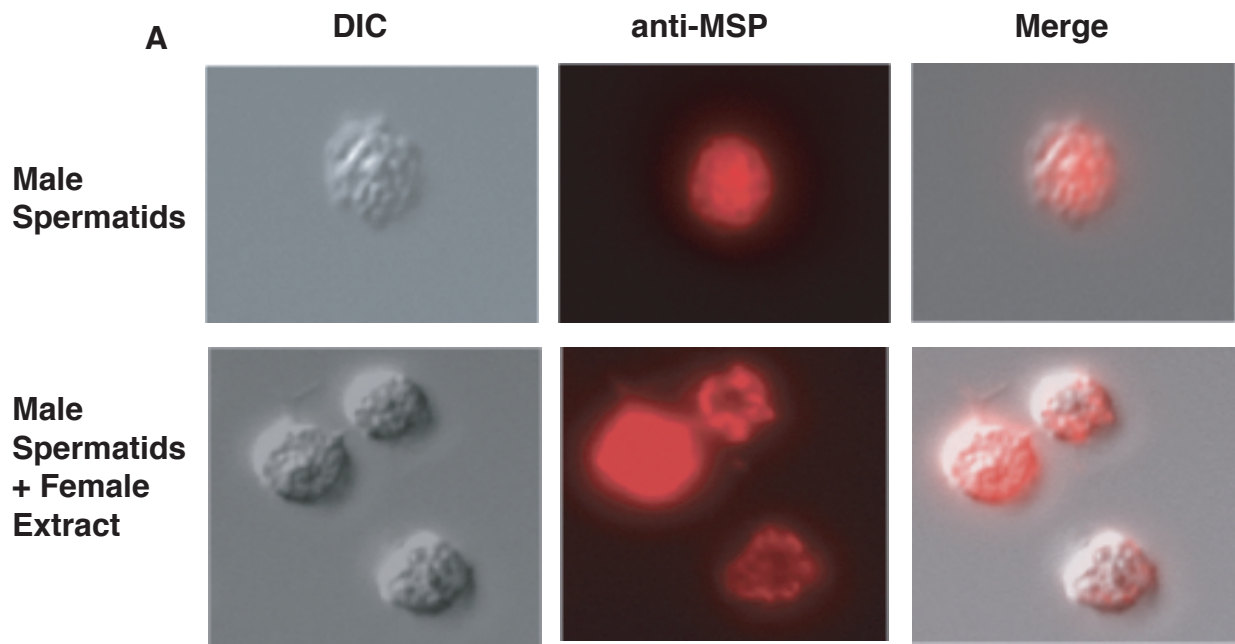
Figure 14. Activation of spermatids *in vitro*. (A) Spermatids isolated from males in the presence of buffer are quiescent, round cells with MSP located throughout the cytoplasm. Spermatids isolated from males in the presence of female extract become irregular in shape, exhiditing small blebs. MSP localization becomes polarized to the cell perifery and localizes within the blebs. Spermatids are approximately 5 microns in size. (B) Quantification of this activity shows greater than 80% of spermatids treated with female extracts show activity.

Immunoflourescence examination of MSP localization revealed that MSP was localized uniformly throughout the spermatids (Fig. 14A). Further, MSP localization within spermatids did not change over time in buffer. Strikingly, when female animals were dissected in the presence of spermatids, a change in spermatid morphology occured. Cells became more irregular in shape, with small blebs at the periphery. In addition MSP localization changed and became polarized to the margin. These results suggested that dissected female animals caused changes in morphology and in MSP localization of spermatids.

The above results coupled with our observations that extracellular MSP localization was not detected in male gonads, prompted us to test the hypothesis that MSP vesicle export was signal dependent and the putative signals triggering MSP vesicle budding are derived from the female animal. To distinguish if the initiating signal was derived from germline or somatic cells of female animals, we tested MSP release in *glp-4 (bn2*ts*)* animals. *glp-4 (bn2*ts*)* animals display a temperature sensitive proliferation defect such that germ nuclei remain arrested in mitotic prophase (Beanan and Strome, 1992). MSP release was detected from sperm of *glp-4 (bn2*ts*)* mutant animals, suggesting signals triggering MSP vesicular export are derived from somatic female cells.

We then generated female extracts and examined spermatid morphology when dissected in the presence of female extracts. Treated cells exhibit small blebs near the periphery and in some cases at a distance away from the cell (Fig. 14 A). Immunoflourescence of MSP localization revealed that these blebs contain MSP (Fig. 14 A). To quantified this effect and determined that approximately 95% of spermatids formed blebs and exhibited polarized MSP localization compared to less than 1% of

spermatids treated with buffer alone (Fig. 14B). Identical extracts generated from male animals showed no activity. These results suggest the activity is female specific, and robust.

Time course of MSP localization in response to female signal

To further characterize the effect of the female extract upon spermatids we performed a time-course analysis of MSP localization. This analysis showed a distinct spatial pattern of MSP and thus we classified the stages of MSP localization into four separate classes (Fig. 15). In class I, occurring immediately after addition of the female extract (0-20sec), MSP begins to localize to the cell periphery forming a ring around the spermatid (Fig. 15). In class II, representing one minute after addition of female extract shows MSP become clustered into large areas around the cell periphery (Fig. 15). In class III, representing two minutes after the addition of female extracts reveals smaller MSP puncta being shed from the cell periphery and sometimes we can detect these puncta at a distance away from the cell (Fig. 15). In class IV, the last stage occurring four minutes and more after the addition of female extract MSP appears to be lost from the cytoplasm, such that intercellular MSP levels are distinguishably lower than previous time points (Fig. 15). This analysis suggests that female extracts signal spermatids to release MSP *in vitro*.

rme-2(b1008) animals contain more activity

To identify the signal/signals that initiate MSP vesicular release we combed through known mutants that have low brood sizes but still contained sperm. We reasoned

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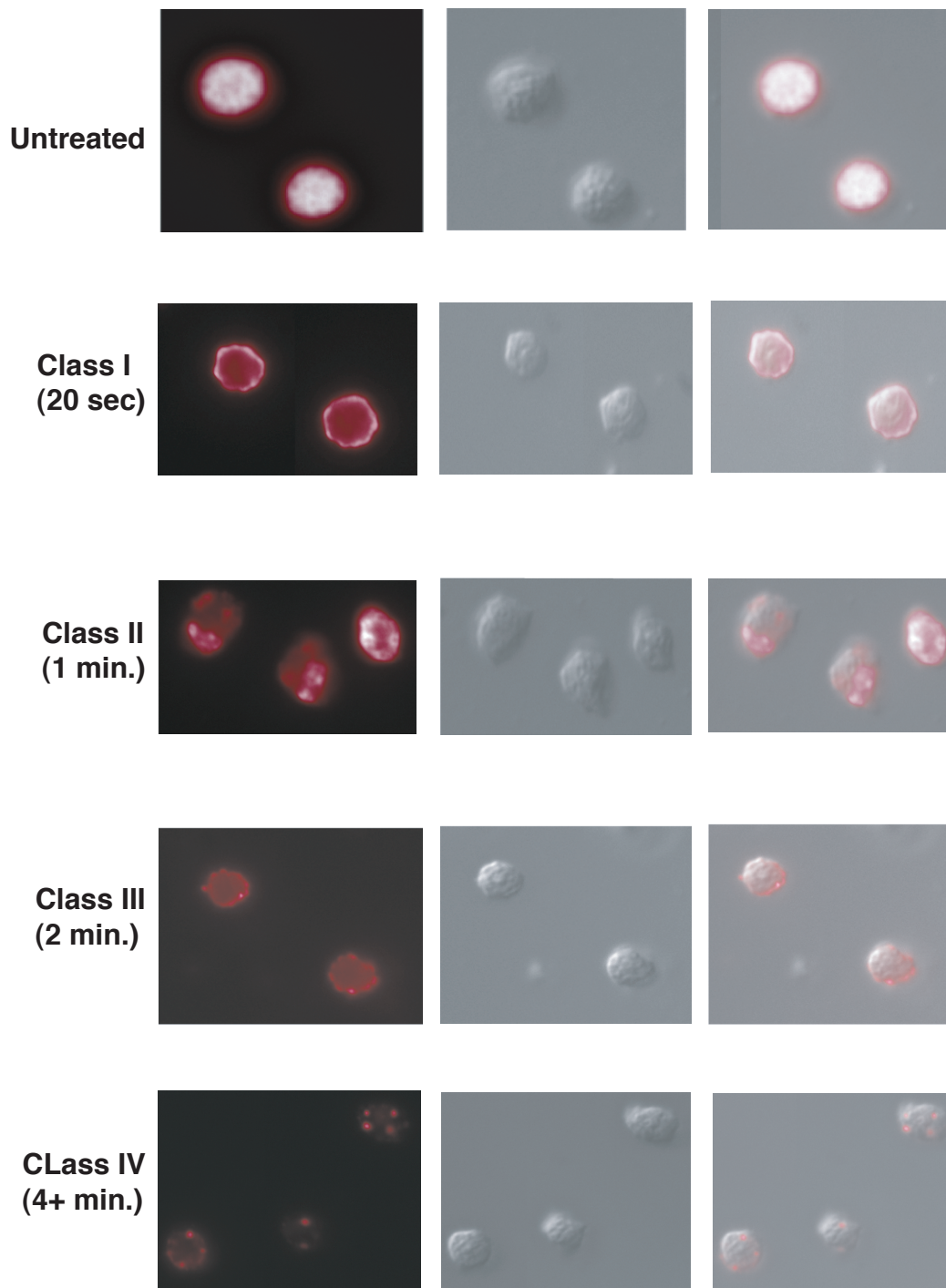
Figure 15. Time course of MSP localization in sperm in response to female signals.
Untreated cells show uniform MSP localization and are quiescent. Addition of active
fractions of female extract causes rapid MSP polarization (20 sec). Surface blebs
containing MSP are then observed (1-2min.) followed by loss of MSP from the cell
cytoplasm (4+ min.). Photos were imaged using the relative exposures, however different
objective lenses were used. Sperm are 5 microns in size.

that if signals initiating MSP release were absent then oocyte meiotic maturation would be reduced in the presence of sperm, due to the lack of released MSP. *rme-2 (bl008)* mutants lack the oocyte yolk receptor, RME-2, which facilitates in the uptake of yolk proteins that are synthesized in the intestine and taken up by growing oocytes (Grant et al., 1999). Thus *rme-2 (bl008)* animals accumulate high levels of apoB-like yolk proteins. These animals exhibit a low brood size although sperm are present within the spermatheca.

To test the hypothesis that *rme-2 (bl008)* mutant animals may affect MSP release from spermatids, we generated extracts from *rme-2 (bl008)* animals. We reasoned that if MSP release was diminished within *rme-2 (bl008)* animals then extracts generated from these animals would not activate spermatids *in vitro*. Suprisingly, extracts generated from *rme-2 (bl008)* animals show a 100-fold increase in activity (Fig 16). Female extracts can promote activity when diluted as much as 1000 times, however extracts generated from a mutant background can be diluted as much as 100,000 times, suggesting this background contains more activity. As mentioned previously *rme-2 (bl008)* mutants accumulate large amounts of yolk proteins, thus this overproduction of yolk may account for the abundance of activity generated from *rme-2* extracts. These results suggest that yolk may be a signal to release MSP from spermatids and thus the MSP export signal is perhaps linked to the nutritional status of the female animal.

To help assess our purification strategy we generated a unit definition for the MSP export signal. One unit is the amount that produces a 50% release response from
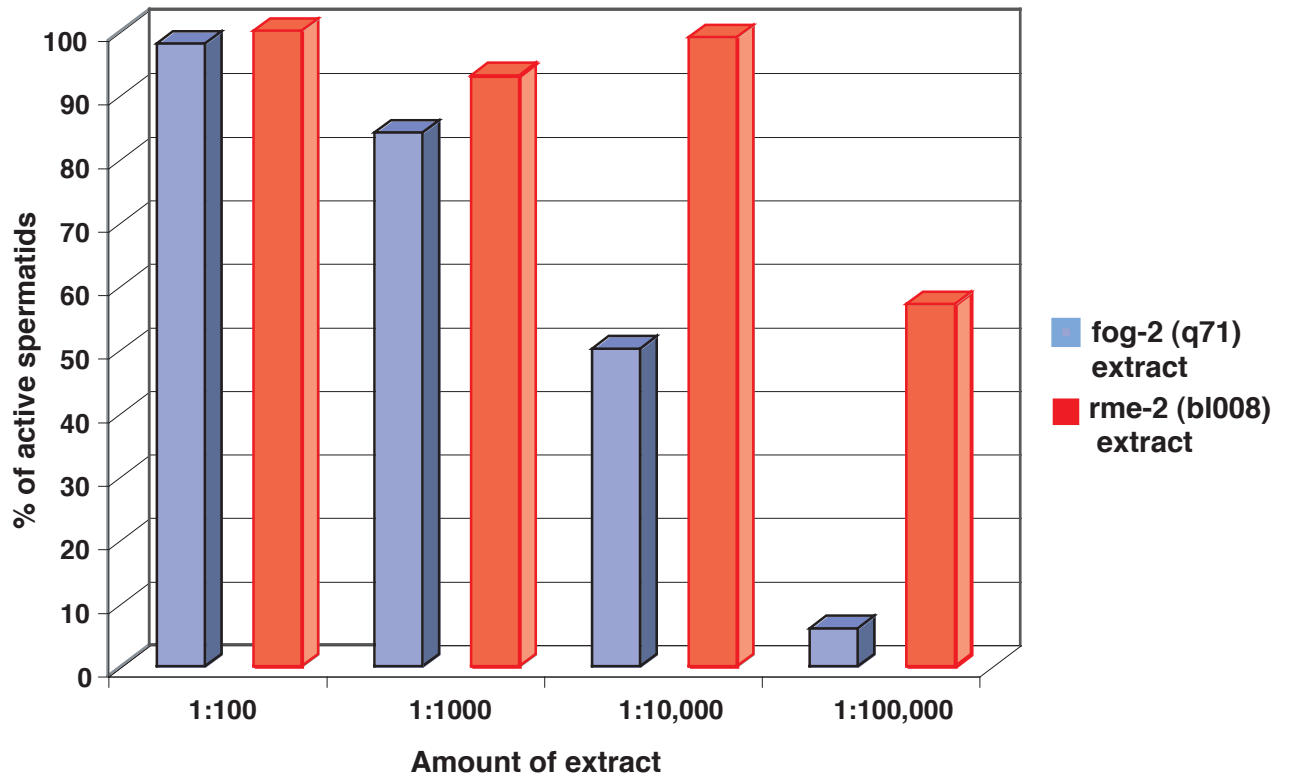
Figure 16. Extracts generated from *rme-2* animals show an increase in activity.
Extracts were made from either *fog-2(q71)* or *rme-2 (bl008)* animals. Extracts were
added to spermatids and assayed for activity at various dilutions. *fog-2 (q71)* extracts
elicite a 50% response in spermatids when diluted as far as 1:10,000, however
extracts from *rme-2 (bl008)* can be diluted to 1:100,000 and elicite a greater than
50% activity.

spermatids in our *in vitro* assay. From 1 ml of packed *rme-2* mutant nematodes, we routinely produce approximately 100,000 activity units. This quantification and unit definition again suggests that activity is abundant.

Activity purification

To identify the signal/signals that initiate MSP release from sperm we have developed a partial purification strategy of activity from both female and *rme-2 (bl008)* animals. Extracts heated at 65°C for 10 minutes remain active but are inactivated when boiled (Fig. 17). The addition of a heating and high-speed spin to remove any insoluble material yields a greater than 10 fold increase in specific activity. We then separated activity on a 10-30% sucrose gradient. The activity fractionates in one broad peak and sediments at approximately 20S under these conditions (Fig. 17). Western blot analysis with antibodies to the yolk proteins shows that the yolk proteins also fractionate in fractions of peak activity (Fig. 17) (Julia Richards). Taken together these results suggest that yolk proteins or components of the yolk protein complex may stimulate MSP release activity.

Discussion

Our hypothesis that MSP export from sperm may be signal dependent comes from several observations. The first is that MSP functions as the major cytoskeletal protein required for spermatozoan locomotion. Thus if release of MSP was not tightly controlled then spermatozoa's motility would soon become compromised, destroying their
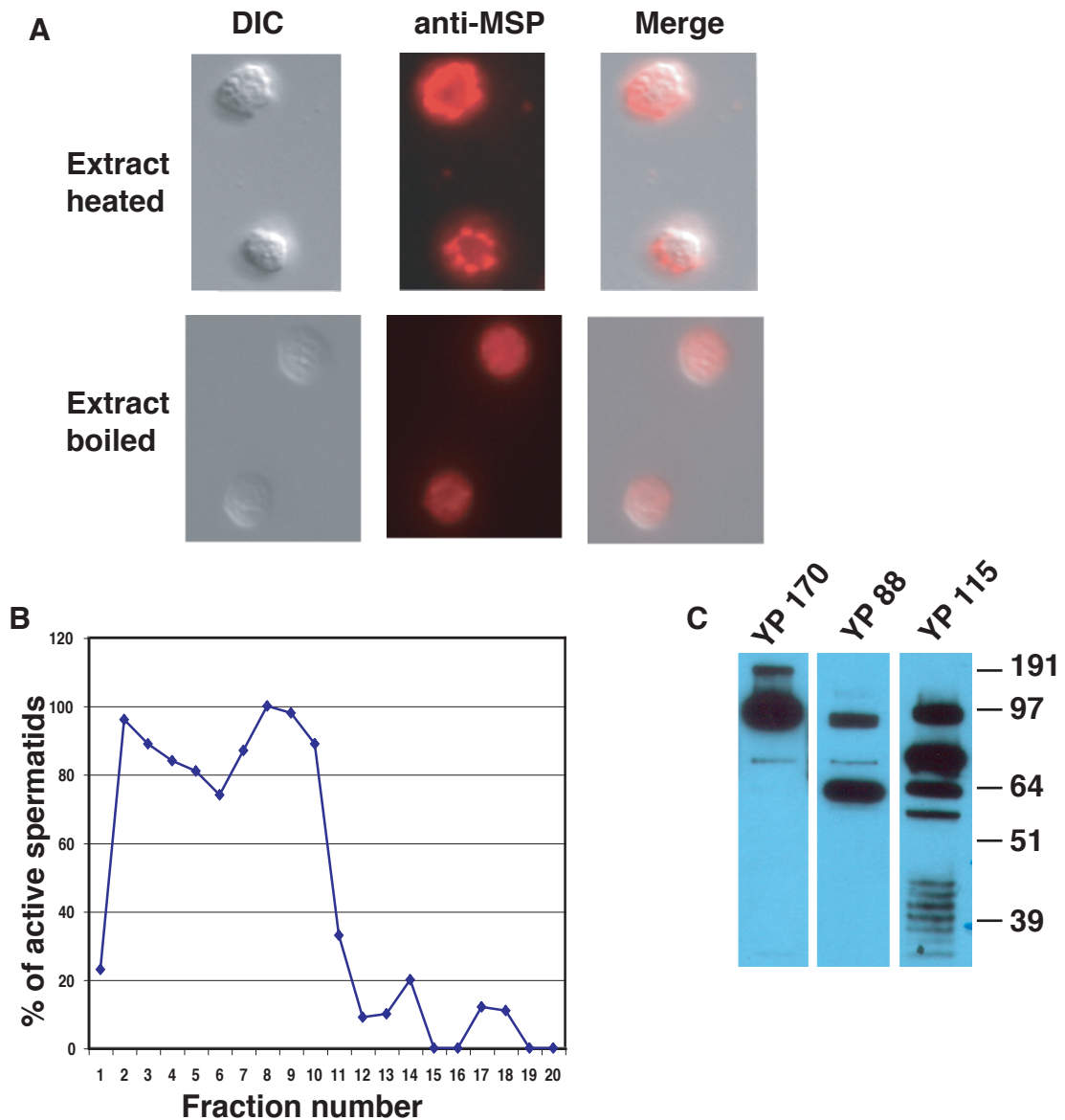
Figure 17. Partial purification of activity from female extracts. (A) Female extracts have been either heated at 65oC (Top panels) or boiled (bottom panels). Activity is heat stable yet abolished when boiled. (B) Line graph illustrating sucrose gradient fractions that show activity. Fraction #1 represents the bottom of the gradient (30%). Fractions are 1ml. Activity migrates in one broad peak between fractions 2-10 representing approximately (20S). (C) Western blot analysis of fraction 8 form a sucrose gradient blotted with yolk antibodies (YP170, YP88, and YP170) detects the presence of yolk proteins (J. Richards and D. Greenstein unpublished results). Several bands are detected suggesting multiple forms of the yolk proteins exist. These proteins are no longer detected when RNAi is performed against the vitellogenin genes encoding these yolk proteins (Vit 6 encodes YP170 and YP88, and Vit 5 encodes YP170). Marker is shown representing kiladaltons.

fertilization ability. Second, the observation that MSP release does not seem to occur in male animals again suggests that MSP release is not the default state but rather must be initiated. Further, male spermatids are quiescent when isolated *in vitro* unless activated by female animal extracts. The observation that quiescent spermatids can be induced to form blebs and rearrange cytoplasmic MSP suggests a release mechanism has been activated. Taken together these observations suggest that release of MSP from sperm may be dependent upon an initiating signal derived from the female animal.

Several lines of evidence rule out an alternative explanation that our *in vitro* assay is measuring an activity other than MSP release. First, if the change in MSP localization were due to the process of spermiogenesis, then a pseudopod would be detected in our assay. This is not the case as pseudopod formation is rarely detected (<5%)within this assay. Since it is known that pseudopod formation can occur in less than 4 minutes our assay would detect them if spermiogenesis were activated (REF). Finally, our purification allows us to separate the low spermiogenesis activity we see in our crude extract, suggesting the activity we are assaying is distinguishable from activity that leads to the formation of a pseudopod.

Yolk protein as an initiator of MSP release

Our results suggest that yolk proteins or components of the yolk complex may be a signal to initiate MSP release from sperm. Co-fractionation of our activity with the yolk complex coupled or results using *rme-2 (bl008)* mutants, suggests this may be the case. Yolk proteins are synthesized within the intestine and secreted into the body cavity (Kimble and Sharrock, 1983). From the body cavity yolk is endocytosised via the RME-

2 receptor by growing oocytes (Grant et al., 1999). Animals in which the RME-2 receptor is mutated accumulate large amounts of yolk proteins within the body cavity. Thus sperm in these animals encounter much more yolk proteins than normal. As a consequence sperm may be constantly signaled to release MSP. We hypothesize that only a fraction of MSP is destined for release from spermatozoa, which would allow enough MSP to remain for locomotion. Excess amount of yolk encountered by sperm raise the possibility that all cytoplasmic MSP destined for release may be released all at once instead of over the life of the sperm. This situation would quickly deplete MSP and thus poison sperm. This hypothesis may explain several phenotypes observed from *rme-2 (bl008)* animals. *rme-2 (bl008)* is slightly egg laying defective, yields an extremely low brood (average brood size is 22 animals) and oocytes often appear stacked (Julia Richards and D. Greenstein unpublished results). Interestingly we have noted their brood size slightly increases as food becomes depleted on the plates. As food becomes exhausted the progeny laid will encounter a lack of food and produce less yolk. Consequently, if these progeny animals synthesize less yolk their sperm should not be overwhelmed by a release signal and sperm will signal over a longer time thus generating a larger brood size. The phenotypes of *rme-2 (bl008)* mutants have not been completely characterized, however preliminary observations suggest that the large amounts of yolk in these animals may act to poison their sperm, altering MSP release.

Interestingly, yolk proteins are detected in fractions that do not contain activity. The simplest, but not favored explanation is that yolk proteins do not constitute the MSP release signal. One explanation for this may be due to oxidization. Lipids may become oxidized during extract formation thus altering their composition and possibly their

ability to signal sperm. It is possible that ideal purification conditions have not been

established resulting in a portion of lipids that become oxidized during preparation. If

this is the case then pools of lipids might exist and only one may signal. Alternatively,

perhaps within the organism conditions exist, creating different oxidizing environments

again resulting in pools of lipids able to signal and those unable to signal. Lipid analysis

by mass spectrometry will be able to address questions such as these.

Environmental influence

It has long been established that fertility is tied to nutrition. Many eukaryotes,

including flies, become sterile in response to caloric restriction (Goberdham and Wilson

2003). *Drosophila* egg production can vary by as much as 60-fold in response to

nutrition (Drummond-Barbosa and Spradling, 2003). In *Drosophila* yolk proteins are

synthesized in the fat body and the ovary when the brain releases juvenile hormone in

response to environmental cues. Similarly *C. elegans* yolk proteins are synthesized in the

intestine in response to nutritional input. Yolk proteins are abundant proteins in

developing embryos, and perhaps the most abundant proteins in *C. elegans* embryos

(Sharrock, 1996). The lipids and proteins derived from yolk are thought to provide

essential nutrients required to support the rapid growth and development of embryos.

Thus the biology of yolk initiating MSP release, ultimately leading to fertilization and

progeny suggest a highly regulated mechanism to ensure the success of newly generated

offspring. If unfavorable environmental conditions were encountered newly formed

embryos might be compromised in their reserved yolk supply and thus might not be

viable. In contrast favorable environmental conditions might ensure successful

propagation. Reproduction of the worm is one of its most important jobs thus signals

regulating this mechanisms are most likely redundant therefore we are currently trying to

identify other signals initiating MSP release.

Future directions

Although these studies are not complete they offer a foundation into the initial

steps of the mechanism governing MSP release from sperm. If yolk or components of the

yolk protein complex initiate MSP release from spermatozoa then we should be able to

immuno-deplete extracts with available antibodies and show a reduced or lack of MSP

release *in vitro*. Further we can utilize RNAi to knock down yolk production causing

sperm to reduce the amount of MSP released *in vivo*. Such a strategy would assist in

performing genetic screens to identify other MSP release signals as well as other

pathways that might regulate this process. In addition we may be able to produce yolk

proteins by over expressing the vitellogenin genes in genetic backgrounds where yolk is

absent, such as in males. Overproduction of vitellogenins may cause release of MSP in

males where it is normally not seen, thus showing yolk proteins are sufficient to cause

MSP release.

The development of an *in vitro* MSP release assay would facilitate a greater

understanding of the MSP release process in a controlled environment while several

factors could be assessed such as temperature, pH, and metabolic requirements. In

addition and *in vitro* assay recapitulating MSP release *in vivo* would allow the isolation of

large quantities of MSP vesicles. Protein components of these vesicles could then be

ascertained. Studies as these create a platform upon which the mechanism governing

MSP release will be elucidated.

CHAPTER IV

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Summary

Previously it was known that *C. elegans* oocyte meiotic maturation and ovulation require the presence of sperm in the reproductive tract (McCarter et al., 1999). Recently, the cytoskeletal protein MSP was shown to be the sperm derived signal triggering oocyte meiotic maturation and gonadal sheath cell contraction over a distance in *C. elegans* (Miller et al., 2001). These findings resulted in the intriguing question as to how MSP is released from sperm. The lack of a recognizable signal sequence coupled with the lack of traditional secretory organelles in nematode sperm eliminated a standard secretion mechanism. I have generated evidence suggesting that release of MSP from sperm occurs by the budding of novel vesicles (Fig 18). These vesicles are likely signaling intermediates used to deliver MSP to distant oocytes and are predicted to be labile. Both spermatozoa and spermatids bud MSP vesicles however their stability and signaling potencies differ. Spermatozoa generate a long-range, short acting signal, whereas spermatids generate a long acting signal. Our EM results suggest that vesicle stability affects their physical and temporal range of signaling. We hypothesize that the MSP vesicle release mechanism is in itself signal dependent, and that the signals initiating the release of MSP are derived from the female animal (Fig. 18).

Inactive spermatid

Female signal

MSP relocalizes forming protrusion

Protrusions bend encapsulating extracellular space

Formed vesicles are released and travel to oocyte surface
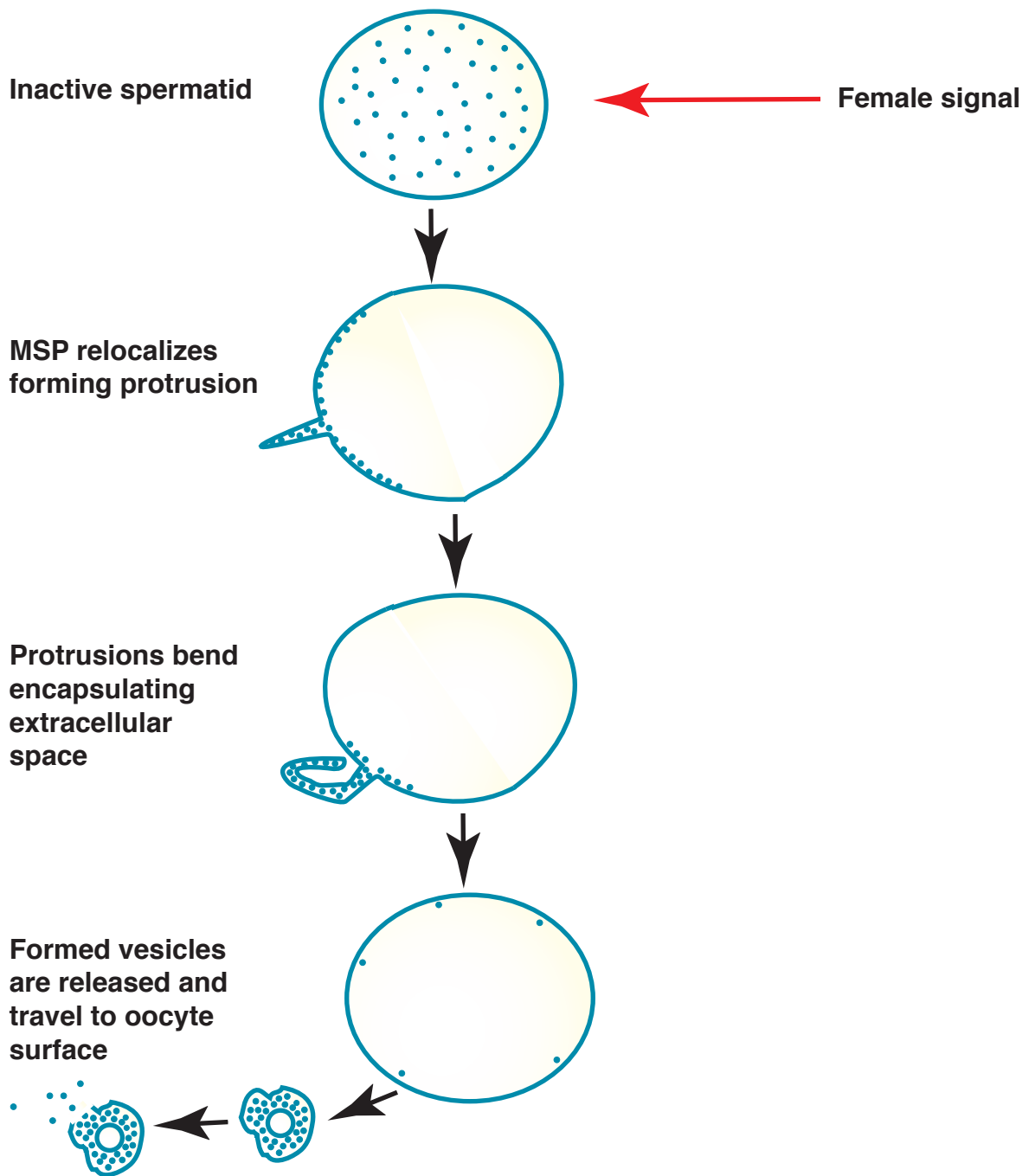
Figure 18. Model of MSP release mechanism. Inactive spermatids contain MSP throughout the cytoplasm. Sperm recieve a signal for the female reproductive tract that triggers sperm to release MSP. In responce to the female signal, MSP localizes to the plasma membrane and forms protrusions. These protrusion bend around encapsulating the extracellular space with in the inner most portion. Vesicles then pinch off forming free MSP vesicles which eventually break open releasing MSP.

Mechanism of Vesicle Budding For Delivering Signals

While the precise mechanism of MSP vesicle budding remains to be determined, our observations and budding model are consistent with many properties displayed by MSP. MSP polymers associate forming filaments that drive protrusion at the leading edge of the nematode pseudopod (Italiano et al., 1996). *In vivo* cell free extracts generate MSP filaments with the addition of MSP, ATP, a pH sensitive soluble factor and a membrane protein suggesting that a relatively simple mechanism yields MSP filament formation (Italiano et al., 1996). We have detected MSP localization along the plasma membrane and in some cases, under high magnification, MSP polymers may be distinguishable at the plasma membrane. We also observed several protrusions from the cell body plasma membrane containing MSP. Thus it seems reasonable that MSP filaments may provide the protrusive force needed to drive membrane extension generating the first step in the formation of an MSP vesicle. Furthermore, MSP filaments are described as flexible and thus able to bend (Italiano et al., 1996; Bottino et al., 2002). This MSP characteristic would permit a membrane protrusion to bend around upon itself capturing extracellular space and forming a double membrane vesicle (Fig. 18).

Our model predicts that the innermost area in the MSP vesicle is derived from extracellular space. The electron density of this inner area appears to be comparable to that of the extracellular space. Further, we consistently observe particulate material within the inner core of MSP vesicles generated in *spe-8(hc50)* (Fig. 13). In *spe-8(hc50)* animals oocytes are not fertilized and often break apart spilling their cytoplasmic contents into the spermatheca lumen. As a result the spermathecal lumen contains an abundance

of particulate material allowing MSP vesicles generated in this space to encapsulate this material.

Our model of a vesicle budding mechanism is in fact analogous to other forms of non-canonical vesicular release such as ectocytosis (Fig. 5). This mechanism of membrane shedding has striking similarities to MSP release including protein concentration at the plasma membrane, membrane protrusion, release within vesicles, and vesicle lability. Proteins such as annexin-I, galectin-1, and galectin-3 have been shown to be released from cells by this mechanism (Hughes et al., 1999; Sato et al., 1993). Thus vesicle budding as a mechanism for protein secretion may be more widespread than currently known.

C. elegans sperm are highly specialized cells and have disposed of common secretion components in meiosis II. Many other specialized cell types including platelets, T-lymphocytes, mammary gland cells and sweat glands also rely on vesicle budding or other non-conventional protein secretion mechanisms to release proteins. It is relatively unclear as to why some proteins are targeted for release by unconventional mechanisms such as budding. One obvious explanation is that specialized cells have evolved other mechanisms and do to the lack of standard secretion components. Alternatively, perhaps secreted proteins that are essential employ redundant secretion mechanisms ensuring protein secretion. If this were the case then one would predict that MSP may be released through the classical secretory machinery if it were localized in a cells containing an ER and Golgi.

Our current data suggest at least two distinct stages of MSP vesicle signaling. The first is the budding stage, in which MSP vesicles form and are released from sperm.

The second stage is the vesicle disintegration stage, in which MSP vesicles travel to distant oocytes and are dissolved to release MSP allowing it to bind VAB-1 Eph receptors on oocyte surfaces. Thus, we predict MSP vesicles are likely intermediates allowing the MSP hormone to be delivered to distant oocytes. Currently we do not understand the mechanisms facilitating MSP vesicle movement and the speed of this movement compared to the half-life of the vesicles. Our observations of extracellular MSP gradients beginning near the sperm, and then decreasing in a gradient fashion toward the oocytes (Fig. 7), suggest that extracellular MSP within the spermatheca travels directionally toward oocytes. Interestingly, we always observe these gradients in this direction and never in the opposite direction, in the diffusible space towards the uterus. The lumenal extracellular space lacks cytoskeletal elements, which typically facilitate classical vesicular movements. Passive diffusion is unlikely to account for MSP vesicle movement due to the lability and relatively short half-life of MSP. Thus a relatively quick mode of transportation is probably responsible for vesicle movement. In addition Brownian movement, the random movement of microscopic particles occurring through collisions, would not exhibit the directionality demonstrated by extracellular MSP in the spermatheca. Perhaps gonadal sheath cell contraction facilitates MSP vesicle movement. The sequential contractions may create a force facilitating movement of MSP vesicles into the gonad arm.

The mechanisms governing vesicle stability are currently unclear. One possibility is that the composition of the vesicles themselves may contribute to their stability. The membrane composition of spermatozoa differs from that of spermatids due to the membranous organelle (MO) fusion process. During spermiogenesis MOs fuse with the

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plasma membrane and release their contents onto the cell surface. In contrast spermatids have not undergone spermiogenesis and have unfused MOs and therefore the contents of their cell surface differs from that of spermatozoa (L' Hernault et al., 1997; Xu and Sternberg, 2003). Alternatively, the environment vesicles are released within (i.e. the spermatheca, gonad arm, and uterus) may determine MSP vesicle stability. MSP vesicles released from spermatids likely remain within the gonad arm whereas MSP vesicles released from spermatozoa can be found in the spermatheca and the uterus. Many characteristics such as pH, osmotic conditions, proteases, and lipases of the lumens of these spaces are unknown. However it is reasonable to assume that these different environments may possess different characteristics and proteins contributing to vesicle stability.

Our data suggest that vesicle stability provides a basis for signaling potency. This model fits the biology of our system. Spermatozoa are localized at a distance from oocytes. Unlike many other systems spermatozoa are unable to approach oocytes due to a distal constriction within the spermatheca. Thus MSP vesicles provide a means for securely delivering the MSP hormone to distant oocytes. Once at the oocyte or in closer proximity to the oocyte the MSP hormone must be released quickly from the vesicles, requiring these vesicles to be somewhat labile in nature. In contrast to the long-range labile vesicles generated by spermatozoa, spermatids need to produce a local, more stable vesicle. In hermaphrodites spermatids are produced first before a switch to oogenesis production occurs, consequently spermatids are located in close proximity to developing oocytes. Although spermatids are adjacent to oocytes, the oocytes are not competent to receive the MSP signal yet. Therefore the MSP signal released form spermatids must

persist allowing young oocytes time to complete development and become competent to receive the MSP signal. Once oocytes gain competency, fertilization can occur quickly without any the delay of spermatids developing into spermatozoa. In addition, oocytes in hermaphrodite animals need to be exposed to the MSP signal prior to the first ovulation, since the first ovulation pushes spermatids into the spermatheca, where they undergo spermiogenesis and become spermatozoa. If spermatids did not signal then the first meiotic maturation and ovulation would not occur. Consequently, spermatids would remain in the gonad arm unable to enter the spermatheca and become spermatozoa capable of signaling. This persistent signal must eventually end otherwise after spermatozoa become depleted meiotic maturation would continue and oocytes would be ovulated in the absence of fertilization, essentially wasting valuable oocytes. Mechanisms governing the abolishment of this signal are unknown but perhaps the presence of spermatozoa facilitates the breakdown or removal of the perduring spermatid signal.

Dual Functions of MSP: Signaling vs. Motility

The dual functions of MSP are intriguing because they are both essential in the reproductive process of *C. elegans*. The motility function of MSP allows spermatozoa to move, without this ability spermatozoa are incapable of fertilization and thus propagation of the species would be halted. This phenotype is demonstrated in *spe-8 (hc50)*. The block in spermiogenesis inhibits *spe-8 (hc50)* spermatids from generating a pseudopod rendering the spermatids non motile. Consequently these spermatids are rapidly cleared from the gonad arm and animals are sterile. Conversely, the signaling function of MSP

facilitates oocyte meiotic maturation, an essential process in reproduction. In the absence of the MSP signal, oocyte meiotic maturation does not occur and animals are sterile. *fog-2 (q70)* female animals, which lack sperm, possess this phenotype. Mutants displaying this sterile phenotype but contain viable sperm have yet to be identified.

An interesting question arises as to why a single protein would possess two critical functions required for the perpetuation of the species. Perhaps the worm has safeguarded this protein, and its important functions, by creating a large multi-gene family (>30 in *C. elegans*). Thus a mutation in one MSP has little effect upon either function. This argument has little weight when one considers that *Ascaris* only contains two MSP genes yet MSPs dual functions are likely conserved. The male female species of *Ascaris* is thought to be ancesteral to the hermaphrodite species of *C. elegans*, thus it is likely that when hermaphrotism evolved MSP genes were duplicated. Several MSP genes would allow the hermaphrodites to produce sperm quickly. An alternative hypothesis as to why a single protein would be required for two critical functions might be due to the streamlined nature of spermatozoa. Spermatozoa delete all unnecessary components not required for fertilization and thus become very efficient, sleek cells. Perhaps condensing two essential functions required for spermatozoa, motility and signaling, into one protein, MSP, is another example as to how spermatozoa become compact and highly efficient cells. A single protein with two distinct functions enables spermatozoa to package only one protein rather than two, leading to efficiency.

The dual functions of MSP coupled with the fact that sperm possess a limited amount of MSP, owing to the lack of protein translation in mature sperm cells, suggests that MSP release must be tightly regulated. If MSP release was not highly regulated and

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an excess of MSP was released, sperm motility would quickly become compromised hindering their ability to fertilize oocytes. Our data support the hypothesis that MSP release is regulated and only occurs when signaled. Male animals or spermatids isolated *in vitro* do not appear to release MSP, suggesting they must receive a signal triggering MSP release.

Signals triggering MSP release may likely be regulated by environmental conditions. Signals of this nature would help ensure successful fertilization and zygotic development. For example, under certain conditions such as the presence of healthy and competent oocytes, fertilization is likely to be successful. In addition under favorable environmental conditions embryonic offspring are more likely to succeed. For example, if food is plentiful, oocytes will be packed full of necessary nutrients to sustain the embryonic divisions and zygotic development. Thus if signals were linked to nutrition then a sensing mechanism would evaluate whether or not conditions are favorable for procreation. Many animals have adapted a similar type of strategy to regulate their reproduction. As a result these animals become infertile when calorically restricted. A similar type of mechanism is even employed by humans, as underweight women often do not menstruate. I have proposed that *C. elegans* employs a similar type of mechanism. In *C. elegans,* I hypothesize that the environmental conditions or the nutritional input regulates the amount of yolk protein generated, in turn triggering MSP release. If adequate nutrition is lacking then the yolk protein levels diminish and MSP is not released form spermatozoa resulting in the absence of oocytes maturation and fertilization. Thus it is possible that signals regulating the reproductive process in *C.*

elegans may be well tuned with the environment preventing oocytes maturation in the absence of ideal conditions.

MSP Conservation and Functions

MSP tertiary structure is that of an immunoglobulin-like fold that has been conserved throughout eukaryotic evolution (Bullock et al., 1996; Baker et al., 2002). MSPs are highly conserved in nematodes functioning as both cytoskeletal and signaling proteins. In addition proteins containing MSP domains, called VAMP-associated proteins (VAPs), are found in fungi, plants and animals. The first function of VAPs arose in Aplysia where they were initially identified as an interactor with the membrane fusion protein synaptobrevin/VAMP. Currently, VAPs have been characterized to have highly diverse functions and have been implicated in multiple roles including neurotransmitter release and vesicle transport. The overall structural organization of VAPs is that of C-terminal transmembrane domain linked by a variable region to an NH2 terminal MSP domain. It is possible that MSP domains within VAPs may facilitate a task similar to that of nematode MSP, either a cytoskeletal or a signaling function. A cytoskeletal function has been implicated in *Drosophila* DVAP-33A, which functions at the neuromuscular junction regulating synaptic bouton budding in a dose-dependent manner by the stabilization of the microtubule cytoskeleton (Pennetta et al., 2002). This role requires DVAP-33A to function as a bridge coordinating the interactions of the cell membrane and the microtubule cytoskeleton during bouton formation. Interestingly, DVAP-33A is located post-synaptically and could facilitate bouton formation by signaling between pre and postsynaptic sites at the neuromuscular junction (Pennetta et al., 2002).

Recently another link between VAP and human neurons has been established. A mutation in the MSP domain of VAPB was shown to cause spinal muscular atrophy and amyolateral sclerosis type 8 in humans (Nishimura et al., 2004). VAPB has been shown to function during ER-Golgi transport and secretion (Soussan et al., 1999). Perhaps mutations in the MSP domain disrupt these functions leading to the accumulation of transport intermediates such as cytosolic membranous aggregates thereby affecting vesicular transport. Additionally, VAPB is localized on membrane structures different from the ER and Golgi organelles and may facilitate the accumulation of proteins at these sites. VAPA and VAPB can form multimeric protein complexes (Soussan et al., 1999; Foster et al., 2000). VAPA has recently been shown to act as a membrane receptor for lipid and sterol binding proteins (Weir et al., 1998; Wyles et al., 2002). Many lipid binding proteins access the ER by the short FFAT motif that directly binds to integral ER VAP protein. Recently it was demonstrated that mutations within the MSP domain of the yeast VAP homolog SCS2 disrupted binding between the FFAT motif and SCS2 affecting the composition of intercellular membranes (Loewen et al., 2003; Loewen and Levine, 2005). These results suggest MSP domains may function in a structural, docking role. Taken together these recent reports suggest that dual functions for MSP domains may not be limited to nematodes but in fact may be more widespread.

Future Directions

These studies have begun to examine signaling events between sperm cells and oocytes required for successful fertilization in the *C. elegans*. Signaling between gametes is complex and extensive thus these studies have just begun to uncover the interplay

occurring between these cells. Our discovery of the mechanism in which sperm bud

vesicles to deliver MSP to distant oocytes generates several exciting questions. Further

understanding this mechanism will require identification of molecular components and

pathways regulating this process. These studies have generated a platform upon which

we can build upon to ultimately understand gametic communication.

A budding mechanism

Our data is comprised of static images of the MSP vesicle budding process.

While these images provide a compelling hypothesis of MSP vesicle budding further

insight and a greater understanding will be generated from live cell imaging. Using vital

dyes we hope to label sperm membranes and visualize the MSP vesicle budding process

in vivo. We would also like to label MSP with GFP protein fusions and visualize the

MSP budding process. Our model predicts that MSP vesicles bud from sperm and are

labile signaling intermediates. Real-time analysis will allow a direct assessment of this

model. Further, we will learn the dynamics, half-life, and spatial regulation of the

vesicles as well as visualize the dynamics of the sperm plasma membrane during the

budding process. In addition visualization of the budding process *in vivo* will allow us to

directly test whether the MSP export signal, yolk, is sufficient to trigger MSP release in

male animals. Currently our data suggests that MSP release does not occur in male

animals. We hypothesize that the signal initiating MSP release is at least in part the yolk

protein, which is not made in the intestine of female animals. Since MSP release does

not occur in males and yolk proteins are not synthesized in male animals, they will be an

ideal background to test whether or not the MSP export signal is sufficient to trigger MSP

release. Thus vesicle budding should occur following injection of the purified signal into males.

As mentioned in chapter III we have begun to develop an *in vitro* assay of MSP vesicle release. Membrane labeling with vital dyes to visualize the budding process *in vitro* will validate this assay. Our hypothesis is that the budding process is signal dependent. Thus by labeling the membranes of spermatids we should be able to initiate plasma membrane shedding in the form of vesicles following the addition of our purified signal. If MSP is released in a membrane-bound form *in vitro*, then the released material should exhibit biochemical properties characteristic of vesicles. For example, released MSP should be protease-resistant in the absence of detergents.

Once we have established that vesicles are generated *in vitro* we can further analyze vesicles by transmission and scanning electron microscopy. Transmission electron microscopy will provide definitive confirmation that MSP vesicles released *in vitro* are identical to MSP vesicles released *in vivo*. Scanning electron microscopy will allow a three dimensional view of the budding process. An *in vitro* assay recapitulating the *in vivo* process will allow us to test several parameters of the budding process such as pH, osmolarity, temperature, and energy requirements in a controlled environment. Ideally, we would like to block the budding process and demonstrate oocyte meiotic maturation does not occur when the budding process is blocked. Further, by blocking the budding process we should be able to identify more budding intermediates using electron microscopy, to further understand the budding mechanism. These studies will further our understanding of the mechanism and the requirements of the MSP vesicle budding process.

Vesicle isolation

Once validated, our *in vitro* assay will allow us to isolate large quantities of MSP vesicles. Mass spectrometry analysis will allow us to understand what proteins constitute the MSP vesicles. It is reasonable to assume proteins required in the formation of MSP vesicles and proteins required for vesicle release will be associated with the MSP vesicles. Once components are identified, mutational analysis may allow us to generate pathways utilized in the MSP vesicle budding process. Ideally, we would like to block MSP release thus directly providing evidence that the MSP vesicle budding process is both necessary and sufficient to cause oocyte meiotic maturation.

Several questions remain concerning vesicle stability. Our model predicts that vesicles are labile and thus break to release their MSP contents. What facilitates this vesicle breakage remains to be determined. It is possible that components of the vesicle are responsible but it may also be the environmental conditions surrounding the vesicle. Thus, to learn more about the factors leading to vesicle stability, we need to understand more about the environment they are formed or released in. Conditions such as osmolarity and pH may be determined by injecting sensing dyes. Gross differences between these environments may shed light upon mechanisms governing vesicle stability. Using our *in vitro* assay we could then test these parameters on vesicles *in vitro*.

Signals initiating MSP vesicle release

Our hypothesis is that the MSP vesicle budding process is signal dependent and that the yolk protein or components of the yolk complex are part of this signal. Due to the importance of initiating sperm to release MSP we hypothesize that more than one

pathway regulates this process. This redundancy would mean that multiple female signals trigger MSP vesicle release. By using RNA mediated interferance (RNAi) against the vitellogenin genes we hope to knockdown the yolk protein production, creating a sensitized background to perform genetic screens to identify other signals and components of the signaling pathways initiating MSP vesicle export. If we can identify the pathways that regulate MSP release we should be able to generate double mutations and block the MSP vesicle export from sperm.

We hypothesize that MSP release is in part regulated by the yolk protein and thus may be linked to the nutrition of the animal. If this is true then we should be able to inhibit the nutritional input and affect MSP release. This might be possible to test by shifting wild-type worms to an inedible food source and measure whether MSP release and fertilization can occur. Along those lines, *eat* mutants display eating abnormalities due to pharyngeal pumping defects and thus double mutants could be constructed and assayed for MSP release (Avery, 1993).

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