GENES REQUIRED FOR THE OOCYTE-TO-EMBRYO TRANSITION IN *C. ELEGANS*

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ABSTRACT OF THE DISSERTATION

Genes Required for the Oocyte-to-Embryo Transition in *C. elegans*

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The reproduction of a genetically distinct organism is a delicate process, and requires, in most organisms, the faithful reproduction of a male and female gamete, which must then find one another and fuse, and then undergo the transition to developing embryo. This thesis is an examination of the molecules required for fertilization in *C. elegans*, emphasizing the molecules involved in the oocyte-to-embryo transition.

The first chapter provides an introduction to the importance of fertility research, the general processes of fertilization with an emphasis on mammalian reproduction, the utility of *C. elegans* as a model organism for studying fertilization, and the basics of reproduction in *C. elegans*.

The second chapter discusses the general processes of the oocyte-to-embryo transition in *C. elegans*, then outlines a module of molecules, which are known to act during this process. Particular focus is given to the elucidation of the *egg-4/5*

genes, which are required for coupling the oocyte-to-embryo transition to the advancement of the cell cycle. The EGG-4/5 proteins represent a new class of molecules identified as protein-tyrosine phosphatase-like, and participate in a novel method of kinase regulation.

The third chapter introduces the importance of the cortical granule exocytosis during the oocyte-to-embryo transition, and later to embryogenesis. A potential new member of the cortical granules is described, *egg-6*, a *C. elegans* gene which had been identified in previous works as being necessary to osmotic integrity. Early molecular work may link the *egg-6* gene to the establishment of polarity in the embryo.

The fourth chapter outlines the phenotypic and partial molecular characterization of a gene required for spermiogenesis, *spe-43.* The process of spermiogenesis in *C. elegans* is not only critical to fertility, but represents a model both for differentiation without *de novo* protein synthesis, and for the evolutionary development of an androdioecious species from a gonochoristic one.

The fifth chapter provides summary discussion the molecules discussed, and their roles in reproduction, with a particular focus on the oocyte-to-embryo transition.

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Chapter 1

An Introduction to Reproduction and the Model Organism *C. elegans*

1.1.1 The importance of fertility research in applications for Reproductive Technology

The reproduction of a genetically new organism requires, in most species, the production of two haploid gametes, which must then find and fuse with one another, and then combine into a new and separate organism. The intricacies of this process are vast. Each step must be executed exactly, from the faithful segregation and packaging of parental DNA, to the initial division of the new organism.

The applications of reproductive research are numerous. Assisted Reproductive Technologies (ART) have accounted for an approximate 3.5 million births since 1978 (Chambers et al., 2009). Techniques used have included ovarian stimulation, intracytoplasmic injection of sperm, and in vitro maturation of oocytes. The costs of this industry are comparably vast; where the average cost in the US of the commonly used In Vitro Fertilization Technique is $12,513 per cycle (Chambers et al., 2009).

Conversely, the need for new contraceptive technologies remains high, particularly in developing nations. Between the years 1995-2000 over 700,000 maternal deaths occurred and more than half of those deaths were linked to unsafe abortions (Aitken et al., 2008). While an estimated 42 million abortions took place in 2009, nearly half of those, some 20 million were performed unsafely

(Shah and Ahman, 2009). Given the high impact of unwanted pregnancy in terms of mortality and financial burden, the development of new contraceptive technologies remains a high priority for many nations (2005; Aitken et al., 2008).

Within the process of reproduction, the requirements for precision and coordination are strict. Correct segregation of the chromosomes is critical not just in the formation of the male and female gametes, but in subsequent cellular divisions of the embryo (Ambartsumyan and Clark, 2008). A mutation or chromosomal abnormality occurring early in embryonic development has the potential to create a mosaic of defective cells. It is estimated that spontaneous miscarriage occurs during 10% of all human pregnancies (Ambartsumyan and Clark 2008). In addition, 20% of all birth defects are linked to gene mutations and chromosomal abnormalities. The quality of the oocyte has also been found to decrease with time, vastly increasing the risks of genetic aberration (Menken et al., 1986).

1.1.2 The process of forming the gametes

The process of forming the female gamete is referred to as oogenesis. In humans, this process occurs early in development with primordial germ cells having developed mature oogonia and primary oocytes by the 25th week of fetal development (Jamnongjit and Hammes, 2005). A primordial follicle derives from epithelial cells to surround the primordial oocyte, and in this niche the oocyte

begins its first meiotic division before arresting in diakinesis of prophase (Jamnongjit and Hammes 2005). This first arrest point is common to most organisms from other mammals to *C. elegans*. In contrast, the chemical signals which maintain the prophase arrest of oocytes vary greatly by organism. In humans the overlying ovary is necessary, as are heightened levels of cAMP (cyclic AMP) deriving from the ovary (Jamnongjit and Hammes 2005).

Maintenance of human oocytes at this stage is critical to reproductive success, as the oocytes can remain static for decades before maturation and fertilization occur (Menken 1986). During their prolonged diakinetic arrest, oocytes accumulate macromolecules and increase in size (Marteil et al., 2009). At this stage, oocytes are surrounded and supported by the follicle cells of the somatic germline. In mammals, the follicles are comprised of three primary layers, the outer theca cells which produce steroids, then the mural granulosa and cumulus granulosa which connect to the oocytes via gap junctions (Jamnongjit and Hammes, 2005; Marteil et al., 2009).

When a mammalian oocyte is selected for maturation, Luteinizing Hormone (LH) is released to stimulate the outer theca and mural granulosa cells (Jamnongjit and Hammes 2005, Marteil et al., 2009). This event triggers dissociation of the cumulus granulosa from the oocyte, allowing the oocytes final maturation process to begin (Jamnongjit and Hammes 2005). Both the cytoplasm and nucleus of the oocyte need to undergo separate maturation processes (Marteil et

al., 2009). In many species including humans and *C. elegans* the maturation process includes Nuclear Envelope Breakdown (NEBD), cytoskeletal rearrangement, and resumption of meiosis (Marteil et al., 2009). In mammals, the oocyte will be arrested a second time at metaphase II of meiosis II until fertilization occurs (Jamnongjit and Hammes 2005, Marteil et al., 2009).

Another common barrier to reproduction is a lack of functional sperm. It is estimated that 55% of couples unable to spontaneously conceive in one year are impeded by male fertility factors (De Kretser and Baker, 1999). This determination is made through analysis of the male semen, followed by classification of normazoospermia, oligozoospermia, or azoospermia (Visser and Repping, 2010). Several factors may contribute to a diagnosis of oligozoospermia or azoospermia, including but not limited to failures in finding the ovum, undergoing capacitation, undergoing the acrosome reaction, fusing with the ovum, or combining with the ovum to form the zygote (Sigman et al., 2009). However, the physical criteria used for determination of sperm quality, as set out by the World Health Organization (WHO) include measurements of pH, sperm concentration, sperm motility, sperm morphology, and sperm viability (Sigman et al., 2009). These judgments are based on broad criteria, and are widely considered inadequate for determination of male fertility levels (Guzick et al., 2001; Visser and Repping). The process of creating the male gamete is referred to as spermatogenesis, and at its base level requires the generation of a simple haploid cell capable of carrying out fertilization. The steps of sperm

development have been well studied in many organisms. However, the signaling pathways which are effectors for spermatogenesis have not been fully elucidated. The basic steps of spermatogenesis are conserved among many species including Homo sapiens, Mus musculus, Drosophila melanogaster, and *Caenorhabditis elegans*, and occur in the following general sequence (Chandley and Bateman, 1962; Gilbert, 2010; Lam et al., 1970; Ward et al., 1981). Sperm are derived from a dedicated pool of stem cells, which mature by undergoing meiosis I and II to form four haploid spermatids (Abou-Haila and Tulsiani, 2000; Ward et al., 1981). In the steps which follow meiosis, the round spermatid must differentiate into a specific shape and gain motility. Mammalian spermatids undergo the formation of an acrosome and an elongation process in the male testis. This maturation process is triggered by hormonal signaling to the sertoli cells, and subsequent activation of g-protein pathways (Allan 2006).

Motility is attained during spermiogenesis, the process of post-meiotic sperm differentiation, when a pair of centrioles is deposited onto the basal lamina of the nucleus of a post-meiotic spermatid (Li et al., 1998). The centrioles then nucleate the axoneme, a bundle of microtubules which comprise the flagella (Gilbert, 2010). While spermiogenesis takes place in the male reproductive tract in mammals, the spermatozoa undergo further maturation steps in the female reproductive tract in a process called capacitation. Capacitation has not been well defined, but is known to involve tyrosine phosphorylation and removal of

sterols from the plasma membrane of the spermatid (Travis and Kopf, 2002; Visconti et al., 1995).

1.1.3 Meeting and Merging in a Big World

After the formation of the male and female gametes comes perhaps the most daunting stage of fertilization. The interactions of sperm and oocytes vary greatly depending on species, encompassing a wide range of environments, and body systems. Sizes, and shapes of germ cells vary accordingly, from the familiar flagellate sperm of mammals, to the amoeboid sperm of the *C. elegans*. Surface molecules on both sperm and egg must be responsible allowing the fusion of the two germ cells. In mammals, the oocyte secretes a zona pellucida, which contains three glycoproteins, ZP1, ZP2, and ZP3 (Bleil and Wassarman, 1980). It is widely considered that one or more components associated with ZP3 are responsible for binding to sperm. It has recently been shown that a zona pellucida associated protein, oviduct-specific glycoprotein (OGP), is necessary for binding of sperm to the oocyte (Lyng and Shur, 2009). It has also been hypothesized that OGP is secreted from the oviduct and absorbed by the zona pellucida (Lyng and Shur, 2009). The corresponding binding factor or factors on the sperm surface have not been fully identified; however, the β-1, 4-galactosyltransferase protein has been shown to bind to ZP3, and trigger the acrosome reaction (Shur, 2008). After the fertilization/fusion event occurs between the male and female gametes, the new embryo must undergo dramatic

restructuring. A barrier must be rapidly constructed around the newly fertilized embryo to prevent penetration of more than one sperm cell, called the block to polyspermy. Mechanisms within the embryo must sense the fertilization event, and trigger the oocyte-to-embryo transition, which includes a myriad of cellular events including resumption of and completion of meiosis, cytoskeletal rearrangements, calcium influx, and cortical granule exocytosis. Though the processes of the oocyte-to-embryo transition are critical for the transition from maternal programming and proteins, to embryonic programming and proteins, little is known about the molecular nature of the process.

1.2.1 The Importance of *C. elegans* as a model organism

The processes of gamete formation, fertilization, and embryonic development are often difficult to examine in humans and higher mammals, which have long reproductive cycles, and comparably few young. During the 1960's, Dr. Sydney Brenner, Nobel Laureate, began searching for a new model organism on which to conduct molecular biological research. He put forward the free-living, non-parasitic nematode, *Caenorhabditis elegans* as an ideal model (Brenner, 1974). The worms are tiny, only 1mm fully grown, reproductively fecund, producing approximately 300 progeny (Figure 1). Their life cycle progresses quickly, from hatching to reproductively mature adult in 3.5 days at 20ºC. They are simple to culture in bulk as they feed on E. coli bacteria and so can be colonized on self-contained Petri dishes. They are highly sensitive to temperature transitions, and

can be grown at lower temperatures to slow their growth or higher temperatures to speed their growth. Temperature sensitive mutations may also be isolated, at which a defect is present at one temperature, but the phenotype does not present at another temperature, allowing for simple isolation and propagation of mutations, which might otherwise be lethal. They possess a transparent cuticle that allows live imaging of their internal processes, through both light and fluorescent microscopy. Transgenic animals are relatively easy to produce, by injection of DNA into the germline, by use of the MOS transposon recombination system, or through microparticle bombardment that can be successfully used to generate arrays (Granger et al., 2004; Kadandale et al., 2009; Praitis, 2006). By this method, gfp and rfp tagged proteins can be introduced and expressed under the direction of either their own, or a tissue specific promoter. Quite uniquely, the lineage of every somatic cell in the organism, both male and hermaphrodite, can be traced from fertilization to adult (Sulston, 1977). In addition, *C. elegans* represents the first multicellular organism to have a completely sequenced genome, and now the genomes of several other species of Caenorhabditis and other nematodes have subsequently been published, making identification and study of orthologues easy (Coghlan et al., 2006; Sulston, 1977). Much pioneering work has been completed in the humble *C. elegans*, including seminal work in apoptosis and cell death, RNAi (RNA mediated interference), and fluorescent protein tagging (GFP) (Chalfie, 1994; Fire, 1998; Hedgecock, 1983). *C. elegans* also present an excellent model for the examination of mitosis and meiosis (Greenstein, 2005; Kimble J, 2005; L'Hernault, 2006).

C. elegans presents an excellent model organism for parsing the molecular underpinnings of the processes of fertilization. Every stage from undifferentiated stem cell, to fertilization competent egg, to developed embryo can be viewed progressively in vivo under light or fluorescence microscopy (Figure 2). Many fluorescent and antigenic markers are available for cellular compartments and components, including nuclear, plasma membrance, golgi, and endosomal (Hadwiger et al., 2010; Sato et al., 2006). Genetic deletion mutants are available for study, in addition to the ease of RNAi in the germline. A primary strength of RNAi in the *C. elegans* germline is the ability to perform RNAi on larval stage hermaphrodites, knocking down their maternal complement of protein, and examining their germline function at adult stages. This allows for the investigation of many genes that would otherwise be embryonic lethal.

In addition to carrying out the same basic developmental processes as mammals and other organisms during the oocyte-to-embryo transition, *C. elegans* undergo the following processes resumption of meiosis, cytoskeletal rearrangement, ovulation, fertilization, the block to polyspermy, completion of meiosis including the extrusion of two polar bodies, extrusion of a chitinous, trilamellar eggshell, formation of a female and male pronucleus, fusion of the two pronuclei, and the initiation of the mitotic divisions (Figure 3) (McNally and McNally, 2005).

1.2.2 *C. elegans* Reproductive Tract and Gametes

As a species, *C. elegans* exist primarily as hermaphrodites, with males arising approximately 1 in 1000 births from homeiotic non-disjunction. Hermaphrodites are self-fertile, while males produce only sperm and are capable of fertilizing hermaphrodites to produce outcross progeny (Figure 1) (Ward and Carrel, 1979). The hermaphrodite germline fills the body cavity in a bi-lobed tube. The germline begins with two DTC's (distal tip cells) one on the anterior and posterior dorsal axis (Figure 1, 2) (Austin and Kimble, 1987). The DTC's are a niche for the germ cells, which proliferate from dedicated progenitor stem cells (Kimble J, 2005). Distal germ cells are maintained at mitosis by LAG-2, a protein ligand secreted by the DTC. Binding of LAG-2 to GLP-1, the corresponding receptor on germ cells corresponds to a signaling cascade, which affects mRNA stability in the early germ cells, and prevents the transition from mitosis to meiosis (Hubbard and Greenstein, 2000; Kimble J, 2005). Regulation of cell cycle in the *C. elegans* germ cells is necessarily precise, and can be clearly visualized by DNA staining in fixed specimens, or by live imaging of fluorescent-tagged histones (Figure 2). Distal germ cells progress in syncitium with one another in an organ called the rachis. As germ cell nuclei begin to differentiate, the germ cells begin to enter prophase of meiosis I in the transition zone (Hubbard and Greenstein, 2000; Kimble J, 2005). Well-defined regions of the rachis correspond sequentially to pachytene, diplotene, and diakinesis of prophase I (Figure 2) (Hubbard and Greenstein, 2000). The arrest of *C. elegans* germ cells in prophase corresponds to the arrest of mammalian oocytes at metaphase I. In this advancing manner,

C. elegans hermaphrodites produce first sperm during their last larval phase of development (L4), and then switch permanently to the production of oocytes. The spermatocytes are stored undifferentiated in each of two spermathecas, one corresponding to the anterior and posterior gonad arms (Figure 1) (L'Hernault, 2006). As oocytes approach the bend of the gonad arm they individuate from the rachis and begin to accumulate yolk (Figure 1).

The male of the species produces only sperm, beginning in its last larval stage. Male germ cells develop in a single gonad arm extending from a single distal tip cell to the seminal vesicle (Figure 1) (Klass et al., 1976). The male spermatids are stored unactivated, and introduced to the hermaphrodite through a mating spicule in the tail region. After their introduction through the hermaphrodite vulva, male sperm quickly out-compete hermaphroditic self-sperm for fertilization of the oocytes (Ward and Carrel, 1979).

In the *C. elegans* hermaphrodite the movement and development of sperm and oocytes are precisely coordinated, reflecting its existence as a primarily androdioecious species. Major sperm protein (MSP) is a core component of the sperm cytoskeleton; the protein is also secreted as a signal to both oocytes and the somatic germline (Harris et al., 2006; Kuwabara, 2003). As oocytes progress sequentially closer to the spermatheca, they are often referred to numerically by position with the oocyte closest to the spermatheca referred to as -1 (Figure 1). Oocytes receive the MSP signal from sperm such that the -1

oocyte is stimulated to undergo nuclear-envelope breakdown (NEBD) and cytoskeletal rearrangement in preparation for ovulation (Harris et al., 2006). The oocyte and sperm also signal to the overlying somatic sheath cells, a type of smooth muscle covering the oviduct, which dilate the spermathecal valve and force the spermatheca to engulf the -1 oocyte (Figure 1). Thus the -1 oocyte is ovulated, an event which occurs approximately every 23 minutes when sperm are abundant (McCarter et al., 1999; McNally and McNally, 2005). Hermaphrodites produce a fixed number of sperm during the L4 larval stage, in the absence of sperm the process of ovulation drops off dramatically occurring approximately once every ten hours (McCarter et al., 1999). However, introduction of sperm through fertilization by a male or artificial insemination causes resumption of robust ovulation rates and fertilization. Returning to our discussion of the cell cycle, the -1 oocyte undergoes NEBD and simultaneously reenters the meiotic cell cycle at metaphase I of meiosis I approximately five minutes before ovulation will occur (Figure 1) (McCarter et al., 1999; McNally KL, 2005).

The first ovulation event pushes the developed spermatids into the spermatheca, and presence of eggs passing through the spermatheca stimulates them to complete activation (Figure 1) (L'Hernault, 2006). As the subsequent oocytes enter the spermatheca they are surrounded by the amoeboid *C. elegans* spermatocytes. Molecules including spe-9 on the surface of the spermatozoa and egg-1 and egg-2 on the egg are necessary for sperm/egg interaction and

sperm entry (Kadandale et al., 2005; Putiri et al., 2004; Singson et al., 1998). When sperm are abundant, fertilization occurs quickly, and the newly fertilized embryo exits the spermatheca to complete embryogenesis.

The process of transferring from newly merged male and female gametes, to a single unified embryo, and from a maternally provided program and set of proteins to an embryonic program and set of proteins, is referred to collectively as the oocyte-to-embryo transition. In *C. elegans*, the process begins just prior to ovulation and fertilization. The MSP signal from sperm is received, and the oocyte rapidly undergoes several physical changes. The nuclear envelope breaks down, and the oocyte DNA reenters meiosis I at metaphase I (McCarter 1999) (Figure 1). In addition, the actin cytoskeleton rearranges, transitioning the oocyte from a cuboid shape to an ovoid one (Figure 1). The mature oocyte will enter the spermatheca where it will be fertilized by a single sperm, then exit to the uterus where is will continue to mature as an embryo (McCarter 1999). An increase in intracellular calcium will correspond to the release of the cortical granules to the cell surface. A chitin-containing, trilammelar eggshell will be secreted to provide support to the developing embryo (Johnston WL, 2006). In wildtype hermaphrodites, six pairs of sister chromatids, corresponding to the six *C. elegans* chromosomes, will complete meiosis I and extrude a polar body containing the excess genetic material(McNally and McNally, 2005). Then six pairs of chromatids undergo the meiosis II division and extrude a second polar body. Finally the female chromosomes decondense at the anterior pole of the

embryo to form the female pronucleus, while on the posterior pole the male chromosomes decondense to form the male pronucleus. Both pronuclei will eventually fuse to form a single diploid nucleus, and the mitotic divisions of embryotic development will begin, the oocyte-to-embryo transition completed.

Figure 1

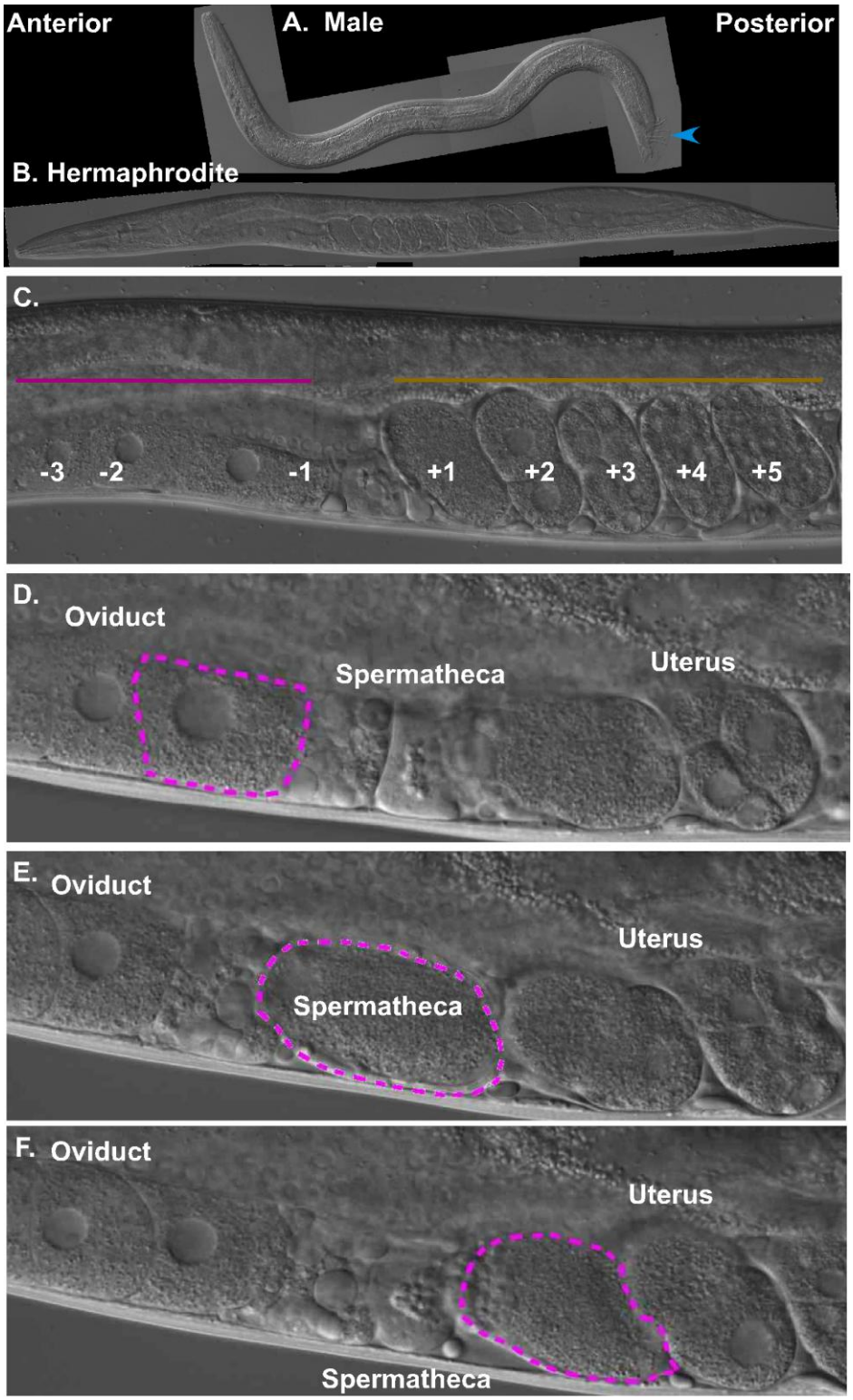
Anterior
A. Male
Posterior
B. Hermaphrodite
C.
-3
-2
-1
+1
+2
+3
+4
+5
D.
Oviduct
Spermatheca
Uterus
E.
Oviduct
Uterus
Spermatheca
F.
Oviduct
Uterus
Spermatheca

Figure 2

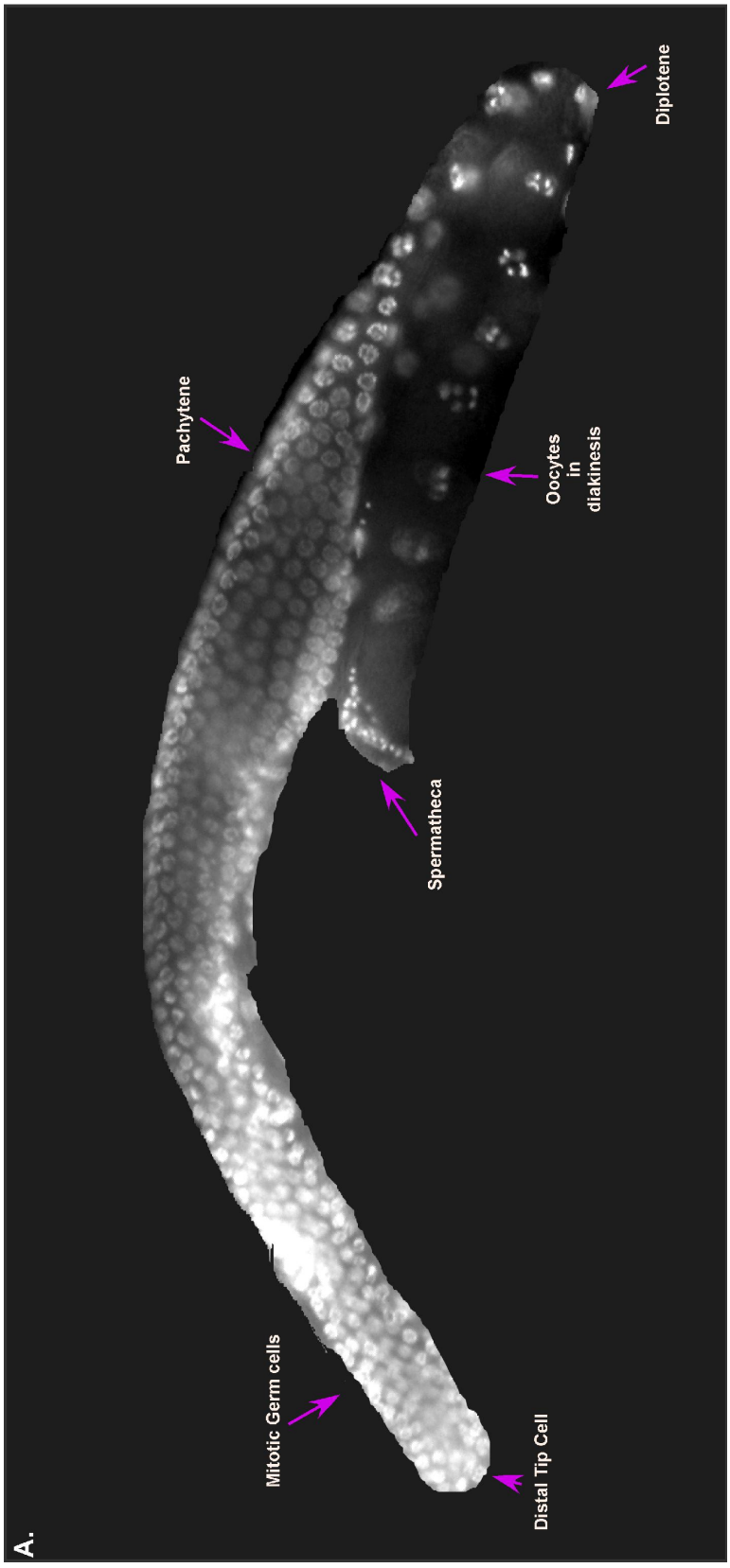
A.
Mitotic Germ cells
Distal Tip Cell
Pachytene
Spermatheca
Oocytes in diakinesis
Diplotene

Figure 1:

A. An adult male. DIC, light microscopy. 20X magnification
B. An adult hermaphrodite. DIC, light microscopy. 20X magnification
C. An adult hermaphrodite, germline centered on spermatheca. 40X magnification**.**
D. An adult hermaphrodite, germline centered on spermatheca. Dots
E. surround proximal oocyte.
F. An adult hermaphrodite, germline centered on spermatheca. Dots
G. surround egg, which has undergone NEBD, cytoskeletal rearrangement,
H. resumption of meiosis, and ovulation into the spermatheca.
I. An adult hermaphrodite, germline centered on spermatheca. Dots
J. surround newly fertilized embryo.

Figure 2

A. The germline of an adult hermaphrodite, fixed and stained with DAPI. The germline extends from distal-tip cell to spermatheca. Regions corresponding to stages of mitosis, and meiotic development are marked. The oocytes individuate from the rachis and begin to accumulate yolk proteins as the round the bend of the germline.

Chapter 2

Egg Activation and EGG-4/5

Acknowledgements for Chapter 2

I would like to acknowledge that portions of this chapter have been previously published in Parry et al., 2009, or are currently in submission to Springer Links for the textbook Cell Cycle and Development.

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2.1.1 *egg-3*, and a module for the oocyte-to-embryo transition in *C. elegans*

Much is known about how the *C. elegans* oocyte is signaled to undergo maturation and ovulation, fertilization, how meiotic and mitotic spindles are formed, how the chitinous eggshell is formed, and how the embryonic polarity is initially formed (Gonczy and Rose, 2005; Greenstein, 2005; Johnston WL, 2006; Oegema and Hyman, 2006). However, the molecular underpinnings of the oocyte-to-embryo transition have not been well elucidated in any species. Recent research in *C. elegans* has outlined some molecules, which may work together to couple the oocyte-to-embryo transition to sperm entry and the advancement of the cell cycle. This gene module includes egg-3, *chs-1*, and *mbk-2*.

The first lynchpin of this gene module is egg-3 (F44F4.2), a gene required for maternal specific fertility (Maruyama R, 2007; Stitzel ML, 2007). This gene was found in a screen for genes that prevent sperm/oocyte fusion, similar to egg-1/2 (Maruyama R, 2007). Knockdown of egg-3 resulted in oocyte specific sterility, such that sperm were phenotypically normal and capable of undergoing fertilization (Maruyama R, 2007). Spermatogenesis and oogenesis were phenotypically normal, and no defects were visible until the one-cell stage of the embryo. An early severe defect is observed in egg-3 when the embryo fails to extrude a polar body at the conclusion of meiosis I and meiosis II (Maruyama R, 2007). As with other fertility defects, the DNA undergoes constant rounds of

mitosis without cytokinesis leading to the formation of endomitotic nuclei (Maruyama R, 2007). In addition, as with other fertility mutants, the chitinous eggshell is not extruded. Further defects are seen during cytoskeletal rearrangement after fertilization. In wildtype embryos, the actin cytoskeleton rearranges after fertilization such that a thick focal cap of actin forms under the plasma membrane directly over the site of sperm entry (Maruyama R, 2007). Subsequently in wildtype embryos, the actin cap disperses throughout the posterior half of the embryo. In egg-3 mutant embryos, an actin cap forms as normal at the site of sperm entry. However, the actin filaments disperse abnormally throughout both posterior and anterior of the embryo (Maruyama R, 2007). The consequences of this abberant dispersal to the embryo are unknown. The protein structure of EGG-3 was analyzed for further clues to its activity, and found that it bore the sequence of a protein-tyrosine phosphatase (Maruyama R, 2007; Stitzel ML, 2007). However, protein-tyrosine phosphatases require absolutely the presence of three amino acid residues for their catalytic activity, an aspartate, a glutamate, and a cysteine, and upon closer examination the EGG-3 protein lacked two of these residues the aspartate and the glutamine. This sequence change indicated that the EGG-3 protein was most likely catalytically inactive, and a member of a small but growing group of proteins referred to as protein-tyrosine phosphate-like (PTPL), or as antiphosphatases (pseudophosphatases). Previously examined molecules of this class include the Pasticcino2 gene in Arabidopsis, which is required for the regulation of cell cycle through direct interaction with cyclin dependent kinase (CDK), mouse and human

genes such as Ptpla which is required for development, and another *C. elegans* gene, sdf-9 which is involved in dauer formation (Bellec and Faure, 2002; Da Costa M, 2006; Jensen et al., 2007; Uwanogho et al., 1999). The structure of PTPL domains suggests two intriguing possibilities, for regulating the activity of other genes. In the first scenario, the PTPL containing protein could bind to a phosphorylated target protein, and then hold that protein as part of a complex with other proteins acting as a molecular scaffold. In the second scenario, the PTPL containing protein could bind to a phosphorylated target protein and then limit the target proteins access to active phosphatases acting as a competitive inhibitor. Determination of EGG-3's role as either scaffold or inhibitor would require further molecular analysis.

2.1.2 The *chs-1* gene, and eggshell extrusion

Gene *chs-1* (T25G3.2) has an integral role in the extrusion of the chitinous eggshell after fertilization, a process that fails in egg-3 mutants (Johnston WL, 2006; Zhang et al., 2005). The gene encodes a large multipass transmembrane protein, chitin synthase, which is responsible for catalyzing the production of chitin from UDP-N-acetylglucosamine (UDP-GlcNAc). This chitin is required as a critical component of the center layer of the trilamellate eggshell. In addition, to the requirement for CHS-1 during eggshell production, knockdown of CHS-1 through gene specific RNAi lead to defects during meiosis and polar body

formation, identical to those seen in *egg-3* mutant hermaphrodites (Johnston WL, 2006).

2.1.3 The *mbk-2* gene, multi-functioning kinase

The gene, *mbk-2*, encodes a kinase important for degrading maternal proteins during the oocyte-to-embryo transition and known to have an identical pattern of protein localization to EGG-3 (Raich et al., 2003). In an elegant example of regulation, *mbk-2* is responsible for phosphorylating and marking for degradation both MEI-1, OMA-1 and OMA-2, and MEX-5 and MEX-6 proteins important for early post-fertilzation processes, which must be destroyed before the transition to later embryonic development (Nishi Y, 2005; Pellettieri et al., 2003; Quintin S, 2003; Stitzel ML, 2007). The *mei-1* gene encodes an orthologue of the catalytic subunit of katanin, which acts as a microtubule severing protein during meiosis but not mitosis. Thus the MEI-1 protein has a function tightly linked to the oocyte-to-embryo transition, as its function is required during the completion of meiosis, but is deleterious to the functioning of the mitotic cycles, which mark the beginning of embryonic development (Pellettieri et al., 2003; Quintin S, 2003; Yang et al., 2003). Similarly, the *oma-1* and *oma-2* genes, which are redundant, encode zinc finger proteins, which are necessary for setting up correct cell fate specification during early embryogenesis (Detwiler et al., 2001; Lin, 2003; Pellettieri et al., 2003). Persistence of the OMA-1 or OMA-2 proteins past the first mitotic division lead to adoption of the incorrect cell fates and embryonic

lethality, again making the precise and timely degradation of OMA-1 and OMA-2 critical to early embryonic development (Lin 2003). Finally, the MEX-5 and MEX-6 proteins are partially redundant proteins required for polarization at the one cell stage of the embryo. The MEX-5 and MEX-6 proteins are localized to the anterior cytoplasm of the embryo where the control the localization of other maternal proteins such as PIE-1, POS-1, and MEX-1 (Nishi et al., 2008; Schubert et al., 2000). Therefore, the highly precise temporal and spatial action of *mbk-2* is necessary to progression through the oocyte-to-embryo transition. However, knockdown of *mbk-2* leads to less severe embryonic defects than depletion of *egg-3* or *chs-1*. In the absence of mbk-2, the embryo completes a phenotypically normal meiosis and extrudes the chitin-containing eggshell. In mbk-2 hermaphrodites, embryos die off from the combined defects caused by aberrant functioning of MEI-1, OMA-1, OMA-2, MEX-5, and MEX-6 (Stitzel ML, 2007).

2.1.4 The intersections and interactions of EGG-3, CHS-1, and MBK-2

The spatial and temporal localizations of the three proteins, EGG-3, CHS-1, and MBK-2 were previously examined using integrated GFP or RFP tagged fusion proteins, and found to be identical during late oogenesis and early embryogenesis, indicating that a direct interaction between the proteins was possible (Maruyama R, 2007; Stitzel ML, 2007). We sought to elucidate whether the localizations of the three proteins were dependent on one another. Using gene specific RNAi, we found that the localizations of EGG-3 and CHS-1 fusion

proteins were interdependent, such that knocking down EGG-3 by RNAi lead to aberrant localization of CHS-1 and vice versa (Maruyama R, 2007). The localization of MBK-2 was dependent on the presence of both EGG-3 and CHS-1 (Maruyama R, 2007). While neither EGG-3 nor CHS-1 localizations were affected by the absence of MBK-2 (Maruyama R, 2007). This result was anticipated because knockdown of MBK-2 leads to less severe defects than knockdown of EGG-3 or CHS-1, as meiosis is completed as in wildtype, and the chitin containing eggshell is formed. Concurrently, it was shown that EGG-3 and MBK-2 have a direct physical interaction in vitro by co-immunoprecipitation of either protein (Stitzel ML, 2007). However, the co-dependence of these proteins does not speak to their precise dynamic localization, which in the case of MBK-2 is critical for its temporal catalytic activity. Therefore, it was critical to determine the trigger for dynamic relocalization of these proteins. A double tagged strain, containing RFP:EGG-3 and GFP:HIS (histone H2B) was constructed, and live imaging with this strain showed that the redistribution of EGG-3, and by extension CHS-1, and MBK-2 occurred somewhere between metaphase I and anaphase I of meiosis I (Maruyama R, 2007). In order to verify the timing of this event, anaphase promoting complex (APC) component mat-1 was depleted by RNAi in GFP:EGG-3, GFP:CHS-1, and GFP:MBK-2 backgrounds (Maruyama R, 2007; Stitzel ML, 2007). Not surprisingly, abrogation of an APC subunit froze the fertilized embryos at metaphase of meiosis I, and caused retention of all three proteins at the cytoplasm underlying the plasma membrane. Therefore, the dynamic redistribution of EGG-3, CHS-1, and MBK-2 relies on the advancement

of the cell cycle (Maruyama R, 2007; Stitzel ML, 2007). Conversely, depletion of EGG-3, CHS-1, or MBK-2 did not have an effect on the depletion of GFP:CYB-1, which is an integral part of the machinery regulating entry into meiosis I. This indicated that EGG-3, CHS-1, and MBK-2 were not required for cell cycle progression during meiosis.

Both EGG-3 and CHS-1 are required for the localization of MBK-2 during development, therefore it remained to determine the precise molecular interactions between the proteins. It was determined that both the EGG-3 and MBK-2 proteins could immunoprecipitated by the other protein, indicating a direct physical interaction (Stitzel ML, 2007). In addition, it was shown that while depletion of APC component MAT-1 lead to retention of MBK-2 at the cortex, simultaneous depletion of MAT-1 and EGG-3 shifted the MBK-2 protein from the cortex to the cytoplasm, allowing it to carry out its biochemical activity (Cheng KC, 2009; Stitzel ML, 2007). The implications of this experiment are two-fold. First, this indicates that the EGG-3 protein plays a direct role in holding the complex at the cortex until signaled by cell cycle progression to redistribute. Second, this indicates that EGG-3 does not directly regulate the biochemical activity of MBK-2, only regulates in spatial and temporal localization. Finally, simultaneous depletion of CDK-1 and EGG-3 did not release the retained MBK-2 from the cortex to the cytoplasm (Cheng KC, 2009). This indicates that CDK-1 does not regulate MBK-2 through the scaffolding molecule EGG-3. The model of MBK-2 activity in conjunction with EGG-3 and CHS-1 lacked a direct link

between the association of these molecules and the regulation of MBK-2's biochemical activity. Therefore, we sought to elucidate a molecule which might interact with EGG-3, CHS-1, and MBK-2, while playing a direct role in regulation of MBK-2 kinase function. We were also interested in the elucidation of further PTPL proteins, similar to EGG-3, which might function during the oocyte-to-embryo transition.

2.2 Materials and Methods

2.2.1 General Methods and Strains

C. elegans strain maintenance and general genetic crosses were performed as described by S. Brenner [S3]. Strains used in experiments included: wild-type variety Bristol, strain N2; *egg-4 (tm1508)*; *egg-5 (ok1781)*; *egg-3(tm1191)*; *dpy-5(e61)*; GFP:tubulin, ruIs57[unc-119(+) pie-1 promoter::GFP::tubulin]; CYB-1:GFP, ekIs2[unc-119(+) pie-1 promoter::CYB-1::GFP]; (gift from E. Kipreos); GFP:MBK-2, axIs1140[pJP1.02] (gift from G. Seydoux); mCherry:EGG-3, asIs2[unc-119(+)pie-1 promoter::mcherry::egg-3], GFP:EGG-3, asIs2[unc-119(+)pie-1 promoter::mcherry::egg-3], GFP:CHS-1, nnIs2[unc-119(+)pie-1 promoter::gfp::*chs-1*]; unc-119(ed3); tcIs1[pRL475+pPDMMO10] (Poma-1:OMA-1:GFP); unc-119(ed3); orIs1[unc-119(+); pie-1::GFP::MEI-1]. Strains were maintained at 20°C or 25°C.

2.2.2 Back-crossing

Back crossing for strains *egg-4 (tm1508); egg-5 (ok1781)* was carried out a minimum of three times and genotypes were confirmed via PCR using primer sets egg-4 fwd: GAAAGCACGCTCAGCAAGAG, egg-4 rev: CATACCGTCTGTAGTTTGGTCGTTCC, egg-5 fwd: TTGTCGAGGAGACGGAGACT egg-5 rev: CGACAGCCGCCACCTTGTTC. The *egg-4 (tm1508)* mutation deletes amino acids 134- 313 and *egg5(ok1781)* deletes

2.2.3 GFP fusions

To create GFP fusions for EGG-4 and EGG-5, genomic DNA for each gene, lacking a start codon and including a stop codon, was amplified from purified *C. elegans* N2 DNA using the primers (GGGGACAACTTTGTACAAAAAAGTTGtggcgttgaacagcgaagtg and GGGGACAACTTTGTACAAGAAAGTTGttagaattcgaagacgctcttc) and inserted by Gateway recombination cloning into intermediate vector pDONR221. Individual clones encoding EGG-4 or EGG-5 were identified by DNA sequencing. Then EGG-4 or EGG-5 sequences were transferred to destination vector pID3.01B (gift from G.Seydoux) using Gateway Technology (Invitrogen). The constructs were then integrated into the *C. elegans* genome at low copy number using the microparticle bombardment method [S4].

2.2.4 DAPI staining

Adult hermaphrodites were washed once in M9, then bathed for 10 minutes in ice cold Methanol Formaldehyde (10ml 16% formaldehyde with 3.3ml of 0.1M K2HPO4 pH 7.2) solution as described in [S5]. Fixed animals were mounted in 1mg/ml DAPI in GEL/MOUNT (biomeda).

2.2.5 Immunohistochemistry

Polyclonal antisera were produced against peptides corresponding to amino acids 132-145 in EGG-4 and EGG-5 by Yenzym Antibodies, LLC (Burlingame, CA). The peptidesequences were as follows: EGG-4 peptide CKPKDEGRREDSGH, EGG-5 peptide CKPKDEGRSEDSGH. The bold amino acids indicate the site of the single amino acid substitution between the two proteins in these sequences. The non-coded N-terminal cysteine residues were included for single point site directed adjuvant conjugation [S6].
Embryos were freeze-cracked, fixed with ice-cold methanol for 5 min at -20C, washed 2X with PBST, and incubated with 1:500 dilution of primary antibody over night at 4C. Samples were washed 4X with PBST, then incubated 4 hours at room temperature with 1:2500 dilution of Alexa Fluor 488. Samples were washed 4X with PBST then mounted with 1 mg/ml DAPI in GEL/MOUNT (biomeda).

2.2.6 RNAi Interference

RNAi interference was carried out by the soaking method [S7, S8]. dsRNA was prepared from cDNA clones (gift of Y. Kohara) yk1630c04 (egg-4) and yk1621b12 (egg-5), yk1216f11(mat-1), yk1254a11(mbk-2), yk719a2(*egg-3*) or from the Ahringer RNAi library [S9]. P0 worms were soaked at 20oC for 24hrs, then recovered on OP50 plates at 20oC or 25oC for 24hrs. P0 worms were examined by Nomarski and fluorescent microscopy.

2.2.7 Chitin Staining

Chitin staining was carried out as described previously (Maruyama et al., 2007). Embryos were freezecracked, fixed with ice cold methanol for 5 min at -20oC, washed 2X with PBST, and incubated with 1:500 dilution of rhodamine-conjugated chitin-binding probe (New England BioLabs) overnight at room temperature. Samples were washed 3X with PBST, then were mounted with 1 mg/ml DAPI in GEL/MOUNT (biomeda).

2.2.8 Yeast-two-hybrid

The DupLEX-A two-hybrid system (OriGene Technologies Inc., MD) was used in accordance with the manufacturer's instructions. Primers were as follows:
Egg-4 Fwd: GGG GAC AAC TTT GTA CAA AAA AGT TGT GAT GGC GTT GAA

CAG CGA AG. Egg-5 Rev: GGG GAC AAC TTT GTA CAA GAA AGT TGG AAT TCG AAG ACG CTC TTC GC. Full-length copies of *egg-4* and *egg-5* were cloned into pDONR221 by Gateway cloning (Invitrogen, CA). Then pDONR221 plasmids corresponding to *egg-4, egg-5, egg-3* [S10],or *mbk-2* (generous gift from G. Seydoux) were transferred into either pEG202 (bait plasmid) or pJG4-5 (prey plasmid). As a positive control *rab-10* and *hum-2* were used as bait (pEG202) and prey (pJG4-5) vectors respectively. Bait vectors pEG202 and LacZ reporter pSH18-34 were transformed into *S. cerevisae* strain EGY48, which contains the Leu2 reporter gene. Transformants were collected from selection plates YNB(Glu) lacking His, Ura. Expression of protein of interest in bait construct was assayed by western blot with primary antibodies against LexA. The EGY48 strain carrying the bait plasmid was then transformed with prey vector pJG4-5 with another gene of interest. As a negative control, bait proteins were transformed with empty pJG4-5 vectors lacking a gateway cassette. Positive transformants were collected from YNB(Glu) plates lacking His, Ura, Trp. Positive clones were tested on three types of plates: YNB (Gal)-his-ura-trp-leu, YNB (Gal)- hisura-trp+x-Gal, and YNB (Glu)- his-ura-trp-leu. Plasmid DNA was isolated from positive yeast clones, and digested with EcoRI and XhoI to release the insert and screened by electrophoresis for insert size. Inserts were then confirmed by sequencing.

2.2.9 Double Mutant Generation

To generate the double mutant (*egg-4 egg-5*), (*egg-4(tm1508) egg-5(+)/egg-4(+) egg-5(ok1781*) males were crossed to *dpy-5(e61) unc-13(e51)/hT2 bli-4(e937) let-(q783)qIs48* hermaphrodites. Resulting F1 progeny were picked to 24 well plates and allowed to self. Eight F2 progeny were screened for infertility from each F1 parent. Of 170 F1 progeny screened, 1 line containing the *egg-4 egg-5* double mutant was obtained. The double mutant was confirmed by PCR.

2.3 Results

2.3.1 A new player in the complex: EGG-4/5

It was clear that the link between the genes discussed above (EGG-3, CHS-1, and MBK-2) which are required for the oocyte-to-embryo transition, was incomplete. While abrogation of EGG-3 caused premature degradation of MBK-2 targets, it did not alter MBK-2's catalytic activity (Stitzel ML, 2007). Although mislocalization of CHS-1 might be key in preventing extrusion of the chitinous eggshell, none of the proteins elucidated thus far could be pegged absolutely as the cause of failure during polar body extrustion (Johnston WL, 2006; Maruyama R, 2007; Stitzel ML, 2007). In addition, it was as yet unknown what factors might link EGG-3, CHS-1, and MBK-2 to the advancement of the cell cycle. Therefore, we wished to elucidate further protein-tyrosine phophatase-like proteins, which might play a role during the oocyte-to-embryo transition; this lead to the identification of proteins EGG-4 and EGG-5 (Cheng KC, 2009; Parry JM, 2009).

2.3.2 The egg-4 and egg-5 Genes Encode Redundant Protein Tyrosine Phosphatase-like Proteins

The egg-4 (T21E3.1) and egg-5 (R12E2.10) genes encode PTPL proteins, and share a high level of sequence identity, sharing 747 of 753 amino acids (99.2% identical) (Figure 1A). In order to determine the function of *egg-4/5*, we obtained deletion alleles of both genes from the National Bioresource Project for the Nematode (Japan) and the North American *C. elegans* Gene Knockout Consortium. The various alleles available are shown in Figure 1. For our studies, we used the egg-4(tm1508) and egg-5(ok1781) alleles and/or RNAi treatment that knocks down both genes simultaneously (Figure 2A). Viable progeny counts showed that each single mutant had a reduced number of hatching larvae compared to wild-type hermaphrodites (Table 1). A double mutant containing both the egg-4 and egg-5 deletion alleles was constructed, and found to be completely sterile (Parry JM, 2009). In addition, RNAi treatment targeting egg-4, egg-5 or both genes resulted in complete sterility. The partial redundancy in function of the genes coupled with their close proximity on the chromosome (0.3 cM apart), their high degree of sequence similarity, and the presence of only a single homologue in other species of Caenorhabditis supports the hypothesis that genes egg-4 and egg-5 represent a gene duplication and that the genes are predominantly redundant. In addition, crossing wild-type males to *egg-4/5* hermaphrodites did not result in the production of viable progeny. These data

support the conclusion that egg-4 and egg-5 are largely redundant and that loss of function of both genes leads to maternal-effect lethality.

2.3.3 Loss of *egg-4/5* Leads to Defects in the Oocyte-to-Embryo Transition

To determine the nature of the maternal-effect lethality associated with loss of *egg-4/5*, we examined the oocytes produced by RNAi-treated and double mutant animals. Germline development, gametogenesis, positioning of gametes, ovulation, and fertilization all appeared to occur normally in *egg-4/5*(RNAi) and double mutant hermaphrodites (McCarter 1999) (Figure 2A; Table 1). However, we found a number of important abnormalities in embryos produced by *egg-4/5* loss-of-function animals.

The block to polyspermy is considered a hallmark of egg activation in many species. A polyspermy event has never been seen in wild-type N2 hermaphrodites. DAPI-stained maternal DNA and sperm DNA can be distinguished based on morphology (Chatterjee et al., 2005). In the majority of embryos produced by *egg-4/5*(RNAi) or double mutant animals, the block to polyspermy was intact and only a single sperm entered each oocyte (Figure 3A). However, in 16% of *egg-4/5*(RNAi) eggs and 25% in *egg-4/5* double mutants, DAPI staining reveals what appear to be polyspermic embryos (Figure 3A). Some of the most striking features of the oocyte-to-embryo transition are associated with changes at the cell surface. In *C. elegans*, a chitinous eggshell

is secreted after sperm entry and its formation is required to support the embryonic development (Ward and Carrel, 1979). Embryos produced by *egg-4/5* hermaphrodites lack any visible rigid eggshell and are indistinguishable (except for sperm entry and the completion of meiosis, see below) from unfertilized oocytes produced by other types of mutants (Figure 3B) (Singson 2008, McNally 2005). Further, *egg-4/5*(RNAi) oocytes lack the defining chitin containing layer of the wild-type eggshell (Figure1). Therefore, we conclude that EGG-4/5 proteins are required for the production of the eggshell after fertilization. We examined filamentous actin (F-actin) dynamics via a GFPmoesin fusion (GFP:MOE, Figure 4A) (Maruyama 2007). In wild-type oocytes, an actin cap forms in the presumptive posterior of the egg cortex several minutes after moving into the spermatheca and marks the site of sperm entry. This actin cap then disperses in a fashion that is limited to the posterior half of the embryo as it moves from the spermatheca to the uterus. This actin cap and its rearrangement are aberrant in *egg-4/5*(RNAi) animals and are not restricted to the posterior half of the one cell embryo during dispersal (Figure 4A). Therefore, this polarized cytoskeletal rearrangement requires EGG-4/5 function.

We observed oocyte chromosomes during meiosis by using mCherry:histone and GFP:tubulin in *egg-4/5*(RNAi) animals (Maruyama 2007) (Figure 4B). Meiotic spindle formation, translocation, and rotation were indistinguishable from wild-type through anaphase I (McNally 2005) (Figure 9B). Chromosomes segregated normally until after anaphase I (Figure 4B). In unfertilized oocytes, meiosis fails

to progress beyond this point (McNally 2005). In *egg-4/5*(RNAi) embryos, meiosis progresses beyond anaphase I but no polar bodies were formed and 12 univalents could be seen at metaphase II (Figure 4B). The degradation of CYB-1:GFP (cyclin B) in *egg-4/5*(RNAi) animals (Figure 9C) suggest that there is no significant meiotic delay. Therefore, EGG-4/5 are required for normal meiosis and polar body formation. The meiotic defects seen in *egg-4/5*(RNAi) animals are very similar to those seen in *egg-3*, *chs-1*, paternal-effect lethal spe-11 mutants and in oocytes depleted in F-actin (Maruyama 2007, McNally 2005).

2.3.4 EGG-4/5 Localize to the Cortex of Developing Oocytes and Then Disperse in Embryos

We employed fusion protein and antibody approaches to determine the subcellular localization of EGG-4 and EGG-5. Transgenic worm strains were created carrying integrated gfp fusions driven by the germline-specific pie-1 promoter (Tenenhaus et al., 1998). Multiple independently derived transgenic lines (four lines for GFP:EGG-4 and two lines for GFP:EGG-5) all had the same distribution pattern, dynamics, and genetic interactions. Both GFP:EGG-4 (Figures 5A and 5G) and GFP:EGG-5 (Figures 5B and 5H) were associated with the cortex of developing oocytes and newly fertilized embryos. In oocytes and newly fertilized embryos, GFP:EGG-4 (Figures 5D–5F) and GFP:EGG-5 (not shown) colocalize with mCherry:EGG-3. Therefore, by extension they also

colocalize with the other egg-activation molecules CHS-1 and MBK-2 during meiotic maturation (Maruyama 2007, Stitzel 2007).

We created a strain of worms carrying both GFP:EGG-4 and mCherry-histone to determine the dynamics of EGG-4 localization relative to the cell cycle. By anaphase I, GFP:EGG-4 and GFP:EGG-5 was degraded or dispersed away from the cortex (Figures 5I and 5J). This movement from the cortex is in sharp contrast to the movement of other oocyte-to-embryo transition molecules (compare embryos in Figures 5A and 5B to 5C). For instance, mCherry:EGG-3 moves to cortical foci at anaphase I (Figure 5C) (Maruyama 2007). We have never seen any of our GFP:EGG-4 or GFP:EGG-5 fusion molecules form foci in embryos (Figures 5A and 5B). Rather, when we follow the movement of these GFP fusions in live animals, the GFP signal abruptly leaves the cortex just before or at early anaphase I. This timing is similar to the timing of when EGG-3, CHS-1, and MBK-2 move from a uniform cortical distribution to cortical foci (Maruyama 2007, Stitzel 2007). The redistribution of EGG-4/5 did not depend on fertilization. Localization and the dynamics of GFP:EGG-4 was normal in spe-9(hc52) mutants and in old hermaphrodites that had completely depleted their sperm (not shown).

However, the redistribution of EGG-4/5 does depend on meiotic progression. Simlar to EGG-3 and CHS-1 dynamics, GFP:EGG-4 stayed associated with the cortex in mat-1(RNAi) animals where oocytes were arrested at metaphase I

(Shakes et al., 2003) (Figure 6B). Furthermore, depletion of cyclin-dependent kinase CDK-1 also caused aberrant retention of EGG-4/5, EGG-3, CHS-1, and MBK-2 to the cortex (Cheng KC, 2009). The *cdk-1* gene is required for meiotic re-entry of the oocyte at metaphase I, and is antagonized by kinase *wee-1* (Burrows et al., 2006*)*. Contrary to the effects of depleting MAT-1 or CDK-1, depletion of WEE-1 caused premature relocalization of EGG-4/5, EGG-3, CHS-1, and MBK-2 away from the cortex (Cheng KC, 2009; Stitzel ML, 2007). Finally, depletion of EGG-4/5 had no effect on the depletion of GFP:CYB-1, and thus no effect on advancement of the cell cycle through meiosis (Parry JM, 2009). The differences in the redistribution of EGG-4/5 from the cortex after fertilization compared to other molecules that moved to cortical foci may reflect important differences in the roles of these molecules during the oocyte-to-embryo transition.

2.3.5 The Localization of EGG-4/5 to the Oocyte Cortex in Developing Oocytes Depends on EGG-3 and CHS-1 but Not MBK-2

We checked EGG-4/5 for interactions with previously identified molecules involved in the oocyte-to-embryo transition. Like EGG-4/5, EGG-3 is a PTPL family protein that is required for the oocyte-to-embryo transition and mCherry:EGG-3 colocalizes with GFP:EGG-4 and GFP:EGG-5 in developing oocytes (Figures 5D–5F) (Maruyama 2007, Stitzel 2007). In egg-3(RNAi) worms, GFP:EGG-4 (Figure 6C) and GFP:EGG-5 (not shown) do not accumulate at the

cortex. Therefore, EGG-3 is required for the proper localization of EGG-4/5 in developing oocytes. CHS-1 is a chitin synthase that is required for eggshell formation and the oocyte-to-embryo transition (Maruyama 2007). CHS-1 was also shown to colocalize with EGG-3, and the proper localization of these two proteins was shown to be interdependent (Maruyama 2007). In *chs-1*(RNAi) animals, GFP:EGG-4 (Figure 3D) and GFP:EGG-5 (not shown) do not localize to the cortex of developing oocytes. Therefore, like EGG-3, CHS-1 is also required for the proper subcellular localization of EGG-4/5. The *mbk-2* gene encodes a DYRK kinase that is required for the oocyte-to-embryo transition and marks maternal proteins for timely degradation (Stitzel 2006). The localization pattern of MBK-2 is identical to EGG-3 and CHS-1. Further, the proper subcellular localization patterns of MBK-2 depend on EGG-3 and CHS-1 but not the other way around (Maruyama 2007, Stitzel 2007, Stitzel 2006). We found that *mbk-2(RNAi)* has no effect on the localization pattern of GFP:EGG-4 (Figure 6E) and GFP:EGG-5 (not shown), indicating that MBK-2 may function downstream of EGG-4/5.

2.3.6 EGG-4/5 Are Required for the Proper Localization of MBK-2

Although MBK-2 is not required for the localization pattern of EGG-4/5, we wanted to check whether EGG-4/5 were required for the localization of MBK-2. In *egg-4/5*(RNAi) animals, GFP:MBK-2 was indeed mislocalized (Figures 7A and 7B). Rather than accumulating at the cortex of developing oocytes, GFP:MBK-2

remains diffusely cytoplasmic and it also does not localize to cortical foci after fertilization. Next we wanted to check whether the EGG-4/5-dependent mislocalization of MBK-2 altered its ability to regulate the degradation of its known target proteins OMA-1 and MEI-1 (Pellettieri et al., 2003; Shirayama et al., 2006; Stitzel ML, 2007). In *egg-4/5*(RNAi) animals, we observed no obvious changes in the degradation patterns of GFP:MEI-1 and OMA-1:GFP (Figure 10A).

2.3.7 EGG-4/5 Are Required for the Movement of EGG-3 and CHS-1 from the Cell Cortex to the Cytoplasm during Meiotic Progression

We wanted to further examine the interactions of EGG-4/5 with EGG-3 and CHS-1. We found that *egg-4/5*(RNAi) did not alter the cortex localization of GFP:EGG-3 or GFP:CHS-1 in developing oocytes (Figures 7C–F). However, in embryos, GFP:EGG-3 (Figure 7D), mCherry:EGG-3 (Figure 10B), and GFP:CHS-1 (Figure 7F) remained associated with a uniform cortex distribution rather than moving to cortical foci at anaphase I. It is important to note that the localization patterns of GFP:EGG-3 and GFP:CHS-1 in *egg-4/5*(RNAi) embryos resembles GFP:EGG-3 in a *mat-1* mutant background where the meiotic cell cycle is arrested and embryos do not reach anaphase I (Figure 10B) (Maruyama 2007). In *egg-4/5*(RNAi) animals, the meiotic cell cycle progresses past anaphase I (Figure 1G and Figure 9C) yet GFP:EGG-3 and GFP:CHS-1 remain uniformly associated with the cortex. We conclude that EGG-4/5 is required to couple the dynamics of

EGG-3 and CHS-1 with meiotic cell cycle progression, holding EGG-3 and CHS-1 at the cortex until the advancing cell cycle signals their release.

2.3.8 Physical Interactions between EGG-3, EGG-4, and MBK-2

This accumulated wealth of epistatic relationships would not be complete without proof of direct physical interactions between molecules. The yeast-two-hybrid system was used to investigate interactions between EGG-4/5, EGG-3, and MBK-2 (Parry JM, 2009). All three molecules were examined in both context of bait and prey. It was determined that EGG-4 and EGG-3, EGG-4 and MBK-2, and EGG-3 and MBK-2 interacted with one another (Figure 7G) (Parry JM, 2009). The interactions between EGG-4 and MBK-2, and EGG-3 and MBK-2 were corroborated by evidence from the Seydoux lab, which showed that these molecules interact in a co-immunoprecipitation assay (Cheng KC, 2009). The interaction results for MBK-2 and EGG-3 are consistent with previously reported binding studies for these two proteins (Stitzel 2007). Although the interactions are weaker than our strong positive control, interactions in vivo could potentially be strengthened by phosphorylation of the various molecules. These results support the idea that direct physical interactions are the mechanism for the colocalization of these proteins.

2.3.9 A Cell Cortex-Associated Complex

Loss of EGG-3, EGG-4, EGG-5, CHS-1, and MBK-2 all led to many defects in the oocyte-to-embryo transition (Maruyama 2007, Stitzel 2007, Singson 2008). Our epistasis studies indicate that there is a complicated interdependence of these maternally provided molecules with regards to their subcellular localization and dynamics (Maruyama 2007, Stitzel 2007). Many of these molecules can physically interact and we propose that they form a complex at the cell cortex during oogenesis. This cortex-associated complex would need to spatially and temporally regulate the output of different effectors relative to the needs of the developing oocyte or embryo and in the context of cell cycle progression. For instance, MBK-2 activity must be properly regulated during oogenesis and during meiotic progression after fertilization (Stitzel 2007). Therefore, it makes sense that different components of this cellular machinery will have some specialization of function. For instance, only EGG-4/5 are known to be required for the block to polyspermy. Like EGG-3, loss of EGG-4/5 can alter F-actin dynamics and polarization after fertilization Maruyama 2007). Unlike EGG-3, loss of EGG-4/5 can also alter the F-actin cap at the fertilization site. After fertilization, EGG-4/5 move from the cortex to the cytoplasm or could be degraded rather than forming cortical foci like EGG-3, CHS-1, and MBK-2. It remains unclear how the unique dynamics of EGG-4/5 are related to their differential effects on the oocyte-to-embryo transition. Certainly their unique localization pattern just after anaphase I strongly suggest that EGG-4/5 are no longer binding to effectors such as MBK-2 and CHS-1 at this critical moment of early development.

2.4 Perspectives

The process of completing the transition between fully developed, mature oocyte, and fertilized developing embryo requires precise spatial and temporal control of molecular events. In short order, the *C. elegans* oocyte is contacted by a protein signal from sperm in the nearby spermatheca to complete the process of maturation, including nuclear envelope breakdown, cytoskeletal rearrangment, and resumption of meiosis I at metaphase I. As maturation is completed the oocyte immediately enters the spermatheca where it should be fertilized by a single sperm, before passing into the uterus. What follows is a highly complex series of maturation events that allow the newly fertilized embryo to complete its transition from maternal program to embryonic program, this is often referred to as the oocyte –to-embryo transition. The complex module of proteins described in these pages, is required for coupling the precise timing of necessary transitory events to the advancement of the cell cycle.

The genes *egg-3*, *chs-1*, and *egg-4/5* are minimally required for production of the chitin eggshell during meiosis, an error-free meiosis and polar body extrusion, and in the case of *egg-3* and *egg-4/5* a normal formation and/or distribution of the cytoskeletal actin cap. All of these genes, *egg-3*, *chs-1*, and *egg-4/5* are also required for the precise spatial and temporal localization of regulatory kinase mbk-2. Because mbk-2 is required to mark several time sensitive maternal proteins for degradation, its localization and activity must be precisely restricted

during the oocyte-to-embryo transition. To this end, several interrelated regulatory mechanisms have been elucidated. First it has been determined that the redistribution of *egg-3*, *chs-1*, mbk-2, and *egg-4/5* depends on advancement of the cell cycle past metaphase I, and that the redistribution is signaled in an EGG-3 dependent manner. It has also been shown that the activity of MBK-2 depends on phosphorylation by cyclin dependent kinase CDK-1, a protein that becomes active during late oogenesis. However, it is critical that MBK-2 does not have access to its targets until the completion of meiosis. Therefore a second regulatory protein, in the form of EGG-4/5, is required for holding MBK-2 and preventing its premature activity. This complex protein module, EGG-3, EGG-4/5, CHS-1, MBK-2, and CDK-1 represents a complex and novel method of regulating the transition between maternal oocyte programming and embryonic programming.

Figure 1

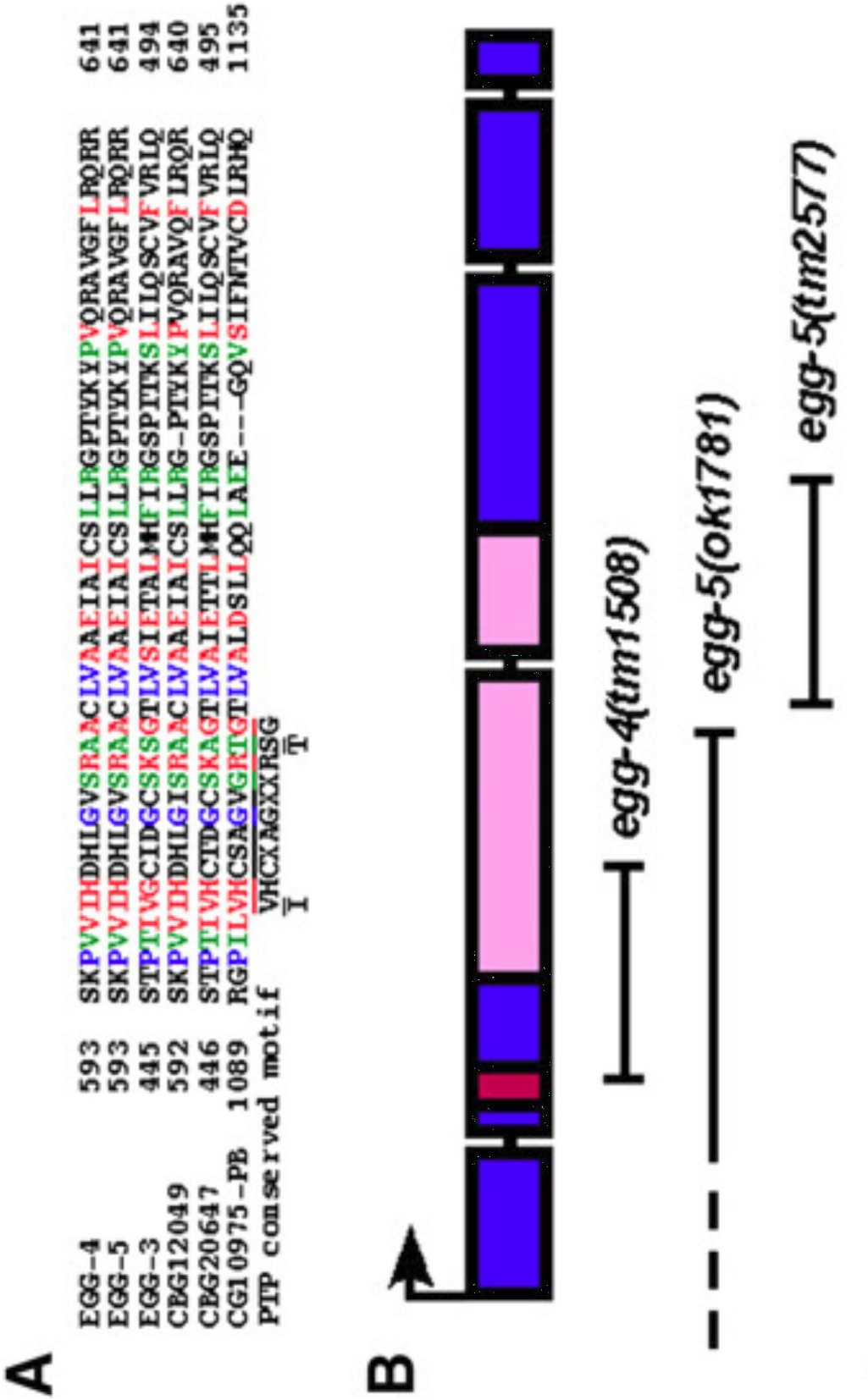

A
EGG-4 593 SKPVVIDHDHLGVSRAACLVAAEIAICSLLRGPTYKYPVQRAVGFLRQRR 641
EGG-5 593 SKPVVIDHDHLGVSRAACLVAAEIAICSLLRGPTYKYPVQRAVGFLRQRR 641
EGG-3 445 STPTIVGCIDGCSKSGTLVSIETALMHFIRGSPTTKSLILQSCVFVRLQ 494
CBG12049 592 SKPVVIDHDHLGISRAACLVAAEIAICSLLRG-PTYKYPVQRAVQFLRQR 640
CBG20647 446 STPTIVHCTDGCSKAGTLVAIETTLMHFIRGSPTTKSLILQSCVFVRLQ 495
CG10975-PB 1089 RGPILVHCSAGVGRTGTLVALDSLLQQIAEE---GQVSIFNTVCDLRHQ 1135
PTP conserved motif VHCXAGXXRSG
I
T
B
egg-4(tm1508)
egg-5(ok1781)
egg-5(tm2577)

Figure 2

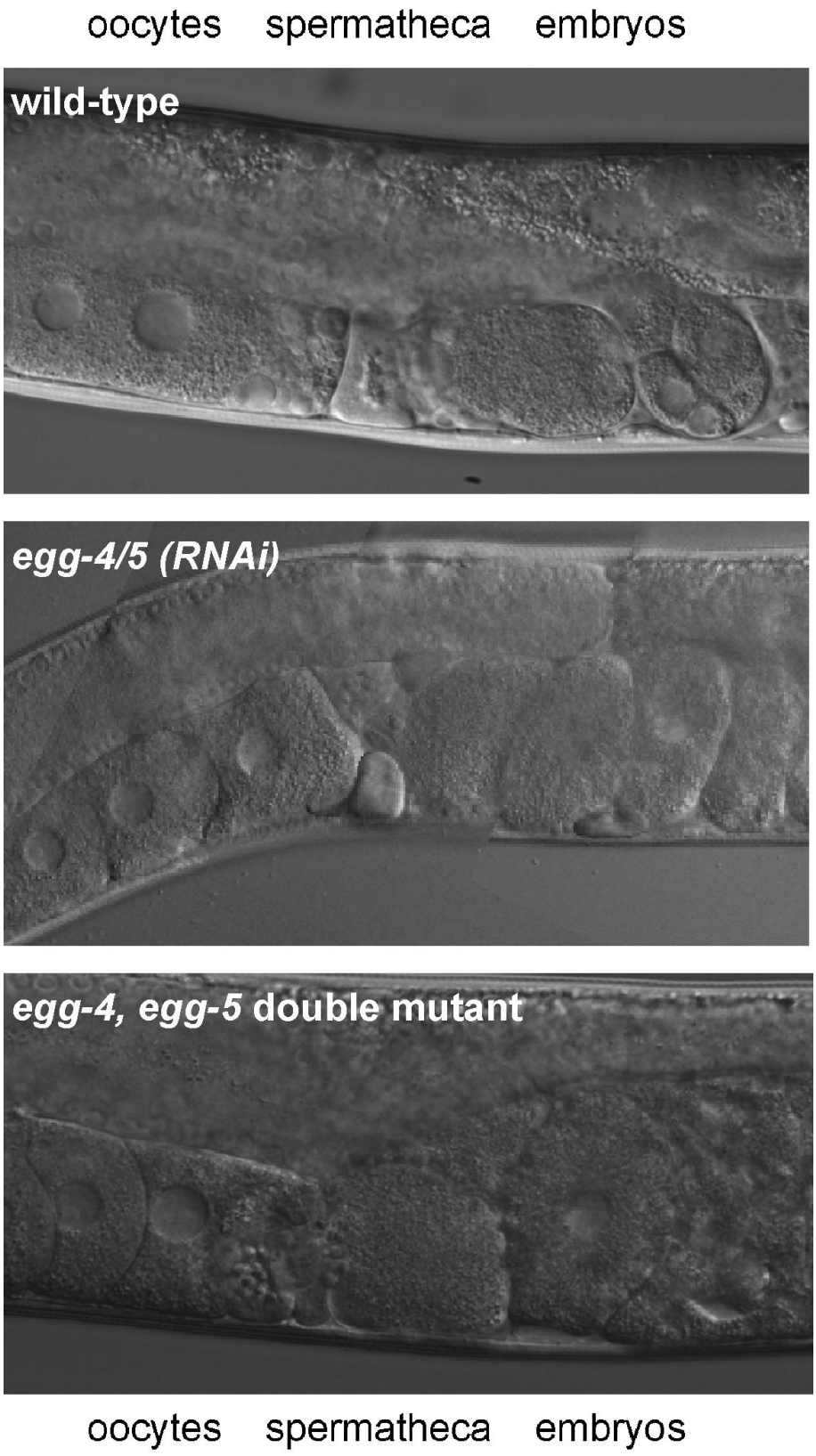

oocytes spermatheca embryos
wild-type
egg-4/5 (RNAi)
egg-4, egg-5 double mutant
oocytes spermatheca embryos

Figure 3

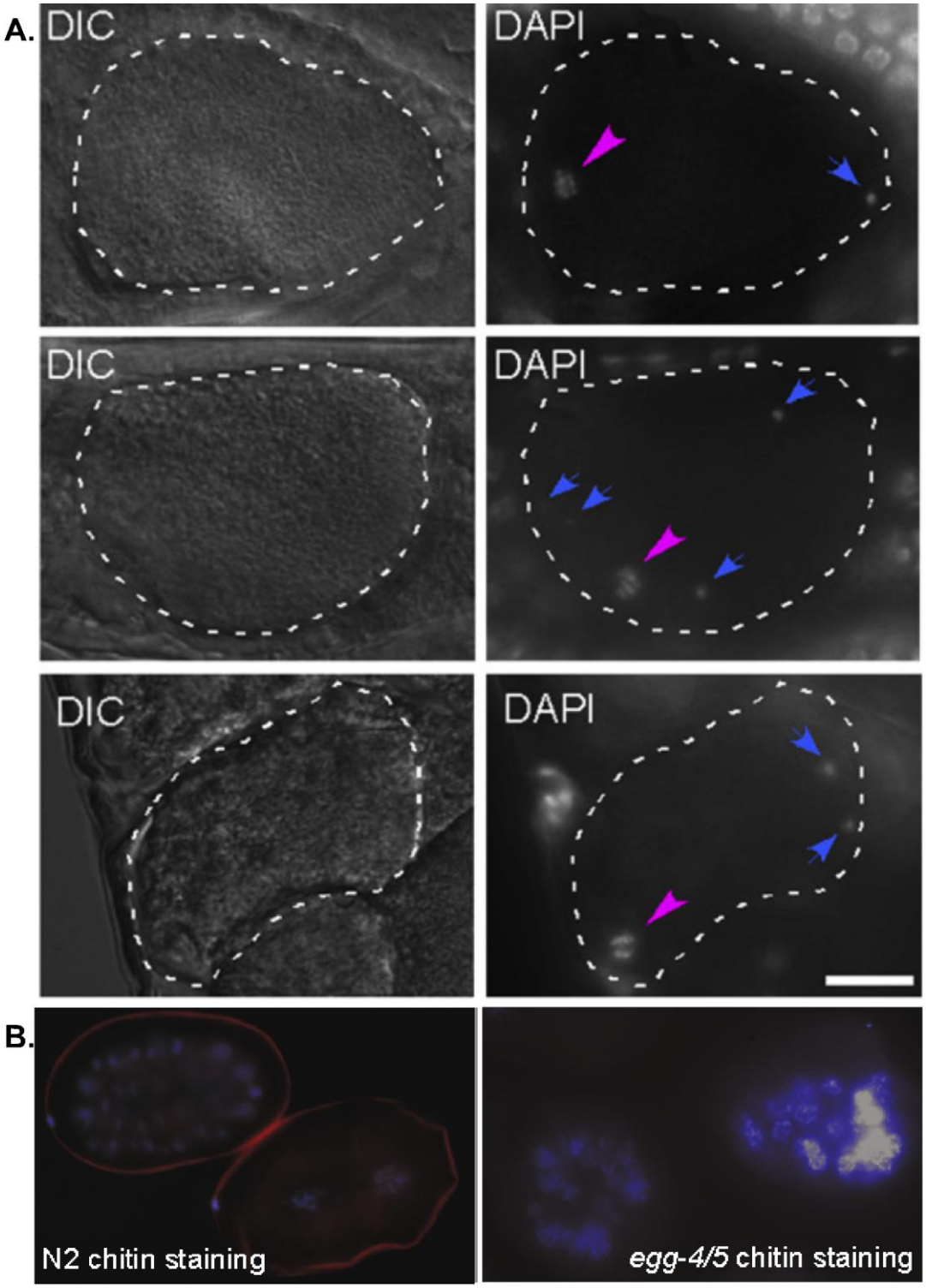

A.
DIC
DAPI
DIC
DAPI
DIC
DAPI
B.
N2 chitin staining
egg-4/5 chitin staining

Figure 4

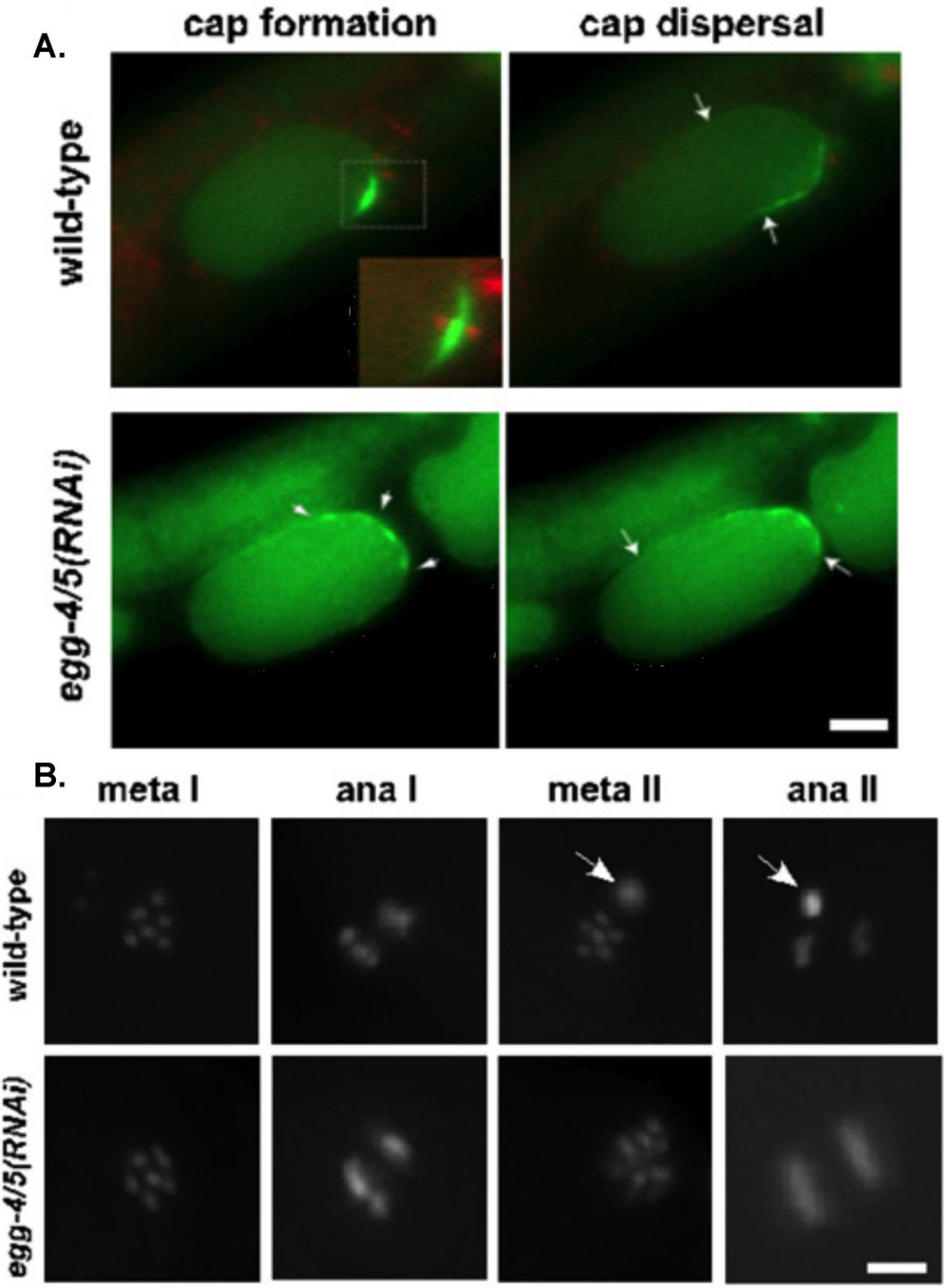

A.
cap formation
cap dispersal
wild-type
egg-4/5(RNAi)
B.
meta I
ana I
meta II
ana II
wild-type
egg-4/5(RNAi)

Figure 5

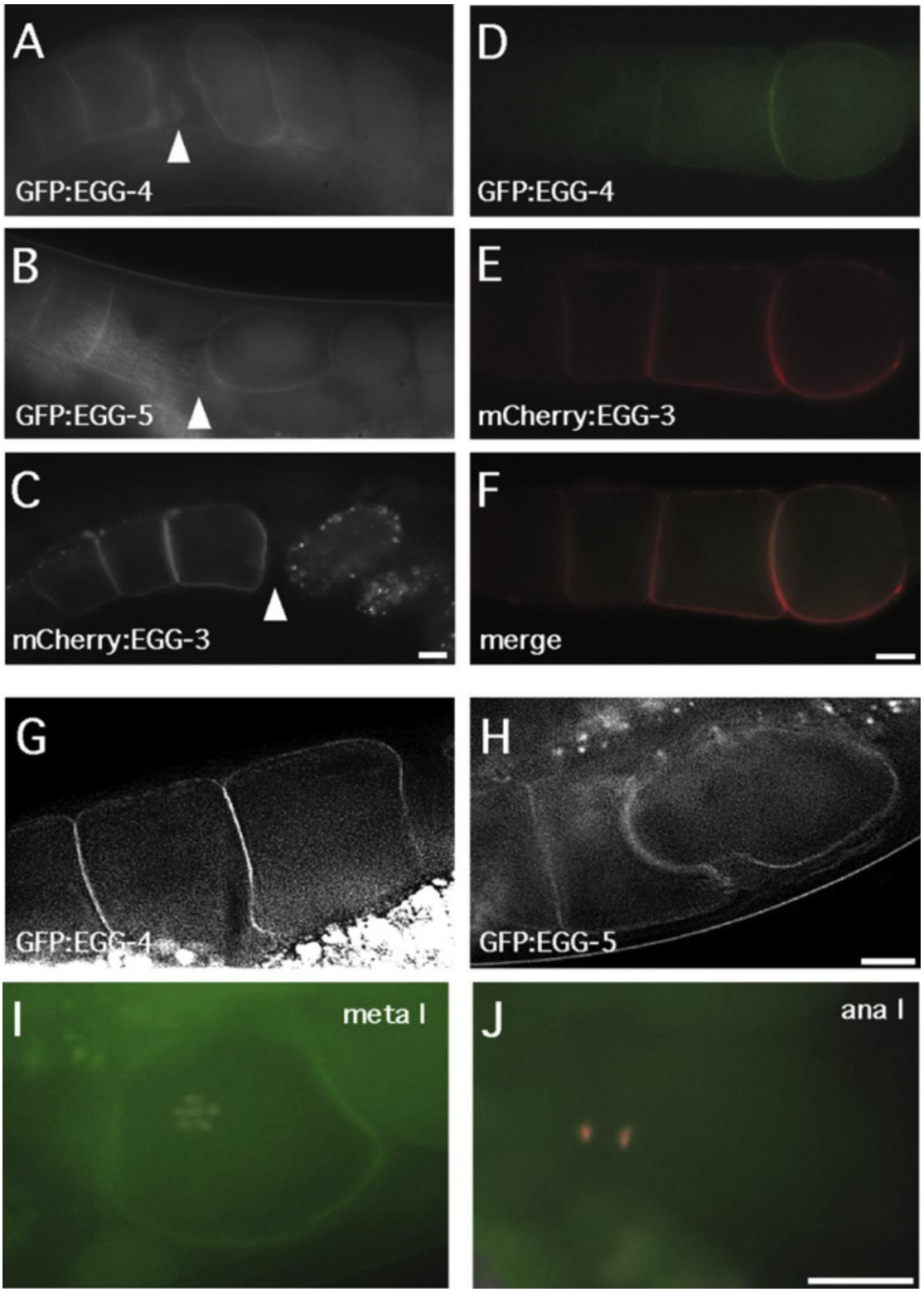

A
GFP:EGG-4
D
GFP:EGG-4
B
GFP:EGG-5
E
mCherry:EGG-3
C
mCherry:EGG-3
F
merge
G
GFP:EGG-4
H
GFP:EGG-5
I
meta I
J
ana I

Figure 6

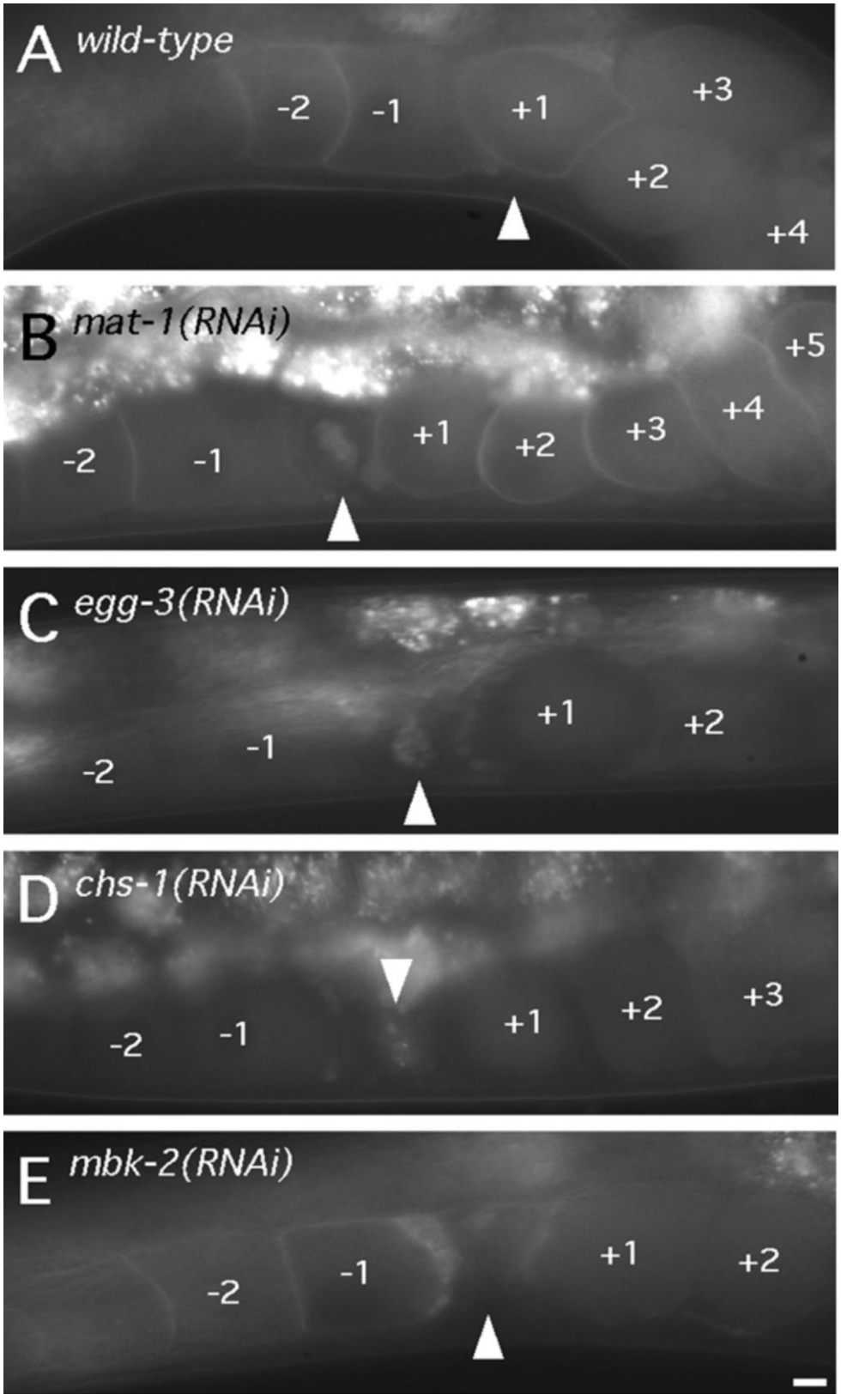

A wild-type
-2
-1
+1
+2
+3
+4
B mat-1(RNAi)
-2
-1
+1
+2
+3
+4
+5
C egg-3(RNAi)
-2
-1
+1
+2
D chs-1(RNAi)
-2
-1
+1
+2
+3
E mbk-2(RNAi)
-2
-1
+1
+2

Figure 7

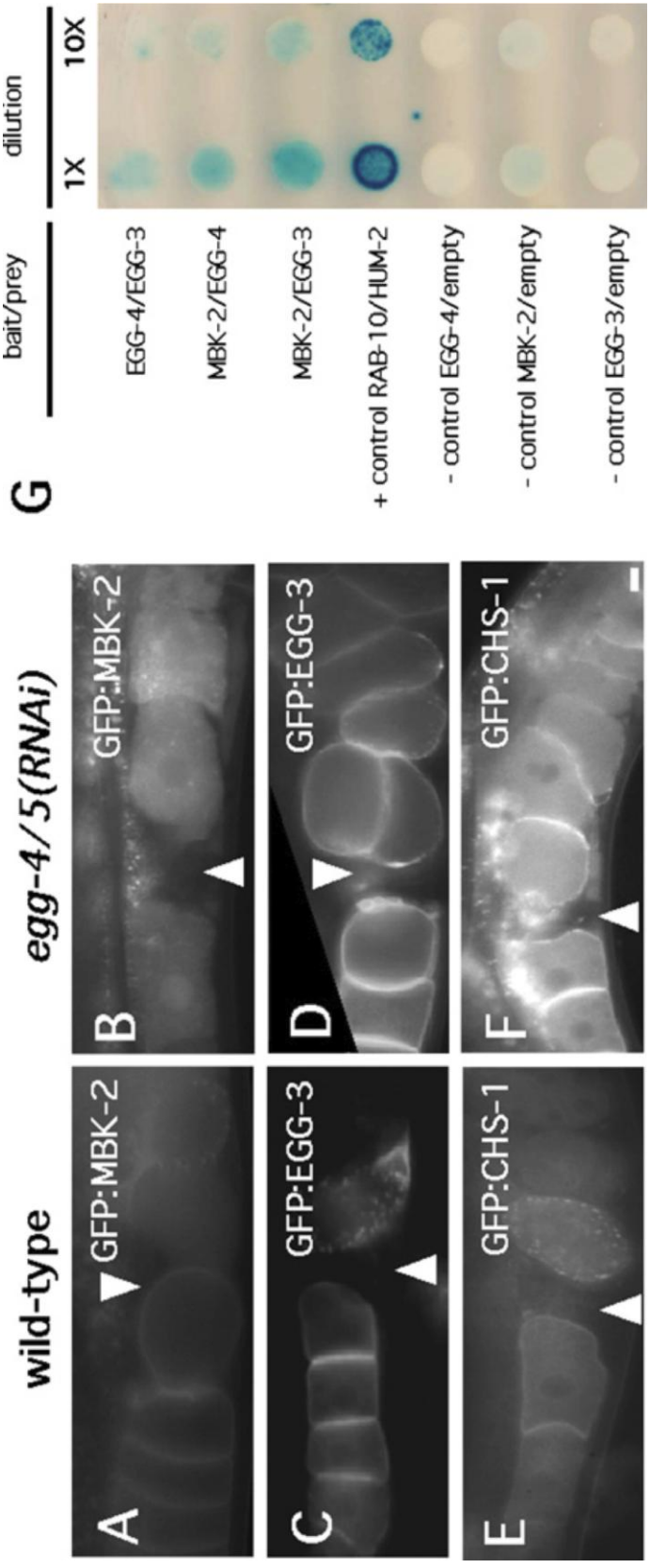

wild-type
egg-4/5(RNAi)
A
GFP:MBK-2
B
GFP:MBK-2
C
GFP:EGG-3
D
GFP:EGG-3
E
GFP:CHS-1
F
GFP:CHS-1
G
bait/prey
dilution
1X
10X
EGG-4/EGG-3
MBK-2/EGG-4
MBK-2/EGG-3
+ control RAB-10/HUM-2
- control EGG-4/empty
- control MBK-2/empty
- control EGG-3/empty

Figure 8

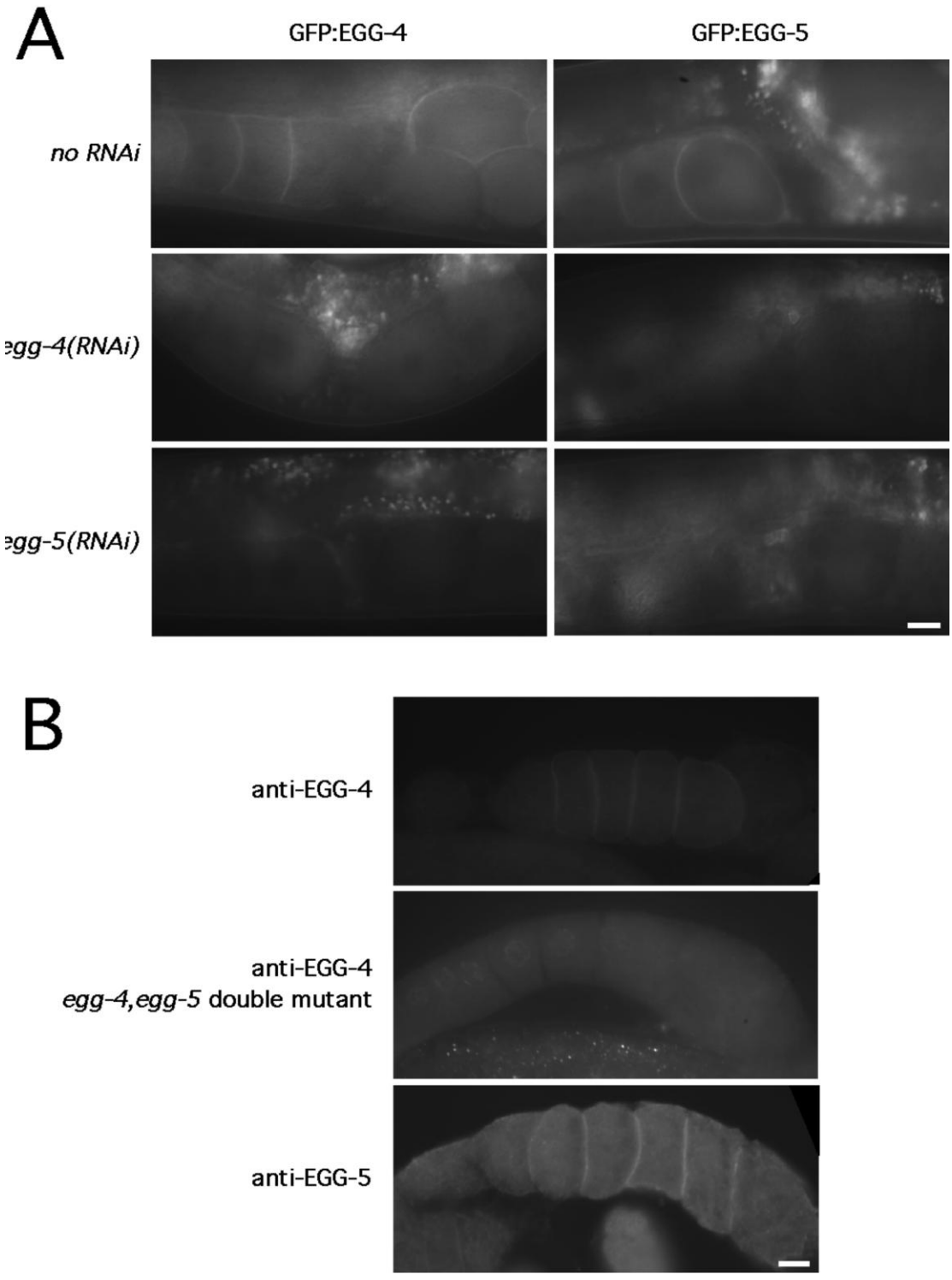

A
GFP:EGG-4
GFP:EGG-5
no RNAi
egg-4(RNAi)
egg-5(RNAi)
B
anti-EGG-4
anti-EGG-4
egg-4,egg-5 double mutant
anti-EGG-5

Figure 9

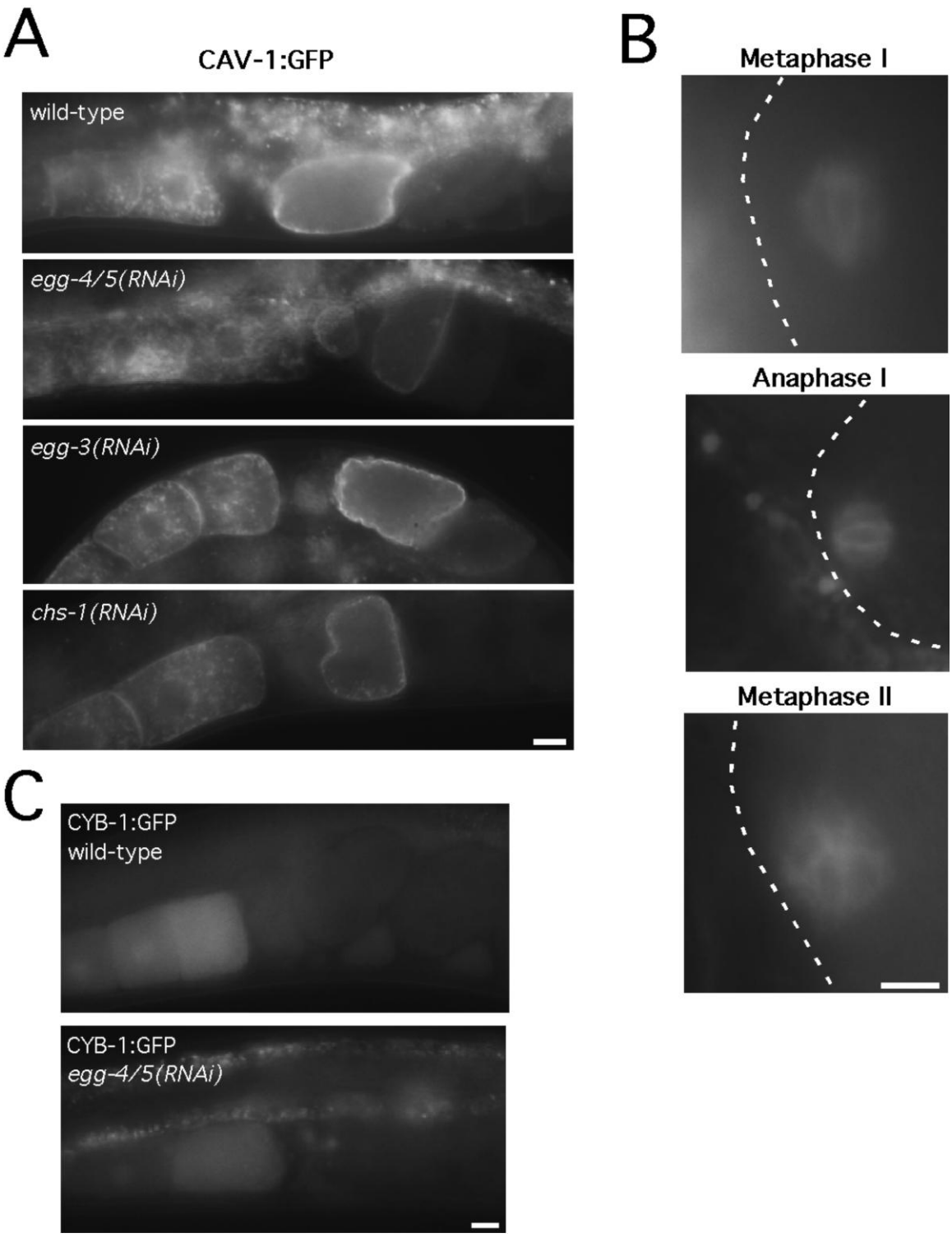
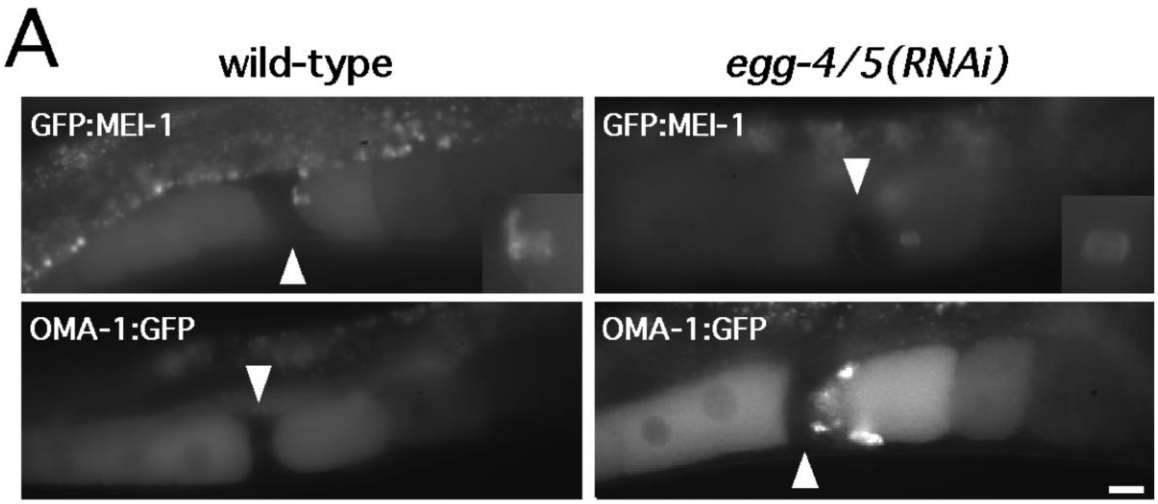

A
CAV-1:GFP
wild-type
egg-4/5(RNAi)
egg-3(RNAi)
chs-1(RNAi)
B
Metaphase I
Anaphase I
Metaphase II
C
CYB-1:GFP
wild-type
CYB-1:GFP
egg-4/5(RNAi)

Figure 10


A
wild-type
egg-4/5(RNAi)
GFP:MEI-1
GFP:MEI-1
OMA-1:GFP
OMA-1:GFP

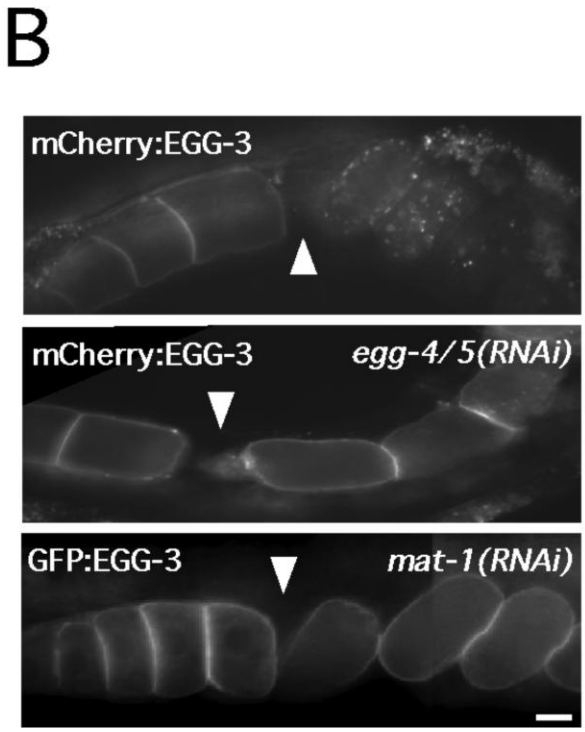

B
mCherry:EGG-3
mCherry:EGG-3
egg-4/5(RNAi)
GFP:EGG-3
mat-1(RNAi)

Figure 1

Table S1. Viable Progeny Produced by *egg-4* and *egg-5* Knockdown or Mutants

Genotype	Temperature ◦C	*Viable progeny % wild-type ± SD, (average number viable progeny ± SD)
wild-type (N2) control for *egg-4***	20	219±21
wild-type (N2) control for *egg-4***	25	147±37
egg-4(tm1508)	20	77±40 (168±88)
egg-4(tm1508)	25	47±24 (69±34)
wild-type (N2) control for *egg-5***	20	184±12
wild-type (N2) control for *egg-5***	25	141±29
egg-5(ok1781)	20	78±12 (143±23)
egg-5(ok1781)	25	23±27 (32±38)
egg-4(RNAi)	20	0
egg-4(RNAi)	25	0
egg-5(RNAi)	20	0
egg-5(RNAi)	25	0
egg-4(tm1508) egg-5(RNAi)	20	0
egg-5(ok1781) egg-4(RNAi)	20	0
egg-4(tm1508) egg-5(ok1781)	20	0

*Viable progeny counts were determined by counting the number of hatched larvae for a minimum of 10 worms. **Control brood sizes were conducted for a minimum of 10 wild-type worms at the same time under identical culture conditions. % wild-type was calculated against control brood counts. SD, standard deviation.

Table 2

Table S2. Reproductive Phenotypes		
Event/Phenotype	Wild-type	*egg-4/5(RNAi)*
Gonad morphology	+	+
Gamete morphology	+	+
Germline nuclear cell-cycle progression	+	+
Yolk uptake/oocyte growth/volume increase	+	+
Disappearance of the nucleolus	+	+
Distal nuclear migration	+	+
Nuclear envelope breakdown (NEBD)	+	+
Cortical rearrangement	+	+
Ovulation	+	+
Outcross progeny production*	+	-
Entry of sperm pronuclei	+	+
Polyspermy**	No	Yes (16%)(25% KO)
Completion of meiosis	Yes	Yes, defective
Polar body formation	+	-
Eggshell formation	+	-
Endomitosis in the gonad arm	-	-
Endomitosis in the uterus	-	+

All events were observed by Nomarski or fluorescence (DAPI stain) microscopy. *egg-4/5(RNAi)* fertility was comparable to wild-type with many outcross progeny being produced. *The ability of *egg-4/5(RNAi)* hermaphrodites to produce outcross progeny was determined by crossing RNAi treated adult hermaphrodites to wild-type males. **Polyspermy rates were determined for RNAi treated and double knockout (KO) animals.

Figure Legends

Figure 1:

A. Alignment of the phosphatase domains of PTPL family proteins. The EGG-4/5 ortholog in C. briggsae (CBG12049), the EGG-3 ortholog in C. briggsae (CBG20647), and a Drosophila melanogaster phosphatase (CG10975-PB). Blue, identical; red, conserved; green, semiconserved.
B. The gene structure of EGG-4 and EGG-5 are identical and therefore depicted with a single schematic. PTP domain, pink; amino acid sequences used to generate antisera, red. The sequences that are deleted by various alleles are indicated as brackets.

Figure 2:

A. DIC images of wild-type, *egg-4/5*(RNAi), and *egg-4/5* double mutant hermaphrodites in the region of the spermatheca. Arrowheads indicate the position of the spermatheca. Maturing oocytes are to the left of the spermatheca and fertilized eggs are in the uterus to the right for each panel. Properly developing embryos can be seen only in the uterus of wild-type animals. Scale bar represents 10 mm.

Figure 3:

A. DIC images of a newly fertilized *egg-4/5*(RNAi), *egg-4/5* double mutant embryos and DAPI staining of the same cells showing oocyte and sperm DNA. Polyspermy is seen in about 16% of RNAi-treated embryos (middle two panels, n = 49) and 25% in double mutants (bottom two panels, n =

29). Pink arrowheads indicate oocyte DNA and blue arrows indicate sperm DNA. Scale bar represents 10 mm.

B. Chitin staining of wild-type and *egg-4/5*(RNAi) embryos. Red staining is chitin; blue staining is DAPI. Scale bar represents 10 mm.

Figure 4

A. Compared to wild-type, actin cap formation and the polarized dispersal of F-actin is defective in *egg-4/5*(RNAi) embryos. A robust actin cap does not form in *egg-4/5*(RNAi) embryos. The dispersal of the F-actin in *egg-4/5*(RNAi) crossed 50% egg length as denoted by the arrows marking the edges of F-actin localization. Wild-type controls 15/15 embryos displayed wild-type actin dynamics. *egg-4/5*(RNAi); 4/5 embryos had abnormal cap formation and 3/6 had abnormal polarized dispersal. Scale bar represents 10 mm.

B. mCherry-histone in wild-type and *egg-4/5*(RNAi) embryos during meiosis. meta I, metaphase I; ana I, anaphase I; meta II, metaphase II; ana II, anaphase II. Arrows indicate the position of polar bodies. Scale bar represents 5 mm.

Figure 5

A. GFP:EGG-4. The arrowheads indicate the position of the spermatheca. Oocytes can be seen to the left of the spermatheca and embryos to the right.

B. GFP:EGG-5

C. mCherry:EGG-3. Protein moves to cortical foci in embryos at anaphase I, in contrast, at this stage GFP:EGG-4 (A) and GFP:EGG-5 (B) become diffusely cytoplasmic. Scale bar represents 10mm
D. GFP:EGG-4 in dissected hermaphrodite gonad
E. mCherry:EGG-3 in dissected hermaphrodite gonad
F. Merge of GFP:EGG-4 and mCherry:EGG-3 in same dissected hermaphrodite gonad. Scale bar represents 10mm
G. Subcellular localization of GFP:EGG-4 (green) with mCherry:HISTONE marked chromosomes (red). At metaphase I the EGG-4 protein is cortical
H. At anaphase I, the EGG-4 protein dissipates or becomes diffusely cytoplasmic.

Figure 6

In all panels the arrowheads indicate the position of the spermatheca. Oocytes are to the left of the spermatheca and embryos are to the right. Oocytes are labeled with negative numbers toward the spermatheca and embryos are labeled with positive numbers from youngest (+1) to oldest.

A. In wild-type animals shown for comparison. GFP:EGG-4 accumulates at the cortex of developing oocytes but is redistributed at anaphase I.
B. The redistribution of GFP:EGG-4 to the cytoplasm is dependent on cell cycle progression. In mat-1(RNAi) embryos, GFP:EGG-4 remains associated with the cortex.

C. The localization of GFP:EGG-4 is dependent on EGG-3. In *egg-3*(RNAi) animals, no GFP:EGG-4 is seen accumulating at the cortex of developing oocytes or embryos.

D. The localization of GFP:EGG-4 is dependent on CHS-1. In chs-1(RNAi) animals, no GFP:EGG-4 is seen accumulating at the cortex of developing oocytes.

E. The localization of GFP:EGG-4 is not dependent on MBK-2. In mbk-2(RNAi) animals, the localization of GFP:EGG-4 is indistinguishable from wild-type. Scale bar represents 10 mm.

Figure 7

A. In all panels (A-F), the arrowheads indicate the position of the spermatheca. Oocytes are to the left of the spermatheca and embryos are to the right. GFP:MBK-2 in wild-type.

B. Rather than accumulating at the cortex and forming cortical foci in embryos, GFP:MBK-2 is diffusely cytoplasmic in an *egg-4/5 RNAi* background.

C. GFP:EGG-3 in a wild-type background.

D. In *egg-4/5(RNAi)* animals, rather than forming foci at anaphase I, GFP:EGG-3 remains associated with the cortex despite meiotic progression.

E. GFP:CHS-1 in a wild-type background.

F. In *egg-4/5(RNAi)* animals, rather than forming foci at anaphase I, GFP:CHS-1 remains associated with the cortex despite meiotic progression. Scale bar represents 10 mm.

G. Binding of EGG molecules in the yeast two-hybrid assay as indicated by stimulation of the blue X-gal color reaction. The RAB-10/HUM-2 combination serves as a strong positive control. Negative controls show that each molecule alone cannot activate transcription of the reporter. Equivalent numbers of cells were plated in each panel.

Figure 8

A. RNAi treatment for *egg-4* not only knocks down GFP:EGG-4 but also knocks down GFP:EGG-5. RNAi treatment for *egg-5* not only knocks down GFP:EGG- but also knocks down GFP:EGG-4.

B. Anti-EGG-5 and anti-EGG-5 shows localization associated with the plasma membrane in developing oocytes. No staining associated with the plasma membrane is seen in *egg-4,egg-5* double mutants. There is a small amount of background nuclear staining seen in the double mutant panel.

Figure 9

A. *egg4/5(RNAi)* does not alter the localization or dynamics of CAV-1:GFP. CAV-1:GFP is a marker for the *C. elegans* cortical granule reaction.

B. The meiotic spindle translocates and rotates similar to wild-type in *egg4/5(RNAi)* worms. The spindle was visualized with GFP-tubulin.

C. CYB-1:GFP is degraded in *egg4/5(RNAi)* embryos.

Figure 10

A. *In egg4/5(RNAi)* worms, GFP:MEI-1 and OMA-1:GFP are degraded in a pattern that is similar to wild-type. The insets show the association of GFP:MEI-1 with the meiotic spindle at higher magnification. In all panels, the arrow head marks the location of the spermatheca. Oocytes are to the left of the spermatheca and fertilized embryos are to the right.
B. EGG-3 does not move to cortical foci in both *egg4/5(RNAi)* and *mat1(RNAi)* embryos. In control worms, mCherry:EGG-3 can be seen accumulating in cortical foci. The GFP:EGG-3, *mat-1(RNAi)* panel is modified from (Maruyama et al., 2007) and shown for comparison.

Chapter 3

The Cortical Granule Exocytosis and EGG-6

3.1.1 Cortical granule exocytosis and cellular changes in the newly fertilized embryo

Once the male and female gamete have found one another and fused, the newly formed zygote must undergo numerous rapid changes in order to ensure its survival. One region of rapid shift is the plasma membrane which is rapidly restructured in response to fertilization. A primary event occurring during restructuring is the cortical granule exocytosis. A possible trigger of this event is the release of intracellular calcium. In mammalian and other systems including humans, the penetration of the sperm causes a rapid increase in intracellular calcium.

Two possible triggers for the influx of intracellular calcium are the ligand/receptor binding of sperm protein to egg protein, or the entrance of a sperm factor during fertilization (Tsaadon et al., 2006). One hypothesis puts forth that binding of an egg receptor to a sperm ligand triggers a signaling cascade which ends in the activation of phospholipase Cζ, which then triggers calcium influx (Tsaadon et al., 2006). Alternatively, sperm derived phospholipase Cζ in mouse models has been found sufficient to trigger intracellular calcium release in embryos (Cox et al., 2002; Tsaadon et al., 2006).

In either case, phospholipase Cζ would trigger a common phosphoinositide signaling pathway leading to activation of IP3 and diacylglycerol, then protein

kinase C, and intracellular calcium rise (Tsaadon et. al., 2006). The critical calcium increase is necessary for following events of egg activation.

A primary event following calcium influx is the release of the cortical granules to the cell surface. Although cortical granules have been identified in many model organisms, their contents have not been fully elucidated. However, it is known that they contain both proteoglyans and glycoproteins (Sato et al., 2008; Tsaadon et al., 2006). In mammals it is suggested that the contents of the cortical granules are responsible for the glycosylation of the egg coat ZP3. As previously discussed, the ZP3 coat is necessary for the binding of sperm and egg in mammals, thusly the cortical granules alter the plasma membrane coat and prevent binding of further sperm (polyspermy) to the newly fertilized embryo.

3.1.2 The cortical granule exocytosis in *C. elegans*

The model organism *C. elegans* also undergoes a cortical granule exocytosis directly after the fertilization event occurs (Bembenek et al., 2007; Sato et al., 2008). The cortical granules in *C. elegans* have been shown to contain both chondroitin proteoglycans and mucin-like glycoproteins (Bembenek et al., 2007; Sato et al., 2008). In addition to restructuring the extracellular matrix (ECM), the cortical granule exocytosis is important for formation of the eggshell which surrounds the *C. elegans* embryo during development and provides mechanical support and a chemical barrier (Johnston et al., 2006, Sato et al., 2006). The

cortical granule bodies in *C. elegans* are defined by the caveolin-1 (CAV-1) protein (Sato et al., 2006; Sato et al., 2008). Caveolins have been indentified in many organisms and are capable of binding cholesterol. Caveolins have been implicated in a number of intracellular processes including signaling and endocytosis (Sato et al., 2006). The caveolin proteins localize intracellularly to caveolae, large bodies which contain glycosphingolipids and cholesterol and form large invaginations on plasma membranes.

The CAV-1 proteins in the *C. elegans* oocyte and embryo have a highly dynamic spatial and temporal localization (Sato et al., 2006; Sato et al., 2008). During development of the distal oocytes attached to the rachis, CAV-1 is found on the plasma membrane (Sato et al., 2006). Later in development, as the oocytes individuate at the round of the bend CAV-1 is found in small cortical vesicals; while in the most mature oocytes approaching the spermatheca, CAV-1 is found in large cytoplasmic ring structures in addition to the small vesicles (Sato et al., 2006). Tellingly, CAV-1 undergoes a rapid relocalization directly prior to ovulation and fertilization, clustering at the cortex of the -1 oocyte, before fusing with the plasma membrane (Sato et al., 2006). After fertilization, CAV-1 is slowly internalized through small vesicles and degraded in the one cell embryo (Sato et al., 2006).

While it is recognized that the *C. elegans* cortical granules, as defined by CAV-1 contain both proteoglycans and glycoproteins, which are components of the

eggshell, other components of the cortical granules have not been identified (Sato et al., 2008). The proteins contained in the cortical granules are critical to restructuring the extracellular matrix of the newly fertilized embryo, for support of future development.

In order to find further components of the cortical granules, critical to the oocyte-to-embryo transition, we searched the gene ontology model developed by the Gunsalus lab. This model predicts proteins which might affect the osmotic integrity of the embryo based on wide-scale RNAi screens, yeast-two hybrid screens, and published data. One critical function of the cortical granule exocytosis is the formation of the eggshell, therefore, components of the cortical granules might be required for the osmotic integrity of the embryo. The gene K07A12.2 was selected for further study, based on the osmotic integrity defect and embryonic lethality observed in previous RNAi experiments.

3.2 Materials and Methods

3.2.1 General Methods and Strains

C. elegans strain maintenance and general genetic crosses were performed as described by S. Brenner [S3]. Strains used in experiments included: wild-type variety Bristol, strain N2; pwIs281 [*CAV-1-GFP*, *unc-119(+)*]; JA1334,

wels11[unc-119(+)TAC-1::GFP]; *par-4(it47);* SS747, bnIs1[pie-1::GFP::pgl-1 + unc-119 (+)];

Strains were maintained at 20oC or 25oC

3.2.2 GFP fusions

To create GFP fusions for EGG-6, genomic DNA for each gene, lacking a start codon and including a stop codon, was amplified from purified *C. elegans* N2 DNA using the primers (GGGGACAACTTTGTACAAAAAAGTTGtggcgttgaacagcgaagtg and GGGGACAACTTTGTACAAGAAAGTTGttagaattcgaagacgctcttc) and inserted by Gateway recombination cloning into intermediate vector pDONR221. A clone encoding EGG-6 was identified by DNA sequencing. Then EGG-6 sequence was transferred to destination vector pID3.01B (gift from G.Seydoux) using Gateway Technology (Invitrogen). The constructs were then integrated into the *C. elegans* genome at low copy number using the microparticle bombardment method [S4].

3.2.3 RNAi Interference

RNAi interference was carried out by the soaking method [S7, S8]. dsRNA was prepared from whole genome lysate using primers identified by Sonnichsen et al., 2005, targeting a 500bp region. P0 worms were soaked at 20oC for 24hrs,

then recovered on OP50 plates at 20°C or 25°C for 24hrs. P0 worms were examined by Nomarski and fluorescent microscopy.

3.3 Results

3.3.1 Identification of the EGG-6 protein in *C. elegans*

The class of proteins motifs known as Leucine Rich Repeats (LRR), is defined by a series of sequence motifs containing 20-29 amino acid residues with leucines, or other hydrophobic amino acids in key positions (Kobe and Kajava, 2001). The general purpose of the repeating motifs is to create a specific three-dimensional protein structure in which each LRR motif corresponds to a β sheet and an α helix connected by a loop (Kobe and Kajava, 2001). The combination of LRR motifs results in a horseshoe shaped molecule in which the β sheets limn the concave side and the α helices the convex side. Protein-protein interactions are facilitated by a wide variety of amino acids placed at specific points in the three-dimensional structure of the LRR containing proteins (Kobe and Kajava, 2001).

The *Caenorhabditis elegans* protein *egg-6* (K07A12.2) is predicted to contain 12 LRR motifs, which are most similar in structure to the Ribonuclease Inhibitor Like family of LRR motifs. It is also predicted to contain a transmembrane domain at the C-terminal end and a signal peptide at the N-terminal end. Thus we predict that EGG-6 would form a type-1 transmembrane protein, with the LRR repeats in

the vesicular lumen or extracellular to the plasma membrane. Given structural evidence, *egg-6* is also likely involved directly in a protein-protein binding interaction. Experimental evidence suggests that the EGG-6 protein is glycosylated at its N-terminus (Kaji et al., 2007). Interactome data indicates that EGG-6 may interact with two proteins, the first, *lin-45,* encodes a Serine/threonine protein kinase RAF required for fertility and vulval cell fates, and the second, *tac-1*, is required for pronuclear migration and mitotic spindle elongation (Boxem et al., 2008). In order to decipher the activity of EGG-6 *in vivo* we used gene specific RNAi depletion, and fluorescent tagged fusion proteins to elucidate the gene's functions and location of activity.

3.3.2 Phenotypic Analysis of EGG-6 RNAi Knockdown

Three previous wide-scale RNAi screens have identified the *egg-6* as having a penetrant embryonic lethal phenotype. Unfortunately, the deletion allele of *egg-6* is fully embryonic lethal and could not be viably recovered. One screen performed by Sonnichsen et. al, used RNAi by injection of short (500 bp) sequences, and identified *egg-6* as osmotic integrity defective (Sönnichsen B, 2005). Targeting the sequence utilized by Sonnichsen et. al, we performed RNAi by the soaking method to knockdown *egg-6*.

Knockdown of EGG-6 brought about embryonic lethality. Soaked P0 hermaphrodites were completely sterile and produced only dead embryos (Figure

1A and B). P0 hermaphrodites produced phenotypically normal oocytes and sperm; while sperm were capable of successfully causing fertilization (Figure 1C). Meiosis progressed as in wild- type, followed by successful polar body extrusion (Figure 1D). The pronuclear formation, fusion, and first division appeared phenotypically normal (Figure 1D). However, embryos uniformly died by the thirty-cell stage which corresponds approximately to gastrulation (Figure 2A).

3.3.4 Phenotypic Similarities to *par, tac-1,* and *lin-45*

The embryonic lethal phenotype of *egg-6*, in which cells die at the 30 cell stage, is highly similar to the phenotype shown by PAR genes, which are defective in establishment of embryonic polarity. We wished to determine whether the terminal phenotypes of the *tac-1* gene, which interactome data indicates may interact with EGG-6, were similar to the terminal phenotypes of *egg-6* and *par*. We acquired deletion alleles of both the *par-4* and *tac-1* genes, and compared their terminal phenotypes to the RNAi knockdown of *egg-6*. In all cases, oocytes and sperm were phenotypically normal and fertilization occurred, yet all embryos died at approximately the thirty-cell stage (Figure 1A, B, anc C). This indicated that EGG-6 might be necessary for the establishment of embryonic polarity and cytoskeletal integrity.

3.3.5 Defects in Establishment of Polarity

The par genes are required for the correct polarization of the cell during embryonic development. The terminal phenotypes of *egg-6* and *par-4* are highly similar leading to the possibility that *egg-6* might play a role in the establishment of polarity. A primary method for examining polarity is to examine the distribution of P-granules, large bodies containing primarily RNA and protein, which localize to the germ cell lineages (Updike and Strome, 2010). The knockdown of *egg-6* by RNAi prevented the proper localization of P-granules to the germ cells in some thirty-cell stage embryos. Further analysis is required in order to determine the severity of the polarity defect in *egg-6* animals. It will be useful to further examine the localization of P-granules in an *egg-6 RNAi* background in order to fully elucidate its phenotype. It should also be determined whether the establishment of polarity in the initial cell division occurs as in wild type. Also the localization of the PAR proteins should be directly examined using GFP tagged PAR proteins, which are available from the *C. elegans* Genomic Center.

3.3.6 Cellular Localization of EGG-6:GFP

In order to establish the cellular localization of the EGG-6 protein *in vivo*, we generated a c-terminal gfp fusion protein. Live imaging of the resulting transgenic hermaphrodites showed a highly dynamic pattern of localization, in which the protein localized to large cytoplasmic ring structures during late oogenesis, followed by fusion with the plasma membrane during nuclear

envelope breakdown (Figure 4A-F). The protein did not persist past the one cell stage (Figure 4A-F). The gfp signal generated by our transgenic hermaphrodites was weak, thus in order to image the localization more robustly we fixed hermaphrodites and performed immunofluorescence using anti-gfp primary antibody. The fixed-cell imaging of EGG-6:GFP revealed a clearer pattern of highly dynamic localization as described above (Figure 4C-F). Furthermore, RNAi knockdown of EGG-6 in the EGG-6:GFP strain followed by immunostaining, led to complete abrogation of the gfp signal (not shown). This indicated both the *in vivo* gfp localization, and immunolocalization were biologically specific.

3.3.7 Comparison of localization with the CAV-1 Protein

It was noted that the localization of EGG-6:GFP bore striking similarity to the localization of the CAV-1 protein (Figures 3A and 3B). As discussed above, the CAV-1 protein marks the cortical granules. Thus if co-localization with CAV-1 were conclusively shown, the EGG-6 protein would likely take part in the cortical granule exocytosis. In order to determine whether the EGG-6 protein was required for localization of the cortical granule proteins, we performed RNAi against EGG-6 in a CAV-1:GFP background. The knockdown of EGG-6 had no effect on the localization of CAV-1:GFP, indicating that EGG-6 is not required for localization of the cortical granules or their exocytosis (Figure 3C and D).

However, this does not rule out a function for EGG-6 in the cortical granule exocytosis.

3.4.1 Discussion and Directions

The *egg-6* gene was selected for further study based on its embryonic lethality at the thirty-cell stage and its lack of osmotic integrity. In addition, the presence of leucine-rich repeats in the EGG-6 sequence present a likely ability in protein-binding.

The EGG-6 protein has been identified as a potential, new component of the cortical granules, based on its highly similar localization pattern to cortical granule marker CAV-1. The cortical granules are an important group of proteins, which are released immediately following fertilization, to the plasma membrane of the embryo. The proteins contained in the cortical granules are critical to restructuring the extracellular matrix of the newly fertilized embryo, for support of future development, including the formation of the eggshell. However, few components of the cortical granules have been identified, so their molecular functions during embryonic development are largely unknown.

Depletion of *egg-6* by RNAi leads to the mislocalization of P-granules in some thirty-cell stage embryos, indicating that generation of polarity at this stage is aberrant. Therefore, the *egg-6* gene represents the first evidence that a

component of the cortical granules may be required for the establishment of polarity during embryonic development.

Figure 1

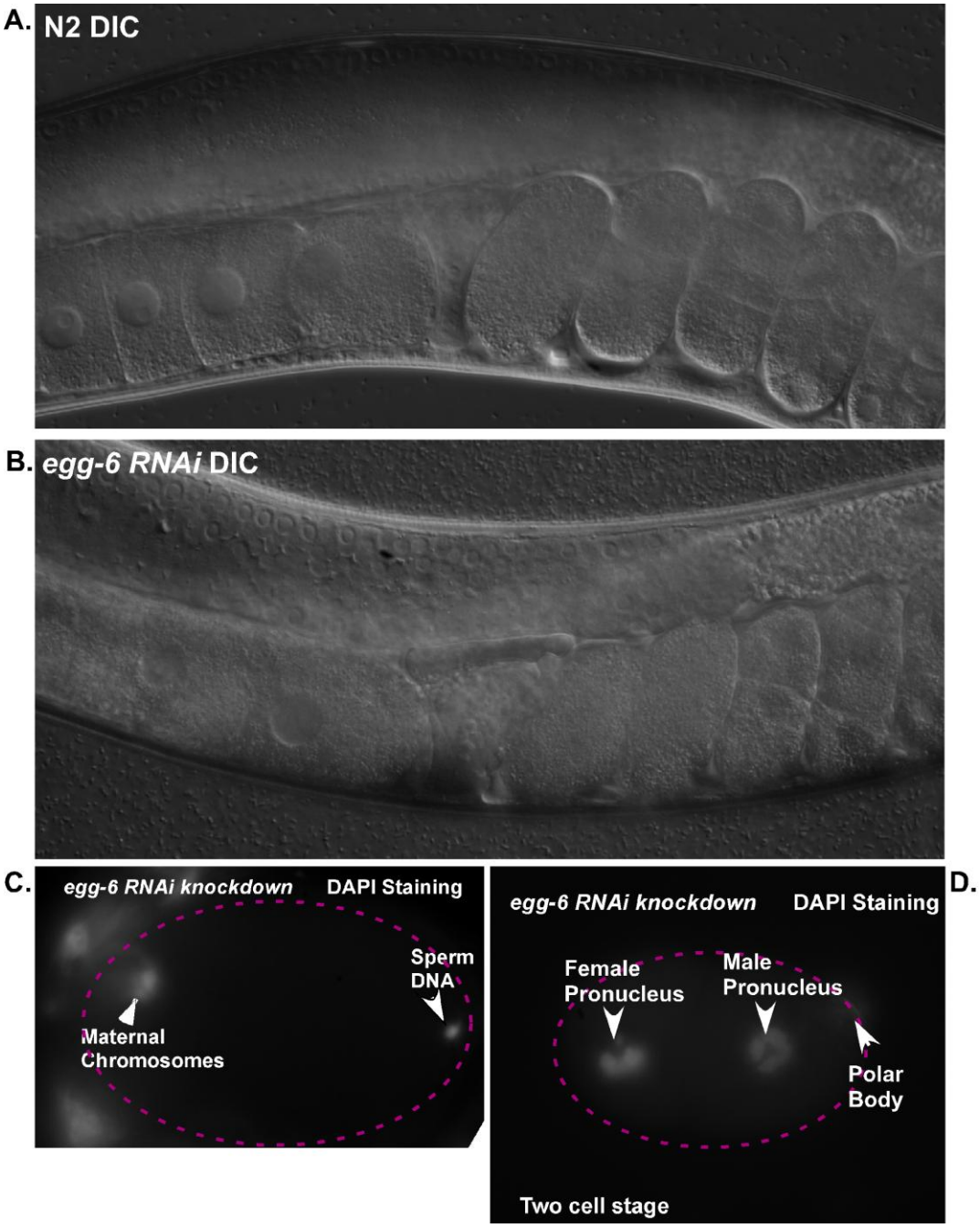

A.
N2 DIC
B.
egg-6 RNAi DIC
C.
egg-6 RNAi knockdown
DAPI Staining
Sperm DNA
Maternal Chromosomes
D.
egg-6 RNAi knockdown
DAPI Staining
Female Pronucleus
Male Pronucleus
Polar Body
Two cell stage

Figure 2

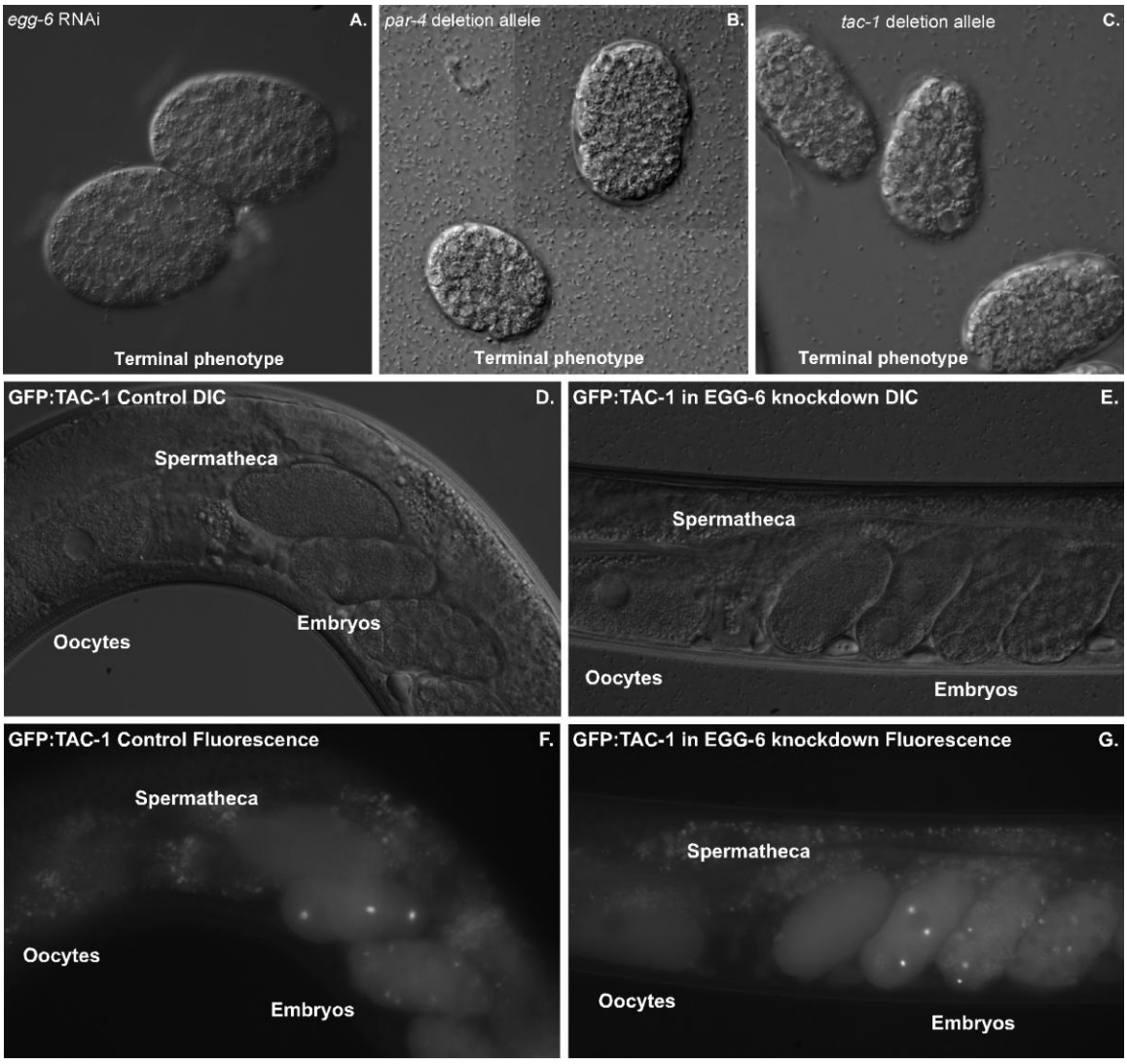

egg-6 RNAi
A.
Terminal phenotype
par-4 deletion allele
B.
Terminal phenotype
tac-1 deletion allele
C.
Terminal phenotype
GFP:TAC-1 Control DIC
D.
Spermatheca
Embryos
Oocytes
GFP:TAC-1 in EGG-6 knockdown DIC
E.
Spermatheca
Oocytes
Embryos
GFP:TAC-1 Control Fluorescence
F.
Spermatheca
Oocytes
Embryos
GFP:TAC-1 in EGG-6 knockdown Fluorescence
G.
Spermatheca
Oocytes
Embryos

Figure 3

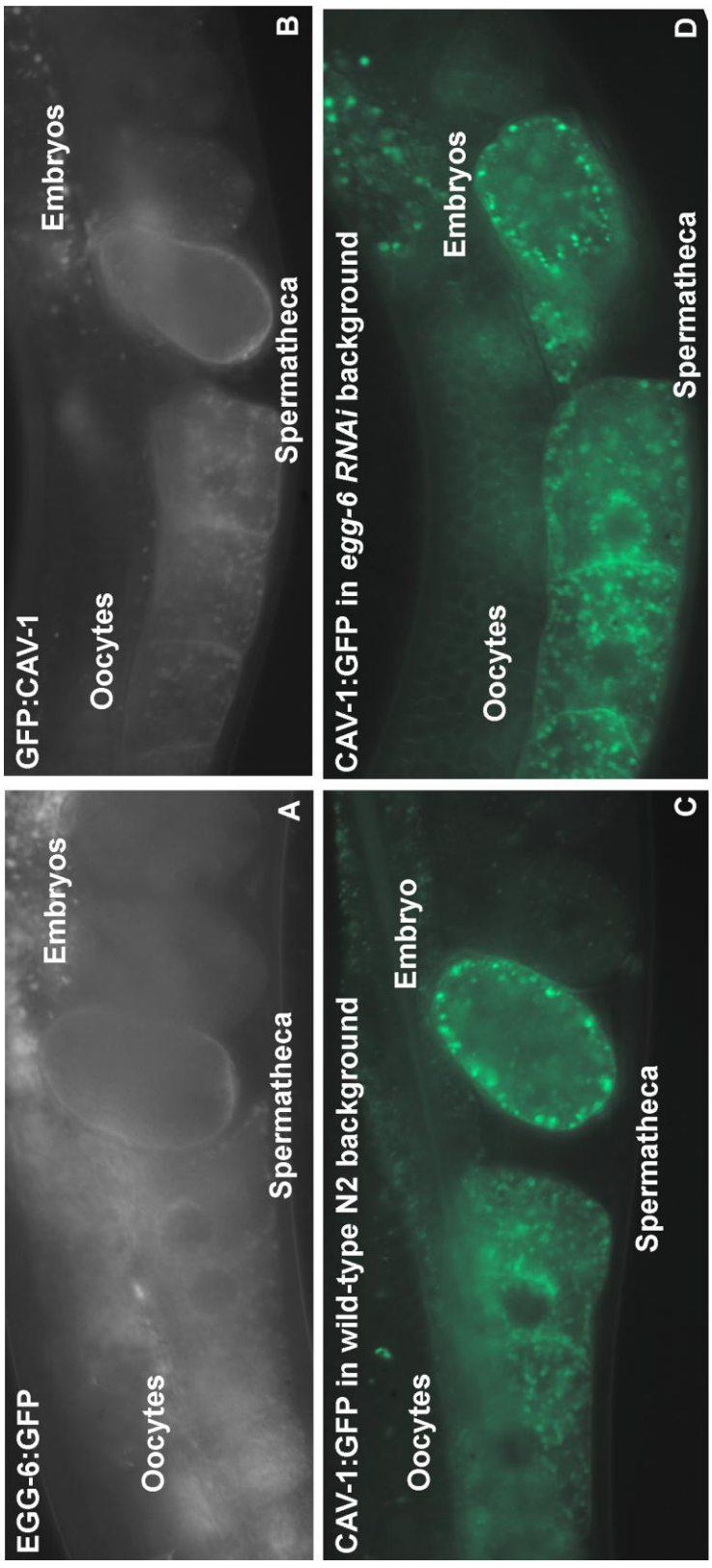

EGG-6:GFP
Embryos
Oocytes
Spermatheca
A
GFP:CAV-1
Embryos
Oocytes
Spermatheca
B
CAV-1:GFP in wild-type N2 background
Embryo
Oocytes
Spermatheca
C
CAV-1:GFP in egg-6 RNAi background
Embryos
Oocytes
Spermatheca
D

Figure 4

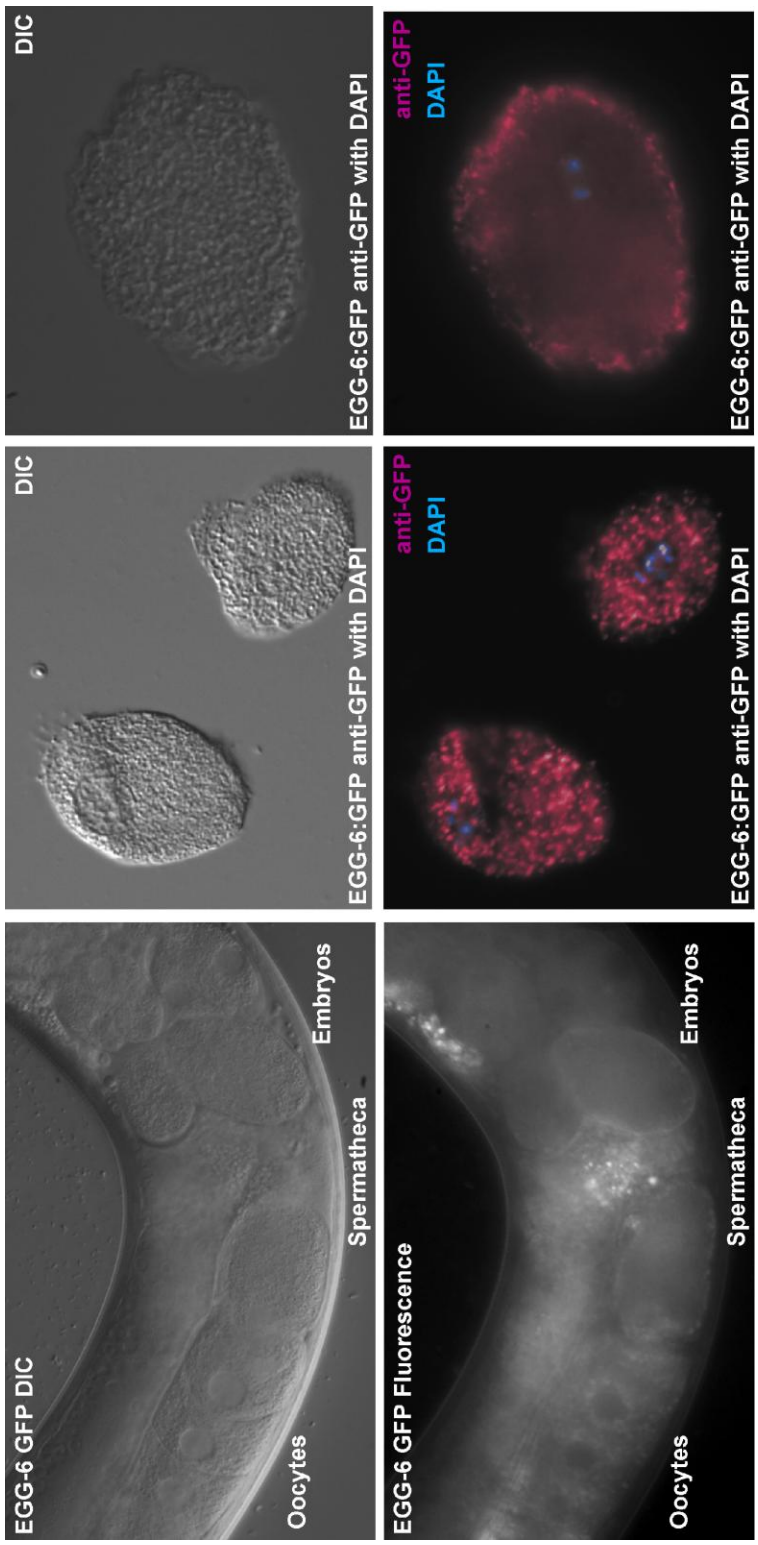

EGG-6 GFP DIC
Oocytes
Embryos
Spermatheca
EGG-6 GFP Fluorescence
Oocytes
Embryos
Spermatheca
DIC
EGG-6:GFP anti-GFP with DAPI
anti-GFP
DAPI
EGG-6:GFP anti-GFP with DAPI
DIC
EGG-6:GFP anti-GFP with DAPI
anti-GFP
DAPI
EGG-6:GFP anti-GFP with DAPI

Figure Legends

Figure 1

A. N2 DIC, wild-type adult hermaphrodite.
B. *egg-6 RNAi* DIC, adult hermaphrodite. Early embryos appear phenotypically normal.
C. *egg-6 RNAi* DAPI staining showing DNA. Sperm entry occurs as in wild-type hermaphrodites.
D. *egg-6 RNAi* DAPI staining. Meiosis, polar body extrusion, and pronuclear formation occur as in wild-type.

Figure 2

A. The terminal phenotype of *egg-6 RNAi* knockdown. The dead embryos arrest at approximately the thirty-cell stage which corresponds to gastrulation.
B. The terminal phenotype of *par-4* deletion mutants. Similar to *egg-6 RNAi*, the embryos arrest at approximately the thirty-cell stage.
C. The terminal phenotype of *tac-1* deletion mutants. Similar to *egg-6 RNAi* and *par-4* mutants, the embryos arrest at approximately the thirty-cell stage.

Figure 3

A. EGG-6:GFP in a wild-type background. The EGG-6 protein forms large punctate bodies in the cytoplasm of the developing oocyte. At NEBD, EGG-6 fuses with the plasma membrane. After fertilization, EGG-6 is diffused or degraded.

B. CAV-1 GFP in a wild-type background. CAV-1 marks the distinct cortical granules in *C. elegans*. The EGG-6 protein shows a highly similar localization to CAV-1.

C. CAV-1:GFP in a wild-type background.

D. CAV-1:GFP in a *egg-6 RNAi* background. Knocking down the EGG-6 protein has no visible effect on the distribution of the CAV-1 protein.

Figure 4

A. EGG-6:GFP in a wild-type background, under DIC, light microscopy.

B. EGG-6:GFP in a wild-type background under fluorescence microscopy.

C. EGG-6:GFP in a wild-type background. Fixed and immunostained with anti-gfp primary antibodies. Anti-gfp (red) and DAPI (blue).

EGG-6:GFP in a wild-type background. Fixed and immunostained with anti-gfp primary antibodies. Anti-gfp (red) and DAPI (blue). When the maternal chromosomes are at anaphase I, EGG-6 has transitioned to the plasma membrane.

Chapter 4

Spermiogenesis in *C. elegans* and the *spe-43* gene

4.1.1 An introduction to spermiogenesis

Before the sperm and egg meet and merge, each gamete must be perfectly formed and fully capable of fertilization. For the male gamete, the spermatozoa, a haploid germ cell must be formed and then gain motility and the capacity for fertilization. Like mammalian sperm, the sperm of *C. elegans,* both male derived and hermaphrodite derived, must undergo meiosis to form the haploid gamete, differentiation to take on the unique characteristics required for species specific fertilization such as acrosome formation in mammals and membranous organelle formation in *C. elegans*, finding and fusing with the female gamete, and integrating with the female gamete to form a new organism. Mutants have been elucidated during each step of *C. elegans* spermatogenesis, including a number that act during the acquisition of cell motility, referred to as spermiogenesis (L'Hernault, 2006). In the case of *C. elegans* the process of spermiogenesis occurs in the absence of new protein synthesis. During the formation of the haploid gamete, a residual body is extruded containing the actin, tubulin, and ribosomes from the immature spermatocytes (L'Hernault 2006). Thus, the maturation of sperm must occur with a limited body of resources.

4.1.2 The precedent for differentiation without new protein synthesis

This requirement for differentiation in the absence of de novo protein synthesis is hardly unique to *C. elegans* spermiogenesis. During mammalian fertilization,

the acrosome reaction must occur in the absence of de novo synthesis. Briefly, the acrosome derives from the golgi body, and is a membranous compartment which sits directly under the head of the sperm and contains a number of glycoproteins and enzymes (Wassarman et al., 2001). The plasma membrane of the mammalian egg is coated in glycoproteins known collectively as the zona pellucida, and it is one of these glycoproteins, mZP3, which binds to the head of the sperm and stimulates the acrosome reaction (Wasserman et al., 2001). Binding of mZP3 activates G proteins to cycle, which brings about an increase in intracellular concentrations of calcium, through the activation of ion channels (Branham et al., 2006). Ion channel activation and calcium influx lower the membrane potential of the sperm and cause the subsequent fusion of the acrosome membranes and release of the acrosomal contents which allow the sperm and egg fusion to occur (Abou-Haila and Tulsiani, 2000; Wassarman et al., 2001).

4.1.3 Living evolution, transition from gonochoristic to androdioecious

While the requirement for cell differentiation without synthesis is not unique to *C. elegans* spermiogenesis, the process does offer a highly informative case of evolutionary divergence. Some species of *Caenorhabditis* such as *elegans* or *briggsae* are androdioecious, having hermaphrodites and males. While other species of *Caenorhabditis* such as *remanei* are gonochoristic having female and male sexes. Thus the divergence of the reproductive systems in androdioecious

and gonochoristic species, which are closely related, offers an excellent tool for studying evolution in development (Baldi et al., 2009). Recent work on the pathway which determines whether germ cells form sperm or oocytes has given insight into the evolution of hermaphroditism in *Caenorhabditis* (Baldi et al., 2009). This work made use of the inherent differences in germ cell differentiation between hermaphrodites which must produce eggs and sperm, and males which produce only sperm. A similar dichotomy in differentiation exists during spermiogenesis in hermaphrodites and males (Geldziler et al., 2005; Minniti et al., 1996; Nance et al., 2000; Nance et al., 1999; Shakes and Ward, 1989). As previously discussed, hermaphrodites produce first sperm and then oocytes. The first ovulation pushes the sessile spermatids into the spermatheca and stimulates spermiogenesis (Figure 1D) (Hubbard and Greenstein, 2000). In males the process of spermiogenesis is necessarily divergent, as the sperm must be transferred during mating in the sessile, undifferentiated form. It has been shown that premature activation of the male sperm prevents proper transfer to the hermaphrodite reproductive tract (Stanfield and Villeneuve, 2006). Accordingly, the molecules which act in the spermiogenesis pathway appear to diverge, which we will discuss in more depth below. Thus, the differences between activation methods in hermaphrodites, females, and males could be easily exploited to study the evolution of fertilization.

4.1.4 The process of producing motile sperm

The process of spermiogenesis occurs in a brief series of well defined events. During manufacture of the primary spermatid, the ribosomes, actin and tubulin are packaged into the residual body (L'Hernault, 2006). The implications of this are two-fold, first that the process of spermiogenesis must necessarily be a simple process without de novo synthesis, and second that the formation of the characteristic pseudopod occurs in the absence of actin and tubulin and must utilize a species specific cytoskeletal protein called MSP (Major Sperm Protein). The MSP proteins comprise a very large class of cytoskeletal proteins in *C. elegans*, which function not only during pseudopod formation, but also as a sperm signal to both the soma and oocyte indicating the presence of sperm (Cheng et al., 2008; McGovern et al., 2007).

Briefly, the stages of spermiogenesis are membranous organelle fusion, MSP polymerization and extension, and MSP coalescion into the pseudopod (Figure 1) (Smith 2006). The membranous organelles, formed from the golgi apparatus during meiosis, fuse with the plasma membrane and deposit their contents on the cell surface. In addition, molecules of Major Sperm Protein (MSP), which are deposited into the cytoplasm during budding of the spermatid from the residual body, are stimulated to polymerize and extend in two distinct steps (Roberts et al., 1986; Roberts and Ward, 1982). In the first step, MSP rapidly polymerizes filaments which are extended from the spermatid in spikes. Then the MSP spikes undergo a rapid collapse and reform as the single, polar pseudopod (Figure 1) (Roberts 1997, Shakes 1989).

4.1.5 Spermiogenesis in *C. elegans*, the known quantities

Previously elucidated members of the spermiogenesis pathway in *C. elegans* have been given the “spe” designation indicating their defect in the larger process of spermatogenesis. These genes include *spe-8, spe-12, spe-19, spe-27,* and *spe-29* (Geldziler et al., 2005; Minniti et al., 1996; Nance et al., 2000; Nance et al., 1999; Shakes and Ward, 1989). Genes of this class are referred to as spe-8 class genes, based on the canonical phenotypes of the spe-8 gene, the first elucidated in the pathway. Worms with mutations in these genes are characterized by self-sterile hermaphrodites, and fertile males capable of siring progeny, the ability to transactivate hermaphrodite spermatids, and the ability to be suppressed by the *spe-6* mutation (Muhlrad 2002). The *spe-6* gene encodes a protein serine/threonine casein type kinase (Muhlrad 2002). It is thought that the SPE-6 protein is necessary to inhibit spermiogenesis until the proper seminal vesicle fluid signal is propagated. At this time the spermiogenesis promoting signal would be carried through the spe-8 class genes and lift the inhibition by *spe-6* (Muhlrad 2002).

Spermatids from these spe-8 class mutant animals also appear normal under light magnification and electron magnification indicating that the fault in fertilization does not lie in one of the steps leading to their production. It has been previously hypothesized that these *spe-8* molecules function in a pathway

with one another, due to their similar phenotypes and additive effects, such as reduced brood sizes, when examined in double mutants (Nance 2000). The activation signal for this pathway is unknown, although the activation of the pathway by proteases may provide a possible clue. It is thought that an unidentified signal would bind to a receptor on the surface of the spermatid beginning sperm activation. Because male worms with mutations in any of the *spe-8* class of genes are fertile, while their hermaphrodite counterparts are sterile, it is thought that separate male and hermaphrodite sperm activators exist. In this model, the male activator would function through a pathway which does not include the spe-8 molecules, or which bypasses, partially or fully, the *spe-8* class of molecules. Some evidence shows that mutants in the *spe-8* class of genes do have a negative impact on male fertility, such as reduced competitiveness and reduced ability to undergo *in vitro* activation in the presence of certain chemicals such as pronase and TEA (Shakes 1989, Nance 1999, Geldziler 2005, Minniti 1996, and Nance 2000). Spermatids may be activated to acquire motility *in vitro* using several chemicals including TEA, pronase, and monensin (Nelson 1980, Ward 1983, Shakes 1989). Already characterized molecules in the *spe-8* class of mutants have provided the strongest clues to the process of spermiogenesis in *Caenorhabditis elegans.*

It is hypothesized that one of the already discovered *spe-8* genes might act as a receptor for the sperm activation signal in hermaphrodites. The receptor would most likely be a transmembrane molecule in the spermatid plasma membrane

such as SPE-12, SPE-19, or SPE-29. However, SPE-12 is thought to sit on top of the plasma membrane rather than being embedded directly into it, and SPE-29 is an extremely small protein. This indicates that SPE-19 is the best known candidate for receptor molecule. It is hypothesized that SPE-19 functions as a receptor molecule for the sperm activation signal. It is also thought that if SPE-19 functions as a receptor molecule, that it will be localized to the spermatid plasma membrane, and that it will be changed after sperm activation, either by cleavage or modifications such as phosphorylation. In addition it is hypothesized that some members of the spe-8 class of molecules function directly in a pathway with one another. It is hypothesized that the pathway for activation might function as follows. A protease inhibitor SWM-1 is stimulated to release its protease upon release of the activation signal (Stanfield 2006). The protease cleaves SPE-19 and SPE-8 or another kinase is stimulated to phosphorylate its cytoplasmic tail. The cleaved and phosphorylated tail of SPE-19 is released and interacts downstream with other members of the *spe-8* class of molecules eventually leading to an intracellular rise in pH. It is clear in this hypothesis that not all members of the activation pathway have been discovered; therefore, further members of the sperm activation pathway must be discovered and elucidated, before the full processes of sperm activation are known in *Caenorhabditis elegans*.

4.2 Materials and Methods

4.2.1 General Methods and Strains

C. elegans strain maintenance and general genetic crosses were performed as described by S. Brenner [S3]. Strains used in experiments included: wild-type variety Bristol, strain N2; spe-43(eb63); spe-8(hc53) dpy-5(e61); spe-6(hc163); spe-8(hc40); MT4150 (unc-117; dpy-4);
The Hawaiian strain CB4856 was used for SNP mapping. spe-43(eb63) was taken from a sperm development screen in the Ward laboratory (Shakes, 1988) Strains were maintained at 20°C or 25°C

4.2.2 Ovulation/progeny counts

To determine rates of ovulation and number of progeny, L4 hermaphrodites were isolated to individual 24-well plates and allowed to self-fertilize at either 20°C or 25°C. Oocytes or eggs were counted daily, and hermaphrodites were transferred to new plates until no new oocytes were produced after 24 hours. Hermaphrodites that died during the experimental window were excluded from the analysis. All errors were generated in terms of standard error of the mean.

4.3 Results

4.3.1 Identification of a new member of the spermiogenesis pathway:*spe-43*

The *spe-43* gene was identified in an EMS mutagenesis screen for genes defective during spermiogenesis. It was immediately classified at a spe-8 class gene, based on its mutant phenotypes including generation of self-sterile hermaphrodites, but fertile males. When compared with wild-type hermaphrodites, the *spe-43* uterus is filled with unshelled, non-developing embryos with endomitotic nuclei (Figures 2A and B). These self-sterile hermaphrodites are capable of producing outcross progeny with fertile males, either wild-type or *spe-43,* indicating that both the soma and oocytes are phenotypically normal. Conversely, the mutant males are predominantly phenotypically normal, and capable of siring outcross progeny with hermaphrodites. Furthermore, germ cell production in the hermaphrodite appears phenotypically normal, and spermatids appear normal under light microscopy.

4.3.2 Ovulation rates in *spe-43* mutants near wild-type levels

One strain of *spe-43* has been isolated thus far, eb63. When grown at 16ºC, 20ºC, or 25ºC the strain is completely sterile, producing no viable progeny (Figures 3A and 3B). Brood counts were performed using N2 worms as a control at both 20ºC and 25ºC. While no progeny were produced by *spe-43* hermaphrodites, ovulations were counted as the production of unshelled, undeveloped oocytes (Figures 3A and 3B). When comparing the numbers of ovulations by wild-type N2 hermaphrodites, counted as production of both eggs

and unfertilized oocytes, and *spe-43* hermaphrodites, counted as production of unfertilized oocytes, it was determined that *spe-43* hermaphrodites ovulate at near wild-type levels at both temperatures (Figures 3A and 3B). The implications of this experiment are two-fold, first that *spe-43* hermaphrodites are capable of producing mature oocytes and undergoing ovulation, and second that the *spe-43* hermaphrodite derived sperm are capable of producing the sperm-derived, MSP signal necessary for wild-type levels of ovulation.

4.3.3 Defects during spermiogenesis, MSP polymerization and extension

When activated *in vitro* with pronase or TEA, hermaphrodite and male spermatids fail to activate fully, and do not form a mature pseudopod. Rather, the spermatids reach the mid-stage of pseudopod development, in which the MSP is polymerized to extend spikes, but these MSP “spikes” do not coalesce to form the characteristic pseudopod. Each spe-8 class mutant produces spermatids which do not fully activate, however, the extent to which the spermatids activate and polymerize MSP varies with each mutation. Spermatids derived by dissection from either *spe-43* hermaphrodites or males, and activated *in vitro* by exposure to either TEA or pronase polymerize and extend long, thin, projections of MSP (Figure 2C-F). However, the spikes never begin to coalesce to form the pseudopod (Figures 2C-F).

4.3.4 Lack of amoeboid sperm prevents traction to the spermatheca

In wild-type hermaphrodites, ovulation occurs once approximately every 23 minutes (McCarter 1999). When this process occurs, the spermatheca expands to engulf the oocyte, and after a time, then contracts to expel the newly fertilized embryo. In fertilization defective mutants of the spe-8 class, the process of ovulation still occurs at near wild-type levels, although no viable progeny are produced. Again, in wild-type animals the movements of oocytes through the spermatheca can push sperm into the uterus, when this occurs the sperm must crawl upstream to reenter the spermatheca. Thus in the spe-8 class mutant *spe-19* sperm are lost from the spermatheca at a much higher rate than in wild-type, as the sperm do not activate to form the pseudopod, and are therefore incapable of tracking back to the spermatheca (Geldziler 2005). In order to determine whether the same process occurs in *spe-43* mutant hermaphrodites, age matched N2 wild-type and *spe-43* hermaphrodites were grown at 20ºC for three days. On each day subsequent to L4 stage, hermaphrodites were fixed and stained with the DNA DAPI dye and imaged under fluorescence microscopy. On the first day of the adult stage, a comparable number of sperm appear in the spermatheca of N2 and *spe-43* animals (Figures 4A and B). However, by the second day of the adult stage, substantially fewer sperm appear in the spermatheca of *spe-43* animals in comparison to N2 animals (Figures 4C and D). Finally, on the third day of the adult stage, no sperm were seen in the spermatheca of *spe-43* hermaphrodites, while a number of sperm still remained in the N2 hermaphrodite spermatheca (4E and F). This indicates that sperm are

indeed lost more quickly from the *spe-43* hermaphrodite reproductive tract. In addition, sperm nuclei were often seen in aberrant locations throughout the *spe-43* hermaphrodite uterus, indicating that sperm were not retracting to the spermatheca (Figure 4F).

4.3.5 Genetic mapping of the *spe-43* gene

A combination of two-point, three-point, and SNP mapping was used to narrow down the physical location of *spe-43* to a region of approximately 750,000 bp (Figures 5A and B). Linkage analysis placed the *spe-43* mutation on the right arm of chromosome IV. Strain MT4150, which includes markers dpy-4 (12.72 cM), and unc-17 (-3.11 cM), was selected for use in three-point mapping. The three-point mapping placed *spe-43* between the unc-17 and dpy-4 markers, recombinant strains were saved for two-point and SNP mapping. Recombinants were crossed to the CB4863 Hawaiin strain to generate SNP mapping strains, 17 strains were isolated for the dpy-4 non unc-17 worms, and 4 strains were isolated for the unc-17 non dpy-4 strains. Eight SNP's spanning the region between unc-17 and dpy-4 were used to determine the genetic position of the *spe-43* mutation to a region of 703,449 bp (Figure 5B). There were no further informative SNP's in this region, therefore sequence SNP's must be used to narrow the region further.

4.4 Perspectives

The study of spermiogenesis in *C. elegans* provides useful biological insights into both the processes of cellular differentiation in the absence of de novo synthesis, and the evolutionary divergence of molecular pathways necessary for the transition from a gonochoristic species containing males and females, to an androdioecious one containing self-fertile hermaphrodites. The *spe-43* gene is a member of the spe-8 class of molecules that are required for the sperm activation of hermaphrodite self-sperm but not male sperm. This dichotomy presents an interesting divergence in the activation pathways of hermaphrodite and male sperm. Future work will be required to determine genes in the spermiogenesis pathway which led to the activation of both male and hermaphrodite sperm, in order to elucidate where the activation pathways diverge and merge.

Figure 1

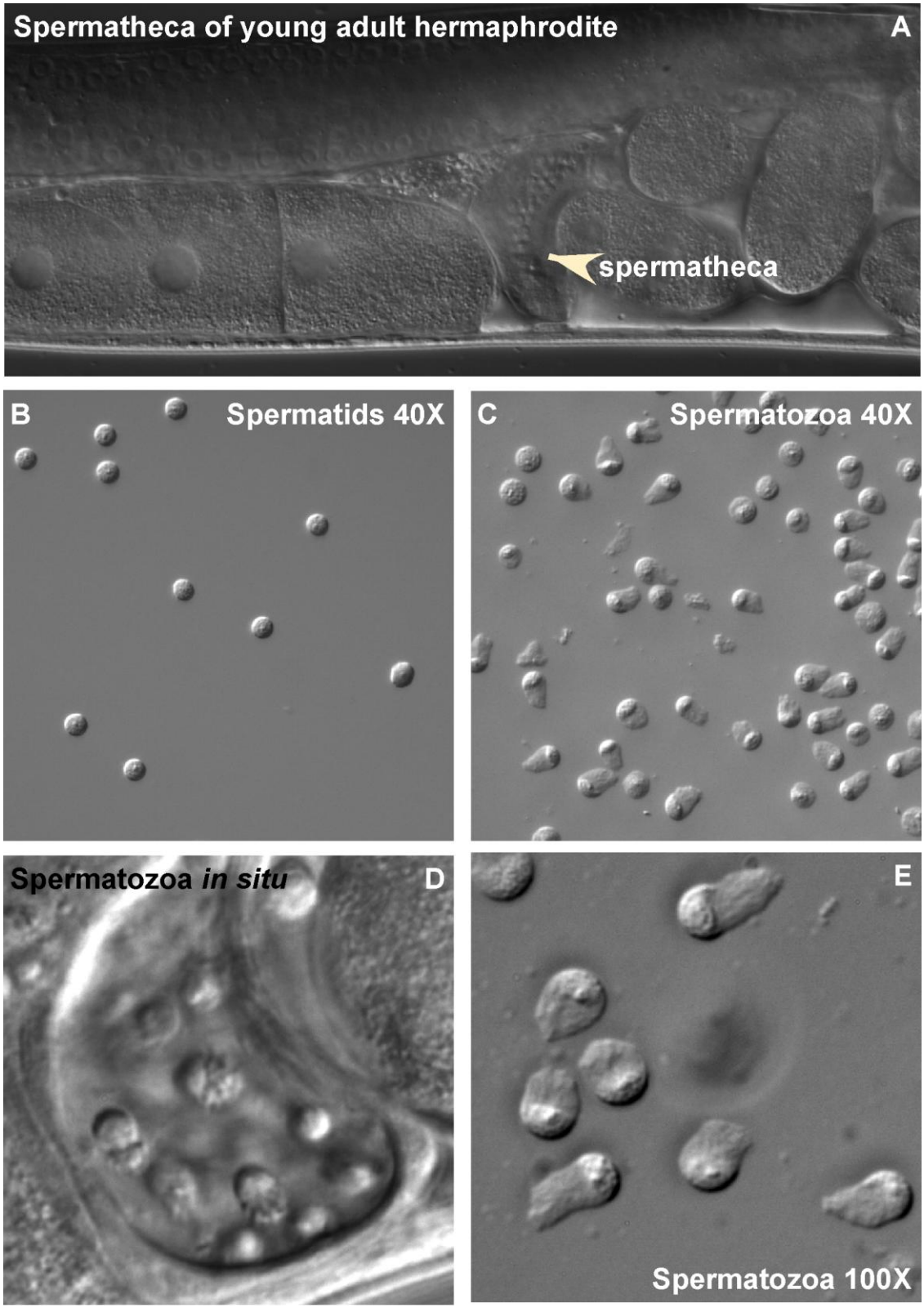

Spermatheca of young adult hermaphrodite
A
spermatheca
B
Spermatids 40X
C
Spermatozoa 40X
Spermatozoa *in situ*
D
E
Spermatozoa 100X

Figure 2

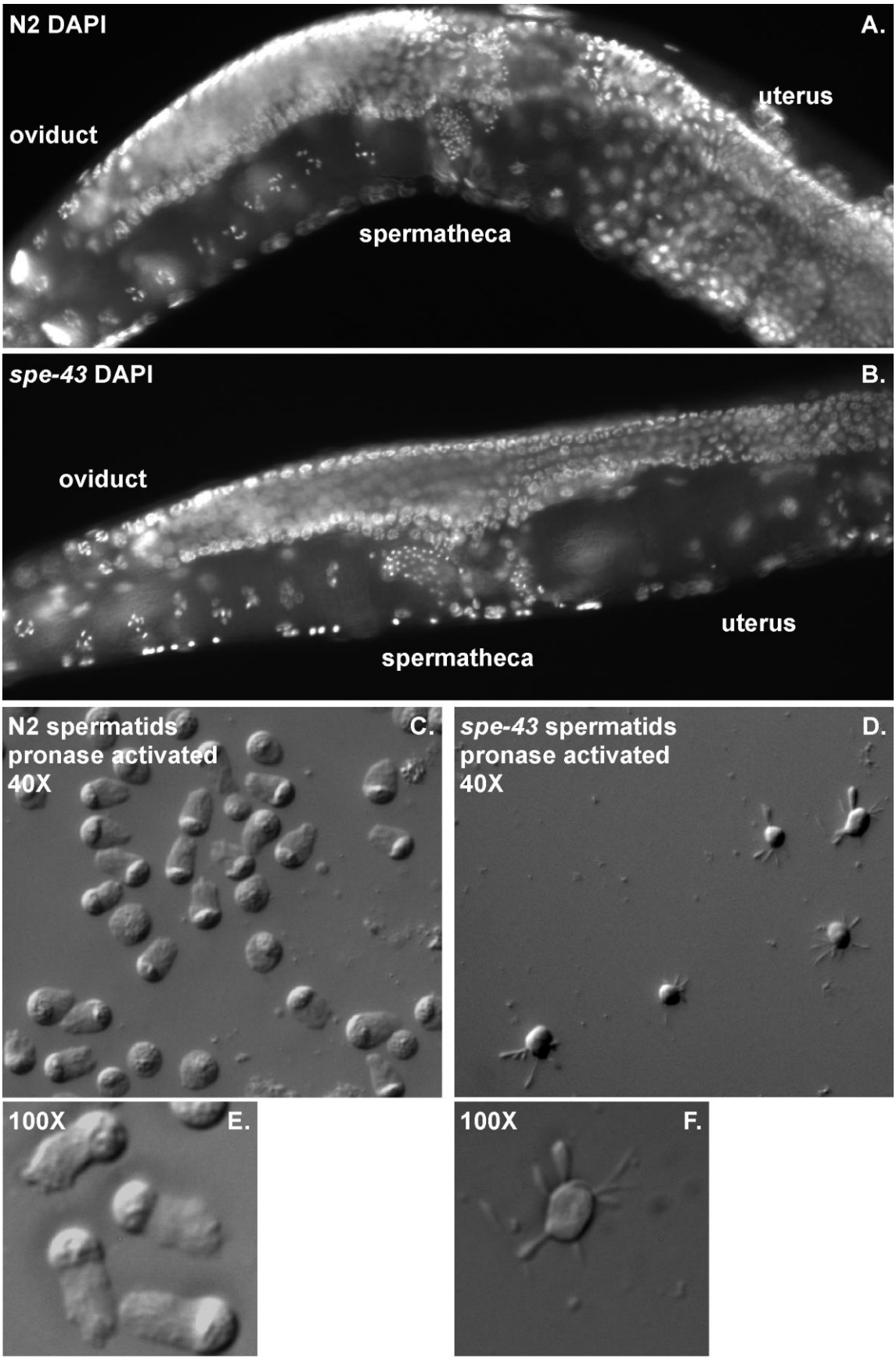
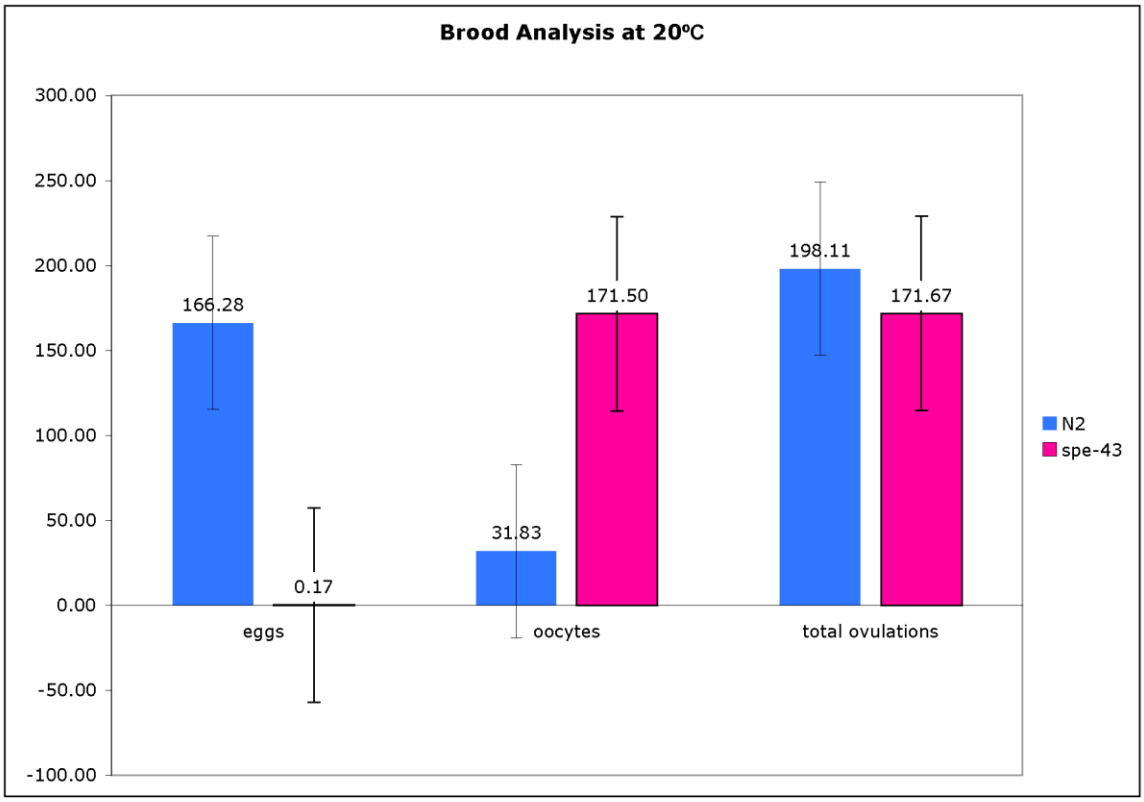

N2 DAPI
A.
uterus
oviduct
spermatheca
spe-43 DAPI
B.
oviduct
uterus
spermatheca
N2 spermatids
pronase activated
40X
C.
spe-43 spermatids
pronase activated
40X
D.
100X
E.
100X
F.

Figure 3


Brood Analysis at 20ºC
300.00
250.00
200.00
150.00
100.00
50.00
0.00
-50.00
-100.00
166.28
0.17
31.83
171.50
198.11
171.67
eggs
oocytes
total ovulations
N2
spe-43

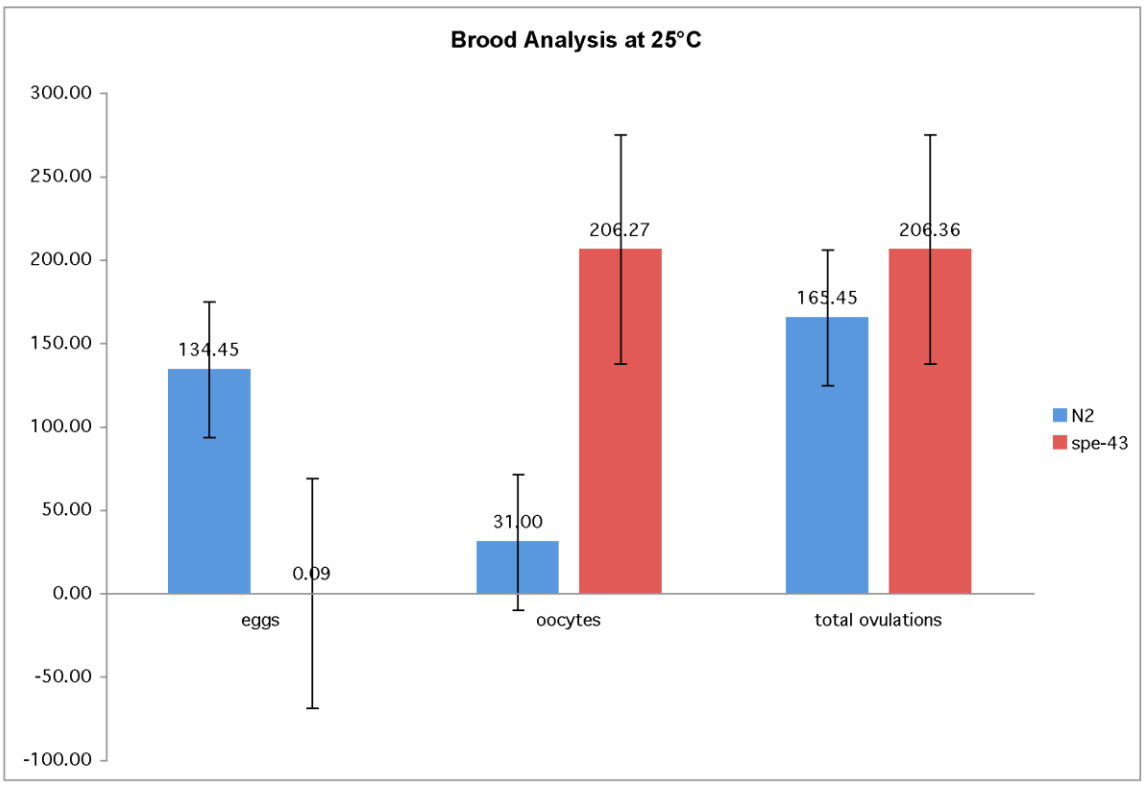

Brood Analysis at 25°C
300.00
250.00
200.00
150.00
100.00
50.00
0.00
-50.00
-100.00
134.45
0.09
31.00
206.27
165.45
206.36
eggs
oocytes
total ovulations
N2
spe-43

Figure 4

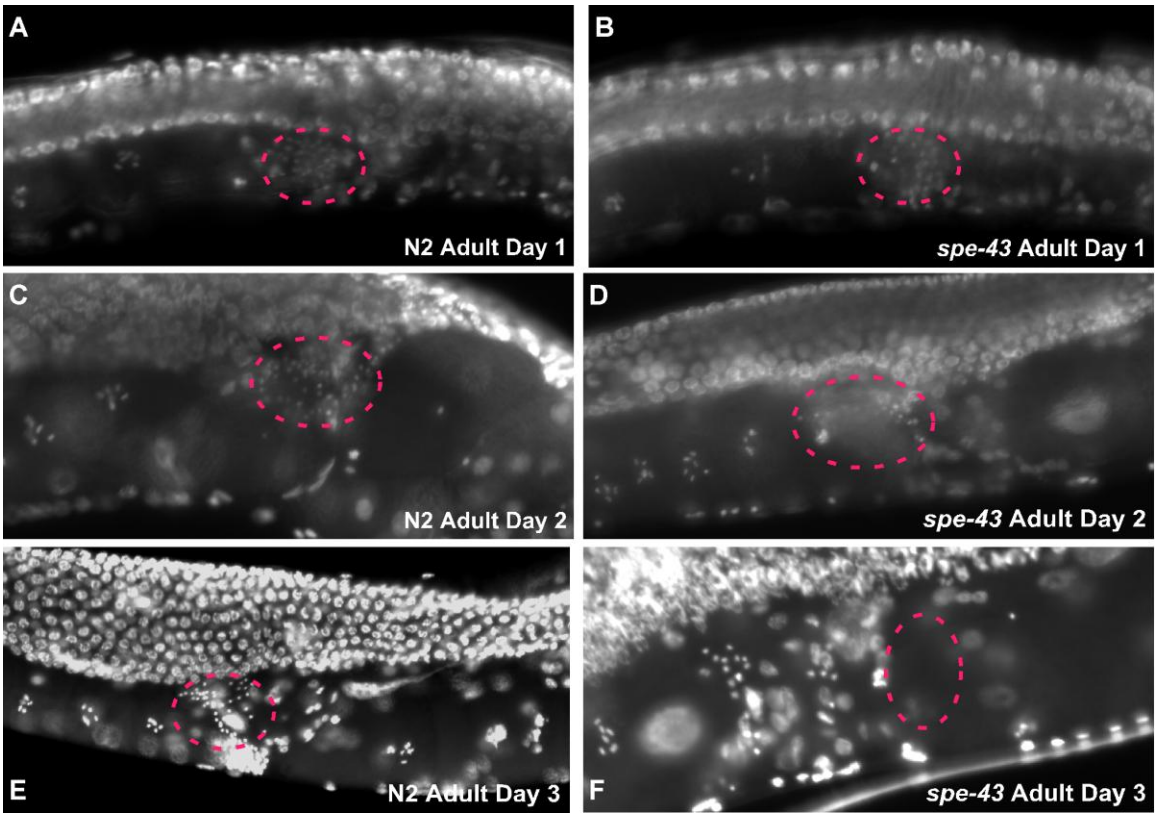

A
N2 Adult Day 1
B
spe-43 Adult Day 1
C
N2 Adult Day 2
D
spe-43 Adult Day 2
E
N2 Adult Day 3
F
spe-43 Adult Day 3

Figure 5

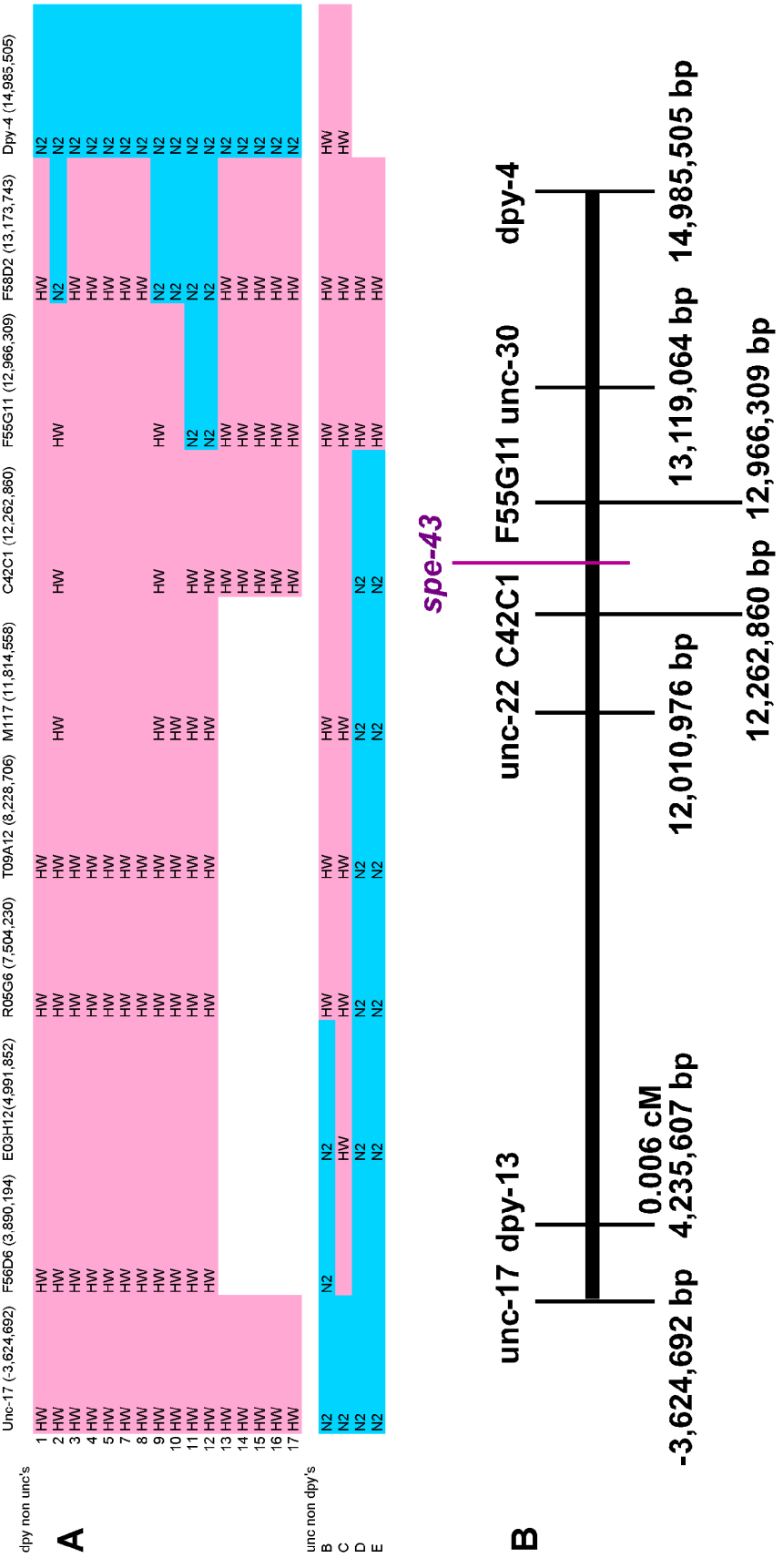

A
dpy non unc's
Unc-17 (-3,624,692)
F56D6 (3,890,194)
E03H12(4,991,852)
R05G6 (7,504,230)
T09A12 (8,228,706)
M117 (11,814,558)
C42C1 (12,262,860)
F55G11 (12,966,309)
F58D2 (13,173,743)
Dpy-4 (14,985,505)
unc non dpy's
B
C
D
E
B
unc-17 dpy-13
0.006 cM
-3,624,692 bp
4,235,607 bp
unc-22 C42C1
12,010,976 bp
spe-43
F55G11 unc-30
12,262,860 bp
13,119,064 bp
dpy-4
12,966,309 bp
14,985,505 bp

Figure Legends

Figure 1

A. Germline of a wild-type, adult hermaphrodite under DIC light-microscopy.
B. Spermatids dissected from a wild-type, young adult male, under DIC light-microscopy. 40X resolution.
C. Spermatozoa dissected from a wild-type, young adult male, and activated with pronase. 40X resolution.
D. Spermatozoa in a live hermaphrodite. 40X resolution.
E. Spermatozoa dissected from a wild-type, young adult male, and activated with pronase. 100X resolution.

Figure 2

A. N2 wild-type adult hermaphrodite germline, DAPI stained. Developing embryos abundant in the uterus.
B. *spe-43 (eb63)* adult hermaphrodite, DAPI stained. Although sperm are abundant in the spermatheca, uterus is full of unfertilized non-developing eggs with endomitotic nuclei.
C. Spermatozoa dissected from a wild-type, young adult male, and activated with pronase. 40X resolution.
D. Spermatozoa dissected from a *spe-43 (eb63)* mutant, young adult male, and activated with pronase. MSP does not coalesce to form pseudopod 40X resolution.
E. Spermatozoa dissected from a wild-type, young adult male, and activated with pronase. 40X resolution.

F. Spermatozoa dissected from a *spe-43 (eb63)* mutant, young adult male, and activated with pronase. MSP does not coalesce to form pseudopod 100X resolution.

Figure 3

A. Brood analysis of *spe-43 (eb63)* at 20ºC. The *spe-43 (eb63)* mutant hermaphrodites ovulate at wild-type levels, but are sterile as compared to N2 wild-type. Error bars are calculated as standard error.

B. Brood analysis of *spe-43 (eb63)* at 25ºC. The *spe-43 (eb63)* mutant hermaphrodites ovulate at higher than wild-type levels, but are sterile as compared to N2 wild-type. Error bars are calculated as standard error.

Figure 4

A. SNP mapping regions for the *spe-43 (eb63)* mutation. The mutation has been mapped to the right arm of chromosome IV. Three-point mapping has given a left-bound at *unc-17* and a right bound at *dpy-4*.

B. The detailed mapping region for the *spe-43 (eb63)* mutation. The mutation has been mapped to the right arm of chromosome IV. The map includes the right and left bounds identified by three-point mapping, SNP's used during SNP mapping, and major points on the right arm of chromosome IV.

Figure 5

A. SNP mapping diagram including SNP designations and region

B. Genetic region containing *spe-43* on the right arm of chromosome IV. Includes major landmarks, as well as strains used for three-point and SNP mapping.

Chapter 5

Discussion and Conclusions

5.1 Summary

The process of fertilization involves precision in minute detail. The gametes, usually male and female must be formed to an exact standard, meet and merge, then undergo a rapid process of combination, the newly formed embryo must change from a fertilized female germ cell to a new organism with its own programming. The model organism *C. elegans* presents an excellent tool for parsing the many intricacies of fertilization. Much like more complex organisms, *C. elegans* form male and female gametes, which must undergo activation before they are competent for fertilization. The gametes must meet, whether during hermaphrodite self-fertilization, or cross fertilization between hermaphrodite and male. Then the newly fertilized embryo must undergo the oocyte-to-embryo transition, moving through many of the same stages as mammals and many other organisms. This work follows the elucidation of molecules involved in three different processes of fertilization, the oocyte-to-embryo transition, the cortical granule exocytosis, and spermiogenesis.

5.2 The oocyte-to-embryo transition revisited

The complex processes of the oocyte-to-embryo transition include resumption of meiosis, cytoskeletal rearrangement, ovulation, fertilization, the block to polyspermy, completion of meiosis including the extrusion of two polar bodies, extrusion of a chitinous, trilamellar eggshell, formation of a female and male

pronucleus, combination of the two pronuclei, and the resumption of the mitotic divisions (McNally and McNally, 2005). A module of molecules has recently begun to be elucidated, which couples the cell cycle to the events of the oocyte-to-embryo transition. The previously described genes, *egg-3* and *chs-1* have now been joined by *egg-4/5* as being necessary for eggshell formation, meiosis and polar body extrusion and the proper localization of regulatory kinase *mbk-2* (Stitzel et al., 2007, Cheng et al., 2009, Johnshton et al. 2005, Maruyama et al., 2007, Parry et al., 2009). Epistatic analysis revealed that the *egg-3, chs-1,* and *egg-4/5* genes were required for the localization of one another, and were all required for the localization of *mbk-2*.

The EGG-3 and EGG-4/5 proteins are also new members of a small but growing class of molecules referred to as protein-tyrosine phosphatase-like proteins (PTPL), which bear the sequence of a protein-tyrosine phosphatase, but lack critical active site amino acids. Thus the PTPL proteins could act as either scaffolding molecules or as competitive inhibitors, either binding a phosphorylated protein and holding it as part of a complex (scaffolding), or binding a phosphorylated protein and preventing it from interactiving with a phosphatase (inhibitor). Research has shown that the EGG-3 protein likely acts as a scaffolding molecule, binding to MBK-2 and holding it as part of a complex with CHS-1 and EGG-4/5, but not altering its biochemical activity. The role of inhibitor of MBK-2 seems to be fulfilled by EGG-4/5. As mentioned above, EGG-4 and MBK-2 were found to interact both by yeast-two hybrid, co-

immunoprecipitation assay, and GST/FLAG pull down assay (Cheng KC, 2009). The specific phosphorylation of the MBK-2 active loop also increased the efficiency of the binding reaction between EGG-4/5 and MBK-2. Additionally, evidence from *in vitro* kinase assays also indicates that MBK-2 kinase activity, phosphorylating target MEI-1, is inhibited by the presence of EGG-4/5 (Cheng KC, 2009). Therefore, rather than acting as a scaffolding molecule like fellow PTPL protein EGG-3, EGG-4/5 seems to behave as a mixed inhibitor for MBK-2 (Figure 1) (Tonks, 2009).

Because *mbk-2* is required to mark several time sensitive maternal proteins for degradation, its localization and activity must be precisely restricted during the oocyte-to-embryo transition. To this end, several interrelated regulatory mechanisms have been elucidated. First it has been determined that the redistribution of *egg-3, chs-1, mbk-2*, and *egg-4/5* depends on advancement of the cell cycle past metaphase I, and that the redistribution is signaled in an EGG-3 dependent manner (Figure 1). It has also been shown that the activity of MBK-2 depends on phosphorylation by cyclin dependent kinase CDK-1, a protein that becomes active during late oogenesis. However, it is critical that MBK-2 does not have access to its targets until the completion of meiosis. Therefore a second regulatory protein, in the form of EGG-4/5, is required for holding MBK-2 and preventing its premature activity (Figure 1). This protein module, EGG-3, EGG-4/5, CHS-1, MBK-2, and CDK-1 represents a complex and novel method of regulating the transition between maternal oocyte programming and embryonic

programming (Figure 1). Given the high degree of complexity inherent in this model, it is predicted that other molecular regulators and effectors must be active during the oocyte-to-embryo transition. Genetic and biochemical analyses are currently underway to seek out new players in this highly dynamic process.

5.3 Cortical Granule Exocytosis Primes the Embryo

The cortical granule exocytosis is a critical process, which occurs just after fertilization in mammals and *C. elegans*. In *C. elegans*, the cortical granual exocytosis is necessary for the restructuring of the ECM, and for extrusion of the chitinous eggshell, which is extruded to provide support to the developing embryo. The cortical granule bodies in *C. elegans* are marked by the protein CAV-1, a calveolin; however, few components of the cortical granules have been elucidated (Sato et al., 2008). In order to find further members of the cortical granules, we searched for genes, which caused defects in osmotic integrity, indicating a lack of structural support.

The *egg-6* gene has been identified in many wide-scale screens, as an embryonic lethal gene and one required for osmotic integrity of the embryo (Sonnichsen et al., 2005). RNAi targeting *egg-6* by the soaking method led to defects in embryonic development, such that embryos ceased developing at the thirty-cell stage. Abrogation of the *tac-1* and *par-4* genes showed a highly similar phenotype. This indicated that the *egg-6* gene might function during

establishment of polarity in the developing embryo. Indeed, depletion of *egg-6* by RNAi prevented the correct localization of the PGL-1, a protein that marks the P-granules in *C. elegans* germ cells in a highly polar manner.

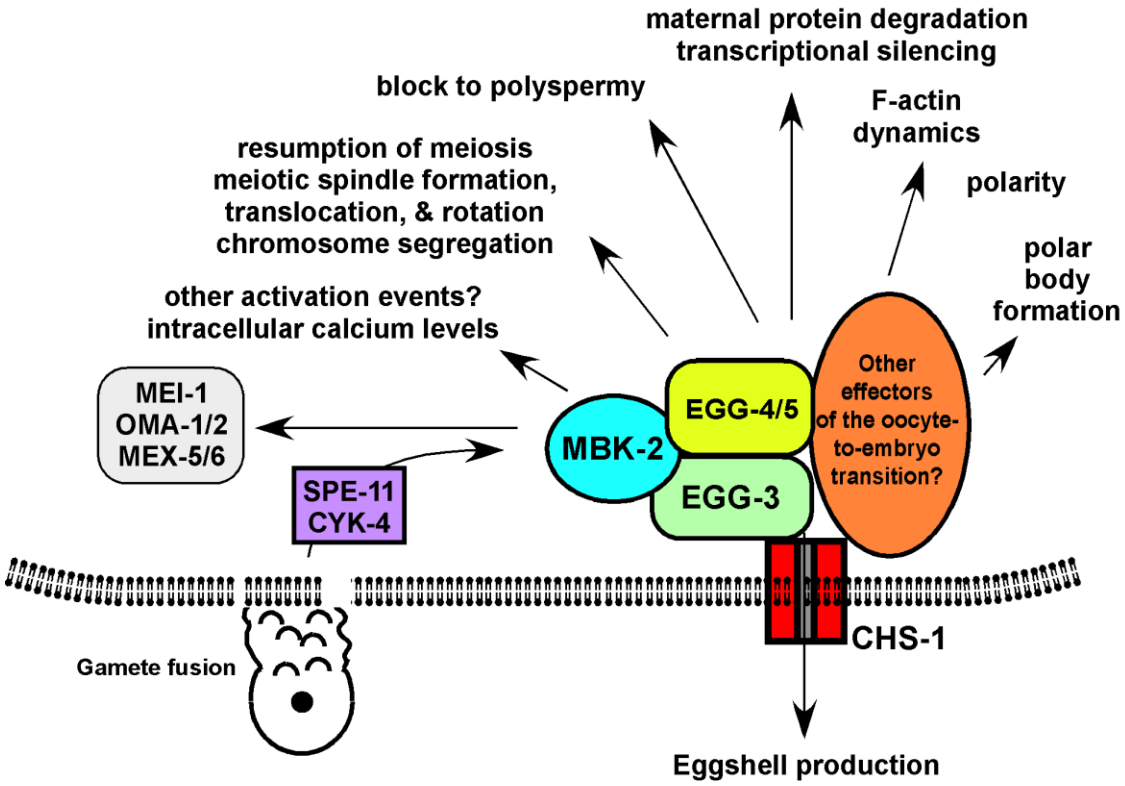
In order to determine the subcellular localization of the EGG-6 protein, a gfp fusion protein was created using the Gateway method followed by microparticle bombardment. The protein localized to highly dynamic, cytoplasmic bodies during oocyte development, then shifted rapidly to the plasma membrane at approximately NEBD. This localization is very similar to the localization of CAV-1; however, we currently lack the ability to determine co-localization with the CAV-1 protein. It will be important to determine whether the EGG-6 protein localizes to the cortical granules, as it would represent the first connection between the cortical granule exocytosis and the establishment of polarity.

5.4 The importance of *spe-43* during spermiogenesis

The highly dynamic process of spermiogenesis is required in most species for the transformation of a non-motile spermatid, into a motile spermatid competent for fertilization. The process of spermiogenesis in *C. elegans* occurs in the absence of new protein synthesis, in response to the presence of oocytes in the spermatheca. In recognition of the subsistence of *C. elegans* as an androdioecious species, the pathways for activation of hermaphrodite and male derived sperm appear to require different biochemical pathways. The spe-8

class of genes include many genes, which are required for spermiogenesis in hermaphrodites, but not in males. The examination of such genes provides interesting insights into the process of cell differentiation in the absence of new protein synthesis, evolutionary divergence between closely related androdioecious species and gonochoristic species, and the overall process of sperm activation in *C. elegans*. The *spe-43 (eb63)* gene was identified in a screen for genes required for spermiogenesis. Like other spe-8 class molecules, it is required for hermaphrodite fertility, but not for male fertility. In the absence of *spe-43*, hermaphrodites ovulate at near wild-type levels, but do not produce progeny. In addition, the spermatids in both *spe-43* hermaphrodites and males fail to activate fully to form a motile pseudopod in the presence of common sperm activators pronase and TEA. Rather, the spermatids extend long spikes of MSP, which fail to coalesce or aggregate. Perhaps due to the lack of motility, the sperm of *spe-43* hermaphrodites are lost from the spermatheca more rapidly than comparably aged N2 wild-type hermaphrodites. The *spe-43 (eb-63)* mutation has been mapped to a region of 750,000 bp on the right arm of chromosome IV, using a combination of two-point, three-point, and SNP mapping. Further mapping, followed by rescue by injection of the gene, will be required to identify precisely which gene the *spe-43* mutation represents.

Figure 1


maternal protein degradation
transcriptional silencing
block to polyspermy
F-actin
dynamics
resumption of meiosis
meiotic spindle formation,
translocation, & rotation
chromosome segregation
polarity
polar
body
formation
other activation events?
intracellular calcium levels
Other
effectors
of the oocyte-
to-embryo
transition?
MEI-1
OMA-1/2
MEX-5/6
EGG-4/5
MBK-2
EGG-3
SPE-11
CYK-4
CHS-1
Gamete fusion
Eggshell production

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Publications

1. EGG-4 and EGG-5 Link Events of the Oocyte-to-Embryo Transition with Meiotic Progression in *C. elegans*. Parry JM, Velarde NV, Lefkovith A, Zegarek MH, Hang JS, Ohm J, Klancer R, Maruyama R, Grant BD, Piano F, Singson A. Current Biology 2009 Nov. 3. 19 (20) 1752-1757
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