LOSS OF THE *cbd-1* GENE CAUSES INTRACELLULAR TRAFFICKING DEFECTS IN *C. elegans*

By

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ABSTRACT OF THE THESIS

LOSS OF THE *cbd-1* GENE CAUSES INTRACELLULAR TRAFFICKING DEFECTS IN *C. elegans*

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Several large scale RNAi screens in *C. elegans* have allowed us to develop a network of genes involved in early embryogenesis that can be broken down into molecular modules. Phenotypic analysis of the *cbd-1* gene, chosen from the oocyte integrity module has helped elucidate its role in intracellular trafficking events important for fertilization and egg activation.

RNAi depletion of *cbd-1* prevented sperm receptor molecules EGG-1 and EGG-2 from accumulating at the oocyte plasma membrane, thereby interfering with fertilization. Similarly, yolk receptor RME-2 did not localize to the membrane and RNAi animals showed reduced yolk uptake in oocytes, causing them to be poorly developed. Yolk uptake via RME-2 is important for CAV-1 localization, which plays a role in Ras/MAPK dependent meiotic progression. RNAi of *cbd-1* caused a mislocalization of CAV-1 and a meiotic arrest at metaphase I. The EGG-3/CHS-1 membrane complex also failed to form, thereby affecting regulation of MBK-2 which did not properly degrade its maternal protein targets.

These RNAi experiments lead us to believe that *cbd-1* functions as a general regulator, controlling trafficking of proteins to the membrane. Proper localization of *cbd-1* targets to the membrane is critical to their function. The targets that we have uncovered thus far are important regulators of meiotic progression, sperm entry, eggshell formation, and egg activation events.

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Chapter 1: Introduction

1.1 Modern Advances in Reproductive Biology

Reproduction is a complex and fascinating biological process required for the survival of many diverse species. Sexual reproduction has the benefits of preventing accumulation of deleterious mutations and enhancing natural selection through increased genetic variation. Reproduction has long been a subject of great curiosity and efforts to improve, reduce, and restore reproductive function have been underway since Aristotle's time. His *Generation of Animals* (350 B.C.E.), the first treatise on reproduction and embryology, describes his method of periodically breaking open fertilized chicken eggs to observe the presence of different organs. From this research, he proposed a binary hierarchy; the male imparts Form to the fetus, while the female contributes Matter, a gendered dichotomy of spirit and flesh, mind and body (Rothman et al, 1996). In the late seventeenth century, naturalist Reinier de Graaf first described ovarian follicle development, fallopian tube function and female ejaculation. Shortly thereafter, using the first microscope, Antoine van Leeuwenhoek observed the first living spermatazoa and hypothesized that they actually penetrated the egg. In 1780, Lazzaro Spallanzani transferred male dog semen into a rutting female, inducing the first case of artificial insemination, a procedure that became widely used in dairy herds (Dawes 1952). British surgeon John Hunter carried out the first artifical insemination in a human soon after Spallanzani's canine experiment; a remarkable success, considering it was not yet known that insemination must coincide with ovulation (Dawes 1952). The discovery of sex hormones and their control over the female reproductive cycle helped bring forth two

main focuses of reproductive research; contraception and assisted reproductive technology (ART).

By the 1930s, it had been found that high levels of androgens, estrogen, and progesterone inhibit ovulation and a procedure had been developed to synthesize progesterone from Mexican yams (*Dioscorea mexicana*) (Goldzieher 1982). With the help of Margaret Sanger, Gregory Pincus and John Rock obtained a small grant to begin hormonal contraception research which yielded Envoid, the first oral contraceptive (Reed 1978). From the time of its first clinical trials in the early 1960s to the present, 200 million women have swallowed the contraceptive pill (Reed 1978). This simple and highly effective medication is arguably one of the greatest advancements in contraceptive research and is the first medicine ever destined for a purely social rather than therapeutic purpose. Rock's primary concern was the family and the creation of a means to let husband and wife live without fear of producing more children than they were in a position to raise (MacLaughlin 1982). It served as a means to separate sex from procreation and more distinctively separated contraception from the sexual act. Unlike previous contraceptives, it could be taken by mouth and did not require use at the moment of intercourse, requiring no special preparations that might interfere with spontaneity or sensation. Women could also use it discreetly without any knowledge on the part of their male partners. Moreover, the birth control pill carried a greater potential than safeguarding against unwanted pregnancy; it became a cornerstone of the sexual revolution that swept industrialized countries in the mid-1960s. The Pill strongly abetted the women's movement by redefining a woman's modern economic role. During the 1950s, the marriage rate had reached an all time high and the average age at which people

married dropped to an all time low (Watkins 1998). By 1959, almost half of all brides walked down the aisle before their nineteenth birthday (Watkins 1998). The oral contraceptive helped women to prolong the age at which they first married, allowing them to invest in educational and career pursuits. Soon after the birth control pill was legalized, college enrollment and graduation rates of women showed a steady increase (Watkins 1998).

The development of the pill was powerfully intertwined with the politics and rhetoric of the Cold War and the threat of overpopulation. Many social reformers of the time had long been concerned with the links between population growth, national economic wealth, social order and military strength (Marks 2001). However, the nature of this discussion radically changed after the Second World War, shifting from equating large populations with economic and military strength to seeing them as a danger to global security (Marks 2001). Far more dangerous than unchecked fertility was the apparent class differential affecting this trend. Statistics implied that those who were reproducing least were the better educated and more economically successful middle and upper classes (Marks 2001). The poor, regarded as the most ignorant, unhealthy, and immoral members of society were continuing to procreate at very high rates (Marks 2001). Seeing differential fertility in biological terms, eugenicists promoted the idea that fitter members of society should be encouraged to breed in greater numbers, while those deemed less fit should have their fertility controlled. Within this context, human reproduction was to be scientifically managed. Much of the publicity for the pill harnessed the wider belief in the power of science. One of the basic assumptions underlying the campaign was that science had caused the problem of population growth

by reducing mortality rates, and therefore it was up to science to solve the problem (Marks 2001). As early as the 1920s, Margaret Sanger had first articulated this view, arguing:

> "Ignorance, poverty, and vice must stop populating the world. To accomplish this, there is but one way. Science must make woman the owner, the mistress of herself. Science, the only possible savior of mankind, must put it in the power of woman to decide for herself whether she will or will not become a mother" (Marks 2001).

The management of biology has supported the liberation of women but has also determined that the decision to have a child is now a rational one. However, what seems rational for our economic situations and lifestyles is not always what is sensible for our biological selves. There is a growing disconnection between the reality of biology and our idea of what our bodies are capable of; between the time we want to have children and the time when our bodies are best at having children. Between 1970 and 2004, the average age at which U.S. women gave birth rose from 21.4 to 25.2 (Gregory 2007). For college educated women, the average age at first birth in 2004 was 30.1 (Gregory 2007). Maternal age is an important factor in egg quality, quantity and later, the embryo quality after fertilization. At birth, each woman has approximately 1,000,000 eggs available for fertilization, which drops to 300,000- 400,000 at puberty and continues to decrease with age (Tilly 1998). An egg needs to be healthy enough to be fertilized and have the essential qualities necessary to divide and grow into a healthy embryo. Poor egg quality may lead to infertility, repeated miscarriage, increased risk for ectopic pregnancy, and chromosomal abnormalities. Women between the ages of 19 and 26 with partners of similar age had approximately a 50% chance of becoming pregnant during any one menstrual cycle if they had intercourse two days prior to ovulation (Dunson 2002). For

women age 27 to 34, the chance of become pregnant was 40%, and for women over the age of 35, the probability dropped to 30 % (Dunson 2002). Likewise, the risk of giving birth to a child with Down's syndrome for a 20 year old mother is 1 in 2000, which increases to 1 in 900 by age 30 and 1 in 100 at age 40 (Goldstein and Reynolds 1999).

Assisted reproductive technologies have made it possible for a large number of otherwise infertile couples to have children. The first child conceived through in vitro fertilization was born in 1978 using a technique that suctioned a single egg with refined laparoscopic equipment (Al-Ghamdi 2008). Currently, FSH is used to stimulate the development of several eggs, which can easily be retrieved transvaginally under ultrasound guidance (Al-Ghamdi 2008). Although the original indication for IVF was tubal disease, it has now expanded to include male factors, endometriosis, ovulatory dysfunction, uterine factors, and unexplained infertility. IVF has expanded to include many techniques and procedures. For example, assisted zona hatching (AZH) can be used to aid in the implantation process of the embryo. Intracytoplasmic sperm injection (ICSI) is beneficial in the case of a male factor infertility where sperm counts are very low. Zygote intrafallopian transfer (ZIFT), which involves transfer of the embryo at the blastocyst stage has been shown to give better implantation and pregnancy rates. Other assisted reproductive technologies that do not necessarily involve IVF can also be used to overcome reproductive difficulties. Egg and sperm donors can be resources for men or women with few or low quality eggs or sperm. A gestational carrier is an option for a patient who does not have a uterus due to congenital absence or surgical removal, a patient who does not have ovaries, or a patient who cannot carry a pregnancy to full term. Preimplantation genetic diagnosis (PGD) uses genetic screens to identify genetically

abnormal embryos, especially when the parents are carriers of a communicable disease. Finally, cryopreservation methods allow eggs, sperm, and embryos to be preserved for later procedures. Assisted reproductive technology procedures performed in the U.S. have more than doubled within the last ten years, with 140,000 procedures in 2006, resulting in the birth of 55,000 infants (Mastony 2009). The development of ART also helped to diversify the concept of the family, providing a means by which same sex couples and single men and women could become parents other than adoption.

Clearly, reproductive sciences stand at the nexus of key issues facing humanity worldwide. Specifically, the development of oral contraceptives and ART have given us the ability to regulate fertility. Most importantly however, the science of reproduction is about the future- information about population dynamics can predict future needs for food, resources, and wildlife management. In addition, scientific advances in reproductive biology have been a catalyst for considerable research and debate in the field of bioethics, increasing public awareness of the interface between science and personal rights and responsibilities (Strauss 2008).

1.2 Fertilization in mammals and non-mammals

During the past two decades a great deal of progress has been made in establishing a molecular pathway for fertilization in mammals. Fertilization is defined as the process of union between two cells, egg and sperm, whereby the somatic chromosome number is restored and the development of a new individual is initiated (Wassarman 1999). It begins in the oviduct with binding of free-swimming sperm to the ovulated egg's extracellular coat, the zona pellicuda and ends a short time later with fusion of egg and sperm plasma membranes to form a single activated cell, the zygote

(Wassarman 1999). Along the way, several recognizable events take place, including the sperm acrosome reaction, penetration of the egg ZP by sperm, and the egg cortical reaction and zona reaction, which denies free swimming sperm the ability to bind to fertilized eggs. Most oocytes pause twice during meiosis, first in prophase I and again in second meiotic metaphase. Oocytes arrested in prophase I enlarge and synthesize RNA and protein as the follicle grows (Wassarman 1999). The oocyte and follicle also become sensitive to the actions of gonadotrophins and FSH and LH surges during mid-cycle (Wassarman 1999). Completion of the first meiotic division takes place when oocytes have undergone extensive growth and cellular interaction with the granulosa and theca cells (Wassarman 1999). The oocyte undergoes asymmetric cytokinesis and extrudes the first polar body containing a haploid chromosome complement. Immediately after the first meiotic division is completed, the second meiotic division is initiated, and oocytes arrest in metaphase II until fertilization provides the signal that reactivates the cell cycle machinery and the embryo divides continuously. The transition from an oocyte developmental program requires a coordinated series of events, collectively known as egg activation. Egg activation includes changes to the egg surface to prevent polyspermy, release of oocyte meiotic arrest, generation of haploid female and male pronuclei, changes in maternal mRNAs and protein populations, and cytoskeletal rearrangements (Wassarman 1999; Singson 2008). In many animals, egg activation is triggered by fertilization, which increases intracellular calcium within the oocyte thereby regulating subsequent molecular events. In other animals, fertilization-independent external signals, including mechanical stimulation and changes in ion concentration can trigger egg activation (Wassarman 1999).

Fertilization in mammalian and non mammalian organisms has many features in common, including a final maturation phase for sperm and eggs, species specific sperm and egg binding, penetration by sperm of one or more extracellular coats surrounding the egg, fusion of sperm and egg, and egg activation (Wassarman 1999). Considering the plethora of reproductive strategies in the animal kingdom, as well as the diverse shapes and sizes of genitals and gametes it is logical that these should be accomodated by different pathways to achieve fertilization. For example, while some sperm are propelled by a long flagellum, others lack a flagellum and exhibit amoeboid-like movement. Similarly, some eggs possess a special site for entrance of sperm, while many others lack such a site and virtually their entire surface can interact with sperm (Wassarman 1999). Also, some features of the gametes and paths to fertilization depend on whether it takes place outside or inside the female. Specifically, the need for species-specific fertilization is abundantly clear for animals that shed unfertilized eggs into water or onto land (Wassarman 1999).

1.3 *C. elegans* as a model organism to study fertilization *in vivo*

Caenorhabditis elegans is a small, free-living soil nematode that lives in many parts of the world and survives by feeding on microbes, primarily bacteria (Hall and Altun 2005). *C. elegans* is amenable to genetic crosses and produces a large number of progeny per adult. It reproduces with a life cycle of about 3 days under optimal conditions. Similar to other nematodes, the life cycle of *C. elegans* is comprised of the embryonic stage, four larval stages (L1-L4) and adulthood. The end of each larval stage is marked with a molt, during which a new stage-specific cuticle is synthesized. There are two sexes, a self-fertilizing hermaphrodite and male. Males arise infrequently (0.1%)

by spontaneous nondisjunction in the hermaphrodite germ line and at a higher frequency through mating (Hall and Altun 2005). Self-fertilization of the hermaphrodite allows for homozygous worms to generate genetically identical progeny, and male mating facilitates the isolation and maintenance of mutant strains as well as moving mutations between strains (Hall and Altun 2005). *C. elegans* is a powerful model organism in which to examine fertilization and egg activation, as it allows us to observe oocyte development, meiotic maturation, ovulation, and fertilization in the intact animals (Greenstein 2005; Hubbard and Greenstein, 2000). The most obvious advantages are that it has a clear cuticle, is highly responsive to RNA interference (RNAi), its entire cell lineage has been mapped and its genome has been sequenced (Hodgkin, 2005; Hubbard and Greenstein, 2005; Oegema and Hyman, 2006; Shaham, 2006). Genetic analysis in *C. elegans* is complemented by the advanced genomics, proteomics and other molecular tools available for the worm (Singson 2008). The reproductive system is one of the most sexually dimorphic tissues in the animal, with many components differing between hermaphrodites and males (Altun and Hall 2005). Hermaphrodites briefly produce a fixed number of sperm before permanently switching to oocyte production, while males can produce sperm throughout their entire life (Brenner 1974). There are several notable differences between nematode and mammalian gametes. *C. elegans* sperm are amoeboid and lack flagella and acrosomes familiar to mammalian sperm (Singson 2008). Oocytes do not have a thick egg coat but have a recently defined thin vitelline layer and undergo a cortical granule reaction (Singson 2008).

1.3.1 Ovulation, fertilization, and egg activation

The self-fertile hermaphrodite has a bi-lobed U-shaped gonad with distal-proximal polarity (Figure 1A). A mitotic cell population located at the distalmost end of the gonad and meiotic cells extend proximally (Figure 1B). The distal germ line is a syncytium. Germ cells have incomplete borders and are connected to one another via a central canal called the rachis (Hirsh et al., 1976). As germ cells move away from the influence of the distal tip cell at the end of each gonad arm, they enter meiosis I and proceed through prophase I to diakinesis becoming active sites of maternal gene transcription (Hubbard and Greenstein 2000) (Figure 2A). Germ cells progress into pachytene and gradually grow; nuclei are characterized by a distinctive "bowl of spaghetti" morphology as homologous chromosomes start to align side by side (Altun and Hall 2005) (Figure 2B). Exit form pachytene requires activation of the mitogen activated protein kinase (MAPK) pathway, thought to be triggered by a signal from the overlying gonadal sheath cells (Church et al., 1995; McCarter et al., 1997). Progression to diplotene occurs in the bend of the gonad arm and cells form a single file towards the proximal arm (Figure 2C). In the proximal arm, oocytes progress to diakinesis, where they arrest until maturation, due to inhibition of MAPK signaling (Figure 2C). During this arrest, oocytes enlarge to fill the entire space within the gonad arm, increasing their cytoplasmic contents and size of the nuclei (Altun and Hall 2005). Oocytes closest the spermatheca swell with yolk granules, formed by endocytosis of yolk protein from the pseudocoelom (Kimble and Sharrok 1983; Grant and Hirsh 1999; Hall et al. 1999). Oocyte maturation takes place in the oocyte closest to the spermatheca, every 20 minutes if sperm are present. Meiotic maturation takes place in an assembly line fashion, such that the most proximal oocyte (often referred to as the –1 oocyte) matures, enters the

spermatheca at ovulation, and is fertilized (Figure 1C). Major sperm protein (MSP) budded off sperm in the spermatheca displaces ephrin bound to oocyte VAB-1 receptors, resulting in relief of MAPK inhibition and oocyte maturation (Johnston et al. 2006). During maturation, nuclear envelope breakdown (NEBD) occurs, cytoskeletal rearrangement causes the oocyte to become spherical, spindles assemble, and bivalent chromosomes begin to align on the metaphase plate (Ward and Carrel 1979; McCarter et al. 1999). MSP also binds non-VAB receptors on oocyte and gonadal sheath cell membranes, stimulating contractions that induce ovulation of the mature oocyte into the spermatheca (Johnston et al. 2006). Oocyte maturation also stimulates dilation of the distal spermatheca, which is pulled over the oocyte. The oocyte is immediately penetrated by a sperm and fertilized. Cell-cell recognition between gametes is likely mediated by SPE-9, SPE-38, SPE-42, EGG-1, and EGG-2 (Singson et al. 1998). The union of sperm and egg at fertilization triggers egg activation and converts the egg into a developing embryo (Singson et al. 2008). In *C. elegans*, egg activation includes an increase of intracellular calcium, completion of meiosis, polar body formation, reorganization of the cortical actin cytoskeleton, and dynamic remodeling of the oocyte surface with secretion of a chitinous eggshell (Singson 2008). Egg activation occurs as the newly formed embryo passes from the spermatheca to the uterus through the spermathecal-uterine valve (Altun and Hall 2005). Eggs are laid prior to the completion of embryogenesis and the hatching of juvenile worms. If the worm is infertile due to mutations that affect gamete function at fertilization, the uterus fills with unfertilized oocytes, which become endomitotic and are also laid by hermaphrodites (Singson 2008).

Reverse genetic approaches have identified several conditional egg sterile mutants that only effect egg function at fertilization or egg activation. The first of these candidate oocyte components of fertilization machinery to be studied were the *egg-1* and *egg-2* genes (Kadandale et al., 2005). These two genes encode type II transmembrane molecules with extracellular domains that contain arrays of eight low density lilpoprotein (LDL)-receptor repeats (Singson 2008). The simple model for EGG-1/EGG-2 is that they function as oocyte surface receptors for sperm (Kadandale et al., 2005). The *egg-3* gene encodes a member of the protein tyrosine phosphatase-like (PTPL) family, which are typically thought to function as competitors for active phosphatases or as scaffolds/adaptors (Maruyama et al., 2007). EGG-3 exhibits a dynamic localization pattern dependent on meiotic cell cycle progression. It is associated with the oocyte plasma membrane/cortex of developing oocytes and later disperses to cortical foci during meiotic anaphase I before being degraded (Maruyama et al., 2007). EGG-3 was found to localize with CHS-1 and MBK-2 (Maruyama et al., 2007). CHS-1 is a large channel protein that catalyzes the polymerization of UDP-*N*-acetyl-glucosamine to produce insoluble chitin that comprises the eggshell (Johnston et al. 2006). MBK-2 encodes a DYRK kinase required to mark maternal proteins, including MEI-1 and MEI-2, and OMA-1 and OMA-2 for degradation during early zygotic development (Pellettieri et al., 2003; Stitzel et al. 2006). The localization of EGG-3 and CHS-1 on the oocyte cortex appear to be interdependent and both are required for proper localization of MBK-2. MBK-2 and EGG-3 have been shown to bind to each other, leading to the conclusion that these three proteins form a complex on the plasma membrane (Maruyama et al. 2007) (Figure 3). Redistribution of EGG-3 and associated proteins in meiotic anaphase is likely

to regulate the access of molecules like MBK-2 to cytoplasmic targets (Govindan and Greenstein, 2007; Stitzel et al., 2007; Singson et al. 2008).

1.3.2 Intracellular Trafficking and Endocytosis in Oocytes

In *C. elegans*, throughout development, cells are continually exporting and internalizing proteins and also undergo dramatic organelle movements. One important model for mechanistic studies of endocytosis in *C. elegans* focuses on oocytes, which internalize yolk proteins and associated lipids by clathrin–mediated endocytosis (Sato 2006). YP170 is a cholesterol binding/transport protein related to human ApoB-100, the major protein component of serum low-density lipoprotein (LDL) (Grant and Sato 2006). The yolk receptor in *C. elegans* is RME-2, an LDL-receptor related molecule expressed specifically in the oocyte. RME-2 contains a typical NPXY internalization motif in its intracellular domain that is known to direct other members of the LDL-receptor family into clathrin coated pits (Grant and Sato 2006). Trafficking of yolk and yolk receptors also depends critically upon the activities of the endocytic Rab proteins, RAB-5, RAB-7, and RAB-11 (Grant and Sato 2006). Upon fertilization, the cell also needs to internalize proteins from the cortical surface such as EGG-3 and MBK-2 and exocytose vesicles that comprise the eggshell and may block polyspermy. Recent work has shown that there is a fusion event in *C. elegans* involving vesicles rich in caveolin (Sato et al., 2006), which is similar to that of cortical granule exocytosis in the slow block to polyspermy. *C. elegans* has two caveolin-like genes, encoding CAV-1 and CAV-2 (Tang et al. 1997). CAV-1 is about 67% similar and 37% identical to the mammalian caveolins-1 and 3, while CAV-2 is more distantly related (Scheel et al. 1999). The exact mechanism by which this effectively blocks polyspermy remains unknown, however it may be more relevant in

eggshell formation. The secretion of a chitinous eggshell in response to sperm entry is necessary to provide a permeability barrier and mechanical support for early embryonic development (Ward and Carrel 1979). Defects in eggshell formation have been linked to defects in cell cycle regulation and meiosis (Bembenek et al. 2007; Shakes et al. 2003).

1.4 Functional genomic analyses in *C. elegans*

Since the completion of the genome project of the nematode *C. elegans* in 1998, functional genomic approaches have been applied to elucidate the gene and protein networks in this model organism (Lee et al., 2004). The architecture and dynamics of molecular networks can provide an understanding of complex biological processes complementary to that obtained from the in-depth study of single genes and proteins. RNAi (Guo and Kemphues, 1995; Fire et al., 1998) is amenable to high throughput approaches that can be used to systematically knock down large numbers of transcripts, thereby relating perturbations of molecular networks to phenotypes (Gunsalus and Piano 2005). The foundation for a phenome map of early embryogenesis has been laid by a host of recent large-scale RNAi studies, which have provided at least a first-pass phenotypic analysis for nearly every protein-coding gene in the genome (Fraser et al., 2000; Gönczy et al., 2000; Piano et al., 2000; Maeda et al., 2001; Piano et al., 2002; Kamath et al., 2003; Simmer et al., 2003; Rual et al., 2004; Fernandez et al., 2005; Sönnichsen et al., 2005). Combining genomic data for gene expression profiles, phenotypic data, and protein-protein interactions has allowed the development of a multiple support network of genes involved in early embryogenesis that can be broken down into molecular modules (Gunsalus and Piano 2005). Recently, a set of 766 genes enriched in the ovary as compared to the soma were identified by microarray analysis (Reinke et al., 2000). A

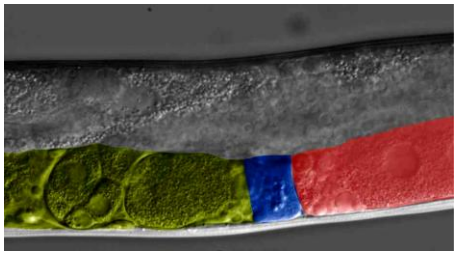
functional analysis of these genes by RNA interference (RNAi) used time lapse microscopy to characterize early embryonic defects for 161 genes in terms of 47 RNAi-associated phenotypes (Piano et al., 2002). To identify candidate genes that affect early events of egg activation we decided to examine the oocyte integrity module (Table 1).

Figure 1: *C. elegans* germline morphology

A

Adapted from Worm Atlas

B



Singson et al. 2008

C

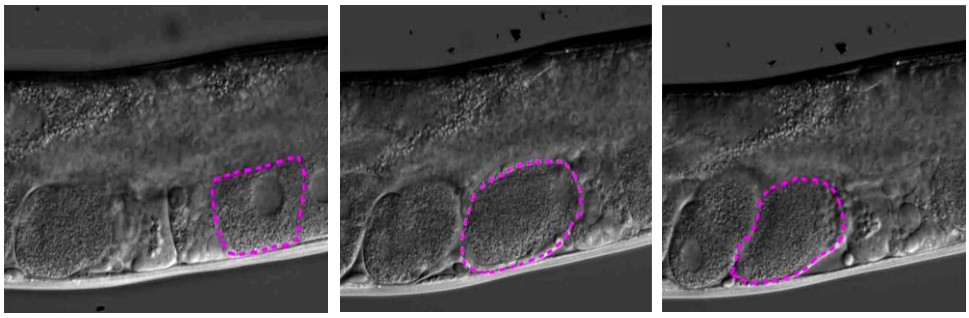


Figure 1: *C. elegans* germline morphology

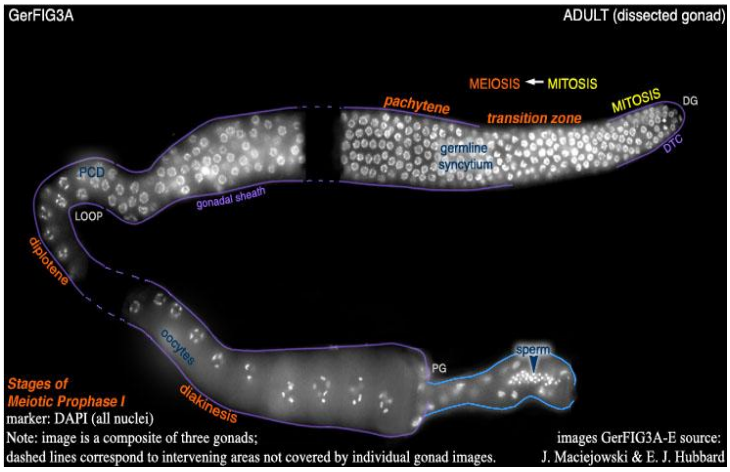
(A) Colored schematic of the bi-lobed gonad. Oocytes are shaded red, spermatheca is shaded blue, and developing embryos are green.

(B) A wild-type hermaphrodite reproductive tract in the region of the spermatheca with developing embryos in the uterus. Oocytes are shaded red, spermatheca is shaded blue, and developing embryos are green.

(C) DIC image of oocyte progression. Highlighted proximal oocyte in the leftmost figure gets ovulated into the spermatheca, where it is fertilized. Upon fertilization it is deposited into the uterus where it undergoes egg activation.

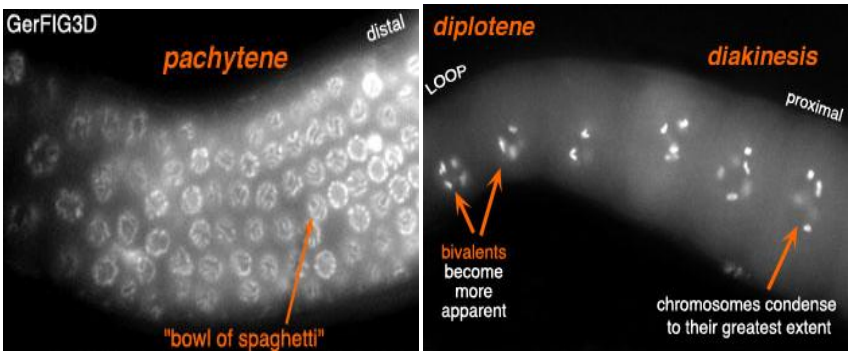
Figure 2: Stages of Meiotic Prophase I

A.



Adapted from Worm Atlas

B. **C.**



Adapted from Worm Atlas

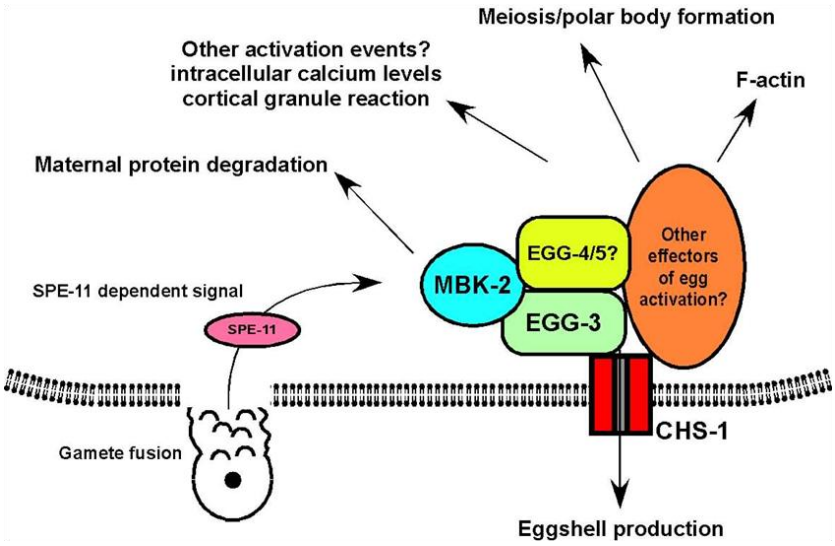
Figure 2: Stages of Meiotic Prophase I

(A) DAPI stain of adult dissected gonad.

(B) Germ cells in pachytene, with nuclei characterized by "bowl of spaghetti" morphology.

(C) Germ cells in diplotene become organized in single file as they enter the proximal arm and progress to diakinesis where they arrest until maturation.

Figure 3: A model for the EGG-3 complex and the regulation of key egg activation events



Singson et al. 2008

Figure 3: A model for the EGG-3 complex and the regulation of key egg activation events

After sperm-egg fusion, egg activation is dependent of the activity of SPE-11.

The EGG-3 complex then regulates eggshell formation, F-actin dynamics, the turnoverof maternal proteins, meiosis/polar body formation, and other egg activation events.

Table 1: Oocyte integrity/meiosis module genes by function

Gene	Name	Identity	Function	Category
C31H1.8	C31H1.8	Unknown	Unknown	Oocyte integrity
C44B12.2	C44B12.2	Unknown	Unknown	Oocyte integrity
C55C3.5	C55C3.5	Unknown	Unknown	Oocyte integrity
F56F3.1	Pqn-45	Unknown	Unknown	Oocyte integrity
H02I12.2	cbd-1	Unknown [peritrophin-A chitin binding domain]	Unknown	Oocyte integrity
K07A12.2	K07A12.2	Unknown	Unknown	Oocyte integrity
T01H3.4	T01H3.4	Unknown	Unknown	Oocyte integrity
T05G5.7	T05G5.7	Uncharacterized conserved protein	Unknown	Oocyte integrity
C27D9.1	C27D9.1	Unknown	Unknown	Oocyte integrity
C25A1.9	C25A1.9	[KOG: protein regulatory phosphatase regulatory subunit]	Unknown	Meiosis
C28C12.2	C28C12.2	Unknown	Unknown	Meiosis
H02I12.5	H02I12.5	Unknown	Unknown	Meiosis

Table 1: Oocyte integrity/meiosis module genes by function.

Genes in the oocyte integrity/meiosis module by function from (Gunsalus and Piano 2005). Highlighted gene, H02I12.2 (*cbd-1*) was used in RNAi based functional analysis.

Chapter 2: Materials and Methods

2.1 *C. elegans* strains

General methods for culturing and handling were carried out as previously described (Brenner, 1974). All experiments were carried out at 20°C unless otherwise indicated. The strains and genetic markers used were as follows: wild-type Bristol strain (N2), CYB-1:GFP, ekls2[unc-119(+) pie-1 promoter::CYB-1::GFP]; mCherry:EGG-3, asIs2[unc-119(+)pie-1 promoter::mcherry::egg-3], GFP:CHS-1, nnIs2[unc-119(+)pie-1 promoter::gfp::chs-1], GFP:EGG-1, unc-119; Is[unc-119 + pie-1::GFP:egg-1], GFP:EGG-2, unc-119; Is[unc-119 + egg-2::GFP], OMA-1:GFP, unc-119(ed3); tcIs1[pRL475+pPDMMO10] (Poma-1:OMA-1:GFP), DH1033 (carrying YP170:GFP).

2.2 RNAi methods

RNAi was performed by soaking (Kamath and Ahringer 2003). cDNA clone for cbd-1 (yk789g03) was obtained from Dr. Yuji Kohara. Clones were PCR amplified with T7 primers and PCR products were used as templates to make dsRNA. L4 worms were soaked in dsRNA solution for 24 hours and recovered. After 24 hours at 25°C on OP50 plates, adult worms were observed by Nomarski DIC and fluorescence microscopy. A control soaking in buffer was carried out in parallel to each RNAi experiment. Efficacy and variability of RNAi was examined by comparing phenotypes manifested in RNAi treated embryos with buffer treated embryos.

2.3 DAPI Staining

RNAi and control animals were washed in M9 and soaked in cold methanol for 5 minutes at room temperature. Methanol was removed and worms were washed in M9. Whole worms were mounted on a 2% agarose pad in 1 μg/ml DAPI in GEL/MOUNT (biomedia). Worms were analyzed using Zeiss Axioplan 2 Imaging Microscope, model 202204. DIC images were taken under a green filter and fluorescent images were taken under a blue filter.

2.4 Live imaging and analysis

For phenotypic analysis, whole worms were mounted with 6mM levamisole in M9 on a 2% agarose pad. Worms were visualized using Zeiss Axioplan 2 Imaging Microscope, model 202204. DIC images were taken under a green filter and fluorescent images were taken under either green (for GFP) or red (for MCherry) filters.

Chapter 3: Phenotypic analysis of *cbd-1* depletion via RNAi

3.1 Background

Fertilization triggers meiotic resumption and the rapid assembly of a chitinous eggshell that surrounds the embryo and is responsible for multiple developmental events at the one cell stage (Johnston et al. 2006). The *C. elegans* eggshell is composed of three layers; an outer vitelline layer, a middle chitin-containing layer, and an inner lipid rich layer. Though detailed studies are lacking, it has been shown that shortly after sperm penetration, the outer plasma membrane-like layer separates from the egg cytoplasm, resulting in a dense outer vitelline layer (Johnston et al. 2006). Underlying the vitelline layer is a structureless zone that subsequently becomes filled with chitin and protein, resulting in the formation of the mechanically resistant middle layer of the shell (Johnston et al. 2006). Specific proteins in the middle layer have not been identified, though proteins with chitin-binding domains are likely candidates. The *cbd-1* gene (H02I12.1), a member of the oocyte integrity module (Table 1), encodes a 1319 residue protein with 12 chitin-binding peritrophin-A domains, which may enable mechanical cross-linking of chitin. Previous studies have shown that RNAi animals have an osmotically-sensitive lethal phenotype, implying that *cbd-1* may participate in eggshell synthesis and early embryonic development.

3.2 RNAi depletion of *cbd-1*

To begin to decipher the function of *cbd-1*, we decided to deplete it using RNAi delivered through a soaking protocol. After soaking wild-type Bristol (N2) L4 worms, it was observed that the worms were sterile, with no embryos. Oocyte morphology was abnormal, remaining small and cuboidal while moving down the distal arm (Fig 4A).

Unfertilized oocytes in the uterus were large and nebulous, implying a defect in eggshell deposition. (Fig 4A). When exiting the spermatheca, embryo movement was runny and slid over top of the neighboring embryo. After examining MCherry:His worms soaked in *cbd-1*RNAi, it was found that the one cell embryo actually contained several nuclei, each arrested in metaphase (Fig 4B). This fusion of oocytes implies that the vitelline envelope is being compromised in these worms. This was consistent with DAPI staining, which did not detect sperm entry.

The molecular nature of the EGG-1 and EGG-2 proteins suggests a model in which their arrays of LDL receptor repeats are displayed on the oocyte surface where they can bind to a sperm ligand (Kadandale et al. 2005). Other LDL receptor repeat-containing molecules are known to function specifically in other oocyte functions such as yolk uptake (Grant and Hirsh 1999). EGG-1:GFP and EGG-2:GFP have been shown to localize to the plasma membrane of developing oocytes, which was consistent with control worms (Figure 5A). EGG-1:GFP and EGG-2:GFP worms subjected to *cbd-1* RNAi showed very little EGG-1:GFP and EGG-2:GFP signal on the oocyte plasma membrane (Figure 5B), implying that they may not be able to mediate gamete interactions at fertilization.

Caveolin are lipid enriched invaginations on the plasma membrane thought to play a role in signaling, cholesterol homeostasis, and clathrin independent endocytosis (Sato and Grant 2006). Previous studies found that CAV-1 is strongly expressed in the germ line and suggest that CAV-1 plays a role in Ras/MAPK dependent meiotic progression (Scheel et al., 1999). The function of CAV-1 is dependent on the association with cholesterol-rich membrane microdomains, providing a link between the membrane

composition of germ cells and meiotic progression (Scheel et al. 1999), processes seemingly affected by *cbd-1*. In the syncytial gonad, CAV-1:GFP localizes to the plasma membrane and to punctuate Golgi ministacks in the cytoplasm. As oocytes form, CAV-1:GFP appears in ring-like membrane compartments called CAV-1 bodies in the cytoplasm (Sato and Grant 2006). In an oocyte about to ovulate, punctuate CAV-1:GFP clusters at the cortex and around the nucleus (Figure 6A). After ovulation and fertilization, CAV-1 bodies fuse with the plasma membrane (Figure 6A). Shortly after, most of the CAV-1:GFP is internalized and degraded in the one-cell stage embryo (Sato and Grant 2006). Fusion of CAV-1 bodies with the plasma membrane occurs normally in *spe-9* and *fog-2* mutants, showing that this is a fertilization independent event (Sato and Grant 2006). *spe-9* mutant sperm cannot fertilize oocytes, while *fog-2* mutants are devoid of sperm (Singson et al. 1998; Kadandale and Singson 2004). CAV-1 plasma membrane fusion is also dependent on the cell cycle. RNAi of *emb-27*, an ortholog of the anaphase-promoting complex produces an accumulation of one-cell stage embryos arrested in metaphase I of meiosis that also fail to degrade CAV-1:GFP (Sato and Grant 2006). *cbd-1* RNAi worms showed a loss of intracellular CAV-1 bodies in proximal oocytes (Figure 6B). This phenotype is similar to that seen in *rme-2* mutants, which lack the yolk receptor and thus fail in yolk uptake by oocytes (Grant and Hirsh 1999). This suggests that yolk uptake via RME-2 is important for correct localization of CAV-1:GFP in oocytes (Grant and Hirsh 2006) and *cbd-1* may function in the intracellular trafficking of these membrane associated protiens.

Considering the small size of oocytes in the proximal arm and defects in CAV-1:GFP localization, we decided to examine yolk content within *cbd-1*depleted oocytes

using a strain expressing transgenes encoding the major yolk protein YP-170 fused to GFP (Grant and Hirsh 1999). During the normal maturation of oocytes within the *C. elegans* germline, yolk proteins secreted by the intestine are actively endocytosed from the pseudocoelomic space and stored within membrane bound compartments. *C. elegans* oocytes internalize yolk proteins and associated lipids by clathrin–mediated endocytosis (Sato et al. 2006). Lipids and proteins derived from yolk are thought to provide essential nutrients required to support the rapid development of the embryo. YP-170:GFP, like endogenous yolk, is a cholesterol binding/transport protein related to human ApoB-100, the major protein component of serum low-density lipoprotein (LDL) (Grant and Sato 2006). *cbd-1* RNAi animals showed decreased accumulation of YP-170:GFP in oocytes and an increased signal in the intestine (Figure 7B). Worms from the control soak showed a phenotype similar to unsoaked worms, confirming that the abberant acculumation of yolk in RNAi animals was not due to removal of the worms from food containing plates during the soaking procedure (Figure 7A).

RME-2, an LDL-receptor molecule is expressed specifically in the *C. elegans* oocyte. RME-2 is first expressed in very early oocytes in the bend region of the gonad where it appears primarily in the ER and Golgi. Soon thereafter it reaches the plasma membrane, and in large nearly full-grown oocytes is primarily found on the surface in clathrin coated pits and recycling endosomes (Figure 8A). After ovulation, RME-2 appears in intracellular vesicles, which diminish as the embryo develops (Figure 8A). Clearly, this redistribution of RME-2 from the cell surface to intracellular vesicles appears to be coincident with the dramatic cellular changes associated with fertilization (Grant and Hirsh 1999). It has been shown that *rme-2* mutants are characterized by

slightly small oocytes devoid of yolk, otherwise normal germ line morphology, high-level pseudocoelomic yolk accumulation, reduced embryo production, and low embryo viability (Grant and Hirsh, 1999), similar to *cbd-1* RNAi animals. RME-2:GFP worms subjected to *cbd-1* RNAi lacked RME-2 accumulation on the plasma membrane and intracellular vesicles (Figure 8B), which is likely responsible for the failure of oocytes to accumulate yolk.

In many species, the egg surface changes dramatically after fertilization and during the egg-to embryo transition. In *C. elegans*, a chitinous eggshell is secreted in response to sperm entry and covers the embryo to provide chemical impermeability and mechanical support for embryonic development (Ward and Carrel 1979). EGG-3, a member of the protein tyrosine phosphatase-like (PTPL) family is essential for regulating several important events during egg activation, such as polarized reorganization of the cortex actin cytoskeleton, eggshell formation, and polar body formation (Maruyama et al. 2007). Localization patterns of EGG-3 and CHS-1 are interdependent and MBK-2 requires EGG-3 and CHS-1 for its proper localization (Maruyama et al. 2007). This suggests that EGG-3 may act as a molecular scaffold regulating egg activation machinery in response to sperm entry through formation of a plasma-membrane associated complex (Maruyama et al. 2007). It has been shown in wild-type oocytes that MCherry:EGG-3 is associated with the plasma membrane of oocytes and newly fertilzed embryos through metaphase, then later moves to cytoplasmic foci during anaphase and is eventually degraded (Maruyama et al. 2007). Control soaked animals were consistent with this phenotype (Figure 9A), however RNAi animals showed reduced accumulation of EGG-3 on the plasma membrane, suggesting a defect in intracelluar trafficking (Figure 9B).

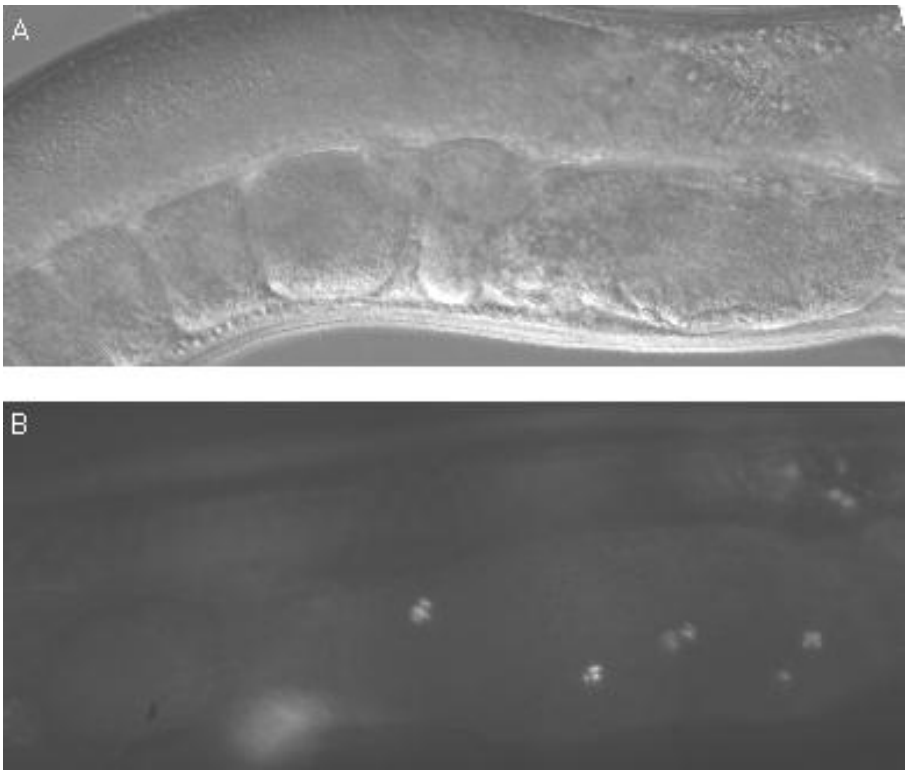
Considering the interdependence of EGG-3 and CHS-1 localization, as well as the poorly formed eggshell of *cbd-1* RNAi embryos, we subjected worms expressing CHS-1:GFP to *cbd-1* RNAi. The *chs-1* gene encodes a chitin synthase that is predicted to have 15 transmembrane domains and catalyzes the polymerization of UDP-N-acetyl-glucosamine to produce chitin (Zhang et al. 2005) The *chs-1* gene is essential for eggshell formation, osmotic integrity, error-free chromosomal segregation during meiosis, and polar body extrusion (Zhang et al. 2005). *cbd-1* RNAi worms showed CHS-1:GFP localization similar to Mcherry:EGG-3 localization in RNAi animals, with reduced accumulation at the plasma membrane in developing oocytes (Figure 10B).

The DYRK kinase MBK-2 is required for the egg-to-embryo transition in *C. elegans*. It directs the ubiquitin-mediated degradation of maternal proteins, including MEI-1 and MEI-2, and OMA-1 and OMA-2, which are required for meiotic maturation. It is thought that EGG-3, along with CHS-1 sequesters MBK-2 at the oocyte cortex where it restricts its interaction until the oocyte enters M phase (Maruyama et al., 2007; Stitzel et al. 2006). OMA-1 is a CCCH-type zinc finger protein that serves as a nexus for signals that regulate the transition from oogenesis to embryogenesis (Shirayama et al. 2006). While OMA-1 promotes oocyte maturation during meiosis, destruction of OMA-1 is needed during the first cell division for initiation of ZIF-1 dependent proteolysis of cell-fate determinants (Shirayama et al. 2006). In the distal gonad, OMA protein levels are low due to GLD-1-dependent translational inhibition (Lee et al. 2004). OMA protein levels increase at the pachytene stage of meiosis I, reach maximum levels in the maturing ooycte, and quickly fall to much lower levels during the first mitosis (Detwiler et al. 2001; Shimada et al. 2002). In OMA-1:GFP control worms, OMA-1 accumulated during

oogenesis and remained high until the one-cell stage, but rapidly declined during the first and second mitosis (Figure 11A). OMA-1:GFP worms soaked in *cbd-1* RNAi retained the GFP signal in unfertilized oocytes in the uterus (Figure 11B).

Considering the meiotic arrest observed in *cbd-1* depleted embryos, we decided to observe dynamics of cyclin B1. Fertilization dependent proteolysis is required for exit from meiotic M phase in mouse and *Xenopus* (McNally et al. 2005). High levels of CYB-1:GFP fluorescence are typically observed in the immature oocytes of the gonad and almost no fluorescence is observed in the mitotic embryos in the uterus. Previous work has shown that in fertilized embryos, CYB-1:GFP levels begin to decrease dramatically soon after spermatheca exit then remain fairly stable for a short period of time (McNally and McNally 2005). These two periods of rapid CYB-1:GFP turnover and CYB-1:GFP stability coincide with the timing of meiosis I and II, respectively, supporting the interpretation that cyclin B levels must decline for the embryo to exit meiosis I, but a residual level of activity must remain in the embryo to propel it to metaphase II. Developing oocytes in the distal arm of control worms showed a strong CYB-1:GFP signal, which disappeared in the first embryo (Figure 12A). *cbd-1* RNAi worms showed high levels of CYB-1:GFP in developing oocytes, as well as unfertilized oocytes in the uterus, which had a small globular shape (Figure 12B).

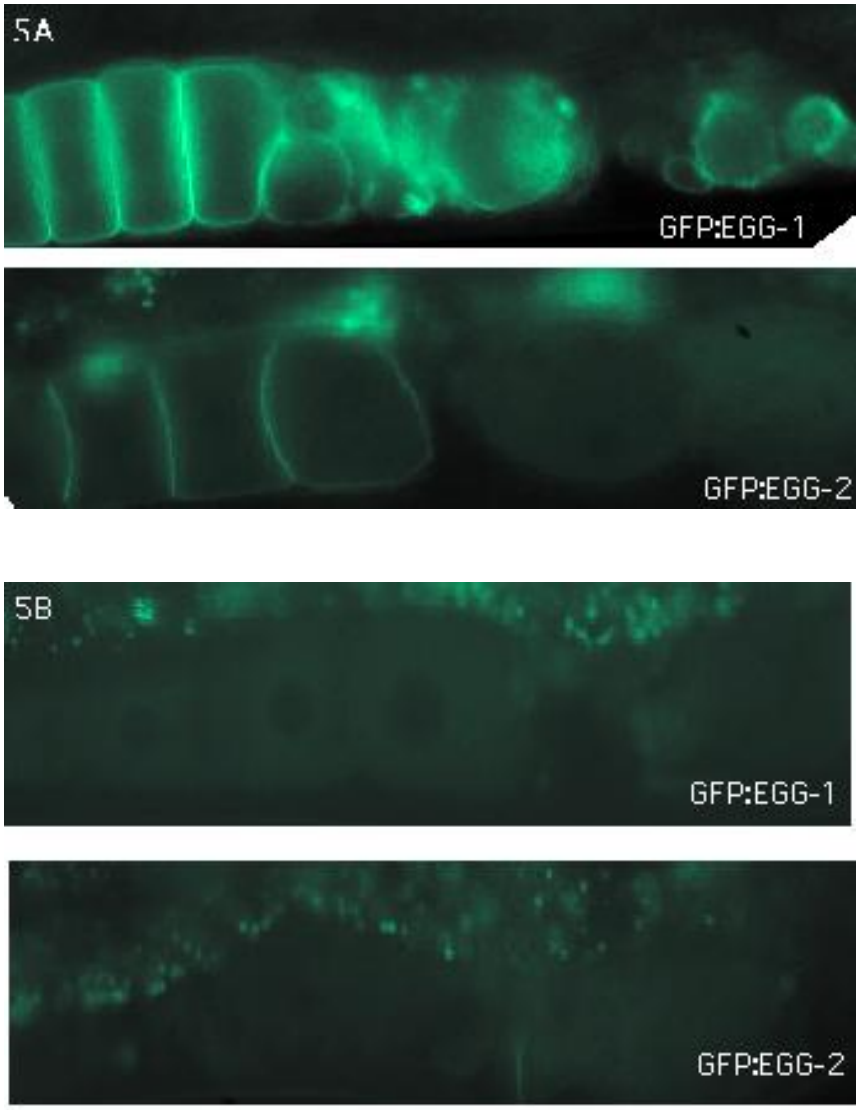
Figure 4: *cbd-1* RNAi depletion affects eggshell deposition and meiotic progression



(A) DIC image showed oocyte morphology was small and cuboidal. Unfertilized oocyte was large and nebulous.

(B) MCherry:Histone showed that the unfertilized oocyte in the uterus contained several metaphase arrested nuclei, implying fusion of several oocytes.

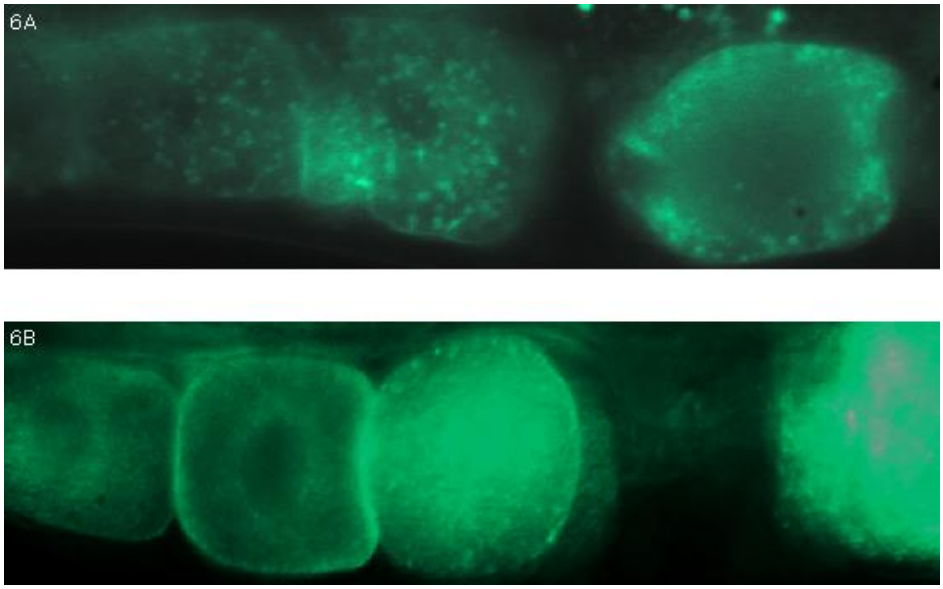
Figure 5: *cbd-1* RNAi depletion affects EGG-1:GFP and EGG-2:GFP localization



(A) Control soaked EGG-1:GFP worms (left) and EGG-2:GFP worms (right) show protein localization at the membrane.

(B) *cbd-1* RNAi worms show very little EGG-1:GFP signal (left) and EGG-2:GFP signal (right).

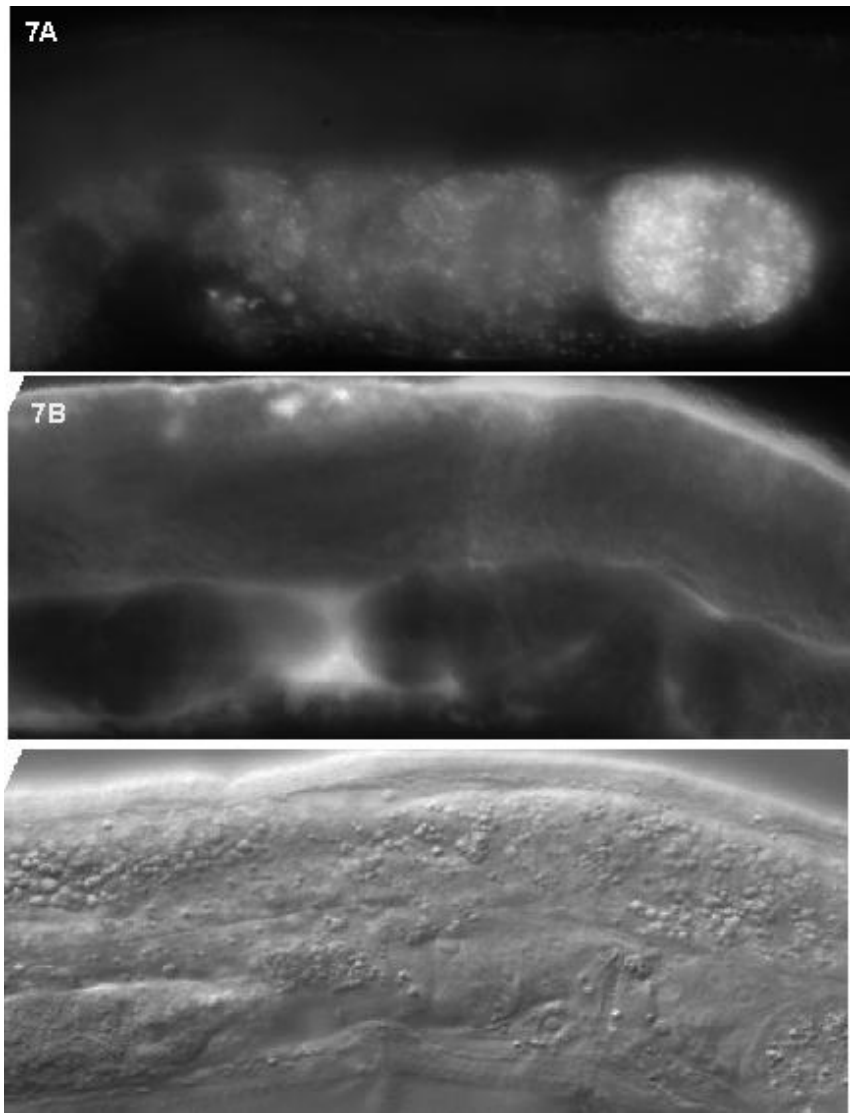
Figure 6: *cbd-1* affects CAV-1 dynamics



(A) In control animals, CAV-1:GFP remains intracellular in proximal oocytes and fuses to the membrane after fertilization and ovulation.

(B) *cbd-1* RNAi animals, showed loss of intracellular CAV-1 bodies in proximal oocytes.

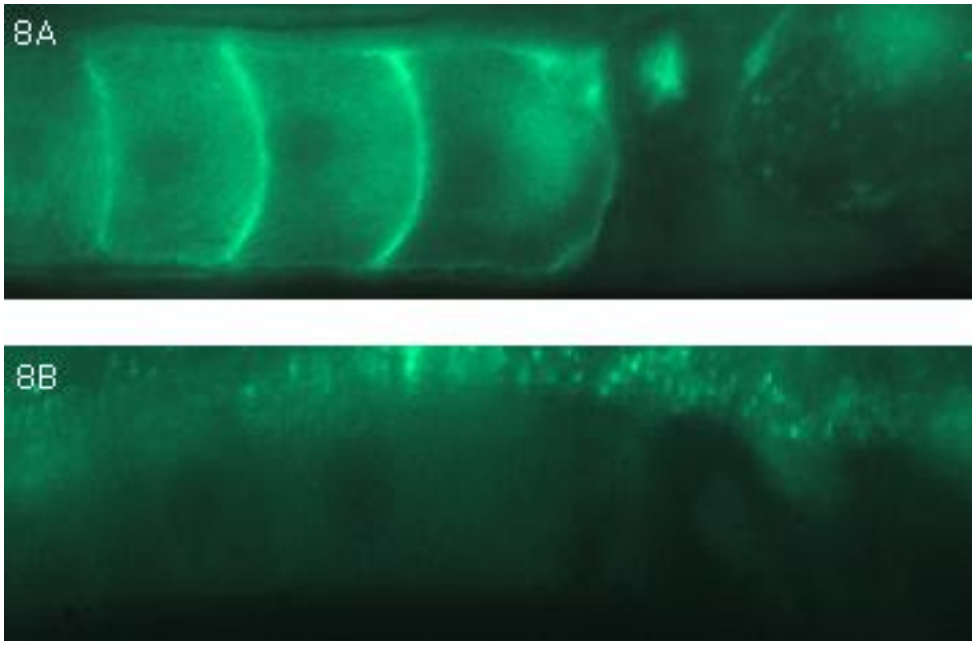
Figure 7: *cbd-1* RNAi worms fail to take up yolk in oocytes



(A) Control soaked YP-170:GFP worms show oocytes enriched in yolk particles.

(B) *cbd-1* RNAi worms show very little YP-170:GFP accumulation in developing oocytes. DIC image (below) shows abnormal oocyte morphology of RNAi animals.

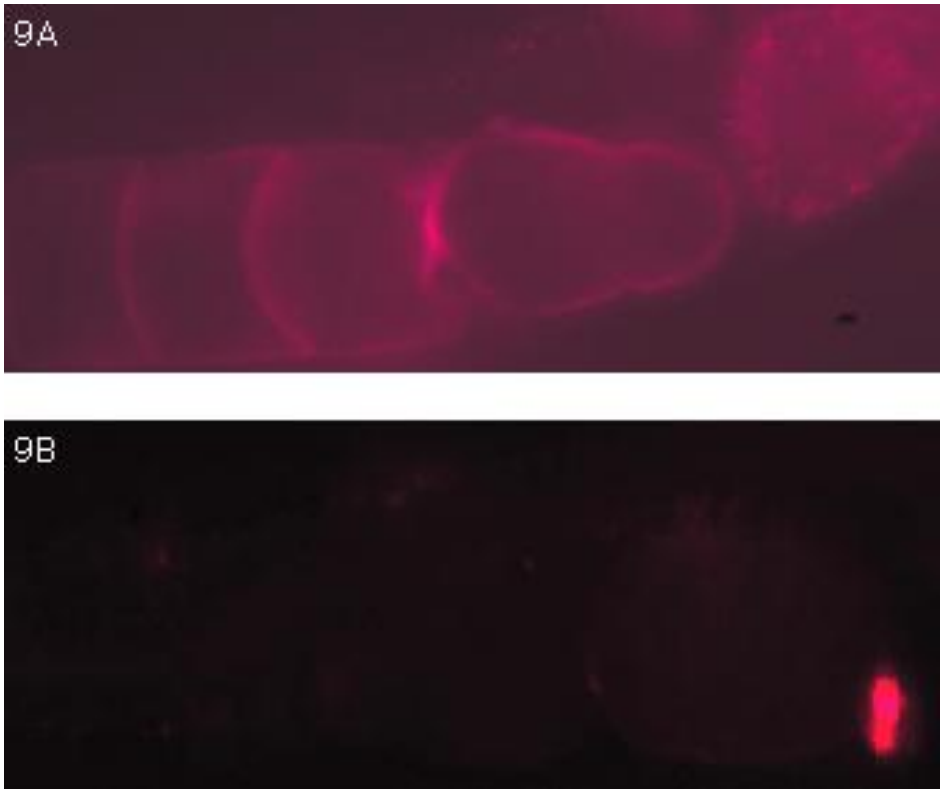
Figure 8: *cbd-1* influences localization of RME-2:GFP



(A) Control soaked worm shows RME-2:GFP accumulation at the plasma membrane of developing oocytes, which appears in intracellular vesicles after ovulation.

(B) RNAi animals show very little RME-2:GFP accumulation in oocytes.

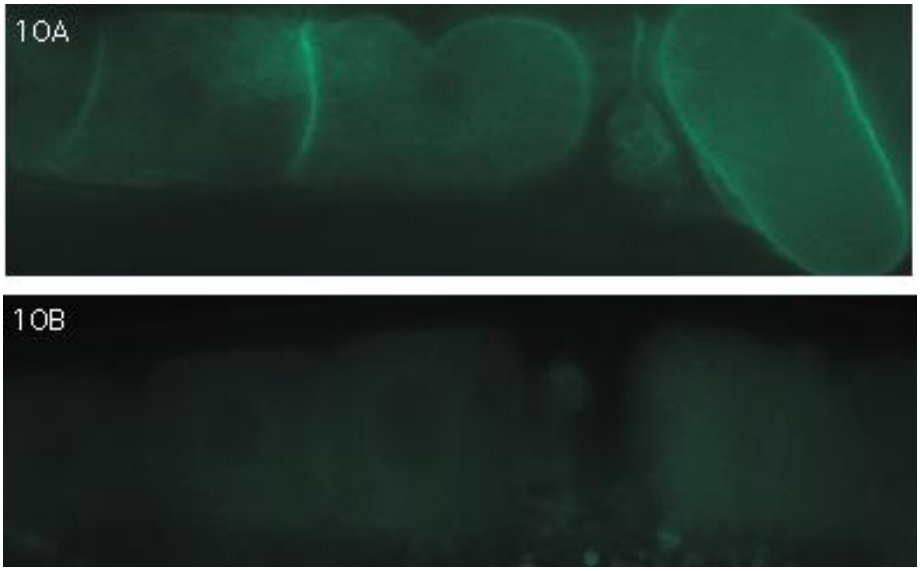
Figure 9: *cbd-1* affects EGG-3 accumulation at the plasma membrane



(A) Control worms show EGG-3:GFP accumulation at the plasma membrane of developing oocytes, which becomes intracellular after ovulation.

(B) RNAi worms lack EGG-3:GFP localization to the plasma membrane in developing oocytes.

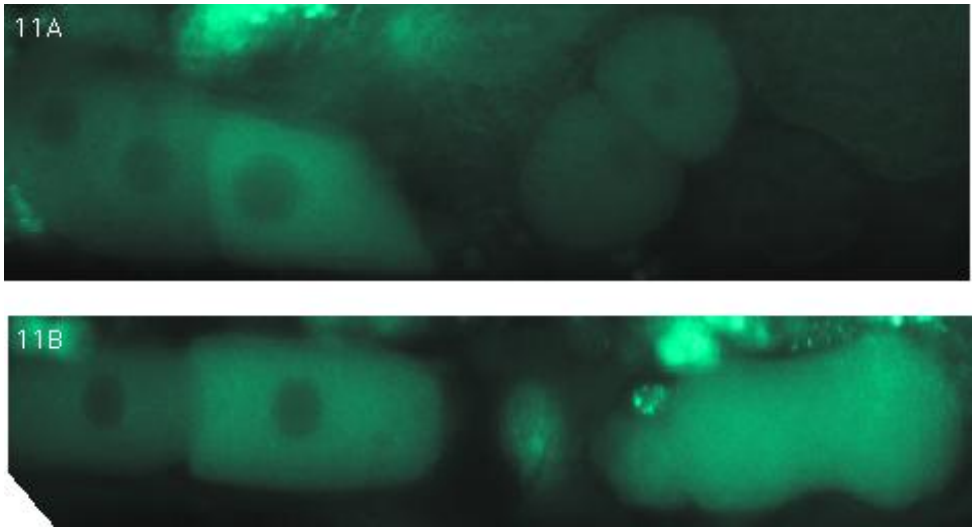
Figure 10: *cbd-1* affects CHS-1 accumulation at the plasma membrane



(A) Control worms show CHS-1:GFP accumulation at the membrane of developing oocytes, similar to EGG-3.

(B) *cbd-1* RNAi worms show a reduced accumulation of CHS-1:GFP at the membrane.

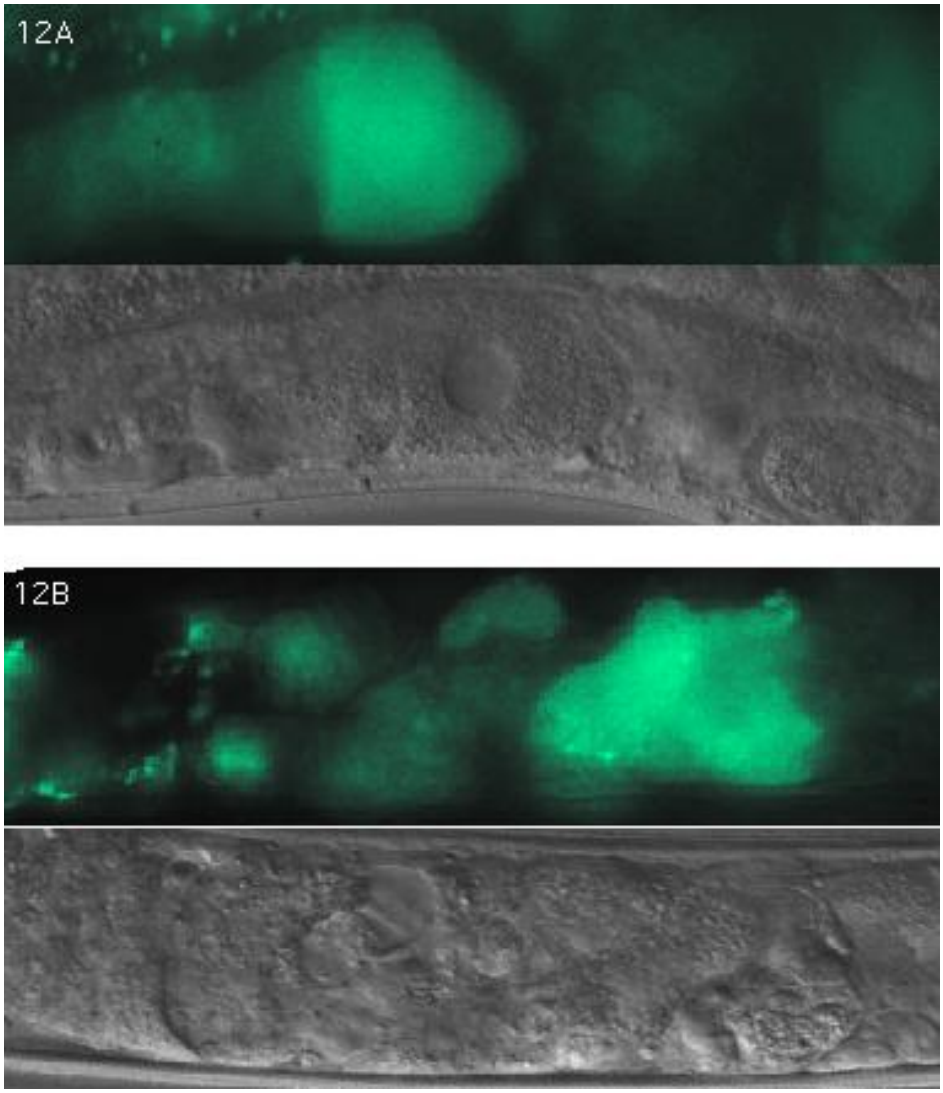
Figure 11: *cbd-1* depletion affects ability of MBK-2 to degrade targets



(A) OMA-1:GFP control soaked worms showed OMA-1 accumulation during oogenesis, which remained high until the one cell stage, but rapidly declined during the first and second mitosis.

(B) RNAi worms show OMA-1:GFP accumulation in oocytes, which is retained in unfertilized oocytes in the uterus.

Figure 12: *cbd-1* influences cyclin B1 turnover



(A) Control soaked animals show CYB-1:GFP accumulation in developing and proximal oocytes, which disappears in the first embryo.

(B) RNAi animals show a CYB-1:GFP signal retained unfertilized oocytes in the uterus. DIC image (right) shows abnormal oocyte morphology of RNAi worms.

Chapter 4: Discussion

4.1 *cbd-1* controls intracellular trafficking events important to oocyte development and egg activation

Clearly, depletion of *cbd-1* through RNAi caused several defects in intracellular trafficking. First, LDL repeat containing sperm receptor molecules, EGG-1 and EGG-2 did not accumulate at the plasma membrane, which likely prevented sperm from entering the oocyte. This is supported by DAPI staining, which did not detect sperm entry in *cbd-1* RNAi animals. Another LDL repeat containing molecule, yolk receptor RME-2 also failed to localize to the plasma membrane when worms were subjected to *cbd-1* RNAi. Observation of YP-170:GFP demonstrated that oocytes of these animals did not properly internalize yolk from the intestine, which likely contributed to their small size and poor development. Considering that yolk uptake via RME-2 is important for CAV-1 localization, it is not surprising that we observed a defect in intracellular trafficking of CAV-1 in *cbd-1* RNAi worms. CAV-1 also plays a role in Ras/MAPK dependent meiotic progression, therefore its mislocalization may have contributed to the metaphase arrest observed in RNAi worms. It is possible that *cbd-1* control of intracellular trafficking may extend to CHS-1, an oocyte transmembrane protein that catalyzes chitin polymerization. RNAi worms failed to accumulate CHS-1 at the plasma membrane, which clearly affected proper eggshell formation, demonstrated by the observation of amorphous masses in the uterus. Though EGG-3 is not itself a transmembrane molecule its localization is interdependent on CHS-1 and was similarly affected by *cbd-1* RNAi. It is possible however, that *cbd-1* may act directly on EGG-3 localization. Regardless, it is assumed that *cbd-1* RNAi prevented EGG-3 and CHS-1 from properly localizing MBK-

2. By holding the protein at the membrane, the EGG-3/CHS-1 complex prevents it from interacting with its cytoplasmic targets. Likewise, the EGG-4 and EGG-5 proteins have been shown to inhibit the phosphatase activity of MBK-2 (Parry et al. unpublished data). *Cbd-1* RNAi worms retained an OMA-1:GFP signal in their embryos, showing a failure of MBK-2 to degrade its targets. It is unusual that the absence of the EGG-3/CHS-1 membrane complex would inhibit MBK-2 activity, since these molecules usually act to physically separate MBK-2 from its targets, however EGG-4 and EGG-5 inhibition may explain the persistence of maternal proteins.

4.2 Perspectives and Future Directions

It is clear that *cbd-1* regulates trafficking of proteins to the membrane that function in sperm entry and egg activation events. Considering the variability of the RNAi procedure, it would be constructive to verify and quantify the depletion of *cbd-1* through an anti-*cbd-1* antibody. It may also be helpful to try another RNAi procedure, such as injection of dsRNA into the gonad, which may produce more consistent results.

Though we have shown that *cbd-1* functions in trafficking of proteins to the membrane, we are not certain of the specificity of its targets or other proteins that it may interact with to perform its functions. We have seen that it clearly affects localization of LDL repeat containing molecules like EGG-1 and EGG-2, however we are unsure of whether it acts on CHS-1, EGG-3 or both. A yeast two hybrid assay to detect physical interactions between these proteins would be useful in finding *cbd-1* targets. Likewise, we are unsure of exactly how CHS-1, EGG-3, and EGG-4/5 regulate MBK-2 activity. A triple mutant (EGG-3/EGG-4/5) would help elucidate these two layers of MBK-2 regulation, though it is still likely that other regulators exist.

Furthermore, it would be interesting to observe the localization of *cbd-1 in vivo* in live embryos. A construct using GFP fused to the C-terminus of *cbd-1* will be bombarded to integrate it into the *C. elegans* genome and create a transgenic line. It would also be interesting to see if this transgene rescued function in a *cbd-1* mutant. If the transgene can partially restore function, a random mutagenesis and screen for affected genes can elucidate more potential targets of *cbd-1* as well as genes that may act in combination with *cbd-1*. This would start to put *cbd-1* in the context of a molecular network.

There must be many molecules that mediate the molecular underpinnings of fertilization in worms and there is a critical need to continue to screen for and isolate mutations that specifically affect sperm or egg function (Singson et al. 2008). Several large scale RNAi screens in *C. elegans* have provided us with tremendous amounts of first –pass phenotypic analysis for nearly every protein-coding gene in the genome (Fraser et al., 2000; Gönczy et al., 2000; Piano et al., 2000; Maeda et al., 2001; Piano et al., 2002; Kamath et al., 2003; Simmer et al., 2003; Rual et al., 2004; Fernandez et al., 2005; Sönnichsen et al., 2005). Detailed phenotypic analyses of these genes, especially those in the oocyte integrity cluser could uncover other regulators of fertilization, egg activation, and embryo development. When reviewing progress in any model organism, it is important to keep in mind how these studies can extend to work in other systems. Reproductive proteins are known to diverge rapidly (Swanson and Vacquier 2002; Vacquier 1998), however understanding the diversity of reproductive strategies can give us insights into the mechanisms of evolution and how nature deals with the universal issues of how cells interact with each other and their environment (Singson et al. 2008).

In this way, work in *C. elegans* will help us better understand the common miracle of fertilization.

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