ABSTRACT

Identification of Germline and Somatic Cell Specific Genes
Essential for Ovulation in *Caenorhabditis elegans*

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The process of ovulation is an integral part of sexual reproduction in *Caenorhabditis elegans*. Various genes and signaling pathways are responsible for the successful process of ovulation to occur. Identification of these key mechanisms will help us gain knowledge into the mechanisms underlying the process of ovulation. *C. elegans,* a free living soil nematode, was used to identify and study the genes that are essential for ovulation.

To facilitate this study we have used RNA interference (RNAi), a reverse genetics process, to restrict the function of the genes. RNAi causes degradation of the target gene by introduction of a double stranded RNA which has sequence specificity to the gene of interest. I have analyzed data pertaining to sterile (Ste) phenotype from the previous genome-wide RNAi screens. This search yielded 259 genes which caused Ste phenotype in the wild type background.

In this screen, we analyzed 259 genes by RNAi in the wild type and various RNAi resistant mutant backgrounds. A detailed study of the gonad morphology was done to

analyze the effect of the depletion of each gene on ovulation. Based on the ability of the mutants to suppress sterility, the genes were categorized as germline or somatic cell specific. In order to study the morphological changes related to gonad arm, we have conducted nomarski microscopy and nuclear staining techniques.

Based on our study, 20 genes were categorized as germline specific for ovulation and 9 genes somatic cell specific. Genes that have been identified as germline specific belong to functional classes which code for protein synthesis, gene expression regulation, protein transport and modification, protein degradation, ATP synthesis and RNA specific functional classes. The somatic cell specific genes mainly constitute cell architecture, signaling and other functional groups.

Identification of Germline and Somatic Cell Specific Genes
Essential for Ovulation in *Caenorhabditis elegans*

by

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DEDICATION

To family and friends

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CHAPTER ONE

Introduction

Caenorhabditis elegans as a Genetic Model

Caenorhabditis elegans (C. elegans), a free-living soil nematode, was used as a model genetic organism by Sydney Brenner (Brenner, 1974). The name *Caenorhabditis elegans* is derived from Greek and Latin words (*Caeno* means recent in Greek; *rhabditis* means rod in Greek; and *elegans* means nice in Latin). The adult worms are mostly self-fertilizing hermaphrodites with a low percentage of males (Brenner, 1974). It has a short life cycle, ease of cultivation in the lab and a large number of mutants have been generated.

C. elegans is an ideal organism to study development because of the invariant cell lineage, allowing the precursor cells for each tissue and structure to be identified precisely (Maine, 2001) . The animals are transparent and this feature helps to study the developmental aspects of the animal. The oocytes can be observed in live animals in real time which is useful for studying the processes of ovulation (Iwasaki et al., 1996).

C. elegans is an excellent model to study the functions of genes because its entire genome has been sequenced (*C. elegans* sequencing consortium, 1998).The genome of *C. elegans* has approximately 19,000 genes in the 97 Megabases with 43% protein similarity with *Homo sapiens* (The *C elegans* sequencing consortium, 1998). *C. elegans* with its advances in genetic, cell biological and molecular biology techniques was the ideal choice for studying the ovulation process (Hubbard and Greenstein, 2000).

Life Cycle of C. elegans

The life cycle of *C. elegans* consists of embryonic, larval and adult stages. There are four larval stages, L1-L4. It takes 8 hours for the egg to transform into an L1 stage, 12 hours from L1 to L2 stage, 8 hours to transform from L2 to L3 larval stage and L3- L4 larval stage and approximately 18 hours from L4 to adult worm. In the absence of food, overcrowding or unfavorable conditions, L2 larval stage worms develop a specific cuticle around the body and enter dauer stage (Riddle, 1988). The worms can remain for up to 3 months in this stage and return to the regular cycle once favorable conditions return.

Development of Germ Cells and Somatic Gonad in C. elegans

In *C. elegans* gonad development originates from a four celled gonadal primordium, Z1-Z4 (Figure 1.1) (Kimble and Hirsh, 1979). The four cells are arranged in an anterior-posterior axis with Z1 and Z2 on the right side and Z3, Z4 on the left side of the worm's midsagittal plane (Kimble and Hirsh, 1979). The proliferation of the Z2 and Z3 depends on the signals from the somatic precursor cells, the Z1 and Z4 (Kimble and White, 1981). During the development of the somatic gonad, the Z1 and Z4 undergo two rounds of mitosis; the first one occurs during L1 larval stage and the second one during L3 and L4 larval stages (Kimble and Hirsh, 1979). During the L4 stage, the germ cells at the proximal end undergo spermatogenesis followed by oogenesis.

Z2 and Z3 act as the progenitors of the germ cells, which constitute an individual germline nuclei and its surrounding cytoplasm (Hubbard and Greenstein, 2000). The Z1 and Z4 give rise to the entire somatic gonad consisting of distal tip cell (DTC), sheath cells, spermathecae, uterus (Hubbard and Greenstein, 2000). The proximal cells of the somatic gonad primordium divide twice, of which two cells later develop to become the

anchor (AC) and the ventral uterine (VU) cells as shown in Figure 1.1 (Kimble and White, 1981).

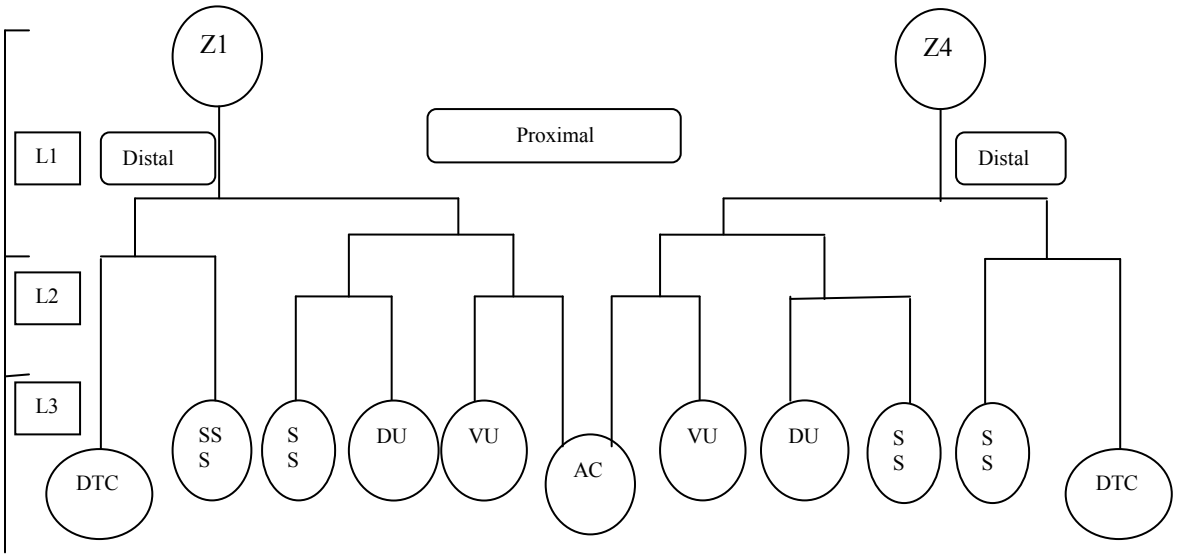

Figure 1.1: Development of hermaphrodite somatic gonad. The lineage from the Z1 and Z4 precursor cells is shown above. The abbreviations are as follows: SS-sheath/spermathecal precursor cell; AC-anchor cell; DTC-distal tip cell; DU and VU, the dorsal and ventral uterine precursor cells.

C. elegans Gonad

The gonad in the hermaphrodite worm consists of two U shaped tubular structures and connects to the vulva in the midbody (Hall et al., 1999). The spermatheca is flanked by the gonad arm distally and proximally to the spermathecal valves which open up in to the uterus (Figure 1.2) (Wissmann et al., 1999). In *C. elegans*, the gonad arm comprises of germ cells which are in a mitotic state in the distal part and undergo meiosis as they move along the gonad arm (Inoue et al., 2006). Oocytes develop in the distal region of the gonad arm and undergo differentiation as they move proximally (McCarter *et al.,* 1999). In the proximal gonad, the oocytes are arranged in assembly line fashion as shown in Figure 1.2. The proximal oocyte (#1) undergoes a typical maturation and

becomes ready for fertilization. Oocyte (#1) will enter the spermatheca to be fertilized, process called ovulation. *C. elegans* hermaphrodites produce an average of 300 sperm before the gonad starts the process of oogenesis (Singson, 2006). This process of self-fertilization is repeated until all the sperm are exhausted.

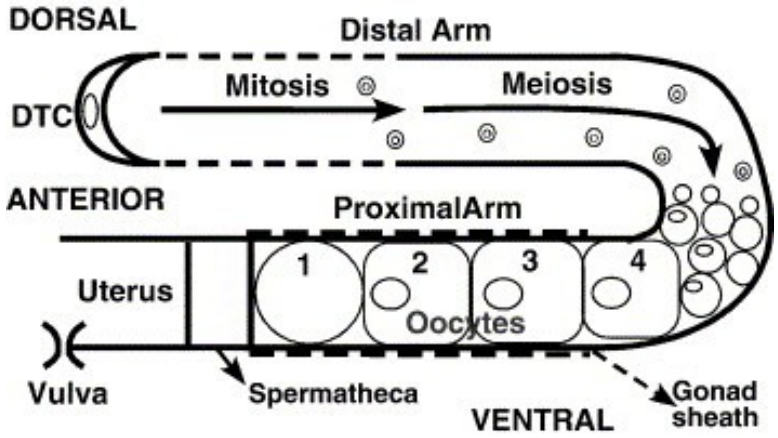

Figure 1.2: Posterior U-shaped gonad arm of *C. elegans* (Xu *et al.,* 2006).

Oocyte Development

The sequence of events during ovulation is oogenesis, maturation, and the final release of the oocyte from the ovary (McCarter *et al.*, 1999). Germ cells in the distal part of the gonad arm undergo mitosis and as they pass through the bend of the gonad arm undergo meiosis to produce mature oocytes (Shimada et al., 2006). Oocytes start to form when the germ cells reach the loop of the gonad (Boxem et al., 1999). As they pass through the gonad arm, the oocytes exit from the pachytene stage, cellularize in the diplotene or diakinesis phase of meiotic prophase I (Inoue et al., 2006). Oocytes remain in the diakinesis stage of prophase I before undergoing meiotic maturation (Hubbard and Greenstein, 2000), which defines the transition between diakinetic prophase and metaphase of meiosis I (Yamamoto et al., 2006). The process of meiotic maturation includes the nuclear envelope breakdown, cortical rearrangement, and meiotic spindle

assembly (McCarter *et al.*, 1999; Miller *et al.*, 2003). Two main signaling interaction events are necessary for oocyte maturation; the first is the oocyte maturation triggered by the sperm and the second is the increase in sheath cell contraction signaled by the mature oocyte (McCarter *et al.*, 1999). Upon receiving the signal from the sperm, oocytes undergo maturation and ovulation, and complete the process of fertilization in the spermatheca located at the proximal end of the gonad (Inoue et al., 2006).

Gonad Sheath

The sheath cells lie between the germ cells and the gonadal basal lamina (Hall *et al.*, 1999). There are 5 pairs of sheath cells forming a single cell layer covering the gonad arm and the germ cells (McCarter *et al.*, 1997). The first and second pairs of sheath cells cover the distal arm and the loop of the gonad while the remaining 3 pairs of sheath cells cover much of the proximal arm of the gonad (Hall *et al.,* 1999). The first 2 pairs consist of very few cytoskeletal filaments whereas the last three pairs of sheath cells are made of contractile filaments (Hall *et al.,* 1999). A schematic representation of the 5 pairs of sheath cells is shown in Figure 1.3. The oocytes are in contact with sheath cells through gap junctions (Hall *et al.*, 1999). In *C. elegans*, gap junctions are required to transmit signals from the sheath cells to the oocytes and to negatively regulate oocyte maturation, by maintaining oocytes in meiotic prophase I when sperm are absent (Whitten and Miller, 2007).

Sheath Cell Contractile Activity

The developing oocytes are enveloped by the sheath cells which undergo periodic contraction. Sheath cell contractions are responsible for the oocyte exit out of the gonad

at the time of ovulation, which indicates interaction between the two structures (McCarter *et al.,* 1997). The contractions of the sheath cell during ovulation force the mature oocyte out of the proximal gonad and the sheath cells pull the dilating distal spermatheca over

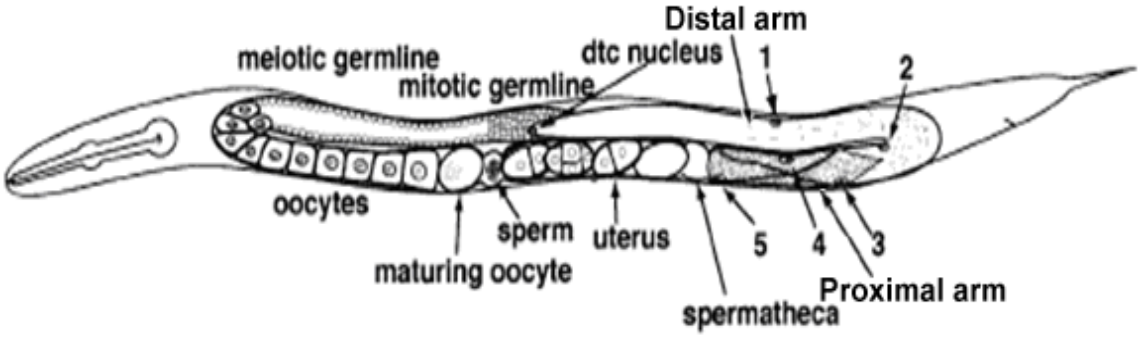

Figure 1.3: Schematic representation of adult hermaphrodite gonad (Hall et al., 1999). The sheath cells are labeled from 1-5. The first two pairs are not visibly contractile whereas sheath cell pairs 3-5 are visibly contractile.

the mature oocyte (McCarter *et al.,* 1997, Hall *et al.,* 1997). The sheath contractions and spermathecal dilation occurs periodically, moderate activity in the early stage which intensifies later and then spermathecal dilation occurs at ovulation (Clandinin et al., 1998). The gonad sheath contractile activity can be divided into two types: basal and ovulatory. Before ovulation, there is a moderate contractile activity in gonad sheath, known as basal contraction. These contractions are triggered by major sperm protein (MSP) (Miller et al., 2001). The contractile activity increases during the late ovulation cycle so that vigorous contractions occur every few seconds, known as ovulatory contraction. This is triggered by a factor, LIN-3, secreted from the maturing oocytes which controls sheath cell and spermathecal dilations (Yin et al., 2004). Germ cells regulate the sheath cell contractile activity by secreting signals (Yin et al., 2004).

Regulation of the Ovulation Process

The MSP act as the signaling molecules, bind to the receptors on the oocyte and proximal sheath cell initiating oocyte maturation and sheath cell contraction (Miller *et al.*, 2003). Variable abnormal morphology-1 gene (*vab-1)* functions cell autonomously in oocytes, to negatively regulate oocyte maturation and ovulation (Miller et al., 2003). VAB-1/Ephrin is the receptor to which the MSP binds to regulate the oocyte and stimulates the sheath cell contraction (Miller et al., 2003). Upon release from the sperm, MSP binds to the *vab-1* and promotes ovulation by acting as a VAB-1/Ephrin antagonist (Miller et al., 2003).

Mutation in *C. elegans* homeobox (*ceh-18)*, a POU-class homeoprotein expressed in sheath cells, result in oocyte defective phenotypes (Greenstein et al., 1994). *ceh-18* mutations disrupt the sheath cells due to which oocytes fail to be in a single row (Rose et al., 1997). *ceh-18* is required for normal sheath cell differentiation and function which in turn is required for meiotic maturation, mitogen-activated protein kinase (MAPK) activation, ovulation and embryogenesis (Rose et al., 1997; Whitten and Miller, 2007). *vab-1 and ceh-18* negatively regulate MAPK activation (Miller et al., 2003). The major sperm protein binds to the *vab-1 and ceh-18* signaling pathway and promotes oocyte maturation and ovulation (Miller et al., 2003).

Yin *et al.,* have shown that LIN-3/LET-23 and MSP/VAB-1 signaling activate phospholipase-C (*plc*) that hydrolyzes phosphatidyl inositol 4, 5-bisphosphate (PIP_2) to generate inositol triphosphate (IP_3) and subsequently activates inositol triphosphate receptor (ITR-1) (Yin *et al.,* 2004). *plc-3* is one of the 6 genes encoding PLC, which hydrolyses PIP_2 to IP_3 (Yin et al., 2004). Depletion of *plc-3*/PLC-**γ,** expressed in the

sheath cells and spermatheca, causes loss of sheath contractile activity and resulted in ovulation defects (Yin et al., 2004). Studies by Xu *et al.,* have shown that mutations in *itr-1 (gf)* and *ipp-5 (lf)* have suppressed sterility caused by *plc-3* RNAi (Xu *et al.,* 2005).

Integrin receptors act as connecting points between the extracellular matrix (ECM) and the actin cytoskeleton and are essential for embryonic development (Cram et al., 2003). Talin loss-of-function causes disorganized gonad myoepithelial sheath cell, disrupted oocyte maturation and ovulation defects (Cram et al., 2003). Integrin αβ PAT-2/PAT-3 heterodimer functions in conjunction with talin to maintain muscle in *C. elegans* (Cram et al., 2003). Loss of *pat-3* β integrin or ECM components cause ovulation defects that are suppressed by a *itr-1* gain-of-function mutation, suggesting that cell-ECM interaction is linked to IP_3 signaling (Xu *et al.*, 2005).

The process of ovulation is also controlled by transcription factors and kinases. The loss of *C. elegans* ortholog of mammalian DP (*dpl-1*), which encodes a subunit of heterodimeric transcription factor EFL-1/DPL-1, causes ovulation defects (Chi and Reinke, 2009). DPL-1expression in the germ cells lead to the rescue of ovulation defective *dpl-1* mutants, which shows that DPL-1 works in germline to regulate ovulation (Chi and Reinke, 2009). Two zinc finger-containing proteins, OMA-1and OMA-2 (Oocyte maturation defective) are required for progression from prophase during oocyte maturation (Detwiler et al., 2001). OMA-1 and OMA-2 might function as RNA binding proteins due to their similarity with zinc finger proteins (Lin, 2003). OMA-1 protein is the focal point for signals that regulate transition from oocyte to embryo (Shirayama et al., 2006). Mutations in cyclin dependent kinases such as *cdk-1* stabilize the OMA-1 protein which promotes oocyte maturation during meiosis (Shirayama et al., 2006).

Gap junctions are required to transmit signals from the sheath cells to the oocytes thereby negatively regulating oocyte maturation (Whitten and Miller, 2007). Gap junctions maintain oocytes in meiotic prophase I when sperm are absent and also promote sperm recruitment to the spermatheca (Whitten and Miller, 2007). An RNAi screen of innexin genes identified INX-14 and INX-22 as components of sheath/oocyte gap junctions (Whitten and Miller, 2007).

Sterility in C. elegans

Sterile (Ste) phenotype in *C. elegans* can be conferred to factors such as defective sperm production, sheath cell contraction, spermathecal and oocyte defects. Sperm induces the process of meiotic maturation and sheath cell contraction which causes the oocytes to enter the spermatheca and the process of fertilization is completed (McCarter et al., 1997; Miller et al., 2003). Sheath cell contractile defects include rate, time and strength of sheath cell contraction at the time of ovulation. This might be due to defective ovulation, abnormal meiotic cycle, oocyte exiting meiotic phase prematurely (Iwasaki et al., 1996).

Oocytes which are unable to enter the spermatheca to start the process of fertilization undergo multiple DNA synthesis (Iwasaki et al., 1996). If oocyte maturation and sheath cell contractions occur normally but spermathecal dilation fails, then oocytes remain in the ovary and initiate DNA replication (Clandinin et al., 1998). These oocytes are termed as endomitotic oocytes and the phenotype is known as Endomitotic (Emo) phenotype (Iwasaki et al., 1996). Lack of sheath cell contractions and functional distal spermatheca causes maturing oocytes to remain in the proximal gonad arm and causes Emo phenotype (McCarter et al., 1997). Oocytes which fail to undergo fertilization and

undergo multiple DNA replications either block the gonad arm or enter the spermatheca forcibly but are destroyed, such phenotype is also known as Emo phenotype (Clandinin et al., 1998). Mature oocyte nuclei have definite boundaries whereas Emo nuclei are larger and no proper definite boundary (Clandinin et al., 1998). Emo oocytes in the proximal gonad arm result in sterile phenotype often resulting in higher frequency of Emo mutants when screening for sterility (Iwasaki et al., 1996).

RNA Interference (RNAi)

Injection of antisense RNA homologous to uncoordinated genes *unc-22* and *unc-54*, lead to a decrease in the endogenous mRNA (Fire et al., 1991). Injection of either sense or anti-sense strand from *par-1* gene into the germline results in the epigenetic inactivation of *par-1* in the progeny (Guo and Kemphues, 1995). Injection of both sense and anti-sense strands of RNA showed a more robust interference compared to the injection of single strands (Fire, 1998). Injection of double stranded RNA (dsRNA) into the head or tail of the organism results in suppression of the gene expression in the progeny from both gonad arms which shows that RNAi can pass through cellular boundaries (Fire, 1998). Injection of dsRNA into the gonads of adults worms resulted in sequence specific gene interference in the somatic cells (Fire, 1998). Injection of either *lin-15a* or *lin-15b* dsRNA did not cause the multivulva phenotype which was seen when dsRNA from both strands was injected, suggesting that the RNAi effect is post-transcriptional in nature (Sharp, 1999). The mechanism by which introduction of double stranded RNA causes a sequence specific degradation of endogenous RNA molecules is known as RNA interference or RNAi (Fire et al., 1998).

Mechanism of RNAi

RNAi can be divided into two stages: initiation stage in which dsRNA is processed into small interfering RNAs (siRNAs) and execution stage during which the silencing complex degrades the homologous mRNA.

Initiation Stage

In this step, the dsRNA upon recognition is cleaved into precise length RNA fragments (Hamilton and Baulcombe, 1999). Zamore *et al.*, 2000 have shown that the dsRNA is cut into 21-23 nucleotide (nt) length RNAs (Zamore et al., 2000). The precise length of 21-23 nucleotide dsRNA are referred as small interfering RNAs or siRNAs and are necessary to avoid unwanted degradation of cellular RNAs (Elbashir et al., 2001a). The 21-23 nt RNAs do not require the presence of the corresponding mRNA suggesting that these RNA are formed by dsRNA processing and not due to homologous mRNA degradation or due to interaction between single stranded (ssRNA) and dsRNA (Zamore et al., 2000).

The cleaved dsRNA had 5'monophosphates and 3' hydroxyl ends which suggest that the processing is done by an RNase-III mechanism (Elbashir et al., 2001a). The cleavage of the dsRNA into 21-23 nt length fragments is carried out by an RNase III enzyme called dicer-1(*dcr-1*) (Knight and Bass, 2001). *dcr-1* loss of function mutants have severe germ line defects which cause sterility which indicates that dicer plays a prominent role in the germ line development in *C. elegans* (Knight and Bass, 2001). This siRNA combines with proteins to form a complex called small interfering ribonucleoprotein particle (siRNP) (Elbashir et al., 2001a).

Execution Stage

The antisense strand of the siRNA interacts with the mRNA and the sense strand protects the antisense strand from degradation (Parrish et al., 2000). RNA strand from the unwound siRNA duplex binds to the RNA induced silencing complex (RISC) (Martinez et al., 2002). Sequence specific pairing between the anti-sense strand and the mRNA occurs, which sets the stage for degradation of the target mRNA (Elbashir et al., 2001a; Martinez et al., 2002) mRNAs are then degraded by the sequence-specific nuclease activity of RISC (Hammond et al., 2000). After the RISC binds to the target RNA, it can also be used to generate new siRNAs of the target mRNA. This requires the presence of RNA dependent RNA polymerase (RdRP) to generate double stranded RNA and dicer to cut the RNA into21-23nt long siRNA (Buratowski and Moazed, 2005).

Genes Essential for RNAi Process

The process of RNAi requires the action of a number of genes and protein complexes. Some of the genes are necessary for the normal development of the organism; others provide resistance against RNAi whereas certain genes cause sensitivity to RNAi. In Table 1.1, a list of genes and protein complexes essential for the RNAi process has been detailed.

RNA-directed RNA Polymerases

The key elements necessary for RNAi silencing belong to the family of RNA-directed RNA polymerases (RdRP's). RNAi involves production of two types of siRNA, the first one containing siRNA derived from the injected dsRNA and the second type resulting from RdRP (Sijen et al., 2001). RdRPs are required for the production of short

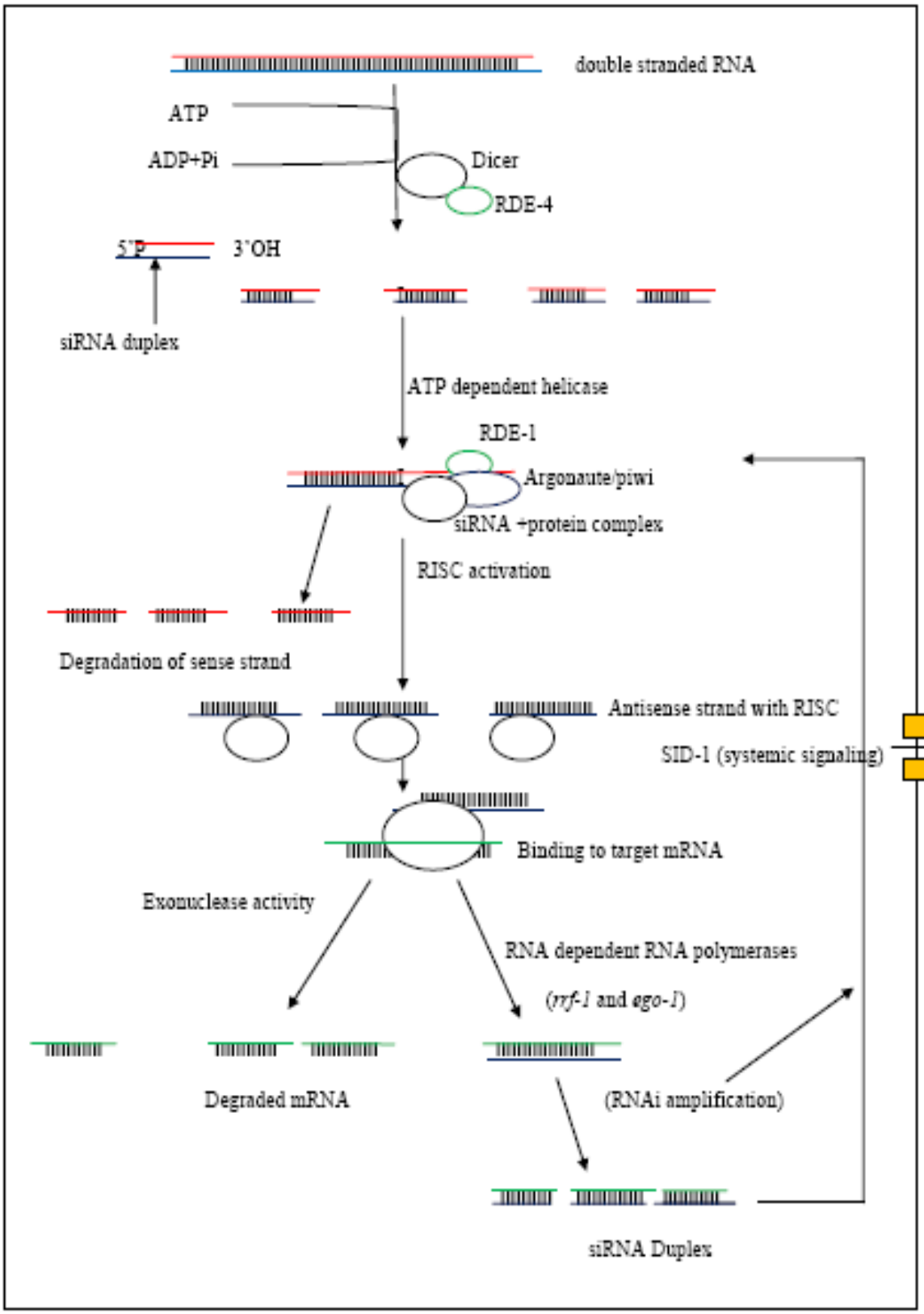

Figure 1.4: Diagrammatic illustration of ds-RNA directed gene silencing mechanism

interfering RNAs (siRNA) to mount an effective RNAi response in *C. elegans* (Sijen et al., 2007). In *C. elegans ego-1, rrf-1, rrf-2* and *rrf-3* are members of the RdRP gene family (*rrf*) (Smardon et al., 2000). *rrf-1* mutant has a 401 amino acid(aa) deletion which includes conserved residues of the RdRP family (Sijen et al., 2001). *rrf-1* deletion causes lack of production of secondary RNAi triggers and complete lack of transitive interference in somatic cells (Sijen et al., 2001). In *rrf-1* mutants, RNAi works in the germline but does not work in somatic tissue without any developmental defects (Sijen et al., 2001). *rrf-2* and *rrf-3* mutants are sensitive to RNAi in both the germline and somatic tissue (Sijen et al., 2001). RRF-1 is required for the production of 22nt RNAs along with worm-specific AGO proteins (WAGO) in the RNAi pathway (Vasale et al., 2010).

Enhancer of *glp-1* (*ego-1*) is essential for germ-line development and also required for effective RNAi for certain genes (Smardon et al., 2000). EGO-1 protein is related to tomato RdRP and Neurospora (QDE-1) proteins which play an important role in post-transcriptional gene silencing (PTGS) (Smardon et al., 2000). Mutants which are defective in *ego-1* gene expression display a weak response or no response to RNAi of some germ-line expressed genes (Smardon et al., 2000). *ego-1* gene activity is required for the proper cell proliferation, development, production of functional oocytes, and plays a temporal role in the transition from spermatogenesis to oogenesis in adults (Smardon et al., 2000).

RNAi Deficient Genes

dsRNA-binding proteins (dsRBPs) are essential for dicer to mount an efficient RNAi response (Parker et al., 2006). Tabara *et al*., reported the identification of RNA interference deficient (*rde*) mutants (Tabara et al., 1999). *rde-4* encodes a dsRNA

Table 1.1. List of genes and protein complexes which are essential for RNAi mechanism.

Gene/Protein Complex	Function
dcr-1	Necessary for cleavage of the dsRNA into 21-23 nt length fragments (Knight and Bass, 2001).
RNA dependent RNA polymerase (RdRp's)	Essential for generating double stranded RNA (Buratowski and Moazed, 2005).
rrf-1 (RdRp family)	Necessary for RNAi amplification mechanism. Leads to loss of transitive interference when depleted. Confers resistance to RNAi in somatic cells (Sijen et al., 2001).
ego-1(RdRp family)	Essential for germ-line development and also required for effective RNAi for certain genes (Smardon et al., 2000).
rde-1	Belongs to Argonaute protein family and is essential for RNAi in *C. elegans*. Confers resistance to RNAi in both somatic and germline (Tabara et al., 1999).
rde-2	Necessary in the RNAi amplification step by acting downstream of siRNA production (Tops et al., 2005). Confers resistance to RNAi in the germline.
rde-4	Encodes a dsRNA binding protein (Tabara et al., 2002).
mut-7	It is for the effector phase and inheritance of RNAi (Grishok et al., 2000).
sid-1,2 and *3*	Involved in systemic RNAi (Winston et al., 2002)
small interfering ribonucleoprotein (siRNP's)	siRNA duplex combines with proteins (Elbashir et al., 2001b).
RNA induced silencing complex(RISC)	Necessary for sequence specific degradation of mRNA (Martinez et al., 2002).
dsRNA-binding proteins (dsRBPs)	Essential for *dcr-1* to bind to double stranded RNA and cleave them into siRNA (Parker et al., 2006).

binding protein which interacts with mRNA identical to the trigger dsRNA (Tabara et al., 2002). RDE-4, a dsRBP found in *C. elegans*, interacts with dicer and binds to long dsRNA then cleaving it into siRNAs (Parker et al., 2006). RDE-4 interacts with DCR-1, RDE-1 and DExH-box helicase to form a complex to direct the RNAi (Tabara et al., 2002).

RDE-4 and RDE-1 form a complex to detect and process the dsRNA and then direct this dsRNA to DCR-1 for cleavage (Tabara et al., 2002). In *rde-1*or *rde-4* mutants, the siRNA remains with DICER complex and there is no RISC formation (Sijen et al., 2007). *rde-1* and *rde-4* mutants are insensitive to RNAi but do not show any abnormality in terms of growth and fertility suggesting that some genes which play a role in RNAi pathway are not essential (Tabara et al., 1999). Systemic RNA-interference deficient (*sid*) genes were identified during genetic screen to identify genes involved in RNAi (Winston et al., 2002). SID-1, expressed in cells sensitive to RNAi, encodes a transmembrane protein and is required for systemic RNAi (Winston et al., 2002).

Studies in mutator (*mut)* and *rde* strains showed reduced RNAi and increase in transposon activity which suggests a role for RNAi in transposon silencing (Tabara et al., 1999). *mut-7* gene is necessary for both phenomenon, indicating that these two pathways could be interconnected probably because gene silencing is a defense mechanism against transposons (Tabara et al., 1999). Studies in *mut-7*, a nonessential homolog of RNase D, have shown that transposon silencing occurs via RNase activity of MUT-7 (Ketting et al., 1999). MUT-7 along with RDE-2 is necessary for the effector phase and inheritance of RNAi (Grishok et al., 2000). Mutations in *mut-7* and *rde-2* genes have shown that the interaction between these genes in the cytosol is essential for RNAi (Tops et al., 2005).

mut-7 and *rde-2* are necessary for RNAi amplification by acting downstream of siRNA production but not required for the initial siRNA response (Tops et al., 2005).

Significance of RNAi Across Taxa

Injection of a few molecules of dsRNA was sufficient to cause a potent interference in the homologous RNA throughout the body and the F_1 progeny indicating the presence of an amplification mechanism and a RNA-transport mechanism (Fire et al., 1998). A gene-specific, dsRNA mediated interference was observed in the cell free translation systems in *Drosophila* embryos (Tuschl et al., 1999). RNAi is similar to that of post-transcriptional gene silencing (PTGS) in plants; both mechanisms require a dsRNA template, are mediated by RdRP, and induce dsRNA to disrupt the homologous RNAs (Parrish *et al.*, 2000; Vaucheret *et al.*, 2001). RNAi effect in some genes is transmitted to the F_2 generation which suggests the action of a gene-linked suppression (Sharp, 1999). The natural function of RNAi is to silence transposon activity and defend against viral attacks in the germline (Ketting et al., 1999). The mechanism of RNAi is conserved in organisms as diverse as fungi, plants and human beings suggest that it might be a part of cellular defense mechanism to protect the genome against viruses (Plasterk, 2002). Mechanisms similar to RNAi play an important role in anti-viral defense, control of mobile genetic elements, heterochromatin formation and regulation of development, molecules *in vivo* (Tavernarakis et al., 2000).

Method of Introducing dsRNA

The various methods developed to introduce dsRNA into *C. elegans* are microinjection (Fire et al., 1998), soaking (Tabara et al., 1998), feeding dsRNA

(Timmons and Fire, 1998), and expression of a plasmid producing hairpin RNA in *C. elegans* was through microinjection (Fire et al., 1998). In this method the dsRNA is injected into the gonad of the adult worms and the progeny produced is analyzed for phenotypes. Feeding and soaking RNAi methods were also developed to introduce dsRNA into *C. elegans* later. Tavernarakis *et al.* used an alternate approach of introducing dsRNA by generating heritable and inducible RNAi in *C. elegans* (Tavernarakis et al., 2000). Transgenic nematodes were constructed with an inverted repeat (IR) gene under the control of a heat shock promoter (*hsp*) in a *C. elegans* expression vector (Tavernarakis et al., 2000).

Genetically Engineered Bacteria for Feeding RNAi Technique

Timmons and Fire introduced dsRNA by feeding *C. elegans* with bacteria engineered to produce the homologous dsRNA (Timmons and Fire, 1998). Removal of RNase III, a dsRNA-specific endonuclease, improves the yield of interfering RNA by reducing the degradation of dsRNA (Timmons et al., 2001). A L4440 plasmid with two T7 polymerase promoters flanking a single copy of the target sequence was transformed into an RNase III deficient bacterial strain [HT115 (DE3)] (Timmons et al., 2001). These engineered bacteria when fed to *C. elegans* strains resulted in phenotypes similar to that of loss-of-function mutants (Timmons et al., 2001).

Advantages

Feeding RNAi is an effective method to deplete endogenous mRNA from the body to study the function of the gene and the resulting phenotype (Corsi, 2006). Once the dsRNA is ingested, it is amplified throughout the cell and the resulting RNAi phenotypes can be monitored in the adult worms (P0) and also in the progeny (F1). The

advantage of feeding RNAi over injection method is that it can be performed on a large number of worms at the same time (Kamath and Ahringer, 2003; Kamath *et al.*, 2001). The bacterial strains can be used to generate dsRNA and makes the process less expensive (Kamath *et al.*, 2001). Feeding RNAi can be applied in mutant worms that cannot be subjected to microinjection technique with dsRNA, efficiently detects Ste phenotypes because the feeding can be done for a prolonged period of time to affect the germ cell or gonad (Kamath *et al.*, 2001); fits for a large scale studies due to the current availability of bacterial RNAi library (Kamath et al., 2003; Rual et al., 2004). The effectiveness of feeding RNAi is similar to that of injection of dsRNA when screening for post-embryonic phenotypes (Kamath et al., 2001).

Disadvantages

Feeding RNAi technique does not work well in nervous tissue, in the male worms and is ineffective in the early larval stages due to impermeability of the eggshell (Kamath *et al.*, 2001; Timmons *et al.*, 2001). The RNAi clones in the chromosome library have not been individually sequenced and might result in extra or missing inserts which would be result in differences in the phenotypes (Corsi, 2006). The percentage of similarity between the dsRNAs and the target mRNAs also plays a role in the effectiveness of RNAi. The RNAi is maximized if the dsRNA injected and the target mRNA have 96% similarity when compared to strands which were only 72% identical (Parrish et al., 2000). RNAi is limited by various factors such as the sensitivity of the tissue targeted, availability of RNAi machinery in the tissue or the stage of the animal and the structure and stability of the target mRNA (Timmons et al., 2001). Identification of *eri-1* locus

suggests that RNAi is negatively regulated which often leads to reduced RNAi intensity, RNAi inhibition in some cell types (Kennedy et al., 2004).

A significant drawback in feeding RNAi is the frequency of false negatives which gives variability in the results obtained from different laboratories or sometimes from the same laboratory (Simmer *et al.*, 2003). RNAi also depends upon the temperature, concentration of IPTG, the developmental stage of the worm and also on the bacteria (Simmer et al., 2003). Phenotypes which have been classified as wild type might not be entirely true because they would display a different phenotype when examined under different conditions (Corsi, 2006). Most of the RNAi experiments are conducted on a large scale and helped identify observable phenotypes but also omit many phenotypes which are not obvious (Corsi, 2006). For the phenotype to be observed depletion of the target mRNA has to fall below a critical level (Timmons et al., 2001).

Dissertation Summary

To understand the role of various genes in the germline and somatic cells in ovulation, we have performed an RNAi screen. We have used the current data available in the wormbase.org and applied feeding RNAi technique in various mutant strains to gain further insights into the function of genes in relation to the ovulation process. With the help of RNAi, DIC microscopy and fluorescence staining, we were able to narrow down the list to 29 genes. These genes belong to various essential functional classes such as protein synthesis, protein degradation, cellular architecture, nucleic acid binding, processing and synthesis and ATP synthesis. Of these 29 genes, we conclude that 20 genes are germline specific and 9 are somatic cell specific in *C. elegans*. Additionally, gene interaction maps have identified connections between various genes.

CHAPTER TWO

Materials and Methods

Identification of RNAi-Induced Sterility

Preparation of Laboratory Reagents

Ampicillin stock solution. Stock solution of 1000X ampicillin (Cat# IBO2040, Shelton Scientific, CT, USA) was prepared by dissolving 2 grams of ampicillin in 50 ml of DI water to give a final concentration of 40 µg/ml. A Sterile 0.22 micron pore size filter was used to filter the stock solution and the filtrate was decanted into 15 ml sterile centrifuge tubes with 4.5 ml of the ampicillin stock solution in each tube and stored at -20^{o}C Isotemp refrigerator (Fisher Scientific Inc, Fairlawn, NJ) until needed.

β-lactose stock solution. A final concentration of 20% w/v β-lactose was obtained by dissolving 10 grams of β-lactose (Cat# 215620, Becton and Dickinson, MD, USA) in 50 ml of DI water. The solution was dissolved by placing it on a hot plate (Model number: PC420, Corning, USA). Once dissolved, the solution was filtered using a 0.22 micron pore sterile filter and aliquot in 4.5ml volumes in 15 ml sterile centrifuge tubes and stored at -20^{o}C till further use.

Luria-Bertani broth (*LB*). LB was used for inoculating the OP50 bacterial used for the regular maintenance of *C. elegans* strains. 10 mg/ml of Tryptone (Cat#J859, Becton and Dickinson, MD, USA), 5 mg/ml of yeast extract (Cat#Y766, Becton and Dickinson, MD, USA), 10 mg/ml sodium chloride (Cat#7760, EM Science, NJ, USA),

and 10 ml of 1 M Tris-HCl pH 7.5 (Fisher Scientific, NJ, USA) buffer were dissolved in 500 ml of DI water. The ingredients were then mixed, aliquot in 100 ml bottles, autoclaved and stored at 4^{o}C.

To grow RNAi bacterial cultures, ampicillin (100 µl) was added to 100 ml of LB broth. Addition of ampicillin helped in growth of bacterial colonies with desired gene of interest.

LB plates. The LB plates were used to grow OP50 *E. coli* stock bacteria for maintaining *C. elegans.* 7.5 g of agar (Cat#A466, Lab Scientific, USA) was added to the ingredients of LB, autoclaved and then poured into 100 mm diameter petri dishes. The plates were allowed to cool overnight and then stored at 4^{o}C refrigerator.

To grow RNAi bacterial colonies, 500 µl of 40 mg/ml ampicillin solution was added to 500 ml of LB media after autoclaving the media and then poured into 100mm petri dishes.

M9 buffer. The final concentration of M9 buffer was set to 3 mg/ml of potassium phosphate monobasic, KH_2PO_4 (Cat# PX1572, EMD chemicals, NJ, USA), 6 mg/ml of sodium phosphate basic, Na_2HPO_4 (Cat#S374, Fisher Scientific, NJ, USA), 5 mg/ml of sodium chloride, NaCl (Cat#7760, EM Science, NJ, USA), 0.1 mg/ml of magnesium sulfate, $MgSO_4$ (Cat#5980, EM Science, NJ, USA). The ingredients except $MgSO_4$ were dissolved in 1L deionized (DI) water and autoclaved. 1 ml of 1M $MgSO_4$ was then added to the solution and stored at room temperature.

Nematode growth media (NGM) plates. NGM plates were used for the growth and maintenance of *C. elegans* strains. The following protocol is for preparing 4 liters of

media. In a sterile weighing dish, 68 g of agar, 10 g of peptone (Lab Scientific, NJ, USA) and 12 g of sodium chloride were added to 4 L of DI water in a 6L Erlenmeyer flask. The mixture was autoclaved at 121^{o}C and 20 psi and then allowed to cool to 55^{o}C. The following chemicals were added to this mixture: 4 ml of 5% cholesterol (J.T.Baker, NJ, USA), 4 ml of 1M magnesium sulfate and 4 ml of 1M calcium chloride (EM Science, NJ, USA) were added. To this media, 100 ml of 1M of KH_2PO_4 buffer (86.7 ml of KH_2PO_4 and 13.3 ml of K_2HPO_4) (pH 6) was added. Using a Unispense dispenser (Wheaton, Millville, NJ), 13.5 ml of media was poured into 60 mm petri dishes and, after solidification, were kept in a 4^{o}C refrigerator.

In order to facilitate the growth of double stranded RNA (dsRNA) producing bacteria, we require plates with the necessary supplements. RNA mediated interference (RNAi) plates were prepared in a similar process to that of NGM plates. After autoclaving the media, 4.5 ml of 20% β-lactose and 4.5 ml of ampicillin (40μg/ml) was added to the mixture. β-lactose acts as an inducer for the T7 polymerase present in the RNAi bacteria and ampicillin helps in preventing contamination.

C. elegans strains

The worm strains were grown at room temperature. The following variants were used: N2 (wild type), *rrf-1(pk1417)* (Sijen et al., 2001), *rde-1(ne219) (Tabara et al., 1999)* and *rde-2(ne221)* (Tabara et al., 1999). RNAi deficient mutant strains (*rde*) show resistance to dsRNA targeting germline specific genes (Tabara et al., 1999). *rde-1* mutant exhibits resistance to RNAi in both somatic and germline specific genes while *rde-2 (ne221)* worms exhibit resistance to only germine specific genes (Tabara et al.,

1999). All the strains were grown and maintained at room temperature. Strains were obtained from Caenorhabditis Genetics Center (CGC), St. Paul, MN, USA.

RNAi bacterial strains

The genes selected to test for Sterile (Ste) phenotype were determined based on previously described phenotypes by RNAi (Harris et al., 2010; Kamath et al., 2003). The RNAi libraries were purchased from Gene Service (http://www.geneservice.co.uk/), Hanxton, U.K. The bacterial libraries were obtained in a 384 well plates and were stored in -80^{o}C freezer (Model number: ULT1386-3-A35, Kendro Laboratory Products, Asheville, NC). The bacteria were cultured on LB plates supplemented with ampicillin (40 μg/ml) and grown overnight in an Isotemp incubator (Fisher Scientific Inc, Fairlawn, NJ) at 34^{o}C. Plates were then removed and stored in 4^{o}C Isotemp refrigerator (Fisher Scientific Inc, Fairlawn, NJ) until further use.

RNAi Feeding Assay

Genes previously defined as causing Sterile (Ste) phenotype in wild type, were subjected to RNAi by feeding RNAi protocol using an RNAi library (Kamath et al., 2003). The predicted gene pairs of interest were tested in the wild type and RNAi resistant worms, *rrf-1(pk1417)*. The RNAi bacteria containing the gene of interest were grown in100 μg/ml LB ampicillin. The RNAi bacteria were placed on a dual action shaker (Lab Line Instrumentation Inc, Melrose, IL) set to 20 rotations per minute and left overnight in an Isotemp incubator (Fisher Scientific Inc, Fairlawn, NJ) at 34^{o} C. On each RNAi plate (NGM supplemented with Ampicillin and β-Lactose) 20 μl of bacterial culture was added using a sterile glass pipette or spreader. The plates seeded with the

RNAi bacteria were left to grow 12-24 hours at room temperature to induce production of dsRNA. The next day, 10 L4-adult stage worms (N2, *rrf-1(pk1417)*, *rde-1(ne219)* and *rde-2(ne221)* were transferred into separate RNAi plates using a Nikon SMZ645 stereoscopic zoom microscope (Nikon Instruments Inc, NY, USA). Aseptic techniques were followed during the transfer of worms to avoid contamination.

The adult worms were allowed to lay eggs for a period of 4 hours and were later removed from the plate or transferring eggs from the regular worm strain plates into RNAi plates seeded with the necessary gene. These plates will be known as stock plates. The eggs laid by the N2, *rrf-1(pk1417)*, *rde-1(ne219)* and *rde-2(ne221)* worms were grown at room temperature for a period of 48 hours. Later a single adult worm was transferred from these plates onto a fresh RNAi plate to supplement the worms with the dsRNA producing bacteria. The worms were grown at room temperature for a period of 48-72 hours. Each plate was then observed under a Nikon SMZ645 stereo microscope for the presence of eggs or progeny. Overlapping sets of RNAi experiments were performed during the continuation of the experiment, resulting in an average of 360 plates per person per week. During the course of the screen the following negative control was performed. N2 worms were fed with *ppd129.36* bacteria which contain a L4440 mock RNAi. A detailed schematic representation of the RNAi experiment has been shown in Figure 4.

Criteria for Characterization of Sterile Phenotype

We have applied the following criteria when scoring the worms in order to characterize sterile (Ste) phenotypes. Generally worms which had less than 10 progeny were categorized as Ste because in a normal wild type the brood size is in excess of 100

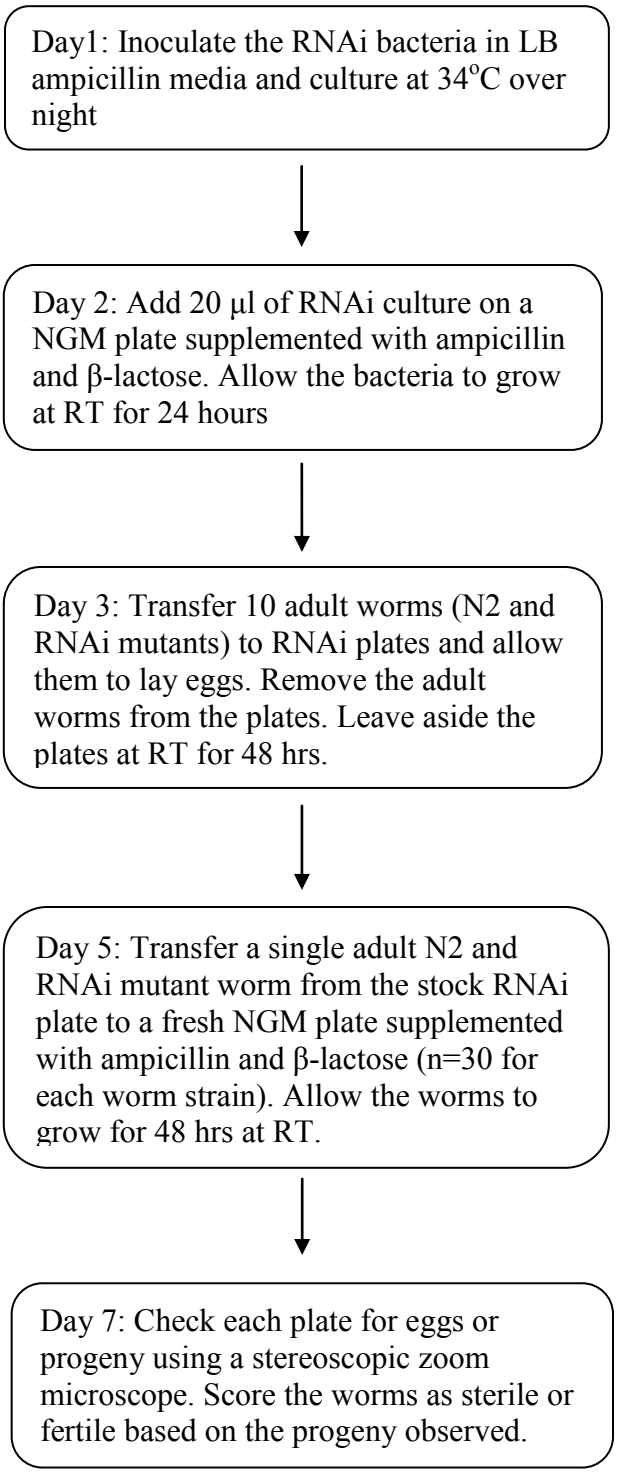

Figure 2.1: Flow chart of feeding RNAi assay

worms (Kamath et al., 2003). In our study, the brood size for characterizing Ste phenotype was set to zero. A Ste phenotype was assigned to the plate if the animals did not have any progeny. RNAi experiments resulting in more than 70% of the phenotype scored were categorized as positive for Ste phenotype. Animals that were dead were not scored for the phenotype.

Study of Gonad Morphology

To visualize the gonad morphology in worms subjected to RNAi, we used differential interference contrast (DIC) microscopy. The adult RNAi treated worms were paralyzed by adding them to M9 buffer containing 2% sodium azide solution (NaN_3, w/v). The worms were then transferred onto a glass slide containing 2% agarose pad. The worms were then observed with a Nikon Diaphot TE2000-U inverted microscope with a 40x plan Fluor objective lens. The images were captured with a Cool Snap cf cooled-CCD digital camera (Roper Scientific, Tucson, AZ) and analyzed by Metavue software (version 7.0, Molecular devices Co, Downingtown, PA).

To identify nuclear accumulation in gonad structure, 4'6-diamidino-2-phenylindole (DAPI) stained RNAi treated worms. DAPI is a fluorescent dye which is used to stain DNA in cells. The adult worms were fixed in 100% methanol and then treated with DAPI (0.1µg/mL, Sigma Chem, Co) for 2-3 hours at RT. The worms were observed using a Nikon Diaphot TE2000-U inverted microscope with a 40x plan Fluor objective lens under a UV filter. The images were captured with a Cool Snap cf cooled-CCD digital camera (Roper Scientific, Tucson, AZ) and analyzed by Metavue software (version 7.0, Molecular devices Co, Downingtown, PA).

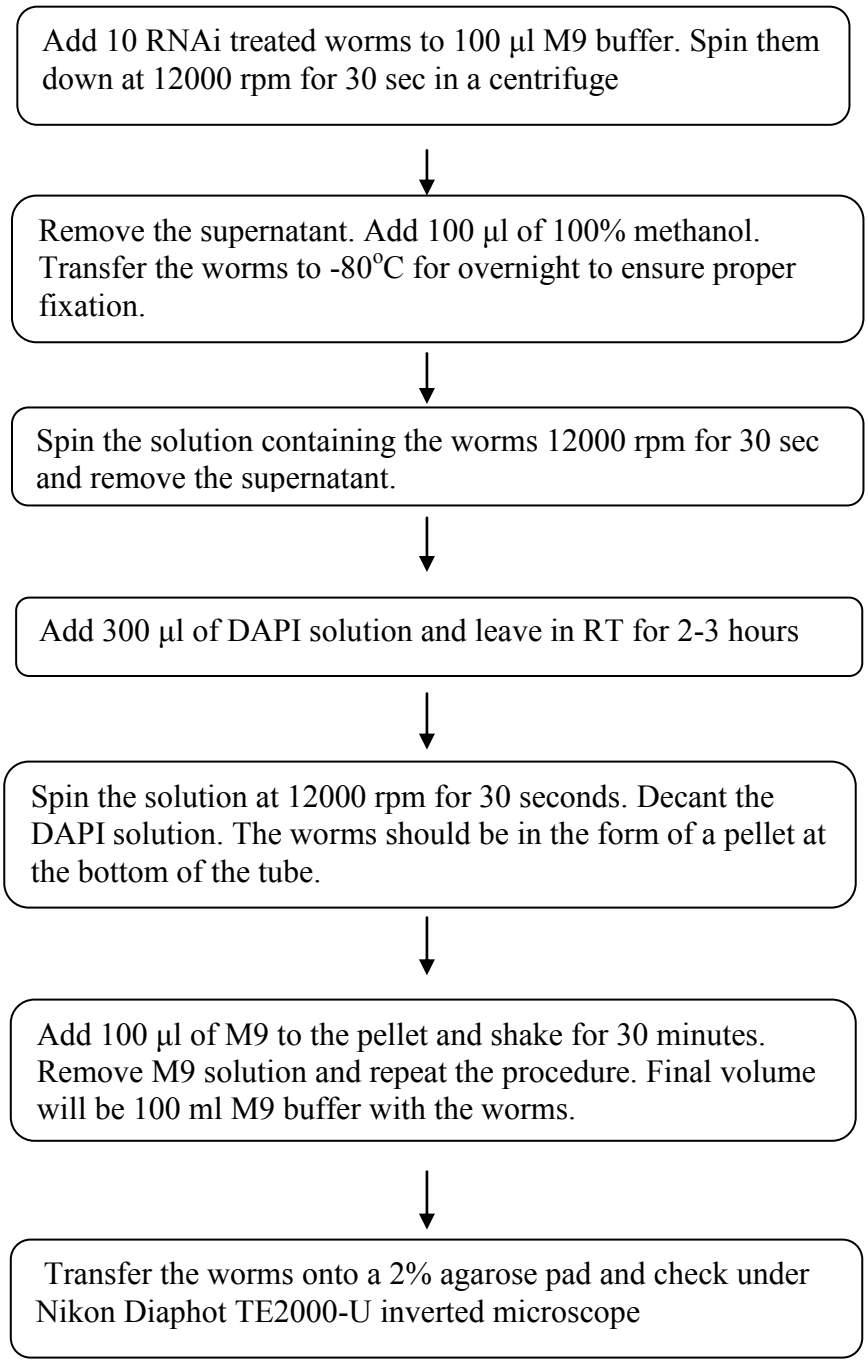

Figure 2.2: Flow chart of nuclear staining protocol.

CHAPTER THREE

Results

Preliminary Screen

The goal of this project is to identify factors from germ and somatic cells that regulate the germ-soma interaction in *C. elegans* gonad. In order to identify ovulation factors, we used feeding RNAi method, to systematically deplete the genes causing sterility. The basic goals of this screen can be summarized as below:

1) Identification of germ cell specific genes that are linked to ovulation using reverse genetics approach.

2) Genes that resulted in reduced sterility phenotypes in *rrf-1(pk1417)* background will be investigated for somatic specificity. In *rrf-1* mutants, RNAi works in the germline but suppressed in somatic tissue (Sijen et al., 2001).

Table 3.1: Number of genes depleted using RNAi from each chromosome (Kamath et al., 2003).

Chromosome	Number of genes
I	52
II	19
III	110
IV	42
V	28
VI	6
Total	259

A three step process was employed to identify the germline and somatic specific genes necessary for ovulation. A schematic representation of the screening process is given in Figure 3.1.

1) We tested for genes causing sterile (Ste) phenotype in wild type and *rrf-1* mutant backgrounds. The next steps involved analyzing the results of this screen and categorize them into the following groups:

1. Germ line specific factors: This group includes genes whose depletion causes Ste phenotype in both the wild type and *rrf-1* mutant background.

2. Somatic cell specific factors: This set includes genes which caused Ste in the wild type background and suppressed Ste in the *rrf-1* background.

2) A second screen was then performed in which the following strains were examined: N2, *rrf-1(pk1417)*, *rde-2(ne221)* and *rde-1(ne219)*. Genes which tested positive for Ste phenotype in the N2 and *rrf-1(pk1417)* mutant were further screened using *rde-1* and *rde-2* mutants. The *rde-1(ne219)* mutant is resistant to RNAi in both germline and somatic cells whereas *rde-2(ne221)* mutant displays resistance to RNAi in the somatic cells only (Tabara et al., 1999).

RNAi that caused Ste in the N2, *rrf-1(pk1417)* mutant background and reduced sterility in *rde-2(ne221)* background were listed as double positive for germline specificity. Genes whose depletion resulted in sterility in N2, *rde-2(ne221)* and suppression of Ste in *rrf-1(pk1417)* were listed as double positive for somatic specificity.

3) The genes of interest from both categories were then scored for ovulation defects, endomitotic (Emo), using DIC microscopy and nuclear staining using 4'6-diamidino-2-phenylindole.

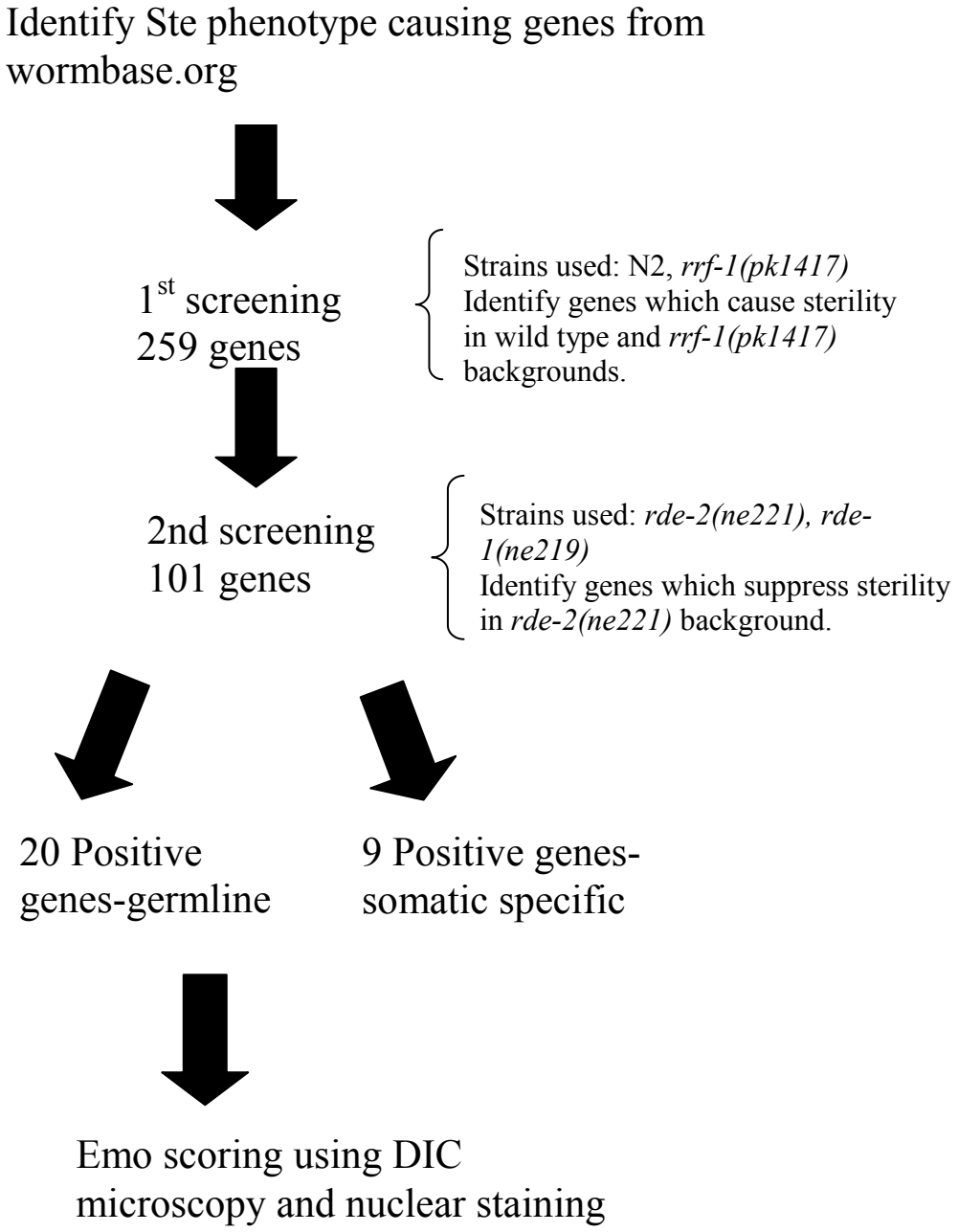

Figure 3.1. Schematic Representation of the Screening Process

Identification of Germ Cell Specific Genes that are Linked to Ovulation Using Reverse Genetics Approach.

To identify the factors regulating fertility in the germline, we have used data collected from previous genome-wide RNAi screens and identified approximately 1200 genes causing sterile (Ste) and sterile progeny (Stp) phenotype after RNAi depletion. From the searches, the 259 genes that show Ste in RNAi analyses were isolated (Appendix A). Using the data, we depleted 259 genes causing Ste phenotype using RNAi, and re-screened for Ste phenotype in the wild type and somatic RNAi resistant *rrf-1* background (Sijen et al., 2001).

We employed a double screening method to identify the genes expressed in the germline and involved in the fertility process. First, we depleted 259 genes in the wild background and found 101(Appendix A and B) genes showing a complete sterile phenotype in N2, no viable progeny producing while displaying no growth defects (Ste). Among the 101 genes, the 69 genes (Appendix B) causing sterility in N2 and *rrf-1(pk1417)* were considered to be potential germline factors for sterility. To confirm for germline specificity, these genes were then tested under germline RNAi resistant *rde-2(ne221)* background (Tabara et al., 1999).

The second screen was aimed at identifying the genes, which can recover loss of sterility in the germline resistant mutant. The 69 candidate genes were tested in *rde-2(ne221)* mutant background, and compared with the wild type background to confirm that these genes act primarily in the germline. This narrowed our initial list to a subset of 20 genes, whose gene expression knockdown showed reduced sterility in *rde-2(ne221)* mutants (Table 3.3). Worms were cultured on plates seeded with bacteria expressing ds-

RNA of corresponding genes and changes in gonad morphology were analyzed by performing fluorescence staining and differential interference contrast microscopy (DIC).

These 69 genes belong to functional categories such as protein synthesis, cell signaling, protein degradation, cell architecture, RNA synthesis and their description is based on previous gene assignments (Kamath et al., 2003). The description of each functional class is given in Table 3.2. In the preliminary screen, 19 genes belong to protein synthesis functional class including ribosome biogenesis genes, 9 belong to protein degradation functional class, 7 genes encompass cell architecture functional class, 12 include RNA synthesis functional class, 5 genes encode for metabolism functions, 3 genes belong to signal transduction class, other functional classes include 3 genes and 8 genes which have insufficient information are classified as unknown.

The 20 genes whose depletion showed germline specificity, we fixed the worms with DAPI and visualized the Emo phenotype using fluorescence staining. To verify these results, we examined for gonad morphology changes in ovulation process. Oocytes in the proximal gonad arm in sterile phenotype often show a higher frequency of Emo (Iwasaki et al., 1996). A majority of these genes resulted in Emo and were confirmed by DIC microscopy and DAPI staining.

In the final subset of 20 germline specific genes, F57B9.5/*byn-1*and T01C3.7/*fib-1* belongs to protein synthesis functional group. Genes such as C23G10.4a/ *rpn-2,* C36B1.4/*pas-4* and C52E4.4/ *rpt-1* encode for protein degradation process. We have identified genes which are part of protein modification and transport- T05C12.7/*cct-1,* C39F7.4/*rab-1,* T19B4.2/*npp-7*and K12H4.4/*phi-20.* Also we have genes encoding for nucleic acid synthesis and binding- R07E5.14/*rnp-4,* T10F2.4, T23G5.1/*rnr-1*,

R144.2/*pcf-11* and T08A11.2/*phi-11*. T05G5.3/*cdk-1*, K08E3.6/*cyk-4* and ZK632.1/*mcm-6* belong to gene expression regulation functional class. The following genes C23G10.8 and Y55H10A.1/*vha-19* not belong to ATP synthesis functional class. R08D7.1 has not been assigned any specific functional class.

Table 3.2. List of all functional classes of genes and their description (Kamath et al., 2003).

Functional classes	Description
RNA synthesis	Includes genes which encode for transcription machinery and splicing
Protein synthesis	Ribosome biogenesis, translation machinery and chaperone encoding genes
Cellular architecture	Cytoskeletal and protein trafficking/vesicle regulation
Metabolism	Genes which encode for energy production and intermediary metabolism
Protein degradation	Components of the ubiquitin-targeted protein degradation machinery
Nucleic-acid binding	Nucleic acid-binding proteins with unknown specificity for DNA or RNA
Signal transduction	Genes which encode for kinases, phosphatases and signal transduction pathway components.
Small molecule transport	Intracellular ion channels and ABC transporters
DNA synthesis	Includes cell cycle and repair genes
Unknown	Genes with insufficient information to assign a function, or genes with no significant matches in any organism.

Table 3.3. List of genes which resulted > 70% sterility in the wild type and *rrf-1(pk1417)* backgrounds, with greatly reduced sterility in *rde-2(ne221)* background. N= Number of animals. Each data entry represents at least the initial phenotypic scoring.

Predicted gene	Locus	Description	Reference	% Sterile (N)			
				N2	*rrf-1 (pk1417)*	*rde-2 (ne221)*	*rde-1 (ne219)*
T05C12.7	*cct-1*	Alpha subunit of cytosolic chaperonin	Leroux and candido 1995	95 ± 2.5(75)	68 ± 5.3(75)	27 ± 8.1(30)	0(30)
C23G10.4a	*rpn-2*	Non-ATPase subunit of 19S complex	Sugiyama 2008	97 ± 2.2(60)	93 ± 3.2(60)	30 ± 8.3(30)	0(30)
C36B1.4	*pas-4*	Proteosome alpha type four subunit	Kahn 2008	85 ± 3.7(90)	58 ± 5.2(90)	29 ± 9.9(21)	0(30)
R144.2	*pcf-11*	Cleavage and polyadenylation factor homolog	Cui 2008	73 ± 4.6(90)	87 ± 3.5(90)	17 ± 6.8(30)	0(30)
T08A11.2	*phi-11*	Ortholog of splicing factor 3b subunit 1	Govindan 2006	80 ± 4.2(90)	71.6 ± 5.8(60)	13 ± 6.2(30)	0(30)
K08E3.6	*cyk-4*	Encodes Rho GAP; regulates anterior-posterior polarity	Jantsch-plunger 200	100(60)	100(60)	12 ± 3.4(90)	0(30)
T05G5.3	*cdk-1*	Cell-cycle progression in M phase for meiotic and mitotic cell cycle	Boxem 1999	92 ± 3.5(60)	95 ± 2.8(60)	13 ± 6.1(30)	0(30)
T23G5.1	*rnr-1*	Large subunit of ribonucleotide reductase	Wormbase	93 ± 4.6(30)	100 (30)	37 ± 8.8(30)	0(30)
ZK632.1	*mcm-6*	Embryonic viability	Updike 2009	95 ± 2.8(60)	98 ± 1.8(60)	33 ± 8.5(30)	0(30)
ZK328.5b	*npp-7*	Nuclear pore protein complex	Galy 2003	84.8 ± 4(79)	71 ± 5(80)	20 ± 7.3(60)	0(10)

Table 3.3 continued

F57B9.5	*byn-1*	encodes a homolog of mammalian BYSTIN-like	Forejt 2001	97 ± 2.5(40)	96 ± 3.4(29)	30 ± 8.3(30)	0(10)
T10F2.4		mRNA splicing factor	Nollen 2004	97 ± 2.2(59)	97 ± 2.2(60)	37 ± 8.8(30)	0(30)
K12H4.4	*phi-20*	signal peptidase complex subunit	Wormbase	100(34)	100(19)	26.7 ± 8(30)	0(10)
R07E5.14	*rnp-4*	encodes for exon-exon junction complex	Longman 2003	100(40)	100(30)	17 ± 6.8(30)	0(10)
R08D7.1		uncharacterized conserved protein	Wormbase	100(40)	96 ± 3.2(30)	13 ± 6.2(30)	0(10)
C23G10.8		ATP synthesis	Wormbase	98.8 ± 1.2(90)	87.8 ± 3.4(90)	10 ± 5.4(30)	0(10)
Y55H10A.1	*vha-19*	Vacuolar H ATPase encodes for ortholog of subunit Ac45	Wormbase	97 ± 2.1(69)	74.5 ± 5.6(59)	18.3 ±4.9(60)	0(10)
C39F7.4	*rab-1*	encodes an ortholog of the small Ras-like GTPase Rab1	Couillault 2004	98 ± 1.6(60)	96 ± 3.2(30)	26.7 ± 8(30)	0(10)
T01C3.7	*fib-1*	encodes a *C. elegans* ortholog of yeast Nop1p	Saijou 2004	100(40)	100(30)	23 ± 7.7(30)	0(10)
C52E4.4	*rpt-1*	encodes a ATPase subunit of the 19S regulatory complex of the proteasome	Wormbase	100(40)	90 ± 5.4(30)	26.7 ± 6.6(45)	0(10)

Protein Synthesis Genes

In this functional class of genes, T05G5.3/*cdk-1*, F57B9.5/*byn-1,* C39F7.4/*rab-1* and T01C3.7/*fib-1* were found to be germline specific for fertility. The *C. elegans byn-1* gene encodes for an ortholog of mammalian BYSTIN-like. Bystin-like (Bysl) gene is located on chromosome 17 in mouse and its ortholog is located on chromosome III in *C. elegans* (Trachtulec and Forejt, 2001). Depletion of *byn-1* mRNA in N2 background resulted in sterility (97 ± 2.5%, N=40). To test whether *byn-1* acts on the germline, we knocked down *byn-1* mRNA in *rrf-1(pk1417)* background, deficient in RNAi in the somatic tissue (Sijen et al., 2001), which showed no significant difference in sterility compared to the wild type (96 ± 3.4%, N=29). This indicates that *byn-1* acts on the germline to cause Ste phenotype. To test this hypothesis, we used *rde-2(ne221)* mutants, in which RNAi is resistant in germ cells (Tabara et al., 1999). The *rde-2* worms showed suppression of Ste in *byn-1* RNAi (27 ± 8.1%, N=29).

To determine whether BYN-1 is essential for ovulation, wild type and *rrf-1(pk1417)* and *rde-2(ne221)* worms were subjected to *byn-1* RNAi. Our finding in N2 (Figure 3.2 A and B) strain indicated that oocytes become trapped in the proximal gonads and underwent extra rounds of replications, because of the ovulation defects. We observed similar Emo oocytes in *rrf-1(pk1417); byn-1(RNAi)* worms (Figure 3.2 C and 3.2 D). However, knockdown of *byn-1* mRNA in *rde-2(ne221)* mutant showed typical oocytes in the proximal gonad arm confirming the germline specificity of *byn-1* (Figure 3.2 E and 3.2 F). The *rde-1(ne219)* strain in which RNAi is suppressed throughout the body was used as a control. The *rde-1(ne219); byn-1(RNAi)* worms had normal oocytes in the gonad arm (Figure 3.2 G and 3.2 H), indicating that RNAi was working properly.

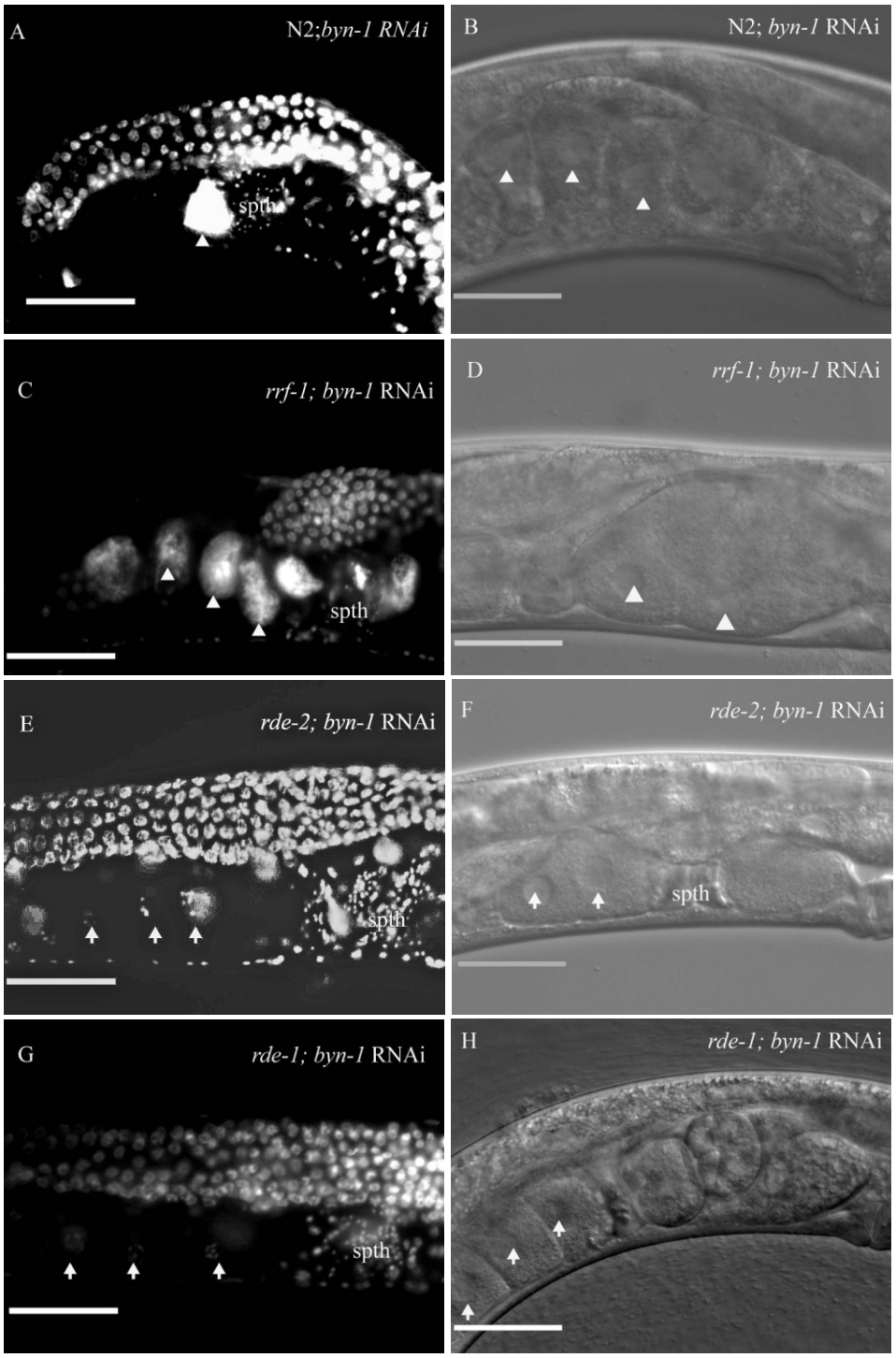

Figure 3.2. Proximal gonads of *byn-1* RNAi animals. (A) Nuclear staining and (B) DIC image of N2; *byn-1* animals showing endomitotic oocytes (Arrow heads). (C) *rrf-1(pk1417); byn-1(RNAi)* animal showing endomitotic oocytes (Arrow heads). (D) DIC image. (E) & (F) DAPI and DIC images of *rde-2; byn-1(RNAi)* worms showing normal oocytes in the proximal gonad (Arrows). (G) and (H) DAPI & DIC images of *rde-1; byn-1(RNAi)* animal with normal oocytes. Spth denotes spermatheca. Scale bar=40 μm.

fib-1, a *C. elegans* homolog of yeast Nop1p is an essential factor for U3 SnoRNP (Saijou *et al.,* 2004). RNAi of *fib-1* resulted in sterility in N2 worms (100%, N=40) and *rrf-1(pk1417)* strain (100%, N=30). In *rde-2(ne221)*, the *fib-1(RNAi)* showed reduction in Ste phenotype (23.3 ± 7.7%, N=30). DAPI staining of *fib-1* depleted N2 animals shows Emo oocytes in the proximal gonad (Figure 3.3 A) and also in *rrf-1(pk1417* animals (Figure 3.3 B). The *rde-2(ne221); fib-1(RNAi)* worms displayed no ovulation defects (Figure 3.3 C). Figures 3.3 C and D show the presence of oocytes in diakinetic stage with 6 bivalent chromatids indicating normal progression of oocytes in the gonad.

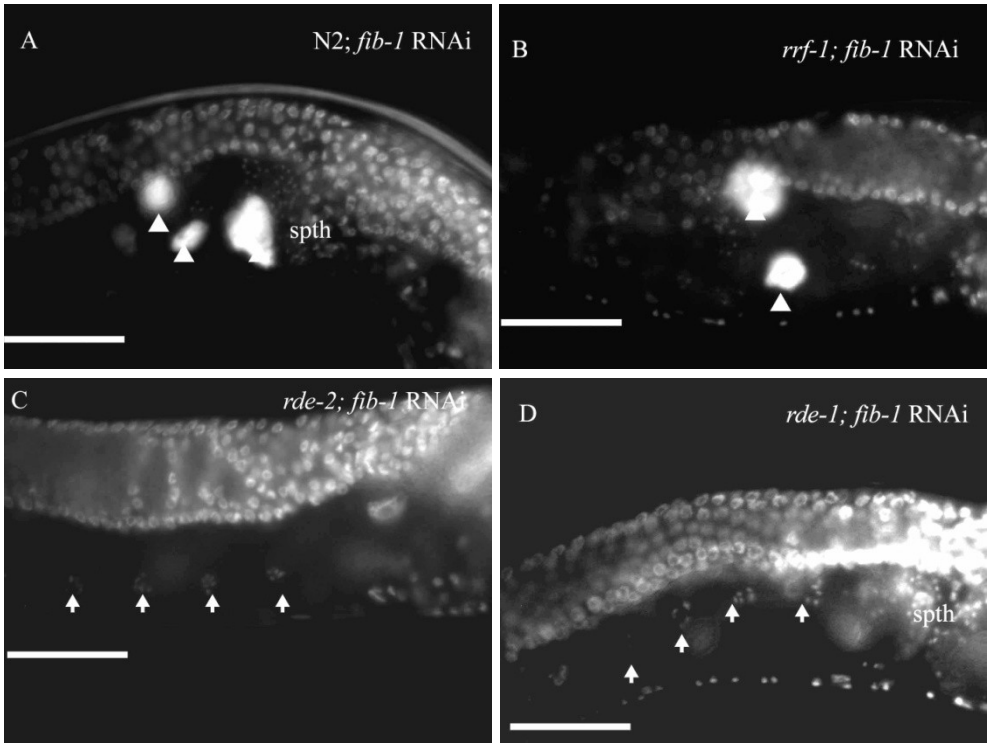

Figure 3.3. Nuclear staining images of *fib-1* RNAi animals. (A) An N2; *fib-1 (RNAi)* animal displaying Emo oocytes (arrow heads) in the proximal gonad. (B) *rrf-1(pk1417); fib-1(RNAi)* animal with accumulated oocytes in the proximal gonad (arrow heads). (C) *rde-2(ne221); fib-1(RNAi)* treated animal showing normal oocytes (arrows) in the proximal gonad. (D) An *rde-1(ne219); fib-1* animal showing normal oocytes (arrows) in the proximal gonad. Spth, spermatheca. Scale Bar= 40µm.

Protein Transport and Modification Genes

We identified four genes belonging to protein transport and modification functional class; T05C12.7/*cct-1,* T19B4.2/*npp-7,* K12H4.4/*phi-20* and C39F7.4/*rab-1.* Chaperonins have the ability of folding proteins and most proteins have to pass through the chaperonin folding mechanism. *cct-1,* an ortholog of human TCP-1, encodes a putative alpha subunit of the eukaryotic cytosolic ('T complex') chaperonin (Leroux and Candido, 1997). In our study, we show that knockdown of *cct-1* caused sterility in N2 (95 ± 2.5%, N=75) and *rrf-1(pk1417)* (68 ± 5.3%, N=75) but in *rde-2(ne221)* the sterility was suppressed 27 ± 8.1%, (N=30). After performing RNAi against *cct-1*,

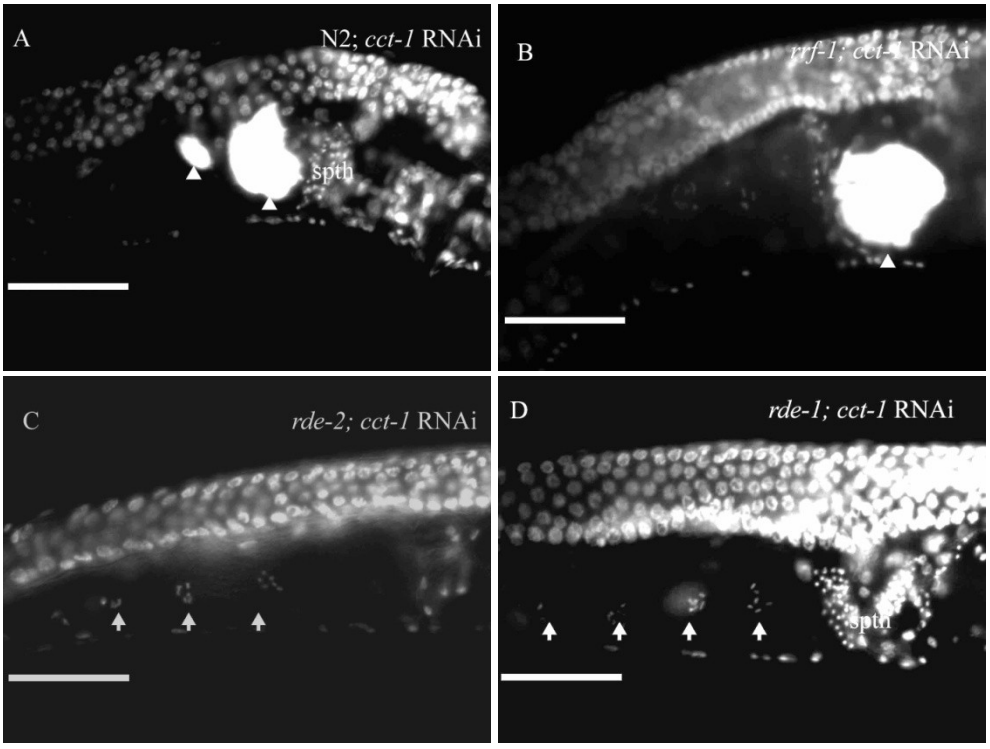

Figure 3.4. Nuclear staining images of *cct-1* RNAi animals. (A) N2 animal showing Emo oocytes (arrow heads) after *cct-1* feeding RNAi. (B) Proximal gonad of *rrf-1(pk1417); cct-1(RNAi)* animal. (C) An *rde-2; cct-1(RNAi)* animal showing normal oocytes (arrows). (D) Proximal gonad of *rde-1(ne219); cct-1(RNAi)* animal with normal oocytes (arrows). Spth=spermatheca, Scale Bar= 40 μm.

nuclear staining was done in worms after 48 hours; we observed endomitotic nuclei in the proximal gonads in wild type (Figure 3.4 A) and *rrf-1(pk1417)* worms (Figure 3.4 B). The *rde-2(ne221)* and *rde-1(ne219)* worms displayed normal oocytes (Figure 3.4 C & D).

phi-20 is a component of signal peptidase complex subunit and is involved in intracellular trafficking, secretion and vesicular transport (Harris et al., 2010). The sterility was 100% (N=34) in N2 and 100 % (N=29) in *rrf-1(pk1417)*. In the *rde-2* mutant, the sterility was 26 ± 8% (N=29). Nuclear staining of *phi-20(RNAi)* in N2 and *rrf-1(pk1417)* showed Emo oocytes (Figure 3.5 A and 3.5 B, respectively) and none in *rde-2(ne221)* (Figure 3.5 C) and *rde-1(ne219)* (Figure 3.5 D).

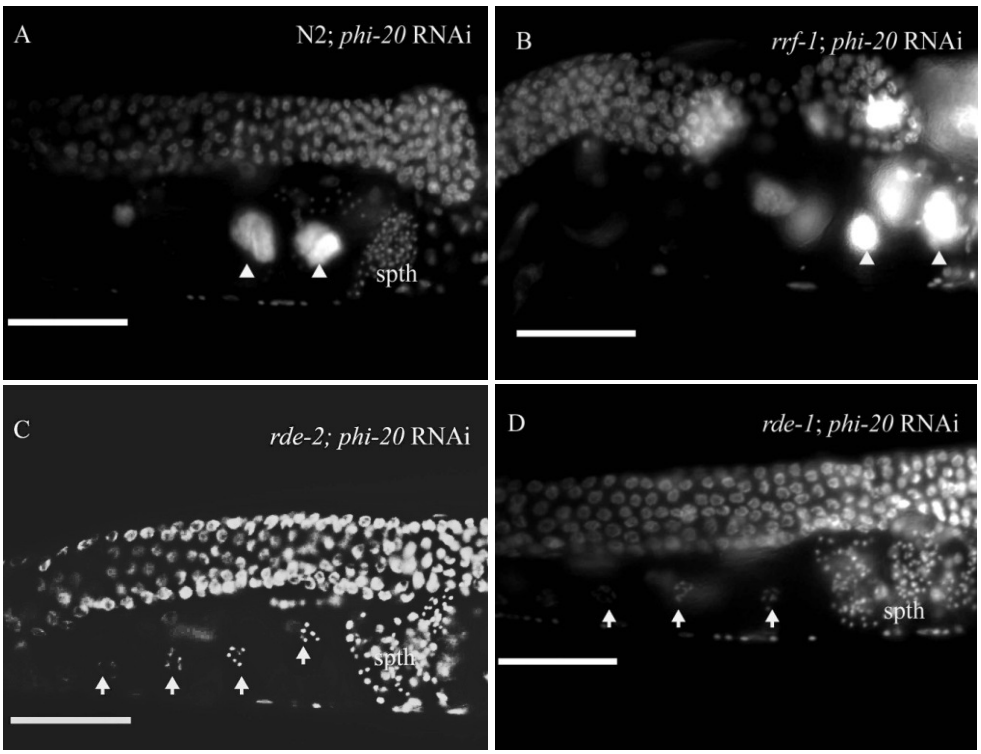

Figure 3.5. Nuclear staining images of *phi-20* fed animals. (A) N2 ; *phi-20* RNAi animal displaying Emo oocytes (arrowheads). (B) *rrf-1(pk1417); phi-20(RNAi)* showing Emo (arrowheads) oocytes in the proximal gonad. (C) DAPI staining of *rde-2(ne221); phi-20(RNAi)* worm showing normal oocytes,. (D) *rde-1; phi-20 (RNAi)* animal with normal oocytes. Arrows indicate normal oocytes. Spth=spermatheca, Scale bar=40 μm.

npp-7 encodes for a subunit of nuclear pore complex protein (Galy et al., 2003). RNAi of *npp-7* in N2 resulted in sterility (84.8 ± 4%, N=79) and in *rrf-1(pk1417)* strain (71 ± 5%, N=80). In contrast the Ste phenotype was reduced in *rde-2(ne221)* background (20 ± 7.3%, N=60). RNAi of *npp-7* in N2 worms showed nuclear accumulation in oocytes in the proximal gonad (Figure 3.6 A). A similar phenotype was observed in *rrf-1(pk1417); npp-7* worms (Figure 3.6 B). The *rde-2(ne221); npp-7(RNAi)* worms did not show any nuclear accumulation (Figure 3.6 C. Figure 3.6 D shows normal oocytes in *rde-1(ne219); npp-7(RNAi).*

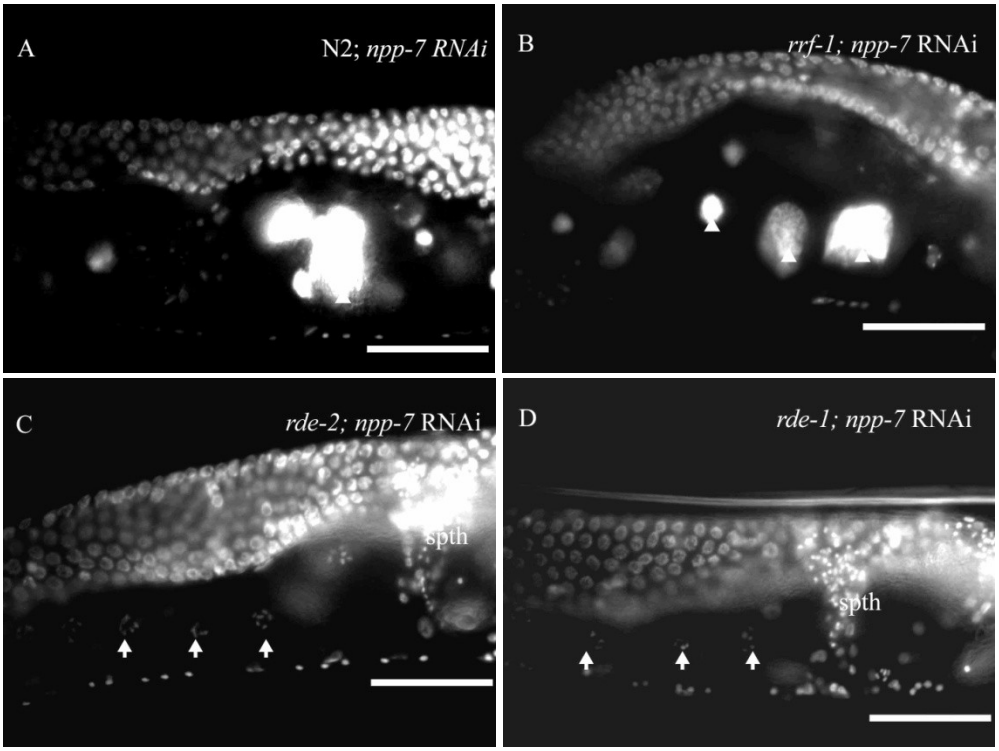

Figure 3.6. Nuclear staining images of *npp-7* RNAi animals. (A) Wild type N2 animal grown on *npp-7(RNAi)* showing Emo oocytes (arrow heads) in the proximal gonad. (B) *rrf-1(pk1417)* ; *npp-7(RNAi)* showing accumulated oocytes in the proximal gonad (arrow heads). (C) Image of *rde-2(ne221); npp-7(RNAi)* showing normal oocytes (arrows) in the proximal gonad. (D) Nuclear staining image of *rde-1(ne219); npp-7* showing normal oocytes (arrows) in the proximal gonad. Spth, Spermatheca. Scale Bar= 40µm.

RAB-1, a member of Rab-GTPase family, plays a role in fusion of COPI vesicles in the endoplasmic reticulum (Poteryaev et al., 2005). *rab-1* encodes for an ortholog of small GTPase (Couillault et al., 2004) and loss of *rab-1* resulted in sterility and embryonic lethality (Kamath et al., 2003). RNAi of *rab-1* in N2 and *rrf-1(pk1417)* caused 98.3 ± 1.6%, (N=60) and 96.7 ± 3.2%, (N=30), sterility respectively. In the *rde-2(ne221)* background, however, the sterility was less severe (26.7 ± 8%, N=30). Emo nuclei in oocytes was seen in N2; *rab-1(RNAi)* (Figure 3.7 A) and *rrf-1(pk1417); rab-1(RNAi)* (Figure 3.7 B) after nuclear staining. The *rde-2(ne221)* and *rde-1(ne219)* showed typical oocytes in the proximal gonad (Figures 3.7 C and 3.7 D, respectively).

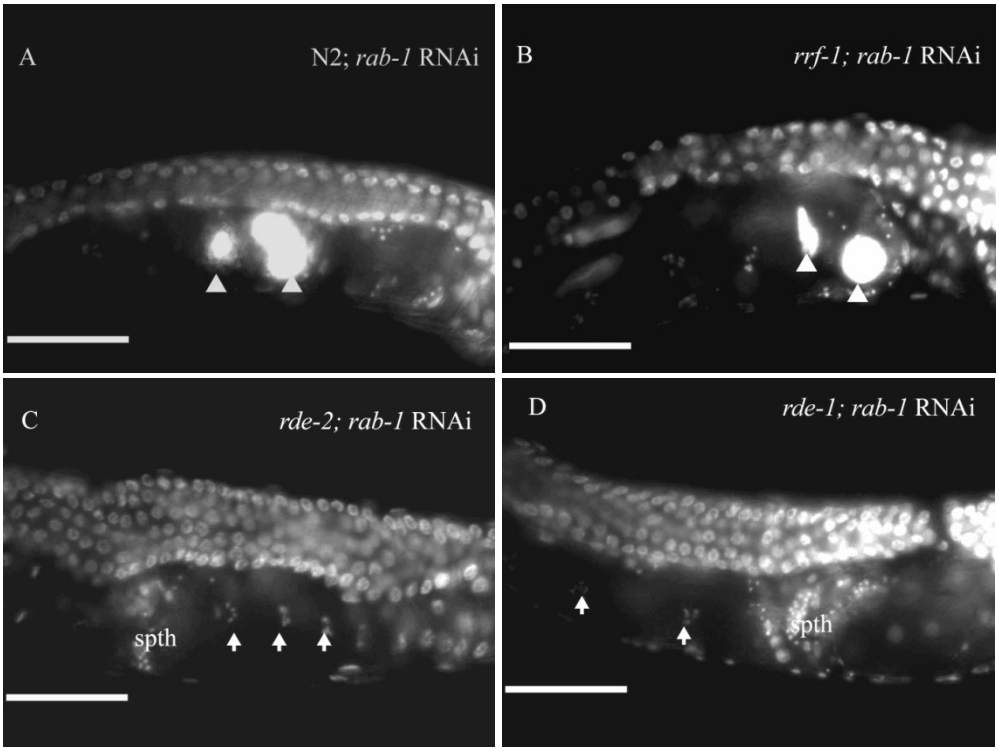

Figure 3.7. Nuclear staining images of *rab-1* RNAi animals. (A) Nuclear staining of N2; *rab-1 (RNAi)* animals showing endomitotic oocytes (Arrow heads). (B) An *rrf-1(pk1417); rab-1(RNAi)* animal showing endomitotic oocytes (Arrow heads). (C)An *rde-2(ne221)*; *rab-1(RNAi)* with normal oocytes (arrows). (D) *rde-1(ne219); rab-1(RNAi)* animal with normal oocytes (arrows). Spth denotes spermatheca. Scale bar=40 μm.

Nucleic Acid Synthesis, Processing and Binding Genes

Our screen has identified many genes essential for RNA functions such as synthesis, splicing and binding processes. Among the subset of 20 germline specific genes, we found five genes, R07E5.14/*rnp-4,* T10F2.4, T23G5.1/*rnr-1*, R144.2/*pcf-11* and T08A11.2/*phi-11* to be essential for fertility in germline. To study the role of the RNA specific genes, we depleted them using RNAi and analyzed the phenotypes. As shown in Table 3.3, RNAi knockdown of *rnr-1, pcf-11*, *phi-11*, *rnp-4*, and T10F2.4 resulted in Ste in N2 and *rrf-1(pk1417)* but reduction of Ste in *rde-2(ne221)* background.

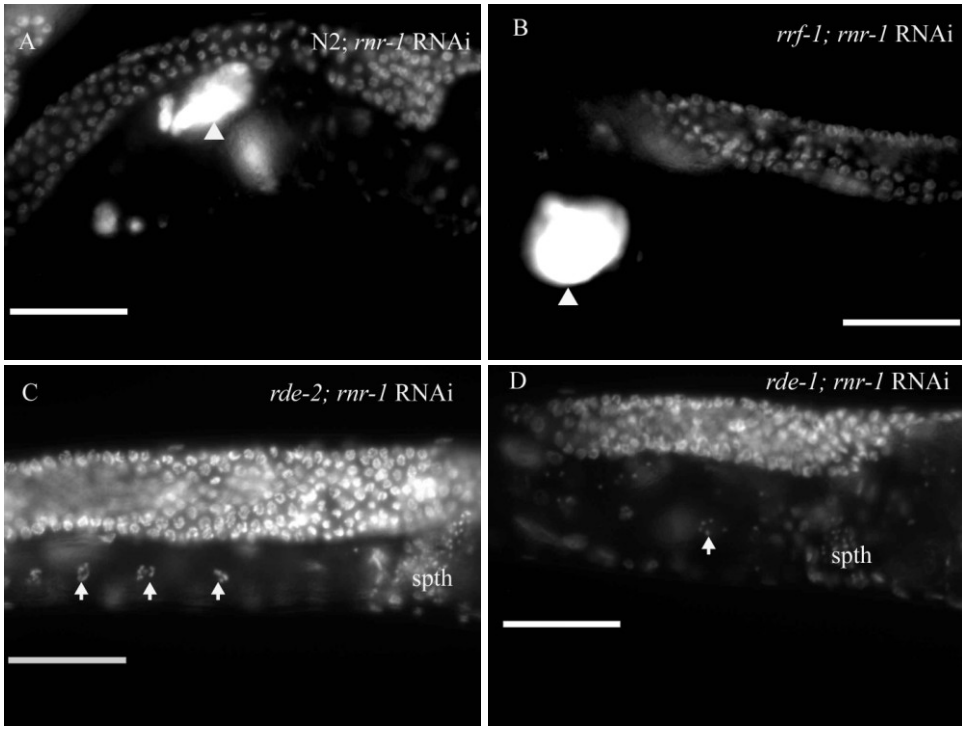

Figure 3.8. Nuclear staining images of *rnr-1* RNAi animals. (A) DAPI staining of wild type animals treated with *rnr-1* RNAi showing accumulation of nuclear material (Arrow heads). (B) Proximal gonad arm of *rrf-1(pk1417); rnr-1(RNAi)* animal showing endomitotic oocytes (Arrow heads). (C) An *rde-2* animal fed with *rnr-1* RNAi showing normal oocytes in the proximal gonad, denoted by arrows (D) *rde-1(ne219); rnr-1(RNAi)* animal showing normal oocytes (arrows) in the proximal gonad (DIC image). Spth denotes spermatheca. Scale bar=40 μm.

rnr-1 gene together with *cye*-1 (Cyclin-E) encodes for ribonucleotide reductase, and is necessary for reproduction and embryonic development (Harris et al., 2010); van den Heuvel 2005). E2F, a heterodimeric transcription factor, regulates the expression of genes essential for DNA synthesis (Brodigan et al., 2003). *rnr*-1 is a target of Rb/E2F regulation and has multiple E2F-binding sites (Brodigan *et al*., 2003; Hong *et al.,* 1998). Loss of *rnr-1* caused 93 ± 4.6%, (N=30) and 100%, (N=30) sterility and in wild type and *rrf-1(pk1417)* respectively. Considerable suppression of sterility was observed in the *rde-2(ne221)* background, 37 ± 8.8%, (N=30). RNAi of *rnr-1 i*n N2 (Figure 3.8 A) and *rrf-1(pk1417)* (Figure 3.8 B), caused Emo phenotype in the proximal gonad. In the *rde-2(ne221); rnr-1(RNAi)* (Figure 3.8 C), the oocytes were in diakinesis stage, showing the 6 chromatids.

rnp-4 encodes a component of exon-exon junction complex and is necessary for viability, late embryogenesis (Kawano et al., 2004; Longman et al., 2003). During the pre-mRNA splicing process RNP-4 binding is necessary to remove introns and imprint the mRNA in the nucleus (Kataoka et al., 2000). These exon junction complexes or EJCs are non-essential components of nonsense-mediated mRNA decay pathway (NMD) (Longman et al., 2007). Depletion of *rnp*-4 resulted in 100% sterility (N=40) in wild type and in *rrf-1(pk1417)* worms (N=30) but the sterility was suppressed in the *rde-2(ne221)* background, 17 ± 6.8% (N=30). Upon knockdown of *rnp-4* gene, Emo phenotype was observed in the proximal gonad arms in N2 (Figure 3.9 A) and *rrf-1(pk1417)* (Figure 3.9 B). Gonad arms with regular oocyte arrangement were observed in *rde-2(ne221); rnp-4(RNAi)* background (Figure 3.9 C) and *rde-1(ne219); rnp-4(RNAi)* (Figure 3.9 D).

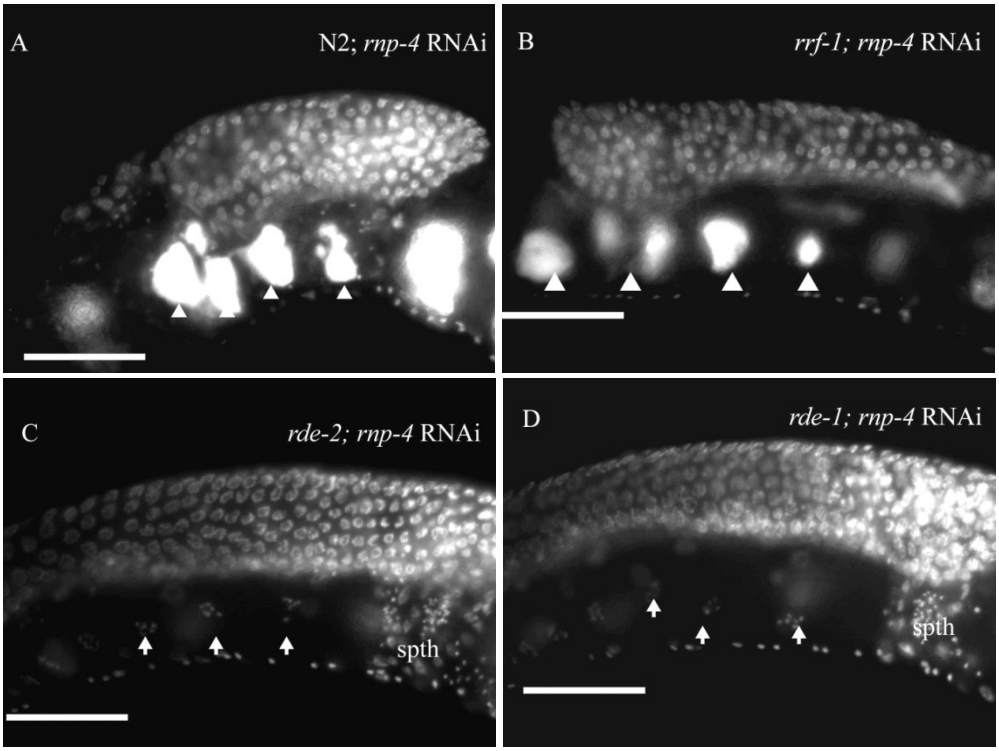

Figure 3.9. The proximal gonads of *rnp-4* RNAi animals. (A) N2; *rnp-4 (RNAi)* treated animal displaying Emo oocytes in the proximal gonad, shown here as arrowheads. (B) *rrf-1(pk1417); rnp-4 (RNAi)* showing Emo oocytes in the proximal gonad. Arrowheads represent endomitotic oocyte nuclei. (C) DAPI staining of *rde-2(ne221); rnp-4 (RNAi)* worm showing normal oocytes, arrows represent normal oocytes. (D) An *rde-1(ne219)* animal fed with *rnp-4* RNAi showing normal oocytes (arrows) in the proximal gonad. spth= spermatheca. Scale bar=40 μm.

The gene *pcf-11,* encodes for a homolog of the cleavage and polyadenylation factor (Cui et al., 2008). The cleavage and polyadenylation factors are part of the protein complex that is essential for identifying the 3' processing signals (Cui et al., 2008). The sterility due to knockdown of *pcf-11* was 73 ± 4.6%, (N=90) in the N2 and 87 ± 3.5%, (N=90) in the *rrf-1(pk1417)*. The Ste was suppressed in *rde-2(ne221)* mutants fed with *pcf-11* dsRNA producing bacteria, (17 ± 6.8%, N=30). Nuclear staining of worms subjected to *pcf-11* RNAi showed Emo in N2 (Figure 3.10 A) and *rrf-1(pk1417)* (Figure 3.10 B) but not in *rde-2(ne221)* (Figure 3.10 C) and *rde-1(ne219)* (Figure 3.10D).

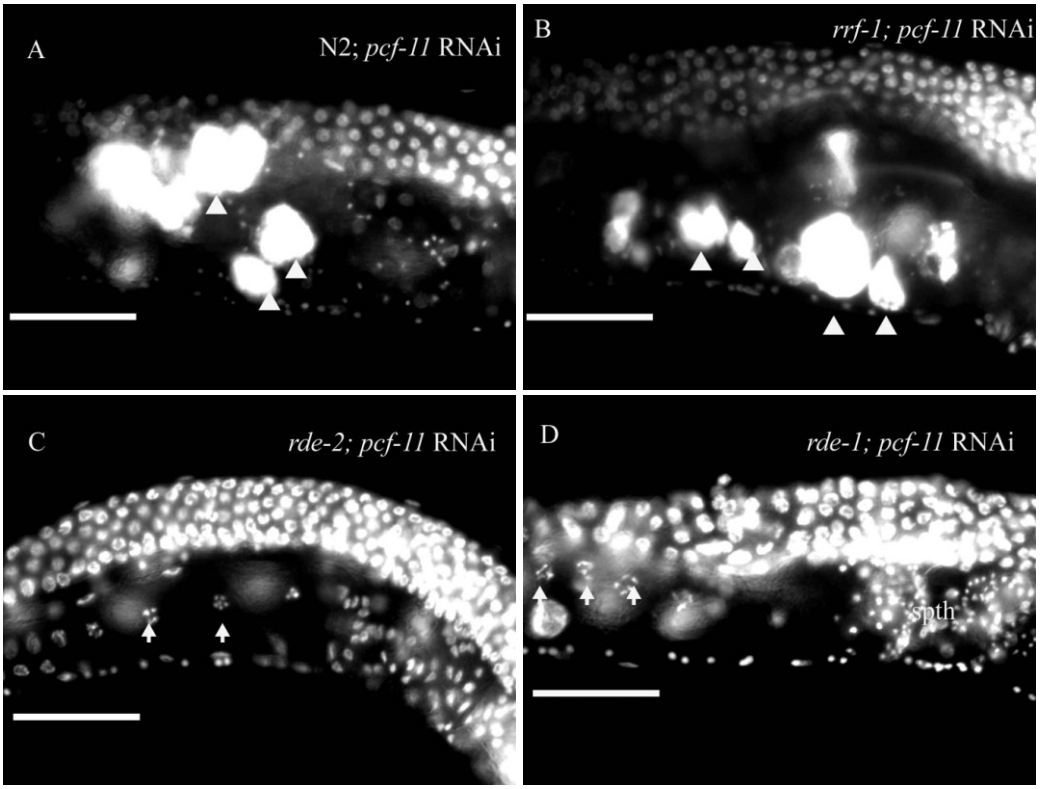

Figure 3.10. Nuclear staining images of *pcf-11* RNAi fed animals. (A) Nuclear staining of wild type animals treated with *pcf-11* RNAi showing accumulation of nuclear material (Arrow heads). (B) Proximal gonad arm of *rrf-1(pk1417); pcf-11(RNAi)* animal showing endomitotic oocytes (Arrow heads). (C)An *rde-2(ne221)* animal treated with *pcf-11* RNAi showing normal oocytes (arrows). (D) *rde-1(ne219); pcf-11(RNAi)* animal showing normal oocytes (arrows) in the proximal gonad (DAPI image). Spth denotes spermatheca. Scale bar=40 μm.

T10F2.4 is one of the four genes in *C. elegans* that encode proteins with a U-box domain. Previous screens of T10F2.4, a *C. elegans* homolog of yeast and human *PRP19*, have shown that T10F2.4 is necessary for embryonic viability (Kamath et al., 2003; Nollen et al., 2004; Simmer et al., 2003). In yeast the *prp19*, a small nuclear ribonucleo-protein (snRNP), is part of the spliceosome structure which excise sequences from pre-mRNA transcripts in eukaryotes (Blanton et al., 1992). The sterility in N2 ; T10F2.4 worms was 97 ± 2.2%, (N=59) and in *rrf-1(pk1417);* T10F2.4 worms was, 97 ± 2.2%, (N=60). The *rde-2(ne221);* T10F2.4 worms showed suppression of Ste, 37 ± 8.8%,

(N=30). Nuclear staining and DIC microscopy have shown endomitotic nuclei in the accumulated oocytes in N2 (Figure 3.11 A & B) and *rrf-1(pk1417)* (Figure 3.11 C & D). The *rde-2(ne221);*T10F2.4 worms displayed typical oocytes in the proximal gonad (Figure 3.11 E & F).

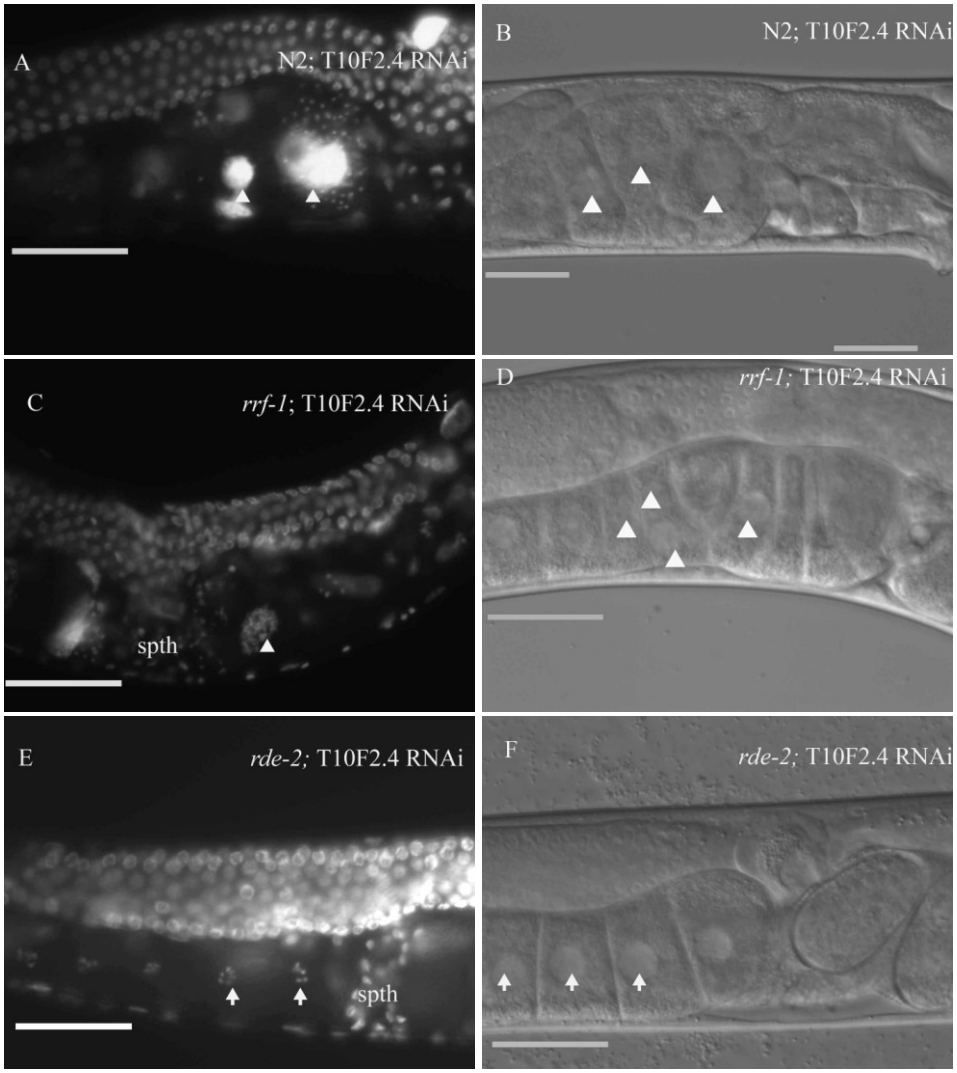

Figure 3.11. Proximal gonads of T10F2.4 RNAi worms. (A) Nuclear staining of N2; T10F2.4 RNAi animal showing Emo oocytes (arrow heads). (B) DIC image of proximal gonad of N2; T10F2.4 *(RNAi)* animal. (C) An *rrf-1(pk1417);* T10F2.4 *(RNAi)* animal showing nuclear accumulation in the oocytes (arrow heads). (D) DIC image of *rrf-1(pk1417);* T10F2.4 *(RNAi).* (E) An *rde-2(ne221);* T10F2.4 RNAi with normal oocytes in the proximal gonad (arrows). (F) DIC image of *rde-2(ne221);* T10F2.4 *(RNAi)* with normal oocytes in the proximal gonad. spth= spermatheca. Scale bar=40 µm.

phi-11 encodes the *C. elegans* ortholog of splicing factor 3b subunit 1, and its product is predicted to be a component of the U2 snRNP required for pre-mRNA splicing (Govindan *et al.,* 2006). N2 and *rrf-1(pk1417)* treated with *phi-11* RNAi showed higher sterility, 80 ± 4.2%, (N=90) and 71.6 ± 5.8%, (N=60), compared to the *rde-2(ne221)* strain (13 ± 6.2%, N=30). After DAPI staining, presence of Emo nuclei in accumulated oocytes was seen in the proximal gonads of N2 (Figure 3.12 A) and *rrf-1(pk1417)* worms (Figure 3.12 B). Typical oocyte morphology was seen in the proximal gonads of *rde-2(ne221)* (Figure 3.12 C) and *rde-1(ne219)* worms after *phi-11* RNAi feeding.

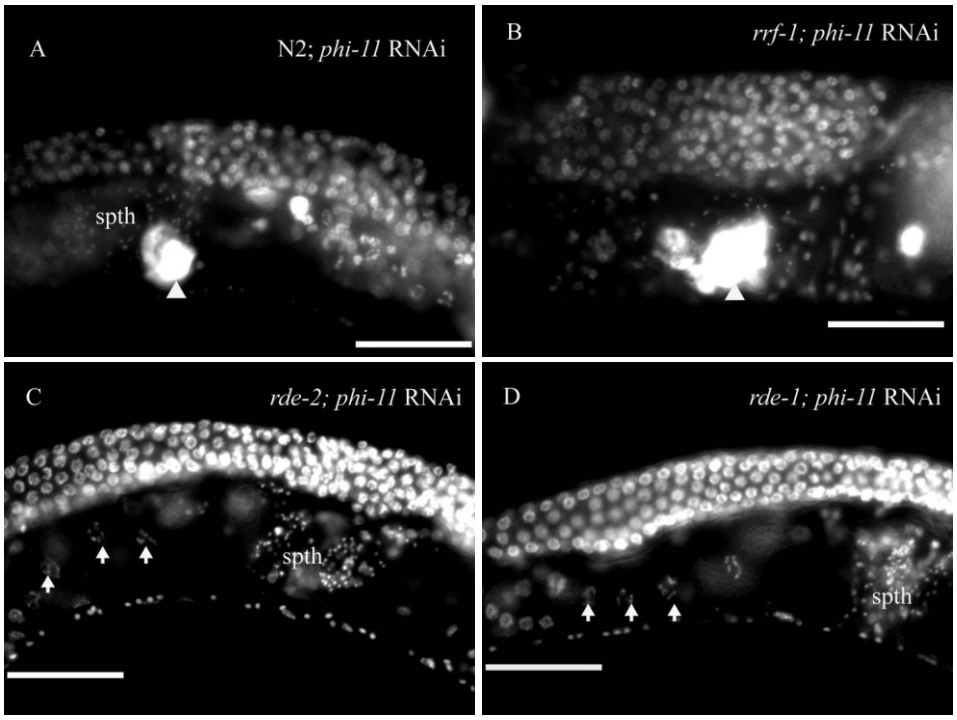

Figure 3.12. Nuclear staining images of *phi-11* RNAi animals. (A)N2 animal treated with *phi-11* RNAi showing nuclear accumulation in the proximal gonad (arrowheads). (B) *rrf-1(pk1417)* animal fed with *phi-11* RNAi showing Emo oocyte nulcei accumulated in the proximal gonad (arrow heads). (C) Nuclear staining of *rde-2(ne221); phi-11 (RNAi)* showing normal oocytes in the proximal gonad (arrows). (D) An *rde-1(ne219)* fed with *phi-11* RNAi showing normal oocytes (arrows) in the proximal gonad. Spth=spermatheca, Scale Bar= 40 μm.

Genes Encoding for Gene Regulation

We also identified *mcm-6*, which controls the DNA replication to once per cell cycle (Updike and Strome, 2009). RNAi of *mcm-6* has resulted in sterility in N2, (95 ± 2.8%, N=60) and *rrf-1(pk1417)* (98 ± 1.8%, N=60) and reduced sterility in *rde-2(ne221)* (33 ± 8.5%, N=30). RNAi of *mcm-6* in N2 and *rrf-1(pk1417)* worms caused Emo in the proximal gonad (Figure 3.13 A), similar phenotype was observed in *rrf-1(pk1417)* as shown in Figure 3.13 B. Knockdown of *mcm-6* in *rde-2(ne221)* and *rde-2(ne221)* did not result in Emo in the proximal gonad arms (Figure 3.13 C and D).

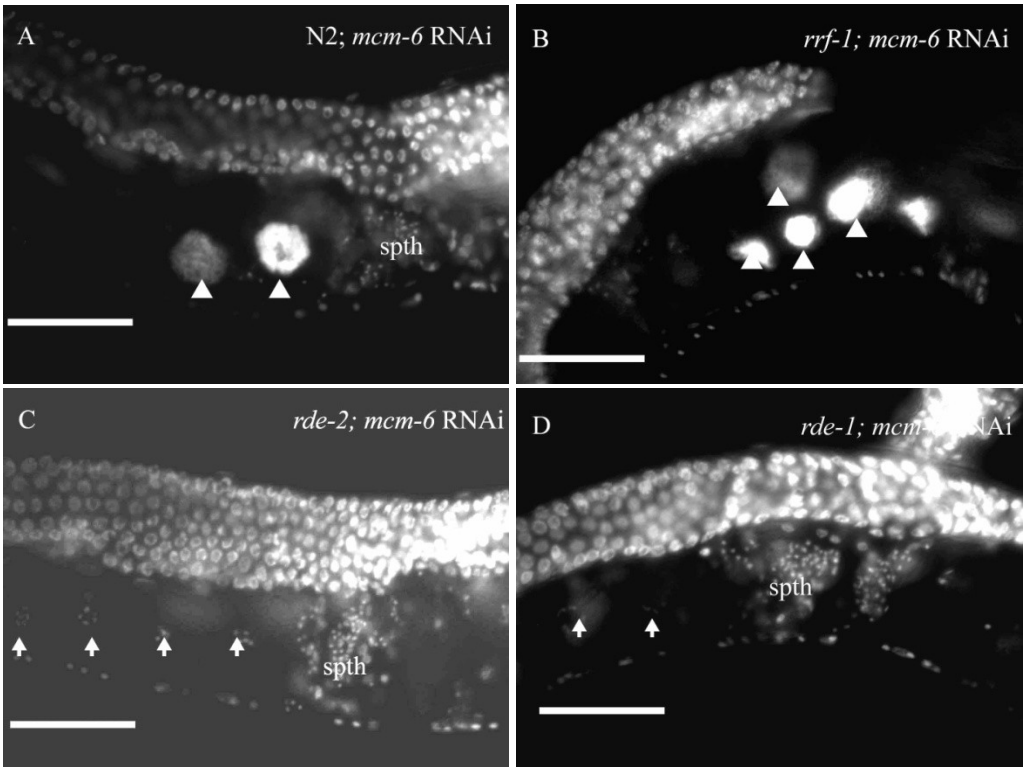

Figure 3.13. Nuclear staining images of *mcm-6* RNAi animals. (A) Nuclear staining of the proximal gonad of N2 strains treated with *mcm-6* RNAi showing endomitotic oocyte nuclei (arrow heads). (B) An *rrf-1(pk1417); mcm-6 (RNAi)* worm showing oocytes with endomitotically replicating oocytes (arrow heads). (C) Nuclear staining of *rde-2(ne221); mcm-6 (RNAi)* animal showing normal oocytes (arrows) in the proximal gonad. (D) An *rde-1(ne219); mcm-6 (RNAi)* animal stained with DAPI showing normal oocytes (arrows). spth, spermatheca, Scale Bar= 40µm.

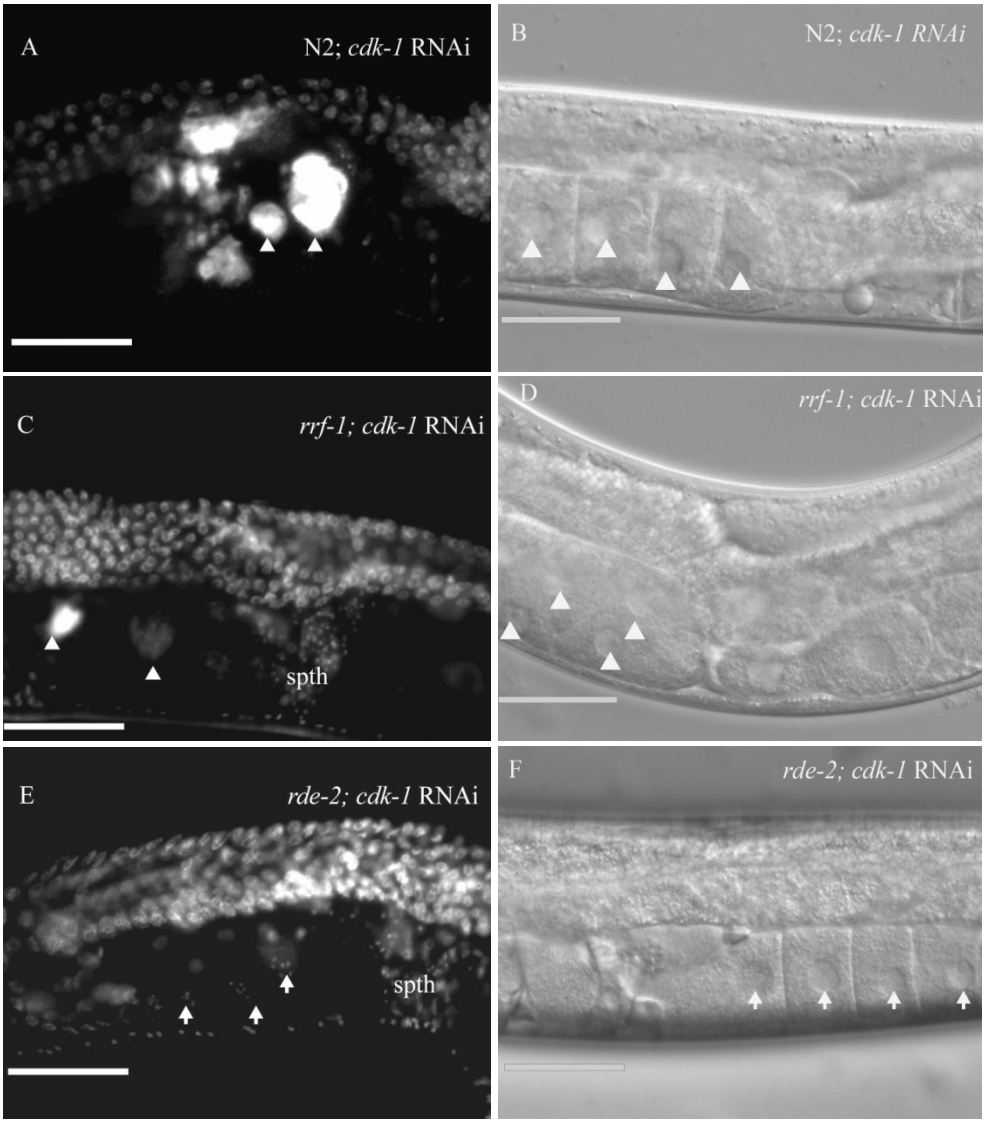

Figure 3.14. Proximal gonads of *cdk-1* RNAi animals. (A) Nuclear staining image of N2; *cdk-1* RNAi showing Emo oocytes (arrowheads) in the proximal gonad. (B) DIC image of N2; *cdk-1 (RNAi)* worms showing Emo oocyte nuclei (arrow heads). (C) *rrf-1(pk1417)* animal treated with *cdk-1* RNAi showing Emo oocyte nuclei (arrow heads). (D) DIC image of *rrf-1(pk1417); cdk-1 (RNAi)* animals showing Emo oocytes (arrow heads). (E) *rde-2(ne221)*; *cdk-1* RNAi showing normal oocytes (arrows) in the proximal gonad. (F) DIC image of *rde-2(ne221)* animals treated with *cdk-1* RNAi showing normal oocytes (arrows) in the proximal gonad. Spth, Spermatheca, Scale Bar= 40 μm.

cdk-1 is a maturation promotion factor (MPF), which promotes chromosome condensation, nuclear envelope breakdown (NEBD) and spindle assembly (Boxem *et al.,* 1999; Burrows *et al.,* 2006). N2 and *rrf-1(pk1417)* mutant worms subjected to *cdk-*

1(RNAi) displayed higher incidence of sterility, 92 ± 3.5%, (N=60) and 95 ± 2.8%, (N=60), respectively. The *rde-2(ne221); cdk-1(RNAi)* resulted in the suppression of sterility, (13 ± 6.1%, N=30). Upon examination of the proximal gonad, we found the presence of endomitotic nuclei in accumulated oocytes in N2 and *rrf-1(pk1417)* worms fed with *cdk-1(RNAi)* (Figure 3.14 A, B, C and D). Oocytes with six bivalent chromatids, indicating the diakinesis stage of meiotic prophase I, were detected in the proximal gonad in the *rde-2* (*ne221*)*; cdk-1(RNAi)* worms (Figure 3.14 E and F).

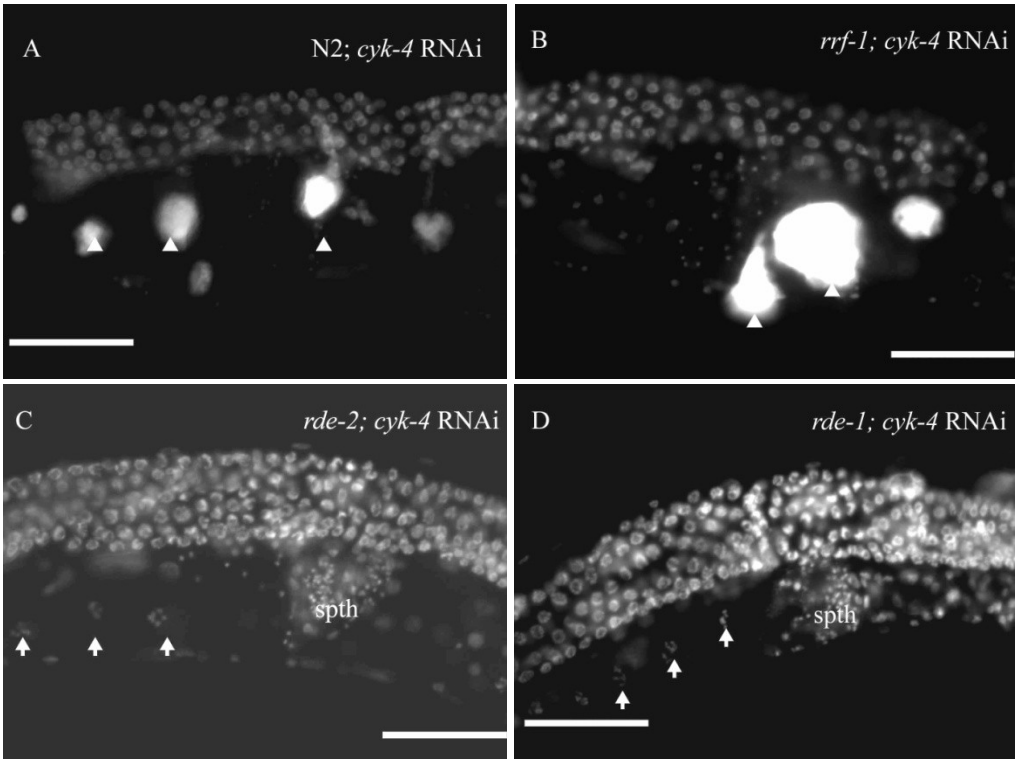

Figure 3.15. Nuclear staining images of *cyk-4* RNAi animals. (A)N2 animal treated with *cyk-4* RNAi showing nuclear accumulation (arrowheads) in the proximal gonad. (B) *rrf-1(pk1417)* animal fed with *cyk-4* RNAi showing enodmitotic oocyte nulcei accumulated in the proximal gonad (arrow heads). (C) DAPI staining of *rde-2(ne221); cyk-4 (RNAi)* showing that *cyk-4* RNAi did not cause emo phenotype in the proximal gonad (arrows). (D) An *rde-1(ne219)* fed with *cyk-4* RNAi showing normal oocytes (arrows) in the proximal gonad. Spth=spermatheca, Scale Bar= 40 μm.

In *C. elegans*, *cyk-4* has been identified as essential for regulating the structure of the mitotic spindle and proper functioning of the contractile ring and in the later stages of cytokinesis (Jantsch-Plunger et al., 2000). Depletion of *cyk-4* caused 100% sterility in wild type (N=60) and in the *rrf-1(pk1417)* mutant background (N=60). In *rde-2(ne221)* worms the sterility is reduced, 12 ± 3.4%, N=60. In the wild type and *rrf-1(pk1417)* background, loss of *cyk-4* caused Emo in the proximal gonad (Figure 3.15 A and 3.15 B). Typical oocytes were observed in the *rde-2(ne221)* and *rde-1(ne219)* background (Figure 3.15 C and 3.17 D).

Genes Encoding for Protein Degradation

Proteasomes are made up of two parts, a 20S protealytic core with α and β subunits essential for proteolysis and a 19S regulatory core with ATPases and non-ATPase subunits, necessary for ubiquitin recognition and de-ubiquitylation processes (Collins and Tansey, 2006). Our screen resulted in identification of proteasome subunits, regulatory non-ATPase subunits and genes encoding for α and β subunits. The preliminary screen resulted in identification of proteasome subunits, both regulatory non-ATPase like *rpn-2* and *rpn-6,* and α and β subunits such as *pas-4* and *pbs-3,* respectively.

pas-4 belongs to the functional class of genes which are responsible for degradation of proteins and encodes a proteasome alpha-type subunit of the core 20S proteasome subcomplex (Kahn et al., 2008). Knockdown of *pas-4* mRNA in N2 and *rrf-1(pk1417)* has caused sterility, 85 ± 3.7%, (N=90) and 58 ± 5.2%, (N=90) respectively. In the *rde-2(ne221)* background, the *pas-4(RNAi)* suppressed sterility (29 ± 9.9 %, N=21). In addition *pas-4* RNAi caused Emo nuclei in accumulated oocytes in N2 (Figure 3.16 A) and *rrf-1(pk1417)* worms (Figure 3.16 B). The *rde-2(ne221); pas-4(RNAi)*

worms suppressed Ste and showed no obvious defects in oocytes, suggests that *pas-4* acts in the germ cells and links to ovulation (Figure 3.16 C).

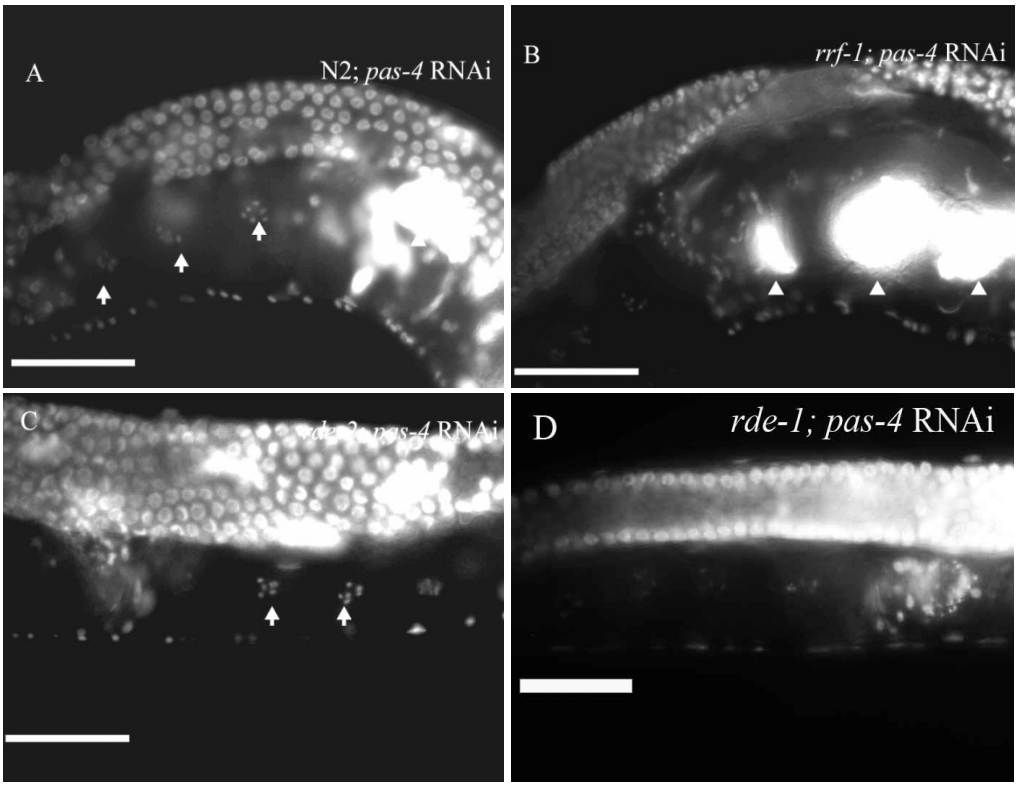

Figure 3.16. Nuclear staining images of *pas-4* RNAi animals. (A)N2 animal treated with *pas-4* RNAi showing nuclear accumulation (arrowheads) in the proximal gonad. (B) *rrf-1(pk1417)* animal fed with *pas-4* RNAi showing Emo oocyte nulcei in the proximal gonad (arrow heads). (C) Nuclear staining of *rde-2(ne221); pas-4 (RNAi)* normal oocytes in the proximal gonad (arrows). (D) An *rde-1(ne219)* fed with *cyk-4* RNAi showing normal oocytes (arrows) in the proximal gonad. Scale Bar= 40 µm.

We also identified *rpt-1* gene, which encodes for ATPase subunit of the 19S proteasome regulatory complex (Davy et al., 2001). RNAi of *rpt-1* in N2 has shown 100% sterility (N=40), while in *rrf-1(pk1417)* it has caused similar degree of sterility, 90 ± 5.4, (N=30). The degree of sterility was suppressed in *rde-2(ne221)* mutants (26.7 ± 6.6 %, N=45). The wild type and *rrf-1(pk1417)* worms subjected to RNAi of *rpt-1*

showed accumulated oocytes in the proximal gonad which underwent endomitosis (Figures 3. 17 A and B). The gonad morphology of *rde-2(ne221); rpt-1(RNAi)* appeared normal, without any defects in the proximal gonad arm (Figure 3.17 C).

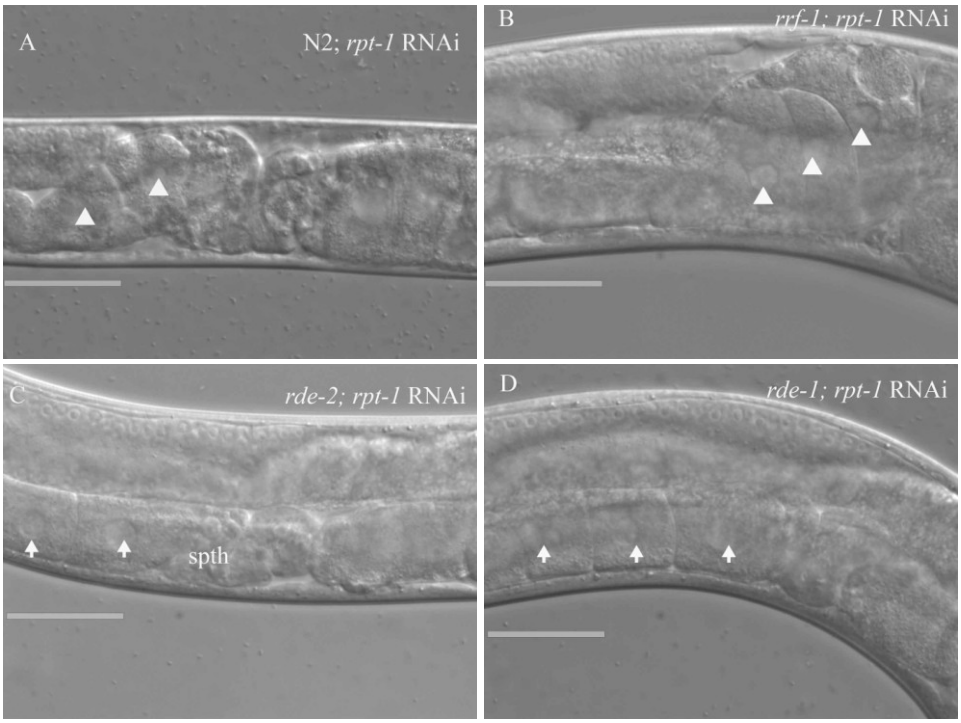

Figure 3.17. DIC images of *rpt-1* RNAi animals. (A) Proximal gonad of N2; *rpt-1 (RNAi)* animal. Arrowheads represent endomitotic oocyte nuclei. (B) DIC image of *rrf-1(pk1417); rpt-1*(RNAi) animal with endomitotic oocyte nuclei (arrow heads). (C) An *rde-2(ne221)* animal fed with *rpt-1* RNAi with normal oocytes in the proximal gonad (arrows) indicating that *rpt-1* RNAi did not cause oocyte accumulation in the proximal gonads (arrows). (D) DIC image of *rde-1(ne219); rpt-1 (RNAi)* with normal oocytes (arrows) in the proximal gonad. spth= spermatheca. Scale bar=40 μm.

Genes Encoding for ATP Synthesis

We have identified two genes, Y55H10A.1/*vha-19* and C23G10.8 that are essential for ATP synthesis. *vha-19*, a member of vacuolar H ATPase encodes for an ortholog of subunit Ac45 (Schoonderwoert and Martens, 2002). Knockdown of *vha-19* resulted in 97 ± 2.1 % sterility in wild type (N=69) and *rrf-1(pk1417)* mutant (74.5 ±

5.6, N=59). The sterility was reduced in *rde-2(ne221)*, (18.3 ± 4.9%, N=30). Nuclear staining has shown the presence of Emo oocytes in the proximal gonad in N2 (Figure 3.18 A and B) and *rrf-1(pk1417)* (Figure 3.18 C and D). DAPI staining of *rde-1* and *rde-2* mutants did not show Emo in the proximal gonad (Figure 3.18 E and F).

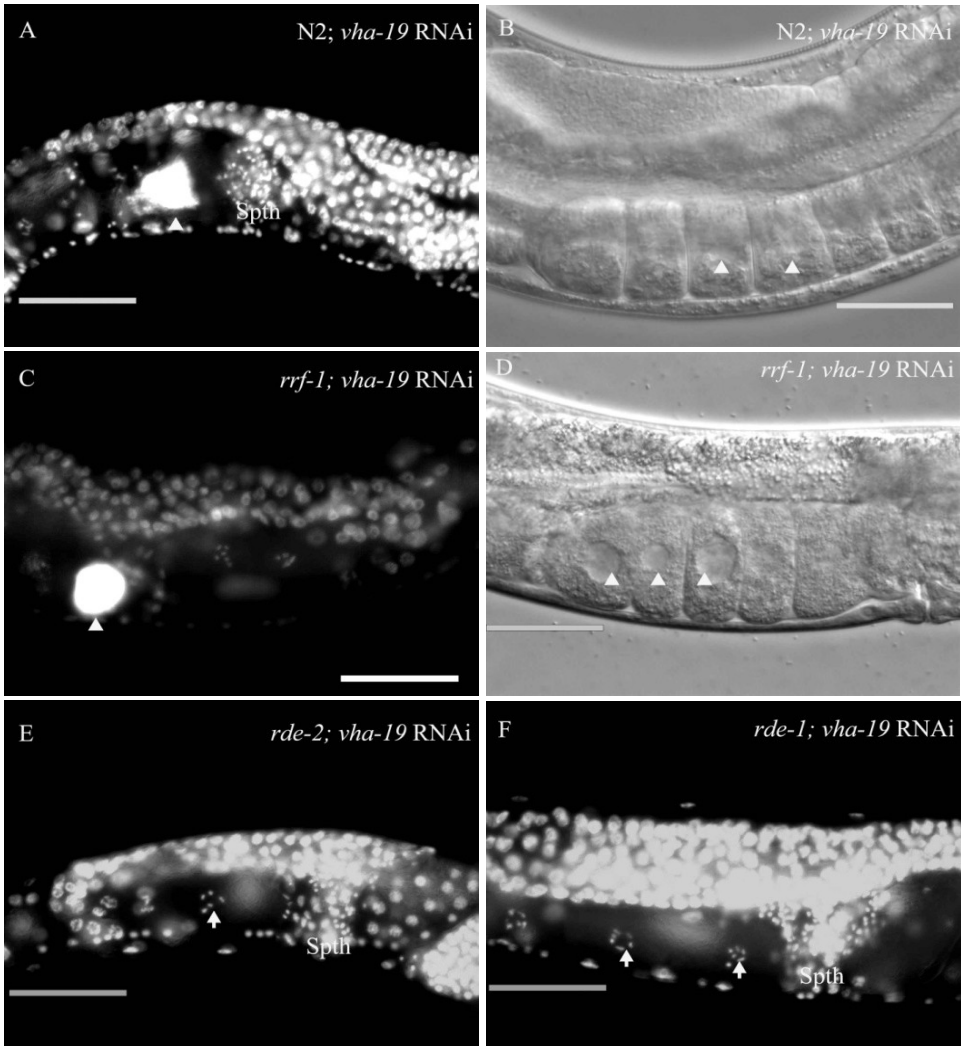

Figure 3.18. Proximal gonads of *vha-19* RNAi animals. (A) DAPI stained image of wild type N2 animal grown on *vha-19(RNAi)* showing Emo oocytes (arrow heads). (B) DIC image of N2 treated with *vha-19(RNAi)*. (C) Image of *rrf-1(pk1417); vha-19(RNAi)* showing Emo oocytes (arrow heads). (D) DIC image of *rrf-1(pk1417); vha-19* showing Emo oocytes (arrow heads). (E) DAPI staining of *rde-2(ne221); vha-19 (RNAi)* showing normal oocytes. (F) An *rde-1(ne219); vha-19* RNAi showing normal oocytes (arrows). Spth, Spermatheca. Scale Bar= 40µm.

C23G10.8 encodes for an uncharacterized protein, which might have a role in ATP synthesis. C23G10.8 RNAi resulted in Ste in the wild type (98.8 ± 1.2, N=90) and in *rrf-1(pk1417)* worms (87.8 ± 3.4, N=90). C23G10.8 RNAi in *rde-2(ne221)* resulted in suppression of sterility (10 ± 5.4 %, N=30), suggesting germline specificity of C23G10.8 for fertility. Figure 3.19 A and B show endomitotic oocytes in the proximal gonads of N2 and *rrf-1(pk1417)* worms respectively. The *rde-2(ne221)* worms show typical oocytes in the proximal gonad (Figure 3.19 C) suggesting a germline specific role for C23G10.8. The *rde-1(ne219)* worms showed normal oocytes in the proximal gonad.

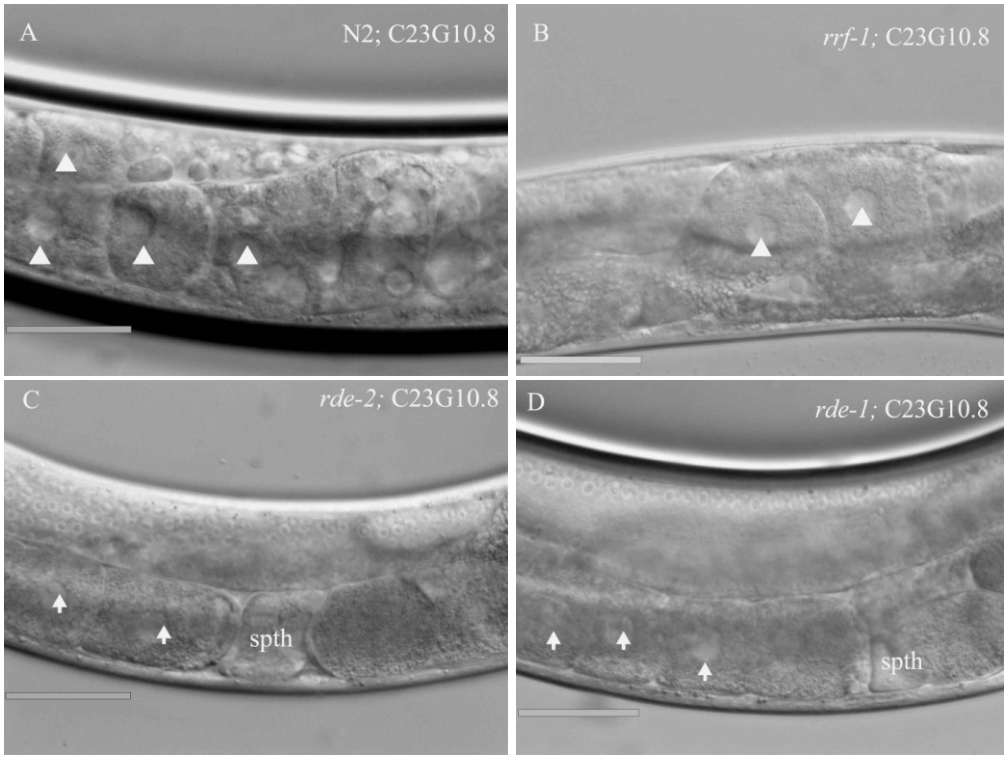

Figure 3.19. DIC images of C23G10.8 RNAi animals. (A) Proximal gonad of N2; C23G10.8 *(RNAi)* animal. Arrowheads represent endomitotic oocyte nuclei. (B) DIC image of *rrf-1(pk1417);* C23G10.8 *(RNAi)* animal with endomitotic oocyte nuclei. (C) An *rde-2(ne221)* animal fed with C23G10.8 RNAi with normal oocytes in the proximal gonad (arrows). (D) DIC image of *rde-1(ne219);* C23G10.8 *(RNAi)* with normal oocytes (arrows) in the proximal gonad. spth= spermatheca. Scale bar=40 µm.

Genes of Unknown Functional Class

R08D7.1 encodes for an uncharacterized protein, similar to splicing factor of pre-mRNA Retention and Splicing (RES) complex in yeast (Dziembowski et al., 2004). RES is an evolutionarily conserved protein that is necessary for intron removal and pre-mRNA retention in the nucleus (Dziembowski et al., 2004). Depletion of R08D7.1 in N2 and *rrf-1(pk1417)* caused 100% sterility, (N=40) and 96.7 ± 8.3%, (N=30), respectively.

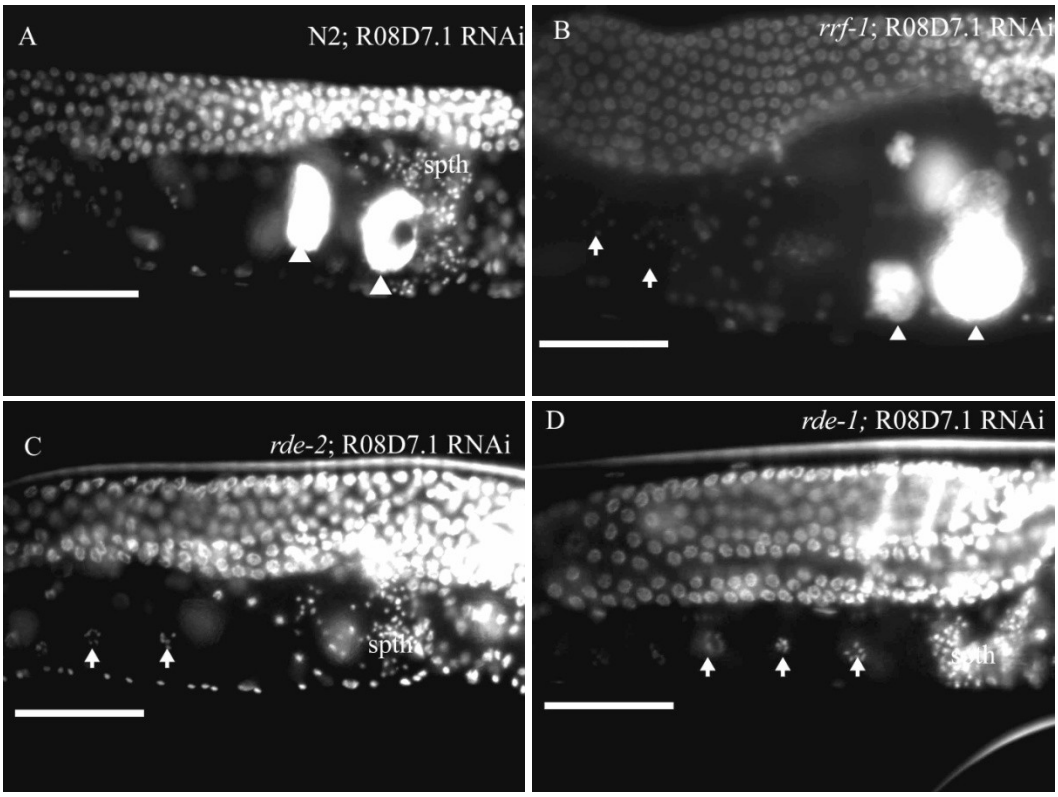

Figure 3.20. Nuclear staining images of R08D7.1 RNAi animals. (A)N2 animal treated with R08D7.1 RNAi showing nuclear accumulation in the proximal gonad denoted by arrowheads. (B) *rrf-1(pk1417)* animal fed with R08D7.1 RNAi showing enodmitotic oocyte nulcei accumulated in the proximal gonad (arrow heads). (C) *rde-2(ne221);* R08D7.1 *(RNAi)* animals showing normal oocytes in the proximal gonad (arrows). (D) An *rde-1(ne219)* ; R08D7.1 RNAi animal showing normal oocytes (arrows) in the proximal gonad. Spth=spermatheca, Scale Bar= 40 μm.

RNAi against R08D7.1 in *rde-2(ne221)* showed reduction of sterility (13 ± 6.2%, N=30). To visualize the germ cell nuclei in the sterile worms, after depletion of the R08D7.1, we examined nuclear morphology of RNAi animals. RNAi against R08D7.1 caused Emo in N2 and in *rrf-1(pk1417)* (Figure 3.20 A and B, respectively), while the *rde-2(ne221)* (Figure 3.20 C) and control RNAi group, *rde-1(ne219)* (Figure 3.20 D) appeared normal.

In summary, we have identified 20 genes that cause defects in gonad morphology resulting in ovulation defects, and act on the germ line exclusively. In all cases, loss of function of these genes has caused Emo in N2 and *rrf-1(pk1417)*, with rescue of normal ovulation in *rde-2(ne221)* confirming germline specificity.

Genetic Interaction Map of Potentially Germline Specific 69 Genes

The 69 fertility genes, potentially germ line specific, share similar RNAi phenotypes with each other and might show gene interactions. The fertility genes were surveyed for interacting genes in *C. elegans* database containing genetic interactions among *C elegans* genes (Zhong and Sternberg, 2006). A protein network program, Pajek (Batagelj, 1998), was used to assemble a network of interacting genes (Figure 3.21) from the *C. elegans* genetic interaction database . Green nodes represent 61 of the 69 fertility genes that showed the interactions and red nodes in the network are the germ cell specific genes determined later in our study. For identities of all nodes, see Appendix B. Genetic interaction network showed that the 89% of the 69 fertility genes are genetically linked to each other and form a set that may work together to control ovulation.

To identify the fertility genes exclusively expressed in germ cells, we simultaneously rescreened the 69 genes in a germline RNAi resistant background. A mutation in *rde-2(ne221)* appeared to inhibit the onset of RNAi in germ cells (Tabara et

al., 1999). For example, a fertile phenotype of an otherwise sterile RNAi in *rde-2(ne221)* means the germline specificity of the gene interfered. By this method, the 20 red nodes were determined as genes expressed in germline and controlling fertility. These genes were scattered in the cluster and orphan group with 8 genes. However, these germ cell specific 20 genes do not include ribosomal proteins and are instead genes interacting with or regulating the ribosomal proteins, suggesting that the germline fertility genes are factors regulating the function of ribosomal proteins.

Green and red nodes clustered by complex interactions are functionally related or involved in cellular functions. For example, ribosomal protein genes are interconnected together and form a 17-gene cluster, implying that ribosomal subunits are a major factor in fertility. The ribosomal encoding genes form a cluster and are linked to genes belonging to other functional classes also. Inside the ribosomal cluster we found 3 genes with a familiar role in ribosome synthesis. The *bys*-like gene in yeast, *enp-1*, is part of 90S pre-ribosomal complex and associates with 20S pre-rRNA, and essential for pre-rRNA splicing and ribosome biogenesis (Stewart and Nordquist, 2005). Another gene *fib-1,* a *C. elegans* homolog of the yeast Nop1p, is essential for 18S rRNA processing in embryos (Saijou et al., 2004). Another gene which is connected to the map is *ubl-1*, which encodes for a protein similar to Drosophila ubiquitin/ ribosomal protein S27a.

The gene interaction map identified a cluster of genes, showing the interaction between genes encoding for large and small ribosomal subunits. Studies have shown that ribosomal genes are expressed four fold higher in the germline compared to soma (Wang et al., 2009). Ribosomal protein synthesis can be regulated at various stages based on the requirement during crucial stages of cellular development. Cell growth

depends on protein synthesis that in turn depends on production of ribosome in the cell (Rudra and Warner, 2004). Reduction of ribosomal proteins of a certain subunit will lead to decrease in the different proteins from the same subunit (Robledo et al., 2008).

Identification of ribosomal genes for germ line specificity indicates the role of post-transcriptional regulation in the development of germline (Updike and Strome, 2009). The ribosomal genes form a cluster and are linked to genes belonging to other functional classes also. Defective ribosome synthesis causes disruptions in the development of somatic gonad and alterations to the soma-germline interaction (Voutev et al., 2006).

This ribosomal protein cluster was then directly linked to the 14 genes such as F01F8.1a/*cct-6*, CD4.6/*pas-6*, C36B1.4/*pas-4*, H28O16.1a/*phi-37*, ZK632.1a/*mcm-6*, K01E5.4/*ran-1*, T05G5.3/*cdk-1*(Boxem et al., 1999), C52E4.4/*rpt-1*, F20B6.2/*vha-12* (Kontani et al., 2005), C34E10.6/*atp-2*, F09F7.3, C26E6.4/*rpb-2,* ZK328.2/*eft-1,* and F25H5.2/*eft-2*. Although these genes are directly interacting with ribosomal proteins, their annotated functions were not limited to protein synthesis. We identified two genes encoding for elongation factor like proteins. F25H5.2/*eft-2* (Ofulue and Candido, 1991) and ZK328.2/*eft-1* (Ofulue and Candido, 1992) are essential for protein synthesis and should maintain the interaction with ribosome. C26E6.4/*rpb-2* (Updike and Strome, 2009) and F09F7.3 are RNA polymerases presumably involved in making mRNA that is another essential factor for protein synthesis. However, there are genes linked to other functions; C52E4.4/*rpt-1*, C36B1.4/*pas-4*, and CD6.4/*pas-6* are the components of proteasome. F20B6.2/*vha-12*, C34E10.6/*atp-2* and H28O16.1a/*phi*-37 genes showed involvement in ATP production meaning that the decrease in energy production is

detrimental in fertility. The chaperonin gene, *cct-6,* encodes two isoforms of a putative zeta subunit of the eukaryotic cytosolic ('T complex') chaperonin (Leroux and Candido, 1995). We identified *mcm-6*, a DNA replication factor gene (Updike and Strome, 2009). T05G5.3/*cdk-1* is involved with cell division. However, genetic interaction data and our results (Table 3.3) suggested that CDK-1 might be important in regulation of protein synthesis during ovulation. CDK-1 might activate multiple targets during cell division, potentially in germ cells. We also identified a member of the Ran family, *ran-1,* as part of this cluster of genes. Genes encoding for Ran family are essential for the formation of spindle and nuclear envelope after cell division in the embryos (Askjaer et al., 2002).

These 14 gene direct linkers to ribosomal proteins appeared to interact with other 23 genes such as C07G2.3a/*cct-5*, T05C12.7/*cct-1*, C39F7.4/*rab-1*, Y38A8.2/*pbs-3*, F25H2.9/*pas-5*, F39H11.5/*pbs-7,* C23G10.4a/*rpn-2*, F59B9.10a/*rpn-6,* F23F1.8a/*rpt-4,* F58A4.3/*hcp-3*, Y55H10A.1/*vha-19*, T20G5.1/*chc-1*, C47E12.5/*uba-1* , F12F6.6/*sec-24.1*, T10F2.4/unknown, C23G10.8/unknown, C50C3.6/*prp-8,* ZK328.5/*npp-10* , Y116A8C.35/*uaf-2*, R07E5.14/*rnp-4*, T27F2.1/*skp-1*, T08A11.2/*phi-11* and R144.2a/*pcf-11*. The 23 genes can be potentially grouped into 6 different functional classes. The 6 functional groups are 1) protein synthesis, 2) RNA synthesis and binding, 3) protein transport and modification, 4) ATP synthesis, 5) protein degradation and 6) gene expression regulation. This second group were also involved in similar functions such as protein degradation (*pas-5, pbs-3* and *7,* and *rpn-2, 6,rpt-4*), protein modification and trafficking (*cct-1*, *chc-1*, *npp-10* and *sec-24.1*), RNA binding (*prp-8*, *phi-11*, *pcf-11, uaf-2*, and T10F2.4), gene expression controls (*rnp-4*, *hcp-3*, and *skp-1*), and ATP synthesis (Y55H10A.1/*vha-19* and C23G10.8). C47E12.5/*uba-1* encodes for the E1 ubiquitin-

activating enzyme in *C. elegans* (Kipreos, 2005). R144.2/*pcf-11* encodes for mRNA cleavage and polyadenylation factor I/II complex (Cui et al., 2008). *npp-10* encodes for a gene orthologous to the human nucleoporin gene (Galy et al., 2003). C39F7.4/*rab-1* is a protein involved in vesicle transport or cell architecture (Poteryaev et al., 2005). The Rab family of GTPases is responsible for vesicular transport (Sato et al., 2006) and RAB-1 may regulate the delivery of proteins. Functions appeared similar to the directly linked group but genes involved in protein synthesis were missing in this group. We identified both ATPase (*rpt-4*) and non-ATPase (*rpn-6*) subunits of the 19S regulatory complex of the proteasome (Kahn et al., 2008). Among the 23 genes showing direct or indirect interactions, 6 protein degradation genes were involved in fertility, suggesting that proper disposal of unwanted proteins are necessary for fertility.

We found 5 genes on the outlier of the interaction map. Residual interacting genes were K08E3.6/*cyk-4,* T19B4.2/*npp-7* and F57B9.2/*let-711*; they are linked to *rpn-2* and appeared to function as cytokinesis and mitotic spindle assembly (Jantsch-Plunger et al., 2000), nuclear pore protein and transcription factor, respectively. *cyk-4* was also linked to *pcf-11*, suggesting that germline factor stimulating cell movement is important for the fertility. Another 2 residual genes identified were F43D9.3/*phi-26* and K02D10.5/*snap-29*. *phi-26* encodes a vesicle trafficking protein Sly1 (Alper et al., 2008) whereas *snap-29* (Soluble NSF attachment protein homolog) is a component of SNARE complex (Harris et al., 2010).

We also found 8 orphan genes which showed no interaction with the 61 genes. Although we failed to link the 8 orphans to the interaction map, their predicted functions in other species might be used to link these genes to the cluster. Out of these 8 genes, we

have identified T23G5.1/*rnr-1*, K12H4.4/*phi-20*, and R08D7.1 which are double positive for germline specificity. *phi-20* appeared to involve in protein trafficking and folding while T23G5.1/*rnr-1*, encodes for ribonucleotide reductase (Mori et al., 2008) and R08D7.1 is similar to pre-mRNA-splicing-factor of RES complex in yeast and *C. elegans* (Dziembowski *et al.,* 2004). *rnr-1* and T12A2.7 can be grouped into RNA regulation. T12A2.7 encodes for a spliceosome-associated coiled core protein (Harris et al., 2010) and the gene *par-2* encodes a protein containing a specialized type of zinc binding motif (Levitan et al., 1994). ATP synthase subunits like *atp-4*, encoding for mitochondrial F0-F1 ATP synthase was not linked to the main interaction map. Also we also found three genes involved in structural functions, laminin/*lam-2* (Kao et al., 2006), RING-finger/*par-2* (Boyd et al., 1996), and ligand-gated ion channel/*lgc-46*, suggesting that these proteins may play important roles in gonad formation and functions; *lam-2* is an ECM protein for gonad morphogenesis (Kao et al., 2006).

Although many types of genes participate in fertility, the protein synthesis, degradation, and transport categories constitute the most abundant class, suggesting that cell functions during ovulation require active and vigorous use of protein regulation machineries. Some of the genes isolated by our RNAi screen may indirectly affect fertility; for example, depletion of ATP production genes may have broad effects on gene expression. In conclusion, fertility genes appeared germline specific in this study can be categorized into at least 6 functional groups such as 1) protein synthesis, 2) Nucleic acid synthesis, processing and binding, 3) protein transport and modification, 4) ATP synthesis, 5) protein degradation and 6) gene expression regulation. We can assume that each of these classes represents a mechanism that regulates ovulation in *C. elegans*.

Table 3.4 List of ribosomal subunit encoding genes in germline. N= Number of animals. Each data entry represents at least the initial phenotypic scorings.

Predicted gene	Locus	Description	Reference	% Sterile (N)			
				N2	*rrf-1 (pk1417)*	*rde-2 (ne221)*	*rde-1 (ne219)*
D1007.6	*rps-10*	Ribosomal subunit 60S small S10 protein	Wormbase	97± 1.7(79)	80± 7.3(30)	40± 8.9(30)	0(10)
Y48G8AL.8a	*rpl-17*	Ribosomal subunit 60S large S17 protein	Wormbase	81.6± 5(60)	97± 3(30)	50± 9.1(30)	0(30)
F40F8.10	*rps-9*	Ribosomal subunit 40S small S9 protein	Wormbase	100 (40)	100(30)	100(30)	0(30)
F54C9.5	*rpl-5*	Ribosomal subunit 60S large S5 protein	Wormbase	100(90)	75 ± 4.1(110)	100(30)	0(30)
C14B9.7	*rpl-21*	Ribosomal subunit 60S large S21 protein	Wormbase	100(59)	78 ± 5.3(60)	100(30)	0(10)
R151.3	*rpl-6*	Ribosomal subunit 60S large S6 protein	Wormbase	100(30)	100(30)	90 ± 5.4(30)	0(10)
Y42D9A.4a	*rpl-7A*	Ribosomal subunit 60S large S7 protein	Wormbase	100(70)	83 ± 6.8(30)	100(30)	0(30)
F37C12.4	*rpl-36*	Ribosomal subunit 60S large S36 protein	Wormbase	87.5 ± 5(40)	87 ± 6.2(30)	43 ± 9(30)	0(10)
R13A5.8	*rpl-9*	Ribosomal subunit 60S large S9 protein	Wormbase	87± 4.3(60)	100(30)	47± 9.1(30)	0(10)

Table 3.4. continued

C27A2.2a	*rpl-22*	Ribosomal subunit 60S large S22 protein	Wormbase	50± 6.5(60)	87 ± 6.2(30)	40± 8.9(30)	0(30)
F56F3.5	*rps-1*	Ribosomal subunit 40S small S1 protein	Wormbase	100(40)	100(30)	100(30)	0(10)
C23G10.3	*rps-3*	Ribosomal subunit 40S small S3 protein	Wormbase	100(60)	83.3± 4.8(60)	100(60)	0(30)
F54E7.2	*rps-12*	Ribosomal subunit 40S small S12 protein	Wormbase	100(30)	97± 3(30)	100(30)	0(10)
F42C5.8	*rps-8*	Ribosomal subunit 40S small S8 protein	Wormbase	74± 6.2(50)	64± 6.5(30)	80± 8(25)	0(30)

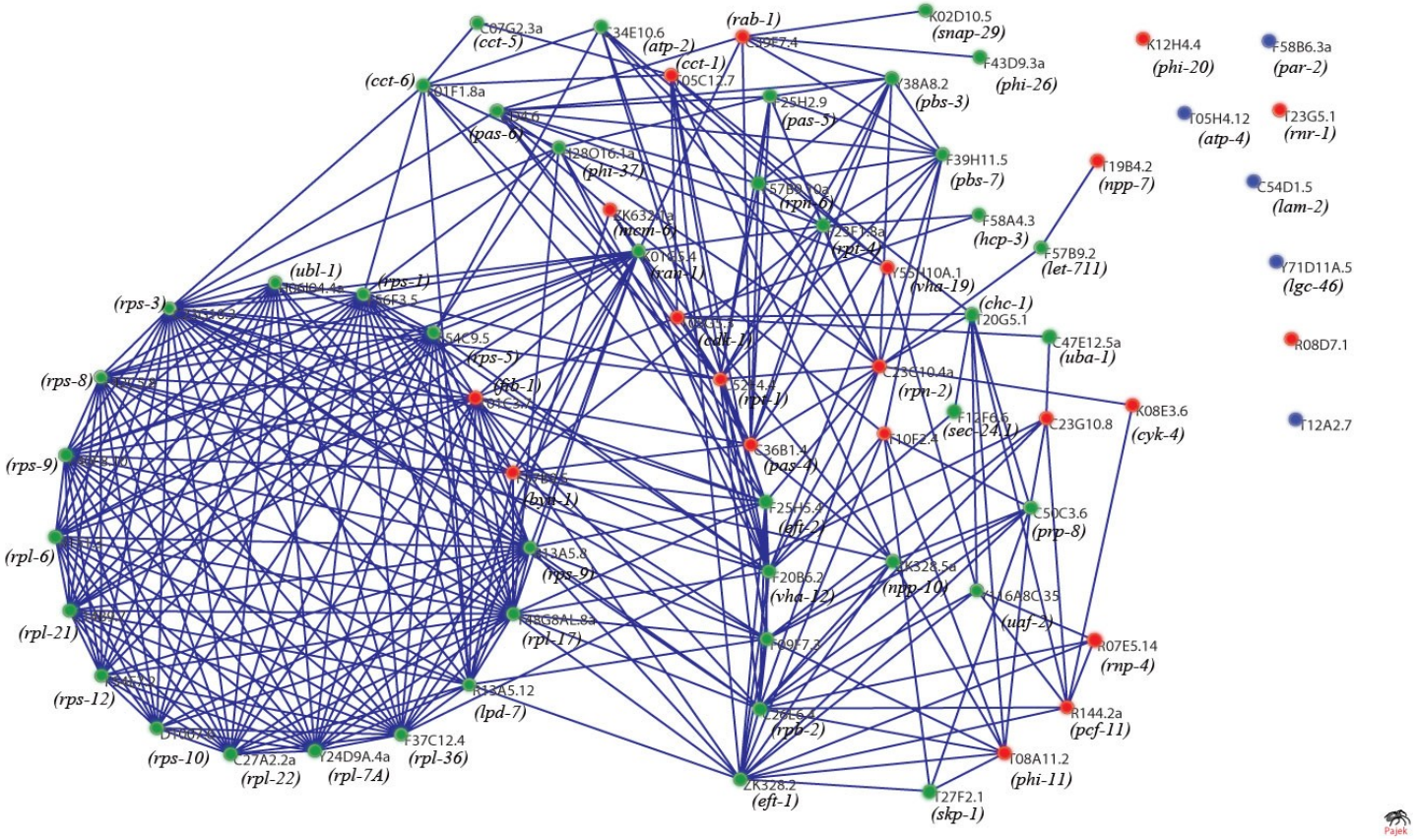

Figure 3.21. Gene interaction map for germline specific genes. The genetic interaction map of germ line specific genes identified from the preliminary screen. Of the 69 genes, 61 exhibited a genetic interaction based on the data from wormbase.org. The gene loci indicated in red represent the genes characterized as double-positive for germline specificity. Identities of the germline specific genes: (1) *npp-7*, (2) *cyk-4,* (3) *rpn-2,* (4) *cdk-1*, (5) *rpt-1*, (6) *cct-1*, (7) *rnp-4*, (8) *phi-11*, (9) *fib-1*, (10) *byn-1*, (11) *pas-4*, (12) *rab-1*, (13) *pcf-11*, (14) C23G10.8, (15) *phi-20*, (16) *rnr-1*, (17) *mcm-6*, (18) R08D7.1, (19) *vha-19* and (20) T10F2.4. The green nodes indicate genes which did not suppress sterility in germline RNAi sensitive background. Blue nodes indicate genes with no interaction.

Genes Essential for Ovulation in Somatic Cells.

I have focused on the 259 genes for preliminary and further studies and decided to conduct RNAi screen on Ste causing genes in N2 background. To confirm if these genes function in somatic cells, we used *rrf-1(pk1417)* mutant to conduct the RNAi along with wild type worms. *rrf-1(pk1417)* mutant encodes an RNA dependent RNA polymerase (RdRp) homologue which is necessary for RNAi in somatic cells (Sijen et al., 2001). If the Ste phenotype is seen in *rrf-1(pk1417)* it would suggest that the gene functions in germline whereas reduction in Ste phenotype would indicate a somatic specificity for the gene.

To define whether these 32 genes are double positive for somatic specificity, we reduced the mRNA of the genes by RNAi in the *rde-2(ne221)* mutant background. RDE-2 is not essential for dsRNA targeting in somatic genes but essential for RNAi against genes expressed in germline (Tabara et al., 1999) . As a control we used the *rde-1(ne219)* mutant background to check for negative regulation. *rde-1(ne219)* is resistant to RNAi in both somatic and germline cells and should not show any sterility (Tabara et al., 1999).

Those genes that have caused Ste phenotype in wild type background but show significantly reduced Ste phenotype (more than threefold decrease) in *rrf-1 (pk1417)* background (Table 3.5). Of these 32 genes (Appendix C), 5 belong to protein synthesis, 3 genes encode RNA synthesis, 5 genes which encode for cell architecture, 6 are involved in signaling pathways, 3 genes in degradation, 2 genes coding for metabolic pathways, 2 genes encoding for neuro and nucleic acid binding functional classes and 6 genes whose functional class is yet to be determined.

Table 3.5 List of genes which resulted in more than 60% sterility in the wild type and *rde-2(ne221)* backgrounds, but greatly reduced sterility in *rrf-1(pk1417)* background. N= Number of animals. Each data entry represents at least the initial phenotypic scorings.

Predicted gene	Locus	Description	Reference	% Sterile (N)			
				N2	*rrf-1 (pk1417)*	*rde-2 (ne221)*	*rde-1 (ne219)*
F54C1.7	*pat-10*	Calcium binding troponin C	Terami 1999	97 ± 2.2(40)	17 ± 6.8(30)	100 (30)	0(10)
Y71G12B.11a	*Talin*	Cell migration and contractility	Moulder 1996	97 ± 2.2(60)	17 ± 4.8(60)	67 ± 8.6(30)	0(30)
Y111B2A.14	*pqn-80*	Prion-like-(Q/N)-domain-bearing protein	Wormbase	100 (120)	40 ± 4.5(118)	100 (30)	0(30)
F38A1.8		Located in muscles and nerves	Wormbase	100(90)	21 ± 4.2(90)	63 ± 8.8(30)	0(30)
F31b12.1a	*plc-1*	Phospholipase C	Yin 2004	80± 4.7(70)	13.3 ± 4.3(60)	70 ± 8.3(30)	0(10)
Y49A3.A2	*vha-13*	Vacuolar H-ATPase subunit A	Syntichaki 2005	100(60)	10 ± 5.4(30)	86.7 ± 4(60)	0(10)
F33D4.2a	*itr-1*	inositol triphosphate receptor	Clandinin 1998	100(72)	30 ± 5.8(72)	70 ± 8.3(30)	0(10)
K08C7.3	*epi-1*	Laminin alpha chain	Kao 2006	100(30)	0(30)	80 ± 7.3(30)	0(10)
W03F8.1	*lam-1*	Laminin beta subunit	Kao 2006	83± 6.8(30)	0(30)	67 ± 8.6(30)	0(10)

Genes Essential for Signaling

There are four members of the laminin family in *C. elegans*, two alpha subunits, one beta and one gamma subunit of which *epi-1* encodes for αB subunit of laminin (Kao et al., 2006). Laminin subunits are essential for cell architecture which makes up the extracellular matrix, cytoskeletal structure and help in regulating signals between adjacent tissues (Huang et al., 2003). Laminin α subunits are located between primary tissue layers and cause abnormal cell-cell adhesions, defects in cell migration when mutated (Huang et al., 2003). Depletion of *epi-1* has caused 100 % sterility (N=30) in wild type but reduced sterility in *rrf-1* mutant background (17 ± 4.8%, N= 60). The *rde-2(ne221); epi-1(RNAi)* worms showed higher degree of sterility, (80 ± 4.8%, N= 30) compared to the *rrf-1(pk1417)* worms. The sterile worms when examined using DIC microscopy and nuclear staining, showed the presence of Emo oocytes in the wild type (Figure 3.22 A and B) and *rde-2(ne221)* background (Figure 3.22 C and D). The *rrf-1(pk1417); epi-1* animals displayed normal oocytes in the proximal gonad (Figure 3.22 E and F). The presence of nuclear staining in the oocytes indicates the failure of oocytes to enter the spermatheca for fertilization.

Inositol 1, 4, 5-trisphosphate (IP_3) is a secondary messenger that plays a role in major cellular processes and *itr-1* gene encodes for an IP_3 receptor (IP_3R). The IP_3R are expressed in the intestine, pharynx, nerve ring, excretory canal and gonad (Baylis et al., 1999). *itr-1* is involved in the normal fertility and plays an important role in the ovulation process (Clandinin et al., 1998). IP_3 diffuses through the cytosol and releases Ca^{2+} by opening the IP_3 gated Ca^{2+}-channel, IP_3 receptors (IP_3 R) on ER (Walker et al., 2002).Ca^{2+} release from ER is required for the contractile activity of the sheath cells

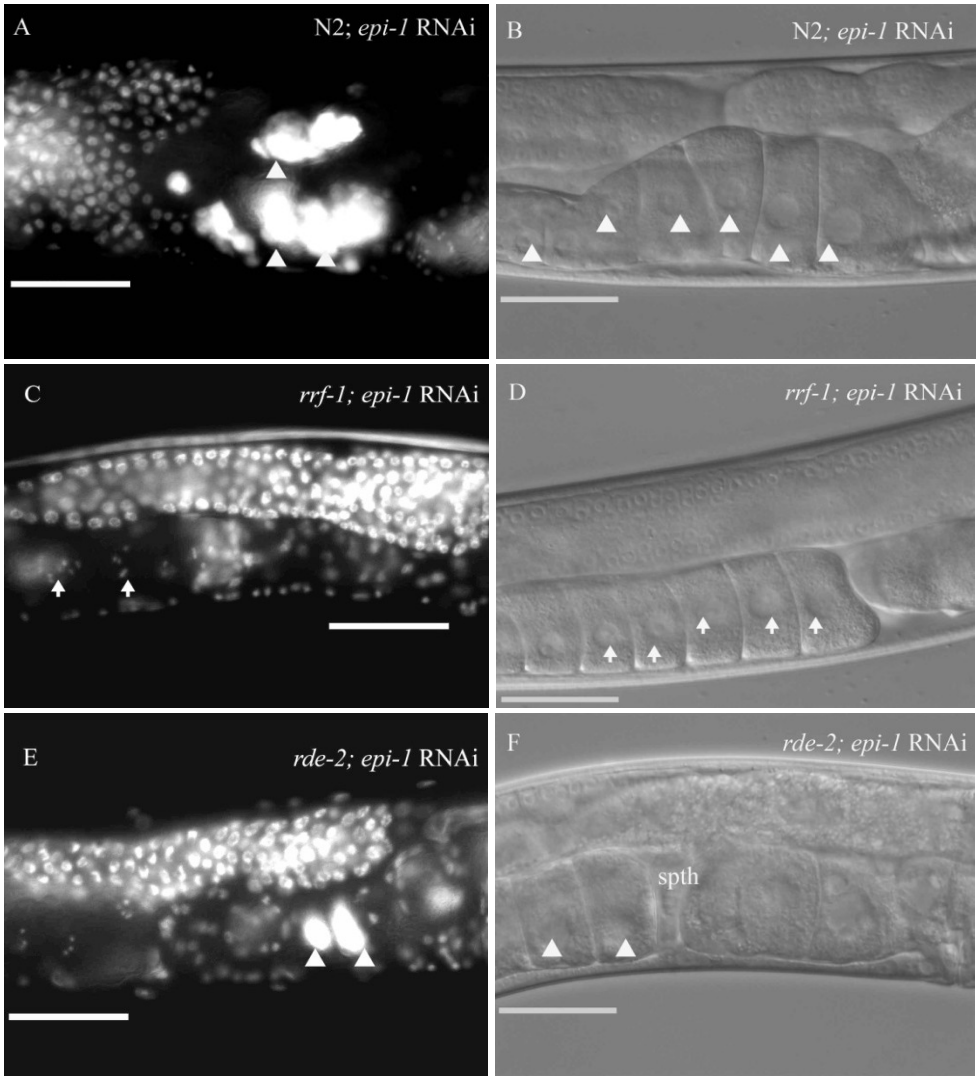

Figure 3.22. Proximal gonads of *epi-1* RNAi animals. (A) DAPI staining of N2; *epi-1* RNAi showing accumulation of nuclear material (Arrow heads). (B) DIC image of wild type animals subjected to *epi-1* RNAi showing endomitotic oocytes in the proximal gonad (Arrow heads). (C) Proximal gonad arm of *rrf-1(pk1417); epi-1(RNAi)* animal showing normal oocytes (Arrow heads). (D) *rrf-1(pk1417); epi-1(RNAi)* animal (DIC image). Arrow heads indicate normal oocytes. (E) DAPI staining of *rde-2(ne221); epi-1(RNAi)* worms showing nuclear accumulation in oocytes in the proximal gonad (Arrow heads). (F) DIC image of *rde-2(ne221); epi-1(RNAi)* showing endomitoticaly replicating oocytes, denoted by arrowheads. Spth denotes spermatheca. Scale bar=40 μm.

during ovulation (Yin *et al.*, 2004). Depletion of *itr-1* or loss-of-function mutations led to the loss of sheath contractile activity (Yin et al., 2004). Knockdown of *itr-1* in N2 and *rrf-1(pk1417)* caused 80 ± 4.7%, (N= 70) and 13.3 ± 4.3%, (N= 60), respectively.In the

rde-2(ne221) background, sterility was 70 ± 8.3%, (N= 30), which indicates that *itr-1* depletion causes ovulation defects due to somatic cell specificity. Nuclear staining and DIC microscopy showed Emo oocytes in N2 (Figure 3.23 A and B) and *rde-2(ne221)* (Figure 3.23 C and D) and normal oocytes in *rrf-1(pk1417)* mutant (Figure 3.23 E and F).

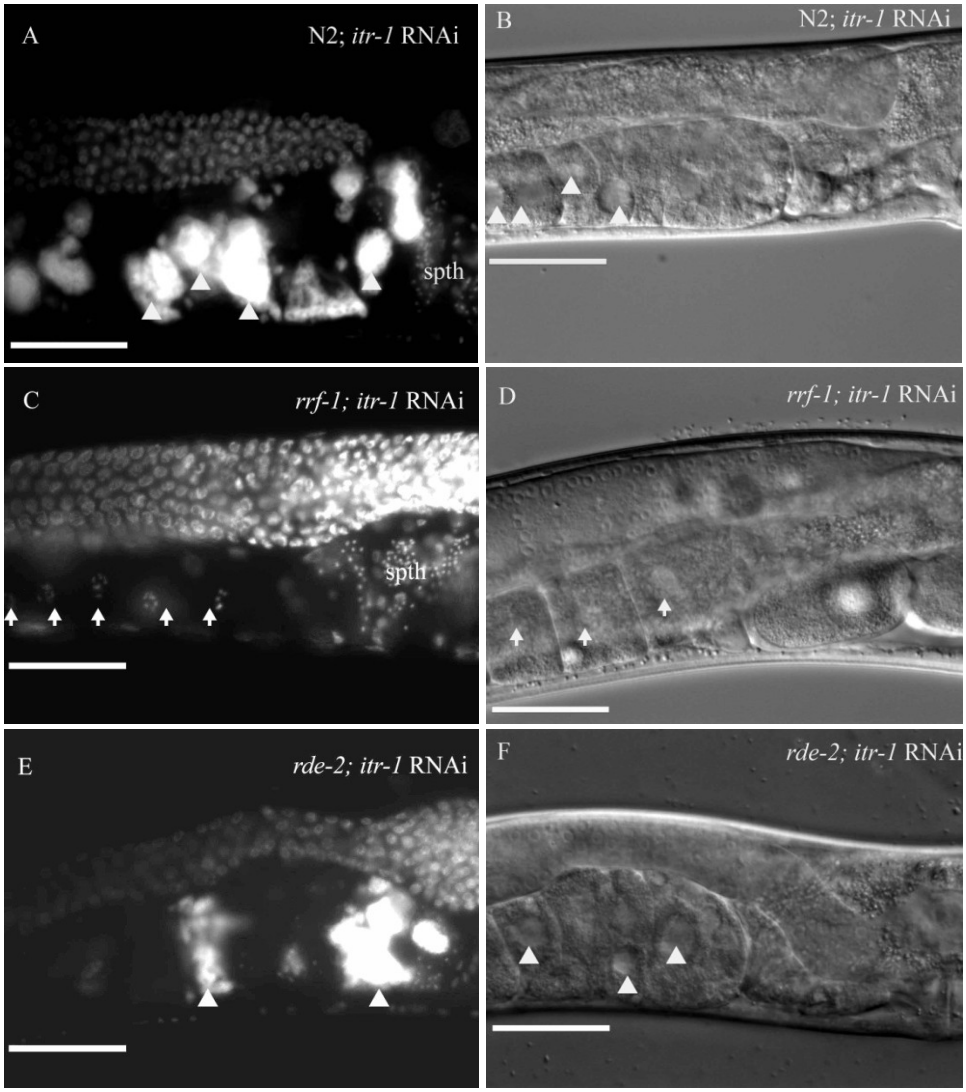

Figure 3.23. Proximal gonads of worms depleted of *itr-1*. (A) N2 animal showing Emo oocytes (arrowheads) after *itr-1* feeding RNAi. (B) Proximal gonad of N2; *itr-1 (RNAi)* animal. (C) An *rrf-1(pk1417); itr-1 (RNAi)* animal showing normal oocytes (arrows). (D) DIC image of *rrf-1(pk1417); itr-1* RNAi animal. (E) An *rde-2(ne221)* animal fed with *itr-1* RNAi with Emo oocytes in the proximal gonad. (F) DIC image of *rde-2(ne221); itr-1 (RNAi)* with Emo oocytes. spth= spermatheca. Scale bar=40 µm.

Phosphatidylinositol 4, 5-bisphosphate (PI-4, 5-P_2) is hydrolyzed by phospholipase C (PLC) and generates inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). In *C. elegans*, 6 genes were predicted to encode phospholipase-C (PLC), which is responsible for the hydrolysis of PIP_2 to IP_3 (Yin *et al.*, 2004). In *C. elegans*, the *plc-1* gene encodes for phospholipase Cε, is expressed in the spermatheca in the early adult stage, and essential for ovulation process (Kariya et al., 2004). Our RNAi screen showed that in wild type and *rde-2(ne221)* worms depleted of *plc-1,* displayed sterility 80 ± 4.7%, (N=70) and 70 ± 8.3%, (N=30), respectively. The sterility in *rrf-1(pk1417)* was significantly reduced, 13.3 ± 4.3%, (N=60). Upon nuclear staining and DIC microscopy, we observed Emo oocytes in the proximal gonad of N2 and *rde-2(ne221)* (Figure 3.24 A, B, E and F). The *rrf-1(pk1417); plc-1(RNAi)* worms showed normal oocytes in the proximal gonad (Figure 3.24 C and D).

Cell Architecture Genes

The cytoskeletal structure in cells is made up of microfilaments and microtubules that are necessary for maintaining the cell motility, architecture and intracellular trafficking. *C. elegans* has two α subunits, one β and one γ that are required for cell adhesions and cell signaling (Kao et al., 2006). Laminin functions in the tissue in the form of heterotrimer model (Kao et al., 2006). Laminin β subunit is encoded by *lam*-1, is expressed between the germ layers and present in all basement membranes (Kao et al., 2006). Loss of *lam-1* gene in wild type caused Ste phenotype (83 ± 6.8%, N= 30) but *rrf-1(pk1417)* mutant has shown suppression of Ste phenotype completely (0%, N= 30). The Ste phenotype in *rde-2(ne221); lam-1(RNAi)* was 67 ± 8.6%, (N=30). The N2; *lam-*

1(RNAi) and *rde-2(ne221); lam-1(RNAi)* worms showed Emo phenotype (Figures 3.25 A, B, E and F). *rrf-1(pk1417); lam-1(RNAi)* displayed normal oocytes (Figure 3.25 C & D).

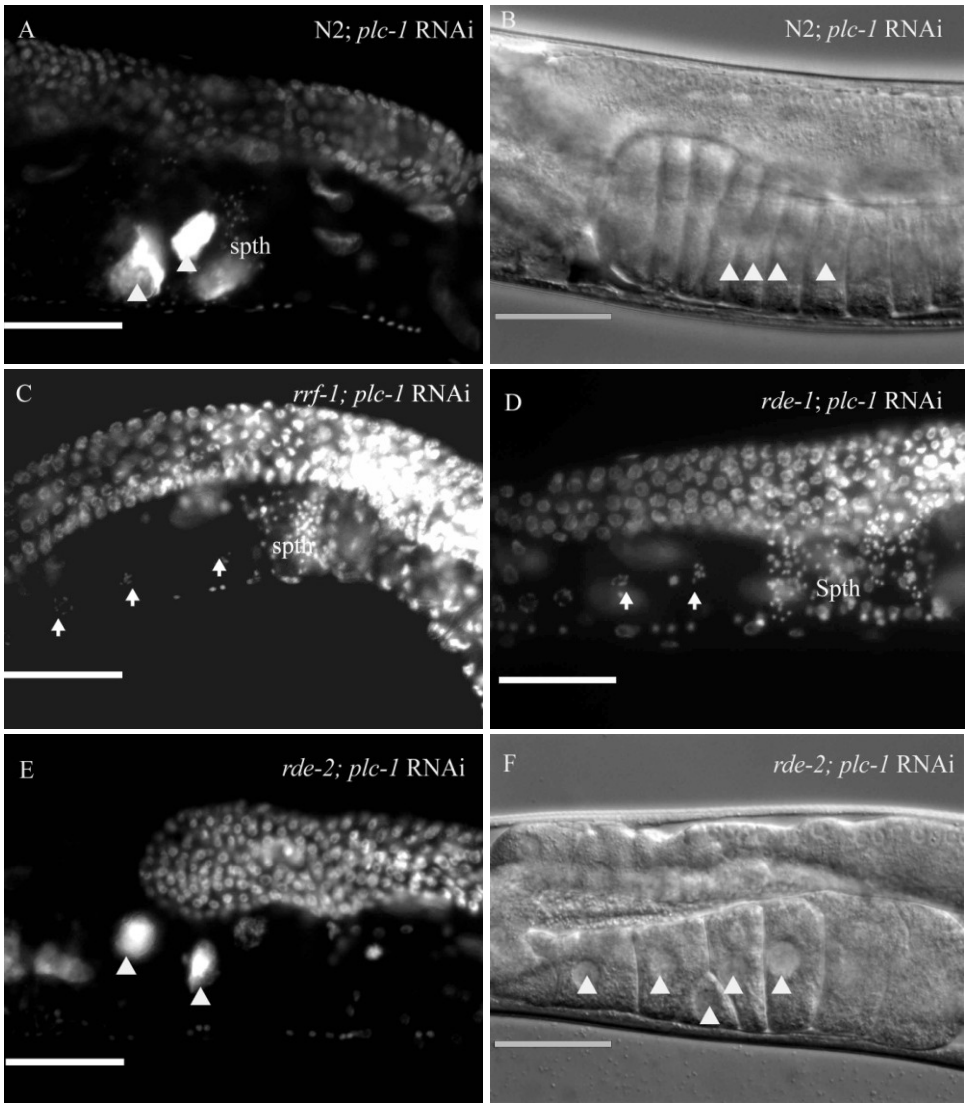

Figure 3.24. Proximal gonads of *plc-1* RNAi animals. (A) Nuclear staining of wild type N2 animal fed with *plc-1* RNAi showing accumulated oocytes in the proximal gonad,. (B) DIC image of N2; *plc-1 (RNAi)* animal showing endomitotic oocyte nuclei in the proximal gonad. (C) DAPI staining of *rrf-1(pk1417)* animal treated with *plc-1* RNAi showing normal oocytes in the proximal gonad. (D) DAPI staining of *rde-1(ne219); plc-1* worms showing normal oocytes in the proximal gonad. (E) An *rde-2(ne221)* animal treated with *plc-1* RNAI showing endomitotic oocyte nuclei in the proximal gonad. (F) DIC image of *rde-2(ne221); plc-1(RNAi)* animal with endomitotic oocytes nuclei in the proximal gonad. Spth denotes spermatheca. Arrowheads represent endomitotic oocytes and arrows represent normal oocytes Scale bar=40 μm.

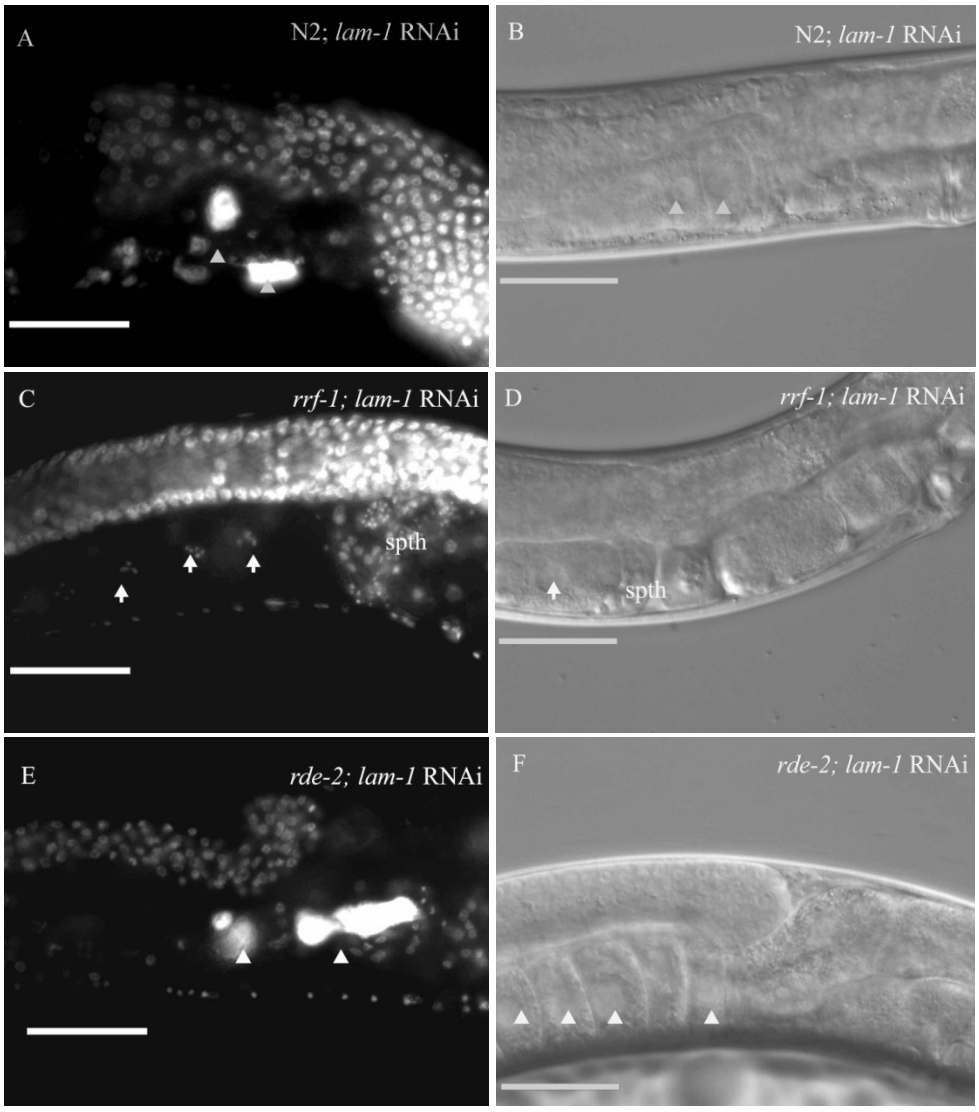

Figure 3.25. Proximal gonads of *lam-1* RNAi animals. (A) Nuclear staining of N2; *lam-1* RNAi showing accumulation of nuclear material (Arrow heads). (B) DIC image of N2; *lam-1* RNAi showing Emo oocytes in the proximal gonad (Arrow heads). (C) Nuclear staining of *rrf-1(pk1417); lam1 (RNAi)* worms showing normal oocytes in the proximal gonad (Arrows). (D) DIC image of *rrf-1(pk1417); lam1 (RNAi)* showing normal oocytes. (E) Proximal gonad arm of *rde-2(ne221); lam-1(RNAi)* animal showing Emo oocytes (Arrow heads). (F) *rde-2(ne221); lam-1 (RNAi)* animal (DIC image). Spth denotes spermatheca. Scale bar=40 μm.

Talin is located in body-wall muscle dense bodies, focal adhesion-like structures and requires the presence of β-integrin for organization (Moulder et al., 1996). The sequence of *C. elegans* talin is 39% identical and 59% similar to mouse talin (Moulder et

al., 1996). Since talin is expressed in DTC, body wall muscles and somatic gonad, *rrf-1(pk1417)*, should suppress sterility. Loss of talin through RNAi has resulted in sterile phenotype in wild type background (97 ± 2.2%, N=60) and the sterility was reduced significantly in *rrf-1(pk1417)* mutant background (17 ± 4.8%, N=60). Our findings were also in positive note in *rde-2(ne221)* mutant which showed sterility (67 ± 8.5%, N=30). These findings were confirmed using nuclear staining and DIC microscopy. The N2; talin *(RNAi)* worms showed Emo (Figure 3.26 A and B), which was suppressed in *rrf-1;* talin *(RNAi)* (Figure 3.26 C and D). The *rde-2(ne221);* talin *(RNAi)* worms showed Emo phenotype in the proximal gonads (Figure 3.26 E and F).

In vertebrates, troponin C is part of the troponin complex, regulates actin-myosin interaction in the muscles, during Ca^{2+} release from the sarcoplasmic reticulum (Terami et al., 1999). In *C. elegans*, troponin C is encoded by *pat-10*, when depleted results in defective calcium-binding and troponin I binding (Terami et al., 1999). RNAi of *pat-10* resulted in high sterility in wild type worms (97 ± 3.2%, N=30) and 100% sterility in *rde-2(ne221)* strain (N=30). Since *pat-10* is expressed in the body wall muscles, depletion in the somatic RNAi resistant *rrf-1(pk1417)* background results in suppression of sterility (17 ± 6.8%, N=30). Nuclear staining and DIC microscopy revealed Emo oocytes in N2; *pat-10 (RNAi)* (Figure 3.27 A and 3.27 B). *rrf-1(pk1417); pat-10(RNAi)* showed normal oocytes in the proximal gonad (Figure 3.27 C and D). The *rde-2(ne221); pat-10(RNAi)* shown in Figures 3.27 E and F had Emo oocytes in the proximal gonad.

The cell architecture genes include F38A1.8 whose gene product has not been identified. F38A1.8 is one of the 13 upstream transcripts conserved across different

nematode species, *C. briggsae*, *C. remanei*, *C. brenneri*, *C. japonica*, *Pristionchus pacificus*, *Brugia malayi* and *Trichinella spiralis* (Sleumer et al., 2009).

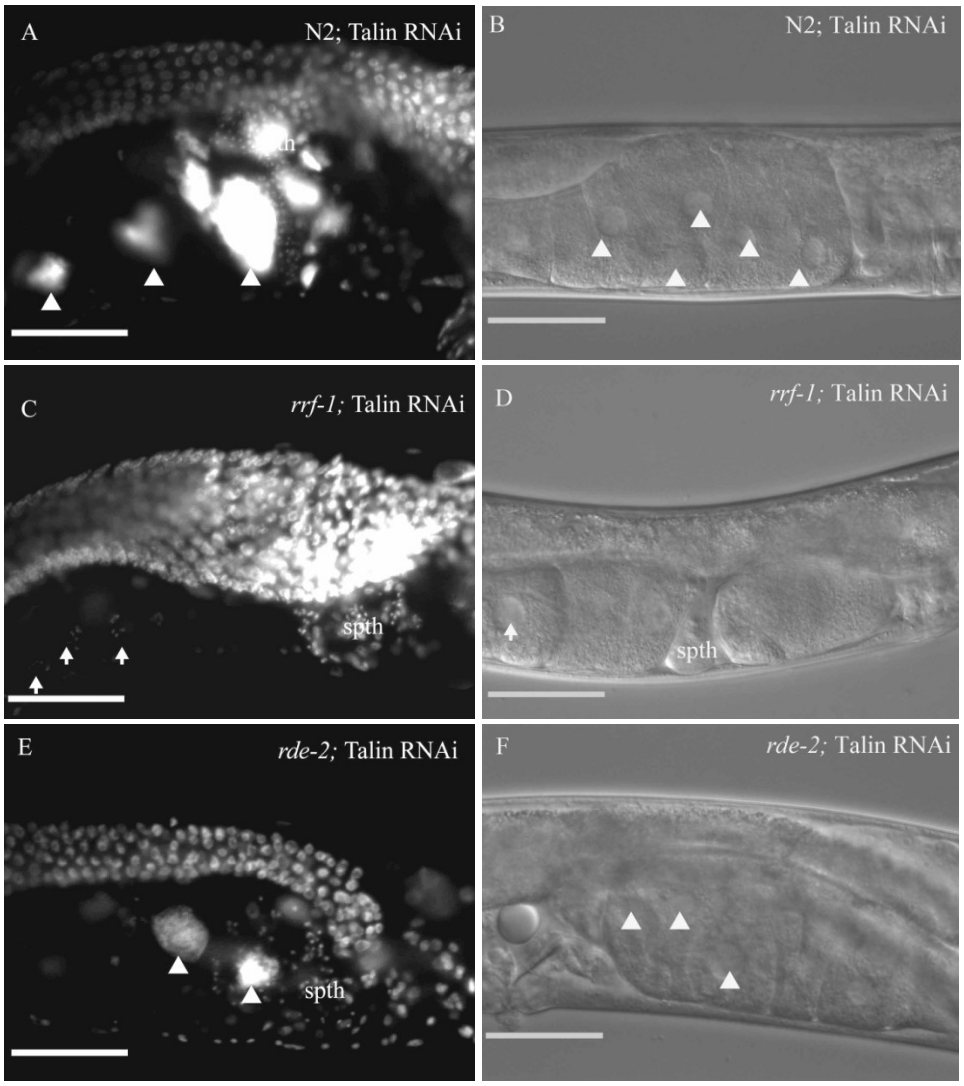

Figure 3.26. Proximal gonads of talin RNAi animals. (A) The nuclear staining image of N2; talin RNAi showing nuclear accumulation (arrowheads). (B) DIC image of N2; talin *(RNAi)* worms showing Emo oocyte nuclei (arrow heads). (C) *rrf-1(pk1417)*; talin RNAi showing normal oocyte in the proximal gonad (arrows). (D) DIC image of *rrf-1(pk1417);* talin *(RNAi)* animals showing normal oocytes (arrows). (E) *rde-2(ne221)* ; talin RNAi showing normal oocytes (arrow heads) in the proximal gonad. (F) DIC image of *rde-2(ne221)*; talin RNAi showing Emo oocytes (arrow heads) in the proximal gonad. Spth, Spermatheca, Scale Bar= 40 μm.

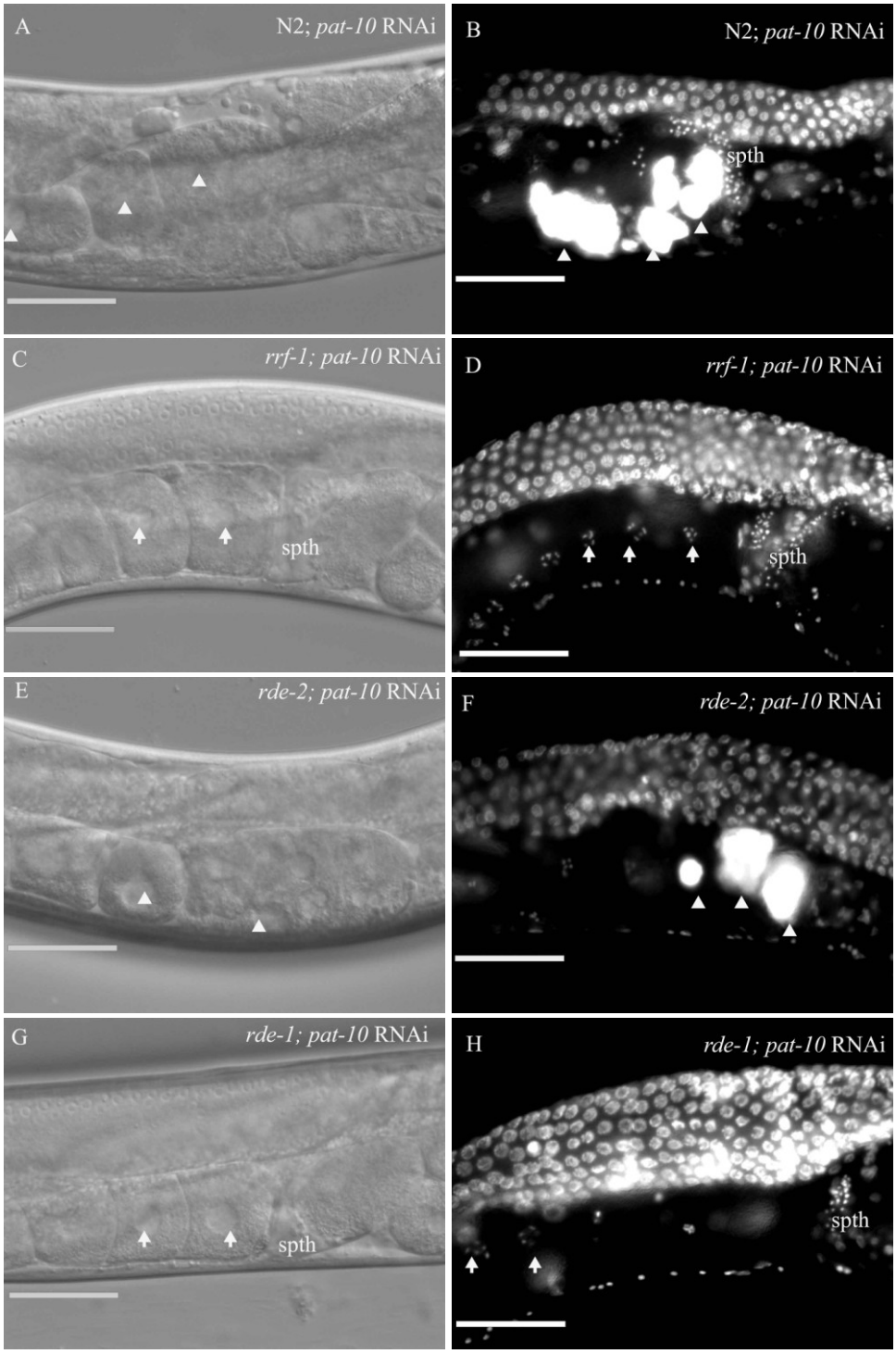

Figure 3.27. Proximal gonads of *pat-10* RNAi animals. (A and B) DIC and Nuclear staining image of N2; *pat-10* animals showing Emo oocytes, respectively. (C) and (D) DIC and nuclear staining images of *rrf-1(pk1417); pat-10(RNAi)* animal showing normal oocytes. (E) & (F) DIC and nuclear staining images of *rde-2; pat-10(RNAi)* worms showing Emo oocytes. (G) and (H) DIC & nuclear staining images of *rde-1; pat-10(RNAi)* animal with normal oocytes. Spth denotes spermatheca. Scale bar=40 μm.

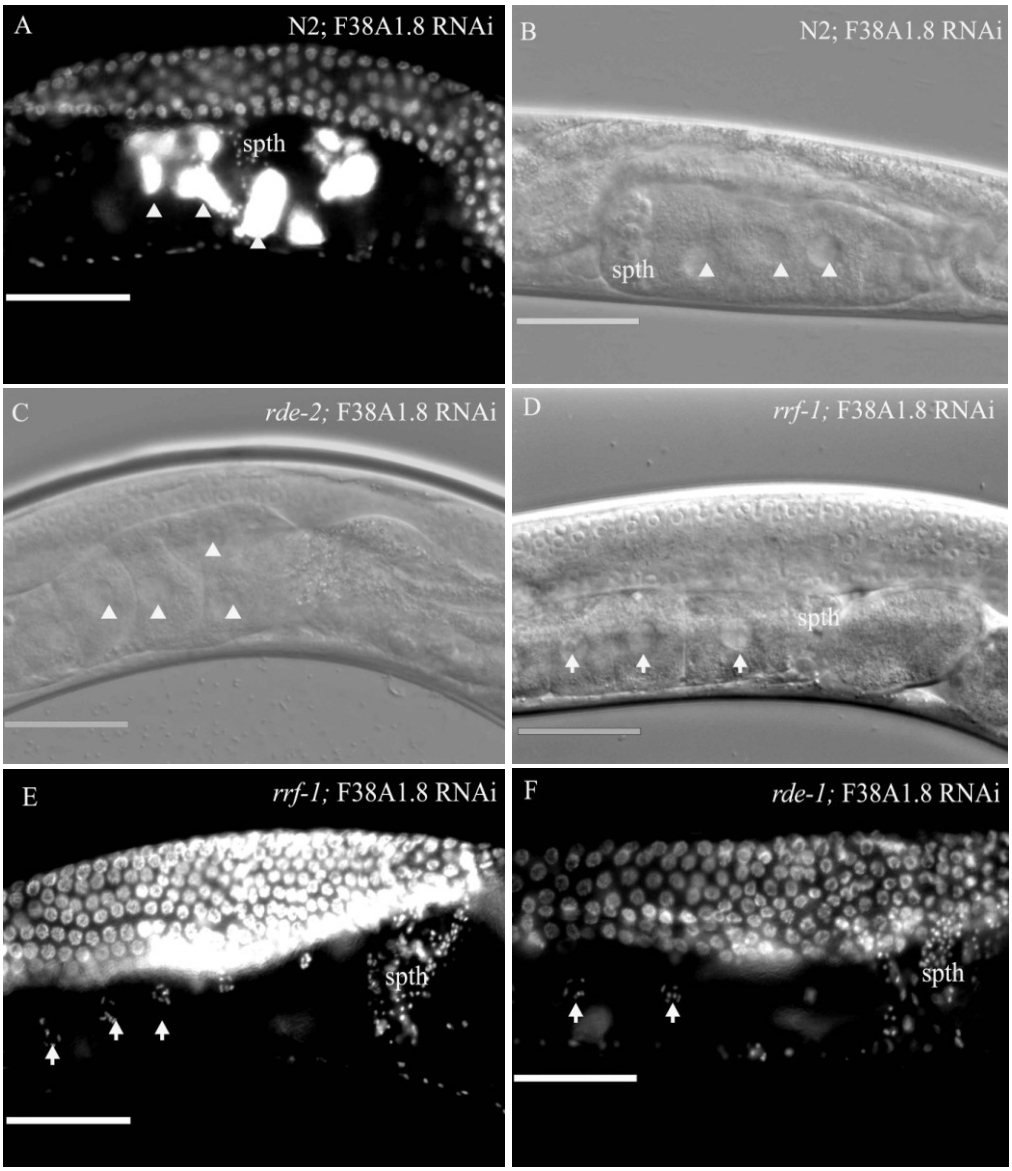

Figure 3.28. Proximal gonads of F38A1.8 RNAi animals. (A) Nuclear staining image of N2 animal grown on F38A1.8 RNAi showing endomitotic oocytes in the proximal gonad. Arrow heads represent Emo oocytes. (B) DIC image of N2; F38A1.8 *(RNAi)* worms showing endomitotic oocyte nuclei (arrow heads). (C) *rde-2(ne221)* animal treated with F38A1.8 RNAi showing normal oocyte in the proximal gonad (arrow heads). (D) DIC image of *rrf-1(pk1417);* F38A1.8 *(RNAi)* animals showing normal oocytes (arrows). (E) Nuclear image of *rrf-1(pk1417)* animals treated with F38A1.8 RNAi showing normal oocytes (arrow heads) in the proximal gonad. (F) DIC image of *rde-1(ne219)* animals treated with F38A1.8 RNAi showing normal oocytes in the proximal gonad. Spth, Spermatheca, Scale Bar= 40 μm.

RNAi of F38A1.8 in N2 background has caused Ste phenotype, (100%, N= 90). Depletion in *rrf-1(pk1417)* resulted in reduced sterility (21 ± 4.2%, N= 90), suggesting that F38A1.8 acts on the somatic cells to regulate ovulation. RNAi against F38A1.8 in *rde-2(ne221)* backgrounds has caused sterility (63 ± 8.8%, N=30). The N2; F38A1.8 *(RNAi)* animals resulted in Emo phenotype (Figure 3.28 A and B). The *rde-2(ne221);* F38A1.8 *(RNAi)* animals also resulted in Emo phenotype in the proximal gonad (Figure 3.28 C). DIC and DAPI images of *rrf-1(pk1417);* F38A1.8 *(RNAi)* animal with normal oocytes are shown in Figures 3.28 D and E.

Genes Encoding for Other Functional Classes

In *C. elegans*, *vha-13* encodes for a vacuolar subunit A of the vacuolar proton-translocating ATPase (V-ATPase). *vha-13* is among the 17 genes that encode for 13 V-ATPase subunits in *C. elegans* (Syntichaki et al., 2005). N2 worms resulted in sterility (100%, N=60) under RNAi conditions for *vha-13* and higher degree of sterility in *rde-2(ne221)* mutants (86.7 ± 4%, N=60). We found that loss of *vha-13* function in *rrf-1(pk1417)* strain suppressed sterility (10 ± 5.4%, N=30). Upon examination of the gonad morphology using DIC and nuclear staining, we confirmed Emo phenotype in N2; *vha-13(RNAi)* (Figure 3.29 A and B) and in the *rde-2(ne221); vha-13(RNAi)* (Figure 3.29 C and D). The *rrf-1(pk1417); vha-13(RNAi)* animals showed normal oocytes in the proximal gonad (Figure 3.29 E and F).

The gene product of *pqn-80* comprises of glutamine/asparagines (Q/N)-rich domain as calculated by algorithm (Michelitsch and Weissman, 2000). Depletion of *pqn-80* in N2 caused 100% sterility (N= 120) respectively. Sterility was reduced in *rrf-1(pk1417)* background, (40 ± 4.5%, N= 118). RNAi against *pqn-80* in *rde-2(ne221)* suppressed

fertility (100%, N=30). Upon examination of gonad structure, we found Emo in N2 (Figure 3.30 A) and *rde-2(ne221)* mutants (Figure 3.30 C). The *rrf-1(pk1417)*; *pqn-80* RNAi showed normal arrangement of oocytes in the proximal gonad (Figure 3.30 B).

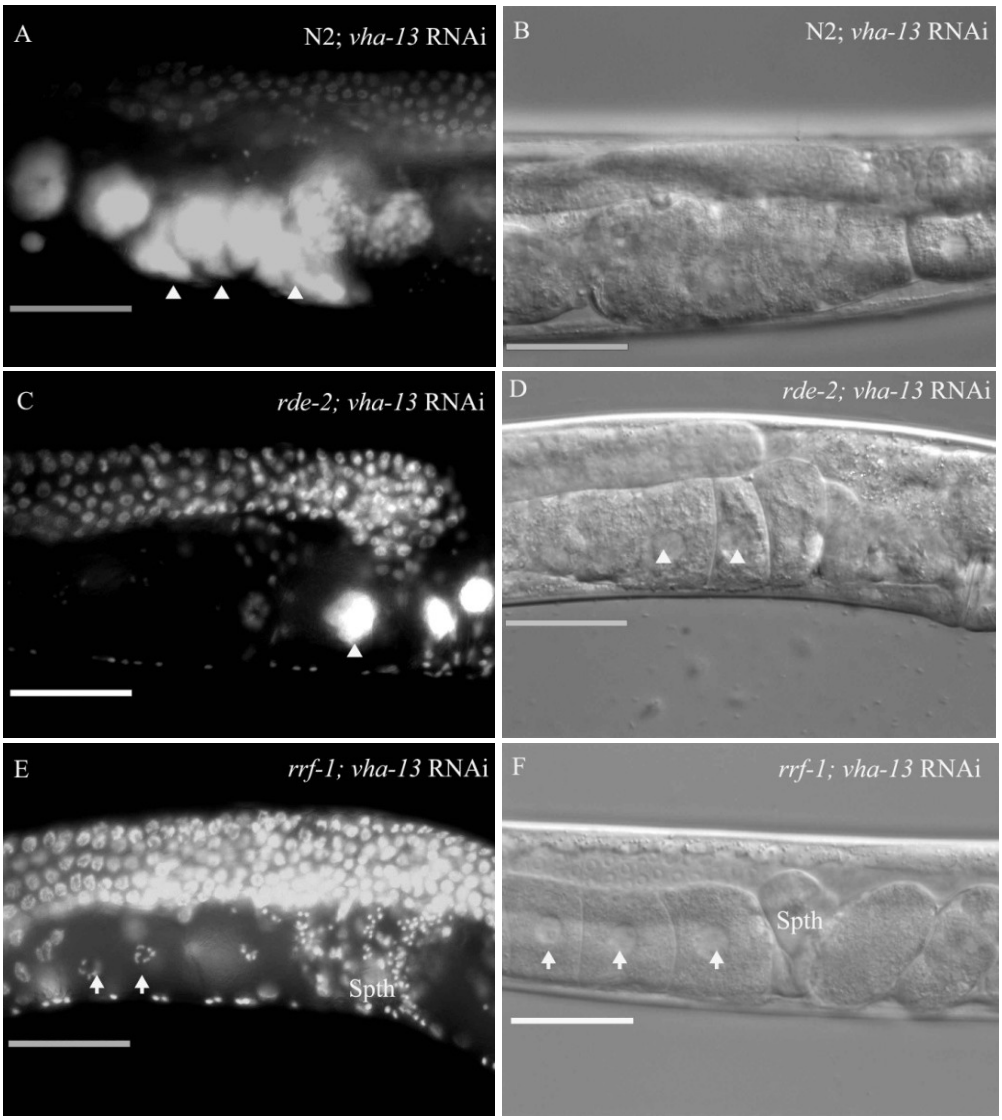

Figure 3.29. Proximal gonads of *vha-13* RNAi animals. (A) N2 animal showing Emo oocytes after *vha-13* feeding RNAi. (B) DIC image of proximal gonad N2; *vha-13 (RNAi)* animal. (C) An *rde-2(ne221); vha-13 (RNAi)* animal showing nuclear accumulation in the oocytes. (D) DIC image of *rde-2(ne221); vha-13 (RNAi)* animal with Emo oocytes. (E) An *rrf-1(pk1417)* animal fed with *vha-13* RNAi with normal oocytes (arrows). (F) DIC image of *rrf-1(pk1417); vha-13 (RNAi)* with normal oocytes. Arrowheads represent endomitotic oocyte nuclei. Spth= spermatheca. Scale bar=40 µm.

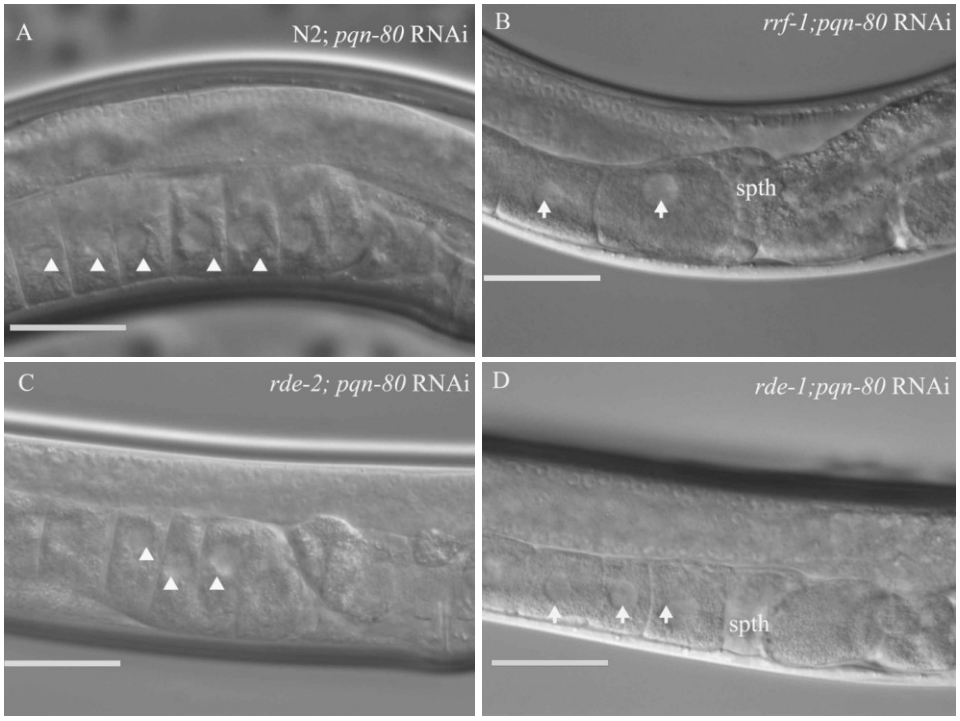

Figure 3.30. Proximal gonads of *pqn-80* RNAi animals. (A) N2; *pqn-80(RNAi)* animal with oocytes accumulating in the proximal gonad denoted by arrowheads (B) Proximal gonad of *rrf-1(pk1417); pqn-80(RNAi)* animal (DIC image). Arrows indicate normal oocytes. (C) DAPI staining of *rde-2(ne221); pqn-80(RNAi)* worms showing nuclear accumulation in oocytes in the proximal gonad (Arrow heads). (D) DIC image of *rde-1(ne219); epi-1(RNAi)* showing endomitoticaly replicating oocytes, denoted by arrows. Spth denotes spermatheca. Scale bar=40 μm.

Gene Interaction Map for Somatic Specific Candidate Genes

A list of 32 genes was sorted from the preliminary screen of 259 genes scored for sterility. These 32 genes showed rescue of sterility in *rrf-1(pk1417)*, a germline RNAi only mutant (Sijen et al., 2001), when compared to the wild type worms. During the secondary screen, we used *rde-2(ne221)* mutant which is germline resistant (Tabara et al., 1999), to identify somatic specific genes. Genes which cause significant sterility compared to *rrf-1(pk1417)* mutant will be classified as somatic specific for fertility. We used a protein network program, Pajek (Batagelj, 1998) to look for interactions between these genes. The nodes in red are the 9 genes which are confirmed as somatic cell

specific and the genes denoted by green have been eliminated after secondary screening with *rde-2(ne221)*. Out of the 9 genes, only Y49A3A.2/*vha-13* is part of a connected network. The rest of the genes (W03F8.5/*lam-1,* F54C1.7/*pat-10*, F38A1.8, F31B12.1a/*plc-1*, Y111B2A.14/*pqn-80*, K08C3.7/*epi-1* and Y71G12.11a/talin) are dispersed and not interconnected to other genes. F33D4.2a/*itr-1* is connected to T01E8.3/*plc-3*.

In the interaction network, four genes belong to ribosomal synthesis (*rps-5*, *rps*-7, *rps-24*, and *rps-28*). We identified two V-ATPase genes (ZK637.8a/*unc-32* and Y49A3A.2/*vha-13*) in the same network. *unc-32* is one of the 4 genes which encode for the *a* subunit of the V_0 complex of the V-ATPases (Pujol et al., 2001) while *vha-13* encodes for the ortholog of subunit A of V-ATPases. The proteasome subunits *pbs-1, pbs-2* and *rpn-1* are connected to a group of ribosomal subunit genes (*rps-5, rps-24* and *rps-28)* and T17E9.2a/*nmt-1,* B0336.2*/arf-1,* F29G9.3/*aps-1*.

Out of the 9 genes which are somatic cell specific, we can categorize them into majorly into cell architecture and signaling transduction functional classes. The corresponding interaction map (Figure 3.31) did not show many significant interactions between the genes. Lack of interactions between the genes suggest that these genes act individually on the somatic gonad especially oocyte and sheath cell structure.

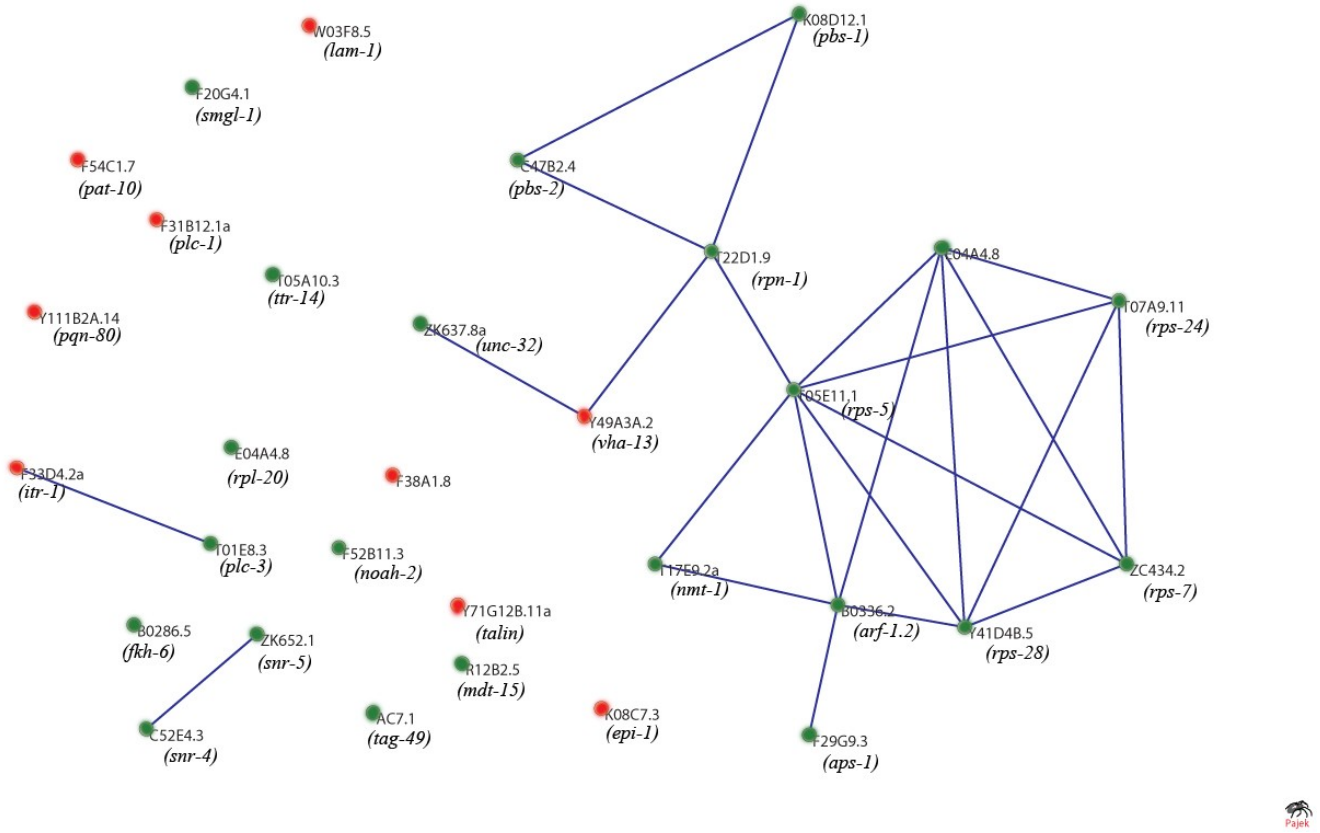

Figure 3.31. Gene interaction map for somatic cell specific genes. The genetic interaction map of germ line specific genes identified from the preliminary screen. The gene loci indicated in red represent the genes which are characterized as double-positive for somatic specificity. Identities of the somatic specific genes: (1) *itr-1*, (2) *pqn-80,* (3) *plc-1,* (4) *pat-10*, (5) *lam-1*, (6) *epi-1*, (7) *vha-13,* (8) *Talin*, (9) F38A1.8. The green nodes indicate genes which did not suppress sterility in germline RNAi resistant background.

Results Summary

We used RNAi and various RNAi sensitive mutants to identify genes involved in the fertility of *C. elegans*. In total, 259 genes that caused Ste were screened, and knocking down >12% of these genes resulted in changes in gonad structure, indicating that loss of these genes results in loss of fertility. Here, we show that ovulation process is spatially different in *C. elegans* and that gene acts differently in germ cell and somatic cells. We then documented the phenotypic changes caused by depletion of genes using differential interference contrast (DIC) microscopy and nuclear staining. The network of genes in clusters helped us in predicting the function of genes and interactions between them in regard to certain physiological functions. In general, genes necessary for basic functions in eukaryotic cells are evolutionarily conserved. We can assume that this network of genes essential for ovulation in *C. elegans* is also conserved. The ribosomal protein subunit genes are essential for the fertility process. Future studies regarding the interaction among the genes should shed more light into the ovulation process. Our screen can be used as a starting point to study the function of these genes in ovulation in other eukaryotic organisms. A comparative analysis between the set of genes identified in our screen with homologs from other species should help in defining their roles in ovulation.

CHAPTER FOUR

Discussion

A pivotal event in the fertilization process in multicellular organisms is the successful completion of ovulation. Ovulation is dependent on signals from various genes and somatic structures. *C. elegans* is an excellent model to study ovulation and identify the genes necessary for an organism due to its transparent body and simple gonad structure. To elucidate the genes essential for fertility in germline and somatic cells, we employed RNAi sensitive mutants and studied defects caused due to gene knockdown. RNAi is now routinely used to study the function of genes by depletion, and later on analyzing the necessary phenotype. We used a feeding RNAi library containing >86% of genes in *C. elegans* (Kamath et al., 2003) to knockdown the expression of genes. Genome wide RNAi screens present advantages over traditional methods like forward mutagenesis, acting as a preliminary point in identifying genes essential for various physiological processes (Updike and Strome, 2009).

Previous RNAi screens have been successful in identifying genes which cause sterility (Ste). But the exact cause of sterility is not known in many of these genes. Our RNAi screen in RNAi sensitive mutants will help in understanding the specificity of the genes in relation to fertility. We performed an RNAi screen, to identify the novel genes that suppress Ste in germline and somatic RNAi specific backgrounds, compared to the wild type. In both wild type and *rrf-1(pk1417)* worms, 259 genes were depleted of their corresponding mRNAs, to test for Ste phenotype. After exposure to RNAi, we scored the plates for high-degree of sterility (> 70%) to avoid embryonic lethality and larval arrest

phenotypes (Waters et al., 2010). This screen resulted in identification of two sets of genes; one set had 69 genes which caused Ste in N2 and *rrf-1(pk1417)* and the other set had 32 genes which significantly suppressed Ste in the *rrf-1(pk1417)* compared to the N2 strain. In order to define the germline and somatic specificity, we rescreened these two sets of genes under *rde-2(ne221)* background, which confers resistance to RNAi in germline background (Tabara et al., 1999). Both *rrf-1* and *rde-2* mutants are viable and do not display any developmental anomalies compared to the wild type. Genes which suppress Ste in *rde-2(ne221)* compared to N2 and *rrf-1(pk1417)* mutant were classified as germline specific. Conversely, those genes which cause Ste in N2 and *rde-2(ne221)* but not in *rrf-1(pk1417)* were termed as somatic specific. In total, 259 genes that caused Ste were screened, and knocking down >12% of these genes resulted in changes in gonad structure, indicating that loss of these genes results in loss of fertility.

Studies have shown that loss of function of genes leading to sterility show high incidence of Emo (Iwasaki et al., 1996). The presence of Emo in the proximal gonads of RNAi animals indicates defects in ovulation. A typical oocyte in a mature wild type animal will have 6 bivalent chromatids, indicating diakinesis stage of meiosis I. We observed oocytes with nuclei which have undergone repeated DNA replication and have become accumulated in the gonad arm without entering the spermatheca for fertilization. Oocytes which display this phenotype will be defined as endomitotic (Emo) oocytes. After completion of the screening process with the RNAi sensitive mutants, we identified 20 genes as germline specific and 9 genes as somatic specific in the ovulation. In these subsets of genes, we analyzed RNAi animals with DIC microscopy to observe changes in

the gonad structure or fixed animals with DAPI stain to visualize nuclear accumulation in oocytes using fluorescence microscopy.

Protein Synthesis Genes

A majority of the genes belong to the functional class of protein synthesis, suggesting the necessity of these genes in ovulation process. The growing oocyte needs synthesis of proteins at a higher level and blocking protein synthesis causes ovulation defects. Genes belonging to *bys*-like family are conserved from across various species, the expression pattern ranges from embryonic to larval stages in *Drosophila* and germline and embryonic tissues in *C. elegans* (Kamath et al., 2003; Stewart and Nordquist, 2005). In mammals, bystin binds to the cell adhesion molecule complex, and is essential for embryo implantation (Suzuki et al., 1998). In *C. elegans,* the mammalian ortholog of Bystin-like, *byn-1* is essential for germline cells (Kamath et al., 2003).

Loss of *byn-1* and *fib-1* genes causes Ste in N2 and *rrf-1(pk1417)* but Ste is rescued in *rde-2(ne221)*. Moreover, loss of function of these genes resulted in Emo in sterile worms confirming defective ovulation. Typically the gonad arm in a mature animal shows oocytes in diakinesis with 6 chromatids, but N2 and *rrf-1(ne221)* worms subjected to *byn-1* and *fib-1* knockdown displayed oocyte accumulation due to Emo (Figures 3.3 and 3.4). As seen in Figure 3.18, *byn-1* along with *fib-1*interacts with a number of ribosomal proteins. In mammals, Bystin family genes are required for 18S rRNA processing and 40S ribosome synthesis (Adachi et al., 2007). *fib-1,* a component of U3 snoRNP, is involved in the 18S rRNA synthesis in *C. elegans* (Saijou *et al.,* 2004). The interactions of ribosomal genes with *byn-1* and *fib-1* suggest the reliance of ovulation on the proper functioning of translational mechanism. Taken together, our results with

the gene interaction mapping, we suggest that *fib-1, byn-1* are essential for pre-rRNA processing and also play a vital role in ovulation, presumably by regulating ribosome assembly in germ cells.

Ribosome Biogenesis Genes

The ribosome genes can be considered part of the protein synthesis functional class. The ribosomes are made up of two subunits, the small 40S and large 60S subunit. Ribosome biogenesis is a complex process which involves ribosome and non ribosome proteins (Perreault et al., 2008) and takes place in cell cytoplasm, and nucleolus (Voutev et al., 2006). The 40S subunit is made up of 30 ribosomal proteins and the large 60S subunit consists of 50 ribosomal proteins (Perreault et al., 2008). We used an RNAi feeding protocol to knockdown gene expression of ribosomal proteins. After the primary RNAi screen, we identified 17 genes which encode for ribosome biogenesis. The complete list of ribosomal encoding genes causing Ste phenotype across various mutant backgrounds is given Table 3.4. RNAi-targeted removal of many ribosomal genes, encoding for large and small subunits, showed sterility across all three strains, indicating that these genes are indispensable for ovulation. The degree of sterility caused due to depletion of ribosomal subunits is severe in the wild type and somatic RNAi resistant background, with insufficient suppression in *rde-2(ne221)* background. It is likely that ribosomal subunit genes are essential as part of the higher demand for protein synthesis that result during ovulation.

Role of Protein Transport and Modification Genes

The knockdown of genes, *cct-1, phi-20, npp*-7 and *rab-1*, which encode for protein transport and modification, causes ovulation defects in N2 and *rrf-1(pk1417*

Chaperonins have the unique ability of folding proteins and most proteins have to pass through the chaperonin folding mechanism. CCT is necessary for distal tip cell migration, oogenesis and embryonic cell division and knockdown of CCT-1 causes sterility (Lundin et al., 2008). Sterility observed in N2 and *rrf-1(pk1417)* upon knockdown of *cct-1* was due to Emo nuclei in accumulated oocytes in the proximal gonad (Figure 3.6). The gene *phi-20* is a conserved component of signal peptidase complex across various species and is essential for intracellular trafficking, secretion and vesicular transport.

Nucleoporin encoding genes are essential for transport of proteins. Studies have shown the need for nuclear pore proteins in embryonic development in *C. elegans* (Galy et al., 2003). We analyzed for ovulation defects in *npp-7* depleted worms and identified Emo oocytes in the proximal gonad in N2 and *rrf-1(pk1417)* (Figure 3.8). The Rab family of GTPases is responsible for vesicular transport (Sato et al., 2006). Previous studies have shown that loss of *rab-1* mRNA results in sterility due to oogenesis defects (Poteryaev et al., 2005). Depletion of *rab-1* causes accumulation of CAV-1 in the germline and embryo (Sato et al., 2006) leading to ovulation defects in *rrf-1(pk1417)* worms (Figure 3.4). The ability of *rde-2(ne221)* to rescue sterility after depletion of *cct-1, phi-20, npp*-7 and *rab-1* indicate the germline specificity of these genes.

Protein Degradation Genes

Protein degradation is essential for maintenance of homeostasis in cells. The proteasomes in a cell work in a spatial and temporal approach to regulate the degradation of proteins (Ghazi et al., 2007). Loss of *rpn-2*, *pas-4* and *rpt-1* genes in our screen has caused Ste phenotype in the wild type and *rrf-1(pk1417)* (Table 3.2). RPN-2 protein is

detected in the nucleus during mitosis, throughout the cytoplasm, in the nuclear membrane and cell cortex (Sugiyama et al., 2008). Prolonged exposure to *rpn-2* RNAi in adult worms causes the embryo to remain at one cell stage without any polar bodies and similar to meiosis arrested embryos (Gonczy et al., 2000; Sugiyama et al., 2008). *pas-4* encodes a proteasome alpha-type subunit of the core 20S proteasome subcomplex (Kahn et al., 2008). Sterility screening and subsequent characterization of *pas-4* worms identified Emo oocytes in the proximal gonad in N2 and *rrf-1(pk1417)* worms (Figure 3.15). *rpt-1* encodes for ATPase subunit of the 19S proteasome regulatory complex (Davy et al., 2001). Knockdown of *rpt-1* resulted in Emo oocytes in N2 and *rrf-1(pk1417)* as shown in Figure 3.16. Expression of RPN-2 in the nucleus might be regulated by 20S catalytic proteasome subunits such as *pas-1* (Sugiyama et al., 2008). The function of these proteins is necessary in order for the cell to function efficiently which indirectly affect ovulation. Confirmation of germline specificity was due to suppression of Ste and absence of Emo oocytes in *rde-2(ne221).*

Role of Nucleic Acid Synthesis, Processing and Binding Genes

Genes encoding for splicing and other RNA synthesis show the importance of RNA processing in the ovulation mechanism. Our results have shown that genes encoding for RNA processes, *pcf-11, phi-11, rnp-4,* T10F2.4 and *rnr-1* are essential in the germline during ovulation. In order to form fully capable mRNAs, addition of 3' polyadenosine tail is necessary during mRNA processing. The cleavage and polyadenylation factors (*pcf*) are part of the protein complex that is necessary for identifying the 3' processing signals (Cui et al., 2008). Lack of *pcf-11* in the germline causes Ste indicating a necessity of normal mRNA processing during ovulation. *phi-11*

encodes for *C. elegans* ortholog of splicing factor 3b subunit 1, and is a component of the U2 snRNP required for pre-mRNA splicing (Govindan et al., 2006). *phi-11* plays a role in the VAB-1 MSP/Eph receptor pathway (Govindan et al., 2006) and functions in the germline to negative regulate the meiotic maturation. Our study showed similar results, the sterility caused due to Emo oocytes in the proximal gonads of N2 and *rrf-1(pk1417)* (Figure 3.13).

rnp-4 is orthologous to Y14 in *S. cerevisiae*, a nuclear RNA binding protein which shuttles between the nucleus and cytoplasm and associated with mRNA export (Kataoka et al., 2000; Longman et al., 2003). Binding of Ce-Y14 is part of pre-mRNA splicing process to remove introns and imprint the mRNA in the nucleus (Kataoka et al., 2000). Knockdown of *rnp-4* causes abnormalities in mRNA export which leads to ovulation defects. U-box proteins are one of the four classes of ubiquitin-protein ligases. T10F2.4 is one of the four genes in *C. elegans* that encode proteins with a U-box domain. Inactivation of T10F2.4 in N2 and *rrf-1(pk1417)* resulted in Ste, which was suppressed in *rde-2(ne221)* (Table 3.3 and Figure 3.10).

Ribonucletoide reductases are necessary for maintaining the dNTP levels during DNA synthesis (Zhao et al., 1998). During germline proliferation, *rnr-1* and *rnr-2* are required for mitochondrial DNA replication, without these genes, sterility has been reported in *C. elegans* (Mori et al., 2008). Genes such as *cye-1* and *rnr-1* encode for ribonucleotide reductase large subunit and are targets for E2F regulation, specifically *cye-1* which is repressed by *lin-35* acting upstream (Boxem and van den Heuvel, 2002). *Lin35b* negatively regulates the transition from G1 to S phase and can lead to abnormal cell-cycle control (van den Heuvel 2005). Our study showed that *rnr-1* works only in the

germline (Table 3.3) to influence ovulation. Since ribonucleotide synthesis is an integral part of DNA replication, there is interaction between the DNA replication factors like *mcm-4* and *rnr-1* (Korzelius et al., 2010). Genes such as *phi-11* and *rnp-4* are involved in pre mRNA splicing and transport between nucleus and cytoplasm. It is difficult to say with certainty how the mRNA specific genes effect ovulation because of the essential nature of mRNA binding and splicing mechanisms in cells.

Gene Expression Regulation Genes

Mini-chromosome maintenance (MCM) genes are essential for binding to the replication origin during G1 phase of the cell cycle and unwinding of the DNA (Labib and Diffley, 2001). In yeast, six MCM proteins form a complex (MCM2-7), which acts as the backbone of the DNA replication pre-initiation complex to guide DNA replication and control cell-cycle progression (Korzelius et al., 2010). MCM-2 and MCM-5 proteins are required for RNA polymerase-II mediated transcription in *C. elegans* (Snyder et al., 2009). The role of DNA licensing factors like MCM is tissue specific, MCM-4 is specific in the epidermis for the growth and viability (Korzelius et al., 2010). In *C. elegans, mcm-6* controls the DNA replication to a single round per cell cycle (Updike and Strome, 2009). Previous work has shown that knockdown of MCM-6 has been shown to cause sterility (Woodward et al., 2006). Knockdown of *mcm-6* results in loss of cell cycle control leading to ovulation defects like Emo.

Cytokinesis comprises of the following changes steps, initiation, cleavage formation and abscission of the furrow which requires the coordination of several pathways (Simon et al., 2008). Paternal *cyk-4* is necessary for the establishment of anterior polarity in *C. elegans* embryos and *cyk-4* donated from sperm is required for

fertilization (Jenkins et al., 2006). Rho-GAP proteins such as *cyk-4* are expressed in germline and up to 100-200 cell stages in embryo (Schmutz et al., 2007). Knockdown of *cyk-4*, encoding for GTP activating protein of Rho family GTPases (Jantsch-Plunger et al., 2000), has resulted in Ste phenotype in germline background only, with Emo in the proximal gonads (Figure 3.17).

In *C. elegans,* meiotic arrest takes place during prophase I for proper oocyte development. The oocytes are released from meiotic arrest due to external signaling from major sperm protein and progress through to the meiotic metaphase I. Cyclin dependent kinases (CDKs) control the transition of a cell from G2 to M stage (Chausson et al., 2004). The process of G2/M transition is promoted by maturation promotion factor (MPF), controlled by *cdk-1*, *wee-1.3* and *cdc-25.2* (Kim et al., 2010). *cdk-1*, a *C. elegans cdc-2* homologue, is essential for the oocyte in the prophase/metaphase stage (Boxem et al., 1999). *cdk-1* acts in tandem with other kinases to regulate the transition of oocyte to embryo (Shirayama et al., 2006). Abnormal prophase to metaphase transition, results in formation of univalents instead of bivalents during diakinesis stage (Inoue *et al.*, 2006). The presence of Emo oocytes in proximal gonads of N2 and *rrf-1(pk1417)* worms (Figure 3.5) depleted of *cdk-1* might be due to inability of the worms to undergo prophase to metaphase transition.

Given the CDK-1 function role in oocyte maturation, we can say that *cdk-1* is germline specific for ovulation and its function may be redundant in somatic cells. During prophase I to metaphase I transition, *cdc-25.2* activates MPF by dephosphorylation and RNAi studies in *cdc-25.2* have resulted in Emo oocytes, which suggest the role of MPF in ovulation (Kim et al., 2010). It is not unlikely that cell cycle

control genes like *cdk-1*, *cyk-4* and DNA replication genes like *mcm-6* have conserved functions which are essential for maintaining proper germline development.

Role of ATP Synthesis Genes

We identified two genes, *vha*-19 and C23G10.8, as germline specific for ovulation. Studies have shown that loss of V-ATPase subunits causes Emo, suggesting the role of V-ATPases in ovulation (Oka and Futai, 2000). Our results in *vha-19* fed animals have shown that wild type and *rrf-1(pk1417)* display ovulation defects and not in the *rde-2(ne221)* background (Figure 3.18). According to current information in wormbase, C23G10.8 is uncharacterized protein. Abnormal gonad morphology was seen after knockdown of C23G10.8, resulting in Emo oocytes in N2 and *rrf-1(pk1417)* (Figure 3.19), which suggests germline specificity, during ovulation.

We have identified R08D7.1, an uncharacterized gene, as germline specific for ovulation. Until now, R08D7.1 has not been shown to be involved in ovulation functions. Knockdown of R08D7.1 caused Emo in the proximal gonads of N2 and *rrf-1(pk1417)* (Figure 3.20). Also, loss of R08D7.1 in *rde-2(ne221)* has suppressed the gonad morphology defects like Emo, confirming R08D7.1 acts on the somatic cells and not on the germ line during ovulation process. R08D7.1 has sequence similarity to Bud13p, which is a component of mRNA retention and splicing (RES) complex and is essential for splicing *in vitro* in yeast (Dziembowski et al., 2004). We can predict that R08D7.1 belongs to RNA specific functional class due to its role in slicing mechanisms.

Significance of Various Functional Groups in Ovulation

In the functional group of protein synthesis, genes encoding for ribosome subunit synthesis are also included. The need for vast quantities of proteins in dividing germ cells shows the importance of protein synthesis genes. The effects of ribosome biogenesis disruption can be seen in germline stem cells (Kudron and Reinke, 2008). The germ cells express higher levels of translation components to either improve the translation efficiency in germ cells, increase the ribosome number in the progeny or modify ribosome composition (Waters et al., 2010).

Changes in ribosome biogenesis machinery or regulators of ribosome synthesis process lead to loss of germ cell proliferation thereby leading to defects in ovulation. Nucleostemin (*nst-1*), a conserved factor present in nucleolus regulates ribosome biogenesis and cell proliferation, loss of *nst-1* in the germline causes failure of germline stem cells proliferation and ribosomal rRNA production (Kudron and Reinke, 2008). Changes in gonad development and interruptions to soma-germline cross talk are caused due to disruptions in ribosome biogenesis (Voutev et al., 2006). It is difficult to separate the necessity of ribosome genes in living cells to other essential process in germ cells (Waters et al., 2010). This can be due to two reasons; the identification of many ribosomal genes suggests a probable relationship between ribosome synthesis and the ovulation process or the essential nature of ribosomes in many general cellular functions.

Studies in mammalian cells have linked cell cycle regulation and ribosome biogenesis, mainly as a check point to stop progression of cell cycle during defective ribosome biogenesis (Pestov et al., 2001). The to and fro transport of molecules between nucleus and cytoplasm occurs through the nuclear pore complex (Moy and Silver, 1999).

In yeast, nuclear pore proteins are essential for export of small ribosomal subunits from the nucleus (Moy and Silver, 1999). Loss of protein transport and modification genes causes accumulation of proteins in the cell leading to ovulation defects. A simple explanation is impairment of protein transport and modification causes disruption to general cellular functions. The proteasome mechanism is essential for the degradation of proteins and for the proper maintenance of cell cycle control, cell differentiation and apoptosis (Collins and Tansey, 2006). One of the reasons that somatic cells are immune to loss of proteasome subunits might be due to redundancy with members of regulatory and non-regulatory family in the somatic gonad. We hypothesize that knockdown of proteasome subunits by RNAi results in the ovulation defects due to the breakdown of protein degradation machinery or lack of ubiquinization in cells.

DNA replication is a highly regulated process necessary to maintain the proper genome number of the cell. To achieve this fidelity during DNA replication, cells have check points to regulate the process. Cell cycle regulators can have tissue specific function especially in germline since cell cycle control targets gametogenesis (Kim et al., 2010). In *C. elegans, cdc-25.1* is expressed in the germline but is not necessary in the somatic cells during post embryonic development (Kim et al., 2009).

Genes Acting on the Somatic Gonad During Ovulation Process

In order to understand ovulation at the tissue level, we performed RNAi screen and screened for genes that caused defects in ovulation. In most of the multicellular organisms the development of oocyte is dependent on the signals from the somatic gonad. The somatic gonad and germline have multiple levels of coordination to maintain the balance during the process of ovulation (Govindan et al., 2006). MPK-1 regulates a

number of processes in the germline in relation to the oocyte; meiotic maturation, growth control, nuclear migration, organization and differentiation (Lee et al., 2007). In mammals the growing oocyte is provided nourishment and other signals by the follicle cells (Voronina and Wessel, 2003).

The following genes, *lam-1*, *itr-1* and talin are expressed in the somatic gonad structure (Kao et al., 2006; Moulder et al., 1996; Yin et al., 2004). Similar results were seen with *lam-1* and *epi-1* RNAi in integrin-IP_3 signaling studies conducted previously, the wild type N2 showing sterility and complete suppression of sterility in *rrf-1(pk1417)* mutants (Xu et al., 2005). Talin disruption by RNAi causes sterility due to distal tip cell migration defects and abnormal oocyte maturation defects like Emo (Cram et al., 2003). *epi-1* (RNAi) in wild type animals has resulted in embryonic and larval lethality along with sterility due to disorganization of basement membrane (Huang et al., 2003). Also, the integrin-related genes like *epi-1* and *lam-1* could be linked to IP3 signaling pathway during ovulation. The gene F38A1.8 belongs to cell architecture functional class but its protein has not been characterized. Reduction of F38A1.8 in wild type and *rde-2(ne221)* background causes oocyte abnormalities. Until now, this gene has not been shown to be involved in ovulation. This phenotype was suppressed in the somatic RNAi resistant mutant, *rrf-1(pk1417)*, indicating a somatic specificity for this gene.

IP_3 diffuses through the cytosol and releases Ca^{2+} by opening the IP_3 gated Ca^{2+}-channel, IP_3 receptors (IP_3 R) on ER (Walker et al., 2002). Ca^{2+} release from ER is required for the rhythmic contractile activity of the sheath cells during ovulation (Yin *et al.*, 2004). LIN-3/LET-23 and MSP/VAB-1 signaling activate PLC-3 that hydrolyzes PIP_2 to generate IP_3 and subsequently activates ITR-1 (Yin *et al.,* 2004). PLC-1 and

PLC-3 produce IP3 in sheath cells and spermathecal cells (Hiatt et al., 2009). The distal spermathecal cells and somatic sheath cells are essential for ovulation in *C. elegans* (McCarter et al., 1997). *plc-1* mutants have shown distinctive ovulation changes, such as oocyte entry and exit defects, improper constriction and dilation of spermatheca (Kariya et al., 2004). We observed ovulation defects in *plc-1(RNAi)* worms in wild type with rescue of sterility in *rrf-1(pk1417)* worms. These ovulation defects might also be caused due to loss of PLC-1 in spermatheca. Studies in *fos-1(RNAi)* animals have shown that FOS-1, an activator protein-1 family member, regulates PLC-1 expression in spermatheca (Hiatt et al., 2009). Since the *plc-1* gene is expressed in somatic gonad, *rde-2(ne221)*; *plc-1(RNAi)* should show sterility confirming the somatic specificity during ovulation. Integrin related genes are linked to IP3 signaling and this cell–ECM interaction in the somatic gonad likely increases IP3 signaling, release of intracellular Ca^{2+} to promote ovulation (Xu et al., 2005). These ovulation defects can be rescued by increased IP3 signaling which causes increase in Ca^{2+} levels (Xu et al., 2005). Our findings indicate that these genes functions predominantly in the somatic gonad during ovulation and that gene activity is probably not required in germline.

Future Directions

The 29 genes identified in our screen, could act in the germline or somatic cells at various stages to regulate ovulation process. These 29 genes out of the 19,000 genes present in *C. elegans* are certainly less than expected, due to factors such as temperature, duration, stringent criteria for Ste and stage of feeding. It is possible that additional genes might be involved in ovulation both in soma and germline. Given our stringent criteria for scoring the worms, we might have omitted few genes which did not result in

complete Ste phenotype or which have not shown significant differences in sterility. Also genes with a maternal function in embryos might have been omitted from our post-embryonic screen. Conducting RNAi screening in *rrf-3* mutant, an RNAi hypersensitive strain should help in identifying genes which cause mild ovulation defects.

The cause of sterility due to these genes might be due to pleiotropic effect. Some of the genes might not act on ovulation alone while causing sterility. A number of genes identified in our screen *cyk-4*, *cdk-1*, *mcm-6*, *npp-7*, *npp-10*, *pas-4*, *rnp-4, rnr-1* and *vha-19* have been previously identified to be factors for stability and function of P-granules in the germ line (Updike and Strome, 2009). In all these genes, we observed nuclear accumulation due to endomitotic replication in the mature oocytes. Additional studies of these genes have to be done to observe spatial and temporal nature of these genes.

Analyzing gene expression levels using reverse transcription PCR (RT-PCR) or quantitative PCR (PCR) should help in confirming the presence or absence of a particular gene in the cell or organism. Removal of a certain protein through RNAi can be proven with specific antibodies for the target protein. Construction of lines expressing the gene of interest and tagged to GFP fusion proteins will help in assessing whether a protein is knocked out by fluorescence microscopy in the particular tissue.

To understand the interactions between genes necessary for ovulation, a comparative study of this data with protein interactions should help in identifying the signaling pathways between the somatic and germline cells. Comparative analysis of RNAi screens from our data and in various species will help us narrow down the genes responsible for ovulation defects. Data obtained from such RNAi screens will help in understanding how gene(s) will work in order to generate a specific phenotype.

APPENDICES

APPENDIX A

Table A.1 List of genes identified as sterile (Ste) phenotype from wormbase.org

Predicted gene(s)					
B0511.10	W02D3.9	C13B9.3	H19M22.3a	ZK1058.2	T11F8.3
C01A2.5	Y105E8A.16	C14B9.7	K01G5.4	ZK328.2	Y55F3AR.3
C01G8.9	Y23H5A.7a	C16A3.9	K02D10.5	ZK328.5b	Y55H10A.1
C04F12.4	Y48G8AL.8a	C23G10.3	K04H4.1	ZK632.1	Y57G11C.16
C12C8.3a	Y71F9AL.17	C23G10.4a	K08E3.6	ZK637.8a	B0250.1
C17E4.9	Y71F9AL.13a	C23G10.8	K11D9.2a	ZK652.1	C05C8.7
C32E8.2	Y71G12B.11a	C23G10.9	K12H4.4	ZK652.4	C15H11.9
C32E8.8	Y87G2A.5	C24H11.7	R07E5.14	AC7.1	C37C3.2a
C36B1.4	ZC308.1	C26E6.4	R08D7.1	C47E12.5	C39F7.4
C36B1.5	ZC308.3	C27D11.1	R08D7.3	C49H3.11	C52E4.3
C47B2.4	ZC434.2	C29E4.8	R10E11.1a	E04A4.8	C52E4.4
C55B7.5	ZK1014.1	C29F9.7	R10E11.8	F12F6.6	C53A5.6
D1007.6	ZK973.10	C30C11.2	R12B2.5	F28D1.7	CD4.6
DY3.2	B0286.5	C34E10.6	R13A5.12	F32E10.4	D1014.3
F20G4.1	C26D10.2	C36A4.4	R13A5.8	F33D4.2a	D1054.14
F20G4.3	C27A2.2a	C50C3.6	R144.2	F38A1.8	D1054.15
F25H2.10	F10B5.1	C56G2.6	R151.3	F38A5.5	F17C11.9
F25H2.9	F13D12.7	F01F1.8	R74.1	F38E11.5	F29G9.3
F25H5.4	F23F1.8	F08F8.2	T05G5.10	F40F11.1	F29G9.4
F26H9.6	F40F8.10	F09F7.3	T05G5.3	F42C5.8	F46E10.1
F27C1.7	F44F4.2	F13B10.2	T08A11.2	F42C5.8	F55A11.2
F36A2.6	F54C9.5	F23F12.6	T10F2.1	F49C12.13	F55C5.8
F39B2.6	F59E10.3	F26F4.10	T10F2.4	F49C12.8	H19N07.1
F39H11.5	M110.4	F30H5.1	T12A2.2	F52B11.3	M03F8.3
F52B5.6	T01E8.3	F37C12.11	T12A2.7	F58E2.9	T01C3.6
F53G12.10	T05C12.7	F37C12.13	T17E9.2a	H02I12.1	T01C3.7
F54C1.7	T19E10.1a	F37C12.4	T20B12.1	JC8.3a	T05H4.12
F55A12.3	T21B10.7	F37C12.9	T20G5.1	K08C7.3	T05H4.6a
F56A3.2	Y25C1A.5	F43C1.2a	K08E5.3	K08D12.1	T10B5.5
F57B10.1	Y38A8.2	F43D9.3	T20H4.3	K11H12.2	T27F2.1
F57B10.3	Y53C12A.1	F48E8.5	T23G5.1	T05E11.1	Y113G7A.3
F59C6.5	Y54E2A.11	F54E7.2	T26A5.9	T07A9.11	Y49A3A.2

Table A.1 continued

H15N14.1	B0336.10	F54H12.1	T26G10.1	T11F8.3	C33D3.1
H28O16.1	B0336.2	F56F3.5	W07B3.2a	T14G10.5	C54D1.5
K02B12.3	B0393.1	F57B9.10	Y111B2A.14	T22D1.9	F18G5.2
K05C4.1	B0412.4	F57B9.2	Y37D8A.10	W03F8.5	F20B6.2
M01A10.3	B0464.1	F57B9.5	Y49E10.15	Y116A8C.35	F31B12.1a
R12E2.3	C02F5.9	F58A4.3	H06I04.4	Y116A8C.42	T05A10.3
T08B2.10	C07A9.2	F58A4.8	H06I04.4	E04A4.8	
T19B4.2	C07G2.3a	H06I04.4	Y71D11A.5	Y24D9A.4a	
T20F5.2	C07H6.5	H06I04.4	Y71H2B.10	Y41D4B.5	
T23G11.3	C08C3.4	H19M22.2a	ZK1010.1	Y45F10D.12	

APPENDIX B

Table B.1. List of the functional classes of germline specific genes and their description. The sequence name and genetic locus are unique identifiers for each *C. elegans* gene (www.wormbase.org). The functional groups are based on previous gene designations (Kamath et al., 2003).

Functional group	Description	Genes
RNA Synthesis	Includes genes which encode for transcription machinery and splicing	*lpd-7, rps-3,rpb-2,prp-8,*F09F.3, T10F2.4, T12A2.7, *phi-11,uaf-2,rnp-4 ,pcf-11, skp-1*
Protein synthesis	Translation machinery and chaperones	*rps-10, eft-2,rpl-22,rps-9,rpl-5,cct-1,cct-5, rpl-21, rpn-2, cct-6,rpl-36, rps-12, rps-1, rpl-9, rpl-6,rpn-6,eft-1,*F42C5.8, *rpl-20*
Cellular architecture	Cytoskeletal and protein trafficking/vesicle regulation	*npp-7,phi-26,chc-1, sec-24.1, rab-1, fib-1, lam-2*
Metabolism	Energy production and intermediary metabolism	*phi-37,atp-2, byn-1, rnr-1, vha-12*
Protein degradation	Components of the ubiquitin-targeted protein degradation machinery	*pas-4,pas-5,pbs-7, rpt-4, pbs-3, rpn-6, ubl-1(c, uba-1), rpt-1, pas-6*
Signal transduction	Genes which encode for kinases, phosphatases and signal transduction pathway components.	*cdk-1,cyk-4,par-2*
DNA Synthesis	Includes cell cycle and repair	*mcm-6*
Others	Consists of genes encoding the 7 transmembrane spanning receptors, chromatin binding, neuro	*npp-10, hcp-3*
Unknown	Genes with insufficient information to assign a function or genes with no significant matches in any organism.	*rpl-17,*C23G10.8, *ntl-1,* Y71D11A.5, *rpl-7A, vha-19, atp-4*, R08D7.1

APPENDIX C

Table C.1 List of the functional classes of somatic specific genes and their description. The sequence name and genetic locus are unique identifiers for each *C. elegans* gene (www.wormbase.org). The functional groups are based on previous gene designations (Kamath et al., 2003).

Functional group	Description	Genes
RNA Synthesis	Includes genes which encode for transcription machinery and splicing	*fkh-6, snr-5, snr-4*
Protein synthesis	Translation machinery and chaperones	*rps-7, rpl-20, rps-5, rps-24, rpl-20*
Cellular architecture	Cytoskeletal and protein trafficking/vesicle regulation	*pat-10, apd-3, unc-32, aps-1,* F38A1.8
Metabolism	Energy production and intermediary metabolism	T17E9.2a, *vha-13*
Protein degradation	Components of the ubiquitin-targeted protein degradation machinery	*pbs-2, pbs-1, rpn-1*
Nucleic-acid binding	Nucleic acid-binding proteins with unknown specificity for DNA or RNA	*pqn-80*
Signal Transduction	Genes which encode for kinases, phosphatases and signal transduction pathway components.	*plc-3, arf-1, plc-1,itr-1, epi-1, lam-1*
Neuro	Consists of genes encoding the 7 transmembrane spanning receptors	*tag-49*
Unknown	Genes with insufficient information to assign a function, or genes with no significant matches in any organism.	*smgl-1,*Talin, *mdt-15,* T05A10.3, *noah-2, rps-28*

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