ABSTRACT

RNAi Screen for Novel Components in *Caenorhabditis elegans* Ovulation and Fertility

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The intercellular and intracellular signaling pathways elucidated through research on the nematode *C. elegans* provide valuable information on the communication systems throughout all organisms. Through the use of RNA interference (RNAi), it is possible to discover additional genes that may play roles in signaling pathways. The inositol trisphosphate (IP_3) signaling pathway maintains the basal and ovulatory contractions of the sheath cells in all *C. elegans* organisms. Utilizing an RNAi feeding protocol to knock down expression of genes, some 155 genes capable of causing sterility in wild-type *C. elegans* were identified. Focusing on these contractions of the sheath cells through control of the IP_3 signaling pathway, a mutant *C. elegans* for the IP_3 receptor, *itr-1(sy290)*, was used. The ITR-1 receptor, located on the endoplasmic reticulum, normally allows for the release of calcium ions when IP_3 binds, and is constitutively active in the *itr-1(sy290)* mutant worm. The mutant *itr-1(sy290)* worms maintain higher concentrations of cytoplasmic calcium, which resulted in a rescue of the sterility seen in the wild-type worms in this study. Due to the potential for pleiotropic effects of many of these sterility causing genes, we looked for known components of the IP_3 signaling

pathway (eg. *plc-3*) and at their sterility scores, as well as the scores most comparable to these known components. This reduced the gene pool down to 24 significant genes. Examination of these genes reveals a wider communication network necessary for proper ovulation in *C. elegans*.

RNAi Screen for Novel Components in *Caenorhabditis elegans* Ovulation and Fertility

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DEDICATION

To my family,

who has stood by me through everything

CHAPTER ONE

Introduction and Background

Sterility and Signaling

In 2006, 56% of women aged 15 to 44 had at least one child (Dye 2008), up slightly from 2004 which showed a 55.4% level (Dye 2005). The number of women not having any births has jumped over the past 30 years from 10% up to 20% (Dye 2008). This high percentage is due to multiple factors, including women waiting to have children until they've finished their education, other personal reasons, and due to infertility of one or both partners (Dye 2008). According to the American Society for Reproductive Medicine, infertility results from factors in women roughly a third of the time, primarily as ovulation disorders, with men counting for another third of the time and a combination of both sexes and unknown causes for the final third (American Society for Reproductive Medicine … [updated 2009]). In order for conception to occur, there are a plethora of factors that need to be in accord. Hormones and other cell signals must relay information to various parts of the body in order to coordinate the process of ovulation in women, maximizing the possibility of fertilization and implantation into the female uterus. Understanding all the signaling factors necessary for ovulation can aid in the eventual treatment of those afflicted with infertility.

All living cells must communicate with their environment via complex signaling pathways to regulate cell behaviors such as differentiation, migration, and adhesion. Proper cell signaling necessitates regulation due to the impact on cell functions, thus ensuring proper responses to the environment. Anomalies in cell signaling may manifest

in a variety of pathological forms including infertility, as described above, as well as tumorigenesis, inflammation and metabolic disorders.

Cell responses are generally mediated by receptor molecules which bind to specific ligands. Examples include insulin binding to receptors on cellular membranes causing the cells to uptake glucose from the blood. Defects in insulin secretion from the beta cells of the pancreas and/or insensitivity of the insulin receptors to the insulin molecule can result in the formation of the disease diabetes (Guillausseau and others 2008). Vertebrate ovaries' need for proper balance between apoptotic and survival signaling for the follicles is another example. Most follicles are apoptosed over time, with approximately 1% making it through to ovulation (Markstrom and others 2002). Disruption of these signals can result in the premature aging and failure of the ovaries, resulting in sterility (Krysko and others 2008). Indeed, evidence has been gathered that organochloride compounds can also disrupt the signaling pathways, leading to sterility (Tiemann 2008). Learning about the components of signaling pathways and how they function can aid those afflicted with diseases and disorders like diabetes and sterility.

Generally, induction of cell signaling pathways requires the formation of receptor-ligand complexes to elevate the level of second messengers inside normally functioning cells. Second messengers then induce massive activation of various downstream effectors. Included in this group are hydrophobic molecules (diacylglycerol, phosphatidylinositols) (Martin 1998) and hydrophilic molecules (Ca^{2+}, cyclic AMP) (Berridge, Lipp, Bootman 2000; Tasken and Aandahl 2004). These second messengers are responsible for many aspects of cellular responses to the environment, from movement of the cells to altered transcription of genes.

Intracellular calcium ions ($[Ca^{2+}]i$) is a second messenger molecule that activates many cellular events by binding to calcium interacting elements within the cell. For example, $[Ca^{2+}]i$ binds to a calcium mediator protein, calmodulin (CaM), which activates many downstream molecules in $[Ca^{2+}]i$-dependent manner (Berridge, Lipp, Bootman 2000). For example, the $[Ca^{2+}]i$-CaM complex activates calcineurin (CNB-1, $[Ca^{2+}]i$ dependent serine/threonine phosphatase) that plays an important roles in locomotion and egg-lying activities in the model organism, *C. elegans*, a soil nematode described in greater detail below (Bandyopadhyay and others 2002; Bandyopadhyay, Lee, Bandyopadhyay 2004; Lee and others 2004).

Caenorhabditis elegans

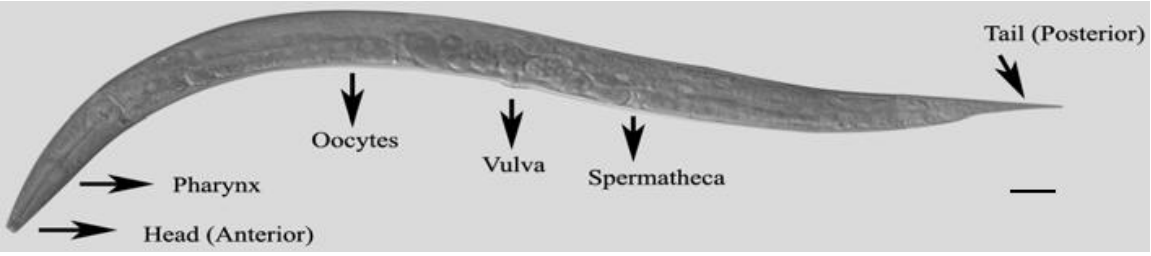

Figure 1.1. DIC image of adult hermaphrodite (magnification 400X), black bar = 40µm.

The model organism *Caenorhabditis elegans* (Figure 1.1) shares many essential biological characteristics that are central problems of human biology, making it an attractive organism to study. At least 83% of the protein sequences in *C. elegans* have orthologous proteins in humans, so genetic and proteomic studies in these nematodes can elucidate the functions of similar genes and proteins in humans (Lai and others 2000). Due to its ease of growth and maintenance, and genomic similarity to humans, this free-living soil nematode is particularly well-suited for genetic, molecular, and cellular approaches to the analysis of development (Brenner 1974; Wood 1988).

C. elegans has only about 1,000 somatic cells, organized into many different cell and tissue types with the complete cell lineage known from zygote to adult (Sulston and Horvitz 1977). There are many characteristics that make this organism amenable to the genetic dissection of developmental processes: short generation time (3.5 days), large numbers of progeny by self-fertilization (300), size (1 mm in length), ease of cultivation (agar plates or liquid culture), easily observable mutant phenotypes (uncoordinated, dumpy, roller, blister, long, multivulva, etc.), hermaphroditic (self-fertilization) and sexual modes of reproduction, and the ability to be revived after freezing (Brenner 1974; Hubbard and Greenstein 2000). Furthermore, the transparency of *C. elegans* allows development to be followed at the cellular level in living animals and also makes possible laser ablation of specific cells to determine their role in development (Kimble 1981).

The *C. elegans* nematode has also proved useful in many molecular techniques. The small genome size of this nematode (approx. 100 megabases), the existence of a set of overlapping cosmid clones spanning almost the entire genome (Kamath and Ahringer 2003), the fact that the entire sequence of the *C. elegans* genome has been completed (C. elegans Sequencing Consortium 1998), all combined with RNA-mediated interference of gene expression techniques have greatly advanced the molecular biology of the worm. Indeed, it has come to the point where almost any gene can be cloned and/or silenced with relative ease. Additionally, the ability of the worms to systematically respond to RNA-mediated interference has been especially useful in recent years, as described in sections below.

C. elegans undergo four larval stages prior to reaching adulthood, termed L1 through L4. During each of these larval stages, there are a number of genes and proteins

that are up and down regulated through the usage of heterochronic genes. The heterochronic genes coordinate these larval transitions through a pathway relying upon reactions with other heterochronic genes as well as various transcription regulators (Ambros 2000). Initially, the concentration of protein LIN-14 is relatively high during the first larval stage, aiding the transcription of larval proteins during this time (Lee, Feinbaum, Ambros 1993; Wightman, Ha, Ruvkun 1993). As the worm progresses, *lin-4* microRNA (miRNA) production increases and binds multiple sites of the *lin-14* mRNA 3' UTR, preventing the translation of more LIN-14 proteins (Lee, Feinbaum, Ambros 1993; Olsen and Ambros 1999; Wightman, Ha, Ruvkun 1993). The *lin-4* miRNA additionally represses *lin-28* mRNA translation through a single 3' UTR binding site (Moss, Lee, Ambros 1997; Seggerson, Tang, Moss 2002). The *lin-28* gene generally affects the larval to adult switch through the repression of some heterochronic genes and increasing the concentrations of others. Genes targeted either directly or indirectly by *lin-28* include *let-7*, *lin-41*, *lin-57* and *lin-29* (Abrahante and others 2003; Ambros 1989; Lin and others 2003; Rougvie and Ambros 1995; Slack and others 2000).

The late larval stages are crucial to the formation of the *C. elegans* gonad. Decreasing the concentration of *lin-28* mRNA causes the *let-7* mRNA concentration to increase in the organism (Reinhart and others 2000). During the later larval stages, *let-7* miRNA prevents translation of the *lin-41* mRNA in some tissues, and appears to degrade the *lin-41* mRNA itself (Bagga and others 2005; Reinhart and others 2000; Slack and others 2000). Loss of LIN-41 transitions the worm from the L3 into the L4 stage, and is necessary for normal, proper progression to fully functional adults.

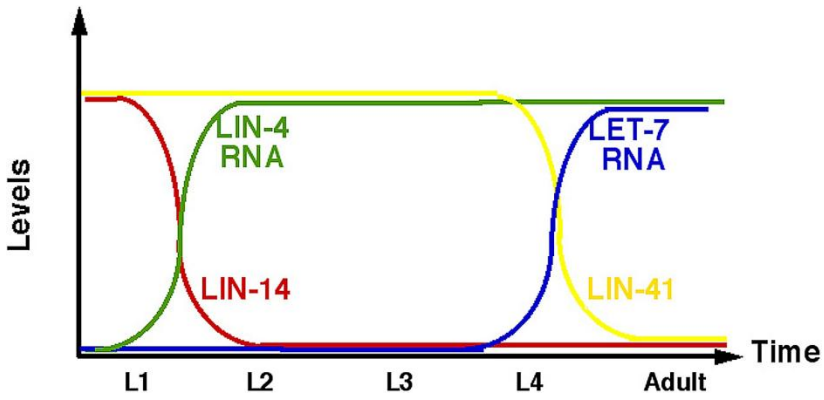

Figure 1.2. Graphical representation of relative levels of some heterochronic proteins over time. (Vella and Slack 2005)

Disruptions in any of the levels of the heterochronic genes can cause drastic effects in *C. elegans*. Often certain larval stages may be bypassed causing a precocious adult worm, or stages may be reiterated, resulting in the duplication of some cells and the stunting of normal growth. One example includes the *lin-41* gene, which requires a drop in protein expression to move *C. elegans* from the L3 larval stage into the L4 larval and adult stages (Slack and others 2000). Null mutations of this gene show a precocious development of adults from the L3 larval stage (Slack and others 2000). Germ cells in these worms refrain from transitioning to proper oocyte production, resulting in a sterile phenotype.

C. elegans Gonad and Ovulation

The gonads of *C. elegans* consist of two U-shaped tubular arms led by a distal tip cell (DTC) and covered with basement membrane, a sheet-like extracellular matrix (ECM) (Figure 1.3). The basement membrane ECM provides structural stability and signaling capabilities (Xu and others 2005). Each gonad arm connects to a spermatheca, which connects to a central uterus (Figure 1.3) (Montell 1999). Germ cells are produced

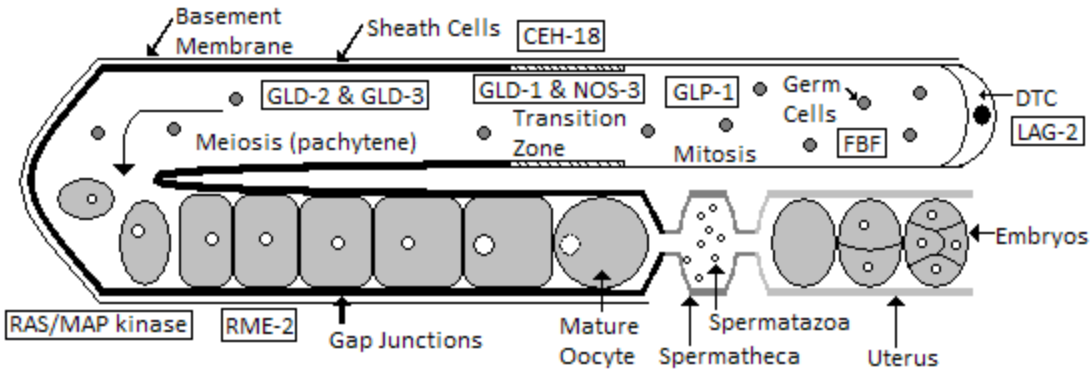

Figure 1.3. Diagram of *C. elegans* gonad with normal oocyte development. The basement membrane surrounds the somatic gonad, and provides stability to the gonad by acting as an anchor, as well as providing signals from the rest of the organism. Mitosis occurs in the distal portion of the gonad, ensuring a steady supply of germline stem cells, under control of the DTC. Movement of the germ cells away from the DTC allows entry to the transition zone (TZ) where the cells begin to undergo meiosis. As the germ cells begin to round the U-shaped gonad, they begin to accumulate yolk proteins secreted into the gonad from the adult worm's stomach, becoming larger during their developmental process and establishing gap junctions with the somatic gonad sheath cells. Spermatazoa, developed during the worm's L2-L3 larval stages, are sequestered in the spermatheca, where the maturing oocyte will enter for fertilization. Fertilized oocytes quickly exit the spermatheca and enter the uterus where they wait to be laid.

near the distal end of the tubular arms. As they move away from the DTC, they become cellularized and undergo the process of oogenesis in the proximal area of the arms (Hall and others 1999). Myoepithelial sheath cells encapsulate the proximal gonad and maintain shape and cause contractions to move the maturing oocytes torward the spermatheca (Hall and others 1999; Hubbard and Greenstein 2000). Gap junctions connect the sheath cells to the oocytes, allowing for communication between the two for proper ovulation control (Hall and others 1999; Miller and others 2001; Whitten and Miller 2007).

Prior to ovulation, oocytes need to mature in preparation for the fusion with sperm, localized in the spermatheca. However, in the distal most portions of the gonad arms, germ cells multiply via mitosis and are kept from entering meiosis through several

signals near the distal tip cell (DTC). Spatially restricted signaling of the germ cells is controlled via three main genes: *glp-1, lag-1,* and *lag-2* (Qiao and others 1995). Genes *glp-1* and *lag-2* both code for transmembrane proteins though protein expression occurs in different cells. LAG-2 expression localizes in the DTC and allows for continued mitotic germ cell proliferation by binding GLP-1 receptors on the germ cells (Henderson and others 1994; Pepper, Killian, Hubbard 2003). The LAG-1 protein is an intracellular DNA-binding transcription factor that functions in response to signals from GLP-1 in the germ cells (Christensen and others 1996). This system of signaling is orthologous to the Notch signaling seen in *Drosophila*, and the GLP-1 receptor is orthologous to the LIN-12 receptor found on somatic cells, which is why this system is often referred to as the LIN-12/Notch signaling system (Greenwald 2005). Loss-of-function mutations in these genes cause all germ cells to enter the meiotic pathway, as the lack of signaling prevents the necessary mitotic pathway genes from being transcribed and translated.

Additional factors influence the mitotic/meiotic decision of the germ cells through their influence on the signaling pathway. The FBF proteins (FBF-1 and FBF-2) are nearly identical RNA-binding proteins that influence the mitosis/meiosis decision by downregulating the translational repressor GLD-1 through binding the *gld-1* mRNA (Crittenden and others 2002). As germ cells move out of the mitotic region, GLD-1 expression is first seen as they leave the influence of the FBF proteins (Hansen and others 2004; Jones, Francis, Schedl 1996). GLD-3, along with GLD-2, is believed to help regulate the FBF proteins by binding them and allowing germ cells to express the mRNAs the FBF normally binds, thus allowing germ cells to exit the mitotic pathway and enter meiosis (Eckmann and others 2002). The GLD-3 protein also influences the

sperm/oocyte decision (Eckmann and others 2004), along with the FBF proteins (Kraemer and others 1999) and NOS-1, an RNA-binding protein also responsible for germ cell viability early in development (Subramaniam and Seydoux 1999).

Another protein necessary for proper meiotic maturation and oocyte progression is CEH-18, which is expressed in the sheath cells. This protein influences oocyte defects when downregulated due to defects in the sheath cells (Greenstein and others 1994; Rose and others 1997). In the absence of major sperm protein (MSP) in the gonad, CEH-18 along with VAB-1 (described below), prevent meiotic maturation and MAPK activation to preserve nutrients and components until needed for procreation (Jud and others 2008; Miller and others 2003). This highlights the influence the somatic tissue has over the germ cells and their fates.

During normal germ cell development, *glp-1* rapidly decreases in the transition zone as the distance between the germ cells and DTC increases, and the germ cells begin to turn the corner of the tubular arm (Crittenden and others 1994). Oogenesis begins in this loop region and continues through the proximal gonad. Germ cells become locked in the pachytene stage of mitosis until they receive signals through the RAS/MAP kinase pathway which allows pachytene exit into diplotene/diakinesis of meiotic prophase I (Church, Guan, Lambie 1995; Schedl 1997). Yolk proteins secreted into the proximal most oocytes from the parental worm's intestine, into the pseudocoelom, then through the sheath cells' gap junctions makes the oocyte larger and more readily identifiable (Hall and others 1999). The nucleolus disappears about 70 minutes prior to ovulation, and the nucleus migrates to the distal portion of the oocyte, though no other sign of polarization is present (Schedl 1997; Ward and Carrel 1979). Maturation of the oocyte proceeds with

nuclear envelope breakdown (NEBD) approximately 5-6 minutes before ovulation (Schedl 1997). The oocyte changes conformation, becoming spherical and less cuboidal at 3 minutes before ovulation (Schedl 1997). Oocyte swelling signals the sheath cells to increase contractions, resulting in the dilation of the distal spermatheca and allowing movement of a single oocyte into the spermatheca for fertilization (McCarter and others 1999). Sheath cell contractions increase from the basal rate of 10-13 contractions per minute up to 19 per minute and higher (McCarter and others 1999). Approximately 4-5 minutes after the oocyte undergoes meiotic maturation, the sheath cell ovulatory contractions are initiated and suppressed quickly after the oocyte enters the spermatheca (McCarter and others 1999). Once in the spermatheca, the oocyte becomes fertilized almost immediately (Ward and Carrel 1979), and meiosis I and II proceed in the uterus. This process occurs repeatedly throughout the *C. elegans*' lifespan, cycling another oocyte from the assembly-line gonad approximately every 23 minutes (McCarter and others 1999).

Normal ovulation control requires several key components. The binding of major sperm protein (MSP) from the spermatheca to VAB-1/Ephrin receptors on the sheath cells triggers contraction of the sheath cells, as well as dilation of the spermatheca (Figure 1.2) (Miller and others 2001; Miller and others 2003). It has been shown that the MSP is responsible for the basal contraction rate of the sheath cells (Miller and others 2001; Miller and others 2003). This basal rate tends to range between 7 and 8 contractions per minute in the wild-type *C. elegans* (McCarter and others 1999). The MSP is also responsible for triggering meiotic maturation in the most proximal oocytes, as oocyte progression halts in its absence, and oocyte microtubule reorganization occurs

when MSP is present (Harris and others 2006; Kosinski and others 2005; Miller and others 2001). Oocyte microtubule reorganization occurs through a signaling network involving *ceh-18* components Gα(o/i) and Gα(s), as well as gap-junction communication with somatic cells of the gonad (Govindan and others 2006; Govindan and others 2009; Harris and others 2006; Miller and others 2003).

A voltage-gated ClC channel, CLH-3b, has been found on the surface of *C. elegans* oocytes and been linked to suppressing ovulatory sheath cell contractions (Denton and others 2004; Rutledge and others 2001). RNAi against the *clh-3* causes an early onset of ovulatory sheath cell contractions (Rutledge and others 2001). Once the oocyte has swollen, CLH-3b activates allowing an influx of Cl^- ions which appears to aid in the suppression of the signals for sheath cells contractions (Rutledge and others 2001).

Once oocytes have properly matured, they secrete LIN-3/EGF, which activates LET-23/EGFR on the sheath cells to increase contractions through the IP_3 signaling pathway (Figure 1.2) (Clandinin, DeModena, Sternberg 1998; Iwasaki and others 1996). LIN-3 is initially expressed as a transmembrane protein and cleaved to allow secretion of the protein, allowing it to bind to LET-23 (Liu and others 1999). Disruption of the *lin-3* or *let-23* genes in *C. elegans* results in spermathecal defects (Yin and others 2004). While no significant differences have been observed in the rates of contractions for these *lin-3* and *let-23* mutants, delays in the initiation of ovulatory sheath cell contractions have been noted (Yin and others 2004). The primary signaling pathway used in the movement of oocytes through the gonad is the inositol 1,4,5-trisphosphate (IP_3) pathway, in which IP_3 binds to its receptor on the endoplasmic reticulum and allows for the release of stored calcium causing the sheath cell contractions and prevents sterility.

Sterility and Its Forms

Sterility is an all encompassing term for any resultant inability to produce progeny, both maternally and paternally. From this and other work, there are several ways in which sterility can be classified. Below we describe the most common forms identified in *C. elegans* (Table 1.1).

Oocytes unable to enter the spermatheca typically result in an endomitotic oocyte (Emo) phenotype. The Emo phenotype is classified as an oocyte in the proximal gonad that results in a lack of spermatheca dilation or proper sheath cell contractions, and hence is unfertilized, yet still exhibits maturation and chromosomal replication (Schedl 1997). As the oocyte cannot be fertilized, the worm is sterile, though this accounts for only two of the forms. While both result in Emo phenotypes, they differ in how they function. Sheath cell contractions help propel maturing oocytes through the proximal gonad, and up to the spermatheca. Spermathecal dilation relies upon a set of signals for it to function properly that differ from the surrounding tissues, mainly the use of PLC-1 in the cleavage of PIP_2 into IP_3 and DAG, as regulated by FOS-1 and JUN-1 (Hiatt and others 2009; Kariya and others 2004). Sheath cell contractions, in contrast, rely on PLC-3, as described in detail below. Allowing differences between these tissues helps regulate the timing of events necessary for proper ovulation and result in similar phenotypes when defects present from these different tissues.

Other forms of sterility include defects in sperm production and defects in oocyte production (Schedl 1997) (Table 1.1). A lack of sperm in the spermatheca will also result in a lack of MSP production, necessary for the basal rate of sheath cell contractions, as described above. In this instance, maturing oocytes may still move through the proximal

Table 1.1. Criteria for classification of the form of sterility for *C. elegans*

Form of Sterility	Criteria
Sheath Cell Contraction Defects	Rate of sheath contractions, Initiation of contractions, Strength of contractions differ significantly from wild-type (Schedl 1997)
Spermathecal Defects	Initiation of spermathecal widening differs significantly from wild-type, Premature closing of spermatheca, Non-functioning (Schedl 1997)
Oocyte Developmental Defects	Oocyte production, Maturation events differ significantly from wild-type (Schedl 1997)
Sperm Production Defects	Sperm production, Sperm location during fertilization differ significantly from wild-type (Schedl 1997)

gonad due to spatial restrictions in the gonad, but the lack of sperm prevents maturation of oocytes (Govindan and others 2009; Nadarajan and others 2009). Additionally, signals necessary for the transition from spermatogenesis to oogenesis allow proper development of oocytes in the worms. Without proper GLD-2 signaling, for example, worms become sterile due to a lack of oogenesis (Kim and others 2009). Additionally, disruptions in heterochronic gene expression can result in a lack of oocyte development, as is the case with *lin-41* (Slack and others 2000).

Whether through disruptions in gamete development, defects in sheath cell contractions or imprecise spermathecal dilation, the resultant sterility remains the same. For the purposes of this dissertation, focus was placed on the sheath cell contractions as controlled by inositol 1,4,5-trisphosphate signaling.

Inositol 1,4,5-trisphosphate (IP_3) Signaling Pathway and Components

The IP_3 signaling pathway generates the sheath cell contractions by releasing the stores of calcium built up in the endoplasmic reticulum. Receptor tyrosine kinases act

within the sheath cells to stimulate the phospholipases responsible for the generation of the IP_3 molecules (Berridge 1993; Majerus 1992). In the case of the *C. elegans*' gonadal sheath cells, the LET-23/epidermal growth factor receptor (EGFR) responds to the secreted LIN-3/epidermal growth factor (EGF) produced by the maturing oocyte to activate phospholipases (Yin and others 2004). This ensures that only oocytes ready for ovulation trigger the necessary sheath cell contraction force to push the oocyte through to the spermatheca where fertilization occurs. Loss of function through deletion or mutation of LET-23 and/or LIN-3 has resulted in the maternally sterile phenotype (Yin and others 2004).

The Rho/Rac-family guanine nucleotide exchange factor VAV-1 has been shown to be essential for proper rhythmic control of contractions in the pharynx, gonadal sheath and defecation cycles (Norman and others 2005). Initial studies of VAV-1 deletion mutations in *C. elegans* resulted in larval arrest and death, due to the inability of the worms to feed properly. Rhythmic contractions are necessary to move food through the pharynx and intestines, similar to the contractions seen to excrete waste products and the movement of oocytes through the gonads. By expressing the *vav-1* trans gene in the pharynx, worms grew into adulthood. Of particular note, a greatly reduced brood size was seen, and could be rescued utilizing known components of the IP_3 signaling pathway (Norman and others 2005).

Each of the genes described above has been shown to play their roles upstream of the calcium release necessary for sheath cell contractions. The protein most directly responsible for the release of the stored calcium from the endoplasmic reticulum is the inositol 1,4,5-trisphosphate receptor (ITR-1). Gain-of-function mutations created in the

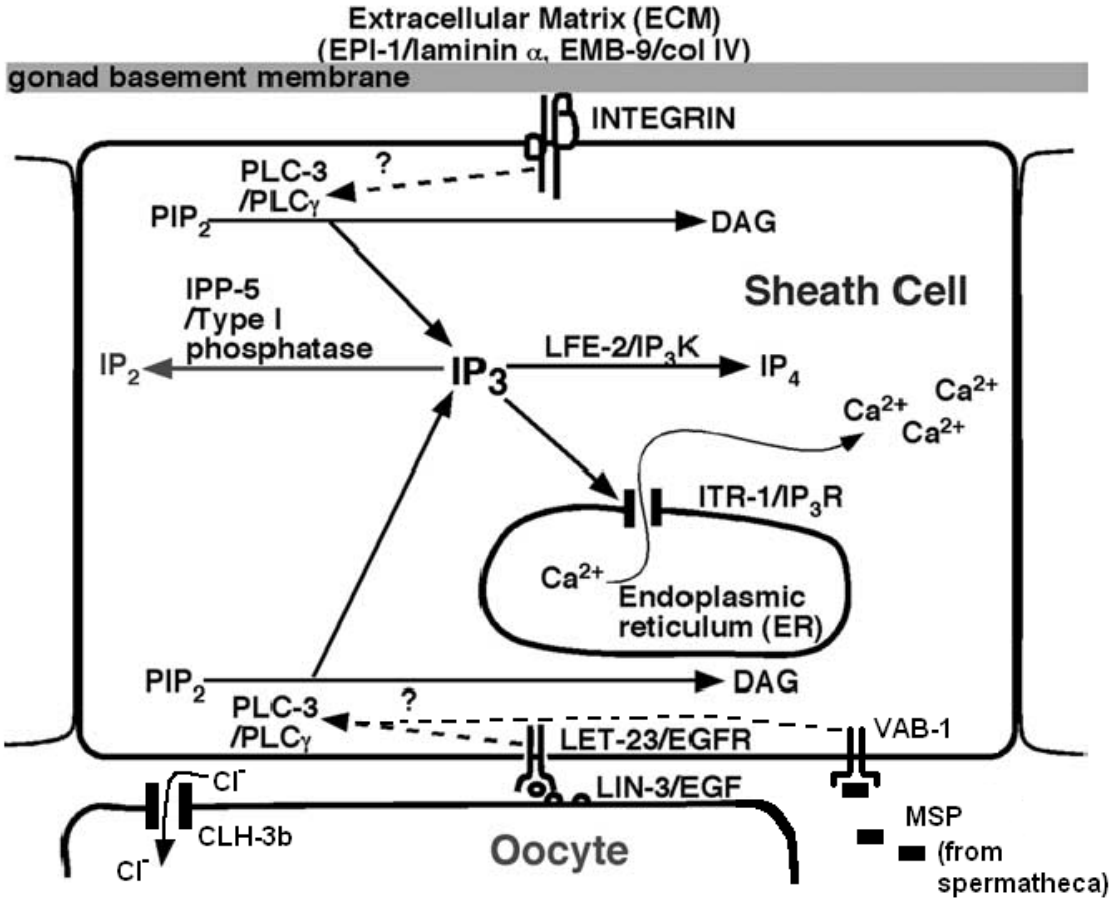

Figure 1.4. The necessary components for contraction within the gonadal sheath cell. Signals from gametes activate the phospholipase, creating IP_3 which then binds to its receptor, ITR-1, on the ER where calcium is released to allow muscle contraction.

itr-1 gene rescue brood-size suppressing/sterility inducing mutations and RNAi of components upstream of calcium release (Clandinin, DeModena, Sternberg 1998). Constitutively active ITR-1 has been shown to suppress sterility in loss-of-function alleles in the LIN-3 and LET-23 genes through increased contractility (Clandinin, DeModena, Sternberg 1998; Xu and others 2005). Additionally, ITR-1 gain-of-function mutants have also been shown to increase the normally low brood size found in VAV-1 mutants (Norman and others 2005). This all occurs after phosphotidylinositol 4,5-bisphosphate (PIP_2) cleavage forms IP_3 and diacylglycerol (DAG), when the IP_3 is free to

move in the cytosol and interact with ITR-1 to open the ER channel (Baylis and others 1999; Berridge 1993) (Figure 1.2).

IP_3 production occurs through hydrolysis of PIP_2 into DAG and IP_3 by the enzyme phospholipase C (PLC) (Yin and others 2004). There are six genes that code for phospholipases in *C. elegans* worms: *egl-8*, *plc-1*, *plc-2*, *plc-3*, *plc-4* and *pll-1* (Yin and others 2004). Of these, only *plc-1* and *plc-3* have shown to significantly affect fertility (Kariya and others 2004; Yin and others 2004). While *plc-1* has shown reduced fertility (Kariya and others 2004), experiments using *plc-3*(RNAi) have shown it causes complete sterility, as well as having an endomitotic oocyte (Emo) phenotype (Yin and others 2004). Gene expression studies utilizing green fluorescent protein (GFP) have shown PLC-3 to be primarily expressed in the gonadal sheath cells and the spermatheca (Yin and others 2004), while PLC-1 was relegated to the spermatheca and vulva (Kariya and others 2004). Both were also seen in the intestines of the nematodes, understandably as contractions must also occur there to move food through the worms for digestion and excretion (Kariya and others 2004; Yin and others 2004).

In order to control the concentration of IP_3 in the sheath cells, and ultimately the rate of sheath cell contractions, enzymes LFE-2 and IPP-5 (Bui and Sternberg 2002) modify IP_3 into other forms unable to trigger ITR-1 (Figure 1.4). The *lfe-2* gene codes for an IP_3 3-kinase, which adds an additional phosphate, negatively regulating the pathway and enabling proper control of spermathecal dilation and ovulation (Clandinin, DeModena, Sternberg 1998). Similarly, *ipp-5* helps negatively regulate the IP_3 pathway, but is a phosphotase, cleaving a phosphate in the 5' position from the IP_3 to produce IP_2 (Bui and Sternberg 2002). Deletions of *ipp-5* result in incorrect closing of the

spermatheca, allowing multiple oocytes through at a single time (Bui and Sternberg 2002).

Endoplasmic Reticulum and Calcium Release. As described above, for the IP_3 signaling pathway to properly regulate the sheath cell contractions necessary for ovulation, calcium must be released from its primary storage site, the endoplasmic reticulum. This cellular organelle provides multiple functions to the cell, including protein synthesis and folding, protein transportation to the Golgi apparatus and cellular membrane, and signal transduction (Berridge 2002). These multiple functions are made possible by the segregation of some duties, with protein synthesis being regulated to the rough ER and signal transduction being a primary function of the smooth ER (Berridge 2002).

In order to safeguard the production of proteins, cells developed an unfolded protein response (UPR). The UPR protects cells by monitoring proteins folded by the chaperones and reacts to an accumulation of mis-folded proteins. Initially, the protein synthesis machinery slows production of any new protein synthesis (Harding, Zhang, Ron 1999), while inducing the creation of additional chaperones to alleviate the building pressure (Kozutsumi and others 1988). Studies indicate that the ER also increases in size to dilute the concentration of proteins in the area as well (Dorner, Wasley, Kaufman 1989). In cases where the backup of proteins cannot be alleviated, cellular functionality gets lost and the ER induces apoptosis (Davis and others 1999; Dimcheff and others 2003).

Apoptosis is accomplished through signals sent to the mitochondria, thus protecting the organism from developing additional problems. Prolonged UPR causes

disruptions to calcium homeostasis and leads to cell apoptosis through activation of caspase-12 (Tessitore and others 2004). This programmed cell death can lead to drastic effects on organisms as a whole, possibly including sterility.

RNA Interference and Mechanism

RNA interference (RNAi) is a cellular response to the presence of double stranded RNA. This type of molecule is rare, most commonly seen in viruses (Blevins and others 2006) and capable of causing interferon responses in both mammalian and non-mammalian species (Reynolds and others 2006; Schultz, Kaspers, Staeheli 2004), though interestingly the interferon response is not found in mouse oocytes and preimplantation embryos (Stein and others 2005). Medically, the ability to utilize this RNAi cellular response has brought about the potential for novel gene therapies (Shuey, McCallus, Giordano 2002).

Initial experiments by Fire et al, in 1998, with RNAi involved injection of dsRNA when the worm was still in the single cell embryo stage (Fire and others 1998). This process dealt with embryonic RNAi, as the embryos themselves were susceptible to the interference. This differs from post-embryonic RNAi, in which the eggs hatch and the worms take up the dsRNA when in the larval stages, or as adults. When mRNA for *unc-22*, which encodes the muscle protein twitchin, was produced, the dsRNA molecules prevented expression of *unc-22* through the RNA-induced silencing complex (RISC), described below. The team also used the dsRNA against *gfp*, which allowed visualization of the decrease in fluorescence. The dsRNA was shown to be able to cross cellular boundaries, from somatic and gonadal tissues, allowing systemic RNAi knockdown. The general expression level of the mRNA needs to be high enough for the

dsRNA to interact with, as low expression levels may escape detection by the mRNA degradation complex (Fire and others 1998).

In order for RNAi to work effectively, dsRNA in length from 21-25 nucleotides work best. The protein DCR-1, commonly referred to as Dicer, is an RNase III nuclease that has been shown to cleave double stranded RNA into these appropriately sized dsRNA fragments (Ketting and others 2001). Mutants in the *dcr-1* gene have been shown to be defective in their RNAi response, at least in the germ line, as well as having developmental abnormalities (Bernstein and others 2001; Ketting and others 2001). The *dcr-1* gene codes for a ribonuclease which aids in the processing of RNAs for the RISC (Ketting and others 2001). These small interfering RNAs (siRNAs) can be formed by Dicer if the initial dsRNA is larger than the optimal length (Bernstein and others 2001; Ketting and others 2001). Once the optimal size has been obtained, the siRNAs are incorporated into the RISC which utilizes the siRNAs as templates for mRNA degradation (Bernstein and others 2001). However, in order for the RISC to function optimally, the siRNA has to be unwound, which is done by a DExH-box helicase (Nykanen, Haley, Zamore 2001; Tabara and others 2002).

C. elegans are of particularly great use with RNAi as they are capable of producing a systemic organismal response to dsRNA. Through all of the various methods of RNAi induction (described below), dsRNA is capable of crossing between cells due to pores specific for dsRNA encoded by the genes *sid-1* and *sid-2* (Feinberg and Hunter 2003; Winston, Molodowitch, Hunter 2002; Winston and others 2007). The only cells that appear rather resistant to RNAi are the neurons, though some work has been

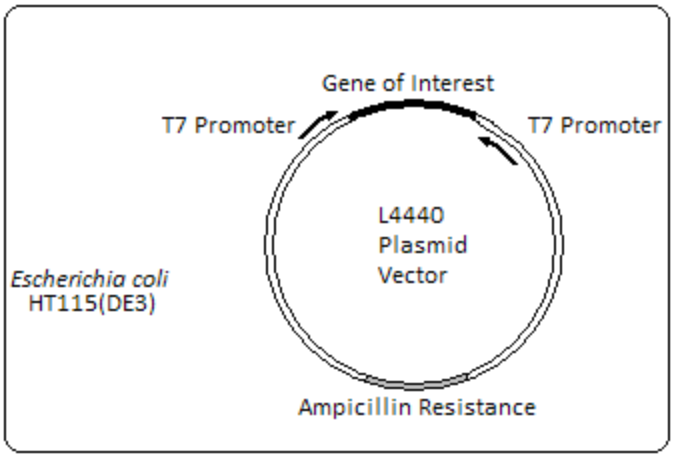

Figure 1.5. The plasmid construct L4440 within an HT115(DE3) *E. coli* bacteria. The plasmid contains the gene of interest to be knocked down via ingestion in between the dual T7 promoters, ensuring copies of the RNA are produced in both directions to produce the dsRNA when in contact with isopropyl-beta-D-thiogalactopyranoside (IPTG) or lactose (Kamath and others 2001).

done in producing worms and cell cultures capable of silencing genes in those cells as well (Krichevsky and Kosik 2002).

dsRNA Producing Bacteria. A bacterial library has been constructed containing 86% of the *C. elegans* genome, allowing researchers to much more easily screen for specific genes using a feeding protocol (Kamath and others 2003). In the case of this library, the L4440 vector was used, which contains T7 promoter regions on both sides of the multiple cloning site, allowing transcription in both directions to produce complementary strands of RNA (Timmons, Court, Fire 2001) (Figure 1.5). Polymerase chain reaction (PCR) products using the Research Genetics *C. elegans* GenePairs primer set for only those genes believed to encode proteins (Kamath and others 2003). The bidirectional transcription of the plasmid allows for the formation of double stranded RNA (dsRNA). Transformation of the plasmids into HT115(DE3), *Escherichia coli* bacteria that are deficient in RNase III activity, proved best in the generation of the

dsRNA (Timmons, Court, Fire 2001). These *E. coli* have inducible expression of T7 polymerase when in the presence of isopropyl-beta-D-thiogalactopyranoside (IPTG) or lactose (Timmons, Court, Fire 2001). This ensures that dsRNA is only produced when researchers' desire.

Methods of RNAi Induction. Early in RNAi experiments, introduction of dsRNA into *C. elegans* was done primarily using injection methods and has continued to be used (Fire and others 1998; Guo and Kemphues 1995; Piano and others 2000; Piano and others 2002). Since the dsRNA is not confined by cellular boundaries, injection site does not matter, unless looking at some mutant worm strains (Tijsterman and others 2004; Timmons and Fire 1998). *C. elegans*' ability to allow dsRNA to cross cellular boundaries has enabled scientists to use other methods of RNAi induction, including soaking the animal in lipid drops containing dsRNA (Fernandez and others 2005; Maeda and others 2001; Tabara, Grishok, Mello 1998), feeding (Kamath and others 2001; Simmer and others 2003; Timmons, Court, Fire 2001) and transgenic modifications using hairpin RNAs the worms then produce themselves (Tavernarakis and others 2000). Each method has its own advantages and disadvantages.

The injection method generally takes a long time and only allows for the injection of several worms. Individual worms are paralyzed and each has to be injected with the RNAi solution, making large sample sizes impractical. Injections for sterility experiments would have to be done in the gonad of the worms in order to test the post-embryonic fertility, so location plays a role as well (Fire and others 1998; Piano and others 2000). Injection of dsRNA into the distal portion of the gonad allows it to be taken up into the forming oocytes when they exit from the syncitium of nuclei seen in the

distal gonad. As the cellular membrane forms around the nucleus, the dsRNA gets incorporated. Oocyte maturation is the product of the activation of certain genes from the nucleus, and the dsRNA is capable of disrupting those genes for which it shares sequence similarity.

An additional possibility for RNAi induction is the soaking method. In this method, worms were added to the dsRNA solution for 24 hours, after which, worms were typically plated onto OP50 seeded NGM plates for a given number of hours to lay eggs (Fernandez and others 2005; Maeda and others 2001). The worms literally soak up the dsRNA, which initiates the RISC proteins. While in the solution, the worms take in the dsRNA orally and it can be spread throughout the worms' bodies via the pore formed by SID-1, as described above. This method allows more worms to be used, and is less time consuming than the injection method, yet has not been shown to be as potent as injection or feeding methods (Tabara, Grishok, Mello 1998). The addition of the chemical spermidine has been shown to increase efficiency however (Maeda and others 2001).

The feeding protocol is the simplest of RNAi protocol to utilize in the lab. *C. elegans* eat bacteria through their mouths and break them down in their pharynx. The uptake of nutrients in the gut also allows the systemic uptake of dsRNA (Timmons and Fire 1998). The data gathered showed efficacy, though less than in the injection method. The feeding protocol has been enhanced through the use of RNase deficient bacterial strains, as well as the inducible expression of T7 polymerase when in the presence of IPTG or lactose (Timmons, Court, Fire 2001).

Studies have shown that the possibility of having false positives of RNAi phenotypes in the worms is only about 0.4%, though there is a larger percentage (10-

30%) in terms of false-negatives, most likely due to changes in conditions during the screenings, both between and in the same labs (Simmer and others 2003). The ability of the feeding protocol to allow researchers to screen a large number of worms, as well as the creation of the dsRNA bacterial library available for purchase, makes the feeding protocol the easier to use.

Previous RNAi Screenings

Genome wide screenings using RNAi have been instrumental in further illuminating *C. elegans* (Kamath and Ahringer 2003; Kamath and others 2003; Maeda and others 2001; Piano and others 2000; Piano and others 2002; Simmer and others 2003). Each of these has shed light onto a great number of gene functions, yet has focused on different aspects.

One of the first genomic studies of *C. elegans* using RNAi was performed by Piano et al. in 2000, and focused primarily on the expression of genes in the ovary. From their data, 81 additional genes were identified to be involved in embryogenesis. Utilizing the injection method, researchers sought worms laying more than 20% dead embryos between 15 and 24 hours post-injection, and made video recordings of them. At least 10 young adult worms were injected with the dsRNA, then moved and singled out for recording and dissection, as needed.

Another paper published that year focused solely on Chromosome I of *C. elegans* (Fraser and others 2000). Utilizing the feeding protocol, the group made a library of RNAi bacteria for the first chromosome. They also used between 10 and 15 worms to do their study and focused on the clearly identifiable phenotypes that resulted. The general

breakdown of the phenotype classes focused on sterility, embryonic lethality and other post-embryonic phenotypes.

In 2001, Maeda et al. published an RNAi screening that utilized the soaking method. The addition of spermidine to the protocol greatly increased the efficiency of the soaking method. Researchers first looked at RNAi for which the loss-of-function phenotypes were already well known. In their methods, the researchers soaked 4 L4 hermaphrodites in the dsRNA solution per RNAi, and after 24 hours transferred them to plates to identify the phenotypes of the worms and their progeny through DIC microscopy and DAPI staining.

Piano's group, in 2002, published another RNAi study, this time looking at gene clustering based on the RNAi phenotypes they observed. The study first looked at the microarray analysis from another group and then performed RNAi on their data (Reinke and others 2000). Injections were handled as Piano's previous RNAi study had done, once again using 10 adult worms per RNAi. The study also looked at the reproducibility of RNAi experiments from other labs. Dosage sensitivity and differences in scoring criteria are thought to be the main causes of discrepancies between the studies.

In 2003, Kamath et al. created an RNAi genome library using the methods utilized in Fraser's 2000 paper. The library comprised about 86% of the *C. elegans* 19,000 genes. The group then utilized the RNAi feeding protocol. L3-L4 stage hermaphrodites were fed RNAi for 3 days. Three worms were then cloned to individual wells and allowed to lay eggs. The small sample size allowed for rapid screening of the genome. However, because of the small sample size, the results may not be as accurate as other screens.

Simmer et al., also from Julie Ahringer's lab, published a paper in 2003 which focused on the use of the worm strain *rrf-3(pk1426)*, which was explained above. More phenotypes were discovered using this *rrf-3* mutant strain, likely due to less competitiveness between the RdRPs, since RRF-3 is knocked out. The results from the study are extensive and cover a large amount of the *C. elegans* genome. However there are some problems with the *rrf-3* mutant strain, mostly being a high-incidence of males, as well as an increase in embryonic lethality and reduced brood size (Simmer and others 2002). For these reasons, some of the results should be held suspect. The sample size used referred back to previous studies done by Fraser et al. and Kamath et al. in 2000, which used 10 to 15 worms per RNAi. Requirements necessary to be labeled "sterile" were that the brood size be fewer than 10 for the wild-type strain, and less than 5 for the *rrf-3* strain.

Dissertation Overview

Studying ovulation in *C. elegans* provides a useful model for the IP_3 signaling pathway. Sterility was examined in wild-type *C. elegans*, along with rescuing that sterility with a gain-of-function *itr-1* mutant nematode. Utilizing the RNAi feeding methodology, the IP_3 signaling pathway was focused on, unlike the broader spectrum analyses from previous RNAi screens. The list of candidate genes generated for the sterility phenotype was based upon previous RNAi data, although this screening used a larger sample size than any previous screens. The use of multiple *C. elegans* strains allowed identification of novel genes of the IP_3 signaling pathway, made possible through these tenuous sterility trials. Discovery of what roles the genes possess and where these genes are primarily located resulted from the use of these worm strains. From these

results, 23 genes have been shown to be rescued when in the *itr-1* background as compared to the wild-type, and cover a range of categories, including ribosomal and proteasomal proteins, transcription factors, signaling proteins and heterochronic genes. These genes appear to play essential roles in the ovulation steps found in the somatic gonad of *C. elegans*. Additional studies will further clarify how these genes interact with the IP_3 signaling pathway to modulate the ovulation process.

CHAPTER TWO

Materials and Methods

Identificaiton and Analysis of RNAi-Induced Sterility

Preparation of Laboratory Reagents and Plates

M9 buffer solution. M9 buffer is used for a lot of washing steps in working with *C. elegans*. Briefly, 3 mg/mL of KH_2PO_4 (EMD Chemicals, NJ, USA), 6 mg/mL of Na_2HPO_4 (Fischer Scientific, NJ, USA) and 5 mg/mL of NaCl (EM Science, NJ, USA) were added together in 1 L of DI water. After autoclaving for 15 minutes, 0.12 mg/mL of 1 M $MgSO_4$ was added to solution once cooled. M9 buffer was stored at room temperature until required.

Nematode freezing solution. Special solution had to be made to allow for the freezing of the worm strains used. About 1 L of solution was made at a time, combining 5.85 g of NaCl, 6.8 g of KH_2PO_4 (EM Science, NJ, USA), 300 g or 240 mL of glycerol (EM Science, NJ, USA), and 5.6 mL of NaOH (1 M, EM Science, NJ, USA), bringing the volume up to 1 L with DI water. The solution was autoclaved for 15 minutes, after which 3 mL of sterile 0.1 M $MgSO_4$ was added and stored at room temperature.

β-lactose stock solution. To induce dsRNA production in the RNAi bacterial lawns used, a 20% w/v solution of β-lactose was prepared to be added to RNAi plates post-autoclaving. Briefly, 10 g of lactose powder (Becton and Dickinson, MD, USA) was weighed and added to 50 mL of DI water in a 100 mL beaker. The solution was

placed on a hot plate and a magnetic stir bar added to aid in dissolving the lactose. When the lactose was fully dissolved, the solution was passed through a sterile filter (0.22 micron pore size) to ensure sterility of the solution. The resultant filtrate was decanted into a sterile 50 mL centrifuge tube and 4.5 mL of this solution was aliquoted into sterile 15 mL conical centrifuge tubes which were subsequently stored in a -20^{O}C freezer until needed.

Ampicillin stock solution. RNAi bacteria from the library utilized contain a gene conferring resistance to the antibiotic ampicillin. Stock ampicillin (40 mg/mL) was added to RNAi plates to aid in the growth of only the bacteria of interest. Ampicillin (Shelton Scientific, CT, USA) was weighed out to 2 grams and added to 50 mL of DI water in a 100 mL beaker. After dissolving with the aid of a magnetic stir bar, the solution was passed through a sterile filter (0.22 micron pore size) to ensure sterility of the solution. Filtrate was decanted into a 50 mL centrifuge tube and aliquoted in 4.5 mL volumes into 15 mL conical centrifuge tubes and stored at -20^{O}C until needed.

RNA-mediated interference (RNAi) plates. Special plates were poured to facilitate the growth of the dsRNA producing bacteria. Typically, four (4) liters was made at a time. In a sterile plastic dish, 68g of agar was weighed out, along with 10g of peptone and 12g of sodium chloride. This was all mixed with 4L of distilled water in a 6L Erlenmeyer flask using a magnetic stir bar. The mixture was then autoclaved.

After the autoclave finished, the molten agar was allowed to cool to approximately 55^{O}C. Cholesterol, magnesium sulfate and calcium chloride were all added, 4mL of each, to the mixture. Then 100mL of 1M KH_2PO_4 buffer was also added

(86.7mL of 1M KH_2PO_4 added to 13.3mL of 1M K_2HPO4). A 20% lactose solution (4.5mL) was the bacterial inducer used for production of the T7 polymerase, and subsequently added to the molten agar. The HT115(DE3) bacteria used has shown the best production of dsRNA when plated on agar with an IPTG concentration of 1mM (Kamath and others 2001; Timmons, Court, Fire 2001). In order to prevent contamination of the plates, ampicillin was added to the molten agar as well, to give a final concentration of 40ug/mL.

Once everything had been added, the agar was poured into plates using a Wheaton Unispense pouring device. Approximately 13.5 mL was poured into a single 13.5mm plate. Normal nematode growth media (NGM) agar plates were made in a similar fashion, simply excluding the addition of the 20% lactose solution and the ampicillin. Plates were allowed to sit overnight before being transferred to plastic boxes and stored in a $4^{O}C$ refrigerator for later use.

Lysogeny broth (LB). Inoculation of bacterial colonies into LB was necessary for growth of the cultures for seeding onto RNAi and NGM plates. Tryptone (5 g, Becton and Dickinson, MD, USA), yeast extract (2.5 g, Becton and Dickinson, MD, USA), NaCl (5 g) and Tris-HCl at pH 7.5 (10 mL, Fischer Scientific, NJ, USA) were all added to 500 mL DI water and mixed with a magnetic sitr bar. Once dissolved, 100 mL of LB was aliquoted into 150 mL bottles and autoclaved. LB was stored at $4^{O}C$ until needed.

In order for the LB to be used to grow cultures of RNAi bacteria, ampicillin (100 µL) was added to 100 mL of LB. This helped ensure only the bacteria of interest were grown.

LB plates. LB plates were necessary for the growth of individual colonies of OP50 stock bacteria to be inoculated in LB. Solution was prepared in the same was as LB, but with the addition of 7.5 g of agar prior to autoclaving and without aliquoting into separate bottles. After autoclaving, the media was poured into 100 mm diameter petri dishes and allowed to solidify while left inverted overnight. Plates were subsequently stored in the 4^{O}C refrigerator until needed.

To grow colonies of RNAi bacteria, ampicillin (500 µL) was added to the LB agar solution post-autoclaving yet prior to pouring into petri dishes. This helped ensure only the bacteria of interest were grown.

C. elegans Strains

The following worm strains were used in this study: wild-type N2, *itr-1/lfe-1 (sy290)* IV, *rrf-1 (pk1417), rrf-3 (pk1426).* All strains were grown and maintained at room temperature (26^{O}C) with *Escherichia coli* OP50 bacteria seeded onto NGM agar plates, with the exception of *rrf-3(pk1426)* which was maintained at 20^{O}C until used in actual experiments, where it was kept at room temperature. The wild-type N2 worms, as implied, maintained a control population against which the other strains could be compared. In order to identify what sterility causing genes may play a role in the IP_3 signaling pathway, we utilized the *itr-1/lfe-1 (sy290)* IV strain containing a gain-of-function mutation in the IP_3 receptor located on the ER. This mutation allows for the constitutive activation of the receptor, allowing calcium to flow more freely from the ER to cause the sheath cell contractions. To clarify the findings from the wild-type and *itr-1* strains, both *rrf-1 (pk1417)* and *rrf-3 (pk1426)* strains were added. These were used in the Emo scoring. Briefly, the *rrf-1(pk1417)* strain contains a mutation which essentially

disables RNAi in the somatic tissues of the worm. As such, only RNAi affecting the sperm and oocytes are susceptible to RNAi, and those affecting the sheath cells would be unhindered. The *rrf-3(pk1426)* strain has been mutated to allow heightened sensitivity to dsRNA, increasing the susceptibility to RNAi in all tissue types. This allowed us to see a stronger resultant phenotype than that seen in the N2 wild-type worms.

Freezing of the worms was done using the standard protocol. Approximately 3 mL of M9 buffer solution is used to float L1 and L2 stage worms from a freshly starved plate. After roughly 3-5 minutes, worms were collected in a sterile glass Pasteur pipette and transferred to a 15 mL centrifuge tube. Sterile 60% sucrose solution (150 µL) was added as well. After mixing, tubes were centrifuged at 3300 rpm in a Fisher Scientific Centrific model 228 centrifuge for 5 minutes. The supernatant was removed post-centrifugation, after which 500 µL of M9 buffer and 500 µL of nematode freezing solution were added. Tubes were gently shaken to ensure mixing of the solutions before aliquoting the solution into two cryovials. Cryovials were then placed into Styrofoam sponge racks, covered with aluminum foil and placed in the -80^{O}C freezer. Styrofoam racks ensure slower freezing and helps protect the worms from forming ice crystals which can result in death of the worms.

RNAi Bacterial Preparations

Libraries of each chromosome (I – V and X) were purchased from GeneService (http://www.geneservice.co.uk/). The RNAi database which provides the well number for the gene of interest was also obtained from the GeneService website. The libraries were all maintained in a -80^{O}C freezer and only briefly taken out to grow colonies.

A sterile 100 µL pipette tip was used to puncture the thin plastic sheet over the 384-well plate the bacterial libraries were kept in and a scraping was taken of the frozen solution. The tip was scraped on an LB/ampicillin agar plate and allowed to incubate at 34^{O}C for 12-14 hours, typically overnight. Plates were afterwards removed, wrapped with parafilm and kept in a 4^{O}C refrigerator until needed. Plates kept for longer than a month were thrown away.

A bacterial colony was then isolated from the LB agar plate with a 100 µL pipette tip and used to inoculate a culture tube containing a small amount of LB/ampicillin broth. The LB broth contained ampicillin, as described above, to prevent any other bacterial growth. The culture tubes were then placed in a 34^{O}C incubator shaker for 12-14 hours, typically overnight. After this incubation period, tubes were placed in a 4^{O}C refrigerator until used later that day.

The LB culture broth was then used to seed four (4) RNAi plates. Approximately 50-100 µL of broth was spread onto a single RNAi plate using a sterile, glass pipette. The RNAi plates were then allowed to sit, agar side down, at room temperature for 12-14 hours, typically overnight, allowing the RNAi bacteria to soak into the plate and grow. The HT115(DE3) bacteria has shown the best production of dsRNA when plated on these RNAi plates and allowed an incubation period at room temperature for overnight (Kamath and others 2001; Timmons, Court, Fire 2001).

The following day, adult *C. elegans* worms, either N2 or *itr-1(sy290)*, were transferred to the RNAi plates and allowed to lay eggs. Approximately four hours after the transference, the plates were checked for a good number of eggs (~30 per plate) and the adults were then killed by flame. Alternatively, eggs could be taken directly from

main worm strain plates and plated directly to the RNAi seeded plates. These RNAi plates with eggs and larvae were known as the stock plates. Once plated onto the RNAi plates, the worms showed greatest RNAi penetrance between 24 and 48 hours of feeding time, due to higher production of dsRNA by the bacteria during that time (Kamath and others 2001).

The same day as the transference, a larger LB/ampicillin broth inoculation was made. As described, this solution once again contained 100ug/mL of ampicillin to prevent other bacteria from growing in culture. The tubes were again placed in the $34^{O}C$ incubator shaker for 12-14 hours, typically overnight. Afterwards, the tubes were removed from the incubator and placed in a $4^{O}C$ refrigerator for use later that day.

RNAi plates were removed from storage in the $4^{O}C$ refrigerator. The experiments required sixty (60) plates total, thirty (30) for each of the worm strains used (N2 and *itr-1*). All sixty plates were seeded with the large LB/ampicillin culture broth prepared 12-14 hours before. These were also then allowed to sit at room temperature, as previously described.

After 12-14 hours had passed, worms from the RNAi seeded stock plates were transferred to the newly seeded RNAi plates. Again, the best RNAi penetrance occurs from 24 to 48 hours, which works well with this protocol (Kamath and others 2001). One worm was placed on each plate, making a total of sixty (60) worms transferred, thirty (30) N2 worms and thirty (30) *itr-1(sy290)* worms. These plates were clearly labeled with what RNAi bacteria they had been seeded with and what worm strain had been placed on them. All the plates were then placed in a plastic container to keep them

together and ordered. This was then placed in a 26^{O}C incubator for approximately 48 hours, until they were ready for sterility scoring.

C. elegans Phenotypic Characterization and Microscopy

Stringent sterility scoring was applied to the worms being observed. Plates containing more than the single worm transferred to each agar plate were counted as fertile, and those containing the single adult worm plated originally were counted as sterile. Sterility could be caused by any of the factors described in Chapter 1. Briefly, these are sheath cell contraction defects, spermathecal defects, sperm production defects and oocyte production defects. Due to the multiple possibilities for sterility and the number of people working on the project, closer examination was required via microscopy.

Adult worms grown on the dsRNA producing bacteria were more closely examined using differential interference contrast (DIC) microscopy. Examination of the *C. elegans* gonads was accomplished by soaking the worms in 20mM sodium azide (NaN_3). These worms were then placed on 2% agarose pads made on thin glass slides and observed on the Nikon TE2000-U inverted microscope with a 40x Plan Fluor objective lens. Ovulation images were obtained at RT with a CoolSnap cf monochrome camera. Images were examined for the presence or absence of oocytes and, if present, what the oocytes themselves looked like. Sheath cell contraction defects often result in an endomitotic oocyte (Emo) production phenotype, as described in Chapter 1. Briefly, the oocyte is unable to move through the spermatheca and become fertilized, instead staying trapped in the proximal gonad where the nucleus undergoes multiple rounds of replication but without cellular division.

Additionally, some worms were recorded using time-lapse photography in the production of ovulation videos. Worms were anesthetized with the dilute anesthetic 0.1% tricaine and 0.01% tetramatisole (TT) at RT for 45 min and fixed to slides of 3% agarose slides. Tricaine/tetramisole blocks major movements of *C. elegans*, including pharyngeal pumping and egg laying, while allowing the gonads to continue sheath cell contractions and oocytes maturation activities (Kirby, Kusch, Kemphues 1990; McCarter and others 1999). Time-lapse images were captured every 15 seconds for roughly 60-120 minutes using the MetaVue software, beginning with oocyte rounding and initial maturation events.

We also utilized 4',6-diamidino-2-phenylindole (DAPI) staining to visualize the Emo phenotype. DAPI binds tightly to DNA and fluoresces when under ultraviolet light. DAPI's ability to pass through cellular membranes makes it useful in the study of whole mount organisms. As the endomitotic oocytes undergo multiple rounds of nuclear replication, more DNA is available for the DAPI to attach to, making the nuclei of such cells more readily apparent. Additionally, as the oocytes stay intact as a single oocyte, divisions are absent unlike the multicellular eggs produced under normal wild-type conditions.

CHAPTER THREE

Results

Cellular Architectural Proteins

Cells consist of a basic architecture upon which their shapes rest. Because of this, cells have their own components required for maintaining their morphologies which are necessary for compartmentalization, communication, transport, and survival. Cellular architectural proteins include components of cytoskeletons and membrane proteins necessary for maintenance of structure and communication between compartments. Our definition of cellular architectural proteins also includes extracellular matrix proteins and receptors because the cell-matrix interaction is essential for maintaining cell architecture.

Cellular architectural proteins often bind together to compartmentalize organelles, allow transport of vesicles from one part of a cell to another, and generally provide the overall structure for the cells. It is understandable that there would be many structural genes necessary for fertility in the worms, as described in Chapter 1. For example, *C. elegans* gonad sheath cells rely upon the muscular contractions to move oocytes forward through the proximal gonad. Disruption in the organization of the cells will in turn disrupt the function of those cells.

Of the cellular architectural proteins believed to cause some form of sterility (Kamath and others 2003), we identified 26 sterility causing genes in our stringent RNAi sterility testing of the wild-type *C. elegans*. Several previously characterized genes and proteins were identified in our screening, including Talin (100%, N = 30) (Calderwood and others 1999; Cram, Clark, Schwarzbauer 2003; Critchley 2000) and *pat-3* (72% ±

8.3%, N = 29) (Lee and others 2001; Xu and others 2005), lending credence to the effectiveness of our screening.

Talin, a cytoskeletal protein that localizes primarily to dense bodies and focal adhesions, binds to various other cytoskeletal proteins including integrin β cytoplasmic tails, vinculin and actin filaments (Calderwood and others 1999; Critchley 2000). It has previously been shown that talin helps direct the migration of the distal tip cell (DTC) ensuring proper gonad morphogenesis, aids the formation of actin cytoskeleton in the gonad sheath cells, and is necessary for the development of mature oocytes in the proximal gonad as an accumulation of oocytes is seen in the loss-of-function phenotypes (Cram, Clark, Schwarzbauer 2003). Phenotypic analysis of talin loss-of-function worms also revealed a general paralysis resulting in an uncoordinated phenotype, highlighting the need for talin in muscle contraction through its interaction with F-actin (Cram, Clark, Schwarzbauer 2003). This interaction highlights the connection between the extracellular matrix integrin proteins and the intracellular F-actin proteins. Given this information, it is unsurprising that RNAi of the talin gene results in sterility of the worms (100%, N = 30). Due to the actin cytoskeleton relying on talin to aid its formation, and that the sheath cell contractions are downstream of the IP_3 pathway, the gain-of-function mutant *itr-1(sy290)* is unable to rescue the sterility seen in the loss-of-function talin worms (100%, N = 30). Since the muscle contractions that provide oocyte movement through the gonad are located in the somatic tissue, the *rrf-1(pk1417)* worms showed a decrease in sterility (33% ± 8.6%, N = 30).

Another previously characterized gene involved in the cytoskeleton that aids *C. elegans* fertility is the β integrin *pat-3*. This *pat-3* gene encodes the sole β integrin

protein in *C. elegans* (Lee and others 2001). During our examination, RNAi of *pat-3* resulted in a primarily sterile phenotype in wild-type *C. elegans* (72% ± 8.3%, N = 29). Additional studies have shown RNAi of *pat-3* to cause early embryonic lethality (Lee and others 2001) and sterility (Xu and others 2005). Localization studies show *pat-3* in the somatic gonad of the *C. elegans* sheath cells with a potential role in the generation of IP_3 (Xu and others 2005). Interestingly, a gain-of-function in the *itr-1* gene drastically rescues the RNAi phenotype of the *pat-3* gene indicating that the integrin is necessary for proper IP_3 signaling upstream of the ITR-1 calcium receptor. Our results in the *itr-1(sy290)* background (3% ± 3%, N = 30) confirmed what had been seen in previous studies and provide evidence that our protocol works for the identification of additional upstream components to the IP_3 signaling pathway (Xu and others 2005). The sterility of *rrf-1(pk1417)* mutant worms largely decreased as compared to the N2 (4% ± 4%, N = 25), further confirming ours and previous studies results indicating a role in the somatic tissues of *C. elegans*, and specifically in sheath cells.

Multiple components of the vesicle transportation system (COPI, COPII, etc) evidenced as sterility causing genes in this study. The gene C13B9.3 (*phi-30*, Protein Homeostasis Interference (Nollen and others 2004)) encodes a delta subunit of the COPI complex responsible for membrane traffic in animal cells. Gene expression profiles have localized the C13B9.3 gene to many muscle cells including the pharynx, intestine, body wall muscles, vulval muscle, spermatheca and gonad sheath cells (Hunt-Newbury and others 2007). Sterility phenotypes have been identified in various RNAi experiments (Gonczy and others 2000; Kamath and others 2003; Simmer and others 2003; Sonnichsen and others 2005) , including our own (87% ± 4.4%, N = 60). The absent rescue of the

Table 3.1. Cellular architectural genes causing sterility (≥ 60% in N2 *C. elegans*) under RNAi conditions. N = number of animals. Each entry represents at least the initial phenotypic scoring.

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
C13B9.3	phi-30	Vesicle coat complex COPI, delta subunit	InterPro	87 ± 4.3 (60)	100 (29)	70 ± 6.1 (57)
C24H11.7	gbf-1	COPI recruitment, Golgi subcompartmentalization	Manolea 2008	83 ± 6.9 (30)	30 ±8.4 (30)	0 (30)
C54D1.5	lam-2	ECM glycoprotein Laminin gamma subunit	InterPro	90 ± 5.5 (30)	100 (30)	97 ± 3.2 (29)
F12F6.6	sec-24.1	Vesicle coat complex COPII, subunit SFB3	Miller 2003	100 (60)	97 ± 2.2 (60)	95 ± 2.8 (60)
F29G9.3	aps-1	Clathrin adaptor complex, small	Boehm 2001	93 ± 4.7 (30)	7 ± 4.7 (30)	3 ± 3 (30)
F30H5.1	unc-45	Myosin assembly protein	Venolia 1999	87 ± 4.3 (60)	95 ± 2.8 (60)	68 ± 6 (60)
F32E10.4	ima-3	Importin alpha; oogenesis	Geles 2001	80 ± 5.2 (60)	12 ± 4.2 (60)	18 ± 5 (60)
F38A1.8		Located in muscle and nerves; Embryonic lethal, maternal sterility	Wormbase	100 (60)	100 (30)	15 ± 4.6 (60)
F38E11.5		Vesicle coat complex COPI, beta-prime subunit	InterPro	97 ± 3.1 (30)	100 (30)	53 ± 9.1 (30)

Table 3.1. Continued

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
F43D9.3	phi-26	Reproduction and molting; Vesicle docking	Wormbase	100 (30)	100 (30)	100 (30)
F54C1.7	pat-10	Body wall, calcium binding troponin C; Muscle contraction	Terami 1999	97 ± 3.1 (30)	97 ± 3.1 (30)	17 ± 6.9 (30)
F59E10.3		Vesicle coat complex COPI, zeta subunit	InterPro	100 (27)	100 (28)	43 ± 9.4 (28)
H15N14.1	adr-1	Deamination of adenosines; RNA binding and processing; Vulval development	Tonkin 2002	60 ± 8.9 (30)	60 ± 8.9 (30)	20 ± 7.3 (30)
ZK1014.1	adr-1	Deamination of adenosines; RNA binding and processing; Vulval development	Tonkin 2002	60 ± 8.9 (30)	43 ± 9 (30)	20 ± 7.3 (30)
K02D10.5	phi-28	SNAP-25 (synaptosome-associated protein) component of SNARE complex	InterPro	100 (30)	100 (30)	100 (30)
R10E11.8	vha-1	Vacuolar H+-ATPase, subunit c	Oka 2000	100 (30)	67 ± 8.6 (30)	43 ± 9 (30)
T01C3.7	fib-1	Fibrillarin RNA-binding protein; Ribosome biogenesis	Saijou 2004	100 (30)	100 (30)	100 (30)

Table 3.1. Continued

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
T19B4.2	npp-7	Nuclear pore complex protein	Galy 2003	82 ± 5 (60)	100 (30)	95 ± 2.8 (60)
T20G5.1	chc-1	Clathrin heavy chain; Yolk oocyte endocytosis	Grant 1999	100 (60)	100 (60)	92 ± 3.5 (60)
W03F8.5	lam-1	ECM glycoprotein Laminin beta subunit	Kao 2006	83 ± 6.9 (30)	0 (30)	0 (30)
W07B3.2	gei-4	Possible intermediate filament	Wormbase	73 ± 5.7 (60)	100 (30)	22 ± 5.3 (60)
Y113G7A.3	sec-23	Vesicle coat complex COPII, subunit SEC23	Roberts 2003	75 ± 8.2 (28)	86 ± 7.6 (21)	73 ± 8.1 (30)
Y25C1A.5		Vesicle coat complex COPI, beta subunit	InterPro	100 (21)	97 ± 3.1 (30)	74 ± 9.1 (23)
Y71G12B.11	Talin	Cell migration and contractility; Co-localizes with integrin in focal adhesions	Xu 2005	100 (30)	100 (30)	33 ± 8.6 (30)
ZK1058.2	pat-3	Integrin beta subunit; Body wall and gonad	Lee 2001	72 ± 8.3 (29)	3 ± 3 (30)	4 ± 3.9 (25)
ZK637.8	unc-32	Vacuolar H+-ATPase, subunit a	Pujol 2001	100 (30)	67 ± 8.6 (30)	17 ± 6.9 (30)

sterility phenotype in the *itr-1(sy290)* background (100%, N = 29) indicates that this gene product plays a role either outside of the IP_3 pathway or downstream of it. Sterile results from RNAi trials in the *rrf-1(pk1417)* mutant *C. elegans* (70% ± 6.1%, N = 57) show a role in germline and oocyte development as well, and that the gene is not relegated solely to the somatic tissues.

Additionally, COPI subunits beta, beta-prime and zeta (genes Y25C1A.5, F38E11.5 and F59E10.3, respectively) (Nickel, Brugger, Wieland 2002) were found to induce sterility under RNAi conditions, showing 100% (N = 21), 97% ± 3.1% (N = 30) and 100% (N = 27) sterility respectively. This data, in conjunction with the results from C13B9.3 (COPI delta subunit), indicate fertility of the organisms relies heavily on the COPI vesicles. These subunits of the COPI vesicle maintained sterility in the *itr-1(sy290)* background (97% ± 3.1%, N = 30; 100%, N = 30; 100%, N = 28 respectively). All COPI genes identified in our study showed relatively high rates of sterility in *rrf-1(pk1417)* RNAi conditions as well (74% ± 14%, N = 23; 53% ± 4.5%, N = 30; 43% ± 9.4%, N = 28 respectively), indicative of a role for the COPI vesicles in germline and oocyte development. As the COPI vesicles are responsible for shuttling proteins throughout the cells, it is unsurprising the number of defects seen under RNAi conditions.

RNAi of several COPII subunits, *sec-23* and *sec-24.1*, yielded significant sterility in all worm strains tested. SEC-23 has been shown to be a component of vesicular transport from the ER to the Golgi (Roberts, Clucas, Johnstone 2003). The function of COPII coat protein SEC-24.1 has largely been elucidated from *Saccharomyces cerevisiae*, in which the protein is involved with the selection of cargo to be transported via the vesicle (Miller and others 2003). Sterility occurred primarily due to defects in

cuticle secretion and oogenesis during development. This explains why the sterility was seen not only in wild-type worms (75% ± 8.2%, N = 28 and 100%, N = 60, respectively), but the *itr-1(sy290)* (86% ± 7.6%, N = 21 and 97% ± 2.2%, N = 60, respectively) and *rrf-1(pk1417)* (73% ± 8.1%, N = 30 and 95% ± 2.8%, N = 60, respectively) strains as well.

Other vesicle related proteins influence sterility in *C. elegans* as seen in this study. The *aps-1* genes codes for an adaptin protein for clathrin protein complexes, which are involved in endocytosis (Boehm and Bonifacino 2001). According to a BLASTP analysis, it is an ortholog of sigma 1 subunit of the adaptor protein complex 1 in *Saccharomyces cerevisiae* which deals with protein sorting in the trans-Golgi network (Phan and others 1994). RNAi of *aps-1* resulted in high sterility in N2 worms (93% ± 4.7%, N = 30), though greatly reduced sterility in both *itr-1* and *rrf-1* strains (7% ± 4.7%, N = 30 and 3% ± 3%, N = 30, respectively). The *aps-1* gene appears to play a role in the sheath cells and has some relation to the IP_3 signaling pathway.

Protein shuttling occurs in more directions than just from the ER to the Golgi and back, or out to the plasma membrane. *ima-3* codes for an α nuclear pore protein that moves proteins in and out of the nucleus as needed (Geles and Adam 2001). During oocyte development, IMA-3 is essential for movement past the pachytene arrest at the loop of the gonad and for spindle formation and nuclear envelope assembly in embryos (Askjaer and others 2002; Geles and Adam 2001; Geles and others 2002). Sterility through RNAi of *ima-3* was high in N2 worms (80% ± 5.2%, N = 60), yet reduced greatly in both *itr-1* and *rrf-1* strains (12% ± 4.2%, N = 60 and 18% ± 5%, N = 60, respectively), indicating a role in the sheath cells and oocyte development.

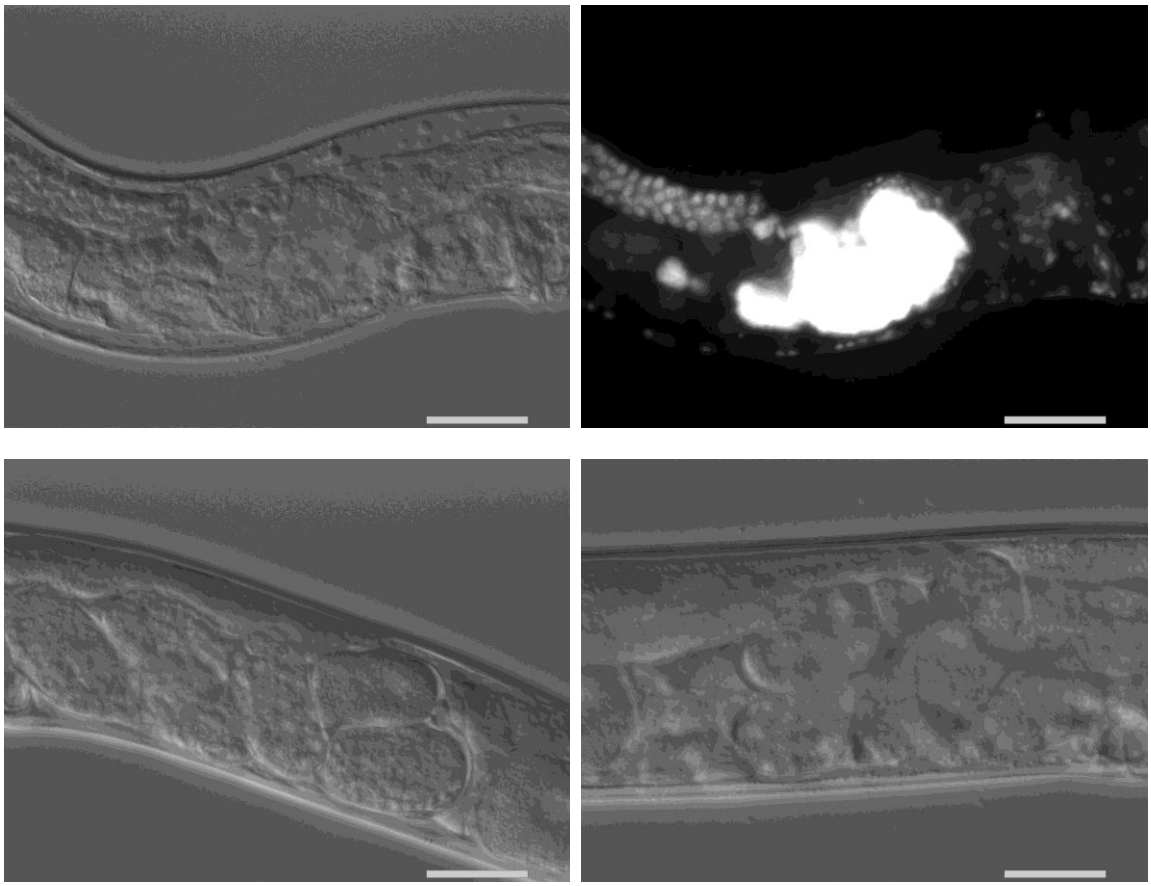

Figure 3.1. Worms fed *aps-1* RNAi bacteria. (A) An *rrf-3(pk1426)* worm showing the accumulation of oocytes in the proximal gonad. (B) DAPI stained *rrf-3(pk1426)* worm with a large mass of nucleic material typical of Emo oocytes. (C) An *itr-1(sy290)* worm with fertilized oocytes in the uterus. (D) An *rrf-1(pk1417)* worm with fertilized oocytes in the uterus. White bars = 40µm.

Another gene found to induce sterility was the *gbf-1* gene (Golgi-specific Brefeldin-A-resistant Factor 1 homolog). This gene appears to have pleiotropic effects as many phenotypes have been identified under RNAi conditions, including defects in endocytosis, embryonic and adult lethality, larval arrest and maternal sterility (Balklava and others 2007; Kamath and others 2003; Simmer and others 2003). Sterility testing resulted in the maternal sterile phenotype in the wild-type *C. elegans* (83% ± 6.9%, N = 30) and though it was partially rescued in the *itr-1(sy290)* background (30% ± 8.4%, N =

30), it was not a large enough difference for us to consider it fully rescued by our standards. Interestingly, *gbf-1* appears to localize in somatic cells only, as the *rrf-1(pk1417)* background lacked the maternally sterile phenotype (0%, N = 30).

The *unc-45* gene codes for a conserved, muscle-specific protein involved in chaperoning myosin assembly and works in conjunction with NMY-2 at the cleavage furrow in *C. elegans* embryos (Venolia and Waterston 1990; Venolia and others 1999). Lethality has been seen in mutant strains showing the impact of the protein on body wall muscle development through interactions with all four myosin chains (Venolia and Waterston 1990). Interestingly, the reduction in myosin resultant from loss of *unc-45* appears to be the outcome of the ubiquitin/proteasome system (Landsverk and others 2007). Overall, the sterility seen across all worm strains in response to our *unc-45* RNAi testing was hardly surprising. Wild-type N2 and *itr-1(sy290)* worms both showed high levels of sterility (87% ± 4.3%, N = 60 and 95% ± 2.8%, N = 60, respectively). Additionally, *rrf-1(pk1417)* mutant strains showed relatively high levels of sterility (68% ± 6.0%, N = 60), although less drastic than N2 and *itr-1* strains.

A lack of definitive information surrounds the F38A1.8 gene, although a BLASTP analysis shows similarities of the gene product to a signal recognition particle receptor subunit alpha in multiple other organisms including humans, and is orthologous to a guanosine triphosphate (GTP) binding protein in *Drosophila melanogaster*. GTPase domains in the protein may propagate signals through the cells. Fluorescence studies showed production in the muscles and nerves of *C. elegans* and phenotypic analyses under RNAi conditions resulted in sterility(Kamath and others 2003; Simmer and others 2003; Sonnichsen and others 2005) and embryonic lethality {{42 Kamath,R.S. 2003; 94

Sonnichsen,B. 2005; 1 Simmer,F. 2003}}. These were consistent with our results in both N2 and *itr-1(sy290)* worms (100%, N = 60 and 100%, N = 30 respectively). However, there was a lack of sterility in the *rrf-1(pk1417)* mutants (15% ± 4.6%, N = 60), indicating a need for this gene in somatic tissues and not the germline.

F43D9.3 has been implicated in vesicle docking and results in molting and reproduction defects in loss of function studies (wormbase.org). BLASTP analysis shows orthology to the *S. cerevisiae* protein SLY1, a hydrophilic protein involved in trafficking vesicles from the ER to the Golgi (Cao, Ballew, Barlowe 1998; Kosodo and others 2003). Sterility from our study was high and maintained across all worm strains (100%, N = 30 for N2, *itr-1(sy290),* and *rrf-1(pk1417)*). The sterility phenotype's lack of rescue by either of the mutant worm strains indicates a role in the germline where the increased sheath cell contractions would not influence the sterility.

The *adr-1* gene works on RNA binding and processing as well as involvement with vulval development (Tonkin and others 2002). Adenosine deamination occurs in the presence of this protein and provides proper fertility of *C. elegans*. There were two gene pairs that focus on the *adr-1* gene: H15N14.1 and ZK1014.1. For the H15N14.1, sterility was relatively high in N2 and *itr-1(sy290)* strains, compared to our negative control and does not appear to play a role in the IP_3 signaling pathway (60%, N = 30 and 60%, N = 30 respectively). It does seem to rely on somatic tissue expression, however, as the *rrf-1(pk1417)* strain decreased sterility (20%, N = 30). Results from the ZK1014.1 were similar across the N2, *itr-1(sy290),* and *rrf-1(pk1417)* worms strains, although a slight but insignificant decrease was seen in the *itr-1(sy290)* strain (60%, N = 30; 43%, N = 30 and 20%, N = 30, respectively).

Several ECM proteins have also been identified as necessary for *C. elegans* fertility. In our study, laminins *lam-1* and *lam-2* were both found to cause sterility when knocked down under RNAi conditions (83%, N = 30 and 90%, N = 30, respectively). Both genes code for subunits of the extracellular matrix glycoprotein laminin in *C. elegans*, with *lam-1* coding for the beta subunit and *lam-2* coding for the gamma subunit. Laminins form part of the basement membrane when in their completed heterotrimeric forms and are responsible for morphogenesis, cell adhesion and cell signaling (reviewed in (Li and others 2003) and (Miner and Yurchenco 2004)). Slow growth (Byrne and others 2007) and embryonic lethal (Kamath and others 2003; Maeda and others 2001; Sonnichsen and others 2005) phenotypes have been identified, as well as the sterility phenotype as seen in our results for both *lam-1* and *lam-2* (above). The emo phenotype evidenced readily under the *lam-1* RNAi conditions in N2 worms, and the RNAi hypersensitive *rrf-3(pk1426)* line as evidenced by DAPI staining (Figures 3.2 A through D). Interestingly, when in the *itr-1(sy290)* background, *lam-1* RNAi was rescued (0%, N = 30) while *lam-2* remained sterile (100%, N = 30), indicating differing functions in relation to the IP_3 signaling pathway, or to the composition of the basement membrane of the gonads. Additionally, the rescuing of the sterility in the *rrf-1(pk1417)* background for *lam-1* indicates the beta laminin is located to the somatic gonad (0%, N = 30), while the maintained sterility of the *lam-2* implies a role in the germline (97%, N = 29).

Of the cellular architectural genes that caused sterility in our study, four of the genes were shown to significantly decrease sterility in the *itr-1(sy290) C. elegans*: *pat-3*, *lam-1*, *aps-1*, and *ima-3*. Each of these genes resulted in at least a 60% decrease in sterility as compared to the wild-type nematodes under the same conditions, implicating

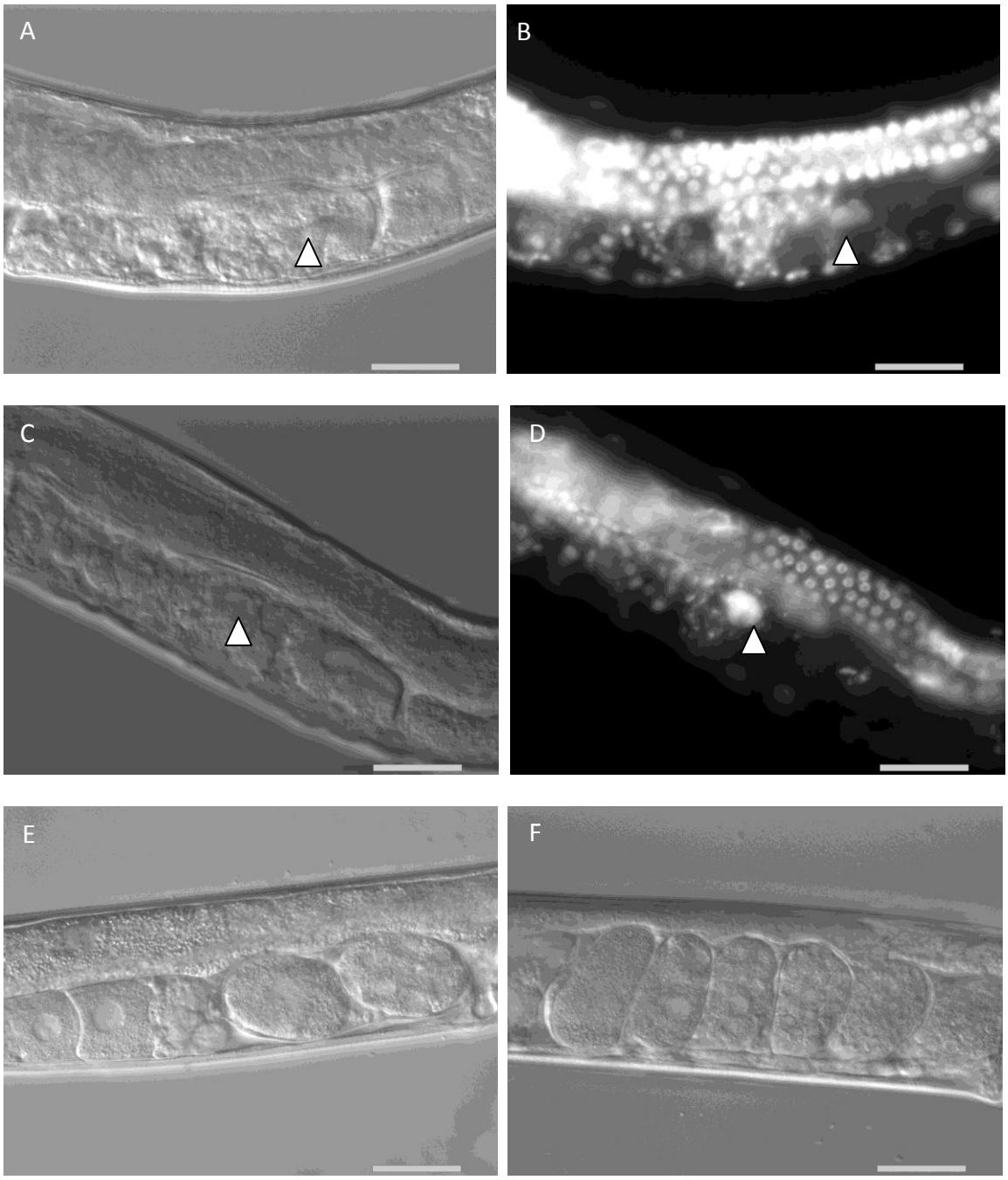

Figure 3.2. Proximal gonads of *lam-1* RNAi animals. (A) DIC image of N2 worm with oocyte accumulation. (B) DAPI staining image of the same N2 worm showing the excessive replication of DNA resulting in an Emo phenotype. (C) DIC image of *rrf-3(pk1426)* worm with accumulation of oocytes. (D) DAPI stained image showing the resultant similar phenotype of the N2 worm under the same conditions. Enlarged nuclei and DNA found in Emo phenotypes are indicated by the triangles. (E) & (F) DIC images of *itr-1(sy290)* worms showing fertilized, viable eggs in utero. White bars = 40µm.

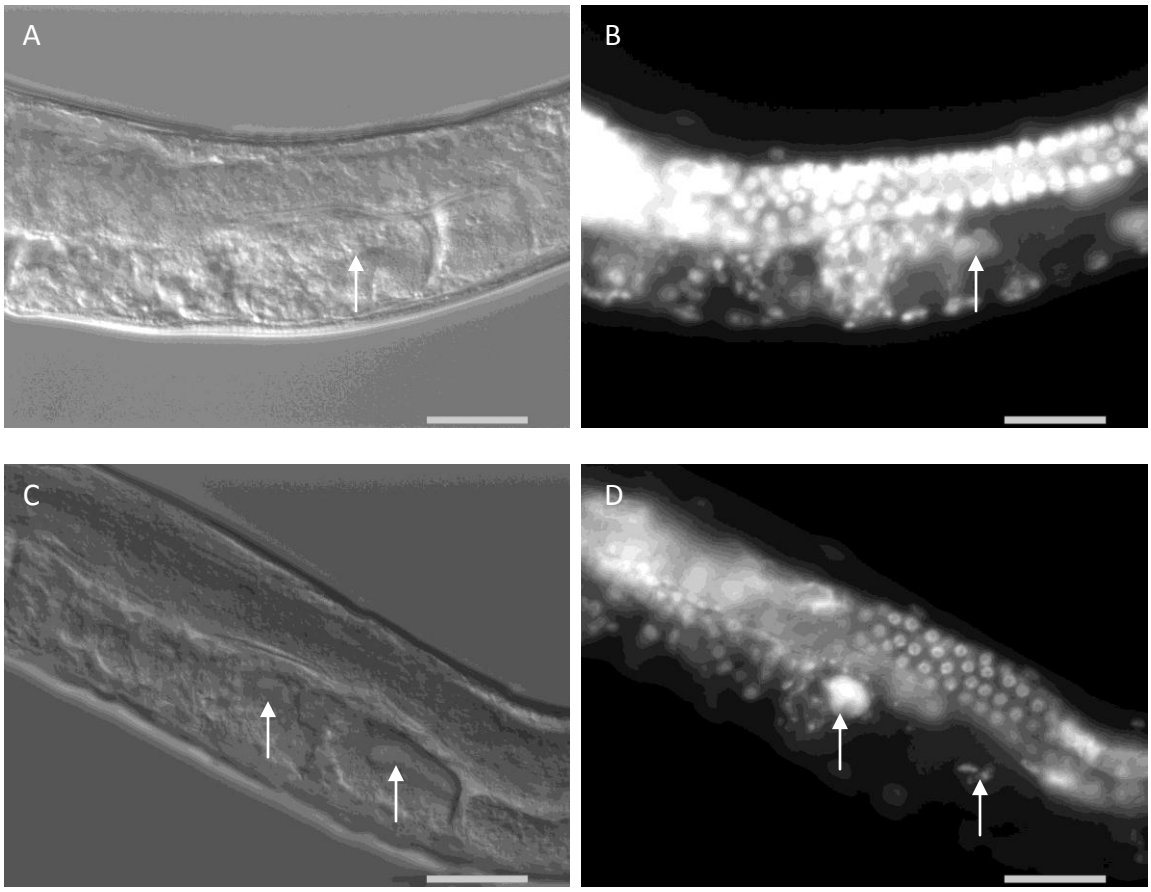

Figure 3.3. Worm strains fed *ima-3* RNAi bacteria. (A) N2 worm showing Emo phenotype. (B) N2 worm with DAPI staining highlighting the excess nucleic acid material in the nucleus. (C) An *rrf-3(pk1426)* hypersensitive mutant worm showing the Emo phenotype. (D) The *rrf-3(pk1426)* worm with DAPI staining. (E) An *itr-1(sy290)* mutant showing fertilized oocytes in the uterus, rescuing the Emo phenotype. (F) An *rrf-1(pk1417)* mutant showing a lack of an Emo phenotype. White arrows point to nuclei of occytes. White bars = 40µm.

these genes somewhere upstream of the ITR-1 protein in the IP_3 signaling pathway. The *pat-3* and *lam-1* genes code for proteins involved in the construction of the ECM, while *aps-1* codes for an adaptor protein for the clathrin coated vesicles (Boehm and Bonifacino 2001), and *ima-3* codes the nuclear pore protein responsible for shuttling proteins in and out of the nucleus (Geles and Adam 2001). Signals from the ECM have been shown to elicit responses in the sheath cells to cause contractions to move maturing oocytes forward through the proximal gonad (Xu and others 2005).

Protein Synthesis Proteins

After mRNAs have been processed and exported from the nucleus to the cytoplasm, translation often occurs to produce the actual proteins. Protein synthesis involves a myriad of components to form the ribosomes and accompanying chaperones that ensure proper protein folding. The ribosomes themselves are made up of two subunits, the large 60S and small 40S ribosomal subunits (Dinman 2009; Rodnina and Wintermeyer 2009). The nucleolus maintains multiple functions in the cell, though may best be known as the site for rRNA processing and ribosome biogenesis (Olson and Dundr 2005). The large subunit can be broken down to its components, which include a5S rRNA, a 28S rRNA, a 5.8S rRNA and roughly 49 proteins, with the rRNAs apparently acting as the catalytic domains for the ribosome (Dinman 2009; Nissen and others 2000; Rodnina and Wintermeyer 2009). The small subunit consists of a single 18S RNA and roughly 33 proteins (Dinman 2009)(Rodnina and Wintermeyer 2009). Beyond the ribosomes themselves, there are a myriad of initiation, elongation and termination factors involved in translating the mRNAs into polypeptide chains (Dinman 2009; Rodnina and Wintermeyer 2009). Additionally, synthetases create the primed tRNAs that bring peptides to the ribosomal complex, and defects in these can cause various physiological problems (Chen, Tuck, Bystrom 2009). Given the number of components needed to manufacture the protein synthesis machinery, it is understandable that the loss of many of these proteins would prove detrimental to organisms.

In order to determine which, if any, of the ribosomal components may be involved in ensuring fertility, we utilized RNAi to eliminate various proteins known to form the subunits of the ribosome. Of the 73 protein synthesis components tested for in our sterility assay, we discovered 53 genes (72.6%) that consistently caused sterility in

N2 worms (Table 3.2). The sterility seen in these worms was also maintained in a majority of the sterility tests on the *itr-1(sy290)* mutant strain.

Within the components of the small subunit of the ribosome, consisting of 33 proteins, we found 20 (60.6%) that consistently caused sterility in N2 wild-type worms. The *itr-1(sy290)* mutant strain also maintained a high sterility count amongst these genes, with a couple of notable exceptions. RNAi conditions using *rps-28* dsRNA producing bacteria showed a 100% (N = 30) sterile phenotype in N2 worms, as compared with only 12% (N = 60) sterility seen in the *itr-1(sy290)* mutants. When *rps-7* translation was halted with RNAi, N2 worms experienced a 77% (N = 60) sterility score while the *itr-1(sy290)* mutants only showed a 10% (N = 60) sterility phenotype. In both the *rps-28* and *rps-7* sterility scoring results, *rrf-1(pk1417)* mutant worms also showed low sterility scores (13%, N = 30 and 10%, N = 30, respectively), indicating a larger role in somatic tissues like the sheath cells, rather than the germ-line cells. With the *rrf-1(pk1417)* mutant strain, worms showed comparable results to the *itr-1(sy290)* mutants (13%, N = 30 and 15%, N = 60, respectively). Lack of sterility in these *rrf-1* mutants, combined with the *itr-1(sy290)* data, suggests that *rps-28* and *rps-7* genes and gene products localize primarily in the somatic tissues and play a role in the ovulation of mature oocytes in the *C. elegans* gonad.

The large subunit, which consists of 49 proteins, had 16 genes (32.7%) that were shown to cause sterility in N2 *C. elegans*. Two RNAi experiments were run against the *rpl-20* gene, due to there being two available gene pair loci for us to use. Both of the gene pairs used in testing *rpl-20*, Y17G9B.e and E04A4.8, showed high incidents of sterility (100%, N = 30 and 80%, N = 30, respectively) in the N2 *C. elegans*. Likewise,

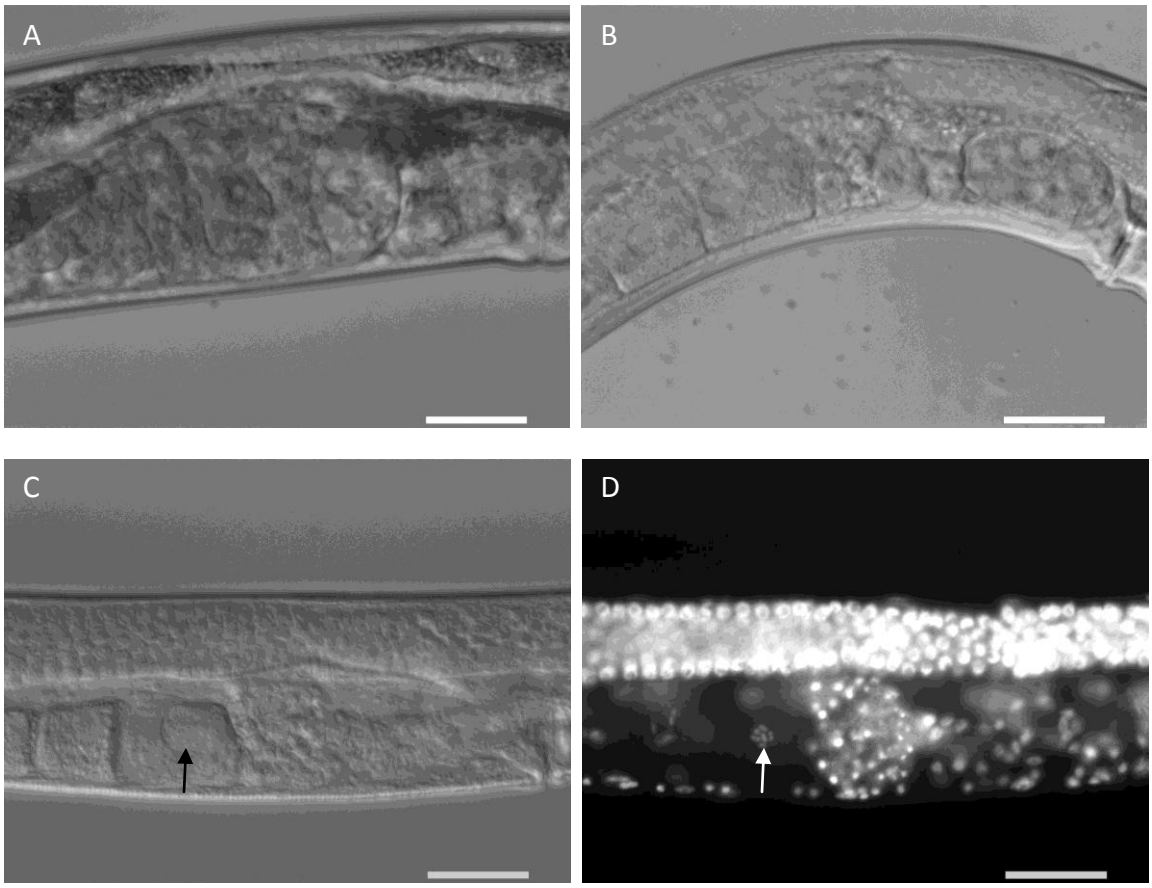

Figure 3.4. Proximal gonads of *rps-7* RNAi animals. (A) The DIC image of wild-type N2 *C. elegans* showing unfertilized oocytes (Emo phenotype) in the proximal gonad. (B) The DIC image of an *itr-1(sy290)* mutant worm showing rescued sterility with a fertilized egg in the uterus. (C) The DIC image of an *rrf-3(pk1426)* mutant worm with enlarged nucleus (black arrow) typical of Emo phenotypes. (D) The DAPI stained *rrf-3(pk1426)* worm showing the beginnings of multicopy DNA (white arrow). Bars = 40µm.

the rescuing of the maternal sterility phenotype in the *itr-1(sy290)* mutants was evident for both gene pairs (3%, N = 30 and 17%, N = 29, respectively), indicating a potential role upstream of the ITR-1 protein in the IP_3 signaling pathway. RNAi results for *rpl-36* show a potential role in the IP_3 signaling pathway as well, as the N2 worms were predominantly sterile (83%, N = 30) while the *itr-1(sy290)* were largely rescued (20%, N = 30). Another component of the large ribosome that may play a role in this pathway is

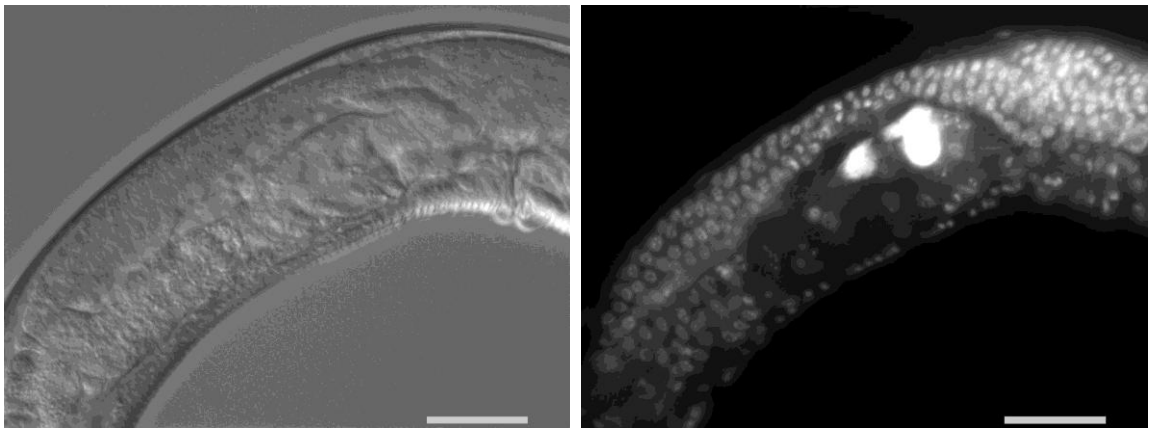

Figure 3.5. *rrf-3* worms fed *ubq-2* RNAi bacteria. (A) DIC image showing compacted oocytes. (B) DAPI stained image showing Emo phenotype. White bars = 40µm.

rpl-7A, which showed a 100% sterile phenotype (N = 30) in N2 worms and only 23% (N = 30) in *itr-1(sy290)* worms.

Many steps compose the process of protein synthesis and each step requires a number of proteins to complete. Multiple enzymes and proteins are necessary to bring the amino acids to the ribosomes, as well as binding the amino acids to each other to form the polypeptide chains that ultimately form the finalized proteins. Chaperone proteins ensure proper folding of nascent proteins in the lumen of the ER. Some of these many proteins have been shown to cause sterility under RNAi conditions.

In order to ensure proper folding of the proteins made by the ribosomes, chaperones located in the lumen of the ER aid the incoming proteins in obtaining their proper configuration. Several chaperone proteins have been shown to induce sterility when knocked down via RNAi as seen in our study. These included proteins encoded by the genes *cct-1* (91%, N = 45), *cct-2* (77%, N = 30), *cct-5* (100%, N = 30), *cct-7* (100%, N = 30), and *cct-8* (100%, N = 30). Each of these proteins forms a part of the chaperonin complex and play an integral role in the folding of actins (Gao and others 1992) as well

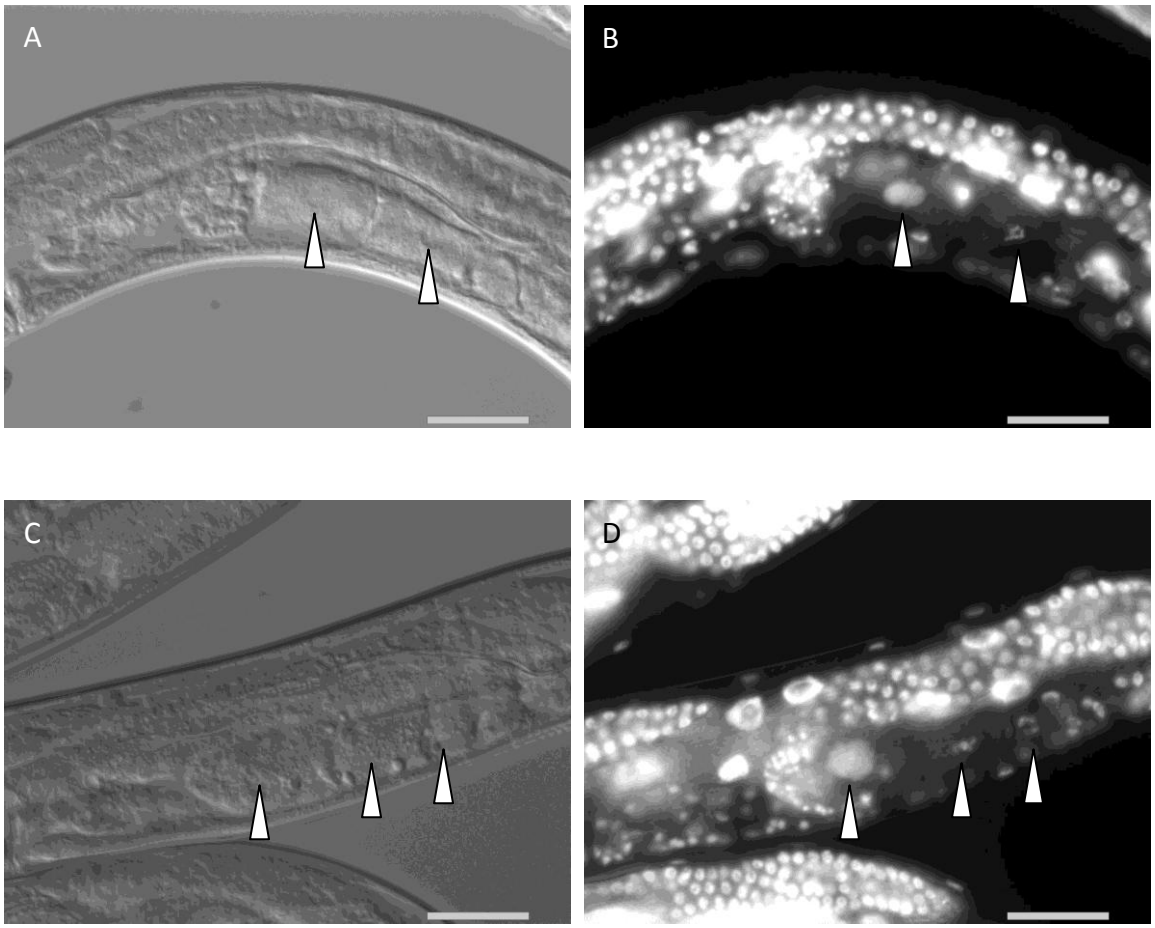

Figure 3.6. Proximal gonads of *rps-28* RNAi animals. (A) (C) The DIC images of wild-type N2 *C. elegans* showing defects in oocyte movement through the proximal gonad. (B) (D) The DAPI stained images of the same wild-type worms showing the endomitotically replicated clumps of DNA. White triangles point to nuclei of oocytes. Bars = 40µm.

as alpha and beta-tubulins (Gao and others 1993; Melki and Cowan 1994; Yaffe and others 1992). Evidence has been reported on the necessity for these and other proteins in embryonic viability and fertility (Fraser and others 2000; Kamath and others 2003; Maeda and others 2001; Simmer and others 2003).

Table 3.2. Protein synthesis genes causing sterility (≥ 60% in N2 *C. elegans*) under RNAi conditions. N = number of animals. Each entry represents at least the initial phenotypic scoring.

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
B0250.1	rpl-2	Large 60S ribosomal subunit L8 protein	Wormbase	61 ± 8.5 (33)	67 ± 8.2 (33)	64 ± 8.4 (33)
B0336.10	rpl-23	Large 60S ribosomal subunit L23 protein	Wormbase	63 ± 5.1 (90)	55 ± 6.4 (60)	50 ± 5.3 (90)
B0393.1	rps-0	Small 40S ribosomal subunit SA protein; Laminin receptor for cell adhesion	Ardini 1998; Ford 1999	83 ± 5.4 (48)	100 (30)	65 ± 6.2 (60)
B0412.4	rps-29	Small 40S ribosomal subunit S29 protein	Wormbase	97 ± 2.2 (60)	97 ± 3.1 (30)	45 ± 6.8 (53)
B0464.1	drs-1	Aspartyl(D)-tRNA synthetase	Interpro	100 (30)	97 ± 3.1 (30)	67 ± 8.6 (30)
B0511.10	eif-3.E	Initiation factor 3 subunit e; May coordinate translation and degradation	Hoareau Alves 2002	100 (60)	80 ± 7.3 (30)	62 ± 5.1 (90)
C07G2.3	cct-5	Chaperonin complex component, TCP-1 epsilon subunit	Leroux and Candido 1995	100 (30)	80 ± 7.3 (30)	80 ± 7.3 (30)
C14B9.7	rpl-21	Large 60S ribosomal subunit L21 protein; Required in embryonic/germline development	Wormbase	100 (30)	93 ± 4.7 (30)	73 ± 8.1 (30)
C16A3.9	rps-13	Small 40S ribosomal subunit S13 protein; Embryonic/germline development	Wormbase	100 (30)	90 ± 3.9 (60)	73 ± 8.1 (30)
C27D11.1	egl-45	Initiation factor 3 subunit a; Develops hermaphrodite specific neurons affecting egg laying	Desai and Horvitz 1989	100 (18)	83 ± 6.9 (30)	70 ± 8.4 (30)
C32E8.2	rpl-13	Large 60S ribosomal subunit L13 protein	Wormbase	92 ± 3.2 (74)	88 ± 4.2 (60)	57 ± 9 (30)

Table 3.2 Continued

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
C49H3.11	rps-2	Small 40S ribosomal subunit S2 protein	Wormbase	100 (30)	100 (30)	60 ± 9 (30)
D1007.6	rps-10	Small 40S ribosomal subunit S10 protein; Lifespan determinant	Hansen 2007	97 ± 1.9 (79)	91 ± 3.2 (80)	52 ± 6.4 (60)
Y17G9B.e	rpl-20	Large 60S ribosomal subunit L18A protein	Wormbase	100 (30)	3 ± 3 (30)	30 ± 8.4 (30)
E04A4.8	rpl-20	Large 60S ribosomal subunit L18A protein	Wormbase	80 ± 7.3 (30)	17 ± 7 (29)	13 ± 5.9 (32)
F13B10.2	rpl-3	Large 60S ribosomal subunit L3 protein; SMG-mediated nonsense suppression	Mitrovich 2000	100 (30)	57 ± 9 (30)	57 ± 9 (30)
F25H2.10	rpa-0	60S acidic ribosomal protein P0	Wormbase	90 ± 5.5 (30)	90 ± 5.5 (30)	33 ± 8.6 (30)
F25H5.4	eft-2	Translation elongation factor 2	Ofulue 1991	98 ± 1.8 (60)	100 (60)	93 ± 4.7 (30)
F36A2.6	rps-15	Small 40S ribosomal subunit S15 protein	Wormbase	76 ± 5.8 (55)	13 ± 4.3 (60)	36 ± 6.5 (55)
F37C12.11	rps-21	Small 40S ribosomal subunit S21 protein; Embryonic/germline development	Wormbase	97 ± 3.1 (30)	100 (30)	50 ± 9.1 (30)
F37C12.4	rpl-36	Large 60S ribosomal subunit L36 protein; Embryonic/germline development	Wormbase	83 ± 6.9 (30)	20 ± 7.3 (30)	87 ± 6.1 (30)
F37C12.9	rps-14	Small 40S ribosomal subunit S14 protein; Embryonic/germline development	Wormbase	100 (30)	100 (30)	100 (30)
F39B2.6	rps-26	Small 40S ribosomal subunit S26 protein; Embryonic/germline development	Wormbase	100 (30)	97 ± 3.1 (30)	73 ± 5.7 (60)

Table 3.2 Continued

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
F40F11.1	rps-11	Small 40S ribosomal subunit S11 protein; Embryonic/germline development; Lifespan determinant	Hansen 2007	97 ± 3.1 (30)	100 (30)	63 ± 8.8 (30)
F40F8.10	rps-9	Small 40S ribosomal subunit S9 protein	Wormbase	100 (30)	100 (30)	100 (30)
F54C9.5	rpl-5	Large 60S ribosomal subunit L5 protein	Wormbase	100 (60)	87 ± 4.3 (60)	100 (60)
F54E7.2	rps-12	Small 40S ribosomal subunit S12 protein; Embryonic/germline development	Wormbase	100 (30)	100 (30)	97 ± 3.1 (30)
F56F3.5	rps-1	Small 40S ribosomal subunit S3A protein	Wormbase	100 (30)	100 (30)	100 (30)
JC8.3	rpl-12	Large 60S ribosomal subunit L12 protein; SMG-mediated nonsense suppression	Mitrovich 2000	61 ± 8.5 (33)	67 ± 8.2 (33)	64 ± 8.4 (33)
M110.4	ifg-1	Translation initiation factor 4F, ribosome/mRNA bridging subunit	Hansen 2007	100 (30)	100 (30)	77 ± 7.7 (30)
R08D7.3	eif-3.D	Translation initiation factor 3 subunit d	Wormbase	100 (30)	50 ± 9.1 (30)	60 ± 8.9 (30)
R13A5.8	rpl-9	Large 60S ribosomal subunit L9 protein	Wormbase	100 (30)	100 (30)	100 (30)
R151.3	rpl-6	Large 60S ribosomal subunit L6 protein	Wormbase	100 (30)	100 (30)	100 (30)
T01C3.6	rps-16	Small 40S ribosomal subunit S16 protein	Wormbase	100 (60)	83 ± 4.8 (60)	63 ± 6.6 (54)
T05C12.7	cct-1	Chaperonin complex component, TCP-1 alpha subunit	Leroux 1997	91 ± 4.3 (45)	67 ± 8.6 (30)	80 ± 6 (45)
T05E11.1	rps-5	Small 40S ribosomal subunit S5 protein	Wormbase	100 (30)	90 ± 5.5 (30)	27 ± 8.1 (30)

Table 3.2 Continued

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
T05H4.6	phi-21	Peptide chain release factor 1	Wormbase	100 (30)	44 ± 10 (23)	62 ± 9 (29)
T07A9.11	rps-24	Small 40S ribosomal subunit S24 protein; Germline development	Wormbase	100 (30)	87 ± 6.1 (30)	97 ± 3.1 (30)
T08B2.10	rps-17	Small 40S ribosomal subunit S17 protein	Wormbase	100 (30)	100 (30)	100 (30)
T10B5.5	cct-7	Chaperonin complex component, TCP-1 eta subunit	Kahn 2008, Lundin 2008	100 (30)	50 ± 9.1 (30)	67 ± 8.6 (30)
T10F2.1	grs-1	Glycyl-tRNA synthetase	Wormbase	84 ± 4.9 (55)	93 ± 3.4 (55)	96 ± 3.9 (25)
T20H4.3	prs-1	Prolyl-tRNA synthetase	Wormbase	100 (30)	100 (30)	50 ± 9.1 (30)
T21B10.7	cct-2	Chaperonin complex component, TCP-1 beta subunit	Leroux 1995	77 ± 7.7 (30)	87 ± 6.1 (30)	77 ± 7.7 (30)
Y105E8C.e	rps-20	Small 40S ribosomal subunit S20 protein	Wormbase	100 (30)	90 ± 5.5 (30)	67 ± 8.6 (30)
Y24D9A.d	rpl-7A	Large 60S ribosomal subunit L7A protein; SMG-mediated nonsense suppression	Mitrovich 2000	100 (30)	23 ± 7.7 (30)	27 ± 8.1 (30)
Y41D4A_3613.a	rps-28	Small 40S ribosomal subunit S28 protein	Wormbase	100 (30)	12 ± 4.2 (60)	13 ± 6.1 (30)
Y45F10D.12	rpl-18	Large 60S ribosomal subunit L18 protein	Wormbase	100 (30)	70 ± 8.4 (30)	50 ± 9.1 (30)
Y48G8A_3945.c	rpl-17	Large 60S ribosomal subunit L17 protein	Wormbase	82 ± 5 (60)	97 ± 3.1 (30)	97 ± 3.1 (30)
Y55F3A_750.d	cct-8	Chaperonin complex component, TCP-1 theta subunit	Wormbase	100 (30)	20 ± 7.3 (30)	7 ± 4.7 (30)
Y71F9A_294.c	rpl-1	Large 60S ribosomal subunit L10A protein	Wormbase	68 ± 4.9 (90)	83 ± 6.9 (30)	63 ± 5.1 (90)

Table 3.2 Continued

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
ZC434.2	rps-7	Small 40S ribosomal subunit S7 protein	Wormbase	77 ± 5.4 (60)	10 ± 3.9 (60)	15 ± 4.6 (60)
ZK1010.1	ubq-2	Ubiquitin/60S ribosomal protein L40 fusion	Jones 1995	70 ± 8.8 (27)	3 ± 3 (30)	100 (27)
ZK328.2	eft-1	GTPase for translation elongation	Ofulue 1992	93 ± 4.7 (30)	60 ± 8.9 (30)	60 ± 6.3 (60)
ZK652.4	rpl-35	Large 60S ribosomal subunit L35 protein	Wormbase	77 ± 7.7 (30)	100 (30)	55 ± 9.2 (29)

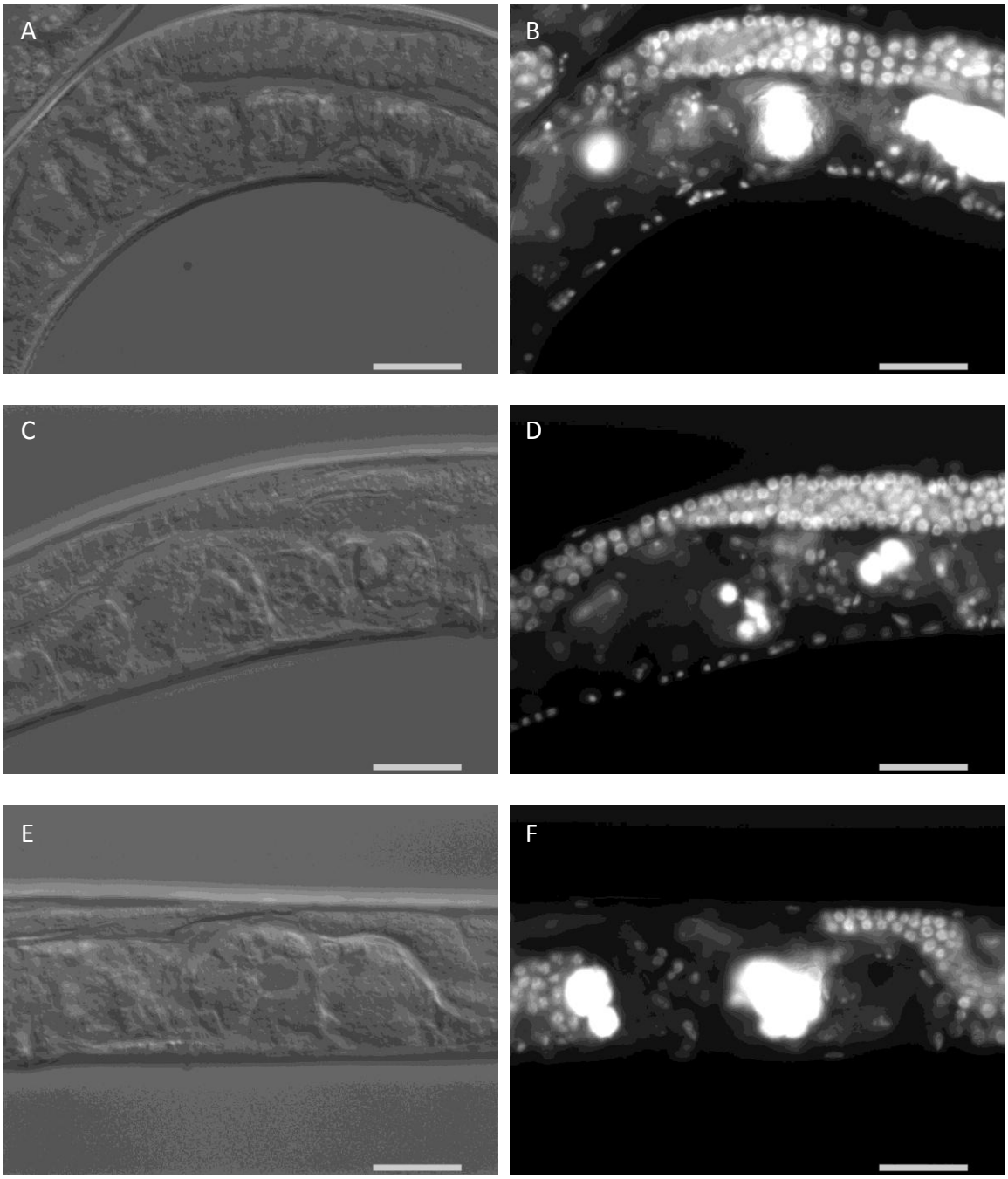

Figure 3.7. Proximal gonads of *rpl-20* RNAi animals. (A) (C) The DIC images of wild-type N2 *C. elegans* showing unfertilized oocytes in the proximal gonad and uterus. (B) (D) The DAPI stained images of the same wild-type worms showing the endomitotically replicated clumps of DNA. (E) The DIC image of an *rrf-3(pk1426)* mutant worm with Emo phenotype. (F) The DAPI stained image of the same *rrf-3(pk1426)* mutant worm showing excess DNA. White bars = 40μm.

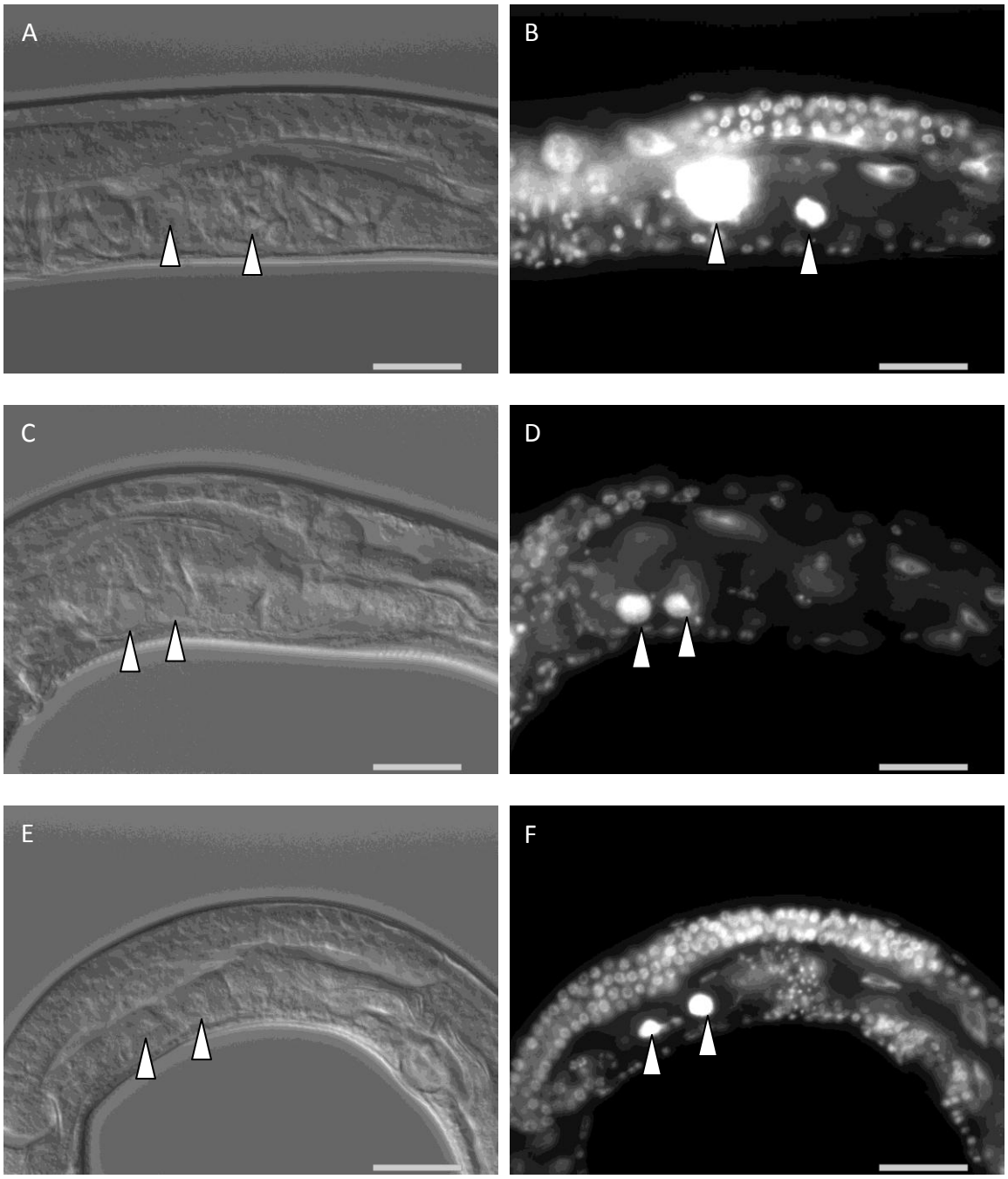

Figure 3.8. Proximal gonads of *rpl-36* RNAi animals. (A) (C) The DIC images of wild-type N2 *C. elegans* showing endomitotic oocytes in the proximal gonad. (B) (D) The DAPI stained images of the same wild-type worms showing the endomitotically replicated clumps of DNA. (E) The DIC image of an *rrf-3(pk1426)* mutant worm with Emo phenotype. (F) The DAPI stained image of the same *rrf-3(pk1426)* mutant worm showing excess DNA. White triangles point to nuclei of oocytes. White bars = 40µm.

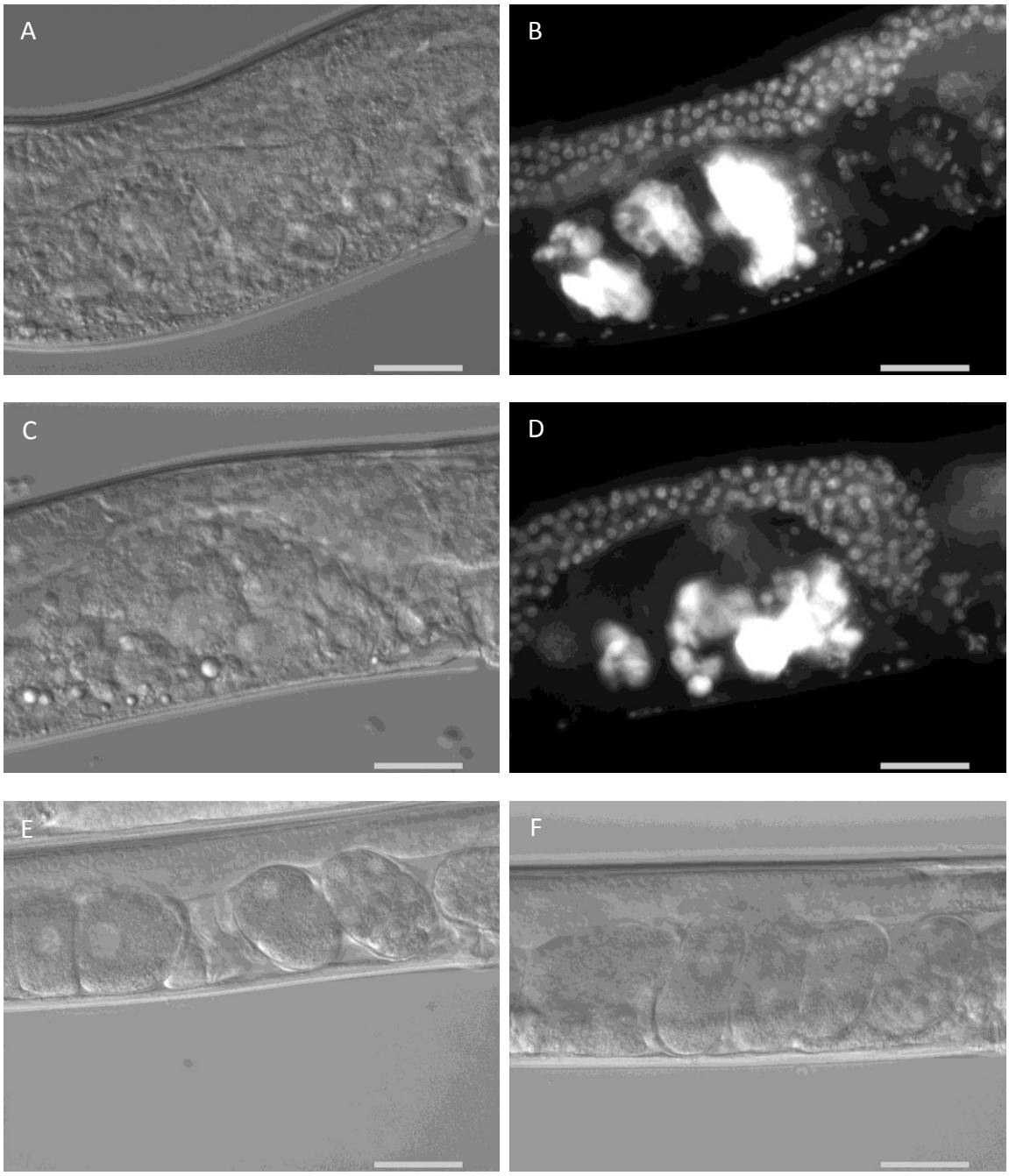

Figure 3.9. Worms fed *cct-8* RNAi bacteria. (A) An N2 worm with compacted proximal oocytes. (B) Same N2 worm with DAPI staining showing accumulated nuclear material. (C) An *rrf-3(pk1426)* worm showing compacted proximal oocytes. (D) DAPI staining of same *rrf-3(pk1426)* worm with accumulated nuclear material. (E) An *itr-1(sy290)* worm with fertilized, viable embryos in utero. (F) An *rrf-1(pk1417)* worm showing fertilized, viable embryos in utero. White bars = 40µm.

As the actin and tubulins are affected by the decreasing CCT family of proteins, we sought whether an increase in cytosolic calcium would reduce the sterility seen in *C. elegans* fed RNAi of the CCT genes. The *itr-1(sy290)* mutant strains showed a decrease in sterility under RNAi conditions for *cct-8* only (20%, N = 30), indicative of a role in the IP_3 signaling pathway. Additionally, CCT-8 protein appears primarily in the germ-line rather than the somatic tissues, as the *rrf-1(pk1417)* strain showed a drastically decreased sterility count under RNAi conditions (7%, N = 30).

The *ubq-2* gene produces a bifunctional protein involved in both protein synthesis and degradation, and is one of only two genes that produce full ubiquitin in *C. elegans* (Jones and others 1995). The N-terminus of UBQ-2 contains a ubiquitin peptide, while the C-terminus has a large ribosomal subunit peptide (RPL-40) (Jones and others 1995). Expression occurs in all somatic tissues, although gonad defects presented in expression study assays (Jones and others 1995). Although the wild-type worms failed to present a sterility score as high as other genes, (70% ± 8.8%, N = 27), the near total rescue in the *itr-1(sy290)* mutants (3% ± 3%, N = 30) made this a significant gene for further evaluation as well. The protein's link to the ribosome, the largest group affected by the excess cytoplasmic calcium seen in the *itr-1* mutants, also appeared significant. During the later stages of gonad development, primarily the L3 stage, ribosome synthesis increases to accommodate the necessary protein production for developing oocytes (Hirsh, Oppenheim, Klass 1976).

Proteasomal Subunit Proteins

Protein synthesis helps maintain the proper homeostasis of cells by producing the necessary enzymes, chaperones and proteins required for everyday survival. On the other

end of the spectrum lies the proteasome, which degrades older proteins that may no longer be effective, as well as mis-folded proteins that cannot be corrected. A special 76 amino acid protein tag called ubiquitin designates the proteins to be degraded. In some species, ubiquitin production increases under stress, though this does not occur in *C. elegans* (Graham, Jones, Candido 1989). Ubiquitin attaches to selected proteins identified by E3 ubiquitin-protein ligases, after the ubiquitin is first activated by an E1 ubiquitin-activating enzyme and being transferred first to an E2 ubiquitin-conjugating enzyme (Hershko and Ciechanover 1998). For degradation of proteins to occur, multiple ubiquitins attach to each other via the E3 ligases. Recognition of ubiquitin labeled proteins occurs when the 26S proteasome identifies four ubiquitin proteins attached to each other on the target protein (Thrower and others 2000).

The 26S proteasome consists of several subunits that join together to form a functional entity, much like the ribosome. The central, 20S core particle consists of four stacked rings (two outer and two inner) that each contain seven proteins of either an α (outer) or β (inner) nature (Peters, Franke, Kleinschmidt 1994). The α rings on the outside provide structural scaffolding for the proteasome and act as a gate for the entry of proteins to be degraded (Smith and others 2007). Once inside the proteasome, protein degradation occurs via protease active sites located on the β proteins that comprise the inner rings (Heinemeyer and others 1997). Additionally, the proteasome contains a 19S subunit that is itself comprised of two subunits, a 10 protein base that binds to the core 20S subunit and a 9 protein lid that binds to the polyubiquitin tag on proteins to be degraded (Baumeister and others 1998; Sharon and others 2006). All together, the 19S subunit allows only polyubiquitinated proteins to flow through the gate of the 20S core

particle to be degraded, as it caps the 20S subunit at either end (Kohler and others 2001; Walz and others 1998).

Of the 21 degradation related genes tested in our study for sterility, 19 were found to maintain a high level of sterility in the N2 wild-type *C. elegans*. Within these 19 remaining sterility inducing genes, significant reductions in sterility (≥ 60%) were seen in 3 genes: *pbs-1*, *rpn-1*, and *rpt-1* (Table 3.3). Each of these genes plays a role in the proteasome, whether as a structural (*pbs-1*, *rpn-1*, *rpt-1*) or enzymatic component (*pbs-1*, *rpt-1*).

The PBS-1 protein affects fertility, embryonic viability, larval viability as well as locomotion (Kamath and Ahringer 2003; Kamath and others 2003; Simmer and others 2003), which stands to reason the results from our RNAi screen. Wild-type *C. elegans* showed a very high sterility under RNAi conditions (97% ± 3, N = 30). The rescue of the sterile phenotype in the *itr-1(sy290)* mutant (33% ± 8.6 %, N = 30) implicates *pbs-1* in the IP_3 signaling pathway. Another possible explanation lies in the excess cytoplasmic calcium playing a role in proteasome function that then allows for reduced sterility.

Components of the 19S regulatory particle have shown larger phenotypic characteristics when depleted, notably *rpn-10* causing the female phenotype in *C. elegans* instead of normal hermaphrodite development (Shimada and others 2006). Both *rpn-1* and *rpt-1* gene products constitute proteins in the 19S regulatory particle of the proteasome. The RPT-1 protein contains an ATPase site while the RPN-1 is a non-ATPase protein, though both are essential for germline development and embryonic viability (Wormbase.org). RNAi of *rpn-1* and *rpt-1* presented high levels of sterility in wild-type worms as well (83% ± 6.9%, N = 30 and 100%, N = 30, respectively).

Table 3.3. Protein degradation genes causing sterility (≥ 60% in N2 *C. elegans*) under RNAi conditions. N = number of animals. Each entry represents at least the initial phenotypic scoring.

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
C36B1.4	pas-4	Proteasome alpha subunit 4 of core 20S subcomplex; Regulates SKN-1	Kahn 2007	80 ± 5.2 (60)	93 ± 4.8 (28)	71 ± 5.9 (59)
F25H2.9	pas-5	Proteasome alpha subunit 5 of core 20S subcomplex; Regulates SKN-1	Kahn 2007	82 ± 5 (60)	27 ± 9.5 (22)	13 ± 6.1 (30)
C47B2.4	pbs-2	Proteasome beta subunit 2 of core 20S subcomplex	Wormbase	97 ± 2.2 (60)	77 ± 5.4 (60)	13 ± 6.1 (30)
T20F5.2	pbs-4	Proteasome beta subunit 4 of core 20S subcomplex; Development	Takahashi 2002	87 ± 6.1 (30)	41 ± 9.1 (29)	60 ± 8.9 (30)
K05C4.1	pbs-5	Porteasome beta subunit 5 of core 20S subcomplex	Wormbase	100 (30)	100 (23)	64 ± 9.1 (28)
R12E2.3	rpn-8	Proteasome regulatory particle of 26S regulatory complex	Wormbase	100 (30)	97 ± 3.1 (30)	53 ± 13 (15)
Y38A8.2	pbs-3	Porteasome beta subunit 3 of core 20S subcomplex; Development	Takahashi 2002	100 (60)	98 ± 1.8 (60)	92 ± 3.5 (60)
C02F5.9	pbs-6	Porteasome beta subunit 6 of core 20S subcomplex	Wormbase	100 (30)	77 ± 7.7 (30)	47 ± 9.1 (30)
F23F1.8	rpt-4	ATPase subunit of 19S regulatory complex	Davy 2001	95 ± 3.4 (40)	60 ± 15 (10)	83 ± 6.9 (30)
C30C11.2	rpn-3	Proteasome regulatory particle of 26S regulatory complex	Wormbase	73 ± 8.1 (30)	57 ± 9 (30)	53 ± 9.1 (30)

Table 3.3 Continued

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
F57B9.10	rpn-6	Proteasome regulatory particle of 26S regulatory complex	Davy 2001	100 (54)	90 ± 3.9 (60)	98 ± 2.1 (46)
F23F12.6	rpt-3	ATPase subunit of 19S regulatory complex; unfolding proteins	Takahashi 2002	67 ± 8.6 (30)	67 ± 8.6 (30)	57 ± 9 (30)
H06I04.4	ubl-1	Ubiquitin/40S ribosomal protein S27a fusion	Wormbase	100 (30)	100 (30)	100 (30)
K08D12.1	pbs-1	Porteasome beta subunit 1of core 20S subcomplex	Wormbase	97 ± 3 (30)	33 ± 8.6 (30)	20 ± 7.3 (30)
T22D1.9	rpn-1	Proteasome regulatory particle of 26S regulatory complex	Takahashi 2002	83 ± 6.9 (30)	0 (30)	0 (30)
C47E12.5	uba-1	Ubiquitin-activating enzyme; embryonic, sperm and germline development	Kulkarni and Smith 2008	90 ± 5.5 (30)	70 ± 8.4 (30)	38 ± 9 (29)
CD4.6	pas-6	Porteasome alpha subunit 6 of core 20S subcomplex; Select proteins to degrade	Davy 2001	100 (30)	100 (30)	87 ± 6.1 (30)
C23G10.4	rpn-2	Proteasome regulatory particle of 26S regulatory complex	Takahashi 2002	100 (30)	43 ± 9 (30)	87 ± 6.1 (30)
C52E4.4	rpt-1	ATPase subunit of 19S regulatory complex	Wormbase	100 (30)	33 ± 8.6 (30)	90 ± 5.5 (30)

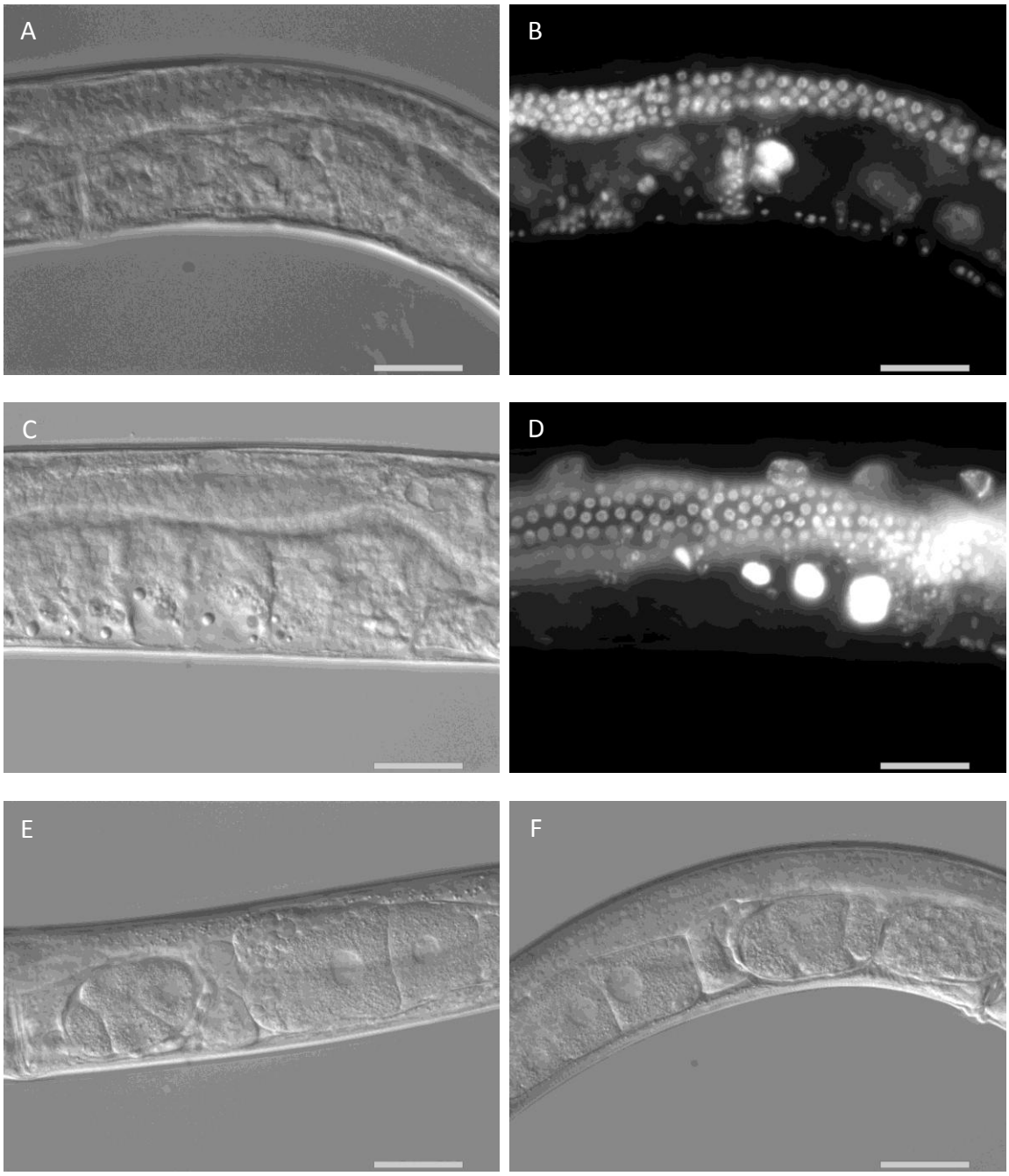

Figure 3.10. Worms fed *pbs-1* RNAi bacteria. (A) An N2 worm with compacted proximal oocytes. (B) Same N2 worm with DAPI staining showing the beginning of an Emo phenotype. (C) An *rrf-3(pk1426)* worm showing compacted proximal oocytes. (D) DAPI staining of same *rrf-3(pk1426)* worm with accumulated nuclear material. (E) & (F) An *itr-1(sy290)* worm with fertilized, viable embryos in utero. White bars = 40µm.

Rescuing of the phenotype in *itr-1(sy290)* mutants (0%, N = 30 and 33% ± 8.6%, N = 30, respectively) presents a possible role for cytoplasmic calcium in the regulatory particle of the proteasome, or these genes as having additional roles in the IP_3 signaling pathway.

Stress appears to play a role in the activation of the proteasome, and vice versa. The *pbs-1* and *rpn-1* promoters contain SKN-1 binding regions which indicate a role in stress response (Kahn and others 2008). The *skn-1* gene codes for a transcription factor required for both embryo and mesodermal development (An and Blackwell 2003; Bowerman, Eaton, Priess 1992). Under RNAi conditions for both of these genes, SKN-1 activation occurs (Kahn and others 2008). Links between these genes and nematode longevity have also been postulated (Oliveira and others 2009; Park, Tedesco, Johnson 2009).

Signaling Proteins

Of course, signaling proteins would be likely prospects for finding genes involved in the IP_3 signaling pathway. During the screening, results were obtained for known components of the IP_3 signaling pathway: *plc-3*, *ppk-1* and *plc-1*. The *plc* genes encode phospholipases which cleave PIP_2 into IP_3 and DAG, with IP_3 going on to bind ITR-1 on the ER and release calcium, controlling muscle contractions in the sheath cells as well as the intestine (Espelt and others 2005; Yin and others 2004). In *C. elegans*, the *plc-1* gene is relegated to the spermatheca and controls ovulation at the spermatheca (Hiatt and others 2009; Kariya and others 2004) and is necessary for normal arrangement of the hypodermal cells during development (Vazquez-Manrique and others 2008). In contrast, *plc-3* is largely expressed in the somatic sheath cells for sheath cell contractions, as described above (Yin and others 2004), though it can rescue the role of *plc-1* in epidermal

Table 3.4. Cellular signaling genes causing sterility (≥ 60% in N2 *C. elegans*) under RNAi conditions. N = number of animals. Each entry represents at least the initial phenotypic scoring.

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
C29F9.7	pat-4	Integrin-linked kinase; adaptor molecule for integrin adhesion complexes	Mackinnon 2002	77 ± 7.7 (30)	100 (30)	68 ± 8.8 (28)
C32E8.8	ptr-2	Membrane protein for cytokinesis in somatic cells	Zugasti 2005	83 ± 4.8 (60)	98 ± 1.8 (59)	100 (29)
F13D12.7	gpb-1	G-protein beta subunit	Zwaal 1996	100 (30)	100 (30)	53 ± 9.1 (30)
F31B 12.1	plc-1	Phospholipase C; Spermathecal dilation	Kariya 2004	77 ± 7.7 (30)	17 ± 6.9 (30)	10 ± 5.5 (30)
F33D4.2	itr-1	Inositol 1,4,5-triphosphate receptor	Clandinin 1998	100 (30)	100 (30)	13 ± 6.1 (30)
F48E8.5	paa-1	Protein phosphatase 2A regulatory subunit	Janssens 2001	100 (30)	87 ± 6.1 (30)	47 ± 9.1 (30)
F55A12.3	ppk-1	Intense sheath cell contractions, spermatheca dilation	Xu 2007	73 ± 8.1 (30)	0 (30)	0 (30)

Table 3.4. Continued

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
K08C7.3	epi-1	Laminin alpha chain, oogenesis	Zhu 1999	100 (30)	40 ± 8.9 (30)	0 (30)
K08E3.6	cyk-4	GTPase-activating protein for microtubules and cytokinesis	Mishima 2002	100 (60)	100 (60)	100 (60)
K12H4.4	phi-20	Signal peptidase complex subunit	Interpro	100 (24)	96 ± 4 (23)	100 (19)
T01E8.3	plc-3	Phospholipase C; Cleaves PIP_2	Yin 2004	76 ± 6.4 (45)	0 (30)	4 ± 2.9 (45)
T05G5.3	cdk-1	Cyclin-dependent kinase for cell cycle progression and divisions	Boxem 1999	100 (30)	100 (30)	100 (30)
Y71D11A.5	lgc-46	Ligand-gated ion channel	Wormbase	92 ± 3.5 (60)	100 (60)	92 ± 3.5 (60)

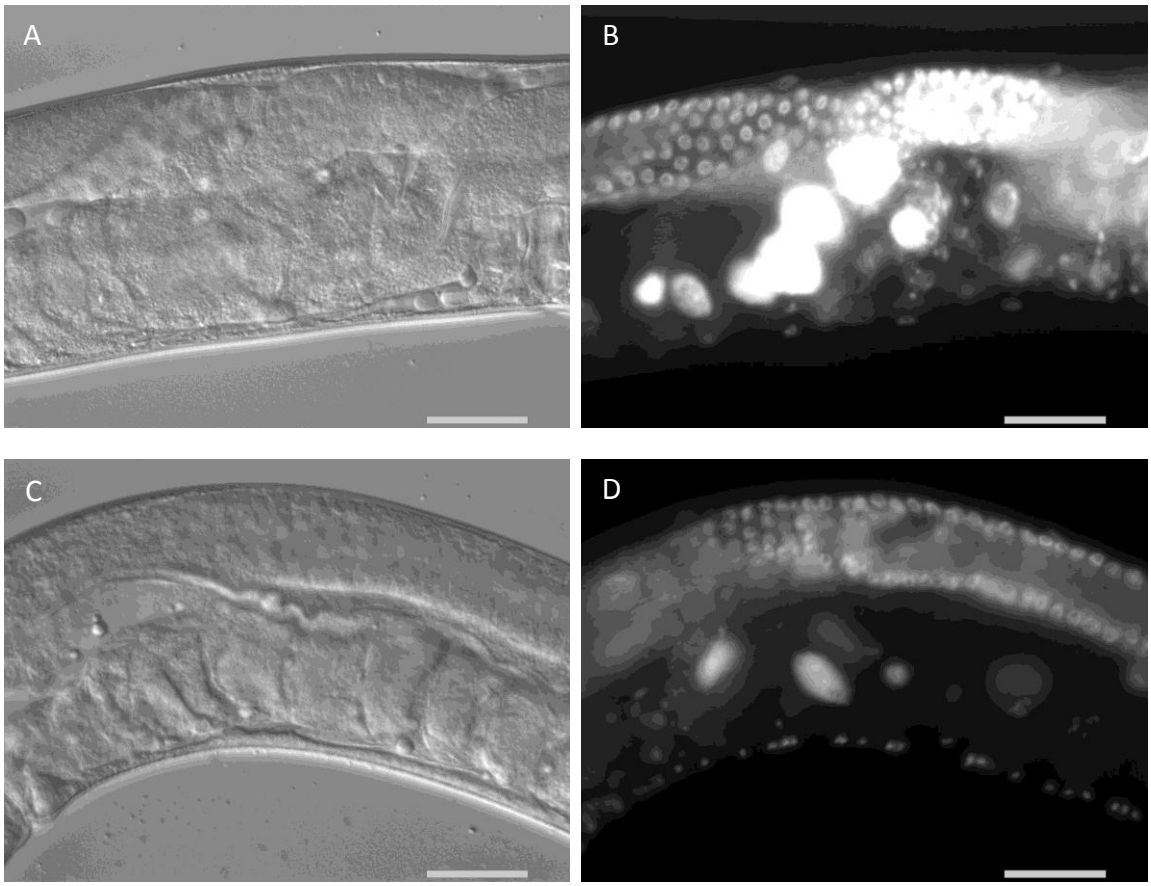

Figure 3.11. Worms fed *plc-3* RNAi bacteria. (A) An N2 worm with compacted proximal oocytes. (B) Same N2 worm with DAPI staining showing the accumulation of nuclear material typical of an Emo phenotype. (C) An *rrf-3(pk1426)* worm showing compacted proximal oocytes. (D) DAPI staining of same *rrf-3(pk1426)* worm with accumulated nuclear material. White bars = 40µm.

morphogenesis (Vazquez-Manrique and others 2008). Most recently, *plc-3* activity and calcium concentrations in neurons for avoidance responses have also been identified (Walker and others 2009). *ppk-1* codes a phosphatidylinositol-4-phosphate 5' kinase necessary for ovulation and myosin (UNC-54) organization, which also interacts directly with the IP_3 receptor, ITR-1 (Walker and others 2002; Xu and others 2007).

Most of the remaining signaling proteins that caused sterility in N2 worms maintained sterility in the *itr-1(sy290)* mutants, indicating they function outside the IP_3

signaling pathway, or may be downstream of ITR-1. A notable exception in sterility scores was *epi-1*, a laminin alpha chain protein of the basement membrane (Zhu and others 1999). *epi-1* also impacts oogenesis through the proper gonad organization and provides staibilty that allows yolk proteins to move to the oocytes during development (Hall and others 1999). Defects in the muscle-lamina connections, similar to those seen in *pat-3* mutations or RNAi, cause disruptions in body wall muscles and sheath cell epithelialization (Hall and others 1999). N2 worms resulted in complete sterility (100%, N = 30) under RNAi conditions for *epi-1*, while the *itr-1(sy290)* mutants showed a decrease in sterility (40% ± 8.9%, N = 30). Additionally, the *rrf-1(pk1417)* worms were completely rescued (0%, N = 30) from the sterility, which shows the *epi-1* is necessary for somatic tissues and in particular the sheath cells. Interestingly, the gonads of *epi-1(RNAi)* worms appeared to undergo proximal mitosis, rather than having an Emo phenotype (Figure 3.12).

Metabolism Related Proteins

All living things undergo metabolic processes in order to live, thrive and reproduce. This includes the breaking down of materials, or catabolism, and the building of others from those components, anabolism. The metabolic related proteins associated with sterility in *C. elegans* have a variety of functions when looking at the group as a whole, though they appear to have links to several of the other categories.

Larval progression up to the adult stage allows the temporal development of the gonads over stages, ensuring all the components come to fruition. The *atp-2* gene codes for an ATP synthase, which is essential for proper development past the L3 larval stage (Tsang and others 2001). During the transition from L3 to L4 and on to adulthood, the

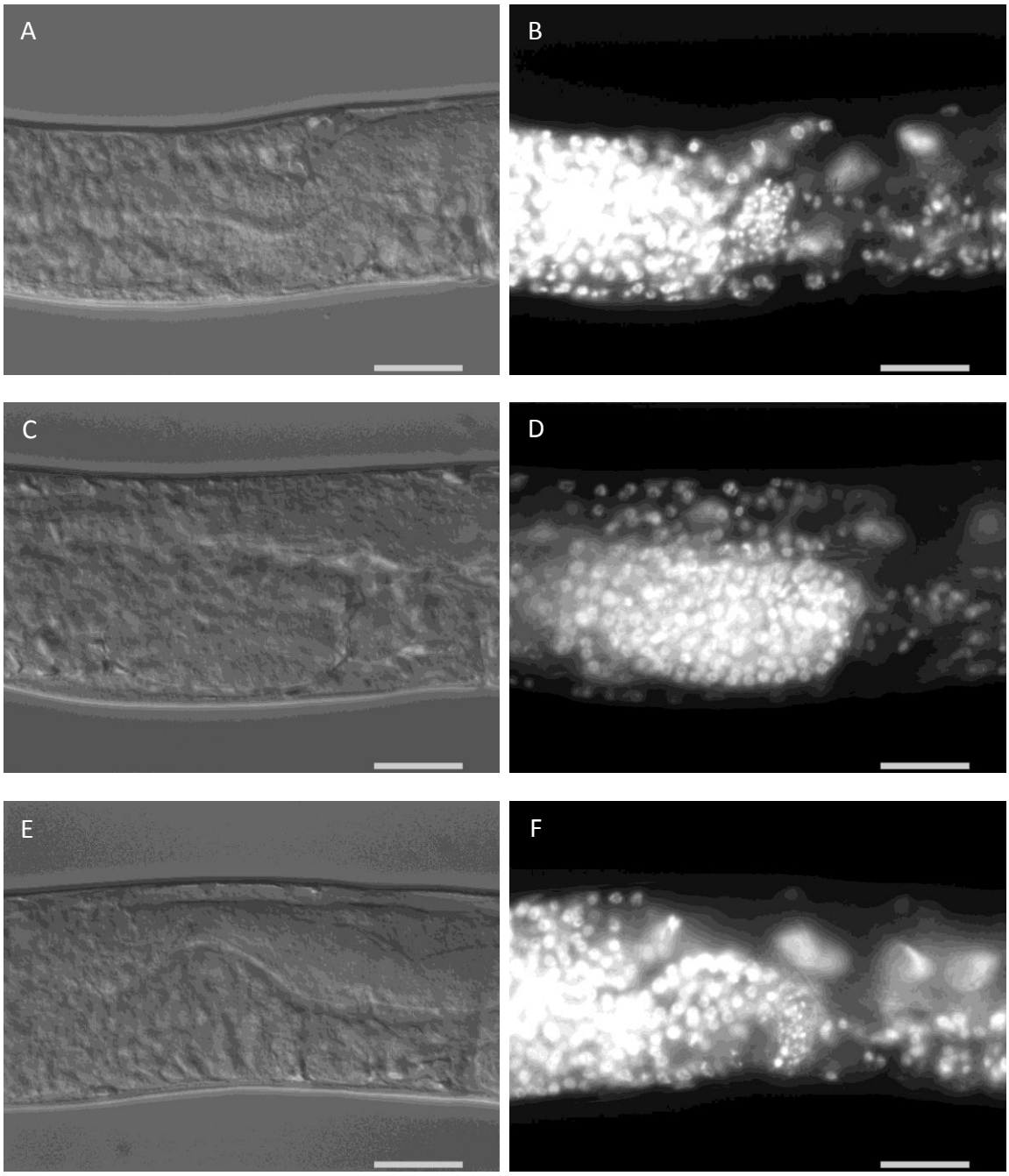

Figure 3.12. Worms fed *epi-1* RNAi bacteria. (A) & (C) DIC images of N2 worms showing a lack of distinct oocytes in the proximal gonad. (B) & (D) DAPI staining of the same N2 worms with apparent mitosis occurring in the proximal gonad. (E) An *rrf-3(pk1426)* worm with a lack of proximal oocytes. (F) DAPI staining of the *rrf-3(pk1426)* worm showing mitosis in the proximal gonad. White bars = 40µm.

worms stop developing spermatids and transition to oocyte development (Slack and others 2000). Without the ability to synthesize ATP, the worms fail to thrive, as the organisms require ATP for most functions. Sterility seen in the worms underscores the necessity for ATP, as N2, *itr-1* and *rrf-1* worm strains maintained high sterility scores (97% ± 3.1%, N = 30; 100%, N = 28; and 87% ± 6.1%, N = 30 respectively).

The F02F8.2 gene codes a 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase that allows conversion of HMG-CoA to mevalonate during sterol biosynthesis and comprises an integral membrane protein in the ER (Hampton, Gardner, Rine 1996). Sterols provide the basic components for hormones as well as components of cellular membranes, such as cholesterol. Sterility in the worms was maintained in N2 and *itr-1* strains (67% ± 8.6%, N = 30 for both) and greatly reduced in the *rrf-1* strain (0%, N = 30) indicating a role in the somatic tissues as opposed to the germ line cells.

Two vacuolar H+-ATPase subunit particles identified in this study also caused *C. elegans* sterility, *vha-12* and *vha-13*. These genes contribute to lysosome formation by acidifying the lysosome, pumping in hydrogen atoms. Excess cytoplasmic calcium has been shown to promote necrotic cell death (Syntichaki, Samara, Tavernarakis 2005). Necrotic cell death requires proper lysosomal biogenesis and function (Artal-Sanz and others 2006). Both genes showed high sterility scores in N2 worms (100%, N = 57 and 100%, N = 30, respectively), though differed in the mutant strains. Interestingly, *vha-12* appears more essential to reproductive health as the high sterility was maintained in both *itr-1* and *rrf-1* mutants (93% ± 3.4%, N = 58 and 100%, N = 57, respectively). However, the *vha-13* gene was partially rescued by the *itr-1* mutant, and almost completely rescued in *rrf-1* worms (43% ± 9%, N = 30 and 10% ± 5.5%, N = 30, respectively). This may be

due to alterations seen in VHA-13 levels under abnormal concentrations of calcineurin levels (Ahn and others 2006).

Fat accumulation provides a source of energy during times of food shortage. The MDT-15 protein regulates levels of numerous metabolic genes, which explains its being placed in the metabolic genes category. MDT-15 regulates fatty acid metabolism through its interaction with another protein, NHR-49, as well as independently of the NHR-49 protein, making it a necessity for dietary importance (Taubert and others 2006). Sterility caused by RNAi of *mdt-15* was high in N2 worms (80% ± 7.3%, N = 30) and remained relatively high in the *itr-1* strain (50% ± 9.1%, N = 30). In the *rrf-1* strain, sterility was reduced greatly (17% ± 6.9%, N = 30), signifying the importance for fat accumulation in the somatic tissues.

The LPD-7 protein has previously been predicted to localize in the nucleolus, the site of ribosome biogenesis, and may be required for maturation of rRNAs and the large ribosomal subunit based on its similarity to proteins in yeast and vertebrates (Lapik and others 2004; McKay and others 2003). This information highlights the need for the ribosomes in fertility of the worms, and links the protein to the protein synthesis genes. N2 worms presented high sterility (100%, N = 30), while the *itr-1* strain was largely rescued (37% ± 8.8%, N = 30). Interestingly, the *rrf-1* strain remained mostly sterile (70% ± 8.4%, N = 30), indicating a need for *lpd-7* in the germline.

Table 3.5. Metabolism affiliated genes causing sterility (≥ 60% in N2 *C. elegans*) under RNAi conditions. N = number of animals. Each entry represents at least the initial phenotypic scoring.

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
C34E10.6	atp-2	ATP synthase for embryonic and larval development past L3	Tsang 2001	97 ± 3.1 (30)	100 (28)	87 ± 6.1 (30)
F08F8.2		HMG-CoA reductase	Wormbase	67 ± 8.6 (30)	67 ± 8.6 (30)	0 (30)
F20B6.2	vha-12	Vacuolar H+-ATPase, subunit B involved in necrosis and cell fusion	Syntichaki 2005	100 (57)	93 ± 3.4 (58)	100 (57)
R12B2.5	mdt-15	Aids fat accumulation, transcription activator for survival to present toxins	Taubert 2006; Yang 2006	80 ± 7.3 (30)	50 ± 9.1 (30)	17 ± 6.9 (30)
R13A5.12	lpd-7	Nucleolus assembly, ribosome biogenesis, fat storage	McKay 2003	100 (30)	37 ± 8.8 (30)	70 ± 8.4 (30)
T23G5.1	rnr-1	Ribonucleotide reductase, alpha	WormBook	93 ± 4.7 (30)	73 ± 8.1 (30)	100 (30)
Y49A3A.2	vha-13	Vacuolar H+-ATPase, subunit A	Syntichaki 2005	100 (30)	43 ± 9 (30)	10 ± 5.5 (30)
Y87G2A.l	vrs-2	Valyl-tRNA synthetase, germline and embryonic development	Wormbase	100 (30)	100 (30)	67 ± 8.6 (30)

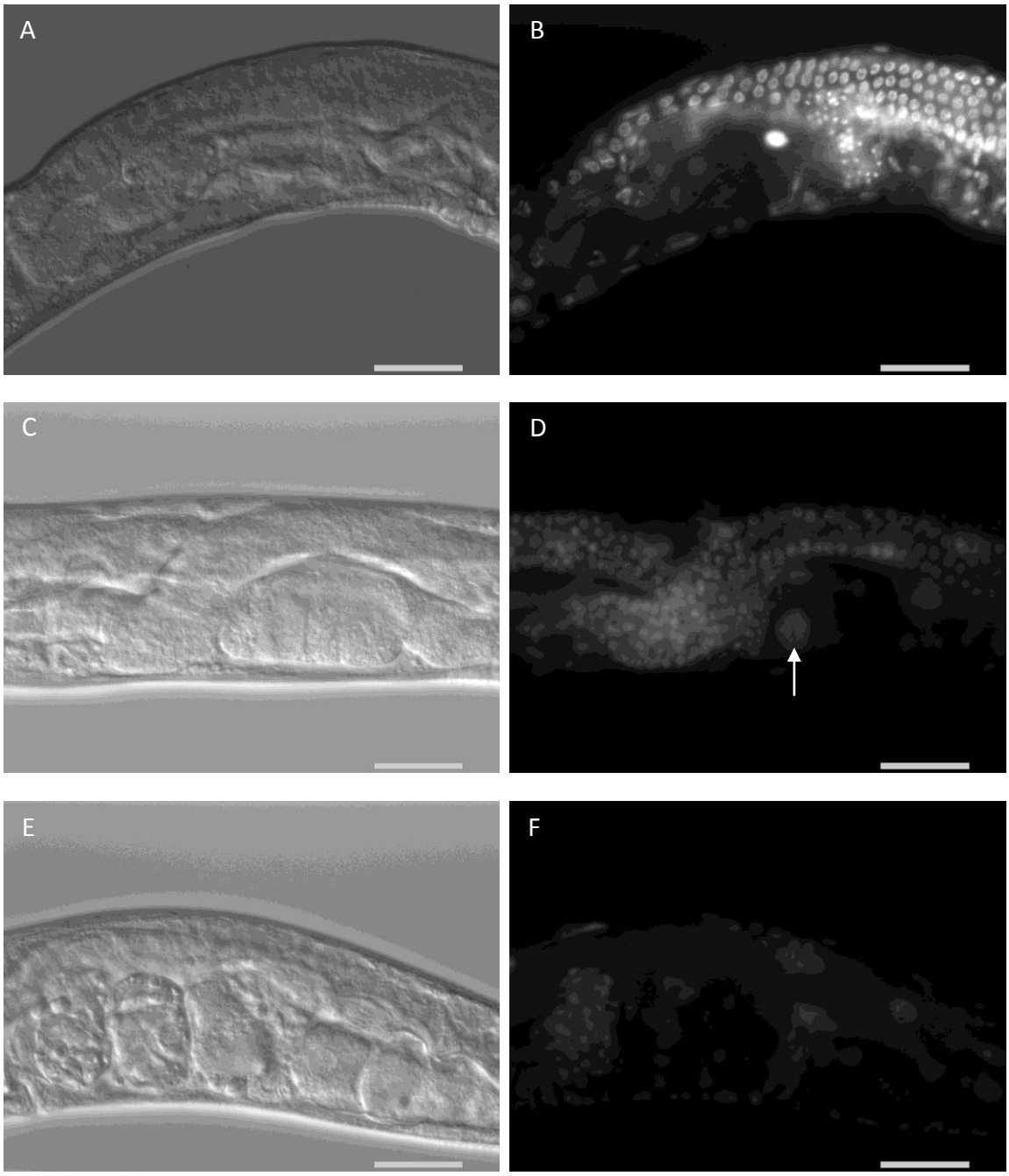

Figure 3.13. Worms fed *lpd-7* RNAi bacteria. (A) An N2 worm with compacted proximal oocytes. (B) Same N2 worm with DAPI staining showing the beginning of an Emo phenotype. (C) An *rrf-3(pk1426)* worm showing compacted proximal oocytes. (D) DAPI staining of same *rrf-3(pk1426)* worm with accumulated nuclear material (white arrow). (E) An *itr-1(sy290)* worm with fertilized, viable embryos in utero. (F) DAPI staining of *itr-1(sy290)* worm, showing multicellular embryo in utero. White bars = 40μm.

The *rnr-1* gene codes a ribonucleotide reductase, predicted to participate in deoxyribonucleotide biosynthesis and deals with the cell cycle control (Brodigan and others 2003; Hong, Roy, Ambros 1998). Sterility under RNAi conditions for *rnr-1* was largely sterile across all worm strains. N2 and *rrf-1* worms showed the highest sterility (93% ± 4.7%, N = 30 and 100%, N = 30, respectively), with the *itr-1* strain showing a slight reduction (73% ± 8.1%, N = 30), though not enough to be considered significant.

Protein production relies upon tRNAs bringing the peptides to the ribosomes for development of the polypeptide protein chain. *vrs-2* codes a predicted cytoplasmic valyl-tRNA synthetase, responsible for attaching valine amino acids to the respective tRNAs (Fraser and others 2000; Maeda and others 2001). RNAi of *vrs-2* resulted largely in sterility. N2 and *itr-1* worms both were completely sterile (100%, N = 30 for both strains), which the *rrf-1* showed a decrease (67% ± 8.6%, N = 30), though again not enough to be considered significant.

RNA Synthesis Related Proteins

RNAs transcribed from the genomic DNA material are responsible, either directly or indirectly, for everything that occurs in the cell. Whether they provide catalytic functions as in the ribosomes, fuse to peptides for the manufacturing of proteins by the ribosomes, or are translated into the various proteins needed by the the cells, the RNAs are invaluable. As such, the proteins involved in processing and controlling the RNA production have essential functions as well, and several appear to play roles in determining the sterility in *C. elegans*.

Table 3.6. RNA synthesis genes causing sterility (≥ 60% in N2 *C. elegans*) under RNAi conditions. N = number of animals. Each entry represents at least the initial phenotypic scoring.

Gene Pair	Locus	Function	Reference	% Sterile (N)		
				N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
C26E6.4	rpb-2	RNA polymerase II	WormBook	100 (49)	99 ± 1 (67)	100 (54)
C52E4.3	snr-4	Bind snRNPs, P granule localization	Barbee 2002	87 ± 6.1 (30)	23 ± 7.7 (30)	17 ± 6.9 (30)
D1054.15	tag-135	Pleiotropic regulator 1	Wormbase	75 ± 5.6 (60)	73 ± 8.1 (30)	48 ± 6.5 (60)
F09F7.3		RNA polymerase III	Wormbase	100 (30)	100 (30)	97 ± 3 (30)
M03F8.3	phi-12	Cell cycle control	Wormbase	100 (30)	17 ± 6.9 (30)	70 ± 8.4 (30)
R144.2	pcf-11	mRNA cleavage, polyA factor	McCracken 2003	100 (30)	93 ± 4.7 (30)	93 ± 4.7 (30)
T08A11.2	phi-11	Splicing factor 3b, subunit 1	Govindan 2006	97 ± 2.2 (60)	97 ± 2.2 (60)	88 ± 4.2 (60)
T12A2.7		Spliceosome-associated coiled-coil protein	Wormbase	100 (30)	97 ± 3 (30)	100 (30)
ZK652.1	snr-5	snRNP biogenesis, embryo and larval viability	Barbee 2002	83 ± 6.9 (30)	27 ± 8.1 (30)	33 ± 8.6 (30)

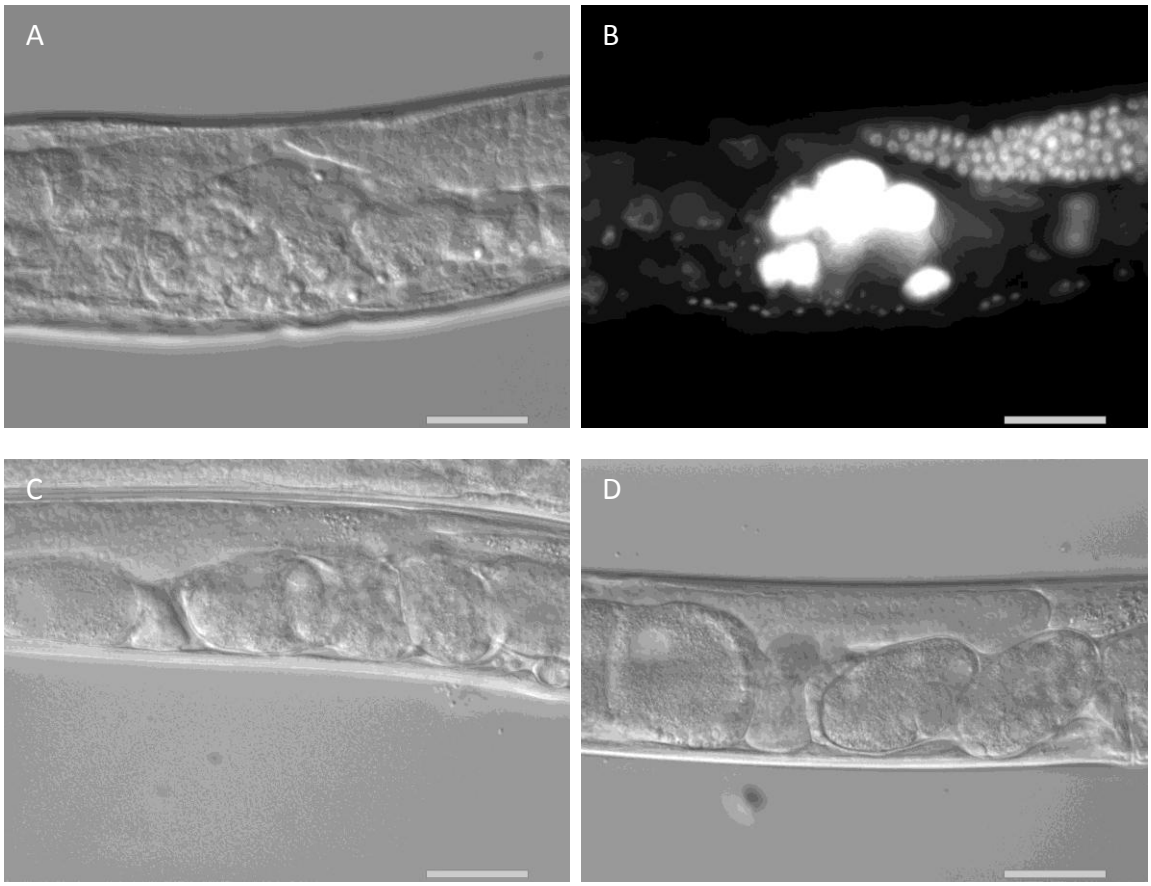

Figure 3.14. Worms fed *snr-4* RNAi bacteria. (A) N2 worm showing Emo phenotype. (B) N2 worm with DAPI staining highlighting the excess nucleic acid material in the nucleus. (C) An *itr-1(sy290)* mutant showing fertilized oocytes in the uterus, rescuing the Emo phenotype. (D) An *rrf-1(pk1417)* mutant showing a lack of an Emo phenotype. White arrows point to nuclei of occytes. White bars = 40µm.

The proteins involved in RNA production and processing that appear to have a role in *C. elegans* sterility in this study form a relatively small grouping of 9 genes, with most appearing to deal with transcription or the spliceosome, and two polymerase genes. Polymerases provide the essential function of creating polymers of nucleic acids, necessary if any protein manufacturing is to occur. The *rpb-2* gene codes the RNA polymerase (Pol) II (B) subunit, and most previous RNAi studies resulted in embryonic lethality (Gonczy and others 2000; Kamath and others 2003; Simmer and others 2003; Sonnichsen and others 2005). The Pol II manufactures mRNAs from the genomic DNA,

which results in the embryonic lethality seen in RNAi conditions. Transcription occurs in oocytes but halts prior to fertilization, although maturation of the oocyte signals for the reactivation of Pol II (Walker, Boag, Blackwell 2007). Under starvation conditions, worms are capable of surviving by limiting transcription until food sources are found, and Pol II has been shown to accumulate to promoters to begin transcription, anticipating the finding of nutrients (Baugh, Demodena, Sternberg 2009). Sterility in this study was nearly complete with N2, *itr-1(sy290)* and *rrf-1(pk1417)* worm strains (100%, N = 49; 99% ± 1%, N = 67; and 100%, N = 54, respectively).

Likewise, the gene encoding the RNA Pol III protein, F09F7.3, has presented with some embryonic lethality but more often with a slow growth phenotype (Byrne and others 2007; Kamath and others 2003). Pol III transcribes the tRNAs, as well as the 5S rRNA and other small RNAs like the microRNAs (Borchert, Lanier, Davidson 2006). This study showed a predominant sterility phenotype in the N2, *itr-1(sy290)* and *rrf-1(pk1417)* worms (100%, N = 30; 100%, N = 30; and 97% ± 3%, N = 30, respectively).

Both *snr-4* and *snr-5* genes code for small nuclear ribonucleoproteins in *C. elegans*. The splicing factors appear to have a special function in segregating P granules, large particles that localize to germ cells (Barbee, Lublin, Evans 2002; Kamath and others 2003). These genes are expressed in embryos and the gonads of adult worms (Barbee, Lublin, Evans 2002). Disrupting these *snr-4* and *snr-5* genes resulted in high sterility in wild-type worms (87% ± 6.1%, N = 30; and 83% ± 6.9%, N = 30, respectively). Decreases were seen for *snr-4* and *snr-5* in both *itr-1(sy290)* (23% ± 7.7%, N = 30 and 27% ± 8.1%, N = 30) and *rrf-1(pk1417)* (17% ± 6.9%, N = 30; and 33% ± 8.6%, N = 30), indicating a possible role for the IP_3 signaling pathway in spliceosome

activity and possibly in P granule segregation. However, the T12A2.7 gene, which is predicted to code for a spliceosome-associated coiled-coil protein for pre-mRNA splicing, showed high sterility across all worm strains (100%, N = 30; 97% ± 3%, N = 30; and 100%, N = 30, respectively). Little additional information on T12A2.7 exists, although it does share some similarity with a mammalian breast cancer protein, DAM1 (Nagasaki and others 1999).

Another BLASTP predicted splicing factor, D1054.15, resulted in sterility under RNAi conditions in worm strains, albeit lower than some other factors. N2 (75% ± 5.6%, N = 60) and *itr-1(sy290)* (73% ± 8.1%, N = 30) showed similar sterility scores, though *rrf-1(pk1417)* was slightly more rescued (48% ± 6.5%, N = 60). The gene name itself, *tag-135*, stands for Temporarily Assigned Gene name, most likely due to the lack of definitive information that surrounds the gene. Even expression profiles have been unable to narrow down its location (Hunt-Newbury and others 2007; McKay and others 2003).

T08A11.2 codes an ortholog of the human splicing factor 3b subunit 1, according to BLASTP results. The splicing factor processes pre-mRNA by associating with the spliceosome, helping form the U2 snRNP through multiple binding sites (Thickman and others 2006). Sterility witnessed in N2 (97% ± 2.2%, N = 60), *itr-1(sy290)* (97% ± 2.2%, N = 60) and *rrf-1(pk1417)* worms (88% ± 4.2%, N = 60) was most likely due to the necessity for properly processed mRNAs.

The mRNA processing requires more than just mRNA cleavage, as a 5' cap and 3' polyadenosine tail is added as well to form fully capable mRNAs. The *pcf-11* gene codes an mRNA cleavage factor that is also associated with polyA tail addition (Cui and

others 2008; McCracken and others 2003). Without this gene, high sterility resulted in N2 (100%, N = 30), *itr-1(sy290)* (93% ± 4.7%, N = 30) and *rrf-1(pk1417)* (93% ± 4.7%, N = 30), highlighting the need for proper mRNA processing.

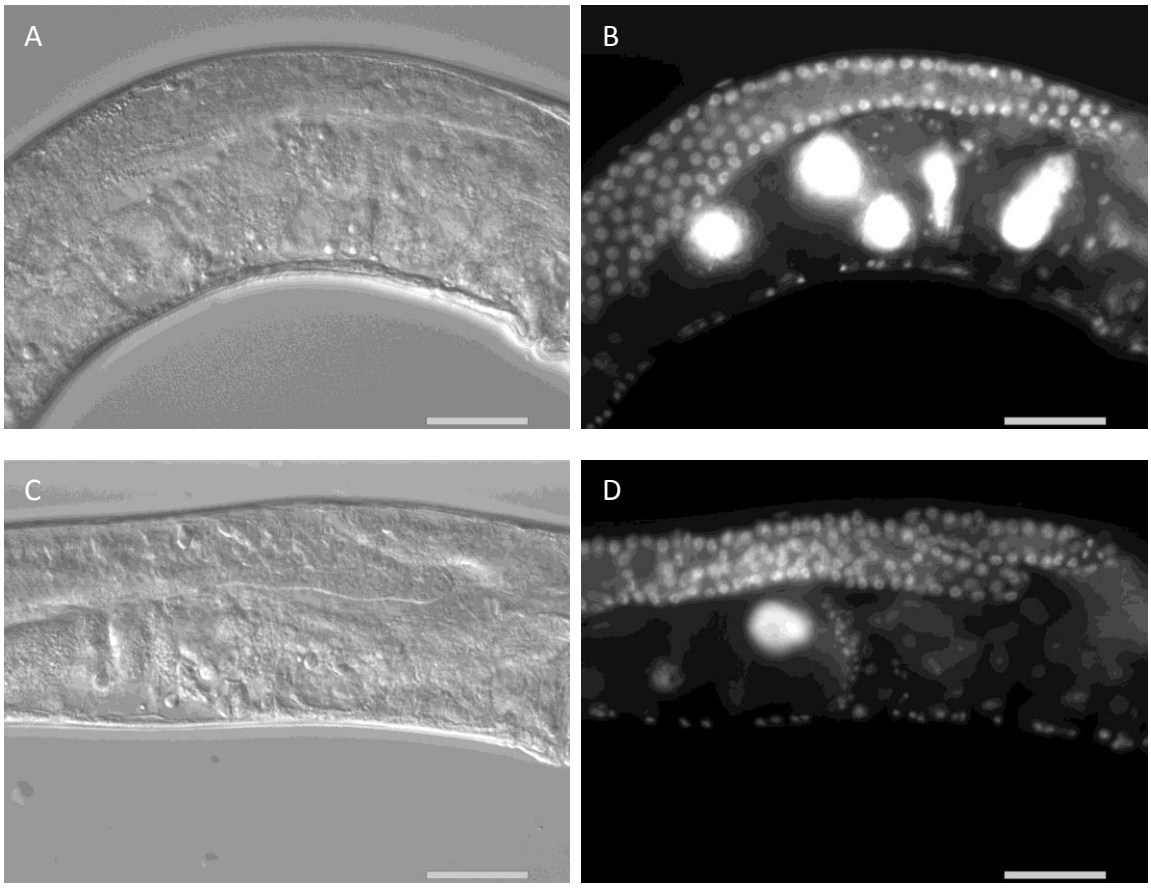

Figure 3.15. Worms fed M03F8.3 RNAi bacteria. (A) & (C) DIC images of N2 worms showing some compactedness of the oocytes in the proximal gonad. (B) & (D) DAPI staining of N2 worms highlighting the accumulation of nuclear material typical of Emo phenotypes. White bare = 40µm.

Of all the various spliceosome components that cause sterility, M03F8.3, which appears to code for a cell cycle control protein, caused sterility in N2 worms (100%, N = 30) but was rescued in *itr-1(sy290)* worms (17% ± 6.9%, N = 30). While the *rrf-1(pk1417)* strain showed a decrease in sterility as well (70% ± 8.4%, N = 30), it was far less pronounced than the *itr-1(sy290)*, indicating a role for M03F8.3 in both somatic and

germline cells, and somehow plays a role in IP_3 signaling or has some relation to cytoplasmic calcium in the sheath cells. BLASTP analysis shows orthology to CLF1 (Crooked neck-Like Factor) in yeast, a protein thought to be involved with the scaffolding of the spliceosome (Vincent and others 2003; Wang and others 2003).

Heterochronic Genes

Developmental molting of the *C. elegans* larval stages relies upon the coordinated expression of certain genes and proteins, as well as the suppression of others. Proper maintenance of these larval transitions occurs through the use of heterochronic genes, as discussed in the first chapter. Briefly, the concentration of protein LIN-14 is relatively high during the first larval stage, and aids the transcription of proteins during this time (Lee, Feinbaum, Ambros 1993; Wightman, Ha, Ruvkun 1993). As the worm progresses, *lin-4* microRNA (miRNA) production increases and binds multiple sites of the *lin-14* mRNA 3' UTR, preventing the translation of more LIN-14 proteins (Lee, Feinbaum, Ambros 1993; Olsen and Ambros 1999; Wightman, Ha, Ruvkun 1993). The *lin-4* miRNA additionally represses *lin-28* mRNA translation through a single 3' UTR binding site (Moss, Lee, Ambros 1997; Seggerson, Tang, Moss 2002). During the later larval stages, *let-7* miRNA prevents translation of the *lin-41* mRNA in some tissues, and appears to degrade the *lin-41* mRNA itself (Bagga and others 2005; Reinhart and others 2000; Slack and others 2000). Loss of LIN-41 transitions the worm from the L3 into the L4 stage, and is necessary for proper progression to fully functional adults.

As stated above, the heterochronic gene *lin-41* enables the development of normal worms to molt from the L3 larval stage into the L4 and adult stages (Reinhart and others 2000; Slack and others 2000). Null mutations in the *lin-41* gene cause worms to bypass

the L4 stage in the development of the seam cells resulting in the precocious development of the adult stage from L3, causing sterility (Slack and others 2000). The *lin-41* mutants that have been constructed show development of sperm but lack oocytes (Slack and others 2000), concurring with our sterility findings using RNAi on *lin-41* mRNA in wild-type *C. elegans* (93%, N = 30) (Figure 3.16 A).

The larval molting from L3 through L4 and into adult stage relies upon the decreased LIN-41 concentration in the worm. Previous studies have shown the LIN-41 acts as a repressor for LIN-29, as it becomes present only in adult worms (Bettinger, Lee, Rougvie 1996). As LIN-29 increases, the hypodermal seam cells of the nematodes undergo their terminal differentiation. This explains why the knockdown of *lin-41* causes the precocious adult fates of larval cells (Slack and others 2000). Additionally, the over expression of LIN-41 reiterates larval stages, since LIN-29 maintains its suppression. Normal oocyte production occurs during the L4 larval stage. Alterations in the heterochronic timing genes around this L4 stage evidence disruptions in normal oocyte production (Figure 3.16 A).

Further examination of the *lin-41* gene through the usage of *C. elegans* mutants has yielded additional information. From this study, constitutively active ITR-1 protein in the *itr-1(sy290)* mutants appears to allow production of oocytes in the proximal gonad, thus reducing sterility (0%, N = 30) (Figure 3.16 B). Additionally, findings in the *rrf-1(pk1417)* mutant strain (0%, N = 30) identified the gene to play a major role in the somatic tissues of the worms instead of the germline, as these worms were also fertile, maintaining consistency with previous expression analyses done on the heterochronic genes (Bagga and others 2005; Bettinger, Lee, Rougvie 1996; Lall and others 2006).

Table 3.7. Additional genes causing sterility (≥ 60% in N2 *C. elegans*) under RNAi conditions. N = number of animals. Each entry represents at least the initial phenotypic scoring.

Gene Pair	Locus	Function	Category	Reference	% Sterile (N)		
					N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
C12C8.3	lin-41	Predicted E3 ubiquitin ligase, transition L3-L4-adult	Heterochronic	Slack 2000	93 ± 4.7 (30)	0 (30)	0 (30)
Y116A8C.35	uaf-2	U2 snRNP splicing factor required for viability	RNA Binding	Zorio and Blumenthal 1999	67 ± 8.6 (30)	90 ± 5.5 (30)	80 ± 7.3 (30)
C23G10.3	rps-3	Small ribosomal subunit S3, KH RNA-binding, lifespan	RNA Binding	Curran and Ruvkun 2007	100 (60)	100 (60)	83 ± 4.8 (60)
R07E5.14	rnp-4	Embryonic viability, fertility, vulva development	RNA Binding	Longman 2003	100 (30)	100 (30)	100 (30)
T27F2.1	skp-1	Transcription cofactor in embryogenesis and molting	Transcription Factor	Kostrouchova 2002	100 (29)	31 ± 8.6 (29)	100 (29)
F29G9.4	fos-1	Anchor cell removal of basement-membrane, invasion	Transcription Factor	Sherwood 2005	80 ± 7.3 (30)	70 ± 8.4 (30)	33 ± 8.6 (30)
B0286.5	fkh-6	Gonad specific for male sex determination	Transcription Factor	Chang 2004; Hope 2003	96 ± 2.2 (80)	95 ± 2.4 (80)	3 ± 1.9 (80)
C33D3.1	elt-2	Initiates and maintains terminal differentiation	Transcription Factor	Hawkins and McGhee 1995	97 ± 3 (30)	87 ± 6.1 (30)	93 ± 4.7 (30)
F57B9.2	let-711	Asymmetric cellular division, gene level regulator	Transcription Factor	DeBella 2006	85 ± 6.9 (27)	97 ± 3.2 (29)	97 ± 3.2 (29)
Y111B2D.b	pqn-80	Prion-like-(Q/N-rich)-domain-bearing protein	Transcription Factor	Wormbase	100 (60)	100 (30)	10 ± 3.9 (58)

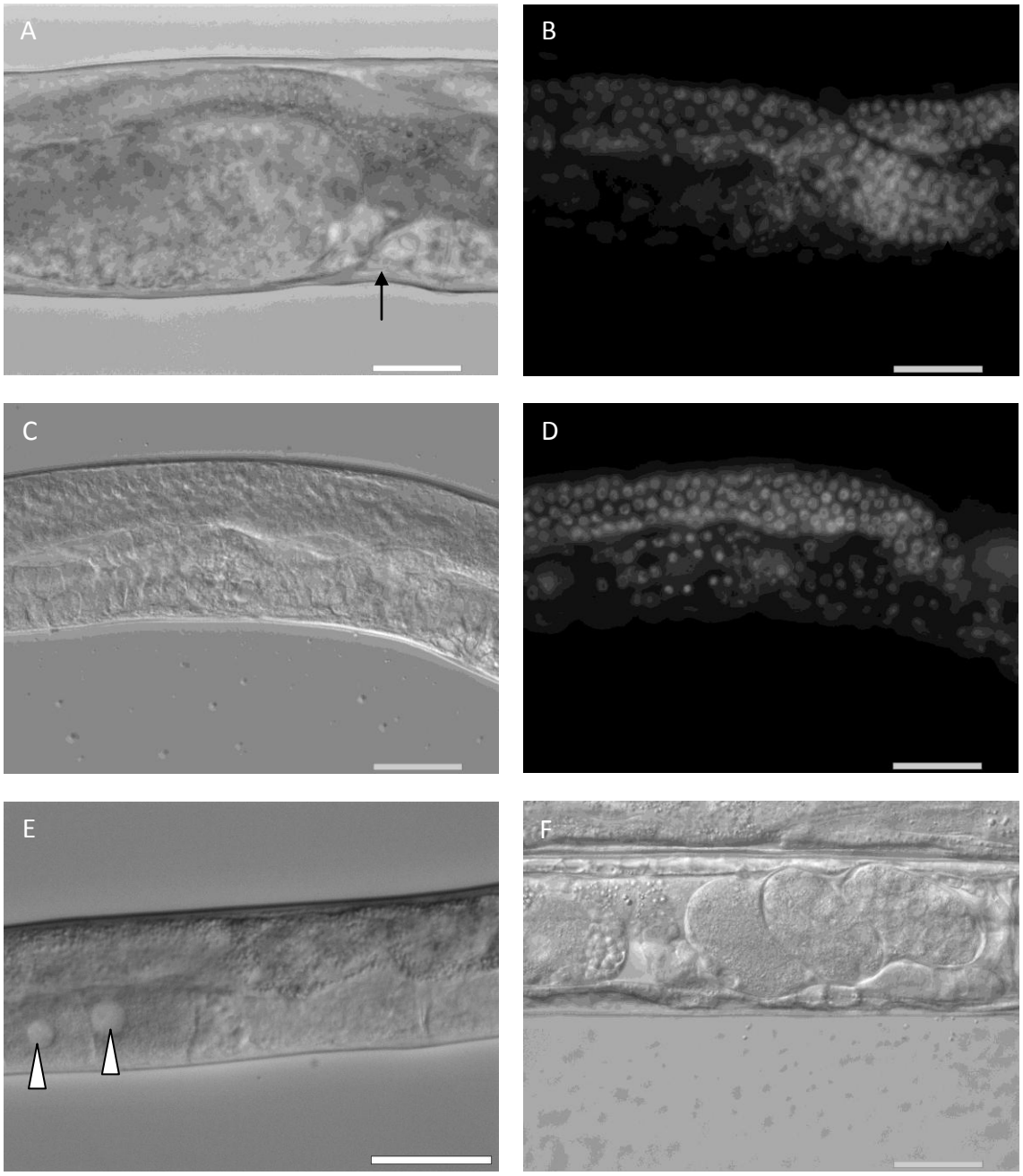

Figure 3.16. Worms fed *lin-41* dsRNA producing bacteria. (A) Wild-type N2 *C. elegans* showing lack of formation of oocytes. White bar = 40µm, black arrow = vulva. (B) A DAPI stained N2 worm showing groups of distinct nuclei, but none for normal oocytes. White bar = 40µm. (C) An *rrf-3(pk1426)* worm displaying a lack of oocytes. (D) DAPI staining of the *rrf-3(pk1426)* worm showing the lack of normal oocyte nuclei. (E) An *itr-1(sy290)* mutant worm showing rescued fertility through development of normal oocytes. White bar = 55 µm. White triangles point to nuclei of oocytes. (F) Another *itr-1(sy290)* worm showing three embryos at various stages in development. White bars = 40µm, unless otherwise specified.

RNA Binding Proteins

Three RNA binding proteins were identified in this sterility screening: *uaf-2*, *rps-3* and *rnp-4*, none of which showed any significant roles in IP_3 signaling or germline versus somatic cells. The *uaf-2* gene codes for an essential component of the spliceosome, in the U2AF protein family, which regulates splicing in the same area as the U2 snRNP, described more above (Zorio and Blumenthal 1999). The *rps-3* gene codes for the small ribosomal protein S3, and also includes a KH RNA-binding domain. Protein K is found in the cell nucleus and binds to pre-mRNAs, and the proteins that share homology with protein K are called K homology (KH) domains (Gaillard, Cabannes, Strauss 1994). With *rnp-4*, the resultant protein expression appears in all somatic and germline cells, and is required for viability in the organism as a component of the exon-exon junction in mRNA splicing as well as being a component in nonsense mediated decay (NMD) of premature stop codons in mRNAs (Kawano and others 2004; Longman and others 2007)

Transcription Factors

In order to ensure the proper mRNAs are transcribed at the appropriate time and in the appropriate place, transcription factors allow a regulatory step in mRNA production. Six transcription factors were identified as sterility causing genes in this RNAi screening. Of these, five of the transcription factors (*fos-1*, *fkh-6*, *elt-2*, *let-711* and *pqn-80*) maintained high sterility scores in both N2 and *itr-1(sy290)* worm strains, though the sterility in the *rrf-1(pk1417)* was more mixed. *let-711* appears to play an essential role in the germline (97% ± 3%, N = 29), while all the others are more somatic specific.

The transcription factor coded by the *skp-1* gene regulates embryogenesis and molting by splicing mRNAs and binding chromatin, as an ortholog of the SKI-binding protein (Kostrouchova and others 2002). N2 and *rrf-1(pk1417)* worms exhibited sterility (100%, N = 29) under RNAi conditions for *skp-1*, while the *itr-1(sy290)* had greatly reduced sterility (31% ± 8.6%, N = 29). SKP related genes *skr-1* and *skr-2* are necessary for oocyte progression past the pachytene arrest in the loop of the *C. elegans* gonad (Nayak and others 2002). SKP-1 and SKP-2 both appear to interact with CUL-1 in the ubiquitin pathway to promote degradation of proteins and allow pachytene exit (Yamanaka and others 2002).

Gene Interaction Map

Gene interactions produce the necessary responses needed to keep individual organisms alive, responsive and healthy. These interactions allow propagation of signals between tissues, cells, and within cells themselves. Gene interaction maps provide a clear way to visualize the larger picture of how various gene products influence each other. This can lead to the identification of novel pathway components or identify links between distinct pathways. In order to better visualize the potential links between significant genes, we generated a genetic interaction map based off genetic interactions as reported on Wormbase. A simple web-based program was used to draw the interaction map of the *itr-1(sy290)* sterility-rescuing genetic interactions.

For a number of genes and proteins from our study, the interactions seen in the map were unsurprising. The largest cluster of genes comprised the ribosomal proteins (purple) which require interactions to assemble into the ribosomal subunits and ultimately the finalized and fully functional ribosome. Likewise, the proteasomal proteins (light

pink) assemble together to form a fully functional proteasome. The genes *plc-3* and *ppk-1* (T01E8.3 and F55A12.3, respectively), as described previously, allow for the cleavage and maintenance of IP_3 in the IP_3 signaling pathway. The inclusion of these genes in the interaction map confirms the significance of the other connections in the IP_3 signaling pathway.

The interaction map identified an interesting link between the various gene groups, however. The predicted gene/protein interaction map constructed from the N2 sterile/*itr-1(sy290)* fertile data showed a central locus in *ima-3* (F32E10.4), an importin α nuclear transport factor with a specific role in oocyte production (Geles and Adam 2001). Nuclear-cytoplasmic transport of proteins progresses cells through the cell cycle, promoting cell growth and development (Affolter, Marty, Vigano 1999). Movement of proteins in and out of the nucleus increases during oocyte development to allow maturation in preparation for ovulation. Importins have been shown to control cell cycle with regard to mitosis, with IMA-3 additionally having particular significance in meiosis (Geles and Adam 2001; Geles and others 2002; Loeb and others 1995). Of the three α importin genes in *C. elegans*, all three exhibit expression in the adult germline, although *ima-3* expression was the only α importin expressed in all stages of development and the only expressed α importin found in the somatic tissues (Geles and Adam 2001). This further confirms that the different α importin classes serve different functions during development and implicates *ima-3* in the IP_3 signaling pathway (Mason, Fleming, Goldfarb 2002).

Ribosome biogenesis requires ribosome processing at various stages in the cytoplasm, nucleus and nucleolus (Voutev and others 2006). This includes rRNA

processing as well as the ribosomal proteins themselves (Moss 2004). Ribosome assembly occurs within the nucleus, specifically the nucleolus. Disruptions in ribosome biogenesis cause defects in *C. elegans* gonadogenesis and germ cell development (Voutev and others 2006). RNAi of *ima-3* may prevent the proper processing or transport of the ribosomal proteins, leading to the sterility witnessed.

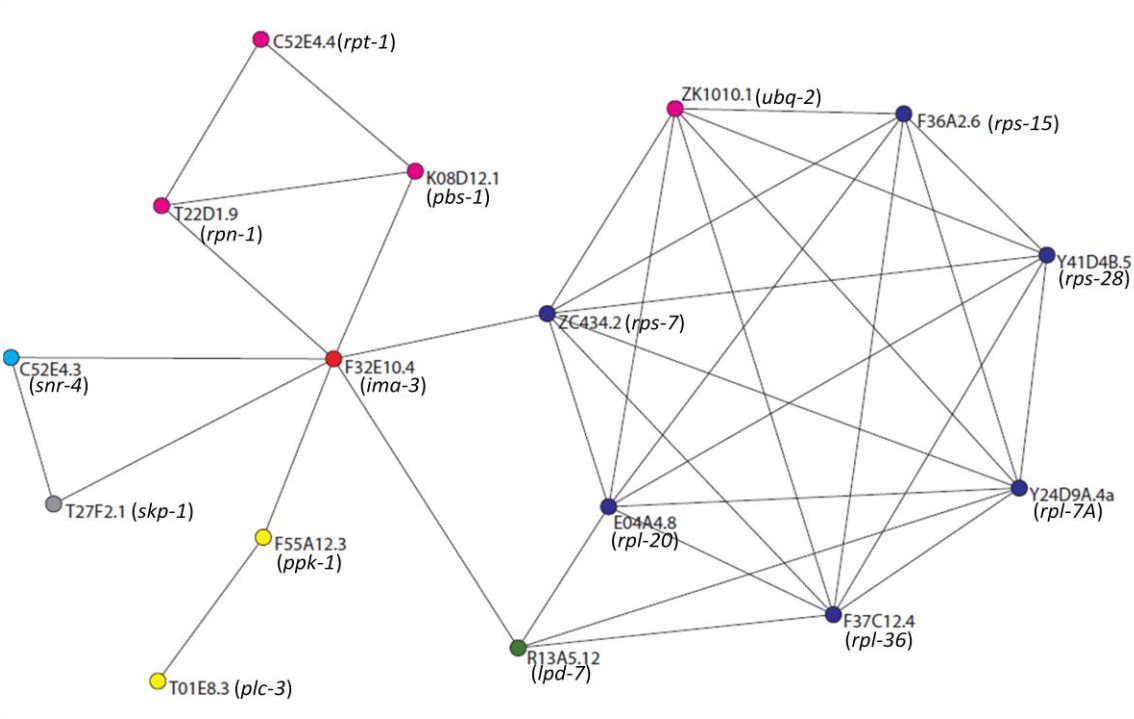

Figure 3.17 - Genetic interaction map of *itr-1(sy290)* sterility-rescuing genes. Of the 23 genes found to be significant, 16 exhibited a predicted genetic interaction based on data from the wormbase.org website. The central (red) locus F32E10.4 (*ima-3*) codes for the sole somatic tissue importin α nuclear transport factor in *C. elegans*.

Evidence exists for a role of α importins in protein degradation. In yeast, the nuclear localization signal receptor, Srp1p, contains elements of the α importin family (Enenkel, Blobel, Rexach 1995). The proteasome component *RPN11* rescued specific mutations in the Srp1p protein, implicating the α importin in the ubiquitin proteasome pathway (UPP) (Tabb and others 2000). Additionally, Srp1p appears to facilitate the

movement of proteins from the ER to the Golgi through its interaction with Sts1p (Tabb and others 2000). The *STS1* gene suppresses mutations in *SEC23*, restoring proper ER-Golgi protein transport (Liang, Lacroute, Kepes 1993). Here we again witness a connection between α importin and the proteasome, through its interactions with *pbs-1* and *rpn-1*.

The *C. elegans* phosphatidylinositol-4-phosphate 5' kinase gene, *ppk-1*, most closely resembles the *mss4* gene in *S. cerevisiae* (wormbase.org). This protein phosphorylates PIP into PIP_2, which leads to cleavage into IP_3 and DAG, as explained previously. A localization study revealed a nuclear localization signal (NLS) in the protein sequence (Audhya and Emr 2003) which allows nuclear-cytoplasmic transport for the protein, which was initially found as a plasma membrane localized protein (Desrivieres and others 1998). Regulation of the phosphoinositides is essential for proper organization of the actin cytoskeleton (Coppolino and others 2002; Desrivieres and others 1998) and the localization of the phosphatidylinositol-4-phosphate 5' kinases provides a measure with which to achieve this regulation.

Cofactors aid in transcription of genes during certain stages of development. Prediction of *skp-1* as a transcriptional cofactor came about from RNAi of the gene and the noticeable lack of expression of several embryonic genes, similar to disruptions in RNA polymerase II (Kostrouchova and others 2002). The *snr-4* gene is predicted to regulate P granules in *C. elegans*, ensuring proper development of the germ cell precursors which must separate from the somatic tissue (Barbee, Lublin, Evans 2002). This small nuclear ribonucleoprotein (*snr*) gets localized to the nucleus for its mRNA processing abilities within the *C. elegans* adult gonads and oocytes (Barbee, Lublin,

Evans 2002). During embryogenesis, nucleo-cytoplasmic transport increases to ensure proper development, so inhibition of these cargo molecules through the nuclear pore may influence the gonad development, resulting in the sterility witnessed in the worms.

Seven of the *itr-1(sy290)* significant genes remained unmapped based on the genetic information from the wormbase.org website: *lam-1*, *epi-1*, *aps-1*, *cct-8*, *plc-1*, M03F8.3, and *lin-41*. Although the genes are unmapped, they remain significant and their functions in *C. elegans* relate to the mapped genes. Both *lam-1* and *epi-1* comprise different laminin chains necessary for proper architecture and development of *C. elegans*' gonads, particularly showing defects in the basement membrane (Kao and others 2006; Xu and others 2005; Zhu and others 1999). *aps-1* encodes a clathrin adaptor molecule responsible for the transport of proteins in lipid vesicles through the cell. The chaperonine encoded by *cct-8* aids the folding of newly formed proteins in the ER and has been implicated in mass RNAi studies as necessary for proper distal tip cell migration and fertility (Cram, Shang, Schwarzbauer 2006; Kamath and others 2003; Sonnichsen and others 2005). The phospholipase, *plc-1*, ensures proper dilation of the spermatheca thus allowing mature oocytes to enter and become fertilized, when proper sheath cell contractions push them forward. M03F8.3 has been predicted to play a role in cell cycle control, and previous experiments have implicated this gene in *C. elegans* molting (Frand, Russel, Ruvkun 2005). A BLASTP analysis from Wormbase showed orthology to the human crooked neck-like protein 1. This crooked neck-like protein works on mRNA processing and splicing as a component of the spliceosome (Chung and others 2002). *lin-41* also plays a role in molting, between the L3 to L4 to adult stages of development, and appears to work in the transition from sperm to oocyte production

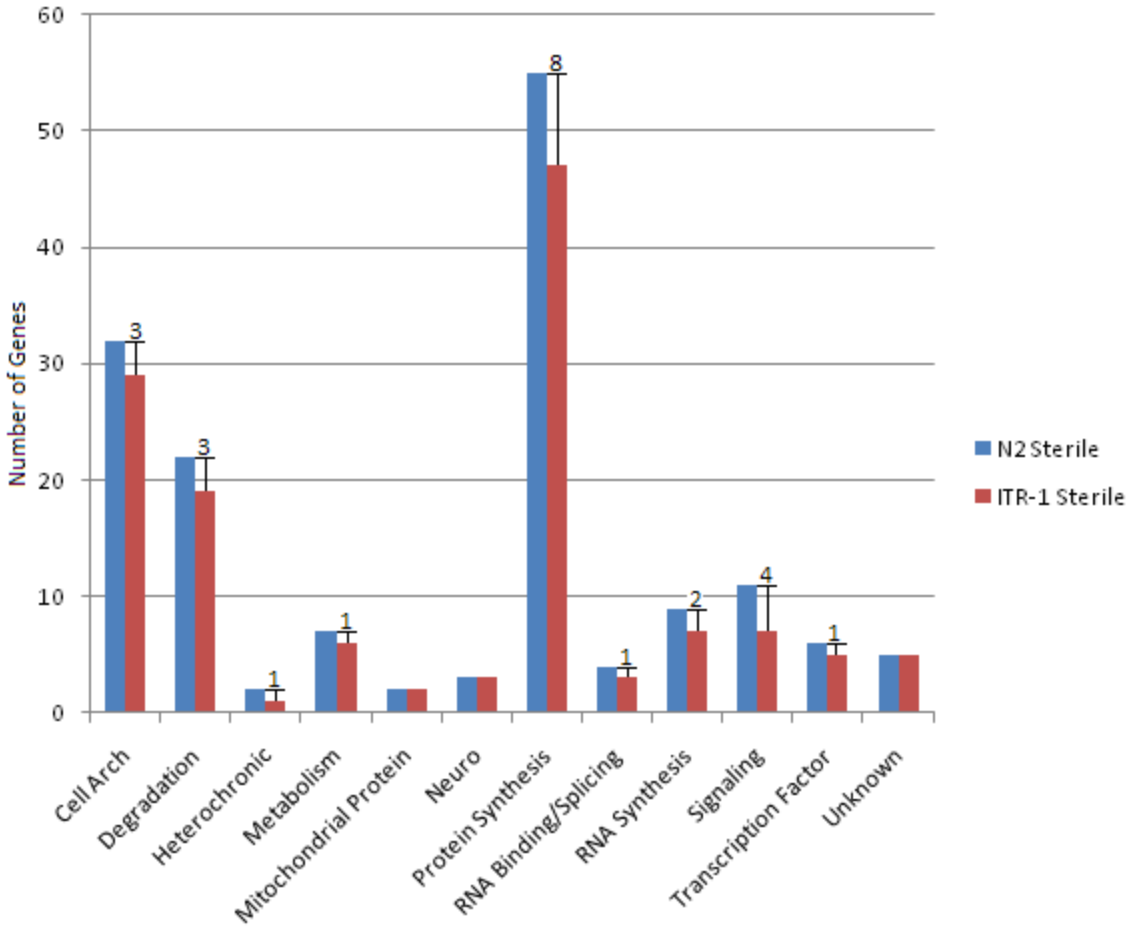

Figure 3.18. Class divisions of genes causing sterility in N2 and *itr-1 (sy290)* worm strains. Groups showing decreases in sterility causing genes were chosen for closer examination, primarily protein synthesis, protein degradation, signaling, cellular architectural and RNA synthesis related genes. Additionally, further analysis was performed on the heterochronic, RNA binding, transcription factor and metabolic classes of genes.

(Slack and others 2000). GFP localization studies show expression in multiple somatic gonad cells where the transitional signals could influence the sperm/oocyte switch (Slack and others 2000).

Results Summary

Of the initial 308 genes tested for sterility in the wild-type nematodes, 155 showed consistently high sterility scores throughout testing. Within the remaining 155

genes causing sterility in the N2 worms, 24 were rescued in the *itr-1(sy290)* mutant worms strain (Table 3.8). Genes rescued in these mutants came from various categories, including protein synthesis, protein degradation, signaling proteins and architectural scaffolding (Figure 3.18). These genes may represent novel components of the IP_3 signaling pathway and novel avenues for further research into the fertility of *C. elegans*.

Table 3.8. Genes causing sterility (≥ 60% in N2 *C. elegans*) under RNAi conditions, rescued by *itr-1* worm strain. N = number of animals. Each entry represents at least the initial phenotypic scoring.

Gene Pair	Locus	Function	Category	Reference	% Sterile (N)		
					N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
F32E10.4	ima-3	Importin alpha; oogenesis	Cell Arch	Geles 2001	80 ± 5.2 (60)	12 ± 4.2 (60)	18 ± 5(60)
W03F8.5	lam-1	ECM glycoprotein Laminin beta subunit	Cell Arch	Kao 2006	83 ± 6.9 (30)	0 (30)	0 (30)
F29G9.3	aps-1	Clathrin adaptor complex, small	Cell Arch	Boehm 2001	93 ± 4.7 (30)	7 ± 4.7 (30)	3 ± 3 (30)
ZK1058.2	pat-3	Integrin beta subunit; Body wall and gonad	Cell Arch	Lee 2001	72 ± 8.3 (29)	3 ± 3 (30)	4 ± 3.9 (25)
K08D12.1	pbs-1	Porteasome beta subunit 1of core 20S subcomplex	Degradation	Wormbase	97 ± 3 (30)	33 ± 8.6 (30)	20 ± 7.3 (30)
T22D1.9	rpn-1	Proteasome regulatory particle of 26S regulatory complex	Degradation	Wormbase	83 ± 6.9 (30)	0 (30)	0 (30)
C52E4.4	rpt-1	ATPase subunit of 19S regulatory complex	Degradation	Wormbase	100 (30)	33 ± 8.6 (30)	90 ± 5.5 (30)
Y17G9B.e	rpl-20	Large 60S ribosomal subunit L18A protein	Protein Synth	Wormbase	100 (30)	3 ± 3 (30)	30 ± 8.4 (30)
E04A4.8	rpl-20	Large 60S ribosomal subunit L18A protein	Protein Synth	Wormbase	80 ± 7.3 (30)	17 ± 7 (29)	13 ± 6 (32)

Table 3.8 Continued

Gene Pair	Locus	Function	Category	Reference	% Sterile (N)		
					N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
F37C12.4	rpl-36	Large 60S ribosomal subunit L36 protein; Embryonic and germline development	Protein Synth	Wormbase	83 ± 6.9 (30)	20 ± 7.3 (30)	87 ± 6.1 (30)
Y24D9A.d	rpl-7A	Large 60S ribosomal subunit L7A protein; SMG-mediated nonsense suppression	Protein Synth	Wormbase	100 (30)	23 ± 7.7 (30)	27 ± 8.1 (30)
F36A2.6	rps-15	Small 40S ribosomal subunit S15 protein	Protein Synth	Wormbase	76 ± 5.8 (55)	13 ± 4.3 (60)	36 ± 6.5 (55)
Y41D4A_3613.a	rps-28	Small 40S ribosomal subunit S28 protein	Protein Synth	Wormbase	100 (30)	12 ± 4.2 (60)	13 ± 6.1 (30)
Y55F3A_750.d	cct-8	Chaperonin complex component, TCP-1 theta subunit	Protein Synth	Wormbase	100 (30)	20 ± 7.3 (30)	7 ± 4.7 (30)
ZC434.2	rps-7	Small 40S ribosomal subunit S7 protein	Protein Synth	Wormbase	77 ± 5.4 (60)	10 ± 3.9 (60)	15 ± 4.6 (60)
ZK1010.1	ubq-2	Ubiquitin/60S ribosomal protein L40 fusion	Protein Synth	Wormbase	70 ± 8.8 (27)	3 ± 3 (30)	100 (27)
C52E4.3	snr-4	Bind snRNPs, P granule localization	RNA Synth	Barbee 2002	87 ± 6.1 (30)	23 ± 7.7 (30)	17 ± 6.9 (30)
M03F8.3	phi-12	Cell cycle control	RNA Synth	Wormbase	100 (30)	17 ± 6.9 (30)	70 ± 8.4 (30)

Table 3.8 Continued

Gene Pair	Locus	Function	Category	Reference	% Sterile (N)		
					N2	*itr-1 (sy290)*	*rrf-1 (pk1417)*
F55A12.3	ppk-1	Intense sheath cell contractions, spermatheca dilation	Signaling	Xu 2007	73 ± 8.1 (30)	0 (30)	0 (30)
K08C7.3	epi-1	Laminin alpha chain, oogenesis	Signaling	Zhu 1999	100 (30)	40 ± 8.9 (30)	0 (30)
T01E8.3	plc-3	Oocyte progression by contraction of sheath cells	Signaling	Yin 2004	76 ± 6.4 (45)	0 (30)	4 ± 2.9 (45)
F31B 12.1	plc-1	Spermathecal dilation	Signaling	Kariya 2004	77 ± 7.7 (30)	17 ± 6.9 (30)	10 ± 5.5 (30)
C12C8.3	lin-41	Regulates L3-L4-Adult transition	Heterochronic	Slack 2000	93 ± 4.7 (30)	0 (30)	0 (30)
T27F2.1	skp-1	Transcription cofactor in embryogenesis and molting	Transcription Factor	Kostrouchova 2002	100 (29)	31 ± 8.6 (29)	100 (29)
R13A5.12	lpd-7	Nucleolus assembly, ribosome biogenesis, fat storage	Metabolism	McKay 2003	100 (30)	37 ± 8.8 (30)	70 ± 8.4 (30)

CHAPTER FOUR

Discussions and Conclusions

RNA interference was performed on a total of 308 different genes believed to cause maternal sterility in N2 wild-type *C. elegans* (Kamath and others 2003). Of these 308 genes, we found 155 that maintained a high level of sterility (≥60%) within these worms. Due to the length of the gene list presented, we shortened the list of target genes on which to focus our efforts by utilizing the *itr-1(sy290)* gain-of-function mutant nematode. Sterility scores remained high in the mutant strain for a majority of the genes experimented upon, although 23 genes presented a greatly reduced sterility score when compared to the N2 results (Table 3.8).

Several possibilities exist for why we observed reduced sterility in the *itr-1(sy290)* mutants as compared with wild-type worms. Increasing sheath cells contractions due to the gain-of-function mutation in the *itr-1* strain remains the most obvious. Blockage of oocyte progression through a disrupted IP_3 signaling pathway provides the most obvious and direct explanation for sterility. In the *C. elegans* sheath cells, calcium binds troponin, a calcium binding protein that also binds to actin molecules, even though the muscle more closely resembles that of smooth muscle tissue (Ono and Ono 2004). Conformational changes occur on the actin filaments to allow myosin heads to attach and ultimately cause the muscle to contraction. The distinction of smooth muscle-like sheath cells comes primarily from the lack of nerves causing contractions. Instead, regulation of sheath cell contractions relies upon chemical

signaling through the gap junctions between the sheath cells and oocytes, and between the sheath cells themselves. Accomplishing this task requires MSP/VAB-1 (Miller and others 2003) for basal sheath contractions and the LIN-3/LET-23 (Clandinin, DeModena, Sternberg 1998) during the ovulation process.

Excess calcium in the cytoplasm through the *sy290* mutation possibly allows the worms increased progeny through other avenues as well. Calcium provides an essential component of enabling various enzymes (eg. calmodulin (Colomer and Means 2007), CaM kinases (Eto and others 1999)) in addition to its role in the IP_3 signaling pathway. One example includes the myosin light-chain kinase (MLCK) (Kamm and Stull 2001). Calcium first binds calmodulin, which activates MLCK to phosphorylate the myosin light chain, allowing the myosin cross-bridges to attach to the actin filament, propagating muscle contraction. This mechanism provides the contractions for smooth muscles, as they lack the troponin that calcium normally binds to in striated muscle to allow for the muscle contractions (Gulati, Persechini, Babu 1990).

The endoplasmic reticulum stores a great concentration of calcium in cells as well as being the site for new protein folding. Intracellular calcium ions $[Ca^{2+}]i$ stored in the ER lumen gets released via the ITR-1 ER surface channel when IP_3 binds to it, causing the sheath cell contractions in *C. elegans* (Baylis and others 1999; Clandinin, DeModena, Sternberg 1998). Calcium concentrations may be linked to new protein production. Ribosomes attach to the ER and feed their growing polypeptides into the lumen of the ER where chaperones ensure proper protein folding, allowing them to function properly. Disruptions in protein production place undue stress upon the ER. In *C. elegans* gonad, disruptions via RNAi of multiple protein production proteins caused sterility. During

oogenesis, new protein production occurs during the growth phase of meiosis to provide the oocytes the necessary proteins for fertilization. Cells in pachytene arrest appear to contribute the majority of the cytoplasmic proteins for the oocytes (Gibert, Starck, Beguet 1984), and defects in clathrin-mediated endocytosis in oocytes result in reduced egg counts (Giuliani and others 2009). Yolk proteins required by oocytes prior to fertilization to ensure proper embryogenesis enter the oocytes from the gonad sheath cells. Synthesis of the yolk proteins occurs in the intestine of the adult *C. elegans* and passes to the sheath cells and ultimately to the developing oocytes through gap junctions (Hall and others 1999). Without proper protein production, oocytes fail to undergo proper ovulation.

Increased cytoplasmic calcium ion levels may also signal an increase in ribosome biogenesis in the nucleolus to accommodate for the disrupted protein production. An increase in ribosome biogenesis would allow for a greater amount of new protein production. Defects in ribosome biogenesis have caused defects in gonadogenesis as well (Voutev and others 2006), which could explain sterility witnessed. Alternatively, the increased calcium concentration may increase translation of genes to compensate for disruptions seen from the RNAi process. Calcium may provide greater efficacy of translation to allow an increase in protein production. Additional work will reveal the mechanism by which the cytosolic calcium concentration regulates the fertility of these nematodes.

Additionally, disruptions in protein degradation through the proteasome cause sterility in *C. elegans*. Disruptions of proteasomal subunits via RNAi identified possible links with cytoplasmic calcium concentrations or the IP_3signaling pathway in the sheath cells. Lack of proper protein degradation causes a backup of proteins being produced in

the ER, resulting in ER stress similar to that of disruptions in protein production. The resultant ER stress generally causes the production of additional chaperone proteins, as well as slowing new protein production and attempting to increase proteasomal activity to clear out mis-folded and inactive proteins (Ron and Walter 2007). Interestingly, *itr-1(sy290)* mutants appear to relieve the sterility from RNAi of several of the ribosomal and proteasomal subunit proteins.

While the ribosomal and proteasomal subunit proteins register some explanation based on the proximity and previously described relationships to the ER and calcium, other possible interactions are less forthcoming. As such, we utilized the significant *itr-1(sy290)* sterility data to compile a protein interaction map, allowing a visualization of how the proteins work in conjunction with each other. This map led to the identification of a link between various pathways, the gene *ima-3* (Figure 3.17). *ima-3* (F32E10.4) codes for an importin α nuclear transport factor with a specific role in oocyte production and is the only importin α expressed in somatic tissue (Geles and Adam 2001). The significance of this transport factor and its relation to calcium concentrations and sterility has yet to be determined.

As mentioned previously, the causes of sterility in *C. elegans* include defects in germ cell production (sperm or oocytes), sheath cell contractions, and spermatheca dilation (Table 1.1). The sterility seen in this study resulted primarily from defects in oocyte production (eg. *lin-41*) and sheath cell contractions (eg. *pbs-1*). Some of the most consistently resulting genes, notably *pbs-1*, were selected for closer examination to better determine the functionality of those genes.

As described previously, *pbs-1* codes a β subunit of the proteasome, affecting fertility, embryonic viability, larval viability and locomotion (Kamath and others 2003; Simmer and others 2003). The β subunit codes for the catalytic portion of the proteasome, responsible for the degradation of ubiquitinated proteins. How RNAi of this proteasomal protein causes sterility remains to be seen, though we have taken movies of the primary ovulation attempts of several N2 worms fed *pbs-1* RNAi bacteria and are currently analyzing the rate of sheath cell contractions in those worms.

Although these 23 genes were rescued, the remaining 132 genes caused sterility in both N2 and *itr-1(sy290)* worms. Some of these genes may participate in the IP_3 signaling pathway but downstream of ITR-1 and the cytoplasmic calcium, explaining why sterility would have been witnessed in both worm strains. Another screening of these 132 genes with additional mutant strains may discover additional components to the IP_3 signaling pathway.

Future Directions

With this current study, we identified 23 genes from various classifications as potential components of the IP_3 signaling pathway. Although we reduced the total number of genes from 308 to these significant 23, much work remains to elaborate the functions of these genes with regard to sterility. A large number of undergraduate students aided in the sterility trials throughout the screening process. Due to the variation between students in their handling of laboratory equipment, their care with organisms and the observations they made on sterility, there lies potential for errors in the results. As such, we attempted to go back and re-examine the most promising looking genes observed without simply relying on initial results, although we were unable to retry all

genes. An additional sterility scoring of the supposed sterility causing genes would be beneficial to ensure the quality of the undergraduates' results.

One aspect of RNAi that needs recognition is the potential for off-target silencing. The possibility exists for mRNAs with similar nucleotide sequences being degraded by the RNAi machinery without the intention of the researcher. RNAi off targets appear to occur primarily when another mRNA has roughly 95% nucleotide sequence similarity (over a 40 nucleotide span) with the target mRNA (Rual, Klitgord, Achaz 2007). One possibility to test this would be to construct additional dsRNA producing bacteria with different sections of the gene of interest. By testing in this way, we could identify if phenotypes differ from one dsRNA to the next, and if so, they could be due to some off-target silencing.

Ensuring RNAi efficacy requires checking for the actual degradation of the mRNA targeted. Through the use of reverse transcription polymerase chain reaction (RT-PCR) techniques, target mRNAs can be converted to cDNA and amplified. The resultant product is then electrophoresed on an agar gel to separate the cDNA bands to identify if the purported RNAi target has actually been knocked down. For the initial purposes of our screening, we utilized the RNAi feeding protocol with the understanding that it was highly effective. However, with the narrowing of the gene pool down to these 23 genes, we must ensure the RNAi efficacy truly remains high under the conditions we tested these genes.

Creation of null or loss of function mutant strains of *C. elegans* provides another avenue towards identifying gene function. With our narrowed list of significant genes, knocking out or down these genes should provide the same phenotypes observed in our

RNAi experiments. Benefits of these mutants include the removal of any potential off-target gene silencing as well as the potential rescuing of the phenotyopes by injection of extrachromosomal plasmids that return the genes and gene products to the mutated worms. The hazard for this type of project lies in the sterility of the worms. Without progeny, a continuous line of mutant worms would not be sustainable, and as such, loss of function mutations would likely work better.

Additional information needs gathering on the 23 significant genes discovered as well. While DIC microscopy was taken of the nematodes' phenotypes, additional mutant strains could provide a better light on the functions of the genes themselves. Due to some N2 worms showing a lack of oocytes, we hypothesize that other factors may be causing the germ cells to continue mitosis rather than exiting and progressing through oogenesis. One possible worm strain is a *lag-2::GFP* strain, which has GFP attached to a *lag-2* promoter, allowing visualization of the distal tip cell (DTC) under green fluorescent light. The DTC maintains a population of stem-cell like, mitotic germ cells thus ensuring a steady supply of oocytes. Since the GFP expresses wherever *lag-2* expresses, if *lag-2* begins expressing in abnormal tissues in the gonad, it could explain the lack of oocytes through the continued mitotic divisions of the germ cells. Evidence of GFP in the somatic gonad outside of the DTC would support this hypothesis.

Localization studies on the 23 significant genes would also provide important insight. In this study, we included sterility data from the *rrf-1(pk1417)* strain which permits RNAi solely in the germ line cells (Sijen and others 2001). Somatic tissues unaffected by the RNAi function normally, so disruptions in sterility in these worms indicate a larger role for those genes in the germ line cells. However, to ensure quality, it

would benefit the characterization of the genes to utilize a strain resistant to RNAi in the germ line only. For this purpose, an *ego-1* (Enhancer of Glp-One) strain could be used. *ego-1* codes an RNA-dependant RNA polymerase necessary for RNAi in the germ line as well as normal germ line development (Smardon and others 2000). Originally, *ego-1* identification came from its interactions with Notch and is required for oogenesis (Qiao and others 1995). By using this strain and comparing the sterility data against the *rrf-1* data, we could better locate the sites of interaction for the genes in question.

For some of our significant genes, the proteins are thought to be more ubiquitously expressed, such as the ribosomal and proteasomal proteins. Creation of GFP fusion lines using the promoters for the 23 significant genes would also allow for better localization studies. In this instance, direct visualization of the sites would be identifiable.

This sterility screening has opened the door to a myriad of potential in the 23 significant genes discovered. The data from the remaining genes contains value as well, though. Although many of the genes tested remained sterile in the *itr-1* mutant, they could have roles in IP_3 signaling downstream of the ITR-1 channel or are unaffected by the cytoplasmic calcium concentration. Some genes were also witnessed to increase the sterility percentages in the *itr-1* mutants when compared to the N2 worm sterility scores. Explanations for this phenomenon would require more study beyond the scope of this current screening and direction.

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