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The function of nonmuscle myosins during oogenesis in *Caenorhabditis elegans*

by

Amanda Pisio

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Abstract

Oogenesis in *C. elegans* requires the activity of type II nonmuscle myosins, complexes containing two heavy and four light chains that bind and move actin. Two different complexes are found in the gonad, differing in their heavy chains, (NMY-1 *versus* NMY-2). These two myosins are redundant during embryonic elongation, and we expected them to also be redundant during oogenesis. Instead, both myosins are required, but their requirement differs at different stages. NMY-2 is required for maintenance and then closure of the ring channels, while both NMY-1 and NMY-2 are required for cytoplasmic streaming. Finally, NMY-1 is required for ovulation. Loss of MLC-4, the regulatory light chain, did not reveal phenotypes other than those seen with the loss of either myosin, meaning that the myosins do not act redundantly during oogenesis. Therefore, the different heavy chains apparently allows differential regulation of separate functions during this process.

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List of Symbols, Nomenclature or Abbreviations

Abbreviation/Symbol	Definition
µg/ml	Microgram per Milliliter
µm	Micrometer
µm/min	Micrometer per Minute
ACY-4	Adenylyl Cyclase 4
ANI-2	Anillin 2
ARP2/3	Actin-Related Proteins 2 and 3
ATP	Adenosine Triphosphate
C. elegans	*Caenorhabditis elegans*
cAMP	Cyclic Adenosine Monophosphate
CEH-18	*C. elegans* Homeobox
CHO cells	Chinese Hamster Ovary cells
CMD-1	Calmodulin
CYK-4	Cytokinesis Defect 4
DAPI	4',6-Diamidino-2-Phenylindole
DIC	Differential Interference Contrast
DNA	Deoxyribonucleic Acid
Drosophila	*Drosophila melanogaster*
DTC	Distal Tip Cell
E. coli	*Escherichia coli*
ECT-2	Epithelial Cell Transforming sequence 2 oncogene
EGL-30	Egg Laying Defective 30
eMLC	Essential Myosin Light Chain
EMO	Endomitotic
Eph Receptor	Ephrin Receptor
ERK	Extracellular Signal-Regulated Protein Kinase
Fem	Feminization of XX and XO Animals
FEM-3	Feminization of XX and XO Animals 3
FLN-1	Filamin
FOG-2	Feminization Of Germline
GDP	Guanosine Diphosphate
GFP	Green Fluorescent Protein
GLP-1	Abnormal Germ Line Proliferation
GSA-1	G Protein, Subunit Alpha 1
GTP	Guanosine Triphosphate
IPTG	Isopropylthio-beta-D-Galactoside
KIN-2	Protein Kinase
LET-502	Lethal 502
LET-60	Lethal 60
LIN-45	Abnormal Cell Lineage
MAPK	Mitogen-Activated Protein Kinase
MEK-2	MAP Kinase Kinase

MEL-11	Maternal Effect Lethal 11
MHCA	Myosin Heavy Chain A
MHCB	Myosin Heavy Chain B
MIG-2	Abnormal Cell Migration
MLC-4	Myosin Light Chain 4
MLC-5	Myosin Light Chain 5
MLCK	Myosin Light Chain Kinase
mM	Millimolar
MPK-1	Map Kinase
MRCK-1	Myotonic Dystrophy-Related, Cdc42-binding Kinase Homolog
mRNA	Messenger Ribonucleic Acid
MSP	Major Sperm Protein
Mts1	Multiple Tumor Suppressor Gene 1
MYO-3	Myosin Heavy Chain Structural Genes
NMY-1	Nonmuscle Myosin 1
NMY-2	Nonmuscle Myosin 2
OMA-1	Oocyte Maturation Defective 1
OMA-2	Oocyte Maturation Defective 2
PAK-1	p21-Activated Kinase
PAR	Partitioning Defective
PBT	Phosphate Buffered Saline and Tween
PIE-1	Pharynx and Intestine in Excess
PUF Proteins	Pumilio/FBF Domain-Containing
RGA-3	Rho GTPase Activating Protein 3
RGA-4	Rho GTPase Activating Protein 4
Rho	Ras Homolog Gene Family
RhoA	Ras Homolog Gene Family, member A
RhoGAP	Rho GTPase Activating Protein
RhoGEF	Rho Guanosine Exchange Factor
rMLC	Regulatory Myosin Light Chain
RNA	Ribonucleic Acid
RNAi	RNA-mediated interference
ROCK	Rho-associated Protein Kinase
ROG-1	Ras Activating Factor in Development of Germline
RRF-1	RNA-dependent RNA Polymerase Family
SH3	SCR Homology 3 Domain
TBB-1	Tubulin, Beta 1
TBB-2	Tubulin, Beta 2
UNC-119	Uncoordinated 119
UNC-54	Uncoordinated 54
UNC-73	Uncoordinated 73
UTR	Untranslated Region
VAB-1	Variable Abnormal Morphology 1
ZIPK	Zipper-interacting Protein Kinase

CHAPTER ONE: INTRODUCTION

The purpose of this thesis was to determine the function of nonmuscle myosin in the gonad of *Caenorhabditis elegans*, specifically to test whether the two proteins encoded by the *nmy-1* and *nmy-2* genes work in the same process, and to determine what upstream or regulatory factors are required for the function of nonmuscle myosins. Nonmuscle myosins are involved in a variety of eukaryotic processes. Current understanding of their regulation does not adequately explain this versatility. With each new nonmuscle myosin process that is studied, novel upstream regulatory proteins are discovered, and their effect on the nonmuscle myosin protein is characterized. Therefore, understanding the role of nonmuscle myosins in all processes in a single organism allows us to understand how similar proteins can be used to produce different functions. Previous work has shown that both NMY-1 and NMY-2 are required for oogenesis but their function during this process was unknown (Piekny, et al. 2003) (Kachur, Audhya and Pilgrim 2008). I hoped to understand first, what processes during oogenesis require nonmuscle myosins, and then to test what upstream factors control those processes. This would show if similar sets of regulatory proteins identified in other processes are also used in this process and how those regulatory proteins are used to localize and regulate the function of nonmuscle myosins.

To determine if both nonmuscle myosins were involved in the same process during *C. elegans* oogenesis and at which step during this process they acted, I proposed to create genetic knockdowns of each gene using RNA interference (RNAi) which can effectively reduce gene function in an otherwise wild type animal. The nematode hermaphrodite gonad continuously produces oocytes, therefore it is possible to simultaneously observe all steps of oogenesis, and within a single organism, to determine which steps are disrupted. My second objective was to determine the localization of the two nonmuscle myosins in the gonad. Each step of oogenesis occurs in its own spatial niche, therefore effector proteins such as nonmuscle myosins will localize to the area of the gonad where they perform a function. By determining the location of both nonmuscle myosins,

either directly or through reporter constructs, the steps of oogenesis that require these proteins can be determined.

In the past few years, nonmuscle myosins have been shown to be extremely versatile proteins that are capable of much more subtle regulation than the simple on/off switch of their skeletal and smooth muscle myosin counterparts; however, how this precise regulation is accomplished is still not well understood. To address the role of nonmuscle myosins in the gonad of *C. elegans* and to understand their upstream regulators, there are three main questions to be addressed. First, the structure of nonmuscle myosin has to be understood in order to comprehend how phosphorylation can cause differences to the myosin's ATPase activity and the stability of myosin filaments. Second, the number of kinases and upstream factors that are known to regulate nonmuscle myosin enforces the concept that spatial and temporal regulation is critical for the proper function of nonmuscle myosin. Finally, we must understand in detail the steps of oogenesis, and defects that can occur when these steps are obstructed through loss-of-function studies. Nonmuscle myosins in the gonad are also controlled by main upstream regulators of the gonad and understanding which of these main pathways are required for myosin function allows the study of direct upstream regulators of nonmuscle myosins. Together these three areas of research are critical for understanding how the localization and loss-of-function studies correlate to individual steps of *C. elegans* oogenesis.

1-1. Regulation and Structure of Nonmuscle Myosin

Type II myosins are distinct from other myosins in their ability to form filaments and create actin structures as opposed to other myosins, such as myosin V, which uses pre-existing actin cables to transport cargo. Type II are the best studied of all myosin classes mostly for their role as the motor protein that causes contractions in muscles. Myosins are actin-dependent motor proteins that can be grouped into at least fifteen classes, but all myosins use the hydrolysis of ATP in order to create movement by interacting with actin (Sellers 2000). To date, all eukaryotes

express at least one type II myosin making them an important class of myosin proteins.

1-1.1. Type II myosins

Type II myosins are functionally subdivided into striated muscle myosin, smooth muscle myosin and nonmuscle myosin. Striated and smooth muscle myosin are required for contraction of muscles in the presence of calcium. Nonmuscle myosins are also able to contract actin filaments but have additional roles such as actin cross-linking and scaffolding for anchoring signaling molecules to actin filaments (Conti and Adelstein 2008). Nonmuscle myosins are thought to play a critical role in nearly all morphogenic processes involving epithelial sheets (Quintin, Gally and Labouesse 2008). They also are required for cytokinesis, cell motility, gametogenesis, and wound healing (Yang, et al. 2012) (M. Vicente-Manzanares 2013) (Betapudi, et al. 2010). Their requirement in such diverse processes indicates complex layers of regulation. Therefore, myosin must be localized to specific regions within cells. The rate of disassociation and strength of contractility of the myosin motor domain, must also be dictated through phosphorylation for a process to occur properly. Nonmuscle myosin contains several structural domains capable of interacting with upstream regulatory factors. While initially there appear to be few differences amongst the gross structure of type II myosins, the ability of nonmuscle myosins to be modified by upstream factors allows them to fulfill such diverse roles.

Type II myosins are made up of two heavy chains, two regulatory light chains and two essential light chains (Figure 1-1). The heavy chain is composed of three domains: an N-terminal head domain contains both the actin and ATP binding sites and is the site of ATP hydrolysis; a linker domain binds light chains; and a C-terminal coiled-coiled tail domain facilitates the interaction between the two heavy chain proteins, which constitutes a single myosin (Heissler and Manstein 2013). The C-terminal domain also allows the myosin complex to bind to other myosin molecules and form long chains, also known as filaments. Two types of

light chains bind to the linker domains: regulatory myosin light chains (rMLC) involved in regulating the motor activity of the myosin through phosphorylation; and essential myosin light chains (eMLC) necessary for stabilizing the catalytic domain of the heavy chain (Lowey and Trybus 2010). Therefore, the type II myosins are protein complexes that require the products of several genes for their function.

The structure of the motor domain of the heavy chain has been solved at the atomic level (Sellers 2000). The motor or head domain is made up of three subdomains connected by flexible linker pieces. A protein-binding SH3 subdomain is connected to an upper and lower subdomain. Between these two subdomains is a cleft that pinches together upon binding actin or nucleotides. It is these upper and lower subdomains that contain the actin binding sites (Sellers 2000). It is here that the hydrolysis of ATP and the binding of actin are coupled to result in unidirectional movement along actin (Heissler and Manstein 2013). The region after the upper and lower subdomain is the linker, which is where the eMLC and the rMLC non-covalently associate with the heavy chain. Together, these two regions are thought to be the lever of the myosin that allows its movement. Phosphorylation on the light and heavy chain can change the accessibility of the actin and ATP binding sites, as well as ATP hydrolysis and manoeuvrability of the lever.

The C terminus tail of the heavy chain consists of an alpha helical coiled-coil motif. The tail region is where the two heavy chains dimerize. This region is needed for myosin to form filaments by using alternating charged amino acids (Heissler and Manstein 2013) (McLachlan and Karn 1982). At the very C terminus of the heavy chain is a short non-helical tailpiece that contains phosphorylation sites, which are required for function of the myosin complex. The structure of type II myosins dictates their ability to form filaments, and bind and move actin. Phosphorylation at numerous subdomains is able to change the stability of myosin filaments and alter the ATPase activity and dissociation of myosin from the actin filaments.

1-1.2. Regulation through Phosphorylation

Phosphorylation is the major form of regulation for nonmuscle myosins. Early studies of smooth muscle myosin showed that phosphorylation of the regulatory light chain was the main on/off switch (Craig , Smith and Kendrick-Jones 1983). Phosphorylation at serine-19, in mammalian type II nonmuscle myosins, causes a conformational change in the heavy chain from an inactive form to an active form. In the inactive form, the tail is curled up between the head domains. In the active form, the tail is stretched out and able to form filaments with other myosins (Moussavi, Kelley and Adelstein 1993) (Figure 1-2). Phosphorylation at a serine on the myosin regulatory light chain occurs on most eukaryotic nonmuscle myosin molecules. However, as domain size differs slightly from species to species, the phosphorylated amino acids in nonmuscle myosin will be referred to in this thesis, by the numbering convention in mammals. In smooth muscle myosins, phosphorylation of rMLC is sufficient to cause contractions and in nonmuscle myosins the inability of the rMLC to be phosphorylated causes cytokinesis defects (Itoh, et al. 1989) (Jordan and Karess 1997). Therefore, the presence of phosphorylation on serine-19 on the rMLC, typically assayed by phosphorylated isotype-specific antibodies, is frequently used as an indicator of levels of myosin activity upon loss of upstream factors.

Nonmuscle and smooth muscle myosins, can also be phosphorylated at threonine-18 and serine-19 by rMLC kinases (Ikebe, Koretz and Hartshorne 1988). The diphosphorylated form increases the ATPase activity compared to the monophosphorylated form (Ikebe 1989). In Chinese hamster ovary cell culture (CHO cells) diphosphorylation decreases the dissociation of myosin from actin filaments creating more stable actomyosin bundles (Vincente-Manzanares and Horwitz 2010). Different phosphorylation states can also dictate localization of the myosin in CHO cells during cell migration; diphosphorylated myosin is found at the lagging edge of the cell while the monophosphorylated form is found throughout (Vincente-Manzanares and Horwitz 2010). In *Drosophila*, mono-phosphorylation causes myosins to form flexible networks that allow expansion of

a tissue, whereas the rigidity of the diphosphorylated form does not allow for that expansion, but instead results in contraction of the tissue. Di-phosphorylation is required during imaginal disc morphogenesis to initially fold the imaginal disc. Then mono-phosphorylation is required to allow the cells to expand. (Aldaz, Escudero and Freeman 2013). Therefore, proper regulation of these two states is critical for the success of this process. Imaginal disc morphogenesis shows that myosins are able to change their strength of contractility from one step in a process to another and that tight regulation of this switch is critical.

Myosin activity is controlled through phosphorylation by the balance of phosphatase *versus* kinase activities, resulting in the level of rMLC phosphorylated on serine-19 and therefore, whether more myosin molecules are in an active or inactive state (Figure 1-3). Different kinases have been demonstrated to be capable of phophorylating rMLC. Myosin light chain kinase (MLCK) is the only kinase that is specific for the rMLC and is activated by calmodulin (Heissler and Manstein 2013). However rMLC can also be phosphorylated by Rho-kinases (ROCK) which are controlled by the G protein, RhoA, which is also affected by calcium levels in the cell (Somlyo and Somlyo 2003). Another kinase that can phosphorylate rMLC on serine-19 is p21-activated kinase (Bresnick 1999). These three conserved kinases are known to phosphorylate the rMLC during multiple different processes in a single organism. This shows that the same regulatory pathways are used in different processes and that there are commonly more than a single kinase regulating nonmuscle myosin during any process.

While many kinases are able to phosphorylate nonmuscle myosin, there has only been one nonmuscle myosin phosphatase identified, serine/threonine phosphatase PP1C, which has been implicated in the regulation of its activity (Grassie, et al. 2011). This phosphatase interacts with myosin through a myosin binding subunit that makes the phosphatase complex specific for the rMLC. This phosphatase subunit can also be regulated through phosphorylation by kinases including ROCK, serine/threonine leucine zipper-interacting protein kinase (ZIPK) and p-21-activated kinase. ROCK is able to phosphorylate the myosin phosphatase and

inactivate it through signaling from Rho (Kimura, et al. 1996). The myosin phosphatase subunit can in turn be phosphorylated by ZIPK in smooth muscles (Endo, et al. 2004). Finally there is some evidence that p21-activated kinase is also capable of phosphorylating myosin phosphatase and inhibiting its function (Takizawa, Koga and Ikebe 2002). Therefore, the single phosphatase is a simple off switch for myosin but complex regulation occurs through the kinases, which phosphorylate not only the myosin itself but also phosphorylate the phosphatase, inhibiting its function.

While the major mechanism of regulation for myosin is thought to be through the rMLC, there is also regulation through the heavy chain itself. There are phosphorylation sites on the heavy chain in the C-terminal non-helical tail domain. While there is evidence that phosphorylation at these sites is important for proper function of mammalian myosins, it remains unclear how this phosphorylation affects the myosins and whether this occurs in other organisms (Vicente-Manzanares, et al. 2009). It has been shown that protein kinase C and casein kinase are capable of phosphorylating the heavy chain which may lead to isoform specific filament formation in mammalian cells (Murakami, Chauhan and Elzinga 1998). Myosins are also regulated via binding of regulatory proteins, as has been seen for mammalian Mts1 which binds to the carboxy-terminal end of the myosin heavy chain and inhibits its activity as well as inhibiting binding of protein kinase C to the myosin (Ford, et al. 1997) (Kriajevska, et al. 1998). It is likely that parts of the myosin complex other than the rMLC are important for regulation. While our understanding of this is incomplete, it may help to explain why in mammalian cells there are so many different genes for the heavy chain as well as different isoforms of those genes. The differences in sequence may allow alternate phosphorylation sites or the binding of specific regulatory proteins.

1-2. Nonmuscle Myosin Play a Crucial Role in Various Different Processes

1-2.1. *C. elegans* Genes that Encode the Nonmuscle Myosin Complex

There are two nonmuscle myosin heavy chains in *C. elegans*, encoded by the *nmy-1* and *nmy-2* genes. There is one gene for each of the light chains. MLC-4 is the regulatory light chain and is proposed to function with both NMY-1 and NMY-2 (Shelton, et al. 1999). The essential light chain is encoded by *mlc-5* (Gally, et al. 2009). NMY-2 has a key role in polarization of the one-cell embryo, cytokinesis, elongation, and is also needed for fertility, indicating that NMY-2 has a functional role in the gonad. NMY-1 has only been shown to function during embryonic elongation; however, *nmy-1* mutants also have a decrease in fertility (Piekny, Johnson, et al. 2003). NMY-1 RNAi show a generalized cell migration defect during embryogenesis and defects in gastrulation that are similar to those seen with calmodulin (*cmd-1*) RNAi, but the specific molecular causes of the defects are unknown (Karabinos, et al. 2003). Although such multifaceted phenotypes implicate nonmuscle myosin in many developmental processes, perhaps the best studied role for nonmuscle myosins in *C. elegans* is during early embryo cytokinesis.

1-2.2. Cytokinesis

Nonmuscle myosins are required for cytokinesis in *C. elegans* as well as many other organisms (De Lozanne and Spudich 1987) (Mabuchi and Okuno 1977). They localize to the contractile ring that is necessary for separating the two cell membranes from each other and provides the motor that drives the constriction of the ring (Figure 1-4). In cultured mammalian cells, rMLC is phosphorylated at serine 1 and 2 at the start of mitosis, holding myosin in an inactive state. As cytokinesis begins, serine 1 and 2 are dephosphorylated and serine 19 is phosphorylated, activating the myosins (Yamakita, Yamashiro and Matsumura 1994). This mechanism prevents premature activation of myosin leading to inappropriate cell division. It also shows that there are more phosphorylation sites than the two previously mentioned. Three kinases have been shown to

phosphorylate rMLC during mitosis: myosin light chain kinase (MLCK), rho-kinase (ROCK), which is also capable of phosphorylating myosin phosphatase and inhibiting it, and citron kinase (Matsumura, et al. 2001). These kinases are critical for proper localization and appropriate activation of myosin.

While the majority of research on cytokinesis has been performed in mammalian cell culture, there has also been research into the role of nonmuscle myosin during the first cell division of the *C. elegans* embryo. NMY-2, the nonmuscle myosin heavy chain, and MLC-4, the nonmuscle myosin regulatory light chain, are required for cytokinesis during both mitosis and meiosis as well as for setting up the polarity of the early embryo through cytoplasmic streaming (Shelton, et al. 1999). Studying nonmuscle myosin in both of these processes allows us to see how signaling pathways identified in mammalian cells are largely conserved in the *C. elegans* system and while many of the same players are active, there are some key differences in the kinases involved in this process (Figure 1-5). One of the most common pathways found in *C. elegans* nonmuscle myosin regulation is activation by a ROCK, LET-502, and repression by the myosin-specific phosphatase subunit, MEL-11. Together these two factors regulate cleavage furrow contraction during cytokinesis in the first few cell divisions of embryogenesis. Mutants of either factor affect the level of phosphorylated MLC-4 which is present. LET-502 is regulated by a Rho, and Rho proteins can be present in either their active GTP bound state or in their inactive GDP state. Whether Rho is inactive or active is, in turn, dependent on both RhoGAPs (Rho GTPase activating proteins, which activate the GTPase function of Rho causing it to hydrolyze GTP into GDP and become inactive) as well as RhoGEFs (Rho Guanine nucleotide Exchange Factors, which stimulate Rho to release GDP so that it can bind GTP and become active). Both RhoGAPs and RhoGEFs therefore control whether Rho is active or inactive. Active Rho then activates ROCK, and ROCK phosphorylates the myosin phosphatase subunit, inhibiting its function. Therefore, active Rho causes phosphorylation of rMLC via ROCK while also inhibiting the only protein capable of dephosphorylating the rMLC (Figure 1-5).

There are many RhoGAPs and few RhoGEFs in *C. elegans*. One important RhoGAP is CYK-4. At the end of cleavage by the contractile ring, CYK-4 downregulates Rho in order to terminate myosin contraction (Jantsch-Plunger, et al. 2000). What makes *C. elegans* interesting is that there is no identified nonmuscle MLCK and calmodulin is not required for cytokinesis, making it even more unlikely that there is an unidentified MLCK present (Batchelder, et al. 2007). This is different from mammalian cells, because MLCK is the only myosin specific kinase and is known to be involved in cytokinesis in other organisms. This raises the question as to whether other kinases are important in cytokinesis in *C. elegans.* Loss of both LET-502 and MEL-11 together results in normal levels of phosphorylation implying the existence of at least a second kinase (Piekny and Mains 2002). Therefore, even though many studies have been conducted on cytokinesis in *C. elegans*, not all the kinases involved in this process may have been identified. This demonstrates a common theme in nonmuscle myosin research, that the entire collection of direct regulators and upstream factors is not complete. Looking at other myosin dependent processes in *C. elegans* could identify kinases required for those processes, which may also be required for cytokinesis.

1-2.3. *C. elegans* Polarization of the First Cell

The first cell division of *C. elegans* is asymmetrical, which means that before the cell can divide it has to determine which side of the embryo is anterior or posterior. This is done through a segregation of specific sets of proteins, called the PAR proteins, to both halves of the first blastomere (Figure 1-6). This process has been found to be dependent on NMY-2. The oocyte is unpolarized in the anterior/posterior direction, and either end can become the posterior pole of the embryo, as the sperm entry point determines the presumptive posterior pole of the embryo (Goldstein and Hird 1996). The sperm contributes not only the male pronucleus to the embryo but there is evidence that a RhoGAP protein, CYK-4, is introduced by the sperm and this initiates the polarization of the actomyosin network (Jenkins, Saam and Mango 2006). This then leads, in turn, to the

polarization of the entire cell (Figure 1-7) (Munro, Nance and Priess 2004). At the posterior side of the cell, there is also a loss of ECT-2, a RhoGEF, as well as the addition of CYK-4 leads to a depletion of RHO-1, the Rho protein, which is thought to cause the destabilization of the actomyosin network due to loss of activation, this causes the network to move anteriorly (Motegi and Sugimoto 2006). This anterior movement of the actomyosin is what causes the cortical cytoplasm to move anteriorly, bringing with it anterior specifying PAR proteins. These anterior specifying PAR proteins antagonize the posterior specifying PAR proteins, moving them into the cytoplasm on the anterior side where they diffuse to the posterior side of the cell, to fill the space vacated by the recently departed anterior specifying PAR proteins (Figure 1-6). The initial rearrangement of the actomyosin network caused a general net flow of cytoplasm: cortical cytoplasm heading anteriorly and inner cytoplasm moving posteriorly which results in a polarized cell. Once the cytoplasmic and cortical rearrangement has begun, the RhoGAP proteins, RGA-3 and RGA-4, act on RHO-1 to create cortical domains of NMY-2 contractile function which sets the size of the PAR domains (Schonegg, et al. 2007). An interesting note is that this is one of the few processes involving nonmuscle myosins where LET-502 and MEL-11 do not appear to play a role (Piekny and Mains 2002). Together this demonstrates not only that separate myosin regulatory pathways are involved in separate processes but that myosin are capable of more than constricting a network and can cause cytoplasmic movement.

1-2.4. Elongation

Another well studied nonmuscle myosin dependent process in *C. elegans* is embryo elongation (Figure 1-8). Elongation is a process where a spherical shaped multicellular embryo elongates into its tubular larval worm shape without the need for an increase in cell number. It occurs though the constriction of circumferential microtubules that are attached to the outer membranes of the nematode (Priess and Hirsh 1986). Elongation is the only *C. elegans* process where the nonmuscle myosin NMY-1 has been characterized in detail. An RNAi

screen showed that loss of NMY-1 resulted in sterility, lethality, dumpy, and uncoordinated phenotypes (Kamath and Ahringer 2003). Dumpy worms are shorter and rounder than their wild type counterparts, indicating that NMY-1 may be required for proper elongation. Defects in the nervous system, musculature or the outside cuticle of the worm can result in an uncoordinated phenotype or changes to the wild type movement of the worm. However, it is possible that in this case uncoordinated phenotype is secondary to the Dumpy phenotype. Additional characterization of NMY-1 genetic knockdowns showed adults have very small brood sizes. The NMY-1 RNAi treated animals also have small pharynxes, an accumulation of bacteria in their gut and occasional gonad arms that fail to migrate properly, though whether these are a secondary effect of the dumpy phenotype or a primary effect of the loss of NMY-1 is unknown (Piekny, Johnson, et al. 2003). Therefore, even though the only developmental process in which NMY-1 has been characterized is embryo elongation, NMY-1 genetic knockdowns indicate that it may have an important role in other processes.

Initial characterization of NMY-1's role in elongation showed that loss of NMY-1 was capable of suppressing *mel-11* mutants and worsening *let-502* mutants making it likely that this cassette also functions in this process (Piekny, Johnson, et al. 2003). During embryo elongation, MEL-11, the myosin phosphatase, is sequestered to the surface of the cell, likely due to phosphorylation by LET-502. Surprisingly, this localization requires the presence of NMY-1. Loss of MLC-4 caused the collapse of filamentous-like structures of NMY-1 into foci, confirming MLC-4 as the regulatory light chain for NMY-1 during this process (Piekny, Johnson, et al. 2003) (Shelton, et al. 1999). *let-502* mutants are unable to contract their tissues during elongation and therefore, fail to go through elongation, while *mel-11* mutants hyper-contract their tissues and frequently burst (Wissmann, Ingles and Mains 1999). Taken together, NMY-1 is required for elongation through phosphorylation and dephosphorylation of its regulatory light chain, MLC-4, by LET-502 and MEL-11. This is another process that utilizes LET-502 and MEL-11.

This cassette works directly on MLC-4, therefore it was important to understand how phosphorylation of the regulatory light chain affects elongation. A form of MLC-4 in which both threonine-17 and serine-18 (the phosphorylation sites correspond to threonine-18 and serine-19 in the mammalian homologue) are changed to asparatate, which mimics phosphorylation, shows a more stable filamentous-like pattern, recapitulating what has been found in mammalian cells. This form was able to rescue the loss of endogenous MLC-4 when provided as a transgene. When threonine-17 and serine-18 were replaced with alanine, a non-phosphorylatable amino acid, it was not able to rescue the loss of endogenous MLC-4 and when only one of the amino acids was replaced with an alanine it partially rescued (Gally, et al. 2009). This shows that just as in mammalian cells, nonmuscle myosin can be phosphorylated at two sites on the regulatory light chain and this di-phosphorylation affects the nature of the myosin complex. Another similarity to mammalian cells is seen when the complex that has the threonine and serine replaced with alanine is used, MLC-4 shows diffuse localization instead of a filamentous-like pattern indicating that phosphorylation of MLC-4 is required for it to form filaments and localize.

In addition to those mentioned above, other kinases also have a role during embryonic elongation in *C. elegans* (Figure 1-5). MRCK-1 appears to work upstream of LET-502 and MEL-11. In the absence of MRCK-1 and LET-502, PAK-1, a p21-activated kinase, is capable of phosphorylating MLC-4 indicating that it is a redundant kinase in this process (Gally, et al. 2009). A RhoGAP, RGA-2, has also been shown to inhibit RHO-1 during elongation which would cause the inhibition of LET-502 and decrease the amount of phosphorylated MLC-4 (Diogon, et al. 2007). The Rac pathway functions very similarly to the Rho pathway, (Rac is also a G protein) acting as a signaling pathway during elongation. UNC-73, a GEF that interacts with both Rho and Rac, and MIG-2, a small G protein with similarities to Rac, are both involved in the Rac pathway and mutants in both enhance the mel-11 mutant phenotype (Wissmann, Ingles and Mains 1999). Studies of elongation revealed two new kinases that are capable of

phosphorylating MLC-4 in *C. elegans* as well as introducing the Rac pathway as an upstream component of the myosin regulatory pathway.

In conclusion, the nonmuscle myosin dependent processes that have been studied in *C. elegans* show that there are some regulatory pathways that are similar between several processes, and others that currently appear to be specific to a single process. LET-502 and MEL-11 seem to be required in many processes and yet during polarization of the first cell embryo they are not required. MRCK-1 appears to be redundant with LET-502 during elongation but does not appear to interact with myosins at any other developmental stage. Therefore, the components of these known cassettes can be analyzed for their potential roles in other previously unstudied nonmuscle myosin dependent processes in *C. elegans*, such as oogenesis.

1-3. *C. elegans* Oogenesis

1-3.1 Steps of oogenesis

C. elegans is a male/hermaphrodite species, meaning that hermaphrodites are capable of self-fertilization or cross-fertilization with males. Before hermaphrodites reach adulthood, they undergo a brief period in the fourth larval stage where they produce sperm. Hermaphrodites then store the sperm in an organ called the spermatheca (Figure 1-9), for use throughout the remainder of their lives to fertilize the oocytes that they subsequently produce after reaching sexual maturity. The *C. elegans* hermaphrodite gonad comprises two mirror image arms that center on a single uterus and ventral vulva where embryos are laid. Each gonad arm has a proximal region in the same plane as the vulva, a loop region where the gonad turns back on itself to run dorsal to the proximal arm, and a distal arm that runs back towards the vulva and ends up lying near the very distal tip of the other distal arm. The core of each gonad arm is made up of germline cells surrounded by somatic sheath cells that on the proximal end, are connected to the spermatheca that connects the oviduct arm to the uterus with the vulva in the

center. From this ovotestis, the same germline precursors produce both oocytes and sperm, but at separate times (Schedl 1997).

At the most distal end of the gonad is the distal tip cell, which is critical for producing signals that maintain a pool of germline stem cells adjacent to it. The distal tip cell has long thin projections that extend out and surround most of the stem cells. The stem cells undergo mitosis to create additional germ cells that eventually move out of the stem cell niche. At this point, these cells begin meiosis and progress through leptotene and zygotene until they are paused during pachytene and remain in this state throughout the distal arm. These cells are positioned along the cortex of the gonad arm surrounded by membrane, except for a ring channel on the internal side, which allows them to connect to a shared cytoplasm that runs through the center of the distal arm called the rachis (Figure 1-9). These pachytene stage cells are thought to act like nurse cells because they are very transcriptionally active (Gibert, Starck and Bequet 1984).

Pronuclear germ cells moving along the arm begin to exit pachytene as they near the loop and progress to diakinesis. I will refer to these as "cells" for convenience, although the nuclei are still part of a syncytium until the ring channels completely close. During this process half the cells undergo apoptosis, and expel their contents into the rachis (Gumienny, et al. 1999). The remaining cells begin to take up large amounts of cytoplasm and grow in size as they continue down the gonad arm. The arrangement of the nuclei changes from one where nuclei are positioned around the cortex of the gonad, to one where they are in a single row along one side. When the cells have reached the appropriate size, they pinch off from the rachis, becoming fully cellularized. The oocyte closest to the spermatheca will undergo meiotic maturation five minutes before ovulation, which involves loss of the nucleolus and migration of the nucleus towards the distal end of the oocyte, followed by breakdown of the nuclear envelope. This is accompanied by a cortical rearrangement that changes the oocyte from a cylindrical shape to a spherical shape (McCarter, et al. 1999).

Ovulation involves relaxation of the valve between the oviduct and spermatheca and contractions of the two sheath cells surrounding the most proximal end of the gonad. Sheath cells undergo contractions circumferentially, squeezing the oocyte into the spermatheca, as well as longitudinally allowing the sheath cells to pull the spermatheca over the oocyte. Fertilization occurs almost immediately upon entry of the oocyte into the spermatheca, and in less than ten minutes after the oocyte has entered the spermatheca, the valve between the spermatheca and the uterus relaxes and the spermathecal tissue surrounding the oocyte contracts in order to push the oocyte into the uterus. Ovulation occurs approximately every 23 minutes at 20 - 23°C if there is sufficient sperm (McCarter, et al. 1999). Detailed descriptions of oogenesis can be found in (Schedl, 1997).

Oogenesis in *C. elegans* resembles the same process in many other organisms, both vertebrate and invertebrates. They all have growing oocytes which are supplied with large amounts of RNA and protein by nurse-like cells. These nurse-like cells usually undergo apoptosis, and their sole function is to supply the oocytes with large quantities of material. In other organisms these nurse cells are also attached to the oocyte via very similar ring channels, stabilized by actin (Mahajan-Miklos and Cooley 1994) (Gondos 1973). Therefore, *C. elegans* oogenesis is not organism-specific but instead, an easily studied process that can then be generalized to oogenesis in many organisms.

1-3.2 Regulation of the Steps of Oogenesis

A combination of genetic and molecular approaches have revealed signaling pathways that are required for the various steps of *C. elegans* oogenesis, and some of the proteins required for this process have been identified (Figure 1-10). Three main signaling pathways are important in establishing different steps of the process. At the most distal end, Notch signaling is critical for maintaining the stem cell population and for the exit from mitosis into meiosis. Throughout the gonad, Ras/MAPK signaling controls multiple steps in the pathway including promoting the proliferative fate, exit from pachytene, meiotic maturation, and

ovulation (Lee, et al. 2007). The final pathway involves the major sperm protein (MSP) signal. This is a signal released by sperm in the spermatheca, either self-made sperm or outcrossed (male) sperm, which acts on the oocytes and surrounding somatic tissue and is needed for the final steps of oocyte production. Therefore, there is a signal from the most distal tip (Notch signaling) a signal from the proximal tip (MSP signaling from the sperm) and an internal signal (Ras/MAPK signaling). These signals lead to the regulation and localization of the effector proteins such as nonmuscle myosins, and differentiate the separate steps during oogenesis. Therefore, upon identifying the steps of oogenesis that require nonmuscle myosins, the major signaling pathways that control those steps can be used as candidates for the initiation of nonmuscle myosin's activation.

1-3.2.1 Proximal Gonad Arm

At the distal end of the gonad, the distal tip cell (DTC) extends projections along the stem cell niche and uses Notch signaling to the surrounding cells to activate the Notch receptor, GLP-1. GLP-1 promotes the proliferative state, but as cells move away from the DTC, they lose Notch signaling. Loss of Notch signaling causes nuclei to enter into meiosis and arrest at (Kimble and Crittenden, 2005). During the pachytene stage, a number of factors are important in maintaining the architecture of the gonad, by keeping the oocytes along the cortex of the gonad and the ring channels open to the rachis. LET-502 and MEL-11 are present in the ring channels (Figure 1-10), along with NMY-2 and ANI-2, an anillin protein that acts as a scaffold and is able to bind actin, myosin and the membrane (Maddox, et al. 2005) (Piekny and Mains 2002). Loss of ANI-2 results in premature cellularization and a shortened rachis (Maddox, et al. 2005). As with many of the factors required at this stage, loss of proteins important for ring channel maintenance results in premature or delayed closure of the ring channel which results in oocytes with varying sizes. This shows that closure of the ring channel is used to control the size of the mature oocyte. Loss of NMY-2 results in loss of internal membranes that in turn leads to pooling of nuclei, showing that ring channels are also necessary for maintaining nuclei along the wall of the gonad

(Green, et al. 2011). The distal arm of the gonad requires Notch signaling for initiation of meiosis and Ras/MAPK signaling for promoting the proliferative state; however, processes occurring in the distal arm do not require MSP signaling. This can be demonstrated by blocking sperm production genetically in hermaphrodites, creating female worms that are incapable of producing their own sperm. Without the presence of sperm, these females do not receive a MSP signal and yet they resemble hermaphrodites in the appearance of their distal arm. However, when crossed with a male, sperm are introduced activating MSP signaling and the females become indistinguishable from hermaphrodites. The distal arm is controlled by two of the three main signaling components and maintenance of the internal architecture by NMY-2 is important for later stages of oogenesis.

1-3.2.2. Progression from Pachytene to Diakinesis

The next step in oogenesis requires the exit from the pachytene stage of meiosis to diakinesis which occurs around the loop region of the gonad. This exit is dependent on the Ras/MAPK signaling pathway, which is also necessary for apoptosis of the nuclei (Gumienny, et al. 1999). The trigger that initiates the Ras/MAPK pathway during oogenesis is currently unknown, though it is thought to involve a receptor tyrosine kinase, similar to its activation in other processes. Loss of a downstream protein tyrosine kinase adapter, ROG-1, results in disruption of progression from pachytene to diakinesis, the same phenotype seen upon loss of Ras/MAPK signaling; however, a gain of function mutation of Ras rescues the loss of ROG-1 placing ROG-1 upstream of the Ras/MAPK pathway (Matsubara, et al. 2007). The Ras/MAPK pathway begins with the Ras protein, LET-60, which phosphorylates LIN-45, a RAF protein, which then phosphorylates MEK-2, a MEK protein, which goes on to phosphorylate MPK-1, an ERK protein (Figure 1-11). MPK-1 then goes on to phosphorylate a large number of substrates and is the initiator of signaling cascades that lead to progression of the next step of oogenesis. While a large number of the substrates phosphorylated by MPK-1 have been identified, none of them are currently

known to lead to the phosphorylation of MLC-4 (Arur, et al. 2009). Phosphorylation of MPK-1 and exit from pachytene does not depend on the presence of sperm, but in very old females that have over 15 oocytes stacked in the proximal gonad, MPK-1 is dephosphorylated and oocytes remain stopped in pachytene (Lee, et al. 2007). This switch from pachytene to diakinesis solely requires the Ras/MAPK signaling pathway and MPK-1 is a major regulator that leads to many downstream changes.

1-3.2.3 MSP Signaling

The Major Sperm Protein (MSP) signal is produced, as the name implies, from a factor associated with the sperm and is necessary for the final steps of oogenesis. The signal is not present in the absence of sperm in the spermatheca and is a method for *C. elegans* to only produce oocytes (which are energetically expensive) when sperm are present to fertilize them. *C. elegans* hermaphrodites normally only produce ~300 fertilized embryos using self-sperm, but if extensively mated to males, can be stimulated to produce four times as many progeny (Murray and Cutter 2011). The MSP signal triggers cytoplasmic streaming in the gonad, the maturation of the oocytes, increased sheath cell contractions as well as more frequent ovulation (Govindan, Nadarajan, et al. 2009) (Miller, et al. 2001). MSP signaling also promotes oocyte growth and microtubule rearrangement (Han, Cottee and Miller 2010) (Harris, et al. 2006). Almost all processes in the proximal gonad arm require the MSP signal to initiate and while oocytes are able to reach full size and are occasionally ovulated without the MSP signal, the speed and efficiency is greatly reduced.

The MSP signal acts on both the maturing oocytes as well as on the sheath cells. In sheath cells it functions through G protein signaling, specifically through ACY-4, which catalyzes the formation of cAMP. cAMP binds to KIN-2 and gap junctions that connect the sheath cells to the oocytes and causes VAB-1 receptors, which are in recycling endosomes in the oocytes, to be localized to the surface where they can bind MSP (Govindan, et al. 2006) (Govindan, Nadarajan, et al.

2009) (Cheng, Govindan and Greenstein 2008). Therefore, defects in signaling from the sheath cells can affect oocyte development. MSP can then bind to VAB-1, an Eph receptor, on the surface of the oocytes where it inhibits ephrin signaling and allows meiotic maturation and MAPK phosphorylation (Miller, et al. 2003). Overexpression of ACY-4 is sufficient to induce meiotic maturation even in the absence of the MSP signal, by inhibiting a negative signal that comes from gap junction communication between the sheath cells and oocytes which normally stops them from undergoing meiotic maturation. MSP also induces sheath cell contractions and this is through a separate pathway than the one that activates meiotic maturation. Sheath cell contraction requires EGL-30 and a gain of function mutation in EGL-30 is capable of causing sheath cell contractions in the absence of the MSP signal (Govindan, Nadarajan, et al. 2009). Loss of any of the components of this pathway inhibits downstream signaling of the MSP signal and freezes the oocytes in diakineses.

1-3.2.4. Cytoplasmic Streaming

Cytoplasmic streaming is the mechanism the gonad uses to transfer RNAs and proteins produced in the distal gonad through the rachis to the developing oocytes in the proximal gonad. Wolke *et al.* (2007) characterized the nature of cytoplasmic streaming in wild type *C. elegans* gonads and found that, similar to polarization of the one cell embryo, cytoplasmic streaming requires an actomyosin network (Figure 1-12). They began by identifying components that were being transferred into the developing oocytes and concluded that all particles in the rachis were being transported. Therefore cytoplasmic streaming is a bulk movement of cytoplasm that transports everything in the rachis towards the developing oocytes. Next, they analyzed the movement of particles in living animals, using DIC optics to determine the speed and direction of this bulk flow. While there is very little movement of particles in the distal end of the gonad, particles in the mid to late pachytene stage move occasionally from the exterior of the gonad towards the interior of the gonad but there is mostly a net movement of particles towards the proximal arm of the gonad. Particles in the loop move faster

and deposit themselves into the three oocytes closest to the loop. Upon entry into the growing oocytes, particle movement ceases. Flow rates are fairly consistent except for a period before ovulation, where streaming slows. Following ovulation, there is a short period where particles are pushed back towards the distal arm before normal cytoplasmic streaming continues. Therefore, gonadal cytoplasm moves at different speeds, depending on which section of the gonad is being examined, and whether ovulation is occurring.

It is estimated that 30 to 40% of the final volume of the oocyte is deposited by streaming (Wolke, Jezuit and Priess 2007). The remainder is composed of yolk particles which are produced in intestinal cells, deposited in the body cavity and then taken up into the oocyte using endocytosis (Kimble and Sharrock 1983) (Grant and Hirsh 1999). Pores penetrate through the sheath cells from gonadal basal lamina in order to transport yolk particles to the oocyte (Hall, et al. 1999). All these components are required for the production of a functional oocyte. Oocytes lacking yolk particles or that do not sufficiently enlarge are unable to support the embryo until it reaches maturity, and result in embryonic lethality following fertilization (Grant and Hirsh 1999) (Rose, et al. 1997).

After studying cytoplasmic streaming in the gonad, Wolke *et al.* (2007) tried to determine the nature of the force that elicited the streaming in the gonad. All known cytoplasmic streaming uses elements of the cytoskeleton, including initial polarization of the *C. elegans* embryo, fountain streaming in the *Drosophila* early embryo and streaming of nutrients and organelles in plant cells (Shelton, et al. 1999) (von Dassow and Schubiger 1994) (Shimmen and Yokota 2004). Therefore, Wolke *et al.* (2007) examined animals following gene knockdown of tubulin, which created gonad defects, including nuclei in the rachis. Under those conditions, cytoplasmic streaming still occurred, with the nuclei in the rachis being carried along toward the proximal gonad arm. Therefore, they concluded that microtubules are not needed for cytoplasmic streaming. They also examined gonads in the presence of actin depolymerizing drugs or a myosin light chain kinase inhibitor, and this resulted in a loss of cytoplasmic streaming, suggesting

that an actomyosin complex is the driving force. The next step was to determine where in the gonad the force was being generated. They isolated different areas of the gonad by injecting large oil bubbles isolating either side of the site of injection, to see where cytoplasmic streaming still occurred and they found that the force behind cytoplasmic streaming is localized to the growing oocytes themselves. While they presented several hypotheses for generation of this force, one hypothesis involves a difference in actomyosin tensions between the growing oocyte and the cytoplasmic core, which causes a net flow of cytoplasm into the growing oocyte. This is similar to what is thought to happen during fountain streaming in the early syncytial embryo of *Drosophila* and therefore, is their favoured explanation (von Dassow and Schubiger 1994).

Additional work has been done on the signaling pathways that affect cytoplasmic streaming. It appears to be controlled by two mechanisms; through phosphorylation of MLC-4 or through activation of GLP-1, because loss of GLP-1 does not increase phosphorylation of MLC-4 but does increase cytoplasmic streaming speeds (Nadarajan, et al. 2009). MPK-1 is also necessary for GLP-1 to increase cytoplasmic streaming speeds, however loss of MPK-1 itself does not affect streaming. Both phosphorylation of MLC-4 and faster cytoplasmic streaming speeds in *glp-1* mutants requires the MSP signal, and both ACY-4 and GSA-1, signaling in sheath cells (Figure 1-13). Overexpression of ACY-4 causes faster cytoplasmic streaming than is seen in wild type indicating a link between MSP signaling and streaming. The MSP signal passes through sheath cells to activate meiotic maturation; therefore, it is possible that meiotic maturation is necessary for cytoplasmic streaming. However, this is unlikely because streaming begins before meiotic maturation, when the MSP signal is first introduced to the system. A second piece of evidence is that loss of OMA-1 and OMA-2 results in failure of cells to undergo meiotic maturation. However, there continues to be cytoplasmic flow, though at a slower speed than wild type; this sustained flow rate without meiotic maturation is the likely reason for the large oocyte size seen in these mutants. This indicates that while cytoplasmic streaming does not require

meiotic maturation, it may require it to achieve the same flow rates seen in wild type hermaphrodites or require OMA-1 or OMA-2 to achieve these rates (Govindan, Nadarajan, et al. 2009). Therefore, cytoplasmic streaming only occurs in the presence of the MSP signal, it occurs before meiotic maturation and does not require meiotic maturation, and there appears to be additional regulation of the rate of cytoplasmic streaming that involves GLP-1.

1-3.2.5. Oocyte Maturation

Oocytes have to change from tiny cells attached to a syncytium at the pachytene stage of oogenesis to the very large fully enclosed cells that will be fertilized. One of the contributing processes is cytoplasmic streaming but there are other processes that must correctly occur. Alleles of *mpk-1,* can be temporally disrupted in order to circumvent the pachytene arrest which is normally displayed in *mpk-1* mutants and reveals defects caused by loss of MPK-1 at other steps in the pathway. In such animals, single file oocytes are missing, and instead one sees oocytes of varying sizes, though generally larger, with smaller oocytes being more proximal than larger oocytes, a phenotype that is never seen in wild type animals. There are also multinucleate oocytes as well as apparently anuclear oocytes. A *let-60* gain of function mutation that causes higher levels of active MPK-1 shows the opposite phenotype with many smaller oocytes but only in the presence of the MSP signal (Lee, et al. 2007). Therefore it appears that MPK-1 at later stages is controlled by the MSP signal, plays a role in oocyte size and is also needed for proper organization of the proximal gonad.

In order for an oocyte to completely mature it has to pinch away from the rachis. As was previously stated, many of the components that make up the ring channel also play an important role in determining the size of the final oocyte but there are also additional signaling pathways which appear to affect the size as well. One of those is GLP-1, and loss of GLP-1 results in larger oocytes. This change in oocyte size is also dependent on MSP signaling making GLP-1 an additional signaling pathway that affects the size of the oocytes but only in the presence of the MSP

signal. Nadarajan *et al.*, 2009 examined *glp-1* mutants and saw both faster cytoplasmic streaming and slower cellularization. Changes in final oocyte size is not seen in mutants affecting cytoplasmic streaming or cellularization alone, meaning that it is the two defects together that result in larger oocytes. Interestingly, delayed cellularization is not dependent on the MSP signal while cytoplasmic streaming is. Therefore, there appears to be a coordination between streaming and cellularization timing so that one can compensate for the other in order to end up with the optimally sized oocyte.

The final step is meiotic maturation which occurs five minutes before ovulation. This process only occurs in the presence of the MSP signal and it is critical that fertilization occur shortly after, otherwise the oocyte begins uncontrollably replicating its DNA, known as an endomitotic phenotype (Emo), the most common phenotype seen in gonads that are unable to ovulate and therefore unable to fertilize their oocytes. This results in multinucleated cells which remain in the oviduct if ovulation does not occur or in the uterus upon ovulation (Iwasaki, et al. 1996). Therefore meiotic maturation appears to prime cells for fertilization and perhaps removes the block against DNA replication.

1-3.2.6. Ovulation

The final step for successful oogenesis is ovulation, which in *C. elegans* also involves fertilization. This process involves both MSP signaling and MPK-1 signaling (Lee, et al. 2007). There are two tissues that have to act in concert in order for ovulation to successfully occur: sheath cells and the spermatheca. Therefore, defects in either tissue results in an Emo phenotype due to a failure in ovulation and oocytes being stuck in the oviduct after undergoing meiotic maturation. The spermatheca is a highly contractile tissue that normally is much smaller than the oocytes that have to fit inside of it. Therefore ovulation requires that the spermatheca stretch in order to make room for the oocyte and contract to expel the oocyte after fertilization.

There are two valves on either side of the spermatheca and ovulation requires the relaxation and contraction of the valves (Figure 1-14). Both MEL-11 and LET-502 are present in the spermatheca and they localize to separate areas, with LET-502 localizing in the valves and MEL-11 localizing between the two valves in the area that has to expand to make space for the oocyte (Wissmann, Ingles and Mains 1999). The valves are important for creating a one way flow of oocytes, so in *let-502* mutants that are unable to constrict their valves, after oocytes are fertilized they either move through to the uterus, or they return to the oviduct, causing a backup of mature oocytes which decreases the future success of ovulation (Wissmann, Ingles and Mains 1999). This misdirection of movement frequently damages the oocytes even if they make their way to the uterus so fertility is greatly decreased. MEL-11 mutants have the opposite phenotype where the valves are unable to relax and so the oocytes are unable to enter the spermatheca and become endomitotic (Emo) in the oviduct (Wissmann, Ingles and Mains 1999). If oocytes are able to enter the spermatheca, when the spermatheca goes to expel the oocyte it contracts so strongly that frequently the spermatheca bursts and the oocyte falls into the body cavity (Kovacevic, Orozco and Cram 2013). UNC-73, a Rac protein, also plays a role in the spermatheca. Mutations in UNC-73 enhance mutations in MEL-11, likely because, in many systems, Rac acts counter to Rho and, therefore, would inhibit rather than promote LET-502, the Rho kinase (Wissmann, Ingles and Mains 1999).

The second tissue required for ovulation is the sheath cells (Figure 1-9). Sheath cells have thin and thick filaments and contain type II muscle myosins, MHCA and MHCB. Contraction of the sheath cells not only squeezes the oocyte into the spermatheca but also pulls the spermatheca over the oocyte at the same time. There are a number of defects that can occur if the sheath cells do not pull as a unit, or if their contractions are compromised. CEH-18 is required for the proper differentiation of sheath cells and mutations in this gene demonstrate the effects of improperly formed sheath cells. The phenotypes seen in *ceh-18* mutants include: failure of ovulation, where oocytes are left in the oviduct and become

endomitotic, or the sheath cells contract asymmetrically, which pinches off a piece of the oocyte. This results in cytoplasmic anucleated "blebs" in the oviduct and in the uterus. The nucleated half of the oocyte occasionally goes on to become a healthy embryo but only if it makes its way to the uterus and if the oocyte still has sufficient cytoplasm. Another defect seen in *ceh-18* mutants, is occasionally more distal oocytes undergo meiotic maturation but this is likely due to defects in signaling from the sheath cells instead of defects in contractions (Rose, et al. 1997). Knowing these phenotypes allow genetic knockdowns of other genes to be classified as sheath cell defects, indicating the gene is required for the proper function of sheath cells.

In conclusion, ovulation requires the role of two somatic tissues, the spermatheca and the sheath cells. Defects in either tissue can result in an emo phenotype which is caused by oocytes undergoing meiotic maturation but not fertilization. When the defects include excessive contractions, oocytes can be damaged. However, unlike other defects in oogenesis, a few oocytes can escape to be properly fertilized and undergo successful embryogenesis. Defects in either of these tissues do not always result in complete sterility.

1-4. Summary of Project Objectives

Nonmuscle myosins are motor proteins that, unlike their muscle counterparts, can be modified to change their degree of ATPase activity and rate of disassociation from actin to adapt to different processes within a single organism. This modification is done through phosphorylation and dephosphorylation of either the myosin regulatory light chain or the myosin heavy chain. Having differing levels of ATPase activity and filament stability allows myosin to have a constricting function during cytokinesis or a tensile function during cytoplasmic streaming in the early embryo. However, the type of function required from nonmuscle myosin during oogenesis remains to be determined. Previous work on NMY-1 showed that it was redundant with NMY-2 during the process of elongation and therefore, it is expected to be redundant with NMY-2 during oogenesis because loss of

either protein results in fertility defects. Looking at several processes revealed a number of different conserved cassettes of regulatory proteins that regulate nonmuscle myosins and many of these cassettes are also expressed in the gonad. However, in order to study nonmuscle myosins in the gonad it is important to understand all of the steps that are required to create a successful oocyte. It is still unclear which steps of oogenesis require nonmuscle myosins. Once those steps are understood, regulatory proteins that are important during other nonmuscle myosin dependent processes can be studied for their potential role during oogenesis. The major signaling pathways (Notch, Ras/MAPK and MSP) that regulate entire steps of oogenesis can then be used to identify additional factors that transfer this upstream signal to the effector myosin proteins.

In order to address which steps of oogenesis require nonmuscle myosins, gonads lacking each of the nonmuscle myosins were analyzed in order to determine at which step during oogenesis defects were first seen and to identify what the spectrum of defects induced. The next step was looking at the localization pattern of the nonmuscle myosins to determine which region in the gonad they localize in order to help determine their role, because as effector proteins their function has to be local. And finally, looking at loss of each nonmuscle myosin when upstream factors were perturbed gave an indication of which upstream factors was necessary for myosin's function. By analyzing the results of these experiments I ascertained whether the two nonmuscle myosins were redundant during the process of oogenesis, which steps in oogenesis required each of the nonmuscle myosins and whether the same upstream signals act on each myosin. I was able to show that NMY-2 is required for the formation of the initial architecture in the proximal arm of the gonad and is also needed for closure of the ring channels. I also showed that NMY-1 is required for cytoplasmic streaming along with NMY-2 but that defects caused by the loss of NMY-1 are most likely defects in ovulation and that NMY-1 requires the MSP signal for its function.

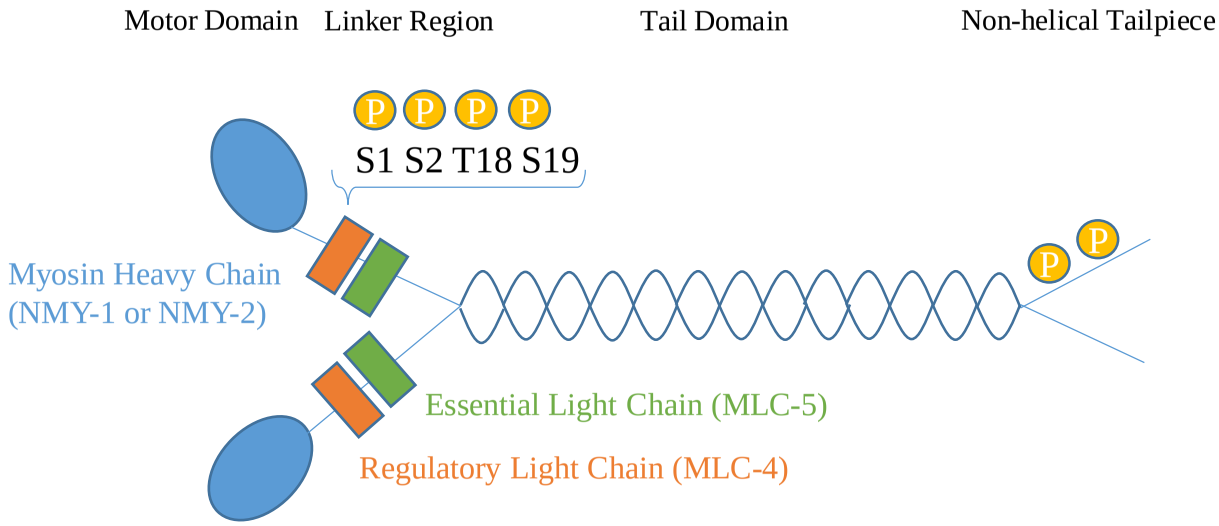


Figure 1-1. **Diagram of type II myosin protein hexamer.** Type II myosins are made up of six different protein subunits: two heavy chains (blue), two essential light chains (green) and two regulatory light chains (red). The heavy chain contains a motor domain that uses the hydrolysis of ATP to bind and move actin, a linker region where the light chains bind, a tail domain that allows the heavy chains to dimerize and allows multiple myosins to form filaments, and a non-helical tailpiece believed to be involved in regulation. *C. elegans* protein names of the nonmuscle myosins are listed after the name of each protein chain in the complex. Known sites of *in vivo* phosphorylation on nonmuscle myosin are indicated by the encircled P's and the numbers below the P's indicate the amino acid position in the mammalian nonmuscle myosin homologue where phosphorylation occurs. S stands for serine and T stands for threonine.

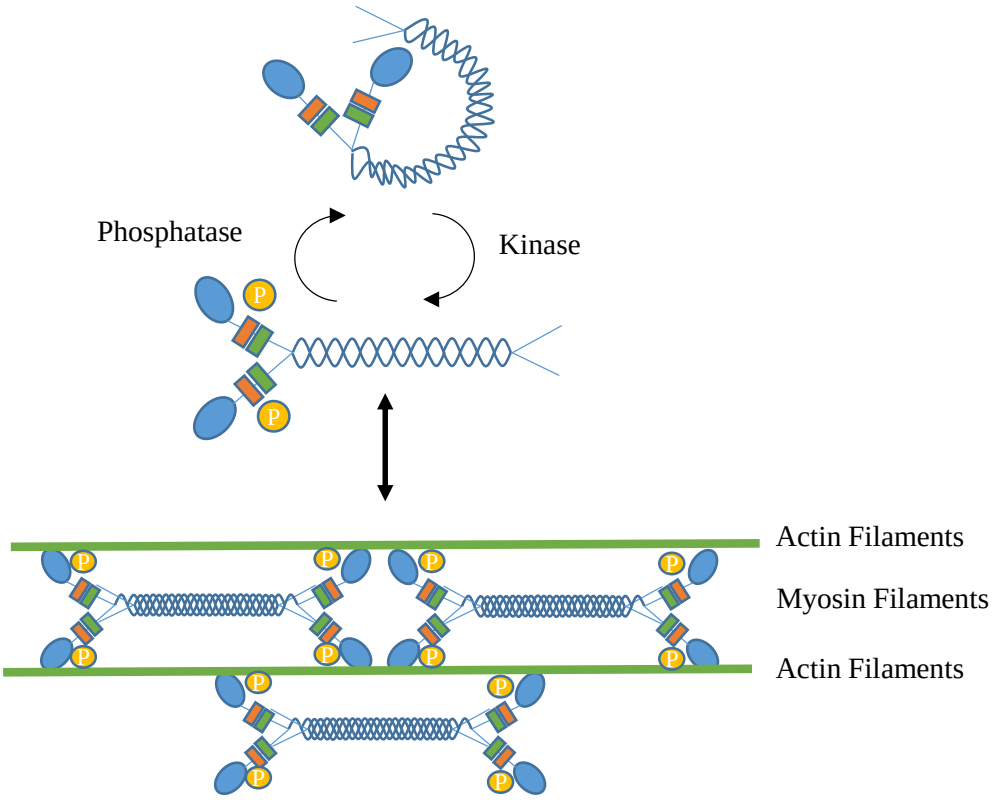


Figure 1-2. **Effect of phosphorylation on nonmuscle myosin.** Unphosphorylated myosin is proposed to remain in an inactive conformation where the head domain binds to the tail region. Phosphorylation of serine 19 on the regulatory light chain causes myosin to switch to the active conformation where it is then able to form filaments with other myosins and interact with actin. Colour scheme is the same as in Fig. 1. Actin filaments shown as green lines.

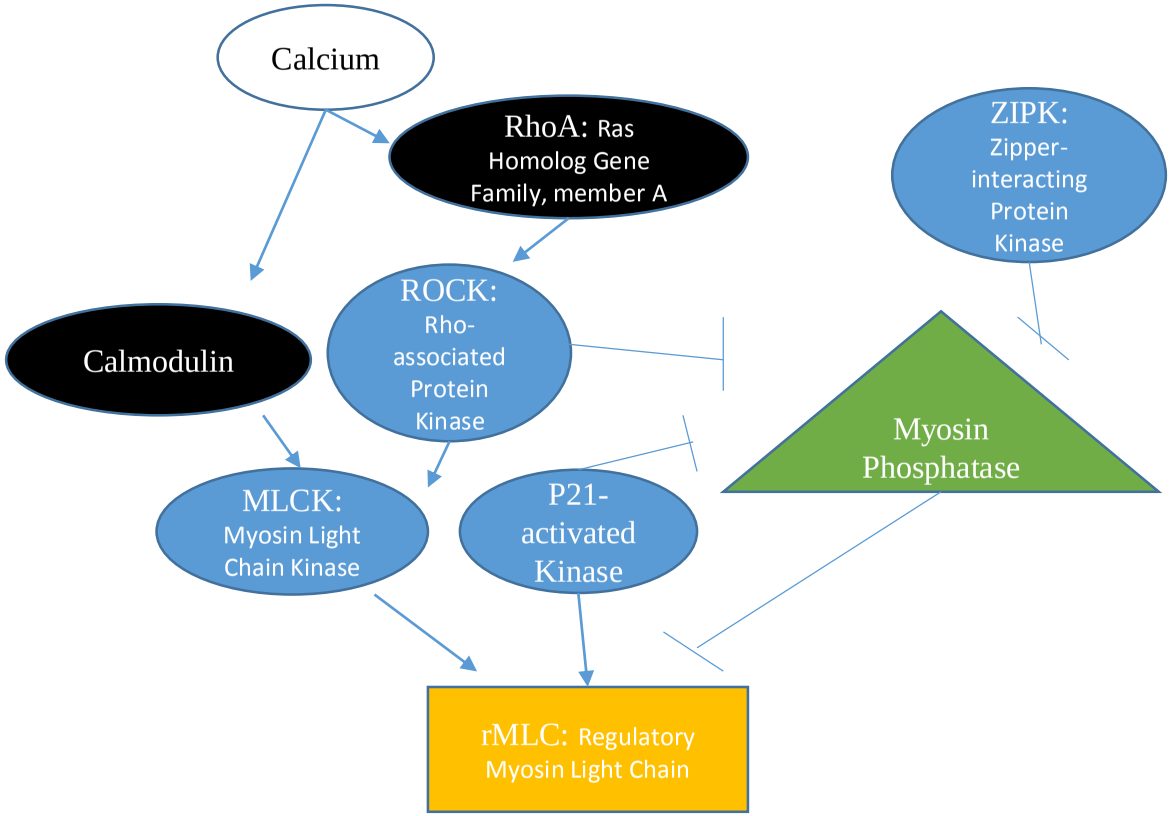


Figure 1-3. **Phosphorylation regulators of the rMLC of nonmuscle myosins.** Myosin kinases and their regulators in mammalian systems are shown. Most of the interactions are known either from studies on cell motility or cytokinesis. Arrows indicate promotion of activity and blunt ended lines indicate inhibition of activity. Blue circles are kinases. Green triangle is a phosphatase. Black circles are modulators. White is an inorganic molecule. Yellow is the rMLC. See text for details.

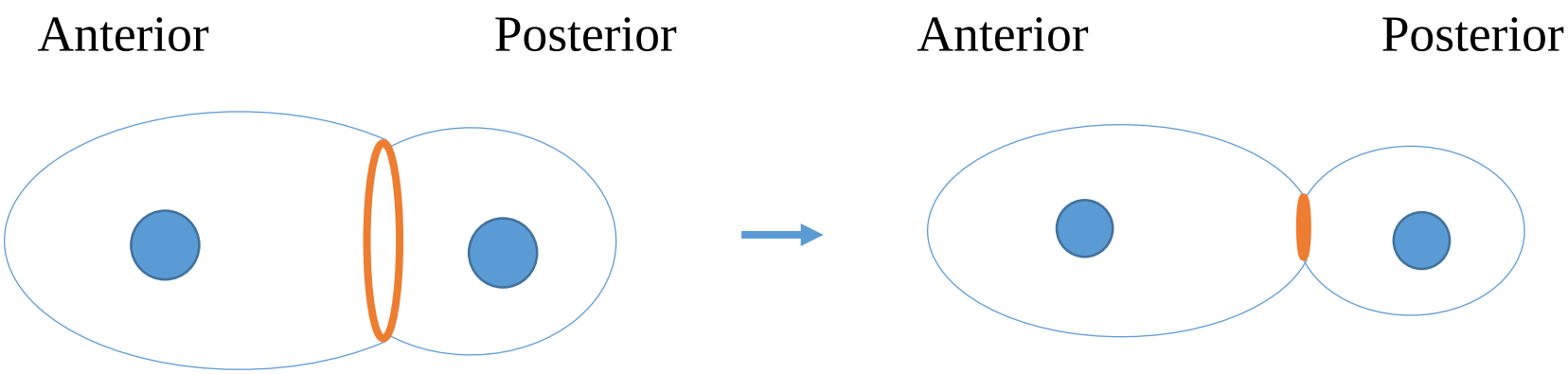


Figure 1-4. **NMY-2 localizes to the contractile ring during cytokinesis.** Diagram represents the first-cell division of a *C. elegans* embryo. NMY-2 is in orange and localizes to the contractile ring between the dividing nuclei. It then contracts pulling the membrane with it until the two cells are separated

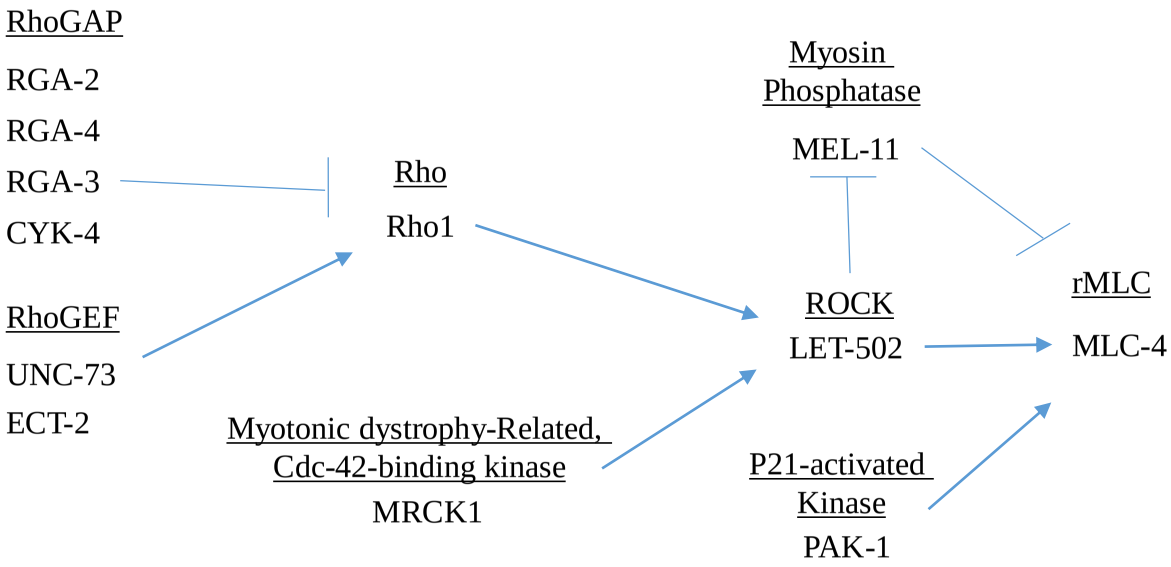


Figure 1-5. **Pathways that directly act on rMLC in *C. elegans*.** Kinases and their regulators that act directly to phosphorylate or dephosphorylate the regulatory light chain of nonmuscle myosins. The class of proteins that each regulator falls into is underlined and their corresponding *C. elegans* protein names are listed underneath. Arrows indicate positive regulation. Bars indicate negative regulation.

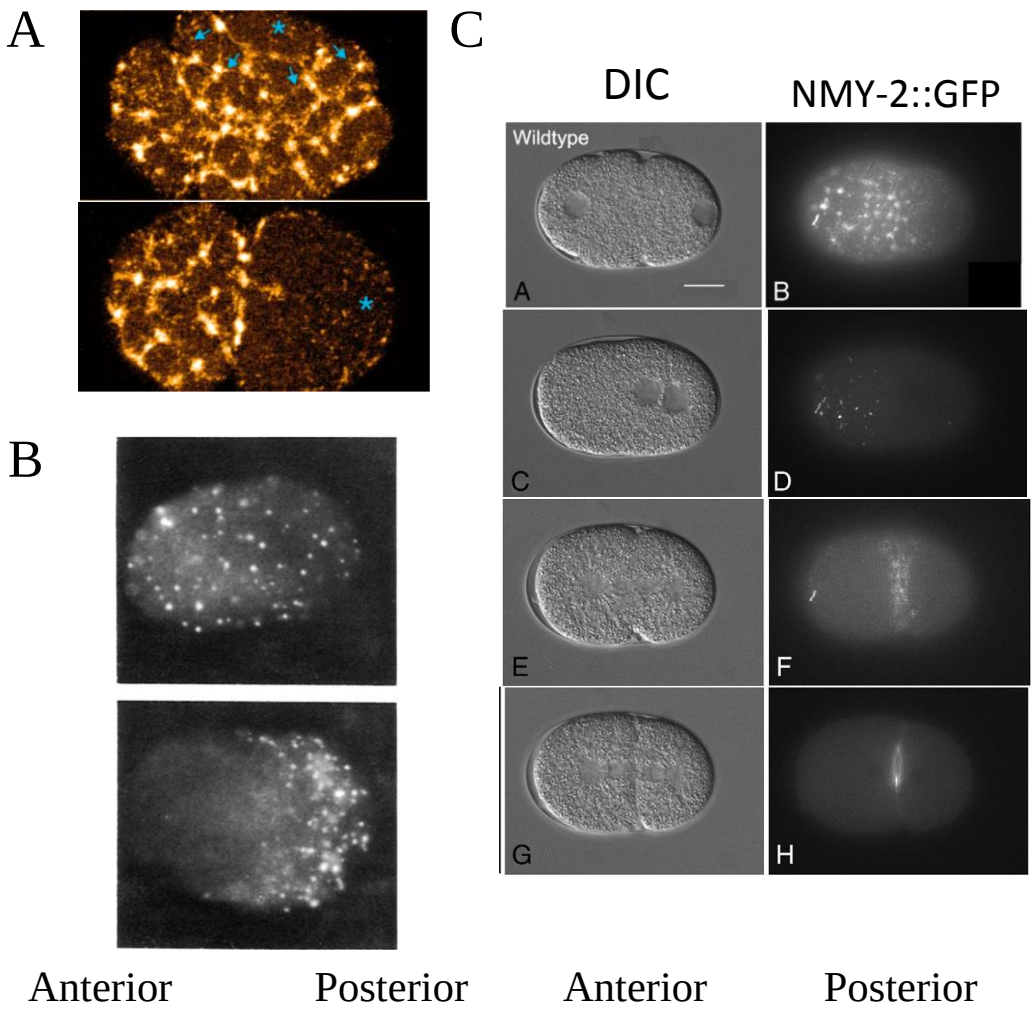

A
B
C
DIC
NMY-2::GFP
Wildtype
A
B
C
D
E
F
G
H
Anterior
Posterior
Anterior
Posterior

Figure 1-6 **Movement of cell components during polarization of the single-cell embryo.** Panel A shows an image adapted from (Munro *et al.*, 2004) of the NMY-2::GFP translational reporter protein forming a meshwork that moves away from the sperm entry point (asterisk) in the direction of the blue arrows. The top image is during cortical flow and the bottom image is after polarization. Panel B, adapted from (Strome and Wood, 1983), shows fluorescently labeled P-granules, one of the PAR proteins. The top image is prior to polarization and the bottom image is after polarization. P-granules initially localize throughout the cell, and after polarization P-granules localize solely to the posterior side of the cell. Panel C shows a figure adapted from (Kachur and Pilgrim, 2008). (A,C,E,G) are DIC images. (B,D,F,H) are fluorescent images showing the location of a NMY-2::GFP reporter protein. (A,B) shows NMY-2 upon entry of the sperm. NMY-2 begins to retreat from the posterior pole. (C,D) shows the end of polarization NMY-2 is in the anterior section of the cell. (E,F) NMY-2 migrates to the contractile ring for the start of cytokinesis. (G,H) during cytokinesis; NMY-2 as part of the contractile ring is closing.

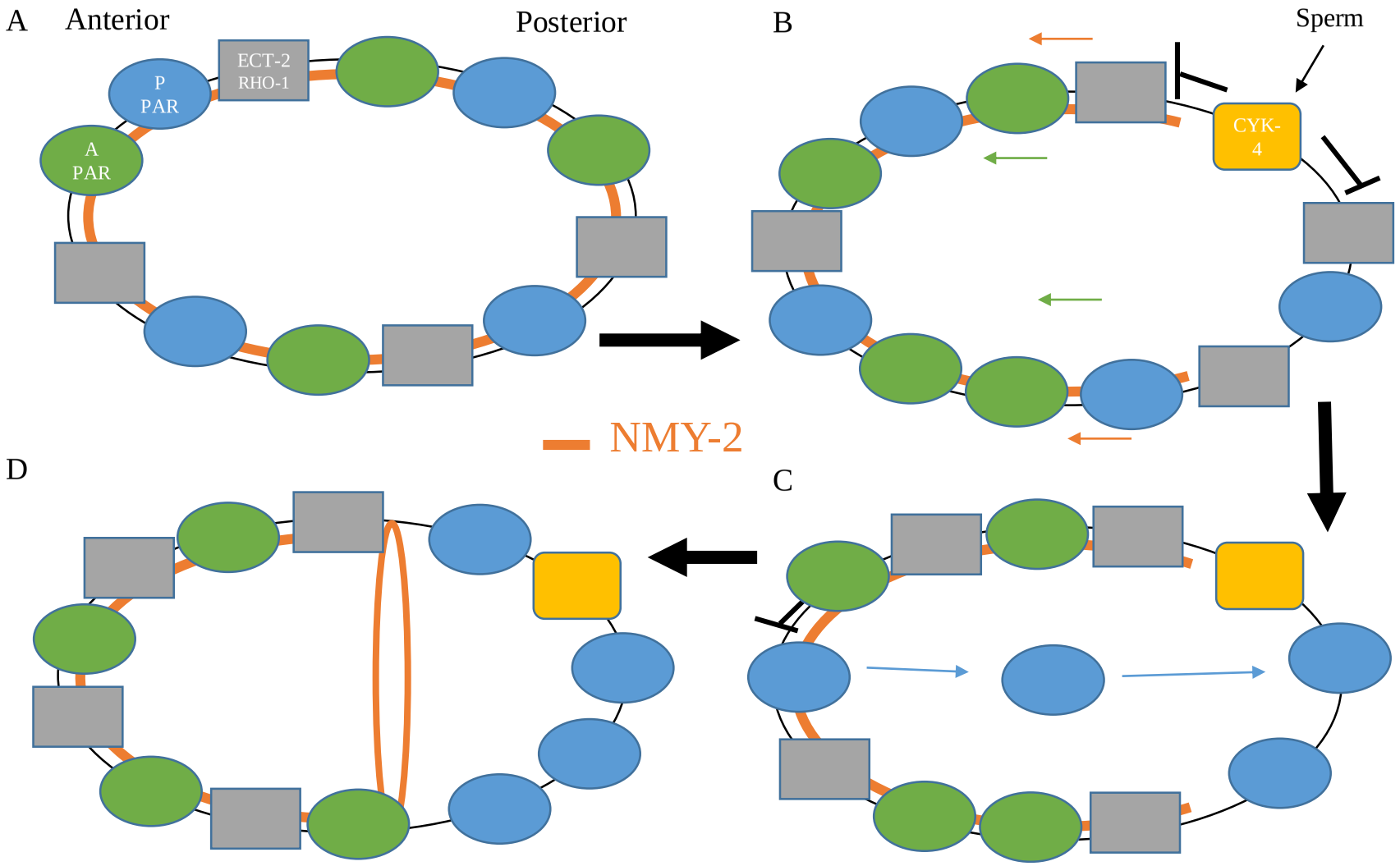

A
Anterior
Posterior
P
PAR
A
PAR
ECT-2
RHO-1
B
Sperm
CYK-
4
NMY-2
C
D

Figure 1-7. **NMY-2 is required for polarization of the single cell embryo in *C. elegans.* A**. NMY-2 initially forms a matrix on the cortex of the cell and all components are equally distributed throughout the cell cortex. **B**. The sperm enters on the posterior side and deposits the RhoGAP, CYK-4 (yellow), which inhibits (blunted line) ECT-2 and Rho-A (Gray). Without activation from the Rho, the actomyosin network (orange) collapses and moves towards the anterior pole bring the anterior specifying PARs (A PAR)(Green) with it. **C.** The A PAR proteins inhibit the posterior specifying proteins (P PAR)(Blue) moving them into the cytoplasm where they diffuse towards the posterior pole. **D.** The cell is polarized with separate factors at each end. This allows cytokinesis to divide the cell with separate PAR proteins in each daughter cell.

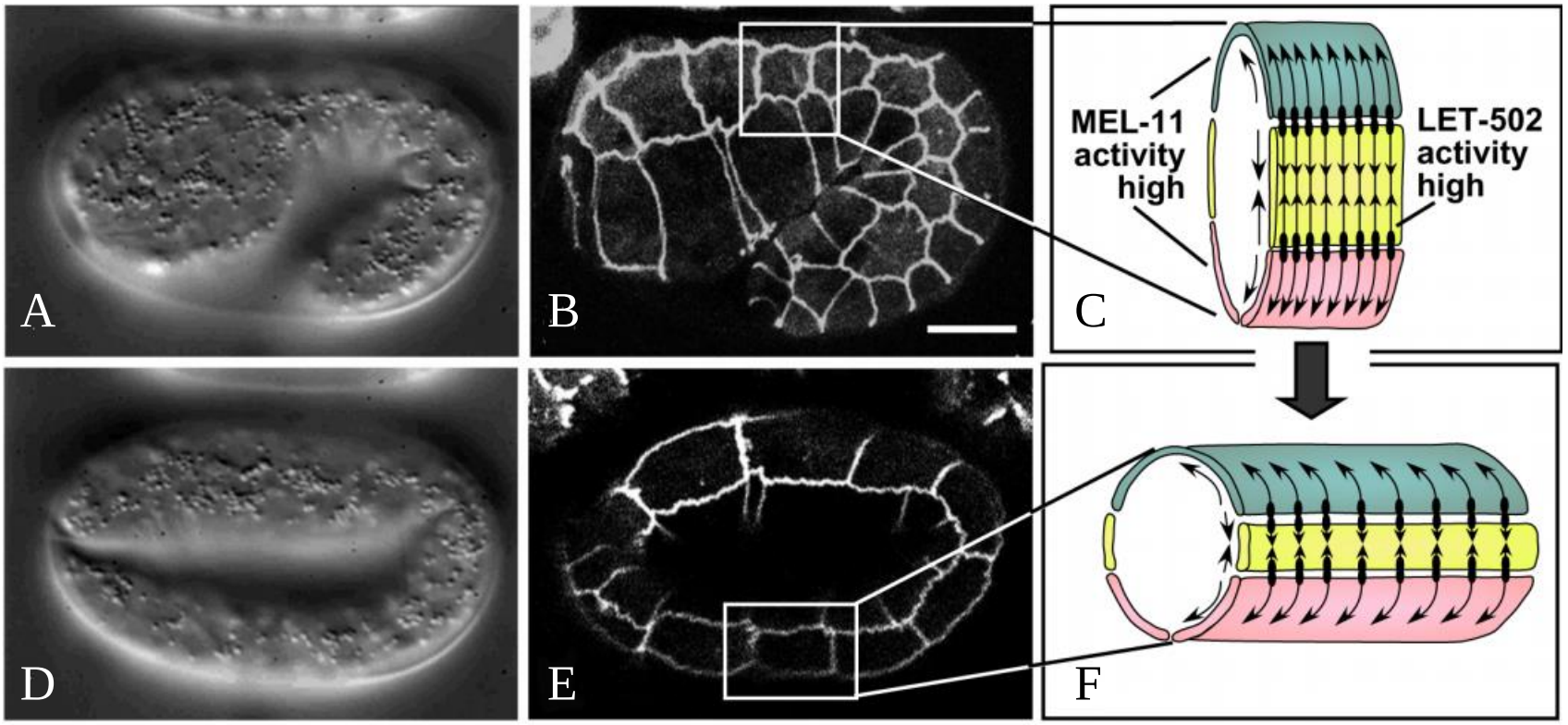


Figure 1-8. **MEL-11 and LET-502 work in concert during elongation of the embryo.** (Adapted from Chisholm and Hardin, 2005)(A,D) are DIC images. (B,E) are fluorescence images from the same embryos expressing a fluorescent protein (DLG-1:GFP) showing the outlines of the cell. (C,F) are schematics of the trunk region indicated by the boxes. Ventral is on the bottom and dorsal on the top. MEL-11 activity, dephosphorylating rMLC, is high in the cells on the top and bottom allowing them to expand. LET-502 activity, phosphorylating rMLC, is high in the middle cells causing them to contract. Scale bar = 5 μm.

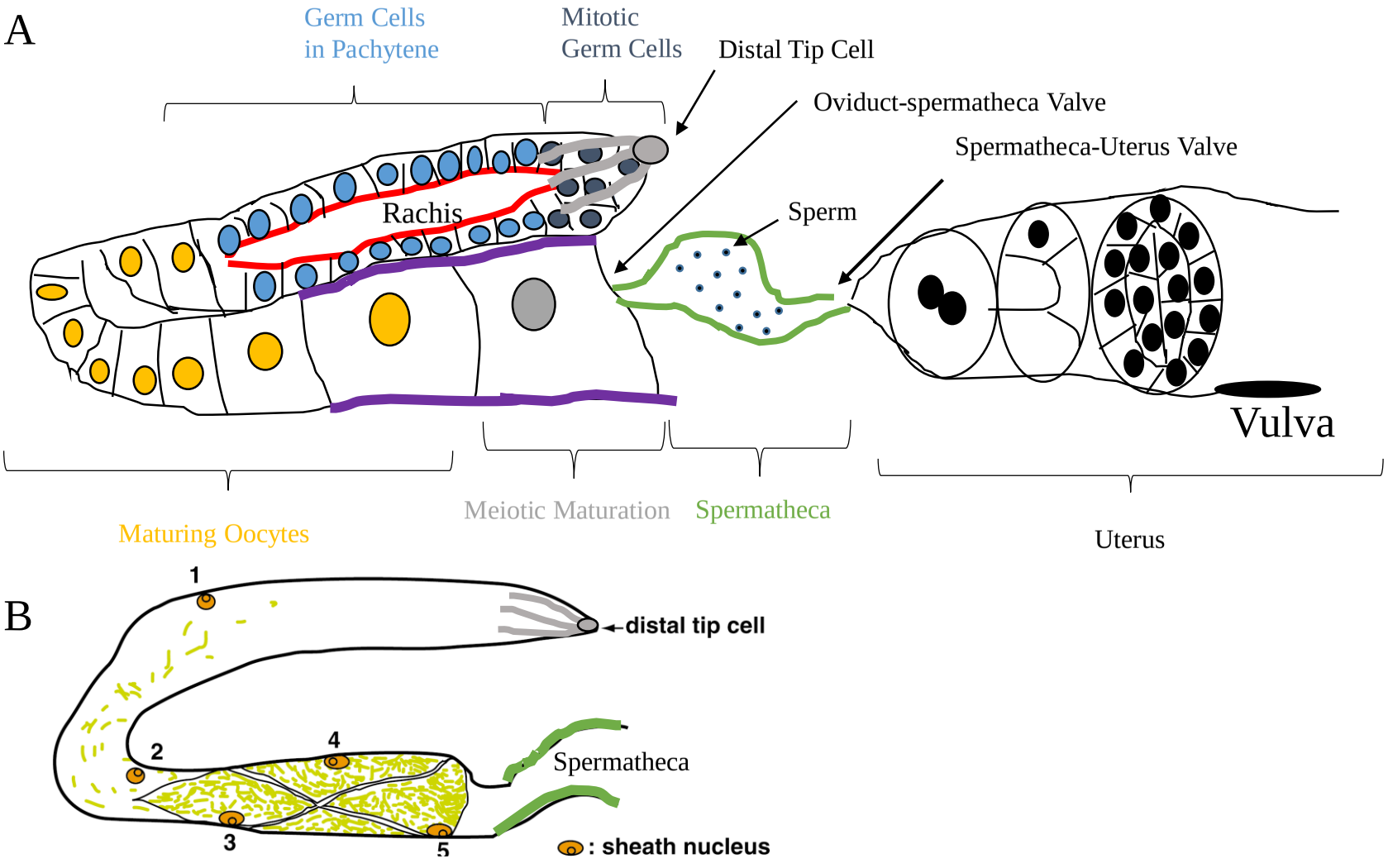

A
Germ Cells
in Pachytene
Mitotic
Germ Cells
Distal Tip Cell
Oviduct-spermatheca Valve
Spermatheca-Uterus Valve
Rachis
Sperm
Vulva
Maturing Oocytes
Meiotic Maturation
Spermatheca
Uterus
B
1
distal tip cell
2
4
Spermatheca
3
5
: sheath nucleus

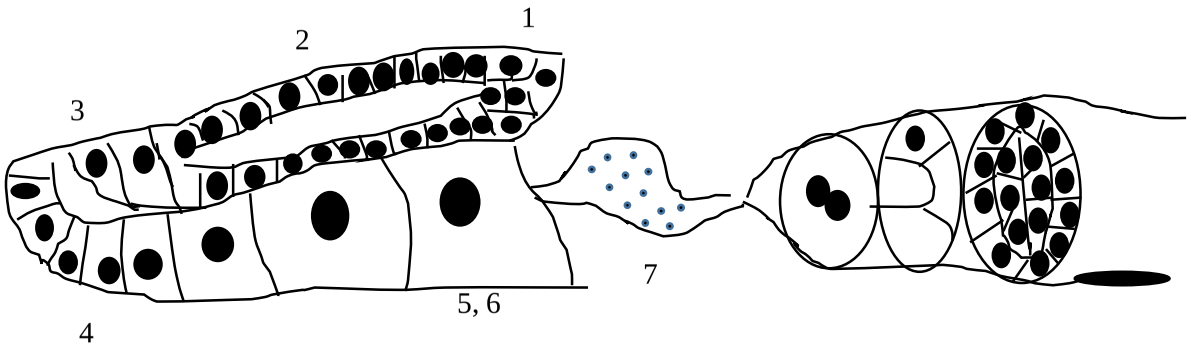
Figure 1-9. **Diagram of *C. elegans* gonad. A**. The *C. elegans* hermaphrodite gonad. Only one of the two syncytial arms is shown. Germ cells begin as mitotic germ nuclei (dark blue) surrounded by projections from the distal tip cell (grey) and then move in a conveyor-like fashion towards the vulva. When nuclei have moved away from the distal tip cell they enter meiosis and arrest in pachytene (light blue) where they are attached through ring channels (red) to the shared cytoplasm, the rachis. As they move around the loop, they exit pachytene and half the nuclei undergo apoptosis while the other half (yellow) begin to take up cytoplasm from the rachis. When they have reached the correct size they pinch off from the syncytium and become fully cellularized. Right before ovulation the oocyte closest to the spermatheca undergoes meiotic maturation (loss of the nucleolus and migration of the nucleus towards the distal end of the oocyte, followed by breakdown of the nuclear envelope) (grey). During ovulation, sheath cells (purple) surrounding the gonad contract, squeezing the oocyte into the spermatheca (green), where it is fertilized before it is expelled into the uterus (black). The embryo will undergo the first few cell divisions before being laid through the vulva. **B.** Somatic cells of the gonad, adapted from Rose *et al.,* 1997. Shows one of each pair of sheath nuclei. Sheath cells (3-5) are contractile while sheath cells (1-2) are not. Yellow color represents presence of myosin heavy chain (*myo-3* and *unc-54* gene product) Grey indicates projections of distal tip cell. Image is in same orientation as in A.

2. Pachytene
- Requires Actomyosin in the ring channels
- NMY-2, MLC-4, ANI-2, LET-502 and MEL-11 colocalize to the ring channels

1. Stem Cell Mitosis
- Requires signaling from DTC
- GLP-1 (Notch signaling) promotes

7. Ovulation
- Requires MAPK signaling
- LET-502 and MEL-11 localize to separate areas of the spermatheca

3. Exit from Pachytene
- Requires MAPK signaling and MSP signaling
- Half of cells undergo apoptosis



6. Sheath Cell Contraction
- Requires MSP signal

4. Cytoplasmic Streaming
- Requires MSP signal
- Requires actomyosin
- GLP-1 affects speed of streaming

5. Meiotic Maturation
- Requires MAPK Signaling and MSP signal

Figure 1-10. **Steps of oogenesis.** The steps involved in *C. elegans* oogenesis and the key requirement for each step. Steps are numbered in the order they occur and each number matches to a location in the gonad shown in the diagram.

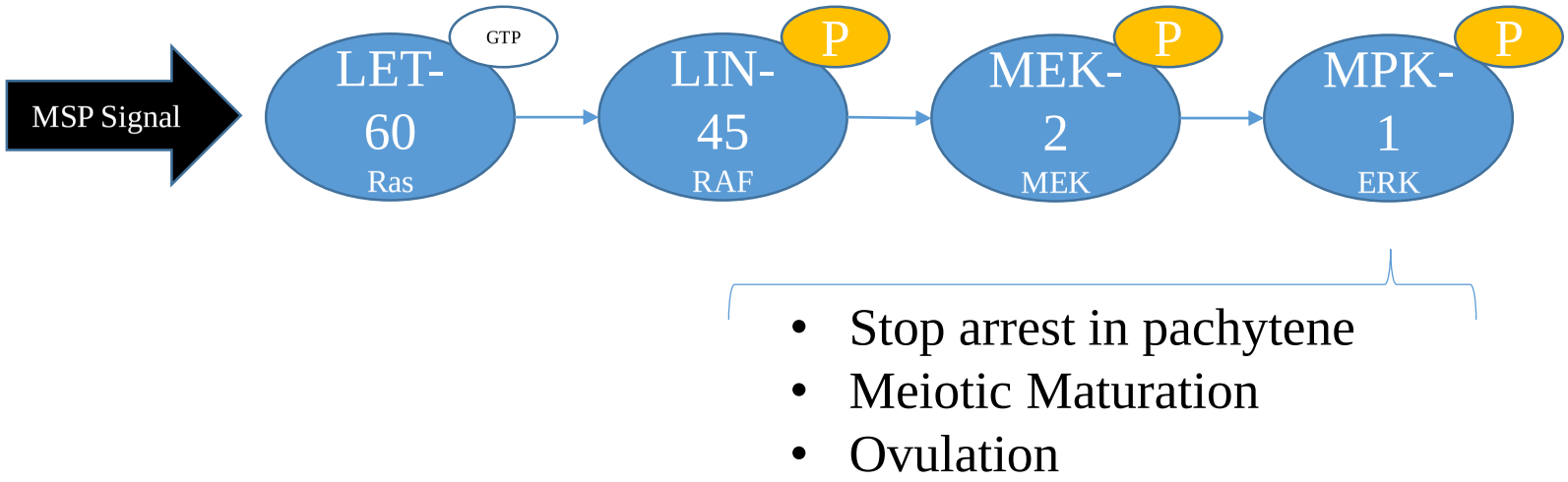


Figure 1-11. **MAP kinase pathway in the *C. elegans* gonad**. The *C. elegans* specific proteins involved in the MAPK signal pathway which regulates steps during oogenesis. The pathway requires the MSP signal (black) and is mostly a series of kinases (blue). The protein name is in large font and the protein family is shown in a smaller font. Non-covalent GTP indicated in white. Covalently bound phosphate in yellow.

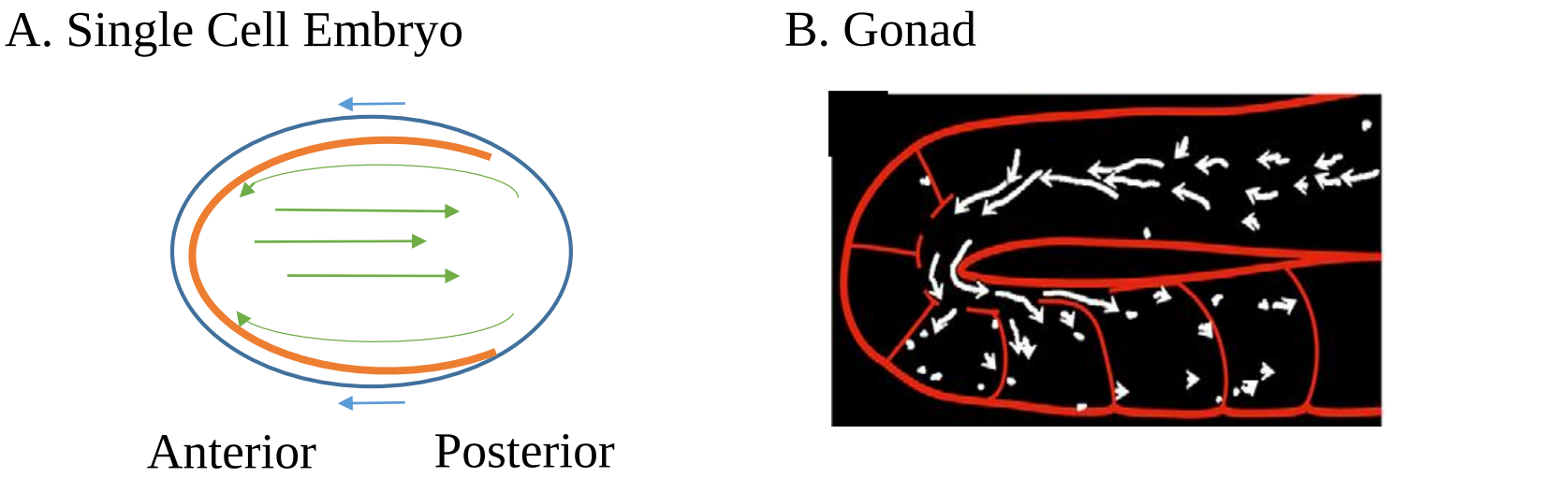


Figure 1-12 **Two examples of cytoplasmic streaming in *C. elegans* that require an actomyosin network**. **A** . The diagram shows the movement of cytoplasm (green arrows) that accompanies the movement (blue arrows) of the actomyosin meshwork (orange). The actomyosin network causes the movement of cytoplasm so that proteins close to the cortex move towards the anterior and other proteins diffuse towards the posterior end of the embryo leading to polarization of the embryo (adapted from Goehring and Grill, 2013). **B** shows cytoplasmic streaming in the gonad. Cytoplasm flows towards the proximal area of the gonad from the pachytene cells into the growing oocytes. This process also requires an actomyosin network in the growing oocyte. The arrows represent individual particles in the gonad followed under DIC optics for two minutes (adapted from Wolke *et al.*, 2007)

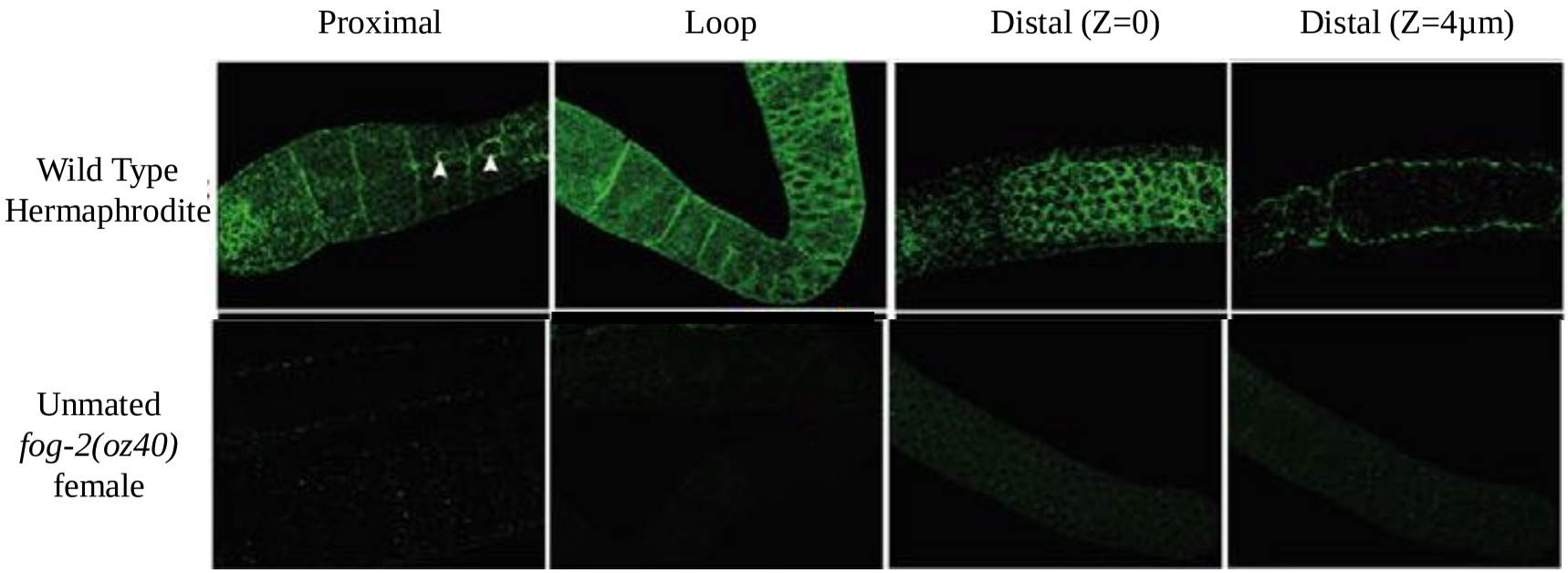


Figure 1-13. **MLC-4 is not phosphorylated in the absence of the MSP signal**. (edited from Nadarajan *et al.*, 2009) Antibody staining using an antibody against the phosphorylated form of MLC-4 on extruded gonads. The top panels show the localization of phosphorylated MLC-4 in wild type hermaphrodites and the bottom panels show its localization in females that do not have sperm, do not receive the MSP signal, and so do not show phosphorylation of the regulatory light chain. From left to right shows proximal to distal locations in the gonad to show that the effect is throughout the gonad.

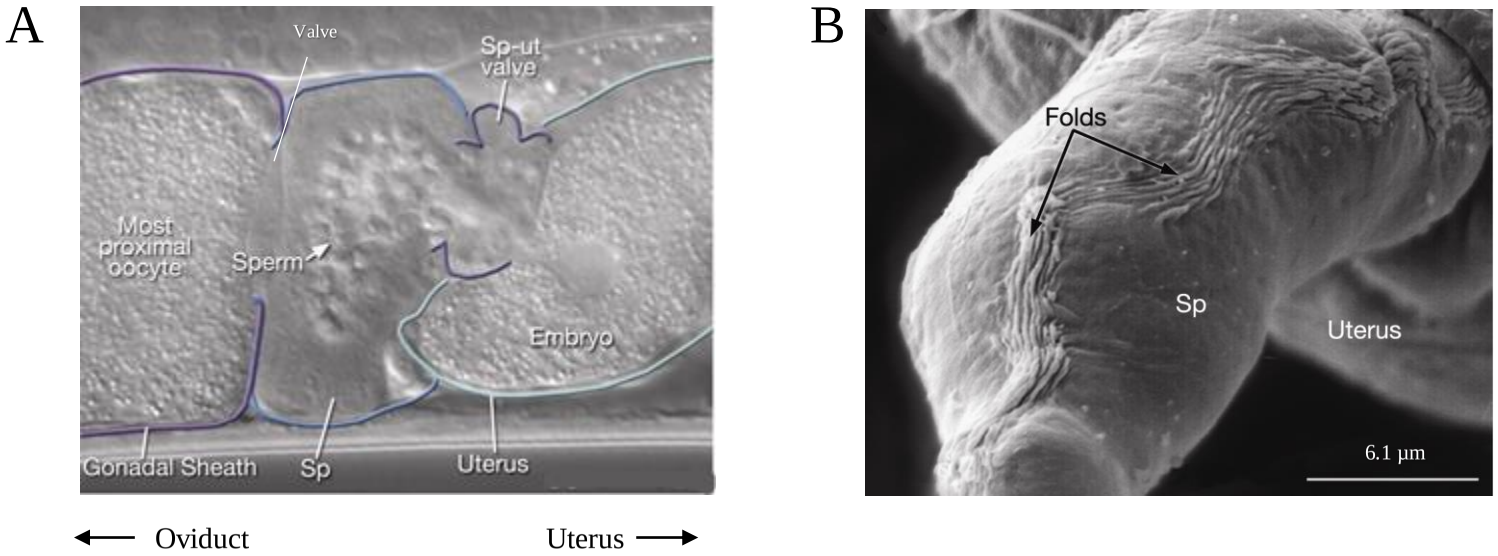


Figure 1-14. **Structure of the spermatheca.** (edited from Lints and Hall 2009) **A** DIC optical image of the spermatheca and surrounding tissues. Both valves are indicated. Sp is spermatheca. Gonadal sheath refers to sheath cells. Sp-ut valve is the valve between the spermatheca and uterus. **B** Scanning electron micrograph of a spermatheca from a dissected gonad. Sp is spermatheca. Folds exist when the spermatheca is empty and allows it to expand when it contains an oocyte.

CHAPTER TWO: MATERIALS AND METHODS

2-1 Strains and Genetics

All *C. elegans* strains were maintained as described (Brenner 1974) and were kept at room temperature except temperature-sensitive mutants were maintained at 16°C. Strains were fed the *Escherichia coli*, OP50. The wild type strain, N2, and the strain CB3844, containing the allele *fem-3(e2006ts)*, were obtained from Medical Research Council Laboratory of Molecular Biology, Cambridge, UK. The membrane::GFP;Histone::MCherry strain, OD95, was obtained from the Caenorhabditis Genetics Center. OC488 was made in the O'Connell lab (NIH, Bethesda) and a kind gift from M. Srayko. The NMY-2::GFP stain, JJ1473, was generously provided by E. Munro (Munro, Nance and Priess 2004). The membrane::MCherry strain, OD70, was created as described (Kachur, Audhya and Pilgrim 2008). NMY-2::GFP;membrane::MCherry strain, DP440, was created by crossing OD70 and JJ1473 together and selecting each generation for worms that carried both transgenes until all worms from each generation had both transgenes. *fem-3* mutant worms carrying the NMY-2::GFP reporter were created by crossing CB3844 to JJ1473 and then back crossing to CB3844 to create homozygous *fem-3* mutants that were heterozygous for the NMY-2::GFP reporter; ten worms from three separate crosses were analyzed.

2-2 RNA-mediated Interference (RNAi)

RNAi was done using the feeding method as previously described (Ahringer 2006). Feeding vectors for L4440 (empty control vector) and NMY-1 were taken from an RNAi feeding library (Kamath and Ahringer 2003). Fragments from the NMY-2 and MLC-4 genes were digested and ligated into an empty L4440 vector. The fragment for NMY-2 starts at bp2489 and ends at bp3570 of the transcript F2OG4.3 unspliced + UTR (www.wormbase.org). This places the fragment in the middle of the 6th exon. The fragment for MLC-4 is a clone of the entire coding sequence of MLC-4.

RNAi feeding vectors were transformed into the *Escherichia coli* strain HT115 and grown overnight in liquid culture. The liquid cultures were concentrated and

then seeded onto RNAi feeding plates (plates containing 25 μg/ml Carbenicillin and 1 mM IPTG). The following day synchronized L1s were placed on the RNAi plates and kept at 25 °C for >48 hours, except those used for cytoplasmic streaming were kept at room temperature or 20 °C.

2-3 Immunofluorescence

Worm gonads were dissected from animals anesthetized with 20 mM sodium azide in M9 buffer, covering the worms with a coverslip and then flash freezing them in liquid nitrogen. Coverslips were then removed and the slides were placed sequentially in -20 °C methanol for 10 minutes, -20 °C acetone for 20 minutes, -20 °C 90% ethanol for 10 minutes, -20 °C 60% ethanol for 10 minutes, and room temperature 30% ethanol for 10 minutes. Slides were then placed in blocking solution (PBT (1X phosphate buffered saline + 0.1% Tween-20) + 5% donkey serum) for >1 hour. Slides were incubated overnight in a humid chamber with the primary antibody in PBT + 20% donkey serum. The following day, slides were washed 3X with PBT and then incubated in the humid chamber for 1 hour with the secondary antibody in PBT. Slides were then washed 4X in PBT with the addition of 1 μg/ml DAPI in the second last wash. Antibleaching solution was added to each slide and they were then sealed and placed at -20 °C until they were imaged. Rabbit anti-NMY-1 polyclonal antibodies were used at 1:200 (Gift from Alison Piekny (Piekny, et al. 2003)) and anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes) was used at 1:100.

2-4 Cytoplasmic Streaming

This protocol was adapted from (Wolke, Jezuit and Priess 2007). RNAi treated worms, 60 to 72 hours after L1s were placed on RNAi plates, were placed in 1 mM levamisole for 5 minutes and then transferred to a drop of M9 on a 4% agarose pad on a slide. The slide was then covered with a cover slip and sealed using petroleum jelly. Images were taken every 15 seconds over a 10 minute time period using differential interference contrast microscopy (DIC). RNAi of NMY-1, NMY-2 and MLC-4 was performed on N2 (wild type) and for each slide N2

worms were compared to OC488 worms that contain a fluorescent marker for identification and had been treated with L4440 RNAi (negative control). This control was used to confirm that the loss of cytoplasmic streaming was due to the RNAi and not to the handling of the slide.

Particles were then tracked using the manual tracker plugin for ImageJ. Particles were tracked over the entire 10 minutes and the average speed of each particle was determined using Microsoft Excel. Particles before the loop, in the loop and after the loop were tracked.

2-5 Microscopy

All images were taken using a Nikon eclipse 80i and a plan apo 60X lens. Fluorescent images were taken with a confocal C2 Si camera. DIC images were taken using a Nikon DS-Qi1Mc camera. All images were edited using ImageJ or Adobe Photoshop.

Strains Used	Genotypes	Phenotypes
CB3844	*fem-3(e2006ts)*	At permissive temperature of 15°C: wildtype. At restrictive temperature of 25°C: XX animals are females, XO are sterile males
DP440	*unc-119(ed3); zuls45; Itls44*	GFP tagged NMY-2 and MCherry tagged membranes
JJ1473	*unc-119(ed3) ; zuls45*	GFP tagged NMY-2
N2	Wildtype	Wildtype
OC488	*unc-119(ed3); bsls20*	MCherry tagged tubulin
OD70	*unc-119(ed3) ; Itls44*	MCherry tagged membrane
OD95	*unc-119(ed3) ; Itls37 ; Itls38*	GFP tagged membrane and MCherry tagged histones

Table 2-1: A list of strains used in this thesis. *fem-3* is a temperature sensitive mutation; at the restrictive temperature XX animals are female. DP440, JJ1473, OC488, OD70, OD95 are strains carrying transgenic reporters. N2 is the wildtype strain. *zuls45 = Pnmy-2::NMY-2::GFP; unc-119(+). ltls44 = Ppie1::PHdomain::mCherry; unc-119(+). bsls20 = unc-119 (+); Ptbb-1::mCherry::tbb-2::tbb-2 UTR. ltls37 = Ppie-1::MCherry::his-58; unc-119(+). ltls38 = Ppie-1::GFP::PH(PLC1delta1); unc-119(+).*

CHAPTER THREE: RESULTS

The question I set out to address was: what steps in the generation of oocytes in *C. elegans* require the activity of each of the two nonmuscle myosin heavy chains? Previous work has shown that NMY-2 localizes to ring channels, connecting the growing oocytes to the common cytoplasm of the rachis. This is consistent with the role NMY-2 has during cytokinesis, in assembling and maintaining the ring channel (Maddox, et al. 2005). The loss of NMY-2 results in a lack of cellularization in the gonad, and NMY-2 is, therefore, a critical component for the formation of the juvenile oocytes. NMY-1 is also important for fertility (Piekny, et al. 2003). NMY-1 was shown to be redundant with NMY-2 during embryo elongation. Therefore, our hypothesis was that NMY-1 plays a redundant role with NMY-2 during oogenesis. If that hypothesis was correct, I would predict that if I genetically knockdown either of the two myosins, I should see either no phenotype or a partial disruption that is worsened by loss of the second myosin. I can also examine the region of the gonad that is affected by loss of either myosin, in order to determine which step or steps of oogenesis require nonmuscle myosins. Finally, by perturbing the main signaling pathways that govern the steps of oogenesis, I can identify which upstream pathway controls the activity of the nonmuscle myosin.

3-1 NMY-1 and NMY-2 are Not Redundant during Oogenesis.

To first determine whether NMY-1 and NMY-2 played redundant roles during oogenesis, I used gene knockdown by feeding bacteria, expressing double-stranded RNA (RNAi) against each of the nonmuscle myosins, to juvenile hermaphrodites that express fluorescent markers for the nuclei and membranes (Figure 3-1). There are three separate methods for inducing RNAi in *C. elegans*: injection, soaking and feeding. Injection of double-stranded RNA directly into the gonad, while efficient, can cause physical damage. My intent was to observe the effects of gene knockdown on the structure of the gonad itself. Therefore, using a method that may damage the gonad when fluid is injected by a needle through both the cuticle and gonad membrane would confuse the results. The feeding method was preferential over the soaking method to avoid preparing large

quantities of double-stranded RNA *in vitro*, which would then be used to soak the worms in.

However, there are some experimental aspects to feeding RNAi that may affect the interpretation of any given trial. There are a number of reasons why RNAi by feeding may fail. IPTG, which induces production of the double-stranded RNA in *E. coli*, is an unstable compound in aqueous solution, and the stock may degrade. The transgenic *E.coli* may become contaminated by OP50, the non-transgenic *E. coli* that is normally used as food by *C. elegans*. Since the phenotype in my assays is expected to be produced in the RNAi-treated animals themselves, rather than in their progeny, and worms at different developmental stages can differ in their RNAi sensitivity, slight differences in worm synchronization may affect the degree of knockdown from animal to animal (Ahringer 2006). Finally, different strains of *C. elegans* can also differ in sensitivity to RNAi due to slight differences in their genetic background. For all these reasons, it was important to establish criteria for including or excluding the results from any particular RNAi trial.

RNAi on a plate was considered to have failed if all worms from a plate, where a phenotype was expected, appeared wild type. Later, once distinct RNAi phenotypes had been observed more than three times, RNAi was considered to have failed in an individual worm if it did not produce a phenotype. In the case of *nmy-1*(RNAi) there was a large variance in phenotypes seen. All worms from a single plate would appear similar but phenotypes would vary between plates. This may have been caused by different levels of mRNA reduction due to differences in the quantity of double-stranded RNA administered. This is common for all three methods of RNAi delivery just described. For this reason, RNAi against NMY-1 was performed on more than ten separate occasions to get an understanding of the variation in phenotype.

It was immediately apparent that both *nmy-1*(RNAi) and *nmy-2*(RNAi) produced distinct phenotypes, which could not be explained by defects in the same process.

Figure 3-1A shows a control gonad with a green membrane marker and a red histone marker. The distal gonad has a honeycomb appearance due to the optical section through the network of ring channels. The proximal gonad has a single row of oocytes that move from smallest on the left (distal) to largest on the right (proximal). Figure 3-1B shows an image using DIC optics of a wild type gonad in the same orientation as Figure 3-1A with the depressions indicating the location of the nuclei. *nmy-2*(RNAi) (Figure 3-1C) produces animals that lack the honeycomb phenotype, and instead of distinct small nuclei (in red), the RNAi-treatment results in a "clumping" of DNA into larger accumulations. Figure 3-1D shows a similar phenotype using DIC, the nuclei again appear clumped together and there are no large oocytes in the proximal gonad. This shows that there are defects in oocyte development from the first step of oogenesis and NMY-2 appears to be required for cellularization of the primordial germ cells. In comparison, loss of NMY-1 affects oocytes as they begin to mature, causing a disruption in the proximal gonad arm, but leaving the distal arm unaffected. Figure 3-1E shows that *nmy-1*(RNAi) is capable of producing a wild type phenotype in the distal region of the gonad, though the optical section is now through the centre of the rachis instead of through the ring channels as in Figure 3-1A. However, the proximal gonad of Figure 3-1E shows a number of differences from wild type. Larger oocytes appear before the loop region, which possibly indicates early cellularization. There are also much larger nuclei in the most proximal part of the gonad indicating that there may be a defect with ovulation, producing endomitotic oocytes that are constantly replicating their DNA. Figure 3-1F shows a gonad from an *nmy-1*(RNAi) worm that was imaged using DIC optics, it shows that there are enlarged oocytes before the loop region. Without the nuclei marker, it is difficult to determine if there are endomitotic oocytes. Therefore, it appears that in the gonad these two non-muscle myosins have at least some separate functions. To rigorously test whether they lack redundancy, the phenotype of a double knockdown must be consistent with the sum of the single nonmuscle myosin RNAi knockdowns.

Loss of the two nonmuscle myosins in the same animal cannot be done genetically, due to the lack of suitable mutations. Doing a double gene knockdown using RNAi is problematic given the inconsistency of RNAi by feeding. Instead, I used RNAi against MLC-4, the regulatory myosin light chain, which is a critical component of both nonmuscle myosin complexes. If these two proteins shared redundant roles then there should be additional defects seen when both are knocked-down and cannot compensate for each other. If they are not redundant, then we would expect loss of both to either be a cumulative effect of their RNAi phenotypes or we would expect it to resemble *nmy-2*(RNAi) because its role appears earlier during oogenesis. *mlc-4*(RNAi), Figure 3-1G, shows the same honeycomb green membrane phenotype seen in wild type worms, however that honeycomb appearance continues past the loop. This indicates a potential delay in cellularization. There is also a large clump of membranes and nuclei in the uterus indicating that there may be a defect in ovulation similar to what is seen with loss of NMY-1. Figure 3-1H shows a very similar phenotype to Figure 3-1 G but using DIC optics. In Figure 3-1H there are some enlarged oocytes, but they are found more proximally in the gonad than is seen in wild type. There is also an expansion of the uterus, as oocytes appear to bunch together. This is distinct from what is seen with *nmy-2*(RNAi), and while at first it resembles *nmy-1*(RNAi), instead of early cellularization, there is a delay in cellularization. Without knowing at what steps in oogenesis NMY-1 functions, it is difficult to ascertain if this is a novel phenotype or perhaps a variation on the *nmy-1*(RNAi) phenotype. Therefore, it was important to determine which aspects of gonad development require NMY-1 activity, in order to explain why MLC-4 resembles neither NMY-1 nor NMY-2.

3-2 The Loss of NMY-1 in the Gonad Causes Defects in the Proximal Gonad Arm

By examining strains expressing fluorescent markers for the membrane, the nuclei and the ring channels it was possible to determine which part of the gonad architecture was disrupted following knockdown of NMY-1. Figure 3-2B shows

that the distal arm of the gonad appeared normal in comparison with the control in Figure 3-2A. However, in the proximal arm of the gonad in *nmy-1*(RNAi) (Figure 3-2B) there is no longer a single row of growing oocytes as seen in Figure 3-2A, but instead, oocytes are on top of other oocytes and they are bunched together. There are also tiny, apparently anuclear, cells in Figure 3-2B indicated by the arrow. In some animals there is a change in the shape of the developing oocyte from the square-edged sphere seen in Figure 3-2E to a more tubular shape seen in the distal arm of Figure 3-2F. In Figure 3-2F these large oocytes are seen not only next to the uterus, as they would in wild type, but also as far as the loop of the gonad. Therefore, there may be premature cellularization of immature oocytes, or an inability for the oocytes to travel through the spermatheca, which causes an accumulation of mature oocytes in the oviduct. Figure 3-2F has a GFP-fusion reporter for NMY-2 as a marker for the ring channels, and shows many more cells than wild type attempting to cellularize. This is indicated by the arrow showing varying sizes of ring channels in green. These different-sized ring channels are not seen in control animals (Figure 3-2E) as only three to four oocytes are usually cellularizing at the same time. Therefore, the defects are isolated to the proximal gonad.

The defects seen with *nmy-1*(RNAi) vary from plate to plate, as mentioned earlier, and therefore a range of phenotypes are shown to illustrate all the areas of the gonad where defects can occur. Figure 3-2C shows a wild type uterus with membranes in green and histones in red. It includes a number of embryos at various stages of development. Depending on the age of the mother, the embryos often form more than a single row but they are always obviously distinct from one another. Figure 3-2D shows embryos in an *nmy-1*(RNAi) uterus. This animal is devoid of any developing embryos and instead is full of a mass of membrane and nuclei. The nuclei are larger than normal and the fluorescence intensity is consistent with an increased amount of DNA per cell. There is also a greater fluorescence intensity from the GFP membrane marker. While some of the oocytes are fully cellularized, there are also large masses of oocytes that are only

partially separated from each other. This implies that oocytes are damaged during ovulation. The occasional oocyte that is successfully fertilized and begins embryogenesis, must have sustained minimal damage or managed to repair that damage.

From these observations we can conclude that NMY-1 is not required for the early steps of oogenesis where the progenitor cells remain attached to the rachis and are highly transcriptionally active. Defects are not seen until the point when some of the progenitor nuclei should be undergoing apoptosis while the remaining nuclei begin to mature by taking up large amounts of cytoplasm and cellularizing. Figure 3-2F shows multiple compartments beginning to cellularize, as indicated by the arrow, which means that either NMY-1 is required to delay cellularization or that NMY-1 is required for the appropriate cells to undergo apoptosis. Nonmuscle myosins are structural proteins and are not known to be involved in signaling, therefore it is less likely that they are needed for apoptosis. However, early cellularization does not explain the large nuclei and extra membrane seen in the uterus.

Another possibility is that NMY-1 is required for proper ovulation, which could account for many of the phenotypes. Defects in ovulation usually result in an endomitotic oocyte (Emo) phenotype, meaning that meiotically mature oocytes remain in the oviduct and undergo many rounds of DNA replication leading to large nuclei. If ovulation fails frequently, then there can be many meiotically mature oocytes in the oviduct (Rose, et al. 1997). Defaults in contraction of the spermatheca result in damaged oocytes being extruded into the uterus. Sheath cell defects can break oocytes into two pieces, which causes anucleated blebs to accumulate in the oviduct (Rose, et al. 1997). Therefore, it is possible, that NMY-1 solely functions in the somatic gonad tissue. In order to determine if NMY-1 had a role in the germline, it was important to see where in the gonad NMY-1 protein is located, and whether it forms a network throughout the gonad or co-localizes to a certain structure.

3-3 NMY-1 Localizes as a Network through the Maturing Oocytes

There are two methods for determining the localization of a protein. Reporter transgenes allow *in vivo* imaging, but in the case of NMY-1, the size of the transcript (8482 bases) makes the manipulation of its sequence difficult. I attempted to clone the NMY-1 genomic sequence into a vector using Gateway cloning but was never able to recover a plasmid containing the full gene. The other method is antibody staining, and an NMY-1 antibody was previously described by Alisa Piekny (Piekny, et al. 2003), who generously shared some of it with me. A drawback of antibody staining is that it can only be used on fixed, isolated tissues, which can distort the gonad structure. This antibody also required methanol fixation, preventing co-staining for actin as methanol affects actin filaments. Methanol fixation also denatures most fluorescent reporter proteins meaning that I could not simultaneously examine any of the various reporter markers described earlier. Another problem with antibody staining is that nonspecific staining can be significant and therefore it was important to use a null mutant of NMY-1 as a negative control. Nevertheless, antibody staining was used to ascertain where NMY-1 localized in the wild type gonad. The localization of NMY-1 will help to determine whether NMY-1 plays a role in the germline, and at which step NMY-1 is required. Figure 3-3J, K, L, M show that when NMY-1 is eliminated from the gonad, the NMY-1 antibody (green) does not stain any extraneous structures, as the faint green color seen is due to background fluorescence. All panels were taken using the same laser intensities and gain, making the controls comparable to wild type staining. The negative controls show a strong decrease in green signal compared to wild type, which confirms that the antibody is only staining NMY-1. Samples were also stained with DAPI (blue). Figure 3-3A shows cytoplasmic staining in the distal region of the gonad but it does not localize to a structure. Figure 3-3B shows the merged image with DAPI, small nuclei on either side of the gonad indicates the distal region. Figure 3-3C is an enlarged image of the distil gonad showing that NMY-1 does not localize to a specific region, such as the ring channels like NMY-2. Figure 3-3D, E shows a

matrix-like localization pattern in the enlarged oocytes that are likely still attached to the rachis based on their distance from the loop region. The loop region was determined by looking for the nuclei that are still relatively small, but that are all aligned along only one side of the gonad. This plane of focus shows NMY-1 staining running throughout the oocytes but excluding the nuclei. This means that the matrix spans the entire cell, because if it was only on the cortex it would not be in the same focal plane as the nucleus of the cell. Figure 3-3F is an enlarged image of the network showing distinct filaments and that the network seems continuous from one oocyte to another. Figure 3-3G, H shows a third plane of view where the cortex of the proximal oocytes is now in view, as can be determined by the absence of visible nuclei. Figure 3-3I is an enlarged view of the dividing region between the network appearance and the diffuse appearance seen in oocytes more proximal. Therefore, NMY-1 localizes in a matrix-like pattern in oocytes that are likely still attached to the rachis in the proximal gonad.

The localization of NMY-1 confirms that NMY-1 likely does not play a role in the creation and maintenance of the progenitor cells. It also makes it unlikely that loss of NMY-1 would be delaying cellularization. As NMY-1 is not a signaling molecule, we would expect it to localize to the ring channels in order for it to promote cellularization. However, there is no apparent localization to the ring channels. It is still possible that NMY-1 is required to allow oocytes to pass through the spermatheca. The matrix-like network that NMY-1 forms could be involved in giving rigidity to the oocyte, thereby allowing the sheath cells to move it directionally through the spermatheca. It is also possible that this matrix performs the opposite task, and changes the oocytes shape, in order for it to fit into the spermatheca. Another possibility is mentioned by Wolke *et al.*, 2007 who examined the process of cytoplasmic streaming in the *C. elegans* gonad. They identified that the motor protein responsible for the streaming had to localize to the growing oocytes and that it was a myosin. The localization of NMY-1 suggests it is a good candidate for this motor protein.

3-4 Loss of Nonmuscle Myosin Stops Cytoplasmic Streaming in the Gonad

Wolke *et al.*, 2007 studied cytoplasmic streaming in the *C. elegans* gonad. They found that streaming occurred only when sperm was present in the spermatheca. They also found that particles travelled at different speeds at different places in the gonad. Particles move fastest (7µm/min) in the rachis just before entering the enlarging oocytes. Wolke *et al.,* 2007 determined the average speed of particles over a two minute period and compared mutants to wild type. Two other papers also used this same two minute time frame to calculate an average particle speed. However, most mutants they examined had compromised signaling pathways but the gonad structure remained intact. Some examples of mutants they examined are female animals that lack MSP signaling or loss of CEH-18, a protein required for signaling from sheath cells. Having the gonad structure intact allowed them to measure the particle speed in the same region of the gonad, thereby making animal to animal comparison easier.

Loss of either nonmuscle myosin, especially loss of NMY-2, severely damages the structure of the gonad, and loss of NMY-1 and MLC-4 causes early or delayed cellularization, respectively. Given this disruption, it difficult to find a single region of the gonad that is comparable between mutants. For this reason particles located before, during or after the loop were examined and particles were tracked for ten minute intervals. Average speeds were calculated but the most distinguishing factor between wild type and the RNAi-treated gonads was the directional movement of the particles.

In wild type or control animals, particles in the distal arm move in a non-directional manner but flow in bulk, meaning that each particle moves in all directions but has a net movement towards the loop region of the gonad. At this time the particles accelerate and move along the rachis, now along the side of the gonad, towards the growing oocytes. It was noted that particles that came close to the edge of the tube slowed down in speed until they once again moved into the

centre of the tube and returned to their increased and directional speeds. (Figure 3-4A)

Based on the localization of NMY-1 and the phenotype of NMY-1 knockdown in the gonad I expected that loss of NMY-1 would inhibit cytoplasmic streaming and loss of NMY-2 would have no effect on streaming. However, RNAi knockdown for all three nonmuscle myosin components had similar effects on cytoplasmic streaming. All RNAi knockdowns examined retained the slow and non-directional movement but at no point in time do they enter into a faster directional movement (Figure 3-4B, C, D). Each RNAi-treated worm imaged was immediately followed by imaging of a wild type worm to be certain that handling of the worms had not caused the effect seen on cytoplasmic streaming. Loss of cytoplasmic streaming has been observed when the worm is stressed. Figure 3-4B shows a gonad treated with RNAi against NMY-1 showing non-directional particle movement consistent with bulk fluid flow as opposed to directional streaming. Figure 3-4C shows a gonad that was treated with *nmy-2*(RNAi) demonstrating the characteristic lack of membranes, and it also shows the lack of directional movement. Figure 3-4D shows a gonad from an animal treated with *mlc-4*(RNAi) and it resembles both *nmy-1*(RNAi) and *nmy-2*(RNAi) animals in its lack of directional movement. Average particle speeds are given in Figure 3-4E, and all RNAi-treatment resulted in slower than wild type speeds. The easiest explanation for this slower than wild type speed is that the particles do not have a period of faster streaming, which is characteristic of wild type animals. These are averages of fifty particle's speeds from three separate RNAi-treated nematodes (Figure 3-4). Therefore, both NMY-1 and NMY-2 are required for cytoplasmic streaming.

Unexpectedly, NMY-2 also showed a lack of cytoplasmic streaming. NMY-2 localizes to the ring channels and is required for the distal gonad architecture, therefore it would not be expected that it should affect cytoplasmic streaming. It is possible that a large change in gonad architecture is sufficient to disrupt cytoplasmic streaming. Wolke *et al.*, 2007 showed that with depletion of tubulin in the gonad there are large architectural defects, including nuclei in the rachis of

the distal gonad. However, even with the depletion of tubulin, cytoplasmic streaming still occurs and the nuclei found in the rachis move with this streaming. This shows that it is not the disruption of the architecture itself, which is responsible for the lack of cytoplasmic streaming.

Govindan *et al.*, 2009 confirmed that cytoplasmic streaming requires the MSP signal. Therefore, it was important to determine whether *nmy-1*(RNAi) and *nmy-2*(RNAi) phenotypes remain the same in the absence of the MSP signal. Nadarajan *et al.*, 2009 observed that in the absence of the MSP signal, MLC-4 is not phosphorylated in the gonad. This observation is consistent with the requirement of the MSP signal for cytoplasmic streaming. MLC-4 has to be phosphorylated at serine-18 in order for the myosin to take an active confirmation. Only in an active confirmation is myosin able to form filaments and localize. Therefore, it would be expected that in unmated females that lack sperm and consequently lack the MSP signal, we would not see localization of NMY-2 to the ring channels. If NMY-2 does not localize without the MSP signal then we would expect that loss of NMY-2 should not have an effect on the phenotype of the unmated female gonad.

3-5 NMY-2 is required for Gonad Architecture in the Absence of MSP Signal

To test the hypothesis that in the absence of the MSP signal *nmy-2*(RNAi) will have no phenotype, RNAi against NMY-2 was performed in a *fem-3* mutant background. *Fem* mutants are incapable of producing their own sperm, so unless XX females mate with normal males there will not be an MSP signal coming from the spermatheca. Unmated *fem-3* mutants look similar to hermaphrodites except without sperm they cannot fertilize their oocytes, and therefore do not have embryos. Also, oocytes in the oviduct of *fem-3* mutants accumulate and take on a flattened appearance, looking like a stack of pancakes (Figure 3-5B). The *fem-3* phenotype does not resemble RNAi against the myosin components. MLC-4 is not phosphorylated without the MSP signal. Therefore, it could be hypothesized that in the absence of the MSP signal, nonmuscle myosins are in an inactive state.

If this hypothesis is true, we would expect to see no difference to the *fem-3* mutant phenotype with RNAi against any of the nonmuscle myosin components.

When gonads of either wild type or *fem-3* mutants are treated with RNAi against NMY-2 they both have the same phenotype, a loss of gonad architecture and a pooling of nuclei, as indicated by the arrow. This means that NMY-2 is active regardless of the MSP signal. This also means that NMY-2 is capable of fulfilling its structural function in the gonad without phosphorylation of MLC-4. This is contrary to our current understanding of non-muscle myosin regulation. Therefore, it is important to confirm both that MLC-4 is not phosphorylated at the serine residue without the MSP signal, and that NMY-2 is able to localize in the absence of the MSP signal.

To test whether NMY-2 is able to localize to the ring channels in the absence of the MSP signal, the GFP fusion reporter for NMY-2 was crossed into a *fem-3* mutant background. If *fem-3* mutant XX animals are raised at the restrictive temperature in the absence of males, then no sperm will be present in the adult. Therefore, the adults will not have the MSP signal. Figure 3-5J shows that even with the lack of an MSP signal, NMY-2 is still capable of localizing to the ring channels and the lateral and basal membranes of the maturing oocytes. The background levels of GFP appear to be elevated, but this is because in crossing it to the *fem-3* mutants, there is now only a single copy of the reporter gene as opposed to the homozygous diploid reporter in the original strain. Therefore, NMY-2 does not require the MSP signal for its localization and activity.

3-6 The NMY-1 Phenotype is Dependent on the MSP Signal

Next, we wished to test whether the results seen for *nmy-2*(RNAi) in a *fem-3* mutant also held true for *nmy-1*(RNAi). Figure 3-5G, H show that in a *fem-3* mutant background, *nmy-1*(RNAi) has no apparent effect and resembles the control (Figure 3-5A, B). While wild type gonads in the absence of NMY-1 show early cellularization, *fem-3* mutant gonads in the absence of NMY-1 resemble the *fem-3* mutant phenotype. This means that gonadal phenotypes observed with *nmy-*

1(RNAi) are dependent on MSP. This could occur directly because MSP signaling activates NMY-1 during cytoplasmic streaming. This could also occur indirectly because there is no ovulation without the MSP signal and so no oocytes are damaged during faulty ovulation. With either explanation, the steps of oogenesis that require NMY-1 must also require the MSP signal.

One reason for why some of the *nmy*-1(RNAi) gonads in a *fem-3* mutant background may now resemble wild type gonads as opposed to the *fem-3* mutant phenotype, may be due to the lack of cytoplasmic streaming. Without cytoplasmic streaming, the creation of mature oocytes may slow. This means that in some of *fem-3*(mutant);*nmy-1*(RNAi) worms observed there has yet to be enough mature oocytes to see the squished oocyte phenotype, which is characteristic of *fem-3* mutants. However, we can conclude that the function of NMY-1 occurs only in the presence of the MSP signal, which is markedly different than NMY-2, which functions independently of this signal.

mlc-4(RNAi) in a *fem-3* background resembles neither *nmy-1*(RNAi) nor *nmy-2*(RNAi). Loss of NMY-1 does not affect the phenotype in a *fem-3* mutant background, but loss of NMY-2 does. Therefore, it was expected that *mlc-4*(RNAi) in *a fem-3* mutant background should resemble *nmy-2*(RNAi). Figure 3-5F shows a gonad that resembles the pachytene state throughout the entire gonad. This is interesting because *nmy-2*(RNAi) gonads show a loss of internal membranes, while all the membranes in the *fem-3*(mutant);*mlc-4*(RNAi) gonads were intact. Instead, in the *fem-3*(mutant);*mlc-4*(RNAi) gonads there appeared to be a lack of cellularization and general oocyte maturation. This observation, along with the requirement of NMY-2 for cytoplasmic streaming, showed that our original prediction for the role of NMY-2 was not supported.

3-7 NMY-2 Localizes to the Ring Channels in the Distal Arm of the Gonad

A GFP reporter of NMY-2 was used to localize of NMY-2 and determine if NMY-2 localizes to the areas needed to fulfill its hypothesized roles in both maintenance of ring channels and cytoplasmic streaming. Figure 3-6A – F shows

the NMY-2::GFP reporter with a membrane mCherry marker in a series of different focal planes. NMY-2 is primarily found in the distal arm of the gonad at the ring channels but also localizes to the proximal gonad at the cortex of the enlarging embryos. Therefore, NMY-2 localizes to the ring channels as previously described. NMY-2 also localizes to the basal and lateral membranes of enlarging oocytes until the oocytes becomes fully cellularized, and then we no longer see NMY-2 in the proximal oocytes. Therefore, NMY-2's localization indicates that it has a role in addition to maintaining the ring channels.

NMY-2's localization is consistent with the hypothesis that NMY-2 is required to maintain the membranes surrounding the primordial germ cells. However, because NMY-2 localizes to a lesser degree at the lateral membranes of those cells, it is more likely that NMY-2 is required for maintenance of these cells and particularly maintenance of the ring channels. Furthermore, the shift in localization of NMY-2 to the lateral and basal membranes as germ cells mature, indicates a change of function at the point when oocytes begin to mature. Therefore, it is possible that NMY-2 plays a role in cellularizing the maturing oocyte or allowing for the oocytes increase in size by helping to add membrane to the oocyte cortex. This also shows, that NMY-2 is present in the growing oocytes where the force causing cytoplasmic streaming originates from. Therefore, NMY-2 might have a direct role in cytoplasmic streaming, such as anchoring the NMY-1 matrix to the cortex of the oocyte.

Another unexpected finding came when examining the localization of NMY-2 in *mlc-4*(RNAi) worms. We would expect that loss of MLC-4 would result in the inability of NMY-2 to form filaments due to NMY-2's requirement for a regulatory light chain. Figure 3-6G, H shows that *mlc-4*(RNAi) in combination with a strain expressing the NMY-2::GFP reporter still has all the characteristics of *mlc-4*(RNAi); there is a delay in cellularization and a large amount of damaged oocytes in the proximal gonad and uterus. However, NMY-2 continues to localize to the ring channels, but does not localize to the lateral and basal membranes of the growing oocyte. There are two explanations for this: either oocytes never

begin maturing due to an NMY-2-independent requirement for MLC-4, or NMY-2 is required for cells to begin enlarging.

3-8 NMY-2 Plays Two Roles in the Gonad of *C. elegans*: Maintaining Ring Channel Structure and Allowing Oocytes to Enlarge and Cellularize

It was not clear why *mlc-4*(RNAi) did not resemble *nmy-2*(RNAi) (Figure 3-7B, D). However, the localization of NMY-2 may help explain the *mlc-4*(RNAi) phenotypes. Firstly, it is likely that RNAi against MLC-4 is not removing all of the MLC-4 protein from the gonad. If NMY-2 is required in the primordial germ cells, which are the active cells translating proteins, then it is possible that the limited amounts of MLC-4 are binding to NMY-2 preferentially over NMY-1. In other words, if MLC-4 is limiting, then some NMY-2 will be active in the distal arm where the synthesis of MLC-4 is expected to take place, but the proximal arm (and NMY-1) will be devoid of free MLC-4. Therefore, NMY-1's activity would be preferentially compromised. This would explain why NMY-2 is still able to localize to the distal gonad and not to the loop region in *mlc-4*(RNAi) animals. It would also explain why in *mlc-4*(RNAi) animals there is no difference from wild type in the distal arm of the gonad, thereby resembling *nmy-1*(RNAi). However, this does not explain why we are seeing a delay in cellularization, where in *nmy-1*(RNAi) there is early cellularization.

Unlike what is seen with *nmy-2*(RNAi), animals treated with *mlc-4*(RNAi) still have a sufficient amount of active NMY-2 for the initial formation of the inner gonad architecture. Therefore, in *mlc-4*(RNAi), later defects caused by the loss of NMY-2 can be observed. While *mlc-4*(RNAi) resembles *nmy-1*(RNAi), there are a few phenotypes specific to the loss of NMY-2 in *mlc-4*(RNAi) animals. The delay in cellularization, which is not seen in *nmy-1*(RNAi), is a result of the loss of NMY-2. This is confirmed by looking at *mlc-4*(RNAi) in a *fem-3* mutant background where NMY-1 is not necessary. In this background, the gonad never cellularizes and oocytes never grow in size. This means that NMY-2 plays a role in cellularization as well as allowing oocytes to enlarge. Therefore, NMY-2 is

required for the initial architecture of the progenitor cells and the ring channels that join them to the rachis, but it is also required for cellularization as the oocytes begin to mature (Figure 3-7E).

3-9 Conclusion

In contrast to our initial hypothesis, I have shown that NMY-1 and NMY-2 do not play redundant roles in the gonad of *C. elegans.* I have shown that each heavy chain is required for different steps of oogenesis but that loss of either heavy chain results in loss of cytoplasmic streaming. This indicates that their role in cytoplasmic streaming cannot be rescued by the other myosin. I have also shown that the additional phenotype seen with *mlc-4*(RNAi) can be explained by a partial loss of NMY-2. Therefore, loss of both myosins does not expose any additional phenotypes that would indicate redundancy. Finally, I have shown that the two myosins do not co-localize in the gonad. Therefore, it is unlikely that the two myosins share any roles in the gonad and are not redundant.

NMY-1 localizes to the proximal arm of the gonad where it is required for the later steps of oogenesis (Figure 3-3D). It is the motor protein responsible for cytoplasmic streaming. In the absence of NMY-1 cytoplasmic streaming stops (Figure 3-4B). NMY-1 may also be needed for proper ovulation. NMY-1 could contribute to the appropriate cell shape needed for the sheath cells to move the oocyte through the spermatheca or NMY-1 could be required in either the spermatheca or sheath cells for proper contractions. Both of the previous hypotheses are supported by the evidence that NMY-1 is not required unless the MSP signal is present (Figure 3-5H).

We now have a more detailed understanding of NMY-2's role in the gonad. NMY-2 localizes to the distal arm of the gonad in the ring channels and in the loop region it moves into the lateral and basal membranes of maturing oocytes before they completely cellularize from the rachis (Figure 3-6D). It is required for the maintenance of the internal structure of the gonad. It is also needed for the appropriate timing of cellularization and maturation of oocytes (Figure 3-7A,C).

fem-3 mutants that lack sperm still undergo the early steps of oogenesis. Also, NMY-2's function does not require the MSP signal. Together these two facts corroborates with the hypothesis that NMY-2 is required for the early steps of oogenesis which occur in the absence of MSP (Figure 3-5D).

The one piece of data that does not fit with our current understanding of nonmuscle myosins is the observation by Nadarajan *et al.,* 2009. Nadarajan *et al.,* 2009 found that in the absence of the MSP signal, MLC-4 (which co-localizes with NMY-2) is not phosphorylated at serine-18. Serine-18 is believed to be the main switch allowing myosin to change from an active confirmation to a non-active confirmation. Our current model is that NMY-2 should not be able to localize or function without this phosphorylation. However, in the absence of the MSP signal, NMY-2 continues to localize to its appropriate location and it continues to be required for maintenance of the internal gonad architecture. In order to answer why MLC-4 is not phosphorylated in the absence of the MSP signal, it will first be important to determine whether the antibody that recognizes MLC-4 phosphorylated on serine-19 in mammalian cells, recognizes the same phosphorylation site in *C. elegans*, serine-18. It will also be important to determine whether in the absence of the MSP signal, MLC-4 continues to localize with NMY-2. If the antibody recognizes a different phosphorylation site it will show that the MSP signal does somehow regulate NMY-2 but the current model that serine-18 is the on/off switch for nonmuscle myosin will remain the same. If MLC-4 does not localize with NMY-2 in the absence of MSP, then either NMY-2 is able to function without a regulatory light chain, which would be novel, or there is another regulatory light chain that has yet to be identified.

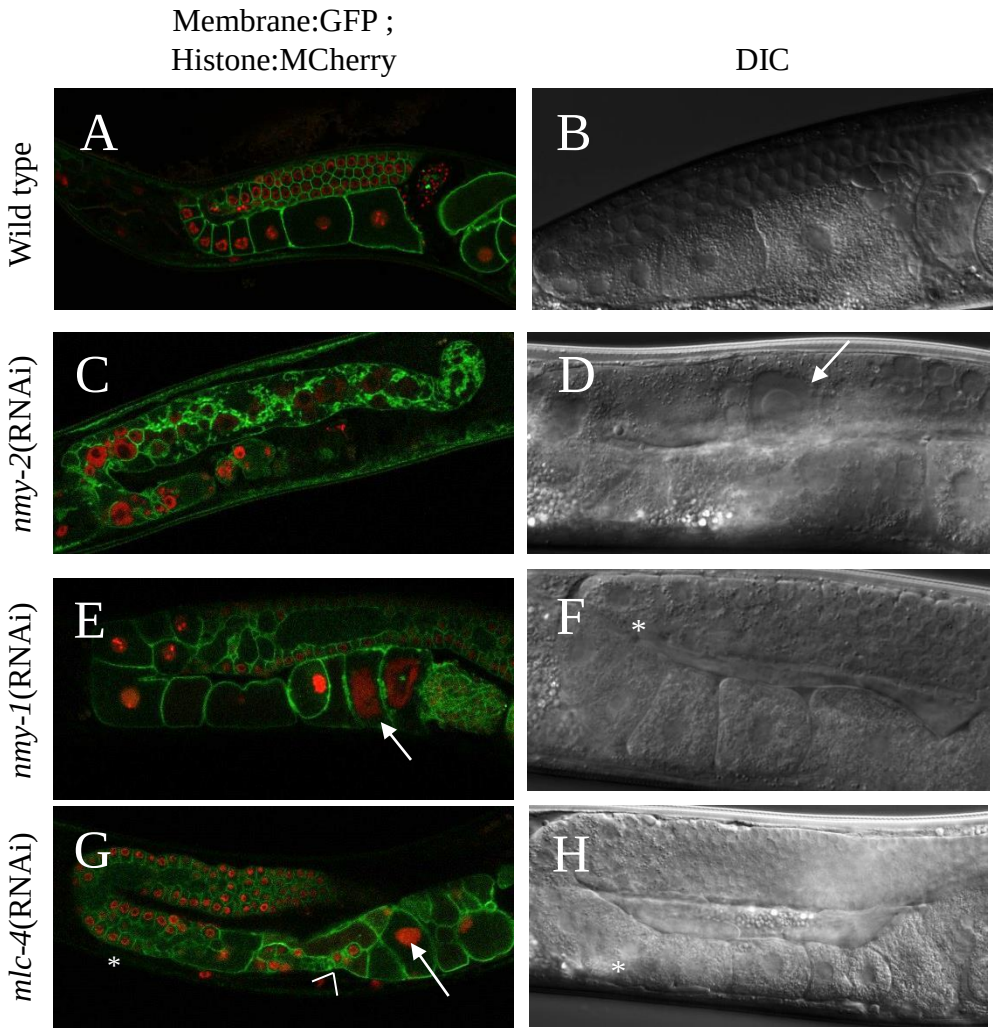

Membrane:GFP ;
Histone:MCherry
DIC
Wild type
nmy-2(RNAi)
nmy-1(RNAi)
mlc-4(RNAi)
A
B
C
D
E
F
G
H

Figure 3-1. NMY-1 and NMY-2 are not functionally redundant during oogenesis. (A,C,E,G) have a green membrane marker and a red nuclear marker. They are also confocal images **(B,D,F,H)** are DIC images. All images have the distal gonad arm on the top and the proximal arm on the bottom with the vulva outside of the image on the right. **(A,B)** are wild type images with a honeycomb appearance in the distal arm and enlarging oocytes in the proximal arm. (**C,D)** shows an *nmy-2*(RNAi) animal, which lacks membrane structure in the distal gonad arm. **(E,F)** shows an *nmy-1*(RNAi) animal, which lacks developed oocytes and shows enlarged nuclei as indicated by the arrow. **(G,H)** shows an *mlc-4(*RNAi) animal, which shows disorganized membranes (arrowhead) and enlarged nuclei (arrow) in the uterus much like the *nmy-1*(RNAi) worms. However, unlike either *nmy-1*(RNAi) or *nmy-2*(RNAi), *mlc-4*(RNAi) produces a proximal gonad arm with the structural characteristics of the distal gonad arm (asterisks). **(D)** An *nmy-2*(RNAi) animal has enlarged and disordered nuclei (arrow). **(F)** An *nmy-1*(RNAi) animal has enlarging oocytes before the loop (asterisks). **(H)** An *mlc-4*(RNAi) animal shows the delay in oocyte enlargement. There are no enlarged oocytes until after the loop (asterisks). All images are from separate animals. A minimum of twenty different animals from three separate RNAi experiments was observed for each RNAi phenotype described. Both confocal and DIC imaging allow for optical sectioning through thick tissue.

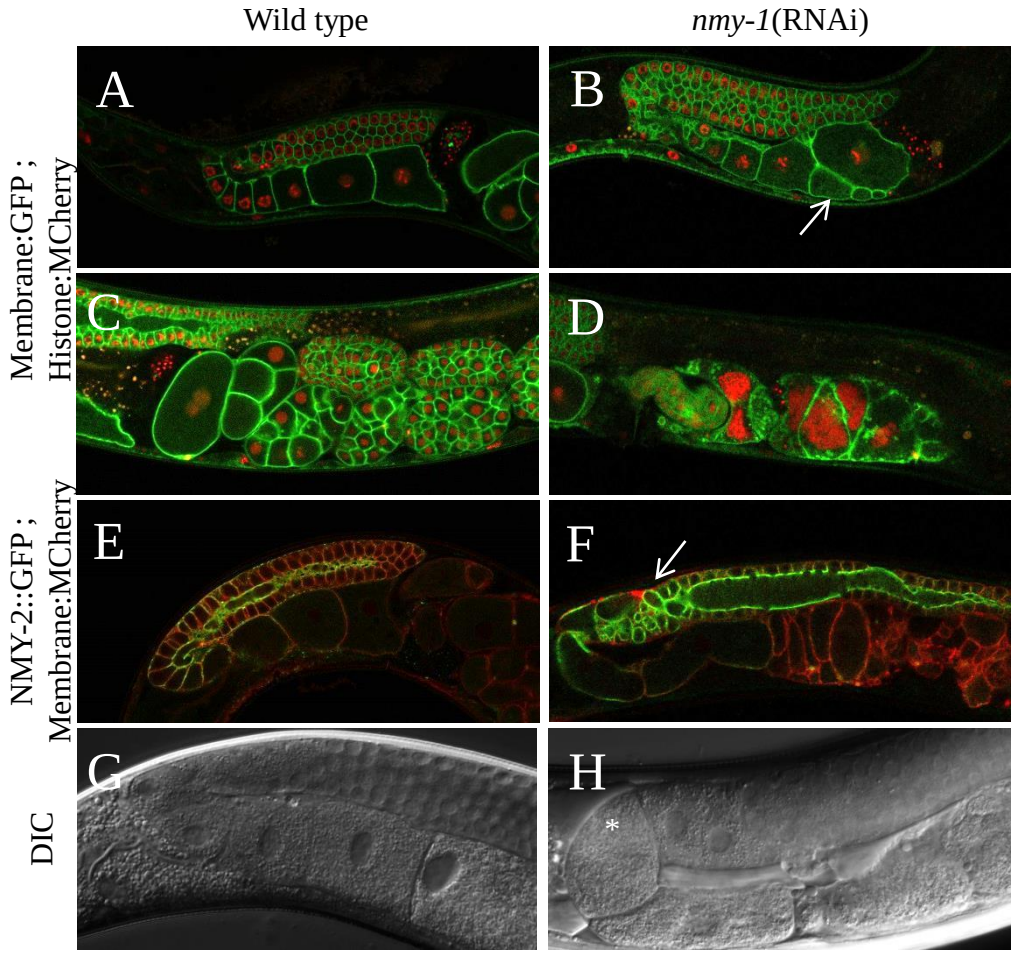


Figure 3-2. The loss of NMY-1 causes defects in the proximal gonad arm. **(A,C,E,G)** are images of wild type gonads. **(B,D,F,H)** are images of *nmy-1*(RNAi) gonads **(A,B)** have a green membrane marker and a red histone marker. **(B)** shows an *nmy-1*(RNAi) gonad with anucleated cells in the proximal gonad as indicated by the arrow. **(C,D)** also have a green membrane marker and a red histone marker but is focused on the uterus with part of the oviduct on the left. **(D)** shows a *nmy-1*(RNAi) animal with a mass of membrane and many nuclei in the uterus instead of **(C)**, which shows developing embryos in wild type gonads**. (E,F)** has NMY-2::GFP as a ring channel marker with a red membrane marker and shows that the loss of NMY-1 does not affect NMY-2 localization. As indicated by the arrow, there are many more oocytes closing their ring channels than in wild type. **(G,H)** are DIC images. **(H)** shows enlarged oocytes in the loop region (asterisks) of *nmy-1*(RNAi) animals, which is not seen in **(G)** wild type animals.

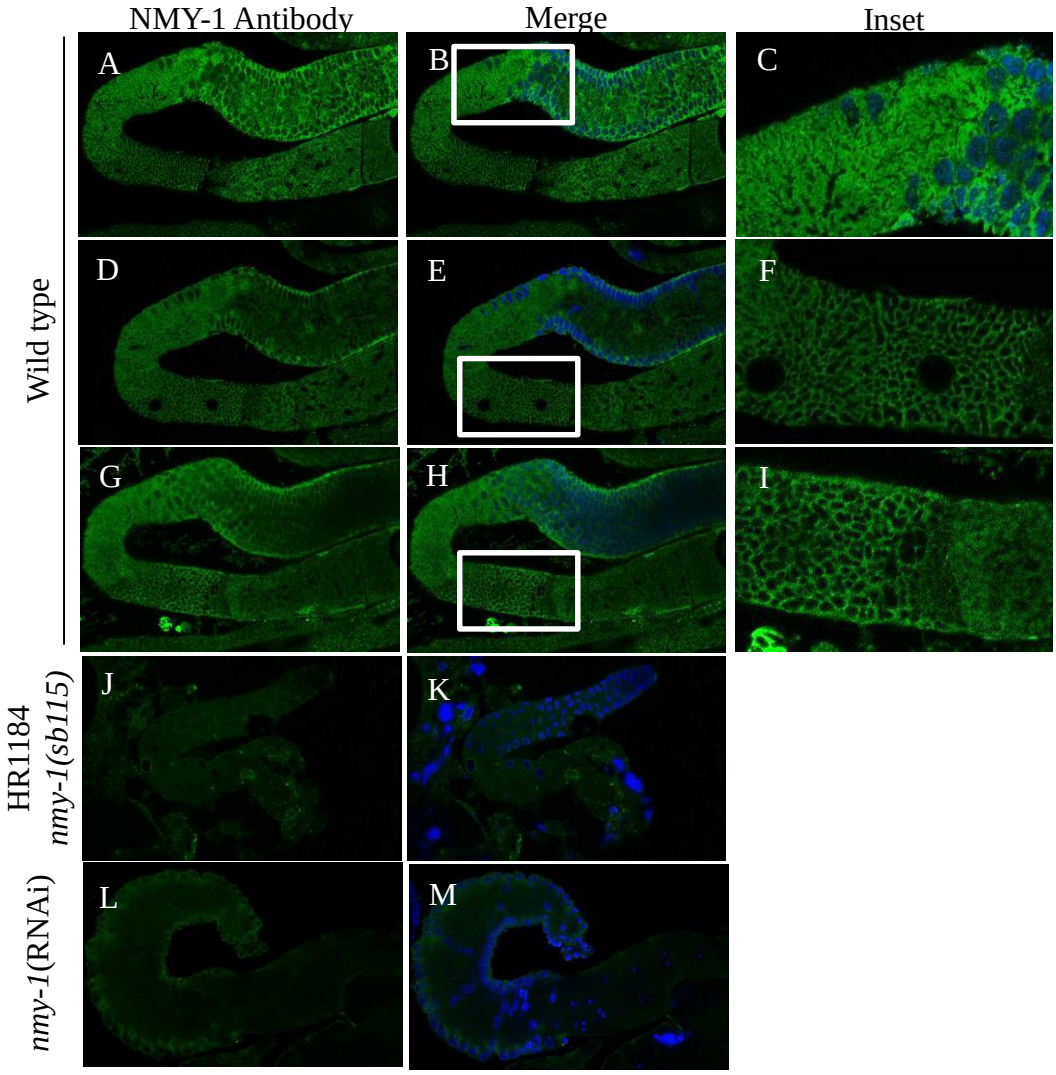
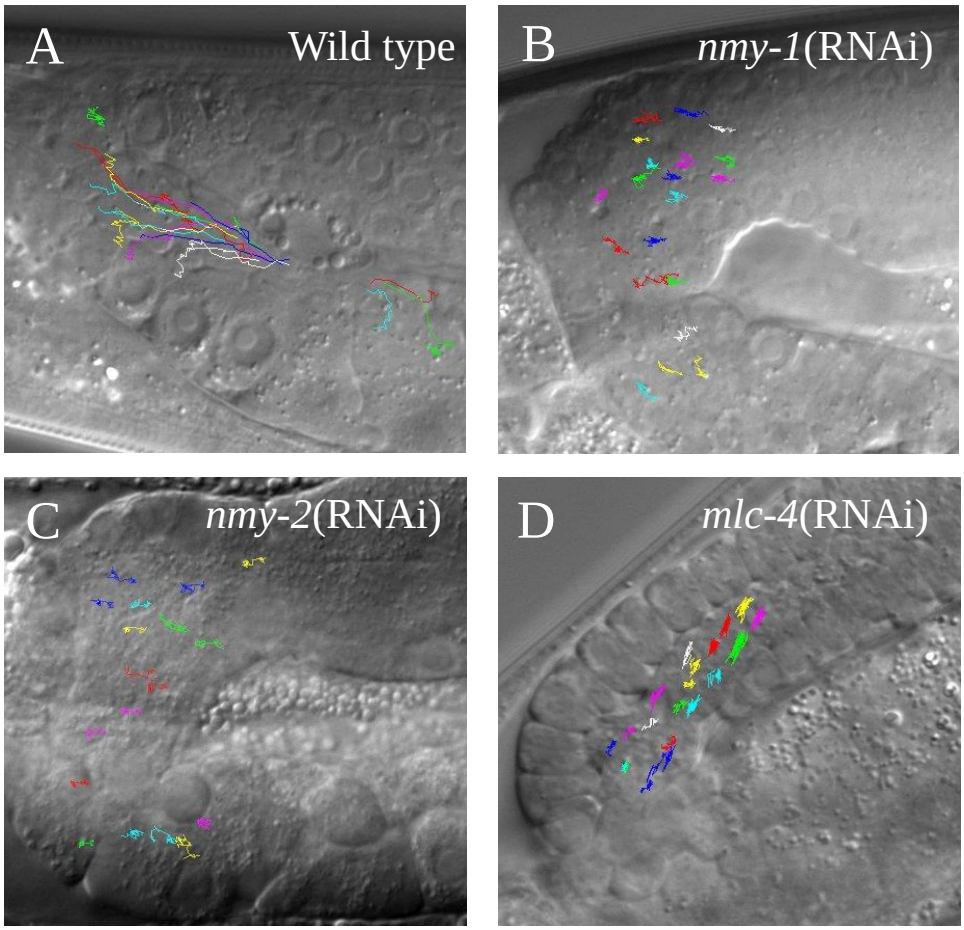


Figure 3-3. NMY-1 localizes to the proximal gonad arm in a network throughout the developing oocyte. (**A,D,G,J,L)** are gonads stained with NMY-1 antibody. (**B,E,H,K,M)** are merged images with DAPI. **(C,F,I)** are insets of merged images. **(A,D,G)** are different focal planes of wild type gonads showing the localization of NMY-1 in a network throughout the developing oocytes. **(J,K)** are gonads from *nmy-1(sb115)*, a null mutant, stained with NMY-1 antibody, confirming specificity of the staining. As a second control, and to assay the degree of protein knockdown by RNAi, **(L,M)** are *nmy-1*(RNAi) gonads stained with NMY-1 antibody. They lack staining showing that there is a large decrease in NMY-1 protein using RNAi. All panels were taken using the same laser intensities and gain making them directly comparable.



E

Actomyosin-dependent Cytoplasmic Streaming		
Genotype		DIC particle speed (μm/minutes ± s.d.)
Wild type		2.23 ± 0.90
nmy-1(RNAi)		1.33 ± 0.36
mlc-4(RNAi)		1.91 ± 0.72
nmy-2(RNAi)		1.39 ± 0.44

Figure 3-4. Nonmuscle myosin components are required for cytoplasmic streaming. A, B, C, D are 10 min particle tracks of **(A)** wild type, **(B)** *nmy-1*(RNAi), **(C)** *nmy-2*(RNAi) and **(D)** *mlc-4*(RNAi). Arrows indicate movement of particles over 10 minute timespan. Wild type shows particle movement towards developing oocytes. This directional movement is disrupted in all RNAi treated animals. **(E)** is a table that shows particle speeds in the four different genotypes. There is a decrease in speed in all RNAi mutants because they lack the fast movement of particles into the developing oocytes. 65 particles in 3 different worms were analysed for each genotype.

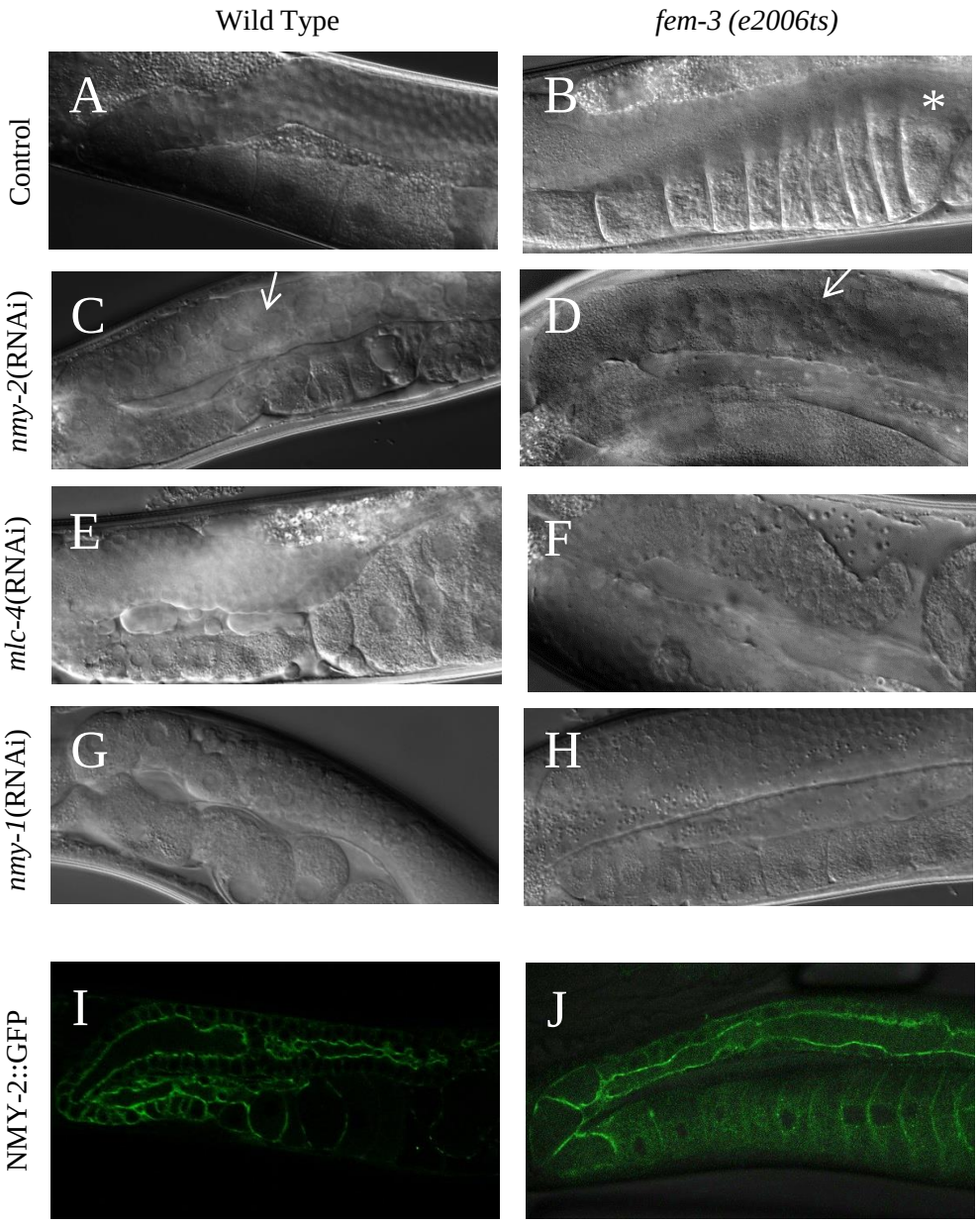

Wild Type
fem-3 (e2006ts)
Control
nmy-2(RNAi)
mlc-4(RNAi)
nmy-1(RNAi)
NMY-2::GFP
A
B
*
C
D
E
F
G
H
I
J

Figure 3-5. NMY-1 requires the MSP signal while NMY-2 functions independently of this signal. Images were taken using DIC. **(A,C,E,G,I)** are images of RNAi in wild type gonads. **(B,D,F,H,J)** are images of RNAi in a *fem-3* mutant background. **(A)** is a wild type gonad. **(B)** is control RNAi into a *fem-3* mutant gonad that shows the stacked oocytes as indicated by the asterisk. **(C,D)** shows *nmy-2*(RNAi) in both genetic backgrounds and the phenotypes are very similar. Both have enlarged pooling nuclei as indicated by the arrows. **(E,F)** shows *mlc-4*(RNAi) in both backgrounds. They also share similar phenotypes. In both cases there is a delay of oocytes enlarging, although in the *fem-3* mutant background there is not a build up of damaged oocytes. **(H)** show that *nmy-1*(RNAi) in *fem-3* mutant background has no apparent effect compared to *nmy-1*(RNAi) in a wild type background **(G)**. This indicates that the *nmy-1*(RNAi) phenotype only occurs in the presence of sperm. **(I,J)** has NMY-2::GFP and shows that there is no change in localization between *fem-3* mutants **(J)** and the control **(I)**.

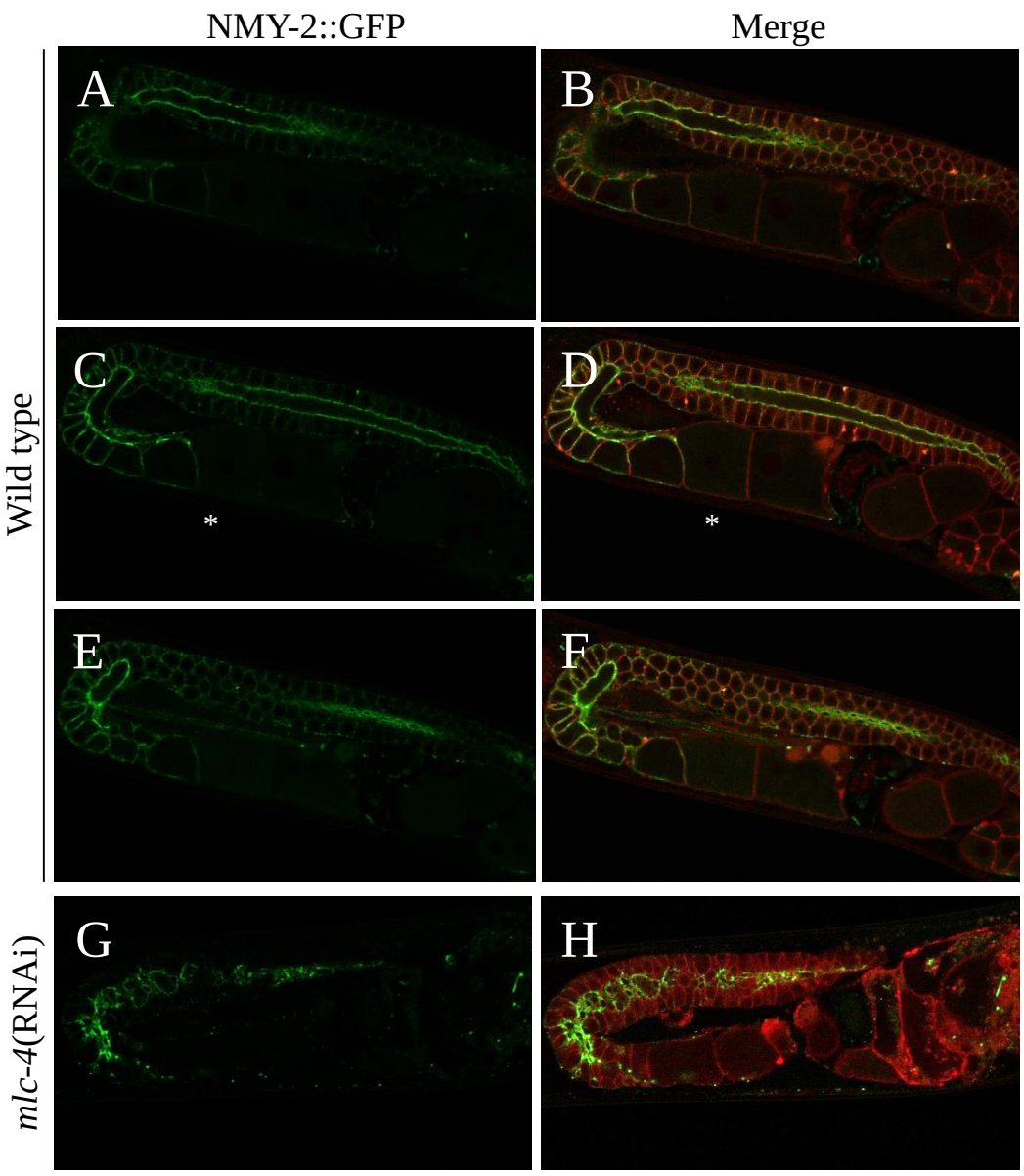
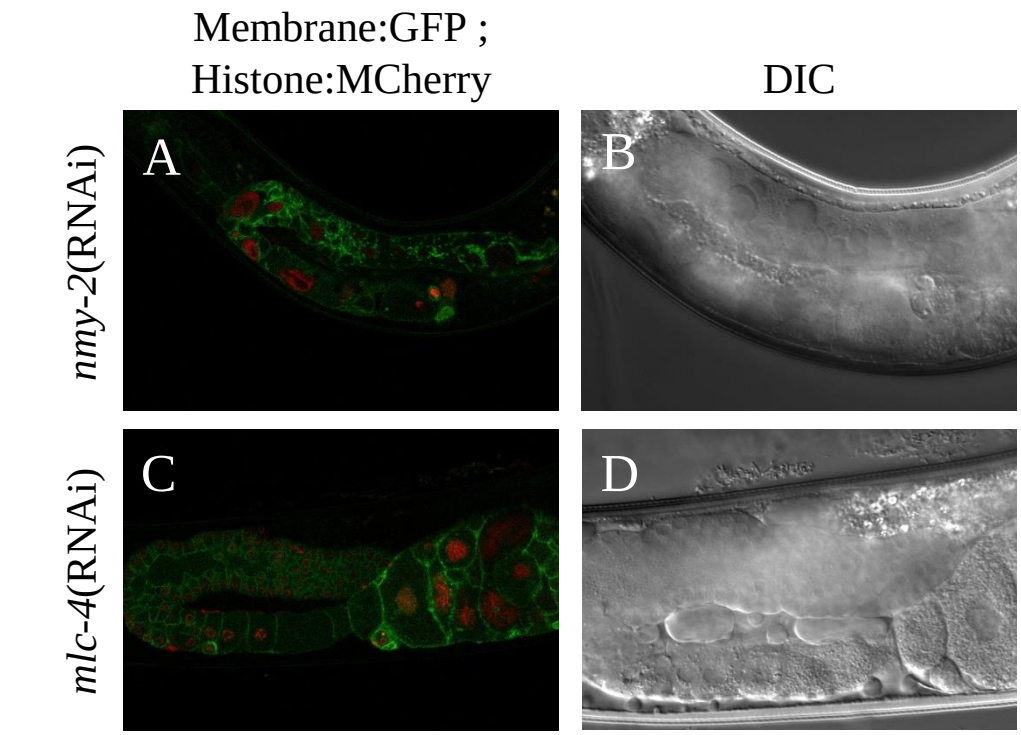

NMY-2::GFP
Merge
A
B
C
D
E
F
G
H
*
*
Wild type
mlc-4(RNAi)

Figure 3-6. NMY-2 localizes to the ring channels in the distal gonad and to the lateral and basal membranes of the developing oocyte. Loss of MLC-4 does not change the localization of NMY-2 in the ring channels but in the lateral and basal membranes the localization of NMY-2 is lost. **(A,C,E,G)** shows NMY-2's localization with an NMY-2::GFP fusion protein and **(B,D,F,H)** is NMY-2::GFP merged with a red membrane marker. **(A,B)** is the deepest focal plane imaged and shows NMY-2's localization to the ring channels. **(C,D)** shows a interior focal plane and shows the rachis along the loop region of the gonad. NMY-2 does not localize to the oocytes that have fully closed their ring channels (after the asterisk) **(E,F)** shows the nearest focal plane and shows that the rachis on the loop region runs along the side of the gonad. NMY-2 localizes to the ring channels and closer to the proximal region of the gonad it also begins to localize to the lateral membranes and basal membranes. **(G,H)** shows NMY-2's localization in *mlc-4*(RNAi) animals. In that background NMY-2 continues to localize to the ring channels, though it is not observed on lateral and basal membranes.



E

1. NMY-2 is needed to maintain ring channels and lateral membranes

2. NMY-2 is required for cellularization and/or expansion of the growing oocytes

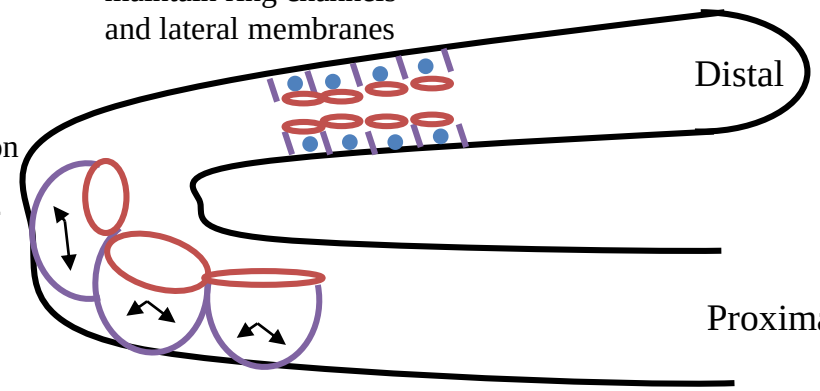


Figure 3-7. NMY-2 is required for maintenance of ring channels and internal gonad architecture as well as cellularization and enlargement of developing oocytes. (A,B) are gonads from *nmy-2*(RNAi) animals. **(C,D)** are gonads from *mlc-4*(RNAi) animals. **(A,C)** contain a green membrane marker and a red histone marker. **(B,D)** are DIC images. *nmy-2*(RNAi) shows a disruption of the internal membranes while *mlc-4*(RNAi) shows a delay in cellularization. **(E)** shows a model where NMY-2 is required for two steps during oogenesis. In the distal arm of the gonad it is required for maintenance of ring channels (red) and internal architecture (purple) while in the loop region of the gonad it is required for the correct timing of cellularization and enlargement of the maturing oocytes.

CHAPTER FOUR: DISCUSSION

The purpose of this thesis was to ascertain at what step of *C. elegans* oogenesis nonmuscle myosins are required, whether they act redundantly during this process, and which upstream regulatory pathway is required for their function. The goal of the thesis was to obtain a better model of how myosin-dependent steps of oogenesis occur and how regulatory factors affect the contractility and stability of nonmuscle myosin. The localization, and effects of gene knockdown of each nonmuscle myosin were examined. RNAi against NMY-1 produces defects in the proximal gonad, while RNAi against NMY-2 produces defects in the distal arm of the gonad. Immunofluorescence showed that NMY-1 localizes to the proximal arm of the gonad. This is consistent with both the mutant phenotype and the conclusion that NMY-1 may act during the later steps of oogenesis. Using a reporter construct driven by the *nmy-2* promoter, I showed that NMY-2 localizes to the distal gonad arm. This is consistent with a role for NMY-2 in the early steps of oogenesis. Therefore, NMY-2 is required for maintenance of the internal architecture of the distal gonad and cytoplasmic streaming. NMY-1 is required for cytoplasmic streaming and ovulation. The knockdown of MLC-4 by RNAi provided the opportunity to analyze the effects of the loss of NMY-2 at later stages. Partial loss of MLC-4 showed that NMY-2 is also required for cellularization. The heavy chains share the same regulatory light chain, which was previously believed to be the major source of regulation. Therefore, the differences in localization of the two heavy chains and the differences in the steps of oogenesis that require each of the nonmuscle myosin must be due to the heavy chains themselves.

4-1 NMY-1 is required for Cytoplasmic Streaming

NMY-1 staining appears in a “matrix” pattern through the developing oocyte (Figure 3-3F). This localization is consistent with NMY-1 acting as the motor protein in the actomyosin complex that is responsible for cytoplasmic streaming in the gonad. Loss of NMY-1 results in a decline or loss of cytoplasmic streaming. As loss of NMY-2 also inhibits cytoplasmic streaming, it is possible that any disruption in the gonad architecture affects cytoplasmic streaming.

However, when Wolke *et al.*, 2007 used RNAi treatment to reduce tubulin levels, it also caused massive disruptions to the gonad architecture and yet cytoplasmic streaming was not affected. Therefore, while NMY-1 stands as the most likely candidate for the motor protein responsible for cytoplasmic streaming, there are also possible hypotheses for the effect of the loss of NMY-2. The complete or nearly complete absence of internal membranes in animals where NMY-2 has been knocked down may block the ability of NMY-1 to form a network, leading to the loss of cytoplasmic streaming. It is also possible that NMY-1 requires a NMY-2 dependent cortical network under tension in order to function. Nevertheless both myosins are required for cytoplasmic streaming.

Assuming that NMY-1 is the motor protein causing cytoplasmic streaming, we can re-examine the models proposed by Wolke *et al.*, 2007 (Figure 4-1). The first model for the generation of cytoplasmic streaming in the gonad is based on the model for streaming and initial polarization of the one cell *C. elegans* embryo. However, they point out that this seems unlikely, because an oocyte is a closed system (the egg shell prevents volume change in the cell) while the developing oocytes are not (elasticity of the gonad is permitted), and in the single cell oocytes NMY-2 localizes to the cortex while NMY-1 forms a matrix throughout the developing oocyte. Another problem with this model is that there is no directional movement of particles along the cortex of the developing oocyte, as is seen in the one cell embryo. The second proposed model was that actin filaments in both the rachis and the opening into the developing oocyte may have motor proteins pulling particles along the filaments and into the oocyte. This also does not explain why loss of NMY-1 affects cytoplasmic streaming because type II nonmuscle myosins such as NMY-1 are not known to be able to carry cargo, and therefore it is unlikely that they are transporting particles. The third model involves growth of the cortex of the oocyte which would pull cytoplasm into the oocyte to fill the growing space. This model is based on the dynamic nature of the leading edge of motile cells, which use a branched network of actin filaments in order to expand the membrane. This process in motile cells is dependent on the

Arp2/3 complex, which has not been shown to have any role in fertility in *C. elegans*. However, the network that NMY-1 forms could indicate that this is how cytoplasmic streaming is driven. The fourth model involves a network of actomyosin that is under differing tension, with less tension in the rachis and more tension in the developing oocyte. This difference in tension is responsible for streaming in other organisms such as during the early syncytial embryo of *Drosophila* (von Dassow and Schubiger 1994). The fourth model may suggest an explanation for the NMY-2 requirement for streaming. The two myosins may be forming separate networks of different tensions. However, because both myosins localize in the developing oocyte, the movement of cytoplasm from a low tension area (one of the myosins) to a high tension area (the other myosin) would occur within the oocyte itself and so could not explain cytoplasm coming from the rachis. It could also be differences in phosphorylation (di-phosphorylation versus mono-phosphorylation on the regulatory light chain) making some of the NMY-1 network under more tension than other parts of the network. Put more simply, the lack of a tensile network creates the low tension area in the rachis and the myosin network creates the high tension area in the oocyte. Now that nonmuscle myosin's involvement in streaming has been identified, the third or fourth models appear the most promising. However, accurate measurements of flow and localization of both myosins within the same animal, which cannot currently be done due to denaturation of the NMY-2:GFP reporter by the methanol fixation required for NMY-1 antibody staining, as well as mathematical modeling of the forces involved may need to be performed to confirm how the force is being generated.

Cytoplasmic streaming requires the MSP signal. While NMY-1 may be required for cytoplasmic streaming, the defects seen in *nmy-1*(RNAi) gonads (Figure 2-5G) do not resemble gonads in unmated females that lack the MSP signal (Figure 3-5B). However, unmated females also do not undergo meiotic maturation, and they ovulate at an extremely low rate, so a long delay in the completion of oogenesis may allow the oocytes to become the correct size in the absence of streaming due to a passive flow of cytoplasm into the growing oocyte. None of the current

models explain how cytoplasmic streaming could be lost and yet oocytes would continue to undergo meiotic maturation and ovulation. There may be problems with oocyte size and viability if the oocytes are being produced at normal speeds but are unable to take up cytoplasm as quickly. However, the opposite may be true because in gonads that express a high copy array of ACY-4, a component of MSP signaling in the sheath cells, MSP signaling occurs in the absence of MSP and there is an increase in cytoplasmic speeds but no change in the size of the oocyte (Nadarajan, et al. 2009). This might indicate that the oocyte is somehow able to sense when it has reached the correct size and then cellularize meaning that in gonads that lack cytoplasmic streaming oocytes may take longer to mature instead of cellularizing at a smaller size. However, most of the phenotype of *nmy-1*(RNAi) gonads cannot be explained by loss of cytoplasmic streaming alone therefore there must be a secondary requirement for NMY-1.

4-2 NMY-1 is required for Proper Ovulation

Loss of NMY-1 does not have any apparent effect on the structure of the distal gonad arm, allowing us to conclude that NMY-1 likely does not play a role in the earlier steps of oogenesis. However, the proximal arm of the gonad shows a variety of phenotypes in *nmy-1*(RNAi) gonads. In many animals, there was an increase in the number of full-sized oocytes in the proximal arm, and sometimes these oocytes could be found throughout the loop region. Another phenotype is the "stacking" of oocytes in the proximal gonad (Figure 3-2B), instead of the single row that is seen in wild type (Figure 3-2A). There are also blebs of apparently nucleus-free cytoplasm in the proximal arm. However, occasional oocytes are laid even though the uterus is filled with multinucleated masses of disorganized membrane. While an unusual phenotype, this is also seen in mutants or RNAi knockdown of proteins required for the proper function of the sheath cells or spermatheca. MPK-1 is required for ovulation, and in its absence, gonads fill with endomitotic oocytes, due to having undergone meiotic maturation and then replicating their DNA repeatedly without being fertilized (Lee, et al. 2007). These resemble NMY-1 knockdown because they can result in mature oocytes

backing up in the proximal gonad arm. Gonads with defects in sheath cells are not able to coordinate ovulation and can asymmetrically contract, causing bubbles of cytoplasm to be split from the oocyte, and left in the gonad as cytoplasmic blebs (Rose, et al. 1997). FLN-1 encodes a stretch-sensitive signaling scaffold, and mutants are unable to sense the presence of oocytes in the spermtheca and therefore do not relax the valve to the uterus. Oocytes are not able to exit the spermatheca, which causes a buildup of oocytes in the spermatheca, causing improper fertilization and damage to the oocytes (Kovacevic and Cram 2010). LET-502 encodes a rho kinase, and mutants are unable to constrict the valves in the spermatheca. While occasional oocytes can exit the spermatheca into the uterus, in many cases they are squeezed from the spermatheca back into the oviduct (Wissmann, Ingles and Mains 1999). This causes damaged oocytes and a buildup of oocytes in the oviduct. Therefore, loss of a protein causing any of these phenotypes is likely required for proper ovulation and may function in the sheath cells or spermatheca.

NMY-1 is expressed both in the sheath cells and the spermatheca and therefore defects in either of these could contribute to the phenotype (Hunt-Newbury, et al. 2007). A recent paper showed that *nmy*-1(RNAi) stopped contractions in the spermatheca and resulted in problems with the oocyte exiting the spermatheca (Kovacevic, Orozco and Cram 2013). This does not prove that there is not also a defect in sheath cell contractions, but it could be a sufficient explanation for the defects seen in the *nmy-1*(RNAi) gonads. Time lapse analysis of ovulation in *nmy-1*(RNAi) gonads would determine if sheath cell contractions occur or not. It would also be important to examine the effects of *nmy-1*(RNAi) in an *rrf-1* mutant background that is deficient in somatic RNAi (Sijen, et al. 2001). This would enable us to differentiate which phenotype is caused by the somatic spermatheca cells and which is caused by NMY-1 in the germline itself. This would also allow us to test whether cytoplasmic streaming requires a NMY-1 actomyosin matrix in the oviduct itself and is not instead disrupted by problems in ovulation. Wolke *et al.*, 2007 did not believe that cytoplasmic streaming required ovulation because

young hermaphrodites before their first ovulation show cytoplasmic streaming and streaming does not require sheath cell contractions because extruded gonads still stream. Therefore the requirement for NMY-1 during cytoplasmic streaming is likely independent from its role in the spermatheca.

4-3 NMY-2 is needed for Establishment and Maintenance of the Gonad Interior Architecture during the Pachytene Stage of Oogenesis and is also required for Cellularization and Enlargement of the Oocyte

Loss of NMY-2 leads to a drastic change in gonad architecture which can be as severe as the loss of most internal membranes. Due to the severe phenotype, it is difficult to tease apart which steps during oogenesis requires NMY-2. It appears it is required either to create the internal membranes that anchor the nuclei to the gonad wall, or to maintain these membranes, because there is no section of the gonad that is not affected by its loss. However, there continues to be nuclei throughout the gonad, indicating that mitosis of the stem cells is likely still occurring within the syncytium. Therefore, NMY-2 is not likely directly involved in promoting proliferation of the germ line, but the loss of the architecture does affect the stem cell niche.

NMY-2:GFP reporter strains show fluorescence strongly associated with the ring channels and perhaps weakly to the lateral membranes, making it unlikely that NMY-2 is maintaining the lateral membranes and more likely that it is maintaining the ring channels. It is difficult to say if the nuclei in the distal arm of the gonad ever enter pachytene due to the endomitotic appearance of the nuclei. One explanation is that the nuclei never enter meiosis and continue to replicate their DNA creating the large nuclei seen in *nmy-2*(RNAi) gonads. Another explanation is that without membranes the oocytes cannot progress past the pachytene stage and consequently, no oocytes can form. With either explanation, NMY-2 is necessary for germ cells to progress to mature oocytes.

The role of NMY-2 in the ring channels that connect cells to a syncytium is not specific to *C. elegans*. In *Drosophila,* nurse cells remain connected to the oocyte

via ring channels and those ring channels also require nonmuscle myosin either for their maintenance or their formation (Jordan and Karess 1997). However, unlike in *C. elegans*, where NMY-2 remains present throughout the entire existence of the ring channel, in *Drosophila* nonmuscle myosins play a role in forming the ring channels but are no longer present in the mature ring channel. Intracellular bridges are also required in mammalian germ cells for spermatogenesis and contain similarities to structures seen during cytokinesis (Greenbaum, Ma and Matzuk 2007). In *C. elegans*, NMY-2, LET-502 and MEL-11 are required for ring channels in the gonad as well as cytokinesis in the early embryo which shows that our understanding of cytokinesis or ring channel formation and closure can be applied to the other process due to their similarities.

The major defects seen at early stages of oogenesis in NMY-2 depleted gonads make it difficult to see if NMY-2 plays a role separate from that of maintaining the ring channels. However, due to the incomplete knockdown of MLC-4 using RNAi, we are able to bypass the first steps of oogenesis. MLC-4 is the regulatory light chain and it is required for the myosin complex to form and function. Therefore, the loss of MLC-4 should mimic the loss of both nonmuscle myosins. *mlc-4*(RNAi) in a NMY-2:GFP reporter strain does not cause loss of NMY-2 localization in the distal part of the gonad, but NMY-2 localization to the lateral membranes is lost during the time that the ring channels should begin to close. RNAi of NMY-1 in a *fem-3* mutant background has no apparent effect, allowing us to conclude that the only phenotype of *mlc-4*(RNAi) in this same background must be caused by loss of NMY-2. In this case, there is a delay in the maturation of germ cells due to a problem exiting pachytene. This indicates that NMY-2 also plays a role in cellularization and the enlargement of the oocyte because at this point its reporter expression is found associated with the entire surface of the oocyte and could be needed for expanding the membrane. This is the first evidence that NMY-2 is required for ring channel closure. Previously it was known to localize to the ring channels and be required for internal membranes (Maddox, et al. 2005) (Green, et al. 2011).

4-4 Both Nonmuscle Myosins can act either as Tensile Actin Connectors or as Constrictors that are changing the Shape of the Actin Skeleton

Initially it was believed that nonmuscle myosin always acted as a contractile unit as myosin does in both skeletal and smooth muscle. However, it can also act to add tension to an actin cytoskeleton, it can act as crosslinker or it can interact with other proteins as a scaffold (Conti and Adelstein 2008). This helps to explain how NMY-2 can be required for the maintenance of the ring channels at one stage, likely through actin crosslinking, and then later can act to close the ring channels, likely requiring its contractile nature, or additionally how NMY-1 might act to produce a tensile network during cytoplasmic streaming and then a contractile force during contractions of the spermatheca. Work in motile mammalian cells shows that nonmuscle myosins function as more than an on/off switch, and that phosphorylation on a range of sites allow for variation in the dynamics of myosins. Examination of the process of elongation in *C. elegans* shows that this is also true in *C. elegans* (Wissmann, Ingles and Mains 1999). Therefore, another interesting avenue would be to look at how phosphorylation sites affect the function of nonmuscle myosins during oogenesis. We would predict that inhibiting the phosphorylation at threonine-18 (threonine-17 in *C. elegans*) would also cause defects during oogenesis though to a lesser degree than loss of the protein itself. To do this transgenes with single amino acid changes for threonine-17 and serine-18 would be inserted into the myosin null mutant genome. The gonads of these animals could then be examined for defects during the process of oogenesis. This would demonstrate the importance of these phosphorylation sites during the process of oogenesis.

4-5 NMY-1 and NMY-2 are controlled by Separate Upstream Factors

An unexpected result from this work was that NMY-1 and NMY-2 did not play redundant roles in the gonad. They share the same regulatory light chain and this has previously been shown to be the main mechanism of regulation. Therefore, there must be another less studied regulatory mechanism that differentiates these

two nonmuscle myosins. This difference cannot be due to transcriptional control, because the gonad is a syncytium and therefore mRNAs can move throughout it and are only transcribed during the pachytene stage. There is evidence that control of translation plays an important role in protein localization throughout the gonad through a variety of mRNA binding proteins such as the PUF proteins (Hubstenberger, et al. 2012). These factors enable the expression of proteins only within certain regions of the gonad and *nmy-1* and *nmy-2* transcripts share a 3' UTR which is only 47% identical. Therefore, it is possible that they are localized by this mechanism. PUF proteins bind to different sequences, with different specificity. The only necessary sequence is a "UGU" which both *nmy-1* and *nmy-2* have over ten copies in each of their 3'UTRs. Even with the few PUF proteins that have consensus binding sequences, sites encoding the consensus sequence cannot necessarily bind the PUF protein and some well-known targets of the PUF proteins are not identified using the consensus sequence (Bernstein, et al. 2005). For this reason, creating a transgene of each of the myosin proteins that is missing its 3'UTR and inserting a single copy of this transgene into the myosin mutant genome would tell us if the 3'UTR is necessary for the function of myosin during oogenesis. Should the 3'UTR prove necessary, then the deletion of the UGU sites would show if it was due to its regulation by the PUF proteins.

There is also evidence that phosphorylation of sites within the non-helical region of the heavy chain is important for function, and NMY-1 and NMY-2 have only 33% amino acid identity in the tail region. NMY-2 has a longer non-helical tail with many more serines available for possible phosphorylation; NMY-1 also has a few serines that are not conserved with NMY-2 (Figure 4-2). Therefore, different phosphorylation sites between the protein may allow for their different function (Vicente-Manzanares, et al. 2009)(Piekny, et al. 2003). However, the phosphorylation sites are unknown in *C. elegans*, as is how they may affect the function of the myosins, making analysis difficult. The non-helical tail domain is one of the larger differences between the two nonmuscle myosins, therefore one future experiment may be creating constructs for both nonmuscle myosins that

have instead, the other nonmuscle myosin's non-helical tail region, to see if the two myosins will localize based on their non-helical tail region or the remainder of the protein. This would elucidate the importance of the non-helical tail region to the nonmuscle myosins during oogenesis in *C. elegans.*

By looking at the steps of oogenesis in which NMY-1 and NMY-2 participate, we can also get an idea of the upstream factors that may be required for those steps. NMY-2 localizes to the distal gonad arm and is involved in the early steps of oogenesis. One of the important signals involved in the distal arm is Notch signalling, specifically by GLP-1. Loss of GLP-1 results in a delay in cellularization which is similar to what is seen in *mlc-4*(RNAi) (Nadarajan, et al. 2009). Therefore it is possible that GLP-1 is involved in signaling to NMY-2 that oocytes have reached the appropriate size to cellularize. It is also possible that GLP-1 signals to ANI-2 which seems to oppose the function of NMY-2 in the ring channels. Loss of ANI-2 results in premature cellularization of the oocytes (Maddox, et al. 2005). Perhaps ANI-2 stops NMY-2 from closing the ring channels until ANI-2 receives a signal from GLP-1 that the oocyte is of the correct size or at the right time point.

NMY-1 may also be affected by GLP-1 signaling, because loss of GLP-1 results in faster cytoplasmic streaming, but only in the presence of the MSP signal. This is similar to what is seen with NMY-1; its role in cytoplasmic streaming as well as ovulation only occurs in the presence of the MSP signal. When *nmy-1*(RNAi) was performed in a *fem-3* mutant background, there was no detectable effect, consistent with the idea that the function of NMY-1 is dependent on the MSP signal. One possibility for how the MSP signal may be transduced to NMY-1, is through the Ras/MAPK pathway, which is involved in initiating a number of steps during oogenesis and also localizes, for no known purpose, to the same cells where cytoplasmic streaming is being initiated (Lee, et al. 2007) (Arur, et al. 2009). Gain of function mutants of the Ras protein in this pathway look phenotypically wild type in a *fem-3* mutant background, showing that this pathway is also dependent on the MSP signal. The MAPK pathway also acts

through a cascade of phosphorylation and while no direct targets of MPK-1 are known regulators of non-muscle myosins, it does not mean that NMY-1 is not further downstream of its direct targets. It would be interesting to see if there continues to be cytoplasmic streaming with the loss of MPK-1. The difficulty would be the arrest of MPK-1 mutants at the pachytene stage, therefore the temperature sensitive mutant would have to be used instead of doing knockdown by RNAi. However, this experiment would show if cytoplasmic streaming occurs downstream of MPK-1 signaling or if it is part of a separate pathway that is activated by MSP signaling.

NMY-1 and NMY-2 are activated by separate upstream signaling pathways and this is best demonstrated by looking at RNAi of NMY-1 and NMY-2 in a *fem-3* mutant background that lacks the MSP signal. *nmy-2*(RNAi) phenotype is identical in both a wild type and a *fem-3* background. However, NMY-1 looks phenotypically wild type in a *fem-3* background but shows defects in wild type. Therefore, NMY-2 does not require the MSP signal +for its function but NMY-1 does. NMY-2 likely is part of a signaling pathway involving GLP-1 and NMY-1 is downstream of the MSP signal and possibly the Ras/MAPK pathway. Therefore, even though they are both regulated principally through their regulatory light chain they are downstream of separate signaling cascades.

4-6 Conclusion

Both nonmuscle myosins in *C. elegans* act during separate steps during oogenesis (Figure 4-3). NMY-2 is required for the early steps of oogenesis where it maintains the internal architecture of the gonad. NMY-2's previously known role has been expanded to include its requirement for cellularization or enlargement of the maturing oocyte. NMY-2 is also required for cytoplasmic streaming but whether or not it plays a direct role in this process is still unknown. NMY-2 does not require the MSP signal for its function and may be involved in a signaling pathway with GLP-1. I have shown that NMY-1 acts during the later stages of oogenesis where it is likely the main motor protein involved in cytoplasmic

streaming. It is also required for contractions in the spermatheca. My experiments concluded that the function of NMY-1 is dependent on MSP signaling and it may act downstream of the Ras/MAPK signaling cascade. This is unlike NMY-2, which is controlled by one of the other three main regulators. Therefore, it can be concluded, that the two nonmuscle myosin in the *C. elegans* gonad are required for separate steps during oogenesis and are not redundant during this process. Understanding the nature of the effector proteins involved at each of the steps of oogenesis, allows us to modify the current models for how each of the steps of oogenesis occur, such as cytoplasmic streaming, to reflect the actual effector proteins involved.

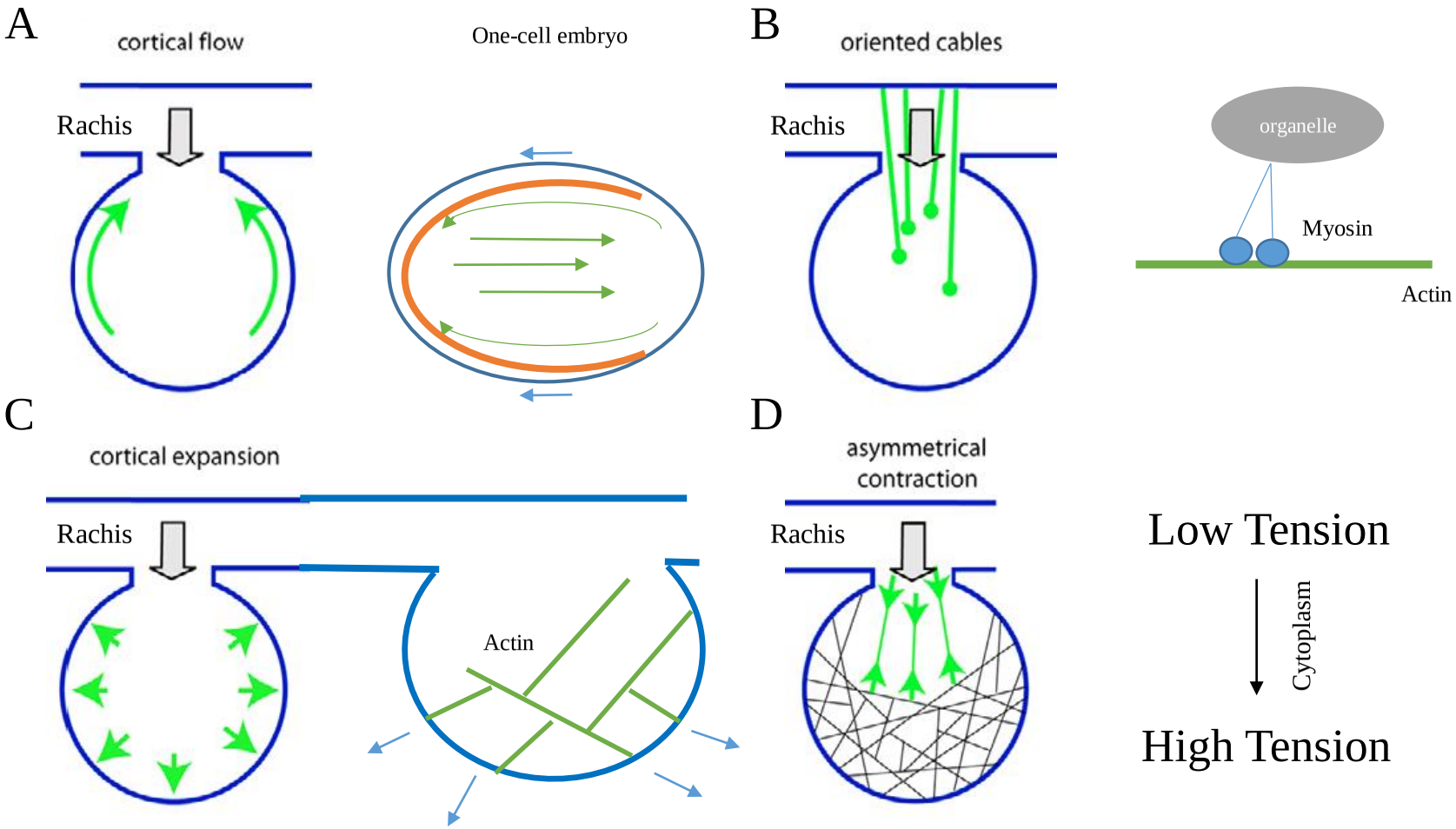

A
cortical flow
Rachis
One-cell embryo
B
oriented cables
Rachis
organelle
Myosin
Actin
C
cortical expansion
Rachis
Actin
D
asymmetrical
contraction
Rachis
Low Tension
Cytoplasm
High Tension

Figure 4-1. **Models for the force driving cytoplasmic streaming.** (Adapted from Wolke *et al.* 2007) Panel A shows cytoplasmic streaming occurring through same mechanism as is used in the one cell embryo. The blue circle represents the growing oocyte and the green arrows represent the force being generated. In the one cell embryo actomyosin network (orange) retracts pulling cytoplasm along the cortex of the cell and interior cytoplasm moves in the opposite direction to compensate. Therefore in the gonad, cytoplasm moves along the cortex towards the rachis due to retraction of the actomyosin network in that direction and cytoplasm is pulled into the cell to compensate. B. Cytoplasmic streaming occurs through motor-driven movement of organelles along actin cables (right hand illustration) into the developing oocyte. The movement and speed of the organelles causes the influx of cytoplasm. C. Actin polymerization (green) expands the cortex of the developing oocyte and cytoplasm enters the oocyte to fill the expanding space. D. There is an area of low tension in the rachis and the opening into the developing oocyte and a high tension level within the oocyte. Cytoplasm moves towards areas of higher tension and therefore into the developing oocyte.

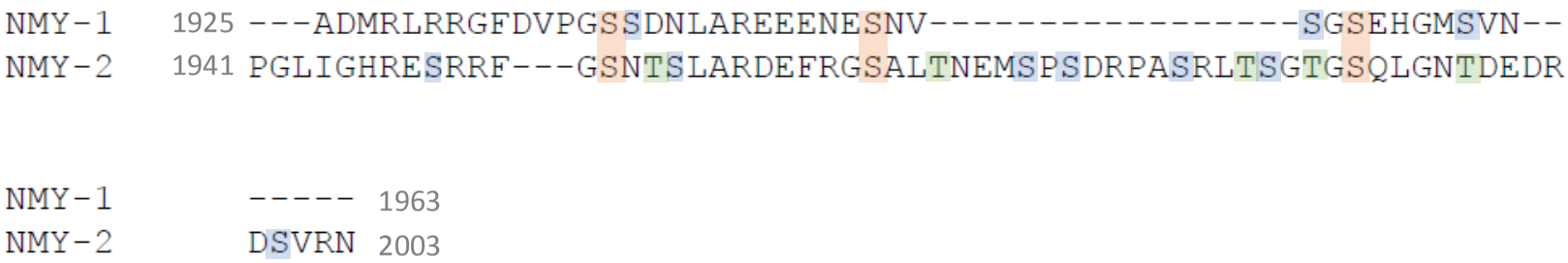


Figure 4-2. **Amino acid alignment of nonmuscle myosin non-helical tail piece in *C. elegans*.** Alignment was performed using Clustal Omega. Amino acids corresponding to the non-helical tail piece for NMY-1 was obtained from Piekny *et al.*, 2003. Amino acids corresponding to the non-helical tail piece for NMY-2 were obtained by aligning the entire protein of NMY-1 to NMY-2 and taking the sequence that aligned with NMY-1's helical tail piece. Numbers on either side of the sequence indicate the first and last amino acid position of the non-helical tail piece. Red coloring indicates serines in common between both proteins. Blue indicates serines that are not shared with the other protein and green indicates threonines that are not shared with the other protein.

2. Pachytene

NMY-2 is required for the maintenance of the ring channels and architecture of the gonad

1. Stem Cell Mitosis

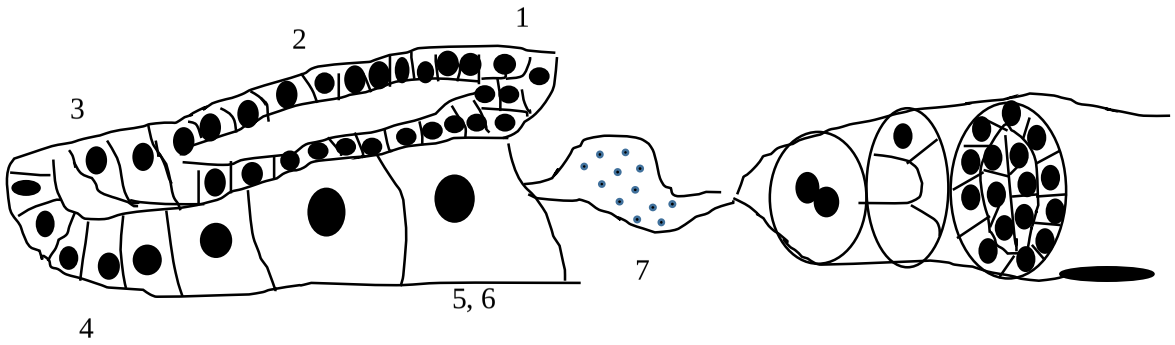
Neither nonmuscle myosin is required.

7. Ovulation

NMY-1 is needed for closure of the two valves on either side of the spermatheca and contractions of the spermatheca itself.

3. Exit from Pachytene

NMY-2 is required for cells to close their ring channels



6. Sheath Cell Contraction

NMY-1 may be also be required for sheath contractions.

4. Cytoplasmic Streaming

NMY-1 is the main motor for this process. NMY-2 is required for NMY-1 to form a matrix or plays its own role in this process

5. Meiotic Maturation

Neither nonmuscle myosin is required

Figure 4-3. **Steps of oogenesis in *C. elegans* requiring nonmuscle myosins.** Processes requiring NMY-2 are shown in red text, those requiring NMY-1 are shown in blue. NMY-2 is required for the early steps of oogenesis while NMY-1 is required for the later steps. Loss of NMY-2 does not show any defects in the stem cell population but does show a loss of membranes during pachytene. When the loss of membranes is rescued there is still a problem with the exit from pachytene. There is also no cytoplasmic streaming with the loss of NMY-2. Loss of NMY-1 stops cytoplasmic streaming and it localizes to the growing oocytes where the force for cytoplasmic streaming is generated. The phenotype seen with the loss of NMY-1 can be fully explained by the inproper ovulation and it has been shown that NMY-1 is needed for proper closure of the valves.

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