The role of ubiquitination during the removal of paternal organelles in *Caenorhabditis elegans*

By

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Abstract:

Ubiquitination regulates distinct pathways such as the cell cycle, endocytosis, transcription and DNA repair. We are interested in understanding whether ubiquitination is required for the strict maternal mitochondrial inheritance observed in most metazoans. There are three classes of enzymes responsible for ubiquitination. The ubiquitin activating enzyme is responsible for an ATP-dependent reaction that initially conjugates ubiquitin onto the enzyme. Ubiquitin is then transferred to the ubiquitin conjugating enzyme (UBC), which has a more specific role in determining the type of ubiquitin lysine chain that forms on the substrate. Ubiquitin ligases bind to substrates targeted for ubiquitination. To determine ubiquitin's role in eliminating paternal organelles, we studied the intermediate step in the pathway to determine the UBC responsible for tagging paternal organelles. In nematodes, paternal mitochondria and ubiquitinated sperm derived membranous organelles (MO) are eliminated through autophagy. Using a transgenic *C. elegans* strain with a GFP::Ubiquitin fusion expressed in the gonad we screened 26 E2s and observed changes in MO ubiquitination. We discovered that ubiquitination on MOs was reduced in simultaneous E2 knockdown of *ubc-18* and *ubc-16*. We used to this system to test the effect reduced ubiquitination had on paternal organelle persistence. Using live cell imaging we discovered that paternal mitochondria persisted during the 2 cell

stage in *ubc-18* knockdowns. On the other hand, sperm derived MOs also persisted past the 8 cell stage in double E2 knockdowns. *ubc-18* drives the recruitment of the proteasome during meiosis I and this localization is important for early removal of paternal mitochondria but not MOs. Ubiquitin is not required for the total elimination of paternal mitochondria. This study provides evidence that a ubiquitin independent pathway is working in conjunction to the ubiquitin dependent pathway. We demonstrate that more than one proteasomal event regulated by ubiquitin receptors RPN-10 and RAD-23 are required for elimination of MOs and paternal mitochondria without disrupting formation of the autophagosomes. Sperm ubiquitination was determined to not play role in tagging paternal organelles, but a possible role of ubiquitination during spermatogenesis and prevention of polyspermy is proposed.

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List of Abbreviations

Ub- ubiquitin
UBC-ubiquitin conjugating enzyme
PME- paternal mitochondria elimination
MO- membranous organelles
GFP- green fluorescent protein
K48- lysine 48
K63- lysine 63
NGM- nematode growth medium
RNAi- ribose nucleic acid interference
APC- anaphase promoting complex
MSP- major sperm protein

CHAPTER 1:

Introduction

Ubiquitination

Ubiquitination is the addition of one or more ubiquitin (Ub) molecules to a substrate[1]. Ubiquitin is a 76 amino acid protein found in most eukaryotic cells[2]. The C- terminus of the glycine in ubiquitin can be conjugated to the ε-amino group of a lysine or α-amino group of the N-terminal amino acid in a substrate protein [3]. Ubiquitination is responsible for directing various signals and processes, the type of Ub modification specifically influences the fate of each substrate [4]. There are three enzymes involved in ubiquitination of substrates. Those enzymes are known as E1, E2 and E3. The E1, ubiquitin activating enzyme is responsible for an ATP-dependent reaction where Ub+AMP product remains bound to the E1 until a thioester bond is formed with the cysteine on the E1[5]. Then, Ub is transferred to a well conserved cysteine in the active site of the E2 enzyme. The E2 enzyme, also known as the ubiquitin conjugating enzyme (UBC), catalyzes the transfer of Ub to a lysine on the targeted substrate forming, an isopeptide bond. Alternatively, the E2 can transfer Ub to a catalytic cysteine found on the HECT family of E3s, which then transfers Ub onto the targeted

substrate forming an isopeptide bond[6]. The interactions between the E2/Ub and E3 substrate designate the type of Ub modification[7, 8] (Figure 1).

Depending on cellular cues, ubiquitin modifications vary. In some cases, a single Ub can be added to a substrate (monoubiquitination and,multi-monoubiquitination), in other cases a chain of covalently bonded Ub molecules are added (polyubiquitination). Polyubiquitination involves the lysine (K) on the ubiquitin already bound to a substrate or the N-terminal methionine residue (M-1). In lysine ubiquitin chains, the ε-amino group of the lysine forms an isopeptide bond with the C- terminal glycine of the new ubiquitin [9]. These chains are linked by any of the 7 lysine or methionine (K6, K11, K27, K29, K33, K48, K63 and M-1) residues present on Ub[10]. K63 and K48 chains are the most characterized chains (Figure 2).

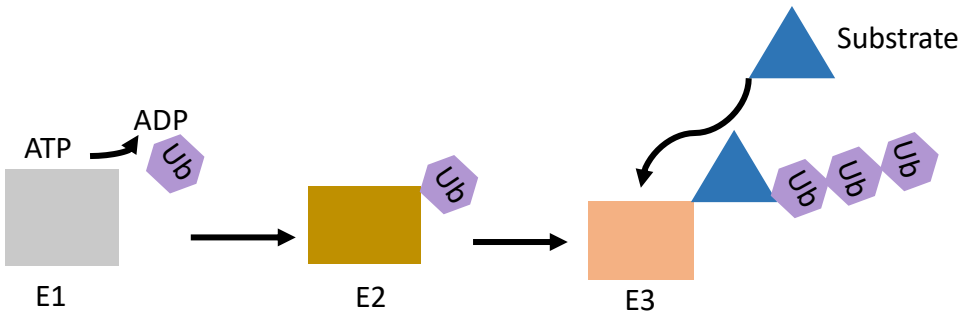


Figure 1- Enzymes involved in the ubiquitination process.

The ubiquitin activating enzyme (E1) is responsible for initiating the ubiquitination cascade by catalyzing the reaction where ubiquitin is "activated". Energy from ATP is used to generate a thioester bond between the E1 enzyme and ubiquitin. The newly activated ubiquitin is then transferred to the ubiquitin conjugating enzyme (E2) via a transthiolation reaction. Finally, the ubiquitin ligase will bind to both the substrate and E2-Ub conjugate and facilitate the transfer of ubiquitin to the substrate. The substrate can be tagged with a single ubiquitin (monoubiquitination) or multiple ubiquitin proteins in a chain (polyubiquitination).

K48 chains and Proteasomal Degradation

K48 ubiquitin chains are well characterized post-translational modifications associated with protein degradation. Commonly, proteins tagged with K48 chains are degraded by the 26S proteasome, a large protein complex that requires metabolic energy to degrade tagged proteins[11]. The proposed model for proteasomal degradation states that minimally four ubiquitin molecules are necessary to be efficiently recognized by the proteasome[12]. However, recent studies have shown that monoubiquitination can also serve as a recognition signal for the proteasome[13] (Figure 2A).

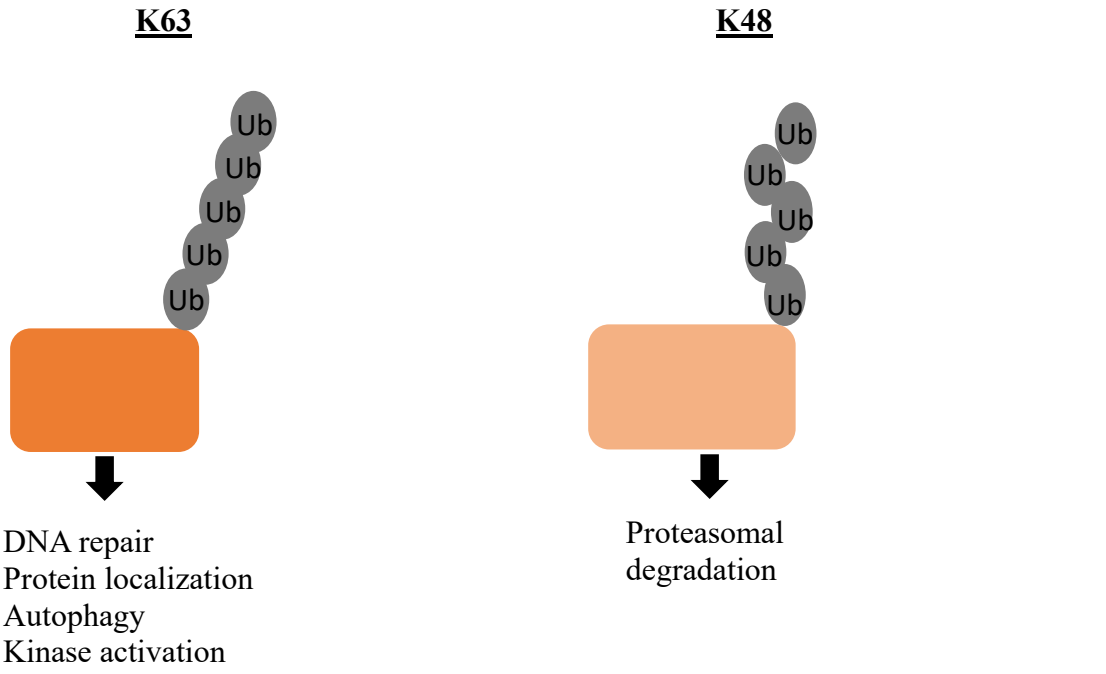
The 26S proteasome complex contains 33 different subunits that are arranged to form a central 20S core particle with one or two 19S regulatory particles[14] (Figure 2C). The core particle is composed of two outer β- rings and two inner α rings, each of which are made up of seven α and β subunits [15]. The α ring serves as channel that restricts the entrance of undamaged proteins into the proteasome. The 19S regulatory particles has six ATPases subunits designated as Rpt1-6 and 13 non-ATPase subunits designated Rpn1-3, 5-13, and 15[16]. Ubiquitin-recognizing subunits, such as Rpn-10 and Rpn13, bind to ubiquitinated substrates in a direct or indirect manner. There are also 3

proteasome-associated proteins, like Rad23, that interact with the proteasome through its ubiquitin-like domain and to ubiquitinated proteins through one or more ubiquitin-associated domains. Redundancy in ubiquitin recognizing particles exists in cells, most of these subunits weakly associate with the proteasome and are not associated with proteasome function but have been found to mediate nonproteolytic functions of ubiquitination[17].

K63 chains

K63 ubiquitin chains have a linear confirmation that has been extensively characterized and associated with several cellular processes such as DNA repair, endocytosis and autophagy [18]. DNA repair factors are regulated by K63 ubiquitin chains binding to DNA or K63 histone ubiquitination [19, 20]. K63 chains can likewise conjugated onto membrane proteins are thought to bring in clathrin associated proteins that signal the internalization of selective cargo and promote endocytosis[21]. Selective cargo for autophagy is also tagged with K63 chains. In the case of autophagy, K63 chains facilitate the recruitment of autophagy receptors that surround the cargo with a double membrane forming the autophagosome[22] (Figure 2A).

A


K63
K48
Ub
Ub
Ub
Ub
Ub
Ub
Ub
Ub
Ub
DNA repair
Protein localization
Autophagy
Kinase activation
Proteasomal
degradation

B

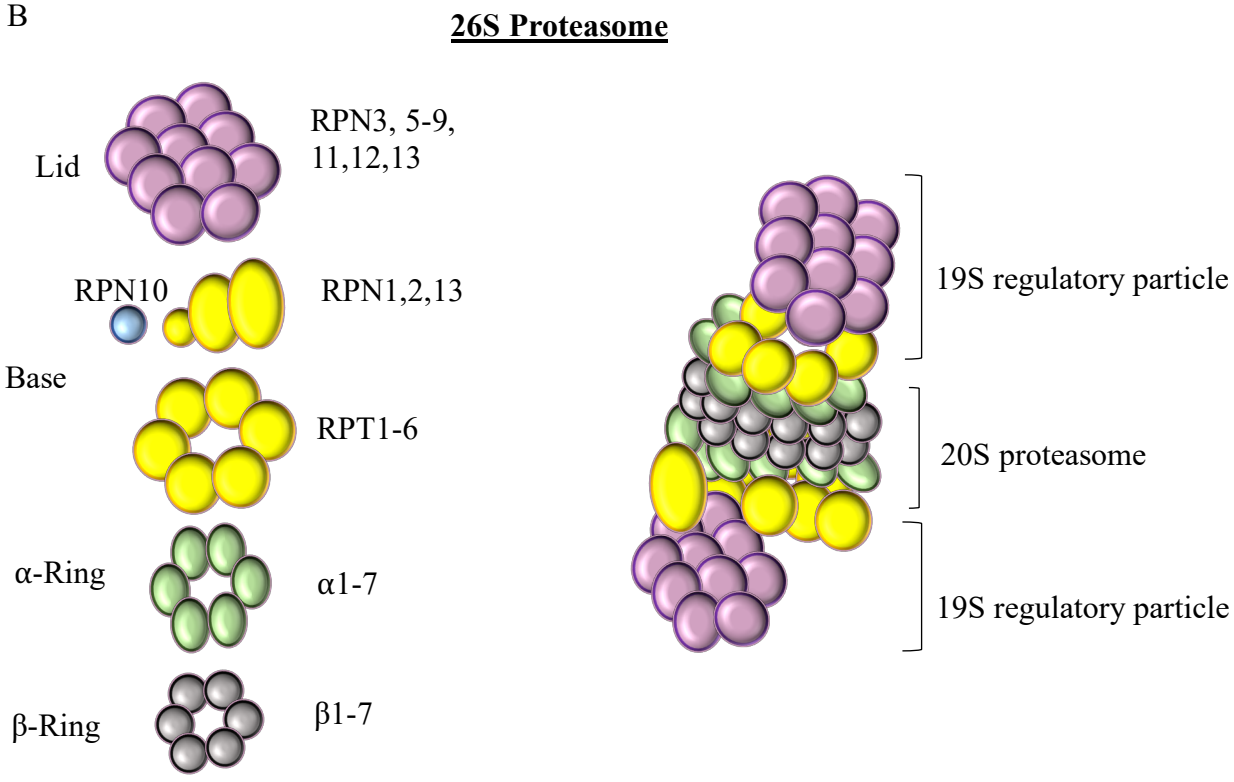

26S Proteasome
Lid
RPN3, 5-9,
11,12,13
RPN10
RPN1,2,13
Base
RPT1-6
α-Ring
α1-7
β-Ring
β1-7
19S regulatory particle
20S proteasome
19S regulatory particle

Figure 2- Substrates tagged with K63 and K48 ubiquitin chains have different fates. **(A)**K63 chains have a linear confirmation that is recognized by effector proteins for processes such as DNA repair, protein sorting through endosomal vesicles, kinase activation and autophagy. On the other hand, K48 chains have a non-linear confirmation that is recognized by different subunits on the proteasome. Once the proteasome is recruited to substrates with K48 chains, theses substrates are usually degraded. **(B)** The proteasome is large protein complex that has different subunits that help recognize targeted particles and other subunits that have protease activity.

Selective Autophagy

Autophagy is the process that involves cargo such as proteins or organelles being surrounded by a double membrane forming the autophagosome, which is later degraded by the lysosome[23, 24]. The autophagosome will surround substrates depending on the signals provided by the cell. Cells will tag cargos for degradation, in a process referred to as selective autophagy[23] Selective autophagy depends on the interactions between the cargo and autophagy proteins. Important selective autophagy proteins include cargo adaptor proteins which have a ubiquitin binding domain (UBD) and an LC3-interacting region (LIR), these domains help adaptor proteins attach to ubiquitinated cargo and begin

the formation of the autophagosome[25]. When selective autophagy is induced, adaptor proteins such as p62, OPTN, Ndp52, NBR1, TAXIBP1 proteins are required for binding LC3. Before LC3 can bind to the adaptor proteins it must be associated with membrane PE (phosphatidylethanolamine) to form the nascent autophagosome membrane around selective cargo (Figure 3 [23, 26]. Ubiquitination can serve as a recognition signal under certain conditions to induce autophagy. K63 chains on substrates are commonly recognized by p62 adaptor protein targeting them from degradation [22]. Cargo adaptor proteins assure that specific proteins or organelles are surrounded by the autophagosome that will eventually fuse with the lysosome and degrade its contents[27] (Figure 3A).

Mitophagy

Mitophagy is a form of selective autophagy where damaged mitochondria are tagged with ubiquitin and elimianted[28]. Healthy mitochondria have a proton gradient across their inner membrane that is maintained by the proton pumps[29]. However, when mitochondria are damaged and the proton gradient is disturbed , mitophagy is triggered. PINK1 (PTEN-induced Kinase 1) and Parkin proteins are important for the initiation of mitophagy. Mitochondrial depolarization triggers stabilization of PINK1, a Parkin and ubiquitin kinase to the mitochondrial

membrane which subsequently recruits Parkin, a ubiquitin ligase to initiate ubiquitination of mitochondrial membrane proteins[28]. Ubiquitin chains attract p62, OPTN or NBR1 which facilitate interactions with LC3 proteins bound to membrane PE to recruit membranes that will form the autophagosome[30-32]. Studies have shown that K63 and K48 chains are both associated with mitophagy[30, 33-35]. K48 chains direct the proteasome to degrade mitofusion proteins, which is a required upstream event of mitophagy [28] (Figure 3B).

During times of cellular stress, mitophagy prevents propagation of the damaged mitochondria. These organelles are also eliminated during early embryogenesis. Following fertilization, the embryo selectively eliminates the paternal mitochondria. Similarities exist between the removal of paternal mitochondria and mitophagy. There is still some debate in the field whether or not paternal mitochondria are tagged for elimination because they are damaged[35-40]. However, evidence strongly supports the involvement of ATG autophagy proteins and LC3 during paternal mitochondria elimination (PME)[33, 41-43].

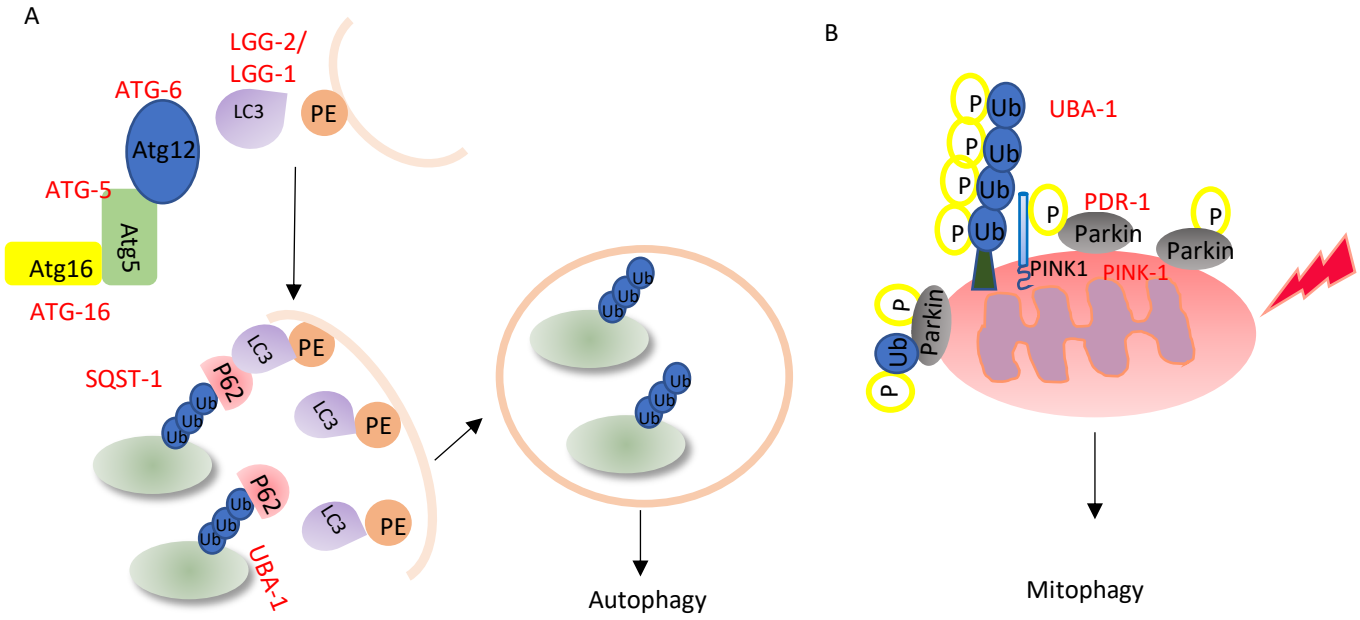


Figure 3- Homologous selective autophagy and mitophagy components are required during PME

(A) Ubiquitination of specific proteins directs the formation of the autophagosome around those targets. Selective autophagy is directed by a series of ubiquitin interacting proteins ATGs that are highly conserved among species. Homology exists in autophagy and mitophagy proteins among different species. The figure above shows some of the mammalian proteins (Black) involved in selective autophagy and mitophagy with *C. elegans* homologues in red. **(B)** The mitophagy pathway also uses ubiquitination as a tag for the formation of autophagosome around damaged mitochondria.

Mitochondrial Inheritance

Mitochondria are essential organelles; their dynamics affect many processes within the cell. Changes in mitochondria dynamics, such as cellular localization and numbers can be triggered as a response to environmental stressors or developmental signals. Developmental signals that cue changes in the cell cycle trigger the segregation of cellular components in order to maintain symmetry in the distribution of double membraned organelles between daughter cells[44]. Early work studying the origin of double membrane organelles determined that the mitochondria had to result from preexisting structures based on the concept that biological membranes are derived from preexisting membranes not de novo[45]. While monitoring the distribution of mitochondria in budding yeast, cytoskeletal rearrangement was required for symmetrical inheritance of the mitochondria[44]. Therefore, pre-existing mitochondria were evenly passed down to daughter cells. Following this observation, studies were directed towards understanding how sexually reproducing animals with two types of mitochondria passed down mitochondria to their offspring. Evidence for strict material inheritance of the mitochondria was found in most animals [36, 46].

Strict maternal inheritance relies on mechanisms responsible for paternal mitochondria elimination (PME) after fertilization. Mechanisms for PME differ between organisms[33, 41-43]. However, the processes of autophagy and ubiquitination have been characterized in all organisms studied (Figure 4).

Mechanisms Involved in the Elimination of Paternal Mitochondria

Ubiquitination is observed during the removal of paternal mitochondria following fertilization in many organisms[33, 35, 42, 47]. In mammals, sperm mitochondria are tagged with ubiquitin and then are eliminated through autophagy[47]. Evidence shows that proteasome activity is required for the elimination of paternal mitochondria in mammals, when proteasome inhibitors were used on early embryos, paternal mitochondria persisted in later embryos[47, 48]. Drosophila also utilizes ubiquitination and autophagy components to eliminate paternal mitochondria, although their process of elimination is different. In flies, PME is initiated prior to fertilization with paternal mitochondrial DNA (mtDNA) being degraded by endonuclease G during spermatogenesis[49]. During fertilization, whole sperm enter the oocyte in a process similar to endocytosis, which requires the degradation of sperm

plasma membrane to allow delivery of sperm DNA into the oocyte. This process is followed by ubiquitination of paternal mitochondria with K63 ubiquitin chains[50, 51]. These tagged organelles are encapsulated by the autophagosome and removed through lysosomal degradation.

The pathway for removing paternal mitochondria observed in *C. elegans* contains all the previously outlined components (Figure 4). Yet, *C. elegans* have two types of paternal organelles that are removed shortly after fertilization, the paternal mitochondria and nematode specific membranous organelles (MOs)[33, 41]. The MOs are Golgi-derived vesicles that are ubiquitinated shortly after fertilization, their function is not well understood. MOs are ubiquitinated with two types of ubiquitin chains, K48 and K63. While K63 chains persist throughout embryogenesis, K48 chains are only observed during meiosis I[52]. During meiosis I proteasomal recruited to the MOs is also observed. Paternal mitochondria cluster together with ubiquitinated MOs in a consistent pattern as they are surrounded by the autophagosome[33, 41, 43, 52] (Figure 5). Ultimately, paternal organelles are removed through lysosomal degradation[53]. The contribution of *C. elegans* sperm ubiquitin during ubiquitination of paternal organelles has not been well characterized. However, in mammalian and Drosophila sperm, ubiquitination of paternal mitochondria has been

observed[35, 47]. There are differences in PME between different organisms. However, *C. elegans* is a sexually reproducing organism that provides with an easy model to genetically manipulate and grow in a lab setting. It is also significant that a simple organism, such as *C. elegans* utilizes homologous components in the PME pathway as is observed in mammals.

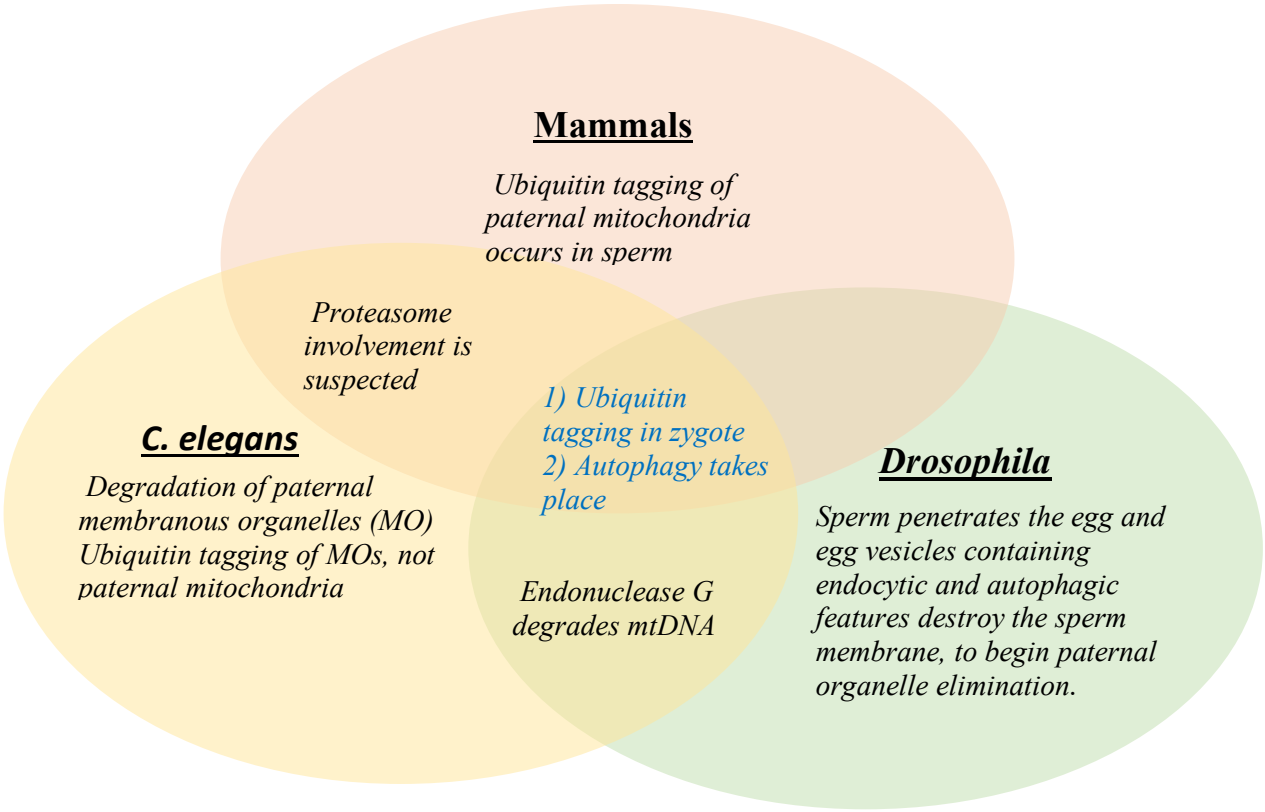


Figure 4- Multiple Mechanisms play a role during PME in invertebrate and vertebrate models

C. elegans , *D. melanogaster* and mammals have been used to better understand the mechanisms involved in removing paternal organelles. This figure highlights unique and overlapping mechanisms found these organisms. Concerning mechanisms involved in the removal of paternal mitochondria, autophagy and ubiquitination are well conserved throughout different species.

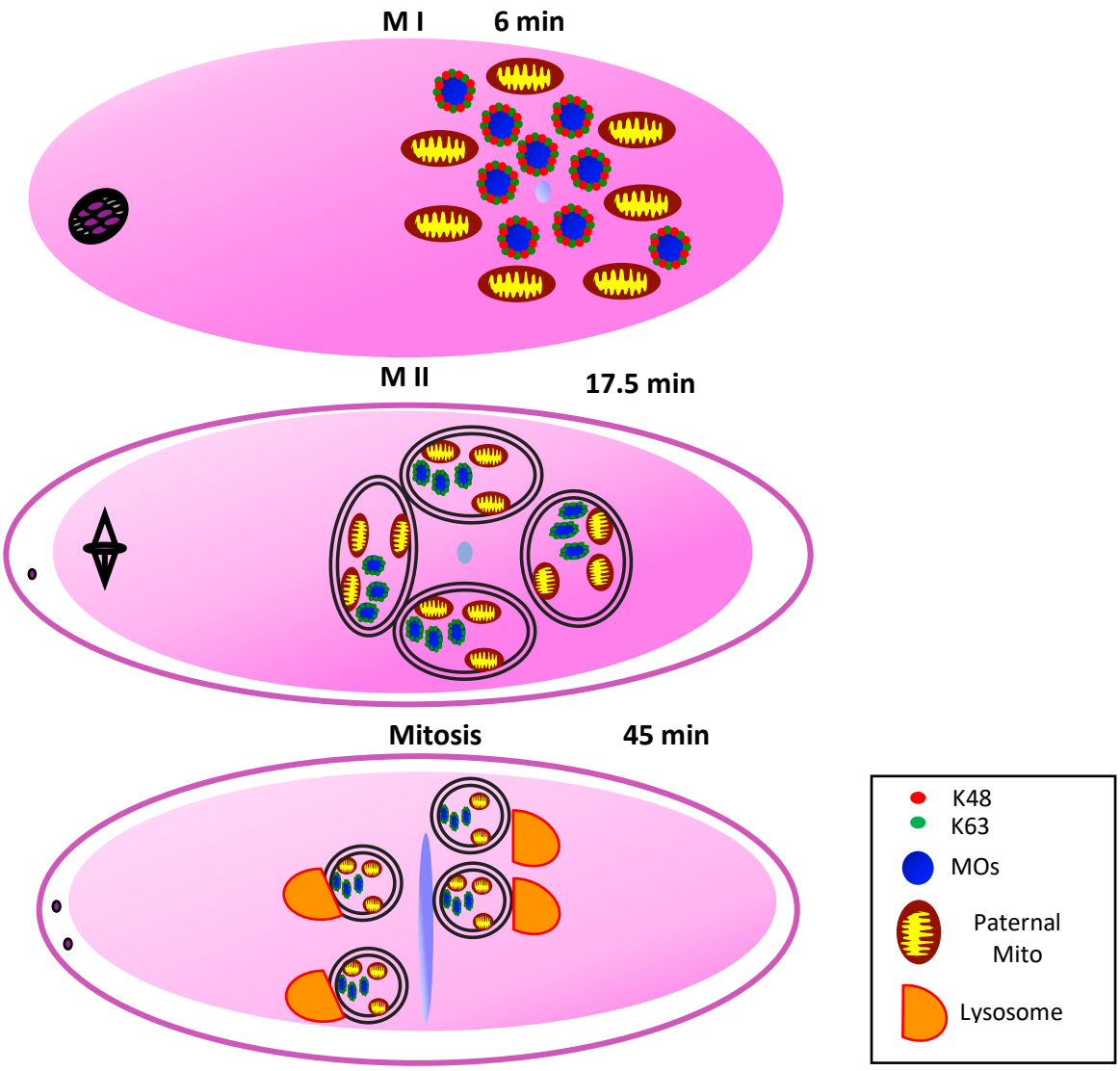

M I
6 min
M II
17.5 min
Mitosis
45 min
K48
K63
MOs
Paternal Mito
Lysosome

Figure 5- Model for *C. elegans* paternal organelle degradation.

After fertilization During meiosis I, nematode specific membranous organelles (MOs) are tagged with K48 (red) and K63 (green) ubiquitin chains. Paternal mitochondria cluster with MOs around the sperm DNA. By meiosis II, K48 chains have disappeared and only K63 chains remain on the MOs. During meiosis II, autophagosomes begin to form around both paternal organelles. In metaphase, the paternal organelles have pericentrosomal localization and begin to fuse with the lysosome for degradation. Post- fertilization times are indicated in this figure.

Project Objectives and Summary

The goal of this study was to determine the role of ubiquitination during post fertilization events which lead to the removal of paternal mitochondria and MOs in *C. elegans* . The current model for PME in *C. elegans* , proposes that MOs are selected for elimination by ubiquitination shortly after fertilization. Autophagosomes surround ubiquitinated MOs and paternal mitochondria due to the clustering of these organelles that occurs during meiosis and II. Therefore, we want to determine if ubiquitination of MOs is driving PME .

After conducting an RNAi screen of the 24 *C. elegans* UBCs, results showed that more than one UBC was responsible for ubiquitinating MOs. A double UBC knockdown screen revealed *ubc-18*/*ubc-16* combination was required for ubiquitination of MOs in the early embryo. Further investigation showed that *ubc-18* was required for the addition of K48 chains on MOs and proteasome localization to these organelles during meiosis I. Inhibition of these events lead to a delay in PME at the 2 cell stage. Additionally, reduction in MO ubiquitination in *ubc-18*/*ubc-16* simultaneous knockdowns lead to persistence of MOs in later embryonic stages. These results suggest the presence of a ubiquitin dependent pathway that involves degradation of MOs and early removal of paternal mitochondria during the first mitosis.

PME and MO elimination was also delayed in the absence of proteasome subunits RPN-10 and RAD-23 without disrupting the formation of autophagosomes. These findings propose that two separate maternal events during development require proteasome-ubiquitin interactions for PME. This study also supports findings that show non-canonical mitophagy component FNDC-1 aids in PME but not the removal of MOs.

CHAPTER 2:

Materials and Methods

Worm strains and maintenance

C. elegans strains were grow on nematode growth medium (NGM) seeded with a bacterial lawn of OP50 E. coli strain and incubated at 20 °C or 25 °C. Wildtype, *ubc-18*(ku354), and *fndc-1*(my14) nematodes were grown at 20 °C. Transgenic nematodes were grown at 25 °C, except VIG19 which was grown at 16 °C. Strains used in this study are listed in Table 1.

Strain Name	Genotype
LN130	*rcIs31 [pie-1p::GFP::Ub + unc-119(+)]; ltIs37[pie-1p::mCherry::his-58]*
LN153	*Is Ppie-1::mCherry::H2B; rcSi2[Pmex-5::rpt-1::GFP + unc-119]II*
WY34	*ubc-18(ku354)* III
KWN774	*him-5::fndc-1(my14)*
VIG19	*mex-5p::GFP::lgg-1*
N2 (Bristol)	Wildtype

Table 1 List of *C. elegans* strains with their genotype used in this study.

Male populations and mating

Male N2 populations were generated by soaking 20 L4 hermaphrodites in 7% ethanol in M9 buffer at 20° C for 20 minutes. F2 generations were screened for males. Males were maintained by mating 10-20 males with 1-2 hermaphrodites on 60 mm mating plates (NGM plates seeded with 100 μL saturated overnight OP50 E. coli culture). Matings between strains were performed by placing 10 hermaphrodites and 20-30 males on mating plates at 20° C overnight for N2 hermaphrodites and 25° C for transgenic hermaphrodites. Embryos from matings were observed 24-48 hours after incubation.

Antibodies

Primary antibodies used in this study at 1:100 concentrations were rabbit anti-K48 ubiquitin (Apu2 from Millipore), rabbit anti-K63 (Apu3 from Millipore), rabbit anti-19S proteasome antibody (Ab2942 from Abcam) , FK2 anti-polyubiquitin (BML-PW8810-0100, Enzo Life Sciences) and mouse monoclonal anti-1CB4 (generous gift from Steve L'Hernault at Emory University). Secondary antibodies used for immunofluorescence at 1:100 concentration were goat anti-mouse FITC and goat anti-rabbit TRITC (Jackson ImmunoResearch Laboratories).

Antibody staining

Presence of K48 and K63 chains were determined using K48 and K63 specific antibodies. Co-localization of each chain with MOs was determined using chain antibodies co-stained with 1CB4 antibodies. Embryos were extracted from one day adults by cutting them open on poly-L-lysine-coated slides. Slides were placed in liquid nitrogen for 5 minutes and then fixed with methanol at -20 °C for 20 minutes. Slides were incubated in primary antibody overnight at 4 °C and then incubated with appropriate fluorescently labeled secondary for 2 hours at room temperature. Primary and Secondary antibodies were diluted in PBST:30% NGS (normal goat serum) and washes were done in PBST (Phosphate Buffered Saline with 0.5% Tween-20). Vectasheild (Vector Labs, Burlingame, CA) plus DAPI was used as the mounting medium. Stained samples were imaged using the LSM 700 confocal microscope equipped with Zeiss software Zen (Black edition).

Mitotracker staining

N2 males were labeled with Mitotracker Red CMXRos (Invitrogen) as described in Hajjar *et al* [52]. 10-15 L4 hermaphrodites were added to mating

plates containing 20 Mitotracker soaked N2 or fundc-1(my14) males. They were allowed to mate for 24 hours at 25°C. Embryos were observed 42- 48 hours after mating using Zeiss LSM 700 confocal microscope[54].

Fluorescence microscopy

For RNAi screen, embryos were observed on the Nikon AZ100 multipurpose zoom microscope. All florescent images were acquired using the Zeiss LSM 700 confocal microscope and analyzed using Zen (black edition) software. For live cell and antibody staining experiments, the 488 nm laser was used to excite GFP and FITC fluorescence, and 555 nm laser was used to excited mCherry, TRITC and Mitotracker red fluorescence. DAPI was excited using a 405 nm laser. Acquisition settings were kept constant between samples to allow for meaningful comparison.

Acquisition settings for experiments:

GFP::Ub assessment : 5% laser, 800 mCherry gain, 718 GFP gain, pixel dwell 25.2μs, pin hole 41 μm.

Detection of ubiquitin chains: 5% laser, TRITC 484 gain, FITC 437 gain, DAPI 600 gain, pixel dwell 12μs, pinhole 39 μm.

MO Counts: 7% laser, FITCI 750 gain, DAPI 650, 12.6 μs pixel dwell, 39 μm pinhole.

Proteasome localization: 7% laser, GFP 700 gain, mCherry 750 gain, 12.6 μs pixel dwell, 37 μm pinhole.

Mitotracker: 5% laser, mCherry 750 gain, 12.6 μs pixel dwell, 47 μm pinhole.

Sperm antibody staining: 5% laser, FITC 750 gain, TRITC 650 gain, DAPI 456 gain, 12.6 μs pixel dwell, 41 μm pinhole.

RNAi by Feeding

RNAi knockdowns were achieved by feeding the worms bacteria expressing dsRNA for each gene of interest. RNAi clones were obtained from the Ahringer library or the Vidal ORF library (*ubc-18*). Simultaneous knockdowns were achieved by feeding worms 1:1 mixture of each RNAi clone. Controls for the RNAi experiments included a L4440 plasmid vector without a gene coding region transformed in the HT115 bacterial strain, as well as the embryonic lethal gene (ubc-2) as a positive control. The positive control was also diluted 1:1 with the empty vector to establish the efficiency of a double knockdown. RNAi clones were streaked from glycerol stocks onto tryptic soy agar with ampicillin (100μg/mL) and tetracycline (10 μg/mL) overnight. Then single colonies were inoculated in tryptic soy broth with ampicillin and tetracycline. Bacterial cultures were grown at 37 °C in shaking incubator for 16 hours. NGM plates containing

0.2% lactose were seeded with bacterial overnights and L1 or L4 worms were transferred to dry plates. Worms were allowed to grow at the appropriate temperature for each strain. In experiments with double UBC knockdowns, F1 one day adults were examined.

Statistical Analysis

Each figure legends explains sample size for each experiment. Two sample z-tests were performed using VasarStats on data from figure 1C. Fisher's exact test was performed on data that was less than 20 embryos and to find significance in embryonic lethality study. Error bars presented in this dissertation represent a 95% confidence interval and were derived using the modified Wald method on GraphPad. Student t-test was used to determine the significance of difference between groups and error bars represent SEM.

CHAPTER 3:

Ubiquitin conjugating enzymes (E2) required for ubiquitination of membranous organelles

Introduction

C. elegans studies in PME looked at two types of paternal organelles that are removed shortly after fertilization, the paternal mitochondria and nematode specific membranous organelles (MOs) [33, 38, 41, 43]. MOs are found in sperm as Golgi derived vesicles that contain major sperm protein (MSP). Release of MSP from the MOs is required for spermatid maturation and fertilization, thou their exact function remains unknown[55]. MOs become ubiquitinated with two different types of polyubiquitin chains following fertilization, K48 and K63 ubiquitin chains [52]. These chains are detected at different times during early development. K63 chains are present from meiosis I until the MOs are degraded. Conversely, K48 chains only appear during meiosis I. The presence of two types of ubiquitin chains suggests two independent ubiquitination events are taking place.

The type of chain is an important factor in determining the fate of a substrate. E2s are the enzymes responsible for directing the type of ubiquitin chain that is elongated onto the substrate[56]. During ubiquitination E2s interact

with E1 and E3 enzymes. E2s affinity for each enzyme is crucial for the formation of polyubiquitin chains. E2-E3 interactions are weak and during polyubiquitination E1 cannot conjugate a new ubiquitin to an E2 that is still bound to an E3[57, 58]. Therefore, the creation of polyubiquitin chains is a series of binding and unbinding interactions between E2 and E3 because E1s are rapidly conjugating ubiquitin to E2s when they are not bound to E3.

There are several mechanisms by which E2s can polyubiquitinate substrates. E2s that are involved in adding the initial ubiquitin to a substrate may lack specificity for a particular lysine on a substrate. This allows these E2s to interact with multiple E3s and allows them to form polyubiquitin chains[56]. Yet, other E2s that are more selective in the addition of ubiquitin to a substrate, potentially only recognize a specific lysine residue. Sets of E2s usually modify substrates with single ubiquitin proteins[59]. E2s can work together to initiate and elongate ubiquitin chains. This mechanism can be observed during polyubiquitination in yeast where APC/C is modified by both Ubc4, which adds ubiquitin to a lysine on the substrate, and Ubc1 which extends the K48 ubiquitin chains[60].

Elongating E2s are very specific to the type of chains they are adding to substrates. Some examples of E2 specificity which are the human E2s, UBE2K, which has been shown to elongate K48, and UBE2N and UBE2V1 which both produce K63 chains[61-63]. In addition to elongating chains after monoubiquitination, there are some E2s that will elongate short chains so that another E2 can further elongate it, increasing the rate of chain formation[64]. Conversely, there are E2s that both initiate and elongate specific chains. Yeast E2 Cdc34 is an example of this, that works with SCF-type E3s to add K48 chains during the cell cycle[65].

The ubiquitination of substrates depends on the interactions of the three ubiquitin enzymes previously outlined (Figure 1). The E1 enzyme can activate numerous ubiquitination events. Knockdown of E1 genes affects overall ubiquitination and is not an effective way to study specific ubiquitination events. In contrast, E3s are very specific to the target substrate or a group of substrates. However, organisms have large number of E3s and finding the specific E3 for a given substrate is work intensive. While E2 provides some specificity to the pathway, the number of genes present in organisms is more manageable. For example, *C. elegans* has about 500 E3s and 25 E2s. Therefore, it was determined that identifying the E2s responsible for ubiquitination of MOs was key in teasing

out the role MO ubiquitination plays in PME. Table 2 summarizes the known *C. elegans* E2s and their gene names. Given the presence of two ubiquitin chains on MOs and the fact that E2s can work in pairs to elongate chains, E2 combinations were assessed. Our results indicate that ubiquitination of MOs is driven by UBC-18 and UBC-16.

Locus ID	**Worm Gene Name**	**Individually Screened**	**Used in Combination Screen**
C35B1.1	*ubc-1*	Yes	Yes
Y69H2.6	*ubc-19*	Yes	Yes
Y71G12B.15	*ubc-3*	Yes	Yes
M7.1	*let-70, ubc-2*	Yes	No
F40G9.3	*ubc-20*	Yes	Yes
R01H2.6	*ubc-18*	Yes	Yes
Y54E5B.4	*ubc-16*	Yes	Yes
B0403.2	*ubc-17*	Yes	Yes
C28G1.1	*ubc-23*	Yes	Yes
C17D12.5		Yes	Yes

Y110A2AR.2	*ubc-15*	Yes	Yes
Y110A2AM.3	*ubc-26*	Yes	Yes
D1022.1	*ubc-6*	Yes	Yes
F58A4.10	*ubc-7*	Yes	Yes
Y87G2A.9	*ubc-14*	Yes	Yes
Y94H6A.6	*ubc-8*	Yes	Yes
Y54G2A.31	*ubc-13*	Yes	Yes
C06E2.3	*ubc-21*	Yes	Yes
C40H1.6		Yes	Yes
F25H2.8	*ubc-25*	Yes	Yes
C06E2.7	*ubc-22*	Yes	Yes
F49E12.4	*ubc-24*	Yes	Yes
F39B2.2	*uev-1*	Yes	Yes
F56D2.4	*uev-2*	Yes	Yes
F26H9.7	*uev-3*	Yes	Yes
F52C6.12	*none*	Yes	No
F29B9.6	*ubc-9*	Yes	No
R09B3.4	*ubc-12*	Yes	No

Table 2. List of *C. elegans* E2s with their gene name and common names.

C. elegans has 25 E2s and 3 E2 variants. 23 enzymes and 3 variants were individually screened for the presence of MO ubiquitination. 21 enzymes and 3 variants were used for double combination screens totaling 276 combinations. Since we were interested in ubiquitination, *ubc-9* and *ubc-12* were excluded from both screens because these enzymes are associated with SUMO (small ubiquitin like modifier) and NEDD8 respectively. *ubc-2* was excluded from combination screen because its embryonic lethality phenotype made it difficult to screen F1 embryos; F52C6.12 was also excluded because its RNAi clone was 80% homologous to *ubc-2* and worms expressed the same embryonic lethal phenotype.

Results:

C. elegans ubiquitin conjugating enzyme screen

Ubiquitinated MOs follow a clear pattern that has been confirmed with MO and ubiquitin antibody staining[33, 52]. MOs surround the paternal DNA during meiosis I and II, as the pronucleus begins to form, the MOs cluster towards the side of the sperm pronucleus. As the pronuclei move towards the middle of the cell, the MOs begin to line up in a pericentrosomal arrangement[41] (Figure 6). Therefore, using a transgenic worm expressing GFP-tagged ubiquitin (GFP::Ub) in the germline is an effective assay to assess MO ubiquitination through an E2 reverse RNAi genetic screen. The transgenic worm also expressed H2B as an mCherry fusion which would allow tracking of the embryonic stage of each embryo (Figure 6). The E2 screen was performed as explained in figure 7.

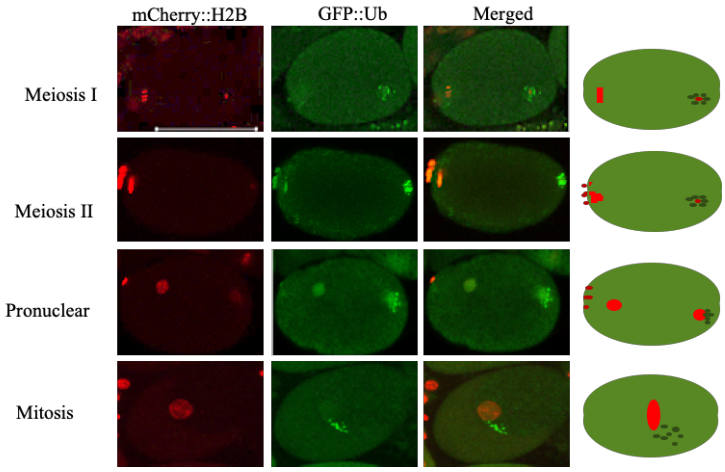


Figure 6 Transgenic *C. elegans* strain used for reverse RNAi genetic screen.

(A) *C. elegans* strain LN130 has GFP::Ub and mCherry::H2B tags in the germline. This strain was used to screen for E2s responsible for ubiquitinating MOs during the one cell stage because ubiquitinated MOs follow a distinct pattern that can be tracked with GFP::Ub. Wildtype embryos have a characteristic distribution of GFP::Ub following fertilization. The cartoon embryos to the right depict the characteristic pattern of GFP::Ub (green) and histone (red). During meiosis I and meiosis II, GFP::Ub is concentrated on the MOs and localizes around the sperm DNA in the posterior region of the embryo (posterior side of the embryo is designated by the point of entry of the sperm). During pronuclear formation, the MOs remain ubiquitinated and become more dispersed in the embryo. 10 one cell embryos were scored for each combination. As the embryo reaches the first mitosis, MOs begin to align in a pericentrosomal pattern. Scale bar represents 50 μm.

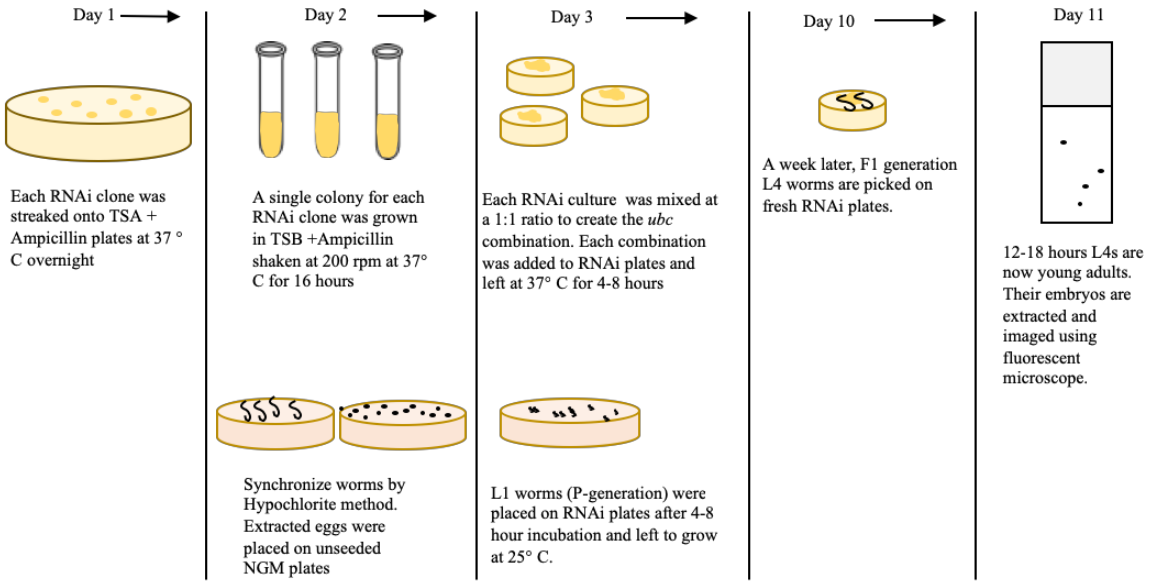


Figure 7- Experiment design of RNAi E2 screen

The RNAi screen was performed using a feeding method in which *C. elegans* were fed RNAi bacterial clones for each E2 gene as well as double combination of E2s. Initial screen analyzed 10 embryos per combination on Nikon AZ100 multipurpose zoom microscope. Combinations were considered hits when 50% or less of the embryos analyzed had GFP::Ub vesicles. The exact procedure outlined above was repeated to confirm hits using confocal microscopy. 20 embryos were scored for each combination for confirmation.

The appearance of GFP::Ub vesicles was normal in single E2 RNAi knockdown embryos (Figure 8A). However, the combination screen revealed some promising hits that were confirmed with confocal microscopy (Appendix 1). An E2 combination was considered a hit when 50% or less of the embryos analyzed expressed normal GFP::Ub. Simultaneous knockdown of *ubc-18* and *ubc-16* showed a consistent 40% reduction in embryos with GFP::Ub labeled vesicles (Figure 8C). Embryos from *ubc-18*/*ubc-16* knockdown worms appeared wildtype except for the absences of GFP::Ub vesicles surrounding the paternal DNA during meiosis(Figure 8D). Double knockdown results collaborate previous work that showed MOs were ubiquitinated with both K48 and K63 ubiquitin chains[52]. Therefore, we hypothesized that at least two E2s would be required for MO ubiquitination. Since the double knockdown reduced ubiquitination in 40% of the embryos observed, these results could indicate the possible presence of other ubiquitin chains, incomplete knockdown through RNAi or the involvement of other E2s.

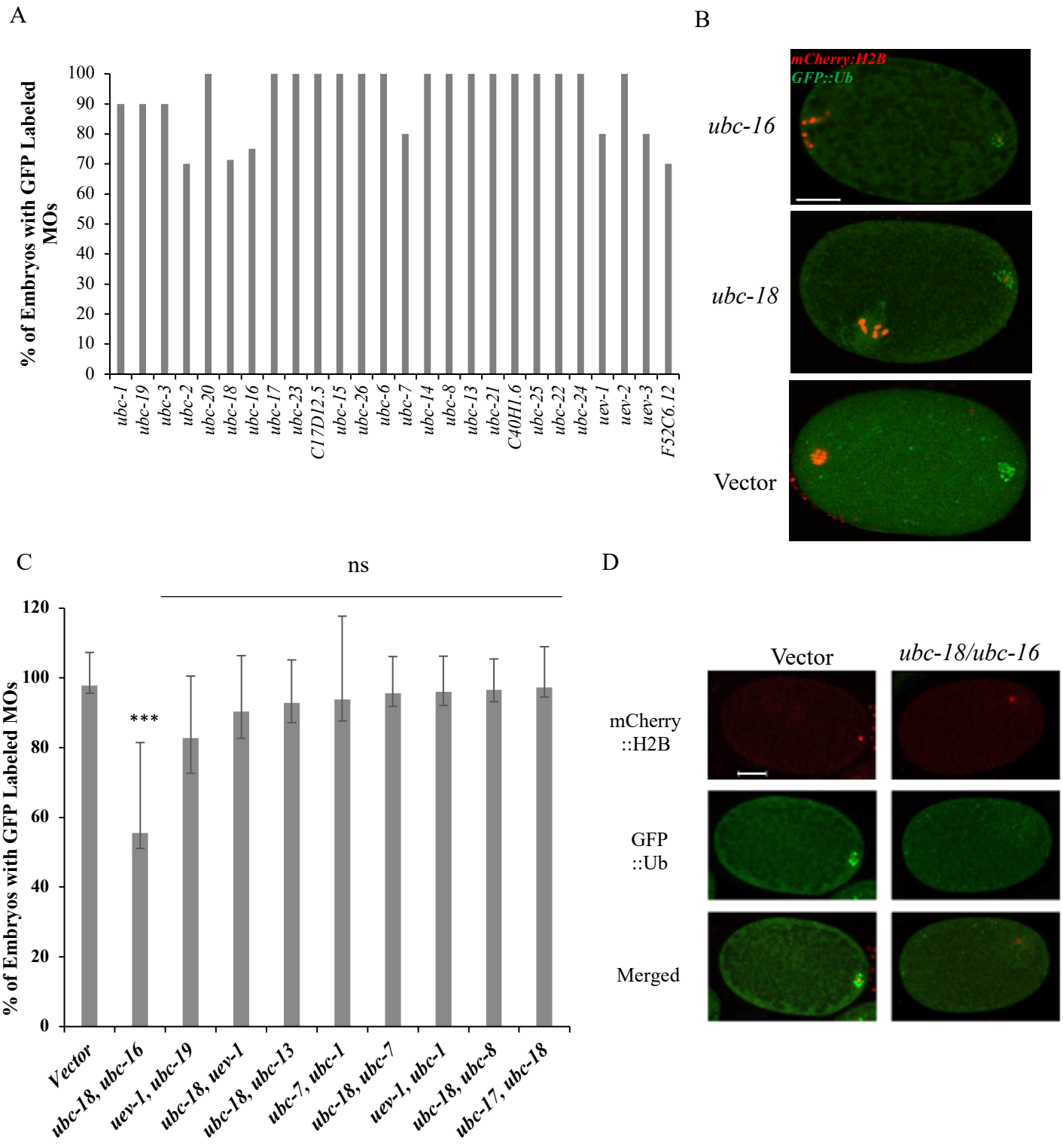

A
% of Embryos with GFP Labeled MOs
100
90
80
70
60
50
40
30
20
10
0
ubc-1
ubc-19
ubc-3
ubc-2
ubc-20
ubc-18
ubc-16
ubc-17
ubc-23
C17D12.5
ubc-15
ubc-26
ubc-6
ubc-7
ubc-14
ubc-8
ubc-13
ubc-21
C40H1.6
ubc-25
ubc-22
ubc-24
uev-1
uev-2
uev-3
F52C6.12
B
mCherry:H2B
GFP::Ub
ubc-16
ubc-18
Vector
C
ns
***
% of Embryos with GFP Labeled MOs
120
100
80
60
40
20
0
Vector
ubc-18, ubc-16
uev-1, ubc-19
ubc-18, uev-1
ubc-18, ubc-13
ubc-7, ubc-1
ubc-18, ubc-7
uev-1, ubc-1
ubc-18, ubc-8
ubc-17, ubc-18
D
Vector
ubc-18/ubc-16
mCherry ::H2B
GFP ::Ub
Merged

Figure 8- Simultaneous knockdown of *ubc-18* and *ubc-16* showed a reduction in the presence of GFP labeled MOs in one cell embryos.

(A) Knockdown of single E2 proteins did affect the presence of GFP::Ub during the one cell stage. **(B)** Embryos from worms fed RNAi bacteria of *ubc-16* and *ubc-18* individually did not show a reduction in GFP::Ub surrounding the paternal DNA. Meiotic embryos are shown**.** Hits from the UBC double knockdown RNAi screen are represented on the graph. Simultaneous knockdown of *ubc-18* and *ubc-16* was the most promising result from the screen and was confirmed using confocal microscopy. The double knockdown showed a 40% reduction in embryos with GFP::Ub vesicles. **(C)**A total of 20 embryos were observed for each condition and statistical differences were determined using a two tailed z test. ***$p < 0.0001$**.** Representative embryos from *ubc-18*/*ubc-16* knockdowns show absence of GFP::Ub vesicles around sperm DNA. Scale bars represent 10 μm.

ubc-18 is required for the addition of K48 chains on MOs

The two types of ubiquitin chains that have been identified on MOs are K48 and K63. These chains appear during different times in the one cell embryo. K48 chains are only present on MOs during meiosis I, while K63 chains are detected during all stages of early development [52]. After identifying two E2s

responsible for ubiquitinating MOs, we examined if each E2 was responsible for a specific ubiquitin chain type using chain-specific antibodies.

K48 chains were only present in 24% of *ubc-18* meiosis I embryos (Figure 9B). Using an MO specific antibody, we were able to show that knockdown embryos showed expected MO clustering around the paternal DNA, without association with K48 chains (Figure 9A). K48 staining is also absent in *ubc-18*/*ubc-16* knockdowns (Figure 9A, B). On the other hand, *ubc-16* (RNAi) did not have an effect on K48 chains in treated embryos (Figure 9A, B). Therefore, the delay in PME observed *ubc-18* /*ubc-16* (RNAi) embryos is only due to the absence of *ubc-18*. Similar results were seen in a *ubc-18* loss of function mutant *(ku354)* (Figure 9

C,D). These results indicate that *ubc-18* is required for the formation of K48 ubiquitin chains on MOs immediately after fertilization.

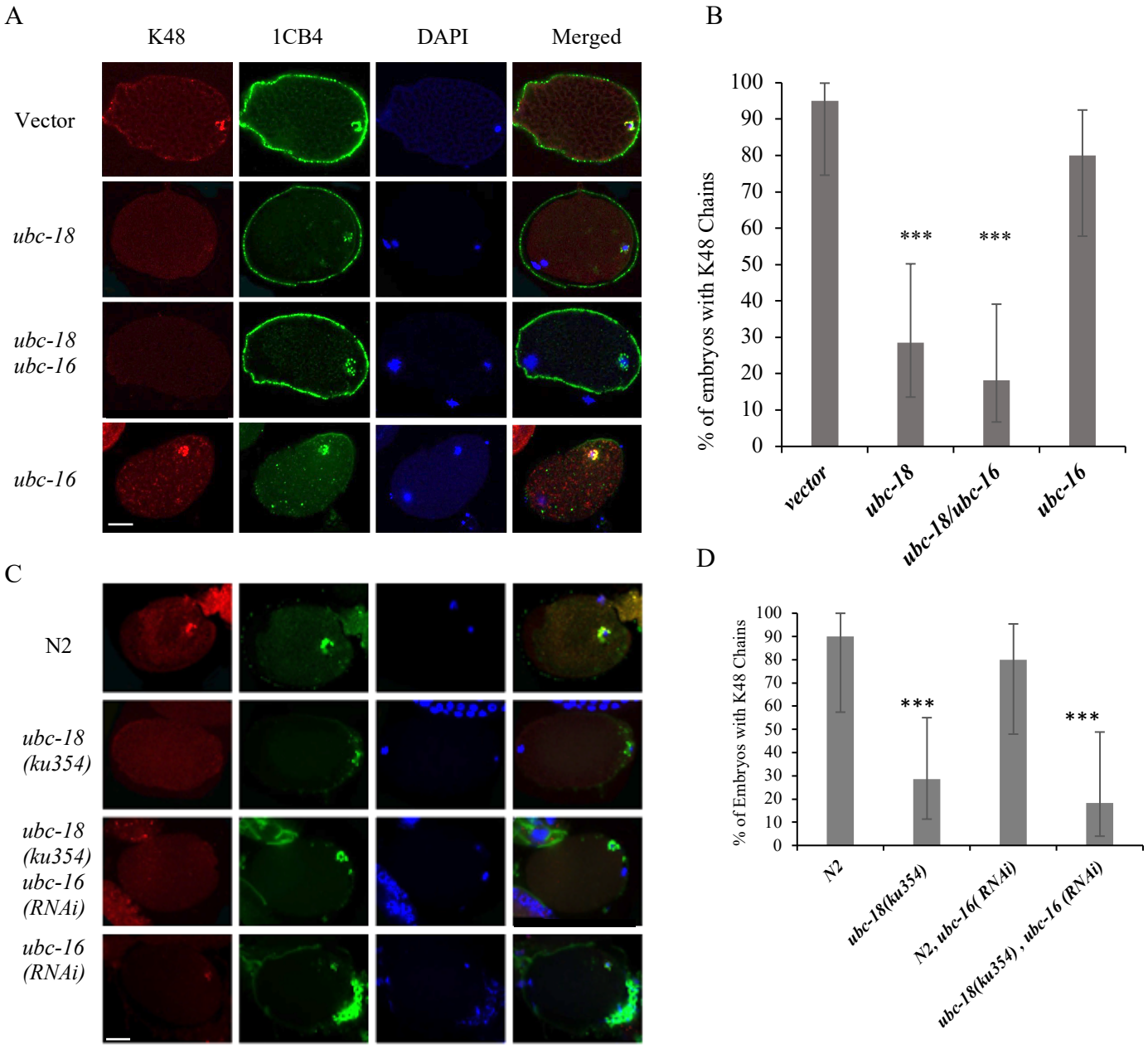


Figure 9- *ubc-18* knockdowns reduce the presence of K48 ubiquitin chains in Meiosis I embryos.

(A) Vector control, *ubc-18, ubc-16* and *ubc-18*/*ubc-16* embryos were stained with K48 chain specific and 1CB4 antibodies. K48 chains colocalize with MOs during meiosis I in control embryos. **(B)**However, this colocalization is disrupted in *ubc-18(RNAi)* not *ubc-16.* K48 chains were observed in 95% of vector embryos, but only 28% of *ubc-18 (RNAi)* embryos. **(C)** To confirm RNAi results, meiosis I embryos from *ubc-18* mutant (*ku354*) were stained with K48 chain linkage specific anti- ubiquitin and MO specific antibodies 1CB4. The results in mutant embryos were comparable to *ubc-18* RNAi knockdown embryos. *ubc-18 (ku354)* embryos showed K48 staining in 28% of the embryos assessed. **(D)** 18% of embryos from *ubc-18(ku354)* worms treated with RNAi of *ubc-16* showed K48 staining matching results from the double RNAi knockdown. For each treatment 20 meiosis I embryos were observed, and statistical significance was calculated by a Fisher's Exact test: ***$p < 0.001$ Error bars represent 95% confidence intervals. Scale bar represents 10 μm.

ubc-18/_ubc-16_ double knockdown does not interrupt the addition of K63 chains on membranous organelles

We hypothesized that reduction in GFP labeled MOs would be a result of an interruption in the addition of K63 chains due to their consistent presence during the one cell stage, as opposed to the transitory presence of K48 chains. The effect *ubc-18* and *ubc-16* had on K63 chains was assessed in one cell wildtype and *ubc-18 (ku354)* embryos (Figure 10A). Our results indicate that *ubc-18* and *ubc-16* do not reduce the presence of K63 chains individually or simultaneously (Figure 10B). MOs colocalization with K63 chains was observed in all embryonic stages prior to the first mitosis. Therefore, the reduction in ubiquitination observed in double knockdowns is not due to loss of K63 chains.

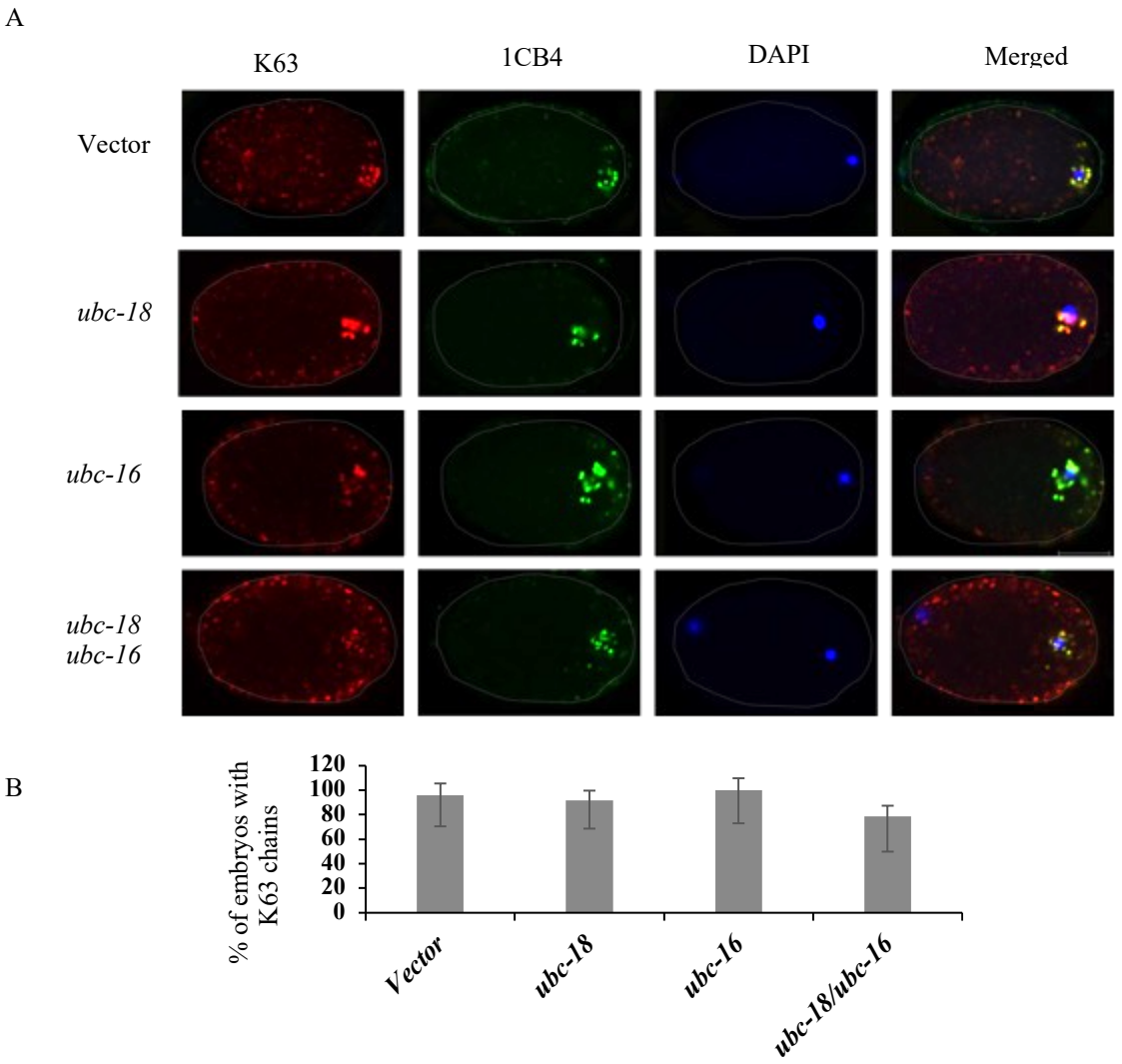


Figure 10- K63 ubiquitin chains at MOs are not reduced in *ubc-18*/*ubc-16* knockdowns.

N2, *ubc-18 (ku354),* N2 *ubc-16 (RNAi)* and *ubc-18 (ku354) ubc-16(RNAi)* embryos were stained with K63 chain linkage specific anti-ubiquitin and MO specific antibodies. All embryos observed showed K63 staining under all conditions tested. For each treatment 20 meiosis I embryos were observed, and statistical significance was calculated by a Fisher's Exact test: Error bars represent 95% confidence intervals. Scale bar represents 10 μm.

K27 ubiquitin chains are detected on MOs

Antibody staining revealed that K63 chains were not reduced in embryos with simultaneous knockdown of *ubc-18* and *ubc-16*. However, GFP::Ub was reduced under the same conditions. Therefore, we decided to look for the presence of other ubiquitin chains on MOs.

Non proteolytic roles of other ubiquitin chains have been identified. K33 regulates T-cell receptor function by regulating its phosphorylation, K29 chains are seen during Wnt/ß catenin signaling, and K27 chains are associated with slowing down proteasomal degradation of mitochondrial protein Miro1 to promote mitophagy[66-68]. We decided to look at K27 chains due to its relationship with mitophagy.

K27 chains localized to MOs during the same stages K63 chains are found (Figure 11) indicating that MOs are ubiquitinated with more than just K48 and K63 chains.

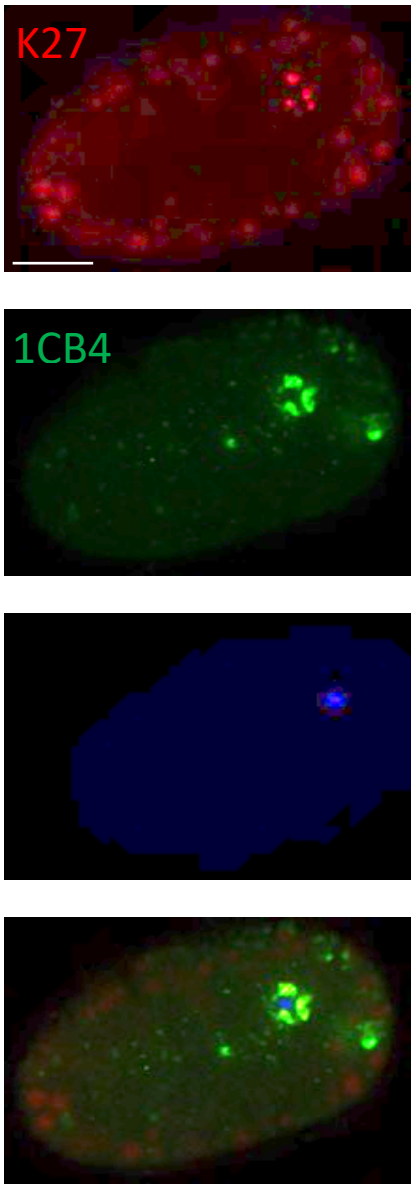


Figure 11- K27 ubiquitin chains are detected on MOs

Using a K27 ubiquitin chain specific antibody one cell embryos were assessed for the presence of K27 chains on MOs. The representative image is of meiosis embryo, with K27 staining colocalized with MO staining. This localization was observed throughout the different stages of the one cell embryo. Scale bar represents 10 μm.

***ubc-18*/*ubc-13* /*ubc-2* triple knockdown reduces the presence of GFP ubiquitin and increases the incidence of polyspermy in early embryos**

Similar to our approach, Fiesel *et al* conducted a screen of human E2s to determine which ones were involved in ubiquitinating damaged mitochondria during mitophagy. The researchers found that upon depolarization of the mitochondria simultaneous knockdown of UBE2D, UBE2L3 and UBE2N reduced its ubiquitination [69]. Their results suggested that the ubiquitination of damaged mitochondria was a process that involved multiple E2s. The combination of multiple E2 knockdowns reduced levels of ubiquitin present but did not completely eliminate ubiquitination. The results from both screens (human and *C. elegans*) highlight the redundancy in the ubiquitination process at the E2 level. The three human E2s that reduced ubiquitination during mitophagy have homologues in *C. elegans* : UBE2D (*ubc-2*), UBE2L3 (*ubc-18*) and UBE2N (*ubc-13*). Therefore, using RNAi we conducted a triple knockdown and observed GFP::Ub expression (Figure 12A). Our results indicate that GFP::Ub was present at very low levels in 78% of the embryos observed(Figure 12B). Double combinations of *ubc-18*, *ubc-2* and *ubc-13* were conducted and we did not observe a reduction in GFP::Ub expression, confirming our results from our previous screen (Appendix 1). Our results were similar to Fiesel *et al* , who reported that

these three enzymes needed to be knocked down simultaneously in order to get the greatest effect.

Another phenotype observed in the triple E2 knockdown *ubc-2, ubc-18* and *ubc-13* was polyspermy. Polyspermy is a rare occurrence during fertilization were one embryo is fertilized by more than one sperm[70]. In *C. elegans* polyspermy was observed embryos with defects in shell producing genes[70]. Our results show that polyspermy occurred in 30% of the embryos treated with the triple E2 RNAi (Figure 12D). Embryos fertilized by two spermatozoa have 2 sets of paternal DNA surrounded by2 different clusters of GFP::Ub vesicles (Figure 12C).

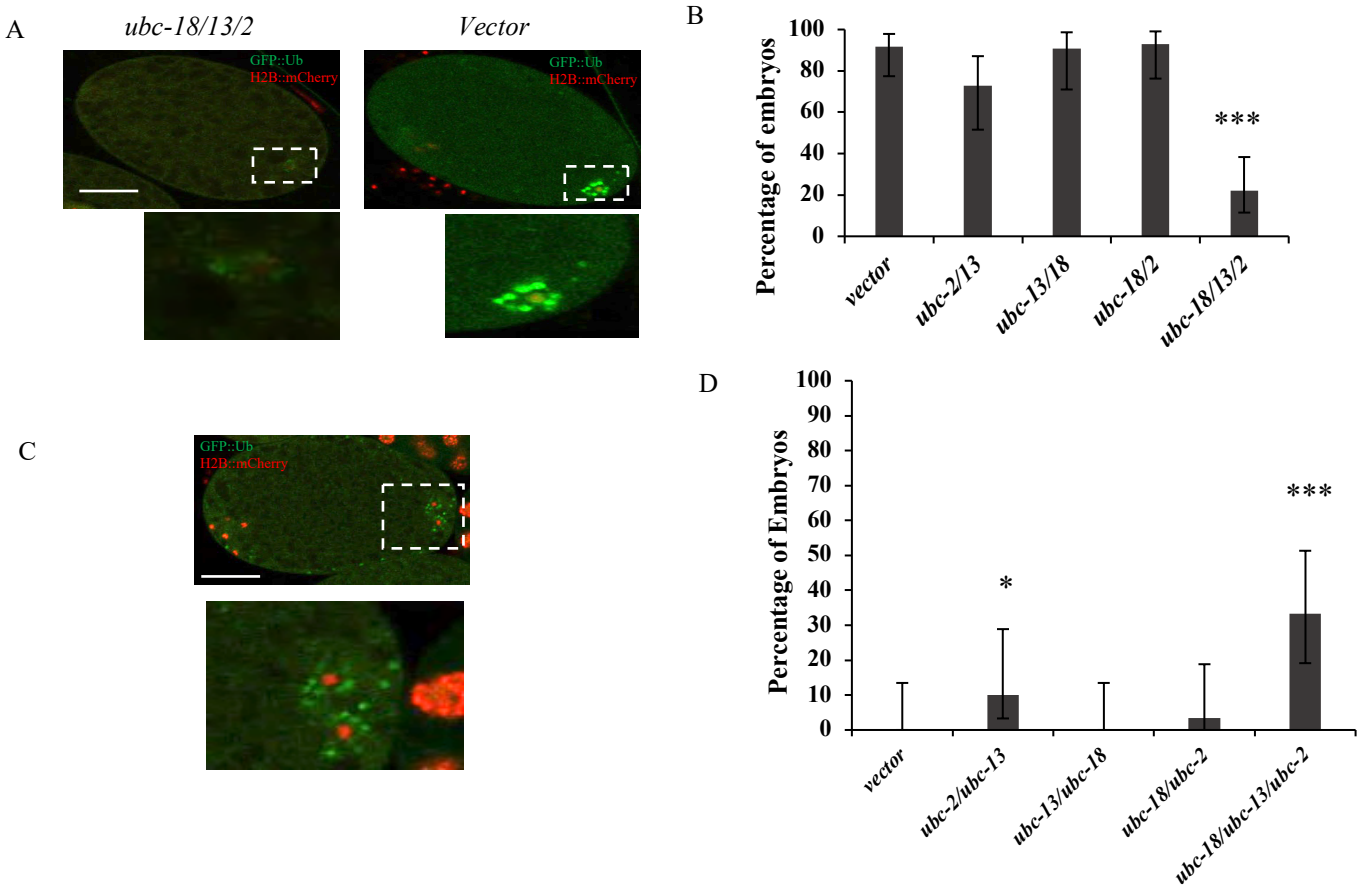


Figure 12- *ubc-18/ubc-13 and ubc-2* knockdowns reduce GFP::Ub in one cell embryos and induce polyspermy.

(A,C) The embryos treated with *ubc-18/13/2* RNAi were halted at meiosis I. The maternal DNA could not continue onto meiosis II due to the role UBC-2 has with the APC complex. **(B)**GFP::Ub expression around the paternal DNA is low in 78% of treated embryos**. (C)** Embryos with polyspermy have two sperm DNA structures surrounded by 2 distinct clusters of GFP::Ub vesicles. These embryos do not survive. **(D)**Embryos from the triple knockdown also showed a 30% increase in polyspermy when compared to the control. For each treatment 30 embryos were observed, and statistical significance was calculated by a Fisher's Exact test: Error bars represent 95% confidence intervals. Scale bar represents 10 μm. $*p < 0.05$. $***p < 0.001$

Discussion:

The goal of this study was to determine the E2s required for ubiquitinating sperm organelles in *C. elegans* . The E2 screens performed assessed GFP::Ub reduction at MOs in one cell embryos. After screening through individual and double combinations of E2s, it was determined that *ubc-18*/*ubc-16* work together to ubiquitinate MOs (Figure 8).

UBC-18 is a homologue of human UBCH7 enzyme, which can only perform transthiolation reactions and is an E2 dedicated for RBR and HECT E3s, [71]. HECT E3s not UBCH7 have been shown to be important for determining the type of ubiquitin chains are formed on substrates[72]. However, studies have revealed that UBC-18 and RBR E3 HHARI (ARI-1) are able to coordinate with another E2 UBC-3/ SCF E3 complex (CUL-1) to ubiquitinate common substrate SKP-1. UBC-18/ARI-1 complex begins the process by monoubiquitinating the substrate in the presence of CUL-1, and UBC-3 is required for further elongation of monoubiquitin to multiple ubiquitin chains[73]. This study provides an interesting insight into the cooperative interactions between multiple E2/E3 complexes.

UBC-16 has different characteristics than UBC-18. UBC-16 is the worm homologue of human UBE2W which is one of the ten E2s identified to interact

with the BRCA1 RING domain of BRCA1/BARD 1 E3 complex[7]. Similar to UBC-18, UBC-16 monoubiquitinates substrates and other E2s such as UBE2N/UBE2V2(UBC-13/UEV-2) can build polyubiquitin chains onto these substrates[7]. UBE3W activity is enhanced by the presence of RING-type E3 ligase[74]. UBC-16 and UBC-18 are shown to interact with different E3 ligases, therefore their roles during MO ubiquitination could be separate. It is important to determine their individual roles and screen for the E3s involved during this process.

Our data indicates that *ubc-18* is required for the addition of K48 ubiquitin chains on MOs during meiosis I (Figure 9). However, considering the timing of K63 chains, we hypothesized that the observed reduction of GFP::Ub in double knockdowns was most likely due to the interruption of the addition of K63 chains because this chain is observed throughout the one cell embryonic stages. Our results indicate that K63 chains are not completely reduced in double knockdowns. A small decrease in K63 ubiquitin chains was observed in embryos with the double knockdown (78%) but it was not statistically significant (Figure 10). We determined that MOs are also ubiquitinated with K27 chains, indicating that ubiquitination of the MOs is not limited to two types ubiquitin chains (Figure 11). The presence of K27chains coincided with K63 chains and, it would

be interesting to see the effect E2 knockdowns have on the addition of these chains.

The presence of K63 chains are associated with autophagy and mitophagy[24, 75, 76]. Therefore, we compared our screen results to those of a published screen performed using human E2s. The goal of that study was to determine the enzymes responsible for ubiquitination during mitophagy in cell culture of human cells. Similar to our results, they were not able to completely eliminate the presence of ubiquitin. However, knockdown of 3 E2s had the most significant effect in levels of mitochondria ubiquitination[69]. Even though our screen looked at ubiquitination of MOs not mitochondria, these results were relevant to our study since mitophagy and PME use some of the same adaptor proteins and signals[30, 38, 77, 78]. Interestingly, the E2s identified in the human screen had worm homologues and one of those was *ubc-18*. When we knocked down the worm E2 homologues, ubiquitination was greatly reduced in 78% of the embryos observed (Figure 12). These results indicate that MO ubiquitination after fertilization is a redundant process that utilizes multiple E2s and possibly multiple E3s but *ubc-18* seems to be an important regulator.

An interesting phenotype observed in the triple knockdown was polyspermy. To my knowledge ubiquitination has not been directly linked to polyspermy. A single study suggested a function for sperm protein SPE-42 as an E3 ubiquitin ligase. Their hypothesis is that the RING finger-like domain of SPE-42 interacts with other proteins to serve as a sperm capacity checkpoint for sperm and egg fusion. The signal could inform the egg that fusion was successful and provide a degradation message to fertilization required membrane proteins and thus preventing polyspermy[79].

The main purpose of this study was to create a system in which specific ubiquitination of MOs was interrupted. This would allow us to determine if ubiquitination of MOs is required for degradation of the MOs and PME. We were able to determine two E2s necessary for ubiquitination of MOs. However, the knockdowns did not completely eliminate the presence of ubiquitin on MOs. The two most characterized chains, K48 and K63 were found on MOs and the discovery of a third chain K27 points to the possibility that more ubiquitination events are taking place on MOs. Further investigation into the role of *ubc-18* and *ubc-16* in the addition of K27 chains is still needed. Knocking down more than 2 E2s could compromise other cellular processes as is seen in the triple E2

knockdown of *ubc-18/13* and *2*. Our study provides insight into the complexity of the ubiquitination pathway after fertilization. We provide evidence that ubiquitination of MOs is driven by a specific subset of enzymes. Our study did not address whether the E2s identified were acting in a direct or indirect manner to affect MO ubiquitination. Analyzing the localization of these enzymes after fertilization would provide further insight into the mechanism.

CHAPTER 4

Ubiquitin-proteasome interactions are required for the elimination of sperm membranous organelles

Introduction:

The ubiquitin proteasome system is used by the cell to maintain proteostasis[80]. K48 ubiquitin chains are conjugated to proteins to signal for proteasomal degradation. It is well accepted in the field that K48 chains signal proteins for proteasomal degradation. However, K48 chains are not a sufficient signal to trigger proteasome degradation. A study by Prakash *et al* first reported that an unstructured disordered region within the target protein along with K48 tagging was necessary to initiate proteasome destruction[81]. Along with these findings, proteomic studies have shown that in the presence of proteasome inhibitor, K48 tagged substrates are more abundant and accumulate more rapidly than other ubiquitin chains, indicating the strong relationship between K48 ubiquitin chains and proteasome[82, 83]. In vitro studies have shown that proteasome can bind other ubiquitin chains such as K11, K27, K6, K29, K33 and K63[82]. Although, in vivo proteasome affinity to K63 chains is diminished[83, 84].

The group of proteasome subunits that are important for ubiquitin-proteasome interaction are the ubiquitin receptors. Rpn10 was the first receptor identified and was found to contains a ubiquitin interacting motif (UIM)[85]. Ubiquitin receptors in multicellular organisms contain more than one UIM. These sites can serve to bind shuttle proteins that have ubiquitin-like (UBL) domains, such as Rad23A/B in mammals and ATG8 proteins in plants[86, 87]. There are several ubiquitin binding receptors in the proteasome complex. Rpn10 and Rpn13 have been characterized as the primary receptors because proteasome with mutations in both these subunits do not bind well to polyubiquitinated substrates[88]. Redundancy exists between ubiquitin receptors, which could explain why knockdowns of these receptors are not lethal to the organisms[89, 90].

Proteasome activity has been liked to different processes, such as autophagy. Autophagy is a process that involves the removal of cellular components through compartmentalization of cargo into membranous vesicles followed by lysosomal degradation. Proteasomal degradation and autophagy are the two major components in cellular protein quality control. There are studies that show that when proteasomal degradation is inhibited, autophagy

components are upregulated[91, 92]. These studies highlight the intricate relationship between autophagy and the proteasome. Proteasome and autophagy degradation are regulated by ubiquitin modifications[4, 23, 24]. Proteasome function has also been linked to mitophagy. Proteasomal degradation of mitochondrial protein mitofusin 1 has been attributed to upstream mitophagy events [34]. The role of proteasome during PME remains elusive. Reports in mammals suggest that proteasome activity is required for the removal of paternal mitochondria[48]. One report in *C. elegans* , provides evidence that mtDNA persisted in embryos lacking ubiquitin recognition particles *rpn-10* and *rad-23*[53].

PME depends on the initiation of selective autophagy [33, 38, 40, 41]. Therefore, studies of PME have focused on how the loss of different components interrupt the formation of autophagosome around paternal organelles. The formation of the autophagosome depends on the recognition of substrates, which is usually requires molecular adaptors such as p62, NBR1, NDP52, VCP, and optineurin. These molecules interact with autophagosome specific proteins that are members of LC3/GABARAP/Gate16 family[25](Figure 3). p62 is recognized as the major cargo receptor of autophagy, this protein interacts with ubiquitin via

its C-terminal UBA domain and ATG8 proteins bind to LC3B through its LC3-interacting region[22, 93].

Our study focused on how ubiquitination of the MOs affects PME and elimination of the MOs . We specifically aimed to address the effect that loss of ubiquitination has on the formation of the autophagosomes during meiosis II. We determined that interactions between ubiquitin and proteasome are necessary for PME.

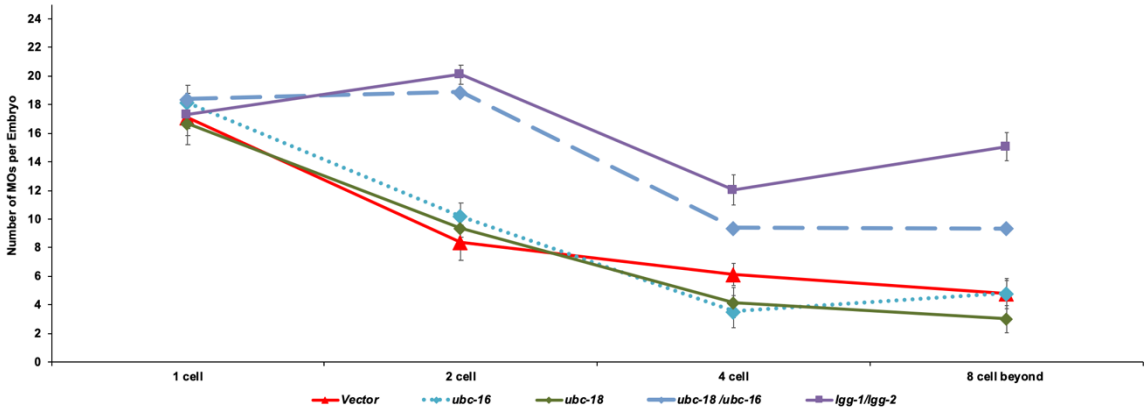
Results

Simultaneous knockdown of *ubc-18*/*ubc-16* induces persistence of MOs in later embryonic stages

Given the importance of ubiquitination in multiple cellular processes, it has been difficult to study the specific role ubiquitin has in the process of paternal organelle degradation. It has been shown that ubiquitination is necessary to promote the elimination of organelles[24, 76]. Therefore, to understand if ubiquitination is required for the elimination of MOs, embryos from *ubc-18*/*ubc-16* knockdown worms were stained with MO specific antibody ICB4. The fate of the MOs was observed through late stages of embryogenesis. During meiosis I, 16-20 MOs can be observed surrounding the paternal DNA

(Figure 13A). MO numbers drop after the first mitosis in control conditions and in *ubc-18* and *ubc-16* individual knockdowns (Figure 13A). However, MO numbers are higher in *ubc-18*/*ubc-16* knockdown in later stages than control conditions (Figure 13A). MO numbers drop during the transition from 2 cell to 4 cell stage (Figure 13A). However, MO numbers did not drop at the same rate in double knockdown conditions as was observed in control and individual knockdowns (Figure 13A). Also, in double knockdown 2 cell embryos, MOs appear to remain clustered and disperse during 4 cell and 8+cell (Figure 13B). As a positive control we did RNAi knockdowns of *lgg-1*/*lgg-2* (LC3/GABARAP homologues in *C. elegans*). *lgg-1*/*lgg-2* are required for selective autophagy and embryos lacking these genes will show persistence of MOs. Therefore, we expected the number of MOs to be higher in *lgg-1*/*l-gg2* knockdown embryos than control embryos[33]. MO numbers of *ubc-16*/*ubc-18* embryos were similar to *lgg-1*/*lgg-2* embryos. These results suggest that ubiquitination of MOs is important for their elimination.

A


Number of MOs per Embryo
24
22
20
18
16
14
12
10
8
6
4
2
0
1 cell
2 cell
4 cell
8 cell beyond
Vector
ubc-16
ubc-18
ubc-18 /ubc-16
lgg-1/lgg-2

B

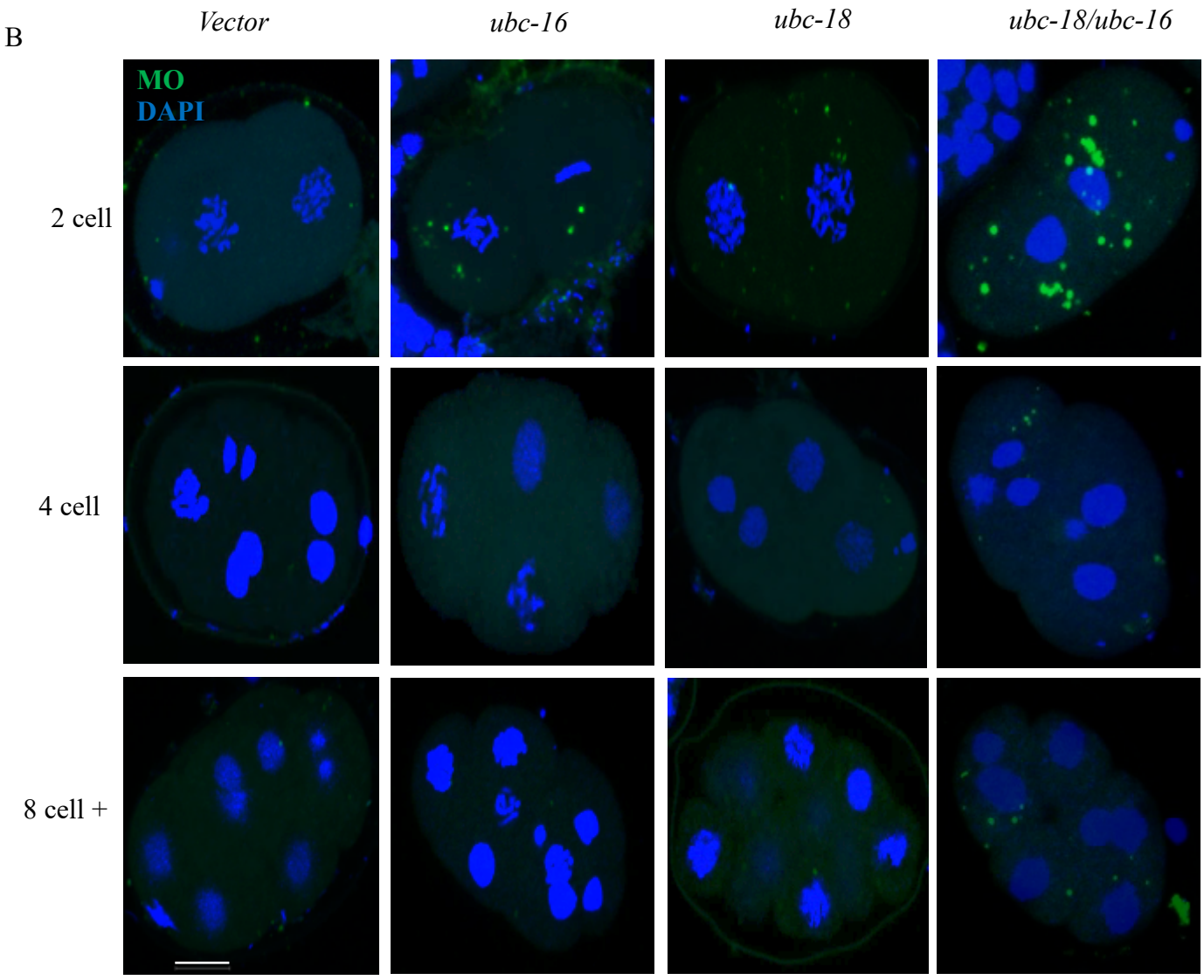

Vector
ubc-16
ubc-18
ubc-18/ubc-16
MO
DAPI
2 cell
4 cell
8 cell +

Figure 13- Ubiquitination of MOs is required for their elimination during embryogenesis

(A) Under control conditions, MO numbers continue to decline as the embryo develops. MO numbers are higher in *ubc-18/16* or *lgg-1/2* treated embryos, indicating that they are not being completely eliminated. MOs were counted in 10 embryos for each stage. Error bars in the graph represent the mean ± s.e.m. Significant differences between embryos were determined by unpaired *t* test. **(B)**Maximum intensity *z*-projections of confocal images of representative embryos stained for MOs using the 1CB4 antibody (green) and DAPI for DNA (blue)**.**

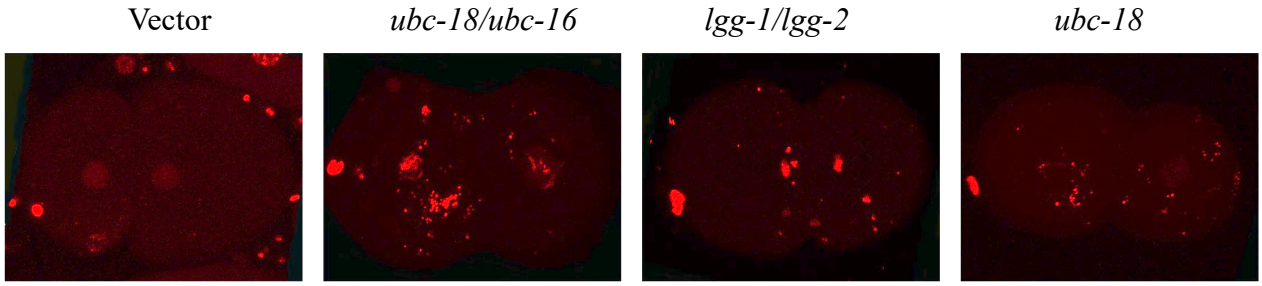
***ubc-18* is required for early elimination of paternal mitochondria**

The removal of paternal mitochondria is dependent on the process of sequestering these organelles in an autophagosome[33, 38, 40, 41, 43, 53, 94]. Ubiquitin has been established as the possible signal required to guide the formation of the autophagosome during early embryogenesis. The initial model of PME in *C. elegans* proposed that ubiquitination of MOs would recruit autophagosome membranes that would also engulf clusters of MOs and paternal mitochondria [33, 43]. Therefore, we hypothesized that ubiquitination of MOs should contribute to elimination of paternal mitochondria. Since we have been able to reduce ubiquitination of the MOs, we tested whether paternal mitochondria elimination is affected.

CMXROS labeled males were mated with hermaphrodites that express mCherry tagged histone 2B in the germline. After the matings, embryos were extracted from the hermaphrodites and imaged with confocal microscopy. Figure 12A shows embryos during the 2 cell stage, shortly after the first mitosis. According to pervious reports, this is the stage where the largest drop in the number of paternal mitochondria is observed. However, in embryos treated with *ubc-18* (RNAi) paternal mitochondria numbers do not drop after the first mitosis

(Figure 14A, B). The simultaneous knockdown of *ubc-18* and *ubc-16* produced the same phenotype. Even though, paternal mitochondria numbers do not drop after the first mitosis, the number of organelles drop in later stages (Figure 13A). Paternal mitochondria numbers in knockdowns of *ubc-18* and *ubc-18/16* reach wildtype numbers after the 4 cell stage. During the 2 cell stage in double knockdowns MO numbers are elevated and drop in later stages similar to what is observed with the paternal mitochondria (Figure 13A and Figure 14A). These observations support the previously discussed model that proposes MOs and paternal mitochondria are eliminated together. These results indicate that *ubc-18* is involved in early removal of paternal mitochondria and suggest that ubiquitination is not the only component in selection of the paternal mitochondria.

A



B

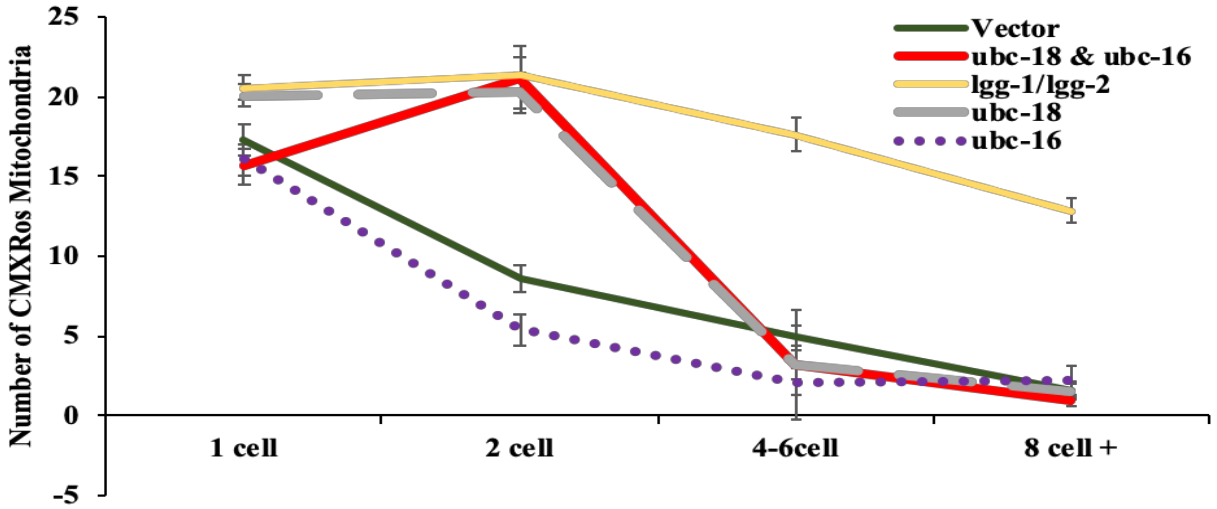


Figure 14- ubc-18 is involved in early removal of paternal mitochondria.

LN130 hermaphrodites were mated with CMXROS soaked N2 males and stained paternal mitochondria numbers were assessed at different stages. Paternal mitochondria numbers in 2 cell embryos were strikingly different in worms treated with *ubc-18/ubc-16* and *ubc-18* RNAi. *lgg-1/lgg-2* RNAi knockdowns were used as a positive control for defective elimination of paternal mitochondria. (n= 20 embryos) Data shown in graph are mean ± s.e.m. Significant differences between mating experiments was determined by unpaired *t* test. ***p<000.1. Scale bars represent 10 μm.

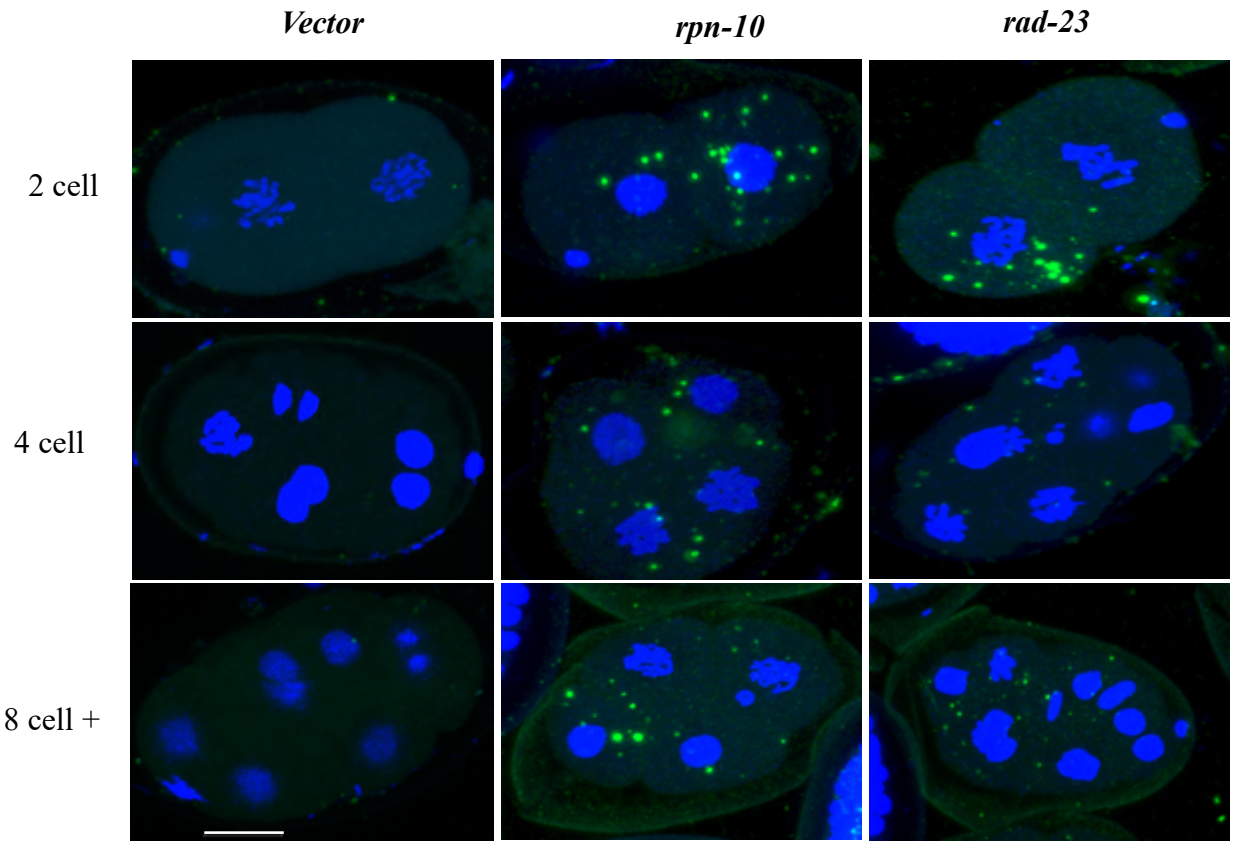
MOs and paternal mitochondria persist in *rpn-10* and *rad-23* knockdowns

Proteasomal protein degradation helps cells regulate different processes. Therefore, proteasome function is critical for cell survival[95]. Understanding the specific role of proteasome in different cellular mechanisms is difficult because proteasome knockdowns or the use of proteasome inhibitors can cause indirect effects. *rpn-10* and *rad-23* are non-essential proteasomal subunits that exhibit wildtype embryonic lethality when knocked down (Figure 16C). Reports from the Xue lab show that *rpn-10* and *rad-23* ubiquitin receptors are involved in the removal of paternal mitochondrial DNA [53]. Therefore, we wanted to test whether elimination of the MOs occurred during RNAi treatments of *rpn-10* and *rad-23*.

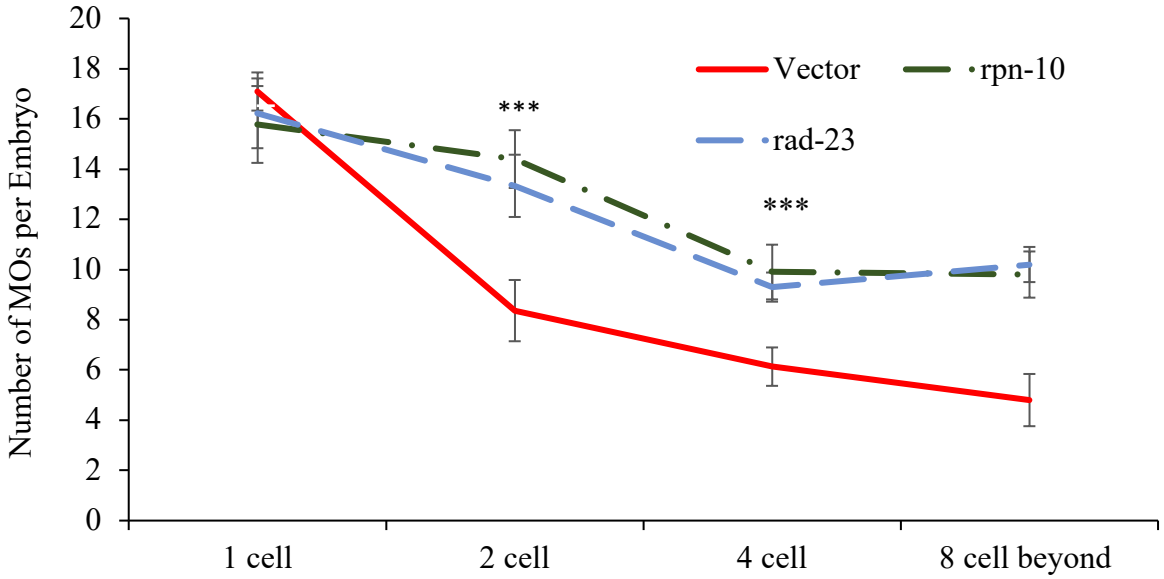
MO numbers drop after the first mitosis and continue to decline as the embryo progresses through development (Figure 15B). However, when *rpn-10* and *rad-23* subunits are deleted, MO numbers remain consistent and MOs can be observed in later stages (Figure 15A, B). MO numbers were higher under proteasomal knockdown condition than E2 combination knockdowns (Figure 13 A, 15A). Since E2 combinations knockdowns did affected the removal of MOs after the 4 cell stage but not affect mitochondria after this stage, we wanted to

explore a phenotype presented by Xue lab. The Xue lab reported that paternal mitochondria DNA was present in larval stages of *rpn-10* and *rad-23* knockdown worms[53]. Therefore, we decided to examine paternal mitochondria visually to confirm their results. Large numbers of CMXROS stained paternal mitochondria were detected in 8-16 cell embryos of *rpn-10* and *rad-23* knockdowns (Figure 15C, D). Paternal mitochondria were not detected in control embryos at this stage (Figure 15C). There was not a significant difference in mitochondria numbers between *rpn-10* and *rad-23* treated embryos (Figure 15D).

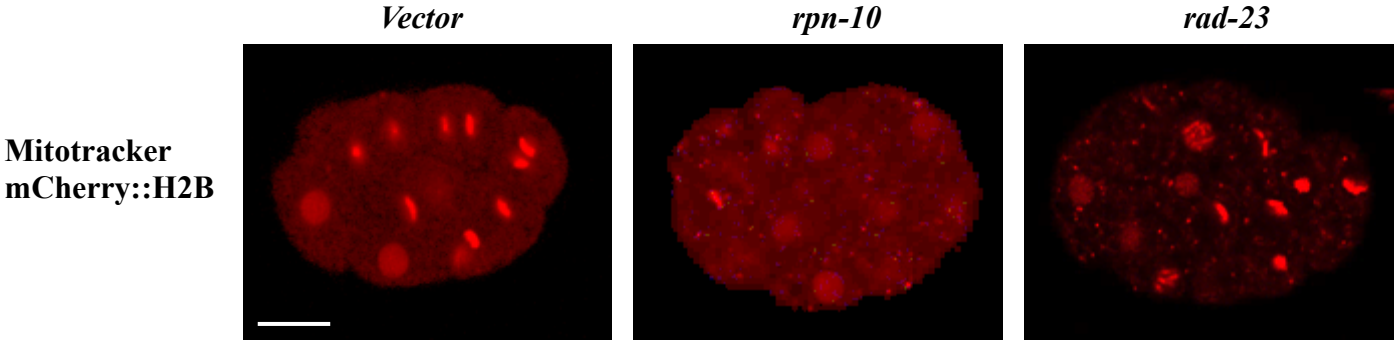
A


Vector
rpn-10
rad-23
2 cell
4 cell
8 cell +

B


Number of MOs per Embryo
20
18
16
14
12
10
8
6
4
2
0
1 cell
2 cell
4 cell
8 cell beyond
Vector
rpn-10
rad-23
***
***

C


Vector
rpn-10
rad-23
Mitotracker
mCherry::H2B

D

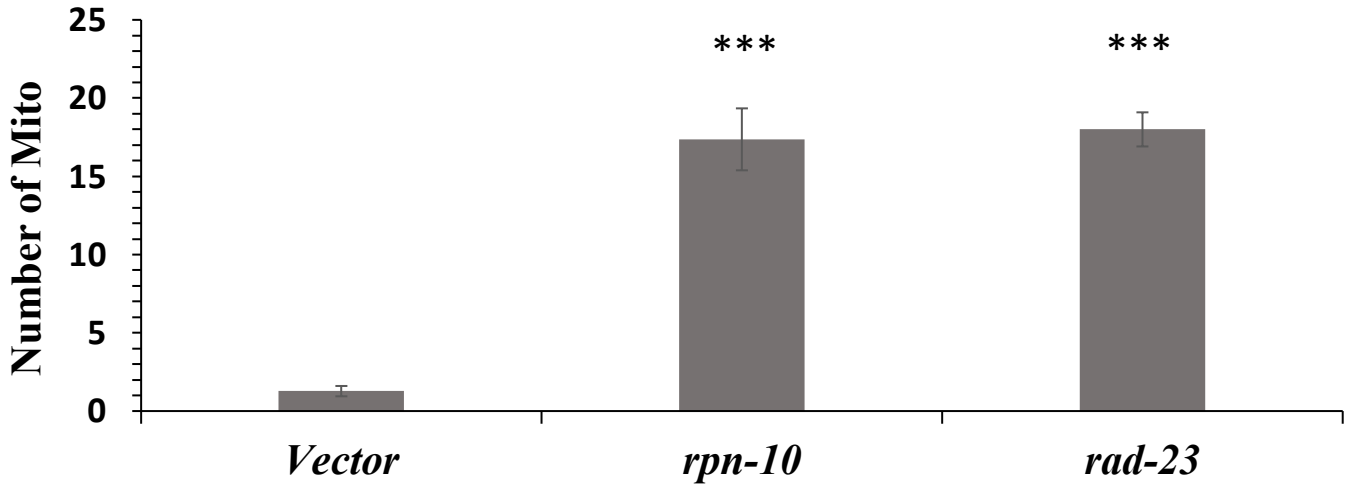

Number of Mito
25
20
15
10
5
0
***
***
Vector
rpn-10
rad-23

Figure 15- Proteasome subunits *rpn-10* and *rad-23* are required for the removal paternal organelles in *C. elegans* .

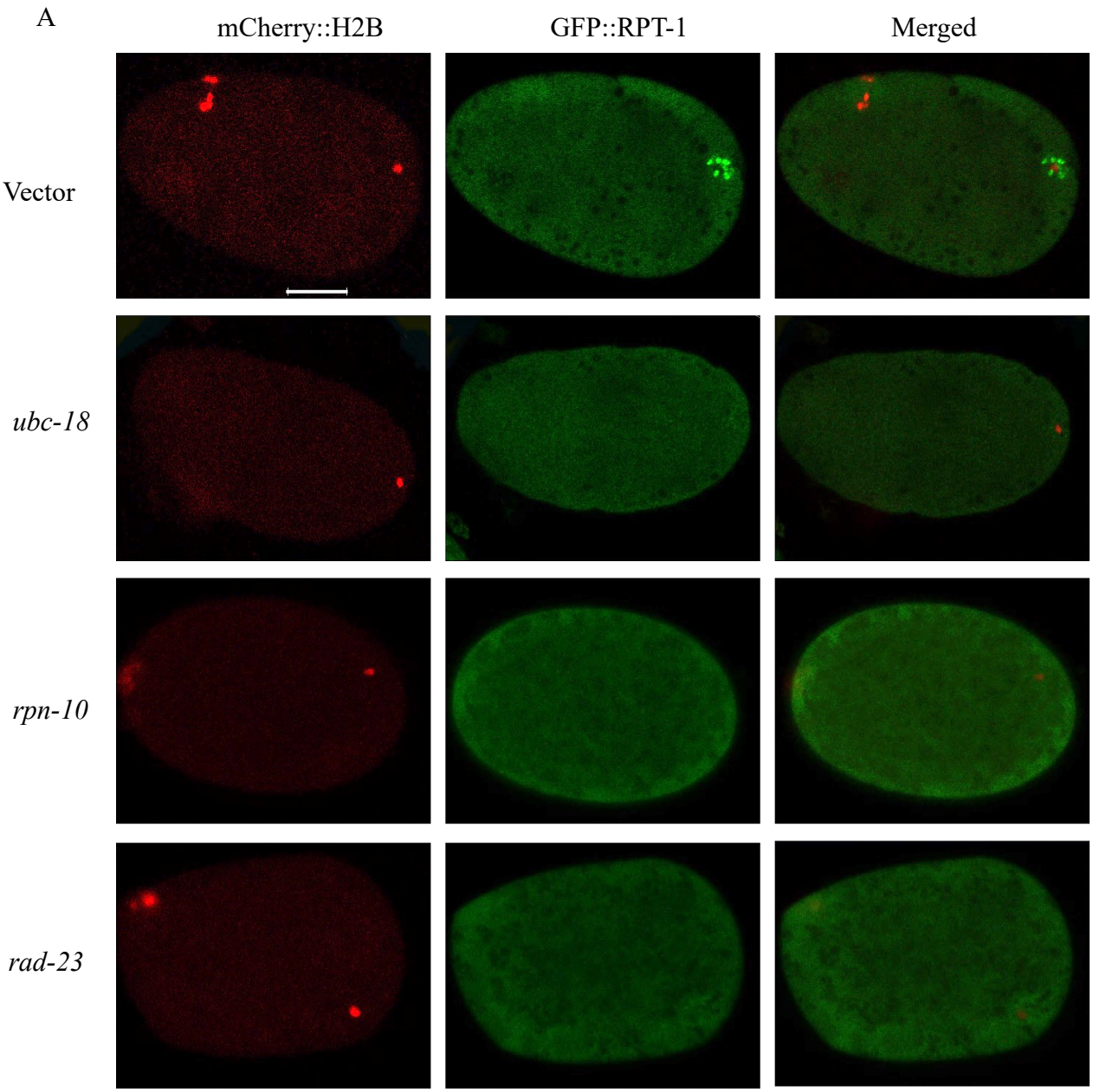
(A)MOs are labeled with 1CB4 antibody (green) and their stages were tracked using DAPI (blue). MOs were detected in later stages in knockdown embryos than the control embryos. **(B)** Data shown in the graph represents the mean ± s.e.m. of 10 embryos. Significant differences between embryos was determined by unpaired *t* test. ***p<000.1. Scale bar represent 10 μm**. (C)**Elimination of paternal mitochondria was tracked in 8-12 cell embryos using CMXROS labeled mitochondria. **(D)** Embryos from worms with proteasome ubiquitin recognizing subunits *rpn-10* and r*ad-23* knockdowns showed larger numbers of paternal mitochondria persist in 2-12 cell embryos when compared to the control (n=20). Statistical significance was calculated by a Fisher's Exact test: ****p* < 0.001 Scale bar represent 10 μm.

Addition of K48 chains driven by *ubc-18* and ubiquitin receptors are necessary for proteasomal recruitment to MOs during meiosis I

The K48 chains and proteasome are detected on MOs only during meiosis I [52]. MOs surround the paternal DNA during meiosis I (Figure 1A), during this time MOs and K48 chains are observed to colocalize (Figure 8A). Our data indicates that the addition of K48 chains to MOs is dependent on *ubc-18* (Figure 8). The addition of K48 polyubiquitin chains to a substrate usually serves as a signal recognized by the proteasome for degradation[12, 96]. Therefore, we used a transgenic worm expressing GFP tagged proteasomal subunit RPT-1 to detect early proteasomal localization in embryos with reduced K48 ubiquitin chains. Early proteasomal localization was interrupted in *ubc-18*(RNAi), which was expected since in the absence of *ubc-18* addition of K48 chains is interrupted (Figure 8) and in most cases proteasome localizes to proteins tagged with K48 chains (Figure 15A, B). Proteasome was not detected in 50% of the meiosis I embryo observed (Figure 16B). These data indicate that loss of *ubc-18* interrupted early recruitment of the proteasome.

Rpn10 and Rad23 are proteins subunits of the regulatory particle of the proteasome known to interact with polyubiquitin chains and are thought to recruit ubiquitinated proteins to the proteasome for degradation[33]. We

documented the effect *rpn-10* and *rad-23* knockdowns had on the elimination of MOs and PME. Since proteasome is only observed on MOs during meiosis, we hypothesized that these ubiquitin receptors might be involved in recruiting the proteasome to the K48 chains on the MOs. When each receptor was knocked down via RNAi, localization of proteasome to MOs was disrupted (Figure 16A, 4B) suggesting that both of these ubiquitin receptors are involved in localizing the proteasome to MOs.


A
mCherry::H2B
GFP::RPT-1
Merged
Vector
ubc-18
rpn-10
rad-23

B

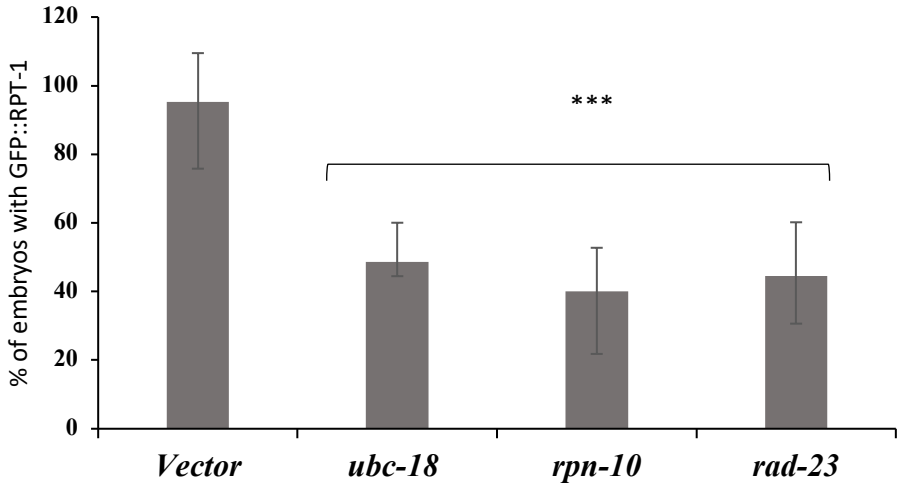


Figure 16- Early proteasome recruitment is interrupted by knockdowns of ubc-18 and ubiquitin recognition particles *rpn-10* and *rad-23*.

Transgenic worms with mCherry::H2B and GFP::RPT-1 fusion were treated with *ubc-18, rad-23, rpn-10* RNAi. GFP::RPT-1 can be observed surrounding the paternal DNA in control embryos. This localization persists until early meiosis II. GFP::RPT-1 localization is reduced during this time but still detectable. However, shortly after the second polar body is released GFP::RPT-1 is not detected. Under RNAi treatments, proteasome does not localize to surround paternal DNA**(A)**. In 50% (n=15) of the treated embryos early proteasome recruitment was interrupted in RNAi treated embryos. Statistical significance was calculated by a Fisher's Exact test: ***$p < 0.001$. Error bars represent 95% confidence intervals.

Autophagosome formation during meiosis II is affected by reduced MO ubiquitination but not by *rpn-10* and *rad-23* knockdowns

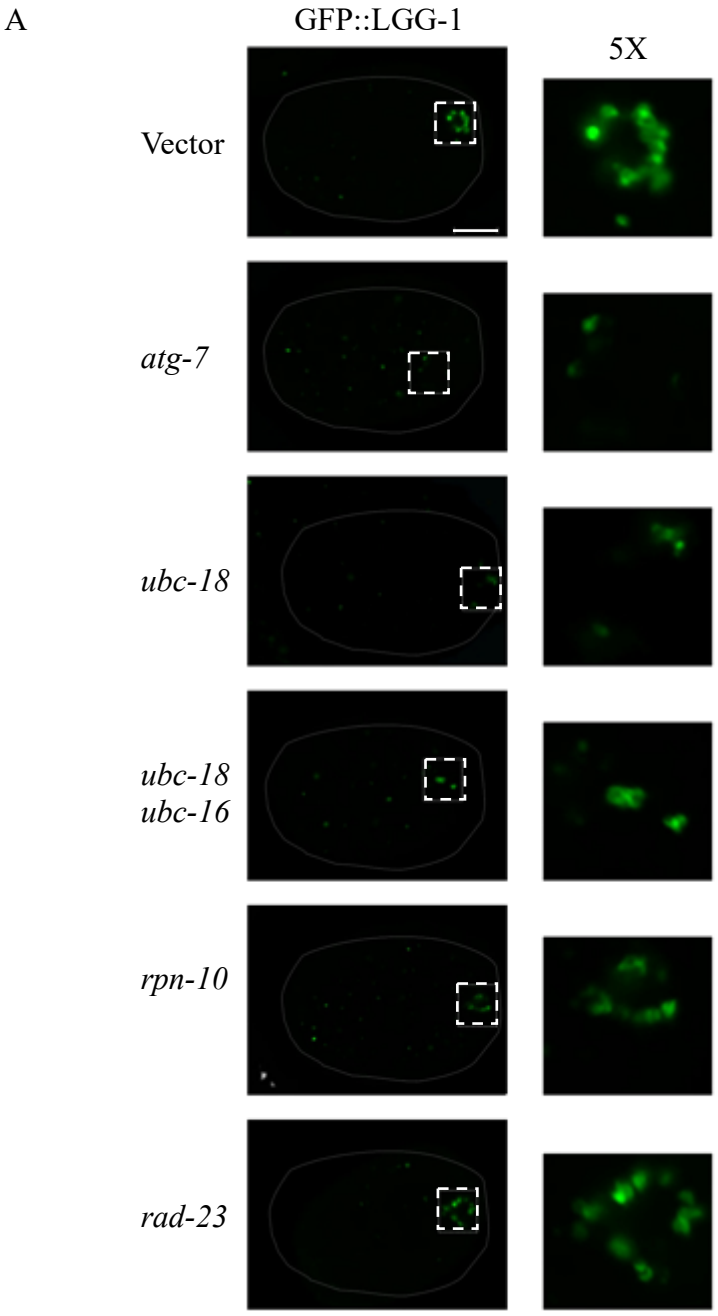
Depolarization of paternal mitochondria can be a contributing factor in their selection for elimination, similar to what takes place during mitophagy[40, 78, 94]. Studies show that the proteasome plays an important role in activating mitophagy components to destroy damaged mitochondria[28, 34, 77, 94]. Therefore, similarities between mitophagy and removal of the paternal mitochondria have been observed [35, 50]. Autophagosome formation is a hallmark for autophagy and mitophagy. During *C. elegans* PME, autophagosomes form around the paternal mitochondria and MOs during meiosis II[41]. As explained in figure 3A, LC3/GABARAB are important components of the autophagosome and *C. elegans* LGG-1 and LGG-2 are homologues of these proteins. Therefore, we decided to observe autophagosome formation using an LGG-1::GFP transgenic worm. There is evidence that during PME, autophagosomes form in a ubiquitin-dependent manner. Consequently, we hypothesize that reduced ubiquitination would affect the presence of autophagosome membranes. Similarly, we expected *rpn-10* and *rad-23*

knockdowns to disrupt autophagosome formation given that they interrupt PME and MO elimination.

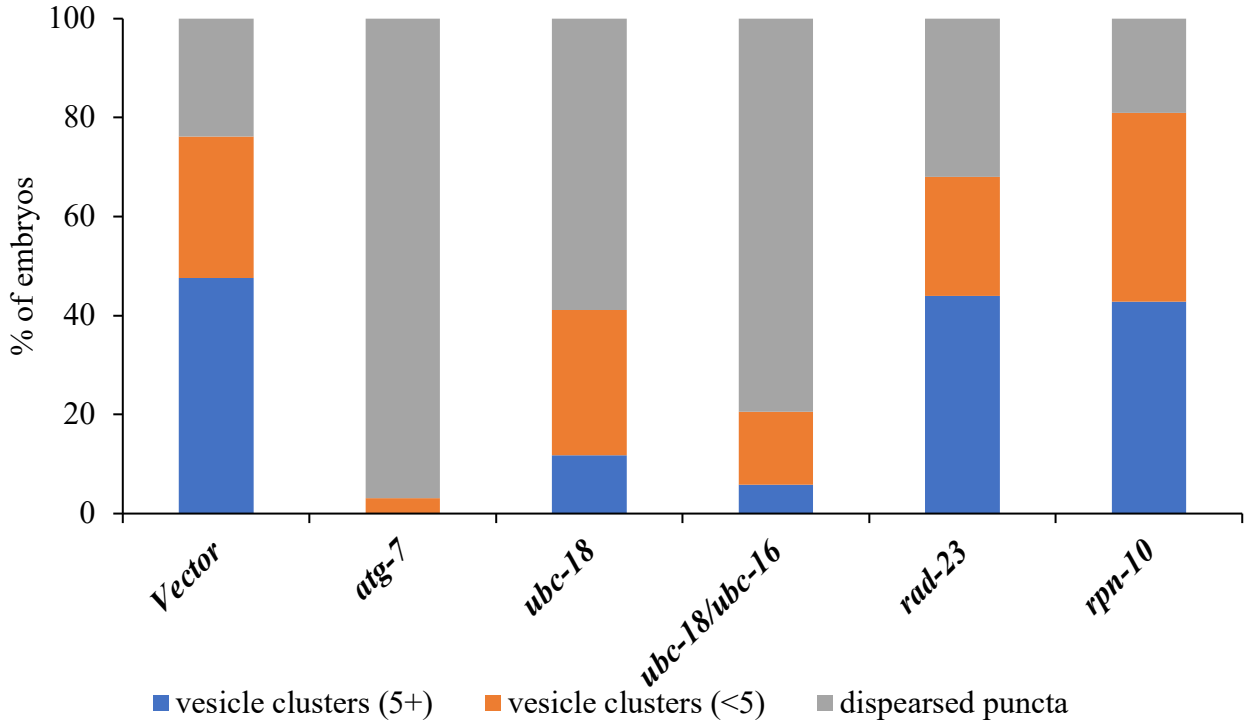
Our results indicate that knockdowns of proteasomal subunits *rpn-10* and *rad-23* do not disrupt the formation of the autophagosome (Figure 15A). One cell embryos were classified based on the, number of LGG-1::GFP puncta and number of vesicles present (Figure 16B). Control embryos showed similar distribution as the embryos with proteasomal subunit knockdowns. On the other hand, *ubc-18*(RNAi) embryos showed more embryos had reduced number of LGG-1 vesicles (Figure 17A). However, the highest reduction in the number of LGG-1 vesicles was observed in the double knockdown of *ubc-18* (RNAi) and *ubc-16* (RNAi) (Figure 17A).

Mutations in the autophagy system are detrimental to organisms, which makes it difficult to assess the effect of paternal organelle persistence under conditions where autophagy is completely interrupted. Since, *rpn-10* and *rad-23* knockdowns provide us with conditions where autophagy is not completely interrupted but paternal organelles persist in later embryonic stages, we tested embryonic lethality. Embryos treated with *ubc-18* and *ubc-18/16* had the most significant difference in embryonic lethality when compared to the wildtype, while *rpn-10* and *rad-23* did not show any significant differences (Figure 17C).

Our results indicate that persistence of paternal mitochondria and MOs is not detrimental to embryonic viability.



B


100
80
60
40
20
0
% of embryos
Vector
atg-7
ubc-18
ubc-18/ubc-16
rad-23
rpn-10
vesicle clusters (5+)
vesicle clusters (<5)
dispearsed puncta

C

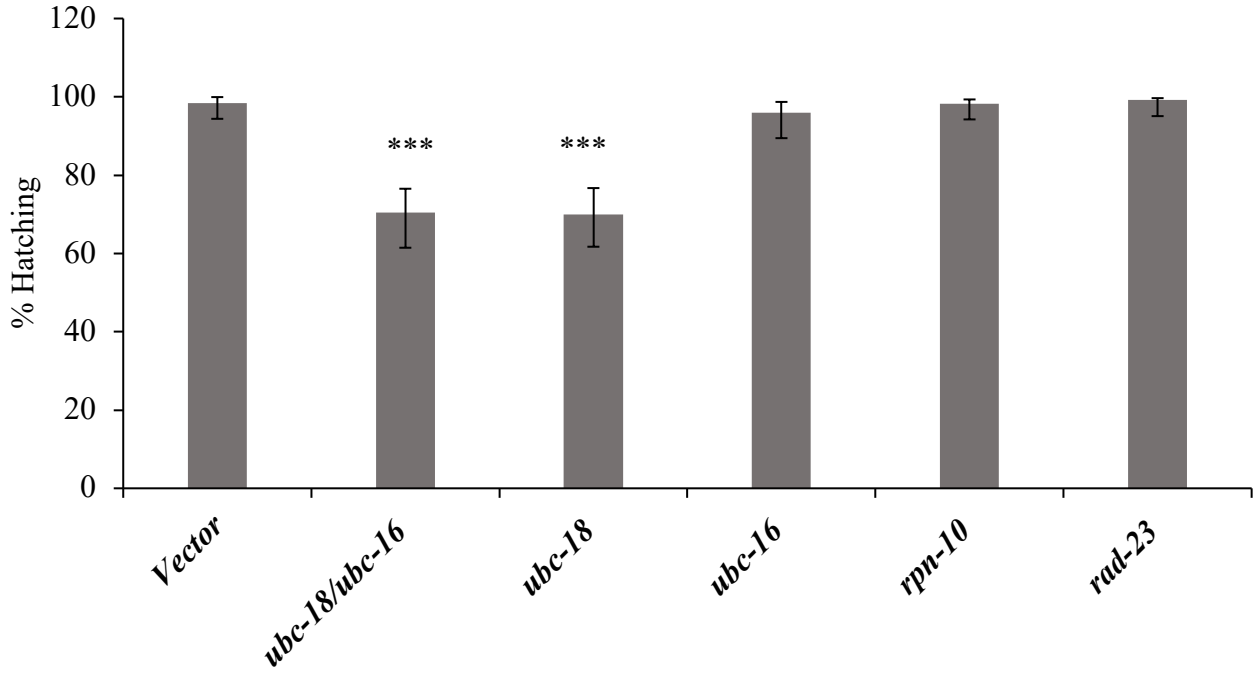

120
100
80
60
40
20
0
% Hatching
***
***
Vector
ubc-18/ubc-16
ubc-18
ubc-16
rpn-10
rad-23

Figure 17- LGG-1 vesicle formation is severely reduced in ubc-18/ubc-16 knockdown embryos but not affected by *rpn-10* and *rad-23*.

(A) One cell LGG-1::GFP embryos were observed after *ubc-18 (RNAi), ubc-18/ubc-16(RNAi), rpn-10(RNAi)* and *rad-23 (RNAi).* Control embryos showed 5 or more bright round GFP vesicles as seen in images above. While the number of vesicles in *ubc-18(RNAi)* and *ubc-18/ubc-16(RNAi)* were reduced. *ubc-18/ubc-16(RNAi)* embryos showed a more severe phenotype than *ubc-18 (RNAi)*. Vesicles in embryos with proteasomal subunits *rpn-10(RNAi)* and *rad-23(RNAi)* were similar to control in numbers and size. *atg-7(RNAi)* was used a positive control for autophagy disruption. **(B)**A total of 20 embryos were observed for each condition and classified based on the distribution of GFP::LGG-1**.** Embryonic lethality was assessed under conditions where paternal mitochondrial elimination was delayed. Worms exposed to *ubc-18* and *ubc-18/ubc-16* RNAi showed a significant decrease in embryonic viability when compared to control worms. **(C)**However, worms with *rpn-10* and r*ad-23* knockdowns did not show a significant decrease in embryonic viability. Statistical significance was calculated between knockdowns by a Fisher's Exact test: ****$p < 0.001$. Error bars represent 95% confidence intervals. Scale bar represent 10 μm.

Discussion

The proposed model for PME in *C. elegans* states that MO ubiquitination drives the elimination of MOs and paternal mitochondria. However, given the multiple regulatory functions of ubiquitin it is a challenge to disrupt the ubiquitin-proteasome system without indirect effects. Our data suggests that double E2 knockdown can reduce the presence of ubiquitin on MOs. Therefore, we wanted to test the effect this had on the removal of MOs.

As expected, reduced ubiquitination delayed the removal of MOs (Figure 13). The persistence of MOs is only observed in the double E2 knockdown, which reduced ubiquitination of these organelles. On the other hand, in embryos treated with *ubc-18* RNAi MOs were eliminated. These two results in conjunction with the proteasome localization data (Figure 15) suggests that MOs are eliminated independent of proteasome recruitment.

During autophagy, ubiquitination signals adaptor proteins form the autophagosome around specific cargo. Studies have shown that the reduction in overall ubiquitination interrupts autophagosome formation during meiosis II[38]. Our findings support this model, autophagosomes are reduced in E2

knockdowns, similar to the phenotype observed in atg-7 knockdowns (Figure 17). Although, atg-7 inhibits all autophagy and has a larval arrest phenotype, E2 knockdowns do not exhibit these phenotypes (Figure 17 C). Therefore, the reduction in autophagosomes seems to be specific to the PME process and not an overall inhibition of autophagy.

Previous work has alluded to proteasomal involvement during the removal of paternal mitochondria[52, 53, 97]. Therefore, we decided to test whether paternal organelles persist under conditions where proteasome recruitment was interrupted. As shown in figure 15, proteasome recruitment is hindered when K48 ubiquitin is reduced in *ubc-18* knockdowns. Since MO numbers are not affected in *ubc-18* knockdowns we could conclude that proteasome is not required for the elimination of MOs. However, PME is delayed in *ubc-18* RNAi treated embryos (Figure 15). It has been observed that paternal mitochondria are clustered with MOs during meiosis I and II, at meiosis II these organelles are surrounded by the autophagosome, autophagosomes arrange in a pericentrosomal manner during the first mitosis and following the first cleavage the numbers of paternal mitochondria and MOs are reduced[33, 41, 52]. Under conditions where K48 chains are not present on the MOs, the PME is delayed at

the 2 cell stage (Figure 14). The reduction in the presence of autophagosomes during E2 knockdowns may explain why PME is delayed (Figure 17). As seen in figure 13, proteasome recruitment by K48 chains does not affect the persistence of MOs. However, it does affect the rate of early PME (Figure 14).

Our results show that MOs persist when proteasome ubiquitin receptors *rpn-10* and *rad-23* are knocked down (Figure 16). Proteasome recruitment is also interrupted in the absence of these receptors. Therefore, the role of the proteasome does not seem to be limited to meiosis I. Persistence of MOs leads us to wonder if *rpn-10* and *rad-23* were involved in general autophagy response. However, studies in *C. elegans* show that autophagy is upregulated in *rpn-10* mutants and the worm is resistant to various stressors[91]. Our results support these findings, *rpn-10* and *rad-23* knockdowns did not show a reduction in the presence of autophagosomes during meiosis II. Knockdown embryos were very similar to untreated embryos, LGG-1::GFP was clustered to the anterior area of the embryo and vesicles were clearly visible under these conditions(Figure 17). Paternal mitochondria persistence in later stages is observed in ubiquitin receptor knockdowns which is more perplexing since autophagosome formation is not interrupted (Figure 16, 17). Considering we observed delayed PME at the 2

cell stage, it may be important to observe mitochondria numbers during the 2 cell stage in ubiquitin receptor knockdowns. Previous reports that link proteasome activity with PME support the theory that PME is driven by damaged mitochondria and proteasome serves to surveil them or to damage them for elimination[53, 78, 97]. However, this does not explain why proteasome is also required for MO elimination.

Ubiquitination is the signal that initially recruits the autophagosomes and proteasome to MOs during meiosis I and is necessary for the initial loss in paternal mitochondria numbers. However, if we disrupt the main ubiquitin receptors on the proteasome, MOs and paternal mitochondria persist in greater numbers. Collectively, our results indicate that proteasome ubiquitin interactions are required for the elimination of MOs and paternal mitochondria.

CHAPTER 5

Paternal Mitochondria are also eliminated in a ubiquitin-independent manner

Introduction

Elimination of paternal mitochondria occurs through a selective process. Evidence in the literature suggests that this process is interrupted by any protein or gene that affects autophagy. In *C. elegans* there is strong evidence that shows that MOs are ubiquitinated and one report that shows paternal mitochondria are ubiquitinated**[33, 38, 43, 52]**. PME studies have focused on the theory that paternal mitochondria are damaged, tagged with ubiquitin, and are eliminated. Therefore, there has been overlap between the mechanisms of mitophagy and PME.

Classic mitophagy involves the PINK1/Parkin system. PINK1 is a kinase that localizes to the mitochondria and is partially imported through TOM/TIM (Translocase of the Outer Membrane/Translocase of the Inner Membrane) complex when mitochondria are polarized. N-terminus end of PINK1 is then degraded by mitochondrial protease inside the matrix and the remainder of the protein is released into the cytosol to be degraded by the ubiquitin-proteasome system**[33]**. Mitochondrial depolarization is the signal that initiates mitophagy, which disrupts the import of PINK1 through TOM/TIM complex. Upon

depolarization, full length PINK1 remains associated with the mitochondria on the outer mitochondria membrane and recruits Parkin, an E3 ubiquitin ligase that conjugates ubiquitin chains to outer mitochondria membrane proteins**[33]**. Parkin and ubiquitin moieties are phosphorylated by PINK, increasing the rate of chain formation by keeping Parkin associated with the mitochondria**[33]**. These ubiquitin chains will be recognized by adaptor proteins such as p62, OPTN, Ndp52, NBR1, and TAXIBP1 that will recruit the autophagosome membrane[27].

The mitochondria can be damaged under cellular stress conditions. There is evidence that the proteasome is responsible for damaging the mitochondria and leading to its depolarization. These studies have shown proteasome degrades outer mitochondrial membrane protein mitofussin in a p97 manner. p97 is an AAAtpase that aids proteasomal degradation of ubiquitinated membrane proteins by extracting proteins from the membrane and feeding them into the proteasome**[28]**. Ubiquitin ligase Parkin has been shown to be responsible for inducing the degradation of outer mitochondrial membrane proteins and intermembrane space proteins through ubiquitin tagging. Studies have also suggested that Parkin can induce rupture of the outer mitochondrial

membrane in a proteasome-dependent manner, the exact mechanism remains elusive. **[33]**.

PINK1/PARKIN induced mitophagy is not the only form of mitophagy found in metazoans. There are other pathways that use autophagy receptors with LIR (LC3-interacting regions) that localize to the mitochondria**[98]**. These proteins can bind LC3 proteins localized to the phagophore membrane, thereby allowing for selection of specific cargo, the most characterized of these receptor mediated pathways are BNIP3, NIX/BNIP3L and FUNDC1**[99-101]**. BNIP3 and NIX induce mitophagy when expressed in Parkin null HeLa cells under hypoxia conditions **[102, 103]**. NIX has a role in hypoxia induced mitophagy and is also responsible for removing mitochondria during red blood cell development. Also in response to hypoxia, outer mitochondrial membrane protein FUNDC1 is dephosphorylated by PGAM5 to allow its association with LC3 and induce mitophagy **[104].** Phosphorylation of FUNDC1 occurs near its LIR domain which turns off the mitophagy inducing function of the protein **[101]**. Unpublished data from the Nehrke lab provides evidence that *fndc-1* a worm homologue for human FUNDC1 is preferentially expressed in sperm

mitochondria and could be involved in PME (personal communication from Keith Nehrke).

Mitophagy is induced when mitochondria are damaged and/or depolarized. There is some evidence that paternal mitochondria are damaged before or after fertilization**[40, 49]**. Reports in *C. elegans* and *Drosophila* show evidence that endonuclease G is required for PME. In *C. elegans* , depolarization of the mitochondria induces translocation of endonuclease G to the mitochondrial matrix to degrade paternal mitochondria DNA. PME is delayed In the absence of endonuclease G **[40]**. This observed phenotype resembles a previously reported phenotype observed during proteasomal inhibition. In both conditions, paternal mitochondria are depolarized. Yet, studies have shown that *C. elegans* PME occurs independently of PINK1, Parkin and p62**[38]**. These findings support the model that paternal mitochondria are not ubiquitinated during PME. Which differs from what during mitophagy.

Depolarized mitochondria are ubiquitinated under stress conditions. However, *C. elegans* studies have been contradictory in regard to ubiquitination

of paternal mitochondria. Therefore, we decided to look at the mitochondria for ubiquitination and ubiquitin independent PME.

Results

Ubiquitination of paternal mitochondria is not detected in the early embryo

It has been reported that mammalian sperm is ubiquitinated before fertilization and this serves as a tag to trigger PME[47, 48]. A recent study in *C. elegans* provided evidence that mitochondria were ubiquitinated at lower levels than MOs and ubiquitination of paternal organelles was responsible for recruiting autophagy receptor ALLO-1[38]. Conversely, other reports have shown that MOs are ubiquitinated and cluster with paternal mitochondria during meiosis I and meiosis II making it difficult to clearly evaluate ubiquitination during these stages[33, 52]. Therefore, it is important to assess ubiquitination of paternal mitochondria to verify if our E2 knockdowns were affecting MO ubiquitination and/or mitochondrial ubiquitination.

Sato *et al* reported that mitochondria were ubiquitinated using a line scan to test for intensities of one cell embryos from a mating of GFP::Ubiquitin and a HSP-6::mCherry transgenic worms. HSP-6 is from the heat shock family of proteins that localizes to the mitochondria. They used a GFP antibody to amplify

their GFP::Ub signal. We repeated their experiment with our strain of GFP::Ub worms mated with N2 males soaked in CMXROS. We decided to observe worms at different stages before the first mitosis to ensure that MOs and paternal mitochondria were not always in their clustered arrangement. During meiosis intensity peaks were close to each other but not colocalized and as MOs and paternal mitochondria become more dispersed, the intensity peaks were further from each other (Figure 18). Line scans correspond to the lines drawn in the images. The lines were drawn over the CMXROS labeled mitochondria and then merged with the GFP::Ub channel to assess co-localization of ubiquitin and mitochondria.

These findings indicate that mitochondria ubiquitination cannot be detected by live cell imaging. The clusters of GFP::Ub observed shortly after fertilization correspond to MOs and these observations have been confirmed with antibody staining (Figure 9,10). So, selection of paternal mitochondria for elimination must follow a ubiquitin independent pattern.

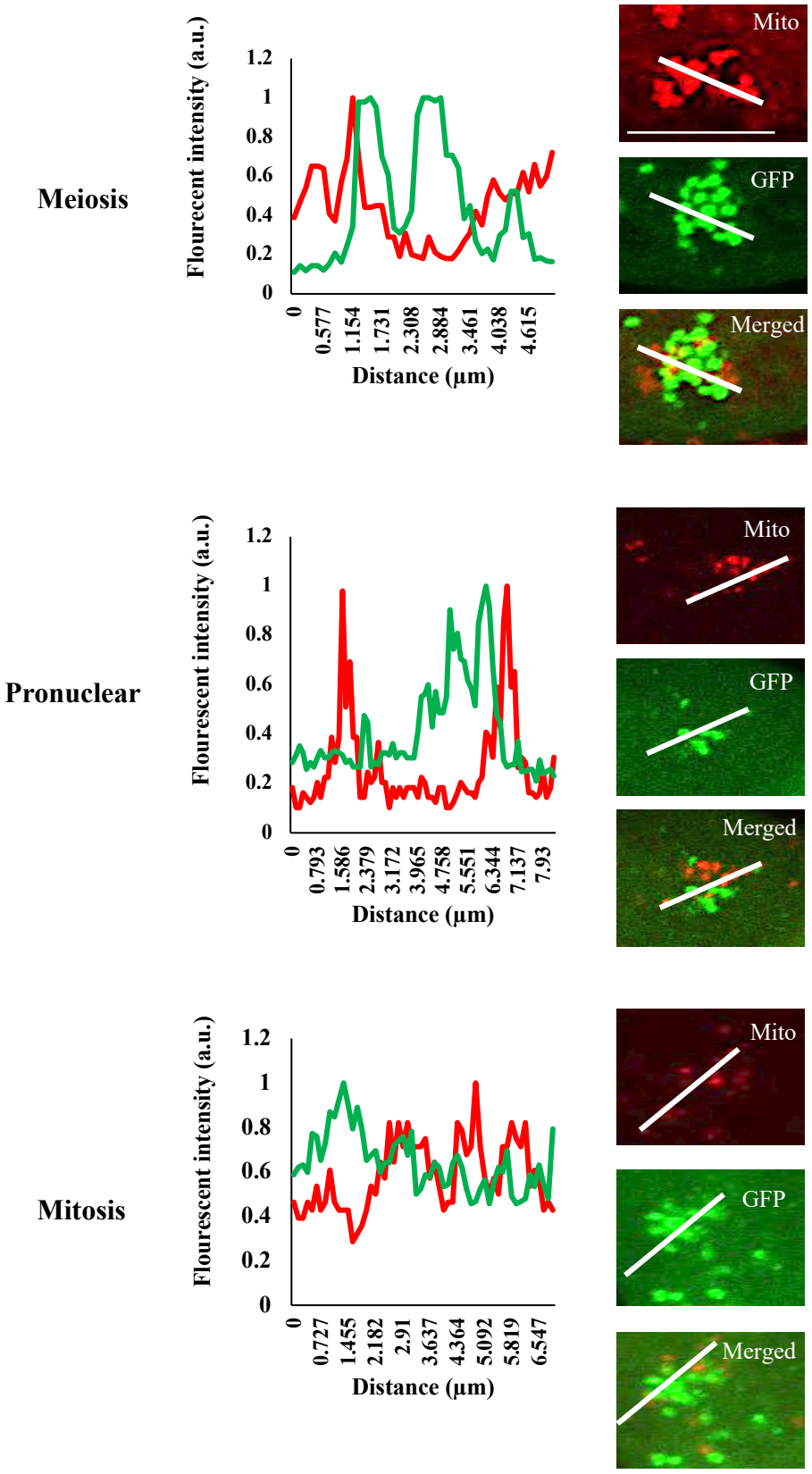

Meiosis
Flourecent intensity (a.u.)
1.2
1
0.8
0.6
0.4
0.2
0
0
0.577
1.154
1.731
2.308
2.884
3.461
4.038
4.615
Distance (µm)
Mito
GFP
Merged
Pronuclear
Flourescent intensity (a.u.)
1.2
1
0.8
0.6
0.4
0.2
0
0
0.793
1.586
2.379
3.172
3.965
4.758
5.551
6.344
7.137
7.93
Distance (µm)
Mito
GFP
Merged
Mitosis
Flourescent intensity (a.u.)
1.2
1
0.8
0.6
0.4
0.2
0
0
0.727
1.455
2.182
2.91
3.637
4.364
5.092
5.819
6.547
Distance (µm)
Mito
GFP
Merged

Figure 18- Paternal mitochondria are not ubiquitinated after fertilization

Line scans of each one cell stage corresponds to lines drawn on the images to the right. 10 embryos were analyzed and presented similar results. Intensity peaks of GFP::Ub and CMXROS tagged mitochondria do not colocalize. However, peaks are close to each other, possibly representing clustering of paternal mitochondria with MOs. Scale bar represents 10 μM.

Paternal mitochondria numbers drop after the first mitosis in *fndc-1* mutants, but paternal mitochondria persist in later embryos

Our previous results show that ubiquitination is required for degradation of MOs but only delays PME at the 2 cell stage. As previously mentioned, ubiquitination is a hallmark for autophagy and p62 or other adaptor proteins are considered the link between the ubiquitin proteasome system and autophagy[105]. Our findings support the model that paternal mitochondria are not ubiquitinated and PME is not dependent on p62 or Parkin. We have evidence that Parkin (pdr-1) is not responsible for ubiquitinating MOs (Figure 19) but MOs are eliminated in a ubiquitin-dependent manner. When MO ubiquitination is reduced, PME is only delayed not halted. So, paternal mitochondria may need another way of signaling the autophagosome. FUNDC-1 was an attractive ubiquitin-independent PME alternative due to its exclusive expression in sperm mitochondria.

fndc-1 mutant males soaked in CMXROS were mated with N2 hermaphrodites, embryos from the mating were extracted and their paternal mitochondria numbers were assessed. We expected to see mitochondrial persistence, which collaborates unpublished data from the Nehrke lab (Figure

20A,B). Paternal mitochondria persisted in the 8+ cell stage in *fndc-1* mutant. We wanted to observe the number of paternal mitochondria during the 2 cell stage to test our findings that suggest that paternal mitochondria are eliminated in a ubiquitin dependent manner during the first mitosis. Mating with N2 males and mutant males did not show a significant difference at the 2 cell but the difference was more significant after the 4 cell stage (Figure 20A). This outcome supports our previous data where early PME is dependent on ubiquitination of the MOs.

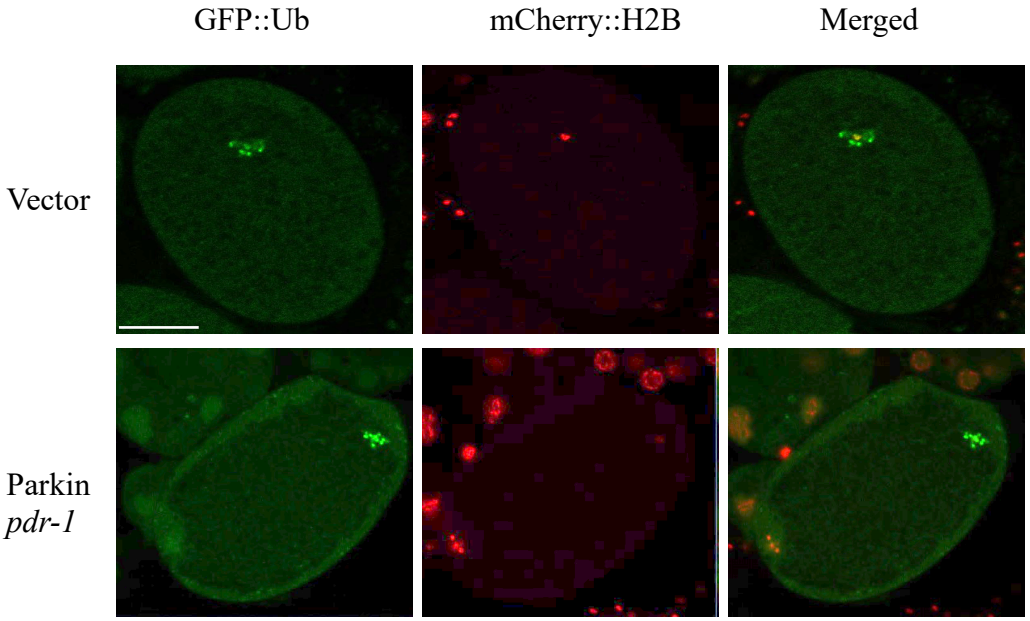
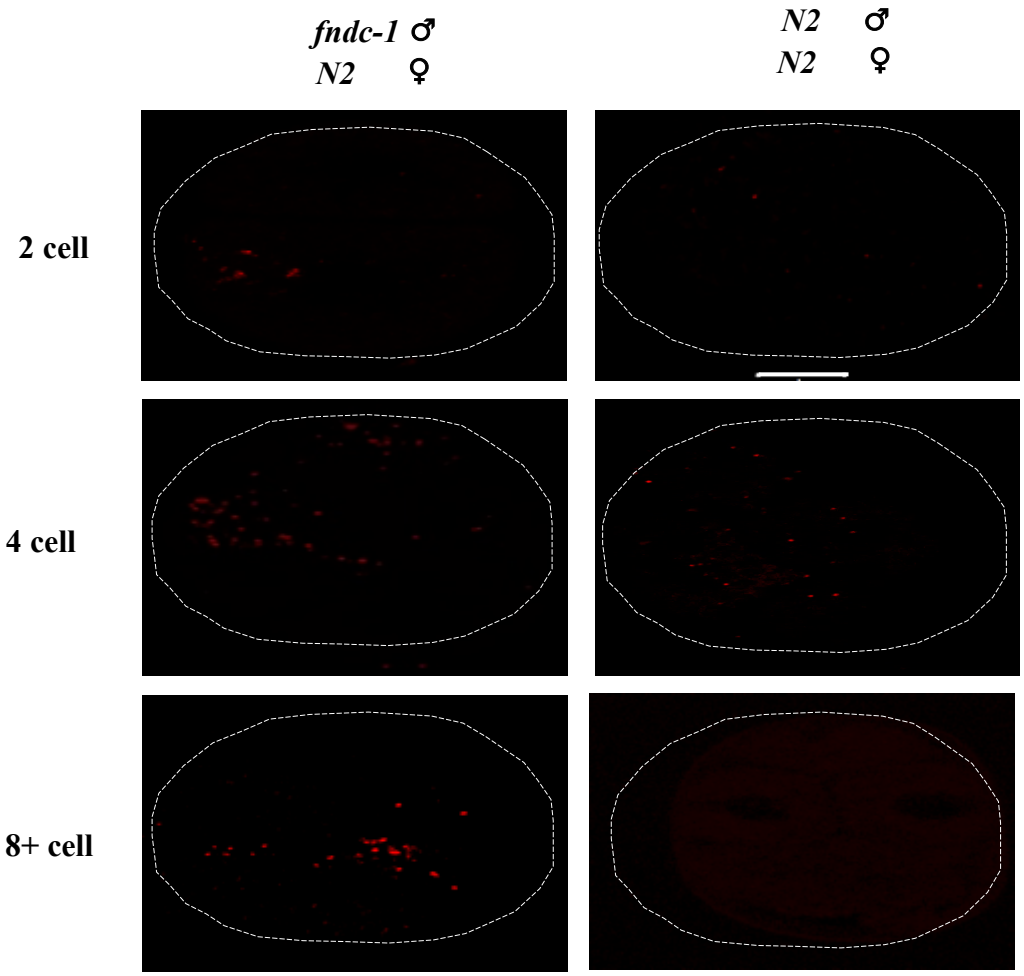


Figure 19- Parkin is not required for ubiquitination of MOs after fertilization

GFP::Ub expression was assessed in *pdr-1* knockdowns. RNAi treated and vector control embryos showed the same expression of GFP::Ub, indicating that *pdr-1* was not responsible for ubiquitinating MOs.

A


fndc-1 ♂
N2 ♀
N2 ♂
N2 ♀
2 cell
4 cell
8+ cell

B

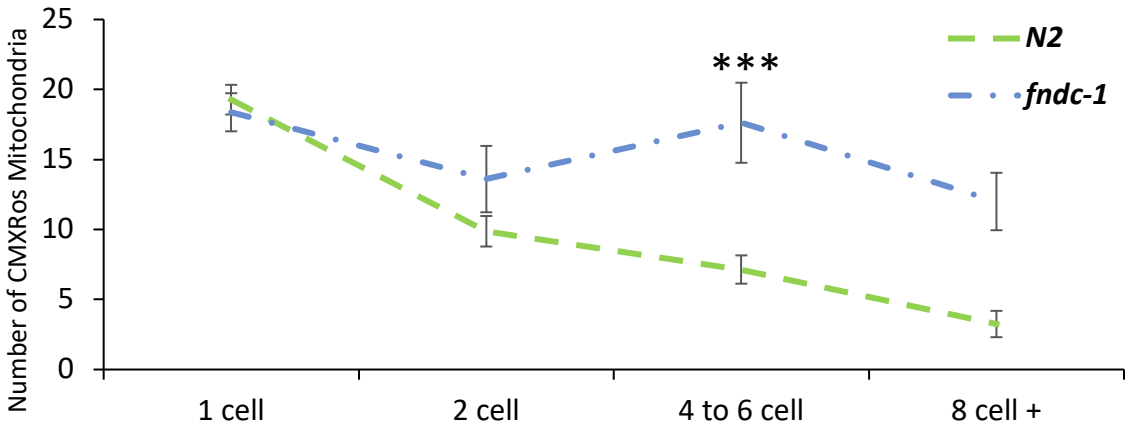

Number of CMXRos Mitochondria
25
20
15
10
5
0
1 cell
2 cell
4 to 6 cell
8 cell +
***
N2
fndc-1

Figure 20- In *C. elegans* Paternal mitochondria are eliminated through non canonical mitophagy

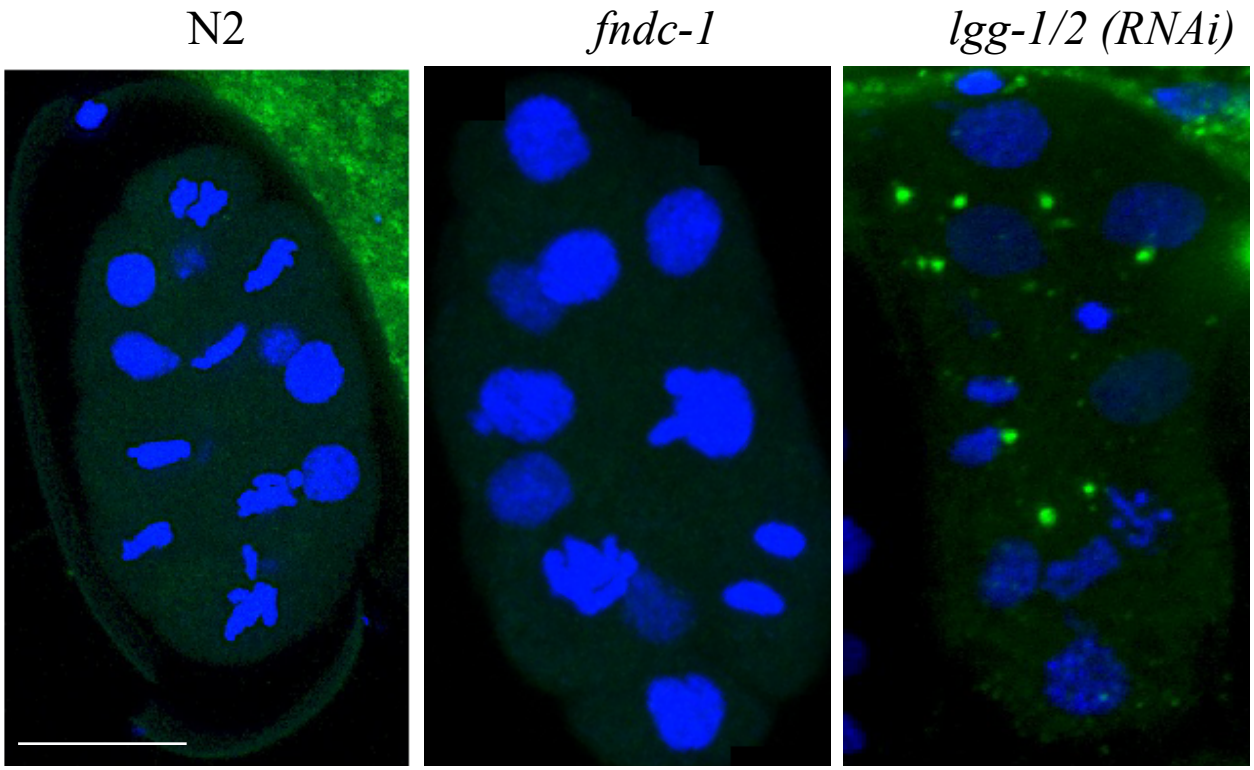
(A) *fndc-1* mutant CMXROS soaked males were mated with N2 hermaphrodites (n= 20 embryos) and a delay in removal of paternal mitochondria was detected until the 8-12 cell stage. **(B)** Data shown in graph are mean ± s.e.m. Significant differences between mating experiments was determined by unpaired *t* test.***p<000.1. Scale bars represent 10 μm.

MOs are eliminated in *fndc-1* mutants

The elimination of MOs and paternal mitochondria is thought to happen simultaneously during PME. Our current model for the elimination of MOs, is that it occurs via autophagy[33, 38, 41, 43, 53]. Our studies support this model by showing that during conditions where ubiquitination is reduced, MOs persist. Our data also show that mitochondria elimination is delayed during these conditions. Conversely, PME was delayed further in *fndc-1* mutants. Therefore, we wanted to test the effect of *fndc-1* deficiency on the elimination of MOs. Embryos from *fndc-1* hermaphrodites were extracted and stained for MOs. MOs were not observed in later stages, which is different from the effect that *fndc-1*mutations had on paternal mitochondria. Embryos after the 8 cell stage were practically clear of MOs (Figure 21A). MO numbers dropped after the first

mitosis and continued to decline, mutant and wildtype numbers were not significantly different (Figure 21B).

A


N2
fndc-1
lgg-1/2 (RNAi)

B

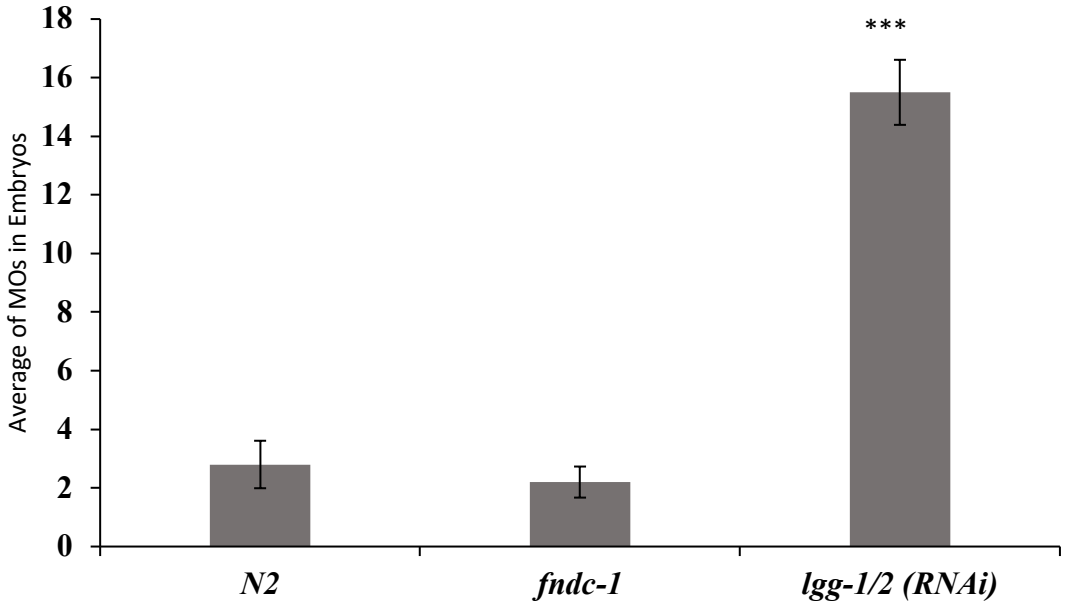

Average of MOs in Embryos
18
16
14
12
10
8
6
4
2
0
***
N2
fndc-1
lgg-1/2 (RNAi)

Figure 21- Sperm derived membranous organelles (MOs) do not persist in *fndc-1* mutant embryos.

MO persistence was assessed in 10 cell embryos using the 1CB4 antibody. DAPI staining was used to visualize DNA and determine embryonic stages. Maximum projection images were observed. The average number of MOs in 10 cell embryos was assessed in two separate trials for n2, *fndc-1* mutants and *lgg-1*/*lgg-2* RNAi treated worms. Asterisks indicate a significant different ($p<0.0001$ by student's *t*-test). Scale bar represents 10 μm.

Discussion

Evidence in mammals suggests that paternal mitochondria are ubiquitinated before fertilization[48]. However, whether *C. elegans* paternal mitochondria are ubiquitinated remains elusive. Early reports addressing post fertilization ubiquitination in *C. elegans* present evidence that MOs are ubiquitinated but not paternal mitochondria, while a recent report suggests both organelles are ubiquitinated, but MOs are ubiquitinated at a higher level[38, 52].

Ubiquitin and MO antibody staining colocalize and follow a specific pattern that is explained in figure 1. Therefore, multiple reports from different labs support the ubiquitination of MOs. GFP::Ub vesicles are used to track MOs during early embryogenesis and it has been reported that paternal mitochondria and GFP::Ub have a distinct pattern, during meiosis I and II these organelles are clustered together and later become more dispersed[52]. The clustering of GFP::Ub and paternal mitochondria might contribute the conflicting reports. After analyzing the fluorescence intensity of GFP::Ub and CMXROS tagged mitochondria of embryos at different one cell stages, we determined that mitochondria are not ubiquitinated (Figure 19). GFP::Ub appeared to colocalize

with GFP::Ub only during stages where MOs and paternal mitochondria were clustered together. The colocalization of ubiquitin and mitochondria was not comparable to the results observed with MOs and ubiquitin. Ubiquitination of the mitochondria has been established as a hallmark for mitophagy and mutations in parkin in *C. elegans* have been shown to lead to heteroplasmy[39]. Recent reports show that parkin mutations do not affect PME in *C. elegans* and our results support the noninvolvement of parkin in the ubiquitination of paternal organelles that occur after fertilization (Figure 20A).

Our results indicated that ubiquitination of MOs was necessary for the initial removal of paternal mitochondria but not PME that occurs after the first mitosis. A ubiquitin independent pathway might be involved in *C. elegans* PME. Unpublished results from the Nehrke lab determined the expression of *fndc-1,* the worm homologue of *fndc-1*, an autophagy receptor important for mitochondrial hypoxia response, using a CRISPR-Cas9 system. Their results indicated that *fndc-1* was exclusively expressed on sperm mitochondria. Therefore, they decided to observe the effect mutations in *fndc-1* had on PME. In collaboration with our lab, we determined that *fndc-1* mutations showed a delay in PME during the 8 cell stage, but also showed a drop in mitochondrial numbers

after the 2 cell stage (Figure 20B). These results together support the hypothesis that there are two pathways involved in *C. elegans* PME.

The current model for PME proposes that paternal mitochondria and MOs are eliminated simultaneously. Our results do not completely support this model. Interruption in the ubiquitination of MOs only delays PME at the 2 cell stage but does affect elimination of MOs. However, knockdowns of *rpn-10* and *rad-23* delay PME and MO elimination. Therefore, we wanted to test the effect of *fndc-1* on the persistence of MOs. *fndc-1* mutants did not have an effect of the elimination of MOs (Figure 21). MO numbers in mutants were similar to wildtype, providing further evidence that MOs and mitochondria are eliminated through different signals. Therefore, we have provided evidence that ubiquitination is driving the elimination of MOs but PME is more highly regulated and *fndc-1* serves to eliminate the mitochondria that are not engulfed by the autophagosomes under conditions where ubiquitination is reduced.

The majority of studies in PME have identified maternal proteins that are required for the selection and elimination of paternal mitochondria. Ubiquitination plays an important role in these models. However, the ubiquitin

independent pathway for PME through paternally supplied *fndc-1* provides an alternative model.

FNDC-1 is an integral outer mitochondrial membrane protein that functions a receptor for hypoxia induce mitophagy. FNDC-1 interacts with LC3 through its LC3 binding motif, this interaction is possible after hypoxia induced dephosphorylation of FNDC-1 occurs[101]. Therefore, the autophagosome membrane surrounds damaged mitochondria without the need of ubiquitination. Our study proposes that FNDC-1 is activated for PME, suggesting that a possible dephosphorylation event occurring in the sperm or embryo activate its LC3 binding activity. Since FNDC-1 is exclusively expressed in paternal mitochondria, it is probable that this is the unique tag required for the specific elimination of paternal mitochondria.

CHAPTER 6

Sperm tissue in *C. elegans* express low levels of ubiquitin

Introduction

Various post-fertilization events require interactions between paternal and maternal proteins. Effects observed from paternal proteins or mRNAs that are supplied by the sperm are known as the paternal effect[106]. So, it is important to look at events that occur in the sperm prior to fertilization. In mammals, sperm mitochondria are ubiquitinated before fertilization and given the importance of ubiquitin during meiosis, expression of ubiquitin is expected in all gonadal tissue[47, 48, 107].

Sperm tissue has dynamic protein expression depending on the stage of spermatogenesis. In *C. elegans* , spermatogenesis begins with the presence of primary spermatocytes that form in the syncytium with a cytoplasmic core known as the rachis, primary spermatocytes undergo meiosis I to divide the 4N nucleus into 2N nuclei[108]. This process is heavily dependent on the anaphase promoting complex (APC), the APC is an E3 ubiquitin ligase complex that is important for regulating the transition from metaphase to anaphase during cell

division[109]. Worms with defects in their APC have primary spermatocytes that will exhibit normal budding from the syncytium and nuclear envelope breakdown but will be arrested at metaphase of meiosis I[110]. This leads to a failure of properly segregated chromosomes [111]. The resulting secondary spermatocytes from meiosis I undergo meiosis II and two haploid spermatids from each 2N spermatocyte is formed. Spermatids are formed by budding off a structure known as a residual body, this is where components such as tubulin, actin and all ribosomes are excluded from the spermatid. Structures observed in the spermatid are its nucleus, mitochondria and MOs[108].

Since spermatids lack ribosomes and their DNA is tightly compacted, they are transcriptionally and translationally inactive[112, 113]. However, spermatogenesis is still regulated at the translational level. An example of two translation regulators expressed in the gonad that are essential to spermatogenesis are, cytoplasmic polyadenylation element binding proteins BPC-1 and CBP-2 and IFE-1 an mRNA cap-binding protein[114, 115]. Post transcriptional modification such as phosphorylation and dephosphorylation also play a role in spermatogenesis. Protein kinases such as SPE-6 are important for the segregation of cellular components during early spermatogenesis and

meiosis, and PP1 phosphatases GSP-3 and GSP-4 are required for segregation of chromosomes during meiosis and for Major Sperm Protein (MSP) disassembly observed during sperm movement[116, 117]. However, the post-translational modification, ubiquitination in *C. elegans* sperm is not well characterized.

A study in *C. elegans* used temperature sensitive E1 (*uba-1*) mutants to study the role of ubiquitination in sperm. Sperm developed normally but were sterile in mutants. Their results coupled with the identification of an E3 ligase homologue spe-16 presented a novel function of ubiquitin during spermatogenesis[118, 119]. Ubiquitination is usually associated with roles in meiosis, histone modification, chromatin remodeling, protein sorting and protein quality control. A hypothesis presented by McDermott-Roe *et al* for the role of ubiquitination is that it may promote proteasomal degradation of proteins that would inhibit fertilization or sperm activation. Sperm proteasome enters the oocyte after fertilization, the exact function of these sperm proteasome is not well understood, but they could be required for successful fertilization[52]. In order to understand the paternal effect during the ubiquitination of MOs it is important to establish the localization of ubiquitin and proteasome in sperm tissue.

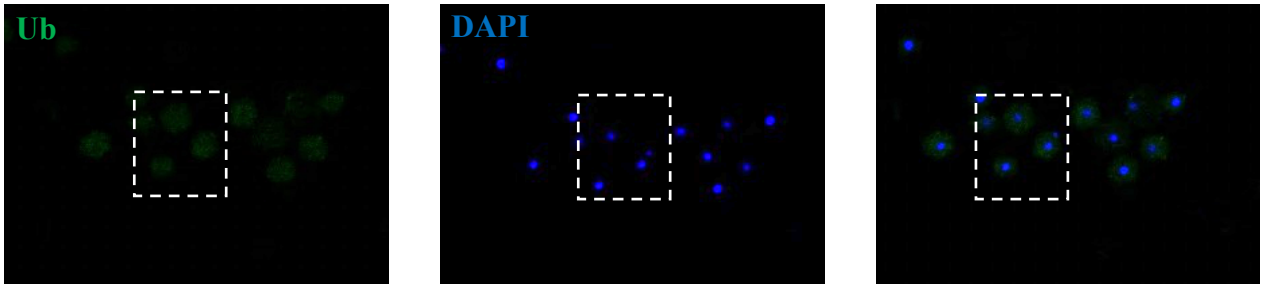
Results

Polyubiquitin chains are detected in spermatocytes

Using antibodies for polyubiquitin (FK2) and ubiquitin (poly and monoubiquitin) we stained the sperm gonad to observe the localization of ubiquitin. We concluded that polyubiquitin was found in primary spermatocytes because ubiquitin antibody and FK2 which only stains polyubiquitin chains localize to the same region in the nucleus (Figure 22B, C). Zoomed in images show a high concentration of ubiquitin in the form of a disc located inside the nucleus but not directly located on the DNA. Ubiquitin staining was detected at low levels in spermatids and rachis region. No distinct staining pattern was observed (Figure 22A, D).

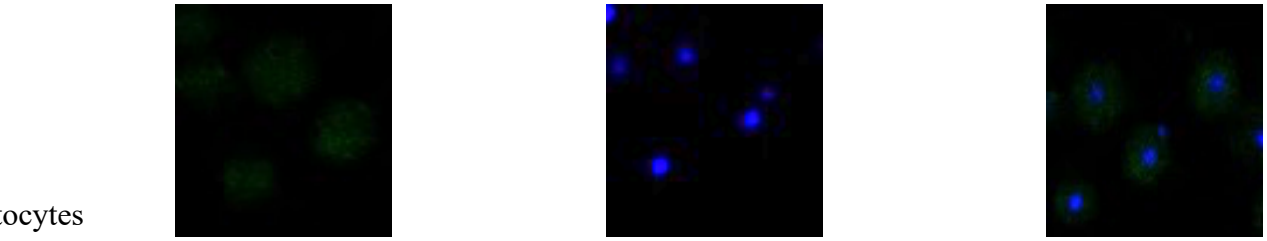
A

Spermatids



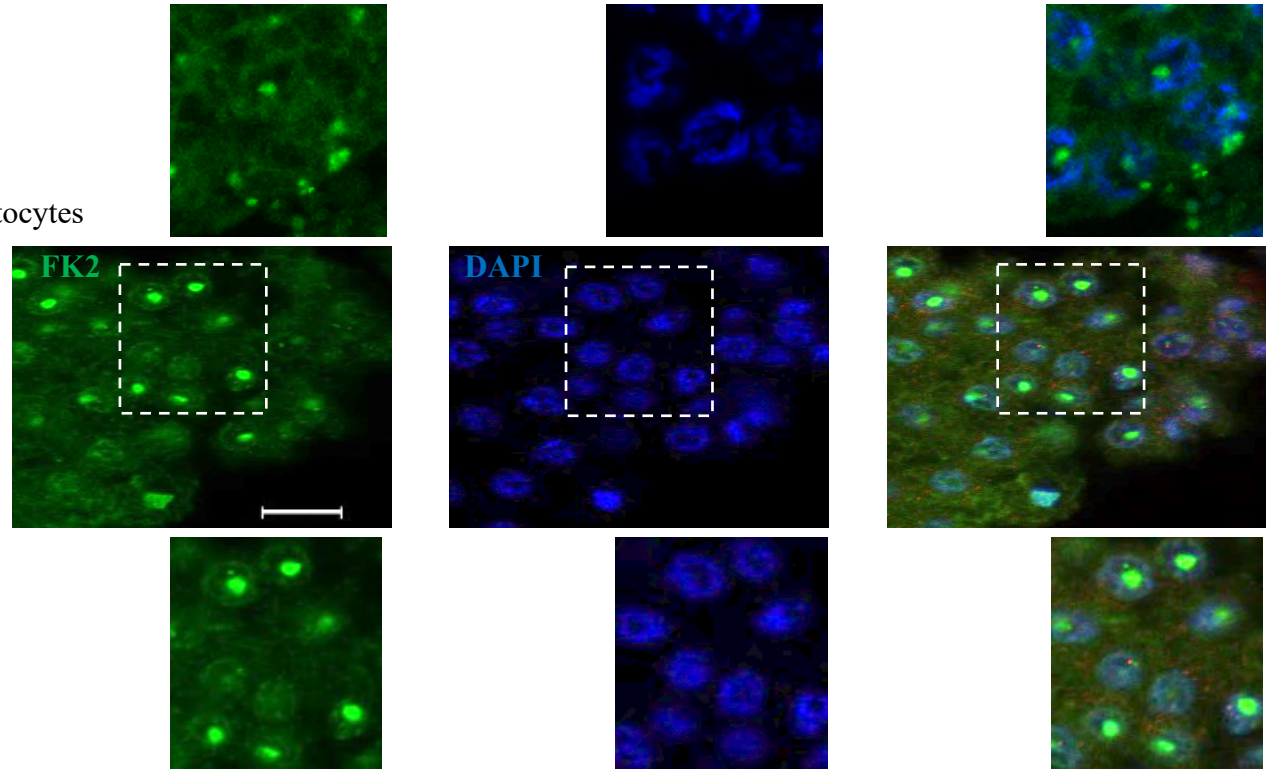
B

Primary Spermatocytes



C

Primary Spermatocytes



D

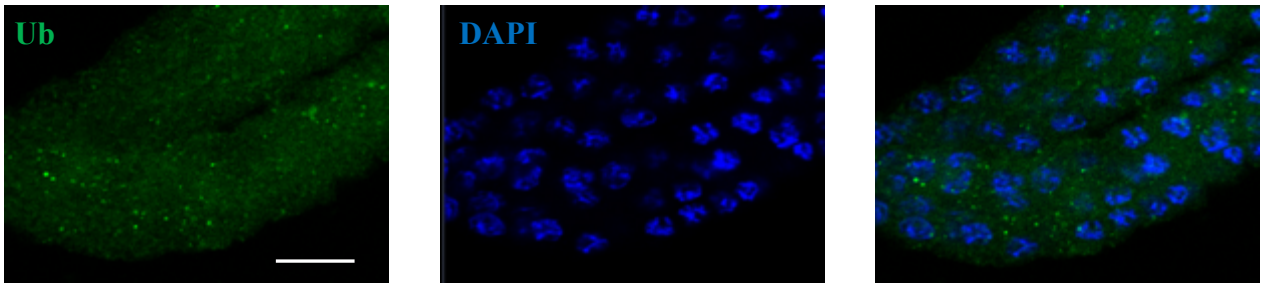


Figure 22- Localization of ubiquitin in sperm tissue.

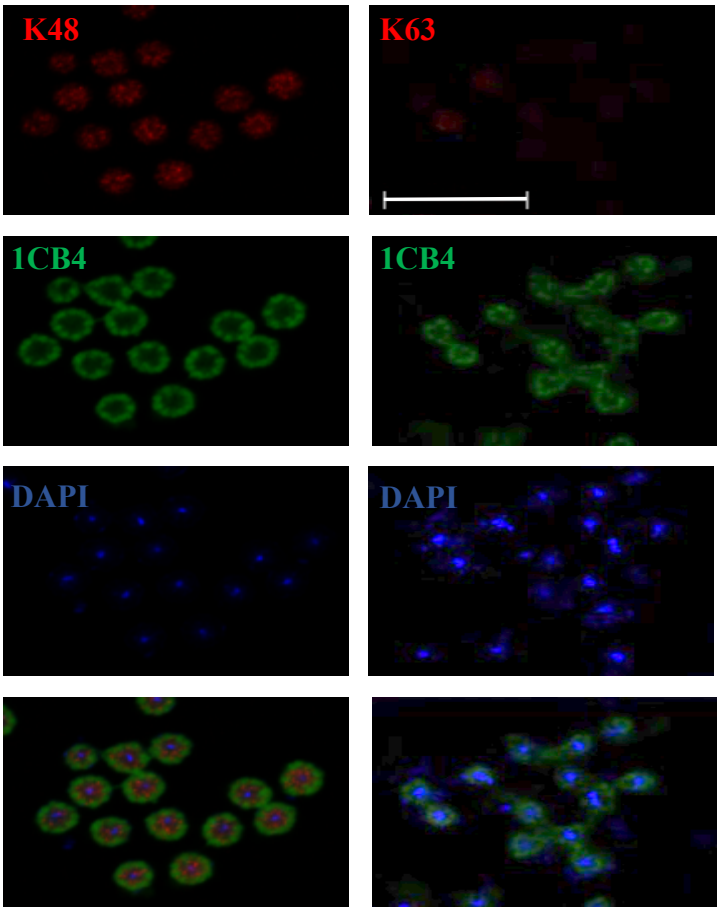
(A) Ubiquitin antibody that detect both polyubiqutin and monoubiquitin chains was used to observe the localization of ubiquitin in different sperm cells. In spermatids, ubiquitin staining levels were low but diffuse in the cytosol. To account for autofluorescence unstained spermatids were observed under 488 lasers. There was a level of autofluorescence detected at a high gain. These parameters were used to exclude background staining**. (B,C)** In primary spermatocyte polyubiquitin localized to the nucleus. FK2 was used to exclusively detect polyubiquitin chains. **(D)** DAPI staining revealed that ubiquitin was adjacent to DNA not colocalized as we observe in the oocytes. Ubiquitin staining was also not detected in the mitotic region of the sperm gonad. Scale bar represents 10 μm.

K48 chains are detected in spermatids but not K63

The two types of ubiquitin chains identified on sperm organelles were K48 and K63. Therefore, we decided to check spermatids for ubiquitin chain staining. K48 staining was detected in spermatids but not K63 (Figure 23A). However, K48 staining was diffuse in the cytosol and not colocalized with MOs. Similar to ubiquitin staining, 46% of the spermatids analyzed were stained with K48 (Figure 23B). Ubiquitin staining appeared weaker than K48 but the lower signal also appeared diffuse in the cytosol, this staining pattern was not observed in K63 stained spermatids.

We also wanted to observe K48 staining in *ubc-18* mutant. The staining was very similar, but staining was detected in fewer spermatids (Figure 23B). The difference was determined to be statistically significant by on tailed z- test.

A


K48
K63
1CB4
1CB4
DAPI
DAPI

B

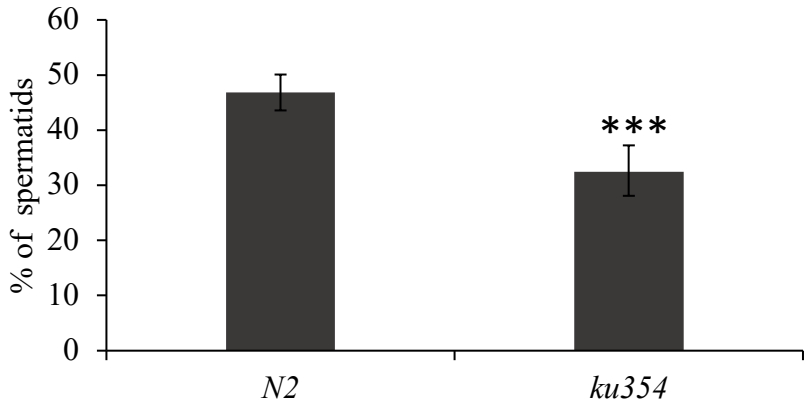

60
50
40
30
20
10
0
% of spermatids
***
N2
ku354

Figure 23- K48 ubiquitin chains are present in spermatids but do not co-localize with MOs.

(A) To test for MO ubiquitination prior to fertilization, colocalization of MO antibody 1CB4 and K48 or K63 ubiquitin chain specific antibodies was assessed. K48 chains were observed in the cytoplasm of spermatids, but do not co-localize with MOs. On the other hand, K63 were not observed in spermatids. To account for autofluorescence unstained spermatids were observed under 488 and 639 lasers. There was a level of autofluorescence detected at a high gain. These parameters were used to exclude background staining. **(B)**Our data indicate that in embryos *ubc-18* is required for K48 chains on MOs, we wanted to test the effect on K48 chains in *ubc-18(ku3654)* mutant males. Mutant male population presented with a 14% reduction in the number of spermatids with positive K48 staining. Scale bar represents 10 μm. z-test was used to test for statistical significance. $p>0.001$ ***. Error bars represent 95% confidence intervals.

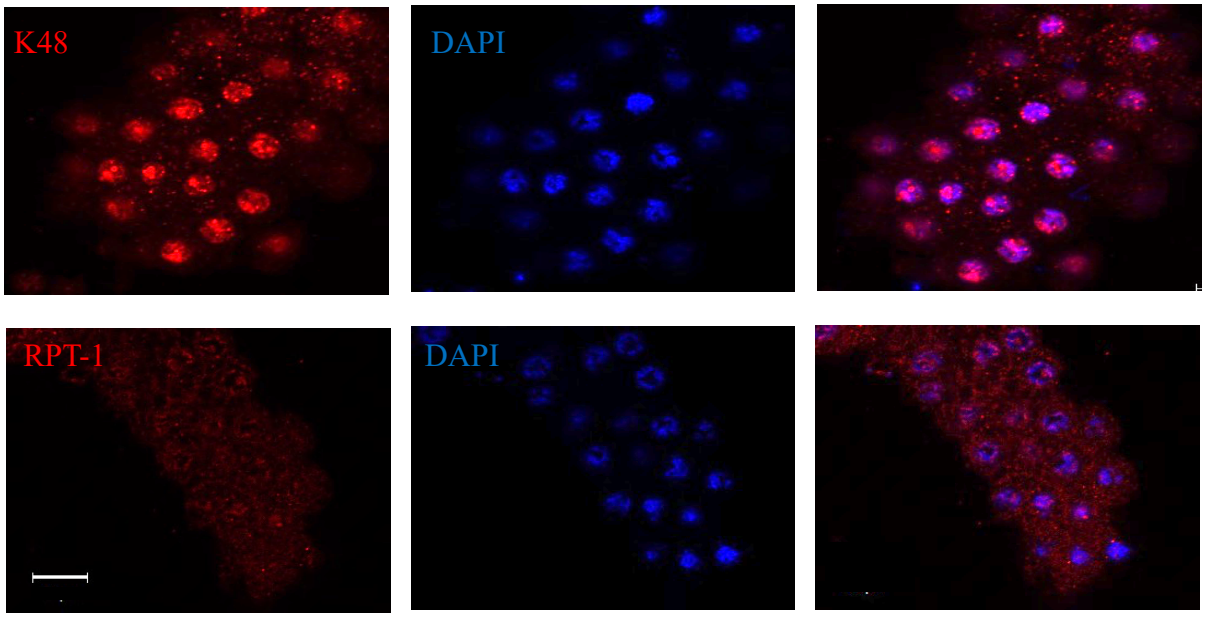
Proteasome co-localizes with MOs in spermatids

K48 ubiquitin chains are usually a signal for the proteasome to degrade target proteins. K48 chains were not detected on MOs but diffuse in the cytoplasm (Figure 24A). Consequently, we wanted to observe proteasome localization. We used an antibody for RPT-1 proteasomal subunit to observe proteasome localization, the same subunit tagged in the transgenic worm used to assess proteasomal localization in the embryo. Our stains revealed that RPT-1 and MOs co-localize in spermatids (Figure 25B). MOs fuse with the sperm plasma membrane to release MSP and these structures can be observed along the periphery of the spermatid. RPT-1 has a more punctate staining but concentrated puncta can be seen along the periphery of the membrane, co-localizing with MOs.

K48 and RPT-1 do no localize to the same regions in sperm tissue. As seen in figure 25A, K48 show distinct puncta staining inside the nucleus of spermatocytes, while RPT-1 shows more of a diffuse stain and some small puncta around the edge of the DNA. K48 staining in the spermatids appears diffuse in the cytoplasm while RPT-1 colocalizes with MOs on the periphery of the cell. On

the other hand, in spermatocytes polyubiquitin antibodies detect nuclear staining that is similar to the staining detected by K48 antibodies. This localization to the nucleus of these cells might indicate a possible role of ubiquitin during spermatogenesis.

A



B

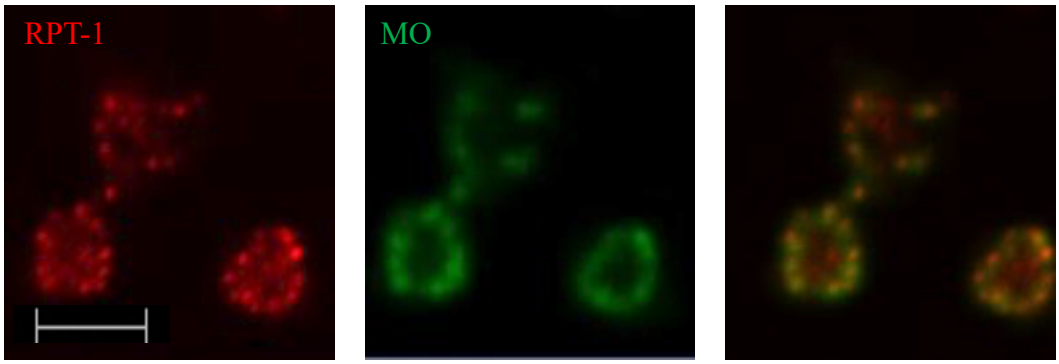


Figure 24- Localization of proteasome subunit RPT-1 in sperm tissue.

K48 ubiquitin antibody stains the nucleus in primary spermatocytes, while proteasomal subunit RPT-1 antibody is absent **(A).** Conversely, RPT-1 is found in the periphery of the spermatids, colocalizing with MOs **(B)**. Scale bar in panel A represents 10 μm, in panel B 5 μm.

Residual Bodies contain high levels of ubiquitin

Given the low levels of ubiquitin detected in spermatids but its detection in primary spermatocytes we wanted to discern whether ubiquitin was lost in the residual bodies following meiosis II. Residual bodies were scored based on the presence of ubiquitin or K48 chains in the residual body compared to budding spermatids. If staining of K48 or ubiquitin appeared to be evenly diffuse between spermatids and residual bodies or absent in residual bodies, these structures were scored as unsegregated. On the other hand, if staining was localized to residual bodies, they were scored as segregated. Figure 26A shows representative images of unsegregated and segregated residual bodies. The ubiquitin staining in the first image is diffuse and evenly distributed. The second image shows concentrated levels of K48 staining in the residual body and absent staining in the budding spermatids. Our data suggests that ubiquitin is being removed through the residual bodies and this could be the reason of the observed low ubiquitin levels in spermatids. FK2 and K48 staining was observed in similar distributions in 24 residual bodies from 3 different stains (Figure 25B).

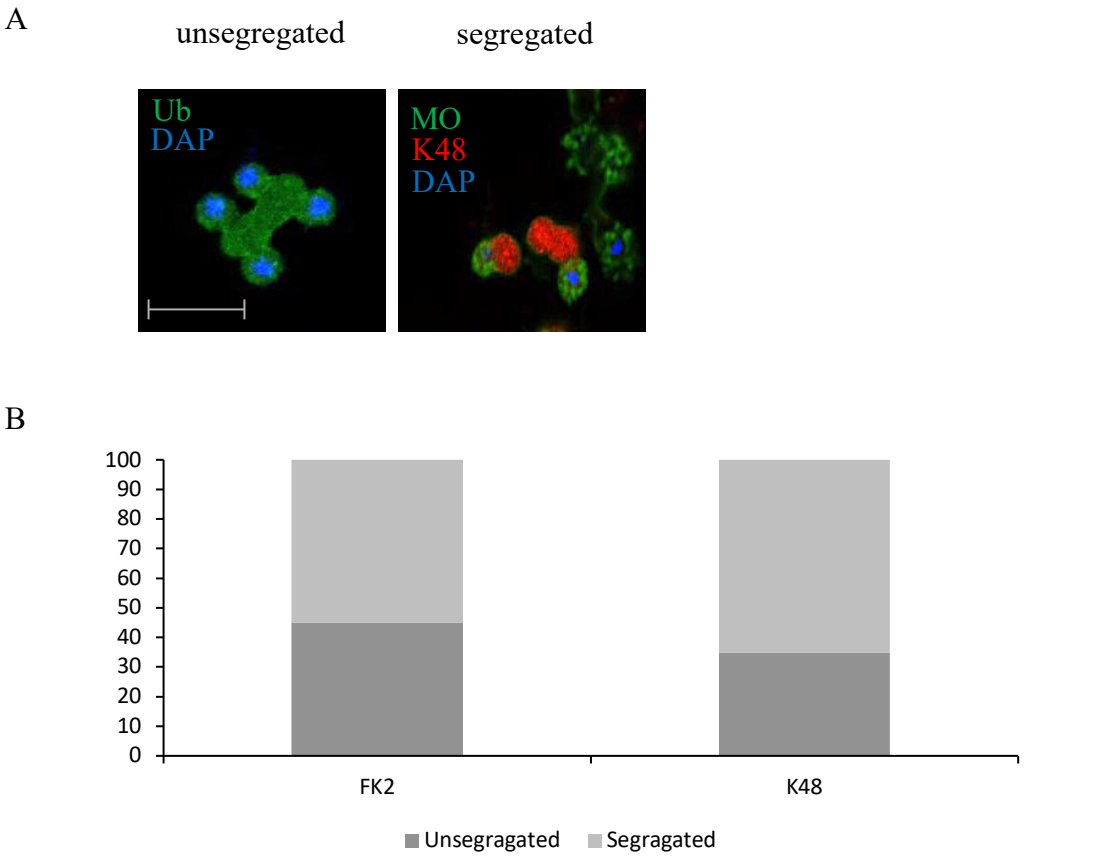


Figure 25- Residual bodies have high levels of ubiquitin.

Low levels of ubiquitin in spermatids led us to test for the elimination of ubiquitin during spermatogenesis. **(A)**Representative images of residual bodies scored are seen here. **(B)**The Residual bodies were scored based on the presence of ubiquitin or K48 stain in the residual bodies, as unsegregated or segregated. 55% of the embryos scored showed segregated FK2 staining in the residual bodies, 45% of residuals were scored as unsegregated**.** K48 ubiquitin chain staining appeared segregated in 65% of the residual bodies observed. Scale bar represent 10 μm.

Discussion

Paternal contributions to post fertilization processes have been observed, some mRNAs and proteins are essential to embryonic viability. Reports about the contribution of sperm to PME have focused on ubiquitination of mitochondria and destruction of mitochondrial DNA by endonuclease G before fertilization. In *C. elegans* , endonuclease G deficient worms have delayed PME. Ubiquitination in spermatids has not been well characterized. Spermatids stained with ubiquitin show very low levels of staining that does not colocalize with MOs (Figure 23A). Therefore, ubiquitination of MOs happens in the embryo not the spermatids. In contrast, ubiquitin staining was observed in the nucleus of primary spermatocytes (Figure 23B). This staining could indicate the involvement of ubiquitin during meiosis I. Recent reports in mice testes have shown that ubiquitin staining is observed in the chromosomes of sperm and is involved in regulating proteins responsible for recombination crossing over events[107]. In yeast, E3 ligase Zip3 recruits the proteasome to chromosomes, this step is essential for meiotic progression[120]. Ubiquitination could be involved in recruitment of the proteasome during early spermatogenesis.

Proteasomal recruitment usually follows after the addition of K48 chains, our results indicate that proteasomal subunit RPT-1 does not co-localize with

K48 chains in primary spermatocytes or spermatids (Figure 24). K48 chains are detected in the cytosol of spermatids, RPT-1 and MO staining is concentrated around the periphery of the spermatid (Figure 23A, Figure 24B). Thus, K48 and proteasome also do not localize in sperm tissue. The presence of other proteasomal subunits still need to be investigated to assess if K48 chains are directing localization of proteasome.

In early embryos K48 chains are added to MOs during meiosis I in a *ubc-18* dependent manner. Since K48 chains were observed in spermatids, we wanted to see if *ubc-18* would have reduced numbers of K48 chains. In fact, 32% of the mutant spermatids observed presented with K48 staining (Figure 23B). Suggesting that *ubc-18* is also involved in addition of K48 chains in sperm or given the low levels of ubiquitin present in spermatids *ubc-18* may be involved in preventing elimination of ubiquitin chains during spermatogenesis. K48 staining was not observed in all embryos. This could be due to the role of K48 ubiquitin in different cellular responses. A group of spermatids maybe responding to different cellular cues. Also, our data suggests that K48 chains are is segregated to about 65% of residual bodies. Therefore, 35% of unsegregated residual bodies could explain why some spermatids still have K48 staining

The presence of ubiquitin in the nucleus during early stages of spermatogenesis, and the fact that not all spermatids show ubiquitin staining and spermatids that are stained have low levels of staining, suggest that ubiquitination is constantly changing during spermatogenesis. During spermatogenesis, most of the sperm's contents are eliminated through the residual body. After scoring residual bodies with we found that at least 50% of the residual bodies observed had high levels of ubiquitin staining (Figure 25B). These results propose that some ubiquitin protein is eliminated during the development of spermatids.

CHAPTER 7

Conclusions and Future Studies

During embryogenesis ubiquitin and proteasome are required to remove paternal organelles

The objective of this study was to determine the role of ubiquitination during PME. Ubiquitination is a post-translational modification used by several pathways to tag proteins for specific purposes such as elimination or trafficking[9, 10, 21, 24, 25, 77, 80, 121, 122]. During PME, there are multiple reports that point to ubiquitin as the initializing signal for paternal mitochondria degradation[33, 35, 38, 43, 48, 53, 97]. The results from this study support this model but also reveal that paternal mitochondria are not ubiquitinated (Figure 20) which oppose recent findings from the Sato lab. Our data suggest that mitochondria are clustered with ubiquitinated MOs during meiosis I and meiosis II. The reported detection of mitochondrial ubiquitination could be due to clustering of MOs and paternal mitochondria. The ubiquitination of MOs is well characterized in the literature[33, 41, 52]. The two types of ubiquitin chains detected on MOs K48 and K63 are important for the elimination of damaged mitochondria. However, these chains were detected on MOs rather than on mitochondria in *C. elegans* . The current model for *C. elegans* PME says that MOs

and paternal mitochondria are eliminated together through ubiquitin driven autophagosome formation (Figure 5). Therefore, a relationship between elimination of MOs and PME exists. Our findings support this aspect of the current model but also reveal new mitochondrial dynamics after the first mitosis. Ubiquitination of MOs by two different chains reveals the possibility of at least two ubiquitination events occurring after fertilization. The initial ubiquitination event is the addition of K48 chains during meiosis I which is driven by E2 enzyme UBC-18 (Figure 10). The E2 responsible for the addition of K63 still remains to be determined (Figure 11). However, simultaneous knockdown of *ubc-18* and *ubc-16* reveal a reduction in ubiquitination after fertilization (Figure 9). These findings suggest that other types of ubiquitin chains may be present on MOs, highlighting that multiple E2 or E2/E3 combinations could be involved the ubiquitination of MOs. Data that show K27 ubiquitin chains on MOs contributes to the proposed model that MOs are ubiquitinated with multiple E2s. Under condition where ubiquitination of MOs is reduced, persistence of these sperm organelles is observed (Figure 14). Autophagosome formation is reduced in the absence of ubiquitination, which would explain the persistence of MOs (Figure 18).

MOs and paternal mitochondria are engulfed in the autophagosome together[33]. Therefore, it was surprising to see paternal mitochondria being eliminated under conditions where MOs persist (Figure 15). The absence of ubiquitination, specifically the addition of K48 chains did not affect PME in later embryonic stages but it did delay PME at the 2 cell stage. The number of paternal mitochondria drop drastically after the first mitosis, in embryos lacking UBC-18 this event is delayed to the 4 cell. This data together suggest that MOs and paternal mitochondria are selected for elimination in a different manner.

In fertilized embryos, the addition of K48 chains is followed by recruitment of the proteasome during meiosis I[52]. This recruitment is interrupted when K48 chains are not present in *ubc-18* knockdowns, which coincides with a delay PME. Suggesting that proteasome recruitment is necessary for the initial removal of paternal mitochondria. It is interesting that proteasome recruitment to the MOs would have an effect on PME and not the removal of MOs. Our data indicate that *ubc-18* knockdowns do not affect MO removal but are responsible for delay in early PME. Our hypothesis is that K48 chains recruit proteasome to clusters of MOs and paternal mitochondria to degrade membrane proteins. This step is necessary for the early removal of

paternal mitochondria. The importance of proteasome is also highlighted in our results that reveal PME and MO elimination are severely delayed in knockdowns of proteasome ubiquitin receptors *rpn-10* and *rad-23* (Figure 16). These ubiquitin interacting subunits of the proteasome also interrupt proteasome recruitment during meiosis I (Figure 16). However, the persistence observed in later stages indicates that another proteasome event is taking place before or during embryogenesis that is required for clearance of paternal mitochondria and MOs. Interestingly, autophagosome formation is not interrupted as is usually the case when PME is interrupted. Our data supports findings that show that autophagy is up regulated in the absence of *rpn-10*[91].

Persistence of paternal mitochondria and MOs without interruption in the recruitment of autophagosome forming proteins leads us to speculate that the indirect or direct involvement of the proteasome in the maturation of the autophagosome, lysosome-autophagosome fusion and/or lysosomal maturation. A possible explanation could be that autophagosomes fail to mature to autolysosomes. There are studies that link vasolin-containing protein (VCP) to proper maturation of vacuoles into autolysosomes[123]. VCP performs its function through interactions with ubiquitin intermediates and Rpn10 a

ubiquitin recognition particle which is found in proteasome-free pools within the cytosol could serve as a shuttle for ubiquitinated substrates or other ubiquitin recognition particles[11, 124, 125]. A relationship during development between Rpn10 and VCP function was observed in *C. elegans* . VCP was necessary for Rpn10 dependent gametogenesis[126]. The proposed mechanism of VCP states that it could functions as an unfloldase that disassembles polyubiquitinated proteins from a CUL2 complex in an ATP-dependent manner. Then delivers these proteins associated with Rpn10 or Rad23 to the proteasome [123, 127]. The same mechanism could be involved in the maturation of autophagosomes containing MOs and paternal mitochondria. In the absence of Rpn10 or Rad23, VCP function could be disturbed and autophagosome-lysosome fusion might not take place. To better define the role of the proteasome and to test our hypothesis, autophagosomes containing MOs and mitochondria should be observed in later stages. The proteasome targets on the mitochondria and MOs still remain to be determined.

Proteasome involvement during PME reinforces the model that proposes PME is induced due to damaged paternal mitochondria. Recent reports have shown that paternal mitochondria are depolarized after fertilization and this is the signal that induced the engulfment of mitochondria by the

autophagosome[40]. However, these reports do not address the lack of ubiquitination in *C. elegans* . On the other hand, the Sato lab reported that ubiquitination of MOs and paternal mitochondria drive the activation of autophagy receptor ALLO-1. Our data contradicts the claim regarding paternal mitochondria ubiquitination. We think that is due to the fact they assessed mitochondria that were clustered with ubiquitinated MOs. They also provided strong evidence that Parkin, PINK1 and Sequestasome mutations do not affect PME in the worm. This study supports the idea that PME is Parkin independent. pdr-1 knockdowns did not affect the presence of GFP::Ub surrounding the paternal DNA after fertilization (Figure 20A). However, our results also show that the 3 E2s identified in a human mitophagy screen reduced the presence of GFP::Ub in *C. elegans* (Figure 12). The knockdowns of *ubc-18*, *ubc-13* and *ubc-2* also produced and an interesting polyspermy phenotype. To our knowledge this is the first report of ubiquitination being involved in preventing polyspermy.

We have established a system where ubiquitination of MOs is reduced by knocking out the E2 enzymes in the embryo. However, the E3 involved remains unknown and requires further study. Having determined the E2s involved will provide information required to select E3s that have a relationship with UBC-18

and UBC-16. For example, a study determined that UBC-18 preferentially interacts well with E3s from the RING/HECT family[107].

Paternal mitochondria but not MOs are also eliminated through a ubiquitin independent manner

There are similarities between mitophagy and PME. PINK1/Parkin mediated mitophagy is not the only pathway damaged mitochondria are eliminated through. Parkin independent pathways are usually tissue and/or stress specific[76, 101]. Work conducted in the Nehrke lab suggests that knockdown of FNDC-1 is required for elimination of paternal mitochondria. Our data supports these findings, but we also establish that in *fndc-1* mutants mitochondria numbers are reduced after the first mitosis providing evidence that there is an early ubiquitin dependent pathway. The number of paternal mitochondria in the *fndc-1* mutants and wildtype are similar at the 2 cell stage but begin to differ in later stages where *fndc-1* mutant embryos have larger numbers of mitochondria when compared to wildtype (Figure 21).

MOs were still eliminated in fundc-1 mutants, providing further evidence to support the model that MOs are eliminated through a ubiquitin independent

pathway. MOs are ubiquitinated and are eliminated independently from paternal mitochondria. According to unpublished data FNDC-1 paternal contribution is required for PME (personal communication with Keith Nehrke).

Ubiquitination occurs in early spermatogenesis and does not contribute to MO ubiquitination

Ubiquitin staining on *C. elegans* sperm tissue revealed that levels of ubiquitin vary as spermatogenesis progresses. We hypothesize that ubiquitination is involved in meiosis I and that is why ubiquitin is detected inside the nucleus. We also provide evidence that ubiquitin could be eliminated in the residual body explaining why ubiquitin is observed at low levels in the spermatids (Figure 22, 25). Our study supports the current model that MOs are ubiquitinated in the embryo not the sperm.

However, our proteasome staining contradicts previous findings that claim proteasome do not localize to MOs[52] (Figure 24). We also show that K48 chains and proteasome subunit RPT-1 are not found together (Figure 23). These findings could suggest that other signals are recruiting proteasome or that other proteasomal subunits are involved during spermatogenesis.

Proposed PME model for *C. elegans*

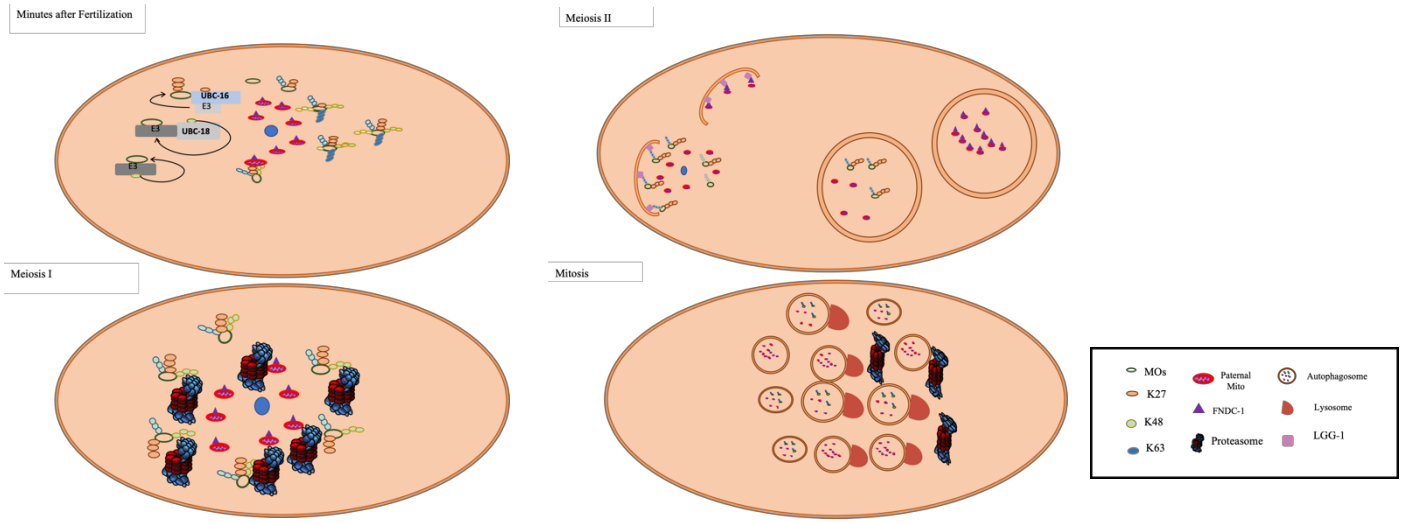


Figure 26- Proposed Model for PME in C. elegans .

MOs are ubiquitinated shortly after fertilization MOs are ubiquitinated with K48 chains by UBC-18 to recruit the proteasome which degrades a protein on the paternal mitochondria. UBC-16 and UBC-18 drive ubiquitination of MOs with other ubiquitin chains, possibly K27. K63 chain elongation could be regulated by other E2 or E2/E3 interactions that remain to be determined**.** During meiosis I, K48 chains recruit proteasome to MOs to degrade proteins required for early elimination of paternal mitochondria. MO ubiquitination recruits the formation of the autophagosome during early meiosis II, to surround the MOs and paternal mitochondria that are clustered nearby. Remaining paternal mitochondria are also selected via their FUNDC-1 membrane protein**.** Proteasome ubiquitin

receptors RPN-10 and RAD-23 interact with the autophagosome to promote fusion with the lysosome and a drop in paternal organelle numbers is observed. Some autophagosomes, MOs and mitochondria are observed after the first mitosis. Proteasome could be involved in further degrading these organelles during later stages of embryogenesis or earlier proteasome interactions driven by *rpn-10* and *rad-23* are necessary for PME and MO elimination.

Appendix 1

Table below are the results from the E2 RNAi combination screen explained in figure 6. Combinations with less than 50% of embryos with GFP::Ub puncta were observed on the LSM 700 confocal microscope to confirm results observed from the screen. Combinations that were above 50% were not scored.

ubc-gene combinations	**% of embryos with GFP::Ub puncta**	**% of embryos without GFP::Ub puncta**	**Were E2 combos screened confirmed on LSM 700?**
ubc-19, uev-2	10%	90%	No
ubc-19, uev-3	10%	90%	No
ubc-7, uev-1	10%	90%	No
ubc-18, ubc-13	11%	89%	No
ubc-13, ubc-22	20%	80%	No
ubc-18, ubc-7	20%	80%	No
ubc-7, uev-3	20%	80%	No
ubc-19, uev-1	30%	70%	No
ubc-1, ubc-7	40%	60%	No
ubc-18, ubc-14	40%	60%	No
ubc-18, ubc-16	**40%**	**60%**	**Yes**
ubc-18, ubc-17	40%	60%	No
ubc-18, ubc-8	40%	60%	No
ubc-20, uev-3	40%	60%	No
ubc-1, ubc-21	50%	50%	No
ubc-1, uev-1	50%	50%	No
ubc-18, uev-1	50%	50%	No
ubc-22, uev-1	50%	50%	No
ubc-14, ubc-13	70%	30%	Not scored
ubc-14, ubc-21	70%	30%	Not scored
ubc-14, ubc-8	70%	30%	Not scored
ubc-15, ubc-13	70%	30%	Not scored
ubc-15, ubc-21	70%	30%	Not scored
ubc-15, ubc-22	70%	30%	Not scored
ubc-15, ubc-24	70%	30%	Not scored
ubc-15, ubc-25	70%	30%	Not scored

ubc-15, uev-1	70%	30%	Not scored
ubc-15, uev-2	70%	30%	Not scored
ubc-17, ubc-13	70%	30%	Not scored
ubc-17, ubc-14	70%	30%	Not scored
ubc-17, ubc-26	70%	30%	Not scored
ubc-17, ubc-6	70%	30%	Not scored
ubc-17, ubc-7	70%	30%	Not scored
ubc-17, ubc-8	70%	30%	Not scored
ubc-20, ubc-13	70%	30%	Not scored
ubc-20, ubc-14	70%	30%	Not scored
ubc-20, ubc-21	70%	30%	Not scored
ubc-20, ubc-22	70%	30%	Not scored
ubc-20, ubc-24	70%	30%	Not scored
ubc-20, ubc-25	70%	30%	Not scored
ubc-20, ubc-8	70%	30%	Not scored
ubc-21, C40H1.6	70%	30%	Not scored
ubc-21, uev-1	70%	30%	Not scored
ubc-21, uev-2	70%	30%	Not scored
ubc-21, uev-3	70%	30%	Not scored
ubc-3, ubc-15	70%	30%	Not scored
ubc-3, ubc-16	70%	30%	Not scored
ubc-3, ubc-17	70%	30%	Not scored
ubc-3, ubc-23	70%	30%	Not scored
ubc-3, ubc-26	70%	30%	Not scored
ubc-3, ubc-6	70%	30%	Not scored
ubc-7, C17D12.5	70%	30%	Not scored
ubc-7, C40H1.6	70%	30%	Not scored
ubc-7, ubc-24	70%	30%	Not scored
ubc-7, uev-2	70%	30%	Not scored
ubc-1, C17D12.5	80%	20%	Not scored
ubc-19, ubc-13	80%	20%	Not scored
ubc-19, ubc-14	80%	20%	Not scored
ubc-19, ubc-15	80%	20%	Not scored
ubc-19, ubc-16	80%	20%	Not scored
ubc-19, ubc-17	80%	20%	Not scored
ubc-19, ubc-18	80%	20%	Not scored
ubc-19, ubc-20	80%	20%	Not scored

ubc-19, ubc-21	80%	20%	Not scored
ubc-19, ubc-22	80%	20%	Not scored
ubc-19, ubc-23	80%	20%	Not scored
ubc-19, ubc-24	80%	20%	Not scored
ubc-19, ubc-25	80%	20%	Not scored
ubc-19, ubc-26	80%	20%	Not scored
ubc-19, ubc-3	80%	20%	Not scored
ubc-19, ubc-6	80%	20%	Not scored
ubc-19, ubc-7	80%	20%	Not scored
ubc-19, ubc-8	80%	20%	Not scored
ubc-1, C40H1.6	90%	10%	Not scored
ubc-1, ubc-13	90%	10%	Not scored
ubc-1, ubc-14	90%	10%	Not scored
ubc-1, ubc-15	90%	10%	Not scored
ubc-1, ubc-16	90%	10%	Not scored
ubc-1, ubc-17	90%	10%	Not scored
ubc-1, ubc-18	90%	10%	Not scored
ubc-1, ubc-19	90%	10%	Not scored
ubc-1, ubc-20	90%	10%	Not scored
ubc-1, ubc-22	90%	10%	Not scored
ubc-1, ubc-23	90%	10%	Not scored
ubc-1, ubc-24	90%	10%	Not scored
ubc-1, ubc-25	90%	10%	Not scored
ubc-1, ubc-26	90%	10%	Not scored
ubc-1, ubc-3	90%	10%	Not scored
ubc-1, ubc-6	90%	10%	Not scored
ubc-1, ubc-8	90%	10%	Not scored
ubc-1, uev-2	90%	10%	Not scored
ubc-1, uev-3	90%	10%	Not scored
C40H1.6, C17D12.5	100%	0%	Not scored
ubc-13, C17D12.5	100%	0%	Not scored
ubc-13, C40H1.6	100%	0%	Not scored
ubc-13, ubc-21	100%	0%	Not scored
ubc-13, ubc-24	100%	0%	Not scored
ubc-13, ubc-25	100%	0%	Not scored
ubc-13, uev-1	100%	0%	Not scored
ubc-13, uev-2	100%	0%	Not scored

ubc-13, uev-3	100%	0%	Not scored
ubc-14, C17D12.5	100%	0%	Not scored
ubc-14, C40H1.6	100%	0%	Not scored
ubc-14, ubc-22	100%	0%	Not scored
ubc-14, ubc-24	100%	0%	Not scored
ubc-14, ubc-25	100%	0%	Not scored
ubc-14, uev-1	100%	0%	Not scored
ubc-14, uev-2	100%	0%	Not scored
ubc-14, uev-3	100%	0%	Not scored
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ubc-15, C40H1.6	100%	0%	Not scored
ubc-15, ubc-14	100%	0%	Not scored
ubc-15, ubc-26	100%	0%	Not scored
ubc-15, ubc-6	100%	0%	Not scored
ubc-15, ubc-7	100%	0%	Not scored
ubc-15, ubc-8	100%	0%	Not scored
ubc-15, uev-3	100%	0%	Not scored
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ubc-16, C40H1.6	100%	0%	Not scored
ubc-16, ubc-13	100%	0%	Not scored
ubc-16, ubc-14	100%	0%	Not scored
ubc-16, ubc-15	100%	0%	Not scored
ubc-16, ubc-17	100%	0%	Not scored
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ubc-16, ubc-22	100%	0%	Not scored
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ubc-16, ubc-24	100%	0%	Not scored
ubc-16, ubc-25	100%	0%	Not scored
ubc-16, ubc-26	100%	0%	Not scored
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ubc-16, ubc-7	100%	0%	Not scored
ubc-16, ubc-8	100%	0%	Not scored
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ubc-16, uev-2	100%	0%	Not scored
ubc-16, uev-3	100%	0%	Not scored
ubc-17, C17D12.5	100%	0%	Not scored
ubc-17, C40H1.6	100%	0%	Not scored

ubc-17, ubc-15	100%	0%	Not scored
ubc-17, ubc-21	100%	0%	Not scored
ubc-17, ubc-22	100%	0%	Not scored
ubc-17, ubc-23	100%	0%	Not scored
ubc-17, ubc-24	100%	0%	Not scored
ubc-17, ubc-25	100%	0%	Not scored
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ubc-17, uev-3	100%	0%	Not scored
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ubc-18, uev-3	100%	0%	Not scored
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ubc-20, uev-1	100%	0%	Not scored
ubc-20, uev-2	100%	0%	Not scored
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ubc-21, ubc-25	100%	0%	Not scored
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ubc-22, C40H1.6	100%	0%	Not scored
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ubc-22, uev-2	100%	0%	Not scored
ubc-22, uev-3	100%	0%	Not scored
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ubc-23, C40H1.6	100%	0%	Not scored
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ubc-23, ubc-14	100%	0%	Not scored
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ubc-26, ubc-8	100%	0%	Not scored
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ubc-26, uev-2	100%	0%	Not scored
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ubc-3, C40H1.6	100%	0%	Not scored
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ubc-3, ubc-7	100%	0%	Not scored
ubc-3, ubc-8	100%	0%	Not scored
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ubc-3, uev-2	100%	0%	Not scored
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ubc-6, C40H1.6	100%	0%	Not scored
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ubc-6, ubc-7	100%	0%	Not scored
ubc-6, ubc-8	100%	0%	Not scored

ubc-6, uev-1	100%	0%	Not scored
ubc-6, uev-2	100%	0%	Not scored
ubc-6, uev-3	100%	0%	Not scored
ubc-7, ubc-13	100%	0%	Not scored
ubc-7, ubc-14	100%	0%	Not scored
ubc-7, ubc-21	100%	0%	Not scored
ubc-7, ubc-22	100%	0%	Not scored
ubc-7, ubc-25	100%	0%	Not scored
ubc-7, ubc-8	100%	0%	Not scored
ubc-8, C17D12.5	100%	0%	Not scored
ubc-8, C40H1.6	100%	0%	Not scored
ubc-8, ubc-13	100%	0%	Not scored
ubc-8, ubc-21	100%	0%	Not scored
ubc-8, ubc-22	100%	0%	Not scored
ubc-8, ubc-24	100%	0%	Not scored
ubc-8, ubc-25	100%	0%	Not scored
ubc-8, uev-1	100%	0%	Not scored
ubc-8, uev-2	100%	0%	Not scored
ubc-8, uev-3	100%	0%	Not scored
uev-1, C17D12.5	100%	0%	Not scored
uev-1, C40H1.6	100%	0%	Not scored
uev-1, uev-2	100%	0%	Not scored
uev-1, uev-3	100%	0%	Not scored
uev-2, C17D12.5	100%	0%	Not scored
uev-2, C40H1.6	100%	0%	Not scored
uev-2, uev-3	100%	0%	Not scored
uev-3, C17D12.5	100%	0%	Not scored
uev-3, C40H1.6	100%	0%	Not scored

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