ZK858.4 CAUSES EMBRYONIC LETHALITY BUT DOES NOT DIRECTLY IMPACT MEX-3 LOCALIZATION IN AN RNAI INDUCED KNOCKDOWN OF E3 UBIQUITIN LIGASES IN *Caenorhabditis elegans*

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Abstract

The ubiquitin proteosome system (UPS) functions in the cell to mark specific proteins for degradation. E3 ubiquitin ligases act as recognition factors and increase the specificity of the UPS. MEX-3 is an RNA binding protein in Caenorhabditis elegans that inhibits the translation of PAL-1, a posterior specifying protein, and contributes to development of the anterior of the embryo. MEX-3 is present throughout the oocyte, 1-cell, and 2-cell embryo. However, MEX-3 is then depleted in the posterior after the second cell division, and PAL-1 is then expressed in the two posterior blastomeres of the 4-cell embryo. MEX-3 is rapidly depleted from the entire embryo after the 8-cell stage. This degradation is location and time specific, and thus hypothesized to be caused by the UPS. MEX-3 is hypothesized to be targeted for degradation by a specific E3 ubiquitin ligase, and knockout of this protein should result in increase in universal MEX-3 expression in the early embryo. This study sought to determine the MEX-3 specific E3 ubiquitin ligase(s). Putative E3 ubiquitin ligases expressed during early embryonic development were knocked out in C. elegans, and phenotypes were determined. Of the knocked-out ligase genes, only one, ZK858.4 caused embryonic lethality at both 15˚ and 24˚ C. However, fluorescence microcopy of GFP::MEX-3 demonstrated that ZK858.4 knockout did not appear to increase global MEX-3 concentrations. Determining which protein targets MEX-3 degradation will provide more insight into the molecular mechanisms of determining anterior/posterior patterning in C. elegans early embryonic development.

Introduction

All multicellular organisms, including humans, begin as a single cell. Following

fertilization, this single cell divides into the many cell types, tissues, and organs of an adult. Every cell bears virtually the same DNA, or genetic material, but, as animal cells divide and arrive at their fate, as blood cells, neurons, intestinal cells, and so on, different types of proteins are made and individual cells begin to look differently, migrate to different places, and behave differently as a result. As an embryo evolves, cells, in general, become more and more specialized, branching further from the initial cell at fertilization, and taking on a more complete, terminal, differentiated state, even though the DNA remains the same.

Although cell culture, biopsy, and genetic studies with human tissue are essential and common practices, studying this process in the human is often unethical and impractical. However, understanding how to regenerate certain tissues and organs and the pathway to certain human diseases requires knowledge of how a single cell divides into so many diverse, collaborative cell types with such efficiency and control. One of the best ways to learn about human development is to study other organisms. A model organism is typically manageable in the laboratory environment and well understood biologically. These organisms, like mice, nematode worms, frogs, and zebrafish, possess DNA and proteins that are evolutionarily conserved throughout the animal kingdom, and because cellular mechanisms evolved from a common ancestor, studying model organisms provides insight into human embryological development.

Early embryonic development of all organisms requires elaborate coordination of timing and localization of events of division and cell fate specification. Developmental biologists study the conserved mechanisms for determining this spatiotemporal patterning. A collection of maternally provided mRNAs and proteins present in the very

early embryo mediates a cascade of events in development, and this molecular machinery is highly conserved in the animal kingdom. Differentiation is the process wherein a less specialized cell develops a specific function and cell type; for instance, a cerebellar, inhibitory Purkinje cell neuron develops from a neuroblast, which also has the potential to become a glial supporting cell, or a host of other neuron subtypes. The organism determines what is front and back, left and right, intestine and germ cell by using transcription factors to enhance or prevent DNA transcription in mRNA, RNA binding proteins to control mRNA translation, and a host of other molecular switches. Studying these processes in a model organism allows for understanding of complex pathways in reproducible, highly controlled experiments.

Ubiquitin-Proteasome System

The ubiquitin-proteasome system (UPS) was first characterized in the Nobel-winning work of Hershko in the early 1980s. Ubiquitin, a highly conserved and universally expressed 76 amino acid polypeptide, marks proteins for specific, targeted protein degradation by the 26S proteasome in cells (Kipreos, 2005). E1, E2, and E3, the three, enzymatic components of the ubiquitin conjugation system, have been studied extensively (Figure 1). E1, the ubiquitin activating enzyme, performs the first step of the reaction. A cysteine residue on E1 is covalently attached in a thiolester linkage to the C-terminus of ubiquitin, and subsequently passed on to the next enzyme in the conjugating system (Haas, et al 1981, Gudgen, et al 2004).

E2, the ubiquitin-conjugating enzyme, covalently attaches the C-terminal glycine of ubiquitin to a Lysine residue on the target protein (Gudgen, et al 2004). Additional

ubiquitins can then be added at any of the seven lysines of ubiquitin to form a polyubiquitin chain. Much of the diversity of the E2 enzymes depends of the number of ubiquitins a particular enzyme adds and the specific ligase location where it performs polyubiquitination. Mono vs polyubiquitination determines the signal function of the molecule; monoubiquitination signals in receptor endocytosis whereas polyubiquitination with four or more ubiquitins marks a protein for translocation to the 26S proteasome and degradation. Polyubiquitin chains conjugated at Lysine-48 are the typical signal for proteasomal degradation.

Although E2 performs the actual transfer, E3, the ubiquitin protein ligase, binds both the E2 and the substrate (Kipreos, 2005). E3 enzymes are the most diverse components of the ubiquitination pathway, and a variety of subtypes are known and understood. The *C. elegans* genome contains about 650 putative E3 ubiquitin ligases. Because of their diversity and binding specificity, E3 ligases are presumed to control the spatial and temporal degradation of specific proteins by the 26S Proteasome (Deshaies, 1999). One such class, the HECT domain E3 ligases (Figure 1b), directly binds to the ubiquitin (via a thiolester on the C-terminus), the substrate (via a domain on the N-terminus), and the E2 ligase (Jackson, et al., 2000), and the ubiquitin is transferred to the HECT domain before attaching to the substrate. Similiarly, U-box proteins and some monomeric RING finger proteins (including C3HC4 type RING fingers) function as E3 ubiquitin ligases by directly binding both the E2 and the substrate (Figure 1c). However, these enzymes do not directly bind the ubiquitin. Another abundant class of E3 ubiquitin ligase is the Cullin-RING finger protein complexes (Figure 1d). These complexes are insensitive to thiol-modifying agents, and thus do not require a direct ubiquitin thiolester

interaction; however, the RING complex requires the zinc-finger motif, as it mediates protein-protein interactions. Currently, Cullin-RING structures are hypothesized to activate the substrate for ubiquitination and recruit the E2 ubiquitin conjugating enzyme (Jackson, et al., 2000). Cullin-RING complexes are unique because the Cullin proteins are small in number, but the multi-protein complex uses a large number of interchangeable species, called "substrate-recognition subunits" (SRS) like F-boxes, BTB domains, and others that can be switched in and out of the Cullin complex, depending on the needs of the cell (Deshaies, et al, 1999, Jackson, et al, 2000).

UPS in Disease – Parkinson's Disease

A cell controls gene expression at many levels: at the level of transcription, via post-transcriptional modifications to mRNA, and by controlling the location and timing of translation. Beyond the initial translation of a protein however, a cell can also control how long a specific protein remains active and determine circumstances to rapidly remove a protein from a cell. This pathway, mediated by the UPS, can be used in a multiplicity of situations, and understanding the specificity of E3 ubiquitin ligases reveals how a universal system of protein degradation can be called to action on a situational basis and restricted to a very small and precise population of proteins.

Parkinson's Disease is a neurodegenerative disorder caused by depletion of dopaminergic neurons, especially those in the *substantia nigra* that project to the *basal ganglia*, resulting in resting tremor, slowness of movement, stiffness, and loss of balance. Parkinson's onset typically occurs after fifty years of age, but some genetic variants of the disease have earlier, more severe onset (Jancovic, 2008). PARKIN, a gene mutated in

some monogenic forms of Parkinson's disease (PD) (Narendra, et al, 2008) and the most common recessive cause of Parkinson's (Kitada, et al, 1998), is an E3 ubiquitin ligase whose role in ubiquitination of mitochondrial membrane proteins has been proposed to play a role in the pathology of PD. Parkin exemplifies how understanding interactions of ubiquitin ligases has vast practical application to diseases and understanding of cell biology.

PARKIN, a ubiquitin ligase encoded by *PARK2*, is implicated in Parkinson's Disease pathology. Parkin is expressed throughout the central nervous system. Parkin-null *Drosophila melanogaster* exhibit a loss of dopaminergic neurons (like those in the *substantia nigra* depleted in human PD), and disordered, swollen mitochondria in neurons prior to degeneration (Narendra, et al, 2008). Initially, localization of Parkin was difficult because it appeared very uniformly in the cytosol. However, in some cells, it also co-localized with a small subset of mitochondria, as well as in the Golgi apparatus, synaptic vesicles and the endoplasmic reticulum (Kuroda, et al, 2006).

Narendra, et al, using the PRK8 mouse monocolonal antibody raised against recombinant human Parkin, examined subcellular localization in human embryonic kidney (HEK293) cells, which express relatively high levels of Parkin (2008). They demonstrated that the colocalization of mitochondria and Parkin was restricted to small and fragmented organelles. Typically, mitochondria maintain an electrochemical gradient across their membrane to facilitate the production of ATP, and thus membrane polarization is essential for mitochondrial function. Translocation of Parkin to the mitochondria resulted from depolarization of the mitochondrial membrane and has subsequently been induced by depolarization by the mitochondrial uncoupler carbonyl

cyanide m-chlorophenylhydrazone (CCCP) in the lab (Narendra, et al, 2008). Following the recruitment of Parkin to the mitochondria, autophagy is induced. Autophagy, or mitophagy in the case of mitochondria, is the process by which cellular components are degraded by the lysosome. Mitophagy eliminates damaged, depolarized mitochondria from the cell. In turn, Parkin mutation leads to a build up of unhealthy, depolarized mitochondria, as demonstrated in the Parkin-null *Drosophila*. CCCP-induced mitochondrial depolarization has been studied significantly in HEK293 and HeLa cells because CCCP treatment is detrimental to neurons and neuronal mitophagy is a much slower, compartmentalized process. Recently, however, many of the results have been replicated in mature cortical neurons (Cai, 2012).

After initiating spatial (mitochondria) and temporal (after mitochondrial depolarization) translocation of Parkin, its function as an E3 ubiquitin ligase becomes clearer. Researchers assessed the concentrations of proteins, especially mitochondrial associated proteins, before and after CCCP depolarization in the presence of Parkin. Using stable isotope labeling by amino acids in cell culture (SILAC) analysis to monitor mitochondrial proteome changes, Chan et, al 2011 calculated that a subset of mitochondrial membrane proteins had altered abundance after CCCP treatment. MFN1 and MFN2, mitofusin proteins, had SILAC ratios of 0.09 and 0.10, respectively, corresponding to the protein level in treated mitochondria divided by the protein level in mitochondria of untreated cells. Healthy mitochondria require a constant dynamic interchange between fusion and fission to maintain their proper morphology and function, and this is facilitated by mitofusins (Zhang and Chan, 2007). TOM70, a mitochondrial import protein and MIRO1, a mitochondrial transport protein, and several other

mitochondrial proteins involved in metabolism and respiration were also reduced after CCCP depolarization (Chan, et al, 2011). The SILAC experiments also verified Parkin enrichment.

Following CCCP treatment and Parkin enrichment, SILAC analysis also demonstrated an increase in lysine(K)-48 linked polyubiquitination (the traditional marker for the recruitment of the 26S proteasome) (Chan, et al, 2011), and this result was verified with immunoblot analysis of mitochondria for K48 linked ubiquitins after CCCP induced depolarization. The HeLa cells expressing Parkin demonstrated an increase in mitochondrial associated K48 ubiquitin after CCCP induction, whereas those without Parkin expression did not show polyubiquitination on the immunoblot, implying direct dependence on Parkin. Microscopy was also performed in Parkin-expressing HeLa cells stained for a subunit of the proteasome, PSMB5, and the mitochondrial marker protein HSP60. Prior to CCCP treatment, PSMB5 was diffusely cytosolic, and following treatment, it was visualized highly associated with HSP60, and thus, mitochondria. This paper indicates that Parkin is recruited to the mitochondria after membrane depolarization and induces ubiquitination of multiple mitochondrial proteins, including MFN1 and MFN2. Following ubiquitination by Parkin, evidence suggests the 26S proteasome is recruited to the mitochondria.

In addition to recruitment of the proteasome, and subsequent degradation of several mitochondrial proteins, Parkin recruitment to the mitochondria seems to, either directly or indirectly, induce autophagy. Narendra, et al, using fluorescence microscopy to visualize mCherry-Parkin and GFP-LC3 (a marker of autophagosomes), demonstrated an overlap of autophagosomes and mitochondria after CCCP induced depolarization in

Parkin-positive HeLa cells (2008). Diminished mitochondrial populations accompanied this co-localization within 24 hours of depolarization. Drugs inhibiting lysosomal function and autophagy, bafilomycin and 3-methyl adenine, rescued the mitochondrial populations, but did not reduce the ubiquitin proteasome effects of depolarization.

The hypothesized induction of mitophagy, and the role of Parkin in the neuron, especially the distal axon, is implicated in Parkinson's disease. Parkin-deficient flies demonstrate swollen mitochondria with disrupted cristae (Greene, et al, 2003). Based on these swollen mitochondria and the containment of defective mitochondria in the axon (no translocation to lysosomes in the cell body), several groups have hypothesized the E3 ubiquitin ligases functions to turn off the fusion pathway. MFN1 and MFN2 are large GTPases that induce mitochondrial fusion, and have been determined to be directly ubiquitinated by Parkin after CCCP depolarization (Tanaka, et al, 2010). After minor depolarizations of the mitochondrial membrane, one mechanism of repair is fusion with a healthy, polarized mitochondrion to compensate for damage. However, after prolonged or excessive damage, Parkin turns off this pathway by degrading the fusion inducing proteins and allowing depolarized, small mitochondria to persist, in turn, inducing mitophagy and elimination of depolarized organelles.

Additionally, recent studies have demonstrated that Miro, a motor protein associated protein that anchors kinesin to the mitochondrial surface (Tanaka, et al 2010, Wang, et al 2011), is degraded in a Parkin-dependent manner after mitochondrial depolarization. Miro dysfunction decreases mitochondrial mobility and may function to quarantine dysfunctional mitochondria as a mechanism to induce autophagy. As a result, Parkin deficient mitochondria have been demonstrated to be more motile after

depolarization in several experiments, alongside experiencing increased fusion.

The Parkin pathway exemplifies how an E3 ubiquitin ligase can directly target a subset of proteins after a cellular event, like mitochondrial depolarization, to induce protein degradation. In this circumstance, the ligase's function is implicated in an autosomal recessive genetic form of Parkinson's disease and better understanding how these proteins regulate and are regulated provides insight to disease pathology and potential therapies. Some proteins, like RBX-1, a RING finger protein that is a subunit in Cullin based E3 complexes (Jia, et al 2011), are implicated in cell cycle control, cell division, and DNA repair, and thus can be studied as potential oncogenes. Other E3 ubiquitin ligases, like EEL-1, a HECT ligase that degrades SKN-1 in *C. elegans*, are present at specific points in development to restrict protein localization, altering the proteome and directing differentiation (Page, 2007).

***Caenhorabditis elegans*: a model organism**

Caenhorabditis elegans, a species of nematode worm, is a model organism that was first studied by Sydney Brenner and colleagues because it is small, easily cultivated in lab, and its transparent cells allow all cells to be easily observed throughout its lifecycle. The organism was selected for study to determine each gene involved in development and to trace the preserved lineage of every cell. All wild-type individuals have the same cell lineage, so researchers can study how mutations effect the development of the organism with single-cell resolution. At this point, the entire genome of *C. elegans* has been sequenced, the cell lineage has been traced, and researchers are continuously determining the function of the genes.

C. elegans is a small (about 1.5 mm long), non-parasitic soil nematode with rapid embryogenesis. The adult is predominantly hermaphroditic and reproduction occurs through self-fertilization and occasional cross-fertilization with the males, which make up about 0.05% of wild populations. After fertilization, the embryo is laid by the hermaphrodite, hatches, and develops through four larval stages. Each adult lays around 300-350 eggs, providing a large number of offspring. Both the large brood size and rapid embryogenesis indicate that *C. elegans* is a "r-selection" based species. R-selected species rely on rapid reproduction to maximize survival, and this evolutionary survival strategy is effective in unstable environments. In addition, the worms can survive poor conditions in the wild, and are convenient in the laboratory, due to the prolonged "dauer" stage: a long enduring larval stage when the worms can arrest development in times of food scarcity, surviving ten times the length of a normal life cycle *C. elegans* (Riddle 1997).

The translucent adult hermaphrodite contains 959 somatic cells, and every cell lineage has been traced, beginning with the first asymmetric cell division. *C. elegans* embryos begin with a one cell stage, wherein the initial founder cell P_0 divides asymmetrically into a larger anterior blastomere, AB, and a smaller posterior blastomere, P_1 (Gönczy, 2005) (Figure 3). This polarity, or difference between the anterior and posterior of the embryo, is initially determined by the location of sperm entry at fertilization, the determinant for the posterior. Every division of a "P" germline blastomere results in the production of a germline precursor and a somatic blastomere. P_1 divides to produces the somatic blastomere EMS and the germline precursor P_2. EMS divides into E and MS. P_2 divides further into P_3 and the somatic C blastomere, and then

P_3 divides into P_4 and the somatic D blastomere. Each individual cell has a unique cell lineage: hypodermis, neurons and anterior pharynx (AB lineage); somatic gonad, muscle, pharynx, and neurons (MS); intestine (E); muscle, hypodermis and neurons (C); muscle (D); and the germ line (P_4).

Directed, asymmetric development this early in the embryo requires precise partitioning of maternally provided proteins and mRNAs. At the earliest stages of development, maternally provided proteins and mRNAs are needed because transcription has not begun, and differentiation is determined by translational control, protein degradation, and protein localization. Maternal effect genes are genes that are transcribed in the mother's cells to provide mRNA and protein to the egg prior to fertilization. Thus, a mother mutant for a maternal effect gene can develop normally, but will produce abnormal progeny. The best studied, and earliest crucial proteins for polarity establishment are the six *par* (partitioning defective) genes. These genes were identified based on maternal-effect mutations that led to defective anterior-posterior partitioning at the one-cell stage. The six PAR proteins differentially associate with the anterior and posterior of the one cell stage embryo and interact with the centrosome and the cytoskeleton of the embryo to influence spindle formation and asymmetric division and distribution of cellular components between the AB and P_1 blastomeres. Disruptions in *par* gene function lead to symmetric divisions. Subsequently, polarity must be maintained between the anterior and posterior, and a variety of maternally provided transcription factors must be asymmetrically distributed to determine the cell fate of the blastomeres.

One such transcription factor PAL-1, a Caudal-like homeodomain protein, is required to determine the fate of one of the posterior blastomeres at the four-cell stage.

Caudal, a posterior determining gene in *Drosophila*, has homologues throughout the animal kingdom, including in mammalian species, that also function in posterior development. Due to this high level of conservation in very diversely developing organisms, homologous transcription factors with the homeodomain DNA binding region are important to study. PAL-1 is a blastomere-specifying gene that is required for development of the somatic cells arising from the P_2 lineage, namely C and D (Hunter & Kenyon, 1996). Ectopic expression of *pal-1* induces C-like cell differentiation, and absence of *pal-1* results in a disappearance of the C and D lineages. Either of these situations leads to improper development and embryonic lethality.

Immunofluorescence demonstrates PAL-1 localization in the early embryo. At the four-cell stage, PAL-1 is detected for the first time in the embryo in the nuclei of the two posterior blastomeres (EMS and P_2) and persists in the descendents of these cells through the twenty-four-cell stage (Hunter & Kenyon, 1996). This localization continued in spite of *pal-1*$^{-/-}$ embryonic genotypes, indicating that maternal mRNA expression was sufficient for the early expression of PAL-1 protein. Localization of *pal-1* mRNA was determined using *in situ* hybridization in the early *C. elegans* embryo. One and two cell stage embryos had a uniform distribution of *pal-1*, and about half of the four cell stage embryos showed a uniform distribution. The other half of the embryos had increased concentration of the mRNA aligned with the enriched protein in the two posterior blastomeres (Hunter & Kenyon, 1996).

This uniform early distribution of *pal-1* mRNA indicates control of expression at the translational level, potentially through an RNA binding protein. MEX-3 is an RNA binding protein that is present throughout the 1-cell and 2-cell embryo, and is then

restricted to the anterior blastomeres at the 4-cell stage. Mutations in *mex-3* lead to muscle development from the AB blastomere, a lineage characteristic of the C blastomere (Draper, et al, 1996). The MEX-3 protein contains two 70-amino acid regions with repeated regions (40% identical) that are related to the KH-domain, a domain involved in binding single-stranded RNA (Draper, et al, 1996, Dejgaard & Leffers, 1996). MEX-3 also binds directly to the pal-1 RNA *in vitro* (Pagano, et al, 2009)

A family of four related hMex-3 genes have been mapped to different human chromosomes and are hypothesized to be post-transcriptional regulators (Buchet-Poyau, et al). Additionally, MEX-3 is homologous to TINO, a KH-domain protein that destabilizes the BCL-2 gene and negatively controls BCL-2 (Donnini, et al, 2004). BCL-2, an apoptosis regulator protein, is implicated in various cancers including breast, prostate and lung carcinomas, and is resistant to many conventional cancer treatments. Thus, understanding the mechanisms for controlling spatial and temporal localization of BCL-2 will impact understanding of human disease.

In *C. elegans*, MEX-3 protein is detected in the oocytes after cellularization and distributed evenly throughout the 1-cell stage embryo, as demonstrated by immunohistochemistry (Draper, et al, 1996). By the end of the 2-cell stage, MEX-3 protein is more abundant in the AB blastomere than P_1, and this difference is increased at the 4-cell stage, with MEX-3 protein predominantly located in the AB daughters. *In situ* hybridization experiments likewise demonstrated *mex-3* mRNA localization. *mex-3* mRNA has a uniform distribution pattern in mature oocytes and early 1-cell stage embryos. By the late 1-cell stage, *mex-3* mRNA begins to be restricted to the anterior half of the embryo, resulting in higher levels of *mex-3* mRNA in the AB blastomere after

the first cell division. At the four-cell stage, *mex-3* mRNA is more abundant in AB daughters than P_1 daughters, and is subsequently degraded rapidly from the embryo.

mex-3 mutant hermaphrodites produce embryos with PAL-1 protein expression in all blastomeres, indicating that MEX-3 plays a role in control of *pal-1* translation (Hunter and Kenyon, 1996). Embryos from *mex-3*$^{-/-}$ hermaphrodites injected with LacZ::*pal-1* 3' UTR RNA expressed the LacZ ubiquitously, whereas embryos from *mex-3*^{WT} hermaphrodites showed reporter expression limited to the posterior, akin to endogenous PAL-1 protein (Hunter & Kenyon, 1996). The 3' UTR is a non-coding segment of mRNA at the 3' end of the molecule that can interact with translation and localization-directing proteins and play a role in the controlling mRNA expression. Overexpression of the LacZ::*pal-1* 3' UTR RNA resulted in *mex-3*$^{-/-}$ like embryonic lethality, presumably because the injected RNA was binding too much MEX-3, leaving too little MEX-3 to regulate the endogenous *pal-1* mRNA. This further indicates that direct interaction of MEX-3 with the 3' UTR of *pal-1* mRNA plays a role in PAL-1 spatial and temporal regulation.

MEX-3 interacts with several proteins in a two-hybrid screen, including MEX-5, MEX-6, and SPN-4 (Huang, et al 2002). Knockout of *mex-5* and *mex-6* resulted in reduced expression of MEX-3 in all cells in the 4 and 8-cell stage embryos, suggesting that these two proteins play a role in stabilizing MEX-3 in the anterior. Knockout of *spn-4*, alternatively, led to increased, non-patterned concentration of MEX-3 in all blastomeres and persistence of the protein up through the 20- to 30- cell stage in some cases, indicating that *spn-4* is required for timely MEX-3 degradation (Huang, et al, 2002).

MEX-3, with its human homologs and important role in cell fate determination as a translational inhibitor of *pal-1* in the anterior, is controlled at multiple points in development. In order to more easily study MEX-3 protein regulation in the *C. elegans* embryo, a transgenic strain was created expressing a GFP::MEX-3 fusion protein. While the GFP::MEX-3 does not show asymmetry at the 4-cell stage, it can replace endogenous MEX-3 at low temperatures, indicating that it is functional (N. N. Huang, personal communication). The lack of asymmetry at the 4-cell stage is likely due to the relatively long half-life of GFP and the lack of the *mex-3* 3'UTR, which re-enforces the protein asymmetry (Y. Kohara, personal communication). GFP::MEX-3 persists in all cells through the 8-cell stage, and like endogenous MEX-3 is subsequently eliminated from somatic descendents and becomes restricted to the germline, indicating a rapid degradation of the protein in somatic cells. We hypothesize that the ubiquitin proteasome system functions to tag MEX-3 for degradation, and, in turn, that there is a specific E3 ubiquitin ligase that targets MEX-3 in the early *C. elegans* embryo. Knockout of this particular E3 ubiquitin ligase should, thus, result in increased embryonic expression of MEX-3, decreased translation of PAL-1, and embryonic lethality.

In order to determine which E3 ubiquitin ligase selectively marks MEX-3 for degradation, hundreds of E3 ligases, known and postulated, (RING type, CULLIN-RING type, HECT domains, etc) were assessed for embryonic lethality and/or and maternal expression in previous experiments. Maternal effect genes were preferentially chosen for knockout, or silencing, because the majority of MEX-3 degradation occurs before significant transcription occurs in early development. Additionally, genes that were largely uncharacterized were preferentially chosen. This narrowed the field of putative E3

ubiquitin ligases by about 90%.

RNAi, or RNA interference, is a regulatory RNA phenomenon wherein small RNAs trigger gene silencing pathways (Jamalkandi, et al. 2011). In 1998, Andrew Fire and Craig Mello introduced long, double-stranded RNA (dsRNA) into *C. elegans*, and effectively knocked out the endogenous gene corresponding to the dsRNA. The Dicer complex processes these long exogenous dsRNAs into small interfering RNAs (siRNAs) (Jamalkandi, et al, 2011). The Dicer complex includes highly conserved proteins like DCR-1, RDE-1, RDE-4, and DRH-1, that recognize dsRNA, process the dsRNA into siRNAs, and target mRNA with homologous sequences for degradation. *rde-1* and *rde-4* were identified as part of the pathway based on "RNA interference deficient" mutants, which are deficient in RNAi but show no other immediately apparent phenotypes (Tabara, et al, 1999).

Fire, et al, demonstrated effectiveness of the dsRNA signal (1998). Using the *unc-22* gene, they injected adult *C. elegans* hermaphrodites with either sense RNA, antisense RNA, or double-stranded RNA to determine which had higher RNAi function. In all experiments, injection of double-stranded RNA containing exon sequences resulted in non-wildtype F_1 phenotypes, indicating that double stranded RNA is the most effective form of RNAi. Additionally, the RNAi effect can migrate through cell borders and can be heritable (Kamath, et al, 2001)).

Yigit, et al, proposed a model in 2006 based on the known functions of RDE and related Argonaute (AGO) proteins implicated in the RNAi silencing pathways (2006). Initial dsRNA injection leads to the cooperation of the Dicer complex components, including RDE-1 to recognize dsRNA and cut it into 21 nucleotide siRNAs. These small

siRNAs, with associated proteins, then begin the process of scanning for target mRNA and initiates amplification of the siRNA signal. Target mRNA likely functions as a template for synthesis of secondary dsRNA, which is then processed by Dicer complexes, amplifying the signal and loading secondary siRNAs onto AGO proteins (like SAGO-1 and SAGO-2), that recognize and degrade mRNA with homology to the dsRNA (Yigit, et al, 2006)).

This naturally occurring response of *C. elegans* to introduced dsRNA provides an invaluable tool for reverse genetics. Since its discovery in *C. elegans*, RNAi and related mechanisms of small RNA based gene silencing have been discovered across the animal kingdom and in plants, as well. RNAi causes robust knockout of target mRNAs and is, thus, an efficient and effective method to silence a gene. In contrast, isolating a genetic mutation in a specific gene can take months in *C. elegans*.

The mobility of the RNAi effect across cells in *C. elegans* allows for multiple modes of introduction. Injection is an efficient mode of dsRNA delivery, and the amplified siRNA signal allows for non-tissue specific injection. Soaking individual worms in dsRNA solution has effectively induced RNAi in many experiments. In addition, by inserting the gene into a plasmid with T7 promoters on either side, *E. coli* expressing T7 polymerase can be transfected with this plasmid, and in turn, will express the dsRNA. *C. elegans* can then be plated on *E. coli* expressing dsRNA as their food source (Kamath, et al, 2001). This feeding technique allows for increased efficiency in screening large numbers of worms and large numbers of genes for genome wide screens.

Based on our hypothesis for MEX-3 degradation and the effectiveness of RNAi, I performed an RNAi screen using both dsRNA injection and dsRNA feeding to identify

E3 ubiquitin ligases that may be responsible for MEX-3 targeting. Consistent embryonic lethality was found in one of the thirteen RNAi knockouts, and this gene, *mel-26*, cause alterations in embryonic morphology, but did not directly affect the localization of MEX-3 in the early embryo. Further studies will be performed to identify any E3 ubiquitin ligases required for MEX-3 breakdown and to characterize the specificity of these enzymes.

Methods

***C. elegans* Plasmid Library**

DNA templates for dsRNA synthesis were taken from the Geneservice Ltd. *C. elegans* library. Colonies containing the plasmids for the genes of interest were streaked on LB-Amp plates with 50 µg/ml ampicillin. They grew at 37˚C overnight and one colony per gene was then grown in liquid LB-amp with 50 µg/ml ampicillin overnight at 37˚C.

Plasmid Isolation

The L4440 Plasmid containing the gene of interest was then isolated from the bacterial culture using a QIAGEN QIAprep Spin Miniprep Kit (Valencia, CA) for purification of plasmids up to 20 µg according to manufacturers protocol. Identity of the gene inserts was identified by digest with one or more restriction enzymes.

PCR

The gene inserts were amplified using the following PCR reaction:

35ul sddH_20, 5ul Biolase buffer, 2ul 50mM $MgCl_2$, 1.5ul 10mM/40mM dNTPs, 3ul forward primer (20 pmol/ul), 3ul reverse primer (20 pmol/ul), and 0.5 ul Biolase DNA

polymerase. 1 µl of a 1:10 dilution of the reaction mixture was added as template.

. The T7 primer sequence used was:

"5' – TAATACGACTCACTATAGGG – 3' "

PCR was then run on a thermocycler for 30 cycles of

94C 30sec
56C 30sec
72C ~1 min per kb

The genes of interest were then verified based on insert length using gel electrophoresis.

dsRNA Synthesis

dsRNA was synthesized using NEB T7 RNA polymerase, NEB Murine RNAse inhibitor, rNTPs, and NEB DNAse in the following amounts: 10 µl 10X NEB Reaction mixture, 5 µl 10/40mM rNTP, 1 µl murine RNAse inhibitor, 1 µl NEB T7 RNA polymerase, and 5 µl (2µg) template DNA product (based on 0.4 µg/µl PCR product) and RNAse-free H_20 to 100 µl volume. PCR produced template DNA, RNAse-free water, RNA polymerase, and rNTPs were incubated at 37˚C for two hours, one more µl of RNA polymerase was added, then incubated for 37˚C for two more hours. 1 µl of DNAse was added to the reaction mixture, followed by a final 10 minute incubation at 37˚C. To reanneal the dsRNA, the reaction mixture was put on a 100˚C heatblock then cooled to room temperature and run out on a gel to verify clean, double stranded product.

RNAi Injection

Worms were grown and fed on NG-OP50 plates according to Brenner 1974. Young Adult hermaphrodites were selected for RNAi injection and prepared according to Wormbook

Reverse Genetics protocol (Ahringer, 2006). After cleaning on an NG-plate without OP-50, young adults were placed on an injection pad made with 2% agarose on glass cover slips. They were mounted in mineral oil to dehydrate the worm and cause them to stick to the pad. Worms were visualized for injection on a Zeiss AX10 Observer A1 microscope. They were injected using a Narashige Micromanipulator and Tritech microinjection apparatus using canned nitrogen gas as the pressure source. Needles were pulled on a Sutter Instrument micropipette puller using borosilicate glass, 10 cm in length with 1.0 mm outer diameter and 0.5 mm inner diameter.

After injections, worms were rescued from mineral oil using rescue buffer and M9, then moved to an NG-OP50 plate for 16-24 hours to allow RNAi to take affect. Injected worms were transferred to individual plates. Embryos from worms at 24˚C were counted every twenty-four hours and the adult was killed after 1 day. Lethality was assessed on the second count (48 hours after placement on individual plate). Embryos from worms at 15˚C were counted every forty-eight hours and the adult was killed after the first two days. Lethality was assessed on the day four count based on the number of hatched embryos.

Fluorescence Microscopy

Negative and Positive control worms were observed on the Zeiss Axiovision Microscope using 40X and 100X (oil immersion) DIC as well as with mercury vapor based UV light to visualize GFP.

HCC21 worms contain a GFP-MEX3 fusion protein expressing the entire MEX3 sequence. HCC22 express GFP-MEX3* without the essential localization regions of the protein.

Worms were prepared for microscopy placed in 45 µl M9 Buffer prepared according to Wormbook protocol with 5 µl Levamisole (to cause spastic paralysis) on a depression slide. A scalpel was then used to cut 30-40 gravid adults in half in the center to release embryos. The preparation was then transferred to a 2% wet agarose pad for use on the microscope.

30-40 HCC21 young adults were injected prior to microscopy for experimental cases. After 16-24 hours to allow RNAi to take affect, the worms were prepared similarly to control. Plates were kept in a humid chamber at 15˚C overnight to observe changes in MEX-3 degradation and localization in embryos overtime and observe terminal phenotype of RNAi knockout lethal embryos.

To calculate when RNAi first began to take effect, worms were injected and then, after 12 hours, embryonic lethality was counted. Worms were put on individual plates and then allowed to lay eggs for two hour time intervals (12, 14, 16, 18, and 20 hours), and then embryonic lethality was counted the following day. The first time interval with 100% lethality was then the time interval determined for RNAi to take effect, and all further microscopy was done that many hours following injection.

Results

With one exception, every gene from the L4440 vector *C. elegans* library was successfully digested with predicted enzymes based on forward and reverse complement sequence using APE software (data not shown). Cosmid # C32D5.11 failed to digest under multiple conditions and did not replicate in PCR at the clear, expected band, even after repeated culture growth and mini-prep. As a result, it was assumed the error was in the transformed bacterial strain bearing the cosmid.

Cosmids "B0393.6" and "B0416.4" were initially screened with RNAi by E. coli feeding, and then subjected to RNAi injection after no significant lethality was seen by feeding. 13 of 14 dsRNAs injected produced no significant embryonic lethal phenotype at either 15˚C or 24˚C (see Table 1 for detailed results).

ZK858.4, or *mel-26*, is a substrate specific adaptor component of a Cullin E3 Ubiquitin ligase. Cul family proteins are scaffolds for diverse subunits of ubiquitin ligase complexes containing multiple polypeptide subunits, with diverse substrate specificity that varies depending on what is bound (deShaies, et al, 1999). One highly studied type is Cul1, which interacts with varied FBox domain proteins. *mel-26*, however interacts with CUL-3, a Cullin known to interact with BTB domains (Wormbase). This domain is an evolutionarily conserved interaction motif found in the N terminus, and it is also known as 'POZ' for *pox*virus and zinc-finger (Ahmad, et al, 1998). The crystal structure of the BTB domain reveals a tightly wound homodimer, with internal α-helix scaffolding and external flanking β-sheets (Ahmad, et al,1998). Prior to this study, several papers indicated that *mel-26* played a CUL-3 independent role in cytokinesis, and that the protein functioned in cleavage furrow ingression (Luke-Glaser, et al 2005). Additionally,

it is postulated to be the E3 ubiquitin ligase responsible, in a CUL-3 dependent manner for breakdown of MEI-1, a microtubule severing complex at the meiosis to mitosis transition (Johnson, et al, 2009).

Because of its diverse roles in the oogenesis and embryonic development, prior RNAi resulted in an array of phenotypes including sterility, embryonic lethality, and dumpy worms. Before dsRNA synthesis and injection, ZK858.4 identity was verified using restriction digest and PCR. At 24˚, there was 99% embryonic lethality amongst offspring from young adults injected with ZK858.4 (*mel-26*) dsRNA. A significant, but not as fully penetrant phenotype was observed at 15˚, with 75% of offspring not developing past the embryonic stage. These results were significant enough to observe under the fluorescence microscope.

Figure 5 shows positive and negative controls for microscopy. GFP::MEX-3 HCC21 strain, the *C. elegans* strain used experimentally in lab has inserted DNA with a green fluorescent protein fused to the *mex-3* sequence. All domains of MEX-3 are present in the transgene, so all potential ubiquitination sites are accessible in the translated GFP fusion protein. During the first through eighth cell stage, GFP is expressed throughout the entire embryo, with punctate expression in the germline, indicating association with P granules (figure 5a). Differential Interference Contrast (DIC) microscopy images provide a clear picture of stage in embryonic development. In HCC21 negative control, with no injection (figure 5a), the GFP expression is eliminated from the embryo, with the exception of the germline rapidly following the eight-cell stage, and by later points in development, non-background fluorescence is absent. In contrast, HCC-22 *C. elegans*, a strain with transgenic GFP::MEX-3* missing the putative

ubiquitination and degradation sites, shows strong fluorescence throughout all stages of the embryo. GFP expression remains consistent from the two-cell stage up through the comma formation stage, late in embryonic development (figure 5b).

Initial microscopy results were variable. DIC images indicated abnormal embryonic morphology, consistent with previously characterized knockouts and hypothesized gene function. This morphological variance made GFP localization difficult to measure. One embryo, (figure 6a) showed highly punctate GFP::MEX-3 at the one-cell stage, and this embryo was also morphologically more rounded than wild type. At the one-cell stage, MEX-3 localization would not be altered by one of the hypothesized E3 Ubiquitin ligases, so this alteration in fluorescence localization demonstrates how abnormal phenotypes are likely due to morphological variance as opposed to direct impact of *mel-26* RNAi on MEX-3 expression (figure 6b). Additionally, multiple embryos appeared to have normal distribution of MEX-3, with fluorescence restricted to the germline after the eight-cell stage (figure 6c)

To account for this morphological disruption, injection was performed to calculate the time until RNAi first took effect, in an attempt to visualize earliest impacts of the knockout without profound morphological variation. 100% embryonic lethality began at the 16 hour mark post injection, so all further dsRNA injection was followed by microscopy at 16 hours. A successful RNAi trial performed within this time unit verified the hypothesized results. ZK858.4 (*mel-26*) injected worms produce embryos with wild-type distribution of MEX-3 when embryonic morphology is consistent with wild type. Figure 7a and 7b demonstrate, at 40x and 100x, the early universal GFP expression, followed by germline restriction, and ultimately total GFP::MEX-3 degradation.

Discussion

Although this study did not find the MEX-3 targeting E3 Ubiquitin Ligase, the lab will continue to pursue RNAi knockout of the remaining candidate genes. One such gene, CO8B11.1 (*zyg-11*), was found to cause embryonic lethality and differential expression of GFP::MEX-3 expression by another student in Dr. Huang's lab, and will be further examined.

After finding a gene that produces the expected lethality and fluorescence expression phenotype, further steps are necessary to verify that the E3 ubiquitin ligase in question functions to specifically target MEX-3 degradation. Several other transgenic strains of *C. elegans* with GFP expressing proteins can be injected with the candidate dsRNA to examine localization. GFP::MEX-5, GFP:: PAL-1, GFP::GLD-1, and GFP::SPN-4 are all functional in early embryonic development and related to the MEX-3 pathway, therefore, if knockout caused altered protein expression in the GFP::MEX-3 strain, but not the others, this indicates a direct interaction between the E3 ubiquitin ligase and MEX-3. Whereas, if fluorescence was altered in one or more of the other strains, the ligase may indirectly influence MEX-3 localization, along with the other proteins of interest.

Biochemical analysis of MEX-3 and the potential ligase can also verify the interaction. Western blots determine protein expression, and WT embryo protein levels of MEX-3 can be compared to knockout embryos to verify that the increased fluorescence is accompanied by a general overexpression of the MEX-3 protein after RNAi. In vitro ubiquitination assays can verify that the E3 ubiquitin ligase is actually adding ubiquitins to MEX-3. Combining an E1, an E2, MEX-3, the candidate E3, and

ubiquitin and putting on a thermocycler, after a period of time, the mixture, when run out on a gel should show a multi-banding pattern characteristic of ubiquitination. The different molecular weights indicate varying amounts of polyubiquitination, and Ub antibodies should colocalize with MEX-3 antibodies.

One control for these experiments could use the MEX-3* mutated protein constructed by Dr. Nancy Huang (correspondence). This mutation of the protein still contains the Zinc-finger RNA binding protein elements, but is missing the ubiquitination/degradation domain. As a result, in the Western Blot, protein concentrations should be equally high in both the E3 ligase knockout and wild type, and both should be higher than concentrations with WT *mex-3*. In the ubiquitination assay, the mutated MEX-3* would not bind ubiquitin, and immunoblotting would lack the characteristic ubiquitin-induced banding pattern.

To further verify the specificity of ubiquitination to this region of the MEX-3 protein by the E3 ubiquitin ligase in question, lysine residues in the degradation region could be selectively mutated to arginine. Arginine has similar polarity to Lysine, with side chain amino groups allowing the proper protein folding; however, the E2 and E3 ubiquitin conjugation process adds only to Lysine residues. If the E3 ubiquitin ligase in question is selectively degrading MEX-3 using the UPS, then full length MEX-3 with modifications to lysine resides should not be ubiquitinated. This can be verified with the aforementioned biochemical assays in vitro, and in vivo, Lysine mutant expression should resemble HCC-22 GFP::MEX-3 expression.

A more thorough exploration of the regulation of MEX-3 in the early embryo will provide insight on the specificity of E3 ubiquitin ligases, details about the early steps of

C. elegans development and potentially provide information about the regulation of homologous proteins in other organisms, including vertebrates. A remarkable amount of conservation exists in early embryonic development. The nematode, *C. elegans*, follows a pattern of asymmetric early cell division that bears little resemblance to vertebrate development; however, the proteins involved in these processes and in the determination of cell fate are highly conserved. In all multi-cellular organisms, the precise patterning of genetic and maternally provided material restricts the fate of certain cell types and provides the necessary framework for cells to become determined. Within *C. elegans*, multiple proteins have early embryonic patterning that resembles MEX-3, and finding the E3 Ligase that targets MEX-3 for degradation will help complete the picture of how these proteins are restricted to the germline.

Several proteins maintain their asymmetry in early cell division by neither degradation nor translational control. MEX-5, a somatic cell-fate determinant, contains a CCCH-finger domain that resembles the germline proteins PIE-1, POS-1, and MEX-1. Ectopic expression of MEX-5 is sufficient to prevent germ cell differentiation, and *mex-5* RNAi results in embryos with muscle excess in the anterior that die without hatching (Schubert, et al 2000). PIE-1, an RNA-binding protein that negatively regulates *skn-1*, is segregated to germline precursors (Gomes, et al 2001). In the zygote, however, both PIE-1 and MEX-5 are diffuse throughout the entire cytoplasm (Daniels, et al 2010). Rates of diffusion in the polarized embryo are different for the two proteins depending on their location; MEX-5 has decreased rates of diffusion in the anterior and PIE-1 has decreased diffusion in the posterior (Daniels 2010). PIE-1 posterior enrichment is dependent on the cycling of PIE-1 between two forms with different diffusion coefficients, and not protein

degradation (Daniels 2009). In this model, the relative amounts of fast and slow moving species varies across the anterior/posterior axis with more slowly diffusing PIE-1 in the posterior compartment, resulting in an increased concentration of posterior protein.

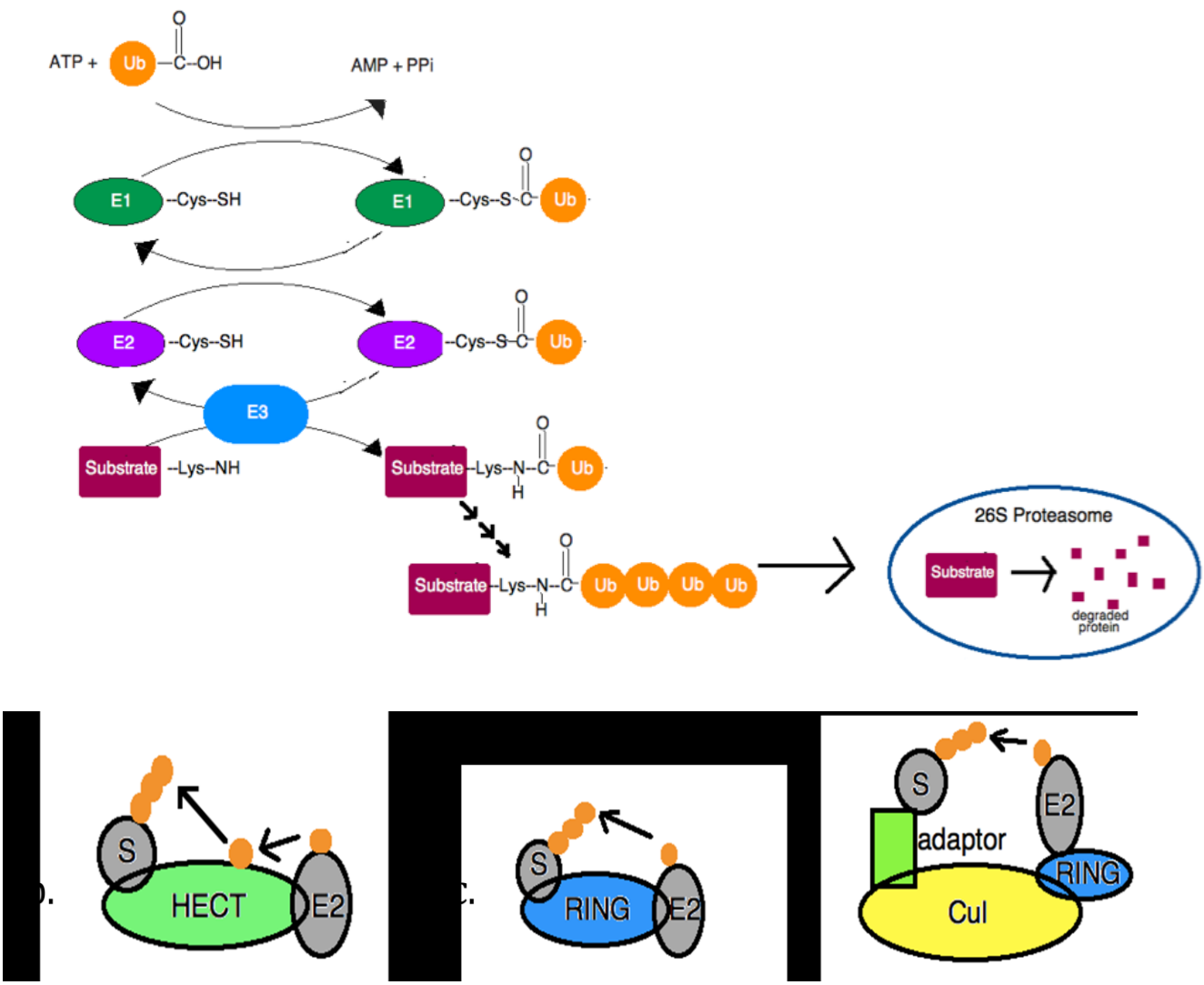
MEX-5, similarly, is controlled by differential diffusion rates between two MEX-5 species, phosphorylated and dephosphorylated in a PAR-1 dependent manner. On the depleted side of the embryo, the posterior, MEX-5 is phosphorylated by PAR-1 into the fast species, allowing fast diffusion and displacement to the anterior. Once in the enriched anterior, MEX-5 is dephosphorylated, and exists as the slow species, decreasing diffusion back to the posterior (Daniels 2010). In this situation, MEX-5 is dependent on one of the early development partitioning genes for its localization, but not in the standard translational or degradation controlled mechanisms.

Other studies have demonstrated that MEX-5, MEX-6, POS-1, PIE-1 and MEX-1, all CCCH-finger containing RNA binding proteins, are regulated by ZIF-1, an E3 Ubiquitin Ligase. It targets and degrades these proteins through their zinc-finger regions in somatic blastomeres (DeRenzo, et al, 2003, Guven-Ozkan, et al, 2010). Although the spatial localization of MEX-5 and PIE-1 is non-dependent on the degradation pathways, the proteins are still targeted for degradation at different points in development, and ZIF-1 seems to be a universal target for proteins of this type. It will be informative to see if an E3 Ubiquitin Ligase that targets multiple related proteins regulates MEX-3. If so, then MEX-3 would likely possess a highly conserved targeted region for ubiquitination.

Each of these maternally provided mRNAs and proteins specifically directs the early embryo to asymmetrical cleavage. These early divisions determine the fate of each of the subsequent divisions, and in turn the entire cell lineage. A more thorough

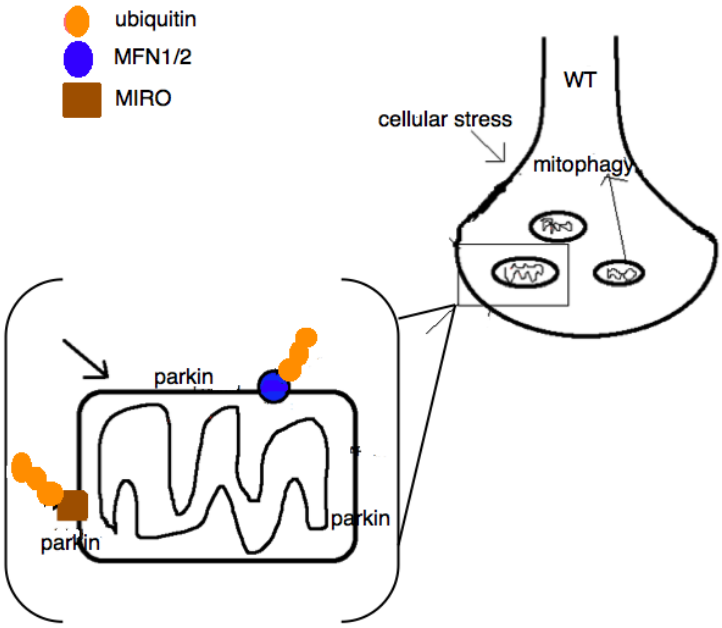
understanding of how each protein involved in anterior posterior development, germ cell differentiation, and somatic cell differentiation will provide a picture of how various mechanisms of gene transcription control, translational control, and degradation interplay to direct development. Unique cases like MEX-5 and PIE-1 regulation reveal how the standard mechanisms are not always the means for regulation. Additionally, E3 Ligases with multiple targets, like ZIF-1, indicate that the method for ubiquitinating substrates like MEX-3, may be very specific at certain points in development, but may also be a cellular event that many similar proteins undergo simultaneously. Further research will only expand the understanding of the role of MEX-3 in inhibiting PAL-1, specifying the anterior, and its potential roles in germline determination.

Figure 1

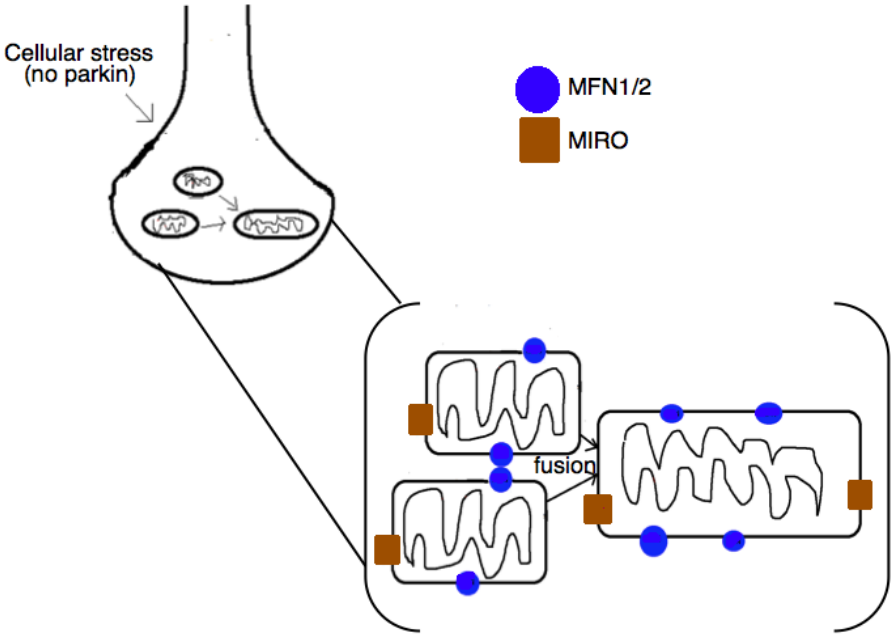


The ubiquitin proteasome system. a. E1 activates the Ubiquitin on a cysteine residue in an ATP dependent manner. Ubiquitin is transferred to the E2 conjugating enzyme, where it also binds to a cysteine residue. E3 interacts with E2 and the substrate to facilitate Ub conjugation to a lysine residue in the target substrate. The ligase attaches a chain of ubiquitins to proteins targeted for degradation that are then targeted for the 26S proteasome where they are broken down. There are several types of E3 ubiquitin ligases: b. HECT domains which receive the ubiquitin from the E2 and then directly transfers the ubiquitin to the substrate (S). c. monomeric RING fingers which bind the substrate and E2 and facilitate transfer by E2. d. Cullin-RING complexes, a multi-protein complex that allows E2 to transfer ubiquitin to substrate (S).

Figure 2



A. WT response to CCCP induced depolarization. Parkin is recruited to the mitochondria, induces UPS-dependent ubiquitination and degradation? of Miro, MFN, and other mitochondrial proteins, prevents fusion, and targets depolarized mitochondria for autophagy.



B. Parkin deficient respond to CCCP induced depolarization. Depolarized mitochondria fuse via MFN GTPase activity, swollen mitochondria collect in axon terminal.

Figure 3 – Cell Lineage

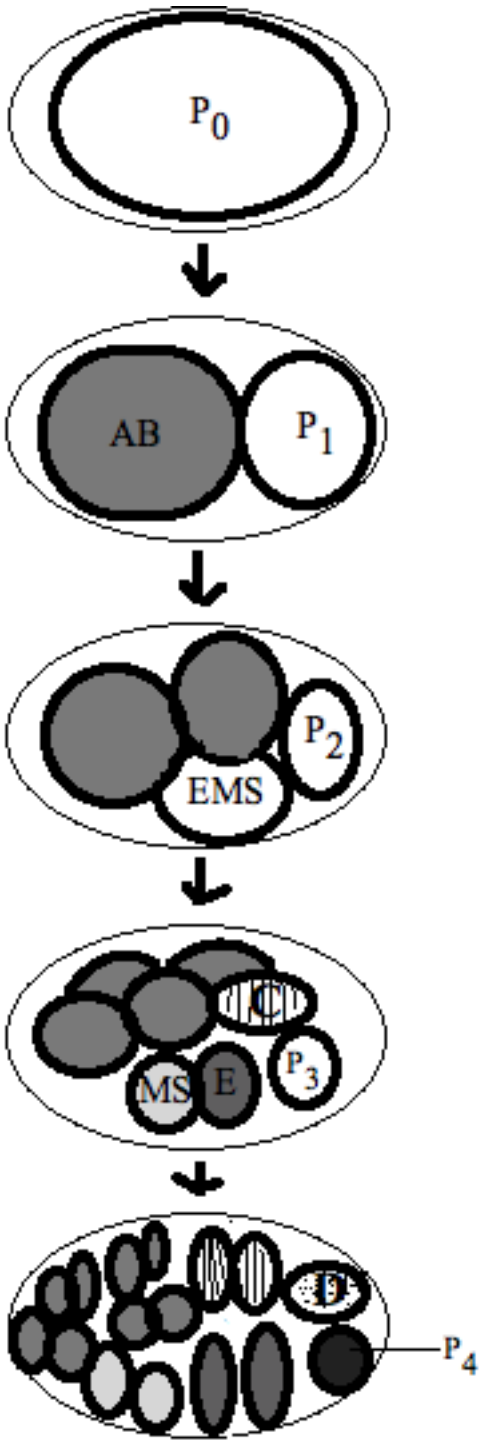
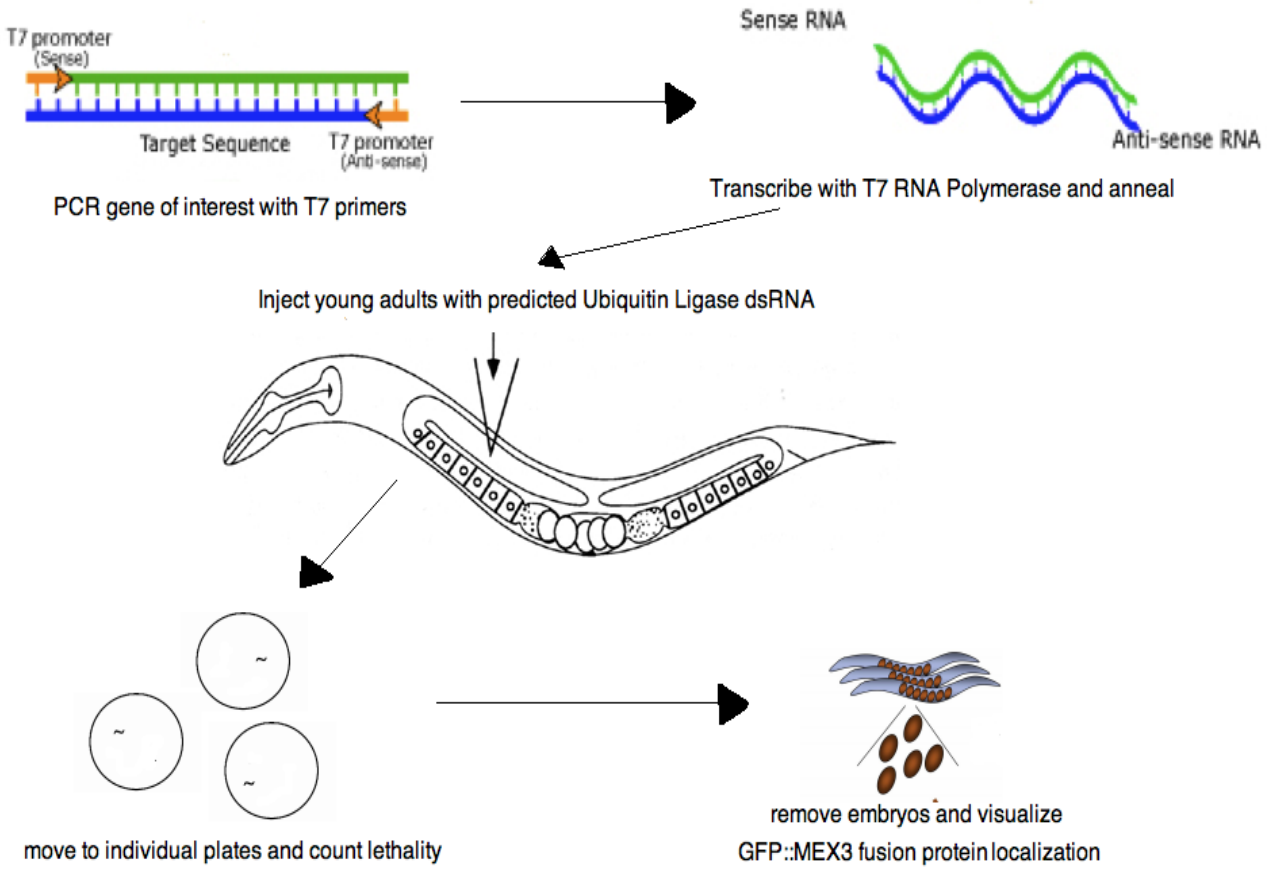


Figure 4 – Methods



]Table 1- RNAi Results

GENE ID	Description & Previous Phenotypes	# Unhatched Eggs/ #Progeny		Embryonic Lethality		GFP::MEX-3 Affected?
		24˚	15˚	24˚	15˚	
ZK858.4 (*mel-26*)	Zinc Finger C3HC4 Type (RING type). No prior embryonic lethality.	169/171	93/124	99%	75%	Not directly
B0393.6	Zinc Finger C3HC4 Type (RING type). Embryonic lethal in 4 prior screens.	2/249	10/395	<20%		-
B0416.4	Zinc Finger C3HC4 Type (RING type). No prior phenotypes characterized	9/301	1/216	<20%		-
CO6A5.9 *(rnf-1)*	Zinc Finger C3HC4 Type (RING type). Y2H interaction with CKI-2 (CDK inhibitor) (Lu & Roy 2007)	13/324	14/111	<20%		-
C32D5.11	PCR and restriction digest unsuccessful. No dsRNA synthesized.	-	-	-		-
F13C5.2	Bromodomain containing protein. Embryonic lethal, larval lethal, and maternal sterile prior phenotypes. Ubiquitous expression from early embryo to adult with higher expression in neurons.	54/742	27/669	<20%		-
F44D12.10	Zinc Finger C3HC4 Type (RING type). No prior RNAi phenotypes characterized	5/236	1/321	<20%		-
F55A11.7	Zinc Finger C3HC4 Type (RING type). No prior RNAi phenotypes characterized.	5/129	13/261	<20%		-
F59B2.6 (*zif-1*)	SOCS-box protein that targets germline proteins for Cullin dependent degradation in somatic cells. Binds germline zinc-finger proteins and an E3 ub ligase subunit (ELC-1). Present in somatic blastomeres after 4 cell stage. Embryonic lethal in one prior experiment.	6/138	5/201	<20%		-
K01G5.1 (*rnf-113*)	Zinc Finger C3HC4 Type (RING type). Expression in	4/291	12/384	<20%		-

	late embryo through adult in ventral nerve cord. Prior phenotypes: embryonic lethal, larval arrest, tumorous germline, sterile.				
RO5F9.1	BTB-POZ domain. Prior phenotypes: embryonic lethal and locomotion variant.	34/269	1/272	<20%	-
T05H10.5A *(ufd-2)*	E4 ubiquitin conjugation factor, catalyzes multiubiquiting chain assembly. Expressed throughout lifetime in body wall muscle, hypodermis and neurons. Prior RNAi phenotypes: embryonic lethal, sex determination variant, sterile F1	2/171	1/154	<20%	-
Y45F10B.9	Zinc Finger C3HC4 Type (RING type). Prior RNAi phenotypes: protein aggregation variant	7/346	4/251	<20%	-
ZC204.11 *(btb-13)*	BTB domain protein. Protein binding function. Embryonic lethal phenotype in one prior experiment, no phenotypes in two other experiments (wormbase)	1/280	2/110	<20%	-

Figure 5: GFP::MEX-3 Microscopy Results - Controls

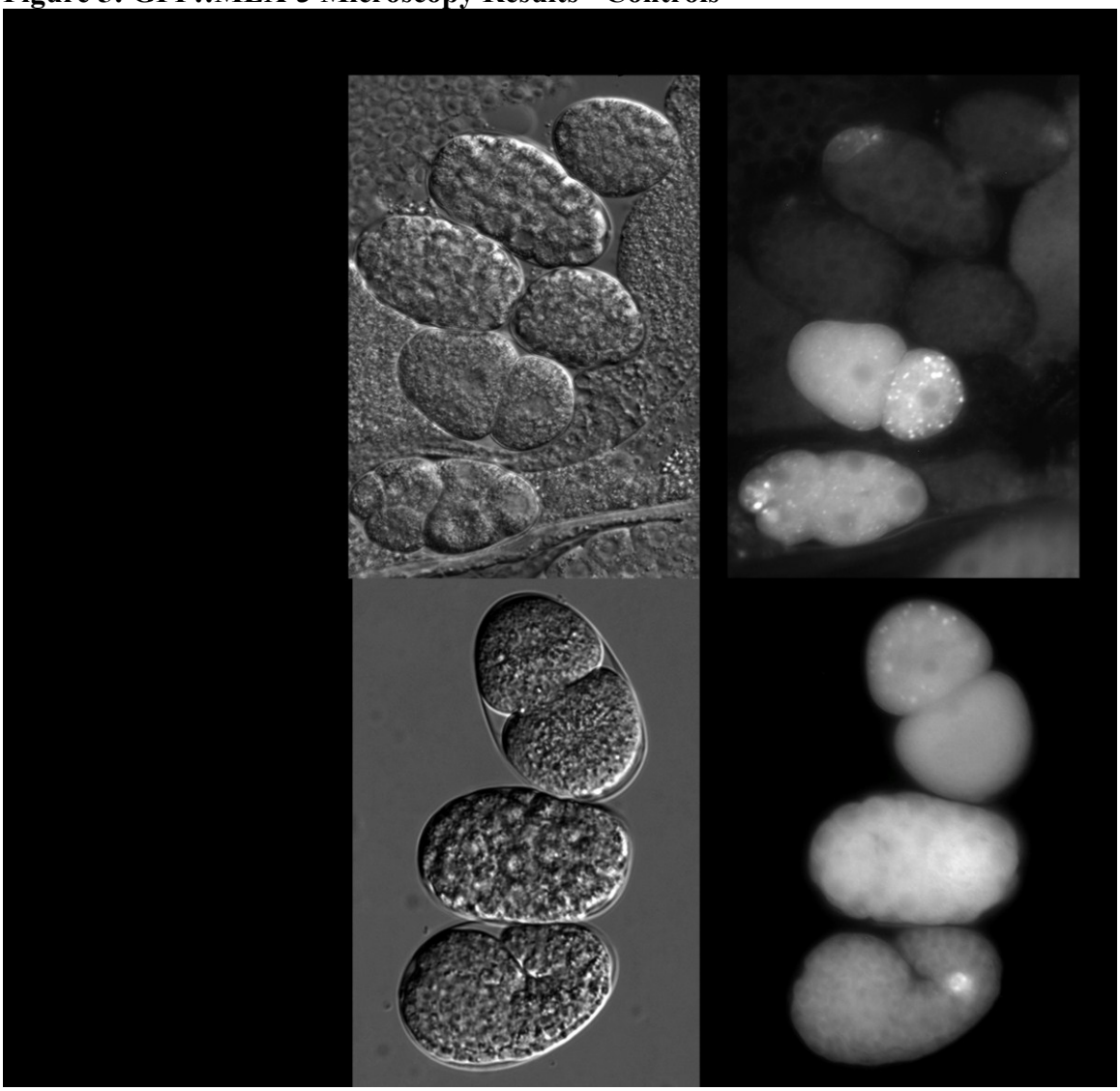


Figure 6

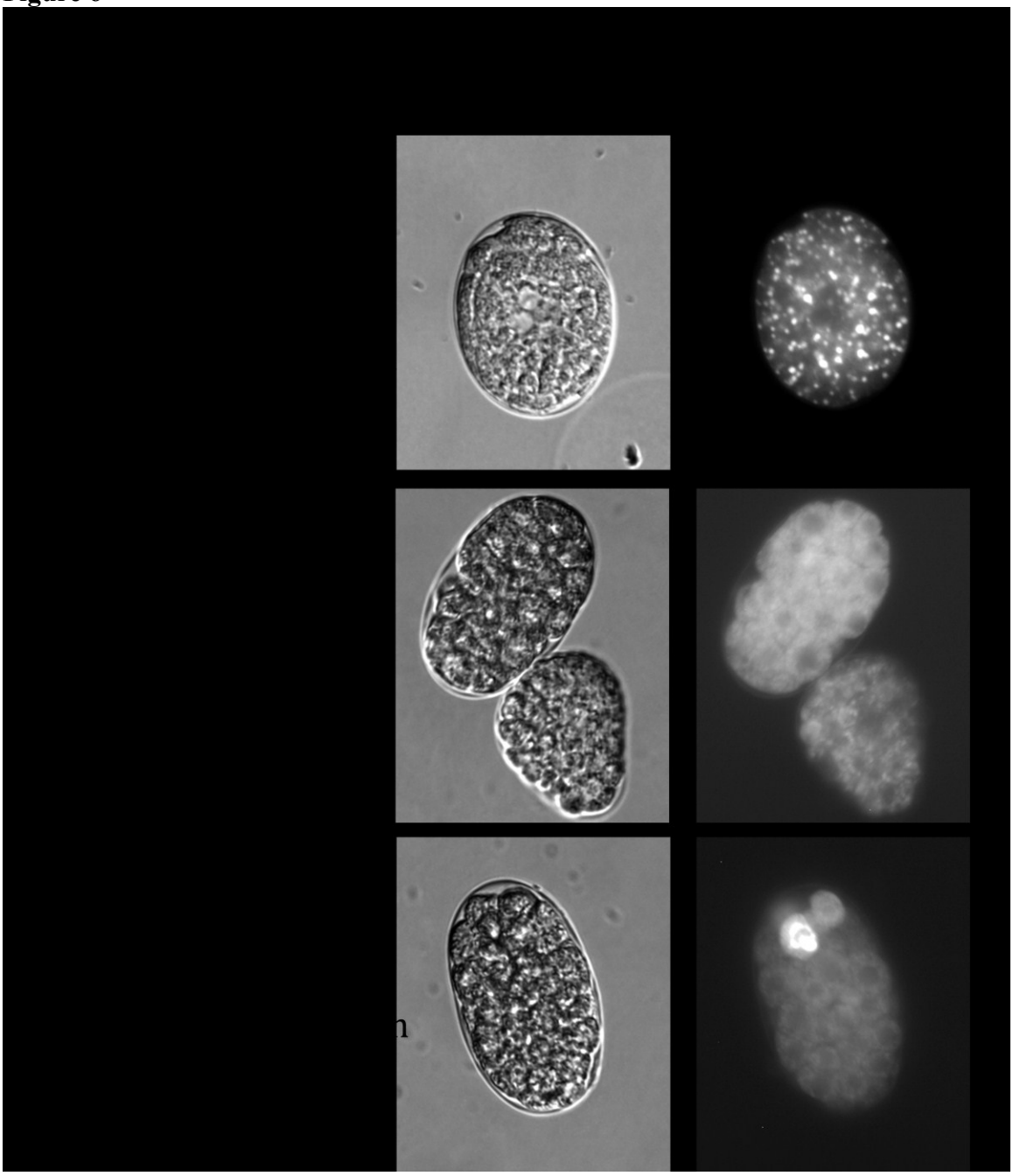
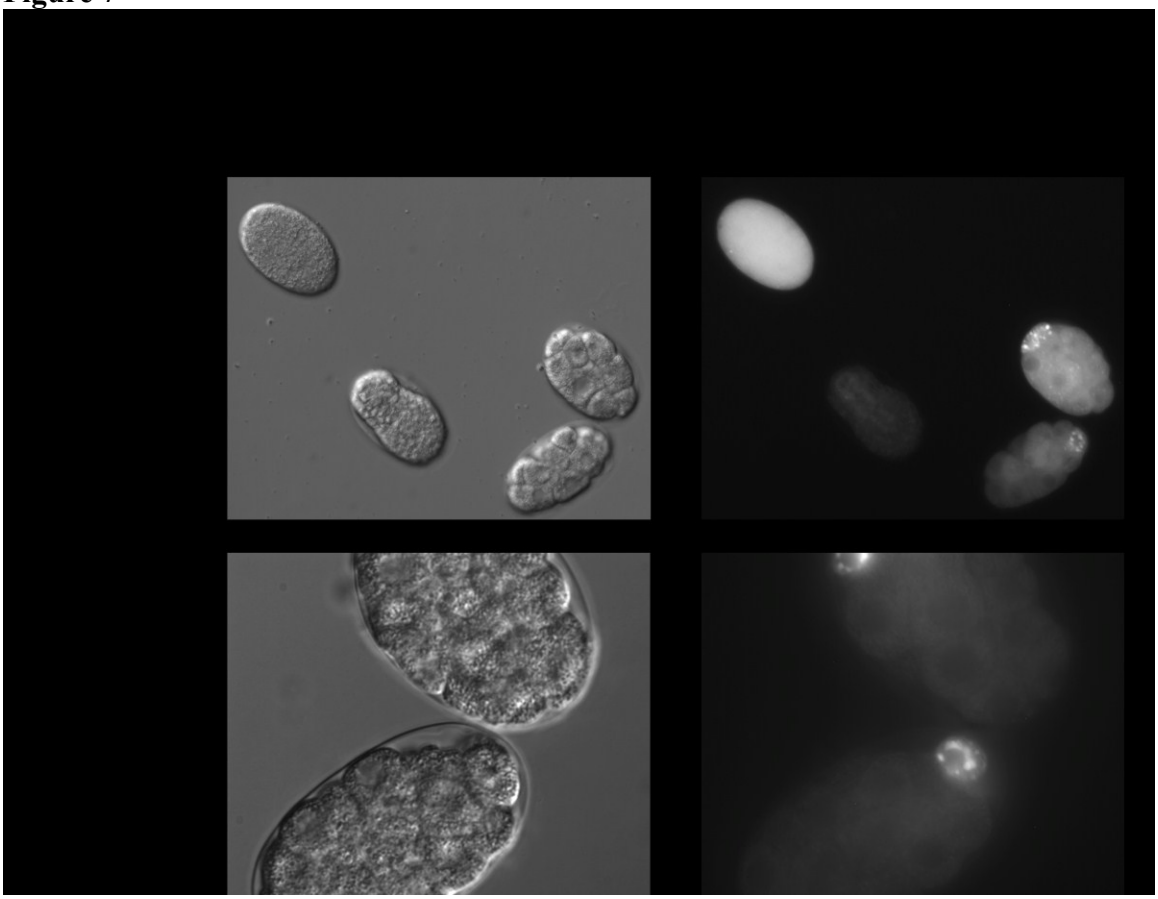


Figure 7



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