SCREENING FOR UBIQUITIN LIGASES INVOLVED

IN *Caenorhabditis elegans* EARLY EMBRYONIC DEVELOPMENT

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Abstract

The protein ubiquitination system is a targeted protein degradation pathway that is an essential component of cell cycle progression in mitosis and meiosis. Recent evidence indicates that the ubiquitin system is required for the degradation of zinc finger proteins that play important roles in embryogenesis. It is possible that the ubiquitin system regulates other proteins involved in early embryonic development by controlling which proteins are degraded, and thereby influencing cell fates.

In *Caenorhabditis elegans (C. elegans)*, there is a single ubiquitin activating enzyme, which has a well-understood function. The twenty-two ubiquitin conjugating enzymes in *C. elegans* have been researched to a moderate extent. Finally, there are believed to be about six hundred ubiquitin-protein ligases. Most of these ubiquitin ligases' exact functions, the proteins they target, remain unknown. Ubiquitin ligases are perhaps the most interesting enzymes in the ubiquitin system because they determine which proteins are targeted for degradation.

Two important proteins involved in the embryonic development of *C. elegans* are posterior alae defective 1 (PAL-1) and muscle excess 3 (MEX-3). PAL-1 is a homeodomain transcription factor protein that is required to specify posterior cell fates. MEX-3 is an RNA-binding protein that binds to *pal-1* mRNA in the anterior cells and restricts the translation of PAL-1 to the posterior cells of the embryo, and thereby influences anterior cell fates. Both *pal-1* mRNA and MEX-3 protein are present throughout newly fertilized embryos, but by the four-cell stage MEX-3 is depleted in

posterior cells and can only bind to *pal-1* mRNA in anterior cells, preventing the translation of PAL-1 in these cells.

It is thought that MEX-3 depletion in the posterior cells is due to it being targeted by unknown ubiquitin ligases and degraded by the 26S proteasome. Research shows that two homologous mRNA binding proteins (MEX-5 and MEX-6) protect MEX-3 from inactivation and degradation in the anterior, allowing for the repression of PAL-1 translation. One major unanswered question is the identity of the ubiquitin ligase(s) that targets MEX-3 for degradation in the posterior of the embryo. This study attempts to answer that question. RNA interference screening of ubiquitin ligases that are expressed during embryonic development has permitted the identification of 20 ubiquitin ligases that do not target MEX-3 for degradation. Screening of additional ubiquitin ligases may lead to a better understanding of the regulation of many key proteins. By understanding more about the interactions between MEX-3 protein and *pal-1* mRNA and how they are regulated, we will learn more about how embryonic development unfolds and what can potentially go wrong.

Introduction

An Introduction to C. elegans

The nematode *Caenorhabditis elegans* is one of the most widely used model organisms. *C. elegans* was the first multicellular organism to have its entire genome sequenced. With access to the sequence of every gene in *C. elegans*, researchers can replicate the DNA of any of those genes and perform experiments to gain a better

understanding of their functions. The success of *C. elegans* research is largely due to the benefits of being biologically simple, having a transparent body, set cell number, small genome, fast life cycle, hermaphroditic mode of reproduction, and because they are easy to grow and maintain (reviewed by Antoshechkin and Sternberg, 2007). An image of *C. elegans* along with a description of its incubation and adult characteristics can be found in Figure 1.

Figure 1. Nomarski optics image of the transparent nematode worm, *Caenorhabditis elegans* (*C. elegans*). Nematodes are anatomically simple. Fully-grown worms have about 1000 cells: hermaphrodites have 959 somatic cells while males have 1031 somatic cells. Adult *C. elegans* reach a size of 1.5mm long and have a life cycle of two to four days, which varies depending on the temperature at which they are incubated. At 15°C the worms grow from embryos to adults in four days and at 25°C they grow from embryos to adults in two and a half days (image provided by J. Naegele).

The fast life cycle of *C. elegans* provides researchers with a superb model organism for studies of genes regulating development. *C. elegans* has a small haploid genome consisting of 8×10^7 nucleotide base pairs, which is about 20 times larger

than the size of the *Escherichia coli* genome. One common method for studying the role of different genes in development is to silence particular genes and then study the effects on embryonic development. A widely used method for silencing genes is RNA interference (RNAi). RNAi exploits a natural cellular response to double-stranded RNA (dsRNA). Cells are thought to have this response to dsRNA because certain viruses inject their own dsRNA into cells to cause changes within the cells that are beneficial for the virus, but very little research has been done on this subject in *C. elegans*. If a cell detects dsRNA, a process is initiated in which RNA complementary to the dsRNA is degraded. RNAi allows researchers to silence a gene in an organism by creating dsRNA of the gene of interest and introducing it into the organism. *C. elegans* is a wonderful candidate for the use of RNAi to understand the functions of specific genes because the effect of RNAi spreads from cell to cell (Fire et al., 1998).

It is possible for thousands of nematodes to be grown on a single Petri dish that has been seeded with *E. coli* for them to feed on. A single hermaphrodite worm can produce between 300 to 350 progeny when self-fertilizing and about 1000 progeny when crossed with a male, allowing for enormous numbers of worms to be grown in a short period of time. The majority of the time in nature, *C. elegans* reproduces by the hermaphrodite's process of self-fertilization, because the ratio of males to hermaphrodites is about 1 to 1000. However, the hermaphrodites will preferentially choose to fertilize their eggs with the sperm of a male if available (Wood, 1998). This allows researchers to cross strains or self-cross, as they see fit.

The trait of preferentially choosing male sperm might be due to the fact that the male to hermaphrodite ratio is so low and that hermaphrodites cannot fertilize each other, so it could have been evolutionarily beneficial for the worms to assimilate new and advantageous DNA when the opportunity presented itself. The ability to cross or self-cross at will was previously only found in plant systems. The discovery of this trait in an animal allowed for researchers to create desired mutant crosses with much greater ease than before (Wood, 1988). The ability to create mutant strains is an essential way to understand the genetics of an organism. It is now possible to create transgenic strains in which a protein of interest is marked by a fluorescent protein tag, allowing researchers to visualize when and where that protein is localized in live animals. These transgenic fluorescent strains can provide essential information on embryonic development by allowing researchers to visualize the effects of knockouts on other proteins that are essential to embryonic development. In my research, I have utilized a transgenic fluorescent strain of *C. elegans* to study the regulation of key proteins involved in embryonic development.

C. elegans Embryonic Development

In *C. elegans*, the lineage of every cell has been traced from the first cell to the last cell formed, which, for hermaphrodites, is the 959th somatic cell. The single cell of a newly fertilized embryo is known as P_0. This cell divides into two cells; a larger anterior blastomere, AB, and a smaller posterior blastomere, P_1. The entry point of the sperm into the egg determines the posterior of the embryo (Sulston et al., 1983). Each "P" cell is a germline blastomere that divides into another germline blastomere and a

somatic blastomere, except for P_4 which divides into two germline blastomeres. P_1 divides into the somatic blastomere EMS and the germline blastomere P_2. In turn, the EMS cell divides into E and MS, both somatic blastomeres, and P_2 divides into P_3 and the somatic blastomere C. P_3 divides into P_4 and the somatic blastomere D. Each of these six founder cells (P_4, AB, E, MS, C, and D) gives rise to a specific cell group in *C. elegans*. The P_4 cell gives rise to the germline; AB gives rise to the hypodermis, neurons, and anterior pharynx; E gives rise to the intestine; MS gives rise to the somatic gonad, muscle, pharynx, and neurons; C gives rise to muscle, hypodermis and neurons; D gives rise to muscle (reviewed by Gönczy and Rose, 2005; Sulston et al., 1983). Our extremely detailed understanding of the embryonic development of *C. elegans* has been vital for tracing where certain proteins are expressed and determining which cells are affected by specific mutations, thus furthering the understanding of how embryonic proteins are regulated by each other to influence the worm's development. (Sulston et al., 1983)

The early embryonic development of *C. elegans* is driven by an assortment of maternally provided mRNAs and proteins that are present in a newly fertilized embryo. Because transcription has not yet begun in the embryo, the maternally provided mRNAs and proteins are required in order for cells to divide properly and take on the appropriate cell fate (reviewed by Evans and Hunter, 2005).

One of these maternally provided proteins is known as muscle excess 3 (MEX-3). MEX-3 is an RNA-binding protein that previous research showed to be required for the translational regulation of a maternally-provided mRNA called posterior alae

defective 1 (*pal-1*) (Hunter and Kenyon, 2006)*.* PAL-1 is a homeodomain transcription

factor protein required for proper cell division and specifying cell fates in the

posterior of the *C. elegans* embryo (Hunter and Kenyon, 2006).

pal-1 mRNA is present throughout newly fertilized embryos, but PAL-1 protein

expression is not seen until the 4-cell stage where it is located in the nucleus of the EMS

and P_2 cells in the posterior, and persists through the twenty-four-cell stage in the

descendants of these cells in the posterior region (Hunter and Kenyon, 1996). This

localized PAL-1 expression is seen in conjunction with MEX-3 levels decreasing in the

posterior at the 4-cell stage (Hunter and Kenyon, 1996; Draper et al, 1996). Hunter and

Kenyon (1996) also observed that in *mex-3*(-) hermaphrodites, PAL-1 protein is detected

in oocytes and all cells of their embryos. Additionally, Bowerman et al. (1997) showed

that when MEX-3 is uniformly distributed throughout the embryo, PAL-1 is usually not

detected, suggesting that MEX-3 represses PAL-1 translation.

MEX-3 is present throughout 1-cell and 2-cell embryos, but is subsequently

restricted to the anterior region by the 4-cell stage. Hunter and Kenyon (1996) were the

first to hypothesize that the restricted anterior expression of MEX-3 is responsible for

repressing PAL-1 translation in the anterior of the worm, resulting is PAL-1 expression

only in the posterior cells starting at the 4-cell stage. The MEX-3 protein contains two

repeated 70-amino acid regions homologous to a KH-domain; a domain involved in

binding single-stranded mRNA (Draper, et al, 1996; Dejgaard & Leffers, 1996). The

findings from these experiments strongly suggest that MEX-3 binds to *pal-1* mRNA and

prevents the translation of PAL-1 protein.

The Ubiquitin Proteasome Pathway

One important mechanism for the regulation of proteins is ubiquitin-mediated proteolysis, also known as the ubiquitin proteasome pathway (UPP). The UPP is a mechanism by which proteins are broken down into amino acids by the 26S proteasome, allowing these amino acids to be reused by subsequent proteins.

Ubiquitin (Ub) is a small protein of about 75 amino acids (varies by species), and the amino acid sequence is highly conserved among eukaryotic species. The attachment of ubiquitin molecules to target proteins marks those proteins for degradation by the proteasome; the 26S proteasome recognizes a ubiquitin-marked protein, and degrades it into amino acids by hydrolyzing bonds between adjacent amino acids.

There are three types of enzymes involved in the UPP: the ubiquitin-activating enzyme, the ubiquitin-conjugating enzyme, and the ubiquitin-protein ligase. In *C. elegans*, there exists only one ubiquitin-activating enzyme, 22 ubiquitin-conjugating enzymes, and perhaps 600 ubiquitin-protein ligases (Reviewed by Kipreos 2005; N. Huang, personal communication). In the first step of the Ub pathway, the activating enzyme uses an ATP molecule to bind an Ub molecule to itself via a thioester linkage. In the next step, the activated Ub molecule is transferred to the conjugating enzyme via another thioester linkage. In the last step, the ubiquitin ligase binds to both the conjugating enzyme and the target protein. The Ub molecule is then either transferred directly to the target protein or first to the ubiquitin ligase, via a thioester linkage, and then to the target protein. This process typically occurs multiple times in order for a

poly-Ub chain to be formed (Reviewed by Kipreos, 2005; Figure 2). For the 26S

proteasome to recognize its target protein, a poly-Ub chain of at least four tandemly-

attached Ub molecules must be attached to the protein (Pickart, 2000). The 26S

proteasome consists of a 20S proteasome at the core and two 19S regulatory

complexes. The 19S regulatory complex cleaves the Ub chain from the substrate, then

unfolds the protein and translocates it in the 20S core (reviewed by Pickart and

Cohen, 2004; Voges et al., 1999; Bowerman and Kurz, 2006; Hershko et al., 1998).
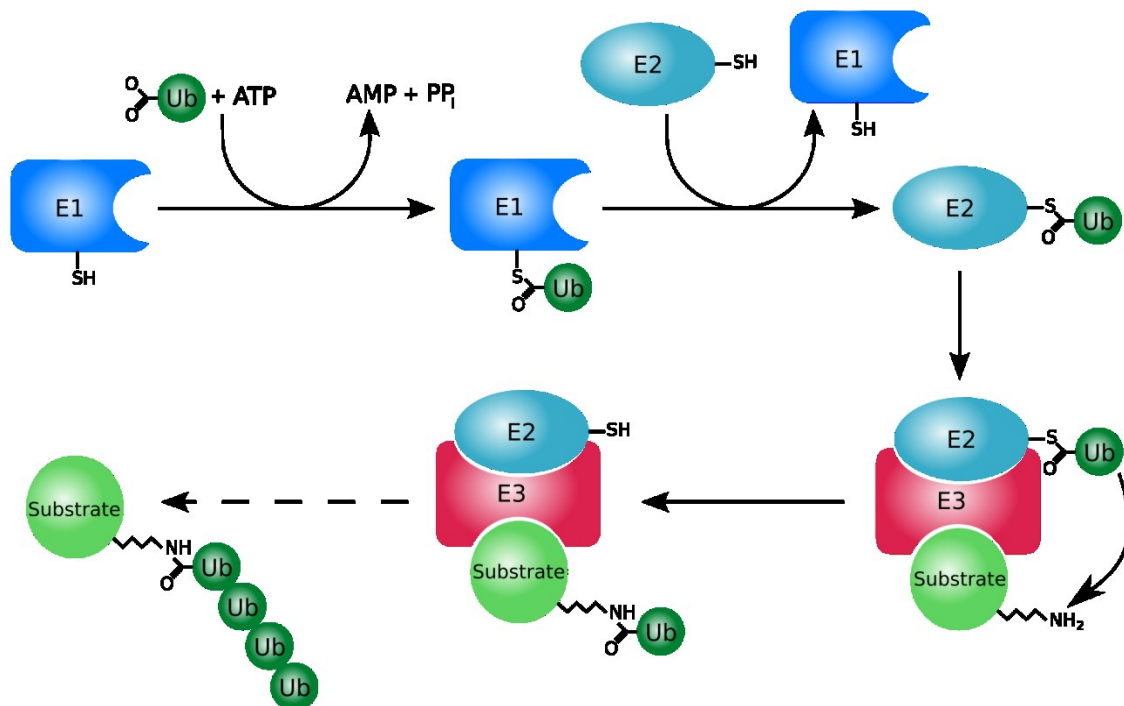

Figure 2. The ubiquitin proteasome pathway (UPP). The activating enzyme (E1) captures a ubiquitin molecule (Ub), then interacts with the conjugating enzyme (E2) and transfers the Ub to it. The ubiquitin ligase (E3) binds to both the substrate protein and the conjugating enzyme. The Ub is then transferred to the substrate. This process occurs several times and a Ub chain is formed on the substrate. The proteasome recognizes the ubiquitin chain and degrades the substrate (image source: http://en.wikipedia.org/wiki/Ubiquitin, created by Roger Dodd at the English language Wikipedia).

Ubiquitin ligases are perhaps the most interesting enzymes in the ubiquitin pathway because each ubiquitin ligase targets a specific protein or set of proteins. By determining the specific ubiquitin ligases and their protein targets, we can gain a better understanding of the processes taking place in embryonic development and what can make those processes go wrong.

Ubiquitin ligases are categorized into four major classes: HECT-domain proteins, U-box proteins, monomeric RING finger proteins, and complexes that contain a RING finger protein (Passmore and Barford, 2004). There are 165 proteins homologous to multimeric ubiquitin ligases. Although, it is not known if all of these proteins function as ubiquitin ligases *in vivo*, most of those that have been tested have confirmed functions as ubiquitin ligases. Nine of these are HECT-domain ubiquitin ligases, four are U-box ubiquitin ligases, and the other 152 are monomeric RING finger proteins.

In addition, there are an extremely large number of multi-subunit RING finger complexes which consist of a small RING finger protein and a combination of other protein components. One of these complexes consists of a RING finger protein, Skp1, CUL1/Cdc53 and an F-box protein. There are at least 326 different F-box proteins in *C. elegans* and 21 Skp1-related genes, but it is possible that not all of these function in ubiquitin ligase complexes (Nayak et al., 2002). Just within this one category of complexes it is possible for there to be hundreds of unique ubiquitin ligases. Consequently, the exact number of ubiquitin ligases is unknown, but it is thought that there are around 600 in *C. elegans* (Reviewed by Kipreos, 2005).

Screen to Identify Ubiquitin Ligases that Target MEX-3 for Degradation

As stated before, in the early stages of *C. elegans* embryonic development, MEX-3 is present throughout the embryo. Then, at the 4-cell stage, levels of endogenous MEX-3 are reduced in the posterior region, while after the 4-cell stage endogenous MEX-3 is rapidly degraded in somatic cells and restricted to the germline cells. This rapid elimination of MEX-3 in the somatic cells suggests that it is being specifically targeted for degradation. Presumably, the ubiquitin proteasome system is responsible for this rapid degradation and there are specific ubiquitin ligases that target MEX-3 for degradation in the early *C. elegans* embryo. Figure 3 shows an illustration of the pathway up to the four cell stage.

Working Model for Regulation of PAL-1 Expression
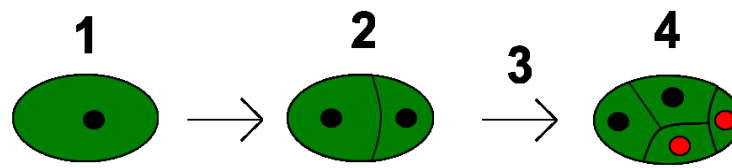
● PAL-1 Homeodomain Protein

■ MEX-3 RNA-Binding Protein

1) At the 1-cell stage, MEX-3 is present throughout the cell.
2) At the 2-cell stage, MEX-3 is evenly distributed throughout the embryo.
3) Ubiquitin ligase(s) (E3) targets MEX-3 for degradation in the posterior.
4) The expression of PAL-1 is restricted to the posterior region; in the anterior region, MEX-3 binds to *pal-1* RNA and prevents its translation.

Figure 3. A working model of the interaction between MEX-3 protein and *pal-1* RNA, the degradation of MEX-3 protein, and the subsequent expression on PAL-1 protein.

The goal of this study was to identify the ubiquitin ligases that target MEX-3 for degradation. In order to identify these ubiquitin ligases, I used RNA interference to sequentially knock out ubiquitin ligases that are expressed during embryonic development. If a ubiquitin ligase is knocked out that does target MEX-3, I expect to see MEX-3 protein present throughout the embryo past the normal 4-cell stage, due to the fact that MEX-3 is not being targeted for degradation, and a subsequent halt in translation of PAL-1 protein resulting in embryonic lethality.

To visualize MEX-3 localization in our experiments, I used a *C. elegans* strain called HCC21. This strain contains a DNA insert that has the coding sequence for green fluorescent protein (GFP) fused to the coding region of *mex-3*, resulting in GFP fluorescence where there is MEX-3 protein. Unlike endogenous MEX-3, which persists throughout the embryo until the 4-cell stage (Draper et al., 1996), GFP::MEX-3 persists in all cells through the 8-cell stage, after which it is rapidly degraded from the somatic cells, persisting in the germ line precursors and on P-granules (N. Huang, personal communication).

The strain does not show GFP::MEX-3 asymmetry at 4-cell stage, but can replace endogenous MEX-3 in ~95% of embryos at 15℃-20℃. Above 20℃, viability rapidly decreases. The absence of GFP::MEX-3 asymmetry is notable because antibody staining shows that endogenous MEX-3 begins to be depleted in the posterior by the 4-cell stage.

The enhanced perdurance of GFP::MEX-3 compared to endogenous MEX-3 could be explained by GFP having a longer half-life than the attached MEX-3. However, the

GFP::MEX-3 transgene can replace endogenous MEX-3 at low temperatures, which suggests that regulation can occur normally (N Huang, personal communication). The failure of the GFP::MEX-3 transgene to replace endogenous MEX-3 at high temperatures could be due to abnormalities in protein degradation, and/or to the lack of the *mex-3* 3'UTR.

Even though the localization of GFP::MEX-3 and endogenous MEX-3 is slightly different, we believe that ubiquitin ligases target both GFP::MEX-3 and endogenous MEX-3 for degradation. We predicted that we would visualize a change in GFP::MEX-3 localization when we knocked out a ubiquitin ligase that was responsible for targeting MEX-3 for degradation. By using the HCC21 strain, any changes that I induced in GFP::MEX-3 localization would be observed under a fluorescence microscope.

I screened a total of twenty ubiquitin ligases that were candidates. The knock out of four of these ligases resulted in a high rate of embryonic lethality. Following the identification of these four, I looked at their effects on GFP::MEX-3 localization. Even though knock down of these ubiquitin ligases caused high amounts of embryonic lethality, they exhibited GFP::MEX-3 degradation patterns very close to those seen in the HCC21 control. I concluded that none of these four ubiquitin ligases were responsible for targeting MEX-3 for degradation.

Materials & Methods

A. Worm Strains

Strain HCC21 [GFP::MEX-3] was used as the reference strain for all RNA interference injection experiments. HCC21 has a mutation in the *unc-119* gene on chromosome III. The strain also has a DNA insert on chromosome II that contains a *pie-1* promoter fused to the coding region of a GFP gene, which is in turn fused to the coding region of MEX-3, and a functional copy of the *unc-119* gene.

As a negative control, I used strain HCC22 [GFP::MEX-3 (N terminus)]. In this strain, the GFP::MEX-3 (N-terminus) protein is stable, due to the absence of the C-terminus of MEX-3. The C-terminus contains many putative regulatory sites that appear to be required for the degradation of MEX-3. I used this strain to compare with the results would be obtained if I knocked out a ubiquitin ligase gene that targeted GFP::MEX-3. In this strain GFP::MEX-3 is persistently expressed throughout the embryo at high levels in somatic cells through the comma stage and in the germ line cells through hatching.

B. Worm Cultures

Worms were grown using standard methods (Brenner, 1974) on Normal Growth Media (NGM) plates seeded with OP50 *E. coli*. Worms were incubated at 15°C or 24°C.

C. **Plasmid DNA Purification**

Double-stranded RNA (dsRNA) was injected into worms to cause RNA interference (RNAi). The first step in creating dsRNA was to purify the desired template DNA. To do this, I first streaked out *E. coli* from a *C. elegans* RNAi library on LB-ampicillin plates and allowed growth overnight at 37°C. The RNAi library is distributed by Source Bioscience: (http://www.lifesciences.sourcebioscience.com/clone-products/mirna--rnai-resources/c-elegans-rnai-library.aspx). Single colonies from these plates were grown in Lysogeny Broth (LB) liquid culture with ampicillin on a shaker at 37°C overnight.

Plasmid DNA was extracted using the QIAprep Spin Miniprep kit (QIAGEN), using manufacturer's instructions. Restriction digests were performed on purified plasmid DNA and run out on an agarose gel with a 1kb ladder to determine if they had the proper sized fragments. The sequences contained within the plasmids from the RNAi library were found by searching PubMed for the primer sequences used to construct the library. The predicted restriction fragment sizes (Table 1) were found by performing hypothetical restriction digests with various enzymes in the software program A plasmid Editor (http://biologylabs.utah.edu/jorgensen/wayned/ape/).

One or more of these enzymes were chosen so that the fragment sizes would be distinct. If the restriction digest resulted in unexpected fragment sizes, the digest was performed again in conjunction with a digest using a different enzyme(s). If the second digests also resulted in unexpected fragment sizes, another single colony was used to repeat the above process. If the second colony also resulted in unexpected

fragment sizes for the restriction digest, the process was started again from streaking out *E. coli* from the RNAi library, and if this failed then it was assumed that the particular well in the RNAi library was contaminated by *E. coli* with a different plasmid DNA. The purified plasmid DNA was digested using the enzymes listed in Table 1 (all restriction enzymes were manufactured by New England Biolabs). The expected fragment sizes and the digestion results are also listed in Table 1.

D. Polymerase Chain Reaction

PCR was performed on the plasmids using 33.75μl of sterile, distilled, deionized water, 5μl 10x *Taq* Buffer (Fermentas), 4μl of 25mM $MgCl_2$, 1μl of 10mM/40mM dNTP mix, .25μl *Taq* DNA polymerase (Fermentas), 2.5μl of both forward and reverse primers, 1μl template DNA for a final volume of 50μl. T7 primers were used as forward and reverse primers to amplify the inserts from the RNAi library because the plasmids are comprised of the L4440 vector with two T7 promoters surrounding the gene of interest (Integrated DNA Technologies); the T7 promoter sequence can be found in Table 2. For the ubiquitin ligases not present in the RNAi library, gene-specific primers were designed for PCR and a Yeast-2-Hybrid cDNA library was used as the template: these gene-specific primers had the T7 promoter sequence added to the 5' end. Digestion reactions were performed on these PCR products using the same methods as those used for the genes from the RNAi library. The enzymes used, expected fragment sizes and digestion results can be found in Table 1.

The PCR cycle conditions were set as the following: 3 minutes at 94℃. 35 cycles of: 1 minute at 94℃, 1.5 minutes at 56℃, 3 minutes at 72℃. 10 minutes at 72℃.

E. Transcription Reaction for dsRNA

Transcription reactions were performed on the PCR products, which had T7 promoter sites, using 5μl 10x RNA Polymerase Reaction Buffer (NEB), 5μl 10/40mM rNTP, 1μl Murine RNAse Inhibitor, 1μl T7 RNA Polymerase (NEB), 1μl PCR product, and 37μl RNAse-free water for a total volume of 50μl. This reaction was kept at 37℃ for 2 hours, and an additional 1μl T7 RNA Polymerase was then added and the reaction was incubated at 37℃ for another 2 hours. Finally, 1μl DNase I (RNase-free) (NEB) was added and the reaction was incubated for another 10 minutes at 37℃ before annealing by placing the tube in a 100℃ heat block and allowing it to cool to room temperature. 5μl of the transcription products were run out on a 2% agarose gel along with a 1kb ladder to verify that the reaction worked and that the dsRNA was the proper length. If the gel showed that the reaction did not work or if the product was the wrong length, the process was repeated.

F. Injections

For each trial, ~20 young adult HCC21 worms were injected with the dsRNA of a particular ubiquitin ligase. The worms were first placed on a communal NGM plate that was not seeded for about 20 minutes in order to reduce the amount of OP50 covering them. The worms were then placed on an injection pad (a drop of dry 2%

agarose on a coverslip) within a drop of mineral oil, so that the worms would stick to the injection pad and be unable to move freely. The injections were performed using a Zeiss AX10 Observer A1 microscope, a Tritech microinjection apparatus with pressurized nitrogen gas, and a Narashige Micromanipulator. The injection needles were pulled from borosilicate glass using a Sutter Instrument micropipette puller. The needles measured 10cm in length, 1.0mm outer diameter, and 0.5mm inner diameter. The dsRNA of the ubiquitin ligase of interest was loaded into a needle by placing small droplets onto the open end of the needle and allowing capillary action to move the liquid up the needle. The needle was then placed in the microinjection apparatus and the dsRNA was injected into the worms. After injecting the worms, they were rescued by placing them in 50μl of recovery buffer and allowing them to recover for 10-30 minutes. I then added 50μl of M9 to allow me to harvest the worms from the solution. The injected worms were moved to communal recovery plates (NGM plates, as described above) and left overnight at 15°C so that RNAi could take effect. Individual worms were then moved to separate NGM plates. Half were incubated overnight at 15°C and half at 25°C to allow the worms to lay embryos. The following day, the adult worms were removed and number of embryos that had been laid was counted. On the 4th and 5th days, the hatched progeny were counted for the worms incubated under temperature conditions of 25°C or 15°C respectively.

G. GFP::MEX-3 localization using fluorescence microscopy

For knockouts that resulted in high rates of lethality, another 30-40 young adult worms were injected with the dsRNA of gene of interest. The worms were then rescued and incubated at 15°C overnight for RNAi to take effect. The next day ~20 worms were placed in a solution of 45µl of M9 and 5µl of 400µM Levamisole and dissected using a scalpel. This solution caused muscle contraction and helped to push embryos out of the dissected worms. A glass pipette was then used to transfer embryos and liquid to a 2% agarose pad and excess liquid was removed using a plastic pipette. A cover slip was placed on top and the slides were observed at several different magnifications using a Zeiss Axioscope microscope fitted with differential interference contrast (DIC) optics for white light imaging, and a mercury vapor bulb for fluorescence imaging of GFP::MEX-3. Both white light and fluorescence images were taken at 100x magnification. The images were then compared to HCC21 negative control images and HCC22 positive control images in order to determine whether or not GFP::MEX-3 localization had been affected by the knockout of the ubiquitin ligase of interest.

Table 1: Expected Fragment Sizes after Restriction Digest

Gene name	RNAi Library location	Enzymes used	Expected fragments (base pairs) F: forward, R: reverse (direction of gene in plasmid)	Digest results
C52D10.7 (*skr-9*)	IV-8M01	*Hind*III	F: 3679+166 R: 2820+1015	Matched expected
C52D10.9 (*skr-8*)	IV-8M05	*Pvu*II	F: 2872+868 R: 3208+532	Matched expected
C53A5.6a	V-9F17	*Pvu*I	F: 2122+1045+705 R: 1940+1045+887	Matched expected
F46A9.4 (*skr-2*)	I-4N09	*Ava*I	F: 3292+542 R: 3164+670	Matched expected
T09F3.1 (*ztf-27*)	II-7C24	*Bgl*II	F: 3297+1521 R: 4185+633	Matched expected
K10G4.5	V-11H07	*Nae*I	F: 3208+500 R: 2523+1261	Matched expected
R52.1 (*sdz-28*)	II-2I14	[1]	[1]	Matched Expected
C06A5.8	n/a PCR	*BstB*I	384+155	Matched expected
C32D5.11	n/a PCR	*Ava*I	349+210	Matched expected
C45G7.4	n/a PCR	*Mfe*I	414+224	Matched expected
F53G2.7 (*mnat-1*)	n/a PCR	*BamH*I	417+234	Matched expected
F55C9.13	n/a PCR	*BsaB*I	239+142	Matched expected
Y119D3B.22 (*fbxa-76*)	n/a PCR	*Xho*I	269+247	Matched expected
Y47G6A.31	n/a PCR	*Nsi*I	336+194	Matched expected
F44G3.14 (*fbxa-143*)	n/a PCR	*Ava*I	558+273	Matched expected
T05F1.13	n/a PCR	*Bgl*II	550+441	Matched expected
T02C1.2	n/a PCR	*Nsi*I	417+360	Matched expected
Y69H2.15	n/a PCR	*Dra*I	846+237	Matched expected
T08G3.13	n/a PCR	*Hinc*II	300+200	Matched expected
Y82E9BL.18	n/a PCR	*Acl*I	369+269	Matched expected
W02G9.2[2]	V-2M20	*Kpn*I	F: 2912+857, R: 3449+320	No digest match[2]
		*Pvu*I	F: 1840+1045+884, R: 2119+1045+605	No digest match[2]
F57C2.2[3]	II-9M23	[3]	[3]	[3]
F58E6.12[4]	V-6H02	[4]	[4]	[4]

[1]Missing records for enzyme used and expected fragment sizes

[2]W02G9.2 was digested twice and neither time resulted in proper fragment sizes, so neither PCR nor transcription reaction was performed on W02G9.2.

[3]MiniPrep did not work so no further steps were taken.

[4]This gene was not actually in the RNAi library and was discarded.

Table 2: Primer Sequences

Gene Name	Forward Sequence	Reverse Sequence	Product Size
C06A5.8	TCGTGGTTTGCATTGCGGACATAC	TTCGTTCTGGCTGAGCAGGACTTT	539
C32D5.11	GCGCTATGCCGCTCAAATTTACGA	ATCACGCATCATGTGGTAGGCTCT	559
C45G7.4	GGAATGCTGCGTGAACCATCACAA	TCTGCTTTCGCTGGGTTACGGTAT	638
F44G3.14 (*fbxa-143*)	AGAAGCCTGCATGTGGCTCTATCA	TTTGGCCCGTTCTTCTCCTTCGTA	831
F53G2.7 (*mnat-1*)	ACAGATCGTTCTTCCAACGCCGTA	TCATCCAGTCTTCGCTTGTTCCGA	651
F55C9.13	TCCCAATTCTCCATCTCCCTGCAA	GGAGCCAGATGTTCTGAGAATGGACT	381
T02C1.2	ATGGTGTCATCCAAGATTTGCGCC	TCAACGGGATTGGACTCTTCGTCA	777
T05F1.13	AGCTCGAGCAACTACCAAATGACG	GTTGCCAAAGTTCCATCAGGTCGT	991
T08G3.13	ATGTGTCGGAGGACAGAAGTTCAC	TAACAGTGTTGCCAATTTCCGGCG	500
Y119D3B.22 (*fbxa-76*)	TAGCCATGGAATTGGATGCGAGGA	AGATCATCTCTGATCCCAACCGCA	516
Y47G6A.31	TGCGGTCACACATTCTGCTACTCT	TTCCACCGAACATCTTTGTGACCC	530
Y69H2.15	TGGAATCCACAGTTGGAACACCGA	TGGTACCTTACAGGTGGCTGGTTT	1083
Y82E9BL.18	ATGCCCACACTTAACCACCTACCA	AGATGTTCAATTTGGTCCGCCTCG	638
T7 Primer	5'-TAATACGACTCACTATAGGG-3'		Varies[1]

[1]When using the T7 primer, the product size varied depending on the size of the plasmid inserts.

Results

Initial Screening of Ubiquitin Ligase Candidates

To identify which ubiquitin ligase(s) were responsible for targeting MEX-3 for degradation, I used RNAi on young adult hermaphrodite *C. elegans* to sequentially knock-out selected ubiquitin ligases. After injecting the worms with dsRNA of the ubiquitin ligase of interest, I incubated them overnight at 15°C, allowing the worms to recover and for the RNAi to take effect. The following day, I cloned out the worms to individual plates, with half at 15°C and the other half at 24°C. I then allowed them to lay embryos overnight. The next day I removed the adult worms and counted the number of embryos that had been laid by each worm. After sufficient time had passed for the embryos to develop and hatch, one day at 24°C and two days at 15°C, I counted the hatched progeny. I conducted two independent trials for each gene in order to ensure that my results were accurate.

I calculated the embryonic lethality rate that resulted from knocking out a particular ubiquitin ligase by first subtracting the number of hatched embryos from the total number of embryos laid to get the number of unhatched embryos. I then divided the number of unhatched embryos by the total number of embryos and multiplied that number by 100 to get the percentage of embryonic lethality. I scored for embryonic lethality because when MEX-3 localization is affected, the embryos should die at some point during development.

Twenty-three genes were selected for screening from the compiled list of 150 ubiquitin ligases that might target MEX-3 for degradation. Out of these twenty-three

genes, three were not able to be screened. Multiple restriction digestions for the gene W02G9.2 did not digest into the proper sized fragments after plasmid purification so I assumed the well in the RNAi library had been contaminated by another gene. For the gene F57C2.2, multiple attempts to isolate the DNA failed so I was not able to test this gene. The gene F58E6.12 was not actually in the RNAi library so it was not tested.

In total, I screened twenty ubiquitin ligases. I found that the majority of these ubiquitin ligases did not result in substantial amounts of embryonic lethality when knocked down, as defined by greater than 20% lethality. As shown in Table 3, when I knocked out SKR-9, SKR-8, C53A5.6a, and SKR-2 I observed greater than 20% embryonic lethality for most of the trials. The first round of injections for SKR-9 resulted in 34.6% lethality at 15℃ and 68.1% lethality at 24℃; the second round of injections resulted in 19.9% lethality at 15℃ and 50.9% lethality at 24℃. For SKR-8, the first injections resulted in less than 20% lethality at 15℃, but 57.6% lethality at 24℃; the second injections resulted in 41.8% and 60.4% lethality at 15℃ and 24℃, respectively. When C53A5.6a was knocked out, the first injections resulted in less than 20% lethality for 15℃ and 24℃, while the second round of injections resulted in 78.9% and 41.7% lethality for 15℃ and 24℃, respectively. The first round of knockouts for SKR-2 resulted in 63.1% and 86.3% lethality for 15℃ and 24℃, respectively. The second round resulted in 84.4% and 91.2% lethality for 15℃ and 24℃, respectively.

Table 3: Incubation/Injection Records, Lethality Rates and Gene Descriptions

Gene name	Temp	1st Injection (embryos laid/hatched)	2nd Injection (embryos laid/hatched)	Percent Lethality 1st trial	2nd trial	Gene Description
T09F3.1 (*ztf-27*)	15℃	306/270	129/124	<20%	<20%	C2H2 type, Zinc finger putative transcription factor family, Chromosome II
	24℃	261/274[1]	274/267	<20%	<20%	
K10G4.5	15℃	51/51[2]	333/312	<20%	<20%	F-box domain, cyclin-like, FTH domain, Chromosome V
	24℃	130/135	260/267	<20%	<20%	
C06A5.8	15℃	41/31	273/249	<20%	<20%	Zinc finger, C3HC4 RING-type, Chromosome I
	24℃	112/99	276/270	<20%	<20%	
C32D5.11	15℃	84/88	294/299	<20%	<20%	Zinc finger, C3HC4 RING-type, Chromosome II
	24℃	155/153	180/181	<20%	<20%	
C45G7.4	15℃	38/41	330/322	<20%	<20%	Zinc finger protein, Chromosome IV
	24℃	30/30	364/345	<20%	<20%	
F53G2.7 (*mnat-1*)	15℃	110/174	246/234	<20%	<20%	Zinc finger, Chromosome II
	24℃	161/194	459/465	<20%	<20%	
F55C9.13	15℃	236/237	259/249	<20%	<20%	F-box domain, Chromosome V
	24℃	185/184	386/353	<20%	<20%	
R52.1 (*sdz-28*)	15℃	120/123	279/307	<20%	<20%	SKN-1 dependent zygotic transcript, BTB/POZ domain, MATH domain, Chromosome II
	24℃	317/336	252/253	<20%	<20%	

Y119D3B.22 (*fbxa-76*)	15°C	270/252	230/219	<20%	<20%	F-box A protein, FTH domain, Chromosome III
	24°C	213/241	349/364	<20%	<20%	
Y47G6A.31	15°C	413/374	283/275	<20%	<20%	Zinc finger, C3HC4 RING-type, Chromosome I
	24°C	450/429	435/456	<20%	<20%	
F44G3.14 (*fbxa-143*)	15°C	385/377	154/183	<20%	<20%	F-box A protein, FTH domain, Chromosome V
	24°C	394/377	296/298	<20%	<20%	
T05F1.13	15°C	384/383	280/263	<20%	<20%	F-box domain, Chromosome I
	24°C	614/459	222/222	<20%	<20%	
T02C1.2	15°C	398/398	271/279	<20%	<20%	Zinc finger, C3HC4 RING-type, Chromosome III
	24°C	319/314	257/239	<20%	<20%	
Y69H2.15	15°C	329/302	229/235	<20%	<20%	BTB/POZ domain, Chromosome V
	24°C	370/399	275/280	<20%	<20%	
T08G3.13	15°C	217/219	284/285	<20%	<20%	BTB/POZ domain, Chromosome V
	24°C	326/342	301/300	<20%	<20%	
Y82E9BL.18	15°C	339/342	236/230	<20%	<20%	FTH domain, F-box domain, Chromosome III
	24°C	341/333	294/299	<20%	<20%	
F46A9.4 (*skr-2*)[3]	15°C	105/284	39/249	63.1%	84.4%	Cyclin A/CDK2-associated protein P19 like. Required for the restraint of cell proliferation, progression through the pachytene stage of meiosis, and the formation of bivalent chromosomes at diakinesis;
	24°C	60/437	19/217	86.3%	91.2%	

						SKR-2::GFP is detected exclusively in the intestine, Chromosome I
C52D10.9 (*skr-8*)[3]	15℃	2/2	148/254	<20%	41.8%	Cyclin A-associated protein. Required for posterior body morphogenesis, embryonic and larval development, and cell proliferation, Chromosome IV
	24℃	25/59	148/373	57.6%	60.4%	
C52D10.7 (*skr-9*)[3]	15℃	17/26	145/181	34.6%	19.9%	Cyclin A-associated protein. Required for posterior body morphogenesis, embryonic and larval development, and postembryonic cell proliferation, Chromosome IV
	24℃	46/114	88/179	68.1%	50.9%	
C53A5.6a[3]	15℃	112/136	48/227	<20%	78.9%	Zinc finger, C3HC4 RING-type, Chromosome V
	24℃	177/167	63/108	<20%	41.7%	

[1]Occasionally I miscounted the number of embryos laid and found a higher number of hatched progeny.
[2]In some of the first injections very few worms survived or laid a substantial amount of embryos because I was still learning how to inject the worms properly.
[3]The four genes in bold were the only ones for which there was a high lethality rate.

DIC and Fluorescence Imaging of GFP::MEX-3 Localization

Following the identification of a ubiquitin ligase that causes embryonic lethality after RNAi, the next step was to determine whether or not the candidate ligase targeted GFP::MEX-3 for degradation. If the ubiquitin ligase did target GFP::MEX-3 for degradation, I expected to observe an apparent change in GFP::MEX-3 localization. More specifically, GFP::MEX-3 should persist in somatic cells for longer than normal.

To see if GFP::MEX-3 localization is affected by the knockout of the four genes identified to cause embryonic lethality, I performed another round of injections, but instead of letting the worms lay embryos and counting the number of hatched

progeny, I let the RNAi take effect overnight, dissected the worms and then examined the young embryos under a fluorescence microscope.

The strain HCC21 has an inserted transgene that expresses GFP fused to MEX-3, which causes GFP to be expressed wherever MEX-3 is expressed. Under a fluorescence microscope I was able to identify where GFP::MEX-3 was localized by observing the presence of GFP in the embryo. Draper et al. (1996) observed that endogenous MEX-3 is located throughout the embryo up to the four-cell stage; at that point, MEX-3 becomes depleted in the posterior cells, but is still clearly visible. After the four-cell stage, MEX-3 is rapidly degraded in the somatic cells and is restricted to the germline. While observing GFP::MEX-3 under a fluorescence microscope, its rapid degradation is seen as a quickly diminishing fluorescence of GFP in the somatic cells after the four-cell stage.

The strain HCC22 has a similar insert as the one found in HCC21, but lacks the C terminus of MEX-3, which is required for degradation. Under the fluorescence microscope, HCC22 embryos show GFP::MEX-3 expression throughout the embryo through the comma stage. If a ubiquitin ligase that is knocked out affects endogenous MEX-3 localization, then I expected to observe similar expression of GFP::MEX-3 throughout the embryo late into the developmental period. Thus strain HCC22 served as a positive control for my experiment and RNAi treatments were compared to the HCC21 negative control as well as the HCC22 positive control. I observed the embryos from the knockout of *skr-9*, sk*r-8*, *C53A5.6*, and *skr-2* under the fluorescence microscope. All four of the knockouts showed that GFP::MEX-3 degradation was

normal, with GFP::MEX-3 rapidly degraded from the somatic cells and restricted to the

germ line after the 8-cell stage. Examples of normal and extended GFP::MEX-3

expression are shown in Figure 4, as well as the images observed from the knockout of

F46A9.4. Images for the other knockouts can be found in the Figures section at the end of the paper.
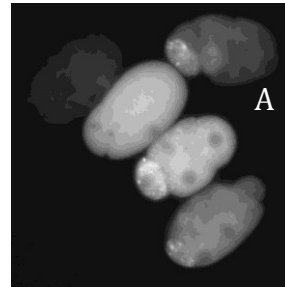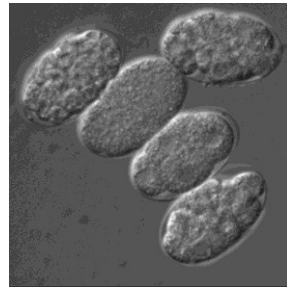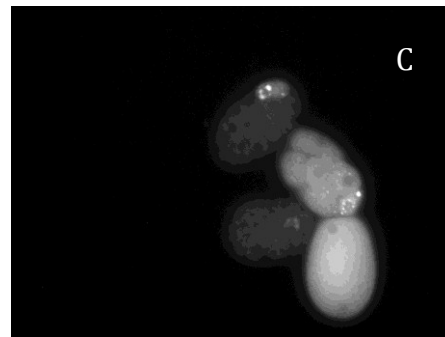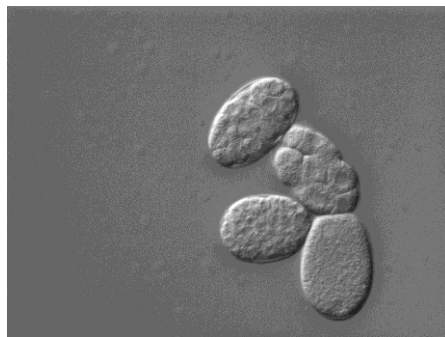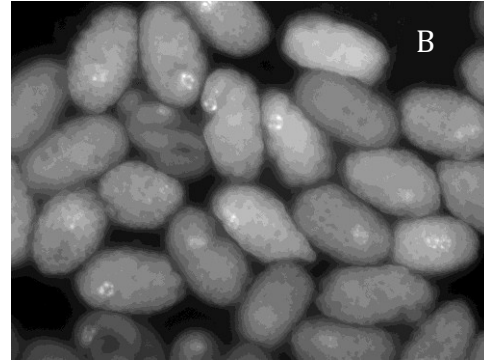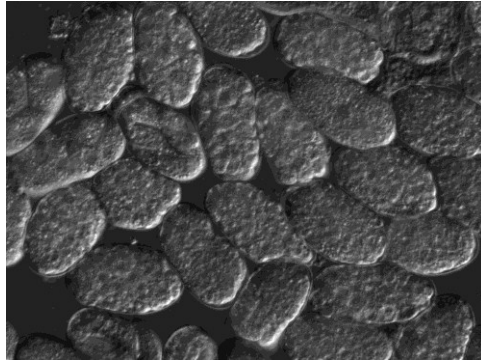

Figure 4. On the left are DIC images and on the right are fluorescence images.
Top images: Normal GFP::MEX-3 degradation, shown in HCC21 negative control.
Middle images: Extended GFP::MEX-3 degradation, shown in HCC22 positive control.
Bottom images: GFP::MEX-3 degradation in F46A9.4 knockout.
A) GFP::MEX-3 begins to be degraded at the 8-cell stage and by the 12-cell stage, expression in the somatic cells is noticeably reduced.
B) GFP::MEX-3 expression persists in the somatic cells until much later in development.
C) GFP::MEX-3 is rapidly degraded from the somatic cells after the 8-cell stage.

Although none of the knockouts showed changes in GFP::MEX-3 localization, I observed other phenotypic differences in the embryos which led me to conclude that the RNAi was effective. There were many embryos which were either more elongated than normal or completely round. In several cases, I observed strange morphologies in the later stages of development. In order to further ensure that the RNAi was effective in the embryos that I observed, I incubated the slides overnight and checked to see if any had hatched the following day.

Discussion

My findings show that of the candidate genes that I screened, none included ubiquitin ligases that specifically target MEX-3 for degradation. I screened through twenty potential ubiquitin ligases and four of these knockouts resulted in embryonic lethality: *skr-9*, *skr-8*, *c53a5*.6, and *skr-2*. However, the images taken under fluorescence microscopy showed that all four of these ubiquitin ligases did not have an effect on the localization of GFP::MEX-3.

One interesting observation that I made of the ubiquitin ligases that caused embryonic lethality was that three of the four are *skr* genes. The *skr* genes are cyclin-A associated proteins that play an important part in cell proliferation. It is possible that other *skr* genes might similarly cause embryonic lethality and perhaps even affect MEX-3 localization. The SKR proteins do not have any substrate recognition domains, so they cannot specify which protein is ubiquitinated, but they might participate in a complex that does target MEX-3. One future path of research could be to test all of the

skr genes to see if they play a role in the regulation of MEX-3. The *skr* genes are all very similar so using RNAi against one might cause RNAi cross-reactivity and multiple *skr* genes would affected. Creating genetic null mutations of these genes could be an alternative method for testing their involvement in the regulation of MEX-3.

The process of screening through the list of potential ubiquitin ligases was done in collaboration with several other undergraduate researchers: Daniela Lopez-Morales, Jessica Olson, Amy Lin, Kathryn Reichard, Anjali Desai, Kroger Schwartz and Peter Heng. Overall, two ubiquitin ligase genes were found that resulted in embryonic lethality when knocked out and also showed a disruption of GFP::MEX-3 localization. These genes are *zyg-11* and *zif-1*. Further research will be conducted to more fully characterize the interactions between *zyg-11*, *zif-1* and the other proteins discussed in this paper, such as the SKR proteins.

Following the identification of a ubiquitin ligase that targets MEX-3 for degradation, further research will be needed to fully characterize whether the ubiquitin ligase directly interacts with and ubiquitinates MEX-3. There are several different possibilities in which the ubiquitin ligase in question may not directly target MEX-3 for degradation. For example, the ubiquitin ligase may target another protein that is responsible for protecting MEX-3 from degradation and a knockout of the ubiquitin ligase in question would result in MEX-3 being protected from degradation and a change in MEX-3 localization. One way of more fully characterizing the interactions between *zyg-11*, *zif-1* and MEX-3 would be to examine the results of knockouts in other transgenic strains of *C. elegans* that express GFP fused the SKR

proteins. If the knockout of these ubiquitin ligases does not cause other proteins to have altered expression in one of these different transgenic strains, this would suggest a specific interaction between these ubiquitin ligases and MEX-3. Immunohistochemistry is another possible way of checking for specific interaction between these ubiquitin ligases and MEX-3: staining embryos with antibodies specific to other embryonic proteins would show any changes in protein localization between a normal embryo and an embryo with *zyg-11* or *zif-1* knocked out.

Furthermore, biochemical analyses could be performed to help to identify whether direct interactions are taking place. For example, one could perform an *in vitro* experiment by adding the putative ligase with MEX-3 protein. One would then use immunoprecipitation for each of the proteins separately and determine if the other was brought down by running a western blot. In addition, if the putative ligase interacts directly with MEX-3 and ubiquitinates it, one should be able to demonstrate this by performing an *in vitro* ubiquitin ligase reaction, then performing immunoprecipitation for MEX-3 and running it out on a western blot, but this time also using an antibody that recognizes ubiquitin. Ultimately, however, it would be important to move from *in vitro* experiments back into the whole organism. One would predict that knocking out the ubiquitin ligases that interact directly with and ubiquitinate MEX-3 might result in a build up of MEX-3 in cells, as it would not be targeted for degradation. It is then possible to determine whether restoring the specific ligase would reverse this process.

By fully characterizing the regulation of MEX-3 in the early embryo, I hope to provide insight for the specificity of the ligases that ubiquitinate MEX-3, and how they interact with proteins to influence embryonic development. This research could potentially help us understand more about the regulation of homologous proteins in organisms other than *C. elegans*, perhaps even in humans. The embryonic development of different organisms has a number of similarities. Many proteins involved in determining cell fates are highly conserved in different multi-cellular organisms, underscoring the importance of this research that could provide insight on what roles certain proteins play in the embryonic development of other organisms.

There are four MEX-3 homologs in humans. One of these homologs is found in intestine epithelial cells during human development. The epithelial cells found on the inside of the intestine are much different from those on the outside. This polarity in the intestine cells is similar to the polarity found in the *C. elegans* embryo. It is possible that *zyg-11* and *zif-1* have similar counterparts in humans. The identification of these ubiquitin ligases that target MEX-3 for degradation in *C. elegans* could be very helpful for finding out if there are also ubiquitin ligases that target the human MEX-3 homologs.
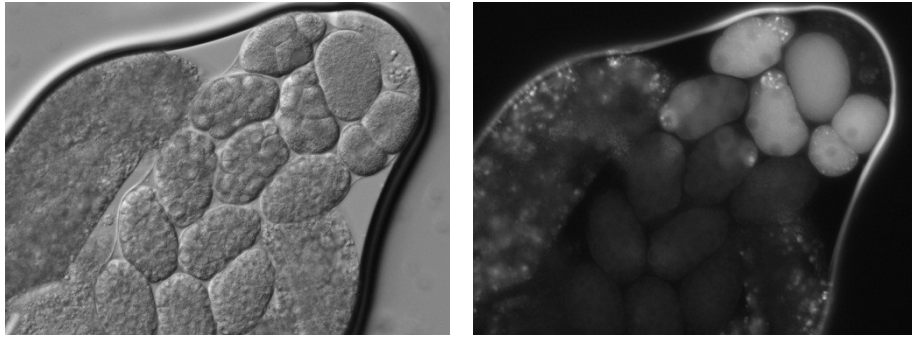
Figures

Figure 5. DIC (left) and GFP::MEX-3 (right) images of embryos with C52D10.7 knocked down. The degradation pattern of GFP::MEX-3 is normal.
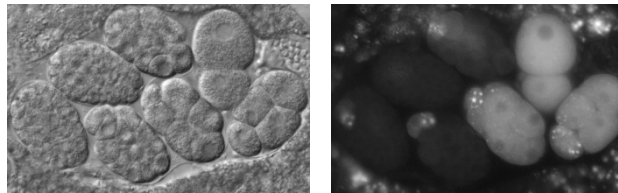

Figure 6. DIC (left) and GFP::MEX-3 (right) images of embryos with C52D10.9 knocked down. The degradation pattern of GFP::MEX-3 is normal.
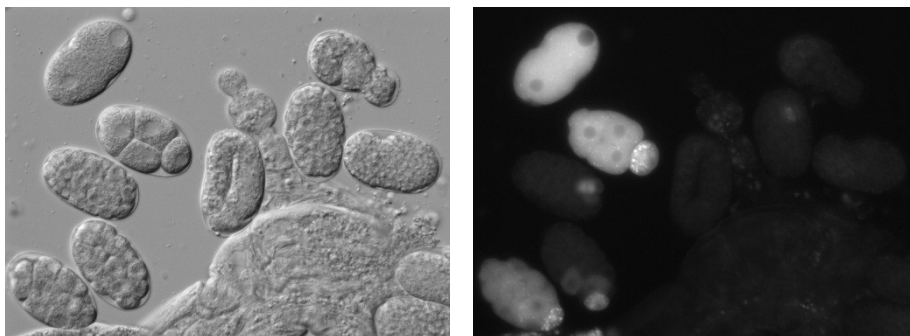

Figure 7. DIC (left) and GFP::MEX-3 (right) images of embryos with C53A5.6 knocked down. The degradation pattern of GFP::MEX-3 is normal.

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