RNAi induced knockout of *zif-1* and of *elc-1* E3 ubiquitin ligases affects MEX-3 protein localization during embryo development of *Caenorhabditis elegans*

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Abstract

PAL-1 is a protein that regulates posterior development of *Caenorhabditis elegans* embryos. Although *pal-1* mRNA is present throughout the entire embryo, the PAL-1 protein is only transcribed in the posterior end of the nematode worm. MEX-3, a RNA binding protein, binds to the *pal-1* mRNA, preventing its translation in the anterior section of the embryo. The MEX-3 protein is essential to maintaining embryo polarity and ensuring that posterior features develop only in the posterior end of the worm. During development, MEX-3 is present throughout the 1-cell and 2-cell embryo stage. MEX-3 is then degraded in the posterior end during the 4-cell stage, allowing the expression of PAL-1 in the two posterior blastomeres. By the 8-cell stage, MEX-3 is depleted from the entire embryo with remnants remaining in germline cells. The ubiquitination pathway is hypothesized to mark MEX-3 for degradation, localizing the protein at various stages of embryo development. This study screened various E3 ubiquitin ligases to determine which ligases are specifically used to mark MEX-3 for degradation during embryo development. Double-stranded RNA was created for selected E3 ubiquitin ligases and then injected into adult worms. This invoked RNA interference (RNAi) of these ubiquitin ligases in the embryos of the adult worms. Knockout of genes D2089.2, F46A9.5, F59B2.6, and Y82E9BR.15 resulted in embryonic lethality. Fluorescence microscopy of GFP::MEX-3 (green fluorescent protein labeled MEX-3) revealed that only F59B2.6 (*zif-1* gene) and Y82E9BR.15 (*elc-1* gene) knockouts affected MEX-3 localization. Double knockouts of *zif-1* and another developmental gene, *mex-5*, support the hypothesis that *zif-1* acts after other regulatory events in MEX-3 localization.

Introduction

C. elegans as a model organism

Caenorhabditis elegans are nematode worms that are approximately 1.5 millimeters in length. These worms are used as model organisms for biological study because of the many advantages they possess. These worms have a short 3-day life cycle, they are able to produce 300-350 progeny from a single worm, and are easy to cultivate, making them a convenient and economical organism with which to work (Riddle et al., 1997). *C. elegans* also contain many observable physical and behavioral features that can be linked to specific genes in the worm's DNA. Such features include their size, their sinusoidal movement, and their reproductive behavior and function (Riddle et al., 1997). This gives scientists the ability to observe changes in the worm when specific genes have been altered or silenced, allowing researchers to identify the function of these genes.

Another benefit of using *C. elegans* as a model organism is the reproductive methods of this worm. *C. elegans* contain both hermaphrodites and males. Hermaphrodites are able to fertilize their own eggs, allowing for the creation of progeny that are genetically identical to the mother if the mother is homozygous for all genes (Riddle et al., 1997). Hermaphrodites can also mate with male *C. elegans* worms, allowing scientist to set up genetic crosses that control the genotype of the progeny (Riddle et al., 1997). This means that selfing and mating can be manipulated in a laboratory setting to either maintain certain traits in a population or to cross new traits into a population by introducing a male worm. With all these characteristics, scientists are able to uncover much about the genetic functions of this worm and apply this knowledge to other organisms containing homologous genes. The study presented in this thesis uses

these advantages of *C. elegans* to study the genes responsible for embryo development as well as to probe how those genes are regulated.

Embryo development

In order for embryos to properly develop, they need to be able to establish polarity. This allows cells to distinguish the anterior end from the posterior end of the embryo. Cells are then able to recognize their spatial location within the organism. As such, cells that recognize that they are in the posterior end of the organism will differentiate into posterior-specific cells while anterior cells will differentiate into anterior-specific cells. *C. elegans* embryos only have 558 cells at hatching. Because of this, failure of cells to differentiate properly in the early embryo may result in abnormalities or embryo lethality later on in development. *C. elegans* embryos specify the anterior and posterior ends of the worm at fertilization. The end where the sperm enters the oocyte is what becomes the posterior end of the embryo (Goldstein & Hird, 1996). This one-celled embryo, known as P0, undergoes unequal divisions, resulting in the formation of a larger somatic cell in the anterior and a smaller P1 germ cell in the posterior. Asymmetric divisions for P1 through P3 occur in the same fashion resulting in an embryo containing five somatic founder cells, and one primordial germ cell (Riddle et al., 1997) (*figure 1*). The five somatic cells are the AB, MS, E, C, and D cells. The germline precursor cell is known as the P4 cell, which later divides into the Z2 and Z3 germ cells.

P-granules are ribonucleoprotein particles that are present in the posterior end of P0, and they help identify the polarity of the embryo. The initial asymmetric cleavage of P0 results in the creation of an AB and P1 cell (*figure 2*). During this time, the P-granules are localized to the P1 cell, indicating the posterior end of the worm (Strome & Wood,

1982). As the embryo continues its unequal divisions, the P-granules continue to be segregated to the cell that is destined to form the germline. The functions of P-granules are not well understood, but they are suspected to be involved in establishing germ-cell fate as well as in setting the polarity of the embryo. MEX-3, a RNA-binding protein, has also been found within these P-granules (Draper et al., 1996).

Maternal mRNAs supplied by the mother are packaged into the oocyte before fertilization. Some of the maternal mRNA is translated into proteins that establish and maintain polarity throughout the developmental process while others are required for basic cellular processes. Some of these genes encode proteins such as the PAR proteins, which play a tremendous role in establishing the initial anterior-posterior polarity, and thus in proper embryo development of *C. elegans* worms (Evans & Hunter, 2005). However, in this study we are concerned about the *mex-3* and *pal-1* genes as well as the roles of the encoded proteins in embryo development.

pal-1 and *mex-3*

The *pal-1* mRNA encodes a protein that is a homeodomain transcription factor that regulates genes required for posterior development in *C. elegans* embryos. A study by Hunter et al. showed that inhibition of *pal-1* mRNA results in disorganized development and embryonic lethality (1996). The study also isolated the P1 blastomere from *pal-1* inhibited embryos and saw that they differed from wild type P2 blastomeres in that the somatic cells, including muscle and epidermis cells, were not developed. However, the two founder germline cells (Z2 and Z3) were present in both *pal-1* inhibited embryos and wild type embryos. This suggests that *pal-1* mRNA is needed for posterior somatic cell development, but not for the establishment of the germline. The study by

Hunter et al. also revealed that *pal-1* mRNA is distributed throughout the embryo in the one- and two-cell stages, and in many four-cell embryos. In later stage embryos, *pal-1* mRNA was found mainly in the posterior of the worm. In contrast, PAL-1 protein is only detected in the posterior end of the embryo starting at the four-cell stage (Hunter et al., 1996). This suggests that although *pal-1* mRNA is present throughout the embryo in the early stages, it is only translated to protein in the posterior section of the embryo starting at the four-cell stage. This leaves anterior *pal-1* mRNA to be untranslated and unstable, resulting in degradation of *pal-1* mRNA anteriorly, and localization of *pal-1* mRNA and PAL-1 protein posteriorly (Hunter et al., 1996).

Another gene that regulates embryo development is *mex-3.* According to a study done by Draper et al., mutations in the *mex-3* gene result in development of body wall muscles in both the anterior and posterior of the embryo (1999). In wild type embryos, body wall muscle develops only from founder cells in the posterior section of the embryo. This suggests that the MEX-3 protein prevents the anterior section of the embryo from adopting posterior cell fates, specifically the development of muscles. The Draper et al. study also found that *mex-3* encodes two KH binding domains, giving the MEX-3 protein the ability to bind to RNA. In addition, the study found that *mex-3* mRNA was evenly distributed throughout mature oocytes and one-celled embryos. The *mex-3* mRNA is then localized to the anterior portion of the embryo after the one-cell stage before it is rapidly degraded from somatic blastomeres after the four-cell stage. However, *mex-3* mRNA remains stable in the cells destined to become the germline, which is typical of maternal mRNAs (Seydoux et al., 1996). As such, MEX-3 protein is only expressed in the descendants of the germline precursor in the posterior end of older embryos.

These two findings suggested that MEX-3 protein maintains asymmetry in the embryo and promotes anterior and posterior cell differentiation by binding to mRNA at specific stages. This is based on the localization pattern of MEX-3 protein, which is opposite to that of PAL-1 protein, and the lack of repression of PAL-1 translation in *mex-3* mutants (Hunter et al., 1996). Draper et al. predicted that MEX-3 protein may bind to *pal-1* mRNA in the anterior and prevent translation, restricting PAL-1 protein translation to the posterior end of the worm (Draper et al., 1996). Another study done by Pagano et al. verifies that MEX-3 protein does bind to *pal-1* mRNA (2009). This supports the idea that MEX-3 protein inhibits translation of the PAL-1 protein in the anterior portion of the embryo.

Additional work by Huang et al. showed that MEX-3 interacts with SPN-4 and MEX-6 which are additional RNA binding proteins (2002). Genetic evidence led to a model for how PAL-1 protein translation is regulated by these multiple proteins. The study hypothesized that in the anterior region of the embryo, the homologous proteins MEX-5 and MEX-6 protect the MEX-3 protein from degradation (Huang et al., 2002; *figure 3*). This stabilizes MEX-3 protein and allows it to bind to the *pal-1* mRNA 3' UTR and prevent its translation in the anterior section of the embryo. In the posterior section however, another regulatory gene, *par-1,* encodes a protein kinase that inhibits MEX-5 and MEX-6, which leaves *mex-3* mRNA vulnerable to inactivation by another protein, PAR-4. Then, MEX-3 protein in the posterior is marked for degradation in a process dependent on the *spn-4* gene, and which presumably involves the process of ubiquitination. Inactivation and degradation of MEX-3 protein allows translation of *pal-1* mRNA in the posterior (Huang et al., *2002*).

Ubiquitination Pathways

Ubiquitin is a molecule consisting of a conserved 76 amino acid polypeptide. The ubiquitination process is the attachment of a chain of multiple ubiquitins to a target protein, marking that protein for degradation (Kipreos, 2005). Ubiquitination involves three enzymes, a ubiquitin activating enzyme (E1), a ubiquitin conjugating-enzyme (E2), and a ubiquitin protein ligase (E3). The E1 requires one ATP molecule to become activated, which allows the enzyme to bind and activate a ubiquitin molecule. E2 then takes the activated ubiquitin from E1. Both E2 and the substrate are then bound by E3 which transfers the ubiquitin from E2 to the substrate. This process is repeated until the target protein has a poly-ubiquitin chain that is able to signal the proteasome to degrade that protein (Kipreos, 2005). *C. elegans* have one ubiquitin activating enzyme, 22 ubiquitin conjugating-enzymes, and hundreds of E3 ubiquitin ligases that have been isolated and identified (Kipreos, 2005). Since each E3 ubiquitin ligase only binds specific target proteins to mark them for degradation, our study is interested in identifying which E3 ubiquitin ligase(s) are required to mark MEX-3 protein for degradation in the posterior end of the embryo during embryo development.

Human Relevance

The purpose of studying model organisms such as *C. elegans* is to develop an understanding of the function of various genes in the worm. This knowledge can then be translated to the understanding of homologous genes and proteins found in humans. As related to the study in this thesis, understanding how MEX-3 protein functions and how it is regulated in *C. elegans* provides insight about how proteins homologous to MEX-3 function in humans. Such research can then be applied in medicine, where it is used to

uncover the cellular and genetic basis for various diseases such as gastric cancer, which seems to be associated with the upregulation of the human homolog of the *mex-3* gene (Jiang et al., 2012).

A study by Buchet-Poyau et al. has identified a family of human genes that are homologous to the *mex-3* gene found in *C. elegans* (2007). There are four genes in the family and they are identified as hMex-3A, hMex-3B, hMex-3C, and hMex-3D. The study found that the four hMex-3 proteins contain KH domains, indicating that they are RNA-binding proteins, just like the MEX-3 protein in *C. elegans*. There is also tissue specific expression of these proteins in humans. The hMex-3D mRNA was expressed in all tissues tested in the study while expression of hMex-3A through 3C mRNA varied from tissue to tissue. Buchet-Poyau et al. also found that hMex-3A and hMex-3B were found in P-bodies, similar to the presence of MEX-3 protein in the P-granules of *C. elegans*. The similar structure and localization of the hMex-3 proteins to the *C. elegans'* MEX-3 protein lead Buchet-Poyau et al. to acknowledge the possibility that the hMex-3 protein works to regulate a human homologue of the *C. elegans' pal-1* gene during human embryogenesis (2007). Buchet-Poyau et al. indicates that the presence of proteins homologous to the *C. elegans* PAL-1 protein are found in other organisms (2007). Homologs of *pal-1* have been well-characterized in mice (Subramanian et al., 1995) and there are also clear human homologs as well. This would indicate that embryo development regulation by some form of a MEX-3 protein is an evolutionarily conserved mechanism (Buchet-Poyau et al., 2007).

Experimental Overview

E3 ubiquitin ligases were chosen to be screened based on previous experiments found in the literature that revealed that inhibiting those certain ligases caused embryonic lethality. Double-stranded RNA (dsRNA) inhibitor molecules were created and used to inhibit the translation of each selected E3 ubiquitin ligase. This inhibition is known as RNA interference (RNAi). The MEX-3 protein needs to be degraded very early in embryogenesis, which is before the zygote can translate its own proteins for this process. As such, the E3 ubiquitin ligase(s) responsible for MEX-3 protein degradation should be maternally supplied. If knocking out an E3 ubiquitin ligase in an adult worm resulted in lethality of more than 20 percent of the worm's progeny, that ligase could have an effect on MEX-3 protein degradation. Ligases that cause embryonic lethality when knocked out will be further screened for its effects on GFP::MEX-3 protein localization during embryo development to determine if it has a direct or indirect effect on MEX-3 degradation. This study identified the E3 ubiquitin ligases responsible for MEX-3 degradation and will contribute to the knowledge of how MEX-3 is regulated in *C. elegans*.

Methods

GeneService, Ltd. RNAi Library

DNA sequences for the selected E3 ubiquitin ligases were found in the GeneService, Ltd. *C. elegans* RNAi Library. E3 ubiquitin ligases were chosen for their ability to cause embryonic lethality when knocked out. For each E3 ubiquitin ligase picked for screening, the corresponding *E. coli* containing the target DNA plasmid was streaked out on LB-ampicillin plates and then incubated at 37^{o}C overnight. The plasmids

consist of the L4440 vector containing dual T7 promoter sites that bracket the gene of interest. One colony was then picked and inoculated into a test tube containing LB broth and 50 μg/ml of ampicillin. The liquid culture was then incubated overnight with aeration at 37ºC.

For genes not found in the library, gene-specific primers tailed with T7 promoter sites were created for a PCR reaction using a cDNA library as a template. These primers will amplify the gene of interest from the cDNA library during PCR reactions, and the T7 promoter sites will allow for transcription of dsRNA.

Purifying Plasmid

Following the protocol provided by the manufacturer, a QIAGEN QIAprep Spin Miniprep Kit was used to isolate plasmids of interest from *E. coli* cells. To verify that the isolated plasmids contained the correct gene of interest, the plasmids were digested with restriction enzymes. The digestion reaction was then run on a 1% agarose gel to determine if the fragment sizes of the reaction matched the theoretical fragment sizes for the gene of interest.

PCR

The isolated plasmids containing the gene of interest were then used as templates for PCR reactions to amplify the sequence flanked by the T7 promoter sites. Reactions contained 5 μl of 10X Taq Buffer (Fermentas), 1μl of 10mM/40mM dNTPs, 5 μl of 20μM T7 Primer (5'-TAATACGACTCACTATAGGG-3'), 4 μl of 25mM $MgCl_2$, 1 μl of 1:10 diluted plasmid template, 33.75 μl of sterile, distilled, deionized water, and 0.25 μl of Taq polymerase (Fermentas). The PCR reaction proceeded as follows:

3 minutes at 94°C

35 cycles

1 minute at 94 degrees

1.5 minutes at 56 degrees

3 minutes at 72 degrees

10 minutes at 72 degrees

5 µl of the PCR product was run using the 1% agarose gel to confirm that the product was of the expected size.

Double-Stranded RNA Transcription

Using the PCR product flanked by T7 promoter sites as a template, dsRNA was created. The RNA transcription reaction contained 5 µl of 10X RNA Polymerase Reaction Buffer (NEB), 5 µl of 10/40mM rNTPs, 1 µl of murine RNAse inhibitor (NEB), 1 µl of T7 RNA polymerase (NEB), 2.5 µL of PCR product, and 35.5 µl of RNAse-free water. The reaction was incubated at 37°C for two hours. An additional 1 µl of T7 RNA polymerase was then added and the reaction was returned to 37 degrees for another two hours. After that, 1 µl of DNAse I (NEB) was added and the reaction was incubated for 10 minutes at 37 degrees. Finally, the reaction was placed in a 100 degree Celsius heat block and cooled to room temperature to allow annealing of the dsRNA. Five µL of the RNA product was run on a gel to confirm that the product was of the expected size.

Worm Strains

The HCC21 *C. elegans* worm strain was used to screen E3 ubiquitin ligases for embryo lethality and to visualize alterations in the localization of GFP::MEX-3 protein.

HCC21 translates both endogenous MEX-3 protein, as well as a transgenic MEX-3 protein that is fused to GFP (GFP::MEX-3). The GFP::MEX-3 protein can replace endogenous MEX-3 protein at temperatures of 20^{o}C and below (N. Huang, personal communication). Both proteins are degraded in somatic cells after the 12-cell stage and remain in the germline cells relatively longer. Thus, the GFP::MEX-3 protein allows our lab to determine if MEX-3 is degraded normally after knocking out an E3 ubiquitin ligase of interest. Worms were grown on Normal Growth plates seeded with OP50 *E. coli* as a food source (NG-OP50 plates).

The HCC22 strain was used as a positive control for fluorescence microscope analysis. The HCC22 strain expresses GFP fused to a truncated version of MEX-3 protein that has the C-terminus removed. This truncated MEX-3 lacks regulatory sites, which makes the protein extremely stable. As such, this protein is not degraded after the 12-cell stage and remains present throughout the entire embryo, all the way to the late stages of development. The persistence of GFP in somatic cells in the late stage embryo indicates that MEX-3 is abnormally localized. This allows our lab to identify mislocalization of MEX-3 in E3 ubiquitin ligase inhibited HCC21 worms when comparing them to the positive HCC22 control strain.

dsRNA Injections for RNAi

Young adult worms were picked from stock plates and placed on unseeded NG plates for five to ten minutes to clean the *E. coli* food source off of their bodies. A drop of mineral oil was then placed on an injection pad and the worms were mounted onto the pad. An injection pad is a thin layer of dried 2% agarose on a glass coverslip, to which the worms will adhere. A Zeiss AX10 Observer A1 microscope, a Narashige

Micromanipulator, and a Tritech microinjection apparatus using canned nitrogen gas as the pressure source, were all used to inject the double-stranded RNA into the young adult worms. Needles were pulled from borosilicate glass capillary tubes (10 cm long, 1.0 mm outer diameter, and 0.5 mm inner diameter) by a Sutter Instrument micropipette puller.

After injection, worms were then rescued using recovery buffer [5mm HEPES pH 7.2, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 66 mM NaCl, 2.4 mM KCl, 4% Glucose (w/v)], put on a shared NG-OP50 plate, and then incubated at 15^{o}C overnight to allow the RNAi to take effect. After incubation, each worm was plated to its own plate and incubated at either 24^{o}C or 15^{o}C . The effects of RNAi can differ at varying temperatures (Ahringer, 2006). A knockout that causes embryonic lethality at 24^{o}C may not show the same phenotype at 15^{o}C. As such, it is important to screen E3 ubiquitin ligases at both temperatures to verify the results of the RNAi. Worms incubated at 24^{o}C were removed from their plates after 24 hours. The embryos on the plates were then counted and then returned for incubation at 24 degrees for another 24 hours. The larvae on the plates were then counted to determine percent viability after knocking out an E3 ubiquitin ligase. Embryonic lethality was then calculated for the embryos laid by the injected worms based on the percent viability of the embryos. Worms incubated at 15 degrees underwent the same procedure except incubation times were 48 hours instead of 24 hours.

Fluorescence microscopy

If knocking out an E3 ubiquitin ligase resulted in embryonic lethality of greater than 20 percent, another round of injections was done for that specific ligase. Thirty to forty gravid worms were injected and incubated at 15^{o}C overnight. After incubation, the worms were placed in 45 µL of isotonic M9 solution and 5 µL of levamisole, which

causes muscle contraction and stiffening of the worm body. Using a scalpel, the worms were cut in half, allowing the embryos to be released into the solution.This was done to obtain embryos at different developmental stages. The solution was then transferred to a 2% wet agarose pad and was covered with a coverslip. The slide was observed at 40x and 100x under a Zeiss Axioscope microscope equipped with differential interference contrast (DIC) optics and a mercury vapor bulb for fluorescence. GFP and DIC pictures were taken of embryos that were grouped together on the slide. These groups usually contained embryos that were at different stages of embryo development. These images were then compared to the uninjected HCC21 negative control and HCC22 positive control to determine if MEX-3 localization, as evidenced by a greater fluorescent intensity in one part or another of the embryos due to the GFP labeling, was affected by inhibiting the E3 ubiquitin ligase of interest.

Results

Nineteen E3 ubiquitin ligases were chosen to be screened for their effects on GFP::MEX-3 protein localization. These ligases were chosen based on information from past studies found in literature which showed that knocking out these enzymes resulted in embryonic lethality. The GeneService bacterial RNAi library provided DNA templates for the genes of interest. Plasmids were extracted and purified using a miniprep procedure. Restriction enzyme digests were then performed to verify that the isolated plasmids contained the E3 ubiquitin ligase sequence of interest. The inserts of the plasmids were then amplified by PCR. Three of these sequences did not produce the expected digest fragment sizes. These genes were F40G9.9, F53F8.3, and K12B6.8. Three other genes, H31G24.3, K08E4.7, and Y82E9BR.15 (*elc-1)*, were not present in the RNAi library. As

a result, gene-specific primers were created for these six E3 ubiquitin ligases that were not found in the library. A cDNA library was then used with these primers in a PCR reaction to amplify the ubiquitin ligase sequence of interest. The difference between the two libraries is that the GeneService RNAi library contains genomic DNA of known sequences in known locations in 384-well plates, while a cDNA library contains various cDNAs mixed into one tube. A digest was performed on the PCR product to verify that the product contained the sequence of interest. Double-stranded RNA (dsRNA) was then transcribed from the PCR products of all 19 genes.

Prior screening of F59B2.6 (*zif-1)* in our lab used a DNA template from the GeneService RNAi library, but did not show embryonic lethality despite results in published literature (DeRenzo et al., 2003). As a result, primers for *zif-1* were designed so that the PCR procedure will amplify a larger portion of the gene from a cDNA library. This allowed for transcription of longer dsRNA and more effective RNA inhibition of *zif-1*.

To screen the E3 ubiquitin ligases, dsRNA corresponding to the genes of interest were injected into adult worms, resulting in RNA interference (RNAi). The screening of these genes resulted in embryonic lethality of 20 percent or greater for 4 of the 19 ligases tested (See *table 1* for complete results). RNAi screens in different labs for the same genes may produce different results, especially for screens targeting hundreds or thousands of different genes. This can likely be attributed to using different methods to induce RNAi, and to errors in the RNAi library used. The current study uses injection of dsRNA to induce RNAi, which is more effective than feeding or soaking. Furthermore, the identity of the targeted genes was confirmed by restriction digest.

RNAi of the D2089.2, F46A9.5 (*skr-1)*, *zif-1*, and Y82E9BR.15 (*elc-1)* ubiquitin ligase genes resulted in embryonic lethality. D2089.2 (RNAi) showed 17% lethality at 24°C and 38% lethality at 15°C for the first trial. Another trial was conducted to verify the positive embryonic lethality results and produced 80% lethality at 24°C and 96% lethality at 15°C. s*kr-1* (RNAi) resulted in 10% and 96.1% embryonic lethality at 24°C and 15°C, respectively, in trial 1. Trial 2 showed 80% and 96% lethality at 24°C and 15°C, respectively. z*if-1* (RNAi) saw lethality of 54% at 24°C and 81% at 15°C for trial 1. Trial 2 saw lethality of 73% and 77% for 24°C and 15°C, respectively. In the e*lc-1* (RNAi), the 24°C group saw 99% embryonic lethality while the 15°C worms saw 100% lethality. No second trial was conducted for *elc-1* (RNAi).

These four genes encoding E3 ubiquitin ligases were then screened for their effects on the localization of GFP::MEX-3 using HCC21 strain worms. HCC21 worms contain a GFP::MEX-3 fusion protein that can replace endogenous MEX-3, allowing our lab to determine if MEX-3 is degraded normally after knocking out an E3 ubiquitin ligase of interest (see *figure 4* for control images). Using fluorescence microscopy to visualize the GFP::MEX-3 protein, D2089.2 (RNAi) and *skr-1* (RNAi) were shown not to affect the localization of GFP::MEX-3 protein despite there being embryonic lethality. Both D2089.2 (RNAi) and *skr-1* (RNAi) showed the same MEX-3 protein degradation and localization as the uninjected HCC21 negative controls (see *figure 5* for D2089.2 (RNAi) and *figure 6* for *skr-1* (RNAi)). Inhibition of the *zif-1* and *elc-1* genes, however, each produced abnormal degradation and localization of GFP::MEX-3 protein when compared to the uninjected HCC21 negative control and the HCC22 positive control (*figure 7*).

Fluorescence microscopy showed that inhibition of *zif-1* caused GFP::MEX-3 to

remain present throughout the entire embryo for a longer period of time than in the uninjected HCC21 control (*figure 8*). However, when compared to the HCC22 positive control, GFP::MEX-3 protein in the *zif-1* knockouts did not persist as strongly throughout the entire cycle of embryo development, eventually fading after the 24-cell stage. The P-granules still localize to the germline cells in the posterior end of the embryo, indicating that some form of polarity is still established and maintained.

When *elc-1* is inhibited, P-granules show abnormal localization in *C. elegans* embryos (*figure 9*). P-granules can be seen gathering in multiple spots in the worm. This indicates that inhibiting the *elc-1* gene causes a defect with establishing polarity in *C. elegans* embryos, so any alterations in cytoplasmic GFP::MEX-3 localization are likely to be indirect effects of this early defect in establishing polarity.

ZIF-1 is a protein that interacts with other proteins containing CCCH zinc finger motifs. According to a study done by DeRenzo et al., there are five proteins found in germline cells that each have two CCCH zinc fingers in common with one another (2003). Those proteins are MEX-1, POS-1, PIE-1, and the MEX-5 and MEX-6 proteins. MEX-5 and MEX-6 are partially redundant proteins that have overlapping functions and that are 70 percent identical to each other (Schubert et al., 2000). During asymmetric divisions in the *C. elegan* embryo, the asymmetrically dividing germline precursor cells (P0, P1, P2, P3, and P4) divide into a somatic daughter cell and germline daughter cell. The P4 cell then divides symmetrically, producing Z2 and Z3 primordial germline cells. The PIE-1, POS-1, and MEX-1 proteins are seen to be at high levels in the new germline cell while MEX-5 and MEX-6 are present at low levels. In the somatic cells, PIE-1, POS-1, and MEX-1 proteins do not persist past the next cell division, while MEX-5 and MEX-6

proteins take two cell divisions to disappear (DeRenzo et al., 2003).

DeRenzo et al.'s study indicates that the ZIF-1 protein is responsible for degrading proteins in the somatic cells, including MEX-5 and MEX-6, and the germline proteins MEX-1, POS-1, PIE-1. The ZIF-1 protein binds to one of the CCCH zinc fingers in each of these proteins, and targets it for degradation. However, the ZIF-1 protein is not the only ubiquitin ligase required to degrade the germline proteins present in somatic cells. According to DeRenzo et al., the ZIF-1 protein acts as a recruitment protein that gathers an ECS ligase. The ECS ligase is composed of the proteins CUL-2, RBX-1, elongin C (ELC-1), and an E2 ubiquitin conjugating enzyme. Complete inhibition of the ECS ligase results in abnormal cell divisions in the early embryo, which precludes meaningful analysis of protein degradation. However, partial inhibition of *zif-1*, *elc-1*, *cul-2*, or the E2 ubiquitin conjugating enzyme by RNAi prevents degradation of the germline proteins. The authors hypothesize that the ZIF-1 protein is required for the complex to be formed (DeRenzo et al., 2003). Similar to the results of the DeRenzo study, our results found that inhibition of *elc-1* resulted in multiple abnormalities during early development before MEX-3 localization is spatially restricted. As this study is primarily focused on the factors that directly regulate MEX-3 localization, the *zif-1* gene was further investigated while the *elc-1* gene was left aside.

Inhibition of the *zif-1* gene through RNAi resulted in longer persistence of GFP::MEX-3 protein throughout the embryo when compared to the uninjected HCC21 negative control. Our lab hypothesizes that the ZIF-1 protein may be directly involved in the degradation of MEX-3, by marking MEX-3 protein bound to the SPN-4 protein for degradation. To help test this hypothesis, worms were injected with a mixture of *mex-5*

dsRNA and *zif-1* dsRNA. Those worms were then compared to worms injected with only *mex-5* dsRNA, which causes RNAi of both *mex-5* and *mex-6* maternal RNA, and to worms injected with only *zif-1* dsRNA.

The *mex-5* (RNAi) embryos showed rapid degradation of MEX-3 (*figure 10*). This is because MEX-5 and MEX-6 proteins normally help protect anterior MEX-3 from the SPN-4 dependent degradation that occurs in the posterior of the embryo (Huang et al., 2002; *Figure 3*). Without MEX-5/6, MEX-3 protein is vulnerable to degradation. In contrast, *zif-1* (RNAi) produces embryos that maintain the MEX-3 protein throughout the entire embryo until the 24-cell stage. This suggests that ZIF-1 protein may be needed to degrade MEX-3 that is unprotected by MEX-5/6 in the posterior section of the worm. In this scenario, ZIF-1 protein would be required for the MEX-3 protein degradation seen in *mex-5* (RNAi) embryos.

The double *mex-5* (RNAi) and *zif-1* (RNAi) was verified to cause embryonic lethality in the worms and then was screened for its effects on GFP::MEX-3 localization using fluorescence microscopy. The *mex-5* and *zif-1* double knockouts showed GFP::MEX-3 protein localization patterns similar to worms injected with only *zif-1* dsRNA (*figure 11*). When compared to *mex-5* (RNAi) (*figure 10*), the *mex-5* and *zif-1* double knockouts resulted in GFP::MEX-3 protein that persisted in the entire embryo longer and at higher intensity than the *mex-5* (RNAi) embryos. Inhibition of the *mex-5* gene showed rapid degradation of GFP::MEX-3, but when *zif-1* and *mex-5* genes were inhibited at the same time, GFP::MEX-3 protein was maintained throughout the embryo, even though the GFP::MEX-3 protein was susceptible to degradation. This is consistent with the hypothesis that ZIF-1 protein is needed for degradation of MEX-3 that is

unprotected by MEX-5/6 (*figure 12)*.

Discussion

The current study finds that ZIF-1 is required for degradation of MEX-3. ZIF-1 protein was previously found to be required for degradation of five proteins in somatic cells in the early stages of embryogenesis. These proteins are MEX-5 and MEX-6, and the germ cell proteins MEX-1, POS-1, and PIE-1 (DeRenzo, 2003). A study by Oldenbroek et al. finds that despite the fact that these six proteins require ZIF-1 for proper localization, the localization of the ZIF-1 protein is itself regulated by four of those six proteins (2012). Those four proteins are MEX-3, POS-1, MEX-5, and MEX-6. Oldenbroek et al. found that SPN-4 protein, which is required for proper MEX-3 protein localization, is also required for ZIF-1 protein localization.

In vitro studies found that POS-1, MEX-3, MEX-5/6, and SPN-4 all bind the 3’ untranslated region (3' UTR) of the *zif-1* mRNA. In vivo studies found that POS-1, MEX-3, and SPN-4 proteins work to inhibit translation of the *zif-1* mRNA while MEX-5 and MEX-6 proteins antagonize repression to allow translation. Oldenbroek et al.'s study shows that POS-1, MEX-3, and SPN-4 binds to different areas of the 3' UTR to prevent translation of the *zif-1* mRNA. On the other hand, the MEX-5 and MEX-6 proteins bind to an area on the 3' UTR that blocks binding of POS-1 and MEX-3 protein, allowing translation (Oldenbroek et al., 2012). Positive and negative regulation of *zif-1* mRNA translation localizes the ZIF-1 protein to somatic cells so that germ cell proteins are degraded there. In germ cells, the ZIF-1 protein is not translated, leaving the germ cell specific proteins here intact.

Oldenbroek et al.'s study shows that neither MEX-3 nor SPN-4 proteins alone are

effective in blocking the translation of the *zif-1* mRNA (2012). Both MEX-3 and SPN-4 proteins together are needed to perform such inhibition. Their study suggests that the repression of *zif-1* translation by MEX-3 and SPN-4 protein occurs only at the 1- and 2-cell stage of embryo development. After that, POS-1 protein prevents *zif-1* translation in the germline blastomere (Oldenbroek et al*.,* 2012). As MEX-3 protein is present throughout the embryo at the 1- and 2- cell stages, this proposal is consistent with the observation that *zif-1* translation is repressed throughout the entire embryo until the 4-cell stage. After the 4-cell stage, the observed inhibition of *zif-1* translation exclusively in the germline blastomere is consistent with localization of POS-1 to the germline.

The results of the current study demonstrate that the ZIF-1 protein is required to degrade the GFP::MEX-3 fusion protein in somatic cells. This is supported by Oldenbroek et al.'s proposal, and we expect that it will also prove true for endogenous MEX-3. Degradation of the GFP::MEX-3 fusion protein in the somatic cells is first detectable at the 8-cell stage, and is most apparent in the 12-cell stage (*figure 4*). If MEX-3 and SPN-4 proteins can only repress *zif-1* translation at the 1- and 2- cell stages, this means that the ZIF-1 protein will be translated in somatic cells by the 4-cell stage. This would allow degradation of GFP::MEX-3 protein by the 8-cell stage. Greater degradation of GFP::MEX-3 protein then occurs by the 12-cell stage as the concentration of ZIF-1 protein becomes greater in the somatic cells of the embryo.

It should be noted that the complete degradation of GFP::MEX-3 protein in somatic cells after the 4-cell stage also occurs for endogenous MEX-3 protein (Draper et al., 1996), and may be controlled separately from the reduction in MEX-3 protein that occurs in posterior blastomeres at the 4-cell stage, concurrent with PAL-1 protein

expression in those posterior blastomeres (Hunter et al., 1996). The reduction of posterior MEX-3 protein at the 4-cell stage is not evident with the GFP::MEX-3 fusion protein, which 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.

In any event, the expression of ZIF-1 protein exclusively in somatic cells starting at the 4-cell stage makes it unlikely that *zif-1* is required for the reduction of MEX-3 protein that is already evident at the 4-cell in the 2 posterior blastomeres, including the germline blastomere. Rather, *zif-1* is required to degrade MEX-3 protein in somatic cells after the 4-cell stage. This is similar to the role of *zif-1* in degrading MEX-5 and MEX-6 after they have fulfilled their function in the somatic cells, and in degrading the germline proteins MEX-1, POS-1, and PIE-1 when they are present in the somatic cells.

It may seem strange that the MEX-3 protein regulates a protein (ZIF-1) that ends up initiating the degradation of the MEX-3 protein itself. However, Oldenbroek et al. shows that such cycles of regulation between two proteins do occur during *C. elegan* embryogenesis. MEX-5 and MEX-6 proteins are targeted by ZIF-1 protein for degradation, but Oldenbroek et al.'s study indicates that MEX-5 and MEX-6 proteins still promote translation of ZIF-1 protein. Thus, MEX-5 and MEX-6 ultimately promote the translation of a protein that helps degrade them. Based on Oldenbroek et al.'s study and the results from the present study, MEX-3 protein inhibits the translation of the ZIF-1

protein until the 4-cell stage. Then after the 4-cell stage, MEX-3 protein may be marked for degradation by the ZIF-1 protein.

In this same screen of E3 ubiquitin ligases, a collaborator found a second gene required for degradation of the GFP::MEX-3 fusion protein (A. Lin, personal communication). This gene, *zyg-11*, encodes a protein that interacts with the CUL-2 and ELC-1 components of the ECS ligase, as does ZIF-1 (Sonneville & Gonczy, 2004). Intriguingly, ZYG-11 is expressed in unfertilized oocytes and early embryos, while ZIF-1 is expressed in somatic cells after the 4-cell stage. This raises the possibility that ZYG-11 is responsible for the reduction in MEX-3 seen in posterior cells at the 4-cell stage, and then ZIF-1 is responsible for degradation of MEX-3 in somatic cells after the 4-cell stage.

Further study of ZIF-1 and MEX-3 interaction

Antibody Staining

To determine whether *zif-1* has the same effect on endogenous MEX-3 protein as on the GFP::MEX-3 fusion protein, *zif-1* RNAi will be repeated on non-transgenic embryos, which will then be stained with an anti-MEX-3 antibody to detect endogenous MEX-3. In particular, this experiment will clarify whether *zif-1* is required for the reduction in MEX-3 seen in posterior blastomeres at the 4-cell stage. In addition, these *zif-1*(RNAi) embryos will also be stained with an anti-PAL-1 antibody, to determine if the excess MEX-3 protein in the somatic cells has any effect on PAL-1 localization.

Yeast two-hybrid

Our results indicate that ZIF-1 protein is required for MEX-3 protein degradation. This is because of the correlation between knocking out the *zif-1* gene and the abnormal persistence of GFP::MEX-3 protein in later stages of embryo development. However,

there is no strong evidence that indicates that ZIF-1 protein directly marks the MEX-3 protein for degradation. Current studies in our lab are taking advantage of the yeast two-hybrid system to test if physical interactions between the ZIF-1 and MEX-3 proteins are present. The presence of physical interactions indicates that there is bonding between ZIF-1 and MEX-3 proteins, supporting the idea that the ZIF-1 protein can bind to the MEX-3 protein and mark it for degradation.

In order for a gene to be expressed, a transcription factor must bind to the DNA strand and then recruit RNA polymerase to initiate transcription. This transcription factor can be physically separated into two domains. One domain is known as the DNA binding domain (DB) while the other is known as the transactivation domain (AD) (Alberts et al., 2002). The DNA binding domain is the portion of the transcription factor that physically binds to a DNA sequence upstream of the gene to be transcribed. The transactivation domain helps recruit other transcription regulators that eventually bind RNA polymerase to begin transcription. The need for both a DNA binding domain and transactivation domain to initiate the transcription of a gene is utilized by the yeast two-hybrid system (Alberts et al., 2002).

In a yeast two-hybrid experiment, the DB domain and AD domain are separated, allowing scientist to fuse one protein of interest (protein X) to one domain and another protein of interest (protein Y) to the other domain (Walhout & Vidal, 2001). This fusion is done using DNA recombination techniques on the sequences encoding the DB and AD domains, and sequences encoding the two proteins of interest. This results in two DNA hybrid sequences, one encoding a DB::protein X and one encoding an AD::protein Y (Alberts et al., 2002). The two resultant DNA hybrid sequences after recombination are

then transformed into a yeast cell. Here, the hybrid sequences are then translated into their respective hybrid proteins. If protein X and protein Y do have a physical interaction, the DB::protein X and AD::protein Y hybrid proteins will come together. This produces a functional transcription factor as the DB and AD domains are now able to interact with one another, initiating the transcription of a reporter gene inside of the yeast cell (Walhout & Vidal, 2001).

The DB and AD domains from a yeast *Gal4p* transcription factor are used for fusion to the two proteins of interest (Walhout & Vidal, 2001). The Gal4p transcription factor normally initiates transcription of the GAL-1 gene. The yeast strain used for a yeast two-hybrid experiment contains the GAL-1 promoter region fused to a reporter gene, allowing researchers to see if an interaction between two proteins of interest has taken place. One reporter gene is HIS3, a gene that allows for biosynthesis of the amino acid histidine. Another reporter gene, lacZ, produces the bacterial enzyme beta-galactosidase, allowing for color detection (blue color) of yeast colonies in the presence of the substrate X-gal. The final reporter gene is URA3, which allows the yeast cell to synthesize the amino acid uracil (Walhout & Vidal, 2001).

If interaction between two proteins of interest occurs, the Gal4p transcription factor will be functional and can bind to the GAL-1 promoter region, allowing for the transcription of these three reporter genes. This means that yeast cells will be able to produce histidine, uracil, and lacZ. This will allow the determination of whether interactions between two proteins of interest have occurred by growing yeast cells on media without histidine, on media without uracil, and on media with beta-galactosidase. Yeast that are able to grow on media without histidine, media without uracil, and yeast

showing blue coloration on the media with beta-galactosidase are each considered positive results. Two out of three positive results are needed to reliably conclude that two proteins of interest interact with one another (Walhout & Vidal, 2001).

Using the yeast two-hybrid, our lab plans on fusing the ZIF-1 protein to the Gal4p transactivation domain (AD). The MEX-3 protein will then be fused to the Gal4p DNA binding domain (DB). Another yeast-two-hybrid system will fuse ZIF-1 to the DB domain and MEX-3 protein to the AD domain. If we are able to obtain 2 out of 3 positive results from the two yeast-two-hybrid systems, this will indicate that the ZIF-1 protein does interact with the MEX-3 protein. Combined with the results from our current study, if there is interaction between the two proteins, this would support our hypothesis that the ZIF-1 ligase does mark the MEX-3 protein for degradation. Our lab already has gene sequences for the MEX-3 protein fused to the AD and DB domains, but needs to perform recombination reactions to fuse the gene sequence for the ZIF-1 protein to the AD and DB domain sequences. However, there have been some delays in this process.

In order to perform the recombination reaction, the gene of interest, in this case the *zif-1* gene, needs to be inserted into the pDONR vector. This vector is then used in a recombination reaction to insert the gene of interest into either a DNA binding domain (DB) vector or a transactivation domain (AD) vector, which will then be transformed into yeast cells. The domain vectors will then be expressed, resulting in translation of the protein of interest fused to the respective transcription factor domain. The *zif-1* gene inserted into the pDONR vector must be the exact cDNA sequence and cannot have any mutations. However, PCR in our lab for the *zif-1* gene from a cDNA library has so far yielded mutant sequences which will result in altered proteins if translated in the yeast

cell. As a result, the lab is still working to obtain the correct *zif-1* cDNA sequence, either through PCR or from another lab.

Implications for *zif-1*

A recent study by Jiang et al. investigated the presence of hMex-3A in cancerous gastric cells, hypothesizing that because hMex-3A is a RNA binding protein, it may be involved in the development of gastric cancer (2012). According to the study, knocking out the hMex-3A protein through small interfering RNA resulted in inhibition of cell proliferation in two types of gastric cancer cells, SNU-16 and AGS, and prevented migration of the cancer. Also, the use of quantitative real-time PCR revealed that the hMex-3A gene was upregulated 63.6 percent in cancer cells when compared to non-cancer cells (Jiang et al., 2012). This supports the proposal that hMex-3A has significant involvement with cell proliferation of tumorous tissue.

In conjunction with the results found in our current study, if the ZIF-1 protein is responsible for marking MEX-3 protein for degradation in *C. elegans* embryos, this would suggest that there may be a similar form of regulation found in humans for hMex-3A. While there are no clear homologs for ZIF-1 outside of nematodes (Altschul et al., 1997), understanding the function of the ZIF-1 protein gives researchers ideas as to how hMex-3A may be regulated in humans. Such insight may allow further investigation of cellular causation of cancer or possible treatments for the cancer. It may be possible to upregulate the expression of E3 ubiquitin ligases to promote degradation of excess hMex-3A proteins in cancerous gastric cells. As such, understanding the developmental process of *C. elegans,* such as the functions of MEX-3, PAL-1, and ZIF-1 proteins, is important not only to medicine, but also because of the role it plays in evolutionary development.

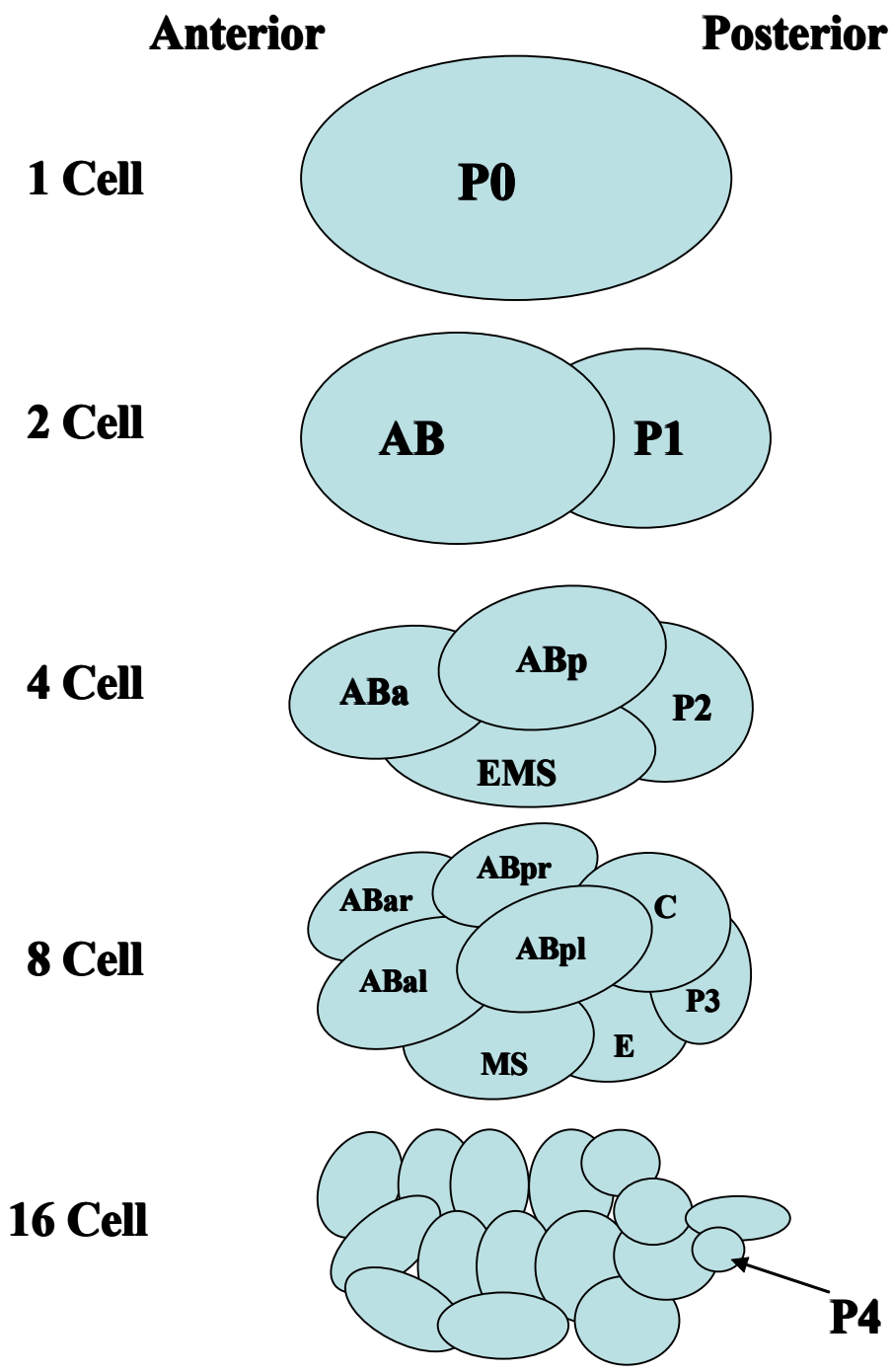

Figure 1. Blastomere cleavage of early *C. elegans* embryos. In this and all subsequent figures, the left side of the diagram is the anterior while the right side of the diagram is posterior. The figure demonstrates the initial asymmetric cleavage of P0 to form the 2-cell embryo. Many subsequent cleavages are asymmetric as well. During the 8-cell stage, the AB, E, MS, C, and D cells represent the somatic lineages. The P0, P1, P2, P3, and P4 cells on the other hand retain germline properties, with P0, P1, P2, and P3 dividing to produce one somatic cell and one germline cell, and P4 giving rise to only germline cells.

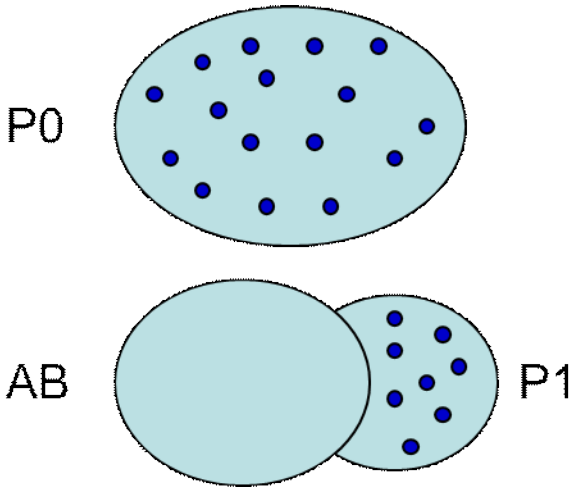

Figure 2. Localization of P-granules. P-granules, represented by the dark blue dots, are present uniformly throughout the 1-celled embryo. During the initial asymmetric cleavage, the P-granules are then localized to the P1 cell in the posterior end of the embryo. Further cleavages localize the granules to the cells destined to become the germline, which means the P2, P3 and P4 cells.

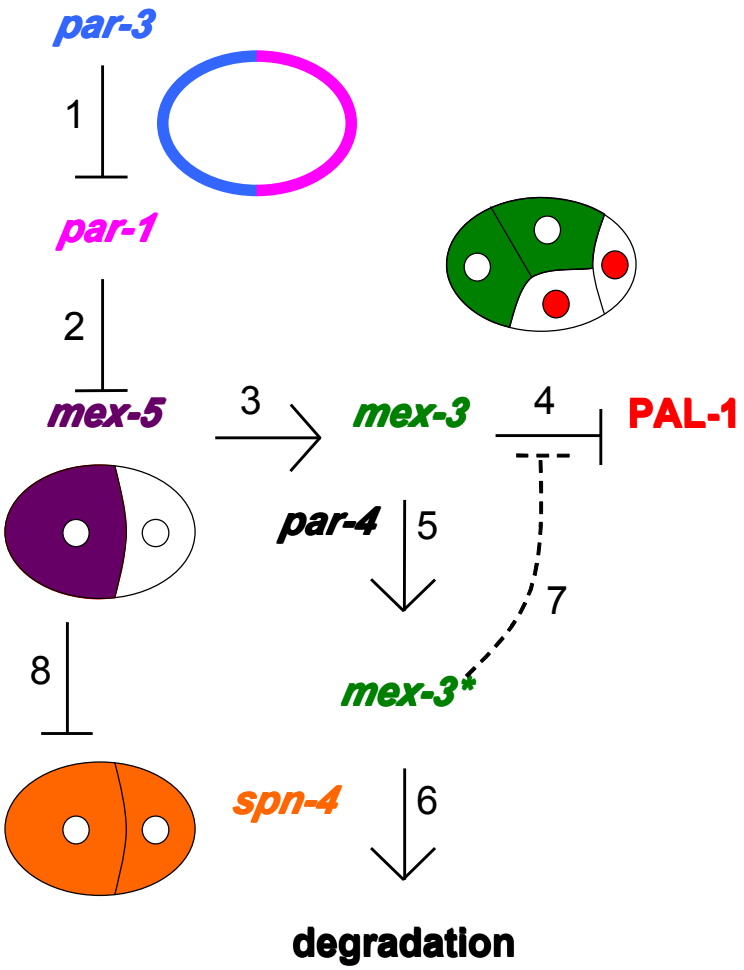

Figure 3. Working model of PAL-1 expression as presented in Huang et al. 2002.

1. Anteriorly localized PAR-3 restricts cortical PAR-1 to the posterior.
2. *par-1* then restricts MEX-5 and MEX-6 to the anterior.
3. *mex-5* and *mex-6* protect MEX-3 from inactivation and degradation in the anterior
4. MEX-3 continues repression of PAL-1.
5. In the absence of *mex-5* and *mex-6* in the posterior, *par-4* inactivates MEX-3(*)
6. MEX-3(*) is subjected to rapid *spn-4* dependent degradation.
7. In the absence of *spn-4*, inactive MEX-3* can interfere with active MEX-3, resulting in ectopic PAL-1 expression.
8. As the embryo develops, *mex-5* and *mex-6* are also required to restrict SPN-4 expression to the posterior

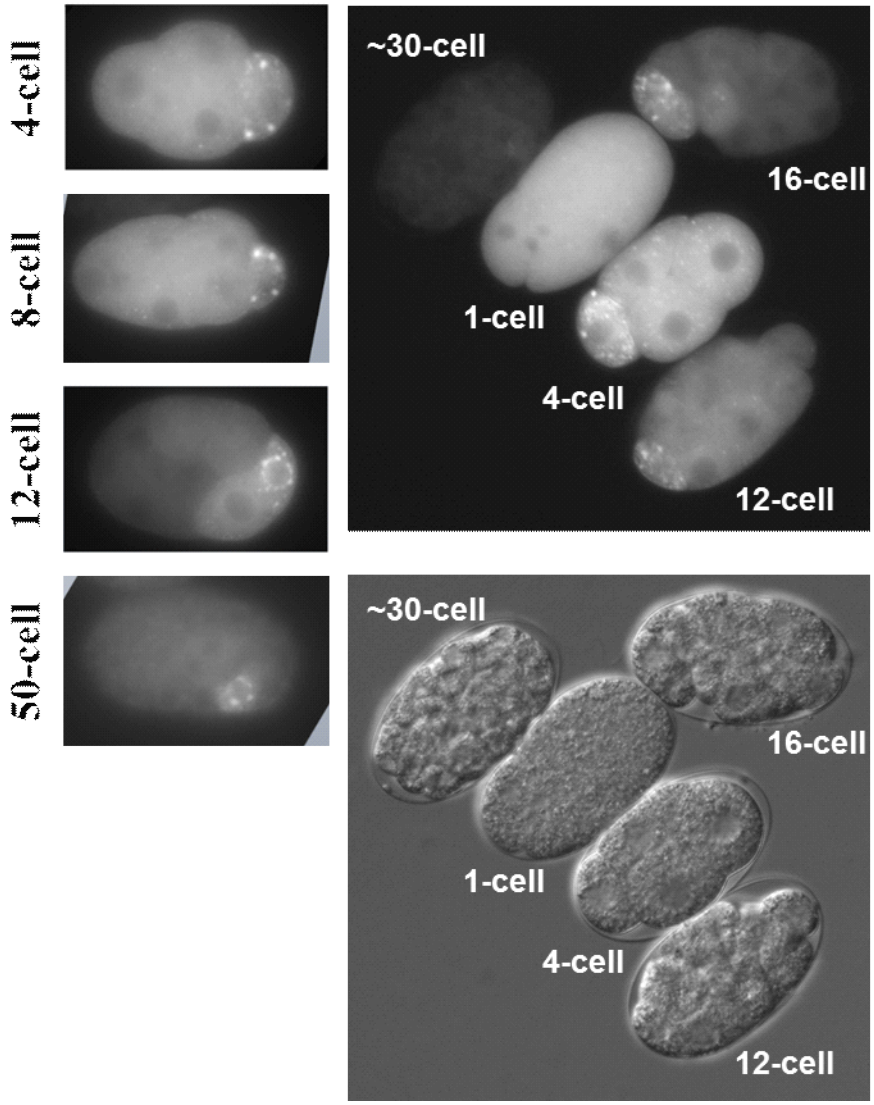

Figure 4. Strain HCC21 (GFP::MEX-3). This strain contains a transgene encoding GFP::MEX-3, in addition to endogenous MEX-3. The fusion protein functions similarly to endogenous MEX-3 protein. This allows identification of where GFP::MEX-3 is localized during various cell stages of the embryo. In the 1-cell to 8-cell stages, GFP::MEX-3 is present throughout the entire embryo. At each division, beginning with the division of the 1-cell stage, P-granules are localized to the cells that are destined to become the germline. After the 8-cell stage, GFP::MEX-3 is rapidly degraded in somatic cells.

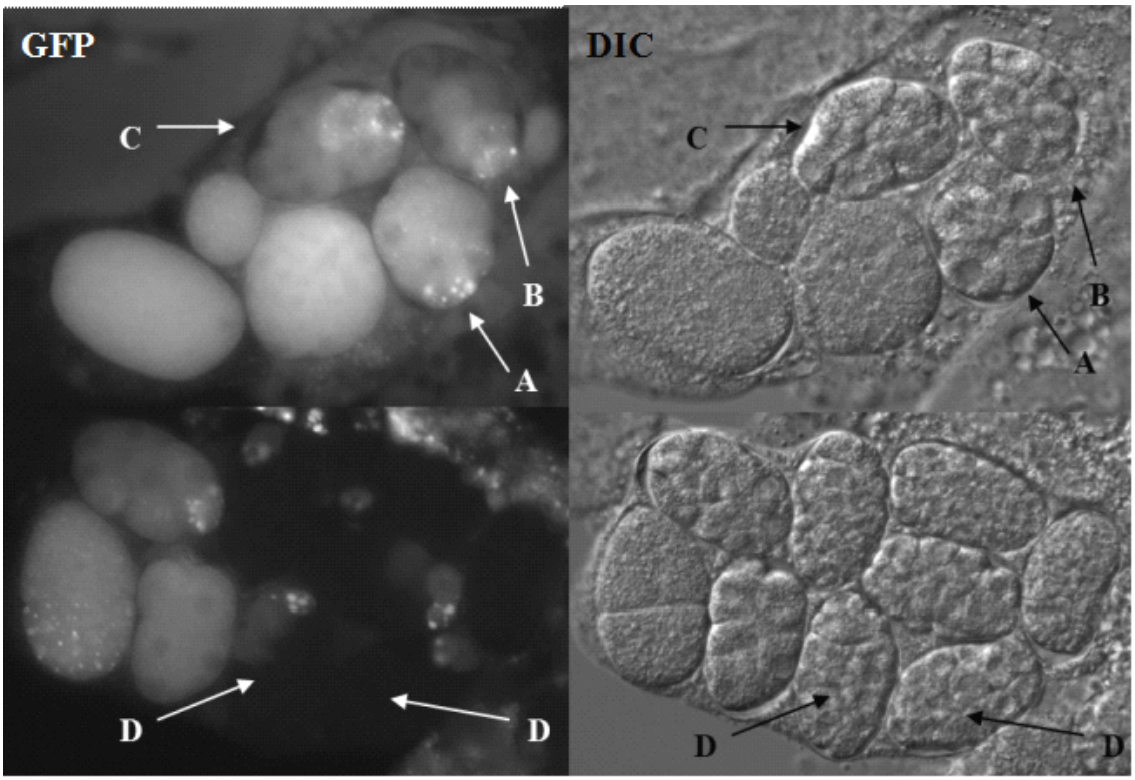

Figure 5. GFP and DIC images of D2089.2 (RNAi) embryos. These worms were incubated over night at 15°C after injections. GFP images show asymmetric GFP::MEX-3 localization in 6-celled (A), 8-celled (B), and 12-celled (C) embryos. Later cell stages (D) do not fluoresce outside of the germline, indicating degradation of MEX-3 protein in the somatic blastomeres by these stages. This GFP::MEX-3 expression pattern is characteristic of the negative control embryos. However, D2089.2 (RNAi) embryos still fail to hatch. This indicates that embryos do fail to properly complete embryo development, but not due to the misregulation of the MEX-3 protein.

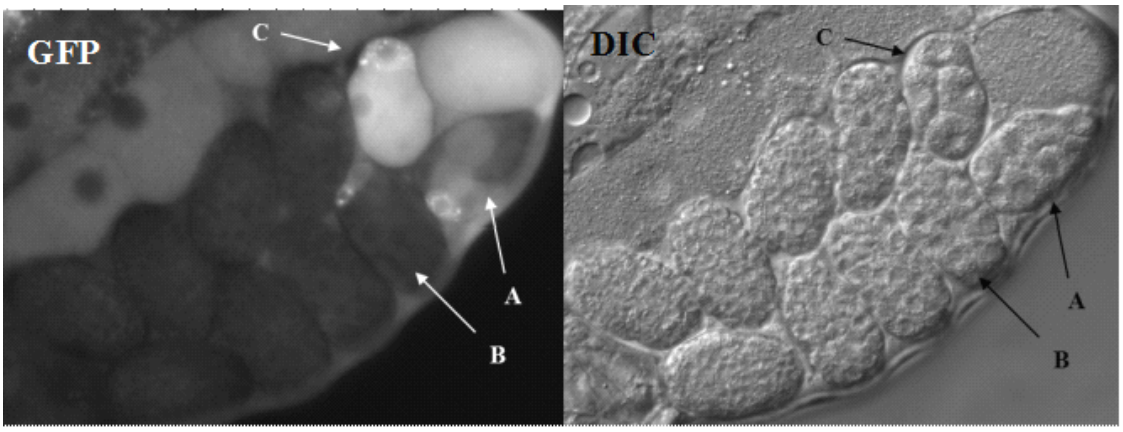

Figure 6. GFP and DIC images of *skr-1*(RNAi) embryos. *skr-1* (RNAi) worms were incubated over night at 15ºC after injections. GFP images of *skr-1* (RNAi) show asymmetric GFP::MEX-3 localization in 6-celled (A) and 12-celled embryos (B). The 4-celled (C) embryos showed asymmetric localization of P-granules. Later cell stages do not fluoresce indicating degradation of GFP::MEX-3 protein in the entire embryo by these stages. This is phenotypic of the negative control embryos. Late staged embryos showed deformations indicating that embryos failed to complete embryo development resulting in embryonic lethality. However, as the GFP images show, this deformation is not a result of misregulated MEX-3 protein.

1-cell to 28-cells

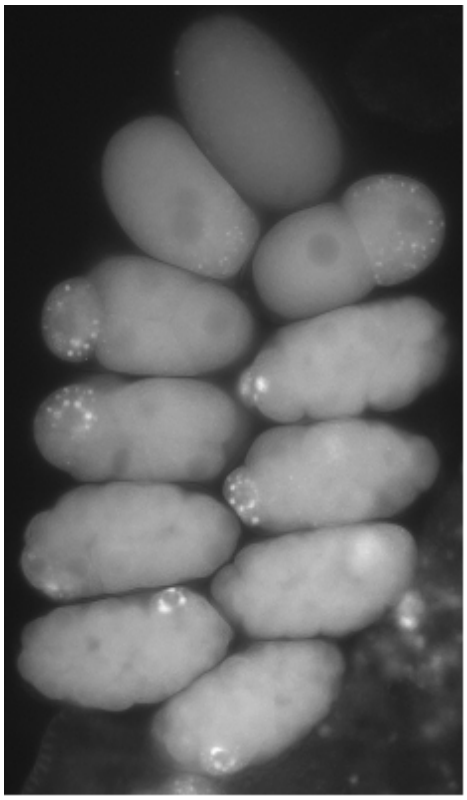

Figure 7. HCC22 strain (GFP::MEX-3 N-terminus). This strain expresses GFP fused to a truncated MEX-3 protein that only has the N-terminus. The C-terminus portion of the protein, which contains many predicted phosphorylation sites required for inactivation and degradation of MEX-3, has been removed. This allows fluorescence of GFP::MEX-3 to be present throughout the entire embryo from the 1-cell to comma staged embryo. If the ubiquitin ligase inhibited by dsRNA injections affects MEX-3 degradation, the GFP::MEX-3 expression in the injected worms should look similar to the GFP::MEX-3 expression found in HCC22.

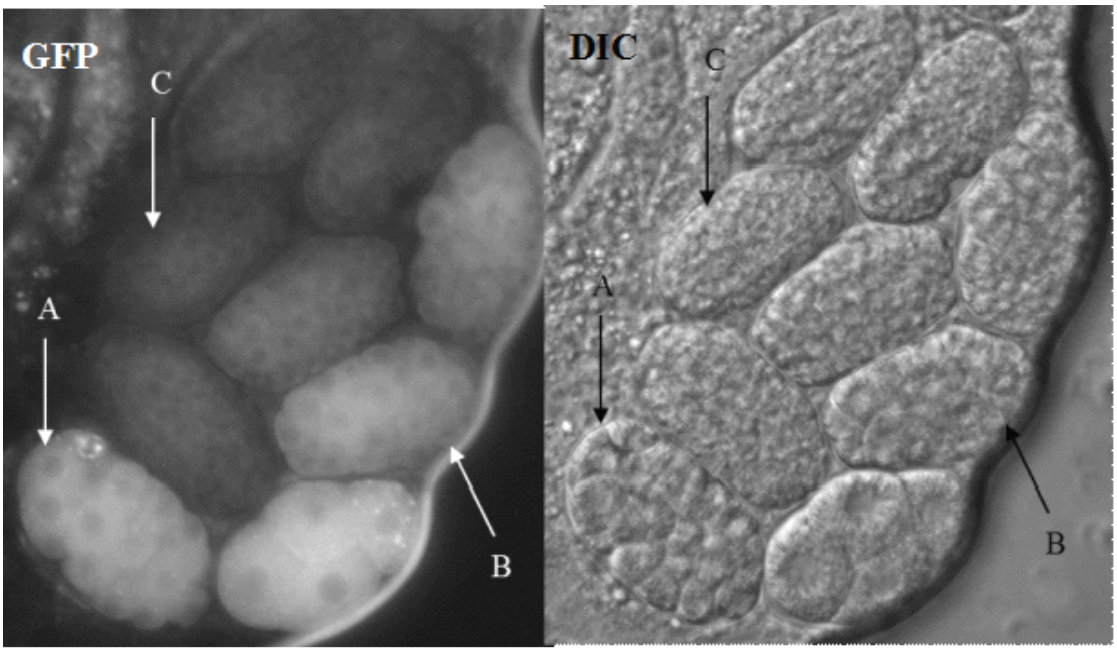

Figure 8. GFP and DIC image of *zif-1* (RNAi) embryos. Injected worms were incubated overnight at 15ºC before samples were prepared. GFP images show GFP::MEX-3 protein localization problems when *zif-1* is knocked out. Embryos at the 16- and 24-cell stage (A and B respectively) showed fluorescence throughout the entire embryo indicating the abnormal presence of MEX-3 protein at these stages. In stages past the 24-cell stage (C), GFP::MEX-3 protein is no longer present throughout the embryo. This suggest that *zif-1* gene may be involved in degrading MEX-3 protein in the early stages of embryo development and is involved in properly localizing that protein

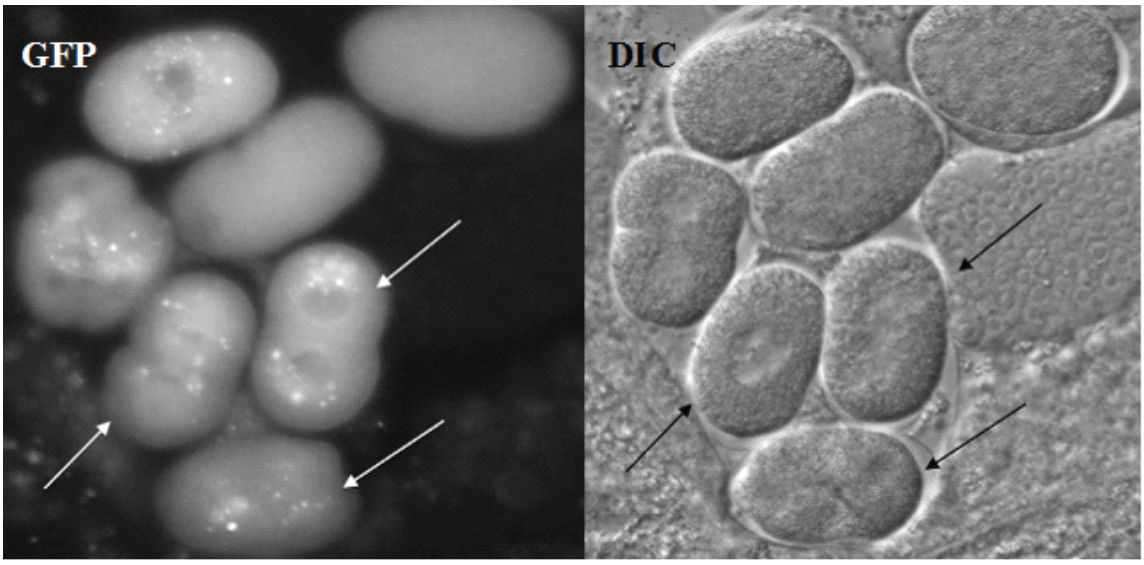

Figure 9. GFP and DIC images of *elc-1* (RNAi) embryos. e*lc-1* (RNAi) worms were incubated over night at 15ºC after injections. GFP images reveal that *elc-1* (RNAi) causes noticeable mislocalization of P-granules as the granules localize to multiple regions of the embryo instead of just the posterior end. This indicates problems with the embryo establishing polarity when *elc-1* is knocked out. MEX-3 protein also shows localization problems indicating that embryonic lethality maybe attributed to the mislocalization of MEX-3 protein, P-granules, and possibly other components.

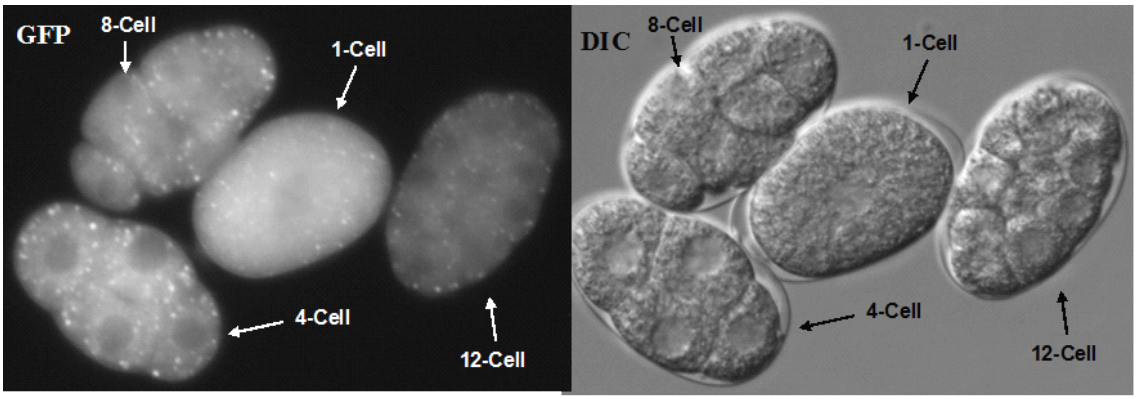

Figure 10. GFP and DIC images of *mex-5* (RNAi) embryos. Injected worms were incubated overnight at 15ºC. Knocking out the *mex-5/6* genes prevents the translation of the MEX-5/6 protein. This means MEX-3 protein is left unprotected and susceptible to rapid degradation. The GFP image shows that after the 4-cell stage, no localization of the MEX-3 protein has occurred as most of the protein seems to have been degraded throughout the entire embryo. P-granules are also mislocalized in the embryos. Without MEX-5/6 protein, localization of MEX-3 protein cannot occur as it is rapidly degraded in early stages of embryo development.

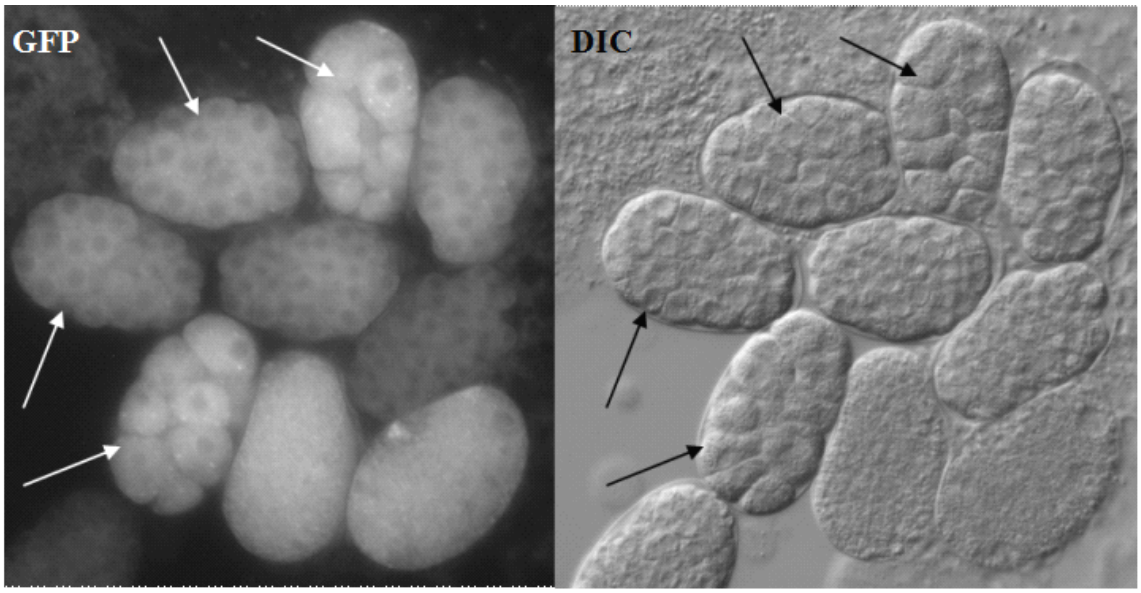

Figure 11. *zif-1* (RNAi); *mex-5* (RNAi) embryos. Worms were injected with a 1 to 1 ratio of *mex-5* dsRNA and *zif-1* dsRNA. Injected worms were then incubated overnight at 15^{o}C. By knocking out *mex-5/6,* the MEX-5/6 protein was not translated and could not protect MEX-3 protein from SPN-4 dependent degradation. This should result in rapid degradation of MEX-3 as seen in the *mex-5 (*RNAi) figure. However, GFP images revealed that *zif-1* (RNAi); *mex-5* (RNAi) embryos show mislocalization of MEX-3 protein. MEX-3 protein is seen throughout the entire embryo in all cell stages up to the 24-cell stage where it then begins to fade. This is characteristic of phenotypes observed with *zif-1* (RNAi) embryos. Such observations suggest that even though MEX-3 is susceptible to rapid degradation due to *mex-5* (RNAi), *zif-1* is still needed to complete the degradation of MEX-3. Arrows indicate some of the late stage embryos that still have mislocalized MEX-3 protein present throughout the entire embryo.

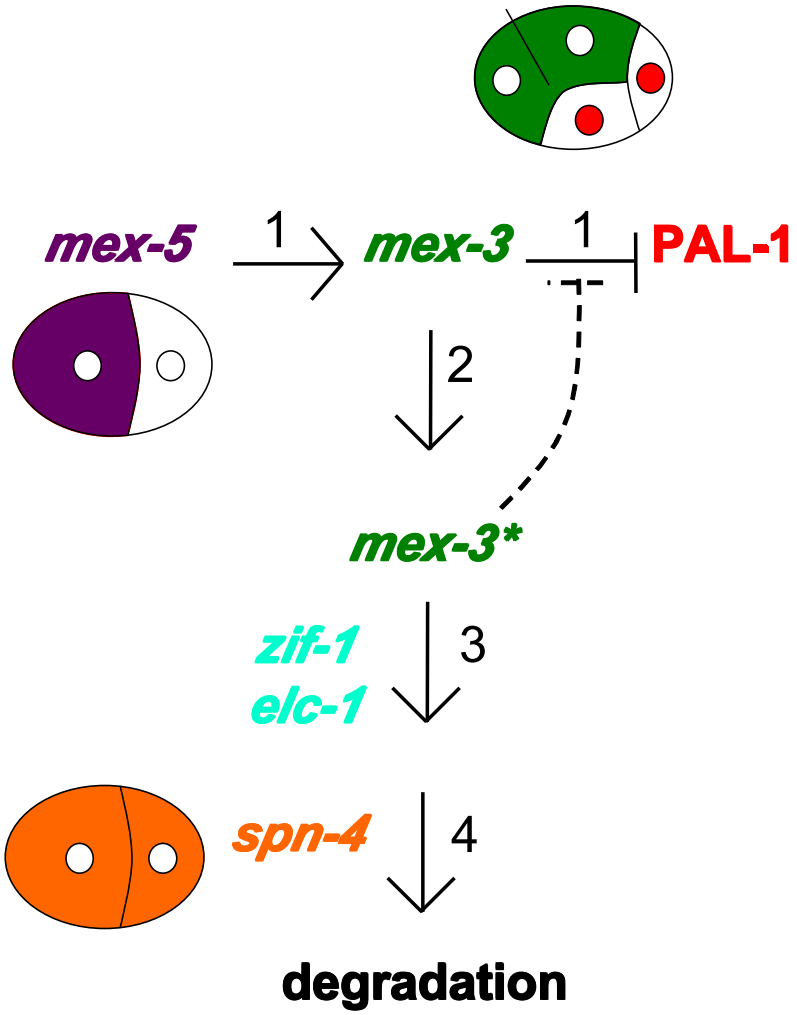

Figure 12. Updated working model of PAL-1 expression

1. *mex-5* and *mex-6* protect MEX-3 from inactivation and degradation in the anterior, enabling the continued repression of PAL-1
2. In the absence of *mex-5* and *mex-6* in the posterior, MEX-3 is inactivated (mex-3*)
3. E3 ubiquitin ligases *zif-1* and *elc-1* mark mex-3* for degradation
4. mex-3* undergoes rapid *spn-4* dependent degradation

Table 1

Hatched embryo and total progeny count, calculated percent lethality, and affect on GFP::MEX-3 localization when knocking out selected genes.

Gene ID	Protein Name or Description	Temperature	Trial 1		Trial 2		
			Hatched Embryos/ Total Progeny	Percent embryo lethality	Hatched Embryos/ Total Progeny	Percent embryo lethality	Was GFP::MEX-3 localization affected?
C17H11.6	Zinc Finger (RING type and C6HC type) motif	24°C	270/280	4.00%	292/299	2.30%	-
		15°C	199/312	36.20%	266/265	0.00%	
C45G7.4	Zinc Finger Motif	24°C	182/180	0%	-	-	-
		15°C	254/249	0%	-	-	
CTel54x.1 (*fbxa-4*)	FBXA-4 protein containing F-box Domain	24°C	238/238	10%	171/164	0%	-
		15°C	222/200	0%	182/175	0%	
F10D7.5a	Zinc finger motif	24°C	155/173	10%	192/199	4%	-
		15°C	207/200	0%	202/189	0%	
F13A7.9 (*skr-11)*	SKR-11 protein	24°C	231/229	0%	-	-	-
		15°C	195/195	0%	-	-	
F26E4.11(*hrdl-1)*	HRDL-1 protein	24°C	246/232	0%	-	-	-
		15°C	252/257	2%	-	-	
F32A6.3 (*vsp41*)	VSP41 protein	24°C	129/144	10.40%	116/117	1%	-
		15°C	190/212	10.40%	237/237	0%	
F40G9.9	F-box domain motif	24°C	68/72	6%	182/189	4%	-
		15°C	17/15	0%	192/189	0%	
F53F8.3	Zinc finger (RING type) motif	24°C	126/140	10%	252/258	2%	-
		15°C	71/76	7%	206/206	0%	
F57C2.1	BTB-20 domain protein	24°C	141/141	0%	185/181	0%	-
		15°C	179/171	0%	202/198	0%	

Table 1 (*continued)*

Gene ID	Protein Name or Description	Temperature	Trial 1		Trial 2		
			Hatched Embryos/ Total Progeny	Percent embryo lethality	Hatched Embryos/ Total Progeny	Percent embryo lethality	Was GFP::MEX-3 localization affected?
F58E6.1	STA-2 transcription factor protein (also involved in apoptosis)	24°C	180/180	0%	-	-	-
		15°C	107/107	0%	-	-	
H31G24.3	BTB/POZ-like motif	24°C	201/220	9%	268/180	7%	-
		15°C	196/192	0%	202/207	2%	
K12B6.8	Protein kinase C-like	24°C	185/185	0%	134/135	0.70%	-
		15°C	128/128	0%	147/147	0%	
K08E4.7	BTB/POZ-like motif	24°C	130/138	6%	107/108	1%	-
		15°C	217/219	1%	109/109	0%	
Y73C8C.8	uncharacterized protein	24°C	195/210	7.10%	-	-	-
		15°C	202/229	11.80%	-	-	
D2089.2	Zinc finger (RING-CH-type) motif	24°C	84/101	17%	36/178	80%	No
		15°C	114/183	38%	7/190	96%	
F46A9.5 (*skr-1)*	SKR-1 protein	24°C	147/163	10%	0/143	100%	No
		15°C	4/102	96.10%	55/162	66%	
F59B2.6 (*zif-1)*	ZIF-1 Zinc finger protein	24°C	46/99	54%	30/110	73%	Yes
		15°C	8/43	81%	30/132	77%	
Y82E9BR.15 (el*c-1)*	ELC-1 protein	24°C	2/218	99%	-	-	Yes
		15°C	0/170	100%	-	-	

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