Analysis of Germline Sepecification in *C. elegans*

"Investigating the role of the PIWI-related protein in *C. elegans* germline development during embryogenesis"

A Major Qualifying Project

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of the
WORCESTER POLYTECHNIC INSTITUTE

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Abstract

During embryo development, cells need to differentiate between germline and somatic cells. Recently, the Mello lab identified an interaction between *prg-1* and *pie-1*, which are important in germline specification and development in the nematode *C. elegans*. This project aimed to confirm the observed results through RNAi injections as well as the of study double mutants to better understand the nature of the interaction. This project tested the hypothesis that PRG-1 physically interacts with PIE-1 by immunoprecipitation (IP), which preliminarily suggests they do not interact. Further studies with the PRG-1 by Western blot analysis found that *prg-1* does not regulate PIE-1 expression levels. Several proteins were also identified that interacted specifically with the PRG-1 protein. Additional experiments are needed to better understand the role PRG-1 and PIE-1 play in functioning together in germline specification, which may have greater implications in understanding epigenetics and stem cell self-renewal.

Acknowledgements

I would like to thank Craig Mello for giving me the opportunity to work in his lab at the University of Massachusetts Medical School and for all of the support throughout my project. I would also like to thank Darryl Conte Jr and Rita Sharma, who truly advised and worked with me on this project and were very patient, understanding, and supportive role models. I would like to thank William Stanney III (WPI class of 2009) for assisting me with the immunoprecipitation and Western blot experiments. I would also like to thank everyone in the Mello lab for making this project a great experience. Last, I would like to thank my major and MQP advisor, Destin Heilman, for his guidance and support in completing my WPI experience and for always believing in me.

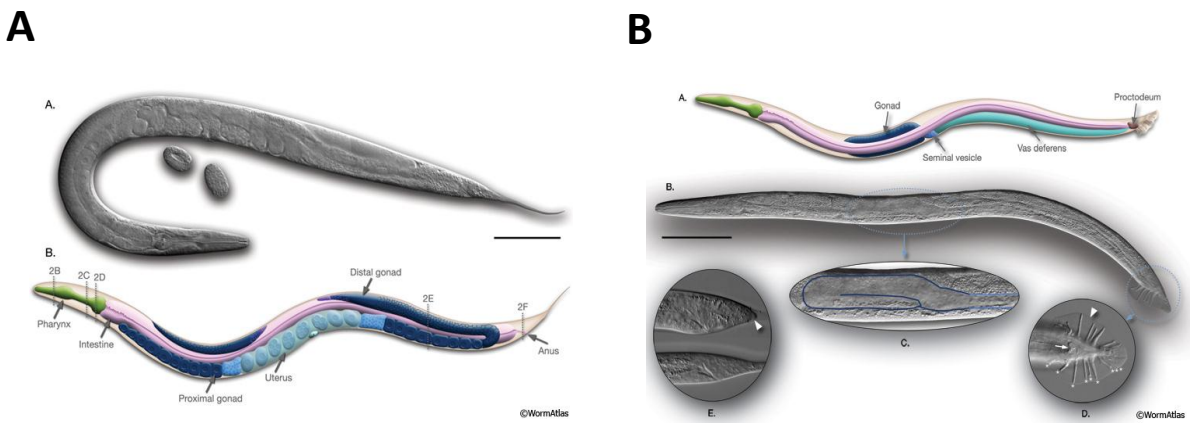
Introduction

This MQP coincides with the work performed by Craig Mello and his colleagues at the University of Massachusetts Medical School on the nematode *Caenorhabditis elegans.* During embryo development, there is a need for cells to eventually differentiate between germline and somatic cells. Germline cells retain the genetic information of the cells, while somatic cells lose their differentiating abilities and remain as a specified cell type. It is then important to understand the controlling mechanism behind cellular differentiation and the factors that are important in regulating the developmental process. This MQP focuses on two genes, *pie-1* and *prg-1*, and their subsequent proteins which are both important in germline maintenance and factors in cellular differentiation. While the function of the PRG-1 protein is not fully understood, recent unpublished data from the Mello lab indicates that there may be a genetic interaction between the two proteins, which may help to better explain the role of *prg-1* in germline maintenance and its potential biochemical mechanism of action in development.

Caenorhabditis elegans as a model for genetic study

Caenorhabditis elegans is a species of soil nematode, whose genome was first sequenced in 1998. *C. elegans* is a good model for studying a broad range of topics, from genetics to development to evolution. Sydney Brenner pioneered the use of *C. elegans* as a genetic model with the goal of understanding the development and structure of the nervous system (Brenner, 1974). *C. elegans* has been, and continues to be, an excellent model due to many factors, including its short lifecycle (approximately 3-4 days at optimal temperature) and high fecundity. They are very easy to grow in large populations and are relatively cheap to maintain, compared to other eukaryotic model systems. *C. elegans* have two possible sexes, hermaphrodite and male, with hermaphrodites dominating the overall population. With only 1000 cells in a wild-type adult, the transparency and relatively simple anatomy of *C. elegans* allows them to be studied in great detail through all stages of development as detailed in the

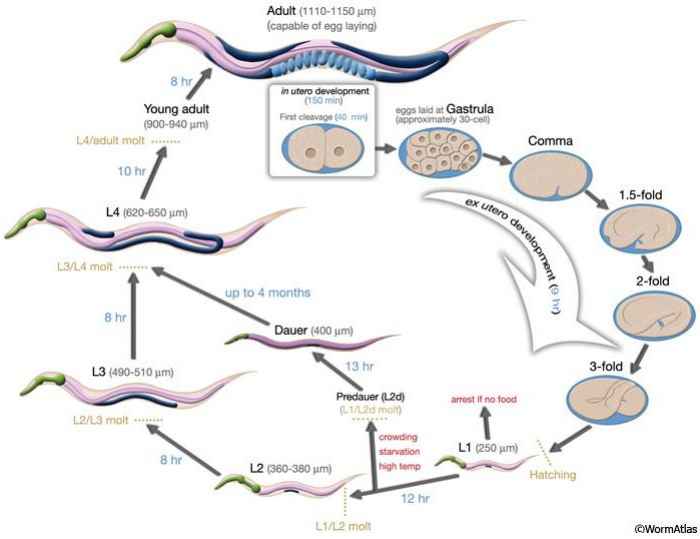
figure below.



Adapted from WormAtlas (Altun & Hall, 2009)

The Anatomy of *C. elegans*: The *C. elegans* anatomy for A) hermaphrodite and B) male worms

The life cycle of *C. elegans* hermaphrodites processes through the embryonic stage, four larval stages, and adulthood as seen in the figure below (Altun & Hall, 2009). The duration of the *C. elegans* life cycle (from a fertilized 1-cell embryo to a fertile adult hermaphrodite) depends upon factors such as temperature, availability of food, and environmental conditions, but ranges typically from 3 to 7 days (Altun & Hall, 2009). Many aspects of the *C. elegans* life cycle can be optimized for study, such as allowing more time to study embryo development by decreasing the temperature. One of the most important aspects of development is embryogenesis, when a single pluripotent cell begins to divide and cells differentiate into the tissues that make up a worm.

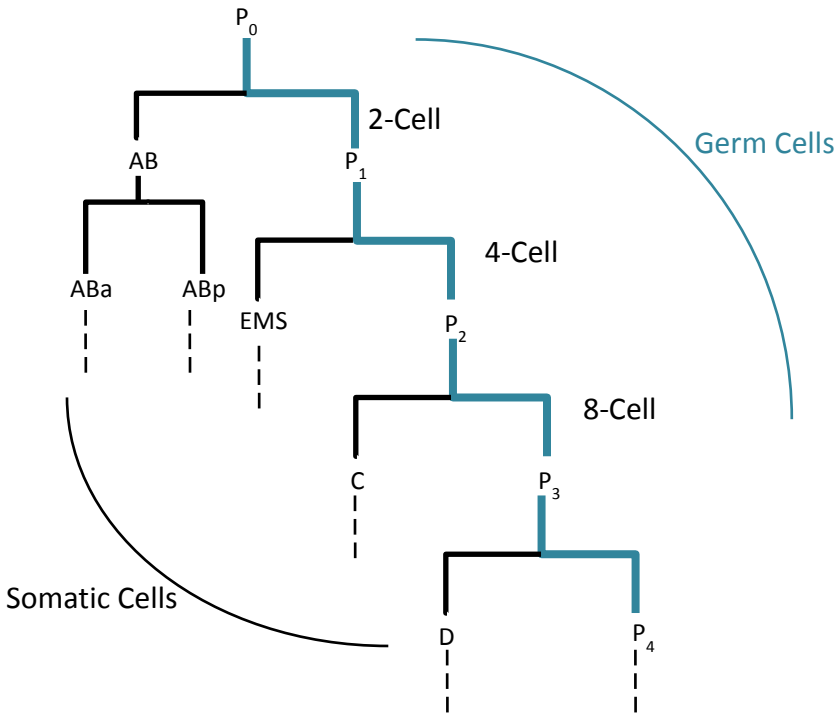


Adapted from WormAtlas (Altun & Hall, 2009)

***C. elegans* Hermaphrodite Life Cycle at 22°C:** The life cycle of the *C. elegans* hermaphrodite from the stages of embryogenesis, larvae (L1-L4), and adulthood and approximate time points at 22°C.

Embryogenesis and Cellular Differentiation

Embryogenesis is the beginning stage of development and involves many important factors to move through the stage before the embryo hatches. An important part of embryogenesis is the pluripotency of germline cells, which allows for germ cells, or the reproductive cells, to remain undifferentiated. Once a cell differentiates and becomes a somatic cell, the fate of that cell is forever determined. Germ-line cells however, maintain that ability to keep dividing and thus preserving the organism's genetic material to the next generation. Embryogenesis in *C. elegans* creates a sequence of unequal divisions for the germ cells (Mello et al, 1996). Following each cell division, one daughter cell will produce only somatic (differentiated) cells while the other daughter will produce both germ cells and somatic cells as described in the figure below.

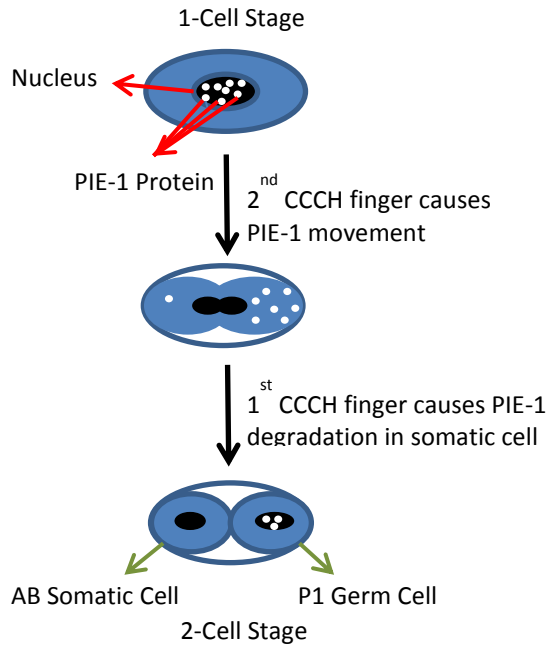


Embryogenesis Cell Fate: *C. elegans* embryo divisions occur through unequal divisions of the germ line blastomeres (P) and somatic cells from the 1 cell to 8 cell embryo stage. The germline is denoted by the bold blue trace and the somatic cells are denoted by the black trace.

Cellular differentiation is the process by which a cell becomes specialized, or somatic, from a pluripotent germ cell. One important question to ask is what factors cause a cell to differentiate. There must be some mechanism or series of events that cause a cell to differentiate itself from the germline. While some components of cellular differentiation are understood, many are not and still leave much to be studied in the field of developmental biology. Specifically, the factors that preserve the germline are of key importance with relation to many other fields of biology. Understanding the factors that preserve an organisms genetic material will help to further understand the developmental process as well as processes that involve immortal cell development and regeneration. In *C. elegans*, the most important factor in germ line specification is the PIE-1 protein, which preserves the pluripotent nature of the germ cell lineage (Mello et al, 1996).

PIE-1 and its role in germline specification

The maternal *pie-1* gene encodes for PIE-1 protein, which is a major factor in germline development. The PIE-1 protein is a conserved zinc-finger (CCCH) protein and localizes to the germline blastomeres both in the nucleus and in the cytoplasmic P-granules (Mello et al, 1996). During cell division, PIE-1 protein is enriched on the posterior side of what will become the new germ cell as detailed in the figure below. This enrichment is dependent upon the 2nd CCCH finger in the PIE-1 protein (Reese et al, 2000). After a germ cell divides into two daughter cells, the level of PIE-1 protein becomes undetectable in the somatic daughter cell due to degradation mediated by the 1st CCCH finger in the PIE-1 protein (Reese et al , 2000).



PIE-1 Localization in Dividing Embryos: The PIE-1 protein is most abundant in the nucleus of the P-cell. During cell division, PIE-1 migrates to the cytoplasm of the daughter cell destined to become the next germline cell. Any remaining PIE-1 protein in the somatic daughter cell is degraded.

The mechanism of action for preserving the pluripotency of the germ lineage is suggested of an inhibitory mechanism that prevents the somatic cell fate. Germline cells differ from somatic cells by

three aspects of RNA metabolism: 1) the protection of maternal RNA from somatic cell degradation 2) the presence of P-granules and 3) the lack of embryonically transcribed RNA. When *pie-1* activity is lost, an increase in RNA transcription is observed in the germ cell, which adopts somatic cell fate (Seydoux et al, 1996). This suggests that PIE-1 protein represses transcription of new mRNA in germ cells, which functions to maintain the pluripotency of the germ lineage (Seydoux et al , 1996).

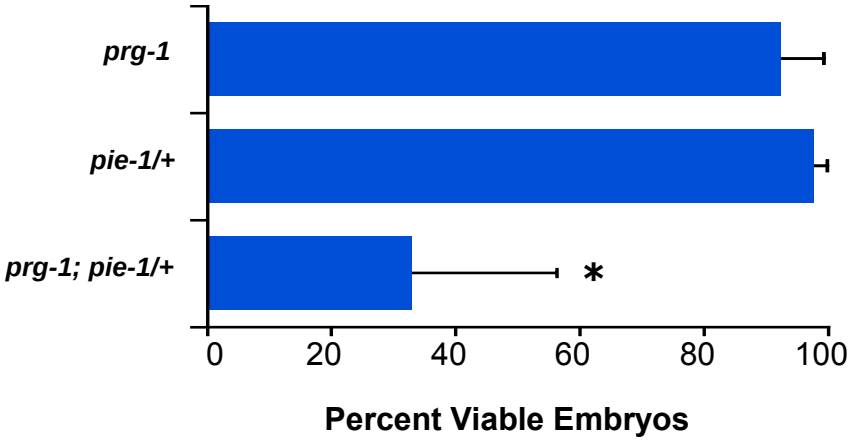
PRG-1 and its Role in Germline Development

prg-1 is a Piwi-related gene that encodes PRG-1, an Argonaute protein related to Piwi proteins required for germline maintenance and fertility (Klattenhoff & Therukauf, 2008). Co-immunoprecipitation studies have shown that PRG-1 and 21U-RNAs, the Piwi-interacting RNAs (piRNAs) of *C. elegans*, form an RNA-protein complex that is required for proper germline development (Batista et al, 2008), as *prg-1* mutants exhibit decreased fertility at elevated temperatures (Batista et al, 2008).

PRG-1 also plays a role in gene silencing, which is likely to be related to its role in germline development. In most animals, Piwi proteins interact with piRNAs to silence transposons (Partha et al, 2008). Transposons are genetic elements that can move from one location in the genome to another, which depending upon the location of insertion may give rise to mutations. Cells employ various methods for silencing transposons, which differ based on the transposon. Studies in *C. elegans* have found that *prg-1* is necessary for silencing the Tc3 transposon, an abundant DNA transposon in *C. elegans* (Partha et al, 2008). Recent studies of *prg-1* found that *prg-1* mutants have increased Tc3 transposase mRNA, but not Tc1 (Partha et al, 2008). This suggests that the RNA-protein complex formed by PRG-1 protein and 21U-RNAs is a specific and powerful suppressor of the Tc3 transposition in the germline of *C. elegans* (Partha et al, 2008).

prg-1 and *pie-1* function together in germline maintenance

The Mello lab is very interested in understanding how PRG-1 regulates germline development. It was hypothesized that since *prg-1* mutants do not eliminate fertility completely, *prg-1* may regulate or function in parallel to other factors important in germline development and fertility (Gordon & Mello, Unpublished Poster). A genome-wide RNAi feeding screen was conducted to knock down the expression of each of the 20,000 genes in *C. elegans* in wildtype, *prg-1(tm872*), and *prg-1(n4357)* worms (Gordon & Mello, Unpublished Poster). The *tm872* and *n4357* alleles are deletion alleles that severely truncate and inactivate PRG-1. From the screen, 22 genes were identified that when silenced by RNAi produced an embryonic lethal phenotype in both *prg-1* mutants, but not in the wildtype worms (Gordon & Mello, Unpublished Poster). A particularly interesting synthetic lethal interactor was the *pie-1* gene, the major germ line regulator. The genetic double also exhibited a synthetic lethal phenotype. A *prg-1; pie-1/+* strain, where the *prg-1* mutation is homozygous and the *pie-1* mutation is heterozygous, exhibits ~70% embryonic lethality, with 1/3 of the dead embryos exhibiting the *pie-1* phenotype as described in the figure below (Gordon & Mello, Unpublished Poster). P-granules were often mislocalized in the *prg-1; pie-1/+* embryos, but localized normally in *pie-1/+* embryos (Gordon & Mello, Unpublished Poster). These phenotypic observations, together with the fact that both PRG-1 and PIE-1 proteins localize to the germ cell in the early embryo, suggest that *prg-1* and *pie-1* function together in germline maintenance (Gordon & Mello, Unpublished Poster).



***prg-1* Causes Embryonic Lethality in *pie-1* Heterozygote:** The prg-1;pie-1/+ double mutant produces a statistically significant decrease in percent viable embryos compared to the single mutations of each gene.

With a genetic interaction observed between *prg-1* and *pie-1*, it is the aim of this project to further investigate that interaction on both the genetic and post-translational level. The experiments in this project revolved around three main questions to further understand the nature of the *prg-1* and *pie-1* interaction. First, a potential method of *prg-1* interaction with *pie-1* could be in the form of regulation, so it is important to consider if the activity of *prg-1* is necessary to regulate the expression of the PIE-1 protein. Another potential method of interaction is on the post-translational level, so studying the interaction between the PRG-1 and PIE-1 proteins is a logical approach to explaining their roles *in vivo*. Finally, few studies have been done to investigate the interaction of PRG-1 with other regulatory proteins, which may also provide insight to the potential role of PRG-1 in germline development. Understanding the genetic interaction between *prg-1* and *pie-1* at a molecular level will help to explain the role of PRG-1 in germ line development, as well as have broader implications in the study of development.

The beginning experiments described here aim to confirm the results of the RNAi feeding screen though the method of injection, where *pie-1* dsRNA was injected into both wildtype and *prg-1(tm872)* worms. A decrease in viable embryos was observed for both *prg-1* mutant and WT, but the decrease was not

statistically significant between the two strains. To test whether the embryonic lethal phenotype of the *prg-1(tm872); pie-1/+* strain was allele-specific, crosses were designed and set up to generate a *prg-1(n4357);pie-1/+* double mutant. The latter experiments in this project focused on the expression nad interaction of PRG-1 and PIE-1 proteins in the early embryo. Early embryos were harvested from both wildtype and *prg-1(tm872)* strains and extracts were subject to a PRG-1 immunoprecipitation (IP). Western blot for PIE-1 protein to detect the interaction of PRG-1 and PIE-1 preliminarily suggests that the proteins do not physically interact. The same Western blot also served to confirm that *prg-1* does not regulate the expression of the PIE-1 protein. Finally, the PRG-1 IP samples were run on a gel and proteins were silver-stained to identify proteins that interact with PRG-1 in the early embryo. Three bands of interest were isolated from the gel and sent off for peptide identification by mass spectrometry analysis.

Materials and Methods

PCR amplification of in vitro transcription templates

The concentration of plasmid DNA was determined using a nanodrop spectrophotometer. Amplifications were done in 50 µL reactions containing 1 µL of 25 ng/µLdsDNA template, 5 µL of 10x Ex Taq buffer, 4 µL of 2.5mM each dNTPs, 2.5 µL of each 10 µM primers CMo10901 and CMo10902, 0.25 µL of 5 U/µLTaKaRa Ex Taq polymerase, and 34.75 µL of deionized water. Samples were amplified by the process: 98^{o}C for 10 seconds, 55^{o}C for 30 seconds, 72^{o}C for 2 minutes, and then repeated for 30 cycles. Following amplification, 5 µL of each sample was diluted with 1 µL of 6x sample loading dye containing bromophenol blue as a tracking dye. Sample were then ran on a 2% agarose gel with a 100 bp and 1 kb ladder at 100V until the tracking dye had migrated two thirds to three fourths of the length of the gel. The concentrations of the amplified products were determined using a nanodrop spectrophotometer.

In vitro transcription of dsRNA

In vitro transcription reactions were performed using the MEGAscript T7 high yield transcription kit (catalog number AM1333). Reactions occurred in 20 µL reactions containing 5µL of dsDNA template, 8 µL of dNTP mix (consisting of 10 µM of each dNTP), 2 µL of T7 enzyme mixture, 2 µL of 10x reaction buffer, and 3 µL of deionized water. Transcription reactions were incubated for 1 hour at 37^{o}C then overnight at 4^{o}C. The DNA template was removed by adding 1 µL of DNase and incubated at 37^{o}C for 15 minutes. The reaction was stopped by adding 70 µL of dionized water and 10 µL of ammonium acetate stop solution. The RNA was extracted by mixing with 100 µL of Ambion 5:1 acid phenol:$CHCl_3$, pH 4.5 in phase separation columns. The tubes spun for 5 minutes at 13000 x g to separate the aqueous phase containing the RNA from the organic phase containing proteins. The aqueous phase was transferred to sterile microcentrifuge tubes and RNA was precipitated with 100 µL of 100% isopropanol at -20^{o}C for 15 minutes. The precipitated RNA was pelleted at 13000 x g for 15 minutes at 4^{o}C and the supernatant was discarded. The RNA pellet was washed with 500 µL of 75% ethanol and spun at 13000 x g for 5 minutes. The excess ethanol was carefully removed and the pellet was allowed to air dry. The pellet was resuspended in 25 µL of 10 mM Tris buffer (pH 8.0). The resulting dsRNA sample was diluted 1:100 in 10 mM Tris buffer (pH 8.0) then the concentration was determined using a nanodrop spectrophotometer. The dsRNA sample was analyzed by electrophoresis in 1% agarose gel (2 µL of dsRNA sample, 1 µL of 6x sample loading dye, and 3 µL of Tris buffer pH 8.0) along with 100 bp and 1 kb ladders.

RNAi by injection

Injection procedure

prg-1(tm872) and N2 hermaphrodites were used for all injections. 20-30 hermaphrodites at the L4 stage were identified 24 hours prior to injection and picked to a single plate seeded with OP50 E. coli. The worms were kept at 15^{o}C overnight and expected to be at the early adult stage the following day. An etched needle was filled with 1 µL of the *pie-1* dsRNA sample and placed into the needle slot of the

instrument and connected to an airflow tube. Agarose injection pads were prepared by placing two drops of 2% agarose gel onto glass slides. Each slide was then covered with another glass slide to spread the gel into a thin layer. After cooling, the top slide was removed and the gel pads allowed to dry for 24 hours at 37°C. The young adult hermaphrodites were picked from the non-food portion of the plate using oil, and then transferred to the dried agarose pad. The oil served as a lubricant to keep the worms from drying out on the agarose pads which allowed the worms to stay in place during the injection. For each strain 10-20 worms were placed onto the agarose pad then injected with the dsRNA. The force of the injection was tested into the oil to ensure that the force would not be too great to rupture the worms. Optimal injections into the worm occurred in the intestines; however injection of the dsRNA could occur anywhere in the body that did not kill the worm. Once all worms on the pad were injected, the worms were recovered from the oil using sterile 1x M9 buffer. The worms were then transferred to a plate seeded with OP50 E. coli and allowed to recover for 3-5 hours at 20°C.

Determination of ideal dsRNA concentration

To determine the ideal concentration for dsRNA injections into *prg-1(tm872)* and N2 worms, *pie-1* dsRNA was diluted to 1 mg/mL, 100 ng/mL, 10 ng/mL, and 1 ng/mL. Both N2 and *prg-1(tm872)* worms were injected with the highest concentration and the lowest concentration to start and were observed for 48 hours following the injection. The concentrations were then adjusted to visually produce the embryonic lethal phenotype in the *prg-1(tm872)* mutant, but not the N2 worms.

pie-1 (RNAi) brood analysis

Once the ideal concentration was determined, N2 and *prg-1(tm872)* hermaphrodites were injected with the ideal concentration of pie-1 dsRNA following the procedure described previously. The recovered worms, along with the same number of control N2 and *prg-1(tm872)* worms were singled to OP50 plates and kept at 20°C. Each plate was labeled with a number to represent the worm (eg. 1,2,3,4, etc.) and a

letter to represent the plate (eg. A, B, C, D, etc.). The injected worms were transferred to new plates every 12 hours and all progeny (embryos plus hatched larvae) were counted immediately following the transfer. All plates were kept at 20°C for another 24 hours after which the number unhatched embryos were determined. The procedure continued until the injected worm stopped producing embryos, approximately 4-5 days. The number of total progeny produced by an injected worm is the sum of hatched larvae and embryos on each plate. The total unhatched is the sum of unhatched embryos on each plate. The percent viability for each worm was determined by the following formula:

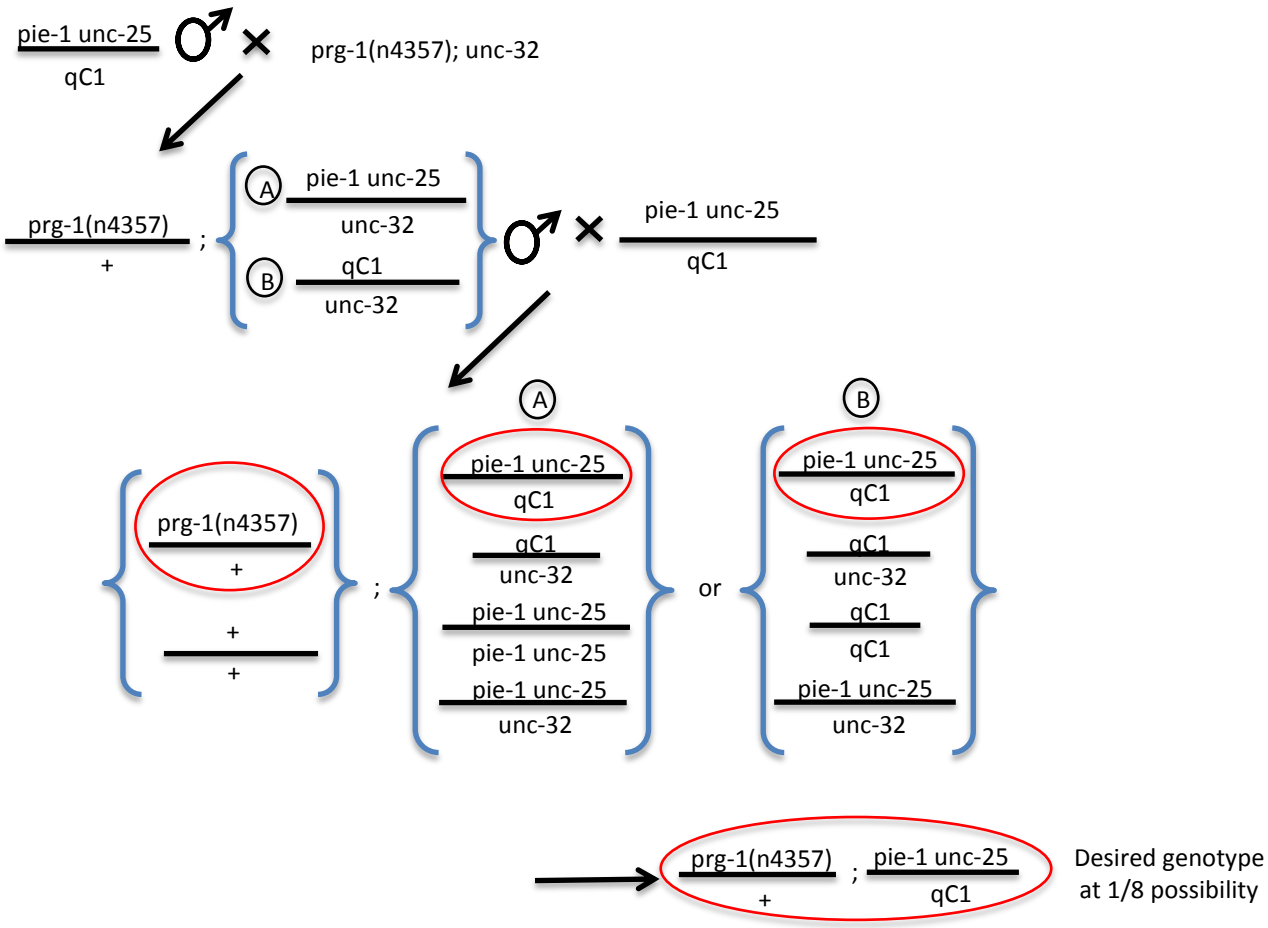
% viable = (total progeny – total unhatched embryos)/total progeny

Determination of the allele specificity of the genetic interaction between *prg-1* and *pie-1*

prg-1(n4357); pie-1 unc-25/qC1 double mutant

The genetic cross map below was used to generate the desired genotype of *prg-1(n4357); pie-1 unc-25/qC1*. A443 (prg-1 unc-25/qC1) males were mated with SX922 (prg-1(n4357); unc-32) hermaphrodites. The first generation cross progeny males were single male mated back to A443 (prg-1 unc-25/qC1) hermaphrodites. The second generation cross progeny were singled to 40 plates and observed for the appropriate pie-1 phenotypic segregation of Unc worms, Dpy worms, and sterile worms. The candidates identified with the *pie-1 unc-25/qC1* were then screened for the prg-1(n4375) mutation by PCR. From the plates identified as candidates, 10 worms were picked from each plate and lysed in 20 µL worm lysis buffer (1 mL lysis buffer and 12 µL proteinase K). The lysis reaction took place at 37°C in the PCR machine. The PCR reaction mixture was set up in 25 µL reactions containing 1 µL plate template DNA , 2.5 µL of 10x Ex Taq buffer, 2 µL of dNTPs mix (2.5mM each), 1 µL of each 10 µM primers CMo16467 and CMo16469, 0.25 µL of 5 U/µLTaKaRa Ex Taq polymerase, and 17.25 µL of deionized water. Samples were amplified by the process: 98°C for 30 seconds, 98°C for 10 seconds, 58°C for 30 seconds, 72°C for

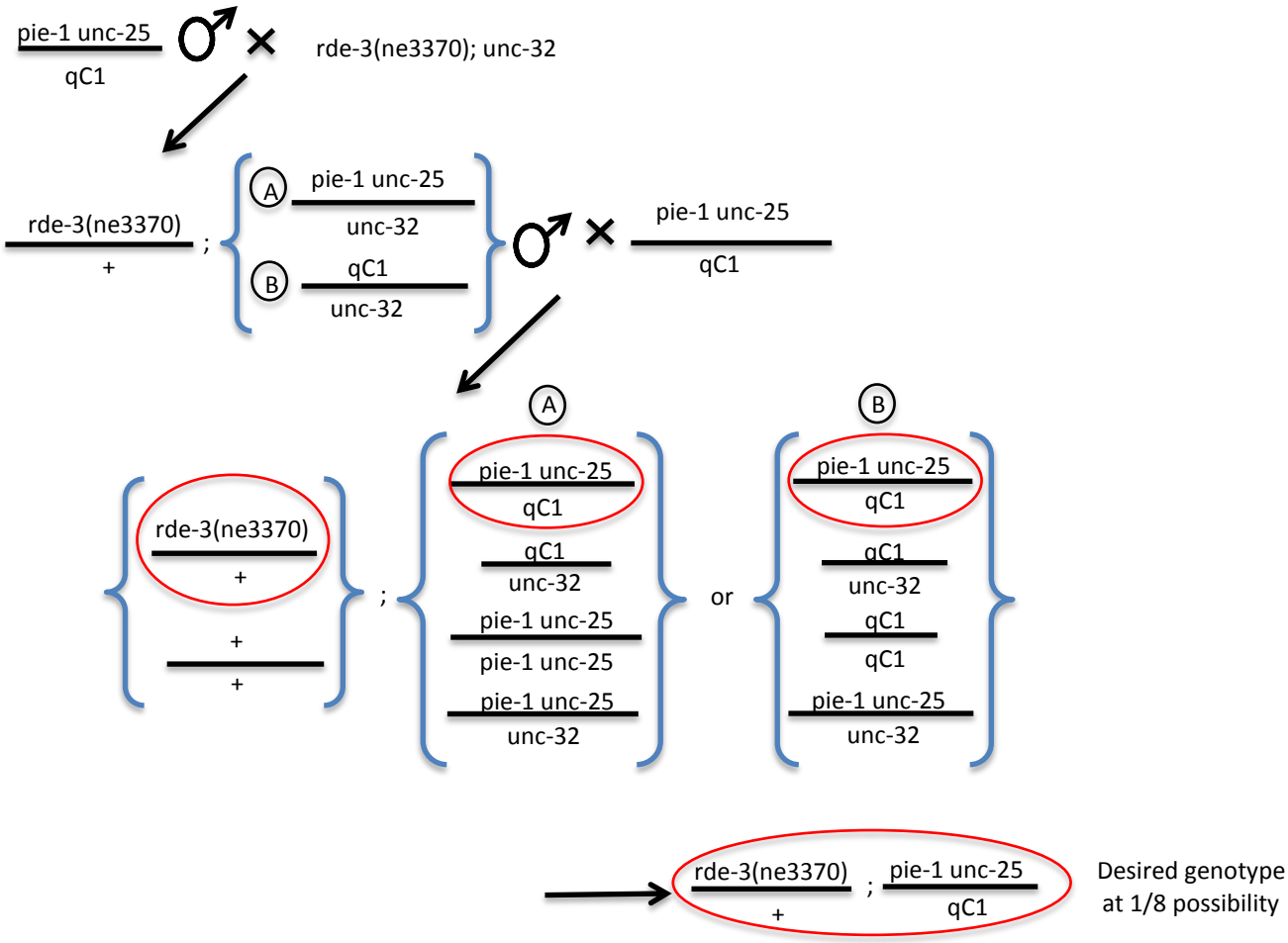
30 seconds, repeated for 34 cycles, 72°C for 5 minutes, then 4°C for the reaction completion. Following amplification, 5 µL of each sample was diluted with 1 µL of 6x sample loading dye containing bromophenol blue as a tracking dye. Sample were then ran on a 2% agarose gel with a 100 bp and 1 kb ladder at 100V until the tracking dye had migrated two thirds to three fourths of the length of the gel.



***prg-1(n4357); pie-1/+* Cross Map:** Cross map designed to execute the prg-1(n4357);pie-1/+ double mutant for observation of embryonic phenotypes. The desired genotype is indicated by the red circles, which can be achieved by back crossing either the A or B males to the original pie-1/+ strain.

rde-3(ne3370); pie-1 unc-25/qC1 double mutant

The cross for *rde-3(n3370); pie-1 unc-25/qC1* was designed and detailed below. The cross was not executed due to timing.



***rde-3(ne3370); pie-1/+* Cross Map:** Cross map designed to execute the rde-3(ne3370);pie-1/+ double mutant for observation of embryonic phenotypes. The desired genotype is indicated by the red circles, which can be achieved by back crossing either the A or B males to the original pie-1/+ strain.

C. elegans early embryo isolation for co-immunoprecipitation (IP) of PRG-1 and PIE-1 proteins

Concentrated OP50 E. coli for large population growth

Terrific broth (TB) growth media was made in 1 L flasks by combining 12 g of bactero-tryptone, 24 g of yeast extract, 8 mL of 50% glycerol, and 900 mL of deionized water. The flasks were autoclaved then inoculated with 10 mL of OP50 E. coli (seeded in liquid broth (LB) shaking for 24 hours at 37°C) and 100 mL of TB buffer. The inoculated flasks were kept shaking at 37°C for 24 hours, then moved to 4°C for an additional 24 hours to settle the bacteria. The TB was aspirated off to leave only the OP50 E. coli at the

bottom of the flasks. The remaining bacteria was transferred to 50 mL conical tubes and washed with 50 mL of 1x M9 buffer then centrifuged at 3000 x g for 10 minutes at 4°C. The pellets (approximately 5 mL per 50 mL tube) were washed 3 more times following the same directions. After the final wash, the 5 mL OP50 E. coli pellet was resuspended in 20 mL of 1x M9 buffer and kept at 4°C.

Large, synchronous population growth of *prg-1(tm872)* and N2 worms

Large populations of both *prg-1(tm872)* and N2 worms were started by seeding 15 cm 50/50 agarose plates with 2 mL of concentrated OP50 and allowed to dry. Once dry, starved *prg-1(tm872)* or N2 plates were chunked to the large plates and the worms grew until adult populations were synchronous. The amount of time it took for the worms to reach the adult stage with embryos depended upon what stage they were plated at. Once a majority of the hermaphrodites reached the adult stage with embryos, the plates were bleached and worms were harvested (note that all centrifugations done at 800 x g for 20 seconds at room temperature). Each plate was washed with 15 mL of 1x M9 buffer and the wash was transferred to a 15 mL conical tube. The tubes were centrifuged as described above. The supernatant was discarded and the worm pellet washed and centrifuged with 15 mL of 1x M9 buffer. The supernatant was discarded and 15 mL of the bleaching solution was added (10 mL 5M KOH, 20 mL 6x hypochlorite bleach, 170 mL deionized water). The worms were bleached while the tubes rocked on a platform for 10 minutes at room temperature. The bleaching was considered done when the solution observed under a microscope contained almost all embryos and no worms. If worms were still intact after 10 minutes of bleaching, the worms were centrifuged and fresh bleaching solution added until only embryos remained. The embryos were centrifuged and the pellets washed three times in 15 mL of 1x M9 buffer. The final pellet was resuspended in 5 mL of 1x M9 buffer and placed on a rocking platform for 24 hours at room temperature to allow the embryos to hatch. Following the incubation, a 1 µL sample was counted for the number of larvae and the total number of worms per 5 mL was determined. The desired amount of worms to plate ranged from 100,000 – 150,000 worms per plate, so the amount

of worm solution to plate was determined based on the concentration determined by counting. The 50/50 15 cm agarose plates were seeded with 2 mL of concentrated OP50 E. coli and allowed to air dry. The worms were plated on the food between 100,000 – 150,000 worms per plate and allowed to air dry. The plates were kept at 20°C and the worms grew until the synchronous population reached the appropriate stage depending upon if further amplification was needed or if early embryos were to be harvested.

Early embryo harvest

Large, synchronous populations were carefully monitored once worms molted into young adults. As soon as embryos were observed, worms were harvested and bleached as described above with a few exceptions. First, all buffers were kept on ice to keep the embryos from further developing. After 3 washes with cold 1x M9 buffer, the early embryos were resuspended in the appropriate amount of water and the number of embryos in a 1 µL samples was determined. The final early embryo pellet was snap-frozen by placing the tubes in a dry ice/ethanol bath and stored at -80°C.

PRG-1 Immunoprecipitation (IP)

Prior to the day of the IP, three aliquots of IP buffer were prepared by combining 1.1 mL Potassium Acetate (KOAc), 200 µL 1M HEPES-Potassium Hydroxide (KOH) pH 7.3, 50 µL Triton X, 10 µL 1M Magnesium Acetate (MgAc), 10 µL TWEEN 20 and adding deionized H_2O to a final volume of 10 mL. One aliquot of the last wash buffer was also prepared by combining 500 µL 1M TrisCl pH 7.5, 300 µL 5M NaCl, 20 µL 1M Magnesium Chloride (MgCl), and adding deionized H_2O to a final volume of 10 mL. Twelve eppendorf tubes were labeled per experimental group (*prg-1* and N2 for 24 total tubes) and all above components were placed in a 4°C walk-in fridge overnight.

Lysate preparation

A Sorvall RC 5B Plus centrifuge (SS-34 rotor) was precooled to 4°C. The entire IP procedure was performed in the 4°C walk-in fridge, unless otherwise noted. At room temperature, a stainless-steel, dounce homogenizer was cleaned with deionized H_2O and hand soap by douncing up and down a few times to clean the chamber, dried, then placed in a bucket of ice to chill. Two tubes of the working IP buffer were prepared by adding directly to the previously prepared aliquot: 10 µL 1M DTT and 2 crushed tablets of protease cocktail, which was then slowly inverted to mix and dissolve the protease tablets. Once dissolved, 100 µL P solution was added the procedure replicated for the second tube of IP buffer. The tubes were placed in a separate ice bucket than the douncer and all materials were transferred to walk-in cooler. The frozen N2 early embryos stored at -80°C were dislodged from the bottom of the tubes by gently tapping and transferred to the dounce. One volume of working IP buffer (1.5 mL) was added to the douncer and the pellet was pulverized by pushing the pestle into the douncer at least 200 times on ice. Once done, the N2 extract was transferred to a pre-chilled eppendorf tube labeled for N2. The douncer was then cleaned by adding 500 µL working IP buffer and repeating the crushing motion several times, which was then discarded. The douncing was repeated for the *prg-1(tm872)* early embryos as well following the same procedure. The extracts were spun at 30,000 x g for 15 minutes at 4°C with the break disengaged. While the extracts spun, 300 µL of Invitrogen Protein G Dynabeads were added to a pre-labeled eppendorf tube for each strain and rinsed on the rotation rack with 700µL of working IP buffer. The embryo extracts were removed from the centrifuge and the supernatants transferred to new eppendorf tubes by pipetting 100 µL at a time, using a new pipet tip each time to avoid transferring the white froth at the top of the extract. Extracted lysates were spun at 30,000 x g for 15 minutes at 4°C with the break disengaged. The tubes containing the protein G beads were placed on a magnetic rack to collect the beads. The wash solution was aspirated and replaced with 750µL of fresh working IP buffer and bead tubes were returned to the rotating rack. Once the second centrifugation

was done, the early embryo supernatants were transferred to fresh tubes.

Determination of protein concentration

The protein concentration was determined for both the N2 and *prg-1(tm872)* purified extracts by preparing a Bovine Serum Albumin (BSA) protein standard from a 10 mg/mL stock. The dilutions were 1.40,1.05, 0.70, 0.35, and 0.14 mg/mL. To each glass cuvette, 25 µL of standard sample was added. A blank was prepared by adding 25 µL of water to a cuvette. The worm extracts were diluted by adding 1 µL of worm extract to 24 µL of deionized water in a cuvette. Once all samples were prepared, working Lowry Component A was prepared by combining 1.1 mL Bio-Rad DC Protein Assay Reagent A (catalog number 500-0113) and 22 µL of Bio-Rad DC Protein Assay Reagent S (catalog number 500-0115). To each cuvette, 25 µL of working Component A and 1 mL of Bio-Rad DC Protein Assay Reagent B (catalog number 500-0114) were added and mixed by pipetting several times. The cuvettes were incubated for 15 minutes at room temperature and an Eppendorf BioPhotometer was programed with the appropriate parameters. After incubation, the blank cuvette was read, followed by the BSA standards in decreasing order, and then the extract samples to determine their protein concentration. In order to proceed to the IP, the concentration of the extracts needed to be equal so they could be comparable in the end. The concentrations of the extracts were then adjusted with working IP buffer to be equal. A 50 µL fraction of each lysate was denatured by adding 20 µL NuPAGE buffer, 2.5 µL DTT, and 7.5 µL dH_2O and boiling for 3 minutes. These input samples were stored at -80^{o}C until the western blots were performed.

Immunoprecipitation of PRG-1

To begin the immunoprecipitation, the tubes with the Protein G beads were placed into a magnetic rack and the working IP buffer removed. The lysate solutions were pre-cleared with these beads to remove any non-specific binding. The Protein G beads were loaded with lysate and placed on a turning rack for 1 hour. During the pre-clear incubation, 50 µL of Protein G Dynabeads were added to new tubes and

rinsed following the same procedure as above. Following the hour incubation, the pre-cleared lysate extact was removed from the original beads using a magnetic rack and transferred to new eppendorf tubes. To the pre-cleared lysates, 11 µL of anti-PRG-1 antibody was added to each tube and incubated for 1 hour on a rotating rack. Following the incubation with anti-PRG-1 antibody, the lysates were transferred to the fresh Protein G Dynabeads (50 µL) and incubated for 1 hour on a turning rack. Following the last incubation with the beads, the lysate supernatants were transferred to new eppendorf tubes. The beads now contained the proteins bound to the anti-PRG-1 antibody. The beads were washed three times with 800 µL working IP buffer for 15 minutes by placing them on a rotating rack then a magnetic rack to remove the supernatant. The working last wash buffer was prepared by adding 10 µL DTT to the last wash buffer tube prepared previously. The beads were then washed in 900 µL working last was buffer and placed on a rotating rack for 15 minutes then a magnetic rack to remove the supernatant. The beads were stored in 50 µL of working last wash buffer and stored at -80^{o}C until Western blot analyses.

Western blots for PRG-1, GLH-4, and PIE-1 protein

Apparatus preparation

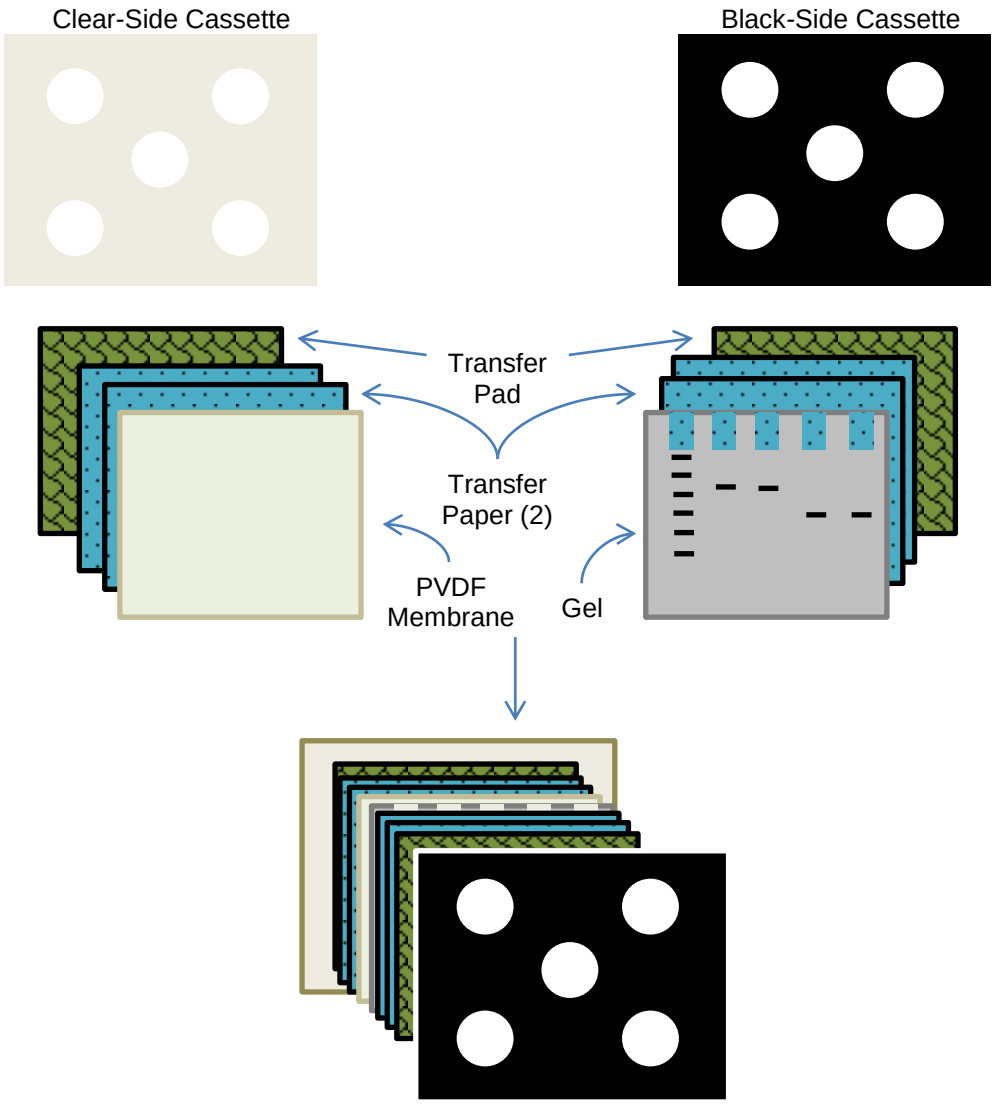
A pre-cast NuPAGE Protein 4-12% bis-tris gel from Invitrogen was placed into the gel apparatus and locked into place. The running buffer was prepared by combining 50 mL 20x NuPAGE MOPS SDS Running Buffer and 950 mL deionized water in a 1 L graduated cylinder, which was then parafilmed and inverted to mix. The inner chamber of the apparatus was filled completely with running buffer and the outer chamber filled half way with buffer. The wells of the gel were rinsed using a pipet to remove any residual gel storage buffer before loading samples.

Sample preparation and loading gel

The IP samples were prepared for loading by adding 20 µL NuPAGE LDS 4x sample buffer (catalog number NP0008) and 10 µL 0.25M DTT to each IP tube. The input samples (previously prepared) and IP samples were then boiled for 2 minutes at 95^{o}C then centrifuged quickly at a fast speed to pellet the beads. To load the gel, 10 µL of Bio-Rad Pre-stained All Blue marker was loaded into the first lane. For the input samples, 100 µg/lane was desired, so 12 µL of the *prg-1(tm872)* and N2 input samples was loaded. For the IP samples, 500 µg/lane was desired, so 8 µL of the *prg-1(tm872)* and N2 IP samples was loaded. The covered was added to the gel apparatus and ran at 50V for 15 minutes, and then the voltage was increased to 100V for 45-60 minutes until the sample dye reached the bottom of the gel.

Gel Transfer

Transfer buffer was prepared by combining 50 mL 20x NuPAGE Transfer Buffer, 100 mL methanol, and 850 mL deionized water in a 1L graduated cylinder, which was parafilmed and inverted to mix. The pre-cast gel was removed from the running apparatus and the mold was cracked. The gel was carefully removed from the mold, and excess gel was trimmed off to leave only the marker and samples. The PDVF membrane was washed in 100% methanol, then dH_2O then transfer buffer. The sandwich pads and 4 sheets of transfer paper were wet in transfer buffer. The transfer sandwich was prepared by the diagram below. The completed transfer cassette was placed into the transfer box along with an ice container to keep the transfer box cool. The completed transfer box was filled with transfer buffer, placed in the 4^{o}C walk-in on a stir plate, and ran at 20V overnight.



Western Blot Membrane Transfer Apparatus: Set up for transferring the gel to a PVDF membrane, which is then placed inside a cassette enclosed by a clear positive side (membrane) and a black negative side (gel). DNA is negatively charged, so when a voltage is applied it migrates from negative to positive, which transfers the DNA bands in the gel onto the PDVF membrane.

Immunoblot with PRG-1 antibody probes

0.1% Tween 20 in Phosphate buffered saline (0.1% PBST) was prepared by combining 100 mL 10X PBS, 1mL Tween 20 and deionized H_2O to a 1 L graduated cylinder, which was parafilmed and inverted to mix. PBST + 4% milk was also prepared by combining 10 g powdered skim milk and 0.1% PBST to250 mL in a glass bottle, which was swirled to mix well. Following the overnight transfer, the membrane was removed from the sandwich and rinsed in PBST. The membrane was then blocked by rocking for 1 hour

in 200 mL PBST + 4% milk. The primary antibody solution was made by adding 8 µL anti-PRG-1 antibody and 2mL PBST + 4% milk. The blocked membrane was placed in between two plastic sheets and heat sealed on 3 edges, leaving 1 edge open to add the primary antibody solution. The primary antibody solution was added and all bubbles were removed by squeezing without spilling any solution. The last edge of the membrane bag was heat sealed and the membrane was incubated with primary antibody rocking for 1 hour at room temperature between two glass plates to distribute the solution evenly over the membrane. Following the primary antibody incubation, the membrane was removed from the bag and washed three times rocking in 200 mL PBST for 10 minutes. The secondary antibody solution was prepared by adding 1 µL anti-rabbit IgG antibody to 5 mL PBST + 4% milk. The secondary antibody solution was added to the membrane following the same procedure with the heat sealed plastic bag. The membrane incubated again rocking for 1 hour at room temperature. Following the secondary incubation, the membrane was again washed three times rocking with 200mL PBST for 10 minutes.

Membrane Imaging

The membrane was prepared for imaging by developing it on a sheet of saran wrap face-down in a mix of 250 µL oxidizing Western Lightening Chemiluminescent Reagent Plus (catalog number PC2649-0202) and 250 µL reducing Western Lightening Chemiluminescent Reagent Plus (catalog number PC2653-0202) for 1 minute. The membrane was the placed faced down and wrapped in saran wrap to produce a smooth, bubble-free casing. The ladder was marked by a glow-in-the-dark substance by dotting each mark of the ladder. The membrane was then imaged for 160 seconds in 10 second increments.

Immunoblot with GLH-4 and PIE-1 antibody probes

The same procedure was followed for immunoblot by stripping the membrane with 10-20 mL Thermo Scientific Restore Western Blot Stripping Buffer (product number 21059) then performing the procedure with anti-GLH-4 (1:100) as the primary antibody and the anti-rabbit IgG secondary antibody.

The membrane was then stripped again and probed using anti-PIE-1 (1:50) as the primary antibody and an anti-mouse IgG secondary antibody.

Silver stain

A gel apparatus was set up following the procedure for setting up the gel apparatus in the Western blot. The gel was loaded with 10 µL and 20 µL of *prg-1(tm872)* and N2 IP samples. The gel ran at 50V for 15 minutes, and then increased the voltage to 100V for 45-60 minutes until the sample dye reached the bottom of the gel. Once completed, the gel was removed from the cassette and rinsed with deionized H_2O (dH_2O). The Invitrogen SilverQuest silver stain kit (catalog number LC6070) was used for this procedure. The gel was fixed in 100 mL of fixative (40 mL ethanol, 10 mL acetic acid, dH_2O to 100 mL) overnight while gently rocking. The following morning, the fixative was decanted and the gel was washed in 30% ethanol for 10 minutes. The ethanol was decanted and the gel incubated 100 mL of sensitizing solution (30 mL ethanol, 10 mL kit sensitizer, dH_2O to 100 mL) while rocking for 10 minutes. The sensitizing solution was decanted and the gel washed in 100 mL dH_2O for 10 minutes. The gel was then stained in 100 mL of staining solution (1 mL kit stainer, dH_2O to 100 mL) while rocking for 15 minutes. After staining, the staining solution was decanted and washed with 100mL of dH_2O for 60 seconds. The gel was the incubated in 100 mL developing solution (10 mL kit developer, 1 drop kit developer enhancer, dH_2O to 100 mL) until bands started to appear and the desired band intensity was observed. Immediately once developing was done, 10 mL kit stopper solution was added directly to the developing solution. The gel was incubated while gently rocking for 10 minutes to allow the development to stop. The gel was then washed in 100 mL dH_2O for 10 minutes. Bands of interest were excised and sent off for protein mass spectroscopy analysis.

Results

pie-1 dsRNA produces embryonic lethality by RNAi injection

Previous unpublished studies performed by the Mello lab first identified a genetic interaction between *prg-1* and *pie-1* through a genome-wide RNAi screen. This project attempted to use the injection method to confirm the results previously observed by feeding. If *pie-1* interacts with *prg-1* as suggested, a decrease in viable embryos should be seen in the *prg-1(tm872)* strain as compared to the wildtype strain. Since the *prg-1* mutant lacks PRG-1 function, a significant decrease in viable embryos would suggest a specific interaction between *prg-1* and *pie-1*.To test the interaction, *pie-1* dsRNA was injected into both wildtype and *prg-1(tm872)* strains. The progeny of both injected strains, as well as control uninjected wildtype and *prg-1(tm872)* worms were observed by transferring the mothers every 12 hours to fresh plates. The progeny of all strains (injected and uninjected) were counted immediately following the transfer of the mother (every 12 hours) and again 12 hours later for both the number of dead embryos and the number of total progeny on the plate (**Figure 1A**). The experiment continued for approximately 3-4 days until the mothers stopped producing embryos. Following the experiment, an overall percent viability was determined for each worm. Three injection trials were performed all following the same procedure and an average percent viability was determined for each strain. A student's T –test suggests that *pie-1* produced a statistically significant embryonic lethal phenotype in both the wildytpe and *prg-1(tm872)* strains when compared to the uninjected strains (**Figure 1B**). However, a statistically significant difference in percent viability between wildtype and *prg-1(tm872)* was not observed (**Figure 1B**). One main objective in reproducing the results obtained previously through injection was to find a concentration of *pie-1* dsRNA that would produce a significant decrease in viable embryos in *prg-1(tm872)* mutants than wildytpe worms. While the results suggests that the *pie-1* dsRNA injections is working to produce an embryonic lethal phenotype in both wildtype and *prg-*

1(tm872), the difference between the two strains is not significant enough to clearly state there is an interaction specifically between *prg-1* and *pie-1*.

The *prg-1* and *pie-1* interaction and allele specificity

The interaction identified previously by the RNAi feeding screen was confirmed by the creation of a *prg-1(tm872);pie-1/+* double mutant, which only observed the interaction of *pie-1* with a specific allele mutation of *prg-1*. This raises the question if the *pie-1* interaction observed was specific to the tm872 allele mutation of *prg-1*. If the interaction between *prg-1* and *pie-1* is allele specific, a decrease in viable embryos would not be seen in a different *prg-1* mutant. To test the possibility of the *prg-1* and *pie-1* interaction being allele specific, a second double mutant was made using another allele mutation of *prg-1*. The *prg-1(n4357);pie-1/+* double mutant was designed and the crosses performed, with the anticipation of studying the embryonic phenotype once the genotype of the double mutant was confirmed. The cross was executed, but the genotype of the double mutant was not confirmed. Due to timing of the experiments, the observation of the embryonic phenotypes was not completed. The double mutant should be made again following the same cross map, and the genotype of the double mutant must be confirmed quickly in order to accurately observe the phenotype of the progeny. The nature of the interaction still remains unconfirmed, which is why it is important to both continue efforts to confirm the observations and as well as focus on the possible methods of interaction and the function *prg-1* and *pie-1* play together in germline development.

PIE-1 does not co-immunoprecipitate with PRG-1

There are many different ways that *prg-1* and *pie-1* may interact to function together in germline development. One possibility for interaction studied in this project is the physical interaction of the PRG-1 and PIE-1 proteins. To test the possibility of protein interaction, PRG-1 and PIE-1 were tested for physical interaction by co-immunoprecipitation. If PRG-1 and PIE-1 proteins interact, the PIE-1 protein

should be detected in the IP for PRG-1 protein in the wildtype strain, but not in the *prg-1(tm872)* strain. This would suggest a physical interaction with the wildtype PRG-1 protein, since the *prg-1* mutant lack PRG-1 protein. To test the interaction by co-IP, wildtype and *prg-1(tm872) strains* were grown to synchronous populations then harvested for early embryos, since the PIE-1 protein is present only in the germline of very early embryos. Lysates were produced from the early embryos, and a small amount of input (pre-IP) samples were saved for later analysis. The remaining lysates were used to immunoprecipitate the PRG-1 protein using an α-PRG-1 rabbit antibody. The input samples and the PRG-1 IP samples were ran on a gel, transferred to a PDVF membrane, and Western blot analysis probed for the presence of PRG-1, GLH-4, and PIE-1 proteins. The PRG-1 IP was successfully executed as the wildtype PRG-1 protein was detected by Western blot in the PRG-1 IP wildtype (WT) sample at a high concentration , and not observed in the PRG-1 IP negative (prg-1 mutant) sample (**Figure 2A**). The *prg-1* mutant (-) sample also served as negative control, since the wildtype PRG-1 protein is not present due to the tm872 allele mutation that creates a smaller truncated protein. The samples were also probed for the presence of GLH-4 protein, which is known to co-immnoprecipitate with the PRG-1 protein. The presence of GLH-4 in the PRG-1 IP WT sample confirms that PRG-1 IP was successful in selectively pulling down the PRG-1 protein (**Figure 2B**). The presence of the PIE-1 protein was confirmed in both the wildtype and *prg-1(tm872)* strains, as seen in the input samples (**Figure 2C**). However, the presence of PIE-1 was not detected in either of the PRG-1 IP samples, which may preliminarily suggest that there is no physical interaction between the proteins (**Figure 2C**). This data suggests that while both the PRG-1 IP and the Western blots were successfully executed, the PRG-1 and PIE-1 proteins may not physically interact, which suggest that another method of interaction was observed.

prg-1 does not regulate PIE-1 expression

Since the PRG-1 IP suggests there is no protein interaction between PRG-1 and PIE-1, another possible method of interaction studied in this project is gene regulation. If one gene regulates another, then the

loss of function in that gene will cause decreased expression of the mRNA produced from the other gene. The presence of *prg-1* may regulate the expression of the PIE-1 protein, which would suggest that *prg-1* is acting upstream of *pie-1* in the genome. To test this possibility, the *prg-1(tm872)* and wildtype input samples (saved from before the IP was performed) were ran on a gel alongside the PRG-1 IP samples then transferred to a PDVF membrane and probed for various proteins describe previously. The final Western blot detected the presence of the PIE-1 protein (~40kD) in both input samples, and at very similar expression levels for both *prg-1(tm872)* and wildtype strains (**Figure 2C**). The presence of PIE-1 in both strains confirms that the protein is not affected by the loss of *prg-1*, since it is detected both in the presence and absence of *prg-1*. This data suggests that the wildtype activity of *prg-1* is not necessary to regulate the expression of the PIE-1 protein, which may suggest that *prg-1* is functioning elsewhere in relation to *pie-1*.

PRG-1 interacts strongly with 40 kD and 200 kD proteins

The genetic interaction between *prg-1* and *pie-1* has yet to be identified, which suggests that there may be other factors contributing to the observed interaction. It is then important to consider if PRG-1 interacts with any other proteins which may provide more insight as to its function in germline development as well as to the connection *prg-1* has to *pie-1*. It is possible that PRG-1 interacts with PIE-1 indirectly, which means there are others proteins that serve to interact between them to produce the observed interaction. It is also possible that PRG-1 and PIE-1 function independently to each other, but interact with the same target to produce the observed result. To determine other proteins that interact with the PRG-1 protein, 10µL and 20µL of the PRG-1 IP samples were loaded and ran on a gel at 50V and then 100V until the dye reached the bottom. The gel was fixed, stained with silver nitrate, and developed to observe all proteins that interact with the PRG-1 protein (**Figure 3A**). Interaction with the WT sample suggests a specific interaction with PRG-1 protein since the PRG-1 protein is absent in the prg-1 mutant. Proteins visible at ~200kD, ~90kD, and ~40kD suggest strong and specific interaction with

the PRG-1 protein, since bands at these lengths are only visible in the WT strain and not the *prg-1* mutant strain (**Figure 3B**). These bands were carefully excised from the gel and submitted for mass spectroscopy analysis at the UMass Medical School. Once the proteins are identified, they may be able to confirm an interaction between PRG-1 with PIE-1 or suggest other proteins that act as bridges connecting PRG-1 to PIE-1 to produce an observed interaction.

Discussion

The germ-cell lineage in any organism serves as a conservation of genetic material that ensures that the most vital information is preserved from generation to generation. Early in embryogenesis, germ cells are distinctly specified from somatic cells. A series of controlling mechanisms ensures that with each cell division, the germline is maintained in a pattern specific to that organism. It is then important to understand those controlling mechanisms that serve to preserve the germ lineage as well as aid in specification of somatic cells. In *C. elegans,* many factors have homologs in other organisms which allow for relatively simple study of germline specification and development and thus make them a good model for understanding other eukaryotic systems. With such overlap, studying the controlling mechanisms in germline specification and development in *C. elegans* has many applications across the fields of biology.

A major factor in germline specification is epigenetics, or the functional modifications that occur to preserve the genetic material. This includes topics such as gene expression or silencing, post translational modifications, and chromatin remodeling. While a portion of germline specification is mediated by genetics, an unknown fraction is mediated by epigenetics which is a key area of focus in the Mello lab. This study focused on the interaction observed between prg-1 and pie-1, both of which play roles in epigenetics in *C.elegans*. PIE-1 is a maternal protein that has implications in epigenetics with blocking new gene expression and a role in preventing chromatin remodeling in early embryos (Seydoux et al, 1996) (Unhavaithaya et al, 2002). PRG-1 is a highly conserved piwi-related Argonaute that interacts

with the piRNAs of *C.elegans*, 21U-RNAs, to form a protein complex important in germline development by an unknown mechanism. PRG-1 also has implications with Tc3 silencing, which may suggest it has an epigenetic effect on germline development as well.

The experiments in this study investigated if the results of the observed interaction could be confirmed, as well as the nature of the genetic interaction between PRG-1 and PIE-1 in germline development during embryogenesis. Here it is shown that *pie-1* produces an embryonic lethal phenotype in both *prg-1* mutant and wildtype worms, however a significant difference between *prg-1(tm872)* and wildtype was not observed, which suggests more experiments need to be performed to confirm the interaction. The study also preliminarily suggests that the PIE-1 and PRG-1 proteins do not physically interact, which may suggest other modes of interaction to explain their function together. Other proteins that interact with PRG-1 were identified by silver stain and sent off for mass spectroscopy analysis, which when identified will shed light on other proteins that interact with PRG-1, and may suggest to other roles PRG-1 plays both in germline development or in relation to PIE-1.

Observing the *prg-1* and *pie-1* interaction by RNAi injection

The injection method was used to recreate the interaction originally identified in the RNAi feeding screen. The injection method into C. elegans has both positive and negative aspects in relation to studying the effect it has on germline development. While injections allow for a rapid method to introduce dsRNA into the organism, it is also an acute force directly into the worm which may cause harm thus affecting the health of the worm. The method is also much more variable than feeding, since factors such as handling, worm life stage, intake amount, force, and timing are all aspects that require careful monitoring and regulation in order to provide reproducible results. The study suggests that injection may not be the best method, and another method should potentially be addressed to reproduce the results obtained in the feeding experiment. It is also important to consider that the

original RNAi feeding screen was a qualitative experiment, only used to visually identify the genes that produced increased embryonic lethality in the *prg-1* mutants than the wildtype worms. The results produced in this study confirm that *pie-1* produced embryonic lethality in both *prg-1(tm872)* and wildtype worms, but since the original experiment did not quantify the difference between *prg-1* mutants and wildtype worms, it cannot be clearly stated if the results confirmed what was observed before. The concentration of *pie-1* injected into the worms suggests that if the injections were to continue, more optimization needs to be done to find the best concentration that gives a statistically significant difference in embryonic lethality in prg-1 mutant when compared to wildtype worms.

Characterizing the *prg-1* and *pie-1* interaction

This study explains the importance of testing the allele specificity of the observed *prg-1* and *pie-1* interaction. With both genes producing important epigenetic states, understanding the relationship that *prg-1* has to *pie-1* will better explain the role they play together in germline development. The protein of interest in this project was PRG-1, since its function in germline development is not fully understood, and recent unpublished data from the Mello lab suggests that *prg-1* may be a master regulator that is required to establish permanent silencing of transgenes (C.C. Mello, personal communication). Previous publications headed by the Mello lab provide insight on *prg-1* interaction with the piRNAs of C. elegans , 21U-RNAs, which is required for silencing of the Tc3 transposon (Batista et al, 2008). The binding of PRG-1/21U-RNA complex to the Tc3 transposon recruits the WAGO/22G-RNA pathway which is dependent upon factors such as RDE-3, MUT-7, and RdRP (RNA-dependent RNA Polymerase complexes) (Gu et al, 2009). The pathway is an amplification cycle producing 22G-RNAs, an endo-siRNA that binds to WAGO (worm-specific Argonaute) proteins (Batista et al, 2008) (**Figure 4A**). Binding of 22G-RNAs to WAGO proteins silences the Tc3 transposon both transcriptionally and post transcriptionally (Batista et al, 2008). The amplification pathway makes PRG-1 a very potent factor in silencing the Tc3 transposon. With a specific function in epigenetics with *prg-1* along with the observation of interaction with *pie-1*, it

suggests that *prg-1* and *pie-1* could be functioning in parallel pathways to affect germline development in embryogenesis. The design of the *rde-3(ne3370);pie-1/+* double mutant served to question if *rde-3*, which is downstream to *prg-1*, has an embryonic lethal phenotype with *pie-1*. If it does, then it would suggest that *prg-1* and *pie-1* function in parallel pathways to affect germline development (**Figure 4B**). Further studies with the *rde-3(ne3370);pie-1/+* double mutant need to be performed in order to better characterize the nature of the interaction, as well as explain the pathways involved to produce the observed effects in germline development.

Connections between the PRG-1 and PIE-1 proteins

Interactions between epigenetic factors on the protein level require that the genes be highly conserved to ensure the accurate production of the proteins to function in preserving the germ lineage. PRG-1 is a member of a high conserved class of Argonautes that have homologs across the phylogenic world. Implications of the PRG-1 protein could spread across many aspects of biology for study outside of the realm of germline development. The identification of interaction between *prg-1* and *pie-1* is significant since each gene and their subsequent protein play major roles in epigenetics by preserving the germline. A connection between the two may explain their role *in vivo*, which was first addressed by studying the physical interaction of PRG-1 and PIE-1. The study preliminarily suggests that the proteins do not interact, but that further studies of protein interaction must be done to confirm or deny interaction. A PRG-1 IP was performed, which needs to be repeated with a higher percentage of early embryos. The experiment in this study later found that only 30% of the embryos used in the IP were considered early embryos, which suggests that the PIE-1 protein may have been present in a concentration too low to be detected. The reciprocal PIE-1 IP should also be performed, which will help to confirm the results obtained through the PRG-1 IP. While the results of physical protein interaction from this study prove to be preliminarily negative, it does not rule out the possibility completely and still requires much study.

A connection between *prg-1* and *pie-1* still exists, which results preliminarily suggest it is not on the protein level. A second connection addressed in this study was designed to observe the interaction through regulation. It was hypothesized earlier in the study that *prg-1* may regulate the expression of PIE-1 protein, which was not found to be true. The input samples saved from before the PRG-IP confirms tthe presence of the PIE-1 protein is present in both the *prg-1* mutant and wildtype embryos at very similar levels of expression. This opens the possibility to reverse the original hypothesis and ask if *prg-1* is downstream of *pie-1* in a pathway, which would suggest that *pie-1* is acting through *prg*-1 to produce its effect on germline development. PIE-1 preserves the pluripotency of the germ line by repressing transcription of new mRNA in the P2 germline blastomere (Seydoux et al, 1996). The SKN-1 transcription factor is present in both the germline blastomere P2 and the somatic blastomere EMS. However SKN-1 is only expressed in EMS and repressed by PIE-1 in the P2 germline cell (Bowerman et al, 1993) (Mello et al, 1992). In the *pie-1* mutant, the absence of PIE-1 protein the P2 germline blastomere adopts an EMS cell fate because SKN-1 is now active in the germ cell (Bowerman et al, 1993). As a result, *pie-1* dead embryos have excess intestine and pharynx. It is possible that PIE-1 may be repressing transcription in the germ cell by PRG-1 dependence and PRG-1 independent mechanisms. With the given results, it would be necessary to test the possibility of this new hypothesis through the design of experiments to address the effect PRG-1 has on PIE-1 and its specific role with SKN-1.

Epigenetics and preserving pluripotency in stem cells

The germ lineage is necessary to preserve the genetic information and produce a properly functioning organism, as previously discussed in this study. In observing the many aspects of the scientific world the germline is essentially an immortal stem cell lineage that preserves pluripotency, or the ability to differentiate into any cell type. By studying germline development during embryogenesis in *C. elegans*, the world will learn how the programing is maintained through epigenetics. Study of the how the programming in germline specification and development works has greater implications in areas such as

stem cell renewal. Understanding the controlling mechanisms in pluripotent cellular differentiation not only affects the field of development, but the fields of stem cell generation and renewal as well. Studies may aid in better understanding and solving the many diseases and injuries that involve the potential use of stem cells. It is the aim of this project to provide a broader implication of a small area of study in hopes that it may provide insight and encourage future research all areas in the scientific community.

Figures

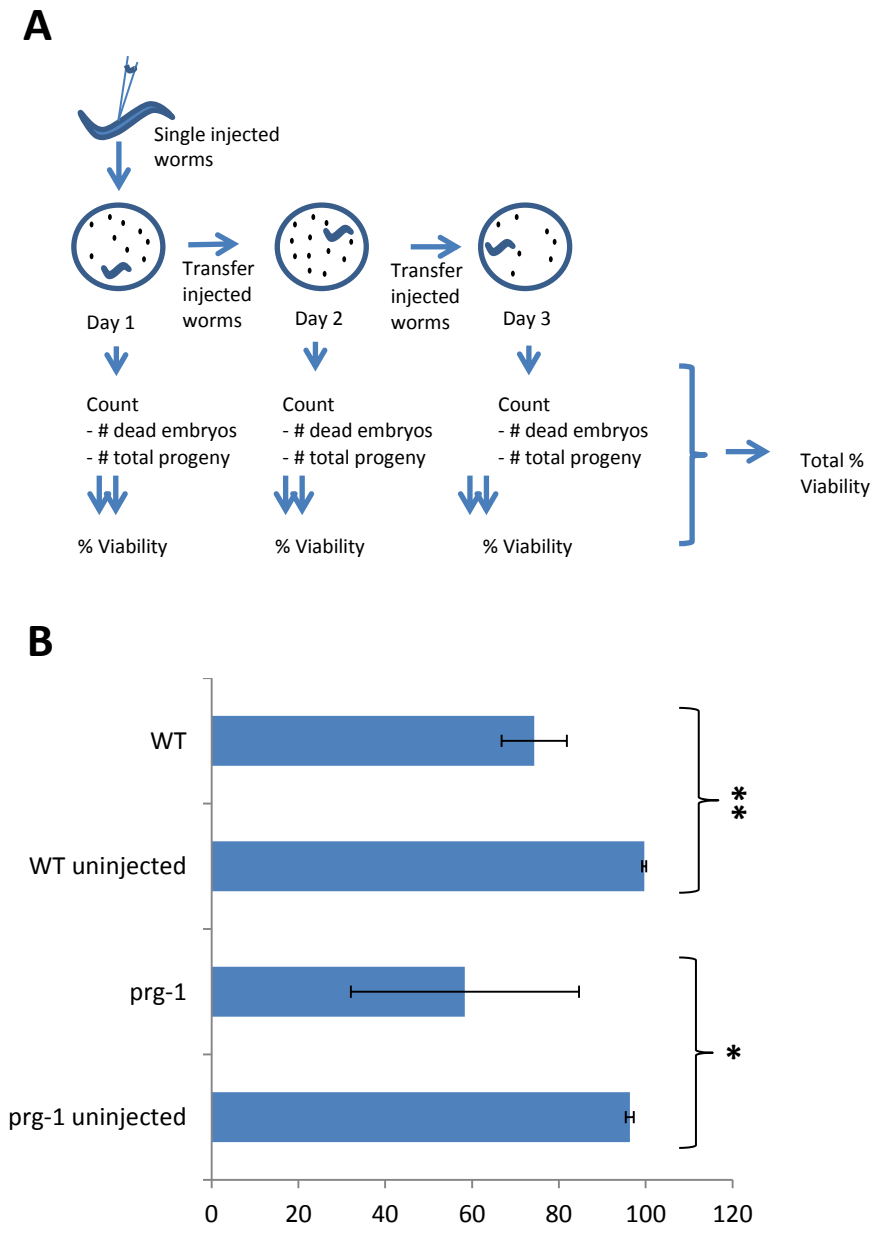
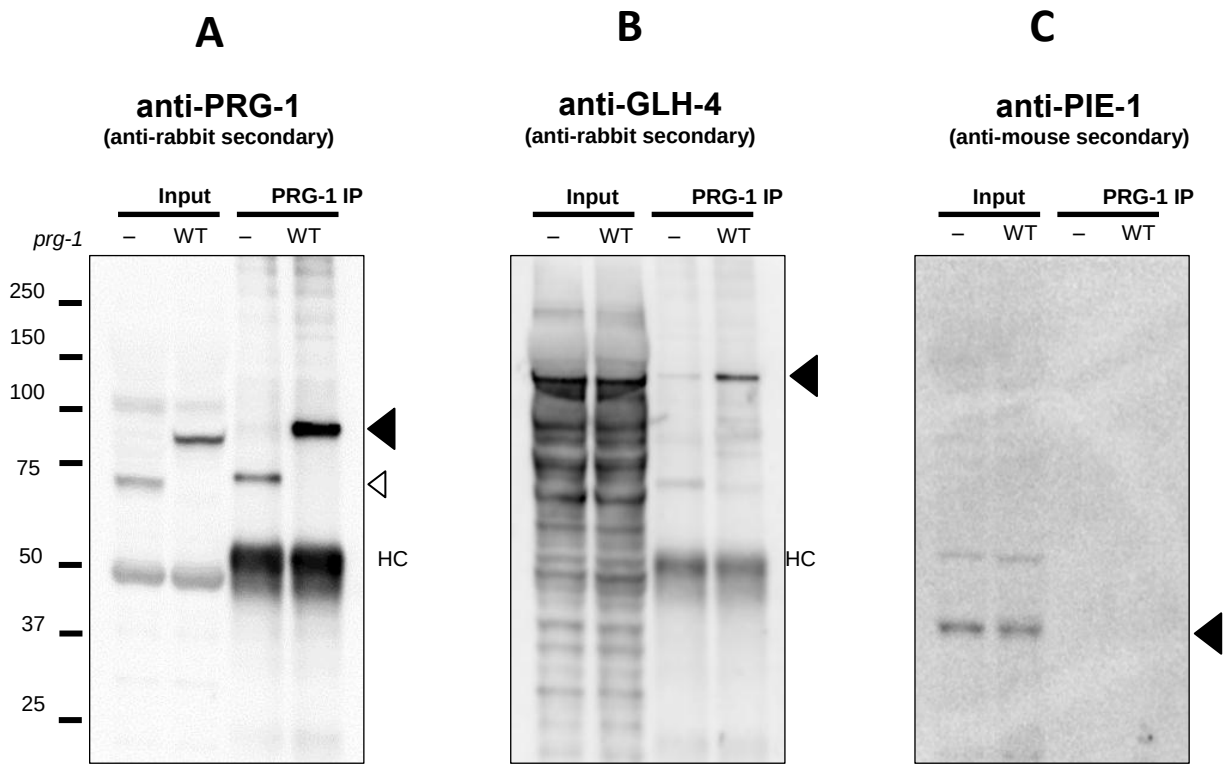


Figure 1. *pie-1* dsRNA Injections.

A) Schematic for the injection experiment to determine the percent viability for n worms. Average n = 10 prg-1 uninjected, 13 prg-1 injected, 10 WT uninjected, and 12 WT injected. B) RNAi injected strains and the uninjected strain controls. **A very statistically significant (P <0.004) difference between the injected and uninjected wildtype (WT) strain. *A statistically significant (P <0.05) difference between the injected and uninjected prg-1(tm872) strain.



◀ = expected protein (PRG-1, GLH-4 or PIE-1, respectively).

◁ = *prg-1* deletion results in truncated PRG-1 protein expressed at lower level.

HC = heavy chain of rabbit anti-PRG-1 used for IP.
Not detected in PIE-1 western, since anti-PIE-1 is a mouse monoclonal antibody.

Figure 2. Western Blot Analysis of PRG-1 IP

A) Immunoblot for the presence of PRG-1 protein, detected between 75-100 kD. B) Immunoblot for the presence of GLH-4 protein, detected between 150-100 kDa. C) Immunoblot for the presence of PIE-1 protein, detected around 37 kD.

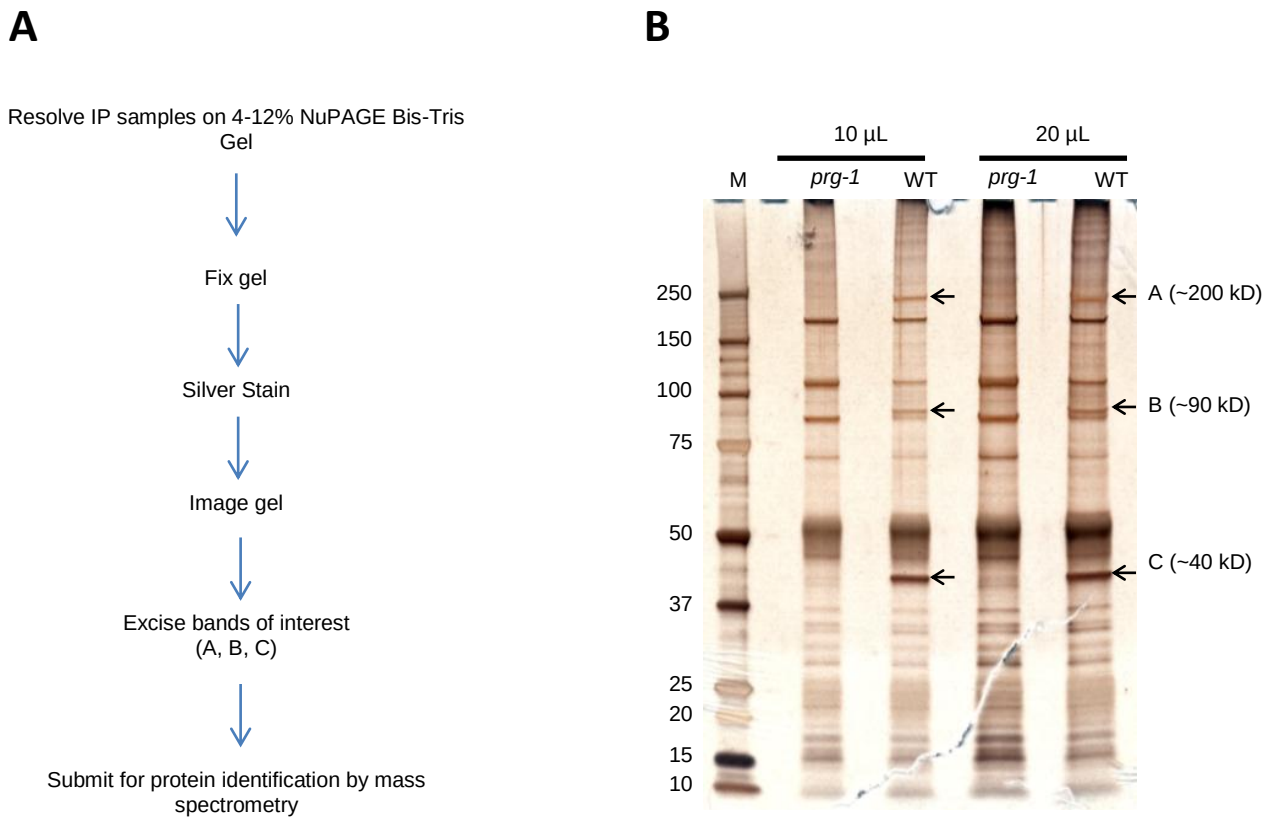


Figure 3. Silver Stain of PRG-1 IP

A) Outline of the silver stain procedure. B) Silver stain of the PRG-1 IP samples with bands of interest excised at the arrows (A = ~200 kD, B = ~90 kD, C = ~40 kD).

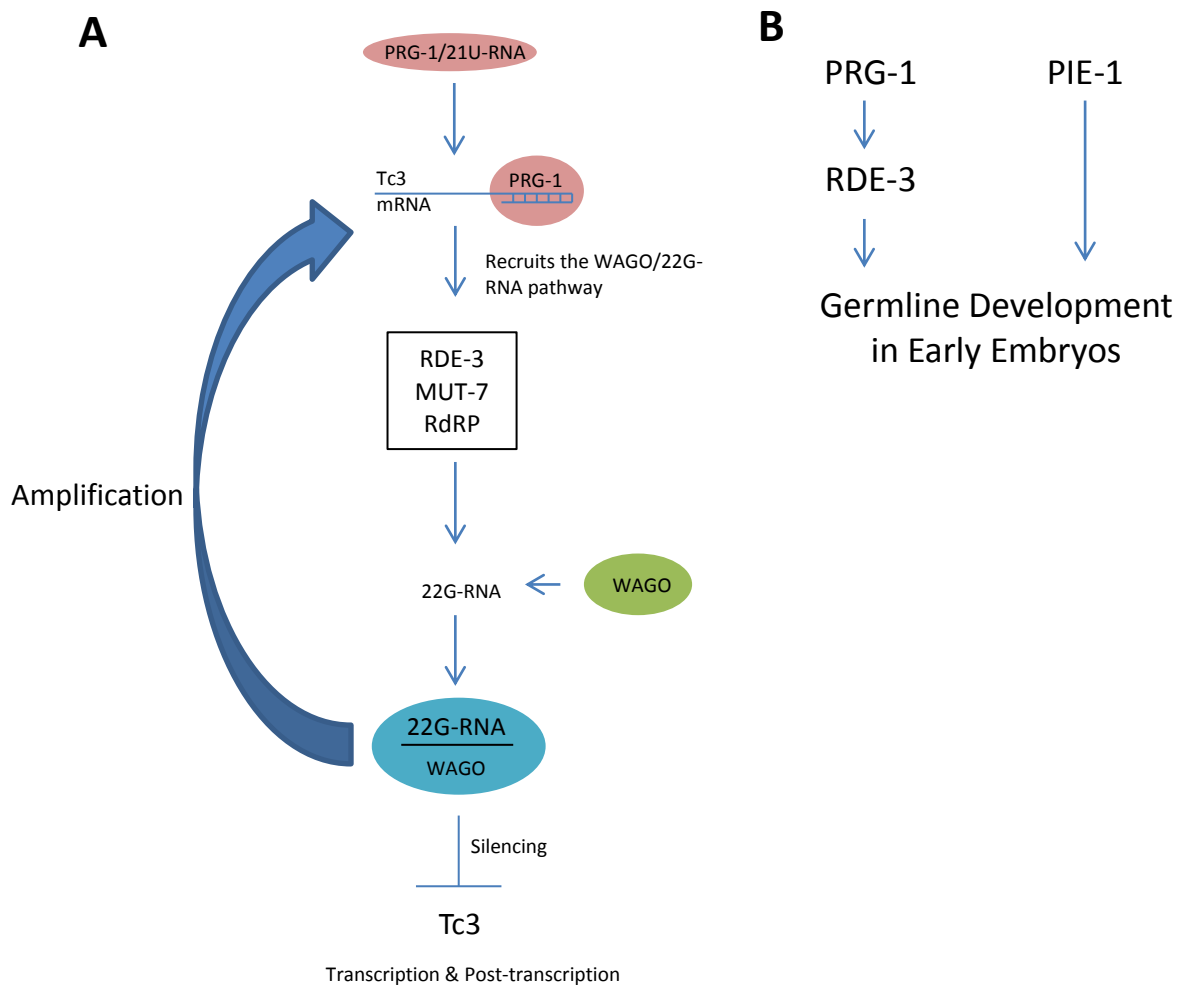


Figure 4. The role of RDE-3 in defining the function of PRG-1 and PIE-1 interaction
a) Diagram of the WAGO/22G-RNA pathway initiated by the PRG-1/21U-RNA complex binding to the Tc3 mRNA. b) Model for hypothesized relationship of PRG-1 and PIE-1 in germline development during embryogenesis.

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