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Investigating subcellular localization of somatic PIWI proteins in the annelid Capitella teleta

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Investigating subcellular localization of somatic PIWI proteins in the annelid *Capitella teleta*

by

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Abstract

PIWI proteins are well known for their various roles and localization in germline development of diverse organisms, yet their specific role and function in somatic tissue remains elusive. PIWI proteins are essential for the biogenesis of PIWI-interacting RNA (piRNA), silencing mechanisms, sexual reproduction, and regeneration. piRNAs are PIWI bound RNAs that play an essential role in transposon silencing. PIWI and piRNAs expression and localization have been discovered in the model organism *Drosophila melanogaster* and mice; however, its localization in the *C. teleta* annelid is currently unknown and the subject remains an active area of research. In the present study, the localization of two PIWI paralogs were analyzed using the process of immunostaining to demonstrate its expression in somatic cells. The presence of PIWI proteins were confirmed using the Western Blot technique and immunofluorescences; observations of PIWI proteins localization were found in the nuclei of early-stage embryos and the cytoplasmic follicles of larvae. This indicates that PIWI proteins are in fact expressed in the *C. teleta* somatic tissue and there is an autonomous function of PIWI in both somatic and germline cells. The results of this experiment provide new insights on the function of PIWI proteins in invertebrate somatic cells and can be applied to build on the knowledge of small RNA biology.

Keywords: PIWI, piRNA, somatic, *Capitella teleta*, germline

Dedication

I would like to dedicate this entire thesis process to my mother and father. The journey to completion was not an easy road to take. There were many sleepless nights, self-doubt, and sacrifices; however, you both always believed in me and supported me throughout this process and every step of my life. You have been my source of inspiration and strength when I felt like giving up. The both of you have been my biggest cheerleaders throughout my four years of college and I would not be where I am today without you. I love you mom and dad.

.

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I would also like to thank Sweta Khanal for all of her constant help and guidance in teaching me how to successfully operate in the laboratory. You were there every step of the way to answer any lingering questions I had and helped guide me in the right direction. Without you, I would have been lost. Thank you so much for everything.

Thank you to the Honors College Keystone Program at the University of Southern Mississippi for showing me that I am capable of so much more. Thank you for pushing me out of my comfort zone and allowing me to soar.

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List of Abbreviations

1. AGO – Arogonaute
2. AUB – Aubergine
3. GMP – Germline Multipotency Program
4. HRP – Horseradish Peroxidase
5. miRNA – microRNA
6. OSC – Ovarian Somatic Cells
7. PBS-T – Phosphate-Buffered Saline with Tween
8. PFA – Paraformaldehyde
9. PGC – Primordial Germline Cell
10. PGZ – Posterior Growth Zone
11. piRNA – PIWI-interacting RNA
12. PIWI – P-Element Induced Wimpy Testis
13. PVDF – Polyvinylidene Fluoride
14. RNA – Ribonucleic Acid
15. SDS-PAGE – Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
16. siRNA – Short Interfering RNA

Chapter 1: Introduction

The P-element induced wimpy testis (PIWI) proteins belong to the Argonaute family that are highly conserved RNA-binding proteins present in various animals (Ku and Lin, 2014). PIWI-interacting RNAs (piRNAs) are characterized by their specific binding to the PIWI proteins. PIWI proteins are required for piRNA biogenesis and function and play an essential role in germline development (Ross et al., 2014). The cells (i.e., eggs and sperm) of the germline forms gametes in sexually reproducing organisms and often does not contribute to the soma, which are all the other cells that are not sex cells (Dannenberg and Seaver, 2018). PIWI proteins have been studied in different model organisms within the germline to observe their function and localization. Previous studies on the *Drosophila* gene PIWI paralogs, the first identified expression of PIWI proteins, determined that their germline function was dependent on the somatic cells in the gonads (Yashiro et al., 2018). While PIWI proteins were commonly perceived as germline specific, their presence has been discovered within somatic cells playing an essential role in sexual reproduction, whole-body regeneration, and genome integrity (Ross et al., 2014; Yashiro et al., 2018).

Although numerous studies have discussed the importance of PIWI protein's role in the germline, this restricted perspective is a consequence of the commonly used model organisms (e.g., *D. melanogaster, C. elegans,* and Mice). Researchers have begun to re-investigate PIWI proteins and the PIWI-piRNA pathway in somatic cells in different organisms, including lower eukaryotes. Studies on mollusks have revealed that PIWI proteins and piRNAs pathways are used to repress transposable elements in the germline and in the soma (Jehn et al., 2018). Studies of the PIWI/piRNA pathway system in the

phylum Mollusca are well-described and provide a mean of comparison for the study presented here, which utilized a model organism in the closely related phylum Annelida. Newly developed invertebrate model species within the phylum Annelida that possess the ability to regenerate have shown PIWI proteins in a new light.

In this project, *Capitella teleta* was used as a model organism to analyze the localization of PIWI proteins within its somatic cells. The *C. teleta* is a segmented annelid whose stable genome, reproduction rate, early development, and stereotypic cleavage pattern makes it an ideal candidate for this study (Seaver, 2016). Annelid embryo tissues were observed at different developmental stages and immunohistochemistry was utilized to localize PIWI proteins within the cells. The expression of PIWI and piRNA biogenesis in somatic tissue and the role of the PIWI-piRNA pathway were subsequently examined. The results presented here illustrate a broader localization for the PIWI protein family in the soma of the *C. teleta* and expand our functional understanding of the piRNA pathway and its expression in tissues.

Chapter 2: Background

2.1: Capitella teleta

The marine polychaete annelid *C. teleta*, formally known as *Capitella sp*., (Parry, 2015; Seaver, 2016) has served as an emerging model organism for developmental and regenerative studies. *C.teleta* is a small, segmented annelid that is a member of the Spiralia family and is commonly found living in organically rich sediments (Seaver, 2016; Hejnol et al., 2006). *Capitella* belongs to the Spiralia family, also referred to as Lophotrochozoa, and is placed in the Sedentaria clade (Hejnol et al., 2009) (Figure 1). Morphologically, *C. teleta* has typical annelid features such as a long, narrow body elongated along an anterior-posterior axis, nine thoracic segments and abdominal segments that continuously regenerates during its lifetime, and a posterior pygidium (Giani et al., 2011; Seaver, 2016). The *C. teleta* body plan is displayed in Figure 3F. In addition to its thoracic and abdominal segments, *C. teleta* undergoes sexual reproduction and exhibits robust posterior regeneration, including regeneration of its ovaries (Giani et al., 2011; Seaver, 2016).

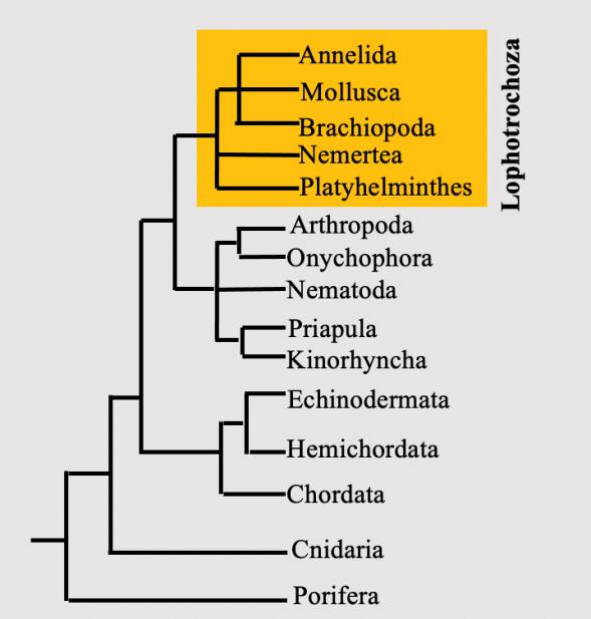

Figure 1: Simplified phylogram of Metazoa. (A) Phylogram of selected metazoan phyla with bilaterian animals with division among the three major superclades: Deuterostomia, Ecdysozoa, and Lophotrochozoa. (Ferrier, 2012).

C. teleta serves as a successful model organism for development due to multiple factors. First, *C. teleta* exhibits sexual dimorphism with the presence of males, females, and hermaphrodites that reproduce sexually (Giani et al., 2011; Seaver, 2016; Dannenberg and Seaver, 2018). The female annelids have visible macroscopic paired ovaries ventrally positioned on the coelomic cavity (Seaver, 2016; Dannenberg and Seaver, 2018). After fertilization, mated females produce a 'brood tube' out of mucus and sand grains that house embryos and early larval stages (Giani et al., 2011; Seaver, 2016) (Figure 2). The female annelid and the offspring remain inside the brood tube until the larvae is ready to emerge. The embryos develop inside the brood for about nine days until they are released (Seaver, 2016). Once the brood is freed from the tube, the competent larvae of *C. teleta* metamorphize into juveniles within an hour (Seaver, 2016) (Figure 3). Next, *C. teleta* embryos undergo spiral cleavage that is mostly seen at the third cleavage, which generates eight blastomeres. (Seaver, 2016; Giani et al., 2011; Ferrier, 2012). Thus, *C. teleta* is especially well suited to explore the presence of PIWI proteins during different developmental stages (i.e., zygotic, larval, and juvenile). Finally, the *C. teleta* species is inexpensive, and easy to manipulate and maintain. Mature *C. teleta* adult annelids can reproduce year-round (Giani et al., 2011) and a lab colony can easily be maintained within plastic bowl containers in an incubator.

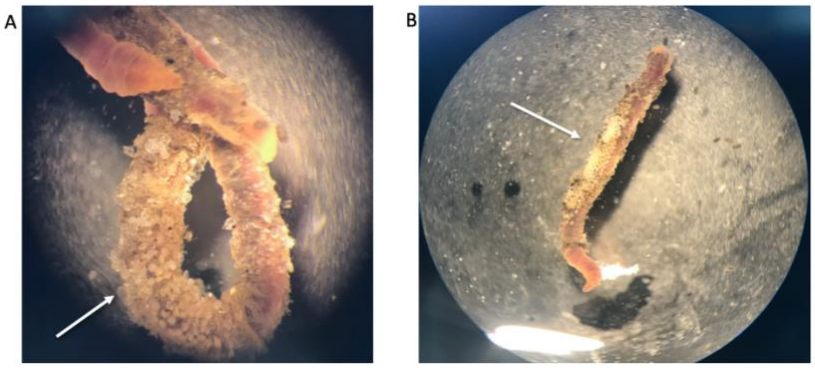

Figure 2: C. teleta brood tube. (A) Anterior microscope image of the female *C. teleta* brood tube. (B) Ventral microscopic image of the female *C. teleta* brood tube.

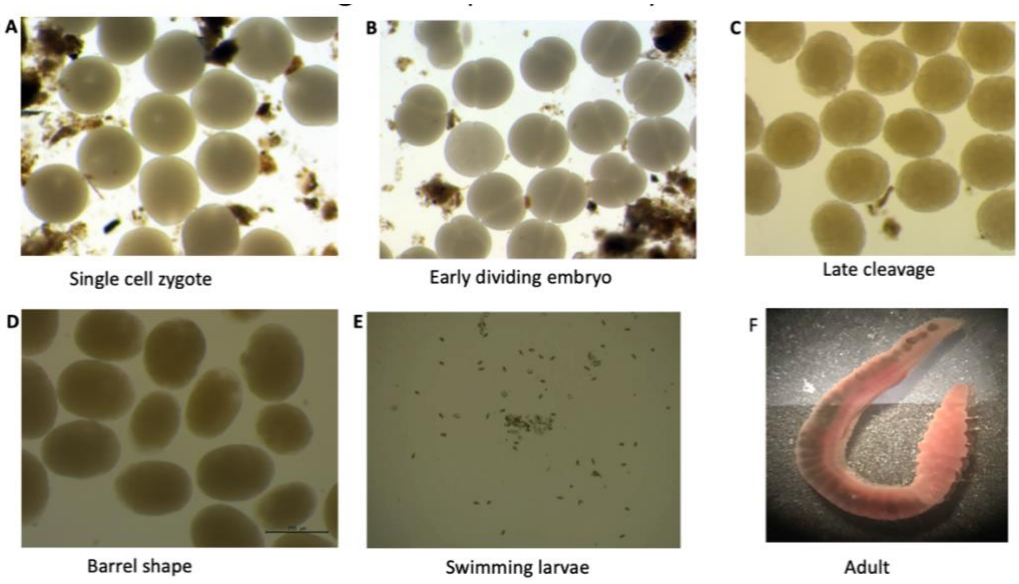

Figure 3: Different developmental stages of *C. teleta*. Microscope images of C. teleta embryos at the (A) singe cell zygote embryonic stage, (B) early dividing embryo, (C) late cleavage, (D) barrel shape, (E) swimming larvae, and (F) an adult annelid image.

2.2: PIWI Proteins and piRNA

As previously mentioned, PIWI proteins were initially discovered as a gene required for germline cell division in *D. melanogaster* germ tissue and have been continuously perceived as germline-specific protein having an essential role in germline formation, sexual reproduction, and transposon silencing (Darricarrère et al., 2012; Wolfswinkel, 2014; Ross et al., 2014; Yuan and Yamashita, 2010). Even though the presence of PIWI proteins in somatic tissues were documented during their first discovery, most studies focus specifically on the different functions of PIWI within the germline of different organisms; therefore, leaving limited research on somatic PIWI proteins. During early embryonic development, the germline completely separates from the somatic cells; however, studies have shown that PIWI proteins are expressed in both the germline and somatic tissue (Yashiro et al., 2018; Dannenberg and Seaver, 2018).

PIWIs can be important in germline defense being that they help to knock out mutations and genetic anomalies in the organism (O'Donnell & Boeke, 2007).

PIWI proteins are members of the Arogonaute (Ago) family that collaboratively bind with microRNAs (miRNAs), short-interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs) in gene expression (Weick and Miska, 2014; Yashiro et al., 2018). Ago proteins are known to comprise the central protein component of RNA-mediated gene silencing complexes and utilize small noncoding RNAs to direct the PIWI protein toward a specific nucleic acid target (Seto et al, 2007). As small RNAs guide Argonaute proteins to specific targets, they are essential in the function of PIWI proteins. PIWI proteins are essential for the biogenesis of piRNAs. piRNAs are similar to PIWI proteins as they are both highly enriched in the germline and are animal specific (Wolfswinkel, 2014; Weick and Miska, 2014; Juliano et al., 2011). piRNA biogenesis pathways differ according to the organism and are transcribed as single-stranded RNA precursors from specific clusters in the genome (Weick and Miska, 2014). The amplification of piRNAs is through an alternated catalytic mechanism of two different PIWI proteins called the ping-pong cycle that occurs in the nuage (i.e., germline organelle) unlike PIWI proteins that have been highly detected in nuclear region of most germ cells (Wolfswinkel, 2014). The ping-

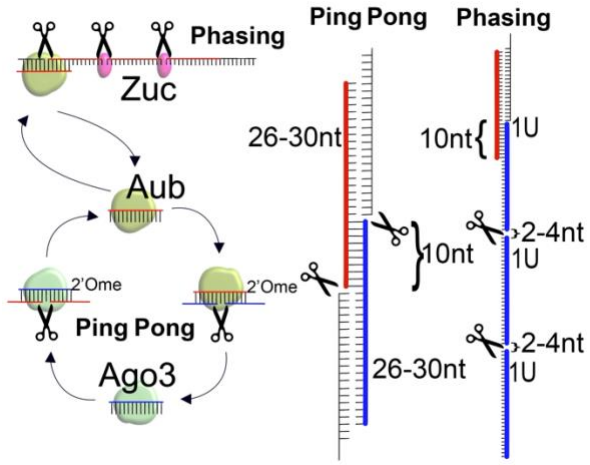

Figure 4: Ping-Pong Amplification Cycle. PIWI proteins are highly enriched in the nuage shown as the cycle above is closely associated with the nuclear pores. The PIWI proteins associate with Aub and Ago proteins that are involved in the ping-pong cycle of piRNA amplification and target transposon RNA for degradation.

pong cycle of piRNA production is a mechanism of piRNA-mediated transcriptional silencing by degradation of transposon RNA and recruitment of histone methylase (Wolfswinkel, 2014; Weick and Miska, 2014; Yakushev et al., 2013) (Figure 4). Thus, piRNAs are dependent on PIWI proteins for their function and biogenesis.

Piwi in Capitella teleta

There are two paralogs of PIWI proteins, piwi1 and piwi2, that are expressed in the genome of *C. teleta*. These genes are expressed in the reproductive structures of adults (gametes and gonads) and in the stem cells of the posterior growth zone (Wolfswinkel, 2014; Dannenberg and Seaver, 2018). The expression of PIWI marker genes is mostly restricted to gametogenesis and early embryonic development; however, their expression pattern and function is different in every organism. For example, in the *D. melanogaster* organism, there are three PIWI proteins (i.e., PIWI, Aub, and Ago 3) (Weick and Miska, 2014). Each PIWI protein has its own unique expression pattern. PIWI is expressed in both germ and ovarian somatic cells (OSCs) and localizes in the nuclei of germ and OSCs, while Aub is expressed in the cytoplasm of germ cells and localizes partially in the nuage (Weick and Miska, 2014). In the *C. teleta*, studies have shown that both *Ct-piwi1* and *Ct-piwi2* RNA are expressed throughout the annelid's life cycle in an almost identical pattern in both somatic and germline cells (Giani et al., 2011). However, the localization of the marker genes in somatic cells is not well described.

2.3: PIWI Proteins Function in Flat Worms and piRNA Expression in Capitella teleta

In planarian flatworms, PIWI proteins are essential for regeneration. Planarians require neoblasts, an adult stem cell population, to express three PIWI proteins (Kim et

al., 2020). Neoblasts are characterized by cell division as they are actively undergoing mitosis and they generate a heterogenous population of pluripotent and multipotent stem cells (Kim et al., 2020; Wolfswinkel, 2014). Pluripotent neoblasts can generate all cell types, particularly those that give rise to the germline (Kim et al., 2020). Moreover, planarians maintain pluripotent stem cells throughout each developmental stage (Kim et al., 2020). Neoblasts share similar characteristics with the germline such as perinuclear granules, known as chromatid bodies or the nuage (Wolfswinkel, 2014), and express the germline multipotency program (GMP) genes (Kim et al., 2020). GMP genes prevent somatic differentiation, regulate stem cell division, and protect genomic integrity (Kim et al., 2020). Moreover, GMP expresses key genes (i.e., PIWI, *vasa*, and *Tudor*) that are members of the piRNA pathway (Kim et al., 2020). Thus, the piRNA pathway plays a significant role in cellular pluripotency, germline maintenance, and stemness (Giani et al., 2011).

2.4: PiP-bodies (piRNA and PIWI Localization in Mouse Sperm/Eggs)

As described above, piRNAs have an association with PIWI proteins and specializes in the protection of genome integrity from the adverse effects of transposable elements (Aravin et al., 2009; Yuan and Yamashita, 2010), and are most prominent in germ cells (Wolfswinkel, 2014). The mouse genome encodes three PIWI-like proteins, MIWI (or PIWIL1), MILI (PIWIL2), and MIWI-2 (PWPWL4), all which play an essential role in spermatogenesis (Thomson and Lin, 2009). MIWI is expressed after birth in pachytene spermatocytes and spermatids (Aravin et al., 2009). MILI and MIWI-2 are the only PIWI proteins needed for transposon silencing in fetal gonocytes (Aravin et al, 2009). MILI is expressed in germ cells and continues to persist in adulthood within male

testes and is present exclusively in the cytoplasm in numerous perinuclear granules (Weick and Miska, 2014; Aravin et al., 2009). In contrast, MIWI2 is abundant in gonocyte nuclei but also appears in cytoplasmic granules (Aravin et al., 2009).

2.5: Expression of PIWI genes in Mollusks

PIWI genes and piRNAs are ubiquitously expressed in mollusks, similar to the expression in *C. teleta* due to lineagae-specfic adapataions. In mollusks, Piwi1 and Piwi2 displayed high expression in the reproductive tract of *Lymnaea stagnalis* (Jehn et al., 2018). The same study also reported that PIWI mRNA expression was significantly high in the male gonad for both Piwi1 and Piwi2 in the *Crassostrea gigas* (Jehn et al., 2018). The expression patterns suggested that the PIWI-piRNA pathway is conserved in the germline and soma in mollusks (Jehn et al., 2018). This shows that the *C. teleta* and molluks have similaries in the somatic PIWI/piRNA expression that was established in an early ancestor.

2.6: Immunohistochemistry

Immunohistochemistry (IHC) is a serological technique that utilizes antibodies to visualize the expression of a specific genes of interest. It utilizes a primary and a secondary antibody. The primary antibodies are raised against the target protein whereas the secondary antibodies are raised against the primary antibody and conjugated with chromophore and fluorophore or an enzyme. For this experiment, primary antibodies, piwi1 (guinea pig) and piwi2 (rabbit), and secondary antibodies, goat anti-guinea pig, goat anti-rabbit, and anti-mouse, were utilized. The antibodies were sourced from the ABclonal Technology. 2B10 and ELAV, which are developmental study hybridoma bank antibodies, were also utilized as an interest to see how the well conserved proteins

worked against the antibodies (Table 1). For our experimentation, the host species utilized for the primary antibody was guinea pig and rabbit. This treatment allows for the PIWI proteins to be marked with the primary antibody that can be treated with the secondary antibody for visualizing the protein of interest localization. The secondary antibody utilized was the AlexaFluor® goat anti-rabbit 568nm antibody, AlexaFluor® goat anti-guinea pig 647nm antibody and the AlexaFluor® goat anti-mouse 488nm antibody. The secondary antibodies are created by using the primary antibody as an antigen in a larger host organism (e.g., goat). These antibodies are labeled with fluorophores to allow visualization using fluorescent microscopy. This will stain green at 488nm. The cells were then mounted in DAPI Fluoromount-G®. This mount stains all the nuclei of the cells and is visualized under florescence as blue.

Primary Antibody	**Secondary Antibody**
Piwi1	Goat Anti-Guinea Pig
Piwi2	Goat Anti-Rabbit
2B10	Goat Anti-mouse
ELAV	Goat Anti-mouse

Table 1: Primary and Secondary Antibodies Utilized

It has been observed that the localization of PIWI proteins have different designations within various of organisms; however, their location in *C. teleta* displays a few similarities. The present research the PIWI paralogs responsible for the development in the germline and soma. Specifically, this examined the PIWI proteins localization within somatic tissue that can utilized to expand on present knowledge and can be applied to agriculture research to manipulate earth worm genetics with piRNAs.

Chapter 3: Methodology

3.1: C. teleta Maintenance and Breeding

C. teleta annelids were obtained from Elaine Seaver at the Whitney Laboratory for Marine Bioscience in Florida. The male and female stocks were kept in clear containers in a gross chamber set at 20°C. The annelids were maintained on mud collected from Biloxi Bay. The mud was previously frozen to eliminate unwanted organisms but maintained the microbes within the mud. The annelids were feed once a week with one tablespoon of mud followed by the addition of fresh seawater and then placed back into the chamber. To acquire early cleavage stage embryos, mature male and female annelids are separated by sex. The mating bowls were set up by combining the annelids, male and female, into fresh vials together to reproduce. It took approximately 12 hours for the annelids to lay eggs. After the females reproduced, the annelids were separated again and placed in distinct vials. The annelids sat for 16 hours until they reach cell division and cell morphology arose. As the annelids reproduced, they were carefully removed from the container to avoid overpopulation. When removing, the annelids were placed under the dissecting microscope to ensure that only healthy annelids were placed into the fresh container of mud to be restocked as a new colony. The overall goal was to collect mature male and female worms to use as mating pairs to produce early-stage embryos.

3.*2: Sample Preparation: Dissection and Collection of Embryos*

Using the dissection microscope and a pair of forceps, the annelids were carefully placed into a petri dish individually to dissect open any brood tubes. The mucus and sand

particles were carefully removed using the forceps to avoid any damage to the annelid. Once the embryos were released, they were gently collected using a pipette and immediately transferred to a 0.5 mL eppendorf tube. The embryos were then subjected to the immunohistochemistry process.

3.3: Immunohistochemistry

The collected embryos were suspended in two separate eppendorf tubes, one for 2B10 and one for ELAV, containing 4% PFA and was placed on the laboratory rockers for 30 minutes at room temperature. The PFA solution was removed, and embryos were washed three times in 0.1%PBS-T solution on a rocker for five minutes. After the third wash, 100µl of 0.1% PBS-T solution and 5µl of Goat Serum was added to the tubes to create a 5% Goat Serum and allowed to block for an hour on a rocker at room temperature. Next, 0.3µl of primary antibody was added directly in the PBS-T Goat Serum and placed on the rocker overnight at 4°C.

Upon removal, a repeat wash cycle with PBS-T was conducted five times for five minutes each. 500µl of 0.1% PBS-T solution and 1µl of the secondary antibodies, AlexaFluor® goat anti-rabbit 568nm antibody, AlexaFluor® goat anti-pig 647nm antibody, and the AlexaFluor® goat anti-mouse 488nm antibody, were added and allowed to incubate in the dark for one hour at room temperature. Upon completion of secondary antibody incubation, the embryos were washed with PBS-T three times. After washing, the embryos within the tubes were ready for slide preparation.

3.4: Western Blot

Once the embryos were collected, the sample was prepared by extracting the proteins from the *C. teleta* embryos and determining the protein concentration. An SDS-

PAGE was used to estimate the molecular mass of the protein to determine its abundance within the protein sample. The sample was ran through 12% resolving gel to separate the polypeptides by size. The sample was also then transferred to a PVDF membrane after activation. It was blocked with 5% non-fat dry milk and the primary antibodies (i.e., piwi1 and piwi2) were incubated by dilution with the blocking buffer on the rocker at room temperature for two hours. Next, the membrane was soaked in the HRP-labeled secondary antibodies (i.e., goat anti-mouse and goat anti-rabbit) for conjugation for two hours. Finally, the membrane was visualized by chemiluminescent detection.

3.5: Slide Preparation and Confocal Microscopy

A glass microscopic slide was cleaned with 70% ethanol using a Kimtech wipe. Using a transfer pipette, a small drop of DAPI Fluoromount-G® was placed on the slide. The embryos were gently collected using a pipette without collecting the extra fluid. Once the embryos were placed on the slide, a paper towel was used to remove excess solution and discarded. To place the coverslip properly over the solution, petroleum jelly was added to each four corners and sealed with nail polish. The slides were allowed to dry and placed in box covered with a paper towel until imaged. Slides were visualized using the Leica Sp8 confocal microscope.

Chapter 4: Results

4.1: Western Blot Results

To estimate relative levels of PIWI proteins in C. *teleta* embryos, Western blotting was performed using the antibodies listed in Table 1, positive controls (e.g., Histone H3 and Vinculin), and protein ladders. The controls were used to verify the changes in protein levels, ensure that the blocking was successful, and acted as a benchmark foe quantification. The PIWI paralogs of the two different developmental stages were compared with the protein ladder to estimate the molecular weight. The molecular weight of Piwi1 was estimated at ~96.7 kDa (Figure 5A) in early embryonic stages and larvae, and Piwi2 was estimated at ~59.7 kDa in both stages (Figure 5B). It can be seen that Piwi1 was expressed greater than Piwi2 in early embryos and larvae. The corresponding antibodies detected each piwi paralog in somatic cells in immunofluorescence analyses.

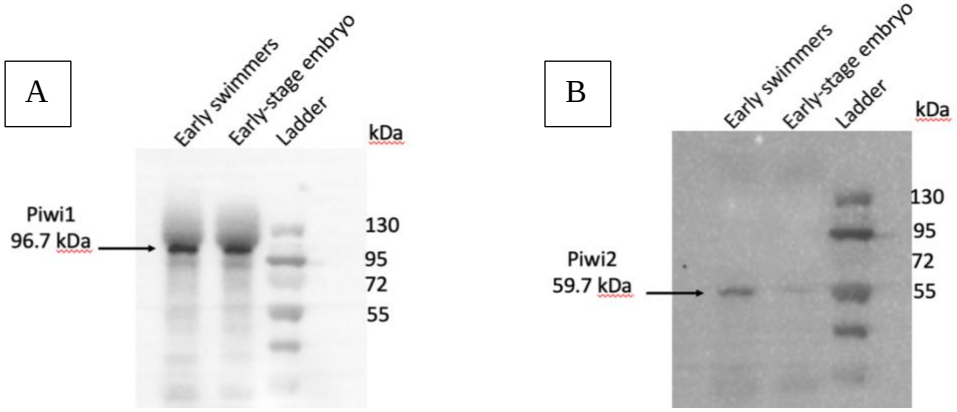

Figure 5: Detection of PIWI proteins in *C. telata* embryos via Western Blot. (A) Expression of Piwi 1 (~96.7 kDa) confirmed in *C. teleta* larvae (lane 1) and early-stage embryo (lane 2) compared to the ladder. (B) Expression of Piwi 2 (~59.7 kDa) confirmed in *C. teleta* larvae (lane 1) and early-stage embryo (lane 2) compared to the ladder.

4.2: Immunohistochemistry Results

Immunohistochemistry was conducted on the embryos with the selected antibodies. Immunofluorescence showed that Piwi1 and Piwi2 were primarily accumulated in the nucleus but were also detected in the cytoplasm. For example, in Figure 6, Piwi1 is visible in nuclei and in cytoplasmic follicles. These results support the hypothesis that PIWI is present in somatic cells within the nuclear region. To further characterize PIWI localization, we also stained larval stage *C. teleta*. Both PIWI paralogs were also expressed in the cytoplasmic region in the larvae stage as seen in Figure 7. This observation confirms that PIWI proteins are detected as cytoplasmic and can be seen in the posterior growth zone of the larvae. In Figure 6C, it can be seen that the Piwi1 is visible in the nuclei and cytoplasmic follicle. These results support the hypothesis that PIWI has present localization in somatic cells within the nuclear region. To further characterize PIWI localization, the two paralogs were also expressed in the cytoplasmic region in the larval stages as seen in Figure 7. This observation confirms that PIWI proteins are detected as cytoplasmic proteins and can be seen in the posterior growth zone of the larvae.

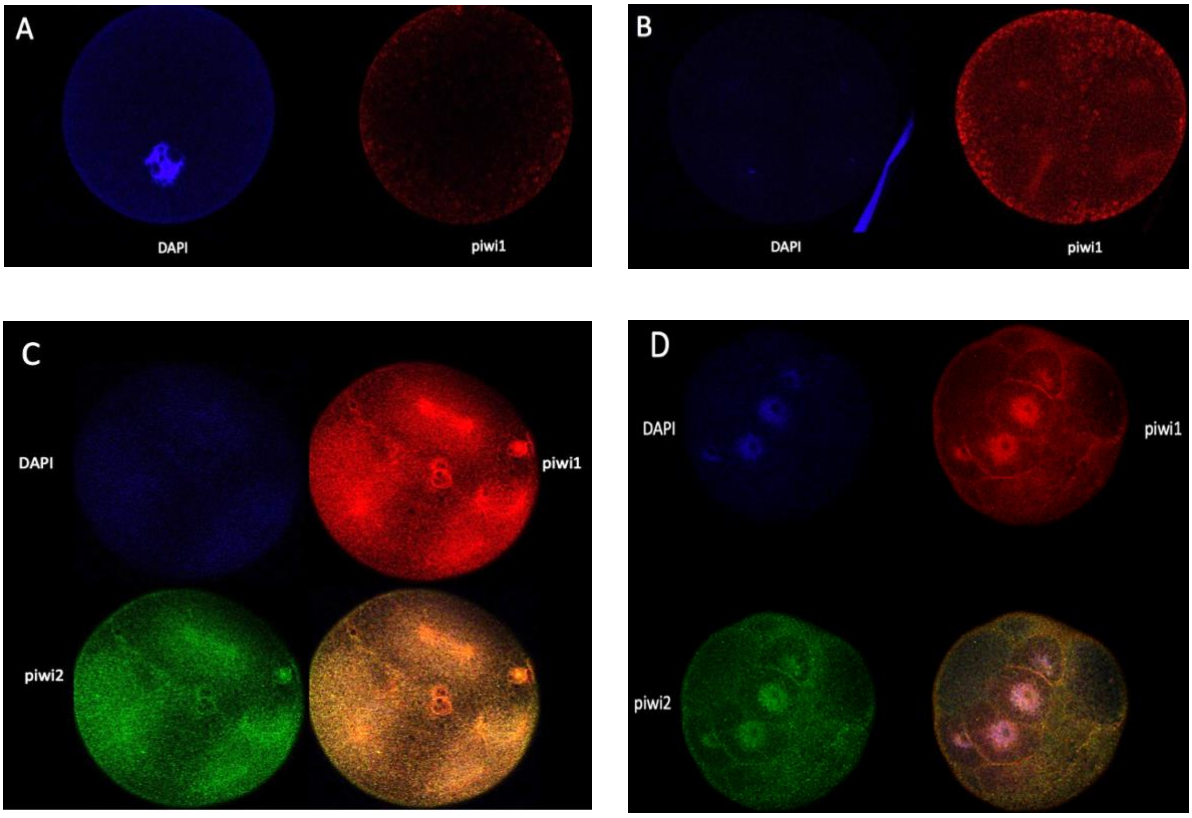

Figure 6: Immunohistochemistry comparison of localization of piwi1 and piwi2 in early Capitella embryos. Embryo slides were created by mating male and female adult annelids. The embryos were dissected at stage 3 cleavage and stained with different antibodies against Piwi1 or Piwi2. Imaginal slides were mounted and imaged using confocal microscopy. Excitation wavelengths used at 488nm (blue), 568 nm (green), and 647nm (red). (A) Staining of single cell embryo using Guinea pig anti-Piwi1 antibody. (B) four cell stage showing expression in the cytoplasm (C) four cell cleavage stage (D) eight cell cleavage stage showing expression prominently in the nuclei and some in the cytoplasm.

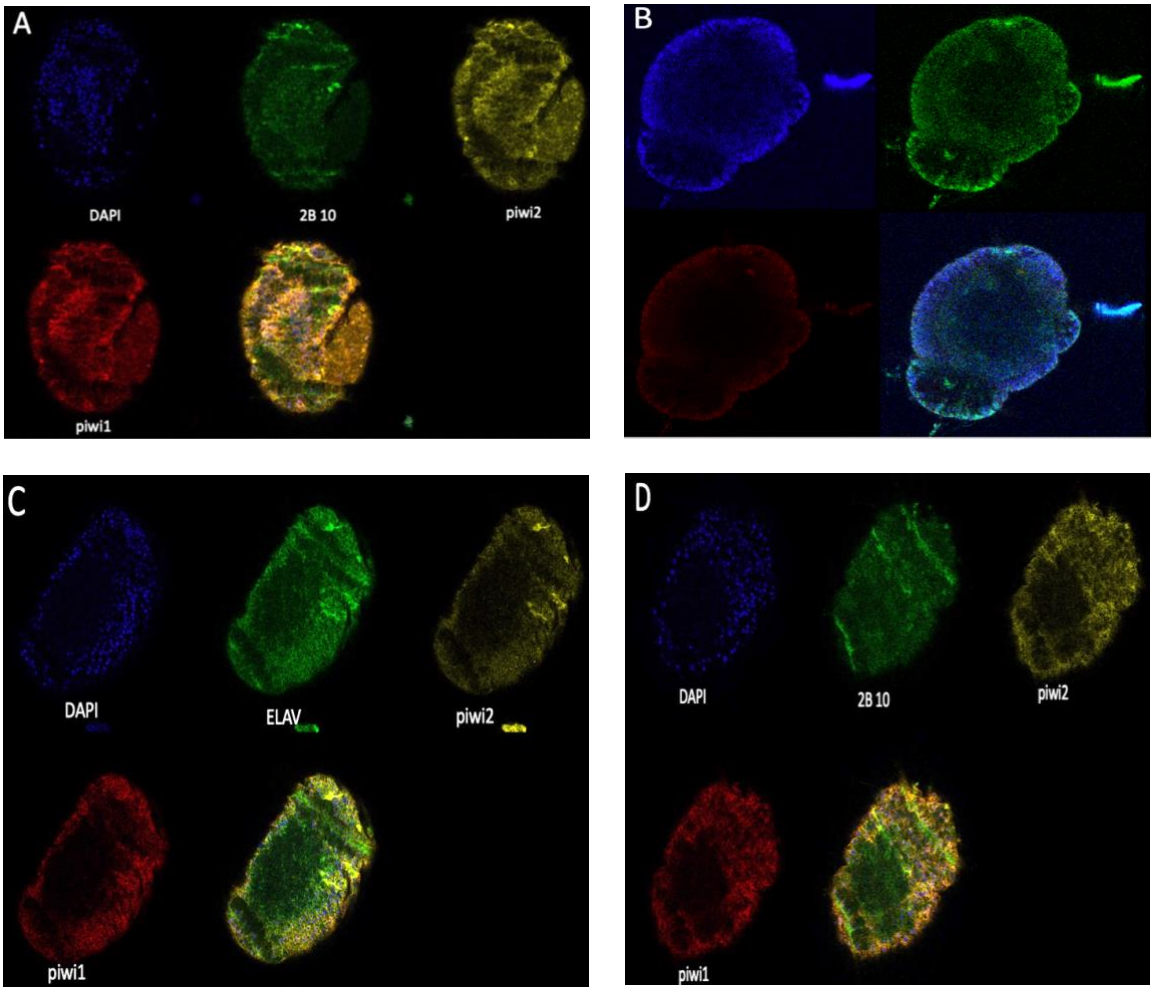

Figure 7: **Immunohistochemistry comparison of larvae stages.** Embryo slides were created by mating male and female adult annelids. The embryos were dissected at stage 3 cleavage and treated with immunohistochemistry utilizing the 4 primary antibodies and secondary antibodies. Images were displayed at 488nm, 568 nm, and 647nm (A) Anterior view of immunohistochemistry comparison of larvae stages (B) Ventral view, with anterior. Both (C) and (D) display immunohistochemistry comparison of larvae stages using DAPI, 2B10 marker protein, piwi1, and piwi2.

Chapter 5: Discussion

The prominent and conserved role of PIWI proteins and piRNAs is to regulate transposon activity. PIWI proteins are complementary to piRNAs and both are involved in cellular biogenesis (Wolfswinkel, 2014). piRNAs are amplified through the alternated catalytic activity of two different PIWI proteins in a mechanism known as the ping-pong cycle (Wolfswinkel, 2014). The ping-pong cycle of piRNA production takes place in the nauage and this is also where most of the PIWI proteins are found. The nuage is RNA enriched that can take many forms such as a diffuse cloud to granules (Weick and Miska, 2014). Previous studies have shown that it is found in the perinuclear region of germline cells and is closely associated with the nuclear pores. Several PIWI proteins have also been detected in the nuclei of germ cells, often during specific development stages. In *D. melanogaster*, PIWI is in the nucleus of germ cell precursors as well as mature germ cells (Yashiro et al., 2018). In the mouse (miwi), PIWI is found in the nuclei of spermatocytes (Aravin et al, 2009). Increasingly, evidence suggests that PIWI proteins and piRNAs function not only in the germline, but also in somatic tissue.

In mice, PIWI-RNA interactions are demonstrated by the MILI and MIWI2 proteins. It was found in a compartmentalization study that the MIWI2 proteins are more present in the gonocyte nuclei yet, they also appear in the cytoplasm (Aravin et. al, 2009). In addition, the MILI protein is present predominantly in the cytoplasm (Aravin et. al, 2009). In the mice, the germline from the beginning of development separates and branches off into the somatic cells which include the normal cells of the body while the germline includes the gonocytes mentioned previously (Aravin et. al, 2009). This is significant because the germline produces the embryos and PIWI proteins have a

significant role in the development of the germline. With the PIWI proteins localizing in the cytoplasm instead of the nuclei more, they will be able to interact with the RNA during transcriptional regulation (Thomson & Lin, 2009).

In the common fruit fly, more specifically, the *D. melanogaster* fly, the localization of PIWI proteins occurs in the nucleus (Thomson & Lin, 2009). In a peer-reviewed article about the PIWI protein in the fly, the focus is placed on the proteins being in the ovarian somatic cells in comparison to the germline cells, although they are also expressed in the germline cells. The significance of the PIWI proteins being localized in the nucleus instead of the cytoplasm is that transcription takes place in the cytoplasm outside of the nucleus and RNAs are responsible for the process where they may be found more in the cytoplasm of somatic cells.

In comparison to localization of PIWI proteins in other organisms, the presence of the cells can be used to draw conclusion with the results from the C. teleta. The results of this study indicate that PIWI proteins are localized prominently in the nuclei and in the cytoplasm of the *C. teleta* during embryonic and larval stages. Piwi1 and Piwi2 are expressed throughout the developmental stages of *C. teleta* that not only includes germline cells, but somatic as well. The two genes showed similar expression patterns to one another. Both genes are broadly expressed during embryonic and early larval stages within the nuclei and gradually become more prevalent in the cytoplasmic region in later stages of development. In larval stages, the localization of the proteins closely corresponds with the primordial germ cells (PGCs) within the descendants of the blastomere in Figure 6 and 7. Since many model systems have fully segregated germlines and lack regenerative capabilities, detailed studies have disproportionately influenced the

views concerning the segregation of the germline from the soma. PGCs are segregated from somatic tissue during early development and are responsible for generating gametes. Moreover, PIWI is commonly known for its role in the germline, but there are examples that shows its expression outside of the germline, such as planarian flatworms that display PIWI proteins expression in the posterior regions (Kim et al., 2020). Our observation adds another example of PIWI proteins being expressed in the cytoplasmic region in larvae.

In conclusion, the results presented in this thesis provide some preliminary but essential understanding of the localization of PIWI proteins within *Capitella*. The results indicate that PIWI is expressed during blastemal growth and later found in the cytoplasmic region and different segments of larvae. Although the functions and localization of PIWI proteins and piRNAs remain unclear, these findings have pointed out that the PIWI-piRNA system has a presence beyond the germline. The information from this study can be applied to agricultural research to help manipulate the genetics (e.g., piRNAs) of earthworms.

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