The Sea Urchin Seawi and Other Argonaute Family of Proteins:

It's a Small RNA World After All

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ABSTRACT OF THE THESIS

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Directors of Development

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A fundamental question in studying embryonic development is to understand the regulation and integration of major developmental events, such as cell proliferation, cell specialization, cell interaction, and cell movement. Regulation of protein transcription, translation, and signaling during development can be modeled by the developmental gene regulatory network (GRN). In the sea urchin GRN, the animal-vegetal axis is established by polarized expression or localization of cell-fate determinants, such as beta-catenin nuclear localization in vegetal cells and bep4 expression in animal cells.

Over the last decade, the discovery of small non-coding RNA as an effector of epigenetic, post-transcriptional, and translational regulation has led

to reconsideration of the GRN. In this thesis, we investigated the role of argonaute/piwi and their association with small non-coding RNA in sea urchin development. We characterized the sea urchin argonaute/piwi proteins, examining their developmental expression profiles, characterizing the proteins present in seawi-associated MT-RNP complexes, and examining the small non-coding RNA populations in eggs, embryos, adult tissues, and MT-RNP complexes.

We show seawi, sea urchin piwi, is expressed in germline lineage and stem cells in development, and seawi predominately associates with piRNA. Within the seawi-associated population of piRNAs, there are specific sets of piRNA sequences, which are complementary to the mRNA of cell-fate determinants bep4 and beta-catenin. These results lead to the potential for small non-coding RNA regulation of GRNs in sea urchin development. We also show that mi- and piRNAs are present in sea urchin sperm. This suggests that sperm has the ability to provide small non-coding RNAs to regulate embryonic developmental events. This finding challenges the current theory of sperm serving only as the delivery system for the paternal genome and provides new insight for a novel paternal regulation.

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ASW	= artificial sea water
ATP	= adenosine triphosphate
BCIP	= bromo-4-chloro-3-indolyl phosphate
BSA	= bovine serum albumin
DTT	= dithiothreitol
EDTA	= ethylene diamine tetraacetic acid
EGTA	= ethylene glycol tetraacetic acid
GRNs	= gene regulatory networks
HB	= homogenization Buffer
IPTG	= isopropyl-beta-D-thiogalactopyranoside
LB	= Luria Bertani broth
NBT	= nitro blue tetrazolium
kDa	= kilodalton
PCR	= polymerase chain reaction
SBTI	= soybean trypsin inhibitor
SDS-PAGE	= sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAME	= p-toluenesulfonyl-L- arginine methyl ester
TBS	= Tris-buffered saline
TBST	= Tris-buffered saline with Tween 20
TRIS	= tris (hydroxymethyl) aminomethane
Tween-20	= polyoxyethylenesorbitan monolaurate
UTR	= untranslated region

CHAPTER 1

Introduction and Literature Review

After fertilization, the zygote (a totipotent stem cell) proliferates through multiple cell divisions before differentiating into different cell types and eventually developing into the adult body. A major goal of studying embryogenesis is to understand the regulation and integration of major development events, such as cell proliferation, cell specialization, cell interaction, and cell movement. The expression of signaling molecules and transcription factors involved in these events can be regulated at multiple levels: transcriptional, post-transcriptional, and translational. Studies of early sea urchin development have served as an excellent model for examining the relationship between molecular regulatory mechanisms and morphogenesis. The timing of early sea urchin development and the ease of experimentally perturbing this development provide an excellent platform for studies of cell specification and morphogenesis.

Sea urchins are members of echinodermata phylum; deuterostomes that are phylogenetically associated with all vertebrate species (Figure 1-1).

Interestingly, the sea urchin is evolutionary more closely related to mammals than other embryological models such as *Caenorhabditis elegans* and *Drosophila melanogaster* (Figure 1-1 Green rectangle). The echinoderm, *Strongylocentrotus purpuratus* (purple sea urchin), has multiple advantages as a model for research in cell and developmental biology. Adult sea urchins are easy to obtain and maintain in the laboratory aquaria, eggs and embryos are easily observed using light microscopy (Figure 1-2A), and millions of gametes can be collected from a single female or male animal. Fertilization is external and early embryogenesis is rapid, embryos can be maintained easily, and millions of synchronously dividing embryos can provide samples for biochemical analysis (Ernst, 1997). Lastly, to date, approximately 90% of the *S. purpuratus* genome has been sequenced and assembled, providing a platform for researchers to analyze gene sequences, genome organization, and data mining (Sodergren et al., 2006).

Sea urchin development and gene regulatory networks

After fertilization, sea urchin embryos undergo rapid nuclear and cytoplasmic divisions, and these divisions result in partitioning the zygote into

smaller blastomeres. The first two cell divisions are meridional and the third division is equatorial, resulting in eight blastomoeres of equal size that are approximately one-eighth the volume of the unfertilized egg. After the fourth cell division there are eight equal size mesomeres at the future animal pole with four macromeres and four micromeres at the future vegetal pole (Figure 1-2B, 16-cell embryo; Figure 1-3A). The fifth division reinforces the asymmetry of the embryo. Micromeres divide into large and small micromeres; the small micromeres will serve as stem cells that give rise to tissues of the adult body plan.

In addition, the embryo must transition from reliance on stored maternal messages to subsequent transcription of embryonic genes starting at the blastula stage (Schier, 2007; Tadros and Lipshitz, 2009). Interestingly, the transition occurs at a point prior to the dramatic onset of tissue differentiation leading to formation of the three germ layers: ectoderm, mesoderm and endoderm. Specification of cell fate in sea urchin embryos involves asymmetric distribution of maternal molecules into specific blastomeres along the animal-vegetal (A-V) axis. Visually the morphogenic axis is clearly established at the 16-cell stage (Figure 1-3A); morphogenic determinants are

segregated asymmetrically at the time of formation of the 8-cell embryo (Angerer and Angerer, 1999; Angerer et al., 2000; Davidson, 1989). Specific blastomeres along this A-V axis are fated to give rise to the ectoderm, the endoderm and the mesoderm. Mesomeres differentiate into ectoderm, macromeres differentiate into endoderm and secondary mesenchyme, and micromeres differentiate into skeletogenic cells and coelomic pouches (Logan and McClay, 1999; Ransick et al., 1996).

In addition to defining the three germ layers, the fifth division leads to specification of the future adult germline stem cells. At this point, the micromeres divide unequally to give rise to four large micromeres (blue) and four small micromeres (green) (Figure 1-3A and B). The small micromeres reside at the vegetal plate of the blastula where they will divide one additional time. These small micromeres descendents are multipotent progenitor cells, or set aside cells (Ransick et al., 1996), which are later carried at the tip of the invaginating gut during gastrulation and are incorporated into the larval coelomic pouches (Figure 1-3B green). The small micromere lineage gives rise to tissues of the adult body plan (Ransick et al., 1996) and to the adult germline (Yajima and Wessel, 2010). Yajima and Wessel (2010) cultured

embryos to adulthood, after removing the small micromeres following the fifth division. The sea urchins developed a normal body plan; however, they possessed small gonads that lacked gametes, indicating the small micromeres serve as the stem cell population need for the adult germline (Yajima and Wessel, 2010).

Eric Davidson and colleagues (Davidson and Levine, 2008; Davidson et al., 2002b; Lee et al., 2002; Lee et al., 2006; Oliveri and Davidson, 2004; Peter and Davidson, 2009) pioneered the work leading to our present standard understanding of how regulation of protein transcription, translation and signaling is functionally integrated in a developing embryo. The essence of their work is described by the developmental regulatory networks (GRNs), which correlate the timing of expression of signaling and transcription factors with embryonic development. The GRNs can be applied to developmental models such as nematode worms, fruit flies, sea urchins and mice (Davidson and Levine, 2008; Lee et al., 2002; Lee et al., 2006; Oliveri and Davidson, 2004; Ririe et al., 2008). GRNs provide a circuit model to describe a control system to regulate gene sets that are expressed in specific spatial and temporal patterns in development, which in turn influence morphogenesis and

differentiation. One of the major features of GRNs in animal development is the importance of morphogens and cell-fate determinants. A morphogen is a signaling molecule that governs the pattern and the position of cells during morphogenesis by using a concentration gradient to signal generation of different cell types in distinct spatial and temporal order (Driever and Nusslein-Volhard, 1988; Lehmann and Nusslein-Volhard, 1991). In fruit flies, classic examples of morphogens are bicoid, nanos and oskar that establish the developmental axes of embryos and set-up the segmentation pattern. (van Eeden and St Johnston, 1999; Zamore and Lehmann, 1996). Nanos protein regulates the establishment of anterior-posterior (A-P) axis of the fruit fly embryo by forming a protein gradient along the A-P axis with nanos expression highest in the posterior of the embryo (Lehmann and Nusslein-Volhard, 1991; Wang and Lehmann, 1991). Nanos regulates translation of downstream segmentation genes to establish a proper body plan (Lehmann and Nusslein-Volhard, 1991; Zamore and Lehmann, 1996). The GRN provides a framework to visualize the complexity of spatial and temporal functional coordination among transcription factors, signaling molecules, and maternal mRNA.

In the sea urchin GRN (Davidson et al., 2002a; Ransick et al., 1996), A-V axis formation is triggered by cell-specific expression of vegetal signaling molecules (VSMs). In particular, VSMs are expressed in cells of small and large micromere lineage, leading to nuclear localization of beta-catenin in the vegetal cells of 16-cell embryo (Figure 1-3A, VSM) and subsequent modification of the protein expression profiles. Preventing Beta-catenin nuclear localization in vegetal cells by overexpressing cadherin disrupts vegetal cell-fate determination and A-V axis formation (Logan et al., 1999; Ransick et al., 2002). Notch expression and signaling in large micromeres is required for secondary mesenchymal fate determination and endoderm differentiation (Sherwood and McClay, 1999) and Wnt-8 signaling, which promotes the mesendoderm fate and downregulates the expression of animal transcription factor (ATFs) such as bep4 and BMP 2/4 in vegetal cells. The competition between nuclear beta-catenin and ATFs further specify the endoderm-ectoderm border (Angerer and Angerer, 2000; Angerer et al., 2000) and to specification of secondary mesenchymal cells during archenteron formation. ATFs that are expressed in animal poles are required for animal hemisphere establishment and ectoderm differentiation (Figure 1-3 A, ATFs)

(Angerer et al., 2000; Kenny et al., 2001; Kenny et al., 1999; Romancino et al., 2001). After the establishment of the A-V axis, there is a sequential signaling cascade to provide cell identity for different germ layers. Therefore, post-transcriptional and translational regulation of the maternal mRNA of such cell-fate determinants is crucial for early development.

Over the last decade, the discovery of small non-coding RNA as effectors of epigenetic, post-transcriptional and translational regulation has led to reconsideration of the GRN. As an example, the piRNA family of small non-coding RNAs has been shown to regulate expression of the maternal morphogen nanos by inhibiting mRNA translation in fruit fly (Rouget et al., 2010). Other examples include miR-430-dependent degradation of maternal mRNAs during the maternal-zygotic transition in zebrafish, and lin-4 and let-7 control of developmental timing of the larval 3 to larval 4 transition. These examples indicate that the large spectrum of roles small non-coding RNAs have in guiding the regulatory pathways that underlie functional application of the GRN to early development. Further, the functionality of small non-coding RNAs is dependent upon the activity of the argonaute family of proteins. Interestingly, Hamill et al. (Hamill et al., 1994) were able to reconstitute a

ribonucleoprotein complex from eggs and two-cell sea urchin embryos that contained five specific mRNAs, one of which was later identified as *bep4* (Romancino et al., 2001) a key player in A-V axis specification. The result is all the more intriguing because the isolated ribonuclear complexes maintained the mRNAs in a translationally-arrested state that could only be lifted by purification of the mRNA away from the complex. The mechanism of this translational arrest remains unknown.

The argonaute/piwi family of proteins and their role in small non-coding RNA function

Argonaute/piwi

As previously described, small non-coding RNAs have epigenetic, post-transcriptional, and translational silencing effects that are important for embryonic development. Large scale cDNA sequencing and genome tiling array studies have shown that around 50% of genomic DNA in humans is transcribed, of which less than 2% is translated into proteins and the remaining 98% is non-coding RNAs (Consortium, 2004). Essential to small non-coding RNAs processing and function is their interaction with the

argonaute protein family.

The argonaute family of proteins is divided into two major evolutionary conserved subfamilies – the argonaute subfamily and the piwi subfamily. Further, the argonaute subfamily can be divided into argonaute 1 and 2 clades and similarly piwi is divided into piwi and piwi-like clades; all of them share highly conserved functional domain structures. Argonaute/piwi proteins are a major component of RNA-induced silencing complexes (RISCs), which silence RNA transcripts by either cleaving mRNA or blocking translation (Parker et al., 2004; Peters and Meister, 2007). Argonaute/piwi have also been shown to regulate DNA methylation, which results in altering gene transcription (Chen et al., 2012; Rajasethupathy et al., 2012; Siddiqi and Matushansky, 2011).

Argonaute, the founding member of the family, was first identified in *Arabidopsis* as playing a role in controlling leaf and flower development (Bohmert et al., 1998). The *Ago1* mutant leaf phenotype resembles the tentacles of small octopus *Argonauta*, thus the name argonaute (Bohmert et al., 1998). Gene inactivation studies revealed that argonaute family members are required for embryonic development and cell differentiation (Peters and

Meister, 2007). Argonaute proteins have been identified from diverse organisms ranging from archaea to humans (Ghildiyal and Zamore, 2009; Sharma et al., 2001; Wei et al., 2012; Zhao et al., 2012).

Piwi, the founding member of piwi subfamily, was first identified by its knockout phenotype in fruit fly -- p-element induced wimpy testis (Cox et al., 1998) – and mutations in piwi resulted in depletion of germline stem cells (Cox et al., 1998; Cox et al., 2000). Mutation of aubergine, another piwi subfamily member, results in disruption of gametogenesis in *Drosophila* (Harris and Macdonald, 2001) and mutation of mouse piwi (miwi) results in spermatogenic cell arrest (Deng and Lin, 2002). These gene inactivation studies suggest that piwi proteins function in maintaining germline stem cells in the embryo and gametogenesis in the adult germline. Piwi expression has been identified across the animal kingdom from nematode worms to humans (Cox et al., 1998; Kuramochi-Miyagawa et al., 2001; Rodriguez et al., 2005; Tan et al., 2002; Wang and Reinke, 2008).

Structurally, argonaute/piwi proteins possess four distinct structural domains: amino terminal, PAZ, middle, and PIWI. Although it has no defined function, the amino terminal domain is thought to be a protein-protein

interaction domain and deletion of the N-terminal domain of ago1 blocks ago1-polyribosome association (Shi et al., 2004). The centrally positioned PAZ domain, consisting of about 100 amino acids, has a RNA binding surface that contains specific positively charged amino acids that are extraordinarily conserved throughout the argonaute family. Structural and biochemical studies of the PAZ domain revealed that it binds small RNA with a 1:1 stoichiometry, and in the specific case of argonaute 2, the PAZ domain can bind the 2-nucleotide 3'-overhang of the siRNA duplex, thus facilitating the anchoring of this guide RNA into the RISC complex (Ma et al., 2004; Yan et al., 2003).

Structural and bioinformatic studies suggest that the PIWI domain – composed of approximately 300 amino acids -- shares similarities with RNase H and the RNase III family of enzymes (Liu et al., 2004), which led to the discovery that argonautes are the sole proteins required to target mRNA for cleavage in RISCs in both the argonaute and piwi subfamilies (Parker et al., 2004; Rand et al., 2004). The N-terminal 138 amino acids of the PIWI domain are responsible for mRNA binding (Kuramochi-Miyagawa et al., 2001). The organization of PIWI and PAZ domain creates two positively charged grooves

that may be nucleic acid binding sites (Liu et al., 2004; Tolia and Joshua-Tor, 2007). Proper structural orientation of the mRNA and small non-coding RNA binding is required for recognizing and silencing specific mRNAs. The middle domain, which has structural homology to the sugar-binding domain of the *lac* repressor (Tolia and Joshua-Tor, 2007), has been shown to be crucial for facilitating PAZ-bound small non-coding RNA recognition and silencing of PIWI-bound target RNA (Jinek and Doudna, 2009; Tolia and Joshua-Tor, 2007).

Argonautes and piwis are associated with multiple RNA and ribonucleoprotein (RNP) complexes, which allows them to perform versatile functions in different cell types. Argonautes have been identified in RISCs (Sontheimer and Carthew, 2004), miRNA associated RNPs (miRNPs) (Easow et al., 2007), stress granules (Leung et al., 2006) and processing bodies (Liu et al., 2005). Cytoplasmic RNPs store specific mRNAs for response to cellular or environment changes, allowing rapid translation of proteins without the need for transcription. Piwis have been identified in polar granules (Megosh et al., 2006) and chromatoid bodies (Kotaja et al., 2006), RNPs specific for the germ cell lineage.

Argonaute and small non-coding RNA

Two of the defining features of small non-coding RNAs are their short length and their association with the argonaute family of proteins in ribonucleoprotein complexes. In animals, small non-coding RNAs include small interfering RNA (siRNA), microRNA (miRNA) and piwi-related RNA (piRNA) (Figure 1-4), each of which interacts with specific argonaute family members. For example, in fruit flies, five genes encode distinct members of the Argonaute family: *ago1, ago2, aubergine* (*Aub*), *piwi*, and *ago3*. Ago1 and ago2 constitute the Argonaute (AGO) subfamily and bind microRNA (miRNA) and small interfering RNA (siRNA), respectively (Figure 1-4; A-a siRNA and A-b miRNA) (Ghildiyal and Zamore, 2009). Aub, piwi, and AGO3 belong to the PIWI subfamily of the Argonaute family, bind to piRNA and are enriched in germline cells (Figure 1-4A-c) (Williams et al, 2002). The next section will discuss how three populations of small non-coding RNAs are generated and what their possible functions are in development.

Small interfering RNA (siRNA)

SiRNAs are 21 nucleotides-long transcripts that mediate RNA

interference (RNAi) (Figure 1-4B siRNA). RNAi is a gene silencing process that is induced by exogenously delivered double-stranded RNA and results in mRNA degradation (Zamore et al., 2000). The siRNAs are incorporated into RISCs, where they recognize complementary target mRNAs and cause the degradation of target mRNAs. In addition to exogenously delivered siRNAs, it is now appreciated that siRNA biogenesis (Figure 1-4 A-a) can be triggered by either exogenous or endogenous dsRNAs, which are later cleaved into double-stranded siRNAs by dicer, an RNase III enzyme. The cleaved products are 21 nucleotide-long double stranded RNA, bear 5' phosphate and 3' hydroxyl groups, and possess two-nucleotide overhangs at the 3' end (Elbashir et al., 2001a; Elbashir et al., 2001b; Zamore et al., 2000). In fruit flies, target mRNA degradation by siRNA is mediated by argonaute 2 in the RISC complex (Hammond et al., 2000). Specifically, the strand that directs silencing is called the guide strand, whereas the remaining strand, which is later destroyed, is called the passenger. To form mature mRNA competent RISC, the argonaute protein cleaves and releases the passenger strand. The target mRNA is then recognized by the complementary guide strand and is cleaved by argonaute 2 (Matranga et al., 2005; Rand et al., 2005).

Loss-of-function, overexpression, and rescue of gene expression are common approaches to study the biological function of genes. Since introducing siRNA into eggs or embryos leads to the degradation of target mRNAs, siRNA has been developed into a powerful tool for *in vivo* loss-of-function studies during embryonic development. In *C. elegans*, for example, thousands of genes have been studied by analyzing knock-out phenotypes during embryogenesis (Fernandez et al., 2005; Sonnichsen et al., 2005). The recently discovered endogenous siRNAs (endo-siRNA) were mapped to specific mRNA and transposon sequences (Fagegaltier et al., 2009; Nilsen, 2008). This endo-siRNA is required for robust development and germline establishment in *Drosophila* and *C. elegans*, respectively. These results suggest that endo-siRNA may play a role in development.

Micro RNA (miRNA)

MiRNAs are endogenously expressed 21-nucleotide RNAs that mainly repress gene translation in animals (Figure 1-4B miRNA). Though mi- and siRNA are both 21 nucleotides long and are processed by dicer, they possess two major differentiating properties. First, siRNAs and miRNAs interact with

different argonautes in RISC, for example in fruit fly, miRNAs are associated with argonaute 1 and siRNAs with argonaute 2 (Tolia and Joshua-Tor, 2007). Second, siRNAs are fully complementary to their target mRNA, which results in degradation of the target mRNA, whereas miRNA partially pairs with its target mRNA, which results in repressing translation of mRNA without transcript degradation (Figure 1-4B) (Lewis et al., 2003).

During miRNA biogenesis (Figure 1-4A-b), primary miRNA transcripts (pri-miRNA) are endogenously transcribed by RNA polymerase II (Lee et al., 2004). In the nucleus, pri-miRNAs are cleaved by drosha and are exported to the cytoplasm by exportin-5 as pre-miRNA (Lee et al., 2003; Wang et al., 2011; Yi et al., 2003). In fruit fly, the endoribonuclease dicer then cleaves pre-miRNA, loads miRNA onto argonaute 1, and destroys the passenger strand of miRNA, which leads to assembly of a mature RISC (Jiang et al., 2005). In worms, fruitflies, mice, and humans, there are a few examples where miRNAs are fully complementary to their target and, as with siRNA, the miRNA functions in cleavage of mRNA (Lai, 2002). Most miRNAs pair with their target through a limited 5' "seed region" of sequences, which are located at positions 2 to 7 in the miRNAs. Binding of the seed region of miRNA to its

target mRNA represses the target mRNA translation instead of cleaving it (Figure 1-4B) (Lewis et al., 2003).

There are multiple examples of miRNA having a role in development. In *C. elegans*, the first discovered animal miRNAs, lin-4 and let-7, control the timing of larval development (Lee et al., 1993; Wightman et al., 1993). In fruit flies, Bantam, miR-2, miR-6 and miR-14 regulate tissue growth by inhibiting apoptosis and promoting cell proliferation (Brennecke et al., 2003; Leaman et al., 2005; Xu et al., 2003). Important signaling pathways, such as the Notch and epidermal growth factor (EGF) pathways, are also under the control of small non-coding RNAs. In fruit flies, miR-2, miR-4, miR-6, miR-7, miR-11 and miR-79 target more than 40 mRNAs that are downstream of the Notch signaling pathway. Misexpression of these miRNAs results in many defects associated with loss of Notch signaling (Lai et al., 2005). In fruit flies, the differentiation of photoreceptor cells relies on EGFR-induced repression on transcription factor Yan. MiR-7 is shown to be the key player to repress the translation of Yan mRNA in photoreceptor cells (Li and Carthew, 2005). In zebrafish, miR-430 promotes the degradation of maternal mRNAs after maternal/zygotic transition (Giraldez et al., 2006).

Piwi-related RNA (piRNA)

PiRNAs are small non-coding RNAs that are 24 to 30 nucleotides long and interact with the piwi subfamily of argonaute proteins (Figure 1-4 A-c and B piRNA). Piwi and piRNA association has been shown to regulate gene expression, maternal mRNA stability, and transposon activity (Houwing et al., 2007; Klenov et al., 2011; Rouget et al., 2010). PiRNAs exhibit regulatory effects on at least two distinct levels, epigenetic regulation of transcription and translational regulation by effecting mRNA stability (Denis et al., 2011; Rouget et al., 2010).

Besides size differences among si-, mi-, and piRNAs, piRNAs have four highly characteristic and conserved properties. As opposed to siRNA and miRNA, piRNA biogenesis is dicer-independent and piRNAs are 2'-O-methylated at their 3' termini. PiRNAs that associate with piwi usually have a 5' terminal uracil nucleotide whereas those that bind to ago3 show no 5' bias (Ghildiyal and Zamore, 2009). Si- and miRNAs target exons and UTRs of mRNA, but piRNA can target exons, UTRs, introns, and other non-coding regions between genes (Aravin et al., 2003; Lau et al., 2009; Lau et al., 2006).

Based on their genome locations, piRNAs can be classified as intergenic,

intragenic, or exonic. Exonic piRNA sequences are complementary to mRNA sequences, so far, have only been identified in frogs and mammals (Aravin et al., 2003; Lau et al., 2009; Lau et al., 2006). To date, the exonic piRNA silencing function has yet to be examined, but since its sequences are complementary to mature mRNA, exonic piRNA are speculated to play a role in mRNA regulation. The other two groups of piRNAs, intergenic and intrageneic, both cluster within non-coding regions of the genome (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006a; Lau et al., 2006; Watanabe et al., 2006).

The current model for piRNA biogenesis was revealed by large-scale piRNA sequencing of piwi- and Ago3-associated piRNA from fruit flies and miwi- and mili-associated piRNA from mice (Figure 1-4A-c) (Brennecke et al., 2007; Grivna et al., 2006a; Lau et al., 2006). PiRNAs with antisense sequences to transposons are bound to piwi, whereas piRNAs with sense sequences or sequences that are identical to target sequences are loaded onto Ago3 and/or piwi-like proteins. The piRNA amplification mechanism is modeled as “ping-pong cycles” (Brennecke et al., 2007). In ping-pong cycles, the antisense piRNA is first generated and used for recognition of target RNA

and results in target RNA cleavage. The cleaved 24 to 30-nucleotide target RNA were then modified into new sense piRNA, which is used to generate more copies of secondary antisense piRNA. Thus, regardless of whether original piRNA sequences are sense or antisense to their target, the ping-pong amplification model creates piRNAs that may have potential roles in RNA and/or DNA regulation.

In embryogenesis, there are three lines of evidence that directly or indirectly show that piRNA and piwi have the ability to regulate mRNAs. Deep-sequencing of piRNAs in *Xenopus* oocytes and eggs suggests that 17% of piRNAs target maternal mRNAs in egg (Lau et al., 2009). Recently in fruit fly, piRNA and piwi proteins were shown to regulate the degradation of maternal nanos mRNA, which is involved in A-P axis specification (Rouget et al., 2010). Two specific piRNAs were shown to regulate nanos mRNA stability and removal of piRNA target sequences from the 3'UTR of nanos mRNA resulted in the disruption of A-P formation (Rouget et al., 2010). Esposito et al. (2011) demonstrated that overexpressing intrageneic piRNAs that target intron 1 of the melatonin receptor 1A (MTNR1A) results in the knockdown of melatonin receptor mRNAs through an, as yet, unknown pathway. This new finding

suggests a novel regulatory mechanism for intragenic piRNAs to function in down-regulating mRNA.

piRNA's role in regulating transposable elements in germlines and gametogenesis

In humans, transposable element sequences occupy roughly 45% of the genome (Consortium, 2004). Three percent of the genome codes for DNA transposons and 42% code for retrotransposons (Wicker et al., 2007; Xie et al., 2006). DNA transposons, which are also called "jumping genes", change their position in the genome of individual cells; Barbara McClintock's discovery of jumping genes in maize earned her a Nobel Prize in 1983. Transpositions are catalyzed by various types of transposase enzymes that copy, cut, and paste transposable elements (Deininger and Batzer, 2002; Singer, 1995). Retrotransposons are replicated through generating an RNA intermediate. The retrotransposon RNA is transcribed into DNA by the enzyme retrotranscriptase. To successful duplicate or propagate themselves, the transposons have to "jump" in cells that are contributed to the next generation, that is germ cells. Therefore, the genome of germ cells is a major target for

transposon amplification. Protection of the genome in germ cells from transposon duplication and insertion is essential for the maintenance of genome integrity.

In mammals, the first large-scale deep-sequencing of piRNAs from rat testes showed that even though 65.8% of piRNAs target non-annotated genome regions, 20% of the unique piRNA sequences are complementary to repeated sequences of transposable elements and less than 1% are complementary to mRNA (Lau et al., 2006), suggesting that the major function of piRNA in adult germline is regulating transposable elements. In fruit fly, piRNA sequences are shown to be complementary to the both the jumping sequences and the coding sequences of enzymes which responsible for transposition, such as stellate, roo and gypsy (Pelisson et al., 2007; Vagin et al., 2006). This sequence complementarity is further demonstrated in nematodes, fruit flies, silkworms and mice (Das et al., 2008; Pelisson et al., 2007; Tatsuke et al., 2009; Xu et al., 2008).

To date, piRNAs have been shown to regulate transposable elements through multiple mechanisms: RNA cleavage of transposon sequences and of mRNAs that code for essential proteins for transposition, and epigenetic

methylation of DNA. As discussed in the earlier argonaute/piwi section, the RNase motif within the PIWI domain is essential for RNA degradation activity. Removing the RNase motif of miwi2 disrupts cytoplasmic localization and piRNA production, leading to increased transposon activity (Aravin et al., 2009). The digested products of transposable element transcripts are detected in wild type fruit fly, and non-digested transcripts are accumulated in piwi and aub mutants (Tchurikov and Kretova, 2011). The novel function of piRNA is an epigenetic effect, which also regulates transposition of transposable element in germline. The loss of mili and miwi2 activates retrotransposon expression by impairing epigenetic DNA methylation in the regulatory regions of retrotransposon LINE-1 (Kuramochi-Miyagawa et al., 2008).

Gametogenesis, separated by gender into spermatogenesis in males and oogenesis in females, is the process by which diploid germline stem cells undergo cell division and differentiation to form mature haploid gametes. During early mouse embryonic development, primordial germ cells (PGCs) begin to migrate to the gonadal ridge. They multiply by mitosis and once they

have reached the gonadal ridge in the late embryonic stage, they are named gametogonia (McLaren, 1998). In adult germlines, gametogonia first undergo a mitotic division and one of the progenitor cells (type A gametogonia) remains as a stem cell, while the other (type B gametogonia) differentiates into a primary gametocyte through meiosis (De Felici, 2010; Dym, 1994; Dym et al., 2009). Each primary gametocyte duplicates its DNA and subsequently undergoes meiosis I and II to produce gametes (Cavicchia and Dym, 1978; Harris, 1978). In males, the spermatids then undergo spermatogenesis to produce spermatozoa (mature sperm) (Cavicchia and Dym, 1978). In female mammals, the primary oocytes are arrested in the diplotene stage of prophase I (the prophase of the first meiotic division). Shortly before birth, all the fetal oocytes in the female ovary have attained this stage. One of the successors from each oogonium later matures into an egg and the remaining divide into polar bodies (De Felici et al., 2005). However, in some marine animals such as the sea urchin, oogenic cells complete both meiosis I and II before being arrested as mature eggs (Chatlynne, 1969; Harris, 1978).

In fruit fly, *piwi* mutation results in reducing gamete production in both

genders (Cox et al., 1998; Cox et al., 2000). Ovaries in fruit fly having mutant *piwi* contain a normal number of germline stem cells at the beginning of oogenesis; however the mature adult ovary has fewer gametes and no longer contains germline stem cells (Cox et al., 2000). In testes, the *piwi* mutants also show male infertility due to severe defects in spermatogenesis (Saito et al., 2006). In zebrafish, frogs and mice, knocking out piwi and piwi-like proteins only affects spermatogenesis but not oogenesis (Kuramochi-Miyagawa et al., 2001; Lau et al., 2009; Tan et al., 2002; Wilczynska et al., 2009). Specifically, in *mili* knockout mice, the spermatogenic cells are arrested at prophase during meiosis II, and in *miwi* knockout mice, cells are arrested at the spermatid stage (Kuramochi-Miyagawa et al., 2001). These data provide evidence for suggesting that piwi/piwi-like proteins and their association with piRNAs are required for maintaining gametogenesis across all animal species.

Seawi and sea urchin embryogenesis

Our lab has previously identified seawi, an argonaute family member (Rodriguez et al., 2005). Seawi is evolutionarily more closely related to

mammalian piwis than either insect or nematode piwis, which serves as an example of the overall evolutionary relationship between echinoderms and mammals (Seto et al., 2007). Seawi is differentially expressed and localized during axis specification, contains the highly conserved PIWI and PAZ protein domains, is a component of reconstituted MT-RNPs, and specifically binds bep4 mRNA in an *in vitro* binding assay (Rodriguez et al., 2005). The identification of seawi and its identification as a component of translationally arrested mRNA-containing complexes known to contain cell-fate determinants (bep4) raises the possibility that sea urchin argonautes/piwis and their associated small non-coding RNAs may have a central role in the function of the urchin GRN. In this thesis, I expand the prior investigation of seawi to now include all members of the argonaute/piwi family (Chapters 2 and 3), initiate an analysis of small non-coding RNAs expressed in egg, embryo and adult (Chapter 4), and explore the possibility that sea urchin small non-coding RNAs function within the context of the GRN (Chapters 5 and 6).

Figure 1-1. The evolutionary relationships among the major animal phyla.

An example of metazoan phylogenetic tree, deuterostome phyla are shown in green, and protostome phyla are further separated into ecdysozoa in red and lophotrochozoa in blue. The sea urchin, echinodermata, is evolutionarily closer to human and mammals than the fruit fly or nematode worm is to humans and mammals. Sea urchin and mammals are circled in green rectangle. Modified from Juliano et al., 2010.

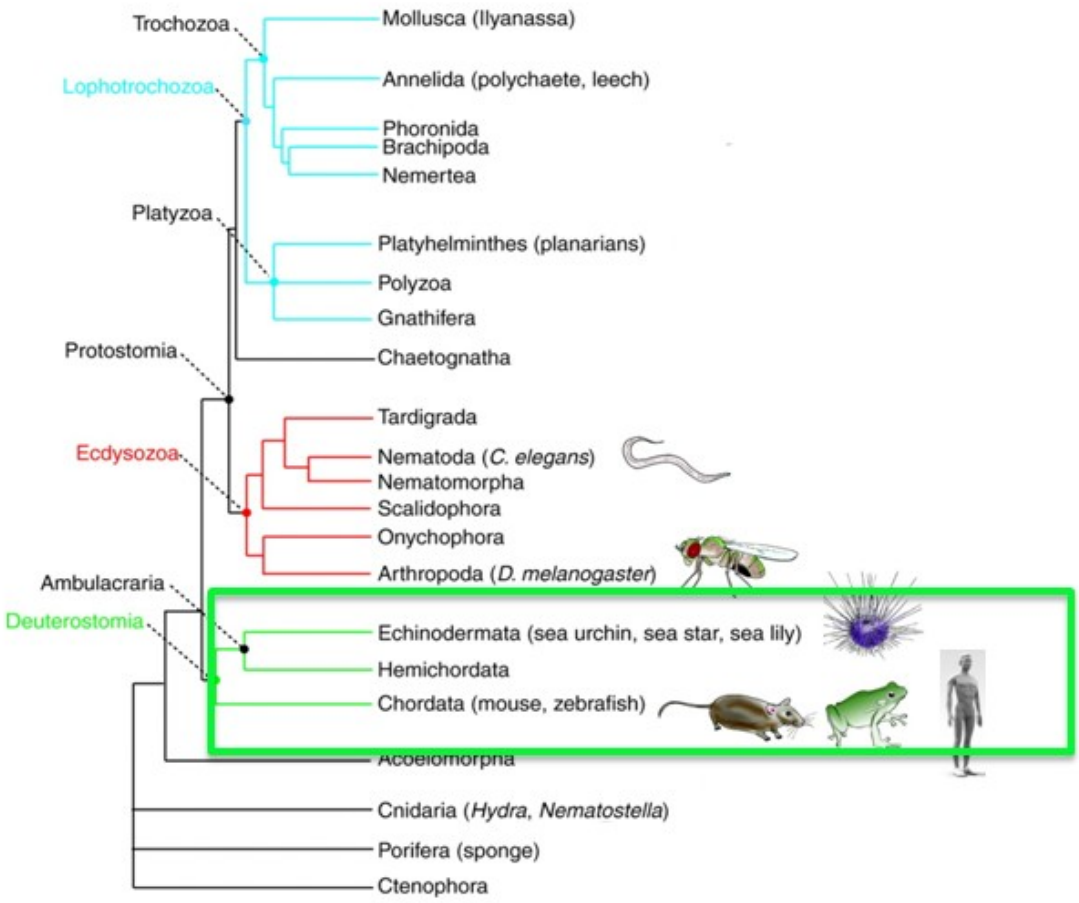
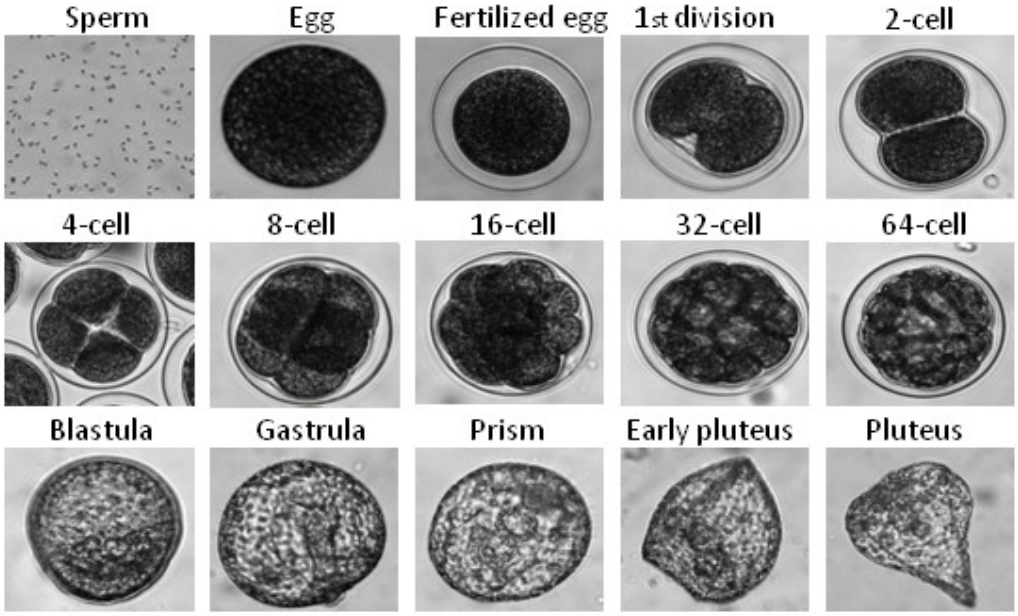

Trochozoa
Lophotrochozoa
Platyzoa
Protostomia
Ecdysozoa
Ambulacraria
Deuterostomia
Mollusca (Ilyanassa)
Annelida (polychaete, leech)
Phoronida
Brachipoda
Nemertea
Platyhelminthes (planarians)
Polyzoa
Gnathifera
Chaetognatha
Tardigrada
Nematoda (C. elegans)
Nematomorpha
Scalidophora
Onychophora
Arthropoda (D. melanogaster)
Echinodermata (sea urchin, sea star, sea lily)
Hemichordata
Chordata (mouse, zebrafish)
Acoelomorpha
Cnidaria (Hydra, Nematostella)
Porifera (sponge)
Ctenophora

Figure 1-2. Sea urchin embryonic development.

(A) Light micrographs of stage of sea urchin embryonic development. (B) A schematic of the early stages of sea urchin development during which the small micromere lineage (red) is used to develope into a stem cell niche. The vegetal fourth cleavage division is unequal, thus giving rise to a 16-cell embryo with four micromeres (red). The micromeres divide unequally to give rise to four large micromeres (blue) and four small micromeres (green). The small micromeres reside at the vegetal plate of the blastula where they divide one additional time. The eight small micromere descendents are carried at the tip of the invaginating gut during gastrulation and upon larva formation, are incorporated into the larval coelomic pouches, the site of adult rudiment formation. The small micromere lineage gives rise exclusively to tissues of the adult. Figure 1-2B is adapted from Juliano et al., 2006.

A.


Sperm
Egg
Fertilized egg
1st division
2-cell
4-cell
8-cell
16-cell
32-cell
64-cell
Blastula
Gastrula
Prism
Early pluteus
Pluteus

B.

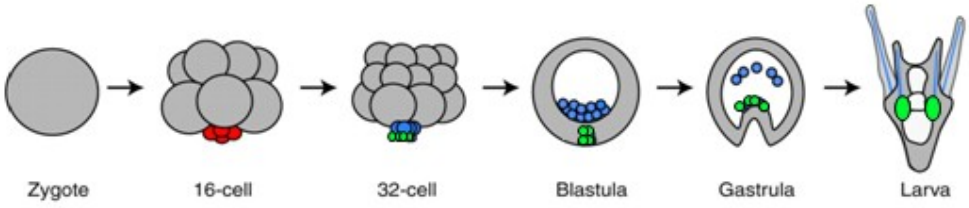

Zygote
16-cell
32-cell
Blastula
Gastrula
Larva

Figure 1-3. Patterning along the A-V axis.

(A) At the 16-cell stage, maternal mechanisms establish distinct combinations of vegetal beta-catenin and animal transcription factor activities in micromeres, macromeres, and mesomeres. Animal transcription factors specify mesomeres as pre-ectoderm (blue). Beta-catenin is required in micromeres (red) for both their signaling function and their differentiation. The combination of beta-catenin and the animal transcription factors begins to specify macromeres as mesendoderm (purple). (B) Between the 16- and the 32-cell stages, micromeres signal overlying macromeres via a Notch pathway and also begin to express SpWnt8. Beta-catenin enters nuclei of macromere progeny cell autonomously. Panel B is adapted from Angerer and Angerer, 2000.

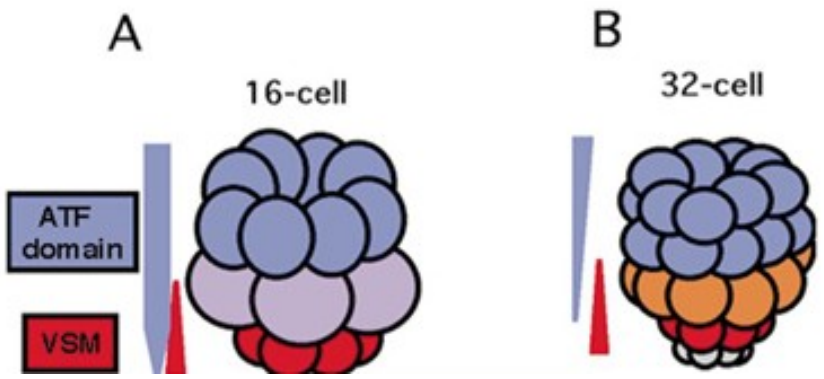
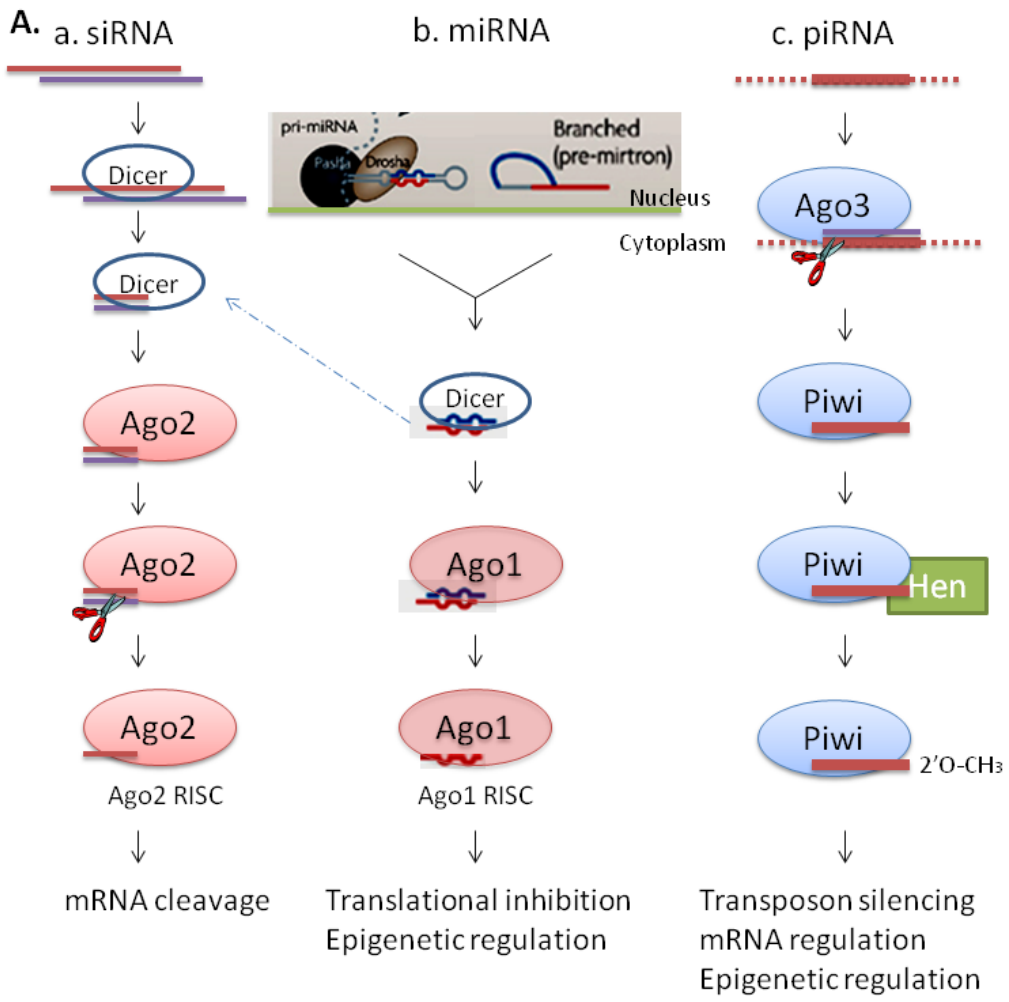

A
16-cell
ATF
domain
VSM
B
32-cell

Figure 1-4. Small non-coding RNA biogenesis and function.

A. The illustration of siRNA (A-a), miRNA (A-b) and piRNA (A-c) pathways. The si- and piRNA sequences are fully complementary to target RNA and results in target RNA cleavage. MiRNA sequences are mainly partially complementary to target RNA and results in translational inhibition. The si- and miRNA require protein dicer for their biogenesis, but piRNA does not. (B) Summary of si-, mi- and piRNA physical properties and general functions.



	piRNA	miRNA	siRNA
Length	24-30 nt	19-22 nt	19-22 nt
Precursor structure	Single long strand	Double stands hairpin	Double stands
Target mRNA recognizing	Fully complement	Seed region complement	Fully complement
Functional outcome of protein sythesis	RNA cleavage Translational inhibition Epigenetic regulation	RNA cleavage Inhibit translation Epigenetic regulation	RNA cleavage

CHAPTER 2

Cloning and molecular characterization of sea urchin argonautes

Previously, Rodriguez et al. (2005) identified and determined the nucleic acid sequence of seawi -- first member of the argonaute/piwi family to be described from the sea urchin *S. purpuratus*. It is known that the argonaute/piwi family is a highly conserved family of proteins that are expressed in diverse species – from worm to human — and that individual cells can express multiple family members. For example, there are 2 argonautes/piwis in the fungus *N. crassa,* 10 in the plant *A. Thaliana,* 27 in the nematode *C. elegans*, 5 in the fruit fly *D. melanogaster*, 7 in the zebrafish, 7 in the mouse and 8 in the human (Carmell et al., 2007; Carmell et al., 2002; Hock and Meister, 2008; Kuramochi-Miyagawa et al., 2001). Consequently, these observations suggest that in addition to seawi, there may/should exist multiple argonaute/piwi proteins in sea urchins.

It is well established that the amino acid sequences are conserved in the overall structural features for argonaute/piwi. Structurally, argonaute

proteins consist of four domains: the amino terminal, PAZ, middle, and PIWI (Carmell et al., 2002; Parker et al., 2004; Song et al., 2004). The PAZ and PIWI domains are so highly conserved that they can serve as a guide to “fish” for other members of the argonaute/piwi family. In this chapter, we molecularly characterized and deduced functional motifs for three additional sea urchin argonautes/piwis. Using computational and bioinformatic tools, domain homologies, potential binding sites, and functional motifs of the newly discovered sea urchin argonautes/piwis were identified and characterized. Lastly, a RT-PCR-based approach was used to determine the temporal expression of the sea urchin argonautes/piwis during early embryogenesis.

Materials and Methods

Collection of *S. purpuratus* gametes, embryos, depleted testes and RNA

Strongylocentrotus purpuratus were purchased from Santa Barbara Marine Biologicals (Santa Barbara, CA) or Pt. Loma Marine Invertebrate Lab (Lakeside, CA), and animals were maintained at 12°C in artificial seawater. Gametes were collected by intracoelomic injection of 0.53 M KCl. Eggs were collected into artificial seawater, washed with divalent-free seawater to remove the jelly coat, and stored in artificial seawater at 4°C until use. Semen was collected directly, using a pipette from the dorsal surface of injected sea urchins.

Sea urchin eggs were fertilized in artificial seawater, the fertilized egg were allowed to settled by gravity and the embryos were transferred to finger bowls containing Jamarin U (Jamarin Laboratory, Osaka, Japan) or sterilized sea water (PETCO, San Diego, CA) at 12 °C. Embryos were cultured and staged using phase contrast light microscopy. Embryos were collected at 16-cell (6 hours), 256-cell (12 hours), blastula (18, 23, and 31 hours), gastrula (43 and 52 hours) and pluteus (75 hours). Gamete-depleted testes and ovaries were prepared by multiple injections of 0.53 M KCl over a two-hour

period. The shell was cut open and gamete-depleted testes or ovaries were removed. Total RNA from depleted testes, eggs and staged embryos was collected with Trizol LS (Invitrogen, Carlsbad CA), according to the manufacturer's protocol. Briefly, eggs (0.25 ml), sperm (0.25 ml), embryos (0.25 ml), testes tissue (0.25 g), depleted testes tissue (0.25 g), ovary tissue (0.25 g) and depleted ovary tissue (0.25 g) were homogenized in 0.75 ml Trizol LS. After chloroform extraction and isopropanol precipitation, the RNA was resuspended in nuclease-free water, quantified spectrophotometrically at 260/280 nm, and store at -20°C.

RT-PCR

One microgram of previously collected RNA was reverse transcribed at 42ºC for 1 hour in a 20 µl reaction mixture using the M-MLV reverse transcription kit (Promega, Madison, WI). The primer sequences and position are described in the following section. PCR cycling conditions were: 1) 95ºC, 5 minutes, 2) 95ºC, 30 seconds; 55 ºC, 30 seconds; 72 ºC, 30 seconds; repeated for 35 cycles, 3) 72ºC, 7 minutes. PCR products were resolved on 1% agarose gels.

Bioinformatics of sea urchin argonaute/piwi genes

Mouse mili and the conserved consensus amino acid sequences for the PAZ domain of mouse argonaute 1 were used to search the *S. purpuratus* genome database (Sodergren et al., 2006). Identified genomic contigs were analyzed by GENSCAN software (Burge and Karlin, 1997) to obtain the coding sequences of putative *S. purpuratus* piwi/argonaute genes. The deduced amino acid sequences were further confirmed by blastp search against the NCBI database. Using these predicted sequences, PCR primers were designed to amplify expressed coding regions of putative piwi/argonaute family members. PCR products were generated using the following primer pairs (The position is designated from the final sequences of respective genes).

Seali:

10340-1f	5'-ATGGCAGGCTTCGGACGC-3' (position 1-18 nt)
10340-1r	5'-CCTTGAACGATGAACGACCG-3' (1283-1302 nt)
Seali-1f	5'- ACTGAGAAACTGGGTGGTCG-3' (1861-1880 nt)
Seali-1r	5'-TCACAGGAAGAAGAGCTTGTCG-3' (2861-2880 nt)
SealiSEQ-4f	5'- GACGAGGCTTTACTTCAC-3' (323-340 nt)

Seali-3r 5’- TCTTCTGGGTGACGCTTCTC-3’ (2178-2197 nt)

Sp-ago1A:

Seago1-1f 5’-ATGTATCAACCACCCTTTCC-3’ (1-19 nt)

Seago1-1r 5’- ATTCCACTGTTTGACCATTC-3’ (1389-1408 nt)

Seago1-2f 5’- GTACTGTAAAGATGAGATTCCAC-3’ (538-559 nt)

Seago1-2r 5’- GGTGCGTAATGTCGGCTCCC-3’ (2853-2872 nt)

Sp-ago 1B:

Seago2-10f 5’- ATGTCGGAGGCCAAAGTGG-3’ (50-68 nt)

Seago2-6r 5’- GAACTCTGCCCTCTATGGTG-3’ (1622-1641 nt)

Seago2-7f 5’-CGCTGTATCAAGAAACTG -3’ (1473-1490 nt)

Seago2-1r 5’- TCATGCAAAGTACATGACC-3’ (2823-2832 nt)

Seago2-15f 5’- ATGTACCAGCCACCACCGC-3’ (1-18 nt)

Seago2-12r 5’- CCCGTCAAACACTGGAAGA-3’ (609-627 nt)

Primers specific for identified argonaute family members are listed as following:

Seawi-1f 5’-GGAGTTTGACAAAGAGCAGG-3’ (position 1300-1320 nt)

Seawi-1r 5’-CTAGAGATAGAAGAGGGTGTCG-3’ (2546-2565 nt)

10340-2f 5’-GGTCCTATGTAACGAGTCAG-3’ (1123-1142 nt)

10340-1r	5'-CCTTGAACGATGAACGACCG-3' (1283-1302 nt)
Seago1-2f	5'- GTACTGTAAAGATGAGATTCCAC-3' (538-559 nt)
Seago1-1r	5'- ATTCCACTGTTTGACCATTC-3' (1389-1408 nt)
Seago2-7f	5'-CGCTGTATCAAGAAACTG -3' (1473-1490 nt)
Seago2-2r	5'- TACCAGGCAGCACCACCACG-3' (1970-1989 nt)

The prepared cDNAs served as template in a series of PCRs utilizing the designed primers and Pfx supermix kit (Invitrogen, Carlsbad CA), and the resulting PCR products were isolated using a gel purification kit (Promega, Madison, WI) and sequenced at the UMDNJ Molecular Resource Facility (UMDNJ, Newark, NJ). The obtained sequences were assembled and compared with predicted sequence data by Clustal W version 2; ClustalW is a multiple sequences alignment computer software. The program generates pairwise alignment and assembles phylogenetic tree. The level of identities and similarities of each domain were generated with NCBI BL2seq software.

Phylogram

Sequences alignment and phylograms were created by ClustalW version 2. Accession numbers of the sequences are Sp_Ago1a [EU733246],

Sp_Ago1b [JX014235], Seawi [NM214600], Seali [EU334659], Dm_Ago1-A [NM166020], Dm_Ago2-C [NM16866], Dm_Ago-3 [EF211827], Dm_Piwi [NM057527], Dm_Aub-A [NM057386], Mm_Ago1 [AY135687], Mm_Ago2 [AY135688], Mm_Ago3 [AY135689], Mm_Ago4 [AY135690], Mm_Miwi [AB032604], Mm_Miwi2 [NM021308], Mm_Mili [AB032605], Hs_Ago1 [NM012199], Hs_Ago2 [NM012154], Hs_Ago3 [NM024852], Hs_Ago4 [NM017629], Hs_Hiwi [AF104260], Hs_Piwi-like1 [NM004764], Hs_Piwi-like2 [NM001135721], Hs_Piwi-like3 [NM001008496] and Hs_Piwi-like4 [NM152431].

Results

Identification of seali, sp-ago1A and sp-ago1B

To identify argonaute family proteins in the sea urchin, we used the amino acid sequences of murine argonaute and piwi orthologues to search the sea urchin genome database. Initially, full length mili (mouse piwi-like) amino acid sequences were used to query the published sea urchin genome database (Sodergren et al., 2006) (Figure 2-1A). Eight genomic contigs were identified, two were confirmed as seawi, and the other six contigs encoded a putative piwi-like gene. By using the putative coding regions and assembling the overlapping regions from the six contigs, two long stretches of deduced seali sequences --1358 nt (position1-1357 nt) and 1119 nt (position 1762-2880 nt) -- were obtained. The deduced sequences were used to design specific primers (see Materials and Methods) for RT-PCR with sea urchin egg cDNA, followed by DNA sequencing (Figure 2-1A). Following multiple cycles of gene walking via RT-PCR and sequencing, we fully sequenced seali, the seawi-like protein. Seali was named and published in the NCBI nucleotide database as EU334659, and in the protein database as NP_001107667 (deduced amino acid sequences shown in Figure 2-2B). Seali, nucleotide sequences contains

open reading frames of 2880 nucleotides, which encodes for protein of 959 amino acids and calculated molecular weight of 107.74 kDa. The protein size of seali is distinguishable from seawi, which contain an open reading frame of 2565 nucleotides, encodes a protein of 854 amino acids, and molecular weight of 96.74 kDa (Rodriguez et al., 2005). Seawi and seali mRNAs are expressed in sea urchin eggs (Figure 2-1C).

We then turn focus on identification of sea urchin argonaute genes. By searching the sea urchin genome database (sp genome v0.5) with the mouse argonaute 1 PAZ domain (Figure 2-1B), multiple contigs were obtained. One of the identified contigs (621) was greater than 200,000 nt long and encoded two putative argonaute family proteins. The putative coding regions were deduced using genescan and specific primers were designed for RT-PCR. Both argonautes were expressed in unfertilized sea urchin eggs. Using multiple cycles of gene walking and sequencing, we identified the full-length sea urchin argonaute 1A and a partial argonaute 1B sequences (position 1038-2832). The remaining ago1B sequences were determined by searching an updated version of sea urchin genome database (sp genome v2.5). These sequences were published as *S. purpuratus* argonaute 1 isoform A (sp-ago1A)

in the NCBI nucleotide and protein database as EU733246 and NP_001124190 (deduced amino acid sequences shown in Figure 2-2C), and as *S. purpuratus* argonaute1 isoform B (sp-ago1B) and protein database as JX014235 (deduced amino acid sequences shown in Figure 2-2D). By using RT-PCR, we show that sp-ago1A and sp-ago1B mRNAs were expressed in sea urchin eggs (Figure 2-1D; 2-8B). Sp-ago1A and sp-ago1B contain 2872 and 2832 nucleotides, which encode 955 and 943 amino acids, and molecular weights of 106.51 and 106.18 kDa, respectively.

Domain analysis of seawi, seali, sp-ago1A and sp-ago1B

The PAZ and PIWI domains are conserved signature domains for all argonaute family members (Carmell et al., 2002). These conserved PAZ and PIWI domain were identified in seawi, seali, sp-ago1A and sp-ago1B sequences (Figure 2-2 PAZ in green and PIWI in red). This confirmed that the discovered mRNAs coded for members of the argonaute protein family.

Argonaute family proteins can be separated by sequence into two subclasses: argonaute and piwi (Carmell et al., 2002). According to our phylogram, sp-ago1A and 1B cluster with argonautes, while seawi and seali

group with the piwis (Figure 2-3, piwi subfamily). Further, seawi is specificly grouped with piwi while seali is grouped with piwi-like proteins. In our phylogram, both sp-ago1A and 1B are grouped with fruit fly ago1, which suggests they are orthologues of different ago1 isoforms (Figure 2-3, argonaute subfamily). In summary, we successfully identified sea urchin argonautes sp-ago1A and sp-ago1B, and sea urchin piwi-like seali, and all of them have the conserved structural features for argonaute/piwi family.

We next compared the four sea urchin argonaute protein sequences to each other and their mouse orthologues to determine the extent of domain similarity and to deduce potential functionality. Seawi domain sequences have a maximum 73% similarity to mouse orthologue miwi, 70% to seali, 49% to sp-ago1A and 55% to sp-ago1B (Figure 2-4A). Seali domain sequences have a maximum 76% similarity to the mouse orthologue mili, 47% to sp-ago1A and 46% to sp-ago1 B (Figure 2-4B). There is a maximum 96% similarity between sp-ago1A and B, and amazingly 93% similarity to their mouse orthologues (Figure 2-5 A; sp-ago1A, and B; sp-ago1B).

First, we find all four sea urchin argonaute domain structures have very high similarity to that of mouse argonautes, some cases even more than their

paralogues. In a previous reported (Rodriguez et al., 2005) and in Figure 2-3, we show that seawi is similar to mammal piwis. Here we further demonstrate that not only seawi but also seali, sp-ago1A and B have high domain similarity to their mammal orthologues. This indicates sea urchin argonautes/piwis may have conserved functionality to other argonaute protein members.

Second, we explored the possibility that sea urchin argonautes/piwis may contain conserved functional motifs in their sequences. We first analyzed the amino acids required for RNA cleaving. Within PIWI domains, there are three conserved amino acids, called the catalytic triad, which are crucial for the RNase III activity (Figure 2-6A). The triad consists of a histidine (H) and two aspartic acid (D) residues (Rivas et al., 2005; Song et al., 2004). In the argonaute subfamily, point mutation to any of the amino acids in the triad of yeast, plant, and human argonautes all result in the loss of catalytic function in degrading mRNAs (Carbonell et al., 2012; Liu et al., 2004; Nakanishi et al., 2012). In the piwi subfamily, point mutation of this triad in miwi and mili both result in reduction of quantity of piRNA, and further leads to spermatogenetic failure and sterility (De Fazio et al., 2011; Reuter et al., 2011). Both argonaute (Figure 2-6 B) and piwi (Figure 2-6C) subfamily proteins in sea urchin contain

the conserved catalytic triad, suggesting that all of the identified sea urchin argonautes/piwis may have RNase activities like argonaute/piwi proteins in other species.

Third, we asked if sea urchin argonautes/piwis also have conserved RNA binding motifs. There are two RNA binding motifs in argonaute proteins: small non-coding RNA binding motif in PAZ domain (Figure 2-7) and mRNA binding motif in PIWI domain (Figure 2-8). The PAZ domain contains a conserved hydrophobic amino acid side chain that stabilizes the beta–barrel motifs that form the RNA binding cleft, and binds small non-coding RNA with a 1:1 stoichiometry, (Lingel et al., 2003; Yan et al., 2003). In human ago2, point mutation of positively charged amino acid in the RNA binding cleft reduce the RNA binding affinity and leads to in attenuated mRNA cleavage activity (Ma et al., 2004). The sea urchin argonautes and piwis have the conserved amino acids for small non-coding RNA binding in PAZ domain (Figure 2-7B). Comparing the overall PAZ domain sequences of sea urchin argonautes and piwis to their fruit fly, mouse and human orthologues, argonautes have a 68% similarity and piwis have 54% (Figure 2-7C). As for amino acids that are crucial for binding, argonautes have a 95% similarity and piwis have 82%

(Figure 2-7D).

The other RNA binding motif is in the N-terminal region of the PIWI domain, where 138 amino acids are responsible for mRNA binding (Kuramochi-Miyagawa et al., 2001). Within this region, three positively charged lysines and other neutral amino acids are required for RNA binding (Wang et al., 2008). *In vitro*, the deletion of this RNA binding motif from miwi eliminates mRNA binding ability (Kuramochi-Miyagawa et al., 2001). Sea urchin argonautes and piwis have the conserved amino acids for RNA binding in PIWI domains (Figure 2-8B). For overall PIWI domain sequences, argonautes have a 63% similarity and piwis have 59% (Figure 2-8C). As for amino acids that are crucial for binding, both argonautes and piwis have a perfect 100% identity (Figure 2-8D). The conservation of these amino acids in sea urchin argonautes/piwis indicates the potential for small non-coding RNA and mRNA binding activity.

In summary, sea urchin argonautes contain the required sequences necessary for having both RNase III and RNA binding activities. The finding of small non-coding RNA binding, mRNA binding and RNase III motifs suggest that the sea urchin argonautes/piwis may function in similar manner as other

family members.

Analysis of the expression of seawi, seali, sp-ago1A and sp-ago1B mRNA in development

To investigate the mRNA expression of argonautes/piwis in embryonic development and adult germline tissue, RT-PCRs were performed with cDNA from sperm-depleted testes, eggs, 16-cell (6 hours), 256-cell (12 hours), blastula (18, 23, and 31 hours), gastrula embryos (43 and 52 hours) and pluteus larva (75 hours). The specific primer pair for each sea urchin argonaute is indicated (Figure 2-9 A; for specific primer sequences see Materials and Methods), and all PCR amplified products were confirmed by nucleic acid sequencing. RT-PCR results suggest that all four sea urchin argonaute/piwi mRNAs are expressed in eggs, depleted testes and all developmental stages (Figure 2-9 B). Although not quantitated, the mRNA expression between depleted testes and egg/embryos are not different in seawi and seali RT-PCR, but it showed an apparent decrease in sp-ago1A and 1B (Figure 2-9B). Moreover, the RT-PCR band intensity of sp-ago1B is significantly lower in late gastrula embryo and pluteus larva, which suggests

that sp-ago1B may mainly be express during early embryogenesis.

Discussion

Structural organization of sea urchin argonautes

This chapter details the molecular characterization of three additional members of sea urchin argonaute family. Besides seawi, we identified and sequenced mRNA for seali, sp-ago1A and 1B. *In silico*, the deduced amino acid sequences revealed that seali, sp-ago1A and 1B contain the requisite generic argonaute conserved domains, phylogenetic analysis shows that seawi and seali are clustered within the piwi subclass, and sp-ago1A and sp-ago1B are clustered within the argonaute subclass.

Examination of the argonaute specific features in the sea urchin sequence can provide insight into their potential properties and cellular functions. Seawi, seali, sp-ago1A and 1B all contain the variable N-terminal, PAZ, variable middle and PIWI domains. Moreover, common structural features responsible for small non-coding RNA binding, mRNA binding, and RNase III motif are extremely conserved across the argonaute/piwi family. Given the importance of these conserved amino acids to their functions, and the highly conserved sequence structure of sea urchin argonaute/piwi to their orthologues, our results suggest that seawi, seali, sp-ago1A and 1B are

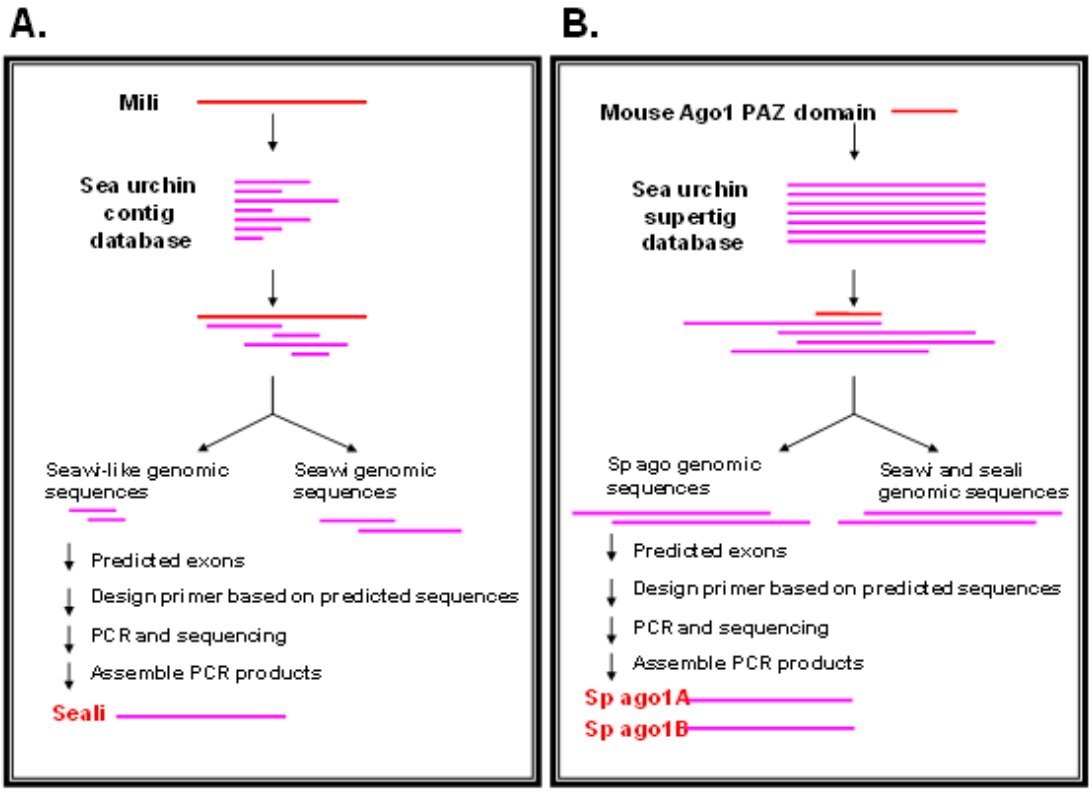
capable of interacting with small non-coding RNAs and mRNAs, and that this interaction can result in cleavage of mRNAs through conserved RNase III motif. The presence of the highly conserved RNase III motif provides suggestive evidence that sea urchin argonautes/piwis should have the ability to degrade RNA. Moreover, in the piwi subfamily, the catalytic triad in the RNase III domain is required for piRNA biogenesis and mutations to amino acids in the triad result in both loss of silencing activity and collapsing in piRNA amplification (De Fazio et al., 2011). Within argonaute sequences, the N-terminal domain is the most variable region and this domain is believed to be responsible for protein interactions and possibly targeting argonautes to specific macromolecular complexes, for example RISCs (Eulalio et al., 2009). Similarly, the high variation between N-terminal domain sequences of sp-ago1A and 1B suggests they might be associated with different RISCs or RNP complexes and may reflect functional specialization of each individual argonaute.

The results in this chapter indicated that seawi, seali, sp-ago1A and sp-ago1B are expressed throughout development and in adult testes, and they potentially associate with specific populations of small non-coding RNA and

mRNA. In animals, both piwi and piwi-like proteins have been previously shown to be essential for driving piRNA functioning in cells (Aravin et al., 2006; Aravin et al., 2003; Farazi et al., 2008; Girard et al., 2006; Lau et al., 2006). Therefore, it is certainly reasonable to postulate that seawi and seali would have a cellular function that is dependent upon interaction with sea urchin piRNAs. In fruit fly, ago1 and ago2 proteins play key roles in miRNA and siRNA silencing pathways, respectively. Indentifying two sea urchin ago1-type isoforms from egg and embryo sample suggests that miRNAs may play key roles in sea urchin embryonic development. Despite considerable effort, no evidence was found to suggest the existence of an ago 2-type gene in the presently published sea urchin genome database. This is an interesting result since taking the observation to its logical conclusion would suggest that sea urchin cells do not express endogenous siRNA and/or they are not capable of exhibiting siRNA activity. Both conclusions may be correct; we did not discover endogenously expressed siRNA in our small non-coding RNA database (see Chapter 4) and microinjected siRNA into sea urchin egg did not result in any level of knockdown of the intended protein target (Ransick, unpublished personal communication).

Figure 2-1. Strategies for identifying argonautes in *S. purpuratus*.

Genomic and standard molecular biological approaches were used to discover and identify the argonaute family members expressed in sea urchins. (A) Based on the published coding sequences of mili, we identified putative coding sequences in the sea urchin genome for the sea urchin seawi-like protein, seali. Further, using RT-PCR and gene walking, we sequenced full-length seali. (B) By searching the sea urchin genome database with the published coding sequences of mouse argonaute 1 PAZ domain, we identified putative coding sequences of sea urchin argonaute subfamily proteins argonaute 1a (sp-ago1A) and argonaute 1b (sp-ago1B). (C) PCR products of seawi PIWI domain, full coding region of seawi and seali. (D) PCR product of full coding region of sp-ago 1A and sp-ago 1A N-terminal domain.


A.
Mili
Sea urchin contig database
Seawi-like genomic sequences
Seawi genomic sequences
Predicted exons
Design primer based on predicted sequences
PCR and sequencing
Assemble PCR products
Seali
B.
Mouse Ago1 PAZ domain
Sea urchin supertig database
Sp ago genomic sequences
Seawi and seali genomic sequences
Predicted exons
Design primer based on predicted sequences
PCR and sequencing
Assemble PCR products
Sp ago1A
Sp ago1B

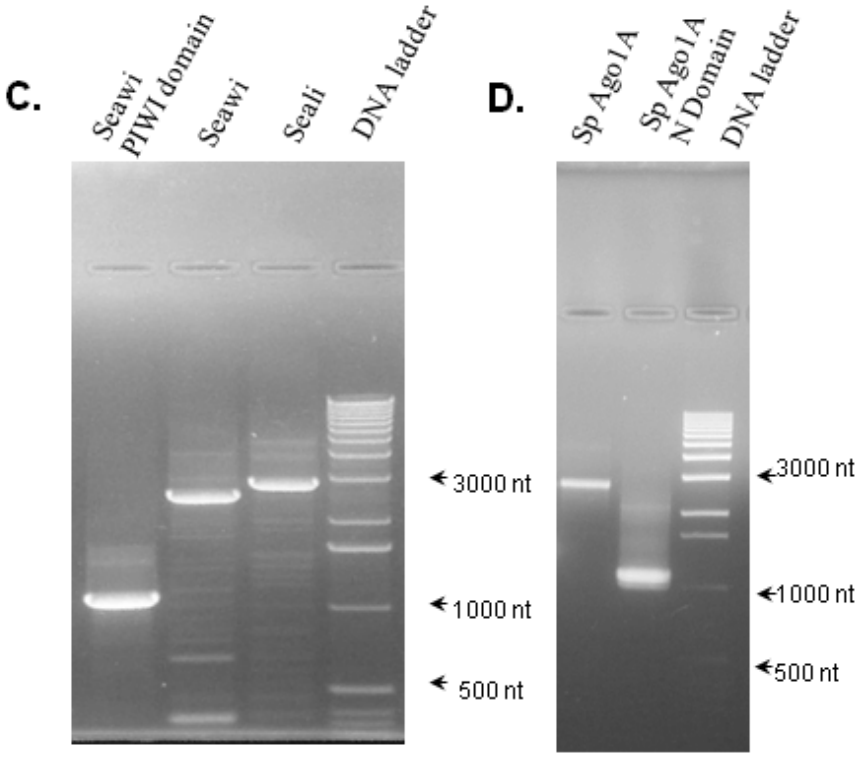

C.
Seawi PIWI domain
Seawi
Seali
DNA ladder
3000 nt
1000 nt
500 nt
D.
Sp Ago1A
Sp Ago1A N Domain
DNA ladder
3000 nt
1000 nt
500 nt

Figure 2-2. Amino acid sequences of seawi, seali, sp-ago1A and sp-ago1B.

Alignment of amino acid sequences of seawi (A), seali (B), sp-ago1A (C) and sp-ago1B (D). The PAZ domain is in green and the PIWI domain is in red letters. Seawi, seali, sp-ago1A and 1B all have highly conserved PAZ and PIWI domains.

A. Seawi

MDRRPGEQAPPPMQQAAPPQEASSGMPVPGRGRSRGGTTQPGPGAVPKQEPRGELTSQIAKLAIGASGEGRVGGAVQDRRR
RRDEIELEPTTRPENFVKQAIAGDKIALIANGFKLKTKPDWQLYQYRVDFEPEILNPRARFALLKGHSALLGKGLTLDMDT
MYSLFKLAEKVTNLSAERKDGSNVNICVTHVATLNPMAPNTLHLYNVLFRRCLKMINMEQVGRNYYDPTAAIDIKQHGLQL
WPGFVTSILQYEYDVMLLSDISHKVLRTQTVWEVMNDLFNKARGRFKEEITKLMIGQIVLTKYNNKTYRVDDIDFDTTPAD
TFETRSGPVSYVDYFKKSYERVIHDVNQPMLVSRPKKREEKKGVGPAYLPPELCFLTGLSDDMRADFNVMKDMGQHTRVGP
QDRCRTLSGFIKKLSSNEEVKTYLDSWGMEFDKEQVKLTGRVLPPEKLFQRGKQFSYNPSNADWSRDTRGNALTDAKILNN
WKIFYTRRDANRGQDFIKSLVRVANPMGMNVRGPEIVELPDDRTETYTRSLQAQIAQDTQIVVVILPTNRKDRYDAIKKTC
VVTHPCPSQVIVSRTLSKQQMLMSVATKIAMQMNCKMGGDLWRVEIPLSNLMIIGIDSYHDSLTKGRSVLGFVASMNKSQT
SFFSSCAFQHAQGEFGANLSTLMNNALKRYYQINEKFPERIIIFRDGVGDSQVNLVVDYELKQIKDTLDKVYPQGTVHKLA
VVVVKKRINNRFFANLRGGLSNPPPGTVIDDVVTKPHLYDYFIISQSVRQGSVSPTSYNVVYDTTGLKPDHMQRLTYKLTH
LYFNWPGTVRVPAPCMYAHKLAFLIGQSVHEVPNNRLADTLFYL

B. Seali

MAGFGRGGRGAALLKLLEEPQRRPGQNGDNTNGDKVEGKPASRPDTAPAPGAPKPGAPKPAFGRGLQMAAMQQQIKKEEPK
PPGSQQSAQQQAAPTSFGRGFSGMPIGRGFTSQQQPTAQKSPARQTPSPARQSPSPPGKSPSPTGKSPSPSMMTSSSAASS
AYPSPAPTPTPSMGPGGDTRDRFANLDEKMGHLSLQGKEIHGTAGREVKAASNYIRIKCVNKAVWQYAVSFDPPIESKKIR
LRMVFDHSDVIGKVRAFDGAVLFLPHKLPQKVTLFESVRLFDQETITIKIILGKEINPQECTQIYNIIFRKVMKILQMKQV
GRHFYNPTTPSIVPQHKLEIWPGYITAISQYEGGLMLHADVSHKVLCNESVLESMQAIYQRIPNNIRFREECVRQIVGTVV
LTRYNNKTYRVDDIDWDSNPQTTFKSRDEEISFIDYYKKSYSLEISDPGQPLLISRIKKKQLNNPGTPVEEEIRLIPELSS
RTGLTDDMRADFRIMKDLAMHTRVTPQQRHQSMLKFIKNVYANEQAAEELSGWGLELQHDLLQLTGRQLPLENIKLGRSSF
KASKEADWGRECTRQHVITAVPLRNWVVVFTRRDAPKAAEFIQMMKQVCPQMGIEIEHPSMVELQDDRTQSYTNMVKKSIN
PQLQLVIAIFPTSRDDRYSAFKKLCCIEAPVPSQVINGRTISQKQKLRSVTQKIALQINCKLGGELWALDVPLSKLMVIGI
DVYHDPNRGKKSIGAFVASMNRDLTSWFSRVCIQTPHQELIGGLKLCFTSSLKKYHDINHALPEKIVIFRDGVGDGQLNVV
ATYEQEQLSQCFSMFGADYKPQLCIVVVQKRINTRIFSVAGGNYENPYPGMVIDHTITRPTWYDFFLVSQHVRQGTVTPTH
YVVVHDSTQLKPDHMQRLAYKLTHLYYNWPGTVRVPAPCQYAHKLAYLVGQNLHKEPSLELCDKLFFL

C. Sp-ago 1A

MYQPPFPPNFPYGMPNYPPNQPGIPGQFPPGGPPGGPPGGPPPGGPPGGGPPGGPPGGPPSGPPGGPPGGPPFTPPPSQQP
PPPGGPPQPPGTPPVAPTELVAMPRRPGFGIDGRPIALRANHFQVKIPPLDLFHYEVNITPEKCPRRINRDVIDTLVTAYK
GRFFQNNLPVFDGRTNMYCKDEIPLDREKQVELEVTLPGEGKDRIFKASIKFEGKVSLSLLEAALKGEVMNIPHEAVQALD
VIMRHLPSLRYTPVGRSFFSPPEEYSHPLGGGREVWFGFHQSIRPSMWKMMLNIDVSATAFYSCQSVIDFLCEVLDIQDLS
DQRRPLSDSHRVKFTKEIKGLKIEITHCGNMKRKYRVCNVTKRSAQTQTFPWQLENGQTVECTVAKYFRERHNLILQYPHL
PCLQVGQEQRHTYLPLEVCNIVAGQRCIKKLTDLQTSTMIKATARSAPDREKEIKSLVHKADFNNDQYVRQFGLSISNEMV
TIEGRVLPAPKIQYGGKLNKPQQAIPNQGVWDMRGKQFHFGVEIEVWAIACFAPQHQCREEALRNFTLQLQKISNEAGMPI
KWQPGFCKYAVGADQVEAMFRHLKKMWPKLQLIIVVLPGKTPVYAEVKRVGDILLGIATQCVQVKNVNRTTAQTLSNLCLK
IKVKLGGVNNILAPNIRPRIFSEPVIFCGADVTHPPAGDDKKPSIAAVVGSMDGHPSRYCASVRIQTHRVEIIQDLQAMVK
ELLVEFYKSTRYKPARIIMYRDGVSEGQFLQVLANEMNAIRDACRSLEEGYEPGITFIVVQKRHHTRLFCAERREQIGRSG
NIPAGTTVDSGITHPLEYDFYLCSHAGIQGTSRPSHYHVLWDDNGFKADELQCLTYQLCHTYVRCTRSVSIPAPAYYAHLV
AFRARYHLVEKDHDRYDHDSVISSANSNGGDGSHHGRDGYGRTPKEMSAAIKVHDDTLKVMYFA

D. Sp-ago 1B

MYQPPPHPNLPYGAPPFPQAPIQRPQAGQPPRQQAIPGPAPIQQQTATTLQGQTTLPPITAGGPPGPPAPAAPPGGPGGPQ
PPRSDLVALPSRPGFGEDGRPIVLRANHFQVKIPSIEIFHYDVTISPDKCPRRVNRDVIDTLVNAYKARYFQNNLPVFDGR
RNMYTKEQLPLENERVELEATLPGEGKDRVFKTQIRYVGKVSLSLLESALKGEVEHMPYDAIQALDVIMRHLPSMRYTPVG
RSFFSPPEEYFHPLGGGREVWFGFHQSVRPSMWKMMLNIDVSATAFYNAQSVIDFLCEVLDIQNISEQRRPLSDSQRVKFT
KEIKGLKIEITHCGNMRRKYRVCNVTKRPAQTQTFPWQLENGQTLECTVAKYFKERHNTILQYPHLPCLQVGQEQRHTYLP
LEVCNIVAGQRCIKKLTDMQTSTMIKATARSAPDREKEIKNLVHKANFNNDRYVRQFGLSISNDMVTIEGRVLPAPKIQYG
GKMSQQNKTQAIPAQGVWDMRGKQFHTGVEIRVWAIACFAPQHQCREEALRTFTAQLQKISNDAGMPIMGQPCFCKYAVGA
DQVEPMFRHLKSTYKGLQLIVVVLPGKTPVYAEVKRVGDTLLGIATQCVQVKNVNRTTAQTLSNLCLKINVKLGGINNILV
PNIRPRVFAEPVIFLGADVTHPPAGDDKKPSIAAVVGSMDGHPSRYCASVRVQNHRVEIIQDLMGMVRELLMEFYRSTRFK
PARIIMYRDGVSEGQFLQVLAQEMTAIRNACRSLEDFYEPGITFIVVQKRHHTRLFCAERREQIGRSGNIPAGTTVDSGIT
HPLEFDFFLCSHAGIQGTSRPSHYHVLWDDNRFKADELQCLTYQLCHTYVRCTRSVSIPAPAYYAHLVAFRARYHLVEKDH
DRYDHDSHQSGTNSNGGDGSSHHGASSYGRSFEDMIRAVKVHDDTLKVMYFA

Figure 2-3. Phylogenetic tree of the argonaute/piwi proteins.

Alignments and phylogram were generated using ClustalW version 2. The alignment was done using the full-length amino acid sequences of argonautes and piwis from the fruit fly, sea urchin, mouse and human (see Materials and Methods). Argonaute/piwi proteins separate according to sequence into two subclasses: argonaute and piwi (*Carmell et al.,* 2002). This phylogram illustrates that sp-ago1A and 1B cluster with the argonaute subclass while seawi and seali grouped with the piwi subclass. Sp-ago 1A and 1B both cluster with fruit fly ago1, suggesting they are argonaute 1 isoforms.

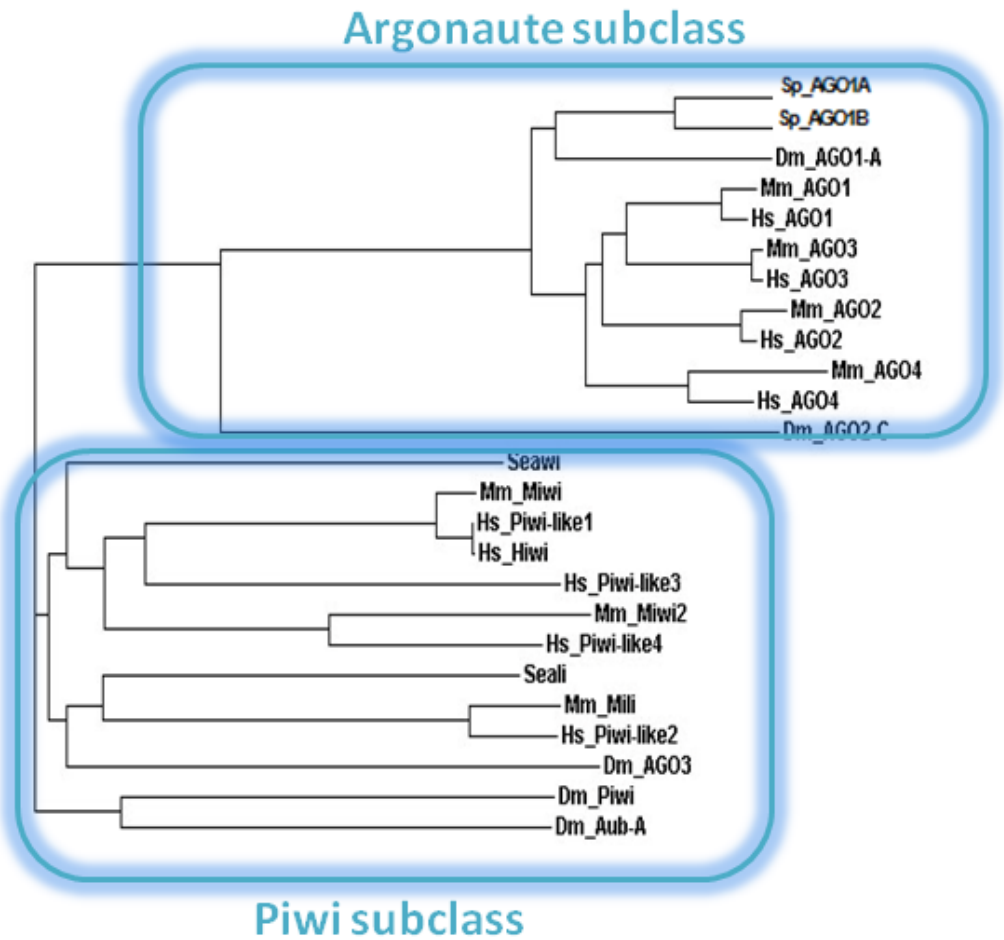
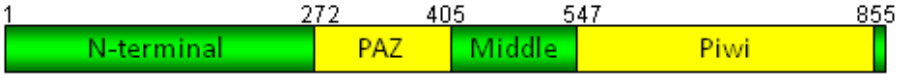

Argonaute subclass
Sp_AGO1A
Sp_AGO1B
Dm_AGO1-A
Mm_AGO1
Hs_AGO1
Mm_AGO3
Hs_AGO3
Mm_AGO2
Hs_AGO2
Mm_AGO4
Hs_AGO4
Dm_AGO2-C
Seawi
Mm_Miwi
Hs_Piwi-like1
Hs_Hiwi
Hs_Piwi-like3
Mm_Miwi2
Hs_Piwi-like4
Seali
Mm_Mili
Hs_Piwi-like2
Dm_AGO3
Dm_Piwi
Dm_Aub-A
Piwi subclass

Figure 2-4. Domain organization of sea urchin piwi proteins.

Domain organization of seawi (A) and seali (B). All sequences have been confirmed by sequencing of PCR products amplified from sea urchin egg cDNA. The level of identities and similarities of each domain with the corresponding regions of sea urchin argonautes/piwis and mouse orthologues are shown (NCBI BL2seq). Sea urchin piwis have high sequences similarity to their mouse orthologues.

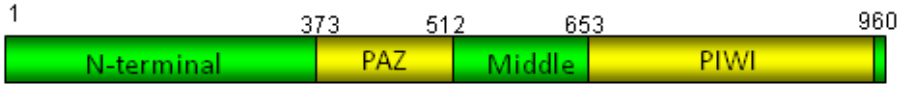
A.

Identities / similarities



Seali	23%/ 38%	47%/ 66%	35%/57%	53%/ 70%
Sp-ago1A	10%/ 14%	16%/ 32%	21%/33%	28%/ 49%
Sp-ago1B	6%/ 9%	15%/ 26%	19%/30%	29%/ 55%
Miwi	37%/ 57%	50%/ 72%	39%/ 58%	58%/ 73%

B.



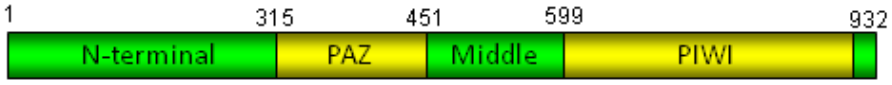
Seawi	23%/ 38%	47%/ 66%	35%/57%	53%/ 70%
Sp-ago1A	10%/ 15%	3%/ 5%	13%/22%	30%/ 47%
Sp-ago1B	16%/ 30%	3%/ 5%	No significant	28%/ 46%
Mili	48%/ 66%	45%/ 70%	32%/ 59%	59%/ 76%

Figure 2-5. Domain organization of sea urchin argonaute proteins.

The level of identities and similarities of each domain with the corresponding regions of sea urchin argonautes/piwis and mouse orthologues are shown (NCBI BL2seq). Sea urchin argonautes also have high sequences similarity to their mouse orthologues.

Identities / similarities

A.



Sp-ago1B	68%/ 76%	92%/ 97%	72%/ 76%	91%/ 96%
Seawi	10%/ 14%	16%/ 32%	21%/ 33%	28%/ 49%
Seali	10%/ 15%	3%/ 5%	13%/ 22%	30%/ 47%
Mouse ago1	60% / 76%	76% / 92%	61% / 76%	78% / 87%
Mouse ago2	61% / 77%	77% / 90%	61% / 73%	80% / 89%

B.

Sp-ago1A	68%/ 76%	92%/ 97%	72%/ 76%	91%/ 96%
Seawi	6%/ 9%	15%/ 26%	19%/ 30%	29%/ 55%
Seali	16%/ 30%	3%/ 5%	Non-significant	28%/ 46%
Mouse ago1	61% / 76%	78% / 93%	69 %/ 81%	78% / 88%
Mouse ago2	61% / 77%	79% / 92%	68% / 79%	80% / 89%

Figure 2-6. Analysis of conserved RNase III sequences in PIWI domains. PIWI domains have been shown to contain the catalytic triad, which is required for RNase activity. The triad consists of two aspartic acids (D) and one histidine (H) residue (*Rivas et. al.* 2005; *Song et. al.* 2004). (A) Schematic of seawi domain structure with the locations of the catalytic triad residues underlined in red. (B) Sequence alignment of the catalytic triad region of sea urchin argonautes/piwis with fruit fly (Dm), mouse (Mm) and human (Hs) argonaute (B) and piwi subfamily (C). The amino acids of the catalytic triad are shown in red. All four sea urchin argonautes/piwis have catalytic triad in their PIWI domain sequences, suggesting their potential RNase activity.

A.

B.

DmAgo1	VIFLGADV	ILYRDGVS	IPAPAYYAHLVAF
DmAgo2	TMYIGADV	IYYRDGVS	YPAPAYLAHLVAA
HsAgo1	VIFLGADV	IFYRDGVP	IPAPAYYARLVAF
HsAgo2	VIFLGADV	IFYRDGVS	IPAPAYYAHLVAF
HsAgo3	VIFLGADV	IFYRDGVS	IPAPAYYAHLVAF
HsAgo4	VIFLGADV	IYYRGGVS	IPAPAYYARLVAF
SpAgo1A	VIFCGADV	IMYRDGVS	IPAPAYYAHLVAF
SpAgo1B	VIFLGADV	IMYRDGVS	IPAPAYYAHLVAF

C.

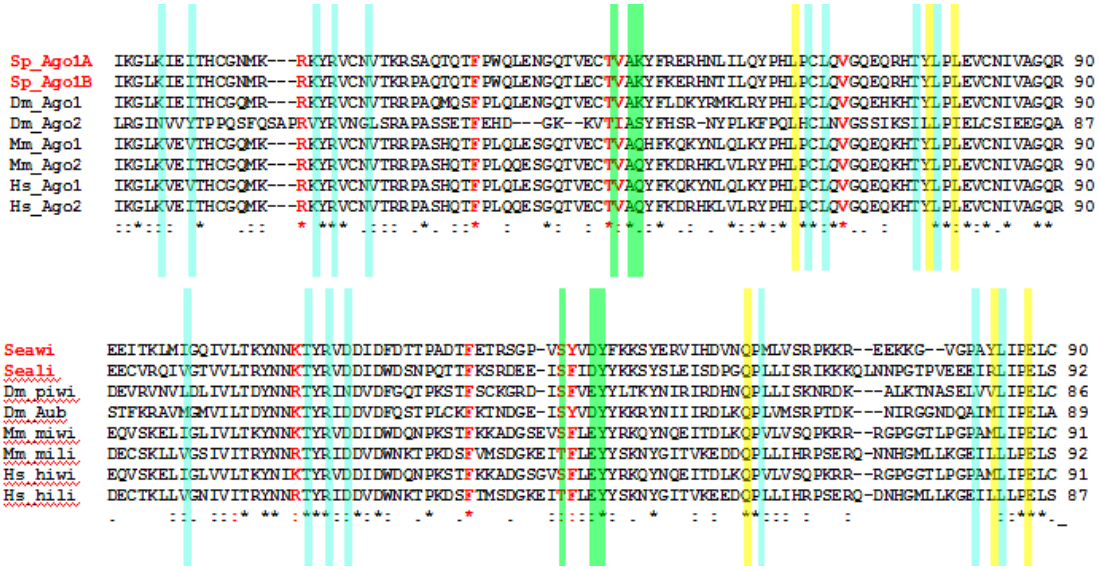
DmAUB	LMTVGFDV	LFFRDGVG	VPAVCHYAHKLAF
DmPIWI	LMTIGFDI	VFYRDGVS	VPAVCQYAKKLAT
HsHIWI/PIWIL1	VMIVGIDC	IVYRDGVG	VPAPCQYAHKLAF
HsHILI/PIWIL2	LMVIGMDV	VVYRDGVS	VPAPCKYAHKLAF
HsPIWIL3	TMFVGIDC	IVYRDGVG	VPAPCHYAHKLAY
HsHIWI2/PIWIL4	LMVVGIDV	IVYRAGVG	VPAPCQYAHKLTF
SpSeawi	LMIIGIDS	IIFRDGVG	VPAPCMYAHKLAF
SpSeali	LMVIGIDV	VIFRDGVG	VPAPCQYAHKLAY

Figure 2-7. Analysis of sequences of PAZ domains.

(A) The red line indicates the general location of small non-coding RNA binding regions in PAZ domains of sea urchin argonautes/piwis. (B) Sequence alignment of PAZ domains of sea urchin argonautes to the fruit fly (Dm), mouse (Mm) and human (Hs) argonaute/piwi proteins. PAZ domain is required for small non-coding RNAs binding. Conserved hydrophobic residues within the central beta-barrel are highlighted in blue, hydrophobic residues associated with the interface between barrels in green, and hydrophobic residues associated with the interface of beta–barrels within the central beta–barrel in yellow. The RNA binding region involves highly conserved residues (shown in red), they are also present in all four sea urchin argonautes/piwis. (C) Comparison of sea urchin argonautes/piwis small non-coding RNA binding region in PAZ domain to the fruit fly, mouse and human orthologues. (D) Comparison of sea urchin argonautes/piwis amino acids that are required for small non-coding RNA binding (in red, yellow, blue and green in B) to the fruit fly, mouse and human orthologue sequences.

A.

B.



C.

Small non-coding RNA bind regions		
	Argonautes	Piwis
Similarity	68%	54%
Identity	29%	19%

D.

Amino acids for small RNA binding		
	Argonautes	Piwis
Similarity	95%	82%
Identity	84%	60%

Figure 2-8. Analysis of sequences of PIWI domains.

(A) The red line indicates the general location of mRNA binding regions in PIWI domains of sea urchin argonautes/piwis. (B) Sequence alignment of the mRNA binding region at the N-terminus of the PIWI domain (*Kuramochi-Miyagawa et al*., 2001). The positively charged lysines and the other neutral amino acids required for RNA binding are shown in red (*Wang et al.,* 2008). (C) Comparison of sea urchin argonautes/piwis mRNA binding region in PIWI domain to the fruit fly, mouse and human orthologues. (D) Comparison of sea urchin argonautes/piwis amino acids that required for mRNA binding (in red in B) to the fruit fly, mouse and human orthologue sequences.

A.

B.

```
Sp_Ago1A   YAEVKRVGDILLGIATQCVQVKNVNRTT-AQTLSNLCLKIKVK 42
Sp_Ago1B   YAEVKRVGDTLLGIATQCVQVKNVNRTT-AQTLSNLCLKINVK 42
Dm_Ago1    YAEVKRVGDTVLGMATQCVQAKNVNKTS-PQTLSNLCLKINVK 42
Dm_Ago2    YDTIKQKAELQHGILTQCIKQFTVERKCNNQTIGNILLKINSK 43
Mm_Ago1    YAEVKRVGDTLLGMATQCVQVKNAVKTS-PQTLSNLCLKINVK 42
Mm_Ago2    YAEVKRVGDTVLGMATQCVQMKNVQRTT-PQTLSNLCLKINVK 42
Hs_Ago1    YAEVKRVGDTLLGMATQCVQVKNVVKTS-PQTLSNLCLKINVK 42
Hs_Ago2    YAEVKRVGDTVLGMATQCVQMKNVQRTT-PQTLSNLCLKINVK 42
           *  :*: .:   *: ***::  .. :.   **:.*: ***: *_

Seawi      YDAIKKTCVVTHPCPSQVIVSRTLS----KQQMLMSVATKIAMQMNCK 44
Seali      YSAFKKLCCIEAPVPSQVINGRTIS----QKQKLRSVTQKIALQINCK 44
Dm_piwi    YSSIKKRGYVDRAVPTQVVTLKTT----KKPYSLMSIATKIAIQLNCK 44
Dm_Aub     YSCIKKRTCVDRPVPSQVVTLKVIAPRQQKPTGLMSIATKVVIQMNAK 48
Mm_miwi    YDAIKKYLCTDCPTPSQCVVARTLG----KQQTVMAIATKIALQMNCK 44
Mm_mili    YGAIKKLCCVQSPVPSQVINVRTIG----QPTRLRSVAQKILLQMNCK 44
Hs_hiwi    YDAIKKYLCTDCPTPSQCVVARTLG----KQQTVMAIATKIALQMNCK 44
Hs_hili    YGAIKKLCCVQSPVPSQVVNVRTIG----QPTRLRSVAQKILLQINCK 44
           *..:**      . *:* :  :.      :   : ::: *: :*:*.*_
```

C.

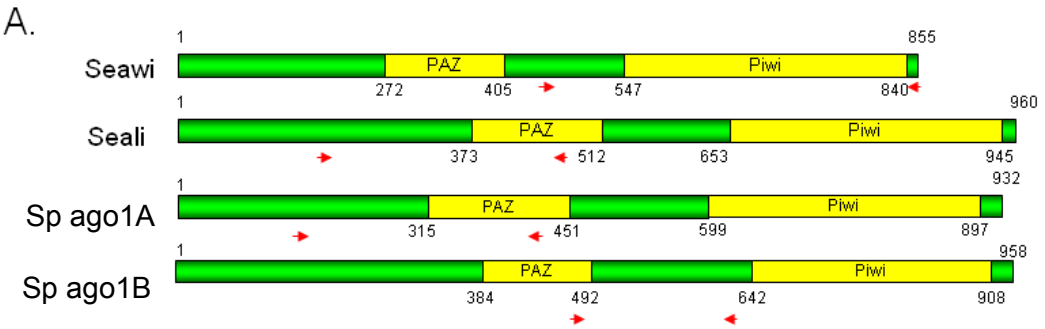
mRNA binding regions		
	Argonautes	Piwis
Similarity	63%	59%
Identity	31%	20%

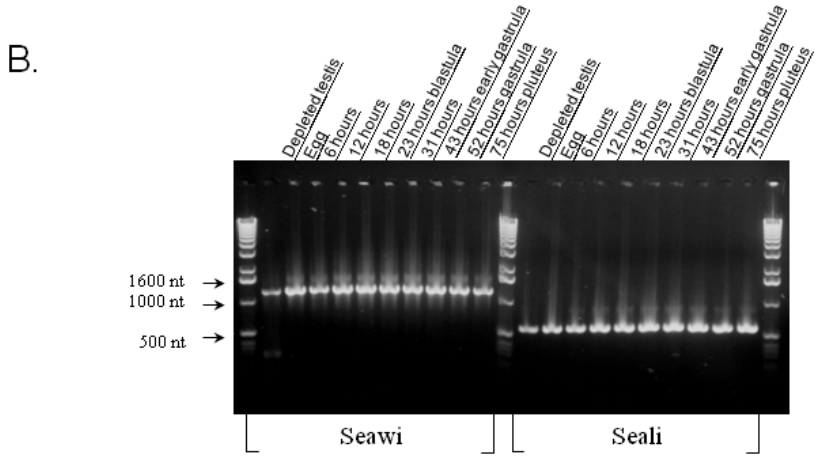
D.

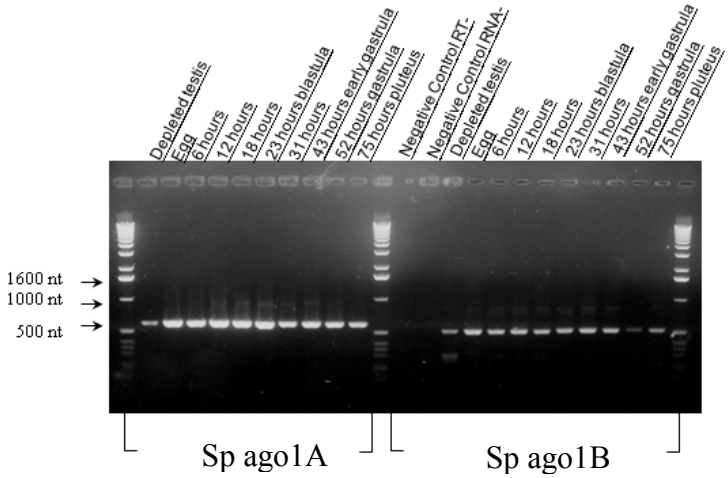
Positively charged amino acids for mRNA binding		
	Argonautes	Piwis
Similarity	100%	100%
Identity	73%	56%

Figure 2-9. Expression of seawi, seali, sp-ago1A and sp-ago1B mRNAs.

(A) Schematic of sea urchin argonaute proteins with primers specific to each family member. Arrows indicate the primer locations and directions for PCR products shown in (B). For all four sea urchin argonautes/piwis, primer pair designs cross at least one intron, PCR products from cDNA and possible contaminated genomic DNA can be separated by size. (B) PCR products of sea urchin argonautes/piwis from sperm-depleted testis, eggs, 16-cell (6 hours), 256-cell (12 hours), blastula (18, 23, and 31 hours), gastrula (43 and 52 hours) and pluteus (75 hours). All four sea urchin argonaute/piwi mRNAs were expressed in sperm-depleted testis, egg and all embryonic stages.


A.
Seawi
1
855
PAZ
Piwi
272
405
547
840
Seali
1
960
PAZ
Piwi
373
512
653
945
Sp ago1A
1
932
PAZ
Piwi
315
451
599
897
Sp ago1B
1
958
PAZ
Piwi
384
492
642
908


B.
Depleted testis
Egg
6 hours
12 hours
18 hours
23 hours blastula
31 hours
43 hours early gastrula
52 hours gastrula
75 hours pluteus
1600 nt
1000 nt
500 nt
Seawi
Seali


Depleted testis
Egg
6 hours
12 hours
18 hours
23 hours blastula
31 hours
43 hours early gastrula
52 hours gastrula
75 hours pluteus
Negative Control RT-
Negative Control RNA-
Depleted testis
Egg
6 hours
12 hours
18 hours
23 hours blastula
31 hours
43 hours early gastrula
52 hours gastrula
75 hours pluteus
1600 nt
1000 nt
500 nt
Sp ago1A
Sp ago1B

CHAPTER 3

Localization of seawi in adult testes and ovaries

Argonaute and piwi subfamily members, in concert with small non-coding RNAs, have been shown to have important roles in regulating multiple aspects of cell, tissue, and organismal development through specific post-transcriptional, translational, and epigenetic mechanisms (see Chapters 1 and 2). It appears that the piwi subfamily of proteins are particularly important for establishment and/or maintenance of both germ-line stem cells and adult germ-line niches. For example, loss of piwi and/or piwi-like protein function in an organism can lead to improper nuclear migration, defective germ cell formation, failure of germ cell maintenance, defective segment polarity formation, and arrest of gemetic cells during spermatogenesis (Cox et al., 1998; Cox et al., 2000; Lykke-Andersen et al., 2008; Meyer et al., 2006). The aforementioned general importance of piwi and piwi-like in oogenesis and spermatogenesis is evidenced across the animal kingdom from fruit flies to man (Carmell et al., 2007; Deng and Lin, 2002; Grivna et al., 2006b; Kalmykova et al., 2005; Kennerdell et al., 2002; O'Donnell and Boeke, 2007;

Qiao et al., 2002; Tan et al., 2002; Wang and Reinke, 2008).

Rodriguez et al. (2005) previously identified that the protein seawi co-localizes with mitotic spindles during the first division, becomes enriched in small micromeres at the 16 cell stage, and is capable of binding bep4 mRNA which codes for an axis-specifying protein. Further, it has been shown that adult sea urchins from micromere-deleted embryos developed gonads and viable gametes (Ransick et al., 1996), but animals raised from small micromere-deleted embryos formed small gonads that lacked gametes (Yajima and Wessel, 2010). Combining prior data on seawi with the observation that piwi proteins have essential roles in germline establishment and maintenance, it would be reasonable to suggest that seawi expression may be enhanced in germ-line progenitors (small micromeres) and seawi should be expressed in adult gonads.

In this chapter, we used immunofluorescence confocal microscopy to characterize seawi expression patterns in gastrula, prism and pluteus embryos and thus identify its localization relative to the known germ-line stem cell niche. Additionally, we used immunoblotting and immunofluorescence confocal microscopy to characterize the expression and localization of seawi

in adult ovaries and testes. These results shed light on potential relevance of seawi expression and localization for establishment and maintenace of the sea urchin germline.

Materials and Methods

Immunohistochemistry and immunoblotting

Adult sea urchin were purchased and maintained as described in Chapter 2. Testes and ovaries were directly removed by dissection. Gamete-depleted testes and ovaries were prepared by multiple injections of 0.53 M KCl into the coelomic cavity over a two-hour period, and the gamete-depleted testes or ovaries were removed by dissection. Testes, gamete-depleted testes, ovaries, and gamete-depleted ovaries were fixed in 4% paraformaldehyde, sucrose infiltrated, rapidly frozen in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC), and sectioned into 10 µm slices by cryostat (Leica Microsystem, Buffalo Grove, IL). Sections were permeabilized and blocked in 5% calf serum and 0.02% Triton X-100 in PBS (blocking solution), and incubated in anti-seawi primary antibodies (antisera 4I4; (Faire and Bonder, 1993) diluted 1:50 in blocking solution, followed by a second incubation in a 1:500 dilution of Texas-red goat anti-rabbit secondary antibodies (Molecular Probes/Invitrogen, Carlsbad, CA) and a 1:1000 dilution of 4',6' diamino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA). Egg, embryos and oocytes for indirect immunofluorescene were prepared

according to Bonder et al. (1989); special thanks to Dr. Alexis Rodriguez for sharing his unpublished images. Images were collected using a Zeiss 510 Meta or BioRad MRC 1024 laser scanning confocal microscope using high NA oil immersion objective lenses. Six micrographs from six testes and six from six ovaries of three individual sea urchins were collected for quantification of seawi fluorescence intensity. The intensity per area in the regions of each cell-type (spermatogonia, spermatocytes, mix population, spermatozoa, oogonia, primary oocytes and secondary oocytes/eggs) and background were collected. The average intensity per area was calculated and the average background intensity was subtracted. Fluorescence intensity values were standardized relative to spermatogonia or oogonia.

Total protein samples of sperm, egg, depleted testes, and depleted ovaries were prepared for SDS-PAGE as described in Bonder et al. (1989). From each sample, 25 µg of protein was separated on a 7.5-15% gradient SDS-PAGE gel and transferred to a nitrocellulose membrane using a GENIE electrophoretic transfer apparatus (Idea Scientific, Minneapolis, MN). After blocking with 5% calf serum with 1% BSA, the nitrocellulose membrane was probed with a 1:500 dilution of anti-seawi 4I4 primary antibodies, followed by

an incubation in a 1:15000 dilution of alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies (Biosource/Invitrogen, Carlsbad CA). The immunoblot was developed in 0.15 M Tris buffer (pH 8.8) with 0.03% BCIP and 0.03% NBT (Fisher BioReagents, Pittsburgh PA) to detect the bound antibodies.

Results

In primary oocytes, seawi is localized throughout the cytoplasm with occasional indication of punctate staining (Figure 3-1A). In secondary oocytes, seawi begins to exhibit enhanced localization to the cortex of the oocyte and increasing association with distinct punta (Figure 3-1B). The enriched cortical positioning of seawi in secondary oocytes foreshadows the cortical rich distribution of seawi in egg (Figure 3-1C). Previously, it was reported that seawi protein expression is enriched in the vegetal blastomeres of the 16 cell stage embryo (Rodriguez et al., 2005) (Figure 3-1D). In a gastrulating embryo, seawi was expressed throughout the embryo, with a distinct enrichment in the small micromere descendants present at the tip of the invaginated archenteron (Figure 3-1E). In prism (Figure 3-1F) and pluteus stage embryo (Figure 3-1G), seawi was clearly enriched in both coelomic pouches. The presence of seawi in the left coelomic pouch is noteworthy since this population of cells is responsible for harboring the stem cell niches for the adult germline.

Seawi's localization to cells of the presumptive germline in embryos and the reported importance of piwi in gametogenesis (Deng and Lin, 2002;

Grivna et al., 2006b; Kuramochi-Miyagawa et al., 2004) provide support for proposing that seawi may play a potential role in the adult germline. Immunoblot analysis showed seawi in the egg and sperm-depleted testes, but did not detect seawi in sperm or egg/oocyte-depleted ovaries (Fig. 3-1H). To determine the localization of seawi in adult testes, individual testis sample from three adult urchins were processed for indirect immunofluoresence staining. The sea urchin testis has a very distinct stratified arrangement with spermatogonia (Sg) located in the outer layer, below the smooth muscle fiber. Spermatocytes and spermatids are located beneath the outer layer, and spermatozoa (Sz) are in the innermost layer (Figure 3-2A). Seawi protein was found most prominently in spermatogonia and in spermatocytes and mixed population cells (Figure 3-2B and C). At low magnification there was no detectable seawi present in mature sperm (Figure 3-2B and C). At higher magnification, seawi is exclusively localized to the cytoplasm of spermatogonia (Figure 3-2D), spermatocytes (Figure 3-2E) and spermatids in the mixed population (Figure 3-2F). Even at high magnification there was no detectable stain in sperm (Figure 3-2G); it is not possible to exclude the possibility that the expression levels in adult sperm could be below the

detection limit of immunofluorecence staining and confocal microscopy. A line scan analysis of single testis sections (Figure 3-3A) identified a gradient of seawi expression along the spermatogenic developmental axis. The fluorescent intensity of seawi signals from images of six testes taken from three sea urchins (Figure 3-3B), were analyzed to quantify seawi expression. Seawi expression was found to decrease along the spermatogenic developmental axis, with greater expression in stem-cell spermatocytes and lowest in mature sperm; mature sperm essentially lack cytoplasm and contain nuclei that have completed meiosis.

As with testes, ovaries have a well-defined structure that allows for cell-type identification. The oogonia are located in the outer layer, primary oocytes are located beneath the outer layer, and secondary oocytes/eggs are in the innermost layer (Figure 3-4A). Seawi was expressed in oogonia (Figure 3-4D), primary oocytes (Figure 3-4E), secondary oocytes (Figure 3-4F) and mature eggs (Figure 3-4G). In sea urchin ovaries, seawi appears to be expressed at a relative constant level from oogonia in the outer-most layer to secondary oocytes/eggs in the inner-most layer (Figure 3-4B and C) however there is differential cellular localization associated with maturation. Seawi was

not detected in non-oogenic cells. A line scan from a single ovarian section further confirmed seawi was expressed at a steady level from oogonia to secondary oocytes/eggs (Figure 3-5 A). Seawi expression images from six ovaries of three individual sea urchins were quantified (Figure 3-5 B). Seawi express similar level in oogonia and primary oocytes, but its expression is slightly increasing in secondary oocytes/eggs.

In this section, we showed seawi is expressed in oocytes, egg and germline progenitor cells throughout embryogenesis. It is also expressed in both testis and ovary in adult sea urchin. Seawi expression in testes is decreased along the developmental axis, whereas levels of seawi reveal relatively constant throughout oogenesis.

Discussion

Seawi in sea urchin embryonic development

Traditionally, maternal mRNA distribution and translation are regulated by binding directly to specific RNA-binding proteins that serve to control spatial and temporal protein expression (Siomi and Dreyfuss, 1997). For example, in fruit flies, *bicoid*, a morphogen that directs A-P axis formation, is shown to function by directly binding to nanos mRNA for translational silencing (Johnstone and Lasko, 2001; Zamore and Lehmann, 1996). Since 2000, small non-coding RNA's and the argonaute family of protein have been frequently identified as key players in regulating aspects of embryogenesis and tissue differentiation. For example, the oskar mRNA sequence appears targeted by miR-280 (Stark et al., 2003). In fruit flies, the 3'UTR of nanos mRNA, there are two piRNA target sequences that are required for translational silencing in fruit flies (Rouget et al., 2010). Translational repression of oskar mRNA in Drosophila occurs primarily through a pathway that requires proteins associated with the small non-coding RNA machinery, such as armitage, aubergine (a piwi protein) and spindle-E (Kennerdell et al., 2002; Tomari et al., 2004). Endogenous small non-coding RNAs that mediate

mRNA silencing are predicted to bind complementary sequences in the 3' UTRs of numerous target transcripts such as oskar, suggesting that the small non-coding RNA machinery may coordinate translational control of gene expression during embryonic development (Kugler and Lasko, 2009; Stark et al., 2003).

Based on our characterization of seawi, seali, sp-ago1A and sp-ago1B, it is reasonable to suggest that these proteins are involved in translational and/or post-transcriptional regulation through the small non-coding RNA pathway. To build upon this hypothesis, the embryonic and adult localization of seawi were determined. Seawi is expressed in oogenic cells and eggs. Seawi displays punctate expression throughout the cytoplasm in primary oocytes. In secondary oocytes, seawi expression was sequestered in the cortex of the cell that sets the seawi localization pattern observed in mature eggs. Previously, Faire and Bonder (1993) provided evidence that seawi (originally identify as dynamin) was closely associated with cortical granules. Seawi positions to the cortex are correlated with cortical granule translocation, and it has been shown that cortical granule movement to the cortex is actin and Rho dependent. The association of seawi with the actin cytoskeleton has

never been established, and consequently the significance of this spacial-temporal association remains to be resolved.

Seawi localizes on mitotic spindles/microtubules during the first division after fertilization (Rodriguez et al., 2005). Microtubules have been suggested to direct maternal mRNA localization to the cortex in *Xenopus* and to the required for establishing A-P mRNA gradients in *Drosophila* oocytes (Johnstone and Lasko, 2001; Kugler and Lasko, 2009; Yisraeli et al., 1990). Segregating mRNAs instead of proteins has the advantage of localizing mRNA translation to the site of the protein's function and may allow the local assembly of the synthesized protein into the proper macromolecular complexes. Localized protein synthesis is, in fact, not unique to developing embryos, but is also found in somatic cells having structural polarity (Donnelly et al., 2011; Rodriguez et al., 2008). During the first division of the sea urchin embryo, maternal mRNAs and their associated protein complexes have to properly segregate into two daughter cells. Seawi processes a mRNA binding motif and previously it has been shown to bind bep4 (Rodriguez et al., 2005), which opens the possibility that the localization to mitotic spindles may reflect a function in segregating maternal mRNA during development.

There is considerable evidence suggesting that germ-line cell fate is determined between the 16 and 32-cell stage in embryos, and this timing is correlated to A-V axis formation (Brandhorst and Klein, 2002; Ransick et al., 1996; Yajima and Wessel, 2010). The deletion of micromeres from 16-cell embryos did not affect adult germline formation (Ransick et al., 1996); however, adults reared from small micromere-deleted 32-cell embryos processed small gonads that lacked gametes (Yajima and Wessel, 2010). Small micromere descendants reside in the coelomic pouches in larval stages and later derive into the sea urchin adult germline (Ransick et al., 1996; Yajima and Wessel, 2010), which indicates that the small micromeres function as the initial germline stem cells.

Seawi is enriched in micromeres in the vegetal pole in 16-cell embryos and seawi remain localized to the small micromere at the tip of the invaginated archenteron in gastrulae and in the coelomic pouches in the prism and pluteus stage. Moreover, seawi expression levels (based on immunofluorescence staining) increase in micromeres right before germline cell-fate determination. This observation suggests that seawi plays a possible role in promoting and/or maintaining the germline stem cells, an observation

that is consistent with data from fruit flies (Cox et al., 1998; Cox et al., 2000).

Seawi in adult gonads

Beside embryonic germline, we establish that seawi is expressed in both adult male and female gonads. Piwi proteins have been shown to play crucial roles in gametogenesis. Loss-of-function of fruitfly piwi, mouse miwi and mili, all result in germ cell arrest during gametogenesis (Cox et al., 2000; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2001). In sea urchin testes, seawi protein is localized to the cytoplasm of spermatogenic cells (sperm progenitors) and the expression level decreases along the developmental axis. This expression pattern raises two possible roles of seawi during spermatogenesis. In *C. elegans*, piwi-like prg-1 is localized in P granules, which are found in progenitor cells for the germline lineage. Loss of *prg-1* causes a marked change in expression of a subset of mRNAs expressed during spermatogenesis, and *prg-1* mutant sperm exhibit defects in egg activation and fertilization (Wang and Reinke, 2008). Given seawi's enrichment in spermatogonia, the possibility exist that it functions in regulating subsets of mRNA required for spermatogenic regulation.

The second possibility is to maintain germ cell genome integrity from destructive genomic insertion of transposable elements. In fruit flies, zebrafish and mice, piRNA that are isolated from testes are complementary to transposon sequences (Carmell et al., 2007; Houwing et al., 2007; Saito et al., 2006). In fruit fly testes, loss-of-function of piwi results in an increase in transposon activity (Kalmykova et al., 2005). Seawi is expressed in highest level in spermatogenic cell, that are undergoing meiosis, a cell process that most susceptible to transposon activity (van der Heijden and Bortvin, 2009). Upon completion of meiosis, seawi would no longer be required for genome "surveillance" and thus it is not expressed in mature sperm.

In sea urchin ovary, seawi expression stays relatively constant from oogonia to primary oocytes and slightly higher in secondary oocytes/egg. Similar to spermatogenesis, seawi may play a role in regulate oogenic mRNA and/or protect the maternal genome during meiosis. Different from testis, seawi is the maternal protein that is carried into egg and embryo stage. Early embryogenesis is marked by rapid DNA replication and rapid division cycles, an intracellular environment that required genomic maintenance. Consequently, maternal stored seawi may function in a genomic surveillance

capacity as has been reported for oogenesis (De Felici, 2010; Wilczynska et al., 2009).

Figure 3-1. Immunolocalization of seawi in oocytes, eggs and embryos.

Seawi is expressed in the cytoplasm of primary oocytes (A) and is enriched in the cortex of secondary oocytes (B). (C) Seawi is enriched in the cortex of the unfertilized egg. (D) In the 16-cell stage, seawi is expressed in all the cells; however, it is enriched in the four micromeres present at the vegetal pole. Expression appears to be punctate in the cytoplasm. (E) In the gastrula stage, seawi continues to be expressed throughout the embryo. Note the distinct enrichment of seawi in the small micromere descendants at the tip of the invaginated archenteron. (F-G) In the prism and pluteus stages, seawi is clearly enriched in coelomic pouches, a known stem-cell niche for the adult body plan and possibly germline. (H) Immunoblotting analysis with anti-seawi antibody. Seawi is expressed in the sperm-depleted testis and egg, but is not detected in egg-depleted ovaries or sperm. Bar in (A) 5μm and (B) to (F) 10μm. The images of seawi localization during embryogenesis (D-G) are courtersy of Dr. Alexis Rodriguez.

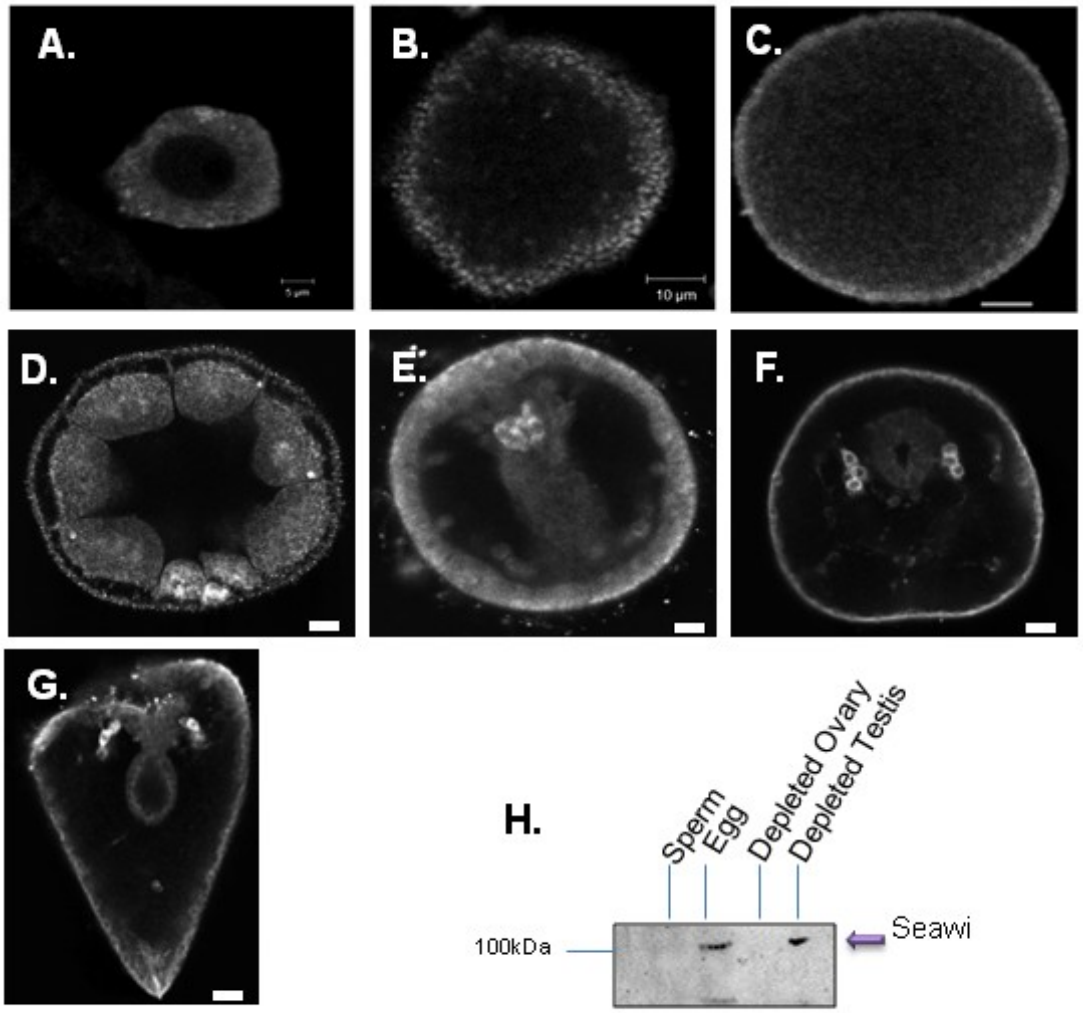

A.
B.
10 µm
C.
D.
E.
F.
G.
H.
Sperm
Egg
Depleted Ovary
Depleted Testis
100kDa
Seawi

Figure 3-2. Seawi localization in adult sea urchin testes.

(A-C) Testis double stained with DAPI (A) and anti-seawi antibody (B); the merged image is shown in (C). (D-G) Seawi is expressed by spermatogonia (D) in the outer layer, spermatocytes (E) and mixed population cells (F) in the middle layer but not in spermatozoa (G) in the most inner layer. Seawi is not co-localized with DAPI, a stain for nucleic acids and thus a nuclear marker. Seawi is cytoplasmically located in spermatocytes and spermatogonia, and there is no detectable evidence for seawi localization in the nucleus. Bar in A to C 10 μm, D to E 2 μm and F 5 μm.

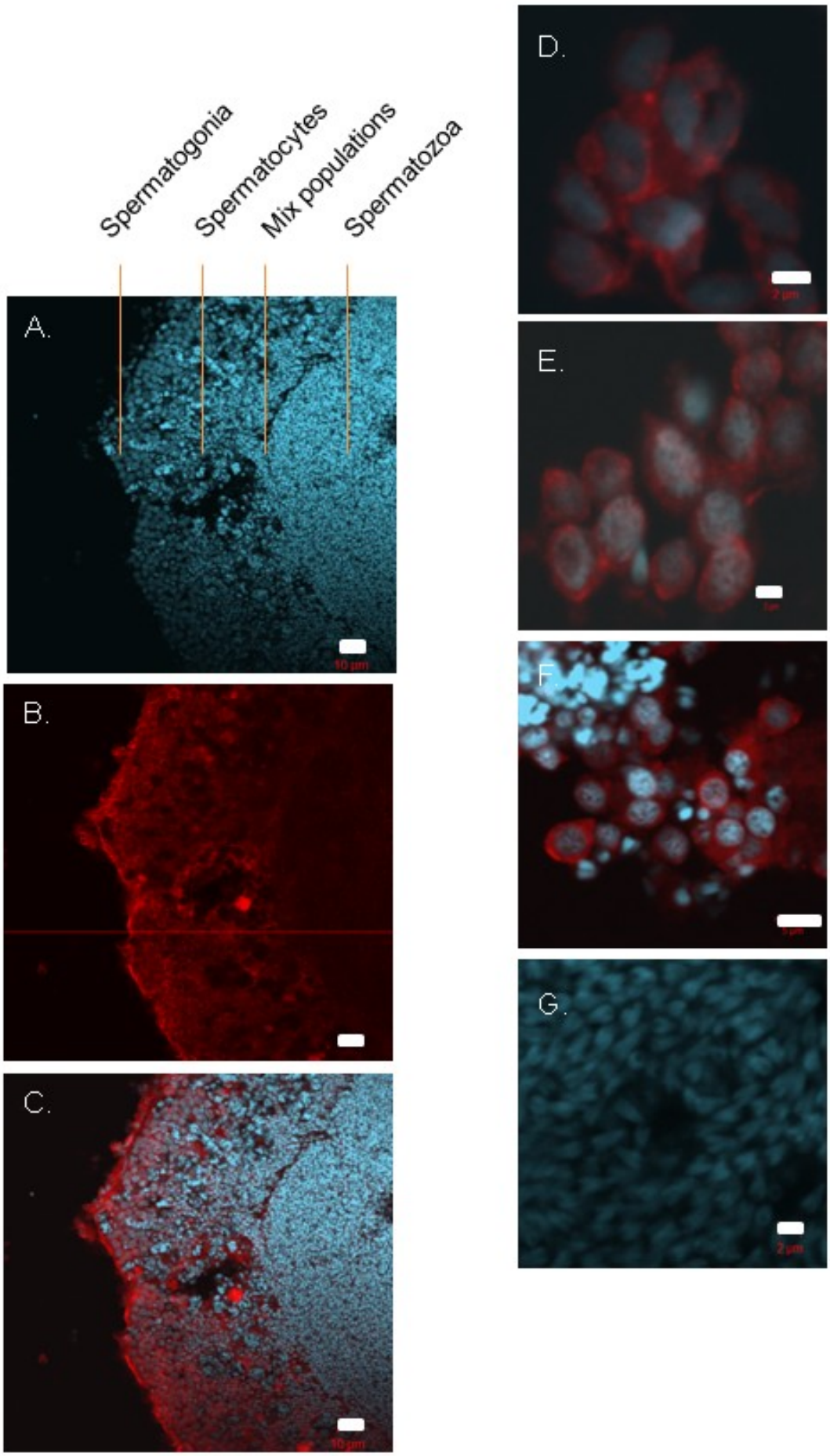
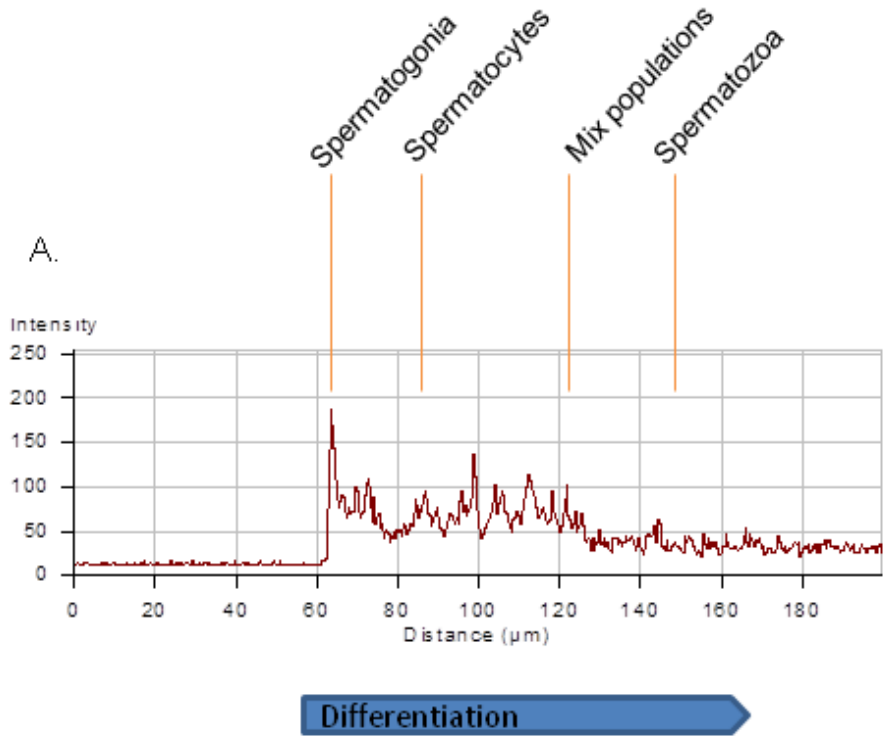

Spermatogonia
Spermatocytes
Mix populations
Spermatozoa
A.
B.
C.
D.
E.
F.
G.

Figure 3-3. Linescan and quantification of the seawi expression in adult testes.

(A) Fluorescence intensity line-scan with correlated line in Figure 3-2 (B). (B) Quantification of fluorescence intensity in different cell populations in testes. Seawi expression decrease along the developmental axis.


Spermatogonia
Spermatocytes
Mix populations
Spermatozoa
A.
Intensity
250
200
150
100
50
0
0
20
40
60
80
100
120
140
160
180
Distance (µm)
Differentiation

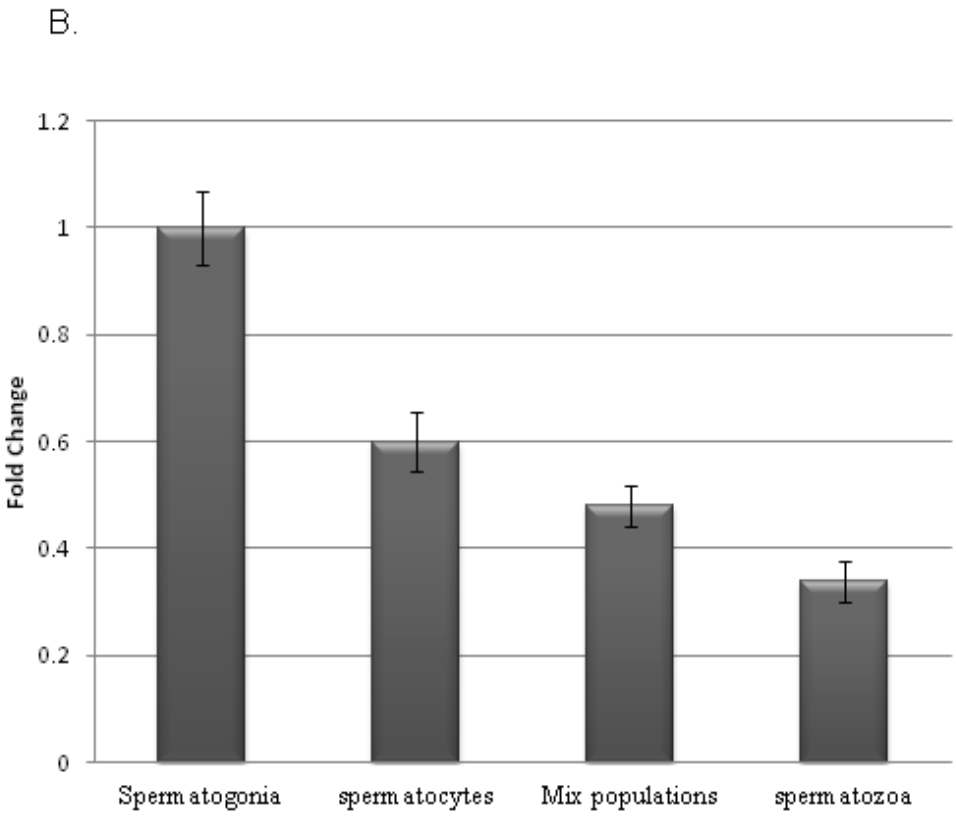

B.
1.2
1
0.8
0.6
0.4
0.2
0
Fold Change
Spermatogonia
spermatocytes
Mix populations
spermatozoa

Figure 3-4. Seawi localization in sea urchin ovaries.

(A-C) Ovaries double stained with DAPI (A) and anti-seawi antibody (B); the merged image is shown in (C). (D-G) Seawi is expressed by oogonia (D) in the outer layer, primary oocytes (E) in the middle layer and by secondary oocytes (G) and eggs in the most inner layer. Seawi is not co-localized with DAPI. Seawi is cytoplasmically located in oogonic cells. Bar in A to C 10 μm, D 2 μm, E 5 μm and F to G 10 μm.

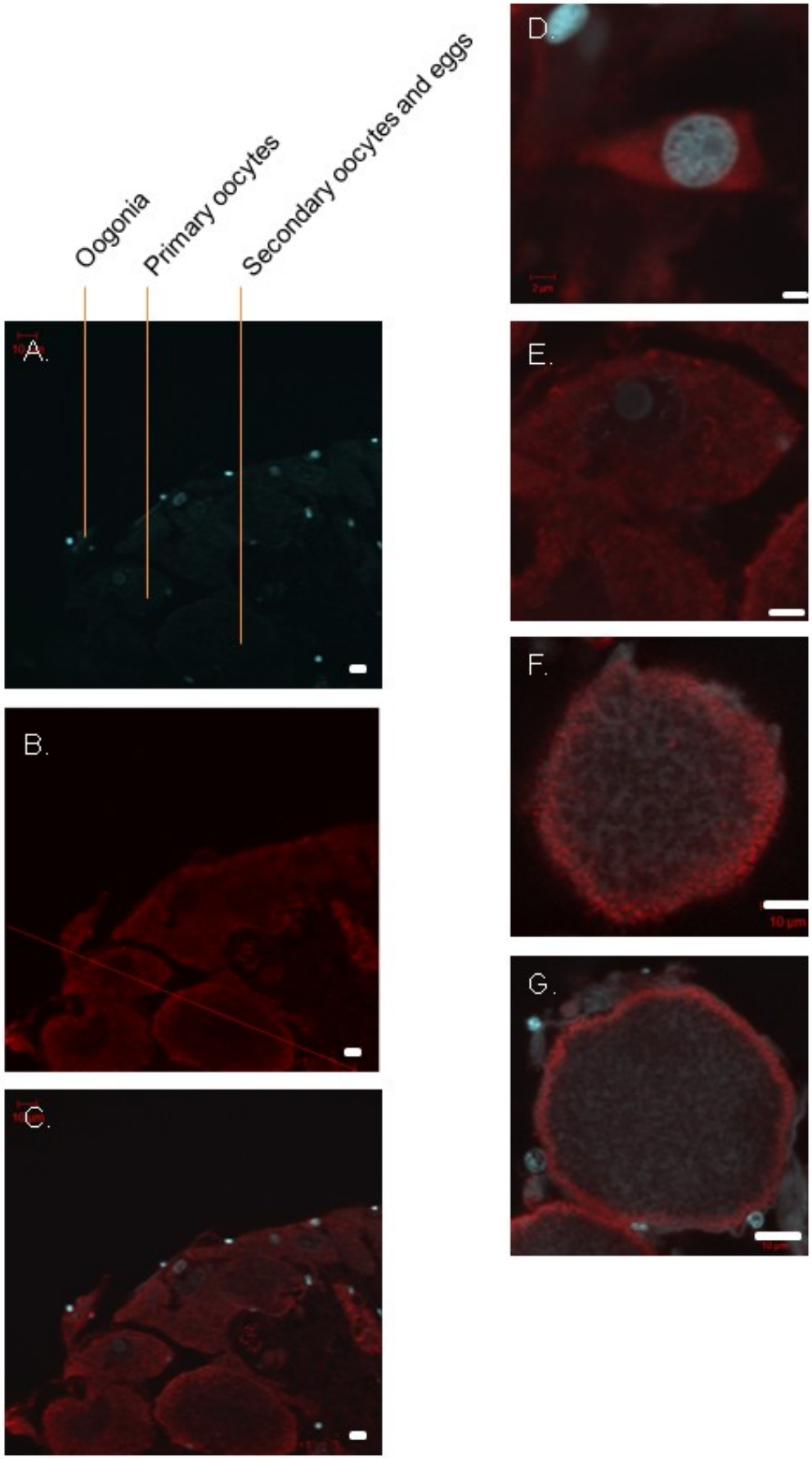
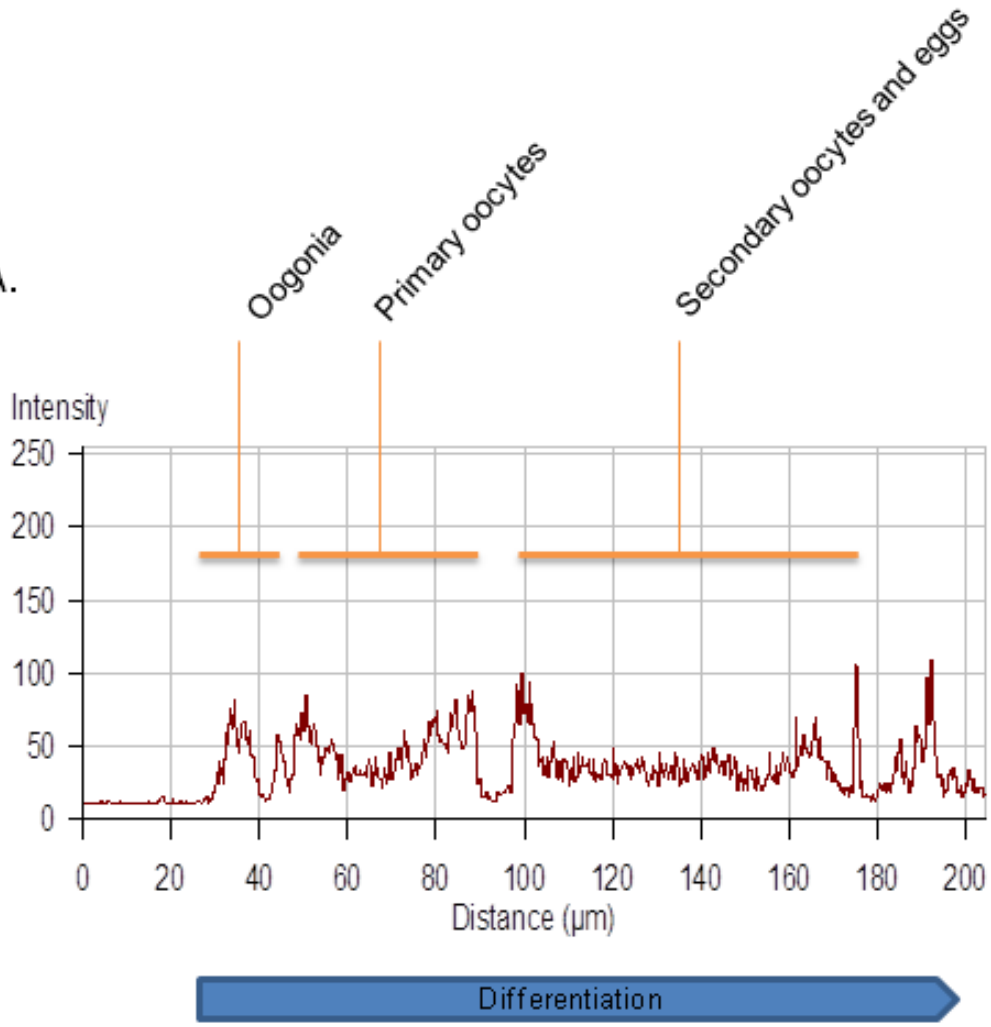

Oogonia
Primary oocytes
Secondary oocytes and eggs
A.
B.
C.
D.
E.
F.
G.

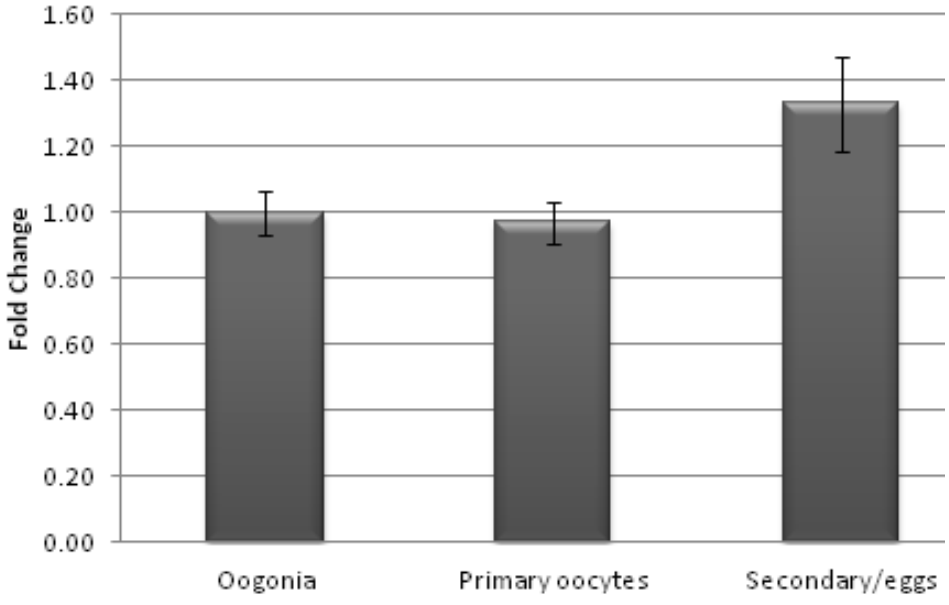
Figure 3-5. Linescan and quantification of the seawi expression in adult ovaries.

(A) Fluorescence intensity line-scan with correlated line in Figure 3-4 (B). (B) Quantification of fluorescence intensity in different cell populations in ovaries. Seawi express constant level with slightly increase in secondary oocytes/eggs. There is no obvious seawi expression gradient along the differentiation axis in adult ovaries.

A.


Oogonia
Primary oocytes
Secondary oocytes and eggs
Intensity
250
200
150
100
50
0
0
20
40
60
80
100
120
140
160
180
200
Distance (µm)
Differentiation

B.


1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Fold Change
Oogonia
Primary oocytes
Secondary/eggs

CHAPTER 4

Sea urchin small non-coding RNAs

Although many examples of the cellular expression of small non-coding RNAs have been identified, various aspects of their origins, structures, associated effector proteins, and biological roles have led to the general recognition of three main categories: siRNAs), miRNAs, and piRNAs (for excellent reviews see (Czech and Hannon, 2010; Ghildiyal and Zamore, 2009; Hutvagner, 2005). The boundaries between the various small non-coding RNA classes are becoming increasingly difficult to discern, but nonetheless some distinctions exist (Figure 4-1). The si-, mi-, and piRNA can be separated based on three major characteristics: length, 3' modification, and precursor structure. The piRNA is 24-30 nt in length and modified with 2-O-methylation at their 3' termini. Moreover, piRNA are generated by RNase III motif in PIWI domain, which leads to a uracil nucleotide as the first nucleotide on the 5' terminus (Beyret et al., 2012). The miRNA and siRNA, on the other hand, are slightly shorter at 19-22 nt in length. SiRNA is modified with 2-O-methylation in fruit flies but not in mammals, and miRNA is not modified at the

3' terminus (Horwich et al., 2007; Klattenhoff and Theurkauf, 2008). Cleaving by enzyme drosha, the mature miRNA is processed from the precursor with hairpin structure (Krol et al., 2004), which is not observed in siRNA precursors (Ghildiyal and Zamore, 2009; Zamore et al., 2000).

As described in Chapter 1, si-, mi- and piRNA play various roles in embryonic and/or germline development. Although the function is still unknown, the sequences of recently discovered endo-siRNA were mapped to retrotransposons, and to some specific mRNA, and they are expressed in both germline and somatic cells (Fagegaltier et al., 2009; Nilsen, 2008). MiRNAs are involved in the spatial and temporal control of gene expression in developmental events in both multicellular and unicellular organisms (Stefani and Slack, 2008). PiRNA has the capability to regulate cellular processing through post-transcriptional, translational regulation of RNA transcripts and epigenetic modification of genomic DNA. Functional studies of piRNAs are mainly based on use of molecular genetic approaches and utilization of loss-of-function mutations in their associated piwi proteins. For example, knocking out the mouse *piwi* genes: *miwi*, *mili* and *miwi2*, results in the disruption of male germline development (Carmell et al., 2007; Deng and Lin, 2002;

Kuramochi-Miyagawa et al., 2004). To protect genome integrity, it has been shown that piwi proteins, together with piRNAs, cleave transposon transcripts in nematode worms (Das et al., 2008), silk worms (Tatsuke et al., 2009), fruit flies (Pelisson et al., 2007) and mice (Xu et al., 2008). Besides transposon transcripts, piRNA has also been shown to regulate morphogen nanos mRNA translation in fruit fly development (Rouget et al., 2010). Piwi and piRNA are also known to epigenetically regulate DNA modification. In CNS neurons of *Aplysia*, piwi and piRNA showed predominately nuclear localization and are required for methylation of nucleotides in CREB promoter, which is a repressor for long term memory establishment (Rajasethupathy et al., 2012).

There were reports of small non-coding RNA, mainly miRNA, in the sea urchin model system. The first identified sea urchin small non-coding RNA is the miRNA *let-7* (Pasquinelli et. al., 2000). While this work was in progress, a number of sea urchin miRNAs were identified and sequenced, and these sequences were conserved in mice and humans. (Campo-Paysaa et al., 2011; Friedländer et al., 2011; Peterson et al., 2009; Wheeler et al., 2009, Song 2012). In addition, inhibition of dicer and drosha, proteins for miRNA biogenesis, leads to embryonic lethality (Song et al., 2011).

Seawi is the first piwi protein to be reported to associate with polyribosomes and possibly silence bep4 mRNA through unknown mechanism (Hamill et al., 1994; Rodriguez et al., 2005). Previously, we showed that seawi, seali, sp-ago1A and sp-ago1B all have small non-coding RNA binding motif in their PAZ domain (see Chapter 2). Seawi is localized to eggs, stem cells, germ-line progenitor cells and adult gonads (see Chapter 3). These results suggest, in addition to miRNA, there may/should exist other sea urchin small non-coding RNAs in eggs, embryos and adult gonads.

In this chapter, we isolated, sequenced and biochemically characterized sea urchin non-coding RNAs from gametes, two-cell embryos and adult gonads. We further examined if any small non-coding RNAs are associated with seawi in eggs and embryos, and using bioinformatic approaches identify possible target transcripts for newly identified sea urchin non coding small RNAs.

Materials and Methods

Immunoprecipitation

Seawi antisera (4I4) 20 µl was mixed with 15 µl magnetic protein A beads (New England Biolabs, Ipswich, MA) at 4°C overnight and the beads were collected and stored at 4°C. Eggs and two cell embryos were collected (see Chapter 2) and lysates were prepared by homogenization (see Chapter 3). Lysates were further subjected to centrifugation at 14000 g in an Eppendorf microfuge 5415c (rotor with 7.3 cm radius) for 10 minutes at 4°C and the resultant supernatant used for immunoprecipitation. Protein concentration was measured by Bradford assay (Bio-Rad, Hercules, CA). Seawi antisera/Protein A beads were added to 100 µl of lysate at 5 µg/µl and rocked gently overnight at 4°C. Beads were collected, washed, and incubated with Proteinase K (Sigma, St. Louis, MO) and store at -80°C.

High-throughput sequencing and small RNA characterization

The following methods were done in colaboration with Dr. Chenjian Li and Dr. Phillip Zamore (The University of Massachusetts Medical School, Worcester, MA). Total RNAs from sperm, eggs, two-cell embryos, testes,

gamete-depleted testes, ovaries and gamete-depleted ovaries, as well as RNAs collected by anti-seawi antisera immunoprecipitation from egg and two-cell embryos and MT-RNP RNAs from two-cell embryo were collected with Trizol LS method (Invitrogen, Carlsbad CA) as previously described in Chapter 2. RNAs were quantified by absorbance at 260/280 nm. Small non-coding RNAs (18–30 nucleotides) were separated on a 15% denaturing urea-polyacrylamide gel (National Diagnostics, Atlanta, GA) and stained with SYBR Gold (Invitrogen, Carlsbad, CA). The 18 to 30 nt region of gels were cut out and “small RNA” was eluted from the gel slices. Half of the collected small RNA was oxidized with 25 mM $NaIO_4$ in 60 mM borax, 60 mM boric acid, pH 8.6, for 30 min at room temperature followed by ethanol precipitation. To generate small RNA libraries, 100 pmole of 3’ pre-adenylated adapter (5’-rAppTCG TAT GCC GTC TTC TGC TTG T/ddC/-3’) was ligated to oxidized or un-oxidized small RNAs in 20 µl using mutant bacteriophage T_4 RNA ligase 2 (Rnl2) (amino acids 1-249, K227Q) (17-23B) (Addgene, Cambridge, MA) at 4°C for 12 hour in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 60 µg/ml BSA, 10% (v/v) DMSO, and 40 U RNasin (Promega, Madison, WI). 3’ ligated product was gel purified, and then ligated to 100 pmole of 5’ RNA

adapter (5'-GUU CAG AGU UCU ACA GUC CGA CGA UC-3') in 20 µl using T_4 RNA ligase (Ambion, Austin, TX) at room temperature for 6 h in 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 10% DMSO, and the ligated products were gel purified. Half the ligated product was used to synthesize cDNA using Superscript III (Invitrogen, Carlsbad, CA) and the reverse transcription primer (5'-CAA GCA GAA GAC GGC ATA CGA-3'), whose sequences are reverse complementary to 3' adapter, and half was used as a minus RT control. The small RNA library was amplified using forward (5'-AAT GAT ACG GCG ACC ACC GAC AGG TTC AGA GTT CTA CAG TCC GA-3') and reverse (5´-CAA GCA GAA GAC GGC ATA CGA-3´) primers, and then purified from a 4% NuSieve GTG agarose gel (Lonza, Basel, Switzerland). There are distinct adapters in known orientation at the 5' and 3' end of small RNA, therefore, the cDNA library is also flanked by two distinct adaptor sequences. Libraries were sequenced using a Solexa Genome Analyzer (Illumina, San Diego, CA), and by filtering with adapter sequences, the strand-specific sequences were obtained (Levin et al., 2010).

Sequence extraction and annotation

For each sequence read, the first occurrence of the 6-mer perfectly matching the 5' end of the 3' adapter was identified. Sequences without a perfect match were discarded. The obtained small RNA sequences were then mapped to the *S. purpuratus* genome (Release version 3.1, SpBase). Genes and transposon annotations were retrieved from SpBase (Release version 3.1). Unannotated sequences were identified using BLAST to query each consensus sequence against the genome. The number of reads for each small RNA was normalized by the number of times that RNA mapped to the genome and by the abundance of small RNAs in the sample. For example, if a piRNA mapped to a location where there were two or more blast-based gene/transposon annotations, the number of reads was normalized by the number of annotations. The quantification of the small non-coding RNA clusters is shown as parts per million, which is calculated by the number of hits divided by total sequence reads. After generating the quantification of small non-coding RNA reads, the size distribution and strand bias were further calculated.

Analysis of small non-coding RNA 5' and 3' termini

From the depleted testes small RNA library, the most abundant oxidized small RNA (piRNA-1 5'-TGGACACCAGATAGTACTCGGCATCCTGC-3') (Figure 4-3B) and the most abundant miRNA (miR-10) (Figure 4-3C) were isolated. For 5' analysis, both small RNAs were radio-labeled in a 20 µl reaction containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, 10%(v/v) DMSO, 10 mg/ml BSA, 2 units/ml RNasin (Promega, Madison, WI), 1.5 mCi/ml [5' ^{32}P] cytidine 5',3' bis-phosphate ([5' ^{32}P]-pCp; Perkin-Elmer, Waltham, MA), and 1 unit/ml T_4 RNA Ligase 1 (New England Biolabs, Ipswich, MA) at 4°C, overnight. The radio-labeled small RNAs were then further purified from a 15% denaturing urea-polyacrylamide sequencing gel and digested with 1.5 U/ml micrococcal nuclease (Takara Mirus Bio, CA) in a 40 ml reaction containing 20mM Tris-HCl (pH 8.0), 5mM NaCl, and 2.5mM $CaCl_2$. The purified, ^{32}P-radiolabeled RNA was hydrolyzed in 200 mM Na_2CO_3 at 100°C for 1 hr, neutralized with an equal volume of 200 mM HCl, then dephosphorylated with 0.5 units/ml calf intestinal alkaline phosphatase (CIP; New England Biolabs, Ipswich MA) in a 200 ml reaction containing 50 mM

Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol at pH 7.9. The results were analyzed on 22.5% denaturing urea-polyacrylamide gel (Horwich et al., 2007; Summer et al., 2009).

For 3' analysis, both small RNAs were oxidized with 80 mM $NaIO_4$ in borax/boric-acid buffer (60 mM borax and 60 mM boric acid at pH 8.6) at room temperature for 30 min and then beta-eliminated with 200 mM NaOH at 45°C for 90 min. The reaction was stopped by the addition of 300 mM NaCl, 1 µg glycogen, and three volumes of absolute ethanol. After 30 min on ice, the precipitated RNA was collected by centrifugation. The purified RNAs were mixed with an equal volume of formamide loading buffer (98% deionized formamide, 10 mM EDTA at pH 8.0, 0.025% (w/v) xylene cyanol, and 0.025% (w/v) bromophenol blue) and resolved on a 22.5% denaturing urea-polyacrylamide gel.

Results

Identification of sea urchin miRNA and piRNA

Isolated RNAs from testes and eggs were loaded onto a urea-polyacrylamide gel and stained with SYBR gold. A distinct band measuring 28 to 30 nucleotides was identified in testes samples; the nucleic acid size that is consistent with piRNAs reported in other species (Figure 4-2, testes with purple arrow) (Aravin et al., 2006; Grivna et al., 2006b; Lau et al., 2006; Saito et al., 2006). The egg total RNA in 18-30 nt range occurs as a smear (Figure 4-2, egg) and consequently for the reported experiments the entire 10 – 30 nt range of RNA was eluted from the gels. For depleted testes samples, sequencing of 18–30 nt RNA yielded over 3.4 million genome-mapping reads (Figure 4-3A, unoxidized) of which ~2.2 million passed the initial 6 nt perfect match screening requirements (see Materials and Methods). The ten most abundant small RNA sequences are shown in figure 4-3B (oxidized) and figure 4-3C (untreated). The sampled oxidized small RNAs have size range from 28 to 29 nt with thymidine (uracil on RNA) as the first nucleotide at 5' terminus. Together with 3' modification that protected them from destruction by oxidation reaction, these three characteristics (length, 5' nt,

and 3' modification) suggest the 28-30 nt small RNAs in the oxidized samples are piRNAs. In untreated sample, the blast search result shows that there are both miRNA and rRNA. Sea urchin miRNAs are 19-21 nt in length and their sequences appear conserved when compared to mammals. Analysis of sea urchin small non-coding RNAs from depleted testes revealed miR-10 to be the most abundant (Figure 4-3C). There are three forms of miR-10, which together represent 1.4% of the total small non-coding RNA population. Following miR-10 are miR-125 and miR-92 (Figure 4-3C). Further analysis using the miRBase (Griffiths-Jones et al., 2006) predicts precursor sequences and hairpin structures for the sequenced nucleic acids suggest that the 19-22 nt pool of small non-coding RNAs are miRNA. Sequencing of the 18-30 nt RNAs from eggs and testes yielded more than 34 million genome-mapping reads whose lengths, sequences, modification to 5' and 3' termini, and predicted precursor structures suggest they correspond to over 200 unique miRNAs and 3 million unique piRNAs.

We further characterized the miRNA and piRNA modifications by using a mobility test on two abundant pi- and miRNA from depleted testes. CIP removes 5' phosphates from nucleic acid strands and this results in a shift

(slower) in mobility (see Figure 4-4A, under SU testes lane CIP+, T4 PNK-). T4 PNK adds a single p32 labeled ATP to the 5' OH end of RNAs which leads to increased (faster) mobility (see Figure 4-4A, under SU testes lane CIP+, T4 PNK+). For both mi- and piRNA, treatment with CIP removed the 5'terminal phosphate that was then added by inclusion of T4 PNK and p32ATP (Fig. 4-4A). Thus, both miRNA and piRNA have a 5' monophosphate (Figure 4-4 A).

To examine modifications on the 3' end, beta-elimination was performed to test for modification (see Figure 4–1) of the 2'-OH group at the 3' terminus of the small RNAs. PiRNA, but not miRNA, was resistant to beta-elimination, suggesting piRNAs do not have a free hydroxyl group in the 2' position of the terminal ribose (Figure 4-4B). Taken together, these data suggest that in sea urchin, piRNAs undergo 2'-O-methylation on their 3' end and miRNA do not. The same chemical modification has also been observed in mouse and fruit fly (Figure 4-1) (Simon et al., 2011).

Using the NCBI database to identify the oxidized small non-coding RNAs or piRNA (Figure 4-3 B), it was revealed that three out of ten hits matched with predicted sea urchin mRNA sequences. Three piRNA sequences were complementary to two predicted mRNAs, XM001192826.1

(Pruitt et al., 2011; Pruitt et al., 2007), which is suggested to have a zinc finger motif (Webb and McMaster, 1993), with RNase H (Goedken and Marqusee, 2001) and reverse transcriptase activities (Huang et al., 1998), and XM001191171.1 (Pruitt et al., 2011; Pruitt et al., 2007), which is predicted to have a Mutator-like element (mule) transposase domain (Babu et al., 2006), a class of metal chelating DNA binding domains from retrotransposons. Both reverse transcriptase and transposase are crucial for transposon replication and transposition. The data not only verify the existence of small non-coding RNAs in the sea urchin but also suggests that piRNAs from sea urchin testes have a conserved role to silence transposable elements in male germline.

Analysis of miRNA and piRNA in sea urchin cells, gonads, and MT-RNP

We prepared and deep-sequenced small non-coding RNAs and examined the overall small RNA populations from total RNA samples of testes, sperm-deplete testes, sperm, ovaries, egg-depleted ovaries and eggs. The length distribution of oxidized (piRNA) and untreated (pi- and miRNA) small non-coding RNAs from male gonads are shown in Figure 4-5 (A unoxidized testes, B unoxidized sperm-depleted testes, C unoxidized sperm, D oxidized

testes, E oxidized sperm-depleted testes and F oxidized sperm). The length distribution of oxidized (piRNA) and untreated (pi- and miRNA) small non-coding RNAs from female gonads are shown in Figure 4-6 (A unoxidized ovaries, B unoxidized egg-depleted ovaries, C unoxidized eggs, D oxidized ovaries, E oxidized egg-depleted ovaries and F oxidized eggs).

In all sea urchin gonad and gamete samples, there are two distinct size populations of small non-coding RNAs, a 19 to 23 nt transcripts and 27 to 30 nt transcripts (Figure 4-5 untreated and 4-6 untreated). Oxidation of samples suggests that only the larger piRNA pool (27-30 nucleotides) possesses a 2'-O-methylation modification (Figure 4–4; 4-5 and 4-6 oxidized). These data indicate there are two small non-coding RNA populations, miRNA and piRNA in all gonads and gametes. Interestingly, pi- and miRNA were detected in populations of mature sperm (Fig. 4-5).

Next, more specifically, we examined if populations of small non-coding RNAs were associated with the protein seawi and/or MT-RNP complexes isolated from eggs or developing embryos. The size distribution of seawi-associated small non-coding RNAs isolated by immunoprecipitation of seawi from egg (Figure 4-7A and D) and two-cell embryos lysates (Figure 4-7B and

E) was determined. Seawi appears to be predominantly associated with ~28 nt piRNAs in egg and two-cell embryos (Figure 4-7A, B, D, E). Characterization of small RNAs isolated from MT-RNPs were showed that MT-RNP only associated with ~28 nt piRNAs with no detectable presence of miRNA (Figure 4-7C and F). Our data demonstrate that seawi and MT-RNPs (possibly through seawi) are mainly associated with ~28 nt piRNA.

Identification of small RNA targets

As discussed (see Chapter1), piRNA biogenesis processed by ping-pong amplification cycles with some sense piRNAs produced in order to generate large amounts of secondary antisense piRNAs (Brennecke et al., 2007), which are used for recognizing specific transcripts. To further investigate the sea urchin piRNA ability to recognize target RNA, we analyze strand bias of piRNA in all our samples.

The strand bias results for piRNA samples from male and female gonads, gametes, seawi IP and MT-RNP are shown (Figure 4-8A-oxidized testes, B-oxidized sperm-depleted testes, C-oxidized sperm, D-oxidized ovaries, E-oxidized egg-depleted ovaries, F-oxidized eggs, G-oxidized seawi

IP pool, and H-oxidized MT-RNP). The seawi IP pool data represent the combination of seawi co-immunoprecipitated piRNA samples from both egg and two-cell embryos. PiRNA sequences that are identical to mRNAs or transposon transcripts are shown as sense strand piRNAs in blue bars and the reverse complement piRNAs are shown in red bars. Large amounts of antisense piRNAs and some sense piRNAs are discovered in all samples, which is in agreement with ping-pong amplification model.

Sea urchin piRNAs are mostly complementary to target RNA, suggesting that their sequences may be used for target RNA transcript recognition. The genome localization of the top 100 most abundant piRNA sequences from various eggs and two-cell embryos were determined using BLAST. As observed for piRNA from species (Aravin et al., 2006; Girard et al., 2006; Lau et al., 2009; Lau et al., 2006), piRNA tended to localize to clusters in the genome for example, 43.8% of the selected sequences from eggs and 57.6% from two-cell embryos aligned with genome scaffold 312 (Figure 4-9A). Another 7.7% of selected sequence read from the eggs and 9.0% from two-cell embryos aligned with genome scaffold 1770 (Figure 4-9B). Even though expressed within the same cluster, a subset of seawi-associated piRNA

species was observed to change in expression between the eggs to two-cell embryo stage (Figure 4-9, compare orange and red squares).

The piRNA cluster region on sea urchin scaffold 312 encodes a putative sodium-dependent inorganic phosphate co-transporter. All the piRNA sequences but one are complementary to introns or intergenic genome regions. A possible function for this sodium-dependent inorganic phosphate co-transporter is the maintenance of high levels of intracellular phosphate during oogenesis to support the growth requirements of the developing oocytes (MacIver et al., 2000; Takeda et al., 1999). Although the detail function of this transporter has yet to be studied, its mRNA in *Drosophila* has been found in oocytes and nurse cells, where piwi protein is expressed (MacIver et al., 2000).

The sea urchin scaffold 1770 encodes recombination activating gene 1 (*rag1*), which initiates the VDJ DNA recombination of immunoglobins or T-cell antigen receptors that affect adaptive immunity in mammals (Schatz et al., 1989). Seawi-associated piRNAs are clustered within intragenic regions between two exons with two exceptions that their sequence directly complementary to *rag-1* exons (Figure 4-9B). *Rag1* is one of the conserved

mediators of immunity that is shared with vertebrates but absent from protostomes (Rast et al., 2006; Thompson, 1995). Noteworthy, the immune effector cells of sea urchins are the coelomocytes, which are derived from cells of the coelomic pouches (Pancer et al., 1999; Smith and Davidson, 1994), which were shown to be enriched for seawi protein (Figure 3-1F, G)

We extended the examination of all piRNA identified from MT-RNP or seawi-IP with emphasis on sequences that might be complementary to bep4 or beta-catenin mRNAs. The genomic view of bep4 (Figure 4-10A) and beta-catenin (Figure 4-10B) is shown. Each exon is shown as a black horizontal bar and each unique piRNA is shown as a blue square. Strikingly, all the seawi and MT-RNP associated piRNA sequences in these regions only match exons of both mRNAs, suggesting there are special exonic clusters of piRNA that may function in regulating mRNA stability and/or translation. The quantification of exonic piRNA clusters show 11.76 ppm (parts per million) for bep4 mRNA and 3.08 ppm for beta-catenin (see Materials and Methods). The low amount of exonic piRNAs explains why, during the large-scale analysis of total piRNAs, the mRNAs targeting exonic piRNAs were not easily identified. Here, we show that seawi is indeed associated with piRNAs that have

sequence complement to cell-fate determinant mRNAs -- bep4 and beta-catenin. Moreover, 22 out of 29 bep4 exonic piRNA sequences from two-cell MT-RNP are also identified in seawi immunoprecipitation samples (Figure 4-10 A).

Discussion

Sea urchin small non-coding RNAs

In this chapter, we report on the first detailed characterization of sea urchin small non-coding RNA in a variety of sea urchin cells and tissues, and protein assemblages. Based on their length, modification and precursor structure, sea urchin small non-coding RNAs were found to exclusively belong to the miRNA and piRNA groups. While it is not possible to completely rule out the possible expression of sea urchin siRNA, the extensiveness of the screening and sequencing (over 30 million transcripts) makes it unlikely that this group of non coding RNAs was missed in the screening. Additionally, the identification process included a protein-screening step which focused the RNA search to mi-/piRNAs associated with seawi and seawi containing MT-RNP complexes. Interestingly, seawi and MT-RNP were identified as having preferential association with ~28 nt piRNA. The observed preferential piwi-piRNA association is consistent with data published for fruit flies, rats, and mice (Aravin et al., 2006; Grivna et al., 2006b; Lau et al., 2006; Saito et al., 2006). Additionally, it was discovered that piRNAs are differentially associated with seawi based on the stage of development.

Sea urchin piRNA in transposable elements and genome rearrangements

In depleted testes, piRNA sequences are complementary to reverse transcriptase and transposase of transposable elements. These two enzymes are responsible for DNA transposon and retrotransposon mobility and replication, respectively. In *rag1* and sodium inorganic phosphate co-transporter genomic sequences, most of the piRNA sequences were complementary to introns or intergenic genome regions, which suggests not having a role in regulation of these two mature mRNAs. The introns and intergenic genome regions are known to contain transposons and its related repetitive sequences (Venner et al., 2009; Wicker et al., 2007). More detailed analysis of what specific transposable elements are located in these regions is needed. But this requires expansion of the database for sea urchin transposable element sequences. Moreover, the timing of splicing of transposable element transcripts is also crucial for piRNA function. However, there is little information on the splicing of the transposable element transcripts (Roberts et al., 2001).

Seawi-associated piRNAs are also complementary to transposon-

derived *rag1* gene sequences in the egg and embryo. The sequences of the *rag1* core region and VDJ recombination recognition sequences (RS) reveal a similar identity to terminal-inverted repeats of the *transib* transposon, suggesting the transposon origin of *rag1* and its RS signal sequences (Kapitonov and Jurka, 2005). It is not unreasonable to suggest seawi and piRNA are present in eggs and embryos to protect genome integrity during development and heredity.

Another possibility may hinge on *rag1* and its ability to rearrange the genome as part of the sea urchin immune system. Coelomic pouch cells not only serve as stem cells for adult body plan and adult germline but those cells also serve as precursors for coelomocytes, which are sea urchin immune cells (Rast et al., 2006; Smith et al., 1996; Smith and Davidson, 1994). This possibility would be interesting to explore by first examining the expression pattern of *rag1* protein and/or attempting to localize *rag1* related piRNA.

PiRNA and axis formation

In Chapter 3, we showed that seawi is localized to punctate structures in the cytoplasm of oocytes and eggs and seawi protein expression is

enhanced in small micromeres and their decedents. The enhanced expression pattern of seawi to germline precursors is similar to other germline related cytoplasmic RNP granules such as germline P granules in *C. elegans* and polar granules in fruit flies (Pitt et al., 2000; Rongo et al., 1997). The common feature of these germline RNP granules is that they asymmetrically distributed to germ cell right during the process of axis formation (Brangwynne et al., 2009; Rongo et al., 1997). Moreover, piwi protein such as aubergine and small non-coding RNA are previously shown to associate with these germline RNP granules (Harris and Macdonald, 2001; Nakamura et al., 1996). While the observations presented in this thesis cannot resolve the *in vivo* association of seawi to RNPs, the enhanced presence of seawi in cells crucial to A-V axis specification is intriguing.

Previously, Hamill et al. (1994) demonstrated that mRNA associated with MT-RNP were not translationally competent unless isolated away from the protein complex. This suggests that components of the MT-RNP complexes may have translational silencing ability for specific mRNAs. Seawi is a major component of MT-RNP complexes, seawi binds bep4 mRNA (Rodriguez et al., 2005) presumably through the conserved amino acid

sequences of the PIWI domain (Chapter 2), and bep4 mRNA is associated with reconstituted MT-RNPs (Romancino et al., 1998). The detailed screening and sequencing of seawi- and MT-RNP-associated exonic piRNAs identified cell-fate determinant bep4 and beta-catenin as potential targets. The discovery of piRNA transcripts targeted to beta-catenin and bep4 mRNA may provide profound insight into a, yet to be described, mechanism for animal-vegetal axis specification. Further, the establishment of the animal-vegetal axis is crucial for later germ layer formation and cell fate specification. The existence of small non-coding RNAs in concert with argonaute/piwi proteins regulating cell-fate determinant mRNAs provide additional understanding of how translational silencing processes may be key components of gene regulatory networks (GRNs). Such a pathway may explain how maternal mRNA of cell determinants can be translationally silenced until its protein is needed and underlines the versatility of using the same protein complexes to regulate different mRNAs in different spatial and/or temporal developmental events. For example, translation nanos mRNA is regulated by piRNA and the piwi family of proteins (Rouget et al., 2010). Interestingly, 3' UTR of nanos mRNAs and targeted piRNA targeted sequences may have origins in the *roo*

and *412* transposons (Rouget et al., 2010). Our finding suggests that sea urchin piRNAs may be directly targeted to exonic regions of mature mRNA of beta-catenin and bep4.

Mi- and piRNA in mature sperm

The regulation of the translation of maternal mRNAs is one of the key concepts in sea urchin GRNs. The proteins and RNAs that are involved in this process are considered maternally inherited (Grainger and Winkler, 1987; Tang et al., 2007). Dogmatically, the principal information contributed by the sperm to the zygote during fertilization is haploid DNA, which does not contribute transcriptionally until maternal-zygotic switches at the mid-blastula stage. The data presented clearly demonstrate that mature sperm carry both mi- and piRNAs, possibly for delivery to the egg. This novel finding provides the exciting conjecture that the sperm delivers regulatory small RNAs that might influence maternal mRNA expression. For example, the screen identified two sperm specific miRNAs, miR-252 and miR-278, and it has been reported the miR-278 is required for energy homeostasis in *Drosophila* development (Teleman et al., 2006).

It may seem unusual that sperm carry piRNA while at the same time there was no detectable seawi localization in sperm cytoplasm (Chapter 3). How could such “naked” piRNA exert an effect on translation in egg? Emperically, it is now widely accepted that exogenously added siRNA into cells become incorporated into RISC complexes and exhibit functionality. Similarly, in sea urchin, sperm introduced piRNA may rapidly associate with cortically localized seawi and maternal mRNA (Moon et al., 1983; Paix et al., 2011). The possibility of paternal piRNA regulating maternal mRNA targets in sea urchin development is a very novel hypothesis that warrants further evaluation.

Figure 4-1. Characteristics of small non-coding RNAs.

The table presents a summary of basic structural and functional properties exhibited by the three major classes of small non-coding RNAs.

	piRNA	miRNA	siRNA
Length	24-30 nt	19-22 nt	19-22 nt
3' termini modification	2-O-methylation	Not modified	2-O-methylation in fruitflies Not modified in mice
Precursor structure	Single long strand	Double stands hairpin	Double stands
Target mRNA recognizing	Fully complement	Seed region complement	Fully complement
Functional outcome of protein sythesis	RNA cleavage Translational inhibition Epigenetic regulation	RNA cleavage Translational inhibition Epigenetic regulation	RNA cleavage

Figure 4-2. SYBR gold staining of sea urchin total RNA.

Total RNA of testes and eggs were loaded onto a polyacrylamide gel and stained with SYBR gold. There is an enrichment of small non-coding RNAs in the 28 to 30 nt range in testes samples. Egg samples did not exhibit a distinct population of small RNA species.

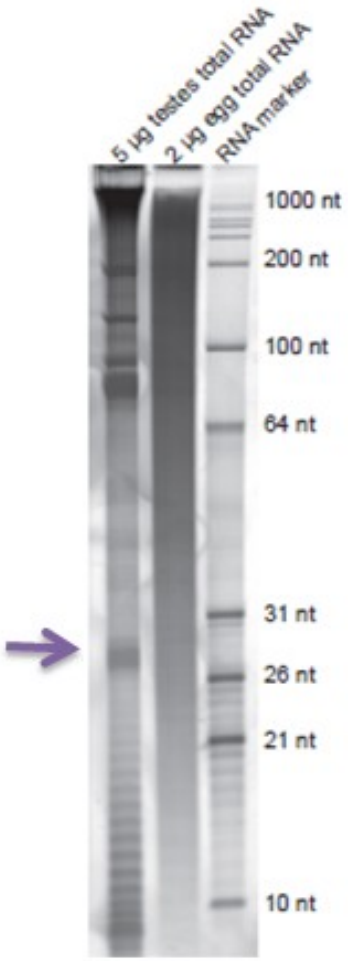

5 µg testes total RNA
2 µg egg total RNA
RNA marker
1000 nt
200 nt
100 nt
64 nt
31 nt
26 nt
21 nt
10 nt

Figure 4-3. Depleted testes small non-coding RNAs.

The overall sequencing and genome matching number are listed in (A). There are more than 3.4 million total sequenced clusters, and 2.2 million sequences were prefect match to 6-mer of the adapter sequences (extracted). After mapping sequencs to sea urchin genome 33,000 were unque small RNA sequences. The ten most abundant small RNAs in untreated (C) and oxidized (B) samples are listed. Annotation is based on a BLAST search against the NCBI nr database. In the oxidized testes RNAs, there is partial information available for sea urchin piRNAs due to poor annotation of the *S. purpuratus* genome and lacking transposon information. However, the most abundant small non-coding RNAs in untreated testes RNA samples are rRNA fragments and miRNAs.

A.

Sample name	Sequenced clusters	Extracted		Genome-matching	
		Inserts	Unique sequences	Inserts	Unique sequences
Oxidized testis RNA	2,860,205	2,093,182	893,894	1,099,922	353,944
Untreated testis RNA	3,424,736	2,238,711	756,914	1,548,662	330,644

B.

Oxidized testis RNA		
Sequences	Reads	BLAST
TGGACACCAGATAGTACTCGGCATCCTGC	2959	Unknown
TGCGTAGAATCCCCGTCGACTTGACAGC	2599	XM001192826.1
TCTAGGACAGAATCTAGCAGAGAATGGC	2234	XM001192826.1
TAGAAAGTCACGTAGCACTTCTGTTGTC	2199	Unknown
TGAATAACAGCTTCGATGGAGGCAGAGC	2051	Unknown
AGGGCTATAAATACTCGAAGGGTACCAGG	1812	Unknown
TGCAAGACATGGAACACGCAGAGCAAGC	1737	XM001191171.1
TGAGCACAGGCTGTAACTCTGGCCTAGC	1689	Unknown
TGGGAGATTGTAGGACTCTCAAATCCTGG	1639	Unknown
TGAACTGTACCTCAAGGGGTAGACGAGGC	1602	Unknown

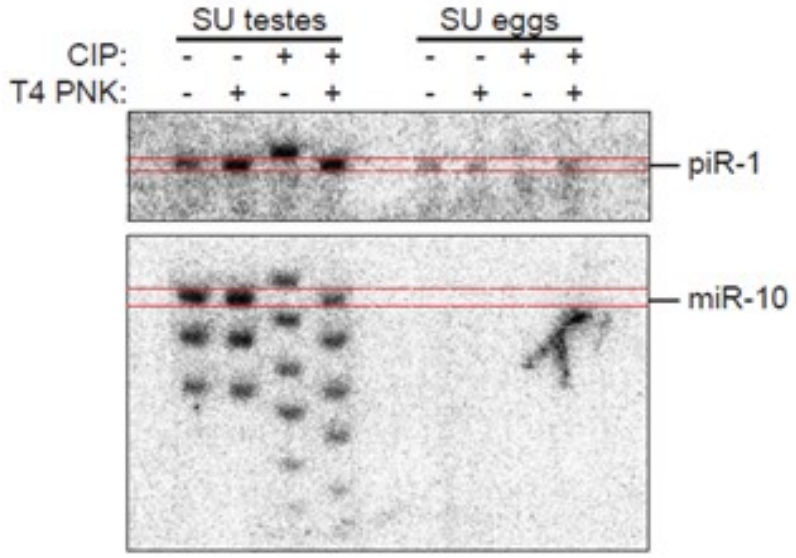
C.

Untreated testis RNA		
Sequences	Reads	BLAST
GGAATACCGGGTGTTGTAGGCAT	38,628	5s rRNA
CTCGACCTCAGATCGGACGAGACCAC	17,567	28s rRNA
AACCCTGTAGATCCGAATTTGTG	16,653	miR-10
AACCCTGTAGATCCGAATTTGTGT	15,139	miR-10
AACCCTGTAGATCCGAATTTGT	14,806	miR-10
TGGGAATACCGGGTGTTGTAGGCAT	13,116	5s rRNA
AACCCGTAAGGTCTTAACTTGT	10,802	Unknown
TCCCTGAGACCCTAACTTGTGA	8,552	miR-125
GGGAATACCGGGTGTTGTAGGCAT	8,039	5s rRNA
TATTGCACTCGTCCCGGCCTGC	6,866	miR-92

Figure 4-4. Biochemical analysis of sea urchin piRNA 5' and 3' ends.

Most abundant piRNA (first sequences from figure 4-3 B) and miR-10 (from figure 4-3 C) was selected for a motility test. (A) Both sea urchin piRNA and miRNA have a 5' monophosphate. CIP treatment (CIP+/ T4 PNK-) removes all 5' phosphates, and T4 PNK (CIP+/T4 PNK+) adds a single phosphate back to 5'. The motility test showed that both sea urchin mi- and piRNA shift back to original size after sequentially treating both CIP and T4 PNK, which suggests both mi- and piRNA have a 5' monophosphate. (B) Sea urchin piRNA, but not miRNA, revealed a 2'-O-methylation modification. Beta-elimination test shows that piRNA, but not miRNA is resistant to oxidation, suggesting that piRNAs have no free 3' hydroxyl group.

A.


SU testes
SU eggs
CIP: - - + + - - + +
T4 PNK: - + - + - + - +
piR-1
miR-10

B.

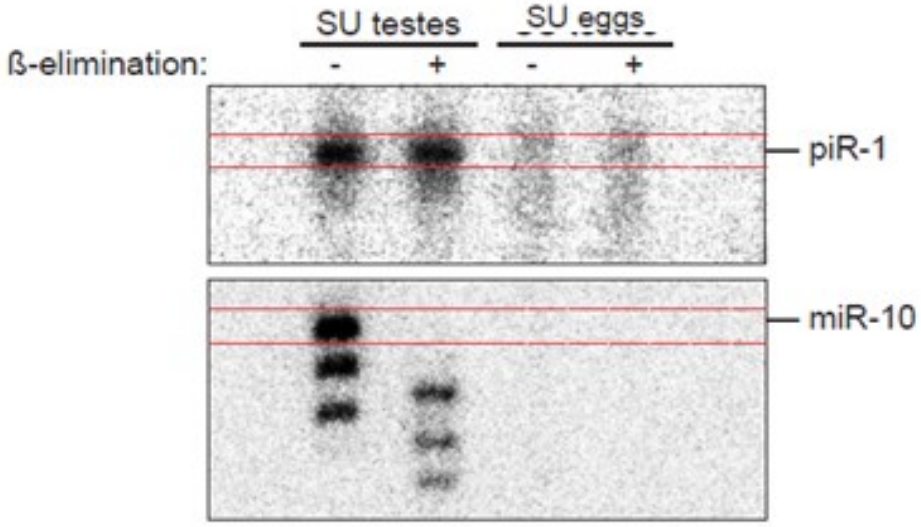

SU testes
SU eggs
ß-elimination: - + - +
piR-1
miR-10

Figure 4-5. Analysis of sea urchin small non-coding RNAs in adult testes.

The untreated (A-C) and oxidized (D-F) samples represent all populations of small non-coding RNAs and piRNAs, respectively. The size distribution of small non-coding RNAs from testes (A and D), sperm-depleted testes (B and E) and sperm (C and F) are shown. Mi- (~22nt) and piRNAs (~28nt) are expressed in all three testis samples. The existence of mi- and piRNAs in sperm suggests paternal contribution through small non-coding RNA to embryo.

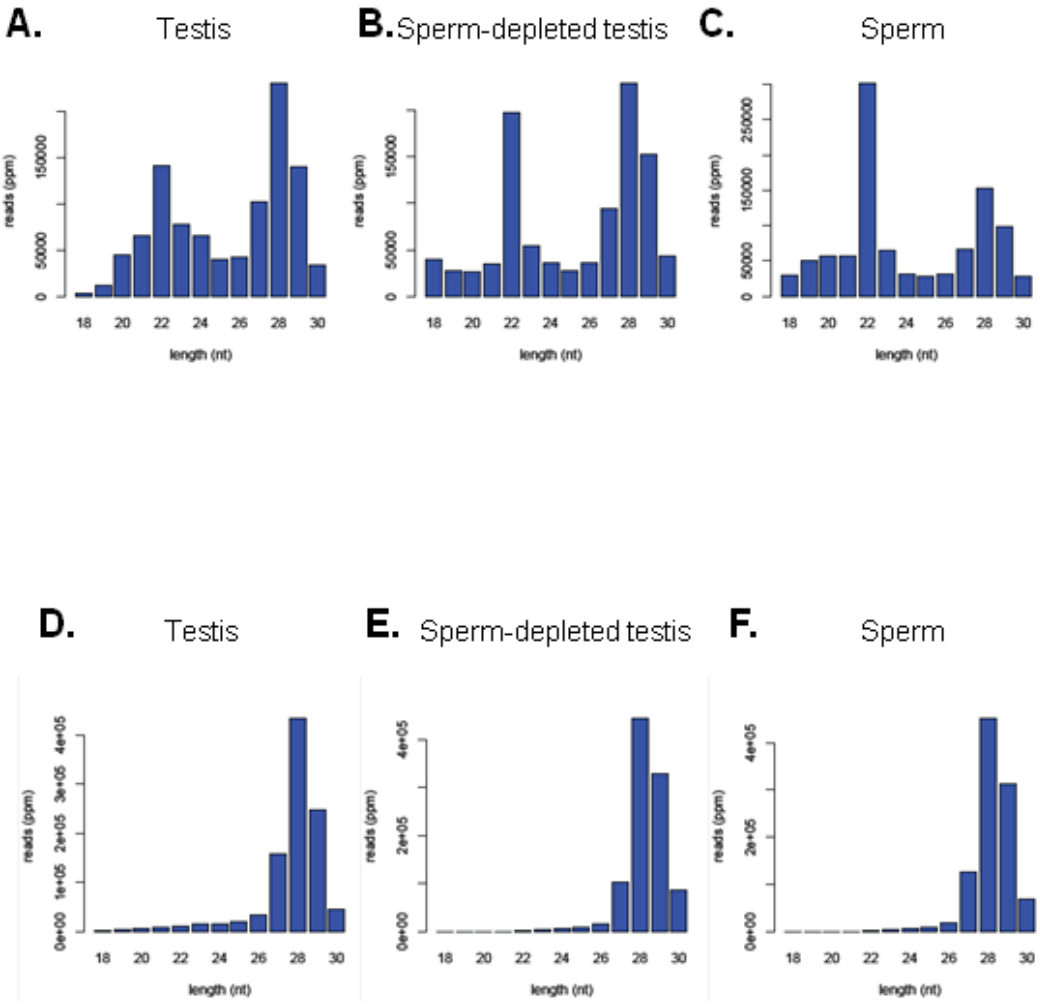

A.
Testis
B.
Sperm-depleted testis
C.
Sperm
D.
Testis
E.
Sperm-depleted testis
F.
Sperm
reads (ppm)
length (nt)

Figure 4-6. Analysis of sea urchin small non-coding RNAs in adult ovaries.

The untreated (A-C) and oxidized (D-F) samples represent total small non-coding RNAs and piRNAs, respectively. The size distribution of small non-coding RNAs from ovaries (A and D), egg-depleted ovaries (B and E) and egg (C and F) are shown. Mi- (~22nt) and piRNAs (~28nt) are also expressed in all three ovary samples.

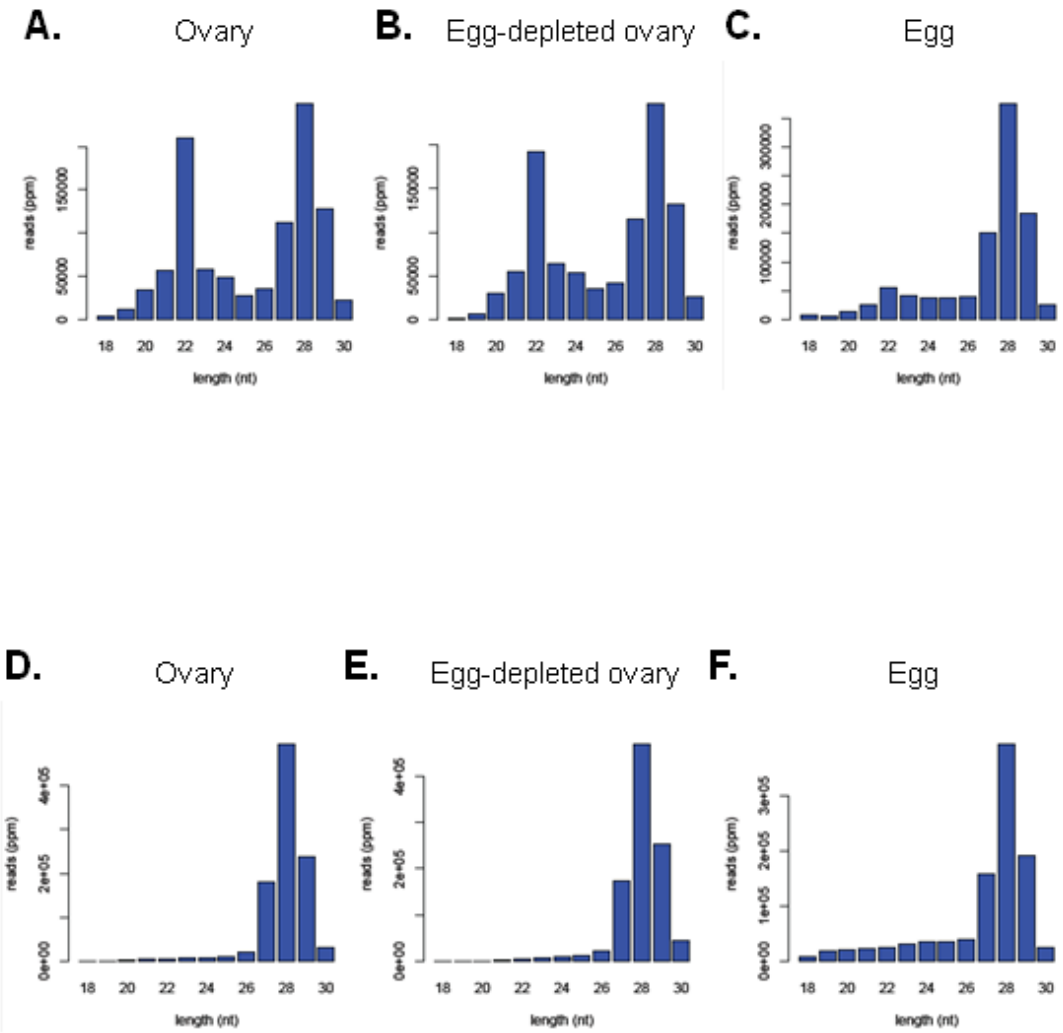

A.
Ovary
B.
Egg-depleted ovary
C.
Egg
D.
Ovary
E.
Egg-depleted ovary
F.
Egg
reads (ppm)
length (nt)

Figure 4-7. Seawi is associated with piRNA in egg, and MT-RNP and seawi both associated with piRNA in two-cell embryos.

The untreated (A-C) and oxidized (D-F) samples represent total small non-coding RNAs and piRNAs, respectively. The size distribution of co-immunoprecipitation of seawi-associated small non-coding RNAs from egg (A and D) and two-cell embryos (B and E). The size distribution of MT-RNP-associated small non-coding RNAs from two-cell embryos are shown in C and F. Based on the size of the co-precipitated small non-coding RNAs, seawi associates with piRNAs in both eggs and two-cell embryos. MT-RNP complexes from two- cell embryos exclusively associate with piRNAs.

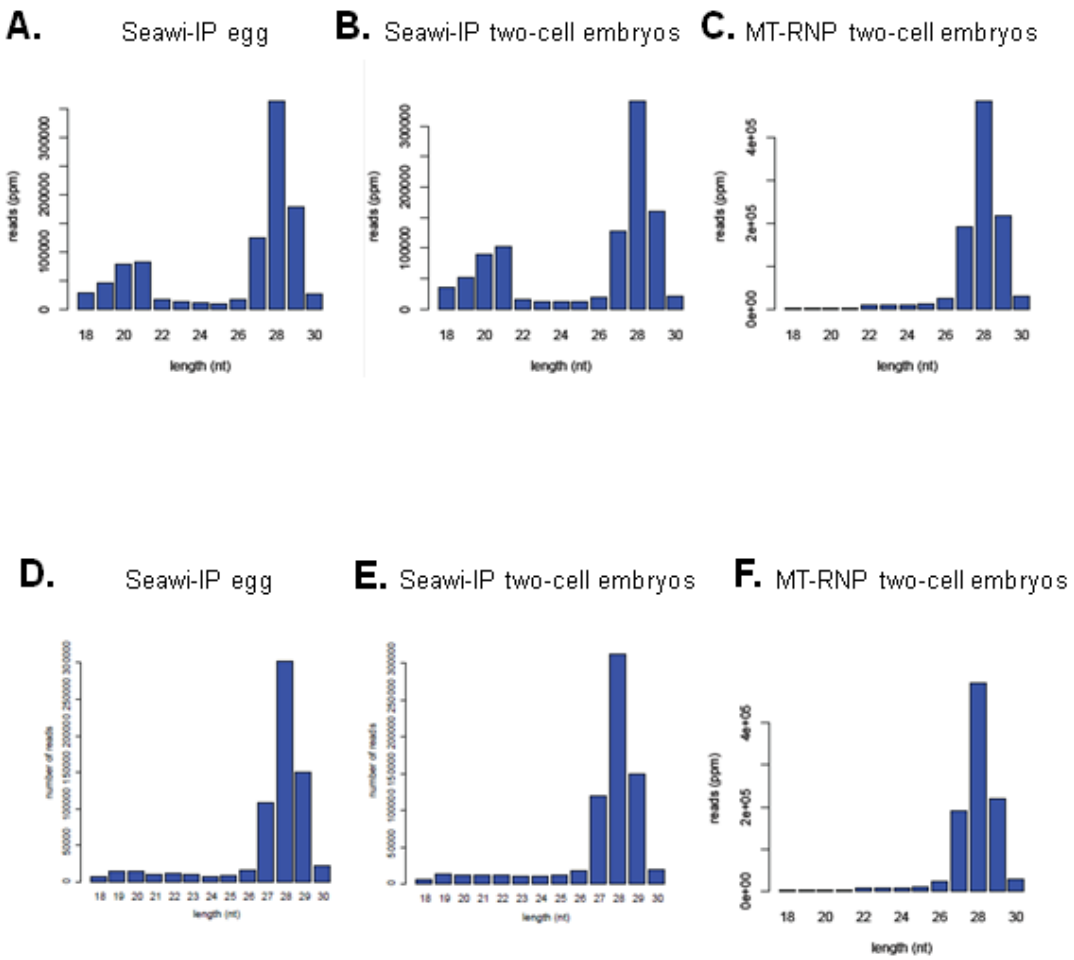

A.
Seawi-IP egg
B.
Seawi-IP two-cell embryos
C.
MT-RNP two-cell embryos
reads (ppm)
length (nt)
D.
Seawi-IP egg
E.
Seawi-IP two-cell embryos
F.
MT-RNP two-cell embryos
number of reads
length (nt)

Figure 4-8. Analysis of strand bias of sea urchin piRNAs.

The size distribution of piRNAs from testes (A), sperm-depleted testes (B), sperm (C), ovaries (D), egg-depleted ovaries (E), egg (F), seawi IP (G) and two-cell MT-RNP (H) are shown. We combined seawi IP from both egg and two-cell embryos data into (G). The piRNA sequence reads that are identical or complementary to target RNA are shown in blue or red, respectively. Sea urchin piRNAs are predominantly shown in red, which suggests their sequences are complementary to target RNA sequences.

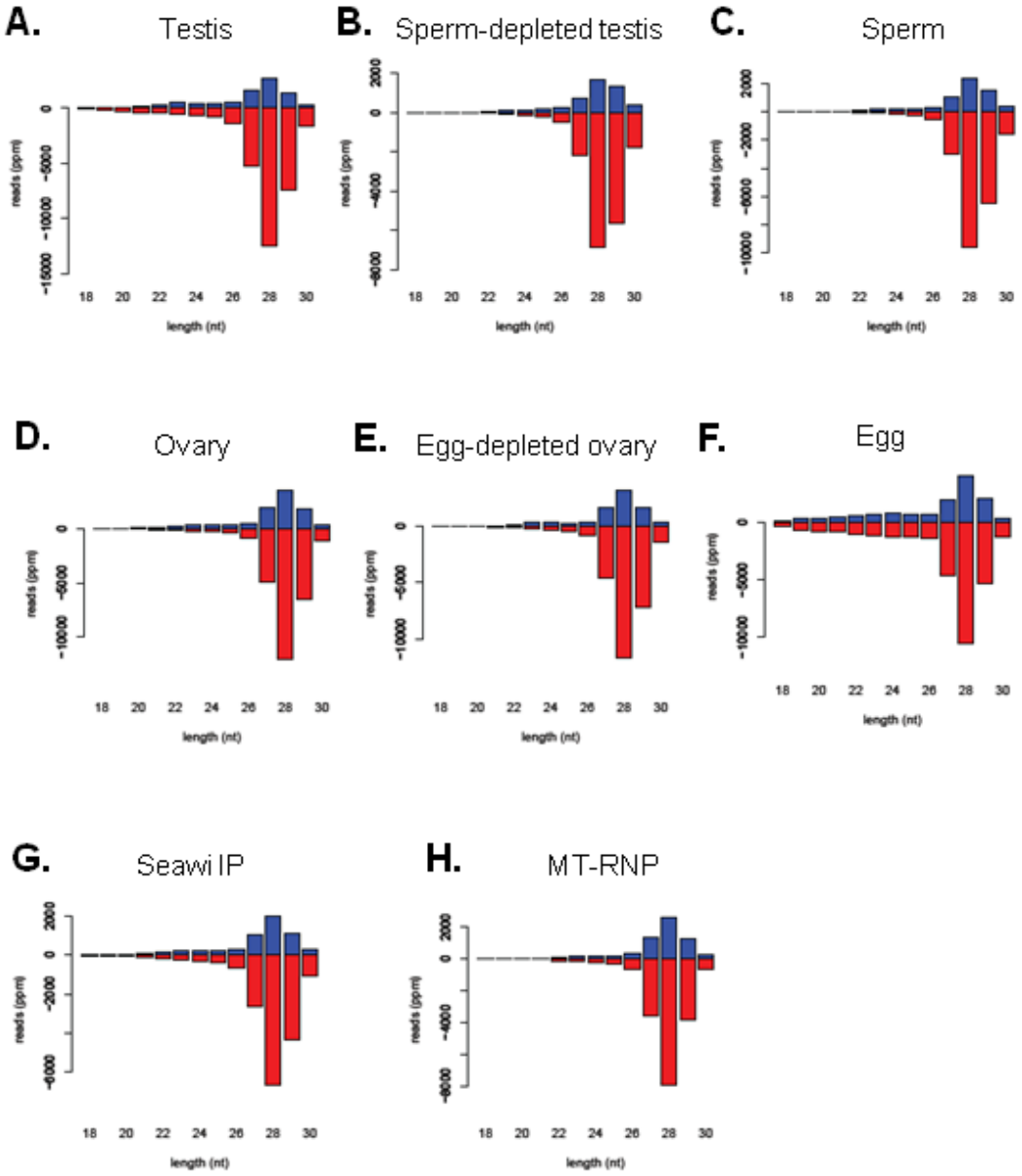
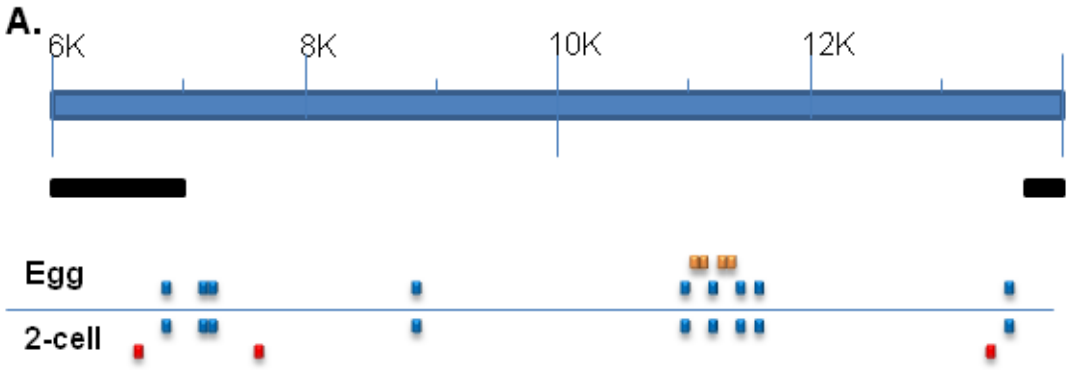

A.
Testis
B.
Sperm-depleted testis
C.
Sperm
D.
Ovary
E.
Egg-depleted ovary
F.
Egg
G.
Seawi IP
H.
MT-RNP
reads (ppm)
length (nt)
18
20
22
24
26
28
30

Figure 4-9. Analysis of most abundant seawi-associated piRNA clusters.

The top 100 most abundant seawi-associated piRNA sequence reads from egg and two-cell embryos were subjected to Blast search against the sea urchin genome. (A-B) Demonstration of the clustering of piRNA sequence reads. Schematics depicting the piRNA clusters on sea urchin genome scaffold 312 (A) and 1770 (B) are shown. Each colored square represents a piRNA sequence; a blue square indicates that the sequence is expressed in both eggs and two-cell embryos, a yellow square denotes an egg-specific piRNA sequence, and a red square specifies a piRNA sequence expressed by two-cell embryos. The most abundant seawi-associated piRNA clusters in intergenic and intronic regions.


A.
6K
8K
10K
12K
Egg
2-cell

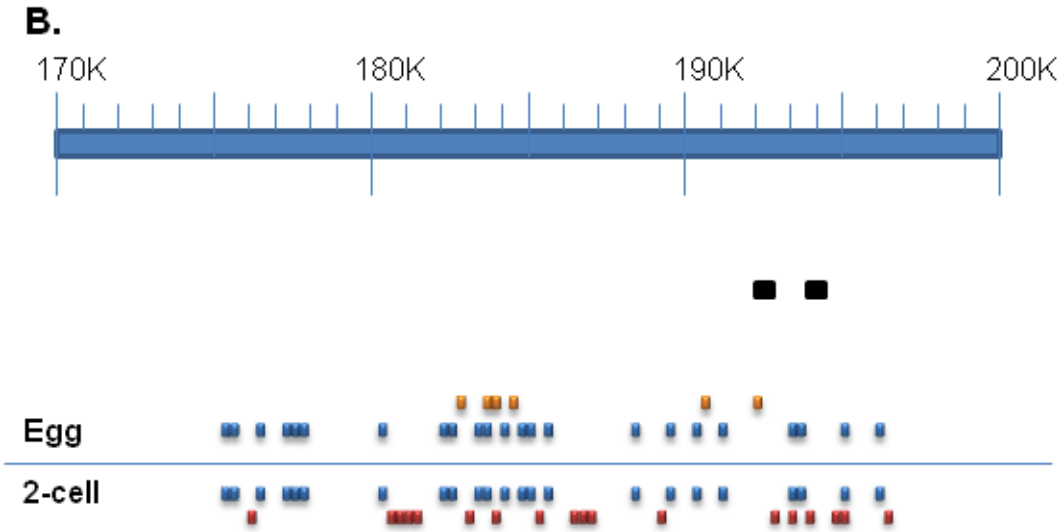
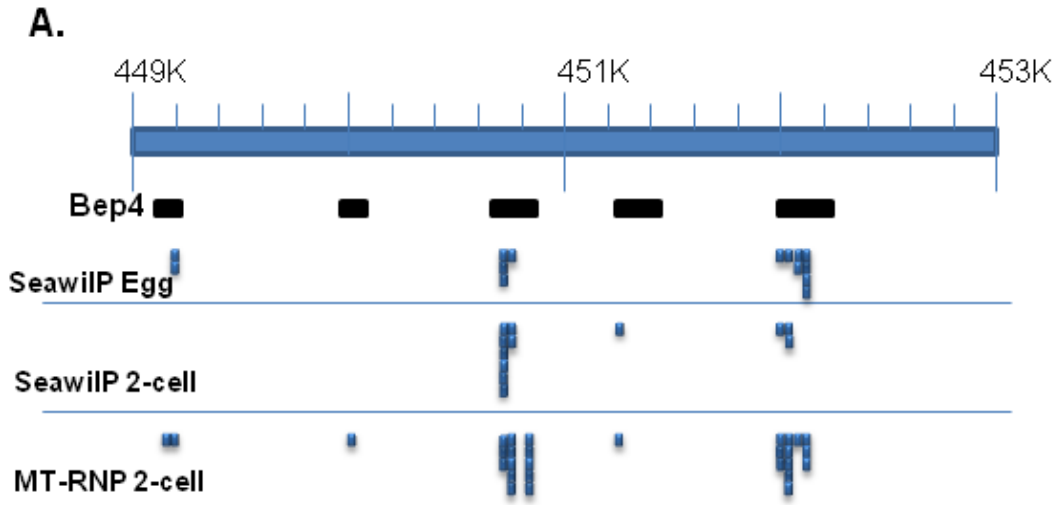
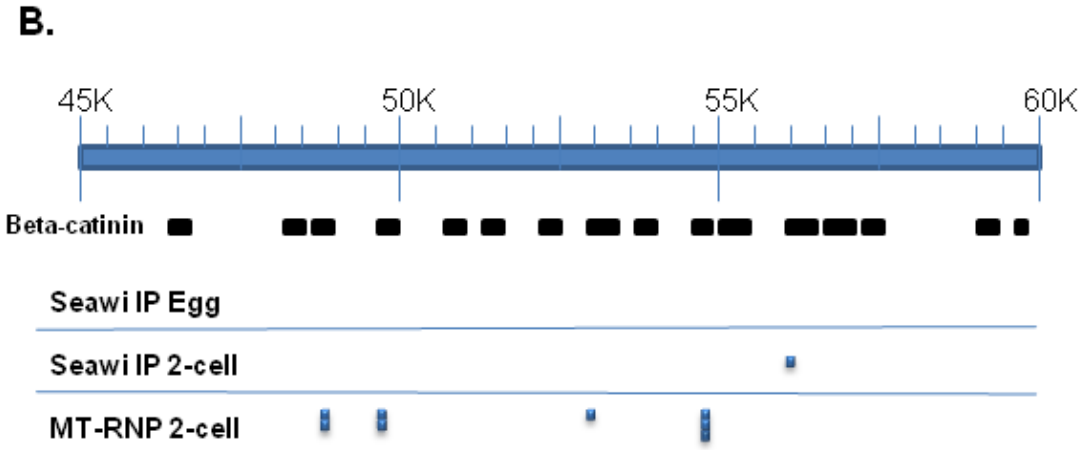

B.
170K
180K
190K
200K
Egg
2-cell

Figure 4-10. Searching seawi-associated piRNAs that specifically target beta-catenin and bep4 mRNAs.

Seawi-associated piRNA sequence reads from egg and two-cell embryos and MT-RNP-associated piRNA from two-cell embryos were subjected to Blast search against the sea urchin genome. (A-B) Demonstration of the clustering of piRNA sequence reads. Schematics depicting the piRNA clusters within sea urchin bep4 (A) and beta-catenin (B) exon sequences are shown. Each blue square represents a piRNA sequence; each black bar represents a stretch of exon sequence on genome. There are specific clusters of seawi- and/or MT-RNP-associated piRNA that are exclusively clustered in exons of beta-catenin and bep4.


A.
449K
451K
453K
Bep4
SeawiIP Egg
SeawiIP 2-cell
MT-RNP 2-cell


B.
45K
50K
55K
60K
Beta-catinin
Seawi IP Egg
Seawi IP 2-cell
MT-RNP 2-cell

CHAPTER 5

Supplement: Proteomics of MT-RNP complexes and anti-seawi antibodies

Argonaute and piwi proteins are reported to be components of a variety of protein-RNA complexes, including miRNPs, processing bodies, stress granules, polar (germ cell) granules, MT-RNPs, chromatoid bodies, and RISCs (Easow et al., 2007; Hamill et al., 1994; Harris and Macdonald, 2001; Jagannath and Wood, 2009; Kotaja et al., 2006; Leung et al., 2006; Rand et al., 2005; Rodriguez et al., 2005; Schwarz and Zamore, 2002; Zeng et al., 2008). Such complexes are further known to be macromolecular assemblages that can function to regulate translational, post-transcriptional, and epigenetic activities in cells, embryos, and adult tissue (Easow et al., 2007; Johnstone and Lasko, 2001; Leung et al., 2006; Liu et al., 2005; Rajasethupathy et al., 2012; Schwarz and Zamore, 2002; Yin and Lin, 2007). For example as discussed in Chapter 3, the expression patterns of seawi are similar to other germline related cytoplasmic RNP granules such as germline P granules in *C. elegans* and polar granules in fruit flies (Pitt et al., 2000;

Rongo et al., 1997).

Suprenant and colleagues (1994; 1989; 1989) isolated MT-RNP complexes from sea urchin eggs and two-cell embryos that contained a specific subset of mRNAs held in a translationally arrested state. Other proteins identified in the complex are alpha & beta tubulin, ribosomes, EMAP, and major vault protein (Hamill and Suprenant, 1997; Rodriguez et al., 2005). Further, electron micrographs of such reconstituted complexes revealed a structure and association with microtubules that was strikingly similar to complexes associated with mitotic spindles (Suprenant et al., 1989). Romancino and colleagues (2001; 1998) discovered that bep4 mRNA, a message that codes for an axis specifying protein, was a component of MT-RNPs. Subsequently, Rodriguez et al. (2005) characterized the protein seawi as a component of MT-RNPs and demonstrated that seawi specifically bound to bep4 mRNA *in vitro*. Further, Rodriguez et al. (2005) would suggest that sea urchin MT-RNPs were complexes analogus to other translationally arrested embryonic RNP complexes.

In this thesis, we extended the analysis of MT-RNP components to include specific populations of miRNA and piRNA. Interestingly, a number of

the piRNA transcripts identified in MT-RNP complexes were also co-precipitated from egg lysates using anti-seawi antibodies (see Chapter 4). In this chapter, we extend the analysis of the protein composition of MT-RNP complexes by using high sensitivity proteomic (MALDI-TOF and LC/MS/MS) and bioinformatic approaches. Additionally, we report on attempts to generate polyclonal antibodies specific to the N-terminal and PIWI domains of seawi.

Materials and Methods

MT-RNP proteomics analysis

Reconstitued two-cell MT-RNP was prepared according to Suprenant et al. (1989) and proteins were separated by SDS-PAGE on 10%. Bands from 200, 100, 77, 35, 33, 31 kDa were excised and peptides sequenced with Matrix-assisted laser desorption/ionization with time-of-flight mass spectrometer (MALDI-TOF). For liquid chromatography with tandem mass spectrometry (LC/MS/MS), material eluted from the 200 kDa gel band was trypsin-digested and separated by liquid chromatography, followed by tandem mass spectrometry (MS/MS) peptide sequencing (Center for Advanced Proteomics Research, University of Medicine and Dentistry of New Jersey, Newark, NJ). LC/MS/MS is higher sensitivity and more thorough form of analysis as compared to MALDI-TOF. All gel procedures were done with virgin materials to limit possible contamination. For peptides identification, the acquired peptide sequences were queried against NCBI's protein database, and specifically searched against sequences from sea urchin. The identity to sea urchin peptides has to be higher than 98% to be retained. The non-annotated peptide amino acid sequences were further searched (tblastn) in

the sea urchin genome database and the putative mRNA sequences from the genome were then used for blast search in Genebank for gene identification.

For bioinformatics analysis, identified genes were input into the Mouse Genome Informatics database (MGI, The Jackson Laboratory, ME)(Eppig et al., 2011; Finger et al., 2011) for identification of putative binding partners. By organizing protein information based on binding partners, identified MT-RNP proteins were grouped into seven categories: microtubule binding, actin binding, other cytoskeleton and protein transport related, mitosis and epigenetic, development, translation/ ribosome related and signaling related proteins.

Generation of anti-seawi domain specific antibodies

To generate antibodies against PIWI domain of sea urchin, full-length seawi was first PCR amplified with platinum high fidelity DNA polymerase (Invitrogen, Carlsbad CA) using the following primers:

seawi-1f 5'-ATGGAGTTTGACAAAGAGCAGG-3' and

seawi-1r 5'-CTAGAGATAGAAGAGGGTGTCG-3'.

PCR conditions are the same as previously described (see Chapter 2)

with annealing temperature reduced to 51.2ºC and elongation time increased to 3 minutes. PCR products (2880 nucleotide) were detected by staining with ethidium bromide, documented, and gel purified by using a gel purification kit (Promega, Madison, WI), and then sequenced (MRF, University of Medicine and Dentistry of New Jersey, Newark, NJ). The purified PCR product was ligated into PCR II-TOPO vector (Invitrogen, Carlsbad CA) and transformed into TOP10 (DH5α) *E. coli* competent cells following manufacturer's protocols (Invitrogen, Carlsbad CA). The colonies with seawi inserted were confirmed by colony PCR with T7 and seawi specific N-terminal domain primer pairs:

T7 5'- TAATACGACTCACTATAGGG-3'

Seawi-8r 5'-TTACACGTCGTACTCGTACTGAAGG-3'

The plasmids were miniprep (Promega, Madison, WI) and sequenced with T7 and Sp6 primers (Genewiz, NJ). Using PCR II-TOPO seawi as template, PCR products of seawi N-terminus domain and PIWI domain are generated with the following primer pairs:

(N-terminal domain)

seawi-11f 5'-CACCATGGATCGTCGGCCGGGAG-3'

seawi-8r 5'-TTACACGTCGTACTCGTACTGAAGG-3'

(PIWI domain)

seawi-12f 5’-CACCATTGTGGTGGTGATCCTGCC-3’

seawi-1r 5’-CTAGAGATAGAAGAGGGTGTCG-3’

All forward primers are specifically designed with 5’-CACC sequences for Champion pET Directional TOPO expression kit (Invitrogen, Carlsbad CA). PCR products were cloned directionally into pET-100 expression vectors (Invitrogen, Carlsbad CA). The PCR products and pET-100 vectors were gel-purified, ligated with TOPO isomerase, and transformed into TOP10 *E. coli*. The resultant plasmids containing seawi PIWI domain coding sequences were sequenced (Genewitz, South Plainfield, NJ) to confirm that the coding sequence had inserted in-frame.

For expression, the vectors were transformed into BL21 *E. coli*. Cultures were grown in LB with ampicillin at 37°C. When OD 600 reading reached 0.6, expression was induced with IPTG (1 mM final concentration). Cells from no induction and cells from 12 hours after induction were collected, and the cell pellets were resuspended in 50mM Tris-HCl and 2 mM EDTA. Resuspended cells were centrifuged and the resultant pellets were resuspend in lysis B buffer (50mM Tris-HCl, 100mM NaCl, 5mM EDTA, 0.1% NaN3, 0.5%

Triton-X 100, 0.1 mM PMSF and 1 mM DTT). After lysing the cells with lysozyme (final concentration 0.1μg/μl), inclusion bodies were purified according to Steinle et al. (2001). Protein samples of each step were loaded stoichiometrically onto 10 or 16% polyacrylamide gels. The final pellet was separated by SDS-PAGE and stained with coomassie blue (2.5 mM coomassie, 30% methanol and 0.5% acetic acid). The bands were excised from the gel and used for MALDI-TOF for sequence verification and for immunizing rabbits (PRF&L, Canadensis, PA).

For MALDI-TOF, the band from 10 or 16% gel stain with coomassie blue was excised. In-gel trypsin digestion was performed by automated TECAN robotic system (TECAN, Switzerland), followed by peptide sequencing. The blastp of the sequenced peptides were performed against NCBI protein database for peptide identity. To quantify the percentage purity of PIWI domain, we applied a semi-quantification method (Duncan et al., 2008). First, we input seawi PIWI domain and the contaminating bacterial membrane protein in to PeptideMass software (Wilkins et al., 1997) to predict possible peptide numbers and peptide sizes from trypsin digest; both proteins were digested into fifteen peptides, so the ratio between sums of the total

intensity represented the percentage of purity.

Since the pET-100 construct encoded a 6X His-tag on the N-terminus of expressed peptides, we also attempted to purify using Ni-NTA with denaturing methods following manufacturer's protocols (Qiagen and Invitrogen). *E. coli* cell pellets were resuspended in lysis A buffers (Invitrogen denaturing buffer A: 8M Urea, 20 mM sodium phosphate buffer, 500mM NaCl, pH adjusted to 8.0; Qiagen denaturing buffer A: 8M Urea, 10mM Tris-HCl, 100mM NaH_2PO_4, pH adjusted to 8.0) and lysing cells with lysozyme (0.1µg/1µl) and sonication (3 times 5 seconds with 20 sec intervals).

Protein samples from multiple purification steps of expressed seawi N-terminal domain and PIWI domain were loaded stoichiometrically to 10 or 16% SDS-PAGE. Immunoblotting were performed (see Chapter 3) with a 1:500 dilution of anti-seawi 4I4 antibodies (Faire and Bonder, 1993) and a 1:15000 dilution of alkaline phosphatase-conjugated goat anti-rabbit antibodies (Biosource/ Invitrogen, Carlsbad CA).

Results

MT-RNP

We apply proteomics to identify and characterize additional proteins present in reconstituted MT-RNP complexes. MT-RNP complexes from two-cell embryo were separated in 10% gel (Figure S5-1A). In these gels, alpha/beta tubulin ran as a single band at approximately 50 kDa. Multiple bands were readily detected at 200, 100, 77, 74, 35, 33, 31 kDa and subjected to MALDI-TOF analysis. The 100 and 77 kDa samples are identified as seawi and EMAP, which agrees with previous findings (Hamill et al., 1994; Rodriguez et al., 2005). Other proteins identified by this screen included actin binding proteins, fimbrin/L-plastin (68 or 70 kDa) and F-actin capping protein CAPZAF (32.6 kDa), translational machinery related proteins, 60S ribosomal protein L7A (31 kDa) and 60S ribosomal protein L6 (32.6 kDa); the signaling molecule RACK and the receptor activating C-kinase (36 kDa) (Figure S5-1B). Some of those proteins have not been characterized in the sea urchin, therefore the putative protein sizes are based on their mouse homologues.

The LC/MS/MS was used for further identification. A total of 73

additional sea urchin proteins were identified associated with MT-RNPs and 71 of those were found to have orthologues in mice. Given the nature of protein isolation methods and the high sensitivity of the analytical methods used a few, or many, of the identified proteins may not be *bona fide* components of MT-RNP. To somewhat untangle the acquired information, we applied Gene Ontology tools from Mouse Genomic Informatics (see Materials and Methods) information on the known functions and potential interacting binding partners to group the proteins into seven categories: microtubule associated (Table S5-2), actin associated (Table S5-3), other cytoskeletal and protein transport (Table S5-4), mitosis and epigenetic (Table S5-5), developmental (Table S5-6), translational/ribosome related (Table S5-7) and signaling related (Table S5-8) proteins. Additionally, several of the classified proteins are worth noting because their presence could have significance to suggested functionalities. For example, the potential presence of spectrin is interesting since the membrane-actin cytoskeleton protein could provide a means to tether MT-RNP within the actin rich cortex. Similar functionalities could be attributed to microtubule binding proteins for tethering to microtubules, motor proteins for intracellular translocation, and factors

required for translation.

The putative MT-RNP complex was reconstructed by using all proteins identified in the screen (Tables S5-2, S5-3, S5-4, S5-5, S5-6, S5-7 and S5-8) including ones that were not previously identified in sea urchin (identified by asterisks in the tables). Black line indicates putative interaction of two proteins by the bioinformatics approach according to the MGI database and the red line indicates mouse homologue protein pairs that were found to associate in other co-IP experiments (Finger et al., 2011). The proteins in green indicate the protein has potential to play multiple roles in the map. The cytoskeleton interaction map with microtubule binding, actin binding, other cytoskeletal binding, protein transport related and signaling related proteins is shown in Figure S5-9A. The translational regulation map was then generated with ribosome related proteins (Figure S5-9B). The two interaction maps from reconstituted MT-RNP complexes suggest the association of microtubules, actin cytoskeleton, and translation machinery and signaling molecules to this seawi containing RNP complexes. In the complexes, we even identified DNA (cytosin-5) methyltransferase (Dnmt-1) that can epigenetically modify DNA.

Anti-seawi domain specific antibodies

To develop additional tools for characterizing the localization and function of sea urchin argonautes/piwis, we attempted to generate specific antibodies for the PIWI and N-terminal domains of seawi (Figure S5–10A). Sequences encoding seawi PIWI fragments were amplified and cloned into a pET-100 expression vector. After nucleic acid sequencing to confirm that the amplified seawi fragments had PIWI inserted in-frame, the plasmids were transformed into BL21 *E. coli* for protein expression. Upon induction with 1 mM IPTG, a band of the expressed fragment was present in the induced sample of each expression (Figure S5-10B Lane 3, Lane 2 non-induced). Immunoblotting with seawi specific antibody 4I4 showed the expressed seawi PIWI domain peptides are recognized (Figure S5-10C).

Since there is a 6x his-tag in frame in the expression vector, the Ni-NTA purification protocol was followed and the flowchart of purification method is shown in Figure S5-11. The Ni-NTA purification was conducted under two different conditions recommended by Invitrogen (Figures S5-10B and S5-14A, denaturing conditions) and Qiagen (Figure S5-14B, denaturing conditions). The expressed fragments ran slightly smaller than their predicted molecular

weights of 41 kDa (38 with 3 kDa-tag) on 10% gel (Figure S5-11B and S5-13A) but ran normally on 16% (Figure S5-13D), which demonstrates a limitation in SDS-PAGE. During the purification that followed Qiagen denaturing condition, little expressed PIWI domain was bound to Ni-NTA (Figure S5-10B, Lanes 11-14), and the majority of the expressed PIWI domain peptides were in the flow through (Figure S5-10B, Lane 6). This result suggests that expressed PIWI was not binding to Ni-NTA columns despite the presence of the his-tag.

Examining the BL21 *E. coli* using phase contrast microscopy revealed the presence of large bright intracellular “speckles” indicating the formation of inclusion bodies. Inclusion body were purified (Figures S5-12 and S5-13) and the procedure monitored by SDS-PAGE and immunoblotting with anti-seawi antibodies (Figures S5-13A and B). The bands from inclusion body purification were excised (Figure S5-13A, red box) for MALDI-TOF peptide sequencing. The sequencing result indicated possible contamination by the *E. coli* outer membrane protein F precursor (gi|26246956 or AAN79539.1), with a size of 39.3 kDa. On a 10% polyacrylamide gel, the purities of the seawi PIWI domain were 64, 33 and 86% in lysis 1 pellet, lysis 2 supernatant and lysis 2 pellet, respectively (Figure S5-13C). The purity of PIWI domain peptides in

the wash 3 pellet (Figure S5-13D, Lane 12 red box) was at 96% (Figure S5-13E). Therefore, the band of insoluble, expressed fragments from the wash 3 pellet was excised from a curtain gel (Figure S5-13F) and used as immunogen for antibody production in two rabbits 27910 and 27911 (Pocono Rabbit Farm and Laboratory, PA). Prior to injection, a preimmune bleed sample was taken from each rabbit. After the injection of the immunogen, immune bleeds were taken at 28-day intervals.

To assess the production of anti-PIWI antibodies, protein samples of purified PIWI domain and sea urchin MT-RNP complexes were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with preimmune or immune antisera. It appears that even the first immune bleeds of anti-PIWI sera from both the 27910 and 27911 rabbits can specifically recognize the expressed PIWI domain (Figure S5-15A - rabbit 27910 and B - rabbit 27911). Immunoblots from second and third bleeds are also shown (Figure S5-16A and B). For both rabbits, our anti-PIWI antisera specifically recognized the immunogen even at 1:50000 dilutions. We next compared four bleeds from both the 27910 and 27911 rabbits on expressed PIWI domain peptides (Figure S5-17A, rabbit 27910 and C, rabbit 27911) and MT-RNP complexes,

which contain endogenous seawi (Figure S5-17B, rabbit 27910 and D, rabbit 27911). By the fourth bleed both rabbits were producing antisera that recognized bacterially expressed PIWI domains (Figure S5-17A and C).

As for recognition of the full coding region of seawi, antisera from both rabbits were tested against protein samples of MT-RNP (Figure S5-17). In MT-RNP complexes, antisera from the second, third and fourth bleeds of rabbit 27911 recognized full-length seawi (Figure S5-17D), and only slight recognition using antisera from rabbit 27910 (Figure S5-17B). Further we immunostained sea urchin testis with all bleeds of antisera from rabbits 27910 and 27911. Seawi antiserum 27911 is superior in localizing seawi in testis as compared to antisera 27910 which essentially exhibited no detectable binding (Figure S5-18A 27910 and B 27911), which is consistent with the detection limits observed by immunoblots.

Sequences encoding the N-terminal domains of seawi were also amplified and cloned into a pET-100 expression vector, sequenced, transformed and expression was induced. A band of the expressed fragment was present in the induced sample of each expression. The expressed fragments ran at their predicted molecular weights of 33 kDa (30.24 with 3

kDa-tag). The expressed N-terminal domain was not successfully purified with Ni-NTA purification methods, nor were inclusion bodies formed. However, together with PIWI domain peptides, we were able to use them to characterize the epitopes of previously generated anti-seawi antibodies. Anti-seawi (4I4) antibodies recognized both N-terminal and PIWI domains on immunoblots (Figure S5-19A, N-terminal and B, PIWI domain). The recognition of the conserved PIWI domain by the 4I4 antibody raises the question of whether this particular antibody might also be binding to other sea urchin argonaute family members. Seali, sp-ago 1A and sp-ago 1B have molecular weights ranging from 106.18 to 107.74 kDa, which is significantly different and easily distinguishable on SDS-PAGE from 97 kDa seawi. Prior published work and the immunoblots presented in this thesis established the monospecificity of 4I4 to seawi (Faire and Bonder, 1993; Rodriguez et al., 2005).

Discussion

MT-RNP proteins, actin filaments and microtubules

Twelve actin-binding proteins were identified as components of MT-RNP complexes. Filamin, firmbrin, lima1 and F-actin capping protein CAPZ all function in stabilizing actin filaments. Moreover, filamin has ability to interact with Rho GTPases, such as Rac1, Cdc42, and RhoA, which have been reported to remodel the actin cytoskeleton (Leung et al., 2009). Seawi is localized to discrete puncta within the egg cortex, which is known to be highly enriched for short actin filaments, maternal RNAs, endoplasmic reticula, and ribosomes (Burgess and Schroeder, 1977; Covian-Nares et al., 2004; Moon et al., 1982; Rodriguez et al., 2005). Upon fertilization, a calcium wave triggers the release of cortical granules and results in forming a fertilization envelope to block polyspermy and translation of maternal mRNA commences (Grainger and Winkler, 1987). During oogenesis, seawi is initially globally localized within the egg and with maturation it is translocated to the cortex of the oocyte in preparation for fertilization. In MT-RNP, two myosin proteins, myosin VI and non-muscle myosin II were identified. Myosin IV and non-muscle myosin II move vesicles toward the pointed and barbed ends of actin

filaments, respectively. In fruit flies, the same two motors are required for vesicles movements in early embryogenesis (Petritsch et al., 2003).

There are twelve microtubule-binding proteins in MT-RNP complexes. In sea urchin, microtubules are formed after fertilization (Bestor and Schatten, 1981) and the association of seawi and/or MT-RNP-like complexes with microtubules may be important for spatial regulation of seawi dependent regulatory activity (Rodriguez et al., 2005). The presence of EMAP, XMAP 215, and several microtubule motor proteins could well function in spatially distributing seawi and seawi associated RNPs during development (Allan, 1996; Brouhard et al., 2008; Fath et al., 1994; Jordens et al., 2001; Matthews et al., 1998; Moore et al., 2008; Tournebize et al., 2000). In summary, the identification of actin/MT motors and actin/MT binding proteins in MT-RNP provides insight into a possible cytoskeletal regulatory mechanism of cortex enrichment of seawi and proper segregation of RNP complexes during division in embryonic development.

MT-RNP and protein synthesis

Argonautes/piwis are involved in post-transcriptional, translational

silencing and epigenetic modification (Tolia and Joshua-Tor, 2007; Yin and Lin, 2007). Twelve translation-related proteins were identified in MT-RNP complexes. Excluded ribosomal proteins, three are serrate RNA effector molecule homolog (srrt or ars2), eukaryotic translation initiation factor 3 (eif3) and receptor for activated protein kinase C 1 (RACK1). All of them are linked to regulate RISCs and small non-coding RNA functions in other animals. In detail, Srrt is a component of the nuclear RNA cap-binding complex that is required for miRNA biogenesis and critical for cell proliferation (Gruber et al., 2009). Srrt directly interacts with drosha and srrt expression contributes to the stability and processing of primary miRNA transcripts (Gruber et al., 2009). Furthermore, depletion of srrt is sufficient to reduce the pri-miRNA processing and miRNA expression levels and is developmentally lethal (Amsterdam et al., 2004). Deletion of srrt in adult mice led to bone marrow failure whereas non-proliferating cells were unaffected, suggesting srrt expression is linked to the proliferative state of the cell (Wilson et al., 2008).

Protein synthesis requires initiation factor eif3, which associates and controls assembly of 40*S* ribosomal subunits on mRNAs bearing either a 5′-cap or an internal ribosome entry site (Siridechadilok et al., 2005). The

mouse eif3 interact with mili, a murine piwi-like protein, which has been reported to be essential for the early prophase of meiosis and involved in promoting germ-line stem cell division and differentiation via translational regulation (Unhavaithaya et al., 2009).

RACK1 binds to KH-type splicing regulatory protein (KSRP), a member of the Dicer complex, and is required for the recruitment of mature miRNAs to the RNA-induced silencing complex (RISC) (Otsuka et al., 2011). In nematode worms and humans, the loss of RACK1 affects the association of miRNA and argonautes with ribosomes, suggesting that this component of the 40S ribosomal subunit can mediate the recruitment of the RISC to the active site of translation (Jannot et al., 2011). In summary, our data suggest that seawi containing MT-RNP complexes also contain proteins for cytoskeletons association, translation regulation, and small non-coding RNA pathway.

Anti-seawi antibodies

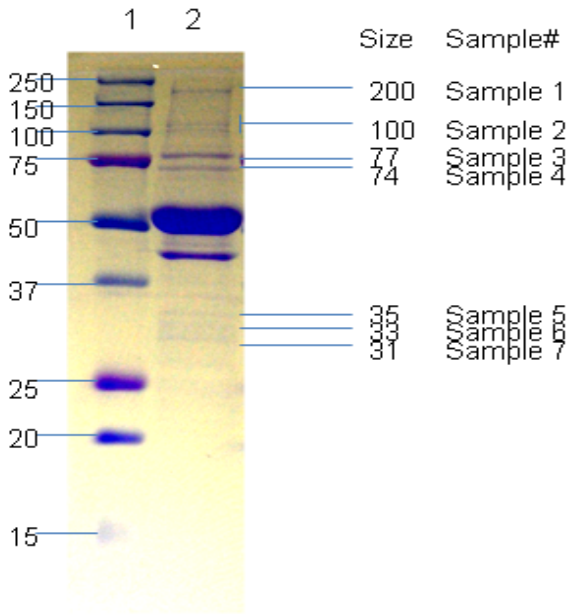
One of our goals was to generate specific antibodies against each sea urchin argonaute/piwi protein. Here, we generated two antisera that used bacterially overexpressed PIWI domain of seawi as immunogen. Both

antisera appear monospecific and recognize the expressed PIWI domain. Seawi antiserum of 27911 appears to be the better of the two preparations since it binds to seawi in both immunoblots and immunostainings. Since the PIWI domain is required for mRNA binding and RNase activity, 27911 may serve as a function-blocking antibody if it is injected into sea urchin eggs.

Figure S5-1. Analysis of proteins in MT-RNP complexes at two-cell embryos.

(A) 10% SDS-PAGE of protein ladder (Lane 1) and MT-RNP complexes (Lane 2). The tubulin bands are at 50 kDa as previously identified (Hamill et al., 1994; Rodriguez et al., 2005). Seven protein bands at 200, 100, 77, 74, 35, 33 and 31 kDa were excised and preceded with MALDI-TOF peptide sequencing. The major proteins for each sample were shown in B. The identification of 100 and 77 kDa bands as seawi and EMAP are in agreement with the previous report (Rodriguez et al., 2005).

A.



B.

	MALDI-TOF	Size	Accession Number
Sample 1	beta-tubulin	50kD	gi|135489
Sample 2	seawi	96.6kD	gi|47551171
Sample 3	EMAP	75kD	gi|47550919
	fimbrin	68kD	gi|115976312
	L-plastin	70kD	gi|115751610
Sample 4	Fimbrin	68kD	gi|115976312
	EMAP	75kD	gi|47550919
	L-plastin	70kD	gi|115751610
Sample 5	beta-tubulin	50kD	gi|135489
Sample 6	alpha-tubulin	42kD	gi|135393
Sample 7	CAPZA F-actin capping protein alpha	32.6kD	gi|115941726
	60S ribosomal protein L7A	31kD	gi|115914093
	60S ribosomal protein L6	32.6kD	gi|72090033
	RACK	36kD	gi|115729641

Table S5-2. Microtubule-associated proteins in MT-RNP complexes.

Gene name, NCBI sequence access number and potential protein binding partners were listed. The listed proteins have microtubule (MT) or tubulin as potential binding partner. Genes with asterisks are unannotated sequences that were identified with BLAST search.

Gene name	Access number	binding partner
EMAP	gi|47550919	tubulin dimer
XMAP215 or mini spindles or cytoskeleton associated protein 5(ckap5)	gi|115744087	MT, hnRNP2, ribonucleoprotein, kif2c
long microtubule-associated protein 1A (mtap1a)*	gi|115623531	MT, actin
CAP-Gly domain-containing linker protein 1 (clip1)*	gi|115649611	MT, Zinc, nuclear acid, IQGAP1, dctn1
dynactin 1 (dctn1)	gi|115751531	dyein, MT
dynamin 1-like (dnm1l)*	gi|115781683	MT, nucleotide, GTP, membrane
kinesin family member 20A (kif20A)	gi|115870896	ATP, MT
tubulin, gamma complex associated protein 6 (tubgcp6)*	gi|115752584	MT
kinesin family member 21A (kif21A)	gi|115737487	ATP, MT
dynein cytoplasmic 1 heavy chain 1 (dync1h1)*	gi|115764779	ATP,MT
microtubule-actin crosslinking factor 1 (macf1)*	gi|115943067	actin, MT, axin1, irp6, clasp1, mapre1
Huntingtin (htt)	gi|146189449	cullin2, beta tubulin, dynein, p53, dynactin

Table S5-3. Actin-associated proteins in MT-RNP complexes.

Gene name, NCBI sequence access number and potential protein binding partners were listed. The listed proteins may associate with actin filament or actin monomer. Genes with asterisks are unannotated sequences that were identified with BLAST search.

Gene name	Access number	binding partner
CAPZA F-actin capping protein alpha	gi|115941726	
spectrin alpha 2	gi|115920116	actin, Ca
spectrin beta 2	gi|72018248	actin, calmodulin, SMAD
filamin A or C	gi|115774591	actin. Small GTPase
myosin 9/10/11/ Myh10*	gi|115678867	actin
myosin VI	gi|47550961	actin, ATP, calmodulin
LIM domain and actin-binding protein 1 (xirp2)*	gi|115774582	actin, zinc
LIM domain and containing protein 1 (lima1)*	gi|115774584	actin monomer and filement, zinc
fimbrin or L-plastin	gi|115976312	actin
prominin 1(prom1)	gi|115611875	cadherin, beta-actin
microtubule-actin crosslinking factor 1 (macf1)*	gi|115943067	actin, MT, axin1, irp1, clasp1, mapre1
microtubule-associated protein 1A (mtap1a)*	gi|115623531	MT, actin

Table S5-4. Other cytoskeleton and protein transport related proteins in MT-RNP complexes.

Gene name, NCBI sequence access number and potential protein binding partners were listed. The listed proteins in (A) may associate with cytoskeleton proteins but not directly associated to actin filament or microtubule. Protein in (B) may associate with macromlecules in transport machinery (B). Genes with asterisks are unannotated sequences that were identified with BLAST search.

A.

Gene name	Access number	binding partner
AHNAK nucleoprotein or desmoyokin*	gi|115918064	S100 calcium binding protein B
neural cell adhesion molecule 1 (Ncam1)*	gi|115610863	
ankyrin 1/3 (ank1/3)	gi|115920155	Spectrin
cubilin (cubn)	gi|115954060	cobalamin(Vitamin B12)
leucine rich repeat containing 16A (lrrc16a)*	gi|115918000	

B.

Gene name	Access number	binding partner
coatomer protein or COPA	gi|115649185	membrane
fimbrin or L-plastin	gi|115976312	actin
adaptor-related protein complex 3, delta 1 subunit (ap3d1)	gi|115951012	
sorting nexin 6 (snx6)	gi|115803059	membrane (phosphotidylinosital)
ankyrin 1/3 (ank1/3)	gi|115920155	Spectrin
cubilin (cubn)	gi|115954060	cobalamin(Vitamin B12)
microtubule-actin crosslinking factor 1 (macf1)	gi|115943067	actin, MT, axin1, lrp1, clasp1, mapre1

Table S5-5. Mitosis and epigenetic related proteins in MT-RNP complexes.

Gene name, NCBI sequence access number and potential protein binding partners were listed. The listed proteins may associate with chromatin or DNA. Genes with asterisks are unannotated sequences that were identified with BLAST search.

Gene name	Access number	binding partner
centromere protein F (Mitosin) (16% coverage) /kinesin K39 (54% coverage)*	gi|72145970	dynein, chromatin/ microtubule
anaphase promoting complex subunit 1 (anapc1) or tsg24*	gi|115622673	Chromatin
LIM domain and actin-binding protein 1 (xirp2)*	gi|115774582	actin, zinc, DNA
CAP-Gly domain-containing linker protein 1 (clip1)*	gi|115649611	MT, Zinc, nuclear acid
LIM domain and containing protein 1 (lima1)	gi|115774584	actin monomer and filement, zinc, DNA
apoptotic chromatin condensation inducer 1 (27%)*	gi|115933386	DNA, RNA
tubulin, gamma complex associated protein 6 (tubgcp6)*	gi|115752584	MT, DNA
mediator complex subunit 13-like (med13l)*	gi|115745092	DNA
DNMT1 protein or DNA-(cytosine-5)-methyltransferase 100%*	gi|72091983	DNA
DNA replication helicase 2 homolog (dna2)*	gi|115655445	DNA, ATP

Table S5-6. Development related proteins in MT-RNP complexes.

Gene name, NCBI sequence access number and potential protein binding partners were listed. The listed proteins may associate with developmetntal process. Genes with asterisks are unannotated sequences that were identified with BLAST search.

Gene name	Access number	binding partner
RACK (receptor for activated C-kinase)	gi|115729641	40S ribosome, receptor tyrosin kinase
major yolk protein precursor	gi|47551123	iron
AHNAK nucleoprotein or desmoyokin*	gi|115918064	S100 calcium binding protein B
alpha-2-macroglobulin (a2m)*	gi|115712705	IL8, IL1, TNF
low density lipoprotein receptor-related protein 1 (lrp1) or a2mr*	gi|282848137	Ca, apolipoprotein, membrane
htt	gi|146189449	cullin2, beta tubulin, dynein, p53, dynactin
intraflagellar transport 172 homolog (ift172)*	gi|72166104	smoothened
lysosomal-associated membrane protein 1 (lamp1)*	gi|72171518	membrane
prominin 1(prom1)	gi|115611875	cadherin, beta-actin
microtubule-actin crosslinking factor 1 (macf1)*	gi|115943067	actin, MT, axin1, irp1, clasp1, mapre1
disco-interacting protein 2 homolog C (dip2c)*	gi|115764735	DNA-(cytosine-5)-methyltransferase

Table S5-7. Translation and ribosome related proteins in MT-RNP complexes.

Gene name, NCBI sequence access number and potential protein binding partners were listed. The listed proteins may associate with ribosomes and/or translation machinery. Genes with asterisks are unannotated sequences that were identified with BLAST search.

Gene name	Access number	binding partner
60S ribosomal protein L7A	gi|115914093	
60S ribosomal protein L6	gi|72090033	
Glutamyl-prolyl-tRNA synthetase or EPRS*	gi|115898425	tRNA, Zn, ATP, RNA
eukaryotic translation initiation factor 3 subunit 1 (eif3)	gi|115729103	40s ribosome
eukaryotic translation initiation factor 3, subunit 2 (eif3)	gi|115903821	piwi-like homologue 2
serrate RNA effector molecule homolog (srrt)*	gi|115933379	drosha, nuclear cap binding protein subunit 1
seawi		
La ribonucleoprotein domain family, member 1 (larp1)*	gi|115725143	RNA
dynein cytoplasmic 1 heavy chain 1 (dync1h1)	gi|115764779	ATP,MT
bat2d or proline-rich coiled-coil 2C(prrc2c)*	gi|115686191	ribosome
bat3 or bag6*	gi|115637319	ribosome
hypoxia up-regulated 1 (hyou1)*	gi|115683720	ATP, nucleotide

Table S5-8. Signaling related proteins in MT-RNP complexes.

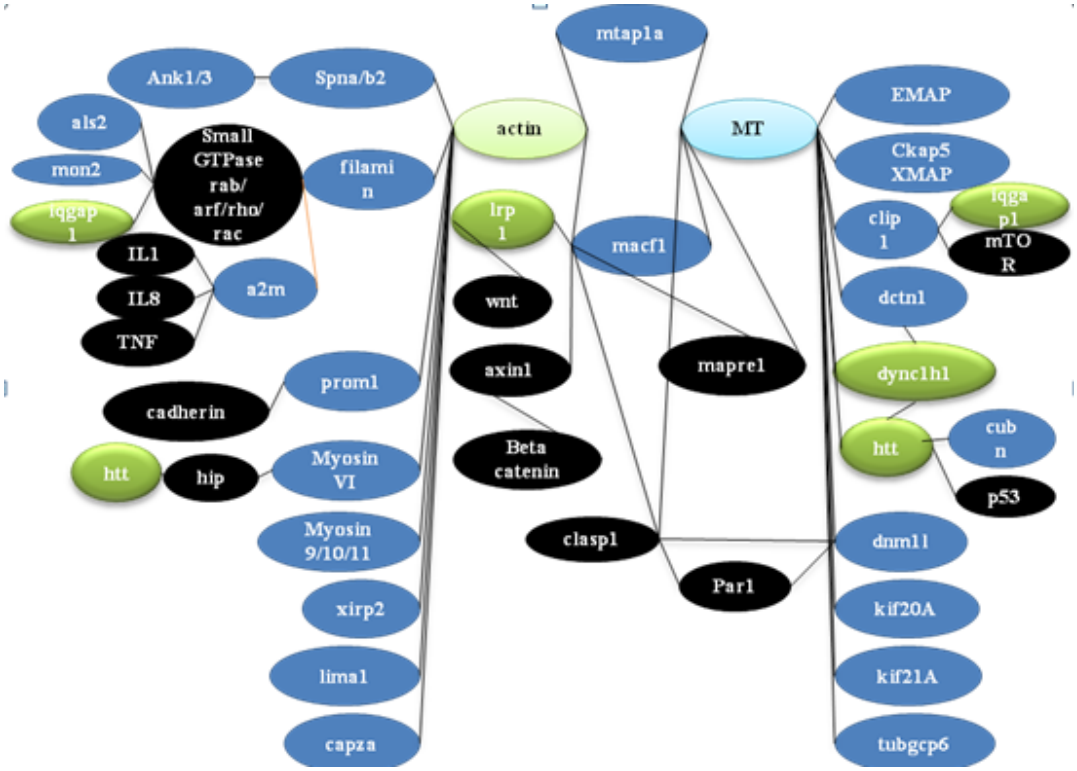
Gene name, NCBI sequence access number and potential protein binding partners were listed. The listed proteins may associate with signaling moelcules. Genes with asterisks are unannotated sequences that were identified with BLAST search.

sodium/potassium ATPase alpha subunit	**gi|184186119**	**ATP, membrane**
dual oxidase 1*	gi|115738144	Ca, membrane
ras GTPase-activating-like protein IQGAP1	gi|115648571	
scavenger receptor cysteine-rich protein precursor*	gi|115687302	membrane
sperm plasma membrane calcium transporting ATPase*	gi|75832170	
neural cell adhesion molecule 1 (Ncam1)*	gi|115610863	
alpha-2-macroglobulin (a2m)*	gi|115712705	IL8, IL1, TNF
amyotrophic lateral sclerosis 2 (als2)*	gi|115667324	rab, rho, rac
scavenger receptor cysteine-rich domain superfamily protein*	gi|115728836	membrane
fertilization envelope protein 9*	gi|282848137	low density lipoprotein receptor-related protein 1 (lrp1) or a2mr
dynamin 1-like (dnm1l)*	gi|115781683	MT, nucleotide, GTP, membrane
mon2 or SF21*	gi|115613324	
solute carrier family 4 (anion exchanger), member 2 (slc4a2)*	gi|115872189	membrane
Plexin A2 (plxna2)	gi|115928293	membrane, semaphorin
microtubule-actin crosslinking factor 1 (macf1)*	gi|115943067	actin, MT, axin1, lrp6, clasp1, mapre1

Figure S5-9. MT-RNP complexes protein interaction map.

(A) Cytoskeleton interaction map. (B) The translational regulation map. The black line indicates interaction of two proteins by predicted domain interactions and the red line indicates two proteins with mouse homologues that were associated in the co-IP experiments. The sea urchin proteins that were identified in these MALDI-TOF/ LC-MS-MS studies is in blue or green. The proteins in green indicate they play multiple roles in the map. Proteins in black were potential binding partners, which were not detected in this proteomics study.

A.


mtap1a
Ank1/3
Spna/b2
als2
mon2
Small GTPase rab/ arf/rho/ rac
filamin
iqgap1
IL1
IL8
TNF
a2m
actin
MT
EMAP
Ckap5 XMAP
lrp1
macf1
clip1
iqgap1
mTOR
wnt
dctn1
axin1
mapre1
dync1h1
proml
cadherin
htt
hip
Myosin VI
Beta catenin
htt
cubn
p53
Myosin 9/10/11
clasp1
dnm1l
Par1
xirp2
kif20A
limal
kif21A
capza
tubgcp6

B.

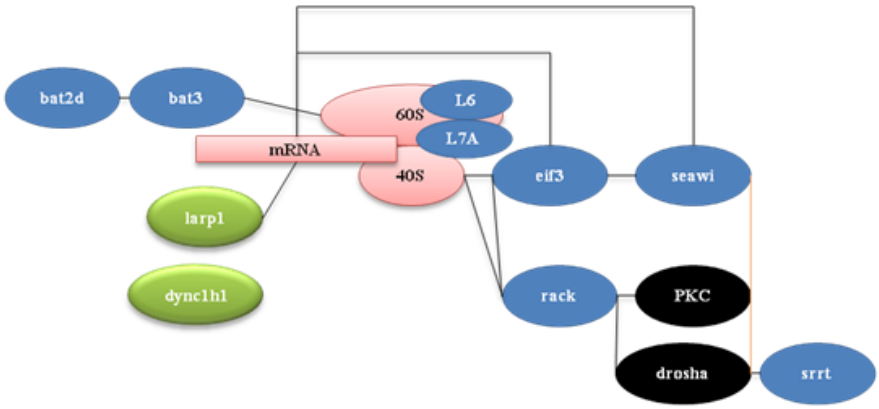
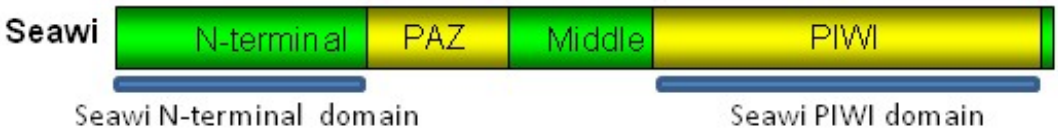

bat2d
bat3
60S
L6
L7A
mRNA
40S
eif3
seawi
larp1
dync1h1
rack
PKC
drosha
srrt

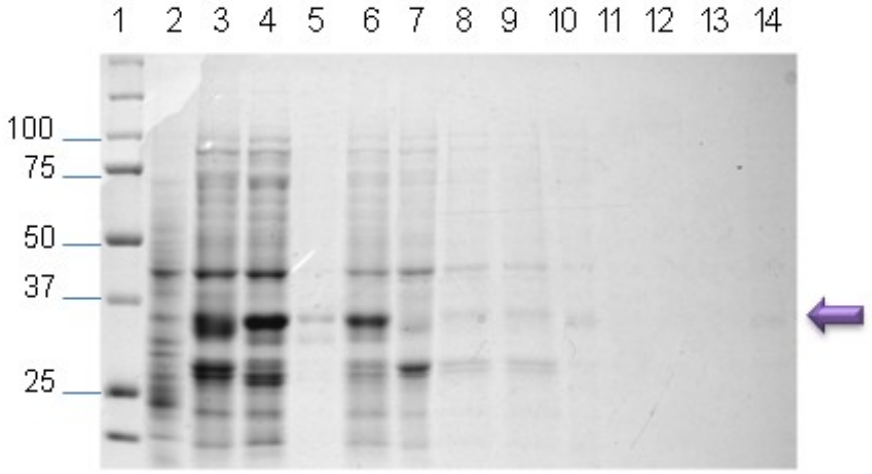
Figure S5-10. Ni-NTA purification of recombinant seawi PIWI domain peptides.

(A) Schematic of region used for the generation of antibodies against seawi PIWI domain. The red line indicates the full coding sequences of conserved seawi PIWI domain. Coomassie blue stain SDS-PAGE (B) and immunoblot of seawi (C) of protein samples from Ni-NTA purification steps. For both B and C, ladder (Lane 1), non-induction (Lane 2), induction (Lane 3), lysis 1 soup (Lane 4), lysis 1 pellet (Lane 5), flow through (Lane 6), beads bound (Lane 7), wash 1 (Lane 8), wash 2 (Lane 9), second beads bound (Lane 10), elute 1 (Lane 11), elute 2 (Lane 12), elute 3 (Lane 13) and final beads bound (Lane 14) are loaded according to figure 3-1 flowchart. Purple arrows indicate the size of expressed seawi PIWI domain peptides.

A.


Seawi
N-terminal
PAZ
Middle
PIWI
Seawi N-terminal domain
Seawi PIWI domain

B.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
100
75
50
37
25

C.

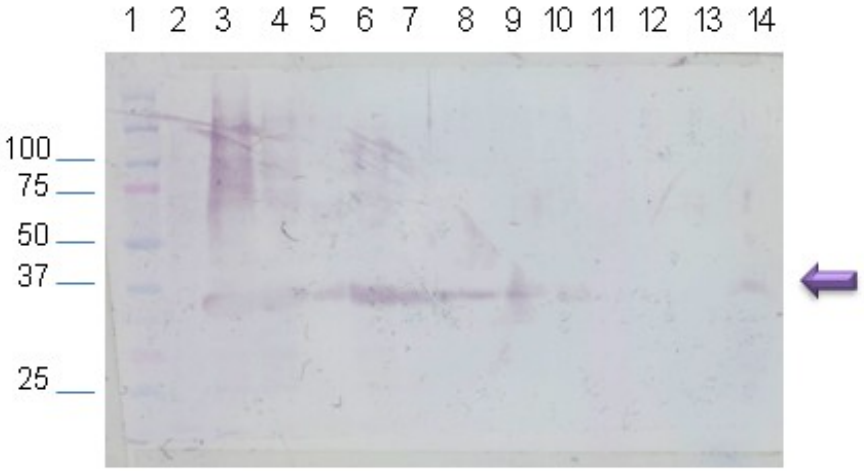

1 2 3 4 5 6 7 8 9 10 11 12 13 14
100
75
50
37
25

Figure S5-11. Flowchart of Ni-NTA purification of recombinant seawi PIWI domain.

Recombinant seawi PIWI domain peptides were induced as described in Materials and Methods. The lysis of bacteria pellets were performed with sonication and lysozyme. After centrifugation, the supernatant was processed with Ni-NTA column binding, wash and elute steps. The samples, highlighted in orange, were stoichiometrically loaded to gel in Figure S5-10B.

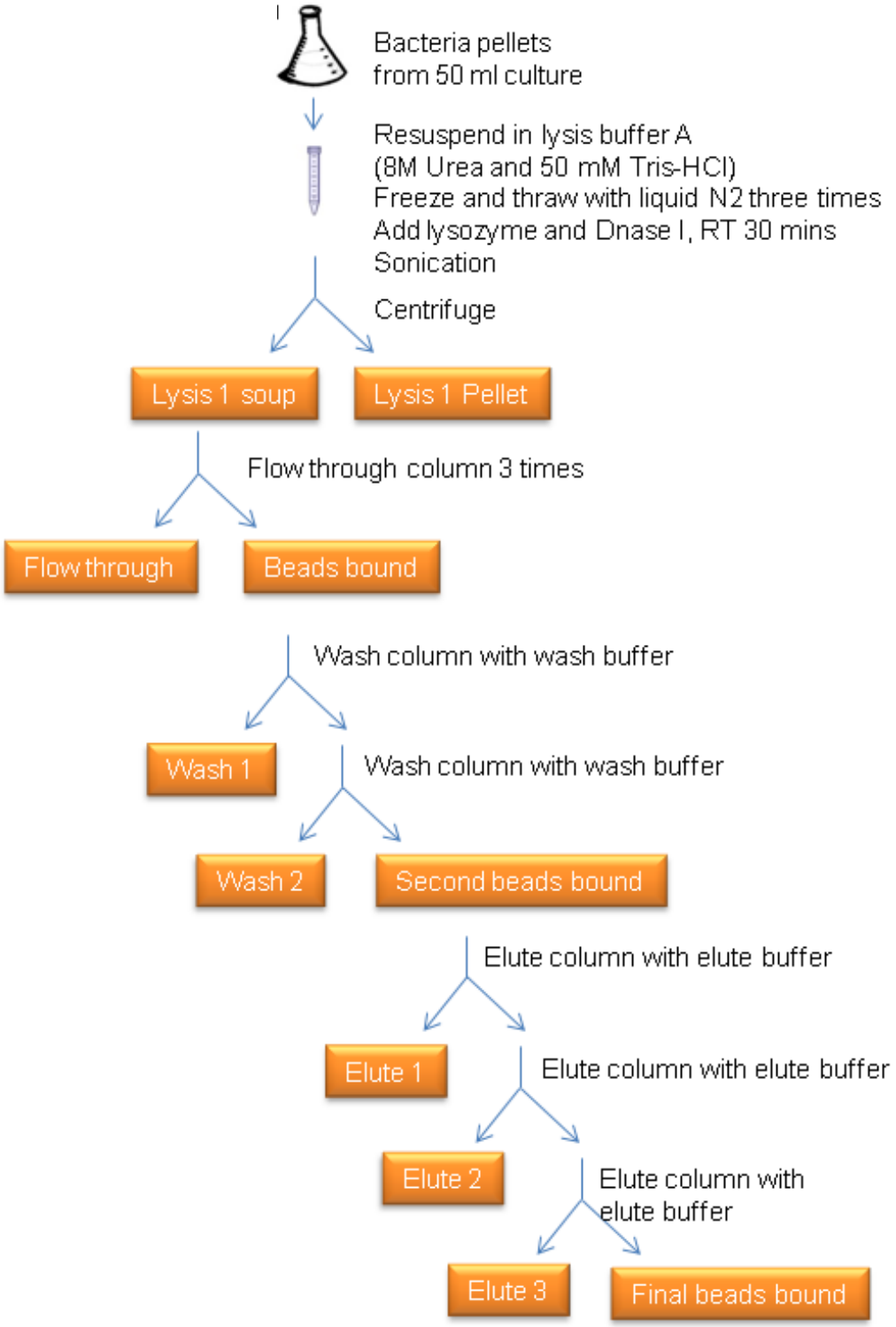

Bacteria pellets
from 50 ml culture
Resuspend in lysis buffer A
(8M Urea and 50 mM Tris-HCl)
Freeze and thraw with liquid N2 three times
Add lysozyme and Dnase I, RT 30 mins
Sonication
Centrifuge
Lysis 1 soup
Lysis 1 Pellet
Flow through column 3 times
Flow through
Beads bound
Wash column with wash buffer
Wash 1
Wash column with wash buffer
Wash 2
Second beads bound
Elute column with elute buffer
Elute 1
Elute column with elute buffer
Elute 2
Elute column with
elute buffer
Elute 3
Final beads bound

Figure S5-12. Flowchart of inclusion body purification of seawi PIWI domain.

Recombinant seawi PIWI domain peptides were induced as described in Materials and Methods. The lysis of bacteria pellets were performed with sonication and lysozyme in non-denaturing condition. After centrifugation, the pellets were processed with multiple washes, and inclusion bodies were collected in the wash 3 pellets. The samples, highlighted in orange, were stoichiometrically loaded to SDS-PAGE in Figure S5-13A.

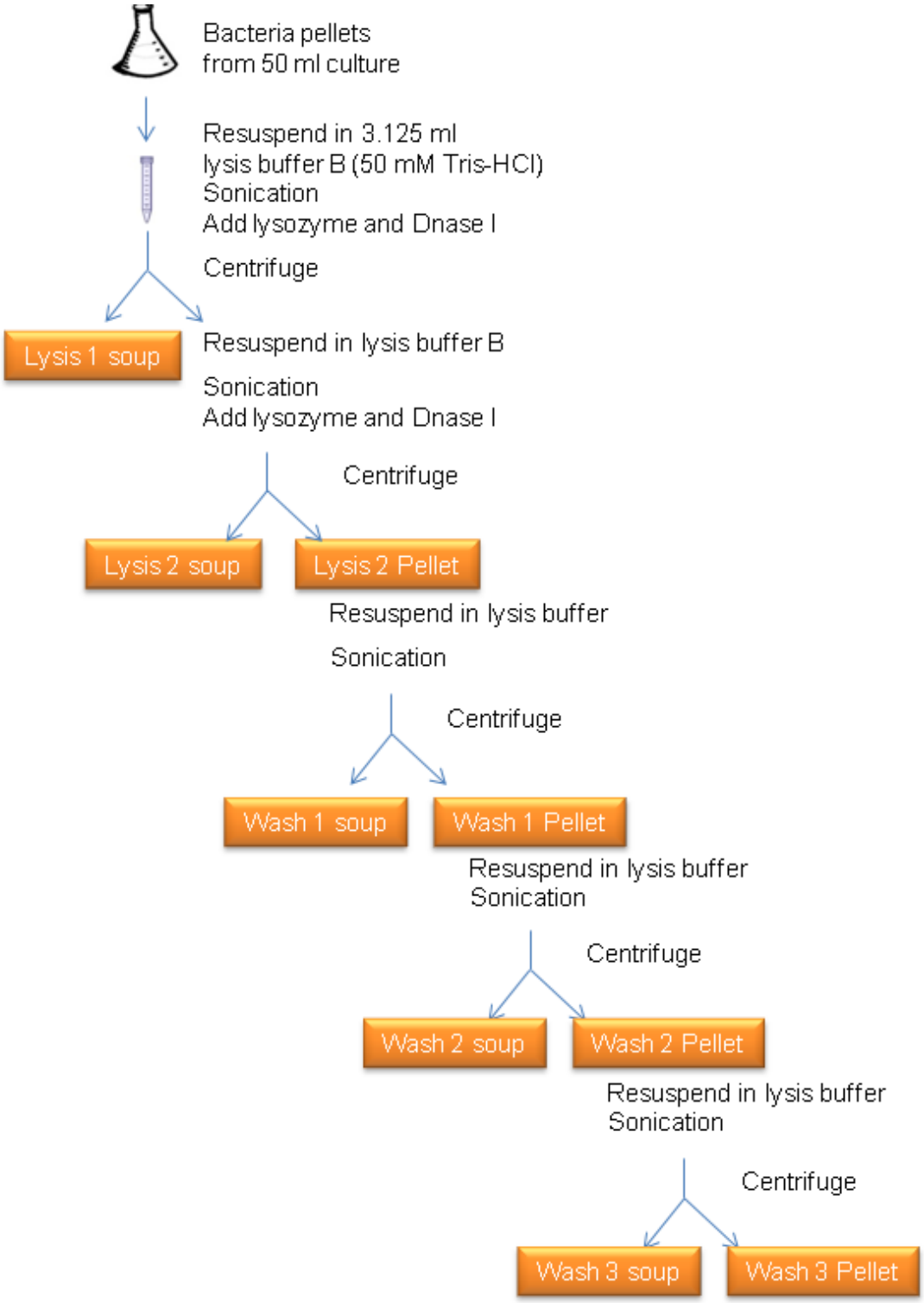
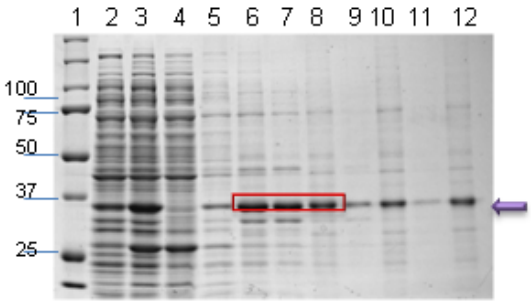

Bacteria pellets
from 50 ml culture
Resuspend in 3.125 ml
lysis buffer B (50 mM Tris-HCl)
Sonication
Add lysozyme and Dnase I
Centrifuge
Lysis 1 soup
Resuspend in lysis buffer B
Sonication
Add lysozyme and Dnase I
Centrifuge
Lysis 2 soup
Lysis 2 Pellet
Resuspend in lysis buffer
Sonication
Centrifuge
Wash 1 soup
Wash 1 Pellet
Resuspend in lysis buffer
Sonication
Centrifuge
Wash 2 soup
Wash 2 Pellet
Resuspend in lysis buffer
Sonication
Centrifuge
Wash 3 soup
Wash 3 Pellet

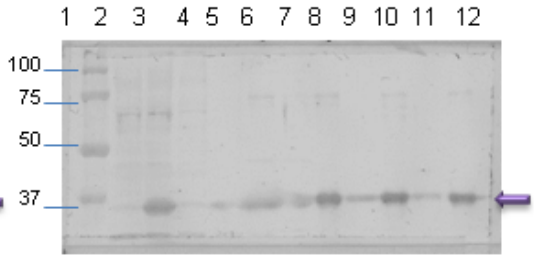
Figure S5-13. Seawi PIWI domain as antigen for polyclonal antibodies generation.

Coomassie blue stain of 10% SDS-PAGE (A), 16% SDS-PAGE (D) and immunoblot of seawi (B) of protein samples from inclusion body purification steps. For A, B and D, protein ladder (Lane 1), non-induction (Lane 2), induction (Lane 3), lysis 1 soup (Lane 4), lysis 2 soup (Lane 5), lysis 2 pellet (Lane 6), wash 1 soup (Lane 7), wash 1 pellet (Lane 8), wash 2 soup (Lane 9), wash 2 pellet (Lane 10), wash 3 soup (Lane 11) and wash 3 pellet (Lane 12) are loaded according to figure S5-12 flowchart. Purple arrows indicate the size of expressed seawi PIWI domain peptides. Red boxes in A and D indicate peptide bands for MALDI-TOF peptide identification, and the purity is shown in C and E. 1 mg of expressed seawi PIWI domain peptides are shown in F, and the purple arrow indicates the size of seawi PIWI domain.

A.



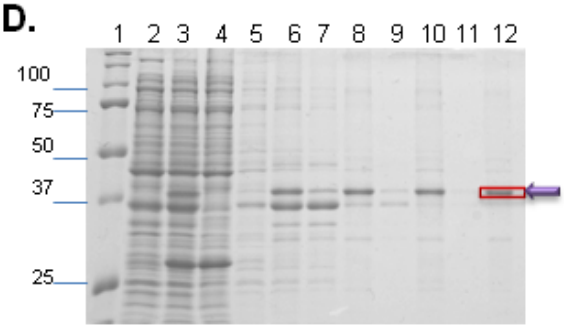
B.



C.

	Lane 6	Lane 7	Lane 8
Seawi-PIWI domain	64%	33%	86%
Outer membrane protein F precursor	36%	67%	14%

D.



E.

	Lane 12
Seawi-PIWI domain	96%
Outer membrane protein F precursor	4%

F.

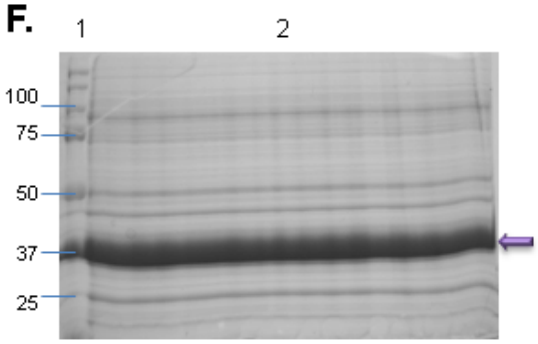
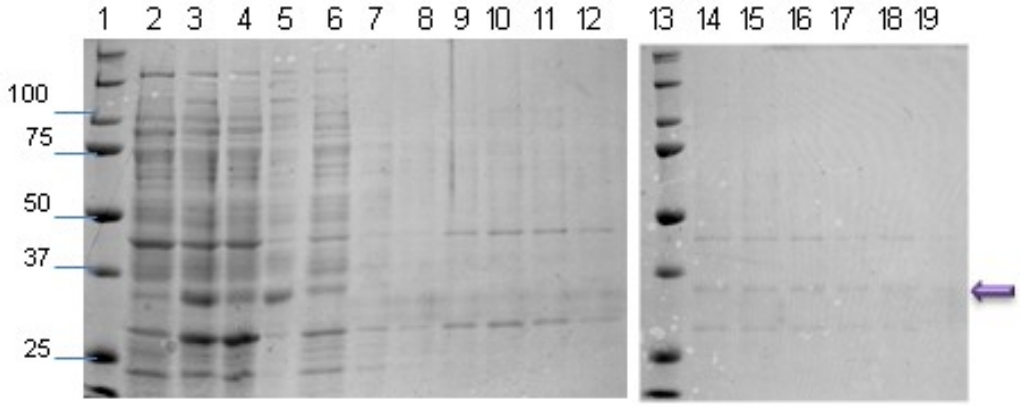


Figure S5-14. Comparison of Invitrogen and Qiagen Ni-NTA purification methods.

Coomassie blue stain 10% SDS-PAGE of protein samples from Invitrogen (A) and Qiagen (B) Ni-NTA purification steps. For both A and B, protein ladder (Lane 1), non-induction (Lane 2), induction (Lane 3), lysis 1 soup (Lane 4), lysis 1 pellet (Lane 5), flow through (Lane 6), beads bound 1 (Lane 7), beads bound 2 (Lane 8), wash 1 (Lane 9), wash 2 (Lane 10), wash 3 (Lane 11), wash 4 (Lane 12), protein ladder (Lane 13), elute 1 (Lane 14), elute 2 (Lane 15), elute 3 (Lane 16), elute 4 (Lane 17), elute 5 (Lane 18) and final beads bound (Lane 19) are loaded according to figure S5-11 flowchart with one extra binding, two extra washes and two extra elutes. Purple arrows indicate the size of expressed seawi PIWI domain peptides.

A.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
100
75
50
37
25

B.

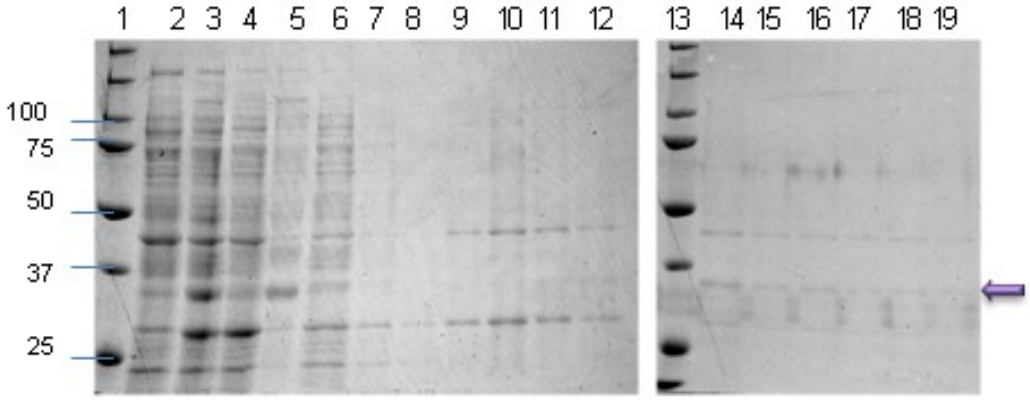
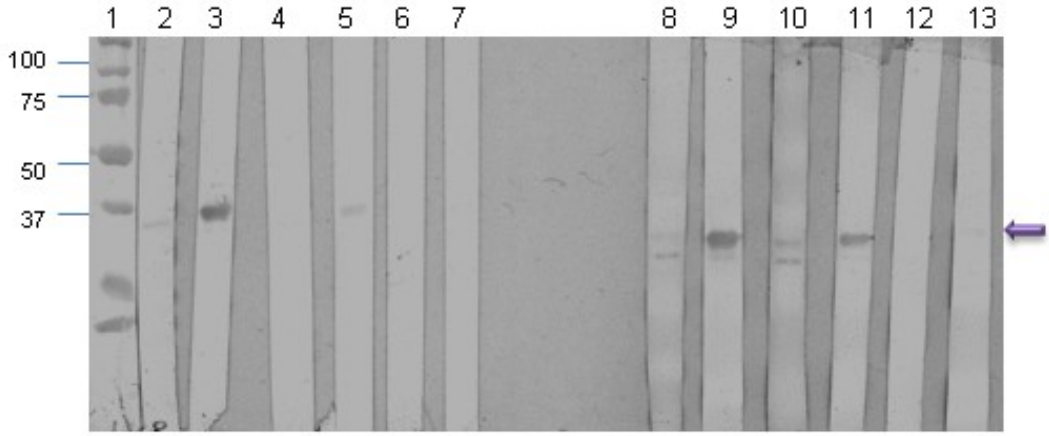

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
100
75
50
37
25

Figure S5-15. Immunobloting of first bleeds of antisera against expressed seawi PIWI domain.

Immunoblotting of antisera from rabbit 2790 (A) and 27911 (B) against seawi PIWI domain. In both A and B, the immunoblotting conditions used either 5% milk blocking buffer (Lane 2-7) or 5% calf serum with 1% BSA blocking buffer (Lane 8-13). Protein ladder was loaded in lane 1. The antisera were prepared in 1:500 (Lane 2, 3, 8 and 9), 1:5000 (Lane 4, 5, 10 and 11) and 1:50000 (Lane 6, 7, 12 and 13) dilutions. Lane 2, 4, 6, 8, 10 and 12 were pre-immune bleeds, and Lane 3, 5, 7, 9, 11 and 13 were first bleed of antisera. Antisera from both 27910 and 27911 recognize expressed seawi PIWI domain specifically even in the 1:50000 dilution.

A.


1 2 3 4 5 6 7 8 9 10 11 12 13
100
75
50
37

B.

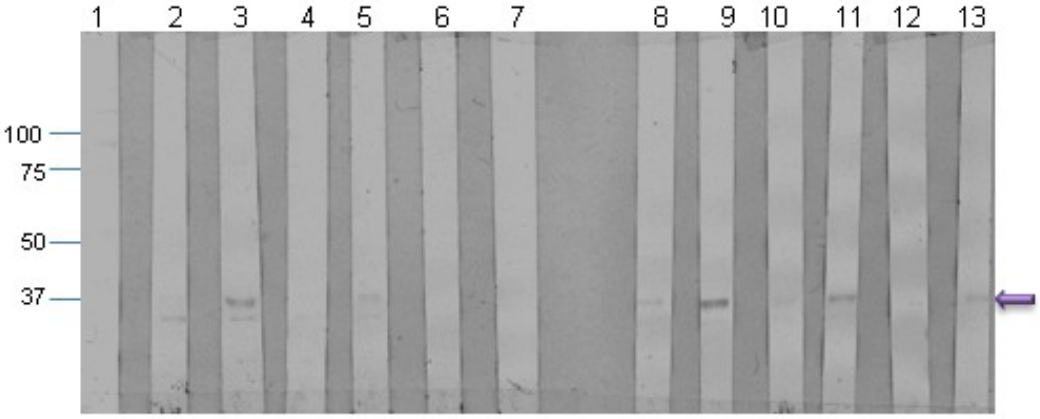
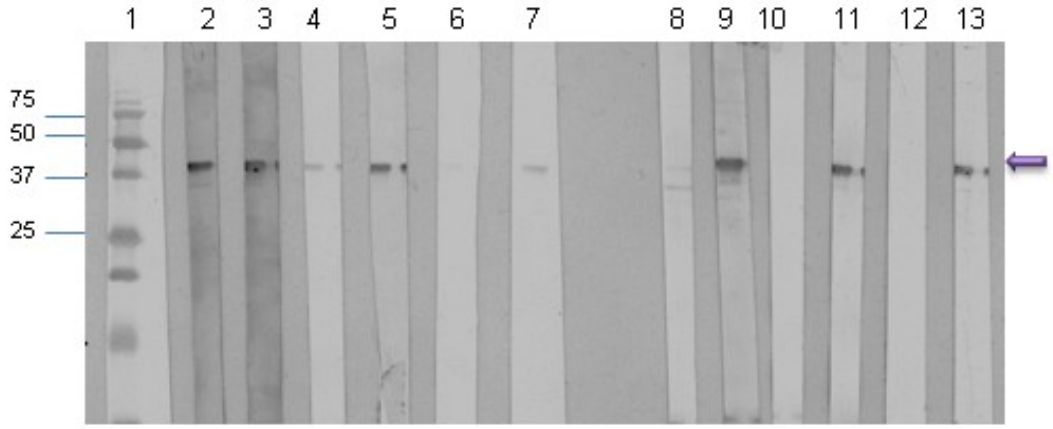

1 2 3 4 5 6 7 8 9 10 11 12 13
100
75
50
37

Figure S5-16. Immunobloting of second and third bleeds of antisera against expressed seawi PIWI domain.

Immunoblotting of second (A) and third bleeds (B) of antisera from rabbit 2790 (Lane 2-7) and 27911 (Lane 8-13) against seawi PIWI domain. 5% milk blocking and 5% calf serum with 1% BSA blocking were applied to antisera from rabbit 27910 and 27911, respectively. (B) Protein ladder was loaded in Lane 7. The antisera were prepared in 1:500 (Lane 1, 2, 8 and 9), 1:5000 (Lane 3, 4, 10 and 11) and 1:50000 (Lane 5, 6, 12 and 13) dilutions. Lane 1, 3, 5, 8, 10 and 12 were using pre-immune bleeds, and Lane 2, 4, 6, 9, 11 and 13 were using antisera. (C) Protein ladder was loaded in lane 1. The antisera were prepared in 1:500 (Lane 2, 3, 8 and 9), 1:5000 (Lane 4, 5, 10 and 11) and 1:50000 (Lane 6, 7, 12 and 13) dilutions. Lane 2, 4, 6, 8, 10 and 12 were using pre-immune bleeds, and Lane 3, 5, 7, 9, 11 and 13 were using antisera.

A.


1 2 3 4 5 6 7 8 9 10 11 12 13
75
50
37
25

B.

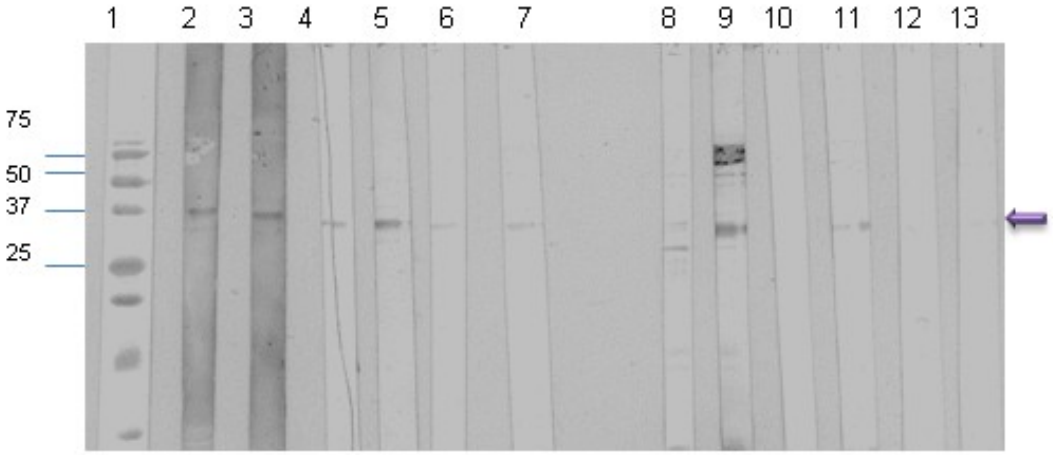

1 2 3 4 5 6 7 8 9 10 11 12 13
75
50
37
25

Figure S5-17. Immunobloting of first, second, third and fourth bleeds of antisera against expressed seawi PIWI domain and seawi.

Membranes with expressed seawi PIWI domain peptides (A and C) and two-cell MT-RNP complexes (B and D) were immunoblotted with antisera from rabbit 27910 (A and B) and 27911 (C and D). Controls were performed using A59 anti-seawi antibody (Lane 1 of A, B, C and D). Lane 2, 3, 4, 5 and 6 were preimmune, first, second, third and fourth bleeds, respectively. Purple arrows indicate the size of expressed seawi PIWI domain (A and C) and seawi (B and D). Antisera from both 27910 and 27911 recognize both seawi PIWI domain and seawi.

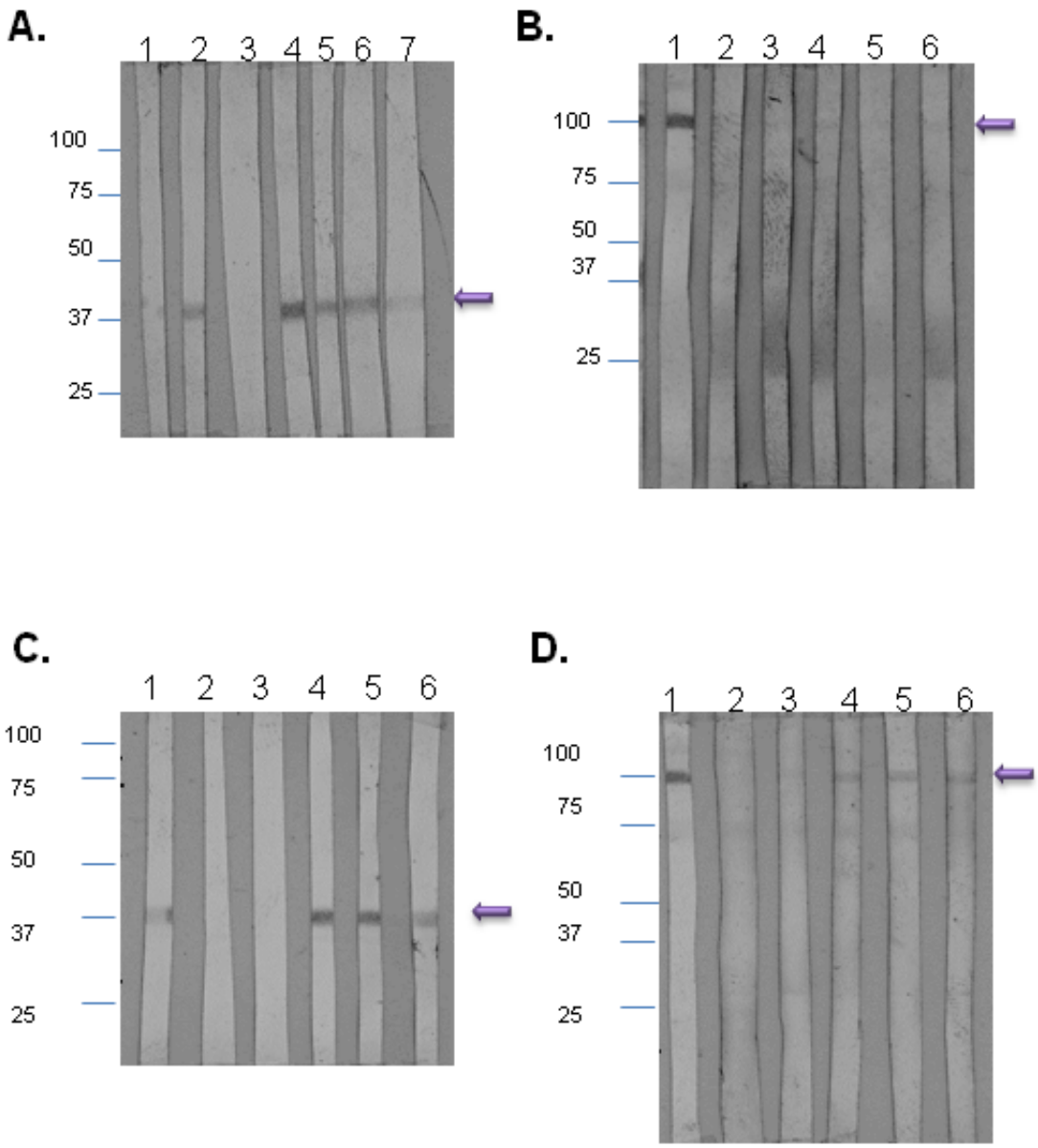

A.
1 2 3 4 5 6 7
100
75
50
37
25
B.
1 2 3 4 5 6
100
75
50
37
25
C.
1 2 3 4 5 6
100
75
50
37
25
D.
1 2 3 4 5 6
100
75
50
37
25

Figure S5-18. Immunostaining of anti-seawi antibodies 27910 and 27911 against sea urchin testes.

Immunostain of cross-section of sea urchin adult testes using antisera from rabbits 27910 and 27911. The fourth bleed of 27910 (A) and 27911 (B) anti-seawi antisera with 1:50 dilution were shown. (C) Immunostaining with secondary antibody alone. Bars in (A) (B) and (C) are 10 μm.

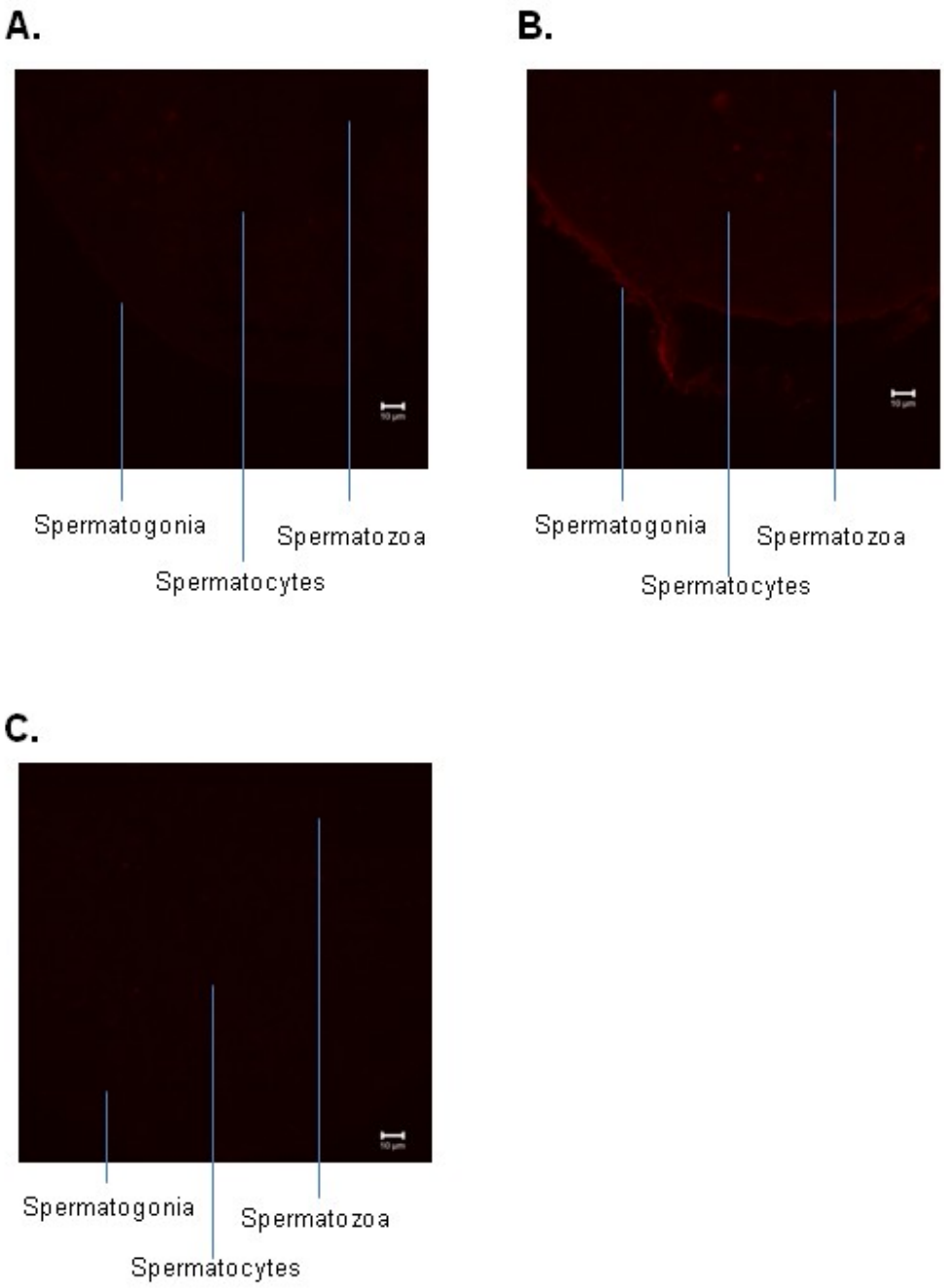
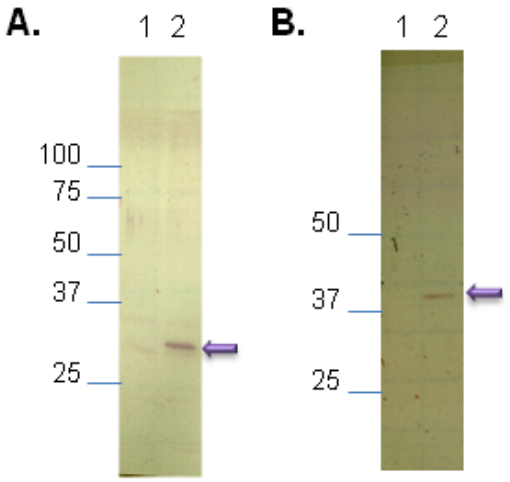

A.
Spermatogonia
Spermatocytes
Spermatozoa
B.
Spermatogonia
Spermatocytes
Spermatozoa
C.
Spermatogonia
Spermatocytes
Spermatozoa

Figure S5-19. Immunoblotting of anti-seawi antibody against expressed seawi N-terminal and PIWI domains.

Immunoblots of anti-seawi antibody 4I4 against seawi N-terminal (A) and PIWI (B) domains. In both A and B, the non-induction (Lane 1) and induction samples (Lane 2) were loaded. This antibody recognized both seawi N-terminal and PIWI domains.


A.
1 2
100
75
50
37
25
B.
1 2
50
37
25

CHAPTER 6

Conclusions and future directions

Functional motifs of seawi, seali, sp-ago1A and sp-ago1B

The work in this thesis describes the molecular and cellular characterization of sea urchin argonautes/piwis and their associated small non-coding RNAs. To initiate the study of sea urchin argonautes/piwis, the complete coding sequence of *sp-ago1A*, *sp-ago1B* and *seali* was determined and published in GenBank (Chapter 2). Together with seawi, the sea urchin argonaute and piwi sequences are highly conserved compared to mammalian orthologues, and contain sequence features, PAZ and PIWI domain for examples, that are unique to argonaute/piwi family members. Sea urchin small non-coding RNAs were identified, characterized, sequenced, and annotated. Seawi was shown to preferentially be associated with piRNA population (see Chapter 4). Recent reports provide detail on the selectivity between specific small non-coding RNA population and argonaute/piwi proteins. For examples, the PAZ domain from the human ago1 and mouse miwi binds preferentially to non-methylated and 2-O methylated small non-

coding RNAs, respectively (Ma et al., 2004; Simon et al., 2011). A single amino acid mutation in the miwi PAZ domain leads to a change in their preferential binding from 2-O methylation to non-methylated small non-coding RNAs (Simon et al., 2011). This subtle structural change can be correlated to the ability of PAZ domains to adapt to different small non-coding RNA populations.

Seawi and its associated piRNA may be involved in A-V axis formation

PIWI domains of all sea urchin argonautes/piwis contain conserved RNA binding and RNA catalytic motifs, which argues for their potential activities in mRNA binding and degradation. As described in Chapter 1, these potential abilities are crucial for small non-coding RNA related post-transcriptional and translational regulation in embryonic development in fruit flies, zebrafishes and mice. Translation initiation factor eif3 and ribosomal proteins were identified in seawi-containing MT-RNP complexes. This observation supports the role of seawi in translational regulation. Moreover, we showed that all four argonaute/piwi mRNAs and seawi protein are expressed throughout embryogenesis, which might indicate they have multiple roles throughout

early embryonic development.

In sea urchins, the A-V axis becomes fully specified by the 16-cell embryo stage. The proper establishment of this axis is crucial for other axes and germ layer specification (Angerer et al., 2000; Davidson, 1989; Logan et al., 1999; Oliveri and Davidson, 2004; Oliveri et al., 2008). At the animal pole of sea urchin embryo, bep4 is expressed and is required for the animalizing cell-fate determination and ectoderm differentiation (Angerer et al., 2000; Kenny et al., 2001; Kenny et al., 1999; Romancino et al., 2001). Applying functional blocking antibody against bep4 to sea urchin embryos leads to nuclear localization of beta-catenin in the animal half of sea urchin embryos and the expansion of the vegetal cell boundaries (Romancino et al., 2001).

Seawi is enriched in micromeres (vegetal cells) at the time of A-V axis formation. Seawi binds to bep4 mRNA (Rodriguez et al., 2005), and a cluster of seawi/MT-RNP-associated piRNA sequences are complementary to cell fate determinant bep4 and beta-catenin mRNAs. These results support the proposed model of Rodriguez et al. (2005) that seawi regulates the formation of A-V axis through post-transcriptional and/or translational regulation of maternal cell-fate determinants bep4 mRNA in vegetal cells and we can now

add that this regulation is guided by seawi and piRNA targeted to bep4 mRNA. Besides bep4 and beta-catenin, our previous result also suggested bep2 and soxB1 mRNAs are presented in MT-RNP complexes (Desai et al., 2004). With our MT-RNP small non-coding RNA database, the results provide possible cell-fate determinant mRNA targets for further evaluation.

Paternal inheritance of mi- and piRNA

One of the most surprising results from the investigations reported in this thesis is the presence of mi- and piRNAs in mature sea urchin sperm. The observation presents the possibility that sperm deliver both genomic DNA and small non-coding RNAs to the awaiting egg. The delivery of mi- and piRNA to the egg would provide paternal factors as contributors to regulation of maternally stored messages. Two of the transcripts are miR-252 and miR-278, which were only detected in mature sperm but not in egg. In fruit fly embryos, the miR-278 transcript has been shown to participate in early development (Teleman et al., 2006). In human sperm tests, besides sperm count and mobility, sperm quality is positively correlated to total RNA quantity; good looking, morphologically normal sperm have significantly greater total RNA

content than abnormal sperm (Roudebush et al., 2004). The current hypothesis for this positive correlation is that specific mRNAs, which are crucial for embryonic development, are inherited from sperm (Hamatani, 2011). Our discovery may add a new twist to the current hypothesis that the positive correlation between total RNA and sperm quality may be due to small non-coding RNA content in sperm.

The epigenetic function of seawi and piRNA

Besides post-transcriptional and translational regulation, piwi proteins and piRNAs are shown to epigenetically modify genomic DNA in the fruit fly (Yin and Lin, 2007), mouse germline (Kuramochi-Miyagawa et al., 2008) and *Aplysia* neurons (Rajasethupathy et al., 2012), and these modification further alter the transcription of genes or transposable elements. In *Aplysia* CNS neuron, in response to serotonin signal, piwi and piRNA increased methylation on CREB promoter, which results in reduction of transcription of CREB mRNAs and leads to changes in long-term memory (Rajasethupathy et al., 2012). Such epigenetic modification of gene expression by piwi and piRNA relies upon association of the DNA methyltransferase (Aravin and Bourc'his,

2008; Rajasethupathy et al., 2012), interestingly dnmt was identified as a component of reconstituted sea urchin seawi-MT-RNP complexes.

The role of seawi and piRNA in stem cell and germline maintenance

In sea urchins, germline cell fate is determined in the small micromeres between the 16 and 32-cell stage embryos (Ransick et al., 1996; Yajima and Wessel, 2010). Small micromere descendants migrate at the tip of the archenteron in gastrulation and differentiate into coelomic pouches in larval stages (Ransick et al., 1996), and later derive into the sea urchin adult germline (see Chapter 3). In other species, it has been well documented that piwi proteins are required for germline maintenance in developing embryos (Cox et al., 1998; Cox et al., 2000; Klattenhoff et al., 2007; Klattenhoff and Theurkauf, 2008; O'Donnell and Boeke, 2007; Tan et al., 2002). The most abundant seawi-associated piRNAs are mapped to non-coding regions: introns or intergenic regions of scaffold 312 and 1770. The scaffold 1770, for example, encodes a transposon derived gene *rag1*, which functions in genome DNA rearrangement. Moreover, the transposable elements are mostly located in introns and intergenic regions (Bennetzen, 2000; Venner et

al., 2009). This would suggest seawi-associated piRNAs in eggs and embryos may prevent uncontrolled transposition and genome rearrangement. Together with seawi localization in germline lineage throughout embryonic development, our results suggest that seawi and its piRNA protect genome integrity in germline in embryonic development.

In sea urchin testes, seawi protein level were found to decrease along the spermatogenic developmental axis, as spermatogonia express the most seawi and mature sperm the least. In ovaries, seawi proteins are expressed in the oogeneic cells but with no decrease along the oogenic developmental axis. In nematode worms, fruit flies, zebrafishes and mice, the loss-of-function of piwi proteins results in spermatogenic cells arrested in testes. Two hypotheses were provided for the function of sea urchin seawi in spermatogenesis, oogenesis and early development (see Chapter 3). First, seawi and its associated piRNA may regulate mRNAs that are essential for spermatogenesis. Second, piwi and its associated piRNA may protect germ cell and blastomere genome integrity by silencing transposable elements. By analyzing piRNA sequences from sea urchin testes (see Chapter 4), we identified piRNA that complement to transposase and reverse transcriptase of

retrotransposons. These two enzymes are required for DNA transposon transposition and retrotransposon replication, respectively. The piRNA results, in agreement with second hypothesis, suggest seawi and piRNA may protect the germ cell genome integrity in sea urchin testes.

Future directions

Seali, sp-ago1A and sp-ago1B in sea urchin development

The full length sequences of seawi, seali, sp-ago1A and sp-ago1B were determined, and the functional motifs – small non-coding RNA, mRNA binding and RNase III -- were identified. To continue the characterization of seali, sp-ago1A and sp-ago1B in sea urchin development, monospecific antibodies against seali, sp-ago1A and sp-ago1B are an obvious next step. By examining amino acid conservations within the four domains, the N-terminal domains are the most variable region among the four sea urchin argonautes/piwis and may function as the best source of immunogen. The entire N-terminal may not necessarily be appropriate for sp-ago1A and sp-ago1B because of the overall degree of conservation but one stretch may be ideal such as the 354 nt region from 5' of N-terminal domain where sp-ago1A

and B only share 16% similarity. An alternative method would be to generate mRNA of GFP fusion seawi, seali, sp-ago1A and sp-ago1B from sea urchin vector (PCMVTNT) through *in vitro* transcription, following by microinjecting into sea urchin egg.

Monospecific antibodies against seali, sp-ago1A and sp-ago1B, would allow for argonaute specific co-precipitation of small RNA, which when coupled with deep sequencing would provide extraordinary insight into argonaute-small RNA temporal association dynamics. Coupling such studies with GFP-fusion protein constructs would potentially enable development of a spatial and temporal argonaute-small RNA association map.

To further examine the importance of functional motifs of each argonaute family member in sea urchin development, mRNA constructs with deletion of small RNA binding, mRNA bind or RNase III motif should be developed and injected into sea urchin eggs and/or specific blastomeres. In addition, a morpholino with specific motif sequences can be co-injected into egg to remove the endogenous seali, sp-ago1A and sp-ago1B mRNAs. After injecting mRNA, cell fates would be followed and observations would be cross-correlated with the both immunolocalization and co-immunoprecipitation

data.

Seawi and its associated piRNA function in A-V axis formation

The data in this thesis establish the associations of seawi, piRNA and bep4 mRNA during sea urchin embryogenesis, determining the *in vivo* function of seawi and its piRNA in A-V axis formation is an important next step. To address this, mRNAs of GFP fusion bep4 and GFP fusion bep4 with deletion of all piRNA target sequences could be generated and microinjected into sea urchin eggs. If our hypothesis is correct, the set of GFP mRNA without piRNA target sequences will show the translation of GFP proteins and *vice versa*. Moreover, the mRNA bep4 without piRNA sequences will show uncontrollable expression; thus lead to disruption of A-V axis formation. The rescue experiment can be done by co-injecting synthesized piRNA with bep4 sequences. An alternative method would be injecting long antisense morpholino nucleotides that are complementary to bep4 piRNA sequences into fertilized eggs. If our hypothesis is correct, this “knock-out” bep4 piRNA will also disrupt the A-V axis formation.

Profiling of small non-coding RNA from staged embryos

In this thesis, the potential of using deep-sequencing method to investigate small non-coding RNAs in sea urchin development is now well established. PiRNA profile changes from egg to two-cell embryo, suggesting the small non-coding RNA profile may change with development. To fully incorporate small non-coding RNAs into the big picture of GRNs, it is possible to deep-sequence small RNA from different embryonic stages and identify their target mRNAs. By incorporating these small non-coding RNAs into existing GRNs, this survey would provide greater insight into developmental events and how they are under the control of small non-coding RNA machinery and how important this small non-coding RNA machinery is during development.

While not extensively discussed in this thesis, sea urchins initially develop bilaterially as larvae, and then later gain pentaradial symmetry in their adult body plan. The bilateral symmetry establishes after the establishment of A-V axis at 16-cell embryos. In late larva, coelomic pouch cells form and are stem cells for pentaradial adult body plan. We show seawi and its associated piRNAs are involved in A-V axis specification and seawi is enriched in

coelomic pouches at larva stage. Our data suggests that seawi and piRNAs may carry the important information about how an egg switches from no symmetry (egg), to bilateral symmetry (micromere at 16-cell embryos) to pentaradial symmetry (adult body plan). Deep sequencing of RNA isolated from small micromeres (Pucci-Minafra et al., 1968) and coelomic pouch cells from larva may lend insight into novel mechanisms for symmetry switches.

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Curriculum Vitae

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EDUCATION

Rutgers University, Newark, New Jersey, 2013
Ph.D. in Biological Sciences, area of specialization: Cell, Development and Molecular Biology

New York University, New York, New York, 2004
M.S. in Biological Sciences, concentration in Genomics and Developmental Biology

Taiwan University, Taipei, Taiwan, 1999
B.A. in Agricultural Chemistry

RESEARCH EXPERIENCE

Doctoral Research: Department of Biological Sciences, Rutgers University, Newark,
New Jersey, 2004-presnt, (Research adviser: Dr. Edward Bonder).

- Characterizing the sea urchin Argonaute/Piwi proteins, examining the developmental expression profile
- Profiling the small non-coding RNA populations in eggs, embryos, adult germline tissues, and MT-RNP complexes
- Characterizing the proteins present in seawi-containing MT-RNP complexes
- Cloning and characterization of sea urchin egg villin

Master Thesis Research: Department of Biology, New York University, New York, New York, 2002-2004 (Research adviser: Dr. Fabio Piano)

- Predictive models of molecular machines involved in *Caenorhabditis elegans* early embryogenesis.
- Large scale RNA interference screening with ovary-enriched ORFeome clones for new genes with roles in the *C. elegans* embryo.
- Target sequences shifting toward 5' end of the RNA interference in the *C. elegans* embryo.

Undergraduate Research: Department of Agricultural Chemistry, Taiwan University, Taipei, Taiwan, 1998-1999 (Research adviser: Dr. Ping-du Lee)

- Purifying glucotransferase from rice by affinity and ion-exchange chromatography.

UNIVERSITY SERVICES AND AWARDS

American Society for Cell Biology (ASCB) Don Fawcett Memorial Award: 2010

Graduate Student Excellence Award: 2008

PUBLICATIONS

1. Cheng MF, Alexander K, Zhou S, Bonder EM, **Chuang LS**.
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PUBLISHED ABSTRACTS

1. **Ling-shiang Chuang**, Chengjian Li, Alexis J. Rodriguez, Susan A. Seipel,

Phillip D. Zamore and Edward M. Bonder.
Sea urchin argonaute proteins and small silencing RNAs
The American Society of Cell Biology Annual Meeting. 2010

2. **Ling-shiang Chuang**, Susan A. Seipel and Edward M. Bonder.
Cloning and analysis of *S. purpuratus* argonaute proteins
The American Society of Cell Biology Annual Meeting. 2008

3. **Ling-shiang Chuang**, Susan A. Seipel, Alexis J. Rodriguez and Edward M. Bonder.
Analysis of sea urchin seawi expression in testis and ovary
The American Society of Cell Biology Annual Meeting. 2007

PRESENTATIONS

1. **Ling-shiang Chuang**, Susan A. Seipel and Edward M. Bonder.
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2. Ramamurthy Mani, Kristin C Gunsalus, Hui Ge, Aaron J Schetter, Debra S Goldberg, Jing-dong J Han, Nicolas Bertin, Ning Li, Jerry Huang and **Ling-shiang Chuang**.
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3. **Ling-shiang Chunag**, Aaron Schetter, Jerry Huang, Kris Gunsalus and Fabio Piano. Predicting and testing protein localization in live *C. elegans* embryos using phenoclustering.
Master poster session. New York University. Spring 2004.

4. Anita G Fernandez, Hsiao-Lan Liang, Caroline Tang, **Ling-shiang Chuang**, Nancy Ying, Marc Vidal, Kris Gunsalus and Fabio Piano.
Toward an RNAi-based phenotypic map in *C. elegans*.
14th International Worm Meeting 2003.