DEVELOPMENTAL DYNAMICS 236:3180–3190, 2007

Genes Involved in the RNA Interference Pathway Are Differentially Expressed During Sea Urchin Development

Jia L. Song* and Gary M. Wessel

RNA-mediated interference (RNAi) is a conserved gene silencing mechanism that involves double-stranded RNA as a signal to trigger the sequence-specific degradation of target mRNA, resulting in posttranscriptional silencing and/or translational repression. Bioinformatic searches in the sea urchin genome database identified homologs of Drosha, DGCR5, Dicer, TRBP, Exportin-5, and Argonautes. Quantitative, real-time polymerase chain reaction indicated that all mRNA accumulate in eggs and in variable levels throughout early development. Whole-mount in situ RNA hybridization showed that all of the important players of the RNAi silencing pathway have abundant mRNA accumulation in oocytes and eggs, but have distinct spatial and temporal expression patterns throughout development. Sequence analysis revealed that each of the four Argonautes examined contain conserved residues important for RNAseH activity within the Piwi domain. This study elucidated that genes involved in the RNAi silencing pathway have dynamic expression and, thus, may have regulatory roles during germ cell development and embryogenesis. *Developmental Dynamics 236:3180–3190, 2007.* © 2007 Wiley-Liss, Inc.

Key words: sea urchin; Drosha; DGCR8 (DiGeorge critical region 8 gene); Dicer; TRBP (HIV-1 transactivating response RNA binding protein); PACT (protein activator of the interferon-induced protein kinase); Exportin-5; Argonautes; RNA interference

Accepted 4 September 2007

INTRODUCTION

RNA-mediated interference (RNAi) is a conserved gene regulatory mechanism that involves double-stranded (ds) RNA as a signal to trigger the sequence-specific degradation of target mRNA, resulting in posttranscriptional silencing and/or translational repression. RNA-directed transcriptional silencing was first identified in plants (Wassenegger et al., 1994; Mette et al., 2000), and subsequent studies revealed that a wide range of eukaryotes from fungi to human use small RNAs and the canonical components of the RNA silencing machinery to carry out conserved gene silencing mechanisms (reviewed in Zamore and Haley, 2005). The RNAi silencing pathway is directed by small RNAs, 21 to 30 nucleotides in length, which can be endogenously synthesized or exogenously introduced. Small regulatory RNAs are categorized by their origin, not by their functions. The biogenesis of both miRNA (micro RNA) and siRNA (small interfering RNA) require dsRNA—specific endonuclease Dicer and small RNA-binding proteins of the Argonaute family.

The ribonuclease III family enzyme Drosha and the dsRNA binding protein DGCR8/Pasha are required for the initial processing of an RNA polymerase II transcribed primary miRNA (pri-miRNA) into approximately 65 nucleotide stem-loop RNA precursors (Basyuk et al., 2003; Lee et al., 2003, 2006). DGCR8/Pasha binds directly to pri-miRNAs by recognizing the flanking single-stranded RNA of the pri-miRNA hairpin structure and

The Supplementary Material referred to in this article can be found at http://www.interscience.wiley.com/jpages/1058-8388/suppmat

Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island

Grant sponsor: NIH; Grant sponsor: NIH/NRSA; Grant number: F32HD046247.

***Correspondence to: Jia L. Song, Box G-L173, 185 Meeting Street, Brown University, Providence, RI 02912.**
E-mail: jia_song@brown.edu

DOI 10.1002/dvdy.21353
Published online 17 October 2007 in Wiley InterScience (www.interscience.wiley.com).

acts as a molecular anchor for Drosha-mediated catalysis (Han et al., 2004, 2006; Yeom et al., 2006). The stem-loop precursor structure is essential for Exportin-5 interaction and export in a Ran-GTP–dependent manner (Yi et al., 2003; Lund et al., 2004).

In contrast to the polymerase II transcribed pre-miRNAs, siRNAs are derived from long dsRNA hundreds to thousands of base pairs. The pre-miRNAs or long dsRNAs are further recognized and processed by Dicer and its cofactor TRBP (HIV-1 transactivating response RNA binding protein) into miRNAs and siRNAs in the cytoplasm, respectively (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Chendrimada et al., 2005). Dicer-cleaved small dsRNAs are then used by the RISC (RNA-induced silencing complex) for target processing. Small RNAs and their associated proteins together regulate transcriptional silencing, translational repression, heterochromatin formation, genome integrity, and mRNA stability (Mochizuki et al., 2002; Bartel, 2004; Verdel et al., 2004; Mochizuki and Gorovsky, 2005; Murchison et al., 2007).

Small RNA-directed mRNA cleavage or translational repression is catalyzed by Argonaute proteins in the RISC. The Argonaute family is divided into AGO-like and PIWI-like subfamilies based on their amino acid similarities (Yigit et al., 2006; reviewed in Tolia and Joshua-Tor, 2007). All Argonaute proteins contain two RNA-binding domains: the PIWI domain, which binds the small RNA guide at the 5′end, and the PAZ domain, which binds the single-stranded 3′end of small RNA (Yan et al., 2003; Haley and Zamore, 2004; Song et al., 2004). The crystal structure of Argonaute from *P. furiosus* revealed that the PIWI domain has an RNAse H fold with conserved aspartate and histidine residues important for its catalytic activity of cleaving the target mRNA (Song et al., 2004).

The sea urchin is a basal deuterostome that shares a common ancestor with the chordates. The embryos of the purple sea urchin, *Strongylocentrotus purpuratus*, consists of only 10–15 cell types with a single cell layer epithelium surrounding several different types of mesenchymal cells (Ettensohn and Ingersoll, 1992; Ettensohn et al., 2004). A diagrammatic representation and fate map of the sea urchin embryo are depicted in Figure 1A. A vast majority of the specific cell fates acquired in this embryo are based on cell interactions (Ransick and Davidson, 1993), and the blastomeres express distinct sets of signaling molecules and transcription factors that regulate these cellular specification and differentiation events (Duboc et al., 2005; Croce et al., 2006; Howard-Ashby et al., 2006a–c; Lapraz et al., 2006; Materna et al., 2006; Walton et al., 2006). However, a regulatory role of small, noncoding RNAs has not been investigated in this organism.

This study examines the spatial and temporal RNA expression patterns of genes that are involved in the biogenesis and function of small regulatory RNAs in the sea urchin RNAi silencing pathway. Their presence and dynamic expressions suggest that the sea urchin may selectively use the RNAi silencing pathway in regulating embryogenesis.

RESULTS AND DISCUSSION

Key Molecules Involved in the RNAi Pathway Are Present in the Sea Urchin and Have a Strong Maternal Component

Genes involved in the RNAi pathway were identified bioinformatically from the sea urchin genome database (http://annotation.hgsc.bcm.tmc.edu/Urchin and www.genbore.org) and partial sequences were cloned for further analysis. *S. purpuratus* contains one putative gene each for *Drosha*, *DGCR8*/*Pasha*, *Dicer*, *TRBP*, and *Exportin-5*, and at least 4 *Argonaute* genes. Each of these predicted proteins contains the known conserved residues for function (Fig. 1B).

Quantitative, real-time polymerase chain reaction (QPCR) analysis indicates that each gene involved in the RNAi pathway has a high mRNA accumulation in the egg that generally decreases during the 72 hr of growth to the pluteus larval stage (Fig. 2). *AGO1* and *Seawi* (Rodriguez et al., 2005; Juliano et al., 2006), however, each has the highest RNA accumulation at the mesenchyme blastula stage. The QPCR results from this study are consistent with the database results from the global genome oligonucleotide microarrays (Wei et al., 2006). The only discrepancies seen between the two technologies are (1) the peak RNA accumulation of *PiwiL1* observed by QPCR is in the egg, whereas by the oligonucleotide microarray, it occurs in the early blastula stage; and (2) *AGO1* observed by QPCR peaks at mesenchyme blastula stage, whereas it is not detected after the blastula stage by the oligonucleotide microarray (Wei et al., 2006). These minor differences may reflect more differences in the batches of wild-type embryos used for these studies than the technologies themselves. Overall, however, the QPCR results shown here are consistent with the results from the whole genome arrays.

Drosha and Its Cofactor, *DGCR8 (DiGeorge Syndrome Critical Region 8 Gene)*/*Pasha*, Have Similar Spatial and Temporal Expression Patterns

The mRNA of sea urchin homologs of *Drosha* and its cofactor *DGCR8*/*Pasha* are most abundant in the oocytes and eggs (Fig. 3). In early blastulae, the mRNA of both genes accumulate throughout the embryo, and later in mesenchyme blastulae, they are enriched in the apical ectoderm, primary mesenchyme cells, and in the vegetal plate. Low levels of *Drosha* and *DGCR8*/*Pasha* mRNA are detected selectively in the endoderm of the gastrulae. In general, this accumulation pattern reflects areas of the embryo most active in cell division, and/or cell fate determinations.

Exportin-5 Is Enriched in the Oocytes

One *Exportin-5* homolog was identified in the sea urchin genome. Its RNA accumulation is highest in the oocytes and decreased dramatically by the 32-cell stage. *Exportin-5* is at low levels and cannot be detected by whole-mount in situ hybridization through subsequent development but is detectable consistently by QPCR (Figs. 2, 3).

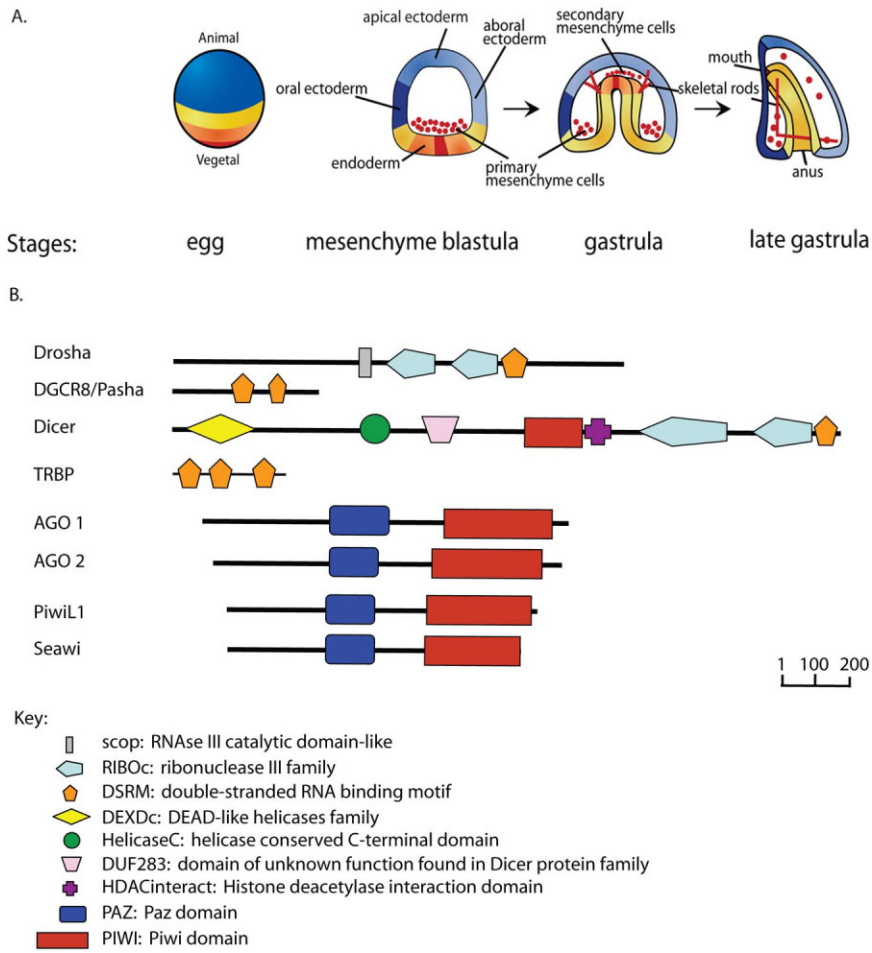

Fig. 1. A,B: The diagrammatic representation and fate map of the embryo during sea urchin development (A; adapted from Gilbert, 2000) and protein domains predicted by the Simple Modular Architecture Research Tool (B; Schultz et al., 1998). Proteins involved in the RNAi silencing pathway contain conserved functional protein domains. The scale bar represents the number of amino acids.

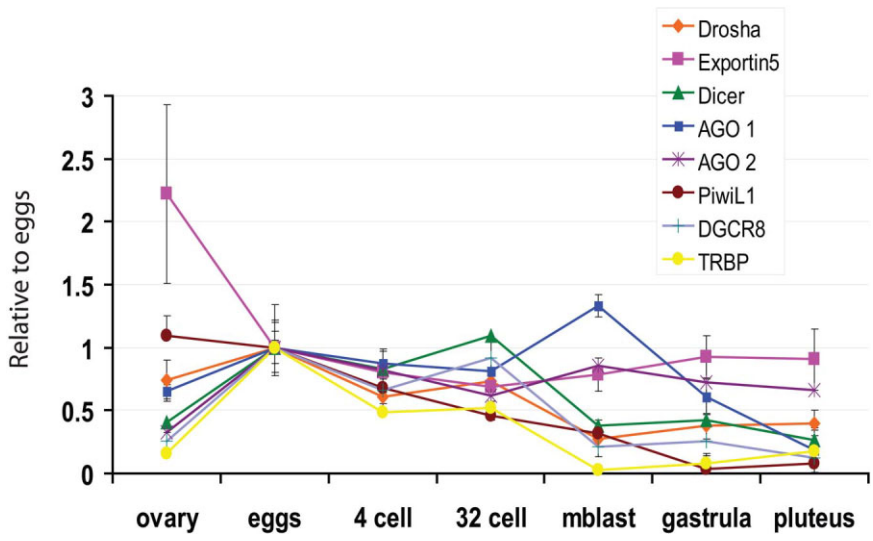

Fig. 2.

Dicer mRNA Is Asymmetrically Enriched in the Egg and Embryos

Only one homolog of *Dicer* was bioinformatically identified from the sea urchin genome database, suggesting that, similar to humans, the sea urchin Dicer may mediate both miRNA and siRNA silencing pathways. *Dicer* mRNA accumulates asymmetrically in one periphery of the oocyte in punctate cytoplasmic structures, similar to some of the *Argonaute* mRNAs (Fig. 3). This asymmetric localization of *Dicer* mRNA is maintained throughout development; in mesenchyme blastula and gastrula stages, its RNA accumulation is enriched selectively in the presumptive oral ectoderm and endodermal epithelium. The transcript then decreases to undetectable levels in the larval pluteus stage 72 hr after fertilization (Fig. 3).

Of special note here is that several sea urchin transcription factors have similar spatial expression patterns as *Dicer*, including the orphan steroid receptor *TF-COUP*, the zinc finger *z55*, and the homeobox transcription factor *E2f3* in that their mRNAs are enriched in the oral ectoderm (Vlahou et al., 1996; Materna et al., 2006; Howard-Ashby et al., 2006a). *TF-Coup* has asymmetric RNA localization in oocytes and eggs, similar to *Dicer*. However, in cleavage stage embryos, TF-COUP RNA is localized lateral to the animal/ventral axis and at 45-degree angle to the oral/aboral axis, whereas *Dicer* RNA is ubiquitous (Vlahou et al., 1996; Fig. 3). *z55* has maternal RNA transcripts in the egg, ubiquitous expression at 7 hr after fertilization, and is restricted to the presumptive oral ectoderm in the blastula stage (Materna et al., 2006). *E2f3* mRNA is not detectable with whole-mount *in situ* hybridization before 24 hr postfertilization (blastula stage) and is localized to the oral ectoderm in blastula and to the oral ectoderm and the endodermal gut in gas-

Fig. 2. Quantitative, real-time polymerase chain reaction (QPCR) measurement of genes involved in the RNA-mediated interference (RNAi) pathway. QPCR results are normalized to ubiquitin and presented relative to the levels in the egg. Standard deviations of triplicates are shown.

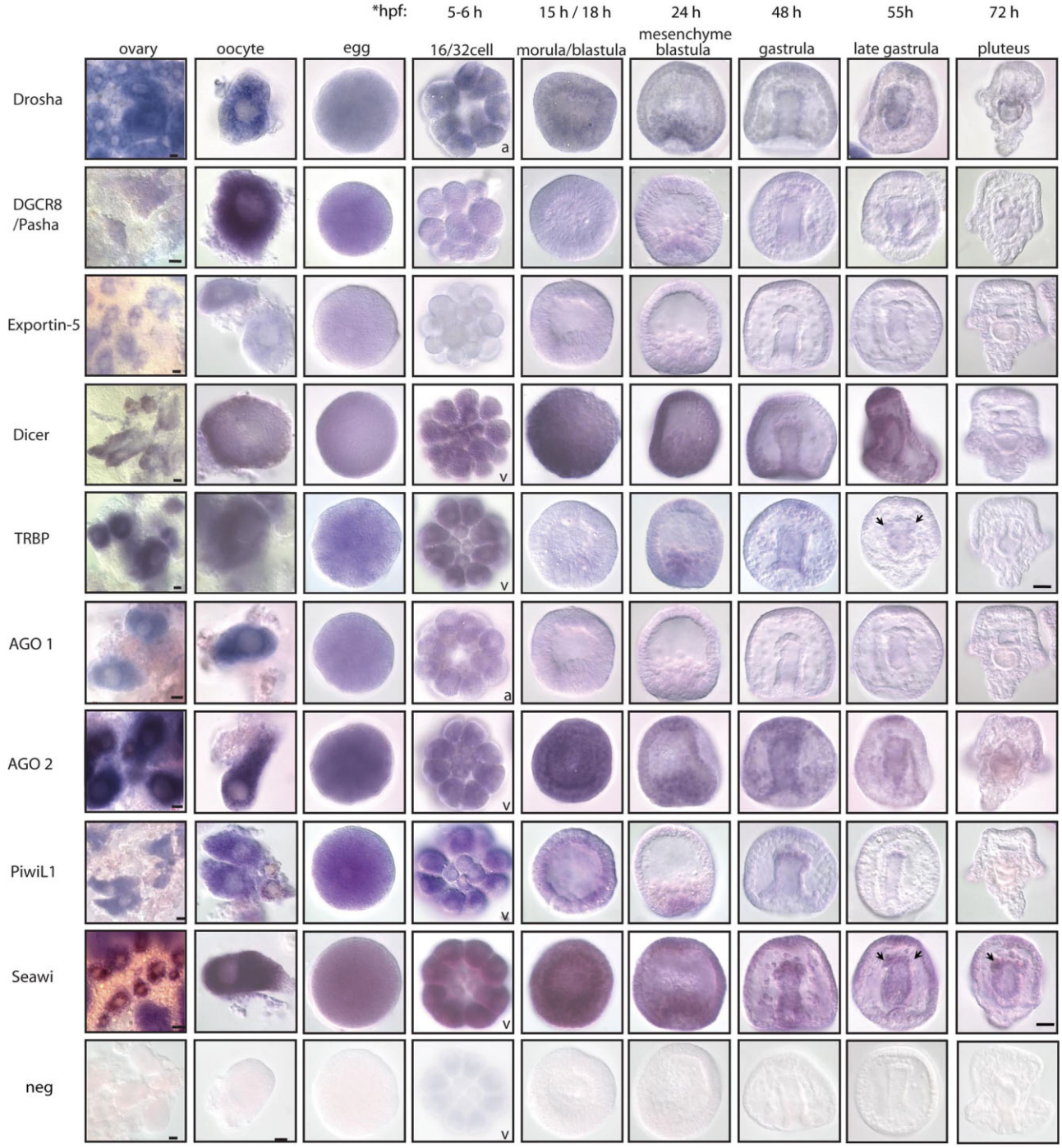

Fig. 3. RNA transcripts detected by whole-mount *in situ* RNA hybridizations. Genes involved in the RNA-mediated interference silencing pathway have dynamic expression patterns during sea urchin development. Embryos are presented in lateral views with the animal pole at the top, except for those marked (v) with ventral side or (a) with animal side of the embryo facing the page. The arrows indicate coelomic pouches. The different developmental stages of the sea urchin are correlated with the time scale indicated by hours postfertilization (hpf). Scale bar = 20 μM.

trula similar to *Dicer* (Howard-Ashby et al., 2006a). Whether these transcription factors functionally interact with Dicer is unclear, but very few genes identified in the sea urchin have this particular pattern of expression.

Phylogenetic analysis indicated that the *S. purpuratus* Dicer clusters with the deuterostome Dicers (Fig. 4). In addition to having the conserved dicer protein domains, the *S. purpuratus* Dicer has an HDAC (histone deacetylase) interaction domain between the PIWI and ribonuclease III domains (Fig. 1). Analysis of Dicer homologs with SMART (Simple Modular Architecture Research Tool; Schultz et al., 1998) indicated that the HDAC interaction domain is unique to *S. purpuratus* and not found in Dicer homologs from

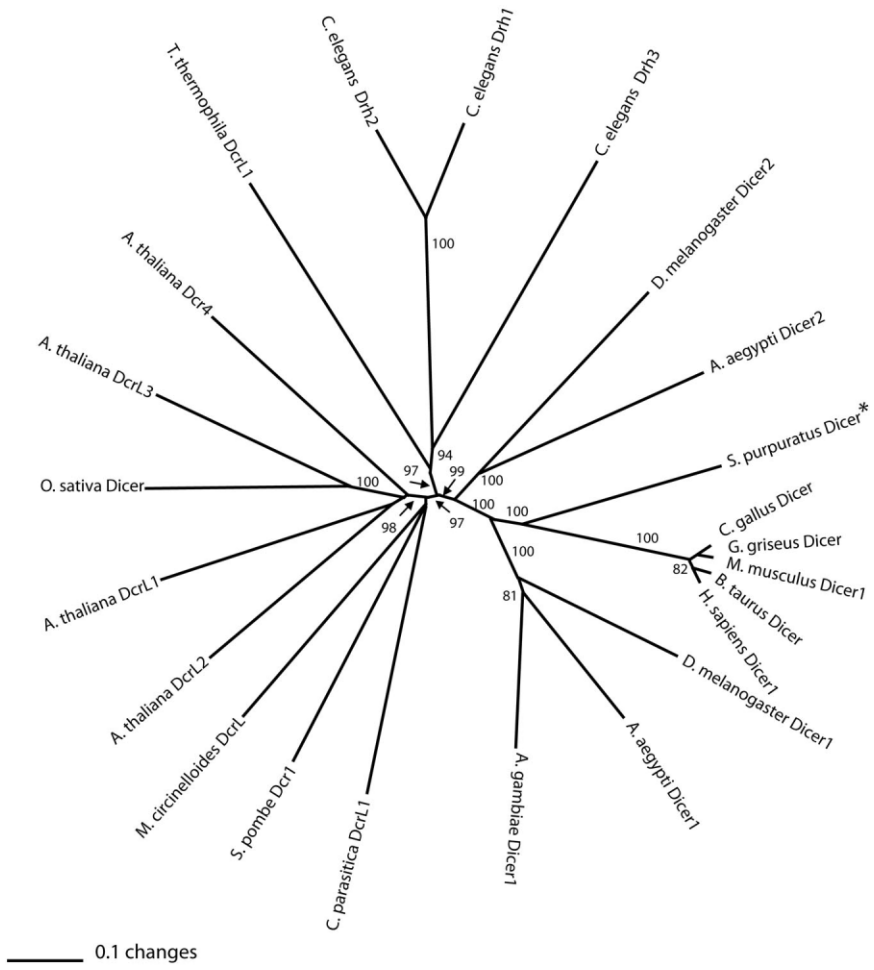

Fig. 4. Phylogenetic tree of Dicer. Amino acid sequences of the sea urchin Dicer gene was aligned using ClustalX (http://www.ebi.ac.uk/clustalw/ and http://www.ch.embnet.org/software/ClustalW.html). Amino acid sequences of the sea urchin Dicer (with asterisk) were aligned with other Dicer proteins from GenBank using the Clustal algorithms within MacVector (Accelrys, Burlington, MA). Neighbor joining trees were computed using PAUP with 1,000 bootstrap replicates (Swofford, 2002). The scale bar represents 0.1 amino acid substitutions per site. The GenBank accession numbers for these genes are available in Supplementary Table S1, which can be viewed at http://www.interscience.wiley.com/jpages/1058-8388/suppmat.

Schizosaccharomyces pombe, *Nematostella vectensis* predicted Dicer, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Ciona intestinalis*, *Xenopus tropicalis*, *Mus musculus*, and *Homo sapiens*.

The RNAi machinery can repress transcription by recruiting histone modifying enzymes to the chromatin. For example, the RNAi-induced initiation of transcriptional gene silencing (RITS) is required for heterochromatic gene silencing at centromeres in the fission yeast (Hansen et al., 2005; Buhler et al., 2006; Moazed et al., 2006; Buker et al., 2007). Dicer-deficient mouse embryonic stem cells are defective in DNA methylation and histone modifications, suggesting a close link between the RNAi pathway and chromatin remodeling (Kanellopoulou et al., 2005). Therefore, the *S. purpuratus* Dicer may function in posttranscriptional regulation through RISC and transcriptional gene silencing by using its HDAC interaction domain to directly recruit chromatin remodeling complexes.

TRBP/PACT mRNA Localization Overlaps With Its Cofactor Dicer

BLASTing the human TRBP and PACT (protein activator of the interferon-induced protein kinase) identified a single homolog in the sea urchin genome database. The *TRBP* mRNA localization overlaps with *Dicer* mRNA localization at the apical ectoderm in mesenchyme blastula through late gastrula (Fig. 3). Because TRBP/PACT has an additional role in immunity within mammals, this may explain why its localization does not solely correspond to that of its cofactor Dicer (Chendrimada et al., 2005; Gatignol et al., 2005). The sea urchin has a large repertoire of potential immune receptors, regulators, and effectors, and a complex innate immune system (Hibino et al., 2006; Rast et al., 2006). Perhaps TRBP mRNA localization in the secondary mesenchyme cells and their descendents reflect a second function.

The sea urchin *TRBP* mRNA also accumulates in the coelomic pouches of the larva (72 hr), where conserved markers of early primordial germ cell development such as *vasa* and *nanos* transcripts are localized (Fig. 3; Table 1; Juliano et al., 2006). In mammalian cells, TRBP is expressed in spermatids where it's involved in translational repression of protamine mRNA translation and regulation of male germ cell differentiation (Lee et al., 1996; Siffroi et al., 2001). The expression patterns of the *TRBP* homolog in the sea urchin suggest that it may have multiple roles, including germ cell regulation and embryogenesis.

Sea Urchins Have at Least Four Argonaute Proteins With Dynamic Expression Patterns

BLAST searches of PAZ and PIWI domain-containing proteins from the sea urchin genome database and molecular cloning identified at least four Argonaute proteins (Fig. 5C). Sea urchin AGO1 and AGO2 are most closely related to the AGO-like subfamily of Argonautes, whereas the sea urchin PiwiL1 and Seawi are most closely related to the PIWI subfamily of Argonautes (Fig. 5A). All four of the sea urchin Argonautes examined contain conserved aspartic acid and histidine residues important for RNAse H activity within the PIWI domain, suggesting that they all have potential slicer activity (Fig. 5B). Seawi was previously found to be a component of microtubule-ribonucleoprotein complexes from two species of sea

urchins, *S. purpuratus* and *Paracentrotus lividus*, and its associated mRNA in the ribonucleoprotein complex were translationally repressed (Rodriguez et al., 2005).

All sea urchin *Argonautes* have high levels of RNA accumulation in oocytes (Fig. 3). Sea urchin *AGO2*, *PiwiL1*, and *Seawi* mRNA have cytoplasmic, punctate localizations in the oocytes; the punctate staining seemed more prevalent in younger oocytes, whereas in larger oocytes, the cytoplasmic accumulation is more perinuclear (Fig. 3). In the 32 cell stage, *PiwiL1* and *Seawi* RNA accumulate in all cells, but seem to accumulate less in the micromeres. The micromeres give rise to the skeletal mesenchyme and small micromeres that end up in the coelom (body wall; Gilbert, 2000). In the mesenchyme blastula stage, *PiwiL1* is localized to the vegetal plate, whereas *Seawi*, *AGO1*, and *AGO2* have a broader mRNA accumulation in the vegetal plate, primary mesenchyme cells, and the apical ectoderm. In the late gastrula stage, mRNAs of all *Argonautes* have decreased significantly, and by the pluteus stage, only the *Seawi* mRNA remains detectable, and is enriched in one of the coelomic pouches, where the future adult rudiment arises (Fig. 3).

The expression profile of *PiwiL1* mirrors that of several germline determination genes, such as *vasa* and *nanos*, in the sea urchin (Juliano et al., 2006). The miRNA pathway has been implicated in the regulation of self-renewal of *Drosophila* germline stem cells and primordial germ cell (PGC) maintenance (Jin and Xie, 2006; Megosh et al., 2006; Park et al., 2007). Depletion of individual members of the RISC complex including PIWI, dFMRP (Fragile X Mental Retardation Protein), or DCR-1, the Dicer protein involved in the miRNA biogenesis, leads to a failure in poleplasm maintenance and primordial germ cell formation in *Drosophila* (Megosh et al., 2006). Similar to *Drosophila*, mammalian Piwi proteins MIWI and MILI are also involved in transposon suppression during spermatogenesis (Aravin et al., 2007). *Miwi2* and *Mili* mutations affect the methylation of L1 repetitive elements (Aravin et al., 2007; Carmell et al., 2007). This finding suggests a conserved pathway of a developmentally regulated transposon repression by piRNA mediated by PIWI subfamily of the Argonaute proteins. The expression patterns of the sea urchin *PiwiL1* and *TRBP* suggest that, similar to *Drosophila*, both piRNA-mediated transposon suppression and miRNA-mediated germline specification may be involved in the maintenance of the germline.

The results of this study indicate that all the important molecules involved in the RNA-mediated interference silencing pathway are present and differentially expressed during sea urchin embryogenesis. These differences in RNA accumulation suggest an important developmental regulatory role of the RNA-mediated silencing pathway in this organism.

EXPERIMENTAL PROCEDURES

Gene Identification and Cloning

All genes discussed in this manuscript were identified and analyzed using the Sea Urchin Genome site (http://hgsc.bcm.tmc.edu/projects/seaurchin/), the Sea Urchin Annotation site (http://annotation.hgsc.bcm.tmc.edu/Urchin/), and the Genebore site (www.genboree.org). Subsequently, part of these genes was cloned by PCR using the *S. purpuratus* ovary cDNA library or by reverse transcription-PCR (RT-PCR) using embryos collected throughout early development. Total RNA was extracted as previously described (Bruskin et al., 1981), and RT-PCR was performed according to manufacturer's directions using the Access RT-PCR kit (Promega Corporation, Madison, WI). The reverse transcription reaction was conducted for 45 min at 48°C, followed by denaturation for 2 min at 94°C. PCR amplification was performed for 40 cycles (denaturation for 30 sec at 94°C, annealing for 1 min at 60°C, and extension for 2 min at 68°C). RT-PCR products were run on 1% agarose electrophoretic gels and PCR fragments were gel purified using QiaQuick spin columns (Qiagen Inc., Valencia, CA) and cloned into pGEM-T EASY vector (Promega Corporation) for nucleotide sequencing.

Animals

Strongylocentrotus purpuratus were collected by Charles Hollahan (Santa Barbara, CA) and housed in aquaria cooled to 16°C in artificial sea water (ASW; Coral Life Scientific Grade Marine Salt; Energy Savers Unlimited, Inc, Carson, CA). Sea urchins were shed by intracoelomic 0.5 M KCl injection. Embryos were cultured at 16°C and collected at various developmental stages.

QPCR

cDNA was prepared from 2 μg of total RNA from embryos and adult ovary by RT-PCR (TaqMan Reverse Transcription Reagents Kit, Foster City, CA). QPCR was performed on the 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) using SYBR Green chemistry (Applied Biosystems). Primer sets were designed to amplify 100–150 bp (Table 2) using Primer3 (Rozen and Skaletsky, 2000). Reactions were run in triplicate, normalized against ubiquitin mRNA, and are presented relative to the egg levels.

In Situ RNA Hybridization

Whole-mount in situ RNA hybridizations were performed as previously described (Minokawa et al., 2004). Partial gene sequences were cloned by RT-PCR using templates from the *S. purpuratus* ovary cDNA library and total RNA extracted from embryos at the 4-cell, 32-cell, blastula, mesenchyme blastula, gastrula, and pluteus stages (Table 3). cDNA products generated from the RT-PCR reactions were cloned into pGEM-T EASY vectors (Promega Corporation). Plasmids containing *Argonaute* genes were linearized with various restriction enzymes to exclude the PAZ and PIWI regions. Linearized plasmids were *in vitro* transcribed, and the antisense probes were labeled with digoxigenin (DIG) using the DIG RNA Labeling kit (Roche Applied Science, Indianapolis, IN). Negative controls were transcribed off plasmid pSPT18-Neo or pSPT19-Neo provided in the DIG RNA Labeling kit.

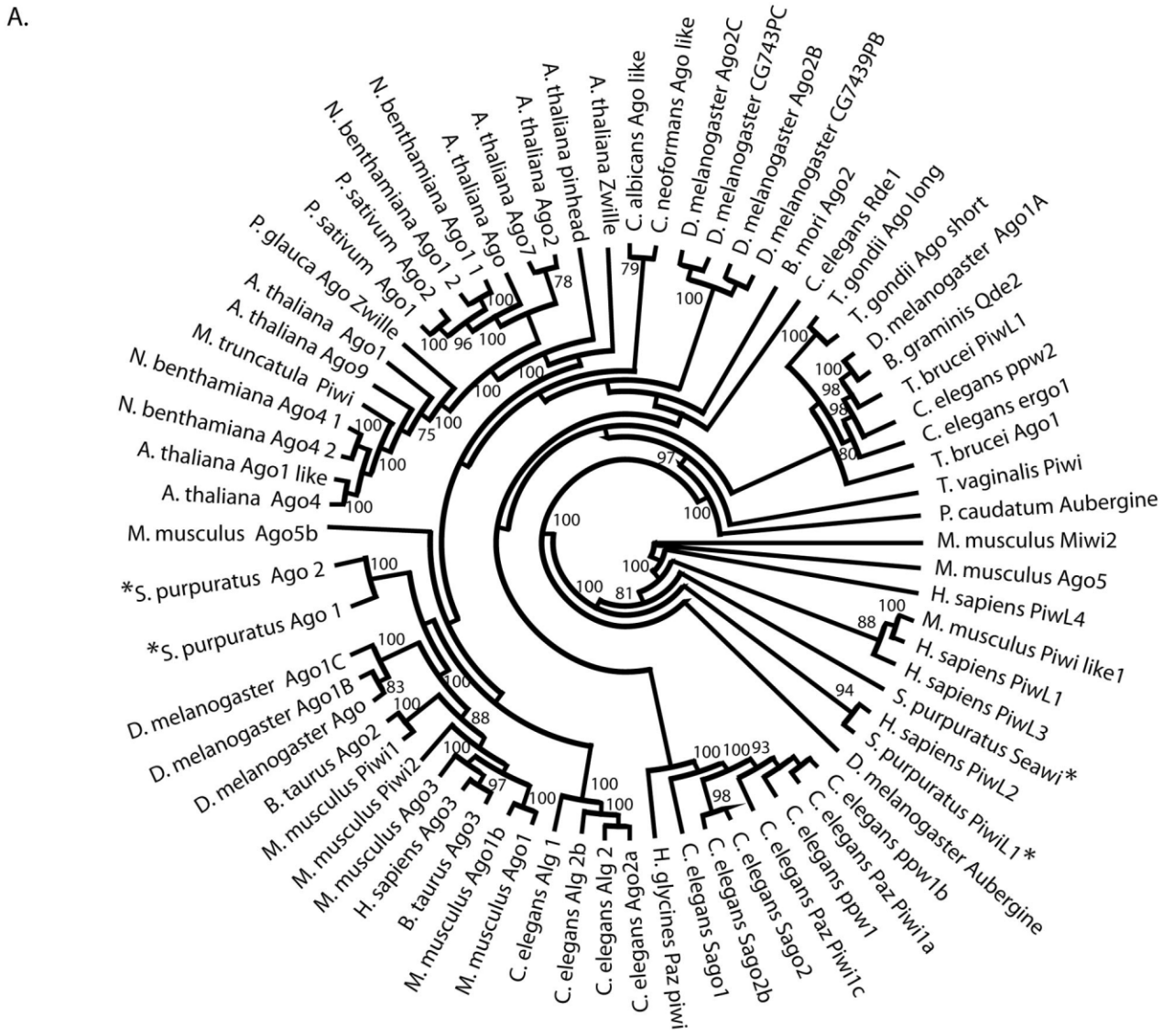

Fig. 5. Phylogenetic tree of Argonautes and sequence analysis. Amino acid sequences of the sea urchin Argonaute genes (with asterisks) were aligned using ClustalX (Thompson et al., 1997) with other Argonautes in GenBank. Neighbor joining trees were constructed using PAUP with 100 bootstrap replicates (Swofford, 2002). Group 3 Argonautes that are specific to *Caenorhabditis elegans* were excluded from this analysis. GenBank accession numbers for the Argonautes are listed in Supplementary Table S2. **A:** Sea urchin Argonaute protein sequences were aligned with three of the *C. elegans* Argonautes that contain slicer activity. **B:** Conserved residues of aspartic acid and histidine residues shaded in grey are found in the PIWI domains of the sea urchin Argonautes. Four sea urchin Argonautes are aligned using Clustal X (Thompson et al., 1997). **C:** Conserved PAZ and PIWI domains are shaded in gray.

Sequence and Phylogenetic Analysis

Protein sequences were aligned using ClustalX (http://www.ebi.ac.uk/clustalw/ and http://www.ch.embnet.org/software/ClustalW.html). Protein domains were analyzed with the Simple Modular Architecture Research Tool (SMART) site (smart.embl-heidelberg.de/). Amino acid sequences of the *Dicer* and *Argonaute* genes were aligned using the Clustal algorithms within MacVector (Accelrys, Burlington, MA). These sequence alignments were analyzed in

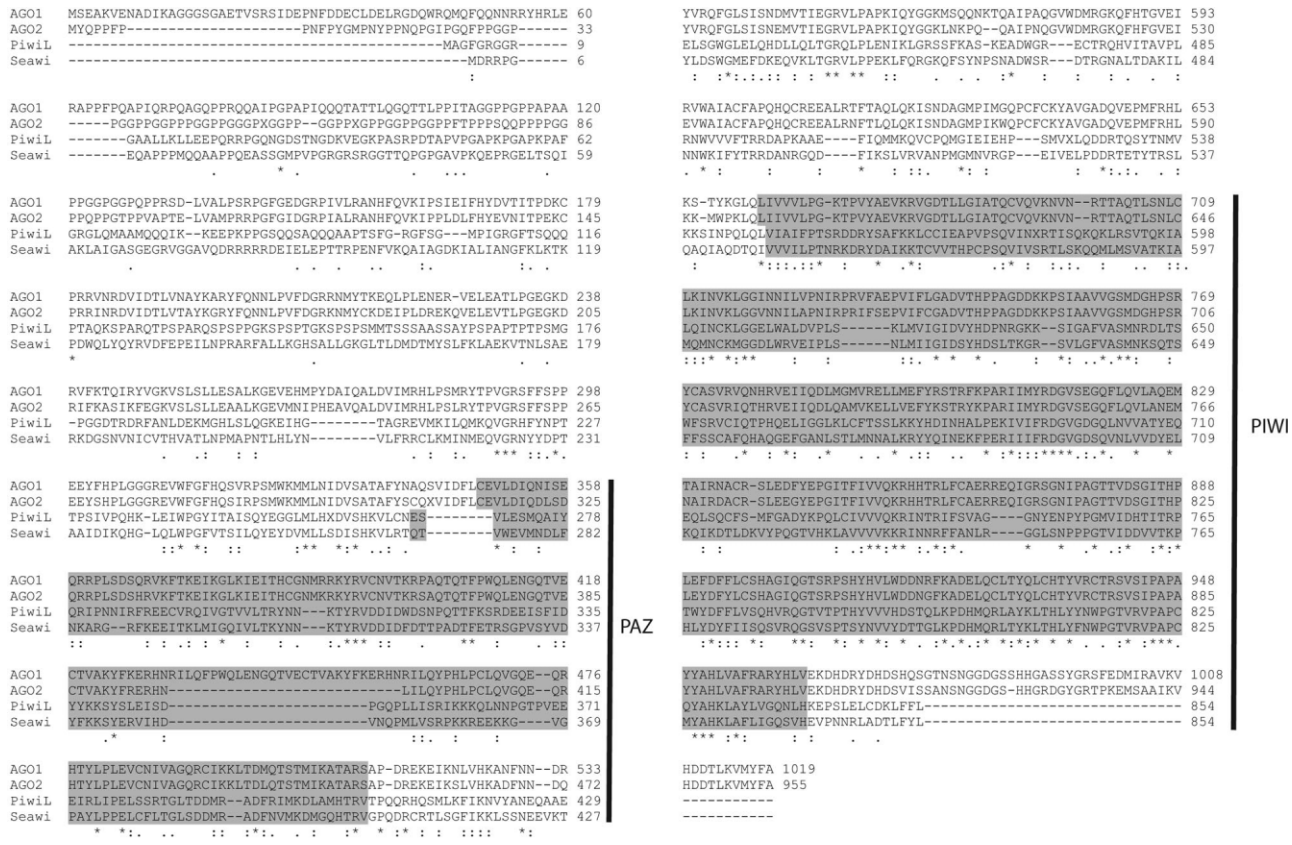

Fig. 5. (*Continued*)

TABLE 1. Summary of Spatial Expression Patterns of Genes Involved in the RNAi Pathway[a]

Gene	Oocyte	Egg	16/32 cells	Blastula	Mesenchyme blastula	Gastrula	Late gastrula	Pluteus
Drosha	Pe; Pa	Pa	Ub	V	PMC; V; AEC	E; AEC	E; AEC	E
Pasha/DGCR8	Pe; Pa	Pa	Ub	Ub	PMC; V; AEC	AEC; E; SMC	E; AEC; CB	ND
Exportin 5	Pe; Pa	Ub	ND	Ub	ND	ND	ND	ND
Dicer	asymmetric	asymmetric	Ub	OEC	OEC	E; AEC; OEC	AEC; E; OEC; CB	ND
TRBP	Pe; Pa	Pa	Ub; less in the micromeres	V	PMC; AEC	AEC; SMC	E; AEC; CP	ND
AGO1	Pe	Ub	Ub	Ub	AEC; PMC; V	ND	ND	ND
AGO2	Pu	Ub	Ub	Ub	AEC; PMC V	E; AEC; PMC; MD; SMC	E; AEC; MD	ND
PIWIL1	Pu	Ub	Ub; less in the micromeres	Ub	V	MD	MD	ND
Seawi	Pu	Ub	Ub; less in micromeres	V	PMC; AEC; V	AEC; E; EC; MD	MD; AEC; E	CP

[a]ND, not detectable; Ub, ubiquitous; Pu, punctuate; Pe, perinuclear; Pa, patchy; E, endoderm; V, vegetal plate; EC, ectoderm; AEC, apical ectoderm; CB, ciliary band; MD, micromere descendents; AEC, aboral ectoderm; OEC, oral ectoderm; PMC, primary mesenchyme cells; SMC, secondary mesenchyme cells; CP, coelomic pouches.

PAUP (Swofford, 2002) using neighbor joining method to establish their relationships. Bootstrap scores were determined from 1,000 or 100 reiterations.

ACKNOWLEDGMENTS

We thank Annette Coleman and Julian Wong (Brown University) for the help with PAUP and Judith Nathanson (Brown University) for her help on the embryo drawing. This work is supported by the NIH awarded to G.M.W. and NRSA awarded to J.L.S.

TABLE 2. PCR Primers Used in Quantitative, Real-Time Polymerase Chain Reaction

Gene	Glean ID[a]	5′ to 3′	
Drosha	SPU_09634	FOR	GAACCGGACGTTCCAAACTA
		REV	GTCGACATTCGCTTGGATTT
Dicer	SPU_28218	FOR	GATCGGTTGCTACCTGGTGT
		REV	GGAAGATCAGCTGCAGGAAG
Exportin5	SPU_018583	FOR	CCTTCCAGCAATCTTCAAGC
	SPU_07337		
	SPU_13561	REV	CTCCTGAATGCAGAGGGAAG
DGCR5/Pasha	SPU_01835	FOR	GATGACGGAGGATGGAAAGA
		REV	GCATGTTGTCCTGTGGAATG
TRBP	SPU_08206	FOR	CCCTGGCCTCATAAACAGAA
		REV	GTAGGTGGGGTCTGGGAAAT
AGO 1	SPU-19389	FOR	GCTCCAGAAGATCTCCAACG
		REV	ACGATCAGCTGCAGTCCTTT
AGO 2	SPU_19390	FOR	GTGGTAACATCCCAGCAGGT
	SPU_20628		
	SPU_23100	REV	ACGACTGGTGCCCTGAATAC
PiwiL1	SPU_23335	FOR	AGCTGGGAGAGAGGTGATGA
	SPU_12335	REV	TACCCAGGCCAGATCTCAAG

[a]Gene assignments corresponding to the sea urchin genome database (http://annotation.hgsc.bcm.tmc.edu/Urchin/ and www.genboree.org).

TABLE 3. PCR Primers Used to Generate RNA *In Situ* Antisense Probes

Gene	5′ to 3′		Length (bp)
Drosha	FOR	TGATGGCTTCCAATGATGAA	1083
	REV	GTCGACATTCGCTTGGATTT	
Dicer	FOR	GCCAGAGCCAGTTTGAGTTC	1707
	REV	GGAAGATCAGCTGCAGGAAG	
Exportin-5	FOR	CTCCTGAATGCAGAGGGAAG	1293
	REV	TCTGCATCACTGGCAAAAAG	
DGCR5/Pasha	FOR	GCCTGATGTAGACATGAAGG	908
	REV	TCAGCTCTGTCACGCTCTGT	
TRBP	FOR	CCATCAGCCAAAGTTCACCA	643
	REV	TGACGAAGCACTGGTAGTGG	
AGO1	FOR	CCTGATGGAATTCTATCGGTC	1004
	REV	TCGAAATTAACCCTCACTAAAGGG[a]	
AGO2	FOR	ATGTATCAACCACCCTTTCCG	1962
	REV	CCTCCCAGTTTGACGTTGAT	
PiwiL1	FOR	AGCTGGGAGAGAGGTGATGA	1305
	REV	TCCCGGTTCATACTGGCTAC	
Seawi	FOR	TTCACAGGTTTCACGGCAAAC	919
	REV	GTACTGGTAGAGTTGCCAGTC	

[a]T3 primer corresponding to the vector sequence of the *S. purpuratus* ovary cDNA library.

REFERENCES

Aravin AA, Sachidanandam R, Girard A, Fejes-Toth K, Hannon GJ. 2007. Developmentally regulated piRNA clusters implicate MILI in transposon control. Science 316:744–747.

Bartel DP. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297.

Basyuk E, Suavet F, Doglio A, Bordonne R, Bertrand E. 2003. Human let-7 stem-loop precursors harbor features of RNase III cleavage products. Nucleic Acids Res 31:6593–6597.

Bernstein E, Caudy AA, Hammond SM, Hannon GJ. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409:363–366.

Bruskin AM, Tyner AL, Wells DE, Showman RM, Klein WH. 1981. Accumulation in embryogenesis of five mRNAs enriched in the ectoderm of the sea urchin pluteus. Dev Biol 87:308–318.

Buhler M, Verdel A, Moazed D. 2006. Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. Cell 125:873–886.

Buker SM, Iida T, Buhler M, Villen J, Gygi SP, Nakayama J, Moazed D. 2007. Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat Struct Mol Biol 14:200–207.

Carmell MA, Girard A, van de Kant HJ, Bourc'his D, Bestor TH, de Rooij DG, Hannon GJ. 2007. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. Dev Cell 12:503–514.

Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, Shiekhattar R. 2005. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 436:740–744.

Croce JC, Wu SY, Byrum C, Xu R, Duloquin L, Wikramanayake AH, Gache C, McClay DR. 2006. A genome-wide survey of the evolutionarily conserved Wnt pathways in the sea urchin Strongylocentrotus purpuratus. Dev Biol 300:121–131.

Duboc V, Rottinger E, Lapraz F, Besnardeau L, Lepage T. 2005. Left-right asymmetry in the sea urchin embryo is regulated by nodal signaling on the right side. Dev Cell 9:147–158.

Ettensohn CA, Ingersoll EP. 1992. Morphogenesis of the sea urchin. In: Rossomando E, Alexander S, editors. Morphogenesis: an analysis of the development of the biological form. New York: Marcel Decker, Inc. p 189–262.

Ettensohn CA, Wessel GM, Wray GA. 2004. Methods in cell biology. In: Ettensohn CA, Wessel GM, Wray GA, editors. Development of sea urchins, ascidians, and other invertebrate deuterostomes: experimental approaches. San Diego: Elsevier Academic Press. p 1–13.

Gatignol A, Laine S, Clerzius G. 2005. Dual role of TRBP in HIV replication and RNA interference: viral diversion of a cellular pathway or evasion from antiviral immunity? Retrovirology 2:65.

Gilbert SF. 2000. Developmental biology. Sunderland, MA: Sinauer Associates.

Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC. 2001. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell 106:23–34.

Haley B, Zamore PD. 2004. Kinetic analysis of the RNAi enzyme complex. Nat Struct Mol Biol 11:599–606.

Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. 2004. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev 18:3016–3027.

Han J, Lee Y, Yeom KH, Nam JW, Heo I, Rhee JK, Sohn SY, Cho Y, Zhang BT, Kim VN. 2006. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell 125: 887–901.

Hansen KR, Burns G, Mata J, Volpe TA, Martienssen RA, Bahler J, Thon G. 2005. Global effects on gene expression in fission yeast by silencing and RNA interference machineries. Mol Cell Biol 25:590–601.

Hibino T, Loza-Coll M, Messier C, Majeske AJ, Cohen AH, Terwilliger DP, Buckley KM, Brockton V, Nair SV, Berney K, Fugmann SD, Anderson MK, Pancer Z, Cameron RA, Smith LC, Rast JP. 2006. The immune gene repertoire encoded in the purple sea urchin genome. Dev Biol 300:349–365.

Howard-Ashby M, Materna SC, Brown CT, Chen L, Cameron RA, Davidson EH. 2006a. Gene families encoding transcription factors expressed in early development of Strongylocentrotus purpuratus. Dev Biol 300:90–107.

Howard-Ashby M, Materna SC, Brown CT, Chen L, Cameron RA, Davidson EH. 2006b. Identification and characterization of homeobox transcription factor genes in Strongylocentrotus purpuratus, and their expression in embryonic development. Dev Biol 300:74–89.

Howard-Ashby M, Materna SC, Brown CT, Tu Q, Oliveri P, Cameron RA, Davidson EH. 2006c. High regulatory gene use in sea urchin embryogenesis: implications for bilaterian development and evolution. Dev Biol 300:27–34.

Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD. 2001. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293:834–838.

Jin Z, Xie T. 2006. Germline specification: small things have a big role. Curr Biol 16:R966–R967.

Juliano CE, Voronina E, Stack C, Aldrich M, Cameron AR, Wessel GM. 2006. Germ line determinants are not localized early in sea urchin development, but do accumulate in the small micromere lineage. Dev Biol 300:406–415.

Kanellopoulou C, Muljo SA, Kung AL, Ganesan S, Drapkin R, Jenuwein T, Livingston DM, Rajewsky K. 2005. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes Dev 19:489–501.

Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH. 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev 15:2654–2659.

Lapraz F, Rottinger E, Duboc V, Range R, Duloquin L, Walton K, Wu SY, Bradham C, Loza MA, Hibino T, Wilson K, Poustka A, McClay D, Angerer L, Gache C, Lepage T. 2006. RTK and TGF-beta signaling pathways genes in the sea urchin genome. Dev Biol 300:132–152.

Lee K, Fajardo MA, Braun RE. 1996. A testis cytoplasmic RNA-binding protein that has the properties of a translational repressor. Mol Cell Biol 16:3023–3034.

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN. 2003. The nuclear RNase III Drosha initiates microRNA processing. Nature 425:415–419.

Lee Y, Han J, Yeom KH, Jin H, Kim VN. 2006. Drosha in Primary MicroRNA Processing. Cold Spring Harb Symp Quant Biol 71:51–57.

Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. 2004. Nuclear export of microRNA precursors. Science 303:95–98.

Materna SC, Howard-Ashby M, Gray RF, Davidson EH. 2006. The C2H2 zinc finger genes of Strongylocentrotus purpuratus and their expression in embryonic development. Dev Biol 300:108–120.

Megosh HB, Cox DN, Campbell C, Lin H. 2006. The role of PIWI and the miRNA machinery in Drosophila germline determination. Curr Biol 16:1884–1894.

Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJ. 2000. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J 19:5194–5201.

Minokawa T, Rast JP, Arenas-Mena C, Franco CB, Davidson EH. 2004. Expression patterns of four different regulatory genes that function during sea urchin development. Gene Expr Patterns 4:449–456.

Moazed D, Buhler M, Buker SM, Colmenares SU, Gerace EL, Gerber SA, Hong EJ, Motamedi MR, Verdel A, Villen J, Gygi SP. 2006. Studies on the Mechanism of RNAi-dependent Heterochromatin Assembly. Cold Spring Harb Symp Quant Biol 71:461–471.

Mochizuki K, Fine NA, Fujisawa T, Gorovsky MA. 2002. Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in tetrahymena. Cell 110:689–699.

Mochizuki K, Gorovsky MA. 2005. A Dicer-like protein in Tetrahymena has distinct functions in genome rearrangement, chromosome segregation, and meiotic prophase. Genes Dev 19:77–89.

Murchison EP, Stein P, Xuan Z, Pan H, Zhang MQ, Schultz RM, Hannon GJ. 2007. Critical roles for Dicer in the female germline. Genes Dev 21:682–693.

Park JK, Liu X, Strauss TJ, McKearin DM, Liu Q. 2007. The miRNA pathway intrinsically controls self-renewal of Drosophila germline stem cells. Curr Biol 17:533–538.

Ransick A, Davidson EH. 1993. A complete second gut induced by transplanted micromeres in the sea urchin embryo. Science 259:1134–1138.

Rast JP, Smith LC, Loza-Coll M, Hibino T, Litman GW. 2006. Genomic insights into the immune system of the sea urchin. Science 314:952–956.

Rodriguez AJ, Seipel SA, Hamill DR, Romancino DP, M DIC, Suprenant KA, Bonder EM. 2005. Seawi—a sea urchin piwi/argonaute family member is a component of MT-RNP complexes. RNA 11: 646–656.

Rozen S, Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132: 365–386.

Schultz J, Milpetz F, Bork P, Ponting CP. 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci U S A 95:5857–5864.

Siffroi JP, Pawlak A, Alfonsi MF, Troalen F, Guellaen G, Dadoune JP. 2001. Expression of the TAR RNA binding protein in human testis. Mol Hum Reprod 7:219–225.

Song JJ, Smith SK, Hannon GJ, Joshua-Tor L. 2004. Crystal structure of Argonaute and its implications for RISC slicer activity. Science 305:1434–1437.

Swofford DL. 2002. PAUP: phylogenetic analysis using parsimony. Sunderland, MA: Sinauer Associates.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882.

Tolia NH, Joshua-Tor L. 2007. Slicer and the argonautes. Nat Chem Biol 3:36–43.

Verdel A, Jia S, Gerber S, Sugiyama T, Gygi S, Grewal SI, Moazed D. 2004. RNAi-mediated targeting of heterochromatin by the RITS complex. Science 303: 672–676.

Vlahou A, Gonzalez-Rimbau M, Flytzanis CN. 1996. Maternal mRNA encoding the orphan steroid receptor SpCOUP-TF is localized in sea urchin eggs. Development 122:521–526.

Walton KD, Croce JC, Glenn TD, Wu SY, McClay DR. 2006. Genomics and expression profiles of the Hedgehog and Notch signaling pathways in sea urchin development. Dev Biol 300:153–164.

Wassenegger M, Heimes S, Riedel L, Sanger HL. 1994. RNA-directed de novo methylation of genomic sequences in plants. Cell 76:567–576.

Wei Z, Angerer RC, Angerer LM. 2006. A database of mRNA expression patterns for the sea urchin embryo. Dev Biol 300: 476–484.

Yan KS, Yan S, Farooq A, Han A, Zeng L, Zhou MM. 2003. Structure and conserved RNA binding of the PAZ domain. Nature 426:468–474.

Yeom KH, Lee Y, Han J, Suh MR, Kim VN. 2006. Characterization of DGCR8/Pasha, the essential cofactor for Drosha in primary miRNA processing. Nucleic Acids Res 34:4622–4629.

Yi R, Qin Y, Macara IG, Cullen BR. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17:3011–3016.

Yigit E, Batista PJ, Bei Y, Pang KM, Chen CC, Tolia NH, Joshua-Tor L, Mitani S, Simard MJ, Mello CC. 2006. Analysis of the C. elegans Argonaute family reveals that distinct Argonautes act sequentially during RNAi. Cell 127:747–757.

Zamore PD, Haley B. 2005. Ribo-gnome: the big world of small RNAs. Science 309: 1519–1524.