Piwi Regulates Vasa Accumulation During Embryogenesis in the Sea Urchin

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Background: Piwi proteins are essential for germ line development, stem cell maintenance, and more recently found to function in epigenetic and somatic gene regulation. In the sea urchin *Strongylocentrotus purpuratus*, two Piwi proteins, Seawi and Piwi-like1, have been identified, yet their functional contributions have not been reported. Results: Here we found that Seawi protein was localized uniformly in the early embryo and then became enriched in the primordial germ cells (PGCs) (the small micromere lineage) from blastula stage and thereafter. Morpholino knockdown of *Sp-seawi* diminished PGC-specific localization of Seawi proteins, and altered expression of other germ line markers such as Vasa and Gustavus, but had no effect on Nanos. Furthermore, Seawi knockdown transiently resulted in Vasa positive cell proliferation in the right coelomic pouch that appear to be derived from the small micromere lineage, yet they quickly disappeared with an indication of apoptosis by larval stage. Severe Seawi knockdown resulted in an increased number of apoptotic cells in the entire gut area. Conclusion: Piwi proteins appear to regulate PGC proliferation perhaps through control of Vasa accumulation. In this organism, Piwi is likely regulating mRNAs, not just transposons, and is potentially functioning both inside and outside of the germ line during embryogenesis. *Developmental Dynamics 00:000–000, 2014.* © 2013 Wiley Periodicals, Inc.

Key words: Piwi; germ line; sea urchin; Vasa

Key findings:

- Piwi knockdown results in Vasa over-expression during embryogenesis.
- Piwi is important for proper PGC proliferation.
- Piwi functions outside of germ line during embryogenesis.

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INTRODUCTION

The P-element induced wimpy testis (Piwi) class of genes regulates piRNA biogenesis and function. These small RNA/protein complexes are responsible for silencing transposable elements in the *Drosophila* germ line and in mammalian germ cells that might otherwise prove deleterious to the germ line (Aravin et al., 2001, 2007; Deng and Lin, 2002; Klattenhoff and Theurkauf, 2008; Lu and Clark, 2010; Mani and Juliano, 2013). More recent reports suggest that the Piwi-piRNA machinery has additional functions outside of the germ line, such as regulating memory storage in the Aplysia central nervous system (Rajasethupathy et al., 2012), and in epigenetic regulation for genome-wide surveillance of various germ line transcripts in worms (Ashe et al., 2012; Bagijn et al., 2012; Lee et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012; Ishizu et al., 2013). Furthermore, piRNAs and Piwi proteins have been suggested to function in select mRNA regulation (Rouget et al., 2010), suggesting an expanded regulatory role beyond transposon sequences. This type of regulation may be particularly important during embryogenesis when cells for the germ line are being selected from a broad cohort, including those that become part of the soma.

The sea urchin is a member of the echinoderms and a sister group to the chordate. We previously found that the sea urchin small micromeres,

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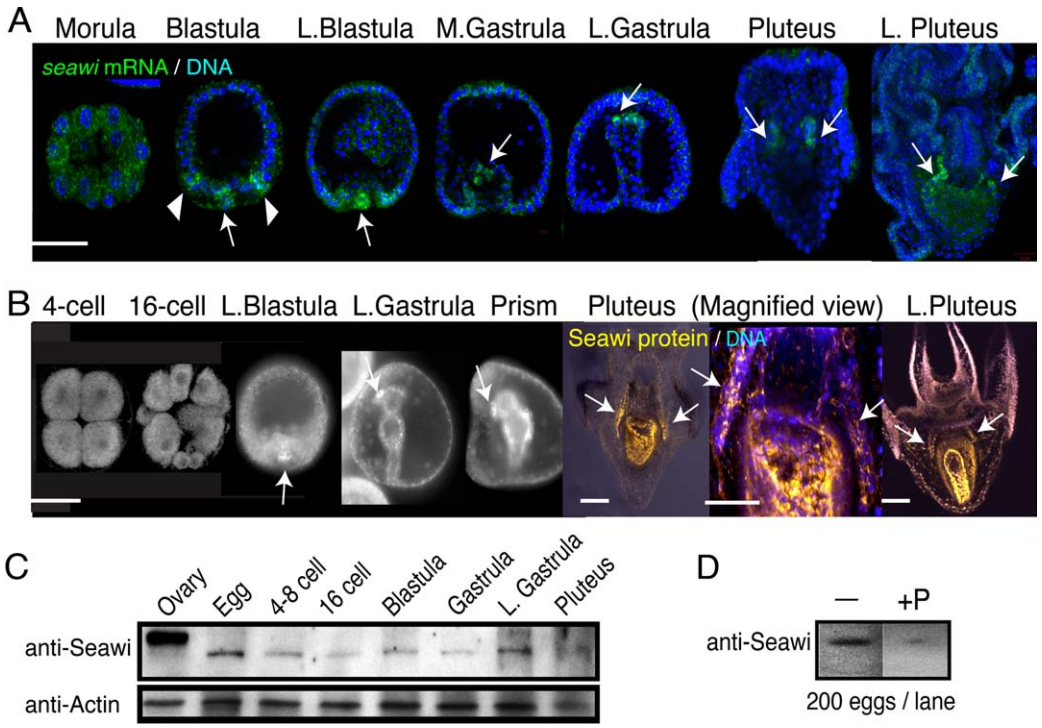

Fig. 1. Seawi mRNA and protein expression patterns during development of the sea urchin. **A:** *Sp-seawi* mRNA (green) was uniformly expressed until blastula stage, and then became gradually enriched into the vegetal plate including the small micromere lineage (arrows) and the future endoderm region (arrowheads) at blastula stage. *Sp-seawi* signal remained enriched in the coelomic pouches in the pluteus stage (arrows). Scale bars = 50 μm. **B:** Seawi protein was uniformly expressed at 4-cell, 16-cell stages and became enriched into the small micromere lineage (arrows) from Blastula, and remained enriched at Gastrula and Prism stages. The Seawi expression was restricted in the coelomic pouches (arrows) in the 1-week-old (pluteus), and 2-week-old (L. Pluteus) larvae. Scale bars = 50 μm. Color images (Seawi in yellow) were taken by confocal laser microscopy with Hoechst (blue) as a counter-staining and the grayscale images were taken by fluorescent microscopy. **C:** Anti-Seawi Immunoblotting. Seawi (approximately 110 kDa) was most abundant in ovary yet consistently expressed throughout development. Actin was used as a quantitative standard. **D:** Anti-Seawi antibody was preincubated with Seawi peptide to inactivate and used for a detection of Seawi immunoblotting. The Seawi signal was reduced with the preincubated-Seawi antibody (+P), whereas the intact antibody (–) showed a signal band around 110 kDa. A total of 200 embryos per lane were loaded.

formed at the fifth embryonic cell division, express a group of conserved germ line genes such as *vasa*, *nanos*, and *seawi* (Rodriguez et al., 2005; Juliano et al., 2006). The small micromeres display several other conserved PGC features, such as slower cell cycling, passive translocation during gastrulation, autonomous specification (after the 16-cell stage), and contribution to germ cell formation in the adult (Tanaka and Dan, 1990; Yajima and Wessel, 2011a, 2012; Wessel et al., 2013). While these results suggest the small micromere lineage is the PGC population or its precursor cells in the sea urchin, this lineage remains somewhat enigmatic. It is complicated by the fact that Vasa and other germ line factors are more widely distributed in the embryo, even in somatic cell lineages. The embryo also recovers its germ line when the precursor cells of the small micromeres (micromeres) are removed at 16-cell stage (Ransick et al., 1996). Removal of small micromeres at the 32-cell stage, however, results in juveniles devoid of gametes (Yajima and Wessel, 2011a), suggesting the small micromere lineage at least contributes to germ line formation in this organism.

Two PIWI homologs have been reported in the sea urchin; one is Seawi (Rodriguez et al., 2005; Juliano et al., 2006), and the other is Piwi-like1 (Song and Wessel, 2007). *Seawi* transcripts are uniformly expressed in the entire embryo during early embryogenesis, yet become gradually restricted to the vegetal plate at the blastula stage and then specifically into the small micromere lineage by the gastrula stage (Juliano et al., 2006). Perhaps germane here is that this expression pattern is very similar to that of Vasa, another conserved germ line marker. Vasa mRNA is uniformly expressed during embryogenesis and then gradually becomes enriched in the small micromere lineage (PGCs) by gastrula stage. In contrast, the Vasa protein becomes highly enriched in the micromeres as early at 16-cell stage and remains enriched in the small micromere lineage thereafter by selective Vasa degradation in the somatic lineage by the Gustavus mediated Ubiquitin-proteosome degradation pathway (Styhler et al., 2002; Piessevaux et al., 2008; Kugler et al., 2010; Gustafson et al., 2011). Here, we investigate the function of Seawi protein and test its functional contributions to the small micromere lineage and on the expression of other germ line markers in the sea urchin *Strongylocentrotus purpuratus*.

RESULTS AND DISCUSSION

Seawi Distribution is Gradually Restricted to the Small Micromere Lineage

Seawi mRNA/ protein expression was investigated by fluorescent *in situ*

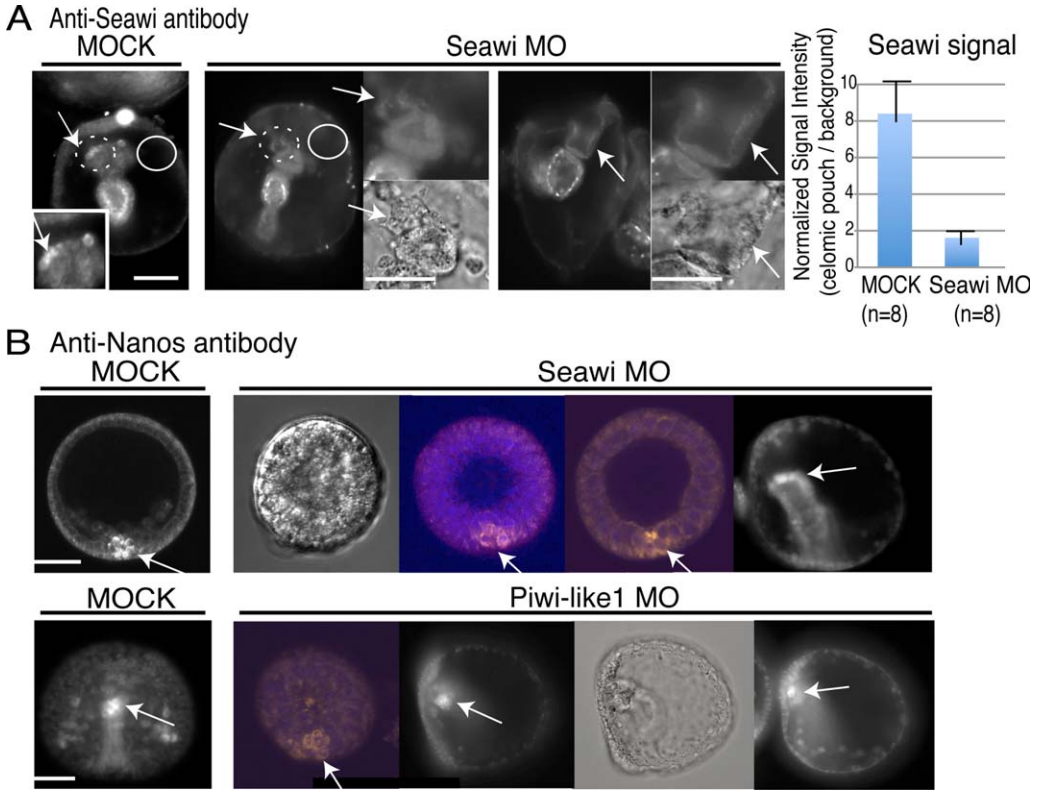

Fig. 2. Seawi protein localization in the small micromere lineage was diminished by *Sp-seawi* morpholino knockdown. **A:** Left panel is control gastrula (MOCK) immunostained with anti-Seawi antibody. Seawi signal was restricted into the small micromere lineage (arrow). Insets are the magnified view of the coelomic pouch area. Right panels are morpholino-injected embryos. Seawi MO diminished the specific signal in the coelomic pouches (arrows). Insets are the magnified views of coelomic pouch (the upper panel is fluorescent image and the lower panel is a bright field). A graph indicates the signal intensity in the coelomic pouch (Dashed-circled) that was normalized by the value of background (ectoderm, Circled) of each specimen. The signal intensity was calculated by *Image J*. Scale bars = 20 μm. **B:** Left panels are control blastula (upper panel, MOCK) and gastrula (lower panel) immunostained with anti-Nanos antibody. Right panels are morpholino-injected embryos. Both Seawi MO (upper panels) and Piwi-like1 MO (lower panels) maintained Nanos signal consistently in the small micromere lineage (arrows) during embryogenesis. Scale bars = 50 μm. Color images were taken by confocal laser microscopy with Hoechst (blue) counter-staining and the grayscale images were taken by fluorescent microscopy.

hybridization, immunolabeling, and immunoblotting (Fig. 1). Both Seawi mRNA (Fig. 1A) and protein (Fig. 1B) were uniformly expressed in the entire embryo during early embryogenesis from 4-cell to 16-cell stages, became enriched to the small micromere lineage from the blastula stage (arrows), and remained enriched until 2-week-old larvae to this lineage, as well as in the entire embryo at low level and in the gut at high level. Strong fluorescence in the gut is likely due to the auto-fluorescence by the algae in the stomach used for larval feeding (Yajima et al., 2013). The anti-Seawi antibody consistently detected a major band around 110 kDa by immunoblotting (Fig. 1C) throughout embryonic development. In the ovary, however, the major Seawi band was larger, and a minor band was also identified around the same size in the late gastrula stage and thereafter, suggesting a different isoform at these stages through splice variation or through posttranslational protein modification. To test the specificity of this antibody to the antigen, an antibody absorption experiment was performed. The antibody was preincubated with Seawi peptide, and this treatment significantly reduced the signal in 110 kDa, suggesting the specificity of this antibody (Fig. 1D).

To test further the spatial specificity of the Seawi antibody, a morpholino (MO) against *Sp-seawi* was designed, constructed, and injected into fertilized eggs. The MO successfully diminished the Seawi antibody signal from the small micromere lineage to 10% of its normal intensity (Fig. 2A, arrows) yet the signal in the gut remained. This suggests the signal in the gut is either maternally derived and long-lived, nonspecific, or a result of the algae diet given to the larvae. We conclude that the Seawi synthesized in the embryo is majorly restricted to the small micromere lineage.

Seawi Regulates Vasa Expression

To test if Seawi regulates other factors in the germ line, Vasa and Nanos expression was assayed in the presence of *Sp-seawi* MO (seawi-MO) or an MO for another PIWI homolog, *Sp-piwi-like1* (piwi-MO). Both Nanos and Vasa also accumulate selectively in the small micromeres but by different mechanisms. In the embryos effectively knocked-down for *Sp-seawi* and *Sp-piwi-like1*, Nanos protein accumulated normally during development both in terms of timing and place (Fig. 2B), whereas Vasa protein level was increased significantly (Fig. 3). In the normal embryo, Vasa is expressed

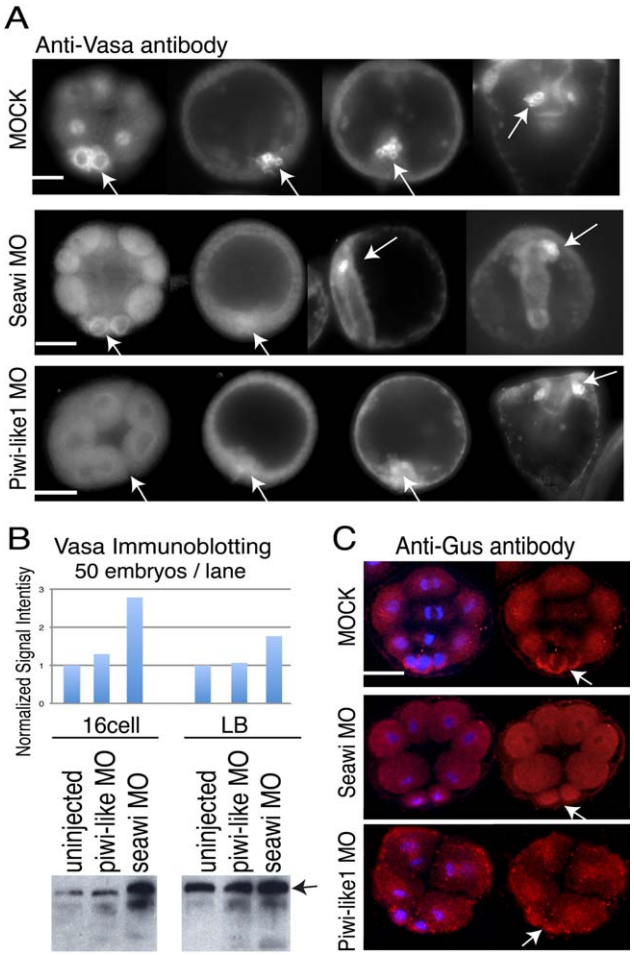

Fig. 3. Seawi knockdown altered Vasa and Gus localization in the small micromere lineage. **A:** Left panels are control early gastrulae (MOCK) immunostained with anti-Vasa antibody. Vasa signal was restricted into the small micromere lineage (arrow). Right panels are morpholino-injected embryos. Both Seawi MO (upper panels) and Piwi-like1 MO (lower panels) over-expressed Vasa and diminished Vasa enrichment in the micromere and small micromere lineage (arrows), yet the signal was resumed in blastula and gastrula stages (arrows). Scale bars = 20 μm. **B:** Anti-Vasa Immunoblotting. Vasa signal (arrow, approximately 85 kDa) was increased in the morpholino-injected embryos. A total of 50 embryos were loaded per lane. A graph indicates the signal value of each immunoblot band. Each value was calculated by *Image J* and normalized to the value of uninjected. **C:** Top panels are a control 16-cell stage embryo (MOCK) immunostained with anti-Gus antibody (red). Gus signal is expressed in the every blastomere yet more enriched in the micromeres (arrows). Middle and Bottom panels are MO-injected embryos. Both Seawi MO and Piwi-like1 MO treated embryos have diminished Gus enrichment in the micromeres (arrows). DNA is stained with Hoechst (blue). Scale bars = 20 μm

in every blastomere but is enriched in the micromeres, and becomes further restricted into the small micromeres later in embryogenesis (Yajima and Wessel, 2011a,b). In the Seawi and Piwi-like1 knockdown embryos, in contrast, Vasa accumulated to abnormally high levels throughout the embryo, to the point that the normal small micromere-enriched expression was not detectable in the 16-cell stage (Fig. 3A,B). This result suggests that Seawi may be important for Vasa degradation in non-PGC lineages. To test this possibility further, we examined Gustavus (Gus) expression in these Piwi-knockdown embryos. Gus is an E3 ubiquitin ligase involved in Vasa turnover and controls Vasa accumulation in the *Drosophila* pole cells (Kugler et al., 2010; Styhler et al., 2002) by recruiting the ubiquitin-proteasome machinery for degradation, protection, or other fate modifications (Piessevaux et al., 2008). In the sea urchin, we previously learned that Gus directly interacts with Vasa and its knockdown leads to ectopic Vasa expression throughout the embryo (Gustafson et al., 2011). Gus, however, appears to have dual functions, one in degradation of Vasa in nonsmall micromeres lineages and the other in the protection of Vasa in the small micromere lineage. In Seawi/Piwi-like1 knockdown embryos, specific micromere enrichment of Gus was diminished (Fig. 3C), suggesting that Piwi proteins may regulate the accumulation of Vasa in the PGC lineage potentially through a Gus-dependent mechanism. We further speculated that Seawi expression/ localization may be regulated also by the same Gus-mediated mechanism because Seawi gradually becomes enriched into the small micromere lineage as Vasa does during embryogenesis. In Gus-knockdown embryos, however, we could not detect a change in Seawi expression patterns either by immunoblotting or immunofluorescence (data not shown). This result suggests that Seawi is expressed and/or localized by a mechanism independent of direct Gus-mediated regulation even though its dynamics are similar to Vasa.

In Seawi/Piwi-like1 knockdown embryos, Vasa enrichment in the small micromere lineage was resumed from the blastula stage and thereafter (Fig. 3A,B, arrows). This timing is concordant with the timing when Seawi protein is restricted to the small micromere lineage and Gus ceases its expression from this lineage (Gustafson et al., 2011). This suggests that the small micromere specific localization of Vasa is transiently regulated by Seawi and Gus functions during early embryogenesis but that Vasa accumulation is regulated by Gus-independent mechanisms later in development.

Seawi Knockdown Results in Proliferation of Vasa-Positive Cells in the Right Coelomic Pouch and Increased Apoptosis

In the normal sea urchin embryo, Vasa expression is exclusively enriched only in the small micromere lineage during gastrula and early larval stages. The four small micromere descendants undergo only 1–2 cell cycles during gastrulation, resulting typically into 8–10 cells and translocate into the coelomic pouches

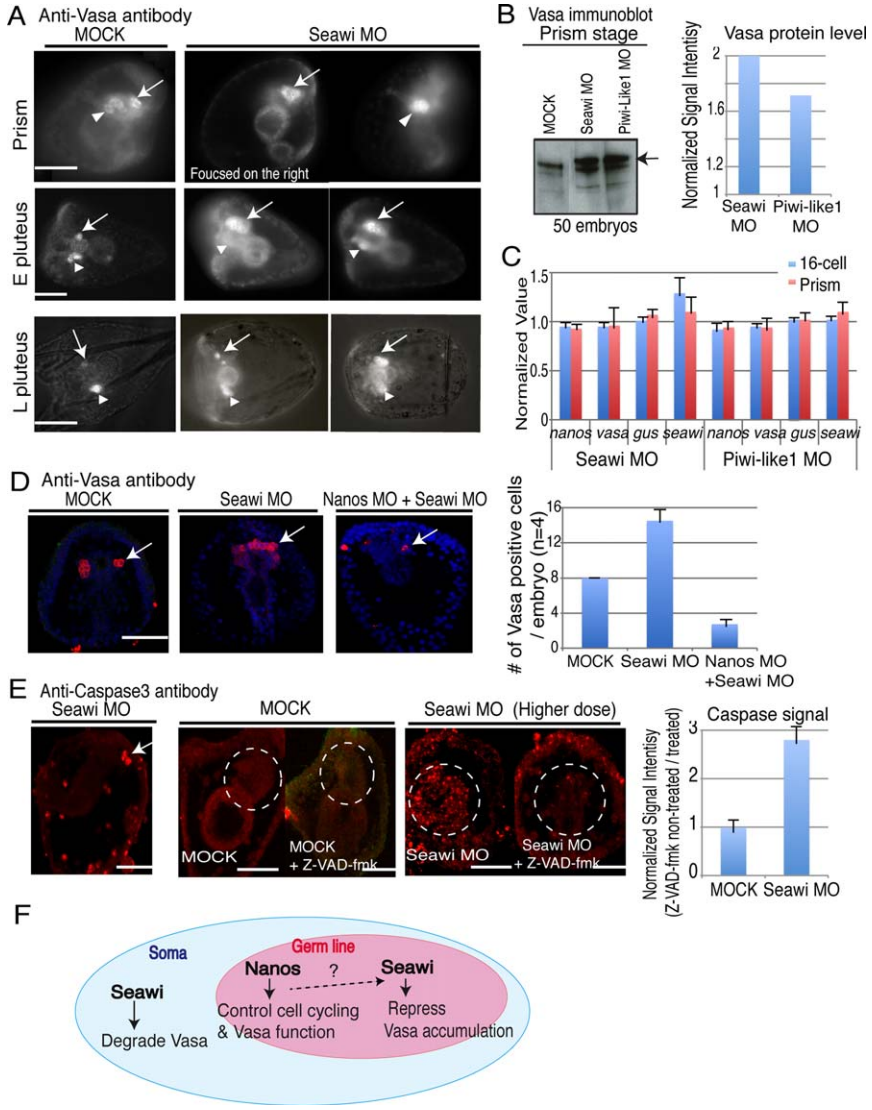

Fig. 4. Seawi knockdown induced the proliferation of Vasa positive cells in the right coelomic pouch at Prism stage. **A:** Left panels are control (MOCK) larvae immunostained with anti-Vasa antibody. Vasa signal was restricted into the coelomic pouches with less enriched in the right coelomic pouch (arrows) compared with the left coelomic pouch (arrowhead). Right panels are Seawi MO-injected larvae. Vasa-positive cells were more accumulated in the right coelomic pouch (arrows) compared with the left side (arrowheads) during prism and early pluteus (Day 3, E pluteus) stages yet shrunk down by the late pluteus (Day 4, L pluteus) stage. The pictures of the Prism embryo are the same embryo with a different focus. Scale bars = 50 μm. **B:** Anti-Vasa Immunoblotting. Vasa signal (arrow, approximately 85 kDa) was increased in the MO-injected embryos. A total of 50 embryos were loaded per lane. A graph indicates the signal value of each immunoblot band. Each value was calculated by *Image J* and normalized to the value of MOCK. **C:** Expression profiles of germ line determinants in the Seawi knockdown embryos by the quantitative RT-PCR analysis. A slight increase of the *seawi* transcripts was observed yet little difference was observed in other gene expressions. Each 1/Ct value was first normalized to that of Ubiquitin, and then the resultant value was standardized to that of the control embryos. **D:** 0.5 mM stock each of Seawi and Nanos2 MO was injected into the fertilized eggs, and the resultant embryos were reduced in number of Vasa-positive cells at Prism stage (Nanos MO +Seawi MO). Arrow indicates a right coelomic pouch area. A graph demonstrates the average number of Vasa-positive cells per embryo injected with MOCK, Seawi MO, or Seawi + Nanos MO. **E:** the Caspase-3 signal (red) was detected in the right coelomic pouch of the Seawi MO embryos (arrow), and increased further in the gut area with the higher dose (1mM) of Seawi-MO (dashed circle) yet the signal was reduced by Z-VAD-fmk, a pan-Caspase inhibitor. A graph indicates the Capase-3 signal value of gut (circled) region in each picture. Each value of Seawi MO injected larvae was calculated by *Image J* and normalized to the value of Z-VAD-fmk treated larvae (n = 3). **F:** A summary diagram of potential functions of Piwi in the soma and germ line. Piwi is involved in the degradation of Vasa in the somatic lineage during early embryogenesis, whereas Nanos specifically represses Vasa function and cell cycling in the PGC lineage and Piwi appears to be involved in this process together.

where the adult rudiment is formed on the left side of the larval body during the late larval stage (Tanaka and Dan, 1990; Yajima and Wessel, 2012). In *S. purpuratus*, typically five out of the eight small micromere descendants transfer into the left coelomic pouch and are thought to resume cell cycling in the late larva during formation of the adult rudiment, whereas the remaining three small micromere descendants in the right coelomic pouch disappear and are thought to undergo apoptosis by late larval stage (Luo and Su, 2012; Campanale and Hamdoun, 2012). In Seawi knockdown embryos, however, we found an over-proliferation of Vasa positive cells in the right coelomic pouch at prism and early larval stages compared with controls (Left:Right = 4.75 ± 0.5:13.3 ± 4.79, n = 10, respectively) (Fig. 4A), and Vasa protein expression was also increased in these larvae (Fig. 4B). To test if Seawi/Piwi-like1 knockdowns affected PGC-related transcripts, we assayed *nanos*, *vasa*, and *gustavus* mRNA abundance by transcript accumulation. We found no significant change in the Seawi-knockdown embryos compared with the controls (Fig. 4C), suggesting that a posttranscriptional mechanism regulates Vasa over-expression. These over-proliferated Vasa-positive cells were then rapidly decreased in number (Left:Right = 5 ± 0.8:4 ± 1.8, n = 10, respectively) by late larval stage (Fig. 4A,L pluteus). To test if these Vasa-positive cells were derived from the small micromere lineage or from another lineage, Nanos2 MO was co-injected with Seawi MO. We previously identified Nanos2 knockdown results in a loss of small micromere descendants by late gastrula stage (Juliano et al., 2010), and thus Nanos2 MO can be used as a molecular tool to specifically remove the small micromere lineage. The double knockdown of Nanos2 and Seawi resulted in a reduced number of Vasa positive cells at Prism stage (Fig. 4D), suggesting the over-proliferated Vasa-positive cells in the Seawi MO embryo was likely caused by excess proliferation of small micromere descendants. In these Seawi-knockdown embryos, activated Caspase-3, an indicator of apoptosis, was detected in the coelomic pouch area as well in the mesenchyme cells

(Fig. 4E, Seawi MO). The apoptotic signal was, however, reduced following addition of Z-VAD-fmk, a pan-caspase inhibitor (Fig. 4E, MOCK and Seawi MO [Higher dose]) (Fujii et al., 2009), suggesting that the Seawi knockdown led to apoptotic cell death broadly in the embryo. Taken all together, the cell cycle control of the small micromere lineage appears to be based on a Nanos-dependent mechanism that trumps Vasa overfunction, and Seawi appears to be involved in this regulation together as well as functioning not just in the germ line but also in somatic lineages during embryogenesis, potentially by altering the Vasa and Gus functions (Yajima and Wessel, 2011b) (Fig. 4F).

Piwi Proteins Regulate Germ Line Factors in the Sea Urchin Embryo

Several recent reports indicate the functional importance of the Piwi-piRNA machinery in multiple biological processes and research in various experimental organisms indicate a fundamental conservation of this biological system among organisms (Ishizu et al., 2013), although the diversity of organisms for this conclusion is small. Recently, over 13,000 piRNA-like small RNAs were identified from the sea urchin gonad (Song et al., 2012; Wei et al., 2012), suggesting that the Piwi-piRNA machinery may mediate gene regulation in sea urchin development. The work described herein is the first report that Piwi proteins regulate other germ line factors, contributes to the proper PGC development, and further functions in the somatic cell development during sea urchin embryogenesis. With these new results, we are now able to pursue specific functional testing of Piwi in early germ line functions in an animal underutilized for such studies.

EXPERIMENTAL PROCEDURES

Animals and Larval Culture

Strongylocentrotus purpuratus were obtained from Pete Halmay (peterhalmay@gmail.com) and Trevor Fay (trevor@montereyabalone.com, CA) and housed in aquaria cooled to 15°C in artificial seawater (ASW; Coral Life Scientific Grade Marine Salt; Energy Savers Unlimited, Inc, Carson, CA). Females were shed by KCl (0.5 M) injection and eggs were collected in Millipore filtered seawater (MFSW; Millipore USA). For embryo culture, fertilized eggs were cultured at 15°C in MFSW and collected at necessary developmental stages. Late larvae (older than a week postfertilization) were cultured in 2-L beaker with gentle stirring at 30 rpm with a rotator (TAITEC, Tokyo, Japan), fed with two types of diatoms, *Chaetoceros gracilis* and *Dunaliella tertiolecta* (UTEX, utalgae@uts.cc.utexas.edu, Texas), and transferred to new MFSW twice weekly (Yajima and Kiyomoto, 2006).

Sp-Seawi-Specific Antibody Production and Purification

A portion of the Sp-Seawi coding sequence representing amino acids 75–554 was PCR amplified from *S. purpuratus* embryo cDNA, cloned into pGEM-T Easy (Promega, Madison, WI) and verified by sequencing. A recombinant protein containing a 6x-histidine tag fused to the Sp-Seawi amino acids 75–554 was expressed in *E. coli* BL21 cells and affinity purified with a Ni-Agarose resin (Invitrogen, Carlsbad, CA). Polyclonal antibodies were generated in a rabbit using the purified recombinant Sp-Seawi protein (ProSci Inc., Poway, CA). An amino-terminal biotin-conjugated synthetic peptide containing the Sp-Seawi AVQDRRRRRDEIELEPTTRPENFVK QAIAG amino acid sequence (GenScript, Spiscataway, NJ) was immobilized on streptavidin-agarose beads (Sigma-Aldrich, St. Louis, MO) and used for affinity purification of the anti-Seawi antiserum. For the antibody absorption experiment, Seawi antibody was preincubated with an excess amount (approximately 10 times more at a molar ratio) of the above Seawi peptide at 4°C for overnight. The preabsorbed antibody was then used for immunoblotting.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting Analysis

Protein samples were prepared in 2× Sample Buffer 100 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 15 mM DTT, and 0.2% bromophenol blue), resolved on a 4–20% gradient Tris-glycine polyacrylamide gel (Invitrogen) and transferred to nitrocellulose membranes. The membranes were analyzed by immunoblotting with rabbit anti-Actin (1:2,000) (Sigma) and rabbit anti-Seawi (1:1,000). As for Vasa immunoblotting, 50 embryos were collected for each lane and immunoblotted against Vasa-antibody (1:1,000) (Gustafson et al., 2011). The experiment was performed three independent times.

Morpholino Design and Injection

Approximately, 6 pl of 0.5–1 mM stock morpholino solution was injected into fertilized eggs. Injection into fertilized eggs was performed as described (Yajima et al., 2007). Morpholinos were made by Gene Tools (Oregon, USA) and were designed in the 5′UTR of *Sp-seawi* (ACATCCTTGGGTTT TAGTGCTTTTC), *Sp-piwi-like1* (CGT CCGAAGCCTGCCATCCTCTCTT), respectively, and *Sp-nanos2* (Juliano et al., 2010). The stock concentration of 0.5 mM MO was found to be most effective in diminishing Seawi signal in the small micromere lineage, and thus this concentration was used in this study unless indicated separately.

Fluorescent *In Situ* Hybridization, Immunolabeling, Chemical Treatment, and Microscopy

Fluorescent *in situ* hybridization was performed as previously described (Yajima et al., 2013) with *Sp-seawi* specific probe (Juliano et al., 2006). Immunolabeling was performed as previously described (Yajima and Kiyomoto, 2006; Yajima and Wessel, 2011b). Briefly, embryos or larvae were fixed with 90% Methanol for 1 hr at −20°C, rinsed with PBS, exposed for the primary and secondary antibody reactions for 5 hr each. Affinity purified primary antibodies were used at the following conditions: anti-Seawi at 1:500, anti-Vasa at 1:500 (Voronina et al., 2008), anti-Nanos2 1:100 (Juliano et al., 2010), anti-activated Caspase3 1:500 (Cell

Signaling, #9661). Cy3 goat anti-rabbit immunoglobulin G (IgG) antibody (Invitrogen) was then used as a secondary antibody at 1:300. Hoechst was used as a counter-staining at a final concentration of 0.1 mg/ml. Z-VAD-fmk (Promega, #G7231) was prepared as described in Fujii et al. (2009). Briefly, a final 40 μM of Z-VAD-fmk was added into a culture at gastrula stage, treated for 12 hr, and fixed for immunofluorescence. Fluorescent images were taken by confocal laser microscopy (Zeiss LMS510) or wide-field fluorescence microscopy (Zeiss Axioplan). Vasa-positive cells in the coelomic pouches were manually counted under the fluorescent microscope.

Quantitative RT-PCR

RT-PCR was performed with the same primer sets used in Juliano et al. (2006) and Gustafson et al. (2011). Embryos or larvae of *S. purpuratus* were collected and subjected to total RNA extraction with RNeasy Mini kit (Qiagen, Valencia, CA). The RNA was made into cDNA with TaqMan RT-PCR kit (ABI, Life Technologies), and 1 μl of each cDNA was used for PCR reactions. PCR primers for each gene were designed previously (Juliano et al., 2006; Gustafson et al., 2011). Quantitative PCR (qPCR) was performed on the 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) with the SYBER Green PCR Master Mix Kit (ABI, Life Technologies). All qPCR experiments were run in triplicate. Data for each gene were normalized against ubiquitin RNA levels and then standardized against the value of Mock-injected samples.

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