UC Irvine
UC Irvine Previously Published Works

Title
PIWI proteins and PIWI-interacting RNAs function in Hydra somatic stem cells

Permalink
https://escholarship.org/uc/item/6583r35g

Journal
Proceedings of the National Academy of Sciences of the United States of America, 111(1)

ISSN
0027-8424

Authors
Juliano, CE
Reich, A
Liu, N
et al.

Publication Date
2014-01-16

DOI
10.1073/pnas.1320965111

License
CC BY 4.0

Peer reviewed
PIWI proteins and PIWI-interacting RNAs function in *Hydra* somatic stem cells

Celina E. Juliano,^a,1 Adrian Reich,^a Na Liu,^a Jessica Götzfried,^a Mei Zhong,^a Selen Uman,^a Robert A. Reenan,b Gary M. Wessel,^a Robert E. Steele,^a and Haifan Lin^a,1

^a^Yale Stem Cell Center and Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06520; ^b^Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, RI 02912; and ^c^Department of Biological Chemistry and the Developmental Biology Center, University of California, Irvine, CA 92697-1700

PIWI proteins and their bound PIWI-interacting RNAs (piRNAs) are found in animal germlines and are essential for fertility, but their functions outside of the gonad are not well understood. The cnidian *Hydra* is a simple metazoan with well-characterized stem/progenitor cells that provides a unique model for analysis of PIWI function. Here we report that *Hydra* has two PIWI proteins, *Hydra PIWI* (Hywi) and *Hydra PIWI-like* (Hyli), both of which are expressed in all *Hydra* stem/progenitor cells, but not in terminally differentiated cells. We identified ~15 million piRNAs associated with Hywi and/or Hyli and found that they exhibit the ping-pong signature of piRNA biogenesis. *Hydra* PIWI proteins are strictly cytoplasmic and thus likely act as posttranscriptional regulators. To explore this function, we generated a *Hydra* transcriptome for piRNA mapping. piRNAs map to transposons with a 25- to 35-fold enrichment compared with the abundance of transposon transcripts. By sequencing the small RNAs specific to the interstitial, ectodermal, and endodermal lineages, we found that the targeting of transposons appears to be largely restricted to the interstitial lineage. We also identified putative nontransposon targets of the pathway unique to each lineage. Finally we demonstrate that hywi function is essential in the somatic epithelial lineages. This comprehensive analysis of the PIWI–piRNA pathway in the somatic stem/progenitor cells of a nonbilaterian animal suggests that this pathway originated with broader stem cell functionality.

---

**Results**

*Hydra* PIWI Proteins, Hywi and Hyli, Are Expressed in Multipotent Stem Cells. Computational searches of the *Hydra* genome (10) revealed four Argonaute proteins: two Argonuate family proteins (*Hy-ago1* and *Hy-ago2*) and two PIWI family proteins (*SI Appendix, Fig. S1B*). The *Hydra* PIWI family proteins were named *Hydra PIWI* (Hywi) and *Hydra PIWI-like* (Hyli) for their PIWI and PIWI-like orthologs (*SI Appendix, Fig. S1B*). We generated polyclonal antibodies against the N-terminal and mid domains of both Hywi and Hyli and demonstrated their specificity with immunoprecipitation experiments (*SI Appendix, Fig. S1 C–G*). The antibodies stained numerous cells throughout the body column, but not in the extremities (*Fig. 1 B and C and SI Appendix, Fig. S2 A–C*). The restriction of Hywi and Hyli expression to the body column, where the stem/progenitor cells reside, was also seen by immunoblot analysis of body columns and heads (*Fig. 1D*). Colabeling with C41 antibody, an interstitial stem cell marker (11), demonstrated that Hywi and Hyli are expressed in interstitial stem cells (*Fig. 1 E–G*).

---

**Significance**

The P-element–induced wimpy testis (PIWI) proteins and their bound small RNAs (PIWI-interacting RNAs, piRNAs) are known to repress transposon expression in the germline, yet they likely have broader regulatory functions. We show that the PIWI–piRNA pathway functions in the stem cells of an early diverging animal. We demonstrate that *Hydra* has two PIWI proteins that are localized in the cytoplasm of all adult stem/progenitor cell types. We identified putative targets of the pathway, both transposon and nontransposon, by sequencing piRNAs and mapping them to a newly assembled *Hydra* transcriptome. Finally we demonstrate that *Hydra* PIWI is essential in the somatic lineages. This study supports the existence of a common regulatory pathway ancestral to both stem and germ cells.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (NCBI BioProject no. PRJNA213790).

1To whom correspondence may be addressed. E-mail: haiфан.lin@yale.edu or celina.juliano@yale.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1320965111/-/DCSupplemental.

PNAS | January 7, 2014 | vol. 111 | no. 1 | 337–342

www.pnas.org/cgi/doi/10.1073/pnas.1320965111

*P* element–induced wimpy testis (PIWI) proteins and their bound small RNAs (PIWI-interacting RNAs, piRNAs) are central players in a regulatory pathway that is essential for germline establishment and maintenance. Loss of PIWI proteins in *Drosophila*, mice, and zebrafish leads to a loss of fertility, due to a disruption in germline stem cell (GSC) formation or maintenance, arrest in meiosis, and other gametogenic defects (1). *Pwi* is also expressed outside the germline, largely in various kinds of stem and progenitor cells. For example, *pwi* genes are expressed in the pluripotent stem cells of planarians, sponges, and tunicates, and are required for regeneration (2). *Pwi* expression is also found in hematopoietic stem cells in humans, mesenchymal stem cells in mice, and somatic stem cells in cnidarians and ctenophores (3–6). However, detailed analyses have been largely confined to the function of the PIWI–piRNA pathway in the germline and the gonadal somatic cells in a few model bilaterians, with a focus on transposon silencing (7). The potential significance of the pathway in stem cells outside the gonad and on nontransposon sequences is largely unexplored. 

*Hydra* is a morphologically simple multicellular organism belonging to the phylum Cnidaria, which is the sister group to bilaterians (*SI Appendix, Fig. S1A*). The adult *Hydra* polyp is composed of three distinct cell lineages: the two epithelial lineages (ectoderm and endoderm) and the interstitial lineage (*Fig. 1A*). The multipotent interstitial stem cells that support the interstitial lineage give rise to three somatic cell types (nerves, nematocytes, and gland cells) and to germ cells (*Fig. 1A*) (8). The epithelial lineages do not have a true stem cell population, but they are mitotic along the entire length of the body column and these progenitor/stem cells are responsible for maintaining the lineage (*Fig. 9*). These cells indefinitely self-renew and retain the capability of differentiating into the nonmitotic cells that function in the tentacles and foot (*Fig. 1A*). In this study we provide a comprehensive analysis of both PIWI protein expression and piRNA sequences in *Hydra*, which demonstrates that the PIWI–piRNA pathway has ancient and broadly conserved stem cell functions, including somatic functions.
Hywi and Hyli are apparently exclusively cytoplasmic (Fig. 2 D–F). These features are identical to the ping-pong signature of biogenesis that was first described in Drosophila (14, 16, 23). Over 90% of piRNAs bound to Hywi have an adenine at their 10th position (14, 16, 23). Our data definitively demonstrate that Hywi and Hyli are absent from the nucleus (Fig. 1 H–J and Fig. 2 E–J). This is in contrast to PIWI proteins in Drosophila and the mouse, some of which are nuclear and likely act as epigenetic regulators (23–25). To test if the cytoplasmic localization of Hywi and Hyli in situ is due to antigen masking or low abundance in the nucleus, we analyzed nuclear and cytoplasmic fractions by immunoblotting and found that Hywi and Hyli are apparently exclusively cytoplasmic (Fig. 2D).

Isolation and Characterization of Hydra piRNAs Reveals Conserved Mechanisms of piRNA Biogenesis. To investigate the function of the PIWI-piRNA pathway in Hydra, piRNAs bound to Hywi and Hyli were isolated by immunoprecipitation and sequenced (SI Appendix, Fig. S5A). Analysis of the size distribution revealed that Hywi and Hyli bind piRNAs of different sizes, which is consistent with PIWI proteins in Drosophila, mice, and zebrafish (Fig. 3A) (14, 16, 23). Over 90% of piRNAs bound to Hywi have a uridine at their 5′ end (SI Appendix, Fig. S5D), and over 80% of piRNAs bound to Hyli have an adenine at their 10th position (SI Appendix, Fig. S5D). Furthermore, we found a complementary 10-base pair overlap between the 5′ ends of Hywi-bound and Hyli-bound piRNAs (Fig. 3B). These features are identical to the ping-pong signature of biogenesis that was first described in Drosophila (14, 26) and also observed in mice and zebrafish (16, 23, 27). Previous sequencing of total Nematostella vectensis and Hydra RNAs identified putative piRNAs (28, 29). Here we have identified bona fide cnidarian piRNAs bound to specific PIWI proteins, thus allowing for comparisons between piRNAs bound to different PIWI proteins. Finally, we show that Hydra piRNAs are 2′-O-methylated at their 3′ ends similar to bilaterian piRNAs (SI Appendix, Fig. S5E) (30, 31). Our data definitively demonstrate that Hywi and Hyli participate in ping-pong biogenesis and prove that this mechanism has a deep evolutionary origin in metazoans.

The Hydra PIWI–piRNA Targets Transposon Transcripts. The prevailing model posits that ping-pong piRNA biogenesis results in decreased transposon expression due to posttranscriptional processing of transposon RNAs into piRNAs (14). To test if Hywi and Hyli function in posttranscriptional transposon repression, we first mapped the piRNAs to the Hydra genome (10). Approximately 50% of the sequenced piRNAs were mapped to unique sites in the Hydra genome. From 55% to 65% of Hydra piRNAs map to repeat sequences that were previously identified by RepeatMasker (SI Appendix, Fig. S5 B and C). Because the total repeat content and SI Appendix, Fig. S2 D–F). In addition, both Hywi and Hyli are expressed in nematoblast nests, which are nematocyte progenitor cells of the interstitial lineage (SI Appendix, Fig. S3) (12, 13). Hywi and Hyli proteins are diffusely distributed in the cytoplasm of interstitial stem cells and are enriched in punctate foci around the nucleus (Fig. 1 H–J). Immuno-electron microscopy demonstrated that both Hywi and Hyli are associated with electron-dense perinuclear structures similar to what is seen in the germlines of several animals, including Drosophila, mice, and zebrafish (Fig. 1 K and L) (14–20).
in the *Hydra* genome is 57%, this mapped population of piRNAs is not significantly enriched for repeat sequences (10).

To better characterize the piRNA targets in *Hydra*, we focused our attention on transcripts that are expressed in the adult. To this end, we sequenced and assembled a *Hydra* transcriptome containing ~27,000 sequences, which we curated to obtain a set of 9,986 transcripts with a significant BLAST (1×e−5) match to the Swiss–Prot database. This allowed for definition of ORFs and transcript orientation. Of the curated transcriptome dataset, 622 transcripts were identified as arising from transposons by BLAST (1×e−5) analysis against the *Hydra* transposons in Repbase. Of our sequenced piRNAs, 1.7 million mapped to the transcriptome when allowing up to a three-base pair mismatch. Among these, 72% of Hywi-bound piRNAs and 58% of Hylbound piRNAs map to transposon transcripts, which is a significant enrichment over the abundance of transposons in the transcriptome (Fig. 3C). Furthermore, significantly more piRNAs map per transposon transcript than per nontransposon transcript (Fig. 3D). The majority of Hywi-bound piRNAs map to transposons in the antisense orientation, whereas the Hyl-bound piRNAs map largely in the sense orientation (Fig. 3 E and F and SI Appendix, Table S1); this sense/antisense bias is consistent with the ping-pong model for piRNA biogenesis and posttranscriptional repression of transposons (14). Although the majority of transposons are lowly expressed, they have a high number of piRNAs mapping to their transcripts (Fig. 3 D and F). This is also consistent with the ping-pong model, which posits that transposon mRNAs are repressed by processing them into piRNAs (14). Taken together, these data strongly suggest that one role of the *Hydra* Piwi–piRNA pathway is to regulate transposon expression.

**Identification of Candidate Nontransposon Piwi–piRNA Pathway Targets.** The processing of mRNAs into piRNAs is also a possible mechanism of posttranscriptional repression for nontransposon genes. We found that both Hywi- and Hyl-bound piRNAs predominantly map to the nontransposon genes of the transcriptome in the sense orientation, which suggests that piRNAs are being made from transcribed RNA similar to other piRNAs in *Drosophila* and mice (Fig. 3E and SI Appendix, Table S1) (32). A group of nontransposon transcripts with more than 10 piRNAs mapping per kilobase were selected as putative targets and subjected to gene ontology (GO) analysis (Fig. 3D and SI Appendix, Table S2 and Table S3). We find significant differences in the enriched GO categories between transcripts with high numbers of Hywi piRNAs mapping to them compared with those with high numbers of Hyl piRNAs mapping to them. This suggests selectivity in the mRNAs that are processed into piRNAs. However, we also found a correlation between the expression level of nontransposon transcripts and the number of piRNAs mapped to them (Fig. 3G). Therefore, some piRNA production may occur from highly expressed transcripts simply due to their high abundance.

To test if the Piwi–piRNA pathway in *Hydra* has targets that are specific to each developmental lineage, we isolated each lineage by FACS for small RNA sequencing. Transgenic *Hydra* were used that express GFP in the endoderm and DsRed2 in the ectoderm (Fig. 2B and SI Appendix, Fig. S4A). The interstitial lineage was collected as the population of cells without fluorescence (SI Appendix, Fig. S4A). We found that the most abundant small RNAs in the interstitial lineage are between 26 and 32 nucleotides in length, with a peak at 28. By contrast, in both the ectodermal and endodermal lineages, the most abundant small RNAs are between 26 and 34 nucleotides, with a peak at 32 (SI Appendix, Fig. S6A). For all three lineages, there is a bias for uridine at the 5′ end of small RNAs between 26 and 34 nucleotides long (SI Appendix, Fig. S6B). To test for potential lineage-specific targets of the Piwi–piRNA pathway, we mapped small RNAs greater than 23 nucleotides from each lineage to the transcriptome. Transcripts that had at least 10 times more mapped piRNAs from one lineage compared with the other two lineages were considered putative lineage-specific targets. Approximately 50% of the targets specific to the interstitial lineage are transposons, whereas only one putative transposon target was enriched in epithelial cells (Fig. 3H). Generally, more piRNAs from the interstitial lineage map to transposons in the transcriptome compared with piRNAs from the epithelial lineages, and this trend was not observed for nontransposon transcripts (SI Appendix, Fig. S6 D and E). These data suggest that transposon regulation is largely specific to the interstitial lineage, which is further supported by the observation that the ping-pong biogenesis signature is significantly stronger in the interstitial lineage (SI Appendix, Fig. S6C). In addition, we identified putative nontransposon targets and subjected these to gene ontology analysis; the results strongly suggest that the pathway has specific functions in each lineage (SI Appendix, Table S4).

**Hywi Has an Essential Function in *Hydra* Epithelial Cells.** To gain insight into the function of the Piwi–piRNA pathway in *Hydra* somatic cells, we sought to knock down *hywi* expression in the epithelial lineages. We modified our previously described operon vector by placing an RNA hairpin in the upstream position and the DsRed2 gene in the downstream position to mark transgenic cells (Fig. 4A) (21). Expression of the two genes is driven by an actin promoter that is not active in the interstitial stem cells, but is active in the differentiated cells of the interstitial lineage and throughout the ectodermal and endodermal lineages (SI Appendix, Fig. S7 A–C). Therefore, the RNAi transgene is predicted to affect *hywi* expression in the epithelial cell lineages, but not the interstitial lineage. Injection of plasmid DNA into early *Hydra* embryos results in random integration and the generation of mosaic patches of stably transgenic tissue (33). We tested two different constructs targeting *hywi* and one control construct with a hairpin from the GFP gene. Hatchlings from these injections...
were scored for transgene (DsRed2) expression in the epithelial cells (Fig. 4B). Fifty-eight percent of control hatchlings showed DsRed2 expression in the epithelial cells, whereas significantly fewer hatchlings from the hywi RNAi injections showed DsRed2 expression in the epithelial cells (15.5% and 25.8%; Fig. 4B). By contrast, the hywi RNAi and control transgenes were integrated into the interstitial lineage at the same rate (Fig. 4B). Fully transgenic ectodermal or endodermal lines are established by asexual propagation and continual selection of buds with the most transgenic tissue (33). From the initial hatchlings expressing the GFP control transgene, we established lines that are fully transgenic in the nontransgenic lineages (Fig. 4C) and SI Appendix, Fig. S7 E and F). Both quantitative RT-PCR (qRT-PCR) and Western blot analysis of transgenic F1 hatchlings demonstrated significant down-regulation of hywi compared with nontransgenic F1 siblings (Fig. 4 D and E and SI Appendix, Fig. S7M). By contrast, the RNA and protein levels of hyli are not significantly affected (Fig. 4 D and E). Hywi is not detected in the epithelial cells by immunostaining, but is still detected in the interstitial and endodermal lineages. Small RNAs greater than 23 nucleotides long were mapped to the transcriptome. Approximately 50% of the putative targets in the interstitial lineage are transposons, whereas no transposon targets were identified as specific to the ectoderm or endoderm. One putative transposon target was identified in the epithelium (combination of ectoderm and endoderm) that is enriched by the first bar. For transposon transcripts, the majority of Hywi-bound piRNAs map in the antisense orientation (white), and the majority of Hyli-bound piRNAs map in the sense orientation (gray). The majority of both Hywi- and Hyli-bound piRNAs that map to nontransposon transcripts map in the sense orientation (34). Approximately 50% of the putative targets in the interstitial lineage are transposons, whereas no transposon targets were identified as specific to the ectoderm or endoderm. One putative transposon target was identified in the epithelium (combination of ectoderm and endoderm) that is enriched over the interstitial lineage.

Discussion
The PIWI–piRNA pathway is best known for repressing transposon expression in the germline to maintain genomic integrity
PIWI proteins accumulate in GSCs and in mouse mesenchyme and Drosophila knockdown hatchlings, perhaps due to piwi knockdown in GV stage embryos. The Target Region has an essential function in germ cell specification. Table S4 shows RNAi constructs with similar RNA and protein levels. The in vitro pathways are similar between Hydra and other organisms. Figure 4 shows the expression of Hywi and Hyli RNAi constructs and the percentage of injected hatchlings that have transgene expression. The % Epithelial Expression is significantly lower for the transgene compared with the control. The % Interstitial Expression is also significantly lower for the transgene compared with the control. The presence of genes with shared expression in the germline and in stem cells has led to speculation that these cells have a common evolutionary origin, with germ cells arising as a lineage-restricted stem cell population. The presence of genes with shared expression in the germline and in stem cells has led to speculation that these cells have a common evolutionary origin, with germ cells arising as a lineage-restricted stem cell population (38, 39). In addition, several lines of evidence suggest that germine genes are also more broadly expressed in metazoan stem cells. For example, piwi, vasa, and nanos are expressed and often required in many multipotent and pluripotent stem cells, both with and without germine potential (2, 40). A handful of expression studies in ctenophores and cnidarians reveal piwi expression in somatic stem cells and progenitor cells, which suggests an ancient role for PIWI in stem cell regulation (2, 40). Our study provides a comprehensive analysis of PIWI proteins and piRNAs in a cnidarian. These data strongly suggest that the PIWI–piRNA pathway has an ancient and conserved stem cell function beyond the germline and sets the stage for a mechanistic understanding of the pathway in adult somatic stem cells.

Materials and Methods
Animals and Culturing Conditions. Hydra magnipapillata strain 105 and Hydra vulgaris strain AEP were cultured by standard procedures (41). See SI Appendix, SI Materials and Methods for details.
Hywi and Hyli Antibody Generation. His-tagged recombinant proteins were made to raise antisera in rabbits (Hywi) or guinea pigs (Hyli). See SI Appendix, SI Materials and Methods for details on protein purification, antibody purification, immunoblotting procedures, immunofluorescence procedures, and immunoelectron microscopy.

Nuclear-Cytoplasmic Fractionation. Fractionation was done using the ProteoExtract Subcellular Proteome Extraction Kit 539790. See SI Appendix, SI Materials and Methods for details.

FACS. For small RNA sequencing, animals were prepared as previously described (42). For immunoblot analysis, transgenic Hydra were dissociated with 0.25% Trypsin–EDTA solution. See SI Appendix, SI Materials and Methods for details.

Immunoprecipitation and piRNA Sequencing. TRizol–LS was added directly to the Protein A bead/antibody complexes to isolate total RNA. Small RNA libraries were prepared using illumina Small RNA Preparation Kit v1.5 following the manufacturer’s protocol. Libraries were gel-purified and sequenced using the Genome Analyzer II. See SI Appendix, SI Materials and Methods for further details about procedures, bioinformatics analysis, and genomic mapping of piRNAs.

Sequencing of Lineage-Specific Small RNAs. Each lineage was collected by FACS, and RNA was isolated using TRizol and used to generate small RNA libraries using the TruSeq Small RNA Sample Prep Kit according to the manufacturer’s protocol. The libraries were prepared using the HiSeq 2000. See SI Appendix, SI Materials and Methods for details.

Assembly of the Hydra Transcriptome and Small RNA Mapping. The transcriptome was assembled using a previously described pipeline (43). piRNA and lineage-specific small RNA mapping was done using Bowtie 0.1.0 (44). Gene ontology analysis of putative PIWI-piRNA pathway targets was done using The Database for Annotation, Visualization and Integrated Discovery (DAVID) (45). See SI Appendix, SI Materials and Methods for details.

Generation of Transgenic Hydra. The generation of transgenic Hydra was performed as previously described (21, 33). See SI Appendix, SI Materials and Methods for details on plasmid construction and injection methods.

Note Added in Proof. While this paper was in production, a report on Hydra Piwi proteins and piRNAs by Lim et al. was also accepted for publication by Developmental Biology (48).

Acknowledgments. We thank Catherine Dana for excellent assistance with Hydra cultures, E. Covington, and Graham Morven for performing immuno-EM. We thank Snea Mani, Stefan Materna, S. Zachary Swartz, and James Gagnon for critical feedback on the manuscript and members of the H.L., R.E.S., and G.M.W. labs for help. Head Sci (USA) 103(16):629–631.