Heat shock protein DNAJA1 stabilizes PIWI proteins to support regeneration and homeostasis of planarian Schmidtea mediterranea

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PIWI proteins are key regulators of germline and somatic stem cells throughout different evolutionary lineages. However, how PIWI proteins themselves are regulated remains largely unknown. To identify candidate proteins that interact with PIWI proteins and regulate their stability, here we established a yeast two-hybrid (Y2H) assay in the planarian species Schmidtea mediterranea. We show that DNAJA1, a heat shock protein 40 family member, interacts with the PIWI protein SMEDWI-2, as validated by the Y2H screen and co-immunoprecipitation assays. We found that DNAJA1 is enriched in planarian adult stem cells, the nervous system, and intestinal tissues. DNAJA1 knockdown abolished planarian regeneration and homeostasis, compromised stem cell maintenance and PIWI-interacting RNA (piRNA) biogenesis, and deregulated SMEDWI-1/2 target genes. Mechanistically, we observed that DNAJA1 is required for the stability of SMEDWI-1 and SMEDWI-2 proteins. Furthermore, we noted that human DNAJA1 binds to Piwi-like RNA-mediated gene silencing 1 (PIWIL1) and is required for PIWIL1 stability in human gastric cancer cells. In summary, our findings reveal a similar transposon-depressing role of PIWI protein in adult stem cells of planarian (17), suggesting conserved functions of PIWI protein in germ line and adult stem cells. Moreover, PIWI proteins are critical for biogenesis of PIWI-interacting RNAs (piRNAs) (10, 12, 18), epigenetic regulation (19–21), and cancer cell survival and metastasis (22, 23). Thus, PIWI proteins participate in multiple vital biological processes through various mechanisms.

Freshwater planarians are capable of regenerating any missing body parts after injury or amputation. Regeneration in the planarian relies on an abundant population of adult stem cells known as neoblasts, which is specifically marked by PIWI family genes (11, 24). Therefore, planarians provide an excellent model for studying the regulation of PIWI proteins as well as its involvement in adult stem cells and regeneration. Both planarian’s abundant adult stem cells, also known as neoblasts, and its unique reproductive system provide distinctive advantages for studying PIWI regulation in the adult soma and in the germ line (25–27). Two homologs of PIWI-like protein were identified in the planarian Schmidtea mediterranea, and smedwi-2 was found to be essential for planarian regeneration (11). Another PIWI homolog, smedwi-3, and piRNAs were later identified in planarian neoblasts (12). Accordingly, PIWI homologs in planarian Dugesia japonica and Dugesia ryukyuensis were identified, respectively (28–30). In D. japonica, DjpiwiB is expressed in neoblasts and inherited into neoblast descendant to suppress transposons (17). Recent studies on S. mediterranea suggested that stem cells and their progeny have different levels of PIWI proteins (24), suggesting that the PIWI level needs to be tightly controlled. However, the underlying mechanisms that control PIWI stability remain largely unknown.

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3 The abbreviations used are: piRNA, PIWI-interacting RNA; Y2H, yeast two-hybrid; QDO, quadruple dropout; FISH, fluorescent in situ hybridization; WISH, whole-mount in situ hybridization; IF, immunofluorescence microscopy; NA, numerical aperture; qPCR, quantitative PCR; TE, transposable element; Xα-Gal, 5-bromo-4-chloro-3-indoly β-D-galactoside; HA, hemagglutinin; FBS, fetal bovine serum.
DNAJA1 stabilizes PIWI proteins for regeneration

Several PIWI partner proteins that facilitate PIWI function have been identified in model organisms. In *Drosophila* and mice, Tudor domain–containing proteins directly interact with PIWI proteins by binding to symmetrically dimethylated arginine residues in PIWI, as catalyzed by PRMT5 and Valois (31–35). This interaction facilitates the function of PIWI proteins in spermatogenesis and transposon silencing. Moreover, Armitage, Zucchini, Squash, Maelstrom, and HEN1 are all found to interact with PIWI and promote the biogenesis of piRNAs (36–39). In *Drosophila*, PIWI recruits heterochromatin protein 1a (HP1a) by directly binding to HP1a, forming a complex that establishes epigenetic modification to repress gene expression (20, 40). Interestingly, the planarian HP1a homolog HP1-1 is required for adult stem cell self-renewal, whereas SMEDWI-2 is also required for stem cell division, even though their interaction has not been reported (11, 12, 41). Furthermore, chaperones and co-chaperones are found to be essential for PIWI functions in *Drosophila* and mice (42–45). In *Drosophila*, heat shock protein 90 (HSP90) and HSP70/HSP90-organizing protein (Hop) bind to PIWI to suppress transposition as well as the expression of genetic variation (42). In addition, co-chaperone Shutdown binds to Trdr1 and HSP90 to assist in the production of piRNAs (43–45). Even though these partners are all critical for PIWI function, PIWI interactors that assist in the production of piRNAs (20, 42-45) are found to interact with PIWI and promote the biogenesis of piRNAs. This interaction facilitates the function of PIWI proteins during regeneration or under environmental stress that have not yet been identified.

The HSP protein family includes a large variety of proteins that respond to stress (46). Among them, HSP40, initially identified as DNAJ in *Escherichia coli* as a temperature-sensitive co-chaperone, is critical for the replication of the bacterial DNA (47). The DNAJ family, consisting of the three subtypes DNAJA, DNAJB, and DNAJC, assists protein folding and degradation to ensure the quality of cellular proteins (48). In the mouse germline, DNAJ type I homolog, DjA1, is critical for spermatogenesis (49). Interestingly, levels of human DNAJA1 and DNAJA2 are high in embryonic stem cells, whereas Mrj, a homolog of human DNAJB6, is required for neural stem cell self-renewal (50, 51), indicating that DNAJ proteins also play roles in stem cells. Moreover, human DNAJA1 stabilizes mutant p53 rather than WT p53, indicating that DNAJA1 promotes cell proliferation through this interaction (52). In planarians, the expression of a DNAJA family gene, Smed-HSP40, was identified in adult stem cells (53). Because DNAJA1 has dual roles in both the germ line and stem cells, the two major places where PIWI also function, DNAJA1 might interact with PIWI proteins.

To identify the PIWI interactor in the planarian *S. mediterranea*, we established a yeast two-hybrid library and utilized full-length SMEDWI-2, N-terminal SMEDWI-2, and C-terminal SMEDWI-2 as baits to fish for potential interactors. These screens resulted in seven prey proteins. One of them, DNAJA1, is required for planarian regeneration and homeostasis. We found that DNAJA1 is expressed in neoblasts, the central nervous system, and the intestine. Most importantly, our results showed that DNAJA1 stabilizes PIWI proteins in the planarian and, thus, is required for piRNA maintenance and other functions of PIWI.

Results

Identification of SMEDWI-2–interacting proteins in the planarian *S. mediterranea*

To identify novel interacting partners for planarian SMEDWI-2, we sought to establish a yeast two-hybrid (Y2H) assay using prey libraries generated from planarian cDNA. The Y2H assay has been among the most popular reverse genetics tools for detecting protein–protein interactions. First, we constructed a yeast two-hybrid prey library using whole planarian cDNA as starting material. Thirty asexual worms were harvested for RNA extraction, and poly(A⁺) mRNA was further enriched and reverse transcribed into cDNA and cloned into plasmid pGADT7 vector to build a plasmid library in the yeast strain Y187 (Fig. S1A). We cultured the yeasts and collected 1.429 × 10⁶ independent yeast clones. The yeast cells were amplified, and at least 7.7 × 10⁸ yeast cells were used in each hybridization. To assess the quality of the cDNA library, we randomly picked 100 yeast clones and amplified the transformed sequences with common primers by PCR. All of the plasmids contained cDNA inserts, with 31, 31, and 17% of cDNAs of 250–500, 500–1000, and >1000 bp in length, respectively (Fig. S1B). Only 21% of the clones contained cDNAs shorter than 250 bp. The results suggest the good quality of the planarian cDNA library.

We next constructed three bait yeast strains in the host strain Y2HG. Gold. Each strain carries a pGBKTK7 plasmid that contains either the full-length SMEDWI-2 sequence (SMEDWI-2-FL) or the SMEDWI-2 N-terminal sequence (SMEDWI-2-NT; amino acids 1–385) or the SMEDWI-2 C-terminal sequence (SMEDWI-2-CT; amino acids 378–833) (Fig. 1A). All three baits expressed planarian proteins, and the baits did not self-activate the reporter for the screen (Fig. 1B and Fig. S1C).

We mated three bait strains with prey libraries separately and observed the formation of typical zygotes (Fig. S1D). We first screened the zygotes on triple dropout plates (culture medium lacking adenine, leucine, and tryptophan) and selected the surviving clones on quadruple dropout (QDO) plates (culture medium lacking histidine, adenine, leucine, and tryptophan). We then picked the clones that grew on QDO plates and cultured the clones on QDO plates with X-α-Gal (Fig. 1C). We finally harvested seven preys containing planarian homologs of HIVEP3, RACK1, KU70, SSCO, collagen, SLCA4, and DNAJA1 (Fig. 1, D and E).

Among the seven putative interactors, DNAJA1 was identified previously as a stem cell–enriched protein in a proteomic analysis (53). Moreover, it was co-expressed with SMEDWI-2 in adult stem cells and displayed a SMEDWI-1-like phenotype (see below), so we chose to focus our study on DNAJA1. Sequence analysis of DNAJA1 showed that it encodes a protein of 411 amino acids, consisting of an N-terminal DnaJ domain and a large DnaJ_C domain that spans the middle and C-terminal regions of the protein (Fig. 1F). Within the DnaJ_C domain, a highly conserved DnaJ_ZF domain was identified by a CXX-CXXPXP motif, which is a symbolic domain for DNAJA family protein (Fig. 1F) (54). Thus, we name this gene *Smed–DNAJA1* (henceforth referred to as DNAJA1 for simplicity). Multiple-sequence alignment showed the high sequence similarity of
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*S. mediterranea* DNAJA1 protein with *D. japonica* DNAJA1 as well as *Homo sapiens* and *Mus musculus* DNAJA1 (Fig. S3).

To verify the interaction between SMEDWI-2 and DNAJA1, we mated yeast Y187 expressing planarian DNAJA1 with yeast Y2HGold™ expressing SMEDWI-2-FL, SMEDWI-2-NT, and SMEDWI-2-CT, respectively. Mated yeast expressing both SMEDWI-2-FL and DNAJA1 grew on QDO plates and turned blue (Fig. 1G). The same results were observed in mated yeast expressing SMEDWI-2-CT and DNAJA1 but not in mated yeast expressing SMEDWI-2-NT and DNAJA1 (Fig. 1G), indicating that DNAJA1 binds to the C terminus of SMEDWI-2.

We next independently expressed HA-tagged planarian DNAJA1 and FLAG-tagged planarian SMEDWI-2 in 293T cells. After harvesting the cells, we incubated cell lysates from 293T expressing DNAJA1 with cell lysates either from 293T expressing empty vector or 293T expressing SMEDWI-2. Then we immunoprecipitated SMEDWI-2 with anti-FLAG antibody and found that DNAJA1 was co-immunoprecipitated (Fig. 1H). These results suggest that DNAJA1 physically interacts with SMEDWI-2 protein.

**DNAJA1 is expressed in SMEDWI-positive cells**

To more precisely define the cells expressing DNAJA1 in the planarian body, we performed fluorescent in situ hybridization (FISH) and immunofluorescence co-staining to compare the expression pattern of DNAJA1 with various cell-type markers. The results revealed that DNAJA1 mRNA was co-expressed with smedwi-1 mRNA, a marker specific for neoblasts (Fig. 2A), and was also enriched in SMEDWI-1 protein-positive cells, which encompass both neoblasts and their early differentiating progenies (Fig. 2A).
Moreover, we confirmed that DNAJA1 mRNA was co-expressed with both smedwi-2 mRNA (Fig. S4A) and SMEDWI-2 protein in the same cells (Figs. 2B). Although highly enriched in neoblasts, smedwi-2 is also expressed in differentiated tissues, such as the central nervous system. We found that DNAJA1 mRNA was expressed in SMEDWI-2–positive cells in both the ventral central nervous system and dorsal germ-lines (Fig. 2, B and C), indicating that the expression of DNAJA1 was not just enriched in neoblasts but also extends to their early progenies.

Exposure to γ-irradiation effectively and specifically ablates planarian neoblasts (55). To further confirm the enrichment of
**DNAJA1 in neoblasts,** we examined the expression levels of DNAJA1 mRNA in γ-ray–irradiated worms by whole-mount in situ hybridization. DNAJA1 transcripts were reduced in γ-ray–irradiated worms, although the majority of signals detected in the intestine and central nervous system remained unaffected (Fig. 2D). We further confirmed the expression of DNAJA1 in the intestinal system by staining DNAJA1 with intestine marker mat (Fig. 2E). Our results showed that DNAJA1 was co-expressed with mat in the intestinal system.

Because DNAJA1 belongs to the heat shock protein family, we next evaluated the expression dynamics of DNAJA1 mRNA under thermal stress. DNAJA1 mRNA was significantly increased when worms were cultured in 30 °C, whereas neoblast-specific genes were slightly altered (worms were cultured in 30 °C, whereas neoblast-specific genes were slightly altered (Fig. 2F), suggesting an expected role of DNAJA1 in response to heat shock. Together, the above data indicate that DNAJA1 is ubiquitously expressed and enriched in cells where SMEDWIs are expressed, suggesting that DNAJA1 and SMEDWIs are functionally connected in regeneration.

**DNAJA1 is required for the stability of proteins controlling planarian regeneration and homeostasis**

To assess the necessity of these seven putative interactors in planarian regeneration, we knocked them down individually by feeding with bacteria expressing dsRNA four times on day 1, 4, 7, and 10. Animals fed with bacteria expressing GFP(RNAi) served as negative control. Interestingly, DNAJA1(RNAi) worms were unable to regenerate a head and only formed a smaller tail. Putative interactor SLAC4(RNAi), SSCO(RNAi), collagen (RNAi), and KU70(RNAi) worms showed no defect in regeneration (Fig. 3A). Rack1(RNAi) led to severe homeostasis defects but no defect in regeneration (Fig. S2A), whereas Hivep3(RNAi) displayed specific defects in tail regeneration but not head regeneration (Fig. S2B). These analyses suggest that DNAJA1 interacts with SMEDWI-2 in adult stem cells to facilitate regeneration.

To further characterize the function of DNAJA1, we examined the phenotype caused by DNAJA1 knockdown in intact worms. Intact DNAJA1(RNAi) worms displayed regression in the head region and curls toward their ventral surface at 30 days after the first RNAi feeding (Fig. 3B). By 35 days after the first RNAi feeding, all of the worms lysed (Fig. 3C). Compared with the phenotype caused by smedwi-2(RNAi) (11), DNAJA1(RNAi) resulted in a weaker phenotype, suggesting that DNAJA1 is not the only partner in the PIWI machinery in adult stem cells. Together, our results revealed that among the seven putative interactors of SMEDWI-2, only DNAJA1, as a validated interactor, is involved in the regeneration process.

**DNAJA1 is required for the stability of proteins controlling planarian stem cell maintenance**

Because the loss of DNAJA1 in planarian resulted in a phenotype similar to that of smedwi-2, we suspected that the stem cell population was affected in DNAJA1(RNAi) worms. To investigate this, we analyzed the stem cell population in DNAJA1(RNAi) and GFP(RNAi) worms by flow cytometry. After the initial RNAi feeding, DNAJA1 was successfully knocked down in DNAJA1(RNAi) worms, as indicated by both in situ hybridization (Fig. 4A) and quantitative RT-PCR (Fig. 4D). The stem cell population in DNAJA1(RNAi) worms consistently decreased during the 27-day period that we monitored (Fig. 4B and C), indicating the requirement of DNAJA1 for adult stem cell maintenance.

To confirm the role of DNAJA1 in stem cell maintenance, we examined the expression of stem cell–specific genes in DNAJA1(RNAi) worms. We monitored the mRNA levels of the stem cell–specific genes and a pigment cell–specific gene, PBGD (56), at corresponding time points in DNAJA1(RNAi) worms. Expectedly, PBGD mRNA was unaffected at all time points examined, yet all four neoblast-specific transcripts (smedwi-1, smedwi-2, hp1-1, and zfp1) were decreased at day 27 (Fig. 4D),...
indicating a role of DNAJA1 in maintaining critical proteins in stem cells. At 27 days after the initial RNAi feeding, SMEDWI-1, SMEDWI-2, and HP1-1 proteins were all significantly reduced (Fig. 4E) as they were in smedwi-2(RNAi) worms who failed to maintain the stem cell population (Fig. S5B), confirming that loss of DNAJA1 resulted in a stem cell loss by this time point.

**PIWI proteins are stabilized by DNAJA1 in planarians**

Because DNAJA1 is a chaperone that interacts with SMEDWI-2 and both proteins are required for stem cell maintenance, we wondered whether DNAJA1 maintains stem cells by protecting the stability of SMEDWI-2. We examined the mRNA fold changes of DNAJA1, smedwi-1, smedwi-2, hp1-1, zfp1, and PBGD in DNAJA1(RNAi) day 19, 23, and 27 (D19, D23, and D27) worms. mRNA levels are normalized to gapdh. Error bars, S.D.; *, p < 0.05; **, p < 0.01; ***, p < 0.001; significance was determined with Student’s t test.

Figure 4. DNAJA1 is required for the stability of SMEDWI proteins and stem cell maintenance. A, representative DFISH + IF results of DNAJA1, smedwi-1, and SMEDWI-2 in GFP(RNAi) or DNAJA1(RNAi) worms. The results show dorsal views. Enlarged areas were indicated with white dashed squares. All panels are single frames. Scale bar, 80 μm. B, flow cytometry analysis of stem cell population at the indicated time point after GFP(RNAi) or DNAJA1(RNAi). At least 10 worms were used for one experiment, and only one of three experiments is shown here. For a complete data set, see Fig. S5A. C, statistical analysis of stem cell population at the indicated time points after GFP(RNAi) or DNAJA1(RNAi). At least 10 worms were used for one experiment, and the average of three experiments is shown here. Error bar, S.D.; significance was determined with Student’s t test. D, mRNA expression -fold changes of DNAJA1, smedwi-1, smedwi-2, hp1-1, zfp1, and PBGD in DNAJA1(RNAi) day 19, 23, and 27 (D19, D23, and D27) worms. mRNA levels are normalized to gapdh. Error bars, S.D.; *, p < 0.05; **, p < 0.01; ***, p < 0.001; significance was determined with Student’s t test.

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levels of *smedwi-1* and DNAJA1 as well as the protein level of SMEDWI-2 at day 23 in *GFP(RNAi)* worms and DNAJA1(RNAi) worms, respectively. The mRNA levels of *smedwi-1* and *smedwi-2* were hardly affected, as assayed by both *in situ* hybridization for *smedwi-1* mRNA (Fig. 4A) and quantitative RT-PCR for both *smedwi-1* and *smedwi-2* mRNAs (Fig. 4D). We next tested whether depletion of DNAJA1 affected the stability of SMEDWI proteins. Interestingly, we found that the level of both SMEDWI-1 and SMEDWI-2 proteins was severely reduced, as indicated by both immunofluorescence microscopy for SMEDWI-2 (Fig. 4A) and by Western blot analysis for both SMEDWI-1 and SMEDWI-2 (Fig. 4E). These results indicate that DNAJA1 promotes the stability of the SMEDWI proteins but not their mRNAs. Furthermore, the reduction of SMEDWI-2 in DNAJA1(RNAi) worms occurred in the cytoplasm, with *smedwi-2* not detectably affected (Fig. 4A), which further indicates that DNAJA1 might bind to SMEDWI-2 in the cytoplasm to maintain its stability.

Because DNAJA1 is responsive to heat shock, we examined whether DNAJA1 protects SMEDWIs under thermal stress in planarians. Interestingly, thermal stress reduced the levels of both SMEDWI-1 and SMEDWI-2 proteins, and loss of DNAJA1 exacerbated the effect of the thermal stress on the proteins (Fig. 4, F and G). This indicates a role of DNAJA1 in protecting SMEDWI proteins from environmental stress.

**DNAJA1 is required for maintenance of the piRNA population in planarians.**

Because *smedwi-2* is required for piRNA maintenance (12), we wondered whether DNAJA1 is also required for piRNA maintenance. Because DNAJA1(RNAi) worms showed a weaker phenotype compared with SMEDWI-2(RNAi) worms, we isolated RNA from *GFP(RNAi)* and SMEDWI-2(RNAi) worms at 13 days after the first RNAi (two feeds) and *GFP(RNAi)* and DNAJA1(RNAi) worms at 23 days after the first RNAi (four feeds). We first analyzed the piRNA population of SMEDWI-2(RNAi) and DNAJA1(RNAi) and found that loss of either *smedwi-2* or DNAJA1 led to a significant decrease of the total piRNA population (Fig. 5A). To clarify the role DNAJA1 plays in piRNA maintenance, we sequenced the small RNAs from the *GFP(RNAi)*, *smedwi-2(RNAi)*, and DNAJA1(RNAi) worms (Table S1). Because the global miRNA expression profile between these groups was similar (Table S2), we assumed that the miRNA pathway is unaffected in *smedwi-2(RNAi)* and DNAJA1(RNAi) worms and normalized all libraries to the level of total miRNAs. Among the small RNAs from the planarian samples (Fig. 5, B and C), we observed a second peak around nucleotides 32–33, which matches the signature of planarian piRNAs. The base composition of these piRNAs showed strong first U bias (Fig. 5D), a typical signature for primary piRNAs. Both *smedwi-2(RNAi)* and DNAJA1(RNAi) caused a significant piRNA reduction, 23.9% (*p = 0.047*) and 18.1% (*p = 0.022*), respectively, compared with their control genotype (Fig. 5, B and C). Transposon-derived piRNAs (Fig. 5E, top two panels) and transposon-targeting piRNAs (Fig. 5E, bottom two panels) were both significantly reduced in DNAJA1(RNAi) and *smedwi-2(RNAi)* worms compared with *GFP(RNAi)* worms, indicating that the observed piRNA reduction is likely a global effect. Altogether, these lines of evidence indicate a role of DNAJA1 in piRNA biogenesis.

**DNAJA1 facilitates *smedwi-2*-mediated regulation of gene expression**

We previously inspected the mRNA expression changes in *smedwi-2(RNAi)* worms at 3 days post-amputation via microarray and found that 391 genes were down-regulated, whereas 494 genes were up-regulated (41). Therefore, we examined whether DNAJA1 is involved in the regulation. Based on the expression -fold change and gene function in 3-day post-amputation *smedwi-2(RNAi)* worms, we first selected 16 down-regulated genes and 15 up-regulated genes and examined their expression in intact worms fed with *smedwi-2* dsRNA only once to mimic an early status of SMEDWI-2 loss. All of the 16 down-regulated genes, except for *MAPK14*, were decreased in intact *smedwi-2(RNAi)* worms (1 feed, day 8) to different extents (Fig. 6A), whereas 12 of the 15 up-regulated genes were increased in intact *smedwi-2(RNAi)* worms (Fig. 6B). To examine whether DNAJA1 is involved in SMEDWI-2–mediated gene expression regulation, we next examined the changes in the expression of these genes in DNAJA1(RNAi) worms. Ten of 15 genes down-regulated in intact *smedwi-2(RNAi)* worms showed reduced levels in day 19 DNAJA1(RNAi) worms, indicating that DNAJA1 is required for the expression of these SMEDWI-2–dependent genes (Fig. 6C). Meanwhile, more than two-thirds of genes that increased in *smedwi-2(RNAi)* worms were up-regulated in DNAJA1(RNAi) worms, indicating that DNAJA1 is required for SMEDWI-2–mediated suppression of gene expression (Fig. 6D). Furthermore, we examined the expression of all of the 16 down-regulated and 15 up-regulated genes in day 27 DNAJA1(RNAi) worms. As expected, we observed a similar gene expression alteration in day 27 DNAJA1(RNAi) worms as compared with *smedwi-2(RNAi)* worms (Fig. 6, E and F), indicating that DNAJA1 is required for SMEDWI-2–mediated regulation of gene expression.

**DNAJA1–PIWI interaction is conserved during evolution**

To explore whether the interaction between DNAJA1 and SMEDWI-2 is conserved in humans, we co-expressed HA-tagged HDJ2, the human ortholog of DNAJA1, with FLAG-tagged PIWIL1, the human ortholog of SMEDWI-2, in 293T cells. To see whether the interaction region in SMEDWI-2 is also conserved, we tested the three PIWIL1 constructs: PIWIL1-FL (full-length PIWIL1) and its truncated forms PIWIL1-NT (containing amino acids 1–554) and PIWIL1-CT (containing amino acids 375–861) (Fig. 7A). HDJ2 and PIWIL1-FL were co-immunoprecipitated with each other (Fig. 7, B and C). In addition, HA-tagged HDJ2 was co-immunoprecipitated with PIWIL1-CT but not PIWIL1-NT (Fig. 7D), which is in line with the finding that planarian DNAJA1 interacts with SMEDWI-2-CT (Fig. 7E). Thus, our data demonstrated that DNAJA1 binds to C-terminal of PIWI proteins.

To further examine whether the function of DNAJA1 in protecting PIWI stability is also conserved, we knocked down...
HDJ2 in the human gastric cancer cell line AGS with three different siRNA sequences and found that down-regulation of HDJ2 resulted in significant decrease of PIWIL1 protein level in AGS cells (Fig. 7F). These results further indicate the functional conservation of DNAJA1 interaction with PIWI proteins during evolution.
Discussion

The PIWI–piRNA pathway is a major eukaryotic small RNA pathway with multifaceted function in gene regulation, transposon silencing, and diverse developmental processes. Identification of PIWI-interacting proteins is an effective routine to decipher the function of the pathway (34). Over the past decade, multiple PIWI-interacting proteins have been identified in Drosophila, C. elegans, mice, and other model organisms. Most of the identified interactors are either involved in piRNA biogenesis or partner with PIWI proteins to achieve a particular regulatory function (34). Despite this progress, little is known about how the stability of PIWI proteins is regulated. In planarians, the function of several PIWI partners has been identified in Drosophila, C. elegans, mice, and other model organisms. Most of the identified interactors are either involved in piRNA biogenesis or partner with PIWI proteins to achieve a particular regulatory function (34). Despite this progress, little is known about how the stability of PIWI proteins is regulated. In planarians, the function of several PIWI partners has been characterized. Our previous work demonstrated the HP1-1, a PIWI interactor, is required for regenerative mitosis through activating the mcm5 gene (41). Moreover, proteins known to interact with PIWI in other organisms, such as VASA and TDRD1, have also been identified in planarians (57–60), even though the interaction between these proteins and PIWI in planarian has not been tested yet. Here, we reported the identification of DNAJA1 in planarians as a SMEDWI interactor with an important and unique function in ensuring the stability of both SMEDWI-1 and SMEDWI-2, under both normal and stressed conditions. Moreover, we show that this interaction, as well as its function in stabilizing the PIWI protein, is well-conserved in human cells. Thus, the interaction between DNAJA1 and PIWI homologues represents a conserved mechanism that regulates PIWI protein stability.

In addition, we demonstrated that DNAJA1 is enriched in SMEDWI-positive cells and is required for stem cell maintenance, regeneration, homeostasis, and piRNA biogenesis in planarians. These findings are consistent with a previous proteomic finding that DNAJA1 is a neoblast-enriched protein (53). Because DNAJA1 sustains the stability of both SMEDWI-1 and SMEDWI-2 proteins yet these two proteins are key players in stem cell maintenance, regeneration, homeostasis, and piRNA biogenesis in planarians, it is likely that DNAJA1 achieves these functions mostly, if not exclusively, via its interaction with PIWI.

Of note, the phenotype caused by DNAJA1(RNAi) is weaker than that caused by smedwi-2(RNAi). This indicates that DNAJA1 is a main but not the exclusive regulator of
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A

PIWIL1-FL:
1
GAGE
ArgoL1 domain
PAZ domain
PIWI domain
861
PIWIL1-NT:
1
GAGE
ArgoL1 domain
PAZ domain
554
PIWIL1-CT:
375
PIWI domain
861

B

|                | pCDNA3.1-HA + PIWIL1-FLAG | HDJ2-HA + pCDNA3.1-FLAG | HDJ2-HA + PIWIL1-FLAG |
|----------------|---------------------------|--------------------------|-----------------------|
| 1.5% Input HA | Immunoprecipitation       | Immunoprecipitation      | Immunoprecipitation   |
|                | HA                        | FLAG                     | IgG                   |

Anti-PIWIL1
100 KD

Anti-HDJ2
58 KD

C

|                | pCDNA3.1-HA + PIWIL1-FLAG | HDJ2-HA + pCDNA3.1-FLAG | HDJ2-HA + PIWIL1-FLAG |
|----------------|---------------------------|--------------------------|-----------------------|
| 1.5% Input HA | Immunoprecipitation       | Immunoprecipitation      | Immunoprecipitation   |
|                | HA                        | FLAG                     | IgG                   |

Anti-FLAG
100 KD

D

|                | HDJ2-HA + PIWIL1-NT(1-554)-FLAG |
|----------------|---------------------------------|
| 1.5% Input HA | Immunoprecipitation             |
|                | HA                              |

Anti-FLAG
80 KD

Anti-HA
58 KD

E

|                | HDJ2-HA + PIWIL1-CT(375-861)-FLAG |
|----------------|----------------------------------|
| 1.5% Input HA | Immunoprecipitation              |
|                | HA                              |

Anti-FLAG
58 KD

Anti-HA
58 KD

F

siRNA in AGS cell

|                  | NC    | HDJ2 | Human Testis |
|------------------|-------|------|--------------|
|                  | 1136  | 1270 | 995          |

100 KD
PIWIL1
58 KD
HDJ2
46 KD
ACTIN
SMEDWI proteins; nor is it an integral component of the PIWI machinery.

It is also noteworthy that DNAJA1 stabilizes SMEDWI-1 and SMEDWI-2 but not HP1-1, another protein enriched in stem cells and important for their maintenance. This reflects that DNAJA1 as a chaperone has a selective clientele.

Beyond its enrichment in stem cells, we found that DNAJA1 is also abundantly expressed in the central nervous system and the intestine in planarians. Interestingly, the SMEDWI-2 proteins are also enriched in the peripheral region of the brain. Previous reports implicated that PIWI is a critical regulator in the brain (61), whereas DNAJA1 protects proteins in neuronal cells (62). These findings point to a possibility that DNAJA1 also mediates PIWI turnover in the central nervous system.

In addition to DNAJA1, we identified six other putative PIWI-interacting proteins in planarians through a yeast two-hybrid assay. Two of them, HIVEP3 and RACK1, are required for regeneration and homeostasis, respectively. RACK1 is a scaffold protein critical for both embryonic and adult stem cells (63). It is also expressed in neoblasts. HIVEP3 is ubiquitously expressed and is an essential regulator of polarity establishment in planarians, which is likely independent of known PIWI functions. Studying how these two proteins interact with PIWI proteins may shed new light on molecular mechanisms underlying regeneration and homeostasis.

**Experimental procedures**

**Planarian culture**

Clonal lines of hermaphroditic and asexual (CIW4) *S. mediterranea* were maintained as described previously (64) in water supplied with 0.21 g/liter Instant Ocean salts. Animals were fed weekly with homogenized calf livers. Animals were starved for 1 week before any experiment. For irradiation, planarians were exposed to 60 grays of γ-irradiation using a sealed source of cesium 137 (Gammacell3000, MDS Dordion, Chalk River, Canada). The animals were kindly provided by P. Newmark (University of Illinois at Urbana-Champaign/Howard Hughes Medical Institute, Urbana, IL), P. Reddien (Massachusetts Institute of Technology/Howard Hughes Medical Institute, Cambridge, MA), and N. Oviiedo (University of California, Merced, CA).

**Yeast two-hybrid assay**

Yeast library was constructed with the Matchmaker Library Construction & Screening Kits (630445, Clontech) according to the manufacturer’s instructions. Details and modifications are described under “Results.” Yeast two-hybrid assays were performed with the Matchmaker Gold Yeast Two-Hybrid System (630489, Clontech) according to the manufacturer’s instructions. Details and modifications are also described under “Results.”

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**RNAi experiments**

We used bacteria expressing dsRNA to induce gene knockdown as described previously (65). The worms were fed four times for screening and DNAJA1 (RNAi) and two times for SMEDWI-2 (RNAi). At least 10 worms were used in each RNAi experiment, and at least three independent experiments were carried out for each gene.

**Sequence analysis of SMEDWI interactors**

We aligned the acquired sequences with online PFAM (http://pfam.xfam.org/), and local ClustalX2. Phylogenetic trees were constructed with ClustalX2 using the neighbor-joining algorithm with 1000 trials of bootstrap and 120 random seeds.

**Whole-mount in situ hybridization (WISH), FISH, and immunofluorescence microscopy (IF)**

WISH and FISH were performed as described previously (56, 66, 67). In brief, worms were killed in 5% N-acetyl cysteine solution (Sigma-Aldrich), fixed in 4% paraformaldehyde, permeabilized in reduction buffer, and dehydrated in a graded series of methanol in PBSTx before bleaching. After rehydration, hybridizations were performed with 0.1–0.5 ng/µl riboprobes. For WISH, we use anti-digoxigenin-AP, 1:4000 (Roche Applied Science). Signal was developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (1:50; Roche Applied Science). For double staining, sequential FISH was performed. For the first round of FISH, anti-digoxigenin-peroxidase antibody (1:500; 11207733910, Roche Applied Science) was used first. Signals were developed with CY3-tyramide and inactivated by adding 4% paraformaldehyde for 60 min. After a >2-h wash, samples were incubated with anti-fluorescein-peroxidase antibody (1:500; 11426346910, Roche Applied Science) overnight. Signals were developed with FITC-tyramide. Within a given experiment, all samples were developed in the fluorescent substrate for the same length of time and imaged using identical exposure conditions. All sections were performed after WISH or FISH. Frozen sections were performed as described previously (56). The sections were placed on charged slides (Premiere, Shanghai, China) and mounted with Mowiol mounting medium before imaging. Antibodies against HP1-1, SMEDWI-1, and SMEDWI-2 were described previously (41). Secondary antibodies were Alexa Fluor 488 and 555 obtained from Molecular Probes, Inc. (Invitrogen). IF was performed after FISH development was finished as reported previously with modifications (68). Samples that underwent FISH were blocked in PBSTx containing 0.25% IgG-free BSA.

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Figure 7. Interaction between human PIWIL1 and human DNAJA1. A, schematic presentation of human PIWIL1 and its truncated variants used for assaying interaction with human DNAJA1. B, human HDJ2 interacts with full-length PIWIL1. Human DNAJA1 homologue HDJ2 and PIWI homologue PIWIL1 were overexpressed in 293T cells, and co-immunoprecipitation using anti-PIWIL1 antibody (ab12337, Abcam) and anti-HDJ2 antibody (ab126774, Abcam) showed that HDJ2 interacts with full-length PIWIL1. C, co-IP experiments visualized by anti-FLAG and anti-HA antibodies revealed that human HDJ2 interacts with N-terminal PIWIL1. D, human HDJ2 does not interact with N-terminal PIWIL1. E, human HDJ2 does not interact with C-terminal PIWIL1. F, Western blot analysis of HDJ2, PIWIL1, and β-ACTIN in AGS cells transfected with negative control (NC) or siRNAs targeting HDJ2. Human testis sample was used to indicate the PIWIL1-negative band.
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(Sigma-Aldrich) and incubated with primary antibodies overnight at 4 °C. After extensive washing with PBS-Tx, samples were incubated with Alexa Fluor 488 – or Alexa Fluor 555–conjugated secondary antibody and mounted using Mowiol mounting medium.

Image acquisition, processing, and quantification

Live animals and WISH samples mounted with Mowiol mounting medium were photographed using a SteREO Discovery.V20 microscope (Carl Zeiss, Jena, Germany) equipped with a Plan Apochromat ×1.0 objective and a digital microscope camera (AxioCamHRc, Carl Zeiss) automated by AxioVision Rel.4.8 software (Carl Zeiss). FISH specimens were mounted with fluorescence mounting medium (Dako, Glostrup, Denmark) or Mowiol mounting medium, and images were captured with a laser-scanning confocal microscope (True Confocal Scanner SP5; Leica; HCX Plan Apochromat confocal scanning ×10/0.4 NA, ×20/0.7 NA, or ×40/0.85 NA objective lens) by LAS AF software (Leica). Images were processed with LAS AF Lite software. All in situ hybridization experiments were performed, imaged, and processed identically (at room temperature, 22 °C) to allow direct comparison between experimental animals and controls.

RNA extraction, qPCR, and gene expression profiling

qPCR was performed as described previously (56), and at least six worms were collected for each biological replicate, and for each experiment, at least three biological replicates were performed. In brief, total RNA was isolated using TRIzol (Invitrogen). CDNA was generated from 500 ng of total RNA with the FastQuant RT Kit with gDNAse (Tiangen, Beijing, China). Gene-specific primers were designed with Oligo Perfect designer (Invitrogen). qPCR was performed with an Ace Q qPCR SYBR Green Master Mix kit (Vazyme, Nanjing, China). At least three biological replicates were performed, and each experiment was performed with triplicate or quadruplicate PCRs. Data are expressed using the comparative cycle threshold method. Relative expression levels were normalized to the levels of gapdh (AY068133) mRNA and plotted with SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Gene arrays applied the Agilent Custom array described previously (41). The same array results of SMEDWI-2 (RNAi) with corresponding controls were used in this project.

Small RNA analyses

Small RNAs were first selected using a length cutoff ranging from 20 to 35 bp. Further, reads were mapped onto the S. mediterranea genome (SmedSxl Genome Assembly version 4.0 downloaded from SmedGD) using Bowtie1 (version 1.2.1.1) (69). We allowed one mismatch during mapping throughout the analyses (parameters “Bowtie -k 1 -v 1 -S -p 10 –no- unal –phred33quals –a sample.Gmapped.read.fq SmedSxl genome_v4.0.fa sample.20to35.fq > sample.bowtie.sam”). Only reads mapped to the genome were retained for downstream analyses. Genome-mapped small RNAs were classified by first identifying rRNAs and then tRNAs and miRNAs, and the remaining were defined as putative piRNAs. Two sets of planarian rRNA reference sequences were obtained; one set consists of the noncoding RNA sequences downloaded from RNAcentral (70) (release 7), and the other is a de novo rRNA prediction analyzed with RNaamer (71) version 1.2 (with hmer version 2.2g) and default settings. Reads mapped to the reference rRNA sequences allowing one mismatch were classified as rRNAs, and the remaining were mapped to planarian tRNAs with Bowtie1. For tRNA identification in planarians, we performed de novo tRNA prediction with tRNAscan (version 1.4) (72) and default settings. tRNA introns where removed. Only tRNA sequences ranging from 50 to 100 nt in length, with no more than 5% Ns considered. Known miRNAs were identified by mapping to the known planarian miRNAs from miRBase (73) (including both precursors and mature miRNAs) using Bowtie1. Another de novo set of miRNAs was identified by running ShortStack (74) with default settings (parameters “ShortStack version 3.8.3 -bowtie_cores 3 -mismatches 1 –sort_mem 8G -readfile sample.fastq.gz –genomefile SmedSxl_genome_v4.0.fa”). Samples were normalized using the total number of miRNAs in the size range of 21–23 bp. To identify transposable element (TE)-associated piRNAs, the putative piRNAs were first filtered with a size cutoff of 30–35 bp. Then these piRNAs were mapped to the TE consensus sequences downloaded from RepBase (75) (http://www.girinst.org/repbase/update/browe.php), allowing up to three mismatches (–v 3). TE-derived piRNAs were sense-derived (samtools (76) view –F 16), whereas TE-targeting piRNAs were mapped to the antisense strands of TE consensus sequences (samtools view –F 16).

Cell culture, gene overexpression, and siRNA

The human cell line HEK293T and AGS were purchased from ATCC (Manassas, VA). HEK293T were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% FBS in a 5% CO2 humidified atmosphere at 37 °C. The gastric cancer cell line AGS was cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum in a 5% CO2 humidified atmosphere at 37 °C.

To overexpress genes, SMEDWI-2, DNAJA1, HDJ2, PIWIL1-FL, PIWIL1-NT, and PIWIL1-CT cDNA sequences were cloned into the pCDNA3.1 plasmids with either FLAG tag or HA tag and were transfected with Lipofectamine 2000 (Invitrogen). The siRNA sequences for HDJ2 are as follows: sequence-1136, 5’-CGCCUAUAUCGGAUUAUATT-3’; sequence-1270, 5’-GGUGGACUUUGAUGCAATT-3’; sequence-995, 5’-GGCGUUCAGAAGCAGAUAATT-3’; sequence-721, 5’-UUUCGCAAAGCUGUCAGGTT-3’.

Protein collection, immunoprecipitation, and Western blotting

60 μl of radioimmune precipitation assay lysis buffer (Solarbio, Beijing, China) were used per worm, and at least six worms were lysed for one experiment. At least three independent experiments were performed. A full 10-cm plate of HEK293T cells or AGS cells was collected for each immunoprecipitation or Western blotting experiment. The worms/cells were lysed on ice for 30 min, and the lysis was centrifuged at 14,000 rpm for 15 min. The supernatant was collected and mixed with 2× SDS-loading buffer and was subjected to Western blot analysis. Immunoprecipitation was performed as reported (77). Briefly, cells were lysed in 1 ml of lysis buffer (20 mM Hepes (pH 7.4), 9884 J. Biol. Chem. (2019) 294(25) 9873–9887
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Briefly, for each experiment, 10 planarians were cut with a razor blade on ice-cold dishes and digested in CMFB (400 mg/liter NaH₂PO₄, 800 mg/liter NaCl, 1200 mg/liter KCl, 800 mg/liter NaHCO₃, 240 mg/liter glucose, 1% BSA, 15 mM HEPES, pH 7.3) supplemented with 1 mg/ml collagenase (V900893, Vetc) for 45 min at room temperature. Cell suspensions were filtered with a 35-μm cell strainer cap (BD Biosciences) and stained with Hoechst 33342 (Life Technologies) and propidium iodide (Life Technologies) and filtered again. Cells were analyzed with MoFlo Astrios (Beckman-Coulter).

Flow cytometry

Flow cytometry was performed as described previously (78). Briefly, for each experiment, 10 planarians were cut with a razor blade on ice-cold dishes and digested in CMFB (400 mg/liter NaH₂PO₄, 800 mg/liter NaCl, 1200 mg/liter KCl, 800 mg/liter NaHCO₃, 240 mg/liter glucose, 1% BSA, 15 mM HEPES, pH 7.3) supplemented with 1 mg/ml collagenase (V900893, Vetc) for 45 min at room temperature. Cell suspensions were filtered with a 35-μm cell strainer cap (BD Biosciences) and stained with Hoechst 33342 (Life Technologies) and propidium iodide (Life Technologies) and filtered again. Cells were analyzed with MoFlo Astrios (Beckman-Coulter).

Statistical analysis

Results are presented as means ± S.D., and statistical analyses were performed in SigmaPlot version 11.0 using Student’s t test for two groups. p < 0.05 was considered significant.

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