Inheritance of a Nuclear PIWI from Pluripotent Stem Cells by Somatic Descendants Ensures Differentiation by Silencing Transposons in Planarian

Highlights

- Three PIWI proteins temporally switch to repress TE during PSC differentiation
- Nuclear DjPiwiB is required for differentiation of neoblasts (PSCs) in planarian
- Depletion of DjPiwiB causes activation of TEs in somatic descendants of neoblasts
- DjPiwiB produced in neoblasts is inherited and acts in their somatic descendants

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In Brief
PIWI proteins are known to mediate transposon silencing in the germline. Shibata, Kashima et al. show that, in the planarian Dugesia japonica, differentiated somatic cells, which themselves do not express PIWI proteins, inherit a nuclear PIWI from their pluripotent predecessors. The PIWI protein is required for transposon silencing during differentiation and regeneration.
Inheritance of a Nuclear PIWI from Pluripotent Stem Cells by Somatic Descendants Ensures Differentiation by Silencing Transposons in Planarian

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http://dx.doi.org/10.1016/j.devcel.2016.04.009

SUMMARY

Differentiation of pluripotent stem cells (PSCs) requires transposon silencing throughout the process. PIWIs, best known as key factors in germline transposon silencing, are also known to act in somatic differentiation of planarian PSCs (neoblasts). However, how PIWIs control the latter process remains elusive. Here, using Dugesia japonica, we show that a nuclear PIWI, DjPiwiB, was bound to PIWI-interacting RNAs (generally key mediators of PIWI-dependent transposon silencing), and was detected in not only neoblasts but also their descendant somatic cells, which do not express piwi. In contrast, cytoplasmic DjPiwiA and DjPiwiC were detected only in neoblasts, in accord with their transcription there. DjPiwiB was indispensable for regeneration, but dispensable for transposon silencing in neoblasts. However, transposons were derepressed at the onset of differentiation in DjPiwiB-knockdown planarians. Thus, DjPiwiB appears to be inherited by descendant somatic cells of neoblasts to ensure transposon silencing in those cells, which are unable to produce PIWI proteins.

INTRODUCTION

Pluripotency is the ability of stem cells to differentiate into all types of somatic and germline cells in an organism. Differentiation of pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs), to somatic cells requires dynamic alterations of gene expression by drastic transitions of epigenetic modifications (Xie et al., 2013). To accomplish this, the chromatin at specific loci in the genome may be “loosened.” This loosening of the genome, or euchromatinization, might, however, lead to concomitant activation of transposable elements (TEs) at these loci, threatening the integrity of the genome. Therefore, TEs should be continuously silenced throughout the differentiation process. Histone modifications may be involved in repressing TEs during PSC differentiation (Leung and Lorincz, 2012). However, the detailed mechanism underlying the control of TEs during PSC differentiation remains unknown.

A number of invertebrates possess high regenerative ability, enabling them to reproduce a whole animal body from a tiny body fragment using adult PSCs (Agata et al., 2007; Sanchez Alvarado and Yamanaka, 2014). Planarian contains abundant PSCs, called neoblasts, throughout most of its body, and so, theoretically, tiny fragments amputated from almost any part of the planarian body would regenerate into complete individuals within a week or so, as long as these tiny fragments contained a sufficient number of neoblasts and sufficient positional information from differentiated tissue to serve as a guide for regeneration (Agata et al., 2014; Agata and Watanabe, 1999; Reddien, 2011; Shibata et al., 2010). The establishment of state-of-the-art experimental techniques has made planarian a highly useful animal model in stem cell and regeneration research (Reddien, 2013; Rink, 2013; Shibata et al., 2010).

Neoblasts in planarian express various genes that are normally restricted to germline cells in non-regenerative animals (Rink, 2013; Shibata et al., 2010; Solana, 2013). One such example is the piwi family of genes. These genes encode germline-specific Argonaute members, PIWI proteins, that repress TEs by specifically associating with PIWI-interacting RNAs (piRNAs) (Iwasaki et al., 2015; Juliano et al., 2011; Malone et al., 2009). piRNAs are small non-coding RNAs enriched in the germline of various animals, and most piRNAs possess sequences antisense to TE transcripts (Iwasaki et al., 2015; Juliano et al., 2011; Malone et al., 2009). Indeed, loss of piRNAs and PIWI proteins in the germline causes TE derepression, leading to infertility of mutant animals (Aravin et al., 2007; Khurana and Theurkauf, 2010). Thus, PIWI-piRNA-mediated gene silencing machinery is indispensable for animal species that rely on a sexual reproductive system.

Neoblasts in planarian Schmidtea mediterranea express three piwi genes, smedwi-1, smedwi-2, and smedwi-3 (Palakodeti et al., 2008; Reddien et al., 2005). Also, small RNAs that show...
RESULTS

DjPiwiB Is Required for Differentiation of Neoblasts into Somatic Descendant Cells

We performed RNAi to knock down individual piwi genes in *D. japonica* and examined how regeneration of the planarians was affected by loss of the PIWI proteins. For this RNAi, planarians were fed food containing double-stranded RNA (dsRNA) twice with a 2-day interval, and then amputated into three fragments on day 7 after the second dsRNA feeding. The regenerative ability of the body pieces was monitored at 0, 3, and 7 days post amputation (dpa). We found that regeneration was particularly defective when *DjpiwiB* was depleted (Figure 1A). When planarians were amputated at 1 day after the last feeding, however, partial regeneration was observed at 6 dpa (Figure 1B), indicating that loss of regenerative ability occurred within 7 days after RNAi. Counting the number of phosphorylated histone

![Image](https://example.com/figure1)

**Figure 1. Effect of *DjpiwiB* RNAi**

(A) Regeneration of planarians after RNAi of various *Djpiwi* genes. dpa, day(s) post amputation.

(B) Regeneration of planarians amputated during the early period after the last dsRNA feeding. Arrows, regenerated eyes. Scale bars, 100 μm.

(C) Immunofluorescence analyses of mitotic activity using anti-pH3 and *DjPiwiA* antibodies in control and *DjpiwiB* KD planarians. Scale bars, 500 μm in whole-body samples. Scale bars, 100 μm in magnified view of boxed regions in whole-body samples.

(D) Number of mitotic cells estimated using anti-phosphorylated histone H3 (pH3)-positive cell count after GFP control (white bars) or *DjpiwiB* (gray and black [head regressed] bars) RNAi. ***p < 0.005.

(E) Relative gene expression levels of neoblast-specific genes in *DjpiwiB* KD planarians. The experiments were performed in triplicate and the SDs were calculated using Microsoft Excel.

(F) Relative gene expression levels of piwi family genes in *DjpiwiA*, *DjpiwiB*, or *DjpiwiC* KD planarians.
H3 (pH3)-positive cells, i.e., cells in the M phase of the cell cycle (Newmark and Sanchez Alvarado, 2000), at 7 days into DjpiwiB(RNAi) treatment revealed that DjPiwiB-depleted neoblasts proliferated normally in non-amputated animals (Figures 1C and 1D). At 14 days into DjpiwiB(RNAi), however, some planarians (n = 8/20) showed noticeable head regression (Figures 1C and 1D). This was accompanied by over a 50% decrease in the number of neoblasts (Figures 1C and 1D). The expression levels of neoblast markers (pca, histone H2b [h2b]), and PRMT (Sakurai et al., 2012; Shibata et al., 2012; Tasaki et al., 2011) were not affected at 7 days into DjpiwiB RNAi (Figure 1E).

RT-PCR confirmed that RNAi of DjpiwiB was specific to this gene and did not affect the expression levels of DjpiwiA or DjpiwiC (Figure 1F). We confirmed that the immunoprecipitated protein was DjPiwiB by western blotting (data not shown), and found by RNA extraction that small RNAs were accumulated in the immunoprecipitation (IP) sample (Figure 2B). The most abundant piRNAs within the immunoprecipitated DjpiwiB complexes appeared to be approximately 32 nucleotides long (Figure 2B). We determined the sequence of 4,177,817 of these small RNAs and mapped them onto 2,629,514,899 base pairs of D. japonica shotgun genome.

Comprehensive Analysis of piRNAs Associated with DjPiwiB
PIWI proteins select gene targets depending on the sequences of piRNAs associated with them in vivo. To determine the sequences of DjPiwiB-associated piRNAs, we immunosolated the DjPiwiB-piRNA complexes from wild-type planarians using an anti-DjPiwiB antibody we raised (Figure 2A). The specificity of the anti-DjPiwiB antibody was confirmed by western blotting and RNAi (see below, and also Figure S2). We confirmed that the immunoprecipitated protein was DjPiwiB by western blotting (data not shown), and found by RNA extraction that small RNAs were accumulated in the immunoprecipitation (IP) sample (Figure 2B). The most abundant piRNAs within the immunoprecipitated DjPiwiB complexes appeared to be approximately 32 nucleotides long (Figure 2B). We determined the sequence of 4,177,817 of these small RNAs and mapped them onto 2,629,514,899 base pairs of D. japonica shotgun genome.
sequence data (2.9-fold estimated genome coverage; Nishimura et al., 2015), and also onto D. japonica expressed sequence tag (EST) sequences (Nishimura et al., 2012, 2015). Seventy-four percent of the reads (3,087,387 reads) were perfectly matched to the D. japonica genome sequence and the D. japonica EST sequence dataset. We then used these small RNAs for subsequent detailed analyses. Although these RNAs showed high sequence diversity (Figure 2C), as do piRNAs of mouse and fly (Aravin et al., 2006; Girard et al., 2006; Saito et al., 2006), about 80% of them possessed uracil as the first nucleotide (1U) (Figure 2D), a conserved feature of piRNAs (Iwasaki et al., 2015). We could not find a ping-pong signature (matching of the first ten bases in the opposite orientation between piRNA basically bound to distinct PIWIs, also a conserved feature of piRNAs; Iwasaki et al., 2015).

Of the DjPiwiB-bound piRNAs, 206,992 matched the planarian EST database (Nishimura et al., 2012), and 34% of them mapped onto EST genes annotated as TEs, with 54%, 19%, and 15% of them classified as polintorn, penelope, and gypsy, respectively (Figure 2E). Although the 1U tendency was observed for both sense and antisense piRNAs that matched TEs, 60% of the TE-sequence piRNAs were in the antisense direction, an enrichment compared with the 48% of total piRNAs that matched all EST-represented genes (Figure 2F), suggesting that a fraction of DjPiwiB-piRNA complexes are capable of targeting TEs in vivo. To see if such targeting indeed occurred, we examined the expression of TEs in DjPiwiB-depleted planarians. We particularly focused on TEs whose sequences were complementary to the DjPiwiB-associated piRNAs that we identified in this study. When DjPiwiB was depleted for 24 hr by RNAi, the expression level of a gypsy transposon corresponding to cDNA clone Djl_aH_208_K05 (DDBJ: FY934805; referred to as gypsy-P1) was 4-fold higher than that of normal cells (Figure 2G). The antisense sequence of gypsy-P1 matched 71 piRNA reads (Table S1). The level of gypsy-P1 expression became even higher with prolonged RNAi treatment and reached an 8-fold increase at 5 days into the RNAi treatment (Figure 2G).

**Loss of DjPiwiB Derepresses TEs Only in Differentiating Somatic Cells**

To examine how the expression of gypsy-P1 was altered in various parts of the animals by DjPiwiB depletion, we performed in situ hybridization experiments using a specific RNA probe hybridizing to the transposon transcripts in the sense orientation. This examination revealed that, on day 3 into DjpiwiB RNAi, gypsy-P1-positive cells were concentrated in the head region, particularly anterior to the eyes, where no neoblasts are normally observed (Shibata et al., 2010) (Figure 3A). The gypsy-P1-positive cells were DjPiwiA negative (Figure 3B), suggesting that derepression of gypsy-P1 occurred only in differentiating somatic cells.

To determine whether those gypsy-P1-positive cells corresponded to cells that had already differentiated before the RNAi treatment, or cells that had differentiated de novo upon DjPiwiB depletion, we tested the expression of gypsy-P1 in DjPiwiB-depleted animals after elimination of neoblasts by X-ray irradiation. X-ray-irradiated DjPiwiB-depleted planarians possessed no gypsy-P1-positive cells (Figure 3C). When planarians were treated with DjpiwiB dsRNA together with U0126, an MEK inhibitor that blocks somatic differentiation from the neoblasts (Tasaki et al., 2011), the number of gypsy-P1-positive cells was drastically reduced as early as 5 days into RNAi (Figure 3D). Furthermore, we analyzed gypsy-P1 expression in regenerating DjPiwiB-depleted planarians, because neoblast proliferation and differentiation increase after amputation to supply new somatic differentiated cells (Wenemoser and Reddien, 2010). Expression of gypsy-P1 was readily detected in the posterior stump region of DjPiwiB-depleted body fragments (Figure 3E) but was detected only at a very low level in intact DjPiwiB-depleted animals at day 3 into RNAi treatment.

RNA-seq of DjpiwiB KD planarians was then performed and revealed that multiple types of TEs whose antisense sequences matched piRNA reads were upregulated. For these TEs, qPCR and/or in situ hybridization showed that they had a similar expression pattern to that of gypsy-P1 in animals with combined DjpiwiB knockdown plus X-ray irradiation or U0126 treatment (Figures S3A and S3B). Based on these results, we infer that the expression of multiple TEs, including gypsy-P1, was activated in DjPiwiB-depleted planarians during somatic differentiation from the neoblasts but not in neoblasts themselves, which possessed full pluripotency.

**Neoblast DjPiwiB, but Not DjPiwiA or C, Is Inherited by Differentiated Somatic Cells**

Although transcription of piwi genes occurs preferentially in neoblasts in D. japonica (Hayashi et al., 2010), some weak expression of DjpiwiB as well as DjpiwiC was also observed in the head region, where neoblasts are absent. We therefore examined which types of cells express DjpiwiB, and found that DjpiwiB was expressed in neoblasts and in brain cells in the head region but not in other types of somatic differentiated cells throughout the entire body (Figures S4A and S4B). However, DjPiwiB depletion induced derepression of gypsy-P1 in de novo differentiated somatic cells in the entire body after RNAi (Figure 3). To understand how this derepression occurred, we compared the pattern of immunohistochemical staining with anti-DjPiwi antibodies before and after Djpiwi KD in the animals. DjPiwiA and DjPiwiC were specifically detected in the cytoplasm of the neoblasts (Figures 4A, 4B, and S2). Notably, DjPiwiC was observed as punctate signals (Figure 4B), suggesting that DjPiwiC might be localized in chromatoid bodies, a neoblast-specific RNA/protein organelle (Shibata et al., 2010). In contrast, surprisingly, DjPiwiB was observed only in the nucleus in almost all cells in the body, namely, in both neoblasts and differentiated somatic cells (Figures 4C–4E and S4C), whereas SMEDWI-2 was preferentially observed in the nucleus of the neoblasts in S. mediterranea (Zeng et al., 2013). Particularly strong DjPiwiB signals were also detected in nucleoli (Figure 4D). Combined in situ hybridization of DjpiwiB and immunostaining of DjPiwiA and DjPiwiB confirmed that, in the trunk region, transcription of DjpiwiB occurred only in neoblasts, although DjPiwiB protein was detected in both neoblasts and differentiated somatic cells there (Figures 4F, S2, and S4).

Immunostaining of DjPiwiB-depleted animals at day 7 into DjpiwiB RNAi revealed that DjPiwiB had by then decreased in 80% of neoblasts, but 90% of differentiated somatic cells in the trunk region were still positive for DjPiwiB (Figure 5A). The immunostaining signals of DjPiwiA and DjPiwiC disappeared from planarians after the respective RNAi (Figure S2), indicating the...
neoblast-specific expression of those proteins and the specificity of the antibodies used as well as of the RNAi. Western blot analysis confirmed the persistence of DjPiwiB in planarians whose neoblasts had already been eliminated by X-ray irradiation (Figure 5B). In contrast, the expression levels of DjPiwiA and DjPiwiC proteins were significantly lowered in those planarians after X-ray irradiation, as expected (Figure 5B). In fact, only DjPiwiB and expressed gypsy-P1 (Figure 5C), whereas many differentiated somatic cells lacked DjPiwiB and expressed gypsy-P1 (Figure 5C). This suggests that, when DjPiwiB was absent, derepression of gypsy-P1 occurred during de novo differentiation. In the trunk region, DjPiwiB mRNA was predominantly expressed in neoblasts (Figure S4) (Hayashi et al., 2010). Therefore, we hypothesized that DjPiwiB protein produced in neoblasts was inherited by somatic descendant cells during their differentiation. The neoblasts are the only cell population that continuously divides in planarian. In contrast, differentiated cells never proliferate. This difference might be one reason why DjPiwiB should be retained during the process of cell differentiation. This hypothesis was further supported by our observation that low-level accumulation of DjPiwiB, we performed immunostaining using anti-poly(ADP-ribose) (PAR), because it is known that poly(ADP-ribose)ylation (PARylation) occurs immediately at DNA break sites after DNA damage (Zhang et al., 2015). X-ray irradiation in D. japonica could induce PARylation (Figure 6A). In DjPiwiB-depleted cells in the head region after KD of DjpiwiB, the fraction of cells with a strong PAR signal was significantly increased compared with the fraction in control GFP KD animals or in DjPiwiB-positive cells of KD planarians (Figure 6B), suggesting that activation of TE might have resulted in DNA breakage.

We found that numerous gypsy-P1-expressing cells showed aberrant DNA staining compared with gypsy-P1-negative cells (Figure 6C). It has been reported that cell death was induced during early regeneration (Pelletieri et al., 2010). We detected degenerating cells, which showed abnormal nuclei and cytoplasm, by electron microscopy observations in both control and DjpiwiB KD planarians at 2 days after amputation (3 days into RNAi) (Figure S6A–A′, B–B′, and C–C′). In some degenerating cells of DjpiwiB KD planarians, however, virus-like particles were observed (Figure S6C′). Also, the upregulation of two apoptosis-related genes in DjpiwiB KD planarians

![Figure 3. Analyses of Cells Expressing gypsy-P1](image)

Two successive dsRNA feedings were performed at 3-day intervals after the first feeding for feeding RNAi.

(A) Expression of gypsy-P1 in the head region of DjpiwiB(RNAi) planarians at 3 days after the last feeding. Green, gypsy-P1-expressing cells; magenta, DjPiwiA. Scale bar, 500 μm.

(B) Cells expressing gypsy-P1 (green) and neoblasts (magenta). Scale bar, 10 μm.

(C) Expression of gypsy-P1 in DjpiwiB(RNAi) planarians with or without X-ray irradiation treatment. The first RNAi feeding of DjpiwiB was carried out for 1 day after irradiation. Five days after the last RNAi feeding, planarians were sacrificed for in situ hybridization. Scale bars, 500 μm.

(D) Expression of gypsy-P1 in DjpiwiB(RNAi) planarians treated with U0126. Planarians were treated with 25 μM U0126 after the first dsRNA feeding, and continuously thereafter. Scale bars, 500 μm. Graph shows the number of gypsy-P1-expressing cells in those planarians. **p < 0.01, ***p < 0.005. The experiments were performed in triplicate and the SDs were calculated using Microsoft Excel.

(E) Expression of gypsy-P1 in intact and regenerating DjpiwiB(RNAi) planarians. Planarians were amputated 1 day after the last dsRNA feeding and fixed 2 days after amputation. Scale bars, 500 μm.

DjPiwiB was observed in somatic cells that had newly differentiated from DjPiwiB-lacking neoblasts (Figures 5D and S5), i.e., in cells that probably succeeded in differentiating without TE derepression at an early time after RNAi. To examine the possibility that gypsy-P1 was actually repressed by inherited PiwiB, we performed immunostaining using anti-poly(ADP-ribose) (PAR), because it is known that poly(ADP-ribose)ylation (PARylation) occurs immediately at DNA break sites after DNA damage (Zhang et al., 2015). X-ray irradiation in D. japonica could induce PARylation (Figure 6A). In DjPiwiB-depleted cells in the head region after KD of DjpiwiB, the fraction of cells with a strong PAR signal was significantly increased compared with the fraction in control GFP KD animals or in DjPiwiB-positive cells of KD planarians (Figure 6B), suggesting that activation of TE might have resulted in DNA breakage.

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was revealed by RNA-seq and qPCR of DjpiwB KD planarians (Figure S6D), suggesting that gypsy-P1-expressing cells might be degenerated, which may cause the differentiation defect. Actually, we found a significant reduction of early progeny of neoblasts, as indicated by the expression of Dj_aH_000_05955HH, a D. japonica homolog of an early epidermal progenitor marker in S. mediterranea (Eisenhoffer et al., 2008; Shibata et al., 2012; van Wolfswinkel et al., 2014), in the regenerating region at 6 days after amputation (7 days into RNAi) (Figure 6D), suggesting that proper early differentiation of neoblasts might be disrupted due to cell death by KD of DjpiwB.

Thus, we propose that inheritance of DjPiwiB from the neoblasts by their descendant differentiated somatic cells is indispensable for repressing TEs during stem cell differentiation (Figures 7A and 7B).

DISCUSSION

Conserved Roles of PIWI Proteins in Germline Development and PSC Differentiation

TE suppression by PIWI proteins and piRNA is a fundamental mechanism to assure production of the next generation via protection of genome integrity in the germline. Our findings here using planarian D. japonica revealed that a nuclear PIWI protein, DjPiwiB, plays a crucial role in TE repression during PSC differentiation to somatic cells, indicating a conserved mechanism of PIWI-mediated TE repression between germline development and PSC differentiation. Planarian regeneration is a type of asexual reproduction, in which neoblasts (like germline cells in non-regenerative animals) are responsible for producing the next generation. Indeed, germline cells in non-regenerative animals and neoblasts express many genes in common (Shibata et al., 2010; Solana, 2013), suggesting commonality of the relevant cellular and molecular features of those cells. This commonality is not restricted to planarians: a considerable number of invertebrate species that undergo asexual reproduction using a regenerative path retain adult pluri- or multipotent adult stem cells in their bodies, and these cells express genes restricted to germline cells in non-regenerative animals, including piwi family genes (Juliano et al., 2015).
and Wessel, 2010; Juliano et al., 2010; Ross et al., 2014). Although no evidence has been reported yet to support it, it seems likely that PIWI proteins have a conserved function of repressing TEs in any cells that are responsible for producing the next generation. Actually, germ cells of the related planarian species *Dugesia ryukyuensis*, which are also derived from neoblasts during sexual conversion from the asexual to sexual state, express three *piwi* family genes, *Drpiwi-1*, *-2*, and *-3* (orthologs of *DjpiwiA*, *B*, and *C*, respectively) (Nakagawa et al., 2012). This may support our speculation about the link between the expression of *piwi* genes and the responsibility of cells for producing the next generation.

**How Do Planarian PIWIs Repress TEs?**

Immunofluorescence analysis of the whole bodies of *D. japonica* using anti-DjPiwi antibodies that we raised detected all three DJPiwi proteins in neoblasts, but only nuclear DJPiwiB, and not the two cytoplasmic PIWIs, was also observed in almost all of the somatic descendant cells of neoblasts (Figures 6A and 6B). This cell-type-dependent PIWI expression is reminiscent of PIWI expression in the fly germline. In the fly ovary, germ cells express three PIWI proteins: one nuclear (Piwi) and two cytoplasmic (Aubergine [Aub] and Ago3) (Brennecke et al., 2007; Gunawardane et al., 2007). In contrast, somatic follicle cells, as well as a cultured ovarian somatic cell line, express only nuclear Piwi (Brennecke et al., 2007; Gunawardane et al., 2007; Saito et al., 2009). Aub/Piwi and Ago3 in germ cells interact with TE transcripts that match the strand opposite to piRNAs, and cleave the transcript using an endonuclease (slicer) activity to repress them at the post-transcriptional level (Brennecke et al., 2007; Gunawardane et al., 2007; Saito and Siomi, 2010). In contrast, nuclear Piwi represses TEs at the transcriptional level via epigenetic modification (Sienski et al., 2012). Germ cells and somatic cells are derived from different origins, namely, germline stem cells and somatic stem cells, respectively, and so the two cells are not kindred. In contrast, somatic cells in planarians are descendants of neoblasts. Thus, these two cases are not absolutely parallel, but it is reasonable to imagine that DJPiwiB might be the counterpart of Piwi, and so it transcriptionally silences TEs in the nucleus, while cytoplasmic DJPiwiA and DJPiwiC might be the counterparts of Aub and Ago3, or vice versa, and implement TE silencing at post-transcriptional levels.
Why Is DjPiwiB, but Not Other PIWIs, Inherited by Somatic Cells during PSC Differentiation?

Neoblasts proliferated normally, and a TE was silenced in them even when DjPiwiB was depleted by RNAi. These results suggest that DjPiwiA and DjPiwiC compensate for the functional loss of DjPiwiB in neoblasts in an epistatic manner, although the method of DjPiwiB-mediated silencing is supposedly different from that of silencing by DjPiwiA and DjPiwiC (Figure 6A). Depletion of DjPiwiB caused a severe defect in regeneration, indicating that the role of DjPiwiB at the onset of neoblast differentiation is crucial (Figure 6B). The differentiating cells expressing gypsy-P1 appeared to die, and perhaps consequently differentiated cells that were needed to regulate the differentiation or maintenance of neoblasts could not be supplied. Another possibility is that other target(s) of DjPiwiB might affect survivability of the neoblasts. These effects might have caused the severe defect or decrease of neoblasts at a later period after RNAi. Interestingly, DjPiwiB appears to be neither transcribed nor translated in the differentiating neoblasts, or in the resulting somatic cells, despite its indispensability for repressing TEs in those cells. This requirement to repress TEs during the somatic differentiation process might be a reason to employ the inheritance of DjPiwiB from neoblasts by their somatic descendant cells that have exited from the cell cycle in *D. japonica*. In sexually reproducing animals, PIWIs are germline specific; namely, *piwi* genes are normally repressed in somatic cells of the animals. Thus, our observation that the differentiating neoblasts, or resulting somatic cells, are transcriptionally silent for *piwi* is reasonable and not surprising. However, this might cause a fatal problem for planarians, and thus the animals might have acquired a system for the inheritance of DjPiwiB from neoblasts to somatic descendant cells during differentiation. However, no DjPiwiA or DjPiwiC is inherited by the fully differentiated somatic cells, although remaining SMEDWI-1 or DjPiwiA was observed in early progeny of neoblasts in both *S. mediterranea* and *D. japonica* (Guo et al., 2006; Yoshida-Kashikawa et al., 2007). Why is DjPiwiB the only PIWI protein selected to remain in descendant cells of neoblasts during differentiation in *D. japonica*? One possibility is that DjPiwiB is a nuclear PIWI, which, as suggested above, might repress gene expression at the transcriptional level via an epigenetic mechanism such as has been observed in fly. Repression of gene transcription by a single protein in nuclei would seem likely to be simpler and safer than translational repression of genes by two proteins via their interaction.
in the cytoplasm. Recently, distinctive functions of SMEDWI-1 and SMEDWI-3 apart from TE repression, namely, the involvement of these cytoplasmic PIWIs in the localization of histone mRNAs in neoblast-specific nucleoprotein granules, have been reported (Rouhana et al., 2014), suggesting that cytoplasmic PIWIs might have particular neoblast-specific functions in addition to TE repression in planarians. Thus, DjPiwiB might be produced in neoblasts, and act in their somatic descendants as epigenetic memory.

**EXPERIMENTAL PROCEDURES**

**Biological Samples**

A clonal strain of planarian *D. japonica*, sexualizing special planarian (SSP) (2n = 16) (Shibata et al., 2012), was cultured at 23°C in highly diluted artificial seawater (0.05 g/l Instant Ocean Sea Salt powder; Instant Ocean). Chicken liver was fed to cultured planarians once every 1 or 2 weeks. Animals were starved for at least 1 week before all experiments.

**X-Ray Irradiation**

Animals were placed on wet filter paper on ice and irradiated with 160 R of X-rays using an X-ray generator (SOFTEX B-5; SOFTEX).

**Preparation of Antibodies**

For production of anti-DjPiwiA, DjPiwiB, and DjPiwiC rabbit polyclonal antibodies, peptides corresponding to a part of each DjPiwi family protein were synthesized and injected into rabbits. Affinity-purified polyclonal anti-peptide antibodies were obtained from the rabbit sera. All procedures were conducted by MBL. The amino acid sequences of the peptides were DjPiwiA (EPALQPETIIDKVGKDG, corresponding to amino acids 27–43), DjPiwiB (KIPMRERRKPGEEDKE, corresponding to amino acids 303–319), and DjPiwiC (FENSEKPTSFKRRREH, corresponding to amino acids 172–188).

**Western Blotting**

Western blotting was performed as described by Tasaki et al. (2011). Anti-DjPiwiA, -B, or -C antibody was used at a dilution of 1/500, 1/1,000, or 1/1,000, respectively.

**Whole-Mount Immunohistochemistry**

Whole-mount IHC was performed as described previously (Yoshida-Kashikawa et al., 2007). The antibodies against DjPiwi proteins and PAR (Treviggen) were diluted 1/1,000. The samples were observed with a confocal microscope (FLUOVIEW FV10; Olympus) or a fluorescence stereoscopic microscope (M205FA T-RC 1; Leica).

**Whole-Mount In Situ Hybridization**

Whole-mount in situ hybridization was performed as described previously (Takano et al., 2007; Umesono et al., 1997). Signals were detected using a TSA kit #2 (Molecular Probes).

**qPCR Analysis of cDNA**

qPCR was performed as described by Sakurai et al (Sakurai et al., 2012). The forward and reverse primer sets for the investigated genes were (5’ to 3’):

- **DjG3PDH**
  - FW: ACCACCAACTGTATTAGCTCCCTTAQ
  - RV: GATGGTCCATCAACAGTCTTTTG

- **pcna**
  - FW: ACCTATCGTGTCACTGTCTTTGACCGAAAA
  - RV: TTCATCATCTTCGATTTTCGGAGCCAGATA

- **h2b**
  - FW: ATTCAAACATCCGTCCGTCT
  - RV: TTTTGTAACAGCCTTCGTTCC

- **PMRT**
  - FW: AGTCAATACGTTAAGAGATACCTG
  - RV: CCTTCCGACCTACCTCATTCG

- **DjpiwiA**
  - FW: CGAATCCGGGAACTGTCGTAG
  - RV: GGAGCCATAGGTGAAATCTCATTTG

- **DjpiwiB**
  - FW: ATGGATCCCATGGCTCCTAATG
  - RV: TGCACAGGGACAGGTACACG

- **DjpiwiC**
  - FW: GGCCTGGAACTGTTAGAGTACCTG
  - RV: CAAACGGTCGCACAATAAATGAC

**Figure 7. Schematic Drawing of Our Working Hypothesis about the Mechanism of Repression of TEs by Inherited DjPiwiB during the Process of Somatic Differentiation from the Neoblasts**

(A) Normal differentiation from a neoblast. (B) Differentiation from a neoblast in DjPiwiB-depleted planarian. T bars indicate repression of TEs.
Feeding RNAi
dsRNA was synthesized basically as described by Rouhana et al. (2013). The primers for the PCR reaction were as follows (5’ to 3’): Zap Linker + TT, GAT CAC TAA TAG CAC TCA CTG GAA TAT CCG GCA CAA GG; M13 Rv, GTT TTC CCA GTC ACG TTG TAA.
Feeding RNAi was carried out as described previously (Rouhana et al., 2013; Sakurai et al., 2012). Control animals were fed dsRNA containing the EGFP cDNA sequence.

Preparation of Planarian Extract for Immunoprecipitation
Two hundred and fifty microliters of Medium Salt Buffer (MSB; 50 mM Tris-HCl, 150 mM NaCl, 0.05% NP-40) containing Mini Complete protease inhibitors (Roche Diagnostics) was added to 20 animals and the samples were quickly frozen in liquid nitrogen. The frozen samples were thawed on ice, homogenized with a plastic pestle, and further dissociated by pipetting with a 200-µl pipette. Then freezing and thawing were repeated once more, and the samples were centrifuged at 18,000 × g for 10 min at 4 °C. The supernatant was collected in a new tube (store 1). After that, 200 µl of MSB solution containing protease inhibitor was added to the pellet, and pipetting, freezing, thawing, and centrifugation were repeated under the same conditions as described above. Then the supernatant was collected in a fresh tube (store 2). This series of operations was repeated once more, and the supernatant was collected in a new tube (store 3). Stores 1, 2, and 3 were mixed and used for IP.

Protein Electrophoresis and Silver Staining
Two hundred microliters of planarian extract was added to the anti-DjPiwiB antibody bound to Protein G Sepharose, and MSB solution was added to a final volume of 1 ml. The mixture was rotated for 3 hr at 4 °C and washed with MSB solution four times for 1 min each with centrifugations at 5,000 × g at 4 °C.

Two hundred microliters of planarian extract was added to the anti-DjPiwiB antibody bound to Protein G Sepharose, 4 Fast Flow slurry (GE Healthcare) was centrifuged and resuspended in 1 ml of MSB solution, mixed well by rotation for 5 min, and centrifuged. Then, the supernatant was removed and 650 µl of MSB solution was added. The solution was separated into 100-µl aliquots, and 50 µl of anti-DjPiwiB antibody (pre-immune serum control) was added to each aliquot and brought up to a final volume of 1 ml with MSB. The mixture was rotated for 3 hr at 4 °C and washed with MSB solution four times for 1 min each with centrifugations at 5,000 × g at 4 °C.

Two hundred microliters of sample buffer was added to beads containing immunoprecipitate and the mixture was boiled for 10 min at 95 °C and centrifuged at maximum speed in a tabletop centrifuge at 4 °C. The lysate was subjected to SDS-PAGE. Silver staining was conducted using a SilverQuest Staining Kit (Invitrogen) according to the manufacturer’s instructions.

Co-immunoprecipitation and Sequencing of DjPiwiB-Interacting RNA
The immunoprecipitate obtained using anti-DjPiwiB antibody was washed with MSB solution, and 150 µl each of H2O and phenol/chloroform was added. After vortexing, the sample was centrifuged for 10 min, and the aqueous layer was transferred to a new tube. The addition of phenol/chloroform and centrifugation were repeated. After transferring the aqueous layer to a new tube, 500 µl of chloroform was added. After mixing, centrifuging, and transferring the aqueous layer to a new tube, RNAs were precipitated by ethanol precipitation. The RNAs obtained were labeled with 32P and detected by electrophoresis. Immunoprecipitated RNAs were sequenced by Hokkaido System Science using an Illumina sequencer. The piRNA-seq data is deposited in DRA: DRA002837.

Analysis of the DjPiwiB-Interacting piRNAs
After adapter trimming with a handmade Ruby script, reads longer than 33 bp were removed. To select reads derived from D. japonica, mapping of piRNA onto the D. japonica shotgun genome and EST sequence dataset was performed with BWA (0.6.1-r104; http://bio-bwa.sourceforge.net/) with default settings (Li and Durbin, 2009). According to the mapping results (allowing for at most two mismatches), piRNAs corresponding to the D. japonica genome or transcripts were used for the subsequent analyses. Nucleotide frequency at each nucleotide position of the piRNAs was calculated and illustrated using the seqLogo package of Bioconductor (http://www.bioconductor.org/packages/2.12/bioc/html/seqLogo.html).

Transposon Annotation of Dugesia japonica Transcriptome
A survey of transposon taxonomic distribution was conducted by matching the EST unigenes to the protein sequences in Repbase (ver. 16.08; http://www.girinst.org/downloads/) using BLASTX software (ver. 2.2.25) with default settings. Only the top hits with E-value less than 1 × 10−6 were used for annotation.

RNA-Seq Analysis of DjpiwiB-Knockdown Planarians
miRNAs were prepared from planarians at 7 days after RNAi of GFP and DjpiwiB. RNA-seq was conducted with GS FLX + (Roche Diagnostics), Miseq (Illumina), or Hiseq2500 (Illumina). All sequence data were deposited in PRJDB4258. De novo transcriptome assembly for making reference sequences using reads obtained with Roche 454 and Miseq (Table S2) was conducted with Trinity with the CuffFly option (version r20140413p1) (Henschel et al., 2012). The assembled reference sequence was deposited as DRA: DRZ007413 and DDBJ: IAAB01000001-IAAB01137201. The reads obtained by Hiseq (Table S2) were mapped onto the reference sequence by aligning and estimating abundance.pl distributed with Trinity, by using Bowtie (Hemat et al., 2011) and xpress (Roberts and Pachter, 2012). The round estimated read counts were used for differentially expressed gene analysis as described by Sun et al. (2013). The analysis result was deposited as DRA: DRZ007434. For details of specific procedures, see Supplemental Information.

Treatment with MEK Inhibitor Followed by Feeding RNAi
MEK inhibitor treatment was carried out as described by Tasaki et al. (2011).

ACCESSION NUMBERS
The accession number for the piRNA-seq result reported in this paper is DRA: DRA002837. The accession number for RNA-seq and the results of the in silico analysis reported in this paper is DRA: PRJDB4258.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.develcel.2016.04.009.

AUTHOR CONTRIBUTIONS
N.S. and K.A. designed the study. N.S., M.K., and T.I. performed the study. M.K. and O.N. performed in silico analysis of the piRNAs. N.S., L.R., K.S., H.S., and M.S. performed IP and isolation of piRNA. S.Y. and K.M. performed the transmission electron microscopy observations. N.S., M.S., and K.A. wrote the manuscript.

ACKNOWLEDGMENTS
We are grateful to Elizabeth Nakajima, Hayoung Lee, Nobuyoshi Kumagai, Yuki Sato, and Takeshi Inoue for critical reading of the manuscript and to Prof. Yoshio Koyanagi of the Institute for Virus Research, Kyoto University, and Prof. Koichi Sano of Osaka Medical College for commenting on the transmission electron microscopy analysis. We also thank all of our laboratory members for their help and encouragement. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas to K.A. (22124001), a Grant-in-Aid for Creative Scientific Research to K.A. (17G0318), Global COE Program A08 of Kyoto University, the Naito Foundation, a Saskawa...