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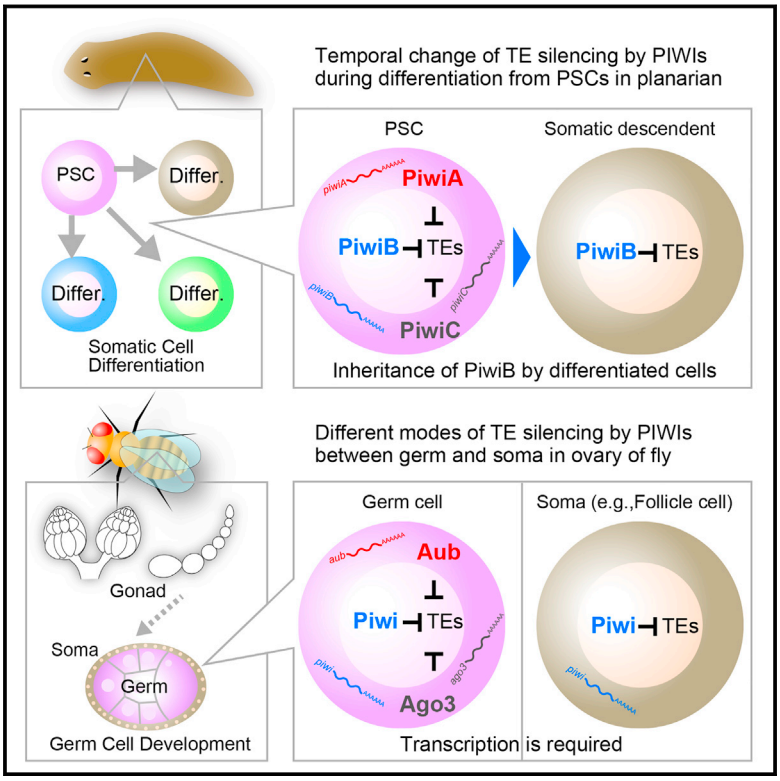
Inheritance of a Nuclear PIWI from Pluripotent Stem Cells by Somatic Descendants Ensures Differentiation by Silencing Transposons in Planarian

Graphical Abstract



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.

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

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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

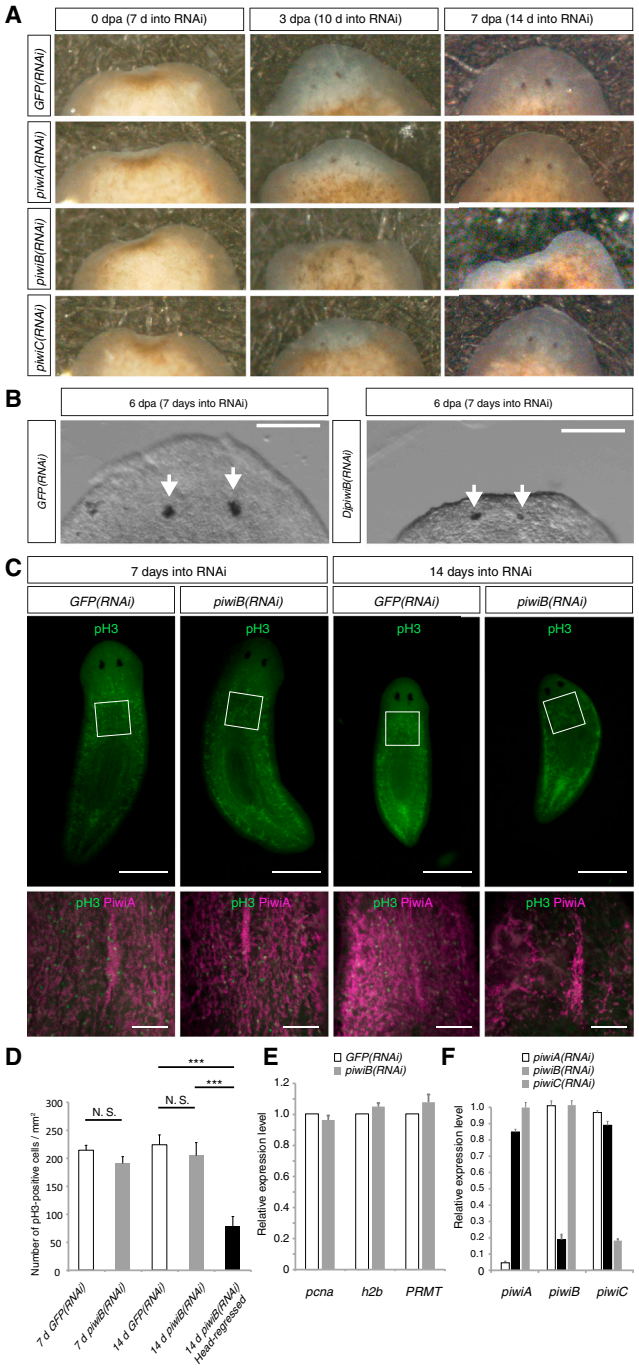

Figure 1. Effect of *DjpiwiB* RNAi

(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 *DjpwiB* 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.

piRNA features have been isolated by size fractionation, and many of these putative piRNAs matched TEs in *S. mediterranea* (Friedlander et al., 2009; Palakodeti et al., 2008). Two other planarians, *Dugesia japonica* and *Dugesia ryukyuensis*, also express three *piwi* family genes predominantly in neoblasts (*DjpiwiA*, *DjpiwiB*, and *DjpiwiC* in *D. japonica* and *Drpiwi-1*, *Drpiwi-2*, and *Drpiwi-3* in *D. ryukyuensis*) (Hayashi et al., 2010; Nakagawa et al., 2012). Although functional analyses of *smedwi* genes using RNAi suggested that *smedwi-2* and *smedwi-3* might be involved in differentiation (Palakodeti et al., 2008; Reddien et al., 2005), the specific roles of PIWI proteins in the differentiation of pluripotent neoblasts to specialized somatic descendants in planarians remain unknown. Furthermore, although piRNAs in *S. mediterranea* might target TEs (Friedlander et al., 2009; Palakodeti et al., 2008), the question of whether TEs are in fact repressed by these PIWI proteins in the planarian PSC system is still unanswered.

To address these questions, we examined how loss of individual PIWI proteins in *D. japonica* affects neoblast differentiation to somatic cells. We found that two cytoplasmic PIWI proteins and one nuclear PIWI protein act in neoblasts in *D. japonica*. Knockdown (KD) of the nuclear PIWI, *DjpiwiB*, by RNAi caused a severe defect in regeneration, whereas RNAi of *DjpiwiA* and/or *DjpiwiC* caused little or no regenerative defect, in accord with the findings reported for *S. mediterranea* (Palakodeti et al., 2008; Reddien et al., 2005). DjPiwiB-lacking neoblasts proliferated and repressed TEs normally, but *gypsy* transposon was derepressed at the onset of differentiation of neoblasts by KD of *DjpiwiB*. All three PIWI proteins were observed in neoblasts, but only nuclear DjPiwiB was inherited by almost all descendant differentiated cells, while transcription of *DjpiwiB* was discontinued in these descendant cells during the differentiation process. This suggests that a DjPiwiB-solo function in differentiating somatic cells ensures the repression of TE(s) during the differentiation process of planarian PSCs.

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

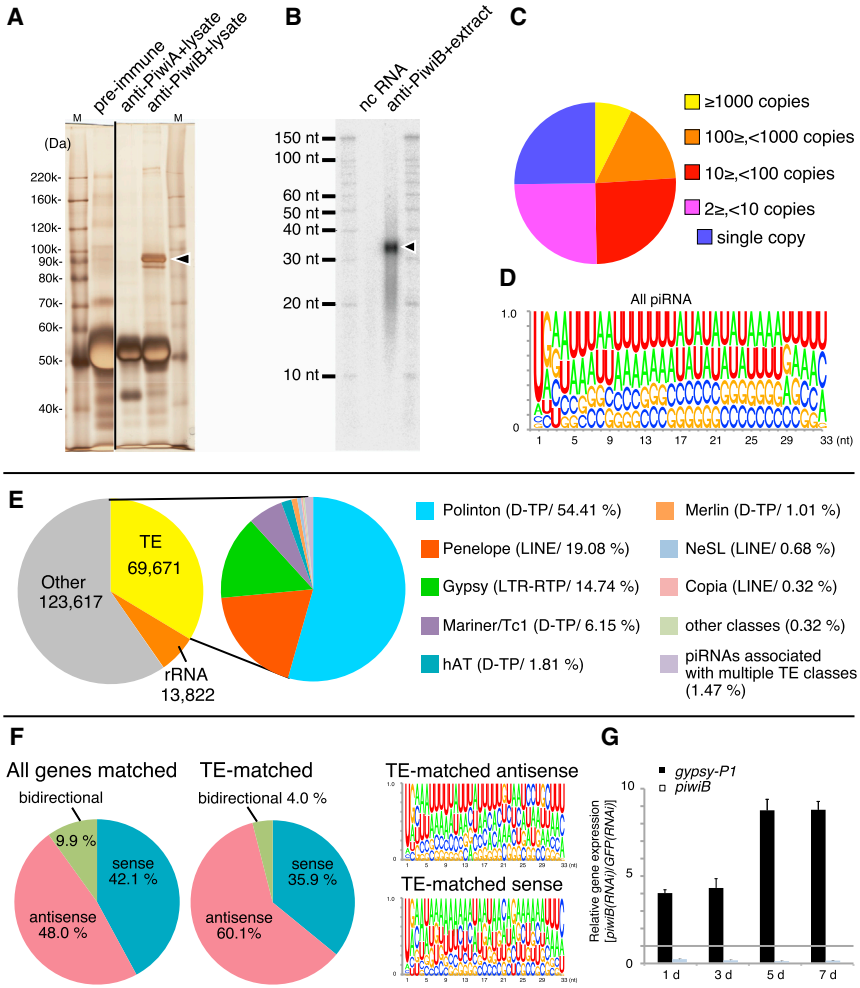

Figure 2. Analysis of DjPiwiB-Interacting piRNAs

(A) Analysis of IP proteins using anti-DjPiwiB, anti-DjPiwiA, and control antibodies by silver staining. Arrowhead indicates immunoprecipitated DjPiwiB protein.

(B) Enrichment of small RNAs by IP using anti-DjPiwiB antibody. ncRNA, RNA immunoprecipitated in the negative control. Arrowhead indicates immunoprecipitated small RNA.

(C) Frequency of representation of various RNA sequences among the total immunoprecipitated RNAs.

(D) Pictogram of nucleotide frequency in piRNA. Nucleotide frequency is represented by letter height.

(E) The fractions of piRNAs matching categories of *D. japonica* EST genes. Numbers indicate piRNA reads matching each category. piRNAs matched to TEs (69,671) were further categorized by types of TEs (pie chart on the right).

(F) Pie charts illustrating the percent of sense- or antisense-orientation piRNAs mapped to all EST-represented genes and TEs. Pictograms showing the nucleotide frequency of TE-matching piRNAs in both antisense and sense orientations.

(G) Expression levels of *DjpiwiB* and *gypsy-P1* detected by RT-qPCR analyses of *DjpiwiB(RNAi)* relative to control *GFP(RNAi)* planarians. The accession number of piRNAs bound to DjPiwiB is DRA: DRA002837. The experiments were performed in triplicate and the SDs were calculated using Microsoft Excel.

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 (*pcna, 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). Also, RNAi of *DjpiwiA* or *DjpiwiC* did not affect the expression level of *DjpiwiB* (Figure 1F). In contrast to the effects of *DjpiwiB* KD, KD of *DjpiwiA* or *DjpiwiC* had no effect on regeneration at 7 dpa (14 days into RNAi) (Figure 1A). However, at a later period (21 days into RNAi), the regenerated heads of *DjpiwiC(RNAi)* planarians regressed, like those in *DjpiwiB(RNAi)* planarians at 14 days into RNAi (Figure S1). These results agree with the reported observations that *smedwi-2* and *smedwi-3* in *S. mediterranea* (orthologs of *DjpiwiB* and *DjpiwiC*, respectively), but not *smedwi-1*, are critical for the regulation of planarian stem cells, suggesting distinct requirements for PIWI members during neoblast differentiation (Palakodeti et al., 2008; Reddien et al., 2005).

We found that DjPiwiB-depleted neoblasts proliferated normally at 7 days after *DjpiwiB(RNAi)*, in accord with findings reported for *S. mediterranea* (Figures 1C and 1D) (Reddien et al., 2005). However, *D. japonica*'s regenerative ability disappeared within 7 days after RNAi of *DjpiwiB* (Figures 1A and 1B). These results suggest that neoblasts in *D. japonica* require DjPiwiB in order to differentiate into somatic cells but not to proliferate.

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 immunoisolated 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 *polinton, 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 *Dj_aH_208_K05* (DDBJ: FY934805; referred to as *gypsy-P1*) was 4-fold higher than that in 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 differentiated 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

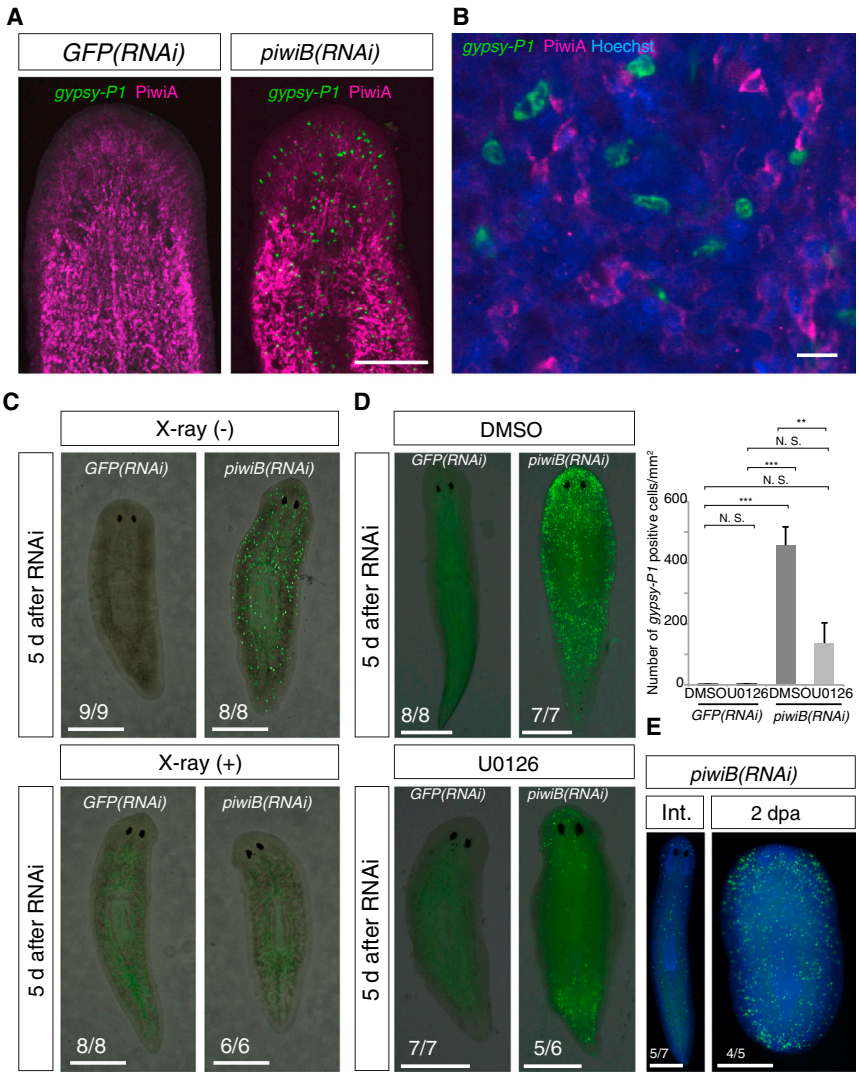

Figure 3. Analyses of Cells Expressing *gypsy-P1*

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)* planarian 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.

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 a few neoblasts that lacked DjPiwiB 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 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 DjPiwiB, we performed immunostaining using anti-poly(ADP-ribose) (PAR), because it is known that poly(ADP-ribosyl)ation (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 (Pellettieri 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

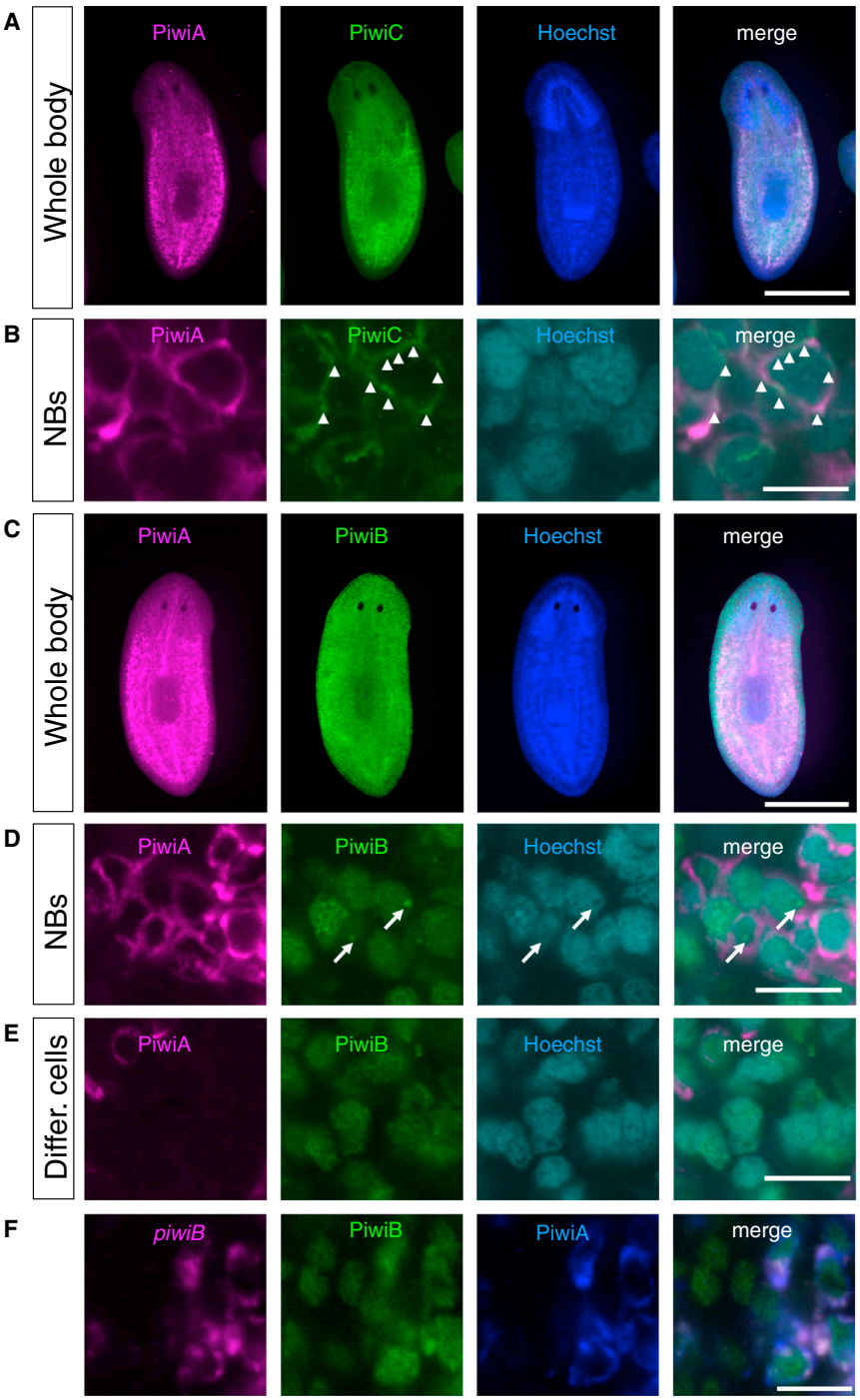

Figure 4. IHC Using anti-DjPiwiB Antibody

(A) Whole-mount co-immunofluorescence analyses using anti-DjPiwiA (magenta) and anti-DjPiwiC (green) antibodies. Hoechst 33342 staining is shown in blue. Scale bars, 1 mm in whole-body samples.

(B) Higher magnification of whole-mount sample of (A). Arrowheads indicate punctate signals of DjPiwiC. Scale bar, 10 μm. NBs, neoblasts.

(C) Co-immunofluorescence analyses using anti-DjPiwiA (magenta) and anti-DjPiwiB (green) antibodies. Hoechst 33342 staining is shown in blue. Scale bars, 1 mm.

(D) Neoblasts (NBs) in whole-body samples. Arrows indicate nucleoli. Scale bar, 10 μm.

(E) Differentiated cells (Differ. cells) in whole-body samples. Scale bar, 10 μm.

(F) Co-staining of *DjpiwiB* mRNA by in situ hybridization and DjPiwiB protein by immunostaining. Blue shows DjPiwiA protein. Scale bar, 10 μm.

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 *DjpiwiB*.

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

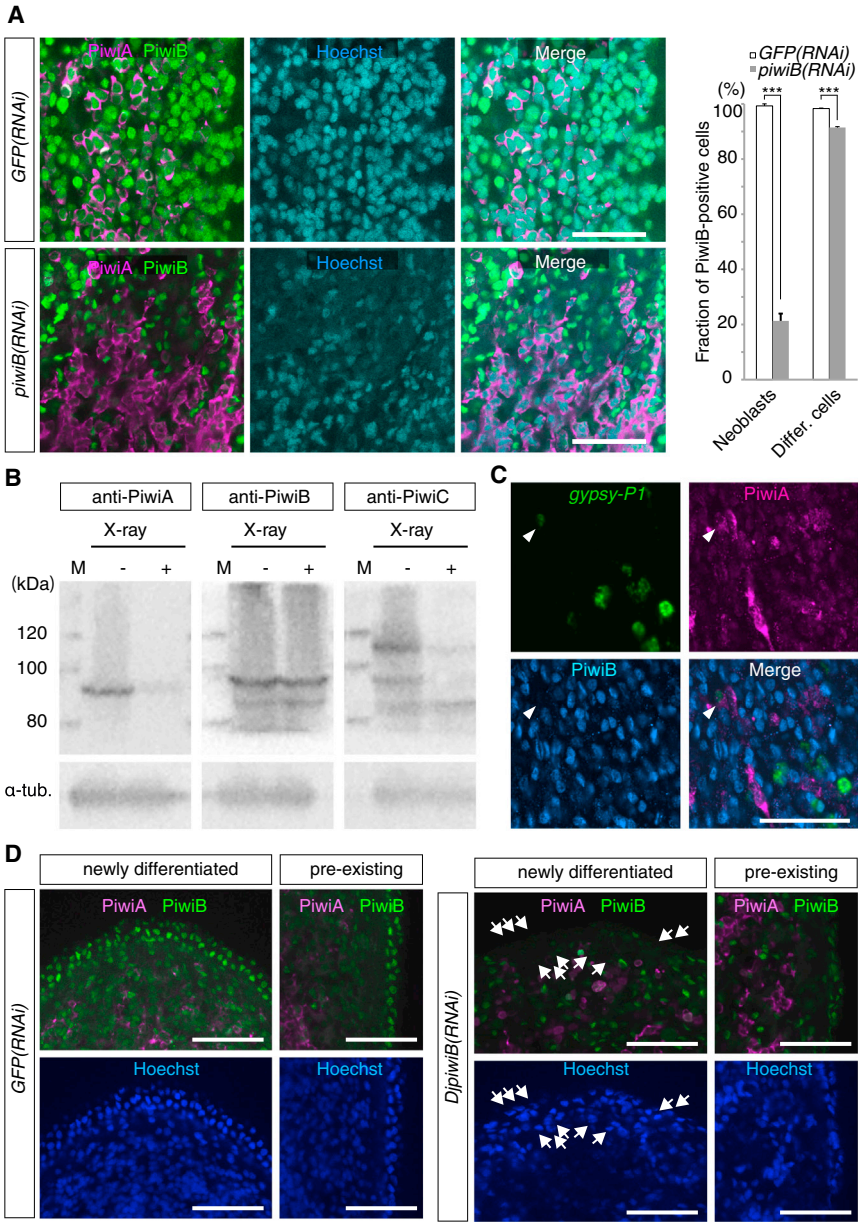

Figure 5. Inheritance of DjPiwiB by Differentiated Somatic Cells

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

(A) Immunostaining of DjPiwiA and DjPiwiB in *DjpiwiB(RNAi)* planarians at 7 days after the last feeding. Scale bar, 50 μm. Graph showing comparison of the number of DjPiwiB-positive neoblasts (left) and differentiated cells (right) between control *GFP(RNAi)* (white columns) and *DjpiwiB(RNAi)* (gray columns) animals. ***$p < 0.005$. The experiments were performed in triplicate and the SDs were calculated using Microsoft Excel.

(B) Western blot analysis using anti-DjPiwiA, -B, and -C antibodies in intact (X-ray –) and X-ray-irradiated (X-ray +) planarians. M, size markers.

(C) Expression of *gypsy-P1* in a neoblast. The arrowhead indicates a cell double positive for *gypsy-P1* (green) and DjPiwiA (magenta). DjPiwiB is shown in blue. Scale bar, 100 μm.

(D) IHC using anti-DjPiwiB antibody in de novo differentiated cells in partially regenerated planarians (see Figure S3). Arrows show DjPiwiB-negative and DjPiwiA-negative cells. Scale bar, 25 μm.

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 of 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.

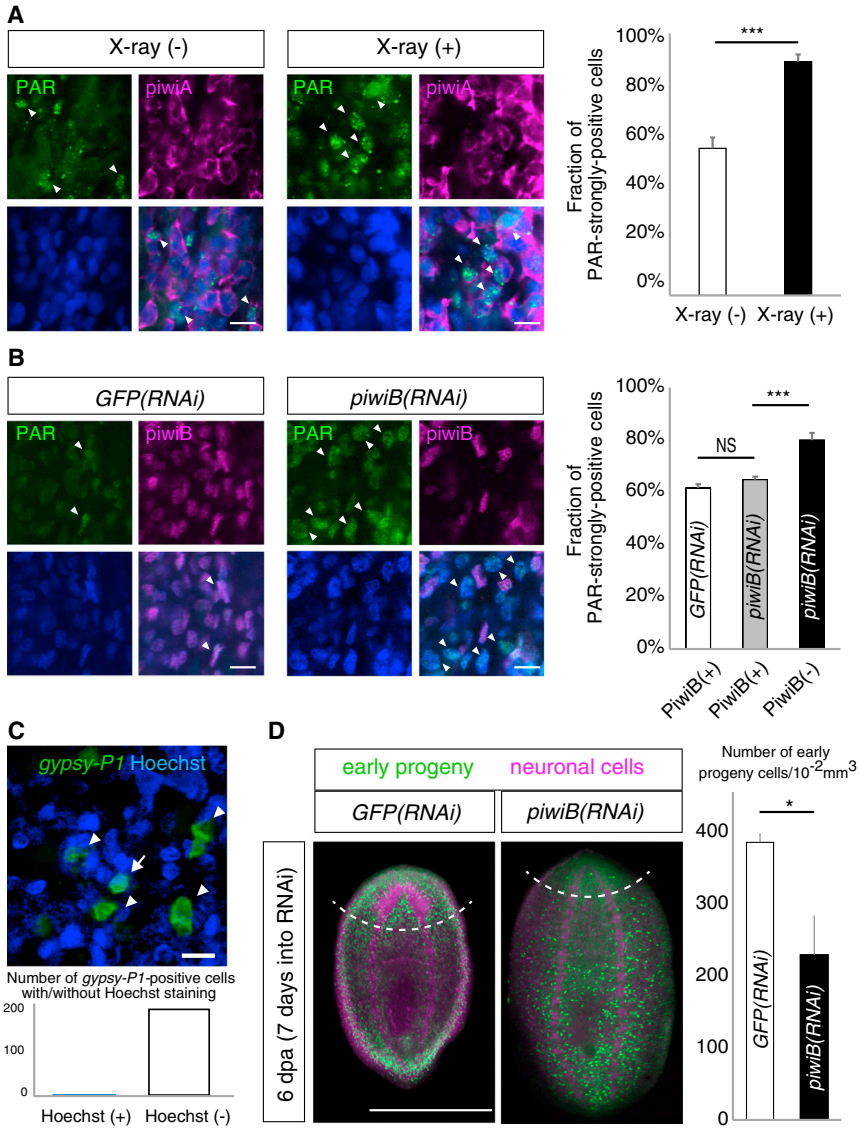

Figure 6. Influence of Depression of *gypsy-P1*

(A) IHC using anti-PAR antibody in intact or X-ray-irradiated planarians. Graph shows the fraction of PAR strongly positive cells in DjPiwiA-positive cells. Scale bars, 10 μm. Graph shows the fraction of PAR strongly positive cells (arrowheads) in DjPiwiA-positive cells. ***$p < 0.005$.

(B) IHC using anti-PAR antibody in control or *DjpiwiB(RNAi)* planarians. Graph shows the fraction of PAR strongly positive cells in DjPiwiB-positive or -negative cells. Arrowheads indicate cells with strong signals. Scale bars, 10 μm. Graph shows the fraction of PAR strongly positive cells in DjPiwiB-positive or -negative cells. ***$p < 0.005$.

(C) In situ hybridization of *gypsy-P1* with Hoechst staining. Arrow indicates double-positive cells. Arrowheads indicate *gypsy-P1* single-positive cells. Scale bar, 10 μm. Lower graph shows the number of cells double positive for *gypsy-P1* and Hoechst, and the number of *gypsy-P1* single-positive cells.

(D) In situ hybridization of early progeny marker (*Dj_aH_000_05955HH*) and immunostaining of synaptotagmin in control and *DjpiwiB(RNAi)* planarians. Dashed lines show regenerated head. Graph shows the number of early progeny marker-positive cells in the regenerated head region. Scale bar, 500 μm. *$p < 0.05$.

The experiments were performed in triplicate and the SDs were calculated using Microsoft Excel.

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

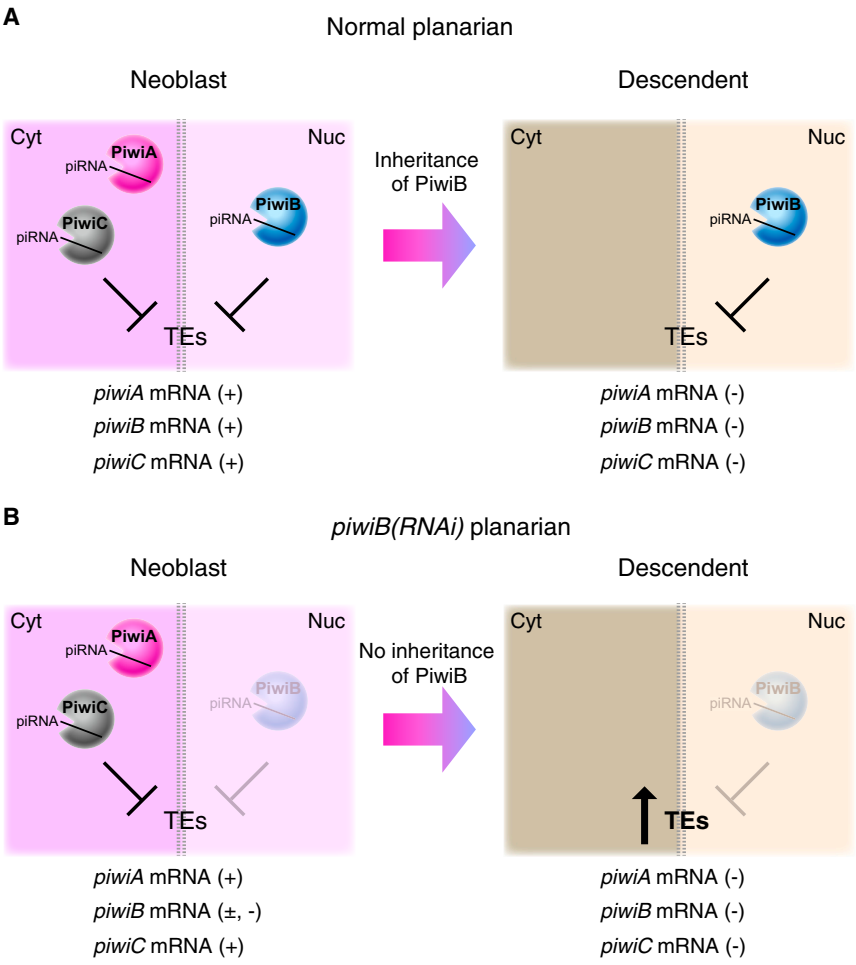

Figure 7. Schematic Drawing of Our Working Hypothesis about the Mechanism of Repression of TEs by Inherited DjPiwiB during the Process of Somatic Differentiation from the Neoblasts

(A) Normal differentiation from a neoblast.

(B) Differentiation from a neoblast in DjPiwiB-depleted planarian.

T bars indicate repression of TEs.

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 (KKPMRRERRKPGEEDKE, corresponding to amino acids 303–319), and DjPiwiC (FENSEKPTTSKFRRREH, 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 (Trevigen) were diluted 1/1,000. The samples were observed with a confocal microscope (FLUOVIEW FV10i; 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: ACCACCAACTGTTTAGCTCCCTTAG
RV: GATGGTCCATCAACAGTCTTTTGC
pcna
FW: ACCTATCGTGTCACTGTCTTTGACCGAAAA
RV: TTCATCATCTTCGATTTTCGGAGCCAGATA
h2b
FW: ATTCAAACATCCGTCCGTCT
RV: TTTTGTAACAGCCTTCGTTCC
PMRT
FW: AGTCAATAACGGTGAAGAGATAACTGG
RV: CCTTCCGACCTACCTCATTCG
DjpiwiA
FW: CGAATCCGGGAACTGTCGTAG
RV: GGAGCCATAGGTGAAATCTCATTTG
DjpiwiB
FW: ATGGATCCCATGGCTCCTAATG
RV: TGCACAGGGACAGGTACACG
DjpiwiC
FW: GGCCTGGAACTGTTAGAGTACCTG
RV: CAAACGGTCGCACAATAAATGAC

Gypsy-P1
FW: GTCTTTTCTGAAACGTTCAACGAAC
RV: CAGCCCATTTACATTTTGTAGGCTT

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 + T7, GAT CAC TAA TAC GAC TCA CTA TAG GGG AAT TCG GCA CGA GG; M13 Rv, GTT TTC CCA GTC ACG 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.

Prior to IP, 300 μl of 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 (or 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 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 1 hr at 4°C and washed five times with 1 ml of cold MSB solution for 3 min each, with centrifugation at 4°C at 5,000 × *g*. Finally, the supernatant was removed completely and the immunoprecipitate was used for protein electrophoresis or collection of RNAs.

Protein Electrophoresis and Silver Staining

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 H_2O 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 ^{32}P 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

mRNAs 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 using align_and_estimate_abundance.pl distributed with Trinity, by using Bowtie (Hatem et al., 2011) and eXpress (Roberts and Pachter, 2012). The rounded 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.devcel.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.

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