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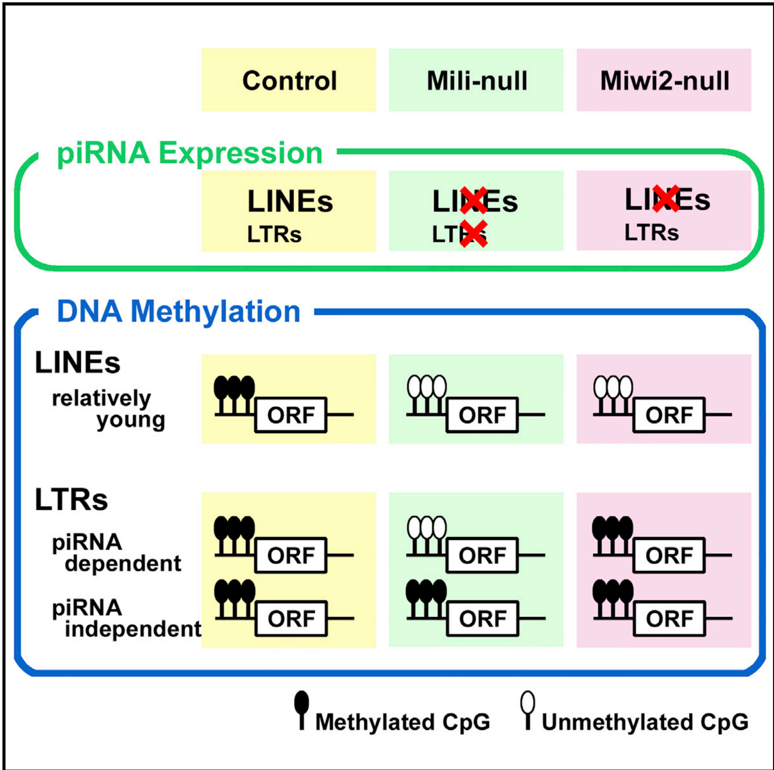
# Comprehensive DNA Methylation Analysis of Retrotransposons in Male Germ Cells

## Graphical Abstract




## Highlights

- A comprehensive DNA methylation analysis of transposable elements is presented
- DNA methylation of LINEs, but not LTRs, is dependent on piRNAs
- Younger LINEs show stronger dependence on piRNAs
- A subset of LTRs shows Mili-dependent, but Miwi2-independent, DNA methylation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## In Brief

piRNAs are crucial for de novo DNA methylation of retrotransposons in embryonic male germ cells. Nagamori et al. conduct a comprehensive DNA methylation analysis that examines the relationship between piRNA production and DNA methylation of LINE1 and LTR retrotransposons, which is more complicated than expected.

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# Comprehensive DNA Methylation Analysis of Retrotransposons in Male Germ Cells

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## SUMMARY

**De novo DNA methylation of retrotransposons is critical for silencing. Here, we use DNA methylation analysis to examine retrotransposons in mouse male germ cells. DNA methylation of long interspersed nuclear elements (LINEs) is dependent on piRNA, and younger LINEs exhibit greater piRNA dependence. In contrast, most long terminal repeat (LTR) retrotransposons produce lower levels of piRNAs and do not show significant piRNA dependence. The relationship between DNA methylation and corresponding piRNA expression of several LTR retrotransposons was reduced in Mili-null cells, but not Miwi2-null cells. These observations raise the possibility of piRNA-dependent DNA methylation without Miwi2. Therefore, it appears that the molecular mechanisms of the gene silencing of retrotransposons are more complicated than previously thought.**

## INTRODUCTION

Transposable elements (TEs) occupy approximately half of mammalian genomes and are divided into two major categories, retrotransposons and DNA transposons (Deininger and Batzer, 2002; Waterston et al., 2002). Retrotransposons are further classified into three subclasses: long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), and long terminal repeats (LTRs.) Among these types, a subset of LINEs and LTRs possesses intrinsic promoter activity (Okamura and Nakai, 2008). Although the vast majority of TEs are inactive due to gene degeneration during evolution, potentially active TEs are silenced by epigenetic regulation, such as DNA methylation (Bourc'his and Bestor, 2004; Kaneda et al., 2004).

In the process of mammalian embryonic male germ cell development, global DNA demethylation takes place in primordial germ cells (PGCs), and subsequent genome-wide de novo DNA methylation proceeds (Lee et al., 2014). TEs are not exceptional in this erasure and re-establishment of the DNA methylation process. Recent studies have shown that a subset of non-coding small RNAs, PIWI-interacting RNAs (piRNAs), participate in the de novo DNA methylation of some classes of retrotransposons, such as LINEs and intracisternal A particles (IAPs) (Aravin et al., 2007; Carmell et al., 2007; Kuramochi-Miyagawa et al., 2008). Mouse Piwi family proteins, mouse Piwi-like (MILI/PIWIL2) and mouse Piwi 2 (MIWI2/PIWIL4), are essential factors for the biogenesis of piRNAs and subsequent de novo DNA methylation of retrotransposons. It is widely thought that piRNAs initially guide the silencing machinery to retrotransposons (Aravin and Hannon, 2008).

The piRNA biogenesis in embryonic male germ cells is divided into two processes, primary and secondary processing (Siomi and Kuramochi-Miyagawa, 2009). MILI-bound piRNA is produced in the primary processing (Aravin et al., 2008; Shiromoto et al., 2013), and MILI participates in secondary processing, also known as the ping-pong amplification cycle (Aravin et al., 2008; Brennecke et al., 2007; De Fazio et al., 2011; Gunawardane et al., 2007). The cRNAs are annealed to MILI-bound piRNAs and cleaved by the slicer activity of MILI (Aravin et al., 2008). These piRNAs are then transferred to MIWI2, and a similar molecular process continues. MIWI2-bound anti-sense piRNAs are considered critically important for silencing retrotransposons based on the following evidence. First, at the time of de novo DNA methylation, the vast majority of piRNAs correspond to retrotransposon sequences (Aravin et al., 2008; Shoji et al., 2009; Vagin et al., 2009). Second, piRNA-bound MIWI2 are, but MILI and the unbound MIWI2 are not, localized in the nucleus (Aravin et al., 2008). Third, DNA methylation of retrotransposons does not take place appropriately in mutant mice in which piRNA production is impaired (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008; Shoji et al., 2009; Watanabe et al., 2011a; Zhang et al., 2012).

Particular subsets of TEs, such as LINE1 and IAP1Δ1, have been reported as the TEs whose DNA methylation can be induced by the piRNA pathway in embryonic male germ cells (Aravin et al., 2007; Kuramochi-Miyagawa et al., 2008; Watanabe et al., 2011b). In contrast, it is known that methylation of some TEs, such as SINEs, is independent of piRNA (Shoji et al., 2009). However, the whole picture of the relationship between DNA methylation and piRNA remains unknown. Methylome

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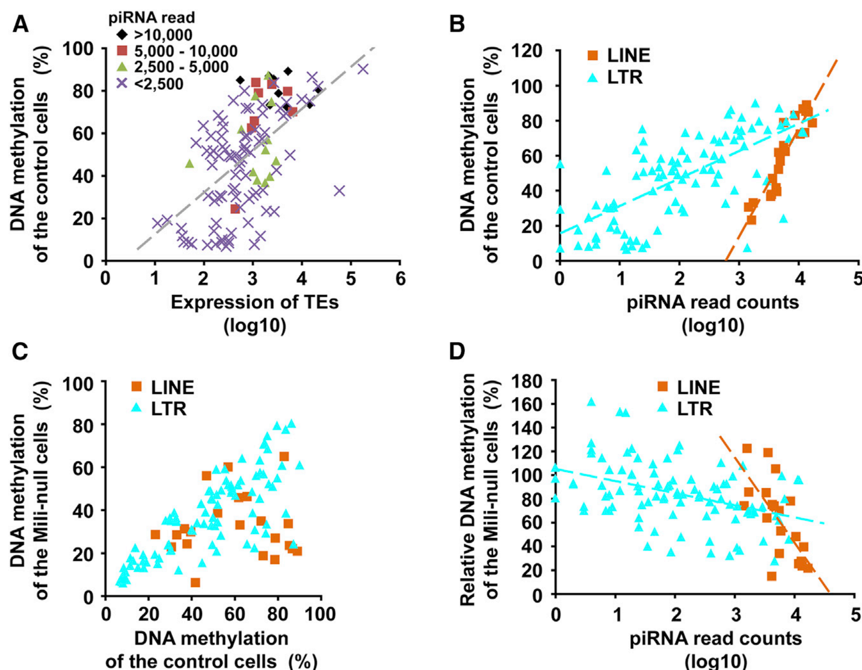


**Figure 1. DNA Methylation, Retrotransposon Expression, and piRNA Production of Retrotransposons in Control and Mili-Null Male Germ Cells**

(A) Relationship between retrotransposon expression and DNA methylation levels of the control (Mili-+/−) germ cells is shown.

(B) Relationship between piRNA read counts of embryonic testes and DNA methylation of the male germ cells from control mice is shown.

(C) DNA methylation in the control and the Mili-null germ cells is shown.

(D) Relationship between piRNA read counts of TEs in control embryonic testes and relative DNA methylation levels in day-10 Mili-null male germ cells. DNA methylation levels at 500-bp windows of TE in day-10 male germ cells were analyzed by PBAT.

RPKM of TEs in E15 gonocytes is represented as the level of TE expression (A) and piRNA read counts (B and D) on E16 testes. LINEs and LTRs are indicated by orange squares and light blue triangles, respectively (B–D). Regression lines of LINEs (orange), LTRs (light blue), and both (gray) are shown. See also Table S2.

analysis of embryonic germ cells would reveal such a relationship, but conventional whole-genome bisulfite sequencing (WGBS) requires approximately $1 \times 10^6$ cells (Kobayashi et al., 2012; Popp et al., 2010), and it is difficult to collect a sufficient number of embryonic germ cells.

In this study, we adopted a strategy termed post-bisulfite adaptor tagging (PBAT), which provides an unbiased methylome analysis using a relatively small number of cells (Kobayashi et al., 2013; Miura et al., 2012). This method successfully obtained methylome data on retrotransposons. Thus, we reveal a relationship between piRNA expression and DNA methylation among the control and Mili-null and Miwi2-null mice and a previously unidentified function of MILI.

## RESULTS

### Verification of PBAT Analysis and the Relationship between piRNA Expression and DNA Methylation of TEs

First, we purified spermatogonia from 10-day-old mice with various genotypes using the fluorescent Oct4-EGFP transgene and confirmed the purity by the patterns of DNA methylation of a paternal imprinted gene, H19, and a maternal imprinted gene, peg1 (Figure S1). Bisulfite-treated DNA libraries by PBAT were produced from 16 individual mice (four Mili-heterozygous [het], four Mili-null, four Miwi2-het, and four Miwi2-null mice). All PBAT reads from each library were mapped to 301 consensus sequences of TEs to compare all the libraries (Table S1). We analyzed the data of the regions with more than 50 reads per 500-bp windows.

To ensure fidelity, we compared the DNA methylation levels of the regions of individual libraries (Figure S2). All samples showed highest correlation within the same genotype (Mili-het, 0.9326–1.0; Mili-null, 0.8374–1.0; Miwi2-het, 0.9336–1.0; Miwi2-null, 0.9467–1.0) (Figure S2). Next, we combined the libraries of the same genotype, then compared with previously deposited data of wild-type sperm (DRA002402), Mili-control, and Mili-null spermatocyte (Molaro et al., 2014). Correlations among sperm, Mili/Miwi2-heterozygous (in our analysis), with Mili-control spermatocyte, and among Mili/Miwi2-null (in our analysis) with Mili-null spermatocyte were very high (Figures S3). These experiments assured our PBAT validation.

In the following analysis, we defined promoters of 1,000 bp upstream from the consensus sequences of LINE and LTR retrotransposons, and we analyzed the data of DNA methylation of the region. Based on the criteria, 115 retrotransposons (22 LINEs and 93 LTRs) were subjected to the following analysis. The relationship between the expression of 115 retrotransposons from purified embryonic day 15 (E15) control male germ cells and the percentages of DNA methylation of corresponding regions from day-10 mice are shown in Figure 1A. A weak correlation was revealed between expression and DNA methylation levels ($y = 19.627x - 7.3199$, $r = 0.5754$), showing that greater DNA methylation was associated with greater retrotransposon expression. Figure 1A also demonstrates that, in general, retrotransposons with relatively high expression of E16 piRNA were correlated with high DNA methylation levels. The piRNA expression was then plotted against DNA methylation levels; the data from two different classes of retrotransposons, LINEs and LTRs, are shown (Figure 1B; Table S2).

Although both LINEs and LTRs showed a correlation between DNA methylation and piRNA expression (LINEs: $y = 61.663x - 171.19$, $r = 0.921$; LTR: $y = 15.685x + 15.643$, $r = 0.6843$), the correlation strength was different. The correlation for LINEs was higher than that for LTRs, suggesting that piRNA is a more important factor for the induction of DNA methylation of LINE retrotransposons. In contrast, DNA methylation levels

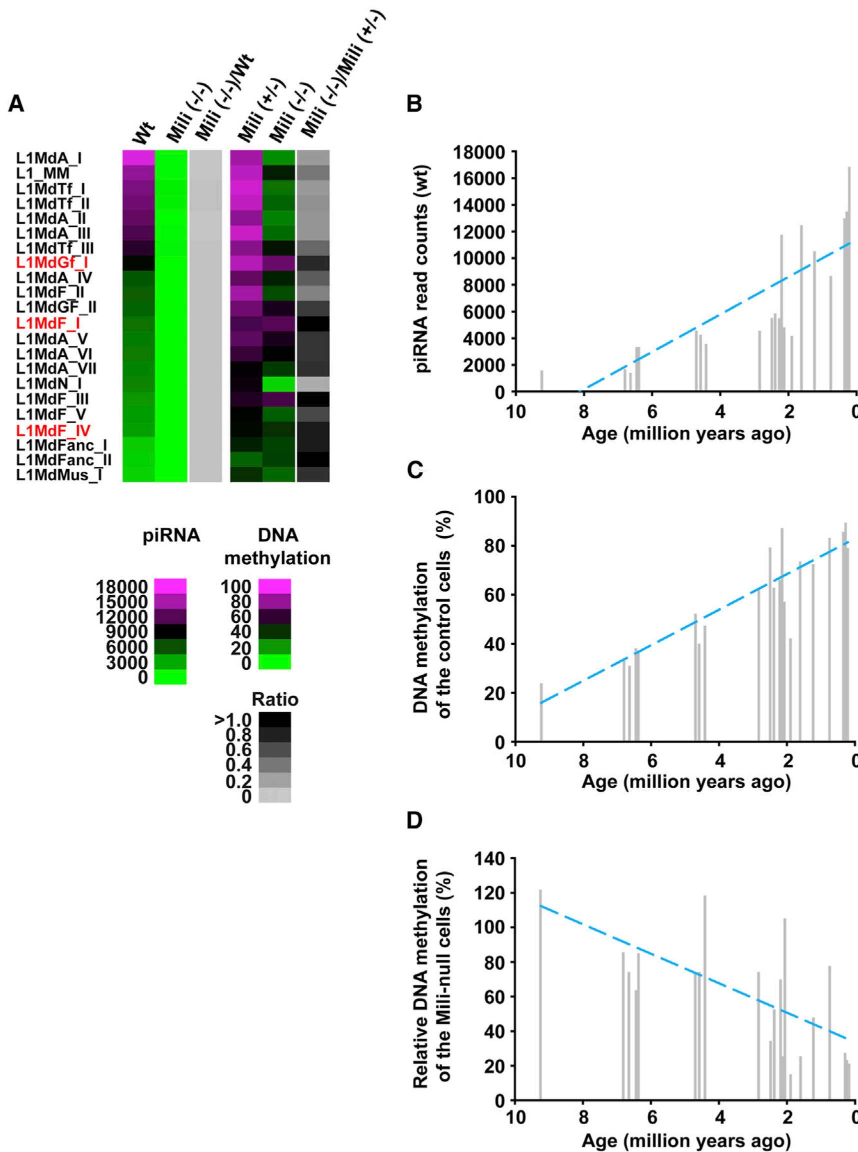


**Figure 2. Relationship among DNA Methylation, piRNA Production, and Evolutional Age of LINE Retrotransposons**

(A) Heatmap analysis of piRNA production and DNA methylation. piRNA read counts from the wild-type and the Mili-null E16 testes, as well as ratios, are shown (left). DNA methylation levels of the control (Mili-+/−) and the Mili-null day-10 male germ cells, as well as ratios, are shown (right). The ratios of piRNA expression and DNA methylation were calculated as follows: those of Mili-null cells were divided by those of control cells, respectively. The piRNA read counts were normalized by the microRNA (miRNA) read number.

(B–D) Relationship between the age of individual LINE retrotransposons and piRNA read counts (B), DNA methylation levels in the control (Mili-+/−) cells (C), and relative DNA methylation levels in the Mili-null germ cells (D). Ages of each LINE family were cited by a previous report (Sookdeo et al., 2013). Regression lines with the age of LINE retrotransposons are shown. See also Table S3.

varied in LTR retrotransposons, several of which exhibited very low expression levels (<100 read counts at E16) of the corresponding piRNAs that were significantly methylated (>40%). These data strongly suggest that a significant proportion of LTRs is methylated in a piRNA-independent manner. Taken together, it is quite reasonable to consider that piRNA-dependent DNA methylation is not a singular molecular mechanism. More specifically, the mechanism of LINEs is likely different from that of LTRs.

## DNA Methylation Levels and piRNA Amounts in LINEs and LTRs in Mili-Null Male Germ Cells

To reveal a comprehensive relationship between piRNA production and DNA methylation of retrotransposons, PBAT analysis was carried out in the Mili-null male germ cells. Significant proportions of the windows showed reduced DNA methylation in the Mili-null mutants (Figure 1C; Table S2), and the patterns of the reduction in DNA methylation in LINEs and LTRs were essentially the same. The relationship between the piRNA read counts of the control male germ cells and relative DNA methylation levels of the Mili null-germ cells is shown in Figure 1D. Considering that piRNA production in the Mili-null male germ cells is severely impaired, the reduction in DNA methylation in the cells should reflect the piRNA dependence of DNA methylation. The correlation of LINE retrotransposons was strong ($y = -71.732x - 329.92$, $r = -0.7067$). In other words, greater amounts of wild-type piRNA were associated with greater piRNA dependence of DNA methylation. Detailed LINE data are shown in Figure 1D as heatmaps of piRNA production and DNA methylation of both the wild-type and the Mili-null male germ cells (Figure 2A).

Next we analyzed piRNA production and DNA methylation from the perspective of the age of LINE retrotransposons. As shown in Figure 2B, the younger LINEs gave rise to more piRNAs ($y = -1403.3x + 11399$, $r = -0.8013$). Consistent with these data, the younger the LINEs were, the higher were the DNA methylation levels (Figure 2C; Table S3; $y = -7.2497x + 82.928$, $r = -0.8922$). Although it was relatively weak, piRNA-dependent DNA methylation also showed a correlation with the age of LINE retrotransposons (Figure 2D; Table S3; $y = 8.5224x + 33.651$, $r = 0.6751$). In contrast to the piRNA-dependent DNA methylation of LINE retrotransposons, LTRs showed lower piRNA dependence (Figures 3 and 1D; $y = -10.197x + 105.17$, $r = -0.4071$; Table S2). However, DNA methylation was significantly high in the LTR retrotransposons (Figure 1B).

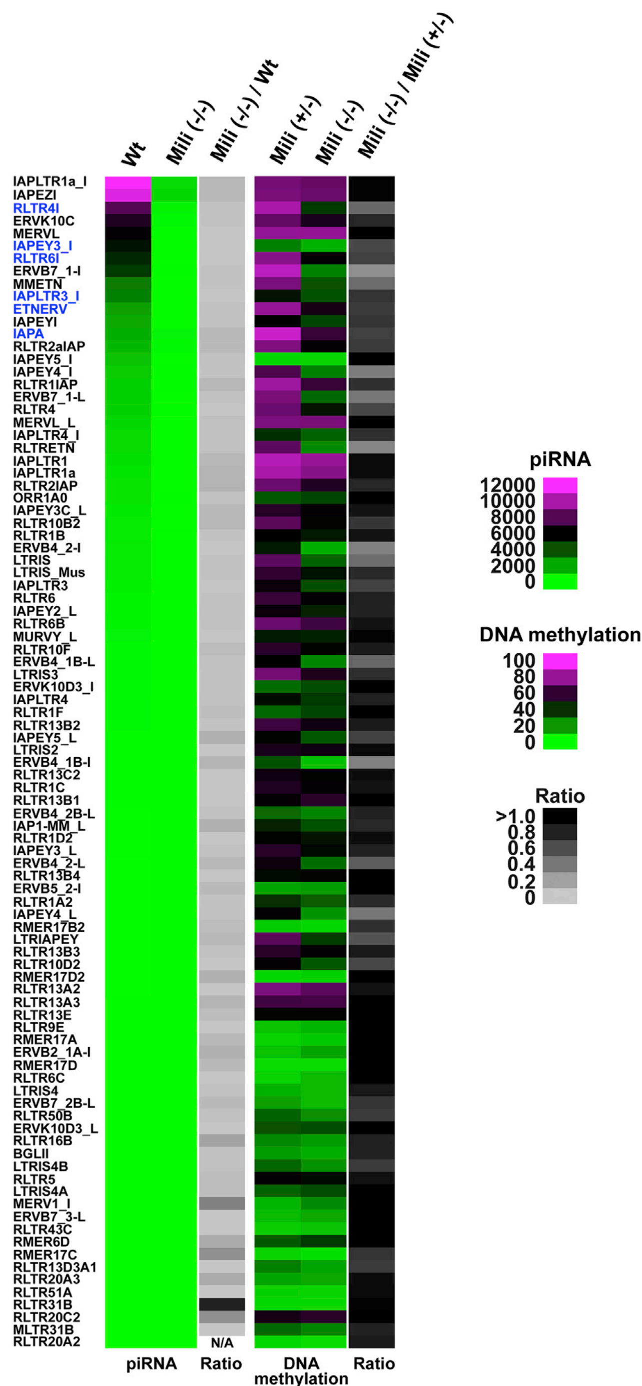


**Figure 3. Relationship between DNA Methylation and piRNA Production of LTR Retrotransposons**

Heatmap analysis of piRNA production and DNA methylation. The piRNA read counts of the wild-type and the Mili-null E16 testes and ratios are shown (left). DNA methylation levels of the control (Mili-+/−) and the Mili-null day-10 male germ cells and ratios are shown (right). The ratios of piRNA expression and DNA methylation were calculated as follows: those of Mili-null cells were divided by those of control cells, respectively. The piRNA read counts were normalized by the miRNA read number. See also Table S2.

## DNA Methylation Levels and piRNA in Miwi2-Null Male Germ Cells

MIWI2 is a protein essential for ping-pong amplification of piRNAs as well as MILI, and it is believed to be directly involved in the suppression of retrotransposon expression in nuclei. Nine LINE retrotransposons showed a reduction in DNA methylation levels in the Miwi2-null male germ cells (Figure 4A). Compared with the data from the Mili-null male germ cells (Figure 1C), it is notable that the number of such retrotransposons was smaller and only LINE retrotransposons were affected. The relationship between the Miwi2-null germ cells and the amount of piRNAs in the control germ cells and relative DNA methylation was plotted (Figure 4B), in a manner similar to that of the Mili-null germ cells (Figure 1D). The inverse relationship of LINEs was similar between the Mili-null and the Miwi2-null conditions (Mili: $y = -71.732x + 329.92$, $r = -0.7067$; Miwi2: $y = -105.26x + 453.7$, $r = -0.8152$). However, the LTR data showed little or no correlation (Mili: $y = -10.197x + 105.17$, $r = -0.4071$; Miwi2: $y = -2.07111x + 92.72$, $r = -0.0927$). Heatmap data from DNA methylation of the Miwi2-null germ cells are shown in greater detail (Figures 4C and 4D). The relationship between the DNA methylation of the Mili and Miwi2 germ cells is shown in Figure S4.

Notably, there were several exceptional LINE retrotransposons that differed from the general tendency. DNA methylation of LINEs was essentially dependent on piRNAs, as described. However, DNA methylation of the LINE retrotransposons, such as L1MdGF_I, L1MdF_I, and L1MdF_IV (Figures 2A and 4C, red), were independent of MILI and MIWI2. These data strongly suggest that DNA methylation of these retrotransposons can be introduced even in the absence of piRNAs. It is conceivable that DNA methylation of individual retrotransposons varies to some extent despite the general rule of piRNA dependence.

DNA methylation levels of LTR retrotransposons from the Mili-null germ cells were similar to those from the Miwi2-null cells (Figures 3 and 4D). However, the similarity of the LTRs was not as high as that of the LINE retrotransposons. The piRNA production of three LTRs with the highest piRNA production in the control embryonic testes (IAPLTR1a_I, RLTR4I, and ERVK10C) was not significantly altered by the Miwi2-null mutation. DNA methylation of RLTR4I was MILI, and presumably piRNA, dependent (at least partially; shown in blue in Figure 3). The other five LTRs (IAPEY3_I, RLTR6I, IAPLTR3_I, ETNERV, and IAPA; shown in blue in Figures 3 and 4) showed a pattern similar to that of RLTR4I. Meanwhile, notably, we reported that a subset of IAP, IAP1Δ1, which consists of only 5% of total IAP genes, showed the reduction of DNA methylation in both the Mili-null and Miwi2-null cells (Kuramochi-Miyagawa et al., 2008). Unfortunately, our analysis cannot discriminate the IAP species because of short reads of the next-generation sequencing.

DNA methylation of these six LTR retrotransposons was piRNA dependent to some extent, but not MIWI2 dependent. It has been reported that, in embryonic male germ cells, piRNA-bound MIWI2 is localized in the nucleus, and it presumably plays important roles in de novo DNA methylation of retrotransposons. In contrast, MILI has been reported to exist only in the cytoplasm, as has MIWI2 that does not bind piRNAs. These data suggest that MIWI2 should serve as an effector of piRNA-dependent

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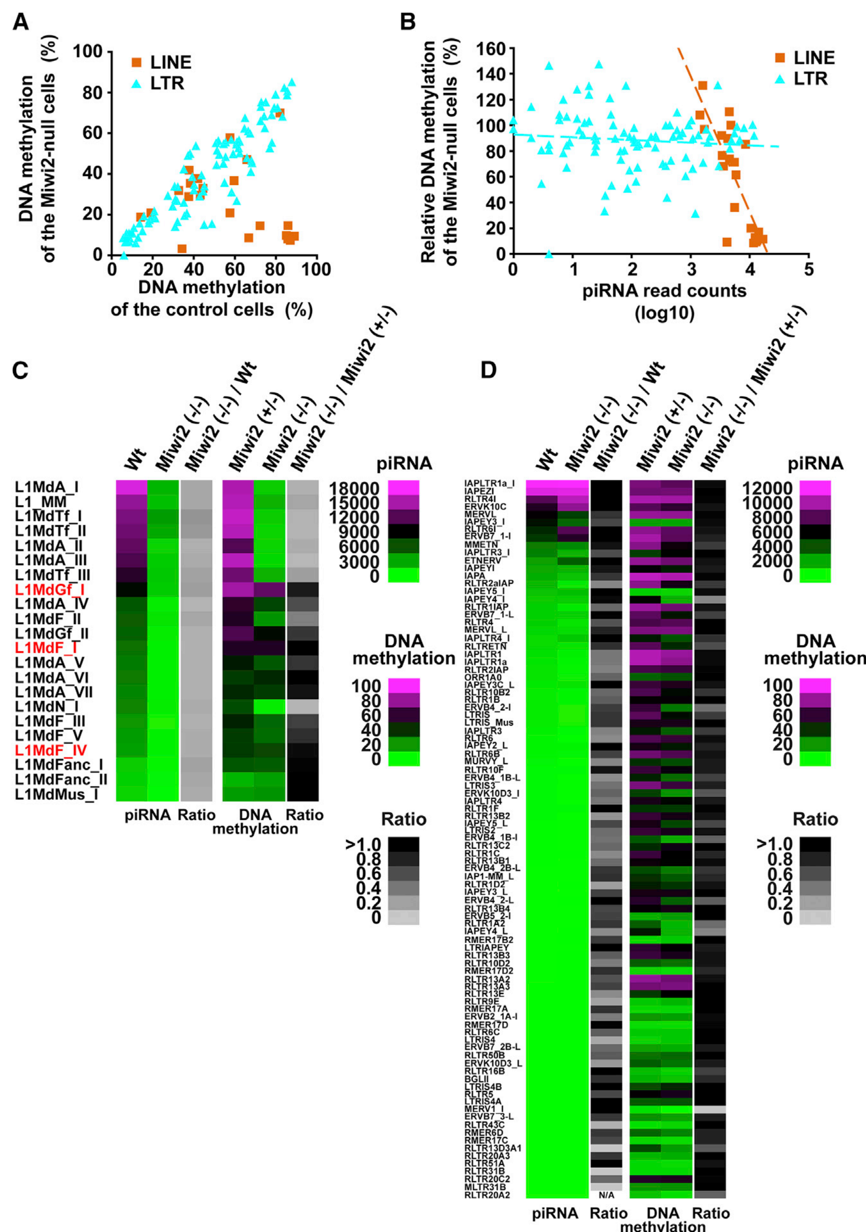


**Figure 4. Relationship between DNA Methylation and piRNA Production of Retrotransposons in Miwi2-Null Male Germ Cells**

(A) Relationship between DNA methylation levels in the control (Miwi2-+/−) and the Miwi2-null germ cells is shown.

(B) Relationship between piRNA read counts of retrotransposons in the control embryonic testis and relative DNA methylation levels in the Miwi2-null day-10 male germ cells. Regression lines of LINEs (orange) and LTRs (light blue) are shown.

(C and D) Heatmap analysis of piRNA production and DNA methylation. The piRNA read counts of the wild-type and the Miwi2-null E16 male testes and the ratios are shown (left). The piRNA read counts were normalized by the miRNA read number. DNA methylation levels of the control (Miwi2-+/−) and the Miwi2-null day-10 male germ cells and ratios are shown (right). The ratios of piRNA expression and DNA methylation were calculated as follows: those of Miwi2-null cells were divided by those of control cells, respectively. The relationship of LINEs (C) and LTRs (D) is shown.

DNA methylation levels at 500-bp windows of retrotransposons in the day-10 male germ cells were analyzed by PBAT. LINEs and LTRs are indicated by orange squares and light blue triangles, respectively (A and B). The piRNA read counts for E16 testes are shown (B–D). See also Table S2.

silencing of some subsets of retrotransposons. However, the six above-mentioned LTR retrotransposons are piRNA dependent, but Miwi2 independent, as discussed earlier. In other words, piRNA-dependent DNA methylation of retrotransposons can take place even under Miwi2-null conditions.

## DISCUSSION

A weak relationship between the expression of E15 male germ cell retrotransposons and DNA methylation levels from day-10 cells was revealed (Figure 1A). These data ostensibly seem opposite, as DNA methylation of promoter regions generally inhibits gene expression. Considering that the higher expression of piRNAs in E16 male germ cells would induce the higher DNA methylation, these data would be reasonable. However, the relationship among DNA methylation levels, retrotransposon gene expression levels, and the amount of piRNAs was unclear. In contrast, the relationship between DNA methylation levels and the amount of piRNAs exhibited a clear correlation, whereby the correlation with LINE retrotransposons was much stronger than that of LTR retrotransposons (Figures 1A and 1B).

DNA methylation of a significant proportion of both LINE and LTR retrotransposons was impaired in the Mili-null germ cells, in which piRNA production was severely impaired (Figure 1C). However, the relationship between the amount of piRNAs in the control germ cells and DNA methylation levels in the Mili-null germ cells was quite different with respect to LINEs and LTRs. DNA methylation of LINE retrotransposons exhibited a strong correlation with impaired DNA methylation in mutant germ cells, suggesting that DNA methylation of LINEs is dependent on piRNAs. However, while a relationship was observed in LTR retrotransposons, the correlation was weak (Figures 1D and 2A).

It has been reported that LINE retrotransposons tend to accumulate genetic abnormalities, such as mutations and deletions, and subsequently become inactivated. Based on this evidence, the ages of individual LINE retrotransposons were estimated (Sookdeo et al., 2013). All the data examined, including piRNA amount and DNA methylation in the wild-type germ cells

(Figures 2B and 2C) as well as piRNA dependence, namely the impairment of DNA methylation in the piRNA-deficient Mili-null germ cells (Figure 2D; Molaro et al., 2014), showed a significant correlation with the ages of LINE retrotransposons. These data imply evolutional mechanisms that suppress the activity of LINE retrotransposons. Specifically, the earlier mechanism is piRNA-dependent suppression, and the later one is genetic alterations.

The correlations between piRNA amounts and DNA methylation levels in the wild-type germ cells (Figure 1B) and the impairment of DNA methylation in the Mili-null germ cells (Figure 1D) indicate that DNA methylation of LINE retrotransposons is mostly dependent on piRNA. In contrast, the dependence was less evident for LTR retrotransposons, showing that DNA methylation of most LTRs was independent of piRNA. Even without a significant number of piRNAs, DNA methylation levels of several LTR retrotransposons was greater than 50% (Figure 1B). Furthermore, DNA methylation of many LTR retrotransposons was not impaired under the piRNA-deficient conditions.

It has been postulated that piRNA-bound MIWI2 acts as an effector for de novo DNA methylation of retrotransposons in embryonic male germ cells based on the following circumstantial evidence (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2010; Shoji et al., 2009). DNA methylation of retrotransposons was impaired in various mutant mice in which the production of piRNAs was reduced. Only piRNA-bound MIWI2 was localized to the nucleus, which was detected by immunohistochemistry. However, as shown in the study described herein, comprehensive analyses of Mili-null and Miwi2-null male germ cells strongly suggested that MILI can function as another effector to induce piRNA-dependent de novo DNA methylation of at least a proportion of retrotransposons.

This study discussed several molecular mechanisms of retrotransposon DNA methylation. Relationships between the amount of piRNAs and age were evident in LINE retrotransposons. In contrast, DNA methylation of LTR retrotransposons did not indicate piRNA dependence. Furthermore, our experiments utilizing physical methods suggest that at least a few retrotransposons can be silenced by nuclear-localized MILI bound to piRNAs. These data indicate that the molecular mechanism of retrotransposon DNA methylation is much more diversified than once believed. Such complicated mechanisms would be a legacy of the long history of an armament race between retrotransposons and animals.

## EXPERIMENTAL PROCEDURES

### Germ Cell Isolations, PBAT, and piRNA Analyses

Germ cells were sorted from 10-day-old Oct4-GFP transgenic mice via fluorescence using BD FACSAria II cell sorter (BD Biosciences). The multiplex PBAT method is described in detail on the CREST/International Human Epigenome Consortium (IHEC) Japan webpage (http://crest-ihec.jp/english/epigenome/index.html) and in the Supplemental Experimental Procedures. The piRNAs were mapped to TE consensus sequence, allowing two mismatches, by CLC Genomics Workbench version 7 (QIAGEN). Detailed methods of data analysis are given in the Supplemental Experimental Procedures. All animal experiments were performed in accordance with the general guidelines of The Institute of Experimental Animal Sciences, Osaka University Medical School.

## ACCESSION NUMBERS

The accession number for all PBAT sequencing data reported in this paper is DNA Data Bank of Japan (DDBJ): DRA003075.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.060.

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