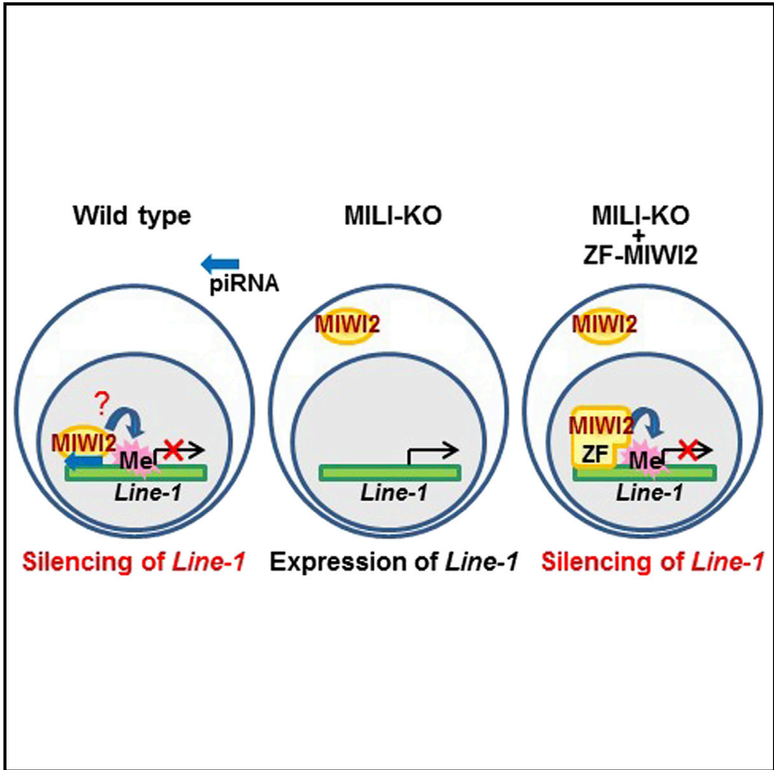
Cell Reports

Report

MIWI2 as an Effector of DNA Methylation and Gene Silencing in Embryonic Male Germ Cells

Graphical Abstract



Highlights

- A zinc-finger protein that recognizes the type A LINE-1 retrotransposon was designed
- De novo DNA methylation without piRNAs was induced by the ZF-MIWI2 fusion protein
- ZF-MIWI2 partially rescued impaired spermatogenesis in piRNA-defective mice
- MIWI2 was associated with proteins involved in de novo DNA methylation

Authors

Kanako Kojima-Kita,
Satomi Kuramochi-Miyagawa,
Ippei Nagamori, ..., Takashi Akazawa,
Norimitsu Inoue, Toru Nakano

Correspondence

tnakano@patho.med.osaka-u.ac.jp

In Brief

Kojima-Kita et al. use zinc-finger technology to argue that MIWI2 functions as an effector of de novo DNA methylation of retrotransposons in embryonic male germ cells.

Kojima-Kita et al., 2016, Cell Reports *16*, 2819–2828
September 13, 2016 © 2016 The Authors.
http://dx.doi.org/10.1016/j.celrep.2016.08.027

CellPress

OPEN ACCESS
CellPress

MIWI2 as an Effector of DNA Methylation and Gene Silencing in Embryonic Male Germ Cells

Kanako Kojima-Kita,[1] Satomi Kuramochi-Miyagawa,[1,3] Ippei Nagamori,[1] Narumi Ogonuki,[4] Atsuo Ogura,[4] Hidetoshi Hasuwa,[5,7] Takashi Akazawa,[6] Norimitsu Inoue,[6] and Toru Nakano[1,2,3,8,*]
[1]Department of Pathology, Medical School
[2]Graduate School of Frontier Biosciences
Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[3]CREST, Japan Science and Technology Agency
[4]RIKEN BioResources Center, Tsukuba 305-0074, Ibaraki, Japan
[5]Department of Experimental Genome Research, Research Institute for Microbial Diseases, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan
[6]Department of Tumor Immunology, Research Institute, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-2 Nakamichi, Higashinari-ku, Osaka 537-0025, Japan
[7]Present address: Department of Molecular Biology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[8]Lead Contact
*Correspondence: tnakano@patho.med.osaka-u.ac.jp
http://dx.doi.org/10.1016/j.celrep.2016.08.027

SUMMARY

During the development of mammalian embryonic germ cells, global demethylation and de novo DNA methylation take place. In mouse embryonic germ cells, two PIWI family proteins, MILI and MIWI2, are essential for the de novo DNA methylation of retrotransposons, presumably through PIWI-interacting RNAs (piRNAs). Although piRNA-associated MIWI2 has been reported to play critical roles in the process, its molecular mechanisms have remained unclear. To identify the mechanism, transgenic mice were produced; they contained a fusion protein of MIWI2 and a zinc finger (ZF) that recognized the promoter region of a type A LINE-1 gene. The ZF-MIWI2 fusion protein brought about DNA methylation, suppression of the type A LINE-1 gene, and a partial rescue of the impaired spermatogenesis of MILI-null mice. In addition, ZF-MIWI2 was associated with the proteins involved in DNA methylation. These data indicate that MIWI2 functions as an effector of de novo DNA methylation of the retrotransposon.

INTRODUCTION

Approximately half of the mammalian genome is composed of transposable elements (TEs) (Lander et al., 2001). Although TEs can affect genome expansion and are thought to be important in evolutionary processes, their expression is potentially harmful due to insertional mutagenesis (Kazazian, 2004). Molecular defense mechanisms involving epigenetic transcriptional regulation were acquired during the evolutionary process to silence TEs (Slotkin and Martienssen, 2007). In mammals, DNA methylation and histone modification represent two major mechanisms (Walsh et al., 1998). For example, DNA methyltransferase-1 (Dnmt1) and ERG-associated protein with an SET domain (ESET) histone methyltransferase (also known as H3K9me3 methyltransferase) are required for retrotransposon silencing in embryonic stem cells (ESCs) (Lei et al., 1996; Matsui et al., 2010).

DNA methylation levels in the genome, including those of retrotransposons and imprinting genes, change dynamically during germ cell development. The vast majority of DNA methylation is lost in the primordial germ cells by embryonic day (E) 13.5 (Lane et al., 2003). De novo DNA methylation subsequently takes place in non-dividing prospermatogonia (i.e., gonocytes) from E16.5 to E18.5 and is completed by the time of birth (Li et al., 2004; Lucifero et al., 2002). The de novo DNA methyltransferases Dnmt3a and Dnmt3b, and the related Dnmt3L protein, play essential roles in this process, and the establishment of the pattern of DNA methylation is a prerequisite for spermatogenesis and other cell lineages (Bourc'his and Bestor, 2004; Kaneda et al., 2004; Kato et al., 2007; Lees-Murdock et al., 2005; Okano et al., 1999; Sakai et al., 2004).

P-element-induced wimpy testis (PIWI) was originally identified as a protein essential for *Drosophila* germ cell development, and it was necessary in the biogenesis of PIWI-interacting RNAs (piRNAs). PIWI family members are evolutionarily conserved in diverse organisms, ranging from *Caenorhabditis elegans* to mammals, and they play important roles in germ cell development (Cox et al., 1998). In mice, the PIWI family proteins MILI and MIWI2 are expressed from the primordial germ cell stage to the round spermatid stage and from the gonocyte until soon after birth, respectively (Kuramochi-Miyagawa et al., 2008). The developmental stage of the concomitant expression of MILI and MIWI2 corresponds to the timing of de novo DNA methylation of TEs, and both MILI and MIWI2 play critical roles in piRNA production, the subsequent de novo DNA methylation, and spermatogenesis (Aravin et al., 2007, 2008).

This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

OPEN
ACCESS
CellPress

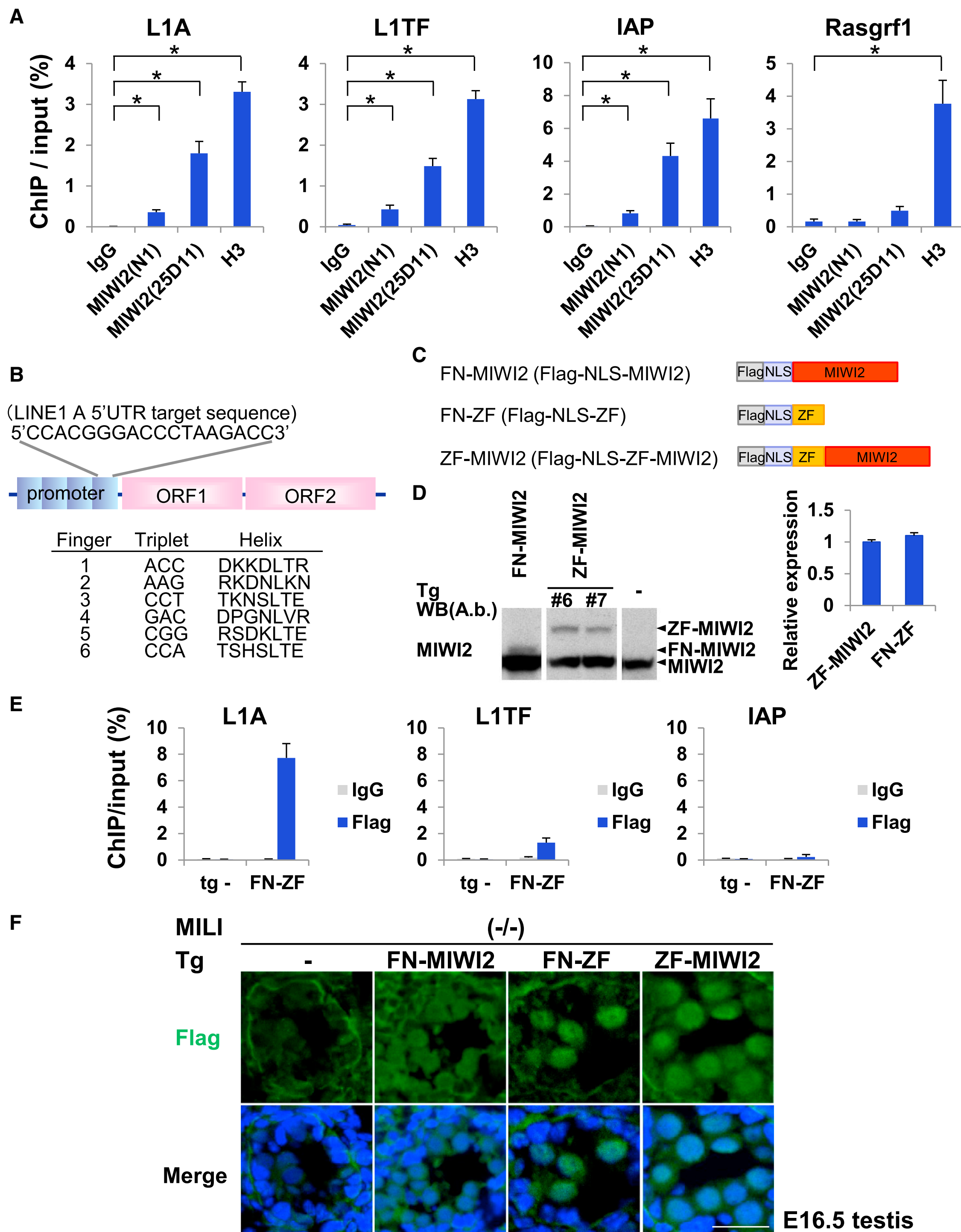


Figure 1. Design of the ZF and Production of Transgenic Mice

(A) ChIP-qPCR analysis of E16.5 testis with two kinds of anti-MIWI2 antibodies (MIWI2-N1 and 25D11). The embryonic testis lysates were chromatin immunoprecipitated with control IgG, anti-MIWI2 antibodies, or Histone H3 antibody (n = 3). The 5′ UTR region of LINE-1 (A and TF types), the long terminal repeat (LTR) region of IAP, and theDMR of Rasgrf1 were analyzed. Error bars denote SD. Statistically significant differences by t test, *p < 0.05.

(B) ZF target type A LINE-1 promoter sequence (18 bp) and amino acid sequences of the recognition helix corresponding to DNA triplets. The triplets from 3′ to 5′ correspond to the helices from the N terminus to the C terminus.

(C) Schematic diagrams show the FLAG-tagged NLS-MIWI2 (FN-MIWI2), FLAG-tagged NLS-ZF (FN-ZF), and FLAG-tagged-NLS-ZF-MIWI2 (ZF-MIWI2).

(legend continued on next page)

The piRNAs belong to a subset of small noncoding RNAs and are almost exclusively expressed in germ cells (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006). They are considered to be required for de novo DNA methylation of long interspersed nuclear elements (LINE-1) and 1Δ1 type intracisternal A particle (IAP) retrotransposons in the gonocytes. The majority of piRNAs expressed at the phase of de novo DNA methylation in the gonocytes correspond to TE sequences (Aravin et al., 2007; Kuramochi-Miyagawa et al., 2008). piRNA production is impaired in many gene-targeted mice, including MILI- and MIWI2-null mice, and all of the mice with impaired piRNA production show defective de novo DNA methylation in the gonocytes (Aravin et al., 2007, 2009; Carmell et al., 2007; Kuramochi-Miyagawa et al., 2008; Pandey et al., 2013; Reuter et al., 2009; Saxe et al., 2013; Soper et al., 2008). Based on this accumulated indirect evidence, it is reasonable to conclude that piRNAs would function as a guide to recruit the gene-silencing machinery to the TE loci. However, to date, no direct molecular evidence exists.

MILI and MIWI2 are both localized in the cytoplasm. MILI is exclusively localized in the cytoplasm but MIWI2 also has been detected in the nucleus. However, nuclear MIWI2 was not detected in the piRNA-defective gene-targeted mice, such as the MILI-null and mouse vasa homolog (MVH)-null mice (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2010). These findings suggest that MIWI2 can be transported into the nucleus only when it is bound to piRNA. Based on this information, we hypothesized that piRNA-bound MIWI2 was an effector of gene silencing, using piRNA as a guide to the specific loci (Le Thomas et al., 2013).

To examine this hypothesis, we used an artificially designed zinc-finger (ZF) protein that specifically bound to the target region in the genome (Mandell and Barbas, 2006). We designed a ZF protein targeted to the common regulatory region of type A LINE-1 elements, and we produced a fusion protein of the ZF and MIWI2 (ZF-MIWI2). Next we investigated whether this fusion protein was recruited to the LINE-1 loci without piRNAs and functioned as an effector of de novo DNA methylation. We demonstrated that the ZF-MIWI2 fusion protein induced de novo DNA methylation of the type A LINE-1 genes and restored spermatogenesis, at least to some extent, in a piRNA-independent manner. In addition, the Dnmts and TDRD9 proteins, which have been reported to be involved in transcriptional repression, were candidates for the piRNA-dependent gene-silencing complex in the gonocytes.

RESULTS

Production of ZF-MIWI2 Transgenic Mice

First a chromatin immunoprecipitation (ChIP) assay was performed to confirm that MIWI2 bound to the genomic regions of LINE-1 and the IAP retrotransposons in the gonocytes. LINE-1 retrotransposons are divided into several distinct types based on their sequences, such as type A and type TF, both of which have similar and unique sequences in their coding and 5′ noncoding regions, respectively. ChIP-qPCR analysis, using embryonic testis lysates, revealed that MIWI2 bound to the regulatory regions of LINE-1 and IAP, but not to the Rasgrf1 negative control (Figure 1A). Two kinds of anti-MIWI2 antibodies (N1 and 25D11) verified the recruitment of MIWI2 to these piRNA target regions. This indicated that MIWI2 was localized to the retrotransposon loci where DNA methylation occurs in the embryonic male germ cells.

A ZF protein, designed using Zinc Finger Tools (Mandell and Barbas, 2006), was targeted to the type A LINE-1 5′ UTR region (5′ CCACGGGACCCTAAGACC 3′). The ZF protein consisted of six ZF domains, each of which specifically recognized and bound to the target nucleotide triplets shown in Figure 1B. The ZF protein was fused to MIWI2, nuclear localization signal (NLS), and one FLAG-tag (ZF-MIWI2 in Figure 1C). MIWI2 with FLAG and NLS (FN-MIWI2) and ZF with FLAG and NLS (FN-ZF) were produced as negative controls. ChIP analysis was undertaken to verify the binding ability and specificity of ZF-MIWI2 to the target type A LINE-1 5′ UTR region in the ESCs. The results showed that the ZF-MIWI2 protein bound to the target type A LINE-1 elements, but not to type TF regions (Figure S1A).

Transgenic mice in which the ZF-MIWI2, FN-MIWI2, and FN-ZF were expressed under the MILI promoter were produced. Although ZF-MIWI2 protein was only weakly detected in the adult transgenic testis, it was clearly detectable in the embryonic and 2- to 3-week-old transgenic testes (Figure S1B). Relative expression levels of these fusion proteins and endogenous MIWI2 protein were analyzed by western blot analysis and ImageJ software, or qRT-PCR assay. The amount of ZF-MIWI2 was about a quarter of endogenous MIWI2, and the amounts of ZF-MIWI2 and FN-ZF were almost the same (Figures 1D and S1C). The expression levels were similar in all of the transgenic mice bearing these three transgenes.

A ChIP assay verified that the ZF protein bound to the target type A LINE-1 5′ UTR region in the transgenic embryonic testes (E16.5) (Figure 1E). These fusion proteins were expressed and localized mainly in the nuclei of the male embryonic germ cells of the E16.5 transgenic mice (Figure 1F).

Induction of DNA Methylation by ZF-MIWI2 without piRNA

The above-mentioned transgenes were introduced into the MILI-null background mice, and, hereafter, those mice are designated as the ZF-MIWI2, FN-MIWI2, and FN-ZF mice, respectively. It was confirmed that piRNAs corresponding to the sequences of type A LINE-1 were hardly detected in the ZF-MIWI2 mice

(D) Expression of FN-MIWI2, FN-ZF, and ZF-MIWI2 in the E16.5 testis lysate of the mice expressing those transgenes. The data of western blotting (left) and qRT-PCR of the E16.5 testes of each transgenic mouse are shown. β-actin was used for normalization of the gene expression in qRT-PCR. Error bars denote SDs (right).
(E) ChIP analysis of E16.5 testis of the transgenic mouse bearing the FN-ZF gene. The 5′ UTR region of LINE-1 (types A and TF) and the LTR region from IAP were analyzed by qPCR (n = 3). Error bars denote SDs.
(F) Immunostaining of the testes of the E16.5 FN-MIWI2, FN-ZF, and ZF-MIWI2 mice with the anti-FLAG antibody (green) and DAPI (blue) for DNA is shown. Scale bar, 20 μm.
Also see Figure S1.

OPEN ACCESS
CellPress

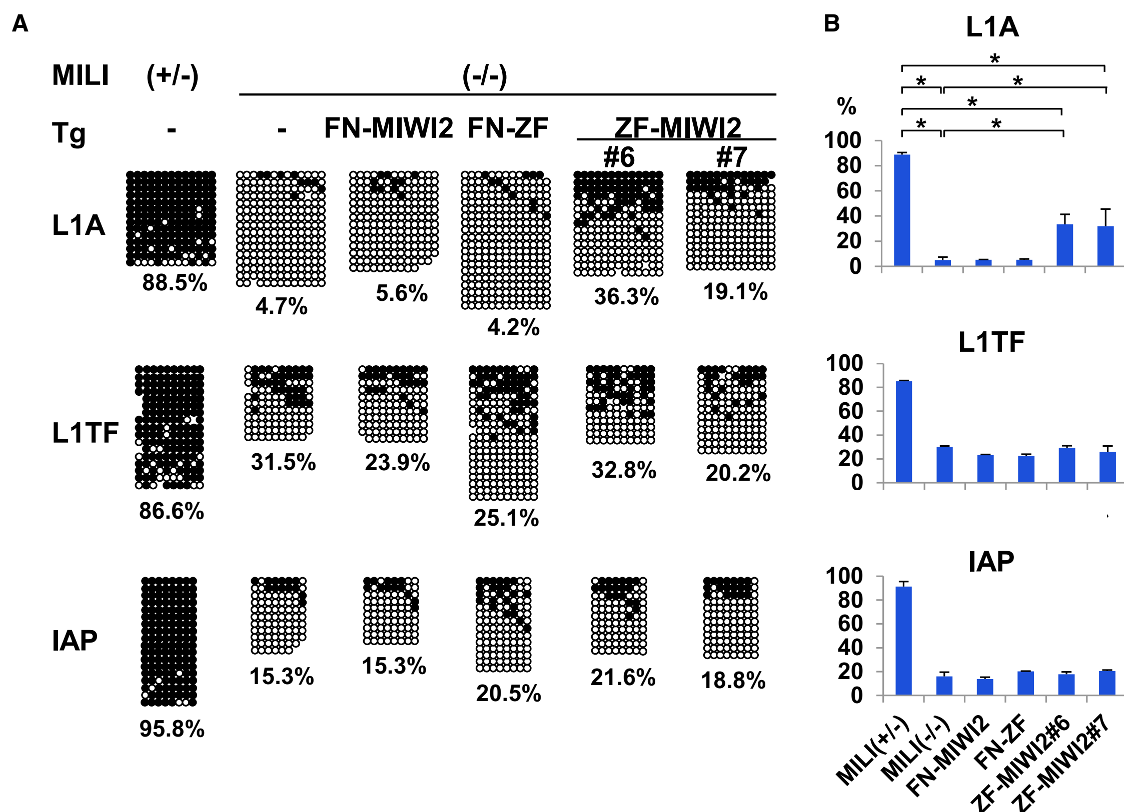


Figure 2. DNA Methylation of the Male Germ Cells of 12-Day-Old ZF-MIWI2 Mice

(A) Representative data of bisulfite sequencing of 12-day-old male germ cells purified by the anti-EpCAM antibody. The 5′ UTR region of LINE-1 (types A [GenBank: M13002] and TF [GenBank: D84391]) and the LTR region from the 1Δ1-type IAP in chromosome 3qD were analyzed using the specific primers, whose sequences are described in the Supplemental Experimental Procedures. Dots and circles represent methylated and unmethylated CpGs, respectively. Gaps in the methylation profiles represent mutated or unreadable CpG sites. The percentages of methylated CpGs are shown below each panel.

(B) Statistical analyses of three independent bisulfite sequencing experiments. Error bars denote SD. Significant differences (*$p < 0.001$ by the u test) between the indicated data of the type A LINE-1 (top panel) are shown.

Also see Figure S2.

(Figure S1D). DNA methylation levels of the LINE-1 and IAP retrotransposons in 12-day-old male germ cells in the transgenic mice were examined by bisulfite sequencing (Figure 2A). The male germ cells were purified with anti-EpCAM antibody (Figure S2), and the purification levels were confirmed by the high methylation status (>90%) of differentially methylation region (DMR)-H19, a paternal imprinted gene (Figure S2B).

A significant reduction in the CpG methylation levels of the retrotransposon genes was observed in the MILI-null male germ cells compared to the high DNA methylation of the control germ cells. DNA methylation levels of type A LINE-1 in the germ cells of the ZF-MIWI2 mice were significantly higher than in the MILI-null, FN-MIWI2, and FN-ZF mice (Figure 2B). To the contrary, the genomic regions of type TF LINE-1 and IAP, which were not targeted by the ZF, were not affected. The male germ cells of FN-MIWI2 and FN-ZF mice did not show any changes in the DNA methylation levels of type A LINE-1 compared to the MILI-null cells. These data clearly demonstrated that DNA methylation of the type A LINE-1 locus was partially rescued by the ZF-MIWI2 protein under MILI-null (i.e., piRNA-defective) conditions (Figure 2).

Candidate molecules associated with ZF-MIWI2 were investigated by immunoprecipitation and western blot analysis. Immunoprecipitation of the E16.5 testis lysate of the control mice revealed that TDRD9 involved in de novo DNA methylation bound to ZF-MIWI2 (Figure 3A). In addition, binding of MIWI2 with Dnmt3a2, Dnmt3L, and TDRD9 was observed in 293T cells expressing those proteins (Figures 3B and S3). The amount of MIWI2 that was co-immunoprecipitated with Dnmt3a2 by anti-FLAG antibody was increased in the presence of TDRD9, as shown in Figure 3B.

Repression of Gene Expression by ZF-MIWI2

Next we examined the expression of the type A LINE-1 gene in the testes of 3-week-old mice using qRT-PCR (Figure 4A). Compared to the testes of the MILI-null, FN-MIWI2, and FN-ZF mice, the expression of type A LINE-1 transcripts was significantly lower in the ZF-MIWI2 mice. Although the expression level in the testis of the ZF-MIWI2 mice was slightly higher than in the control MILI heterozygous testis, the difference was not statistically significant. In contrast, the levels of type TF LINE-1 and IAP transcripts were not affected by the expression of ZF-MIWI2.

Figure 3. Proteins Associated with the ZF-MIWI2 Protein

(A) Binding of ZF-MIWI2 to TDRD9 in the E16.5 testes of the ZF-MIWI2 mice. The embryonic testes lysates were immunoprecipitated with the anti-FLAG antibody and subjected to western blotting with the described antibodies.

(B) Co-immunoprecipitation assays for MIWI2, Dnmt3a, Dnmt3a2, TDRD9, and Dnmt3L in transfected 293T cells.

Also see Figure S3.

The testes of FN-MIWI2 and FN-ZF mice did not show a reduction in the expression levels of the retrotransposons, including type A LINE-1. These data were confirmed by northern blot analysis (Figure 4B). Although the data varied slightly, expression of type A LINE-1 in the ZF-MIWI2 testes was decreased compared to the MILI-null, FN-MIWI2, and FN-ZF testes, consistent with the data from the qRT-PCR.

To investigate whether the reduced level of the type A LINE-1 transcripts in the ZF-MIWI2 testes was attributable to transcriptional suppression, a ChIP assay to examine the recruitment of RNA polymerase II was undertaken (Figure 4C). Recruitment of RNA polymerase II to type A LINE-1, TF LINE-1, and IAP genes was observed in the testes of the MILI-null and FN-MIWI2 mice. However, recruitment to the type A LINE-1 loci was significantly reduced in the testes of the ZF-MIWI2 mice, similar to those of the control MILI heterozygous mice. These data supported the notion that the ZF-MIWI2 protein influenced the silencing of ZF-targeted type A LINE-1 genes by transcriptional processes.

Spermatogenesis in the Testes of ZF-MIWI2 Mice

The size, weight, and H&E staining results of the testes of the ZF-MIWI2 mice were similar to those of the MILI-null, FN-MIWI2, and FN-ZF mice (Figures 5A, 5B, and S4A). In contrast, immunostaining using an antibody against MIWI, which was expressed only in the later stages of spermatogenesis, showed a significant difference between the ZF-MIWI2 and control mice. MIWI was not detected in the testes of the FN-MIWI2 and FN-ZF mice or in the MILI-null testis. However, a small but significant number of germ cells of the ZF-MIWI2 mouse expressed MIWI. Staining patterns of MIWI revealed protein in chromatoid bodies, characteristic of a dense structure in the cytoplasm of male germ cells after the pachytene stage (Figures 5C and S4B). Although expressions of MIWI and CREM were not detectable in the 24-day-old MILI-null testis, those were detected, albeit weakly, in the ZF-MIWI2 mouse testis of same age. Meanwhile, qRT-PCR analysis of 7-week-old ZF-MIWI2 mouse testis did not show the expression of Protamin1 (Prm1), Protamin2 (Prm2), and Transition protein1 (Tnp1), all of which were mainly expressed in post-meiotic spermatogenesis (Figure S4C) (Foulkes et al., 1992; Okada et al., 2007). These data showed that spermatogenesis of some of the male germ cells in the ZF-MIWI2 mouse proceeded to a later stage beyond that of pachytene spermatocytes, but not to the fully matured one.

DISCUSSION

We recruited MIWI2 protein to type A LINE-1 loci without piRNAs using ZF-targeting technology. DNA methylation of the type A LINE-1 retrotransposon was successfully induced by the MIWI2 fusion protein. Subsequently, expression of the type A LINE-1 retrotransposon was silenced without piRNAs, and partial rescue of spermatogenesis was observed. Our results showed that MIWI2 initiated the molecular mechanisms necessary for DNA methylation. An immunoprecipitation assay revealed that MIWI2 was associated with TDRD9 in the embryonic testis. Dnmt3a2 and Dnmt3L, a binding partner of Dnmt3 required for the de novo DNA methylation of retrotransposons in the embryonic testis, also were bound to MIWI2.

Transcriptional Repression of the Retrotransposon by ZF-MIWI2 without piRNAs

It was hypothesized that the piRNA-MIWI2 complex entered the nucleus and that piRNA functioned as a guide to the retrotransposon loci for de novo DNA methylation. It is probable that MIWI2 has a role in methylation in this model but this has not been proven (Kuramochi-Miyagawa et al., 2008). The ZF-MIWI2 fusion protein induced DNA methylation of the promoter region of the type A LINE-1 retrotransposons and the almost complete suppression of their expression under the MILI-null condition (Figures 2 and 4). ChIP analysis of RNA polymerase II revealed that the suppressed expression was due to transcriptional suppression. The role of ZF-MIWI2 in DNA methylation led to a search for the proteins associated with MIWI2. It is likely that the proteins involved in de novo DNA methylation, namely DNMT3a2 and DNMT3L, form a complex with MIWI2 (Figure 3). However, it is unclear whether the association with these DNMTs is sufficient for methylation.

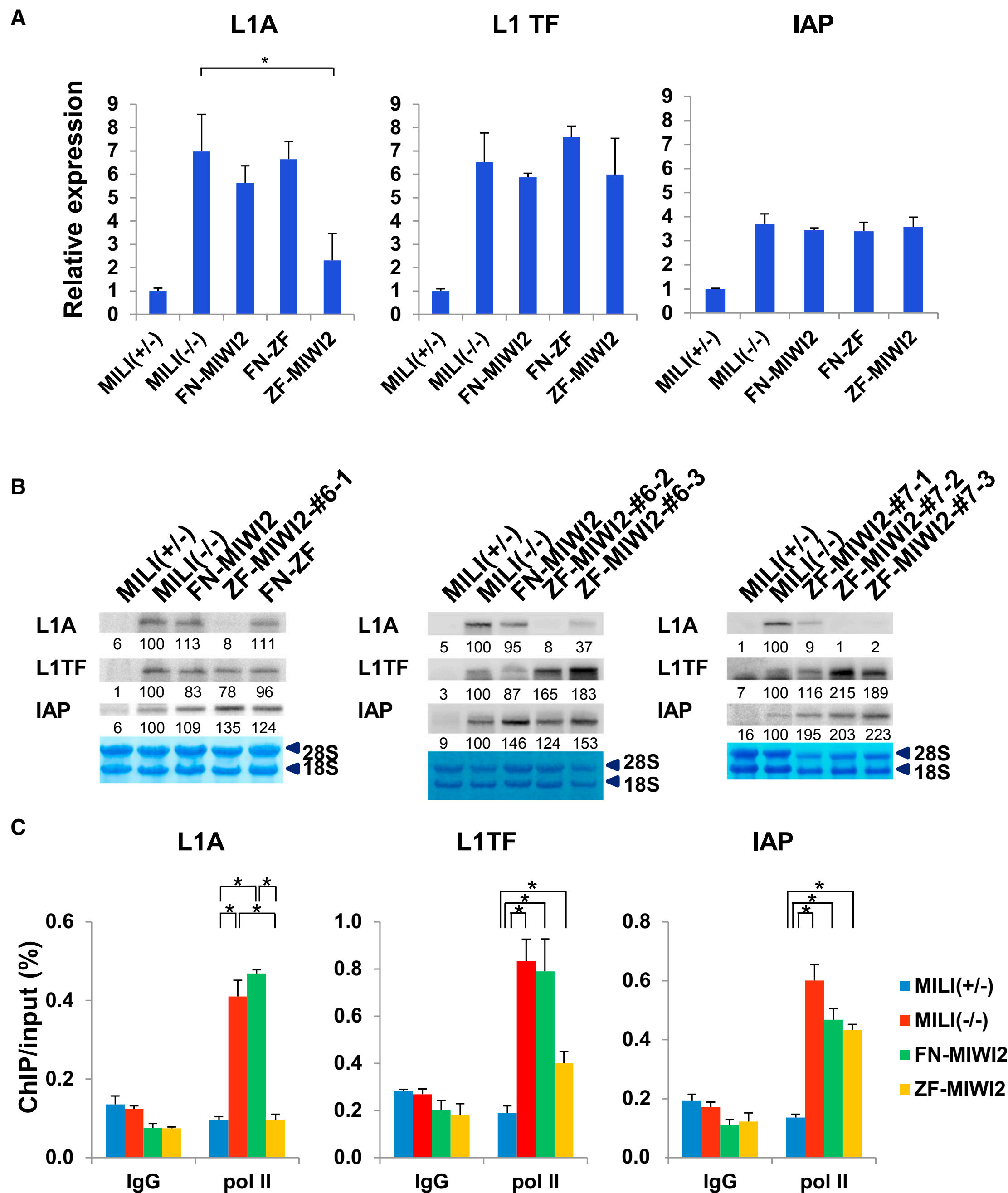


Figure 4. Expression of and RNA Polymerase II Recruitment to the LINE-1 and IAP Retrotransposon Genes in the ZF-MIWI2 Testes

(A) qRT-PCR data for the LINE-1 and IAP retrotransposons in the testes of 3-week-old control MILI-heterozygous, MILI-null, FN-MIWI2, FN-ZF, and ZF-MIWI2 mice. Error bars denote SDs. β-actin was used for normalization of the gene expression in qRT-PCR. Significant differences by t test, *$p < 0.05$.

(B) Northern blot analysis of LINE-1 and IAP-1Δ1 retrotransposons in the testes of 3-week-old control MILI-heterozygous, MILI-null, FN-MIWI2, FN-ZF, and ZF-MIWI2 mice. The 5′ UTRs of types A and TF LINE-1 and the 3′ UTR of IAP were used as probes. The intensities of the bands were analyzed using ImageJ software, and the relative ratios are shown under each lane.

(C) ChIP-qPCR analysis of the 3-week-old testes of control MILI-heterozygous, MILI-null, FN-MIWI2, and ZF-MIWI2 mice with the antibody against total RNA polymerase II. The 5′ UTR region of LINE-1 (types A and TF) and the LTR region from IAP were analyzed (n = 3). Error bars denote SDs. Statistically significant differences by t test, *$p < 0.05$.

Also see Figure S5.

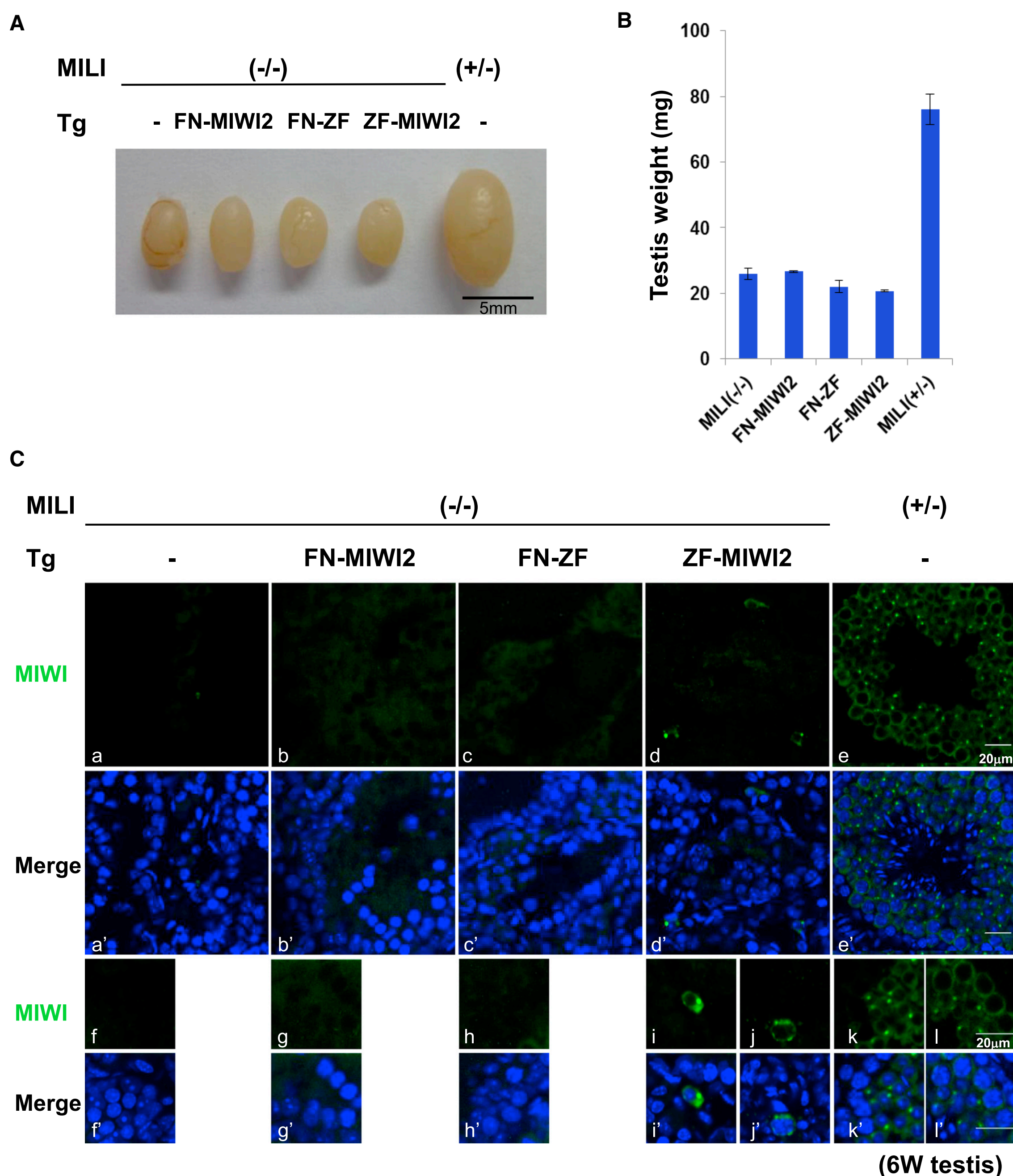


Figure 5. Spermatogenesis in the ZF-MIWI2 Mice

(A) Testes of 6-week-old control MILI-heterozygous, MILI-null, FN-MIWI2, FN-ZF, and ZF-MIWI2 mice are shown. Scale bar, 5 mm.

(B) Weights of the testes from the above-mentioned mice. The data from the MILI-heterozygous testes show significant differences compared the other groups ($p < 0.001$ by t test). Error bars denote the SD.

(C) Immunohistochemical staining in the testes of 6-week-old control MILI-heterozygous, MILI-null, FN-MIWI2, FN-ZF, and ZF-MIWI2 mice. Sections were stained with the anti-MIWI antibody (green) and DNA was visualized by DAPI staining. Scale bar, 20 μm.

Also see Figure S4.

PIWI-related retrotransposon gene silencing is associated with H3K9me3 modification of the promoter regions in *Drosophila*, which lack regulation of DNA methylation (Sienski et al., 2012). The level of H3K9me3 modification of retrotransposon genes was decreased in the MIWI2-null testes (Pezic et al., 2014). The H3K9me3 modifications of the type A LINE-1 promoter region was analyzed in 12-day-old ZF-MIWI2 male germ cells by ChIP assay (Figure S5). However, the difference in the

levels of H3K9me3 at the type A LINE-1 locus between the germ cells of the ZF-MIWI2 and MILI-null mice was not significant. Considering that only piRNA-bound MIWI2 can enter the nucleus, and not non-bound MIWI2, some conformational change should be induced by the binding process. The ZF-MIWI2 protein should lack such a conformational change, which is probably why modification of H3K9me3 was not detectable. Compared to the restored DNA methylation and/or histone H3K9me3 levels, the degree of transcriptional suppression appeared to be much higher. This discrepancy might be explained by the occupation of the promoter region by the fusion protein and the subsequent hindrance of the recruitment of RNA polymerase II.

Partial Rescue of Spermatogenesis in ZF-MIWI2 Mice

Spermatogenesis in the MILI-null mice is arrested at the pachytene spermatocyte stage by apoptosis, and the cells never reach the later differentiation stage with the expression of MIWI (Kuramochi-Miyagawa et al., 2001, 2004). A few, but significant, numbers of MIWI-expressing germ cells were observed in the ZF-MIWI2 testes (Figure 5). This suggests that DNA methylation of type A LINE-1 genes and/or the suppression of the gene is critical to avoid complete apoptotic cell death at the pachytene stage. Round spermatids identified in the ZF-MIWI2 testis were subjected to intracytoplasmic sperm injection, but fertilization did not take place (data not shown).

Tudor domain-containing proteins (TDRDs) are an evolutionarily conserved family of proteins expressed during the development of germ cells (Arkov et al., 2006; Chuma et al., 2006). The null mutant TDRD1, TDRD9, and TDRD12 mice showed impaired spermatogenesis and abnormal LINE-1 expression, similar to that of the MILI- and MIWI2-null mice (Pandey et al., 2013; Reuter et al., 2009; Shoji et al., 2009). Meanwhile, TDRD5-null mice showed less impaired regulation of the LINE-1 gene and a milder spermatogenesis phenotype that is partial progression through the meiotic prophase to the round spermatid phase (Yabuta et al., 2011). Similarities in the relationship between retrotransposon regulation and the phenotype between the TDRD5-null mice and the ZF-MIWI2 mice would support the above hypothesis.

It is likely but not yet proven that the embryonic piRNAs are directly involved in de novo DNA methylation and gene silencing of retrotransposons. As discussed, it is conceivable that piRNA functions as a guide to recruit the molecular machinery involved in gene silencing. However, the mechanisms remain unknown due to practical difficulties, for example, the collection of sufficient numbers of embryonic germ cells and the purification of the nuclear piRNA-MIWI2 complex from these cells. Thus, an alternative strategy was employed to tether the MIWI2 protein to the type A LINE-1 locus using ZF technology. It should not be excluded that this artificial experimental system did not fully replicate the function of MIWI2. However, our data strongly suggest that MIWI2 has a key role in de novo DNA methylation and the subsequent gene silencing. The mechanisms underlying apoptosis of the pachytene stage spermatocytes in piRNA-defective mice are also unknown. Partial rescue of DNA methylation of only a single class of LINE-1 retrotransposons removed the developmental barriers to some extent, implying that DNA methylation levels are critical for normal spermatogenesis. Thus, our data obtained using a novel strategy provided new insights into the understanding of the physiological roles of piRNAs and the related DNA methylation in embryonic male germ cells.

EXPERIMENTAL PROCEDURES

Plasmids, cell culturing, transient transfection methods, H&E staining, and primers are described in the Supplemental Experimental Procedures. Other methods are summarized below, with additional details included in the Supplemental Experimental Procedures.

ZF Protein Design

To design the ZF protein, we used Zinc Finger Tools (http://www.zincfingers.org/software-tools.htm) (Mandell and Barbas, 2006). The 18-bp region of the 5′ UTR in the type A LINE-1 gene was utilized as a target for the ZF protein.

Generation of Transgenic Mice

Construction of the FLAG-NLS-ZFP-MIWI2 (ZF-MIWI2), FLAG-NLS-ZFP (ZF), and FLAG-NLS-MIWI2 (FN-MIWI2) transgenes are described in the Supplemental Experimental Procedures. Animal care was in accordance with the guidelines of Osaka University.

Preparation of Mice under MILI-Deficient Conditions

MILI-heterozygous mutant male mice carrying *FLAG-NLS-MIWI2*, *FLAG-NLS-ZFP*, or *FLAG-NLS-ZFP-MIWI2* transgenes were crossed with *MILI*-homozygous mutant female mice.

Isolation of Germ Cells

Germ cells were sorted via immunostaining of the epithelial cell adhesion molecules (EpCAMs) using the BD FACSAria system (BD Biosciences).

Western Blotting

Immunoprecipitates or lysates were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). After blocking, the filters were incubated with the corresponding antibodies. HRP-anti-mouse IgG and HRP-anti-rabbit IgG (Pierce) were used as the secondary antibody, and the signal was detected using ECL Western Blotting detection reagents (GE Healthcare).

ChIP-PCR Assay

The individual methods for using ESCs, 3-week-old testes, isolated germ cells, and embryonic testes are described in the Supplemental Experimental Procedures.

Bisulfite Sequencing

Sorted germ cells (day 12 after birth) were bisulfite-treated using the EZ DNA Methylation-Direct kit (Zymo Research). Sequences of the PCR primers and the PCR conditions are described in the Supplemental Experimental Procedures.

Northern Blot Analysis

Total RNA samples were prepared from testes using ISOGEN (Nippon Gene) and stained with 0.02% methylene blue. The subcloned PCR products were labeled with [α-^{32}P]-dCTP and used as probes.

Immunohistochemical Staining

Cryosections blocked with 10% normal goat serum and 3% BSA in PBS for 0.5 hr at room temperature were subjected to immunofluorescence staining. After treatment with the corresponding antibody overnight at 4°C, the sections were treated with secondary antibody. Immunostained cryosections were examined under a confocal microscope (LSM5Pascal, Carl Zeiss).

Immunoprecipitation and SDS-PAGE

The 3-week-old or E16.5 testes were homogenized, and the lysate was subjected to freeze-thawing and treated with benzonase. After centrifugation,

the supernatant was subjected to immunoprecipitation and SDS-PAGE. The gel was subjected to western blotting. The transfected 293T cells were treated with another lysis buffer before western blot analysis of the lysate.

qRT-PCR

Total RNAs prepared from E16.5 and 3-week-old testes were treated with Turbo DNase (Life Technologies) and subjected to RT-PCR using ThermoScript RT-PCR (Invitrogen) and random hexamers. The qPCR was analyzed using the ViiA 7 Real-Time PCR system (Life Technologies) and specific primers.

ACCESSION NUMBERS

The accession number for the deep sequencing data reported in this paper is DDBJ: DRA004810.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.08.027.

AUTHOR CONTRIBUTIONS

K.K.-K. performed the experiments and analyzed the data. K.K.-K., S.K.-M., and T.N. designed the experiments and wrote the paper. I.N. supported the analysis of deep sequence and H.H. supported the generation of transgenic mice. T.A. and N.I. discussed the experiment with K.K.-K. N.O. and A.O. performed the intracytoplasmic sperm injections.

ACKNOWLEDGMENTS

The authors thank Dr. S. Chuma of Kyoto University for the anti-TDRD9 antibody, Dr. S. Tajima of Osaka University for the anti-Dnmt3a antibody and the FLAG-tagged-Dnmt3a and Dnmt3a2 expression plasmids inserted into the pcDNA4 vector, and Ms. Y. Esaki for assistance in generating the Tg mice. We also thank Ms. N. Asada and NPO Biotechnology Research and Development for the technical assistance and Ms. M. Imaizumi for the secretarial work. This work was supported in part by a Grant-in-Aid for Scientific Research Grant on Innovative Areas, "Epigenome Dynamics and Regulation in Germ Cells" (26112511), Research (A) (15H02509), Research (B) (24370074) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT)/Japan Society for the Promotion of Science (JSPS) and Core Research for Evolutional Science and Technology (CREST) (J150701424) from the Japan Agency for Medical Research and Development (AMED).

Received: November 18, 2015
Revised: June 17, 2016
Accepted: August 8, 2016
Published: September 13, 2016

REFERENCES

Aravin, A., Gaidatzis, D., Pfeffer, S., Lagos-Quintana, M., Landgraf, P., Iovino, N., Morris, P., Brownstein, M.J., Kuramochi-Miyagawa, S., Nakano, T., et al. (2006). A novel class of small RNAs bind to MILI protein in mouse testes. Nature *442*, 203–207.

Aravin, A.A., Sachidanandam, R., Girard, A., Fejes-Toth, K., and Hannon, G.J. (2007). Developmentally regulated piRNA clusters implicate MILI in transposon control. Science *316*, 744–747.

Aravin, A.A., Sachidanandam, R., Bourc'his, D., Schaefer, C., Pezic, D., Toth, K.F., Bestor, T., and Hannon, G.J. (2008). A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. Mol. Cell *31*, 785–799.

Aravin, A.A., van der Heijden, G.W., Castañeda, J., Vagin, V.V., Hannon, G.J., and Bortvin, A. (2009). Cytoplasmic compartmentalization of the fetal piRNA pathway in mice. PLoS Genet. *5*, e1000764.

Arkov, A.L., Wang, J.Y., Ramos, A., and Lehmann, R. (2006). The role of Tudor domains in germline development and polar granule architecture. Development *133*, 4053–4062.

Bourc'his, D., and Bestor, T.H. (2004). Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. Nature *431*, 96–99.

Carmell, M.A., Girard, A., van de Kant, H.J., Bourc'his, D., Bestor, T.H., de Rooij, D.G., and Hannon, G.J. (2007). MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. Dev. Cell *12*, 503–514.

Chuma, S., Hosokawa, M., Kitamura, K., Kasai, S., Fujioka, M., Hiyoshi, M., Takamune, K., Noce, T., and Nakatsuji, N. (2006). Tdrd1/Mtr-1, a tudor-related gene, is essential for male germ-cell differentiation and nuage/germinal granule formation in mice. Proc. Natl. Acad. Sci. USA *103*, 15894–15899.

Cox, D.N., Chao, A., Baker, J., Chang, L., Qiao, D., and Lin, H. (1998). A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev. *12*, 3715–3727.

Foulkes, N.S., Mellström, B., Benusiglio, E., and Sassone-Corsi, P. (1992). Developmental switch of CREM function during spermatogenesis: from antagonist to activator. Nature *355*, 80–84.

Girard, A., Sachidanandam, R., Hannon, G.J., and Carmell, M.A. (2006). A germline-specific class of small RNAs binds mammalian Piwi proteins. Nature *442*, 199–202.

Grivna, S.T., Beyret, E., Wang, Z., and Lin, H. (2006). A novel class of small RNAs in mouse spermatogenic cells. Genes Dev. *20*, 1709–1714.

Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E., and Sasaki, H. (2004). Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature *429*, 900–903.

Kato, Y., Kaneda, M., Hata, K., Kumaki, K., Hisano, M., Kohara, Y., Okano, M., Li, E., Nozaki, M., and Sasaki, H. (2007). Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. Hum. Mol. Genet. *16*, 2272–2280.

Kazazian, H.H., Jr. (2004). Mobile elements: drivers of genome evolution. Science *303*, 1626–1632.

Kuramochi-Miyagawa, S., Kimura, T., Yomogida, K., Kuroiwa, A., Tadokoro, Y., Fujita, Y., Sato, M., Matsuda, Y., and Nakano, T. (2001). Two mouse piwi-related genes: miwi and mili. Mech. Dev. *108*, 121–133.

Kuramochi-Miyagawa, S., Kimura, T., Ijiri, T.W., Isobe, T., Asada, N., Fujita, Y., Ikawa, M., Iwai, N., Okabe, M., Deng, W., et al. (2004). Mili, a mammalian member of piwi family gene, is essential for spermatogenesis. Development *131*, 839–849.

Kuramochi-Miyagawa, S., Watanabe, T., Gotoh, K., Totoki, Y., Toyoda, A., Ikawa, M., Asada, N., Kojima, K., Yamaguchi, Y., Ijiri, T.W., et al. (2008). DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. Genes Dev. *22*, 908–917.

Kuramochi-Miyagawa, S., Watanabe, T., Gotoh, K., Takamatsu, K., Chuma, S., Kojima-Kita, K., Shiromoto, Y., Asada, N., Toyoda, A., Fujiyama, A., et al. (2010). MVH in piRNA processing and gene silencing of retrotransposons. Genes Dev. *24*, 887–892.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., et al.; International Human Genome Sequencing Consortium (2001). Initial sequencing and analysis of the human genome. Nature *409*, 860–921.

Lane, N., Dean, W., Erhardt, S., Hajkova, P., Surani, A., Walter, J., and Reik, W. (2003). Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. Genesis *35*, 88–93.

Le Thomas, A., Rogers, A.K., Webster, A., Marinov, G.K., Liao, S.E., Perkins, E.M., Hur, J.K., Aravin, A.A., and Tóth, K.F. (2013). Piwi induces piRNA-guided transcriptional silencing and establishment of a repressive chromatin state. Genes Dev. *27*, 390–399.

Lees-Murdock, D.J., Shovlin, T.C., Gardiner, T., De Felici, M., and Walsh, C.P. (2005). DNA methyltransferase expression in the mouse germ line during periods of de novo methylation. Dev. Dyn. *232*, 992–1002.

Lei, H., Oh, S.P., Okano, M., Jüttermann, R., Goss, K.A., Jaenisch, R., and Li, E. (1996). De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development *122*, 3195–3205.

Li, J.Y., Lees-Murdock, D.J., Xu, G.L., and Walsh, C.P. (2004). Timing of establishment of paternal methylation imprints in the mouse. Genomics *84*, 952–960.

Lucifero, D., Mertineit, C., Clarke, H.J., Bestor, T.H., and Trasler, J.M. (2002). Methylation dynamics of imprinted genes in mouse germ cells. Genomics *79*, 530–538.

Mandell, J.G., and Barbas, C.F., 3rd. (2006). Zinc Finger Tools: custom DNA-binding domains for transcription factors and nucleases. Nucleic Acids Res. *34*, W516–523.

Matsui, T., Leung, D., Miyashita, H., Maksakova, I.A., Miyachi, H., Kimura, H., Tachibana, M., Lorincz, M.C., and Shinkai, Y. (2010). Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. Nature *464*, 927–931.

Okada, Y., Scott, G., Ray, M.K., Mishina, Y., and Zhang, Y. (2007). Histone demethylase JHDM2A is critical for Tnp1 and Prm1 transcription and spermatogenesis. Nature *450*, 119–123.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247–257.

Pandey, R.R., Tokuzawa, Y., Yang, Z., Hayashi, E., Ichisaka, T., Kajita, S., Asano, Y., Kunieda, T., Sachidanandam, R., Chuma, S., et al. (2013). Tudor domain containing 12 (TDRD12) is essential for secondary PIWI interacting RNA biogenesis in mice. Proc. Natl. Acad. Sci. USA *110*, 16492–16497.

Pezic, D., Manakov, S.A., Sachidanandam, R., and Aravin, A.A. (2014). piRNA pathway targets active LINE1 elements to establish the repressive H3K9me3 mark in germ cells. Genes Dev. *28*, 1410–1428.

Reuter, M., Chuma, S., Tanaka, T., Franz, T., Stark, A., and Pillai, R.S. (2009). Loss of the Mili-interacting Tudor domain-containing protein-1 activates transposons and alters the Mili-associated small RNA profile. Nat. Struct. Mol. Biol. *16*, 639–646.

Sakai, Y., Suetake, I., Shinozaki, F., Yamashina, S., and Tajima, S. (2004). Co-expression of de novo DNA methyltransferases Dnmt3a2 and Dnmt3L in gonocytes of mouse embryos. Gene Expr. Patterns *5*, 231–237.

Saxe, J.P., Chen, M., Zhao, H., and Lin, H. (2013). Tdrkh is essential for spermatogenesis and participates in primary piRNA biogenesis in the germline. EMBO J. *32*, 1869–1885.

Shoji, M., Tanaka, T., Hosokawa, M., Reuter, M., Stark, A., Kato, Y., Kondoh, G., Okawa, K., Chujo, T., Suzuki, T., et al. (2009). The TDRD9-MIWI2 complex is essential for piRNA-mediated retrotransposon silencing in the mouse male germline. Dev. Cell *17*, 775–787.

Sienski, G., Dönertas, D., and Brennecke, J. (2012). Transcriptional silencing of transposons by Piwi and maelstrom and its impact on chromatin state and gene expression. Cell *151*, 964–980.

Slotkin, R.K., and Martienssen, R. (2007). Transposable elements and the epigenetic regulation of the genome. Nat. Rev. Genet. *8*, 272–285.

Soper, S.F., van der Heijden, G.W., Hardiman, T.C., Goodheart, M., Martin, S.L., de Boer, P., and Bortvin, A. (2008). Mouse maelstrom, a component of nuage, is essential for spermatogenesis and transposon repression in meiosis. Dev. Cell *15*, 285–297.

Walsh, C.P., Chaillet, J.R., and Bestor, T.H. (1998). Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nat. Genet. *20*, 116–117.

Yabuta, Y., Ohta, H., Abe, T., Kurimoto, K., Chuma, S., and Saitou, M. (2011). TDRD5 is required for retrotransposon silencing, chromatoid body assembly, and spermiogenesis in mice. J. Cell Biol. *192*, 781–795.