博士論文

Induced DNA methylation and gene silencing by targeting MIWI2 to retrotransposon in spermatogenesis.

(精子形成過程における MIWI2 による DNA メチル化を介したレトロトランスポゾン制御)

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2015 年 3 月 25 日　修了

Abstract

The expression of long interspersed elements-1 (LINE1) and intracisternal A-particle (IAP) retrotransposons is regulated by *de novo* DNA methylation during spermatogenesis. The mouse PIWI family proteins, MILI and MIWI2, bind to piRNA (PIWI interacting RNA), corresponding to retrotransposon genes, and play roles in gene silencing in the embryonic testis. Unlike MILI, piRNA-associated MIWI2 localizes mainly to the nucleus. It is thought that the MIWI2/piRNA complex acts as an effector or guide for *de novo* DNA methylation in retrotransposon regulatory regions. To elucidate the function of MIWI2 in the nucleus, I created a fusion construct consisting of MIWI2 fused to a zinc finger (ZF) protein targeted towards the regulatory region of type A LINE1 elements and tagged with a nuclear localization signal (NLS). A transgenic (Tg) mouse was subsequently created, expressing the ZF-MIWI2 fusion protein, which bound directly to type A LINE1 target loci in the absence of piRNA. The Tg mice were crossed with MILI-deficient mice, which show a reduction in DNA methylation in the regulatory regions of retrotransposon genes and an up-regulation of the corresponding transcripts. In MILI-deficient Tg mice, the de-repression of retrotransposon genes was partially rescued in only the ZF-targeted type A LINE1 regions. Furthermore, the impaired spermatogenesis observed in the MILI-deficient mice was also partially rescued by crossing with the Tg mice. These data suggest that the induction of DNA methylation and the silencing of ZF target genes by ZF-MIWI2 are independent of the presence of piRNA, and that MIWI2 plays a key role in the recruitment of some proteins involved in the epigenetic repression of target loci in the nucleus.

Contents

Cover…1

Abstract…2

Contents…3

Introduction…4-9

Results…10-20

Discussion…21-25

Experimental procedures…26-35

References…36-41

Achievements…42-43

Introduction

Approximately half of the mammalian genome is composed of transposable elements (TEs) (Lander et al., 2001). TEs have effects on genome expansion and are thought to be important for organism evolution. Most TEs are truncated and inactive, but some mouse TEs are active (Kazazian, 2004). To protect the host genome from TE-mediated mutagenesis, molecular defense systems involving epigenetic transcriptional regulation have been acquired during the evolutionary process (Slotkin and Martienssen, 2007). In mammals, genomic DNA methylation (Walsh et al., 1998) and histone modification are two such mechanisms. It is reported that Dnmt1 (DNA methyltransferase-1) and ERG-associated protein with SET domain (ESET) histone methyl transferase (also known as H3K9me3) are required for retrotransposon silencing during early embryogenesis (Lei et al., 1996; Matsui et al., 2010; Xu et al., 1998).

The DNA methylation levels of retrotransposon and imprinting genes change dynamically during spermatogenesis. The DNA methylation patterns of primordial germ cells are mostly lost by E13.5 (Lane et al., 2003). *De novo* DNA methylation is subsequently established in non-dividing prospermatogonia (i.e., gonocytes) from E16.5 to E18.5, and completed by birth (Li et al., 1993; Li et al., 2004; Lucifero et al., 2002). The *de novo* DNA methyltransferases Dnmt3a and Dnmt3b, and the related protein Dnmt3L play important roles in this process, which is essential for spermatogenesis (Bourc'his and Bestor, 2004; Hata et al., 2002; Kaneda et al., 2004; Kato et al., 2007; Lees-Murdock et al., 2005; Okano et al., 1999; Sakai et al., 2004; Webster et al., 2005) (Fig.1).

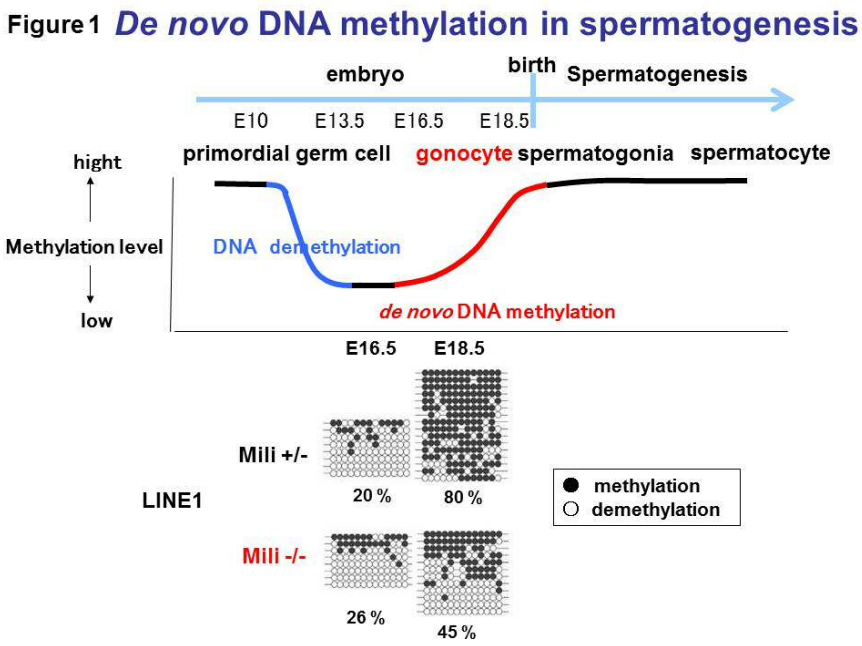

Figure 1 *De novo* DNA methylation in spermatogenesis.

(Top) DNA methylation level of the regulatory region on retrotransposon genes in spermatogenesis. (Bottom) DNA methylation level of the regulatory region on LINE1 in MILI deficient- and control-germ cells.

Piwi is essential for self-renew and evolutionarily conserved in diverse organisms. PIWI family proteins are belongs to Argonaute family. Argonaute family proteins have the conserved two domains, PAZ domain, which binds to small RNA, and PIWI domain, which has slicer activity (RNase H like). This family consists of two sub families. One is Ago sub family which ubiquitously expresses in cells and binds to siRNA and miRNA (21-23nt). Another is PIWI sub family which specifically expresses in germ cells and binds to piRNA (PIWI interacting RNAs) (25-31nt) (Fig.2).

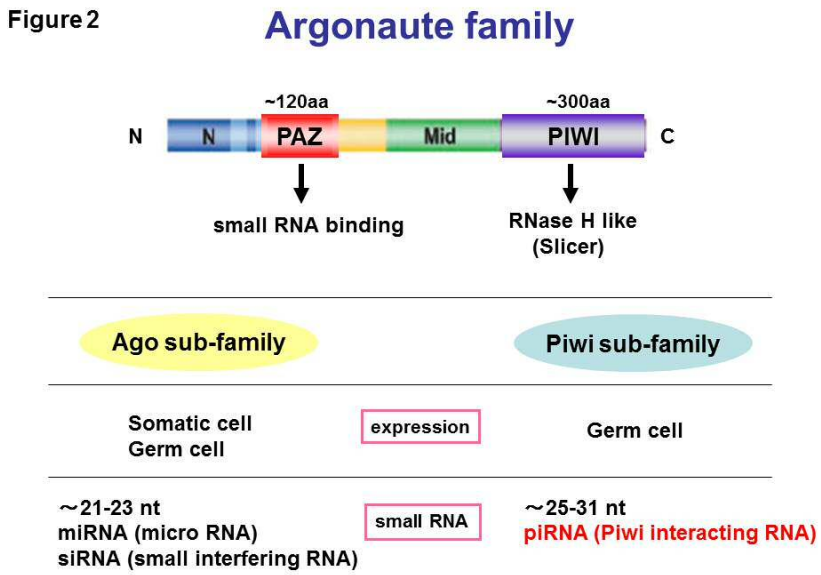

Figure 2 The classification of Argonaute family members.

(Top) Schematic diagram of Argonaute family protein. (Bottom) The characteristics of Ago-sub family and Piwi sub family proteins.

The mouse PIWI sub family proteins, MILI and MIWI2, are expressed from the primordial germ cell stage (E12.5) to the round spermatid stage, and at the early stage of spermatogenesis from the gonocyte (E15.5) until soon after birth, respectively (Kuramochi-Miyagawa et al., 2008) (Fig.3).

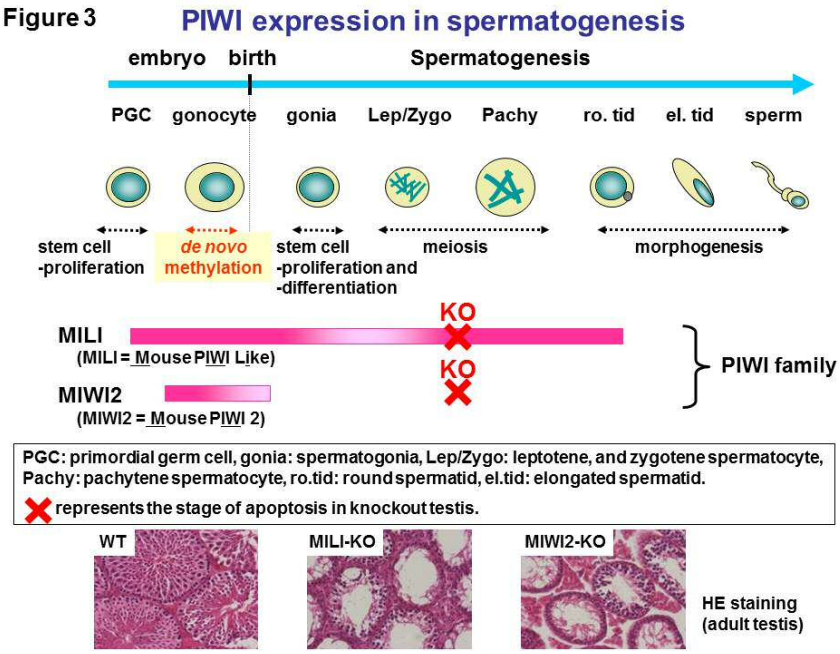

Figure 3 PIWI expression in spermatogenesis.

(Top) Schematic diagram of PIWI expression in spermatogenesis. (Bottom) HE staining of wild type, *MILI*$^{-/-}$and *MIWI2*$^{-/-}$testes.

In normal embryonic gonocytes, a large number of piRNAs exist. These are corresponding to retrotransposons, such as LINE1 (long interspersed nuclear elements) and IAP (intracisternal A particle), which belong to the non-long terminal repeat (non-LTR) and LTR retrotransposon families, respectively (Aravin et al., 2006). A decrease in the expression of piRNAs related to these retrotransposons was observed in MILI- or MIWI2-deficient mice (Aravin et al., 2007; Carmell et al., 2007; Kuramochi-Miyagawa et al., 2008). In addition, MILI and MIWI2 have been shown to bind to piRNAs. These data suggest that MILI and MIWI2 play essential roles in piRNA production in embryonic development, leading to the proposal of a next model. piRNA production can be largely divided into two processes, primary biogenesis and secondary biogenesis which is called the ping-pong cycle. MILI is involved in both

processes, while MIWI2 is related with secondary biogenesis only (Aravin et al., 2008; Aravin et al., 2009; Shoji et al., 2009) (Fig.4).

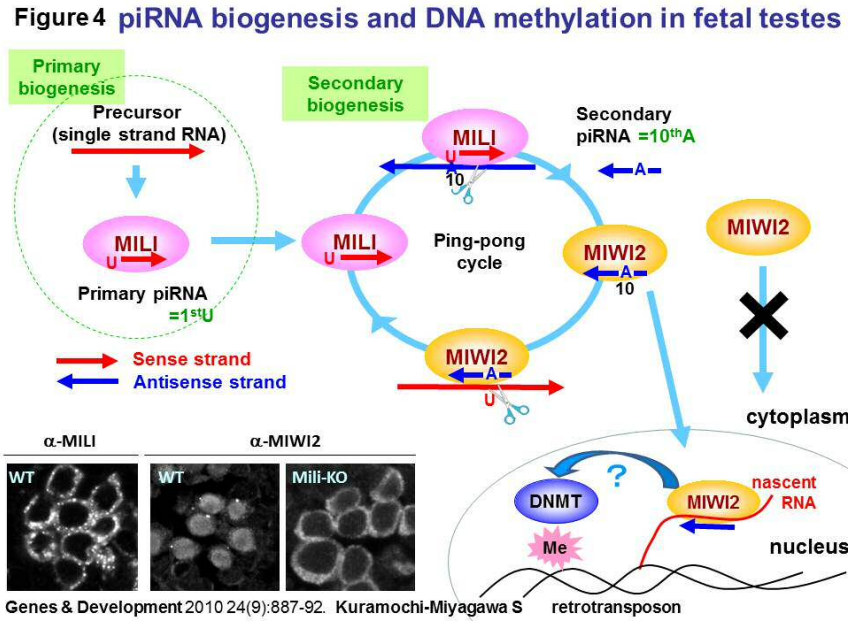

Figure 4 piRNA biogenesis and DNA methylation in fetal testes.

(Top) Schematic diagram of piRNA biogenesis in gonocyte. (Bottom) Immunohistochemical staining of germ cells using anti-MILI and -MIWI2 antibodies in wild type and *MILI*$^{-/-}$ fetal testes. (Kuramochi-Miyagawa et al., 2010)

In general, retrotransposon genes, such as LINE1 and IAP, are regulated by *de novo* DNA methylation in their regulatory regions during embryonic spermatogenesis. The reductions of DNA methylation and accumulation of these transcriptions were observed in MILI- and MIWI2-deficient mice (Aravin et al., 2007; Carmell et al., 2007; Kuramochi-Miyagawa et al., 2008) (Fig.1). MILI and MIWI2 are thought to be important for retrotransposon gene silencing via *de novo* DNA methylation in embryonic stage. In wild-type embryonic testes, MILI is localized to the cytoplasm, including the pi-body, while MIWI2 is localized mainly to the nucleus and the piP-body in cytoplasm (Shoji et al., 2009). However, in MILI-deficient mice, the localization of

MIWI2 without piRNA was in the cytoplasm, not the nucleus (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2010) (Fig.4). Then, it is conceivable that a part of MILI-deficient phenotype, such as the decrease of DNA methylation in regulatory region of retrotransposon, is caused by the loss of function of MIWI2 in nucleus. It is suggested that MIWI2 could be key player for *de novo* DNA methylation of retrotransposon. However, the mechanism of these PIWI-related events in nucleus remains unclear.

To reveal the function of MIWI2 in *de novo* DNA methylation of retrotransposons, I made use of the zinc finger (ZF) tool (Mandell and Barbas, 2006). Because ZF protein recognizes and binds with high specificity to a target region in the genome, I designed a ZF protein targeted towards the regulatory region of type A LINE1 elements. Next, a ZF-MIWI2 fusion protein was constructed, containing the ZF protein, a nuclear localization signal (NLS) and MIWI2 with a FLAG-tag. I subsequently generated a transgenic mouse (Tg) expressing ZF-MIWI2, which bound directly to the target region of type A LINE1 retrotransposons in a piRNA-independent manner.

In this paper, I elucidated the function for ZF-MIWI2 in the induction of DNA methylation and demonstrated its effect on target gene silencing by using MILI-deficient Tg mice. And it was suggested that these effects by ZF-MIWI2 caused to spermatogenetic progression. This study contributes novel insight into the roles and mechanisms of piRNA and MIWI2 in the nucleus.

Results

Production of ZF-MIWI2 transgenic mice

The piRNA-associated protein MIWI2 is thought to act as an effector and guide of *de novo* DNA methylation of the regulatory regions of retrotransposons in embryonic germ cells. To reveal the function of MIWI2, I performed a ChIP assay to establish whether MIWI2 bound to genomic retrotransposon regions. LINE1 retrotransposons are divided into several distinct types based on their sequences. These include type A and TF type, which have similar and unique sequences in their coding and 5′-noncoding regions, respectively. The ChIP assay, using an embryonic testis lysate, revealed that MIWI2 binds to the regulatory region of LINE1 and IAP, but not Rasgrf1 (Fig. 5A). These data indicate that MIWI2 is a key player in *de novo* DNA methylation of retrotransposon regulatory regions in the nucleus. Furthermore, piRNAs corresponding to retrotransposon sequences might act as a guide to the locus of DNA methylation. If MIWI2 can bind to the specific locus in a direct manner, this *de novo* DNA methylation may occur even in the absence of piRNA.

To test this hypothesis, I designed a ZF protein targeted towards the type A LINE1 5'UTR region (5'CCACGGGACCCTAAGACC3'), using the tools available at the URL http://www.zincfingertools.org (Mandell and Barbas, 2006). Zinc finger domains specifically recognize target nucleotide triplets and bind to the DNA by inserting an alpha-helix into the major groove of the DNA double helix (Fig. 5B). The ZF protein was fused to MIWI2, and to the NLS and FLAG tags. Using the ChIP assay, I tested whether ZF-MIWI2 bound to the target type A LINE1 5'UTR region. The results clearly indicated that in ES cells transfected with the ZF-MIWI2 expression plasmid,

ZF-MIWI2 binds specifically to the target type A LINE1 elements, but not to TF type regions (Fig. 5C).

Tg mice were generated, expressing ZF-MIWI2, which binds directly to type A LINE1 target regions without binding piRNA. As a negative control, I generated Tg mice expressing 1) NLS-MIWI2 (Tg FN-MIWI2), in which MIWI2 entered the nucleus, but was not fused to the ZF protein, and 2) NLS-ZF (Tg ZF), in which only the ZF protein bound to the target locus (Fig. 5D). These fusion proteins were expressed and localized mainly to the nucleus in the Tg embryonic testes (E16.5) (Fig. 5E). Expression levels were similar between ZF-MIWI2 #6 and #7 (Fig. 5F). I subsequently used an in vivo ChIP assay to verify whether the ZF protein bound to the target type A LINE1 5'UTR region. The ZF protein bound specifically to the target region in the Tg embryonic testes (E16.5) (Fig. 5G).

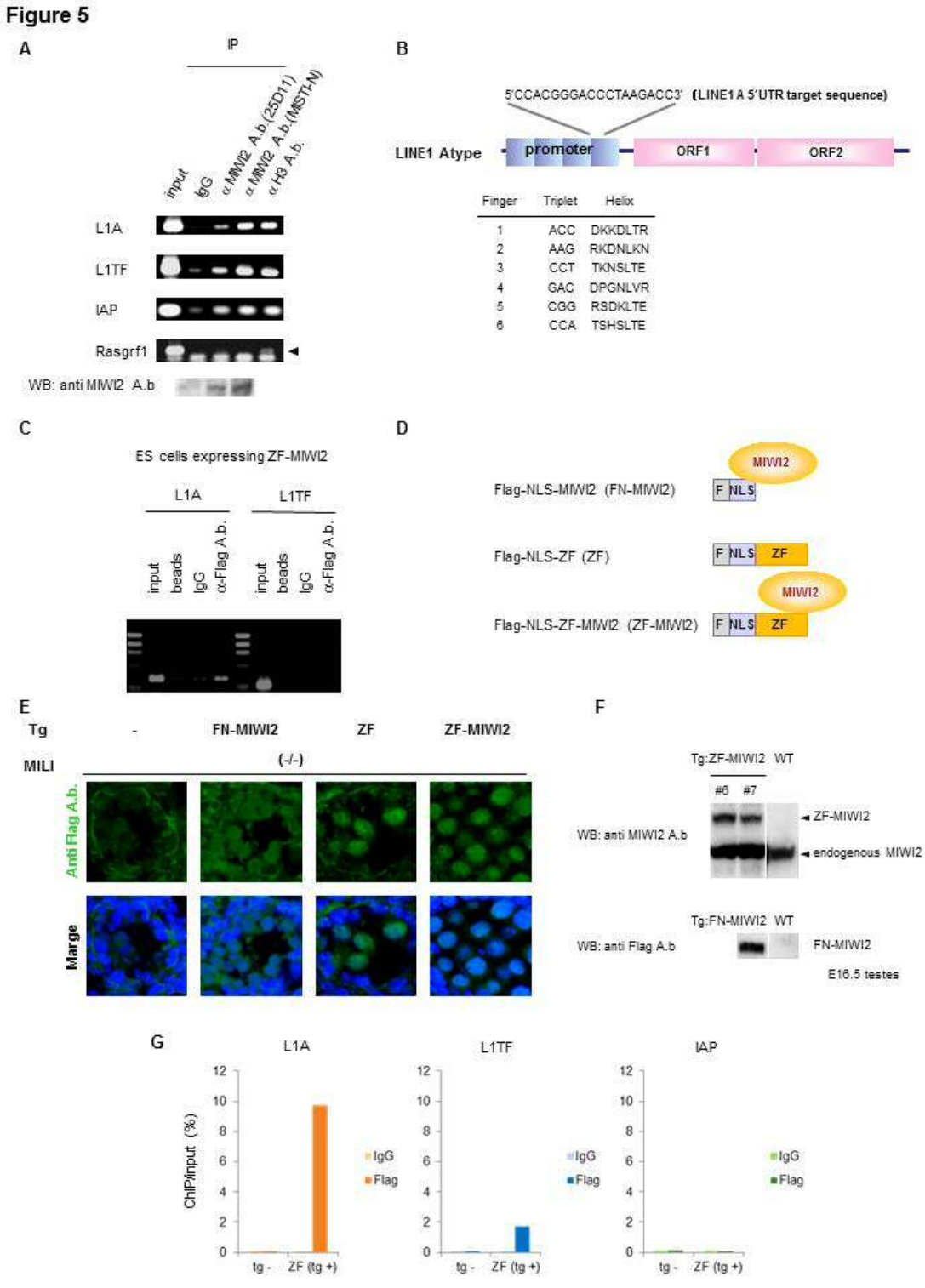

Figure 5 ZF-mediated targeting of MIWI2 to the LINE1 A type promoter region

(A) (Top) The ChIP with Anti-MIWI2 (MISTI-N, 25D11) in embryonic testis (E16.5). The embryonic testis lysates were Chromatin-ImmunoPrecipitated with anti-MIWI2 antibodies, and subsequently subjected to Western blotting

with the anti- MIWI2 (25D11) antibody (Bottom). 5'UTR region of LINE1 (A and TF type), the LTR region from the IAI-type IAP in chromosomes 3qD and DMR region of Rasgrf1 were used as primer. (B)(Top) The 18bp ZF target LINE1 A type promoter sequence. (Bottom) Each DNA triplet is shown with the corresponding amino acid sequence of its recognition helix. (C) ChIP with anti-Flag antibody using ES cells expressing ZF-MIWI2. ZF binds selectively to target LINE1 A type but not to TF type region. (D) Schematic diagrams of Flag-tagged NLS-MIWI2, Flag-tagged NLS-ZF and Flag-tagged-NLS-ZF-MIWI2. "F" indicates the Flag tag. (E) Immunostaining of FLAG-tagged proteins in embryonic testes under MILI-deficient condition (E16.5). FN-MIWI2, ZF and ZF-MIWI2 are stained with the Alexa Fluor 488-conjugated anti-mouse immunoglobulins (H+L) after binding with anti-Flag antibody (green color) and DNA was visualized by DAPI (blue color). (F) Protein expression of ZF-MIWI2 (Upper panel) and FN-MIWI2 (lower panel) in Tg embryonic testes (E16.5). Lysate from Tg ZF-MIWI2 (#6, #7) and Tg FN-MIWI2 embryonic testes is used. (G) The percentage of ChIP with anti-FLAG antibody relative to input chromatine as quantified by qPCR analysis in Tg ZF embryonic testis (E16.5). 5'UTR region of LINE1 (A and TF type) and the LTR region from the IAI-type IAP in chromosomes 3qD were analyzed.

Induction of CpG methylation of DNA by ZF-MIWI2

To explore the possibility that ZF-MIWI2 induces DNA methylation, the DNA methylation status of the target region was examined using Tg mice (Tg ZF-MIWI2, Tg FN-MIWI2, Tg ZF) crossed with MILI-deficient mice. I isolated male germ cells from the testes of 12-day-old mice and subjected their genetic material to bisulfite sequencing at the promoter region of the retrotransposon genes. The high purity of the germ cells was confirmed by the high methylation status (>90%) of DMR-H19, a paternal imprinted gene (data not shown). A significant reduction in the CpG methylation level of retrotransposon genes was observed in MILI-deficient male germ cells (LINE1 A: 5.0%, LINE1 TF: 30.1%, IAP: 15.9%), compared to almost complete methylation in

control germ cells (LINE1 A: 88.8%, LINE1 TF: 85.1%, IAP: 91.4%). In MILI-deficient Tg ZF-MIWI2 germ cells, the DNA methylation status of the ZF targeted type A LINE1 locus was slightly rescued, compared to MILI-deficient male germ cells (Tg ZF-MIWI2 #6, 7). Furthermore, the methylation status of the region not targeted by the ZF protein (LINE1 TF type, IAP) was not affected by ZF-MIWI2. The level of methylation of these retrotransposon genes remained low in MILI-deficient Tg FN-MIWI2 germ cells, MILI-deficient Tg ZF germ cells as well as in MILI-deficient germ cells. These data indicate that CpG methylation of the ZF targeted type A LINE1 locus is slightly induced by ZF-MIWI2, even in the absence of piRNA binding (Fig. 6).

Figure 6

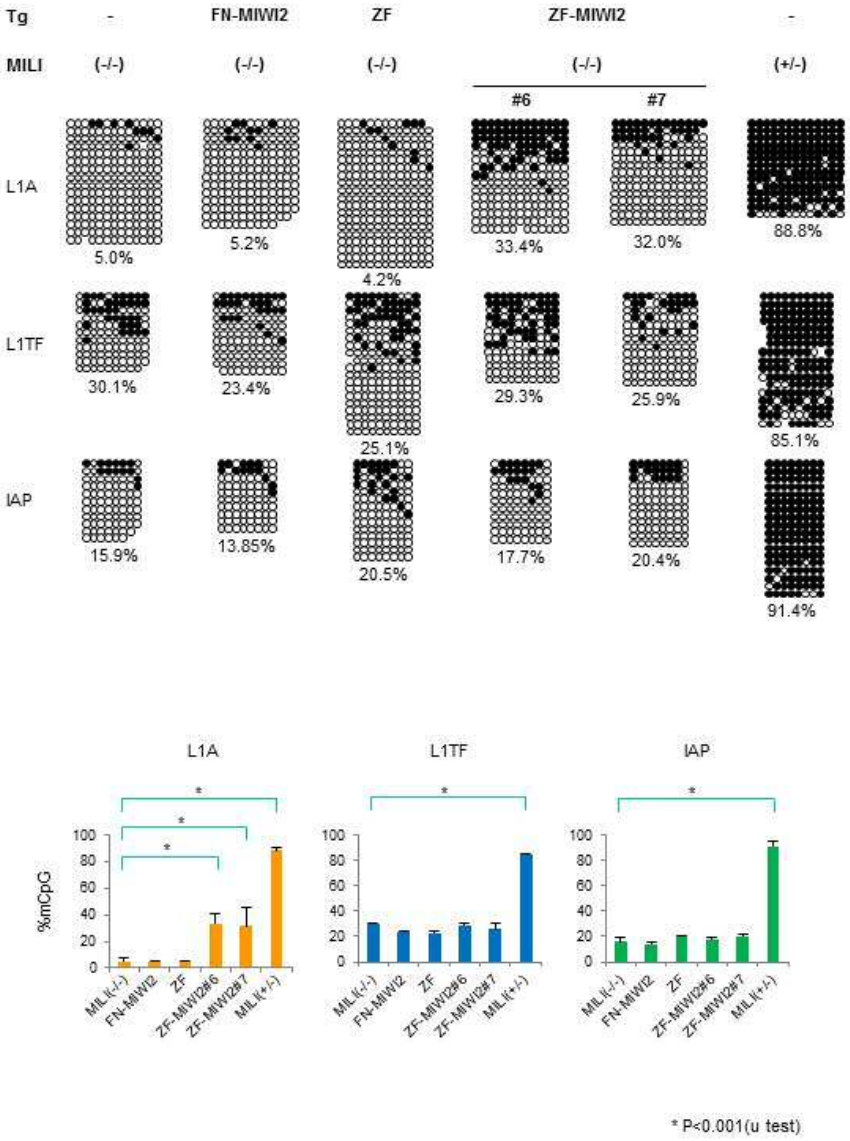

Figure 6 DNA methylation effects by ZF-MIWI2 fusion protein in MILI-deficient testis.

(Top) Bisulfite sequencing of LINE1 (A and TF type) and IAP. Germ cells were collected from p12 testis using anti Epcam antibody as a marker. 5'UTR region of LINE1 (A and TF type) and the LTR region from the IΔI-type IAP in

chromosomes 3qD were analyzed. Filled and open 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. n≥3. (Bottom) The results of three independent experiments and statistical analysis are summarized (u test). Error bars denote SD.

Repression of ZF target LINE1 expression by ZF-MIWI2

Next, I examined the expression of the ZF targeted type A LINE1 gene in the testis of three-week-old mice, using Northern blotting (Fig. 7A). The transcripts of the two LINE1 types, type A and TF type, were up-regulated in the MILI-deficient testis, as was the expression of IAP. In MILI-deficient Tg ZF-MIWI2 testes, the transcripts of type A LINE1 retrotransposons were almost undetectable (Tg ZF-MIWI2 #6, #7). Conversely, the accumulation of TF type LINE1 and IAP transcripts was unchanged in Tg ZF-MIWI2 mice. In the testes of MILI-deficient negative control transgenic mice (Tg FN-MIWI2 and Tg ZF), the expression of these retrotransposon transcripts was similar to that in MILI-deficient testes. A ChIP assay was performed to assess the recruitment of RNA polymerase II protein to the target type A LINE1 region in MILI-deficient Tg testes (Fig. 7B). RNA polymerase II protein recruitment to the regulatory region of retrotransposon genes was observed in both MILI-deficient Tg FN-MIWI2 testes and MILI-deficient testes. In MILI-deficient Tg ZF-MIWI2 testes, however, little recruitment of RNA polymerase II protein to target type A LINE1 A regions was observed. Histone H3 ChIP was used as a positive control in these experiments. These data suggest that ZF-MIWI2 influences the silencing of ZF targeted type A LINE1 genes by transcriptional processes.

Figure 7

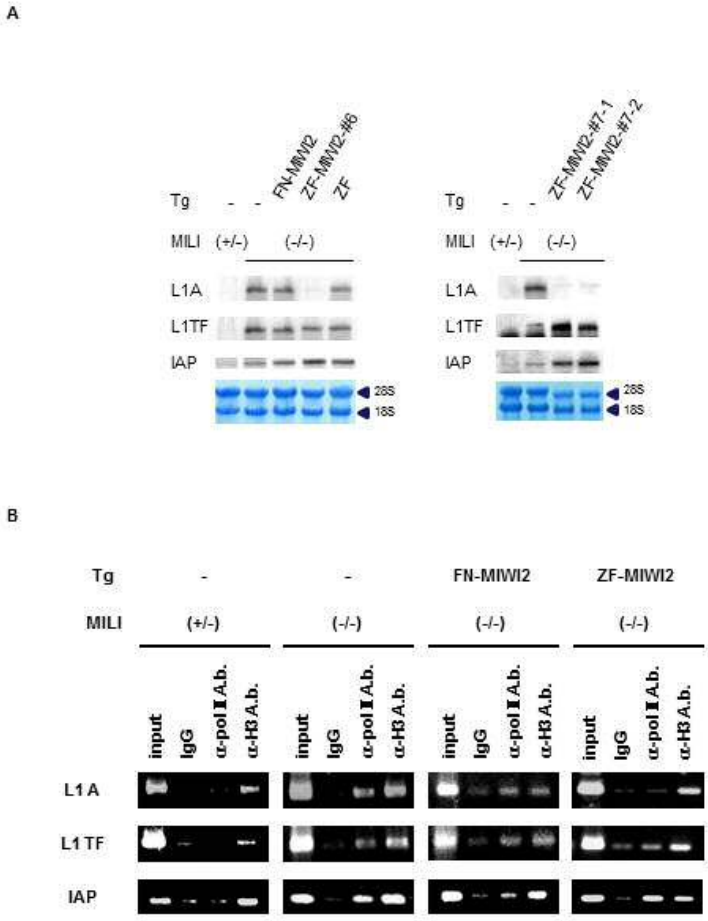

Figure 7 Expression of the LINE1 and IAP retrotransposons and Pol II Recruitment in the Tg ZF-MIWI2 testes.

(A) Northern blotting analysis of LINE1 and IAP retrotransposons in the testes of 3-wk-old Tg FN-MIWI2-, Tg ZF-, Tg ZF-MIWI2 (#6,#7)- *MILI*$^{-/-}$ and *MILI*$^{+/-}$ mice. The 5′UTRs of A and TF type LINE 1 and the 3′UTR of IAP were

used as probes. (B) ChIP with antibody against total Pol II using testes of 3-wk-old Tg FN-MIWI2-, Tg ZF-MIWI2-*MILI*$^{-/-}$ and *MILI*$^{+/-}$ mice. 5'UTR region of LINE1 (A and TF type) and the LTR region from the IAP-type IAP in chromosomes 3qD were analyzed.

Spermatogenesis in MILI-deficient Tg ZF-MIWI2 testes

Next, I observed spermatogenesis in the Tg testes (Tg ZF-MIWI2, Tg FN-MIWI2, Tg ZF) under MILI-deficient conditions. The size of each Tg MILI-deficient testis was as small as that of MILI-deficient testis, compared to control testis (Fig. 8A). To investigate spermatogenesis in more detail, I performed immunostaining using an antibody against MIWI, which is normally detected at the later stages of spermatogenesis, from pachytene spermatocyte to elongating spermatid. In Tg FN-MIWI2 and Tg ZF testes under MILI-deficient conditions, as well as in MILI-deficient testes, MIWI was not detected. Impaired spermatogenesis was observed at the pachytene stage in these testes. However, a few germ cells expressing MIWI were observed in MILI-deficient Tg ZF-MIWI2 testes. The germ cells expressing MIWI appeared to be round spermatid cells in the process of forming chromatoid bodies (Fig. 8Bd, 8Bi, 8C). These data suggest that spermatogenesis might proceed to a later stage, beyond pachytene spermatocytes, in these testes. Some effects of ZF-MIWI2, such as the partial rescue of de-repression of retrotransposon genes, might therefore be important for spermatogenetic progression to the round spermatid stage.

Figure 8

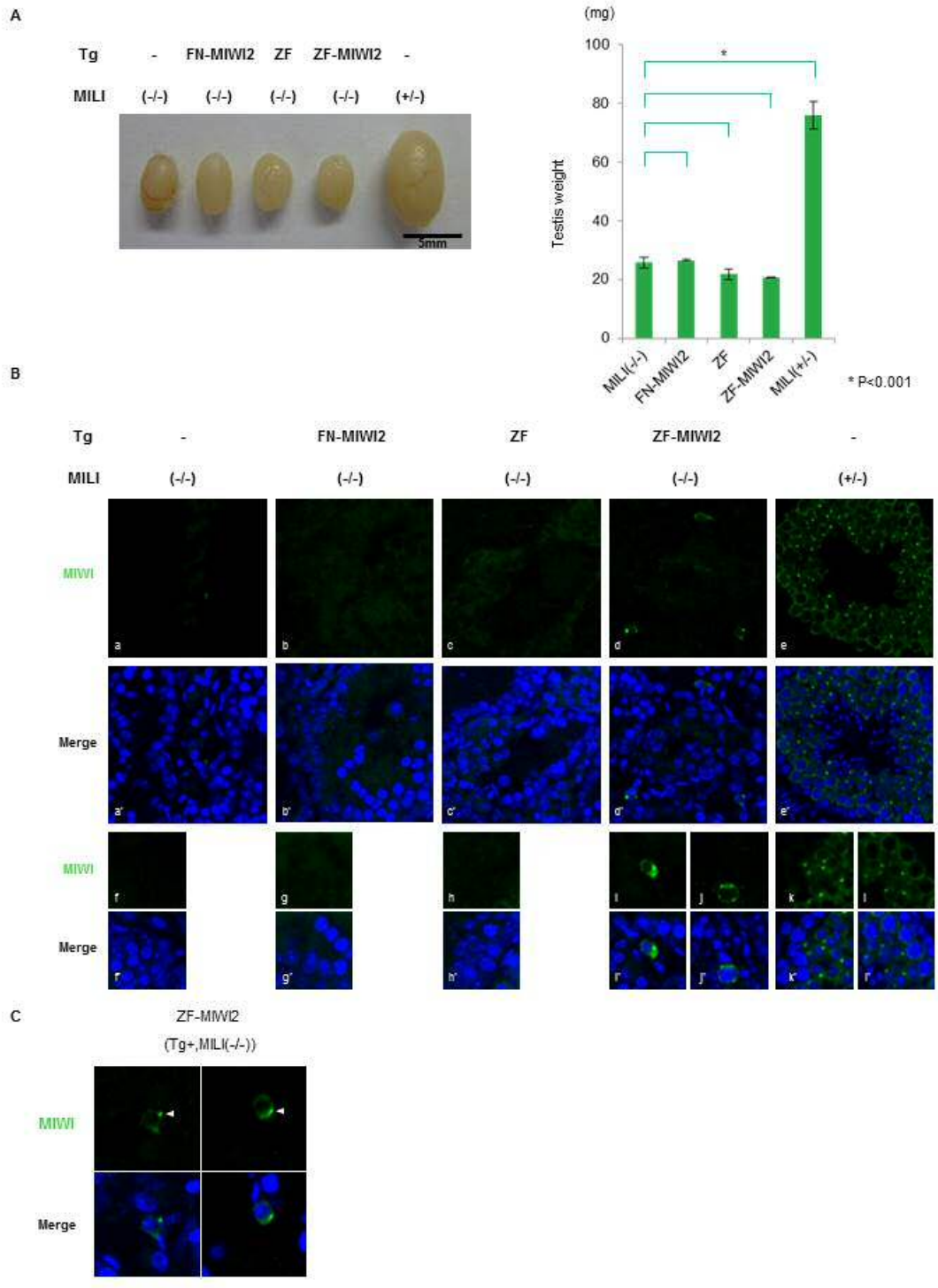

Figure 8 Spermatogenesis in Tg ZF-MIWI2 testes under MILI-deficient condition.

(A) (Left) Comparison of the testes from 6-week-old *MILI*$^{+/-}$, *MILI*$^{-/-}$,Tg FN-MIWI2-, Tg ZF-, Tg ZF-MIWI2- *MILI*$^{-/-}$ mice. Bar, 5 mm. (Right) Weights of testes from above-mentioned mice. Error bars denote SD. (B)

Immunohistochemical staining of *MILI*$^{+/-}$, *MILI*$^{-/-}$,Tg FN-MIWI2-, Tg ZF-, Tg ZF-MIWI2-*MILI*$^{-/-}$ testes. Sections from mouse testes were stained with the Alexa Fluor 488-conjugated anti-rabbit immunoglobulins (H+L) after binding with the anti-MIWI antibody (green color) and DNA was visualized by DAPI (blue color). (C) Immunohistochemical staining of round spermatid in Tg ZF-MIWI2-*MILI*$^{-/-}$ testes. Sections from mouse testes were stained with the Alexa Fluor 488-conjugated anti-rabbit immunoglobulins (H+L) after binding with the anti-MIWI antibody (green color) and DNA was visualized by DAPI (blue color). Arrow head indicates chromatoid body.

Discussion

Induction of DNA methylation by ZF-MIWI2 in a piRNA-independent manner

Previous studies have reported a decrease in piRNA and a reduction in DNA methylation in the regulatory region of retrotransposon genes (LINE1 and IAP), as well as accumulation of the corresponding transcripts in MILI-and MIWI2-deficient mice (Aravin et al., 2007; Carmell et al., 2007; Kuramochi-Miyagawa et al., 2008). Most retrotransposons are maintained in a transcriptionally silenced status by DNA methylation (Walsh et al., 1998). Based on these reports, it is suggested that MILI and MIWI2 are involved in retrotransposon gene silencing by *de novo* methylation via piRNA. Furthermore, MIWI2, but not MILI, localizes mostly to the nucleus in embryonic germ cells. Together, these data suggest that the MIWI2/piRNA complex acts as an effector or guide of the *de novo* DNA methylation of retrotransposon regulatory regions. In this study, I employed a ZF targeting strategy to address the possibility of a piRNA-independent effect of MIWI2 in the nucleus. In the ZF targeted type A LINE1 region, DNA methylation was induced by a gene-targeted ZF fused to MIWI2, but not by the same ZF not fused to MIWI2, even when it localized to the nucleus by means of its own NLS. These data indicate that, under these conditions, even if MIWI2 localizes to the nucleus without piRNA, it does not induce DNA methylation. In contrast, endogenous MIWI2 in the presence of piRNA in the wild-type embryonic testis does induce DNA methylation of the target region. DNA methylation itself is therefore independent of the presence of piRNA. I therefore conclude that piRNAs corresponding to the retrotransposon genes may play a role as a guide, leading MIWI2 to the target region. This report is the first to demonstrate MIWI2-mediated DNA methylation at the target retrotransposon region. I also show

that MIWI2 is involved in the recruitment of some proteins involved in epigenetic gene silencing in the nucleus.

Partial progressive spermatogenesis in MILI-deficient Tg ZF-MIWI2 mice

Spermatogenesis in MILI-deficient mice is arrested at the pachytene spermatocyte stage, at which point apoptosis is induced, before the cells would usually begin expressing MIWI (Fig.1). Interestingly, a few germ cells expressing MIWI were observed in MILI-deficient Tg ZF-MIWI2 testes. This suggests that the impaired spermatogenesis observed under MILI-deficient conditions is partially rescued in Tg ZF-MIWI2 mice.

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). In mice that are mutants in piRNA-biogenesis-related TDRDs, such as TDRD1, TDRD9 and TDRD12, spermatogenesis is arrested at the meiotic prophase and LINE1-retrotransposon transcripts are upregulated in a manner similar to that seen in MILI- or MIWI2-mutants (Pandey et al., 2013; Reuter et al., 2009; Shoji et al., 2009). However, spermatogenesis in TDRD5-deficient mice proceeds through the meiotic prophase to the round spermatid phase, regardless of de-repression of LINE1 (Yabuta et al., 2011). This is somewhat similar to the phenotype of MILI-deficient Tg ZF-MIWI2 mice. In this TDRD5-focused research paper, it was suggested that the degree of de-regulation of LINE1 is milder in TDRD5-deficient testes, compared with testes deficient in other proteins (*Tdrd1, Tdrd9, Tdrd12, Mili, Miwi2)* (Yabuta et al., 2011). Considering these data together, I believe that the partial rescue of spermatogenesis under MILI-deficient conditions is a result of partial retrotransposon silencing by

ZF-MIWI2.

Transcriptional repression by ZF-MIWI2

Upregulation of target genes was drastically rescued by MILI-deficient ZF-MIWI2, despite the only partial rescue of DNA methylation (Figs. 6 and 7). It is reported that PIWI-related retrotransposon gene silencing is associated with not only DNA methylation of the targeted CpG sites but also the presence of H3K9me3 in the promoter. Recently, it was demonstrated that the level of H3K9me3 modification of retrotransposon genes was decreased in MILI- and MIWI2- deficient testes (Pezic et al., 2014). To investigate PIWI-related histone modification, I analyzed the density of H3K9me3 modifications at the ZF target region by ChIP assay, using Tg male germ cells isolated 12 days after birth (Fig.9). However, the difference in H3K9me3 levels at the ZF targeted type A LINE1 locus between Tg ZF-MIWI2 germ cells under MILI-deficient conditions and MILI-deficient germ cells was very small. These data suggest that ZF-MIWI2 does not have a direct effect on H3K9me3 methylation of the ZF target region. However, it is reported that histone H3K9 dimethyltransferase G9a co-suppresses LINE1 in spermatogonia, although it is not essential for retrotransposon silencing (Di Giacomo et al., 2014; Tachibana et al., 2008; Tachibana et al., 2002). Thus, histone modification is important for the epigenetic transcriptional regulation of retrotransposon genes. Some form of histone modification, other than H3K9me3, might be essential for retrotransposon silencing. In addition, it is possible that silencing of the ZF targeted gene by ZF-MIWI2 might represent a secondary effect of an unknown gene silencing mechanism. In future studies, I will aim to identify the molecules involved with retrotransposon gene silencing that associate with ZF-MIWI2.

Figure 9

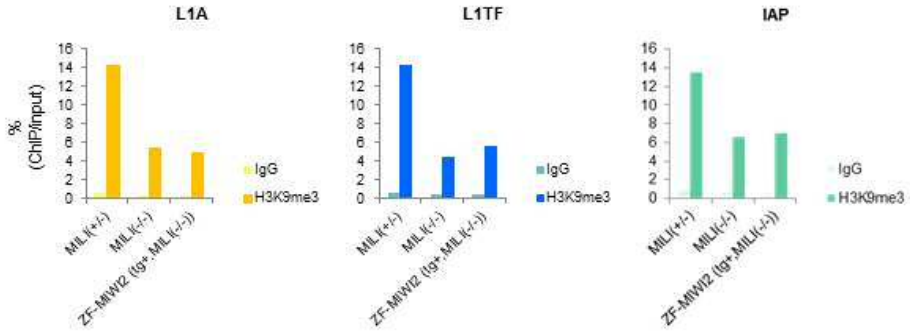

Figure 9 ChIP with anti-H3K9me3 antibody using germ cells. Germ cells were collected from *MILI*$^{+/-}$, *MILI*$^{-/-}$, Tg ZF-MIWI2-*MILI*$^{-/-}$ p14 testes using anti Epcam antibody as a marker. The testis lysates were Chromatin-Immuno-Precipitated with anti-H3K9me3 antibodies, and subsequently subjected to q-PCR. 5'UTR

region of LINE1 (A and TF type) and the LTR region from the IAI-type IAP in chromosomes 3qD were used as primer.

Experimental procedures

Zinc finger (ZF) protein Design

To design ZF Protein, I used Zinc finger tools at URL http://www.zincfingertools.org. I chose the 18bp of 5' UTR in LINE1 A type gene as a target for ZF Protein. To generate the amino acid sequence for a ZF Protein, each zinc finger repeat is modeled between the N-and C-terminal backbones. ZF has the sequence of each variant helix depended on triplet for target sequence. The linker sequence is placed between ZF repeats and the construct containing all fingers are flanked by the N-and C-terminal fixed sequences (Mandell and Barbas, 2006).

The amino acid sequence of N-terminal backbone : YKCPECGKSFS

The amino acid sequence of C-terminal backbone : HQRTH

The fixed amino acid sequence of N-terminus : LEPGEKP

The fixed amino acid sequence of C-terminus : TGKKTS

The amino acid sequence of linker : TGEKP

The amino acid sequence of designated ZF protein is as follows;

LEPGEKPYKCPECGKSFSDKKDLTRHQRTHTGEKPYKCPECGKSFSRKDNLKNHQRTHTGEKPYKCPECGKSFSTKNSLTEHQRTHTGEKPYKCPECGKSFSDPGNLVRHQRTHTGEKPYKCPECGKSFSRSDKLTEHQRTHTGEKPYKCPECGKSFSTSHSLTEHQRTHTGKKTS (176aa)

Plasmids

The Flag-tagged NLS-ZFP-MIWI2, Flag-tagged NLS-ZFP and Flag-tagged NLS-MIWI2 expression plasmids were constructed by inserting the Flag tag and NLS

(SV40: PKKKRKV) at the N-terminus of Zinc finger protein fused MIWI2, Zinc finger protein only or MIWI2 into the pcDNA4 vector (Invitrogen, Carlsbad, CA).

Generation of transgenic mice

Construction of the Flag-NLS-ZFP-MIWI2 (ZF-MIWI2), Flag-NLS-ZFP (ZF) or Flag-NLS-MIWI2 (FN-MIWI2) transgenes has been described as follows. At first, the *Mili* promoter region including noncoding 1st exon and 1st intron to just after ATG start site in 2nd exon (5.7 kbp; 70,432,369 – ctcaATGgatcc (BamHI) 70,426,691 in Ch14 by GRCm38/mm10) was constructed into SalI-BamHI sites of pEGFP-N1 plasmid. Then EGFP sequence was replaced to ZF-MIWI2, ZF, or FN-MIWI2 by BamHI and NotI sites. The inserts including *Mili* promoter and polyA of the constructs were isolated and purified. Some fertilized eggs obtained from mating of BDF1 males and females were used for pronuclear injection of the DNA to generate transgenic mice. (generated by NPO for Biotechnology Research and Development, Research Institute for Microbial Diseases, Osaka University.) Founder mice were mated with C57Bl/6 mice to generate lines. Produced transgenic mice were identified by polymerase chain reaction (PCR) with specific primers as follows;

[For FN-MIWI2 or ZF-MIWI2 transgenes Detection]

5' Primer: 5'-TGGAACAGAACCAACAGTCG-3'

3' Primer: 5'-GAAGTCCTGTCTGCCTTTGC-3'

[For ZF transgene Detection]

5' Primer: 5'-TGGAACAGAACCAACAGTCG-3'

3' Primer: 5'-TTCCGTTAGCTTATCACTTCT-3'

Preparation of mouse under MILI-deficient condition

MILI heterozygous mutant male mice carrying *Flag-NLS-MIWI2, Flag-NLS-ZFP, Flag-NLS-ZFP-MIWI2* transgene were crossed with *MILI* homozygous mutant female mice. The specific PCR primers for genotyping of *Mili* are as follows;

[For Endogeneous Detection]

5' Primer: 5'-AGG TCC TGT GTA GAG CCA AG-3'

3' Primer: 5'-AAG TGA CAA GGT GCG AGT CT-3'

[For KO Detection]

5' Primer: 5'-CCT ACC CGG TAG AAT TGA CC-3'

3' Primer: 5'-AAG TGA CAA GGT GCG AGT CT-3' (same sequence for endogenous detection)

Isolation of Germ cells

Whole testis (D12 after birth) was removed and treated with 1 mg ml^{-1} collagenase type II and DNase1 15min at 37°C. And it was suspended with 0.25%tripsin 10 min at 37°C and washed with Hank's Stock Solutions (HBSS) (nacalai tesque, Japan). Anti-EpCAM (PE) antibody (CD326) was added in the cell suspension with 5% BSA/PBS. After rotating 90 min at 4°C, the immune complex was washed with HBSS. The germ cells were sorted using immunostaining of EpCAM using a BD FACSAria system (BD Biosciences, Franklin Lakes, NJ). The purity of germ cells were verified by rerunning the sample after sorting and was always found to be more than 95%.

Cell culturing, transient transfection

The ES cells were maintained in the absence of feeder cells in Glasgow modification of Eagle medium (GMEM) supplemented with fetal calf serum, 2-mercaptoethanol, and LIF. The ES cells were transfected with each plasmid using Polyethylenimine (Cosmo Bio, Tokyo, Japan) according to the manufacturer's recommendations. At 27 hr post-transfection, the cells were harvested by centrifugation for 10 min at 3000 r.p.m. at 4°C.

Western blotting

The immunoprecipitates or lysates were separated by SDS–PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). After blocking, the filters were incubated with anti MIWI2 antibody (25D11) or anti-Flag M2 antibody (Sigma Chemical Co., St. Louis, MO) or anti- -actin antibody (Sigma). Anti MIWI2 monoclonal antibody (25D11) was produced with GKGRQDFEELGVC (69-80 aa peptide sequence of MIWI2) as antigen by Dr. Yasuyuki Kurihara (Yokohara National University). HRP-anti-mouse IgG (Pierce, Rockford, IL) was used as the secondary antibody, and the signal was detected using the ECL Western Blotting detection reagents (GE Healthcare, UK).

ChIP-PCR assay

(For ES cells)

ES cells were treated with 1%PFA for 8 min at room temperature. After quenching the PFA crosslinking reaction with 0.2M glycine, the fixed cells were washed with PBS. The cells were suspended in radio immunoprecipitation assay (RIPA) buffer (20 mM HEPES-NaOH(pH 7.5) 150 mM NaCl, 1 mM EDTA (pH 8), 1% NP-40, 0.5%

deoxycholate and 0.1% SDS) and sonicated to an average DNA fragment size of around 500 bps. Solubilized chromatin was clarified by centrifugation for 10 min at 15,000 r.p.m. at 4 °C. The chromatin was incubated with anti-Flag M2 antibody (Sigma Chemical Co) over night at 4 °C. Immune complexes are bound to pre-blocked protein G Sepharose 4 Fast Flow beads (GE Healthcare, UK) for 3 h at 4 °C. The pre-blocked beads were treated with salmon sperm DNA and BSA at 4 °C for 1 h. The immune complex bound to protein G beads were washed with RIPA buffer, high-salt wash buffer (20 mM Tris–HCl (pH 8), 500 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate and 0.1% SDS), LiCl wash buffer (250 mM LiCl, 20 mM Tris–HCl (pH 8), 1 mM EDTA, 1% NP-40 and 0.5% deoxycholic acid (DOC)), and with Tris–EDTA. Immune complexes bound to protein G beads were suspended in elution buffer (20 mM Tris–HCl (pH 8), 300 mM NaCl, 1 mM EDTA and 0.5% SDS) and incubated over night at 65 °C. After incubation, the samples were treated with 30 μg ml^{-1} RNase A for 1 h at 37 °C, and 100 μg ml^{-1} proteinase K over night at 56 °C. DNA was extracted with phenol/chloroform and precipitated with ethanol + Dr.GenTLE (Takara) as a carrier. Precipitated DNA was re-suspended in 50 μl of water and analyzed by PCR using the specific primers mentioned-below.

(For 3W testis)

Whole testis (3W after birth) was removed and treated with 1 mg ml^{-1} collagenase type II and DNase1 and 0.25%tripsin 15 min at 37°C. The cell suspension was washed and reated with the same method above-mentioned. The chromatin was incubated with anti- polII antibody ((N-20) sc-899, santa cruz, USA) over night at 4 °C. Immune complexes were bound to protein G Dynabeads (life technologies, Norway) for 1.5 h at 4 °C. The immune complex bound to protein G beads were washed with RIPA buffer,

high-salt wash buffer, LiCl wash buffer, and with Tris–EDTA. Immune complexes bound to protein G beads were treated with the same method above-mentioned. Precipitated DNA was re-suspended in 50 µl of water and analyzed by PCR using the specific primers mentioned-below.

(For isolated germ cells and embryonic testes)

E16.5 testis was removed and treated with 1 mg ml^{-1} collagenase type II and DNase1 and 0.25%tripsin 15 min at 37°C. The cell suspension or isolated germ cells were treated with 1%PFA for 8 min at room temperature. After quenching the PFA crosslinking reaction with 0.2M glycine, the fixed cells were washed with PBS. The cells were suspended in lysis buffer (50mM Tris pH(7.5), 140mM NaCl, 10µM EDTA, 1% SDS) and sonicated. After addtion of RIPA buffer, Solubilized chromatin was clarified by centrifugation for 10 min at 15,000 r.p.m. at 4 °C. The chromatin was incubated with anti-H3K9me3 antibody (ab8898, Abcam, Japan), anti-MIWI2 antibody ((25D11) or MISTI-N which described previously (Kuramochi-Miyagawa et al., 2008)), anti-H3 (ab1791, Abcam, Japan) or anti-Flag antibody (FLA1, MBL, Japan) over night at 4 °C. Immune complexes were bound to protein G Dynabeads for 1.5 h at 4 °C. The immune complexes bound to protein G beads were washed with RIPA buffer and Tris–EDTA. Immune complexes bound to protein G beads were suspended in elution buffer and incubated over night at 65 °C. After incubation, the samples were treated with 30 µg ml^{-1} RNase A for 1 h at 37 °C, and 100 µg ml^{-1} proteinase K over night at 56 °C. DNA was extracted with phenol/chloroform and precipitated with ethanol + Dr.GenTLE (Takara) as a carrier. Precipitated DNA was re-suspended in 30 µl of water and analyzed by PCR using the specific primers mentioned-below.

Bisulfite Sequencing

Sorted germ cells (D12 after birth) were bisulfite-treated with the EZ DNA Methylation-Direct Kit (Zymo Research, Irvine, CA). Sequences of the PCR primers are mentioned-below. The first and second rounds of PCR amplification of IAP (GenBank accession no. M17551) and the *H19* (GenBank accession no. U19619) were carried out with Ex Taq (Takara Bio, Shiga, Japan). The PCR conditions were as follows: the first round of 2 min at 94°C followed by 30 cycles consisting of 30 s at 94°C, 30 s at 50°C (for H19) or 1min at 55°C (for IAP), and 1 min at 68°C, and the second round of 2 min at 95°C followed by 15 cycles consisting of 30 s at 95°C, 30 s at 50°C (for H19) or 1 min at 56°C (for IAP), and 1 min at 72°C. Fully or seminested PCR was performed to amplify the *H19* (GenBank accession no. U19619) differentially methylated regions (DMRs). The PCR of LINE1 Atype (GenBank accession. no. M13002) and LINE1 TFtype (GenBank acc. no. D84391) were carried out with AccuPrime Taq DNA polymerase (Invitrogen) under the following conditions: 2 min at 94°C followed by 30 cycles consisting of 30 s at 94°C, 30 s at 55°C (for Line1 Atype) or 30 s at 50°C (for Line1 TFtype), and 1 min at 68°C,. The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), cloned into the pGEM-T Easy Vector (Promega, Madison, WI), and then sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Northern blot analysis

Total RNA samples were prepared from testes using ISOGEN (NIPPON GENE CO., LTD.) and stained by 0.02% methylenblue. RNA Northern blot analysis was performed at 65°C in 0.2 M $NaHPO_4$ (pH 7.2), 1 mM EDTA, 1% BSA, and 7% SDS. The

membranes were washed with a 0.2× SSC, 0.1% SDS solution at 65°C. The subcloned PCR products were labeled with [α-^{32}P]-dCTP and used as probes. The sequences used for PCR primers were as follows: the 3′-noncoding region of IAP (GenBank accession no. X04120), nucleotides 4489–4793; and the 5′-noncoding region of LINE1 A type (M13002), nucleotides 531–1642 and LINE1 TF type (D84391), nucleotides 874–1156.

Immunohistochemical staining

Testes of the *MILI* homozygous and heterozygous mutant male mice, and *MILI* homozygous mutant male mice carrying *Flag-NLS-MIWI2, Flag-NLS-ZFP, Flag-NLS-ZFP-MIWI2* transgene were dissected and fixed in 4% paraformaldehyde for 2 hr at 4°C (for 6W testes) or 2% paraformaldehyde for 1hr at 4°C (for E16.5testes). After washing in PBS contained 10% and 20% sucrose, the testes were embedded in OCT compound. The cryosections blocked with 10% normal goat serum and 3% BSA in PBS for 0.5 hr at room temperature were used for immunofluorescence staining. After treatment of the anti-MIWI polyclonal antibody (G82; Cell Signaling; USA) or anti-Flag M2 antibody (SIGMA) over night at 4°C. Alexa Fluor 488-conjugated anti-rabbit immunoglobulins (H+L) (Molecular Probes, Eugene, OR) was used as the secondary antibody for 1 hr at room temperature. Nuclei were counterstained with 1 μg ml^{-1} DAPI. Immunostained cryosections were examined under a confocal microscope (LSM5Pascal, Carl Zeiss Co.,Ltd., Japan).

Primer (for Bisulfite sequencing)

Primers Sequence (5' – 3') are as follows;

H19 (GenBank acc. no. U19619), product size: 423 bp

1st (outside) forward: GAGTATTTAGGAGGTATAAGAATT

1st (outside) reverse: ATCAAAAACTAACATAAACCCCT

2nd (inside) forward: GTAAGGAGATTATGTTTATTTTTGG

2nd (inside) reverse: CCTCATTAATCCCATAACTAT

LINE1(A) (GenBank acc. no. M13002), product size: ～310 bp

Forward: TTATTTTGATAGTAGAGTT

Reverse: CAAACCAAACTCCTAACAA

LINE1(Tf) (GenBank acc. no. D84391), product size: ～270 bp

Forward: GTTAGAGAATTTGATAGTTTTTGGAATAGG

Reverse: CCAAAACAAAACCTTTCTCAAACACTATAT

IAP, product size: 259 bp (ch3)

Ch3-1st forward: GTTTGTAATGGTGGGAGA

1st reverse: AAATAAAATATCCCTCC

2nd forward: TTGTGTTTTAAGTTGGTAAATAAATAATTTG

2nd reverse: CAAAAAAAACACCACAAACCAAAAT

Primer (for ChIP-PCR)

Primers Sequence (5' – 3') are as follows;

LINE1(A) (GenBank acc. no. M13002),

Forward: CTACCTTGACAGCAGAGTC

Reverse: CTCTCCTTAGTTTCAGTGG

LINE1(Tf) (GenBank acc. no. D84391)

Forward: TAGGAAATTAGTCTGAACAGGTGAG

Reverse: TCAGACACTGTGTTGCTTTGGCAG

IAP (ch3)

Forward: CAAGATGGCGCTGACATCCT

Reverse: TTCGCAGTTCTGGTTCTGGA

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Achievements

Associations between PIWI proteins and TDRD1/MTR-1 are critical for integrated subcellular localization in murine male germ cells

Genes to Cells (2009) 14, 1155–1165　Kanako Kojima, Satomi Kuramochi-Miyagawa, Shinichiro Chuma, Takashi Tanaka, Norio Nakatsuji, Tohru Kimura and Toru Nakano

以下の論文においては、サンプル採取などに関与しました。

DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes

GENES & DEVELOPMENT 22:908–917 (2008)

Satomi Kuramochi-Miyagawa, Toshiaki Watanabe, Kengo Gotoh, Yasushi Totoki, Atsushi Toyoda, Masahito Ikawa, Noriko Asada, Kanako Kojima, Yuka Yamaguchi, Takashi W. Ijiri, Kenichiro Hata, En Li, Yoichi Matsuda, Tohru Kimura, Masaru Okabe, Yoshiyuki Sakaki, Hiroyuki Sasaki, and Toru Nakano

MVH in piRNA processing and gene silencing of retrotransposons

GENES & DEVELOPMENT 24:887–892 (2010)

Satomi Kuramochi-Miyagawa, Toshiaki Watanabe, Kengo Gotoh, Kana Takamatsu, Shinichiro Chuma, Kanako Kojima-Kita, Yusuke Shiromoto, Noriko Asada, Atsushi Toyoda, Asao Fujiyama, Yasushi Totoki, Tatsuhiro Shibata, Tohru Kimura, Norio Nakatsuji, Toshiaki Noce, Hiroyuki Sasaki, and Toru Nakano

GPAT2, a mitochondrial outer membrane protein, in piRNA biogenesis in germline stem cells.

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