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Induction of DNA methylation by artificial piRNA production in male germ cells

(雄性生殖細胞における人為的 piRNA 産生を介した DNA メチル化の誘導)

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Abstract

Global DNA demethylation and subsequent *de novo* DNA methylation take place in mammalian male embryonic germ cells. PIWI (P element induced wimpy testes) - interacting RNAs (piRNAs), which are germline-specific small RNAs, have been postulated to be critically important for *de novo* DNA methylation of retrotransposon genes, and many proteins, including PIWI family proteins play pivotal roles in this process. In the embryonic mouse testis, two mouse PIWI proteins, mouse PIWI-like (MILI) and mouse PIWI2 (MIWI2), are involved in the biogenesis of piRNAs through the so-called ping-pong amplification cycle, and long single-stranded RNAs transcribed from the gene regions of piRNA clusters have been proposed to be the initial material. However, it remains unclear whether transcription from the piRNA clusters is required for the biogenesis of piRNAs. To answer this question, I developed a novel artificial piRNA production system by simple expression of sense and antisense EGFP mRNAs during the embryonic piRNA biogenesis phase. EGFP expression was silenced by piRNA-dependent DNA methylation, indicating that concomitant expression of sense and antisense RNA transcripts is necessary and sufficient for piRNA production and subsequent piRNA-dependent gene silencing. In addition, I demonstrated that this artificial piRNA induction paradigm could be applied to an endogenous gene essential for spermatogenesis, DNMT3L. This study provides not only novel insights into the

molecular mechanisms of piRNA production, but also presents an innovative strategy for inducing epigenetic modification in germ cells.

Table of contents

1. General introduction --5~12

2. Results--13~27

3. Discussion ---27~33

4. Materials and methods --34~43

5. Tables---44~68

6. References --69~76

7. Acknowledgments --77~77

8. Acheivement --78~80

General introduction

RNA interference (RNAi) is a gene silencing system mediated by Argonaute family protein members and small RNA molecules [1] [2]. Argonaute proteins harbor two well-conserved domains, PAZ and PIWI (Figure G1). The former is required for binding of Argonaute protein to RNAs, while the latter has RNase H activity (RNA slicer activity) [2] . In general, Argonaute family proteins bind to the target RNAs and achieve gene silencing via transcriptional and post-transcriptional regulations [3]. Transcriptional regulations involve changing of chromatin status by recruitment of epigenetic modifiers, such as DNA methyltransferases (Dnmt) and histone lysine 9 (H3K9) methyltransferases, to the target loci in the nuclear. DNA cytosine methylation and H3K9 methylation are representative gene silencing marks of chromatin. In contrast, post-transcriptional regulations mean that Argonaute proteins directly cleave the target RNAs or inhibit translation together with decapping enzymes and poly deadenylation complexes [3] [4] [5] [6] .

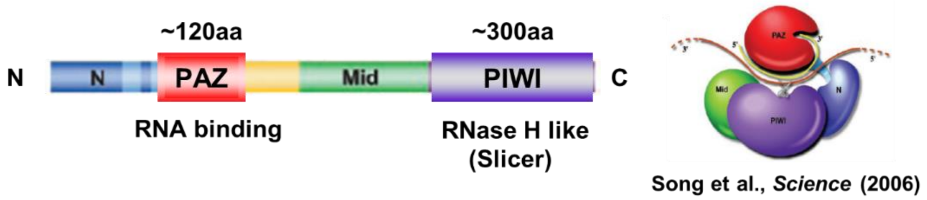

Figure G1. Protein domains (left) and structural image (right) of Argonaute family

Argonaute family is distinguished into two subfamilies: AGO and PIWI (Figure G2). These two subfamilies are different in classes of associated small RNAs, way of gene silencing, and expression patterns in multicellular organisms. AGO subfamily proteins bind to small interfering RNAs (siRNAs) and micro RNAs (miRNAs) whose length are about 21-23 nt, and mainly participate in post-transcriptional silencing. On the other hand, PIWI subfamily proteins associate with PIWI-interacting RNAs (piRNAs) whose length are about 25-31 nt. Notably, PIWI subfamily proteins take part in both post-transcriptional and transcriptional gene silencing. In addition, AGO subfamily is ubiquitously expressed, while the expression of PIWI subfamily is limited within germ-cells in mammals [7].

	small RNA	expression
AGO sub-family	miRNA siRNA（～21-23 nt）	Ubiquitous
PIWI sub-family	piRNA（～25-31 nt）	Germ cell specific

Figure G2. AGO and PIWI sub-family, their small RNAs and expression pattern

Although numerous kinds of cell types exist, only germ-cells can transmit the genetic information to the next generation. Therefore, genetic stability of germ-cells is quite important for the preservation of species. Several studies have showed that PIWI family proteins play an important role in protection of germ-line genome from endogenous invaders, called retrotransposons [8] [9] . Retrotransposons are mobile genetic elements which consist about 40% of rodent genome, and induce insertion mutagenesis thorough reverse transcription [10] [11] (Figure G3, left). Loss of mouse Piwi proteins causes hyper-expression of retrotransposons, such as IAP and LINE-1 (Figure G3, right), and genetic instability in germ cells, thus leading to the failure of germ line development [12] [13] [14] .

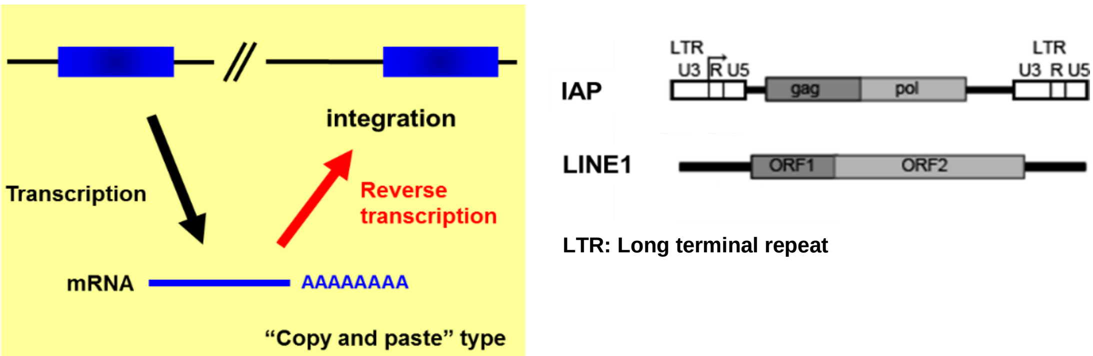

Figure G3. Transposition mechanism of retrotransposons (left), and schematic structures of IAP and LINE-1 (right)

Development of murine germ-cells includes several dynamic changes of epigenetic information. During mice spermatogenesis, global DNA demethylation takes place in primordial germ cells (PGC), at embryonic day 9.5 to 12.5 [15]. In the next developmental stage, called embryonic gonocytes (embryonic day 15 to 19), DNA methylation of retrotransposon and imprinting genes are re-established by Dnmt3a and Dnmt3-like (Dnmt3L) [16] [17] (Figure G4). It has been reported that two mouse PIWI proteins, Mouse PIWI like (MILI) and Mouse PIWI2 (MIWI2) are critical for introduction DNA methylation to LINE-1 and IAP retrotransposons. The promoter regions of these retrotransposons are greatly hypomethylated in MILI and MIWI2-deficient mice embryonic germ-cells. In addition, the amount of embryonic piRNAs related to the retrotransposon sequences were drastically reduced under such conditions. These data clearly indicated that MILI and MIWI2 participate in de novo DNA methylation of the retrotransposons via piRNA production [12] [18].

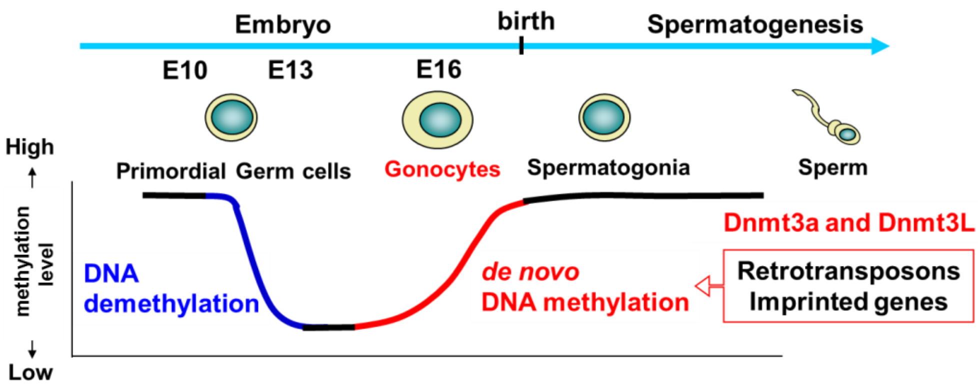

Figure G4. A dynamic changing of DNA methylation status during spermatogenesis

Recent studies have given shed light on how piRNAs are produced by PIWI proteins. Biogenesis of piRNAs in mouse embryonic testes can be divided into largely two steps: primary and secondary processing. During primary processing, sense long RNAs are transcribed presumably from piRNA clusters, which are retrotransposon-enriched region in the genome. These long RNAs are cleaved and loaded into MILI protein as primary piRNAs. In the secondary processing, MILI slices the anti-sense target RNAs and produce secondary piRNAs. The secondary piRNAs are then incorporated into another PIWI protein, MIWI2. Such piRNA production system participated by MILI and MIWI2 proteins is called ping-ping cycle model [8] [19] . It has been shown that MIWI2 not MILI translocates to the nuclear and presumably induces de novo DNA methylation thorough recruitment of DNA methyltransferase protein complexes during piRNA biogenesis [20] (Figure G5). However, the underlying mechanism(s) how MIWI2 takes part in this process is largely unclear.

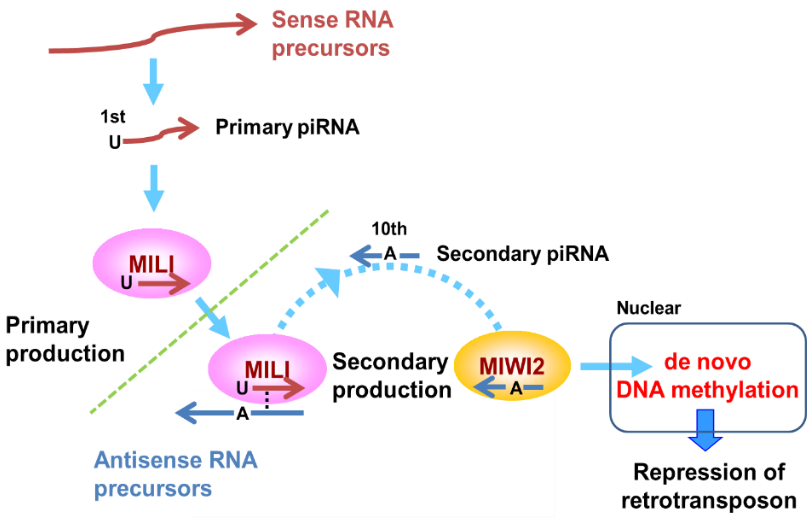

Figure G5. Model for embryonic piRNA biogenesis and *de novo* DNA methylation

Several papers strongly indicated that piRNA production requires long transcripts from piRNA clusters and retrotransposon sequences [21] [22], however, little is known whether these features are actually essential for piRNA biogenesis. In order to address this question, I established a novel piRNA production system using transgenic mice expressing both sense and antisense EGFP mRNAs at the phase of piRNA production (Figure G6). Reduced expression of EGFP and hypermethylation of the transgene were observed in the male-germ cells of the transgenic mice. Additionally, artificial piRNAs related to EGFP sequence were produced in the transgenic mice embryonic testes. These results clearly demonstrate that sense and antisense transcripts are necessary and sufficient for piRNA biogenesis.

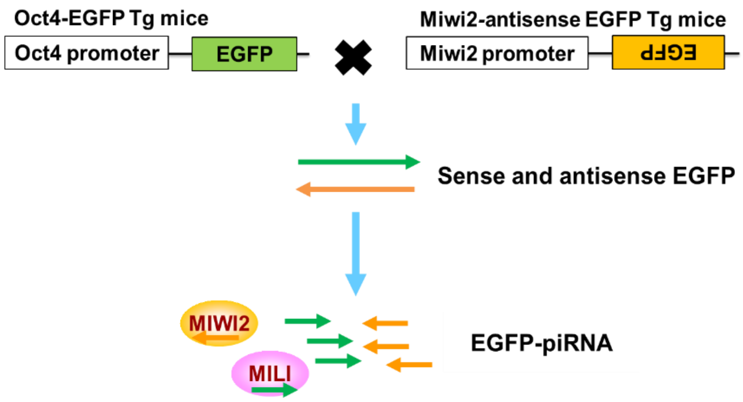

Figure G6. Experimental design of the artificial piRNA production system

The artificial piRNA production system is successful in the case EGFP transgene, however, it is unclear whether this paradigm can be applied to interference of endogenous genes expressed in embryonic germ-cells. To test this possibility, I established a transgenic mouse which expresses antisense Dnmt3L in embryonic testes. The reason why I choose this gene is that phenotypes of Dnmt3L-KO mice are already known [17]

[23] [24]. This point will make it easy to determine whether the above-mentioned aim is achieved or not. The transgenic mice expressing antisense Dnmt3L showed similar phenotypes to these of Dnmt3L-KO mice. Well-consistent with this result, Dnmt3L expression was silenced by piRNA-mediated DNA methylation, showing that this experimental system can be used for repression of endogenous genes *in vivo*.

In this study, I emphasize two important findings. One is a new insight into embryonic piRNA biogenesis. It has been assumed that transcription from piRNA clusters and retrotransposon sequences are essential for piRNA production. Contrary to the general brief, my research strongly suggests that these features are irreverent to piRNA production and the key is sense and antisense transcripts.

The second, a novel method for gene silencing by piRNA-mediated DNA methylation, is much more important. This simple experimental system makes it easy to inhibit gene expression via DNA methylation during spermatogenesis, which would lead to functional analysis of the target genes. Moreover, I believe that this system is also useful for experiment of trans-generational epigenetic inheritance. Recently, more and more reports suggest that epigenetic abnormalities, such as aberrant DNA methylation patterns, caused by environmental changes are transmitted to the next generation through germ-cells and affect the phenotypes of the offspring [25] (Figure G7, left). These reports indicate that epigenetic status at a certain gene is escaped from genomic reprogramming after fertilization and influence the gene expression in the next offspring [26] [27] (Figure G7, right). However, there has been no strategy for induction of gene-specific DNA methylation, thus it is quite difficult to examine whether aberrant epigenetic status in germ-cells is directly linked to the next progenitor's phenotypes. My experimental system will solve this problem and give a new insight into the study of epigenetic inheritance.

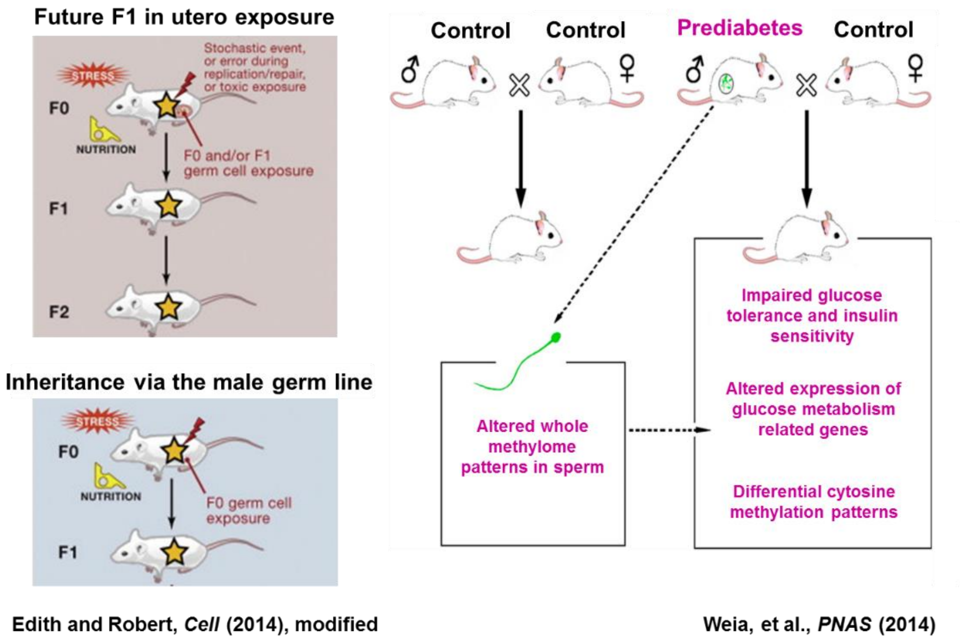

Figure G7. Trans-generational inheritance of acquired traits caused by certain environmental changes (left), and altered DNA methylation patterns in the sperms from prediabetes male mice (right)

Results

DNA methylation of EGFP transgene by concomitant expression of antisense transcripts

Comprehensive sequencing of piRNAs and genomic mapping of embryonic piRNAs suggest that long transcripts from piRNA clusters are required as the precursors for piRNAs [21] [22] [28] [29] [30] [31]. However, how they are utilized as the substrate of piRNA, and even whether they are a prerequisite for piRNA production have not yet been elucidated. It is also unknown why piRNAs corresponding to retrotransposons are preferentially produced. My hypothesis, that neither long transcripts nor sequence preference is important for piRNA production, challenges the aforementioned two unproven general beliefs. In this study, I adopted a simple experimental system wherein sense and antisense enhanced green fluorescent protein (EGFP) transgenes were expressed in embryonic male germ cells during *de novo* DNA methylation. I used this paradigm to assess the induction piRNA-dependent DNA methylation.

In the Oct4-EGFP mouse (Figure 1A left) testis, EGFP expression was detectable from embryonic day 7[32] to at least 2 weeks after birth (Figure 1B and C). Three lines of Miwi2-asEGFP transgenic mice (#1, #6, and #8), in which antisense EGFP mRNA expression was controlled by the 2.5 kb Miwi2 promoter (Figure 1A right), expressed

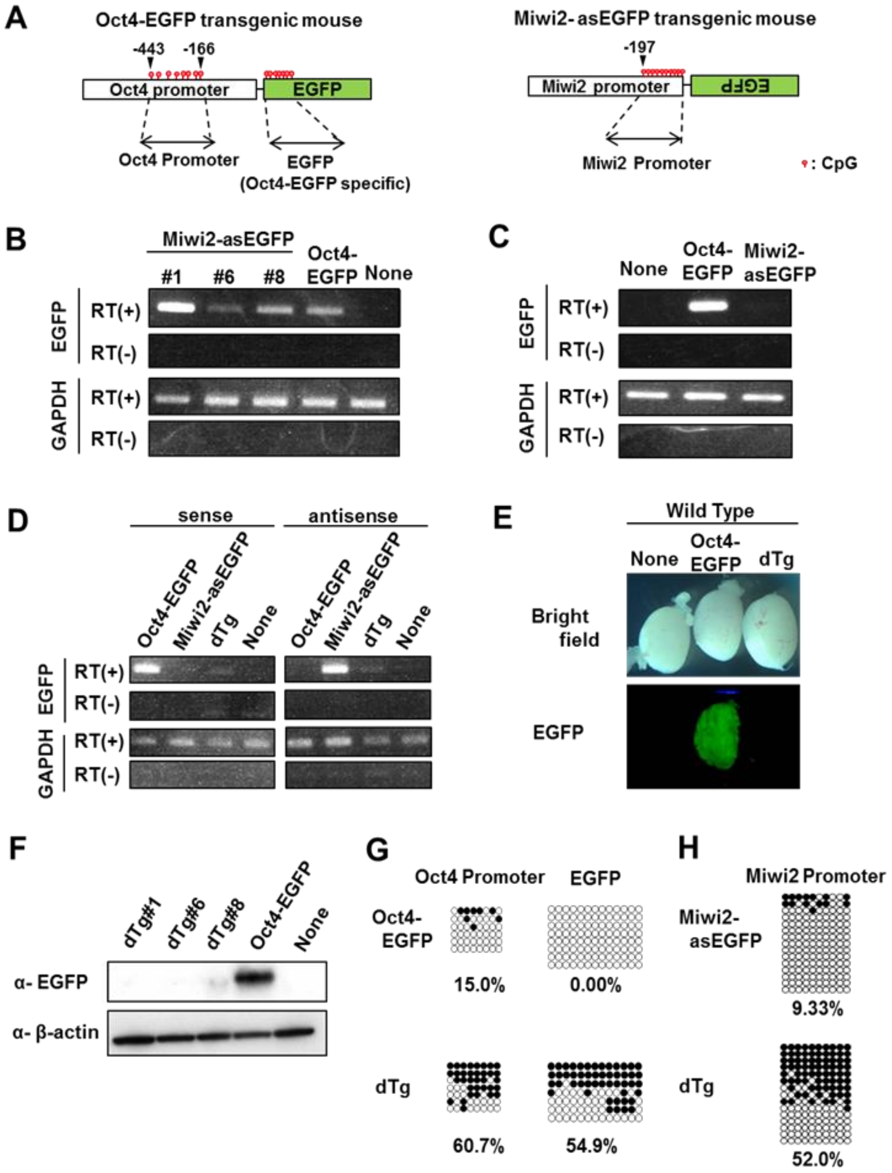

Figure 1. DNA methylation-mediated gene silencing by the expression of antisense EGFP

(A) Schematic structure of two transgenes, Oct4-EGFP and Miwi2-antisenseEGFP (Miwi2-asEGFP). Red circles represent CpG sites and arrowed bars show the region subjected to bisulfite sequencing analysis. (B) RT-PCR analysis of antisense EGFP in embryonic day 16.5 testes harvested from three lines of Miwi2-asEGFP transgenic mice (#1, #6, and #8). (C) RT-PCR analysis of sense and antisense EGFP mRNAs in day 14 testes. GAPDH was used as a control. (D) RT-PCR analysis of sense (left) and antisense (right) EGFP strands using strand-specific primers. RNA was prepared from embryonic day 16.5 testes and GAPDH was used as an internal control. (E) Bright field and fluorescent photographs of day 14 testes. (F) Western blotting of dTg #1, #6, #8, Oct4-EGFP, and wild-type (None) day 14 testes with anti-EGFP antibodies. β-actin was used as an internal control. (G) Bisulfite sequencing analysis of the Oct4 promoter and EGFP. Genomic DNA from day 14 EpCAM-positive germ cells was used. White and black circles represent unmethylated and methylated cytosine, respectively. (H) Bisulfite sequencing analysis of the Miwi2-asEGFP transgenes. Genomic DNA from E-cadherin-positive germ-cells was used to analyze the Miwi2-promoter.

antisense EGFP RNAs in embryonic day 16.5 testes (Figure 1B). The antisense EGFP transcript was only expressed in the embryonic testis of the Miwi2-asEGFP mouse, consistent with the expression of MIWI2 [12] [18] (Figure 1B and C). I primarily used transgenic line #1 in subsequent experiments, because of its high expression of antisense EGFP RNAs.

In double transgenic mice bearing both Oct4-EGFP and Miwi2-asEGFP transgenes, expression of EGFP was silenced at embryonic day 16.5 (Figure. 1D left), and at day 14 after birth (Figure. 1E). Not only the representative EGFP antisense transgenic line (#1), but also the other lines (#6 and #8) showed essentially same silencing (Figure 1F). This suppression is unlikely to be a result of a direct effect of the antisense EGFP transcript, because antisense EGFP was minimally or not at all expressed in male germ cells at day 14 (Figure. 1C).

Next, I examined the DNA methylation status of the Oct4-EGFP transgene by bisulfite sequencing (Figure 1G). In male germ cells of double transgenic mice, methylation of the Oct4-EGFP promoter and the EGFP-coding region was significantly higher than that of the Oct4-EGFP mice. Although the expression of EGFP was utilized as a marker to visualize EGFP gene silencing, sense and anti-sense EGFP transgenes can be considered equivalent from the point of view of gene expression control. Next, I examined the expression and DNA methylation of the Miwi2-asEGFP transgene (Figure 1D right and H). Similar to the results for the Oct4-EGFP transgene, silenced expression of antisense EGFP and high DNA methylation of its promoter were detected in the double transgenic male germ cells. These data clearly demonstrate that expression of both sense and antisense transgenes was silenced by DNA methylation of their promoters.

Involvement of artificially induced EGFP-related piRNAs in the gene silencing of EGFP transgenes

A critical question to answer was whether or not DNA methylation and subsequent gene silencing were dependent upon the piRNA pathway. To resolve this, I examined the expression of EGFP in double transgenic mice under Mili- and Miwi2-deficient conditions (described as $dTg^{Mili\text{-}Null}$ and $dTg^{Miwi2\text{-}Null}$ mice, respectively). Gross examination and Western blotting analysis clearly demonstrated that the expression of EGFP, which was abrogated in the double transgenic mice, was recovered under Mili and Miwi2 null conditions (Figure 2A and B). Levels of methylation of the Oct4-EGFP promoter in the dTg/Mili-Null and dTg/Miwi2-Null mice were quite low, compared to the simple double transgenic mice (Figure 1G and 2C). These data demonstrate that gene silencing of Oct4-EGFP was dependent on MILI and MIWI2, i.e., the piRNA

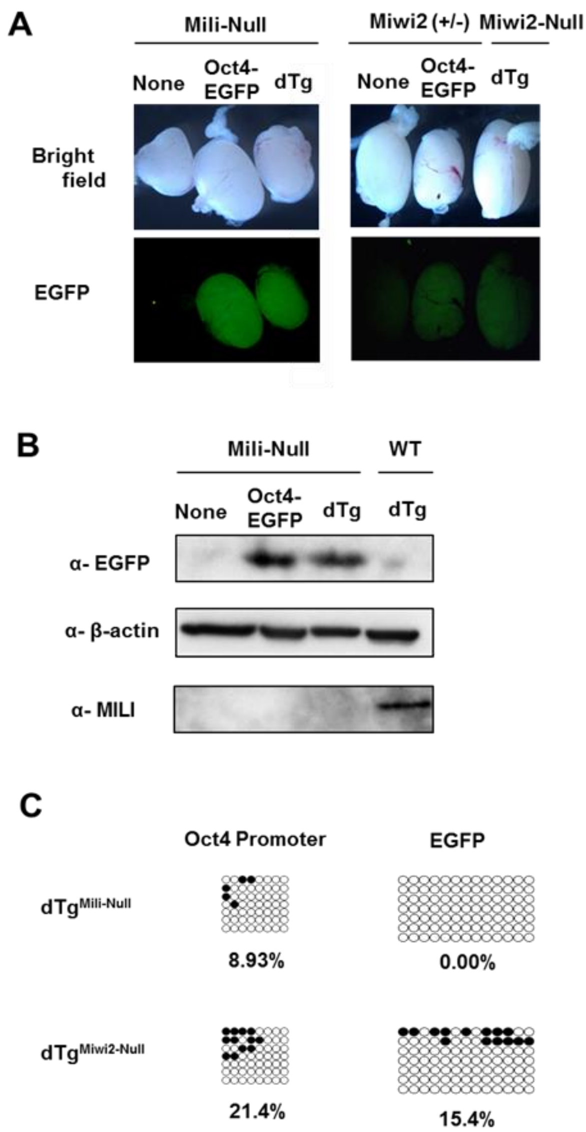

Figure 2. piRNA pathway-dependent silencing and DNA methylation of EGFP transgene

(A) Bright field and fluorescent photographs of Oct4-EGFP and double transgenic mice testes under the MILI and MIWI2 null conditions. Left: data of the mice bearing no transgene (None), Oct4-EGFP transgene, and both Oct4-EGFP and Miwi2-asEGFP transgenes (dTg) are shown. Right: Bright field and fluorescent observations of day 14 testes of Miwi2-+/-, Oct4-EGFP/Miwi2-+/-, and dTg/Miwi2-Null mice. (B) Western blot analysis of day 14 Oct4-EGFP and double transgenic testes under the MILI null condition, using anti-EGFP and anti-MILI antibodies. β-actin was used as an internal control. (C) Bisulfite sequencing analysis of day 14 EpCAM-positive germ cells of double transgenic mice under MILI and MIWI2 null conditions.

pathway.

To examine the production of EGFP-related piRNAs, I carried out deep sequencing analysis of small RNAs in the embryonic germ cells of double transgenic mice. A total of 552 reads of EGFP-related RNAs were obtained from the RNA sequence data (11747822) of 18–45 nt in length. The length of EGFP-related small RNAs showed a single peak of 25–31 nt (Figure 3A), which was consistent with the length of piRNAs. Both sense- and antisense-piRNAs related to EGFP were mapped to the entire EGFP sequence (Figure 3B). A strong sequence bias, namely, uracil in the first position (1st U) and adenine in the tenth position (10th A), has been reported as a signature of piRNA production [8] [33]. As shown in Figure 3C, the majority of both sense and antisense EGFP piRNAs demonstrated a high 1st U bias (54% [113/209] and 88% [302/343], respectively). A strong 10th A bias was only evident in sense EGFP-piRNAs (64% [134/209]).

The distributions of 1st U and 10th A piRNAs against sense and antisense EGFP transgenes are shown in Figure 3D and E. These piRNAs were screened for the ping-pong signature (10 base matching between 1st U and 10th A piRNAs with reverse orientations, and the results are shown in Figure 4. Approximately 50–60% of the piRNAs matched the ping-pong signature (Figure 4A and C). Representative ping-pong

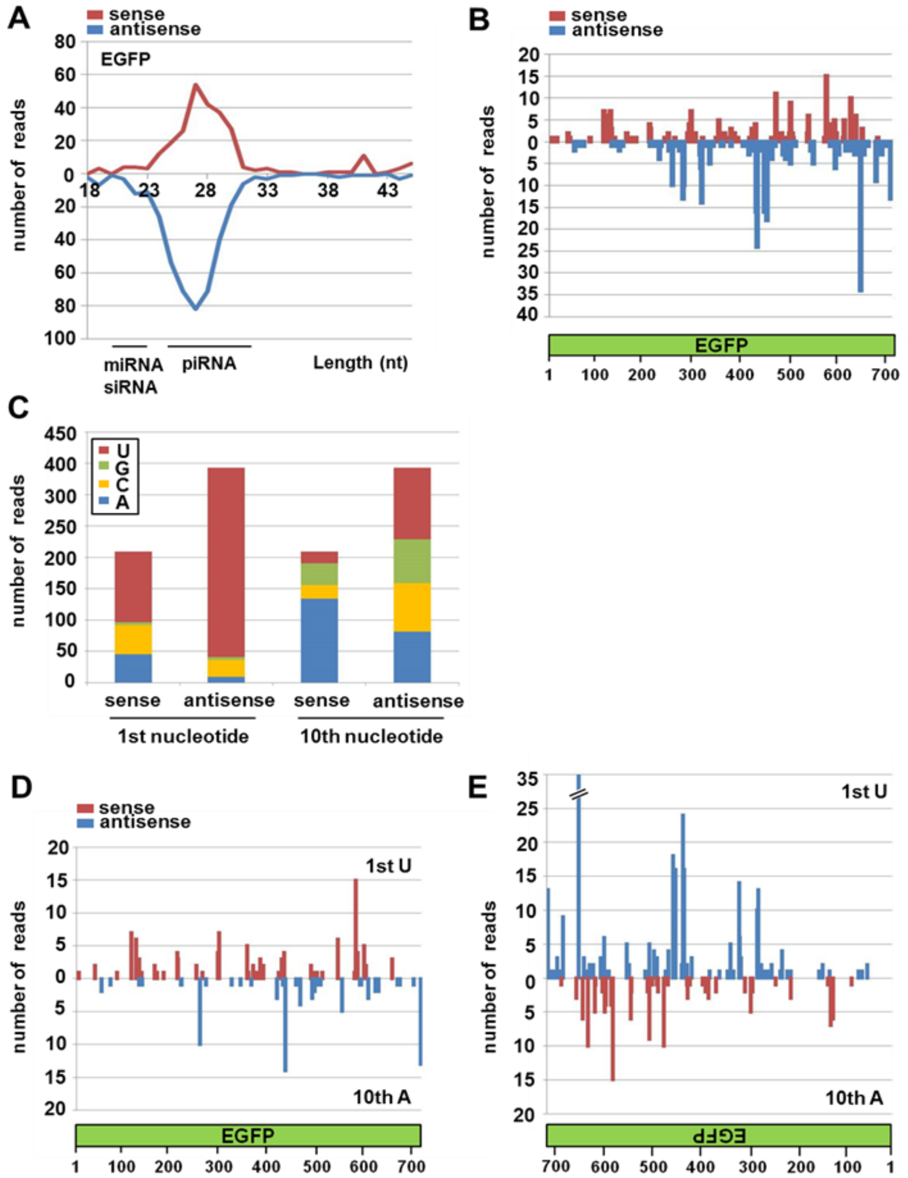

Figure 3. Deep sequencing analysis of small RNAs in EGFP double transgenic embryonic male germ cells

(A) Length distribution of the small RNAs corresponding to EGFP. (B) Mapping of EGFP piRNAs. Red and blue bars indicate sense and antisense EGFP piRNAs, respectively. (C) Numbers of the first and tenth nucleotides in EGFP piRNAs. (D) (E) Mapping of 1st U and 10th A EGFP piRNAs. Sense 1st U and antisense 10th A piRNAs corresponding to the sense EGFP are shown in (D). Antisense 1st U and sense 10th A piRNAs corresponding to the anti-sense EGFP are shown in (E).

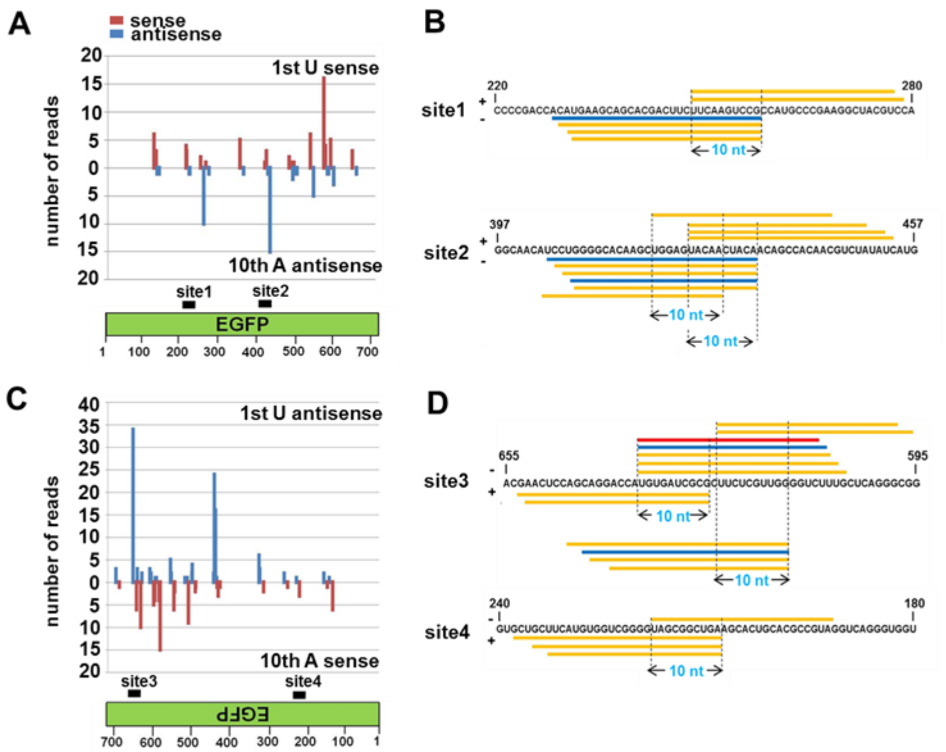

Figure 4. Ping-pong signature in EGFP piRNAs

(A) (C) A 10-base overlap between 1st U and 10th A EGFP piRNAs, corresponding to sense and antisense EGFP (A and C, respectively). Overall, 58% of 1st U sense (66/113), 58% of 10th A antisense (47/81), 50% of 1st U antisense (152/302), and 66% of 10th A sense (89/134) EGFP piRNAs were positive for the ping-pong signature. (B) (D) Detailed mapping of a 10-base overlap of EGFP piRNAs corresponding to sense and antisense EGFP (B and D, respectively). EGFP piRNAs are represented by bars, colored according to their sequence read number.

signature data for sense and antisense EGFP sequences are shown in Figure 4B and D, respectively. These data clearly show that piRNAs for EGFP were produced via the ping-pong amplification cycle.

Silencing of Dnmt3L gene through DNA methylation introduced by the expression of antisense Dnmt3L

Next, I aimed to establish whether this piRNA-dependent germ cell-specific gene silencing was applicable to endogenous genes. I selected Dnmt3L (DNA methyltransferase 3-like) as a model gene, because it is expressed in embryonic male germ cells and null mutant mice show defective DNA methylation of retrotransposon genes and impairment of spermatogenesis [23], similar to the Mili or Miwi2 null mice [13] [12]. I produced Miwi2-asDnmt3L transgenic mice expressing the antisense mRNA of Dnmt3L under the control of the Miwi2 promoter. These transgenic mouse lines (#3 and #6: described as asDnmt3L#3 and asDnmt3L#6, respectively) had significantly smaller testes than control mice (Figure 5A and 6A).

I used the asDnmt3L#3 line in further experiments, because it showed the more severe impairment of spermatogenesis (Figure 6B). DNMT3L proteins were drastically reduced in asDnmt3L embryonic testes and spermatogenesis was severely impaired

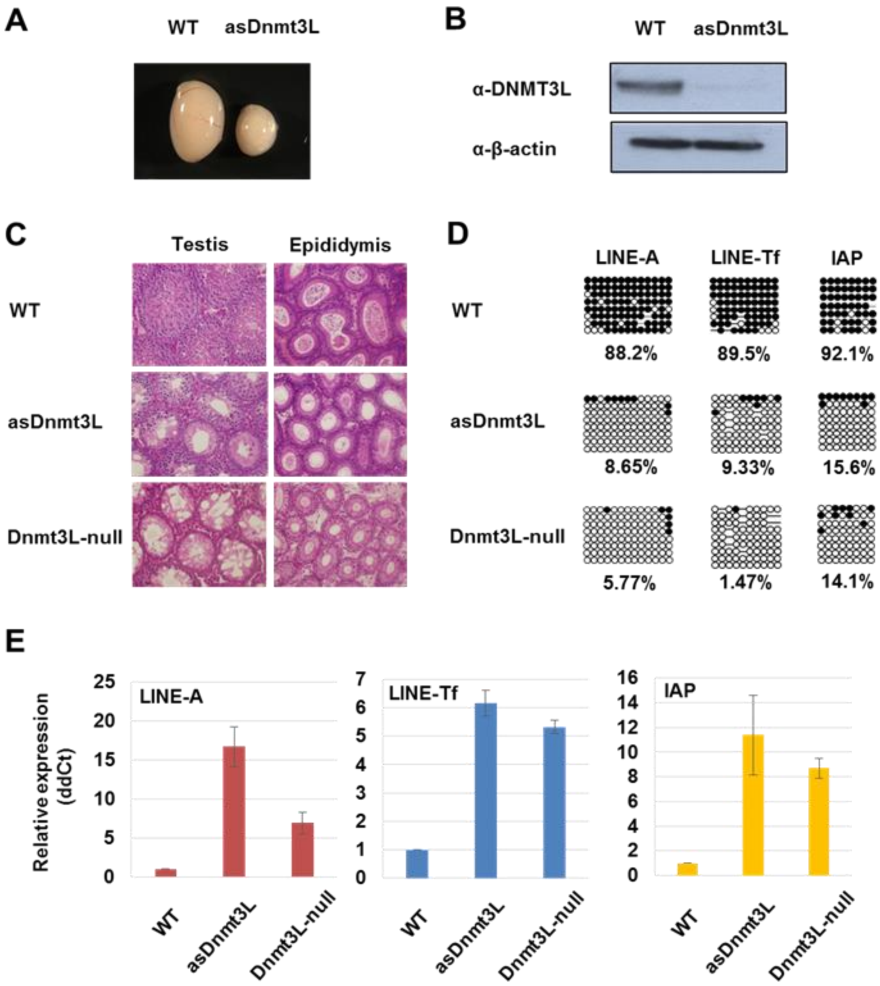

Figure 5. Impaired spermatogenesis, silencing of Dnmt3L gene, and increased retrotransposon expression in the anti-sense Dnmt3L transgenic mouse

(A) Testes of 5week-old wild-type and asDnmt3L mice. (B) Western blotting analysis of embryonic day 16.5 wild-type and asDnmt3L testes, using anti-DNMT3L and anti-β-actin antibodies. (C) Hematoxylin-eosin staining of 5-week-old wild-type, asDnmt3L, and Dnmt3L-null mice. (D) Bisulfite sequencing analysis of LINE-A, LINE-Tf, and IAP retrotransposon promoter regions. Genomic DNA was extracted from the EpCAM positive germ cells of 2-week-old wild-type, asDnmt3L, and Dnmt3L-null mice. (E) Quantitative RT-PCR analysis of LINE-A, LINE-Tf, and IAP retrotransposon expression. RNAs were extracted from whole testes of 2-week-old wild-type, asDnmt3L, and Dnmt3L-null mice. β-actin was used as an internal control.

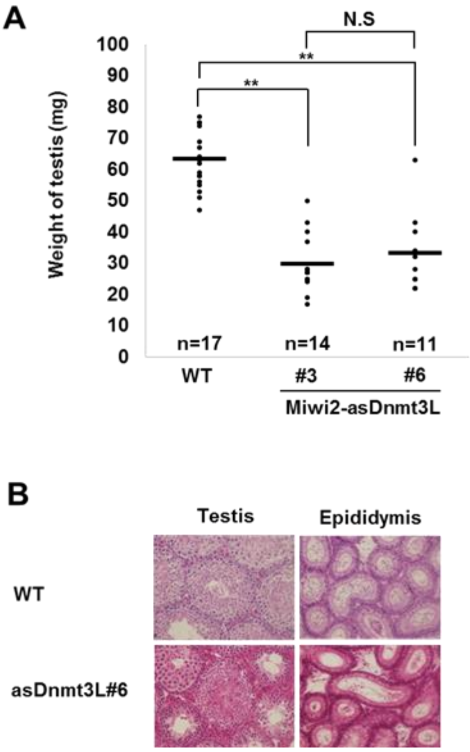

Figure 6. Partially impaired spermatogenesis of Miwi2-asDnmt3L#6 mice

(A) Weights of testes of the Miwi2-asDnmt3L mouse. Testes were harvested from 5-week-old wild-type, Miwi2-asDnmt3L#3, and Miwi2-asDnmt3L#6 mice, respectively. Black bars indicate average weights, with the numbers of samples described below. Statistical analysis was performed using a Student's t test (**$p< 0.01$). (B) Hematoxylin eosin staining of Miwi2-asDnmt3L#6 testes and epididymis.

(Figure 5B and C). DNA hypomethylation of the promoter regions of LINE-1 and IAP retrotransposons, and subsequent abrogation of gene silencing, were observed in asDnmt3L male germ-cells (Figure 5D and E). This phenotype was essentially identical to that of the Dnmt3L-null mice, strongly suggesting that piRNA-mediated gene silencing of Dnmt3L takes place in asDnmt3L embryonic testes. The DNA methylation status of control regions of the Dnmt3L gene, spanning from the promoter to the second exon [34] [35] [36], was significantly increased in the asDnmt3L male germ cells (Figure 7A). It is quite likely that the observed phenotype is manifested by DNA methylation in a piRNA-dependent manner.

Comprehensive analysis of Dnmt3L related piRNAs

Next, I carried out deep sequencing analysis of small RNAs in the asDnmt3L embryonic male germ cells. Although there were very few Dnmt3L-related piRNAs in the control male embryonic germ cells, a significant number of piRNAs were observed in the transgenic mice (Figure 7B, C, and 8A-C). Mapping of Dnmt3L-associated piRNAs demonstrated that these piRNAs were produced from various regions of the Dnmt3L mRNAs (Figure 7D). In addition, both sense and antisense Dnmt3L piRNAs harbor high 1st U (43.6% [5323/12205] sense; 63.2% [14069/22266] antisense) and 10th

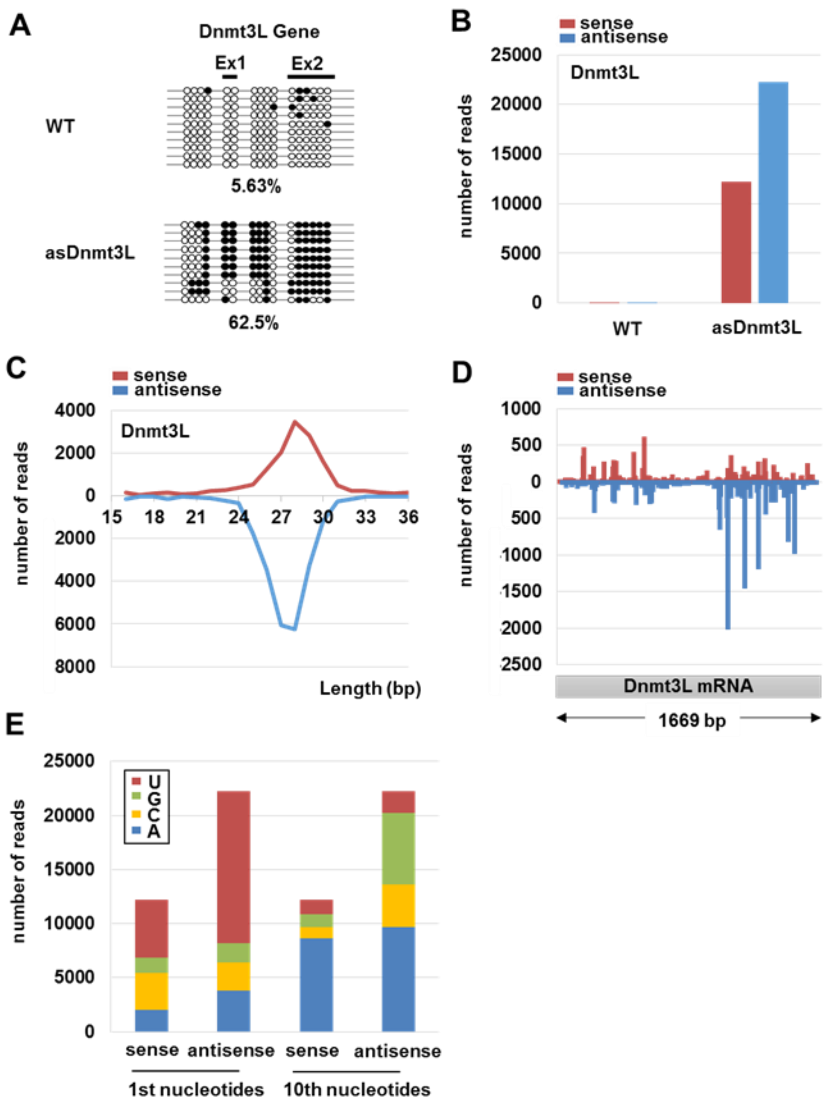

Figure 7. Induction of Dnmt3L gene piRNA production and DNA methylation by the expression of anti-sense Dnmt3L mRNA

(A) Bisulfite sequencing of the control region of the Dnmt3L gene. Genomic DNA was extracted from EpCAM-positive germ cells of 2-week-old wild-type and asDnmt3L mice. Black bars indicate exons in the Dnmt3L promoter. (B)-(E) Deep sequencing analysis of small RNAs in asDnmt3L embryonic male germ cells. Numbers of small RNAs with 25–31 nt length corresponding to the Dnmt3L sequence (B). Length distribution of small RNAs corresponding to the Dnmt3L sequence (C). Mapping of Dnmt3L piRNAs (D). Red and blue bars indicate the sense and antisense strands, respectively. Numbers of the first and tenth nucleotides in Dnmt3L piRNAs (E).

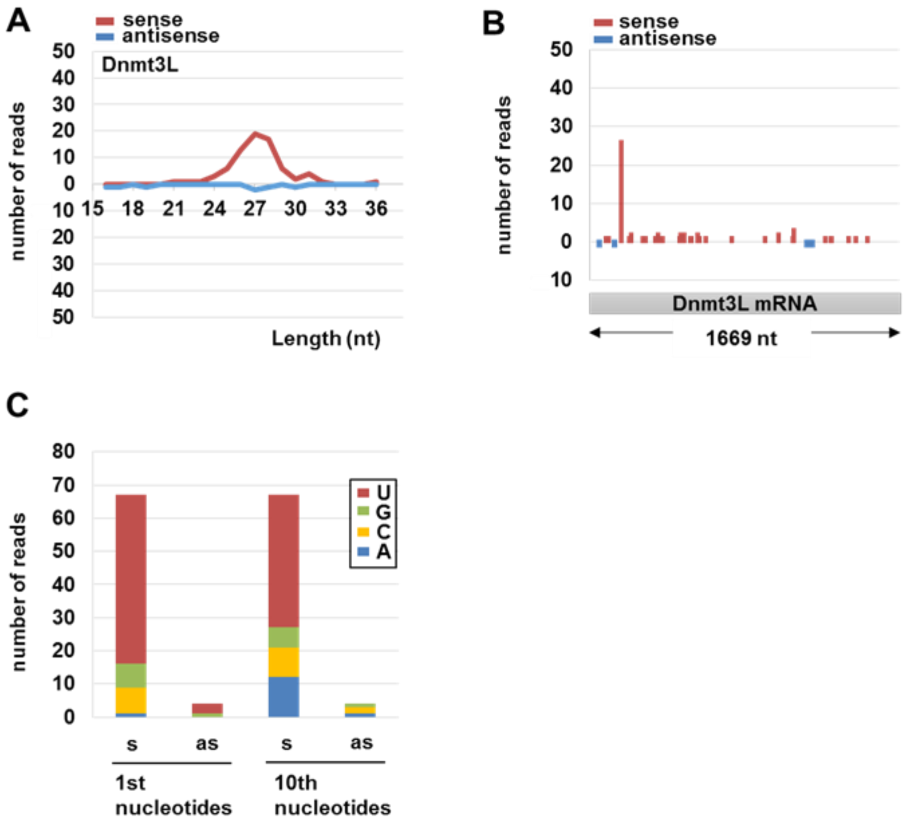

Figure 8. Pre-existed Dnmt3L piRNAs in WT embryonic testes

(A)-(C): Dnmt3L piRNAs in wild-type embryonic day 16.5 testes. (A) Length distribution of Dnmt3L-associated small RNAs. Range of length is from 16 to 36 nt. (B) Mapping of Dnmt3L piRNAs. Red and blue bars indicate sense and antisense strands, respectively. (C) Numbers of the first (1st U) and tenth (10th A) nucleotides.

A bias (70.7% [8625/12205] sense; 43.4% [9670/22266] antisense) (Figure 7E), and approximately 90% of Dnmt3L piRNAs harbored the ping-pong signature. Overall, 95% of 1st U sense (5068/5328), 94% of 10th A antisense (9043/9670), 97% of 1st U antisense (13630/14069), and 92% of 10th A sense (7919/8625) Dnmt3L piRNAs were positive for the ping-pong signature. These data clearly demonstrate that expression of anti-sense Dnmt3L mRNA induces the production of corresponding Dnmt3L piRNAs and subsequent DNA methylation.

Discussion

Possibility of post-transcriptional silencing by the expression of antisense RNAs

In this study, I showed that artificial piRNA production and subsequent DNA methylation were triggered by the simultaneous expression of sense and anti-sense RNAs in the embryonic testes. Although DNA methylation is a representative gene silencing mark, I cannot exclude the possibility of mRNA degradation and/or translational silencing of EGFP and Dnmt3L genes. This is because there exist anti-sense RNAs, which can potentially introduce the post-transcriptional suppression. Transcriptional repression and post-transcriptional degradation are mutually un-exclusive, however, my results clearly showed that significant DNA methylation and subsequent transcription took place both in the cases.

Timing of the de novo DNA methylation of endogenous Dnmt3L and retrotransposons

Loss of Dnmt3L expression had impact on the DNA methylation of LINE-1 and IAP retrotransposons. In the asDnmt3L mice male germ-cells, the retrotransposon genes were significantly hypomethylated, on the other hand, the endo-Dnmt3L gene was highly methylated (Fig. 5D and 7A). Although it seems to be a contradiction, I consider that the timing of Dnmt3L expression and the methylation of the retrotransposons would be the key to answer this question. Dnmt3L begins to be expressed from embryonic day 13.5. DNA demethylation of LINE-1 begins at embryonic day 10.5 and completed at embryonic day 16.5 [18]. Considering that Dnmt3L is expressed before the *de novo* DNA methylation of the retrotransposons, my consideration is quite reasonable. If it is not the case, the results which I obtained cannot be explained.

Spreading of the piRNA-mediated DNA methylation to the surrounding regions

The promoter regions of both Oct4-EGFP and Miwi2-asEGFP were hypermethylated in the dTg male germ-cells (Fig 1G and H). DNA methylation was introduced into the introns of endo-Dnmt3L gene either in the case of asDnmt3L mice

(Fig 7A). Because there were few piRNAs related to the transgene promoters and Dnmt3L introns (data not shown), it is quite likely that piRNA-mediated DNA methylation was spread to the surrounding regions. Although the underlying mechanisms are still unknown, this experimental system would give a new insight into how piRNAs induce DNA methylation to their target loci.

In order to ask whether the DNA methylation of the surrounding regions affects the gene expression, I tried to perform quantitative PCR analysis of the genes near from the Miwi2-asEGFP transgene integration sites and the endo-Dnmt3L locus. However, because there were no expressed genes around these sites (data not shown), it was impossible to perform this experiment.

Comparison between the EGFP and Dnmt3L-related piRNAs

The absolute reads number of Dnmt3L piRNAs was much larger than that of EGFP piRNAs (34471 and 552, respectively). Even taking the reads of miRNAs as an internal control (the reads of the dTg and asDnmt3L cells were 1204399 and 8092774, respectively), the relative amount of Dnmt3L piRNAs was still 9 times higher compared to that of EGFP piRNAs (Figure 9). Meanwhile, the characteristics of Dnmt3L piRNAs and EGFP piRNAs were a little different. Although sense and antisense Dnmt3L

Number of reads

		dTg#1
EGFP-piRNA	sense	209
	antisense	343
miRNAs		1204399
25-31 nt total RNA		1891875

		asDnmt3L#3
Dnmt3L-piRNA	sense	12205
	antisense	22267
miRNAs		8092774
25-31nt total RNA		11554738

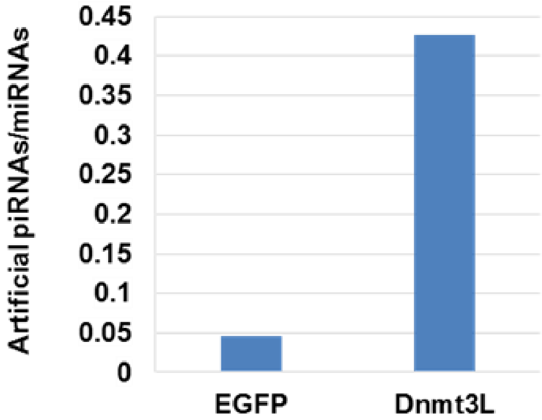

Figure 9. Comparison of the amounts of EGFP and Dnmt3L-piRNAs

Left: tables showing the actual reads number of the artificial piRNAs, miRNAs, and total RNAs with the length of 25 to 31 nt. Right: relative amounts of EGFP and Dnmt3L piRNAs normalized by the miRNAs.

piRNAs showed the tendency of 10thA bias (Figure 4E), only antisense but not sense EGFP piRNAs possessed this preponderance (Figure 2C). These differences would be due to the amount of RNA transcripts, the balance between sense and antisense transcripts, and/or the sequence differences of the two kinds of genes.

Irreverence of transcription from piRNA clusters for piRNA production

My data support two important concepts. One is that retrotransposon sequences and transcription from piRNA clusters are irrelevant to the piRNA biosynthesis of embryonic mouse male germ cells. Recently, it was demonstrated that EGFP-related piRNAs were produced in flies, mice, and cultured silkworm ovary cells, as shown by inserting an EGFP sequence into their piRNA clusters, suggesting that the locus of the gene is important for piRNA production [37] [38] [39]. These papers highlighted the importance of the piRNA cluster region in piRNA production. However, *in silico* piRNA cluster analysis [40] of my Miwi2-asEGFP mice indicated that the genomic insertion sites of the transgenes were not in typical piRNA clusters (Table 1 and 2). Thus, my data demonstrate that the transcription from the piRNA cluster is not a critical factor for piRNA biogenesis if sense and anti-sense RNAs are co-expressed. However, it is possible that piRNA clusters are the sites at which presumably long anti-sense transcripts are produced under some circumstances.

There were EGFP related piRNAs in the Oct4-EGFP and Miwi2-asEGFP transgenic mice embryonic testes (8 in 22098877 and 273 in 18792650 of the RNAs with 18 to 45 nt length, respectively). Similarly, although the numbers were quite low, there were endogenous sense piRNAs including the piRNAs corresponding to Dnmt3L in the control embryonic male germ cells (Figure 8A-C). Such “seed” EGFP and Dnmt3L piRNAs may have some roles at the initial step of ping-pong cycle in the dTg and Dnmt3L antisense transgenic mice. However, considering that there were much more abundant sense piRNAs in these transgenic mice embryonic testes, both sense and anti-sense transcripts should have been necessary for the efficient piRNA production even in the case.

Utility of the artificial piRNA induction system

The second important finding is the utility of piRNA-dependent silencing of endogenous gene(s). My simple experimental system for inducing artificial piRNAs and subsequent DNA methylation-dependent gene silencing provides a novel procedure for induction of DNA methylation to inhibit gene expression during spermatogenesis. To judge whether the artificial piRNA system can be applied to other genes, now I am producing transgenic mouse lines which express antisense RNAs of various genes in the embryonic germ cells under the control of Miwi2 promoter. Moreover, I believe that sperm containing abnormal DNA methylation patterns introduced by piRNA represent a useful tool for the study of transgenerational epigenetic inheritance.

Materials and methods

Transgenic mice

The Oct4-EGFP transgenic mouse line established by Yoshimizu et al [32] was used in this study. The Miwi2 promoter region (2.5 kbp) was amplified by polymerase chain reaction (PCR) using C57BL/6J genome DNA taken from the tail as a template. The PCR primers for the Miwi2 promoter region were designed based on a genomic DNA database (NCBI NC_000075.6).

EGFP and Dnmt3L cDNAs were amplified by PCR using a template of the pEGFP-N1 vector (GenBank Accession #U55762) and the C57BL/6J cDNA library of embryonic day 16.5 testes, respectively. The PCR primers for Dnmt3L cDNA referred to the 1–1699 nucleotides of Dnmt3L mRNA (NCBI NM_019448.4). The PCR procedure was performed using Ex Taq (TaKaRa). EGFP and Dnmt3L cDNA were conjugated under the Miwi2 promoter with reverse orientation. These transgene cassettes were injected into fertilized eggs to establish Miwi2-asEGFP and Miwi2-asDnmt3L transgenic mice.

Expression analysis of EGFP transcripts by RT-PCR

Total RNA was extracted from embryonic day 16.5 or day 14 after birth testes using ISOGEN (NIPPON GENE). After DNase treatment, reverse transcriptase reactions were performed using 500 ng RNA (ThermoScript, Invitrogen) with random hexamers.

The expression of EGFP and GAPDH was detected by PCR using EGFP- or GAPDH-specific primers with KOD FX (TOYOBO).

Quantitative RT-PCR analysis of retrotransposon genes

Total RNA was extracted from day 14 testes using RNeasy Plus Mini Kit (QIAGEN), according to the manufacturer's protocol. After DNase treatment, reverse transcriptase reactions were performed using 500 ng RNA (ThermoScript, Invitrogen) with random hexamers. PCR reactions of β-actin, LINE-A, LINE-Tf, and IAP were performed using gene-specific primers and FastStart Universal SYBR Mix (Roche). Data were analyzed using an Applied Biosystems 7900HT Fast real-time qPCR system (ABI).

Western blotting

Day 14 testes and embryonic testes were homogenized in lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 2.5 mM MgCl2, 0.1% NP-40, 1 mM DTT) containing a protease inhibitor cocktail. Equal amounts of protein were resolved by SDS-PAGE, prior to transfer to a PVDF membrane. After blocking in 5.0% skim milk, the membrane was incubated with primary antibodies (mouse anti-EGFP, MBL, 1:1000; mouse anti-β-actin, MBL, 1:1000; rabbit anti-MILI, MBL, PM043, 1:1000; and rabbit anti-DNMT3L antibodies, kindly gifted from Dr. S. Tajima, Institute for Protein Research of Osaka University, 1:5000) at 25°C for 1 h. Secondary antibodies (anti-mouse IgG-HRP, 1:3000; and anti-rabbit IgG-HRP, 1:2000) were applied under the same conditions. Chemiluminescence was detected using ECL (Amersham, Bioscience) according to the manufacturer's instructions.

Isolation of germ cells from day 14 testes using magnetic beads

Purification of male germ cells was based on a previous report [41]. Testes were removed and fixed with 70% ethanol for 20 s. After washing with phosphate-buffered saline (PBS) three times, testes were minced with surgical knives. Minced testes were treated with DNase (Benzonase, Invitrogen) and collagenase in Hank's balanced salt solution (HBSS, Gibco) for 10 min. After three washes with HBSS, tissue debris was removed by filtration with a nylon membrane (BD Falcon, Bedford, MA). A total of 0.5 µg rat anti-E cadherin antibody (TaKaRa) was incubated with a 15 µg 50% slurry of Dynabeads M-450 (Invitrogen) in HBSS for 30 min, and then washed with HBSS three times. The antibody–Dynabead complex was rotated with the cell suspension at 4°C for 1 h. After washing with HBSS three times, male germ cells were prepared.

Isolation of germ cells from day 14 testes using fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) was used to isolate male germ cells, as previously described [42]. Testes were suspended in HBSS and incubated at 37°C for 20 min with Collaganase and DNase. After three washes with HBSS, the testes were treated with 0.25% of Trypsin at 37°C for 10 min. DMEM (Dulbecco's Modified Eagle's Medium, Gibco) containing 10% fetal bovine serum was added to stop the trypsination reaction. Then the sample was incubated with DNase at 37°C for 5 min. After washing with HBSS twice, cell pellets were suspended in 5% bovine serum albumin (BSA)/PBS solution. A total of 5.0 μg anti-EpCAM antibody conjugated with PE (PE anti-mouse CD326, BioLegend) was incubated with the cells with rotation at 4°C for 2 h. To remove cell debris, cell pellets suspended in 5% BSA were filtered using a nylon membrane (BD Falcon, Bedford, MA) after three washes with HBSS. The cells were collected using an FACS Aria (BD Biosciences) instrument.

Bisulfite sequencing analysis

Total genomic DNA (1.0 μg) was subjected to bisulfite treatment with an Epitect bisulfite sequencing kit (QIAGEN). The target sequences including transgene Oct4 promoter, transgene Miwi2 promoter, and EGFP coding regions, were amplified using Ex Taq polymerase (TaKaRa) with bisulfite sequence primers according to the manufacturer's protocol. The amplified PCR products were purified from agarose gel using a QIAquick Gel Extraction Kit (QIAGEN), and conjugated into pGEM-T easy plasmid vectors (Promega). The resulting plasmids were introduced to DH5α cells. Transformed bacteria were selected as ampicillin-resistant clones. DNA sequencing was performed using an ABI 3130xl genomic analyzer (ABI).

To analyze the DNA methylation status of LINE-A, LINE-Tf, IAP, and Dnmt3L genes, I used genomic DNA from EpCAM-positive cells sorted by FACS, as described above. The bisulfite sequence primers for the Dnmt3L gene were as described previously [34]. Total genomic DNA (1.0 μg) was subjected to bisulfite treatment using EpiTect Plus DNA Bisulfite Kit (QIAGEN). Retrotransposon genes were amplified using Ex Taq polymerase (TaKaRa), and the Dnmt3L promoter region was amplified using EpiTaq (TaKaRa) and specific bisulfite sequence primers. Purification and subcloning of PCR products and subsequent DNA sequencing were carried out as described above.

Small RNA cloning and sequencing

Total RNA was prepared from mouse embryonic day 16.5 testes with ISOGEN (NIPPON GENE). The RNAs were gel-fractionated to obtain RNA molecules that were 18–45 nt in length. The purified RNAs were subjected to library construction using the Digital Gene Expression for Small RNA Sample Preparation Kit (Illumina, San Diego, CA). Sequencing of small RNA was performed using an Illumina Hiseq2000 sequencer. Sequencing analysis was conducted by Hokkaido System Science Co., Ltd.

Bioinformatics analysis

To collect EGFP and Dnmt3L piRNAs, EGFP- and Dnmt3L-related small RNAs were extracted from the RNA library, including non-mismatches, by CLC Genomics Workbench (CLC bio Japan).

Histological analysis

Testes were fixed with a 50 × volume of Bouin solution overnight with rotation at 4°C. After rotation in 70% EtOH for 30 min and then 100% EtOH for 30 min, the testes were incubated with 1% benzoyl peroxide in dibutyl phthalate and methyl methacrylate mixed solutions (1:3) overnight at room temperature. Testes were embedded in dibutyl phthalate and methyl methacrylate mixed solutions containing 1% N-N dimethylaniline and 2% benzoyl peroxide, and placed on ice for 2 h. Embedded sections were stained with Hematoxylin (Mayer's Hematoxylin solution, Wako) and 1% eosin (Eosin Y, Wako).

Strand-specific RT-PCR

Preparation of RNA and reagents for the reverse transcriptase assay was performed as described above. A total of 500 ng DNase-treated RNA was incubated with GAPDH primers and sense-EGFP- or antisense-EGFP-specific primers in a 10 µL reaction mix at 65°C for 5 min. After incubation, the mixtures were immediately placed on ice, and then a reverse transcriptase-containing mixture was added. The reverse transcriptase reaction was performed with the following conditions: 65°C for 60 min, 85°C for 5 min, and 4°C overnight. After the reaction, this cDNA library was treated with RNase H at 37°C for 20 min. Expression of GAPDH and sense- and antisense-EGFP was detected by PCR with the primers described above.

In silico piRNA cluster analysis

To identify the piRNA cluster in embryonic day 16.5 testes, RNAs in the 25–31 nt range were mapped to mouse genome mm9 using the CLC genome browser. Uniquely mapped reads were analyzed by protract [40]. The piRNA clusters calculated under these conditions are shown in Table 2.

Capture sequence analysis

To identify the insertion sites of Miwi2-asEGFP transgenes, capture sequence analysis was performed. Genomic DNA prepared from Miwi2-asEGFP transgenic mice was fragmented using an S220 acoustic solubilizer (Covaris) to a length of 150–200 nt. Target DNAs containing the junction sequences between the transgene and the genome were purified and concentrated using the SureSelect Target Enrichment System (Agilent Technologies), according to the manufacture's protocol. The bait RNA sequences for the SureSelect Target Enrichment System are described in below. Sequencing analysis of captured DNA fragments was performed using an Illumina HiSeq 2000 sequencer. These sequencing steps were conducted by Hokkaido System Science Co., Ltd. The sequences obtained were analyzed by BLAST, and then annotated using the BLAT UCSC genomic browser for mm9.

Ping-pong signature

I evaluated EGFP and Dnmt3L piRNAs for the ping-pong signature by calculating the percentage of EGFP and Dnmt3L piRNAs mapping to an element with a complementary ping-pong partner.

PCR primers

Name	Primer sequence	Application
Miwi2 promoter F	GTCGACCAGATCACTTGACTTTACTGGCC	DNA cloning
Miwi2 promoter R	CTCGAGCCTGCAGGCAATTGGTTCCTGGGTGCCAGC	DNA cloning
EGFP F	TGCGGCCGCATGTGAGCAAG	DNA cloning
EGFP R	CGGAATTCTTACTTGTACAGCTCGTC	DNA cloning
Dnmt3L F	GCGGCCGCACACCCTCAACCCCATC	DNA cloning
Dnmt3L R	ACCGGTAACAATCCTATGATATATTTG	DNA cloning
GAPDH F	ACCACAGTCCATGCCATCAC	RT-PCR
GAPDH R	TCCACCACCCTGTTGCTGTA	RT-PCR
EGFP F	AACTTCATCTGCACCACCG	RT-PCR
EGFP R	TGCTCAGGTAGTGGTTGTCG	RT-PCR
GAPDH	TCCACCACCCTGTTGCTGTA	Strand-specific reverse transcription
sense EGFP	TTACTTGTACAGCTCGTCCATG	Strand-specific reverse transcription
antisense EGFP	AACGGCCACAAGTTCAGCGTGTCC	Strand-specific reverse transcription
Actin F	CGGTTCCGATGCCCTGAGGCTCTT	Quantitative RT-PCR analysis
Actin R	CGTCACACTTCATGATGGAATTGA	Quantitative RT-PCR analysis
IAP 1d1 F	AACGCTGCTGCTTTAACTCC	Quantitative RT-PCR analysis
IAP 1d1 R	ATTGTTCCCTCACTGGCAAA	Quantitative RT-PCR analysis
LINE-A F	CAGCTGAGTCGCCTGACAC	Quantitative RT-PCR analysis
LINE-A R	CTCTCCTTAGTTTCAGTGG	Quantitative RT-PCR analysis
LINE-Tf F	CTGTACCACCTGGGAACTGC	Quantitative RT-PCR analysis
LINE-Tf R	TGCTGGCAAGCTCTCTTACA	Quantitative RT-PCR analysis
Tg-Oct4 promoter F	GGTTTTTTAGAGGATGGTTGAGTG	Bisulfite sequence analysis
Tg-Oct4 promoter R	CCCTTACTCACCATAATAAAC	Bisulfite sequence analysis
Tg-Miwi2 promoter F	GATTTATGTAATGTTAATAGGTGT	Bisulfite sequence analysis
Tg-Miwi2 promoter R	CCCTAAACAAAAACCCCAAC	Bisulfite sequence analysis
EGFP F	TATTGGTTGTTATTATGGTGAGT	Bisulfite sequence analysis
EGFP R	CTCCAACTTATACCCCAAAATATTA	Bisulfite sequence analysis
H19 outside F	GAGTATTTAGGAGGTATAAGAATT	Bisulfite sequence analysis
H19 outside R	ATCAAAAACTAACATAAACCCCT	Bisulfite sequence analysis
H19 inside F	GTAAGGAGATTATGTTTATTTTTGG	Bisulfite sequence analysis
H19 inside R	CCTCATTAATCCCATAACTAT	Bisulfite sequence analysis
IAP 1d1 outside F	GTTTGTAATGGTGGGAGA	Bisulfite sequence analysis
IAP 1d1 outside R	AAATAAAATATCCCTCC	Bisulfite sequence analysis
IAP 1d1 inside F	TTGTGTTTTAAGTGGTAAATAAATAATTTG	Bisulfite sequence analysis
IAP 1d1 inside R	CAAAAAAAACACACAAACCAAAAT	Bisulfite sequence analysis
LINE-A F	TTATTTTGATAGTAGAGTT	Bisulfite sequence analysis
LINE-A R	C(AG)AACCAAACTCCTAACAA	Bisulfite sequence analysis
LINE-Tf outside F	GTTAGAGAATTTGATAGTTTTTGGAATAGG	Bisulfite sequence analysis
LINE-Tf outside R	CCAAAACAAAACCTTTCTCAAACACTATAT	Bisulfite sequence analysis
LINE-Tf inside F	TAGGAAATTAGTTTGAATAGGTGAGAGGT	Bisulfite sequence analysis
LINE-Tf inside R	TCAAACACTATATTACTTTAACAATTCCCA	Bisulfite sequence analysis
Dnmt3L Gene outside F	ATTTTATGTGTGAGGTTTAGAGTTTTT	Bisulfite sequence analysis
Dnmt3L Gene outside R	ACCTAAAAATCTCACAAAATTTCAAC	Bisulfite sequence analysis
Dnmt3L Gene inside F	GTTTTGAGTTTTATAGAATTTTATAATTTTT	Bisulfite sequence analysis
Dnmt3L Gene inside R	AAAAACTATCAACATCAAAACTAAAAC	Bisulfite sequence analysis

Oligonucleotides for capture sequence

bait	Bait RNA sequences referred to Miwi2 promoter
1	ACCCTTTCTGCTACCCTGTGTTAGTTAGCCAAAAGCTGTTGACTGGGAATTTGGAGCATCCCTCCAGAAATACAAGGCTTCATCTCATAGAAAACTGCTCCAGAGTGACCAGCTCCTCTA
2	CTACCCTGTGTTAGTTAGCCAAAAGCTGTTGACTGGGAATTTGGAGCATCCCTCCAGAAATACAAGGCTTCATCTCATAGAAAACTGCTCCAGAGTGACCAGCTCCTCTAGGAAGGTCAC
3	TTAGTTAGCCAAAAGCTGTTGACTGGGAATTTGGAGCATCCCTCCAGAATACAAGGCTTCATCTCATAGAAAACTGCTCCAGAGTGACCAGCTCCTCTAGGAAGGTCACAGCAGACCTC
4	AAAAGCTGTTGACTGGGAATTTGGAGCATCCCTCCAGAAATACAAGGCTTCATCTCATAGAAAACTGCTCCAGAGTGACCAGCTCCTCTAGGAAGGTCACAGCAGACCTCTTGACATAGT
5	GACTGGGAATTTGGAGCATCCCTCCAGAAATACAAGGCTTCATCTCATAGAAAACTGCTCCAGAGTGACCAGCTCCTCTAGGAAGGTCACAGCAGACCTCTTGACATAGTACCCACAGGC
6	TTGGAGCATCCCTCCAGAAATACAAGGCTTCATCTCATAGAAAACTGCTCCAGAGTGACCAGCTCCTCTAGGAAGGTCACAGCAGACCTCTTGACATAGTACCCACAGGCCTCTGGTACA
7	CCTCCAGAAATACAAGGCTTCATCTCATAGAAAACTGCTCCAGAGTGACAGCTCCTCTAGGAAGGTCACAGCAGACCTCTTGACATAGTACCCACAGGCCTCTGGTACAATACTAACTT
8	TACAAGGCTTCATCTCATAGAAAACTGCTCCAGAGTGACCAGCTCCTCTAGGAAGGTCACAGCAGACCTCTTGACATAGTACCCACAGGCCTCTGGTACAATACTAACTTTACTGACAGA
9	CATCTCATAGAAAACTGCTCCAGAGTGACCAGCTCCTCTAGGAAGGTCACAGCAGACCTCTTGACATAGTACCCACAGGCCTCTGGTACAATACTAACTTTACTGACAGACAAGCATGTG
10	AAAACTGCTCCAGAGTGACCAGCTCCTCTAGGAAGGTCACAGCAGACCTCTTGACATAGTACCCACAGGCCTCTGGTACAATACTAACTTTACTGACAGACAAGCATGTGGTGACTTGTC
11	CAGAGTGACCAGCTCCTCTAGGAAGGTCACAGCAGACCTCTTGACATAGTACCCACAGGCCTCTGGTACAATACTAACTTTACTGACAGACAAGCATGTGGTGACTTGTCCGTATTGACA
12	AGCTCCTCTAGGAAGGTCACAGCAGACCTCTTGACATAGTACCCACAGGCCTCTGGTACAATACTAACTTTACTGACAGACAAGCATGTGGTGACTTGTCCGTATTGACACAAGTGCTTT
13	GGAAGGTCACAGCAGACCTCTTGACATAGTACCCACAGGCCTCTGGTACAATACTAACTTTACTGACAGACAAGCATGTGGTGACTTGTCCGTATTGACACAAGTGCTTTGTCTCCAGTA
14	AGCAGACCTCTTGACATAGTACCCACAGGCCTCTGGTACAATACTAACTTTACTGACAGACAAGCATGTGGTGACTTGTCCGTATTGACACAAGTGCTTTGTCTCCAGTAAAGTCACCCT
15	TTGACATAGTACCCACAGGCCTCTGGTACAATACTAACTTTACTGACAGACAAGCATGTGGTGACTTGTCCGTATTGACACAAGTGCTTTGTCTCCAGTAAAGTCACCCTCGAAGTTCTC
16	ACCCACAGGCCTCTGGTACAATACTAACTTTACTGACAGACAAGCATGTGGTGACTTGTCCGTATTGACACAAGTGCTTTGTCTCCAGTAAAGTCACCCTCGAAGTTCTCCCTCATCCCT
17	CTCTGGTACAATACTAACTTTACTGACAGACAAGCATGTGGTGACTTGTCCGTATTGACACAAGTGCTTTGTCTCCAGTAAAGTCACCCTCGAAGTTCTCCCTCATCCCTCTGGCTATGC
18	ATACTAACTTTACTGACAGACAAGCATGTGGTGACTTGTCCGTATTGACACAAGTGCTTTGTCTCCAGTAAAGTCACCCTCGAAGTTCTCCCTCATCCCTCTGGCTATGCCTCTCTTAAG
19	TACTGACAGACAAGCATGTGGTGACTTGTCCGTATTGACACAAGTGCTTTGTCTCCAGTAAAGTCACCCTCGAAGTTCTCCCTCATCCCTCTGGCTATGCCTCTCTTAAGAGTTTACAAG
20	CAAGCATGTGGTGACTTGTCCGTATTGACACAAGTGCTTTGTCTCCAGTAAAGTCACCCTCGAAGTTCTCCCTCATCCCTCTGGCTATGCCTCTCTTAAGAGTTTACAAGACTGTATATC
21	GTGACTTGTCCGTATTGACACAAGTGCTTTGTCTCCAGTAAAGTCACCCTCGAAGTTCTCCCTCATCCCTCTGGCTATGCCTCTCTTAAGAGTTTACAAGACTGTATATCCGTTGCCTGA
22	CGTATTGACACAAGTGCTTTGTCTCCAGTAAAGTCACCCTCGAAGTTCTCCCTCATCCCTCTGGCTATGCCTCTCTTAAGAGTTTACAAGACTGTATATCCGTTGCCTGAGTTACAAATA
23	CAAGTGCTTTGTCTCCAGTAAAGTCACCCTCGAAGTTCTCCCTCATCCCTCTGGCTATGCCTCTCTTAAGAGTTTACAAGACTGTATATCCGTTGCCTGAGTTACAAATAAACACCATGT
24	GTCTCCAGTAAAGTCACCCTCGAAGTTCTCCCTCATCCCTCTGGCTATGCCTCTCTTAAGAGTTTACAAGACTGTATATCCGTTGCCTGAGTTACAAATAAACACCATGTTTGATTTAAA
25	AAGTCACCCTCGAAGTTCTCCCTCATCCCTCTGGCTATGCCTCTCTTAAGAGTTTACAAGACTGTATATCCGTTGCCTGAGTTACAAATAAACACCATGTTTGATTTAAATCTAGCTATT

Table 1. Insertion sites of Miwi2-asEGFP transgenes

Miwi2-asEGFP Tg	The junction sequence between the genome and the transgene	Strand	integration sites
#1	CAGGGTGATTCTTACACATCACCAGATTCGGAAGCAACACCAGGCTGCTTGACT	+	chr17:57820104
#6	TGAAAGTACAGACACCACGGAAGTCCACTTAGTGTCCAGTGAGTTTTATTGGGG	+	chr2:154985502
#8	GTCACACCTGCCCAGCTGCTCAGGTGCGCCTGGCACCTTGCAATGCTCTCCTGT	+	chr8:90145336

Black: Genome
Red: Transgene Miwi2-promoter

Table 2. piRNA cluster analysis

Number	Chromosome locus	Range	Cluster score
1	chr15	74451806-74506517 (54712 bp)	288
2	chr7	80236500-80306199 (69700 bp)	279
3	chr14	24883334-24954845 (71512 bp)	270
4	chr10	75288584-75361982 (73399 bp)	259
5	chr11	103270468-103313542 (43075 bp)	258
6	chr10	62114624-62168346 (53723 bp)	252
7	chr6	127726991-127800732 (73742 bp)	243
8	chr8	94710866-94735835 (24970 bp)	232
9	chr10	82802000-82964895 (162896 bp)	230
10	chr19	5788874-5865130 (76257 bp)	228
11	chr7	77020407-77099473 (79067 bp)	207
12	chr1	157898791-157948865 (50075 bp)	201
13	chr8	119717171-119746089 (28919 bp)	198
14	chr1	133902000-133941369 (39370 bp)	194
15	chr3	94385611-94428711 (43101 bp)	188
16	chr15	79747475-79797093 (49619 bp)	181
17	chrM	1-16165 (16165 bp)	179
18	chr17	27400582-27534917 (134336 bp)	170
19	chr9	3000098-3047719 (47622 bp)	170
20	chr17	66500752-66589367 (88616 bp)	163
21	chr17	5734046-5793397 (59352 bp)	160
22	chr15	82129428-82206235 (76808 bp)	154
23	chr3	34752158-34777840 (25683 bp)	149
24	chr3	96949086-96980323 (31238 bp)	149
25	chr8	112553156-112605461 (52306 bp)	149
26	chr7	60144682-60170363 (25682 bp)	146
27	chr12	79436389-79471789 (35401 bp)	141
28	chr16	22766631-22800497 (33867 bp)	140
29	chr15	84989713-85026168 (36456 bp)	137
30	chr10	127094390-127150669 (56280 bp)	136
31	chr1	120555596-120581535 (25940 bp)	133

32	chr4	73871620-73896894 (25275 bp)	133
33	chr10	30523062-30540504 (17443 bp)	131
34	chr11	60589590-60614961 (25372 bp)	131
35	chr8	112634570-112656484 (21915 bp)	130
36	chr13	53487889-53515695 (27807 bp)	129
37	chr15	97176990-97227545 (50556 bp)	129
38	chr10	51153852-51172707 (18856 bp)	128
39	chr11	107843891-107871324 (27434 bp)	125
40	chr16	17570636-17610264 (39629 bp)	125
41	chr2	180048321-180077198 (28878 bp)	122
42	chr9	100857688-100913032 (55345 bp)	118
43	chr17	25543993-25593898 (49906 bp)	116
44	chr2	127496778-127545635 (48858 bp)	115
45	chr8	8984415-9009836 (25422 bp)	108
46	chr7	20432882-20548750 (115869 bp)	105
47	chr17	35787058-35881288 (94231 bp)	103
48	chr1	158555448-158596354 (40907 bp)	101
49	chr4	149329877-149357812 (27936 bp)	100
50	chr11	74621143-74718984 (97842 bp)	97.1
51	chr3	103025903-103063280 (37378 bp)	96.9
52	chr5	144499750-144562105 (62356 bp)	96.4
53	chr11	96542213-96577263 (35051 bp)	93.7
54	chr11	59952424-60002146 (49723 bp)	93.2
55	chr7	28959997-29066753 (106757 bp)	93.2
56	chr16	22009482-22053665 (44184 bp)	92.1
57	chr7	29123909-29184862 (60954 bp)	92.1
58	chr7	30889586-30931373 (41788 bp)	90.7
59	chr5	24712475-24757944 (45470 bp)	90.4
60	chr13	95105298-95130952 (25655 bp)	89.7
61	chr11	97917071-97952139 (35069 bp)	89.2
62	chr1	133456477-133554953 (98477 bp)	88.2
63	chr6	125506091-125554004 (47914 bp)	87.9
64	chr13	12350224-12368747 (18524 bp)	87.7
65	chr3	16954534-16984773 (30240 bp)	87.5
66	chr4	135168240-135206897 (38658 bp)	87.5

67	chr6	92099593-92140259 (40667 bp)	87
68	chr11	94594852-94625857 (31006 bp)	86.2
69	chr10	94818107-94859659 (41553 bp)	85.2
70	chr11	3029805-3101885 (72081 bp)	84.3
71	chr16	90504606-90554369 (49764 bp)	83.5
72	chr6	51530342-51563024 (32683 bp)	83.1
73	chr7	16833504-16897012 (63509 bp)	81.7
74	chr2	168904591-168979519 (74929 bp)	81.2
75	chr5	126350103-126375304 (25202 bp)	80.8
76	chr10	66134729-66191405 (56677 bp)	80.1
77	chr16	59231505-59253144 (21640 bp)	79.6
78	chr5	135622564-135660792 (38229 bp)	79.2
79	chr2	30501822-30524201 (22380 bp)	78.1
80	chrX	70253587-70284683 (31097 bp)	78
81	chr3	129157995-129226743 (68749 bp)	77.2
82	chr2	154810117-154855144 (45028 bp)	77.1
83	chr2	156006389-156051261 (44873 bp)	76.3
84	chr10	85195796-85236641 (40846 bp)	75.4
85	chr3	88521753-88569899 (48147 bp)	75.3
86	chr5	145381848-145509525 (127678 bp)	75.1
87	chr19	29729123-29767545 (38423 bp)	74.8
88	chr2	164630124-164776153 (146030 bp)	74.7
89	chr4	153089095-153150693 (61599 bp)	73.6
90	chr7	19811081-19850107 (39027 bp)	73
91	chr14	7475469-7523181 (47713 bp)	72
92	chr10	80506906-80548464 (41559 bp)	71.9
93	chr17	45565375-45595209 (29835 bp)	71.4
94	chr7	20053381-20099473 (46093 bp)	71.1
95	chr11	69818709-69863275 (44567 bp)	70.8
96	chr1	93437325-93462091 (24767 bp)	70.4
97	chr7	36537700-36555055 (17356 bp)	70.4
98	chr1	184737119-184815319 (78201 bp)	70.1
99	chr1	135018070-135049821 (31752 bp)	68.6
100	chr13	65380142-65419106 (38965 bp)	67.8
101	chr12	77545311-77581001 (35691 bp)	67.7

102	chr18	63815392-63862226 (46835 bp)	67.6
103	chr7	116846564-116880305 (33742 bp)	66.6
104	chr2	118715164-118753096 (37933 bp)	66.2
105	chr17	71085378-71122956 (37579 bp)	65.1
106	chr2	129998643-130032531 (33889 bp)	64.7
107	chr11	120148659-120229904 (81246 bp)	64.4
108	chr5	137153065-137197591 (44527 bp)	63.9
109	chr2	164232839-164302716 (69878 bp)	63.8
110	chr4	87266706-87294538 (27833 bp)	63.7
111	chr12	101390319-101428375 (38057 bp)	63.6
112	chr7	52330923-52399573 (68651 bp)	63.4
113	chr11	75376529-75399353 (22825 bp)	63
114	chr9	102992922-103031441 (38520 bp)	63
115	chr1	127071504-127086681 (15178 bp)	62.6
116	chr13	106288331-106323796 (35466 bp)	62.5
117	chr6	128374910-128417057 (42148 bp)	62.5
118	chr8	38445764-38452910 (7147 bp)	62.4
119	chr5	106796285-106860761 (64477 bp)	62.2
120	chr13	12602582-12629319 (26738 bp)	62.1
121	chr2	167290914-167361076 (70163 bp)	62.1
122	chr5	135395145-135439407 (44263 bp)	62
123	chr12	88099416-88179019 (79604 bp)	61.7
124	chr13	51682310-51741210 (58901 bp)	61.7
125	chr4	82358456-82393744 (35289 bp)	61.3
126	chr15	75169846-75201620 (31775 bp)	60.6
127	chr10	85300648-85343938 (43291 bp)	60.3
128	chr11	120401029-120451518 (50490 bp)	60.3
129	chr12	73636107-73664733 (28627 bp)	60.3
130	chr14	61858733-61888528 (29796 bp)	60
131	chr7	117190267-117204802 (14536 bp)	59.7
132	chr8	124340970-124377897 (36928 bp)	59.7
133	chr3	51013196-51060748 (47553 bp)	59.4
134	chr5	150023890-150059762 (35873 bp)	59.2
135	chr8	123558817-123579841 (21025 bp)	59.2
136	chr11	117525909-117559355 (33447 bp)	58.4

137	chr17	27945963-27979275 (33313 bp)	57.9
138	chr13	41895910-41928789 (32880 bp)	57.6
139	chr17	88137447-88172733 (35287 bp)	57
140	chr10	86019749-86099845 (80097 bp)	56.5
141	chr4	94920969-94946061 (25093 bp)	56.5
142	chr19	45849294-45902964 (53671 bp)	56.3
143	chr14	57480820-57510327 (29508 bp)	56.2
144	chr12	52573645-52665183 (91539 bp)	55.7
145	chr19	41647262-41677320 (30059 bp)	55.3
146	chr4	146351861-146402051 (50191 bp)	55.2
147	chr8	48532018-48571695 (39678 bp)	54.9
148	chr14	55132714-55175148 (42435 bp)	54.4
149	chr17	88649736-88682843 (33108 bp)	54.4
150	chr13	55187131-55235711 (48581 bp)	54.2
151	chr2	170119392-170136138 (16747 bp)	54.2
152	chr13	99750442-99812662 (62221 bp)	53.9
153	chr9	62585433-62616995 (31563 bp)	53.8
154	chr19	47301817-47333617 (31801 bp)	53.7
155	chr9	103058350-103095641 (37292 bp)	53.6
156	chr15	83007952-83054045 (46094 bp)	53.5
157	chr3	112998322-113043595 (45274 bp)	53
158	chr16	3742650-3808396 (65747 bp)	52.9
159	chr16	55937553-55975083 (37531 bp)	52.9
160	chr16	64706359-64715383 (9025 bp)	52.9
161	chr4	155051023-155107810 (56788 bp)	52.6
162	chr8	8579125-8600448 (21324 bp)	52.6
163	chr17	48286813-48364137 (77325 bp)	52.1
164	chr13	52208059-52222763 (14705 bp)	52
165	chr12	3852260-3913344 (61085 bp)	51.9
166	chr1	154220258-154256011 (35754 bp)	51.8
167	chr11	114649127-114692160 (43034 bp)	51.7
168	chr9	24493810-24533209 (39400 bp)	51.6
169	chr2	158364935-158394148 (29214 bp)	51.5
170	chr17	36115622-36158653 (43032 bp)	51.2
171	chr15	81629197-81672417 (43221 bp)	50.9

172	chr7	51403081-51447019 (43939 bp)	50.9
173	chr8	87352847-87397221 (44375 bp)	50.9
174	chr1	88364392-88439933 (75542 bp)	50.8
175	chr4	137862163-137897592 (35430 bp)	50.5
176	chr8	125561758-125585300 (23543 bp)	50.3
177	chr10	80624455-80659536 (35082 bp)	50.2
178	chr18	83065633-83102538 (36906 bp)	50.2
179	chr10	98661847-98689048 (27202 bp)	49.8
180	chr10	120684233-120709934 (25702 bp)	49.8
181	chr4	146560909-146668238 (107330 bp)	49.7
182	chr5	77692714-77723772 (31059 bp)	49.7
183	chr14	70376087-70415109 (39023 bp)	49.6
184	chr8	87197022-87233709 (36688 bp)	49.3
185	chr11	79494349-79523554 (29206 bp)	49.2
186	chr17	36205029-36259557 (54529 bp)	49
187	chr6	49424202-49450528 (26327 bp)	48.8
188	chr12	74093379-74124923 (31545 bp)	48.5
189	chr10	41145486-41172641 (27156 bp)	48.4
190	chr14	56583748-56622234 (38487 bp)	48.4
191	chr15	98802692-98836734 (34043 bp)	48.4
192	chr14	122487912-122513605 (25694 bp)	48.2
193	chr6	116351111-116359272 (8162 bp)	48.2
194	chr5	144088948-144114858 (25911 bp)	48.1
195	chr10	55346533-55356158 (9626 bp)	48
196	chr14	45555050-45564352 (9303 bp)	48
197	chr8	123178785-123205551 (26767 bp)	48
198	chr1	154079379-154152127 (72749 bp)	47.7
199	chr15	96379355-96413528 (34174 bp)	47.7
200	chr2	172672071-172724885 (52815 bp)	47.6
201	chr5	24499897-24540339 (40443 bp)	47.6
202	chr4	152987082-153026601 (39520 bp)	47.5
203	chr7	52103735-52164636 (60902 bp)	47.5
204	chr6	142935495-142965252 (29758 bp)	47.4
205	chr8	114259109-114281636 (22528 bp)	47.4
206	chr19	12719274-12742568 (23295 bp)	47.3

207	chr11	3165944-3200937 (34994 bp)	47.1
208	chr6	95068625-95100834 (32210 bp)	47.1
209	chr8	119312234-119342196 (29963 bp)	47.1
210	chr10	25602839-25623686 (20848 bp)	46.5
211	chr14	57436516-57471565 (35050 bp)	46.5
212	chr8	74729535-74780808 (51274 bp)	46.4
213	chr7	54530058-54587785 (57728 bp)	46.2
214	chr17	47621873-47660133 (38261 bp)	46
215	chr2	30385974-30424194 (38221 bp)	46
216	chr5	65275466-65326074 (50609 bp)	45.8
217	chr19	28748261-28761132 (12872 bp)	45.7
218	chr4	51873701-51912760 (39060 bp)	45.7
219	chr6	120820803-120842615 (21813 bp)	45.5
220	chrX	73500325-73556670 (56346 bp)	45.5
221	chr4	139499393-139538604 (39212 bp)	45.4
222	chr7	25240640-25341417 (100778 bp)	45.3
223	chr12	70630038-70674122 (44085 bp)	45.2
224	chr14	54170735-54209184 (38450 bp)	45.2
225	chr16	16600000-16627981 (27982 bp)	45.2
226	chr7	134895575-134938068 (42494 bp)	45.2
227	chr19	3699559-3732422 (32864 bp)	45.1
228	chr11	51802153-51821833 (19681 bp)	44.8
229	chr15	102099482-102126182 (26701 bp)	44.8
230	chr14	21443953-21463805 (19853 bp)	44.7
231	chr5	150888545-150927583 (39039 bp)	44.7
232	chr1	174306350-174314027 (7678 bp)	44.6
233	chr2	97496964-97523021 (26058 bp)	44.6
234	chr2	158038916-158069970 (31055 bp)	44.5
235	chr11	93981838-94010491 (28654 bp)	44.2
236	chr1	134287721-134324573 (36853 bp)	44.1
237	chr14	8213180-8250149 (36970 bp)	43.9
238	chr7	104458259-104495705 (37447 bp)	43.7
239	chr4	131804999-131834463 (29465 bp)	43.5
240	chr2	172825106-172864391 (39286 bp)	43.3
241	chr18	82847777-82889676 (41900 bp)	42.9

242	chr5	135928034-135971014 (42981 bp)	42.8
243	chr17	89752989-89770333 (17345 bp)	42.5
244	chr3	36978810-36999716 (20907 bp)	42.5
245	chr14	34780335-34806698 (26364 bp)	42.4
246	chr17	56113095-56146932 (33838 bp)	42.2
247	chr3	9901488-9923179 (21692 bp)	42.1
248	chr10	22445117-22481548 (36432 bp)	42
249	chrX	39382660-39408712 (26053 bp)	42
250	chr5	108450291-108481165 (30875 bp)	41.8
251	chr15	88849726-88874609 (24884 bp)	41.7
252	chr16	8517035-8551814 (34780 bp)	41.7
253	chr10	86139001-86167074 (28074 bp)	41.5
254	chr18	74350387-74384707 (34321 bp)	41.3
255	chr6	30874288-30970842 (96555 bp)	41.1
256	chr8	123022528-123081745 (59218 bp)	41.1
257	chr12	79047466-79069213 (21748 bp)	41
258	chr12	20694599-20731830 (37232 bp)	40.9
259	chr1	193718269-193777954 (59686 bp)	40.8
260	chr16	32142665-32169655 (26991 bp)	40.8
261	chrX	131218461-131230901 (12441 bp)	40.8
262	chr7	31458617-31490872 (32256 bp)	40.7
263	chr16	18491803-18519411 (27609 bp)	40.5
264	chr7	135009840-135064069 (54230 bp)	40.5
265	chr5	134561396-134619179 (57784 bp)	40.3
266	chr6	70906467-70933599 (27133 bp)	40.2
267	chr15	6623186-6652481 (29296 bp)	39.9
268	chr15	37274422-37306915 (32494 bp)	39.6
269	chr7	132573539-132608509 (34971 bp)	39.6
270	chr12	43452139-43468953 (16815 bp)	39.3
271	chr2	152201759-152251632 (49874 bp)	39.3
272	chr7	27916715-27959306 (42592 bp)	39.2
273	chr8	93392585-93434279 (41695 bp)	39.2
274	chr18	18977536-19018118 (40583 bp)	39.1
275	chr15	81280593-81299314 (18722 bp)	39
276	chr5	33084881-33115716 (30836 bp)	39

277	chr9	72468984-72507060 (38077 bp)	39
278	chr16	93994921-94022507 (27587 bp)	38.9
279	chr17	35957536-35990076 (32541 bp)	38.7
280	chr12	112935910-112975453 (39544 bp)	38.4
281	chr10	33376911-33421584 (44674 bp)	38.1
282	chr3	101100059-101139266 (39208 bp)	38.1
283	chr10	79482929-79524692 (41764 bp)	37.9
284	chr11	102189240-102228742 (39503 bp)	37.9
285	chr11	73486921-73511703 (24783 bp)	37.8
286	chr3	87924224-87996830 (72607 bp)	37.8
287	chr7	19700390-19728815 (28426 bp)	37.7
288	chr7	50434462-50470382 (35921 bp)	37.7
289	chr1	58685198-58725414 (40217 bp)	37.6
290	chr3	87761612-87799540 (37929 bp)	37.6
291	chr18	24676263-24717114 (40852 bp)	37.4
292	chr2	35358925-35388195 (29271 bp)	37.4
293	chr2	84923960-84969291 (45332 bp)	37.3
294	chr13	14105468-14147644 (42177 bp)	37.2
295	chr6	134846366-134896927 (50562 bp)	37.2
296	chr18	13105666-13134859 (29194 bp)	37.1
297	chr6	86416848-86444293 (27446 bp)	37.1
298	chr9	62279148-62306655 (27508 bp)	37.1
299	chr6	125033280-125058782 (25503 bp)	37
300	chr9	27150146-27163758 (13613 bp)	37
301	chr13	62383245-62453505 (70261 bp)	36.8
302	chr3	108227844-108263378 (35535 bp)	36.8
303	chr1	8382491-8402847 (20357 bp)	36.7
304	chr19	46107514-46138707 (31194 bp)	36.7
305	chr6	137894764-137924520 (29757 bp)	36.7
306	chr11	95620862-95642049 (21188 bp)	36.5
307	chr2	22860304-22925937 (65634 bp)	36.5
308	chr12	8574448-8623131 (48684 bp)	36.4
309	chr13	40857731-40877635 (19905 bp)	36.3
310	chr7	30370173-30433638 (63466 bp)	36.1
311	chr4	39322790-39338953 (16164 bp)	36

312	chr13	63652163-63683304 (31142 bp)	35.9
313	chr13	97997773-98022471 (24699 bp)	35.9
314	chr18	90001646-90037710 (36065 bp)	35.8
315	chr3	10106192-10129110 (22919 bp)	35.8
316	chr4	133698364-133774954 (76591 bp)	35.8
317	chr9	114297968-114332280 (34313 bp)	35.8
318	chr10	118565979-118599064 (33086 bp)	35.7
319	chr15	79359970-79391412 (31443 bp)	35.7
320	chr16	7723387-7777652 (54266 bp)	35.7
321	chr2	138342327-138406052 (63726 bp)	35.7
322	chr6	113661057-113686455 (25399 bp)	35.7
323	chr7	29311857-29348057 (36201 bp)	35.7
324	chr14	20289784-20317931 (28148 bp)	35.6
325	chr14	114493734-114519867 (26134 bp)	35.6
326	chr16	93681020-93725052 (44033 bp)	35.6
327	chr3	66886503-66907633 (21131 bp)	35.6
328	chr17	69711291-69742765 (31475 bp)	35.5
329	chr19	44651321-44687734 (36414 bp)	35.5
330	chr10	68760370-68799229 (38860 bp)	35.4
331	chr11	90819940-90850538 (30599 bp)	35.4
332	chr12	25030032-25067841 (37810 bp)	35.4
333	chr16	3317537-3353944 (36408 bp)	35.4
334	chr2	32064285-32099205 (34921 bp)	35.4
335	chr2	163245618-163257348 (11731 bp)	35.4
336	chr5	149784451-149828869 (44419 bp)	35.4
337	chr6	54631765-54653117 (21353 bp)	35.4
338	chr8	112247386-112278459 (31074 bp)	35.3
339	chr1	181636823-181663903 (27081 bp)	35.2
340	chr6	7650738-7682657 (31920 bp)	35.2
341	chr9	75317448-75355733 (38286 bp)	35.2
342	chr4	133436384-133475913 (39530 bp)	35.1
343	chr13	67730435-67763010 (32576 bp)	35
344	chr14	18245508-18280976 (35469 bp)	35
345	chr16	31423562-31457966 (34405 bp)	35
346	chr13	24558350-24580812 (22463 bp)	34.9

347	chr17	68599080-68623917 (24838 bp)	34.9
348	chr6	132912073-132928950 (16878 bp)	34.9
349	chr16	10692873-10716879 (24007 bp)	34.8
350	chr2	166910736-166950650 (39915 bp)	34.8
351	chr16	56033938-56062706 (28769 bp)	34.6
352	chr17	89656785-89665856 (9072 bp)	34.6
353	chr3	16675806-16708815 (33010 bp)	34.6
354	chr7	29403866-29445184 (41319 bp)	34.5
355	chr1	74694491-74727910 (33420 bp)	34.4
356	chr10	126412313-126451393 (39081 bp)	34.4
357	chr13	50765214-50827631 (62418 bp)	34.4
358	chr10	75226766-75251153 (24388 bp)	34.3
359	chr8	73878700-73921879 (43180 bp)	34.3
360	chr18	68053192-68067268 (14077 bp)	34.2
361	chr15	80755151-80779054 (23904 bp)	34.1
362	chr15	89370774-89400737 (29964 bp)	34.1
363	chr7	134267892-134301660 (33769 bp)	34.1
364	chr15	79574214-79592933 (18720 bp)	34
365	chr4	154780268-154812531 (32264 bp)	33.9
366	chr5	72951157-72961620 (10464 bp)	33.9
367	chr5	125408519-125444964 (36446 bp)	33.9
368	chr18	36982674-37045244 (62571 bp)	33.8
369	chr4	74693102-74729352 (36251 bp)	33.8
370	chr13	51545767-51569522 (23756 bp)	33.7
371	chr16	4337399-4362346 (24948 bp)	33.6
372	chr11	105304440-105329198 (24759 bp)	33.3
373	chr4	146017811-146044914 (27104 bp)	33.3
374	chr9	5639560-5662183 (22624 bp)	33.3
375	chr1	167995453-168024634 (29182 bp)	33.2
376	chr9	120632294-120648586 (16293 bp)	33.1
377	chr17	18683617-18711251 (27635 bp)	33
378	chr5	34082476-34110661 (28186 bp)	33
379	chr1	52358809-52397292 (38484 bp)	32.9
380	chr14	121778984-121805139 (26156 bp)	32.9
381	chr16	17243988-17263426 (19439 bp)	32.9

382	chr17	87984189-88015725 (31537 bp)	32.9
383	chr10	116895017-116947560 (52544 bp)	32.8
384	chr11	3212585-3235632 (23048 bp)	32.8
385	chr13	117936138-117965948 (29811 bp)	32.8
386	chr17	30713450-30730354 (16905 bp)	32.7
387	chr14	74706754-74723548 (16795 bp)	32.6
388	chr15	82967885-82996094 (28210 bp)	32.6
389	chr14	76812223-76834851 (22629 bp)	32.4
390	chr9	51645988-51667829 (21842 bp)	32.3
391	chr1	88324138-88350418 (26281 bp)	32.2
392	chr14	3255439-3294465 (39027 bp)	32.2
393	chr10	79844963-79883862 (38900 bp)	32.1
394	chr5	33560830-33599958 (39129 bp)	32.1
395	chr7	34598187-34624870 (26684 bp)	32.1
396	chr8	12015591-12032402 (16812 bp)	32.1
397	chr19	3751784-3782505 (30722 bp)	32
398	chr2	170427123-170471598 (44476 bp)	32
399	chrX	12915224-12937815 (22592 bp)	32
400	chr11	86689880-86719241 (29362 bp)	31.9
401	chr14	79872254-79892059 (19806 bp)	31.9
402	chr9	58391068-58420746 (29679 bp)	31.9
403	chr1	181570513-181597975 (27463 bp)	31.8
404	chr11	102927240-102971156 (43917 bp)	31.8
405	chr13	49880022-49900006 (19985 bp)	31.7
406	chr2	94635832-94664446 (28615 bp)	31.7
407	chr4	53951182-53978839 (27658 bp)	31.7
408	chr4	136094124-136111113 (16990 bp)	31.7
409	chr19	10053360-10066153 (12794 bp)	31.5
410	chr4	141711472-141741849 (30378 bp)	31.5
411	chr15	89772354-89793388 (21035 bp)	31.4
412	chr8	58025613-58058567 (32955 bp)	31.4
413	chr19	35151657-35183930 (32274 bp)	31.3
414	chr2	157195892-157218929 (23038 bp)	31.3
415	chr5	43582862-43625700 (42839 bp)	31.2
416	chr7	71253317-71286436 (33120 bp)	31.2

417	chr8	73378588-73449440 (70853 bp)	31.1
418	chr10	17019795-17062687 (42893 bp)	30.9
419	chr12	45053501-45077534 (24034 bp)	30.9
420	chr13	7810595-7835204 (24610 bp)	30.9
421	chr5	111722253-111759460 (37208 bp)	30.9
422	chr17	36061207-36100077 (38871 bp)	30.8
423	chr6	69069254-69110007 (40754 bp)	30.8
424	chr19	29918509-29937339 (18831 bp)	30.7
425	chr4	41203085-41232077 (28993 bp)	30.7
426	chr1	84850548-84884400 (33853 bp)	30.6
427	chr13	67970027-68003156 (33130 bp)	30.6
428	chr15	78330621-78354656 (24036 bp)	30.6
429	chr13	57039439-57072558 (33120 bp)	30.5
430	chr16	76908261-76928631 (20371 bp)	30.4
431	chr2	35075953-35100290 (24338 bp)	30.4
432	chr5	30353495-30395127 (41633 bp)	30.4
433	chr10	34681254-34709430 (28177 bp)	30.3
434	chr4	145222953-145252228 (29276 bp)	30.3
435	chr2	85775326-85871396 (96071 bp)	30.2
436	chr3	142329588-142365713 (36126 bp)	30.2
437	chr7	65638802-65664371 (25570 bp)	30.2
438	chr13	21901766-21925891 (24126 bp)	30.1
439	chr5	77340772-77385620 (44849 bp)	30.1
440	chr4	32857939-32898216 (40278 bp)	30
441	chr5	4188593-4208453 (19861 bp)	30
442	chr3	69720232-69747973 (27742 bp)	29.9
443	chr3	95421004-95479798 (58795 bp)	29.9
444	chr14	56407647-56434170 (26524 bp)	29.8
445	chr19	4935090-4973949 (38860 bp)	29.8
446	chr5	26377727-26419817 (42091 bp)	29.8
447	chr14	14755241-14778718 (23478 bp)	29.7
448	chr14	41866901-41886229 (19329 bp)	29.7
449	chr15	96795629-96818389 (22761 bp)	29.7
450	chr15	98739139-98775828 (36690 bp)	29.6
451	chr18	61990315-62005389 (15075 bp)	29.6

452	chr1	172866151-172881820 (15670 bp)	29.5
453	chr11	8803320-8839775 (36456 bp)	29.5
454	chr14	32071907-32108780 (36874 bp)	29.5
455	chr3	58199700-58221407 (21708 bp)	29.5
456	chr17	74862073-74888286 (26214 bp)	29.3
457	chr7	30510934-30537405 (26472 bp)	29.3
458	chr5	136613432-136647055 (33624 bp)	29.2
459	chr6	41147271-41169003 (21733 bp)	29.2
460	chr14	7152461-7189732 (37272 bp)	29.1
461	chr12	83662609-83684996 (22388 bp)	29
462	chr10	3236930-3273040 (36111 bp)	28.9
463	chr11	5676777-5701724 (24948 bp)	28.9
464	chr14	80475386-80499649 (24264 bp)	28.9
465	chr15	64540966-64546017 (5052 bp)	28.8
466	chr19	5387764-5424447 (36684 bp)	28.3
467	chr19	16073027-16100113 (27087 bp)	28.3
468	chr4	145320473-145357052 (36580 bp)	28.3
469	chr5	22053677-22078507 (24831 bp)	28.3
470	chr5	136083069-136123630 (40562 bp)	28.3
471	chr11	17051191-17068303 (17113 bp)	28.2
472	chr4	146189405-146219029 (29625 bp)	28.2
473	chr7	30097592-30117273 (19682 bp)	28.2
474	chr11	102145118-102172352 (27235 bp)	28.1
475	chr18	83107666-83126416 (18751 bp)	28.1
476	chr11	76344419-76371268 (26850 bp)	28
477	chr15	36971836-36993952 (22117 bp)	28
478	chr16	33745506-33773897 (28392 bp)	28
479	chr10	113637826-113658469 (20644 bp)	27.9
480	chr13	45568820-45589989 (21170 bp)	27.9
481	chr10	127393969-127417911 (23943 bp)	27.8
482	chr13	64254114-64264815 (10702 bp)	27.8
483	chr15	3742967-3770118 (27152 bp)	27.8
484	chr16	15191264-15206477 (15214 bp)	27.7
485	chr5	3636830-3674878 (38049 bp)	27.7
486	chr14	34059682-34091240 (31559 bp)	27.6

487	chr14	106843305-106871740 (28436 bp)	27.6
488	chr2	92497836-92530719 (32884 bp)	27.6
489	chr1	83961258-83986524 (25267 bp)	27.5
490	chr11	97041809-97060104 (18296 bp)	27.5
491	chr5	135301067-135337733 (36667 bp)	27.5
492	chr12	8517519-8532031 (14513 bp)	27.3
493	chr17	84864321-84872009 (7689 bp)	27.3
494	chr17	27031172-27064128 (32957 bp)	27.2
495	chr9	51777848-51794153 (16306 bp)	27.1
496	chr4	146310932-146327733 (16802 bp)	26.9
497	chr8	18089491-18108609 (19119 bp)	26.8
498	chr9	25649156-25685221 (36066 bp)	26.8
499	chr18	80009598-80039822 (30225 bp)	26.7
500	chr12	33572297-33605397 (33101 bp)	26.6
501	chr10	14308904-14333114 (24211 bp)	26.4
502	chr11	88734580-88778158 (43579 bp)	26.4
503	chr2	84722262-84748553 (26292 bp)	26.4
504	chr17	21109257-21126689 (17433 bp)	26.3
505	chr17	23938102-23957756 (19655 bp)	26.3
506	chr12	23183013-23230598 (47586 bp)	26.2
507	chr13	100982778-101009322 (26545 bp)	26.2
508	chr2	20301727-20326797 (25071 bp)	26.2
509	chr7	30946683-30985246 (38564 bp)	26.2
510	chr8	120277137-120299519 (22383 bp)	26.2
511	chr9	95141141-95151761 (10621 bp)	26.2
512	chrY	1998294-2039158 (40865 bp)	26.2
513	chr17	46794596-46826666 (32071 bp)	26.1
514	chr4	12059829-12079523 (19695 bp)	26
515	chr17	83914092-83927738 (13647 bp)	25.9
516	chr9	121455368-121476890 (21523 bp)	25.9
517	chr12	111933553-111952768 (19216 bp)	25.8
518	chr10	83875395-83907052 (31658 bp)	25.7
519	chr11	61221420-61243762 (22343 bp)	25.7
520	chr2	162633597-162651540 (17944 bp)	25.7
521	chr2	165708347-165770155 (61809 bp)	25.7

522	chr2	65591393-65630297 (38905 bp)	25.6
523	chr6	122571167-122594870 (23704 bp)	25.6
524	chr15	19919484-19952179 (32696 bp)	25.5
525	chr2	176463259-176485488 (22230 bp)	25.5
526	chr5	134425370-134523218 (97849 bp)	25.5
527	chr4	41748448-41783555 (35108 bp)	25.2
528	chr5	110058262-110093887 (35626 bp)	25.2
529	chr7	151029876-151065222 (35347 bp)	25.2
530	chr17	88347936-88378297 (30362 bp)	25.1
531	chr19	42216995-42244835 (27841 bp)	25.1
532	chr8	127292636-127323987 (31352 bp)	25.1
533	chr12	55224072-55248211 (24140 bp)	25
534	chr13	33774948-33804735 (29788 bp)	25
535	chr4	147409399-147432340 (22942 bp)	25
536	chr13	23421131-23449514 (28384 bp)	24.9
537	chrX	30508911-30564218 (55308 bp)	24.9
538	chr13	50390605-50423501 (32897 bp)	24.8
539	chr13	52138571-52166532 (27962 bp)	24.8
540	chr3	87621009-87660018 (39010 bp)	24.8
541	chr2	96608277-96628399 (20123 bp)	24.7
542	chr10	125147919-125159960 (12042 bp)	24.6
543	chr7	109044342-109073067 (28726 bp)	24.6
544	chr1	136150235-136173169 (22935 bp)	24.5
545	chr12	20297334-20318890 (21557 bp)	24.5
546	chr15	87794291-87802578 (8288 bp)	24.5
547	chr18	43649216-43684795 (35580 bp)	24.5
548	chr5	44517763-44541336 (23574 bp)	24.5
549	chr9	7661848-7684000 (22153 bp)	24.4
550	chr16	31216335-31229594 (13260 bp)	24.3
551	chr2	165601842-165622293 (20452 bp)	24.3
552	chr5	123577849-123605408 (27560 bp)	24.3
553	chr6	48259746-48295695 (35950 bp)	24.3
554	chr16	90668558-90689163 (20606 bp)	24.2
555	chr4	56428850-56450993 (22144 bp)	24.2
556	chr7	31811562-31829061 (17500 bp)	24.2

557	chr8	61748881-61753796 (4916 bp)	24.2
558	chr10	17972393-17992572 (20180 bp)	24.1
559	chr12	106702862-106722197 (19336 bp)	24
560	chr15	77787352-77806317 (18966 bp)	23.9
561	chr19	56177734-56208850 (31117 bp)	23.9
562	chr4	144843977-144864392 (20416 bp)	23.9
563	chr11	117170118-117195607 (25490 bp)	23.8
564	chr12	22752228-22780847 (28620 bp)	23.8
565	chr15	95834203-95858513 (24311 bp)	23.8
566	chr17	22517030-22534437 (17408 bp)	23.8
567	chr5	23703204-23746356 (43153 bp)	23.8
568	chr7	107812748-107836441 (23694 bp)	23.8
569	chr15	7490143-7498220 (8078 bp)	23.7
570	chr16	30254885-30276776 (21892 bp)	23.7
571	chr5	122624065-122659896 (35832 bp)	23.7
572	chr1	104698596-104715600 (17005 bp)	23.6
573	chr10	118525754-118549995 (24242 bp)	23.6
574	chr10	125681253-125718744 (37492 bp)	23.6
575	chr11	68775254-68793197 (17944 bp)	23.6
576	chr13	104179652-104192890 (13239 bp)	23.6
577	chr5	116183058-116211855 (28798 bp)	23.6
578	chr10	93069379-93081441 (12063 bp)	23.5
579	chr11	115883781-115903392 (19612 bp)	23.5
580	chr13	23830441-23864529 (34089 bp)	23.5
581	chr4	133366867-133391424 (24558 bp)	23.5
582	chr1	173397294-173452925 (55632 bp)	23.4
583	chr17	43014503-43048252 (33750 bp)	23.4
584	chr18	36035624-36058461 (22838 bp)	23.4
585	chr3	103173352-103202766 (29415 bp)	23.4
586	chr5	114477349-114502878 (25530 bp)	23.4
587	chr10	83935428-83957731 (22304 bp)	23.3
588	chr14	101136075-101161995 (25921 bp)	23.3
589	chr2	84548060-84575486 (27427 bp)	23.3
590	chr5	25379543-25402239 (22697 bp)	23.3
591	chr8	83537811-83542391 (4581 bp)	23.3

592	chr1	167090527-167141649 (51123 bp)	23.2
593	chr10	120566670-120594262 (27593 bp)	23.2
594	chr17	91944473-91976167 (31695 bp)	23.2
595	chr18	42633281-42654210 (20930 bp)	23.2
596	chr5	123497141-123527503 (30363 bp)	23.2
597	chr8	129467564-129489484 (21921 bp)	23.2
598	chrX	15040000-15069891 (29892 bp)	23.2
599	chr1	136422479-136445161 (22683 bp)	23.1
600	chr14	47407016-47431466 (24451 bp)	23.1
601	chr17	35421099-35443404 (22306 bp)	23.1
602	chr6	146464738-146490066 (25329 bp)	23.1
603	chr13	42073233-42089044 (15812 bp)	23
604	chr13	62914488-62931846 (17359 bp)	23
605	chr5	24243682-24286188 (42507 bp)	23
606	chr19	9535227-9575190 (39964 bp)	22.9
607	chr2	84343024-84363674 (20651 bp)	22.9
608	chr9	115049773-115082220 (32448 bp)	22.9
609	chr1	88968164-88981362 (13199 bp)	22.8
610	chr13	98615959-98635267 (19309 bp)	22.8
611	chr5	70937985-70961428 (23444 bp)	22.8
612	chr7	26584972-26605570 (20599 bp)	22.8
613	chr14	9562345-9596136 (33792 bp)	22.7
614	chr12	21965509-22007988 (42480 bp)	22.5
615	chr8	72051859-72090740 (38882 bp)	22.5
616	chr12	85286302-85304571 (18270 bp)	22.4
617	chr7	30660162-30684909 (24748 bp)	22.4
618	chr7	46905653-46916339 (10687 bp)	22.4
619	chr13	4562800-4572167 (9368 bp)	22.3
620	chr13	55268500-55306629 (38130 bp)	22.3
621	chr16	23431280-23447702 (16423 bp)	22.3
622	chr12	85427457-85450782 (23326 bp)	22.2
623	chr19	55694804-55718615 (23812 bp)	22.1
624	chr7	10900495-10922583 (22089 bp)	22.1
625	chr3	34542305-34570188 (27884 bp)	22
626	chr10	7679200-7698584 (19385 bp)	21.9

627	chr5	100464656-100486572 (21917 bp)	21.9
628	chr2	150960785-150983644 (22860 bp)	21.8
629	chr3	7656952-7684322 (27371 bp)	21.8
630	chr3	121262624-121282565 (19942 bp)	21.7
631	chr5	22844197-22886927 (42731 bp)	21.7
632	chr5	128189322-128231882 (42561 bp)	21.7
633	chr2	134401765-134428919 (27155 bp)	21.6
634	chr12	18692274-18718951 (26678 bp)	21.5
635	chr16	91238791-91270441 (31651 bp)	21.5
636	chr1	175486349-175518934 (32586 bp)	21.4
637	chr13	22397047-22406497 (9451 bp)	21.4
638	chr15	100655664-100672227 (16564 bp)	21.4
639	chr16	89691714-89712136 (20423 bp)	21.4
640	chr6	149212304-149257478 (45175 bp)	21.4
641	chr11	96776784-96797398 (20615 bp)	21.3
642	chr13	62172505-62207509 (35005 bp)	21.3
643	chr14	32924586-32936968 (12383 bp)	21.3
644	chrY	1817254-1877785 (60532 bp)	21.3
645	chr14	104013661-104037467 (23807 bp)	21.2
646	chr4	130550067-130580004 (29938 bp)	21.2
647	chr6	43863199-43876802 (13604 bp)	21.1
648	chr2	175093731-175116756 (23026 bp)	21
649	chr5	48224175-48240501 (16327 bp)	21
650	chr11	97639599-97667641 (28043 bp)	20.9
651	chr18	33953521-33970009 (16489 bp)	20.9
652	chr13	29345173-29361652 (16480 bp)	20.8
653	chr16	91078272-91103781 (25510 bp)	20.8
654	chr4	8599563-8617709 (18147 bp)	20.8
655	chr6	126192902-126213204 (20303 bp)	20.8
656	chr11	6425733-6450218 (24486 bp)	20.7
657	chr2	177595117-177616257 (21141 bp)	20.7
658	chr5	129264291-129294264 (29974 bp)	20.7
659	chr6	140246948-140274633 (27686 bp)	20.7
660	chr11	7741154-7788749 (47596 bp)	20.6
661	chr11	86619984-86648181 (28198 bp)	20.6

662	chr12	77315637-77344867 (29231 bp)	20.6
663	chr13	52068059-52089250 (21192 bp)	20.6
664	chr19	42854236-42866792 (12557 bp)	20.6
665	chr5	75493661-75512269 (18609 bp)	20.6
666	chr16	6676898-6703491 (26594 bp)	20.5
667	chr16	22926958-22950696 (23739 bp)	20.5
668	chr17	49538608-49545870 (7263 bp)	20.5
669	chr11	98799091-98824069 (24979 bp)	20.4
670	chr19	8943815-8965146 (21332 bp)	20.4
671	chr8	19853139-19871905 (18767 bp)	20.3
672	chr1	112210912-112234480 (23569 bp)	20.2
673	chr10	75649251-75666984 (17734 bp)	20.2
674	chr14	117128172-117136993 (8822 bp)	20.2
675	chr3	11546112-11554089 (7978 bp)	20.2
676	chr5	134641837-134666608 (24772 bp)	20.2
677	chr5	138079006-138111252 (32247 bp)	20.2
678	chr5	143041255-143069882 (28628 bp)	20.2
679	chr11	19933876-19951567 (17692 bp)	20.1
680	chr15	76718948-76742471 (23524 bp)	20.1
681	chr2	128687969-128701865 (13897 bp)	20.1
682	chr3	89576630-89597839 (21210 bp)	20.1
683	chr5	138636161-138670087 (33927 bp)	20.1
684	chr17	33178560-33199143 (20584 bp)	20
685	chr5	130617913-130637402 (19490 bp)	20
686	chrX	98437724-98469413 (31690 bp)	20
687	chr1	171945499-171953363 (7865 bp)	19.9
688	chr10	127521029-127540210 (19182 bp)	19.9
689	chr4	9634920-9664095 (29176 bp)	19.9
690	chr4	11353754-11377272 (23519 bp)	19.9
691	chr4	112022069-112049730 (27662 bp)	19.9
692	chr16	91974904-92006945 (32042 bp)	19.8
693	chr10	126712058-126733452 (21395 bp)	19.7
694	chr17	22265391-22276480 (11090 bp)	19.7
695	chr3	85758313-85780818 (22506 bp)	19.7
696	chr7	36808040-36813463 (5424 bp)	19.7

697	chr18	8293108-8320313 (27206 bp)	19.6
698	chr8	95975544-96009431 (33888 bp)	19.5
699	chr11	29008035-29031016 (22982 bp)	19.3
700	chr12	18076419-18110951 (34533 bp)	19.3
701	chr2	152002297-152023330 (21034 bp)	19.3
702	chr4	41941351-41978267 (36917 bp)	19.3
703	chr17	35607876-35634395 (26520 bp)	19.2
704	chr6	18059313-18091356 (32044 bp)	19.2
705	chr9	118254247-118277266 (23020 bp)	19.2
706	chr5	148941049-148964734 (23686 bp)	19.1
707	chr1	185839112-185854974 (15863 bp)	19
708	chr10	80387752-80444588 (56837 bp)	18.9
709	chr16	31576005-31595195 (19191 bp)	18.9
710	chr3	9833178-9849934 (16757 bp)	18.9
711	chr1	178498445-178517395 (18951 bp)	18.8
712	chr4	40540520-40607731 (67212 bp)	18.8
713	chr5	142195825-142203686 (7862 bp)	18.8
714	chr5	142704359-142728632 (24274 bp)	18.8
715	chr1	115506948-115525744 (18797 bp)	18.7
716	chr7	26639473-26657804 (18332 bp)	18.7
717	chr14	110226962-110240988 (14027 bp)	18.5
718	chr15	35868149-35887694 (19546 bp)	18.5
719	chr18	3031099-3064343 (33245 bp)	18.5
720	chr10	78862108-78914358 (52251 bp)	18.4
721	chr5	136714237-136736377 (22141 bp)	18.4
722	chr8	109198071-109212691 (14621 bp)	18.4
723	chr10	79739399-79758578 (19180 bp)	18.3
724	chr10	99060236-99075461 (15226 bp)	18.3
725	chr15	90944595-90955057 (10463 bp)	18.3
726	chr5	3236450-3257538 (21089 bp)	18.3
727	chr14	70136573-70157888 (21316 bp)	18.2
728	chr13	50440716-50459121 (18406 bp)	18.2
729	chr17	45695354-45714700 (19347 bp)	18.1
730	chrX	146909541-146933503 (23963 bp)	18.1
731	chr6	58097840-58133068 (35229 bp)	18

732	chrY	1764542-1789424 (24883 bp)	18
733	chr4	83166525-83187379 (20855 bp)	17.9
734	chr10	46256985-46270546 (13562 bp)	17.8
735	chr11	116638979-116662059 (23081 bp)	17.8
736	chrX	8290680-8363092 (72413 bp)	17.8
737	chr2	75461465-75499834 (38370 bp)	17.7
738	chr4	147207767-147244322 (36556 bp)	17.6
739	chr6	70631902-70651346 (19445 bp)	17.6
740	chr7	16575682-16599916 (24235 bp)	17.6
741	chr7	52225327-52246190 (20864 bp)	17.5
742	chr9	107943260-107966386 (23127 bp)	17.5
743	chr16	21974477-21996754 (22278 bp)	17.4
744	chr18	75159916-75180011 (20096 bp)	17.4
745	chr4	42587339-42620899 (33561 bp)	17.4
746	chr8	57943963-57966656 (22694 bp)	17.4
747	chr5	146076341-146097996 (21656 bp)	17.3
748	chr14	15066628-15085549 (18922 bp)	17.2
749	chr6	117578775-117591854 (13080 bp)	17.2
750	chr9	87171694-87197268 (25575 bp)	17.2
751	chr10	73582307-73601809 (19503 bp)	17.1
752	chr16	14323155-14344219 (21065 bp)	17.1
753	chr19	3220520-3243560 (23041 bp)	17.1
754	chr3	18736505-18755956 (19452 bp)	17.1
755	chr4	118570787-118591200 (20414 bp)	17.1
756	chr3	115609283-115633312 (24030 bp)	17
757	chr13	4337930-4356267 (18338 bp)	16.9
758	chr9	71265567-71282197 (16631 bp)	16.9
759	chr6	94981207-95020745 (39539 bp)	16.8
760	chr17	66353826-66369729 (15904 bp)	16.7
761	chr2	37196258-37218443 (22186 bp)	16.7
762	chr2	100951444-100973728 (22285 bp)	16.7
763	chr5	136220610-136249455 (28846 bp)	16.7
764	chr15	8856183-8878638 (22456 bp)	16.6
765	chr19	59677215-59686692 (9478 bp)	16.6
766	chr13	103606712-103624309 (17598 bp)	16.4

767	chr4	145127022-145181426 (54405 bp)	16.4
768	chr7	11344552-11376574 (32023 bp)	16.4
769	chr4	115367964-115394096 (26133 bp)	16.3
770	chr13	3605613-3625816 (20204 bp)	16.2
771	chr18	26150985-26173379 (22395 bp)	16.2
772	chr9	11206773-11224499 (17727 bp)	16.2
773	chr6	38307561-38331122 (23562 bp)	15.9
774	chr16	11670930-11687910 (16981 bp)	15.8
775	chr19	32511462-32530231 (18770 bp)	15.8
776	chr2	5468040-5486774 (18735 bp)	15.7
777	chr4	123386977-123422251 (35275 bp)	15.6
778	chr5	141555495-141584588 (29094 bp)	15.6
779	chr7	28802161-28822652 (20492 bp)	15.6
780	chr7	89373022-89391887 (18866 bp)	15.6
781	chr12	111133180-111155724 (22545 bp)	15.5
782	chr7	138267906-138282919 (15014 bp)	15.5
783	chr9	11803476-11830525 (27050 bp)	15.5
784	chr10	57565491-57578716 (13226 bp)	15.4
785	chr6	31578704-31595597 (16894 bp)	15.1
786	chr6	60828783-60861398 (32616 bp)	15.1
787	chr4	78141025-78163138 (22114 bp)	15
788	chr4	114940395-114970362 (29968 bp)	15
789	chr6	4124569-4141910 (17342 bp)	15
790	chr7	16973272-16993046 (19775 bp)	15
791	chr10	58843479-58854046 (10568 bp)	14.7
792	chr17	54020916-54043618 (22703 bp)	14.7
793	chr5	129720015-129757077 (37063 bp)	14.7
794	chr5	104359570-104379681 (20112 bp)	14.6
795	chr17	26403266-26422162 (18897 bp)	14.5
796	chr18	6656070-6680609 (24540 bp)	14.5
797	chrX	121393885-121399724 (5840 bp)	14.5
798	chr14	113685657-113705954 (20298 bp)	14.4
799	chr17	19030404-19075757 (45354 bp)	14.3
800	chr12	105386299-105397595 (11297 bp)	14.2
801	chr9	21314231-21329849 (15619 bp)	14.2

802	chr4	42457459-42498444 (40986 bp)	14
803	chr9	109320919-109328578 (7660 bp)	14
804	chr2	154175362-154197979 (22618 bp)	13.9
805	chr12	44758723-44779698 (20976 bp)	13.8
806	chr9	74186214-74190814 (4601 bp)	13.8
807	chr10	34347572-34367500 (19929 bp)	13.7
808	chr6	57231099-57256865 (25767 bp)	13.7
809	chrX	115682977-115697868 (14892 bp)	13.6
810	chr10	54244040-54262204 (18165 bp)	13.5
811	chr8	3336436-3351406 (14971 bp)	13.4
812	chr9	77443738-77460283 (16546 bp)	13.3
813	chr15	22672290-22686368 (14079 bp)	13.1
814	chr8	15739075-15765564 (26490 bp)	13
815	chr18	46471822-46494054 (22233 bp)	12.9
816	chr9	35881870-35903751 (21882 bp)	12.9
817	chr9	13067962-13088319 (20358 bp)	12.5
818	chr10	52368109-52386948 (18840 bp)	12.1
819	chr1	128835830-128840897 (5068 bp)	11.9
820	chr17	48411546-48428795 (17250 bp)	11.3
821	chr3	52917543-52939816 (22274 bp)	11.2

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Achievement

論文

1. Itou D, Shiromoto Y, Shin-ya Y, Ishii C, Nishimura T, Ogonuki N, Ogura A, Kuramochi-Miyagawa S, and Nakano T. Induction of DNA methylation by artificially produced piRNAs in male germ-cells. ***Current biology***, in press (2015).

2. Itou D, Kuramochi-Miyagawa S, and Nakano T. Distinct roles for MVH in embryonic piRNA production, in preparation (2015).

学会発表

1. Induction of DNA methylation by artificially produced piRNAs in male germ-cells.

   雄性生殖細胞における人為的 piRNA を介した DNA メチル化の誘導

   第 16 回　日本 RNA 学会年会、2014 年 7 月、愛知県名古屋市

   ○伊藤大介、城本悠助、宮川（倉持）さとみ、仲野徹

2. Induction of DNA methylation by artificially produced piRNAs in male germ-cells

   雄性生殖細胞における人為的 piRNA を介した DNA メチル化の誘導

   新学術領域研究「生殖細胞のエピゲノムダイナミクスとその制御」

2014 年度　若手勉強会、2014 年 7 月、茨城県つくば市

〇伊藤大介、城本悠助、宮川（倉持）さとみ、仲野徹

3. MVH helicase activity is required for DNA methylation of LINE-1 retrotransposon

MVH ヘリカーゼ活性は LINE-1 レトロトランスポゾンの DNA メチル化に必須である　第 36 回　日本分子生物学会年会　2013 年 12 月、兵庫県神戸市

〇伊藤大介、宮川（倉持）さとみ、仲野徹

4. De novo DNA methylation by artificially produced piRNA in murine testes

CSHL meeting, Epigenetics and Chromatin, September 2012, U.S.A New York

〇Daisuke Itou, Yusuke Shiromoto, Satomi Kuramochi-Miyagawa, Toru Nakano

5. Induction of sequence-specific DNA methylation by RNA interference in mice testis

第 34 回日本分子生物学会　2011 年 12 月、神奈川県横浜市

〇Daisuke Itou, Satomi K-Miyagawa, Yusuke Shiromoto, Toru Nakano

6. MVH in piRNA biogenesis

International symposium on Epigenome Network, Development and

Reprogramming of Germ Cells, November 2010, Fukuoka, Japan

〇Daisuke Itou, Satomi K-Miyagawa, Toru Nakano

受賞

1. 第 16 回　日本 RNA 学会年会　優秀発表賞
2. 新学術領域研究「生殖細胞のエピゲノムダイナミクスとその制御」

   2014 年度若手研究会　ベストプレゼン賞

研究費獲得状況

2013 年 4 月～　日本学術振興会　特別研究員 DC2