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Supporting Online Material for

Role for piRNAs and Noncoding RNA in de Novo DNA Methylation of the Imprinted Mouse *Rasgrf1* Locus

Toshiaki Watanabe,* Shin-ichi Tomizawa, Kohzoh Mitsuya, Yasushi Totoki, Yasuhiro Yamamoto, Satomi Kuramochi-Miyagawa, Naoko Iida, Yuko Hoki, Patrick J. Murphy, Atsushi Toyoda, Kengo Gotoh, Hitoshi Hiura, Takahiro Arima, Asao Fujiyama, Takashi Sado, Tatsuhiro Shibata, Toru Nakano, Haifan Lin, Kenji Ichiyanagi, Paul D. Soloway, Hiroyuki Sasaki*

*To whom correspondence should be addressed. E-mail: toshwatatoshiakiwatanabe@gmail.com (T.W.); hsasaki@bioreg.kyushu-u.ac.jp (H.S.)

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Supporting Online Material

Materials and Methods

Isolation of spermatogonia

Spermatogonia were isolated from postnatal testes at 6-11 days post partum (dpp) of 3-8 individuals by fluorescence activated cell sorting (FACS) using Ep-CAM immunofluorescence (S*1*) or using fluorescence from a Oct-4/EGFP transgene (S*2*). Postnatal testes were treated with 0.25% trypsin/1 mM EDTA for 10 min, and testicular cells were suspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). After filtration using a cell strainer (40 μm, BD Falcon), testicular cells were collected by centrifuge. For Ep-CAM immunofluorescence, testicular cells were incubated with rat monoclonal anti-Ep-CAM antibody (G8.8, Santa Cruz), and then with Alexa Fluor 488 anti-rat IgG (Invitrogen). Fluorescence-positive cells were collected by FACS.

Isolation of fetal germ cells and somatic cells

Testes were collected from C57BL/6J fetuses at 16.5 dpc and were treated with 0.25% trypsin /1 mM EDTA. The cells were suspended in DMEM supplemented with 10% FCS. After filtration using a cell strainer (40 μm, BD Falcon), cells were cultured on a tissue culture dish for 3 hr. Attached cells (somatic cells) and floating cells (germ cells or prospermatogonia) were respectively collected.

Bisulfite Sequencing

Bisulfite treatment of DNA was performed as described previously (S*3*). In brief, DNA

was denatured with NaOH and then treated with 9 M sodium bisulfite from BisulFAST kit (TOYOBO). DNA was collected using a microcolumn, desulphonated and eluted using EZ DNA methylation kit (Zymo Research). The primer sequences used are listed below. The amount of DNA used for PCR was 0.65 ng for oocytes and embryonic male germ cells, 1.2 ng for blastocysts, and 7.5-25 ng for other tissues and cells.

Small RNA library preparation

To prepare small RNA libraries, 5-10 testes were collected from wild-type and mutant fetuses at 16.5 dpc and total RNA was extracted using ISOGEN (Nippon Gene). Because all mutant mice had a similar genetic background (largely C57BL/6J, with some genomic portions from 129), we used *MitoPLD*+/+ as a wild-type control. Digital Gene Expression for Small RNA Sample Prep Kit (Illumina) was used for library construction, and the clones were sequenced using Genome Analyzer II (Illumina).

Analysis of small RNAs

After removing the linker sequences, 20-33-nt small RNA sequences were extracted and mapped to the genome (mm9) using SeqMap (S*4*), and only perfectly matched sequences were extracted. Presumptive degradation products from abundant RNAs (rRNA, tRNA, snoRNA, snRNA, snoRNA, scRNA and srpRNA) were removed as described in (S*5*), and the remaining reads were grouped and counted according to their length (length distribution analysis, Fig. 2A). To compare small RNA levels between the samples, small RNA counts were normalized by using total miRNA counts as a reference. To identify small RNAs mapping to the *Rasgrf1* DMR (mm9 chr9: 89769659-89779158) (Fig. 3A,B), all small RNA sequences were examined allowing up

to two nucleotide differences (where the number of indel should not exceed one). Small RNA hits were counted for every 100-nt window and normalized by the miRNA counts. To know the exact origin of small RNAs that had a sequence homology to the *Rasgrf1* RMER4B copy, they were again mapped to the genome with a perfect match criterion (table S3). The numbers of the small RNAs mapping to the *Rasgrf1* RMER4B copy, with or without mismatches, in wild-type and mutants are summarized in table S1. For identification of ping-pong sites in other RMER4B copies, unique sequence small RNAs mapping to any RMER4B copies were first identified, and all small RNAs were examined for homology to these RMER4B copies allowing two mismatches including indels. If the small RNAs identified in the second screen were on the opposite strand of the unique small RNA identified in the first screen, and if their first ten nucleotides were complementary each other, they were regarded as a ping-pong pair (fig. S6).

RT-PCR, 5'RACE and modified 5'RACE

Total RNA was isolated from various tissues and cells using ISOGEN (Nippon Gene). Otherwise noted, C57BL/6J mice were used to prepare tissues and cells. For RT-PCR and qPCR, total RNA of 100 ng – 1 μg was treated with DNase I and then reverse transcribed. The mRNA level of *Oct4* or *beta-actin* was used for normalization. The 5' end of pit-RNA was determined by 5'RACE using GeneRacer (Invitrogen) according to the manufacture's instruction. Total RNA of 5 μg from 16.5-dpc fetal testes was used. For modified 5'RACE, total RNA of 5 μg from 16.5-dpc fetal testes was directly ligated to the linker RNA supplied in GeneRacer. After reverse transcription, PCR was performed using a gene-specific primer and a linker primer. The reaction conditions were as described in the manufacture's instruction of GeneRacer, except that the calf

intestine alkaline phosphatase treatment and tobacco acid pyrophosphatase treatment were omitted. The sequences of the primers used are listed below.

Supplementary Text

Characterization of pit-RNA at the *Rasgrf1* DMR

To examine the spacial and temporal regulation of pit-RNA, we performed qPCR on total RNAs prepared from several tissues (fig. S5A). We did not detect pit-RNA in five 3-dpp somatic tissues that we examined. During testis development, pit-RNA was expressed at 12.5 dpc and 16.5 dpc but undetectable at 2 dpp or adult stage. DNA methylation of the *Rasgrf1* DMR during testis development was previously reported (S6): it is very low (5-10 %) in primordial germ cells at 12.5 dpc, and *de novo* methylation proceeds in prospermatogonia to an almost full methylation (80-100 %) state by 18.5 dpc. The expression pattern of pit-RNA is consistent with the mode of *de novo* methylation of the *Rasgrf1* DMR.

The pit-RNA transcript had a single transcription start site within a direct repeat essential for *Rasgrf1* imprinting (S7) (Fig. 4D). A series of strand-specific RT-PCR assays revealed that pit-RNA is transcribed until at least 2-kbp downstream of the direct repeat (fig. S5B). We were not able to determine the exact 3' end of pit-RNA due to the repeat-rich nature of the region. An allelic expression analysis using a polymorphism between C57BL6/J and JF1 strains revealed that pit-RNA expression is not imprinted in 16.5-dpc testes (fig. S5C). The biallelic expression of pit-RNA is consistent with its *cis*-acting role in *de novo* methylation since both alleles of the *Rasgrf1* DMR should be methylated in the male germline for this gene to be imprinted. We also examined the imprinting status of the chr7 piRNA cluster (a presumptive precursor for the primary piRNAs), but its expression was biallelic in 16.5-dpc testes (fig. S5D).

Do other copies of RMER4B also require the piRNA pathway for methylation?

To know whether the PIWI-piRNA-dependent methylation is common to all RMER4B copies, we first searched for copies that are potentially targeted by piRNAs other than the one in the *Rasgrf1* DMR. By applying the method that we used to find the ping-pong pairs in the *Rasgrf1* DMR, we identified two other copies that have a ping-pong signature sites (fig. S6). These sites were potentially targeted by the piRNAs from the chr7 piRNA cluster with some mismatches.

We then examined the methylation status of the RMER4B copies homologous to the *Rasgrf1* RMER4B copy, including one of the two having a ping-pong signature, in *MitoPLD*-/- spermatogonia. In all seven RMER4B copies that we examined, methylation was fully established in the mutant spermatogonia (fig. S7), suggesting that PIWI-piRNA is not essential. The results suggest that the copy in the *Rasgrf1* DMR is unique among the RMER4B copies in its requirement of the piRNA pathway for methylation.

Are other retrotransposons differentially methylated in somatic tissues?

Our study shows that the piRNA pathway is involved in DNA methylation of the *Rasgrf1* DMR in the male germline, which is inherited by the offspring as the paternal-specific methylation. Previous studies showed that L1Md and some IAP retrotransposons become methylated in the male germline in a PIWI-piRNA-dependent manner (S*8, S9*). By contrast, the bulk of L1 retrotransposons stays hypomethylated in oocytes (S*10*). Then, are these retrotransposons differentially methylated in the offspring? A number of studies showed that these retrotransposons are generally highly methylated in somatic cells. We examined the methylation status of individual L1Md

and IAP copies in adult liver genomic DNA and found that they are almost completely methylated (fig. S8). Furthermore, we examined the allelic methylation status of two IAP and seven RMER4B copies, including the one in the chr7 piRNA cluster, using strain-specific polymorphisms. Differential methylation was not observed at any of these copies in adult liver DNA (fig. S9A,B). These results together indicate that most copies of these retrotransposons are not differentially methylated in the offspring.

The *Rasgrf1* RMER4B copy is resistant to the epigenetic reprogramming in early development

The above studies showed that the RMER4B copy at the *Rasgrf1* DMR is unique among the RMER4B copies in that it is methylated in a parental-origin-specific manner in somatic cells. It was shown that, during early development, the bulk of L1 and IAP retrotransposons are substantially demethylated, and then become remethylated (S*10, S11*). Do RMER4B retrotransposons undergo similar changes during development? If so, does the *Rasgrf1* RMER4B copy follow that fate, or escape from these changes?

We designed a primer pair that amplifies the bulk of RMER4B copies (and some RMER4A copies, see legend for fig. S10), and analyzed the methylation changes during development by bisulfite sequencing (fig. S10). The RMER4B retrotransposons were highly methylated in spermatogonia and sperm (89.5% and 86.6% methylation, respectively), whereas they were hypomethylated in oocytes (25.3%). At the blastocyst stage, the bulk of RMER4B retrotransposons showed only 17.0% methylation, suggesting that the paternal RMER4B copies are demethylated during preimplantation development. This is in contrast to what is observed for the RMER4B copy in the *Rasgrf1* DMR, which is protected from this demethylation (S3). In 12.5-dpc whole

embryos, the bulk RMER4B was highly methylated (76.2%) and stayed at a similar level in adult tissues such as the liver (86.4%). This high level of methylation was observed on both parental alleles (fig. S9). Thus most RMER4B copies are subject to the global *de novo* methylation occuring in early postimplantation development. Again, the *Rasgrf1* RMER4B copy is unique in that its maternal allele is protected from this strong wave of methylation.

Together, these results indicate that not only the piRNA-dependent *de novo* methylation during male germ cell development, but also the resistance to the epigenetic reprogramming during early development, is critical for the differential methylation and imprinting of *Rasgrf1*. It is noteworthy that the postfertilization processes may be also regulated by the direct repeat (S*7, S12*).

Supplementary Figure Legend

fig. S1 DNA methylation status of the *H19* and *Dlk1-Gtl2* DMRs in *Mili-/-* and *Miwi2-/-* spermatogonia. DNA was isolated from spermatogonia from 9-11-dpp testes and analyzed by bisulfite sequencing. The portion of the *H19* DMR examined here is located between regions E and F in fig. S2 (top). The portion of the *Dlk1-Gtl2* DMR examined is located between regions B and C in fig. S2 (middle). *Mili+/-* spermatogonia and 12.5-dpc whole embryos are the controls.

fig. S2 DNA methylation status of the *H19*, *Dlk1-Gtl2* and *Gpr1-Zdbf2* DMRs in *MitoPLD-/-* spermatogonia. Colored bars indicate the regions differentially methylated between oocytes and sperm (gametic DMRs) (S*13*). The green bars marked as gametic DMR contain regions critical for imprinting. DNA from 6-8-dpp spermatogonia was analyzed by bisulfite sequencing. Repeat sequences identified by RepeatMasker are shown by colored boxes: green, LINE; purple, SINE; white, simple repeat; gray, LTR; light gray, DNA transposon. CpG sites are indicated by short vertical bars. The results obtained with wild-type spermatogonia and 12.5-dpc whole embryos are also shown.

fig. S3 The Chr7 piRNA cluster generates RMER4B piRNAs. (A) LTR, LINE and SINE retrotransposons identified by RepeatMasker are shown at the top. Those on the plus strand are shown by blue boxes and those on the minus strand are shown by red boxes. Small RNAs uniquely mapping to this cluster are counted in 100-nt windows and the numbers are shown in the graph. An enlarged view of the region containing the RMER4B copy is shown at the bottom. (B) Alignment of the sequences of the regions around sites 1 and 2 (see Fig. 3B) of the RMER4B copy from the *Rasgrf1* DMR and the

corresponding region of the copy from the chr7 piRNA cluster. The red horizontal bar indicates the sequence of the most abundant chr7-derived piRNA that targets pit-RNA.

fig. S4 A model for piRNA-directed methylation of the *Rasgrf1* DMR. The piRNAs generated from the chr7 piRNA cluster are bound by MILI and/or MIWI2. The MILI- and/or MIWI2-piRNA complexes then target the nascent pit-RNA. DNMT3a and DNMT3b respectively form a *de novo* DNA methyltransferase complex with DNMT3L, which is responsible for methylation of the *Rasgrf1* DMR (S*6, S14*). The complexes are recruited to the DMR by a yet unknown mechanism.

fig. S5 Characterization of pit-RNA at the *Rasgrf1* DMR. (A) Fetal testis-specific expression of pit-RNA. qPCR was performed with total RNAs from indicated tissues. Somatic tissues were from 3-dpp male mice. The expression level of pit-RNA was normalized using that of beta-actin. (B) Structure of pit-RNA. A series of strand-specific RT-PCR was done with total RNA from 16.5-dpc testes. The results shown at the bottom are for pit-RNA and those at the top are for RNA of the opposite strand. AK029869 is a non-coding EST from adult testes, which was also detected in the fetal testes. (C) Biallelic expression of pit-RNA. The sequences of the pit-RNA transcripts from three 16.5-dpc fetuses from C57BL/6J (B6) females crossed with JF1 males were determined. Polymorphisms at chr9: 89774398 and 89774368 (mm9) were used to distinguish between the B6- and JF1-derived pit-RNA sequences. Numbers of the RT-PCR products from the respective alleles are shown. (D) Allelic expression status of the RNA in the chr7 piRNA cluster. Sequence traces of the RT-PCR products generated from 16.5-dpc testis RNA with primers flanking the polymorphism rs46357180 at chr7:

6,552,735 (highlighted in yellow) (mm9). Control samples were from inbred FVB/n mice (FVB) [A], B6 mice [B] or a 1:1 mixture of the two inbred samples to simulate a scenario where both alleles were equally expressed [C]. The F1 test progeny were from B6 mothers and FVB fathers [D], or from the reciprocal cross [E].

fig. S6 Other RMER4B copies showing a signature of the ping-pong cycle. piRNA sequences are represented by bars and are colored according to the number of reads obtained. Plus strand hits are shown above the sequence and minus strand hits below it. Sequence mismatches are indicated by black portions of the bars. Unique hit piRNAs are indicated. Ten-nt overlaps characteristic of a ping-pong pair are also indicated.

fig. S7 DNA methylation status of seven RMER4B copies in *MitoPLD*-/- spermatogonia. RMER4B copies that showed a high sequence similarity to the *Rasgrf1* RMER4B copy or to the RMER4B piRNAs were selected and examined. The genomic positions of the copies are indicated at the top. The RMER4B copy in the chr7 piRNA cluster was included in the analysis (third from the right). One of the two RMER4B copies predicted to be targeted by piRNAs (fig. S6) was also included (fourth from the left).

fig. S8 DNA methylation status of individual L1 and IAP retrotransposon copies in adult liver DNA. The genomic positions of the copies are indicated.

fig. S9 DNA methylation status of seven RMER4B copies (A) and two IAP copies (B) in adult liver DNA. The maternal and paternal alleles are distinguished using polymorphisms identified between C57BL/6J and JF1 mice. The genomic positions of

the copies are indicated. The RMER4B copy in the chr7 piRNA cluster was included in the analysis (third from the right in (A)). One of the two RMER4B copies predicted to be targeted in fig. S6 was also included (fourth from the left).

fig. S10 Developmental changes in DNA methylation of RMER4 copies. A primer pair that detected bisulfite-converted products from the bulk of RMER4 (comprising both RMER4A and RMER4B) LTR sequences was used. With this primer pair, RMER4B was more frequently amplified than RMER4A (For example, when adult liver DNA was analyzed, 8 clones were from RMER4A and 78 clones were from RMER4B). To identify the original copy of individual PCR amplicons, the sequences were BLASTed against an in-house database containing all genomic RMER4 sequences where non-CG cytosines were converted to thymine. Then, only CpG sites in the identified RMER4B copies were analyzed (Those in the RMER4A copies were excluded). The percentage of methylated CpG in each stage is shown at the top. Numbers of the CpGs, methylated CpGs, and clones analyzed are shown in a table at the bottom, together with the percentage of methylated CpG.

Primer Sequences

Bisulfite Sequencing

(Fig. 1a *Mili*)

Rasgrf1 bisF1	GAGAGTATGTAAAGTTAGAGTTGTGTTG
Rasgrf1 bisF2	TAAAGATAGTTTAGATATGGAATTTTGGG
Rasgrf1 bisR	ATAATACAACAACAACAATAACAATC

(Fig. 1a *Miwi2* and *MitoPLD*)

Rasgrf1 F K	GGAATTTTGGGGATTTTTTAGAGAGTTTATAAAGT
Rasgrf1 R K	CAAAAACAACAATAATAACAAAAACAAAAACAATAT

(Fig. S1)

H19 bisF1	GAGTATTTAGGAGGTATAAGAATT
H19 bisR1	ATCAAAAACTAACATAAACCCCT
H19 bisF2	GTAAGGAGATTATGTTTATTTTTGG
H19 bisR2	CCTCATTAATCCCATAACTAT
Dlk1-Gtl2 bisF1	GTGTTAAGGTATATTATGTTAGTGTTAGG
Dlk1-Gtl2 bisF2	ATATTATGTTAGTGTTAGGAAGGATTGTG
Dlk1-Gtl2 bisR	TACAACCCTTCCCTCACTCCAAAAATT

(Fig. 1b)

Rasgrf1 F A	GAGGTAAATTTTTTTGAGGGGAAGTT
Rasgrf1 R A	ACTCCACATAAAAAACATAACTTCCCAC
Rasgrf1 F B	GTATGTTATTGTGTTTTTAAGGTTGTTTTTTAAA
Rasgrf1 R B	CTAAAAAAACCTAACTCATACAAACAAAAAAAAC
Rasgrf1 F C	TGGTTTTTAGATTAATGATTTTTTAATTGATGATAATTGGGT
Rasgrf1 R C	ATTCTATCCTTTAAAATAACCAATAAAAACACAATACACT

Rasgrf1 F D	AGTGTATTGTGTTTTTATTGGTTATTTTAAAGGATAGAAT
Rasgrf1 R D	AAACCATCACAAAAAACCACACAACTC
Rasgrf1 F E	GAGTTGTGTGGTTTTTTGTGATGGTTT
Rasgrf1 R E	AAATCCTAAATTTCTAATAAAAAACCAATACCCA
Rasgrf1 F F	GTATGTTTTTTTTGAAGTAATGATTATTTTTGTTG
Rasgrf1 R F	TCCCTAATAACCAAAAAAAAACATTTTAAATCCCC
Rasgrf1 F G	GGGATTTAAAATGTTTTTTTTTGGTTATTAGGGAT
Rasgrf1 R G	CTCAAAACAACATAAACCTAACATAATAACAAAAT
Rasgrf1 F H	GTTATTATGTTAGGTTTATGTTGTTTTGAGTTAATTATTTTA
Rasgrf1 R H	ACATTCTCAACAAAAACAATAACCTACCTA
Rasgrf1 F I	TGTTTTTGTTGAGAATGTATGTGTTTAAGAGAGT
Rasgrf1 R I	ACCCAACTCTACATAAACCAAACAAAATAATAATACC
Rasgrf1 F J	GGTATTATTTTGTTTGGTTTATGTAGAGTTGGGT
Rasgrf1 R J	AACTTTATAAACTCTCTAAAAAATCCCCAAAATTCC
Rasgrf1 F K	GGAATTTTGGGGATTTTTTAGAGAGTTTATAAAGT
Rasgrf1 R K	CAAAAACAACAATAATAACAAAAACAAAAACAATAT
Rasgrf1 F L	GTTATTGTTGTTTTTTTTTTTTATTGTTTTGTTTTAGTTGTTA
Rasgrf1 R L	CAAAAACAATAAAAAAATAAACAATAAAAAAACAAAAACAATAC
Rasgrf1 F M	TTGTTGTTTTTGTTTTATATTGTTTTTGTTTTAGTTGTTA
Rasgrf1 R M	CAAATCTAATCTTCCTCATCAAAATATCTCCAACTA
Rasgrf1 F N	TAGTTGGAGATATTTTGATGAGGAAGATAGATTTG
Rasgrf1 R N	AACCATCCTAATTAACAAAACAAAACCC
Rasgrf1 F O	TTTTTGTTTGTTTTTAGTATGTTAAGATTGTAAATGTGAATT

Rasgrf1 R O	TTCCCAACCACATATAAATTCACATACCTT
Rasgrf1 F P	AAATTTGAATTTGGAATATTTATTAGTTTGGGGGA
Rasgrf1 R P	ACCCTAAAAACAAAACAAATCAAAAACTCTCT
Rasgrf1 F Q	TTGTTTTTAGGGTTTTTTGTAAGTTTATTGGA
Rasgrf1 R Q	ATAACCCCACATTTATTCAAATACAAAAACC
Rasgrf1 F R	TGGGAGGAAGGATTGTGTATATATGGAT
Rasgrf1 R R	ACTTCCAAAACACTCTCTCTACTTTCTCTA
(Fig. S2)	
H19 F A	TGAATATTTTATGATGGAATTGTTTTTAGATTAGG
H19 R A	CCTTCCTTACTTAAAAAACTCAAAACACC
H19 F B	AGTGGGTGGATAATTAGGTGGTTGA
H19 R B	ACAAACACACCATTCCCATAAAACACTAC
H19 F C	GAATGGTGTGTTTGTAGGTTGGAT
H19 R C	AACAAATAAATCTCCTTCTTCTCTCTTAACCTC
H19 F D	GTTATTGTTGTGGGTGGATTTTAAGTTATGATATTG
H19 R D	ACCACACCAATAAATAACTTACATATAATAACCAACC
H19 F E	AATGGTTGAATTTTAGTTTTTGTTTTTATGGTT
H19 R E	ACCAATACAATCCCACATACTTTATCATAAAA
H19 F F	GAATTTAGATAAGTGGTGGAGAAAGAGTTTTGT
H19 R F	CATATCCATATCCCAACTATATCACTCTTTTATAA
H19 F G	GATGGGATATATAGATAGGATATATAGATGGGAT
H19 R G	CATTATCCCCTCCATATAACACCTAC
H19 F H	ATGGGTATAGAGAGAAAAGAGGGAGT
H19 R H	CTTTTACAACCAAAACAAAAACAATAATAAAAC

IG-DMR F A	AGGTTAAGATTTAATGATAGAAGGTTAGGTT
IG-DMR R A	TCTCCATTCTCTCCAACCAATAAAAAC
IG-DMR F B	ATATGGATGTATTGTAATATAGGTTAGGTGTT
IG-DMR R B	AATACACCATAACATAAACATAAAAATCCACAA
IG-DMR F C	TTATTATTTTGTGGTTATTTTTATTTAGAGAAAGTTTTATTGTTGT
IG-DMR R C	TCCTTAAAACCCTCAAAAAACCAAATTATAATCTC
IG-DMR F D	AGGAAGGGTAGTAGGAATTTGTT
IG-DMR R D	ATCCAACCCAACCAAATCCATAAAC
IG-DMR F E	TGGAGGGTTAAGTGGGTTAGTTTTAAG
IG-DMR R E	CCCTAAATCAACTATAAAAATATATATACCAACAA
Gpr1-Zdbf2 F A	TTTGTTTGGTATGTGTAGAAGATTT
Gpr1-Zdbf2 R A	ACAAATCATACTATCTACAAAAAAAC
Gpr1-Zdbf2 F B	GATTTAGATTTAGTTGGTTAGTTTTATAT
Gpr1-Zdbf2 R B	CAAACTTAACTACAAATACCTTTATTACC
Gpr1-Zdbf2 F C	GAAGTAAGGTAAGTTGTATATAGG
Gpr1-Zdbf2 R C	AAATCAAACTACAAACTCCAAACTA
Gpr1-Zdbf2 F D	TAGATGAGAGTTAAGGTATAGTAAATTA
Gpr1-Zdbf2 R D	ATAAACTCTCCAAAACCAAAAAAAA
(Fig. S7 and S9)	
RMER4B chr2:71016632 F	TTGTAAATAGGAGTAAGATGAAAATGTAGA
RMER4B chr2:71016632 R	AAAAACCACTCCTCCAACTAAAAAC
RMER4B chr15:89892349 F	TGGGATAAGTTTGATTTTTTTTGTTAG
RMER4B chr15:89892349 R	AAAAACCATCTATATTTTATAAATCACC
RMER4B chr16: 23130024 F	GTTAGTTTTTTGTGGGTTTTTTATA

RMER4B chr16: 23130024 R AAACTAATCTCATACAATAACTTTATTCAA

RMER4B chr2:129208392 F GTTGTGGGGTTGTTTTAGATTTGTT

RMER4B chr2:129208392 R AAAACCATCTACTCATTCCTCCATTC

RMER4B chr7:6568152 F TAATTTGTTTTATGTTTTAGTAGGGGTATG

RMER4B chr7:6568152 R AATTCRAAATCTTTTACCTACACAATCC

RMER4B chr13:114120078 F GTTTATTAGGGGTGTTAAGTTTTTTGG

RMER4B chr13:114120078 R CAAATAACAAAACTTAAAAACTTTAACCAC

RMER4B chr7:128329236 F GAAATTAGTATTTAGTAATTATTAAGTAGT

RMER4B chr7:128329236 R TACTTCAAATTATCCACAACAACTA

(Fig. S8)

IAPEY_LTR chr3:133,335,819 F AGAATGAAGAGAAGTTATTTGATGTTTATA

IAPEY_LTR chr3:133,335,819 R TCATACCACTATATTAAATTATATCCCAAT

IAPEY2_LTR chr7:63,839,069 F ATTTTTAAAATGTTTAAAAATGTAAAGTGT

IAPEY2_LTR chr7:63,839,069 R ATCAAAAATCTTATCCCCTAAAAAC

IAPEY_LTR chr7:135,704,525 F TGATTGGTGTTTGGGTATTAATTAAA

IAPEY_LTR chr7:135,704,525 R CCCAATCCCTAACACTACACAATAC

IAPEY3_LTR chr1:184,030,968 F TGATTTAGGTTGTAGTAGTATGGGTT

IAPEY3_LTR chr1:184,030,968 R CTTTATATCAATAAATTACCTAATCCAATC

IAPLTR2_Mm chr1:121,924,402 F TTTTGAAGATGTAAGTAATAAAGTTTTGT

IAPLTR2_Mm chr1:121,924,402 R ACTCTATATAACCAAAACTCAAAAATCC

IAPLTR2b chr2:26,064,096 F GTTTGGGAGTTTTAGTGAGATTTTG

IAPLTR2b chr2:26,064,096 R CCAAACTTAACAACATACCCCTTTA

IAPLTR1_Mm chr4:154,520,338 F TATTTTTTTATAAAAGAAGATGGGGATATA

IAPLTR1_Mm chr4:154,520,338 R AAAAATTTAACCTCTATCTCCACTCTC

IAPLTR1_Mm chr4:154,527,175 F	GTTTTGGAATGAGGGATTTTTTT
IAPLTR1_Mm chr4:154,527,175 R	CATATAACTTCTTATTTTTCCCCCAA
L1Md_A chr15:64632110 F	GTGTTGGGTAAGATTTTGTTGGTA
L1Md_A chr15:64632110 R	CACAAAACTCTAAAAACTCATAAAAAAAA
L1Md_Gf chr8:89457528 F	GGTTTTTGTTTATTTTAAATAAAGGTT
L1Md_Gf chr8:89457528 R	ACCTCAAAAACTCTATAACTCTTACC
(Fig. S9)	
IAPEY3_LTR chr1:184124503 F	TGATTTAGGTTGTAGTAGTATGGGTT
IAPEY3_LTR chr1:184124503 R	CTTTATATCAATAAATTACCTAATCCAATC
IAPLTR2b chr2:26097649 F	GTTTGGGAGTTTTAGTGAGATTTTG
IAPLTR2b chr2:26097649 R	CCAAACTTAACAACATACCCCTTTA
(Fig. S10)	
RMER4Bbulk F	GGTTGTTTTAGATTGTTTATAGTAGTTG
RMER4Bbulk R	CAAAATATCTAATTTCTTCTTATTATTTCT

Expression analysis of pit-RNA (Fig. 4A)

Rasgrf1 DMR RNA F	ATACGGGCAACCTTGGGATCATAGGCA
Rasgrf1 DMR RNA R	CAAAATTCTTACTACACATGGCACA
Oct4 F	GGAGAAGGTGGAACCAACTC
Oct4 R	AGATGGTGGTCTGGCTGAAC
GAPDH F	ATGACATCAAGAAGGTGGTG
GAPDH R	CATACCAGGAAATGAGCTTG

Modified RACE analysis of pit-RNA (Fig. 4B)

Rasgrf1 Modified RACE 1stPCR	GTCCTCTGACTTGAATAATATGCCCGTT
Rasgrf1 Modified RACE 2ndPCR	TACCTTGCCTGGTTTATGTAGAGCTG

5’RACE analysis of pit-RNA (Fig. 4C)

Rasgrf1 5’RACE 1stPCR	TGCAGAATATGGGGCTGCTTCACATTG
Rasgrf1 5’RACE 2ndPCR	AATTTTTCTCCATGCTCTAGCAGGCATGTG

q-PCR analysis of pit-RNA (Fig. 4D)

Rasgrf1 DMR RNA F	ATACGGGCAACCTTGGGATCATAGGCA
Rasgrf1 DMR RNA R2	GGAGGCGTTTATCTGAATAGTCGCAT
Oct4 f	GGAGAAGGTGGAACCAACTC
Oct4 r	AGATGGTGGTCTGGCTGAAC

Expression analysis of pit-RNA (fig. S5A)

pit-RNA qPCR F	AGCTGGGCTTACAAACTTCG
pit-RNA qPCR R	GCAAAAAGGAGGCGTTTATC
beta-actin qPCR F	ACAGCTTCTTTGCAGCTCCT
beta-actin qPCR R	ATTCCCACCATCACACCCTG

Structure of pit-RNA (fig. S5B)

Rasgrf1-1-5	CCGGACTTCCCTGTTGTAAT
Rasgrf1-1-3	AGCTTGTGCTCACAGGTTAGA
Rasgrf1-2-5	CACGGGCTTTTCTCTGTTTT
Rasgrf1-2-3	GGCCTGGCATAGTAGCAGAA
Rasgrf1-3-5	CTGCTGCTCAATCGTCTCTCTTCTG
Rasgrf1-3-3	CAACAGAAGCTGAAGGAGACG
Rasgrf1-4-5	GGAAGCTGGACTTCTGAGTGCTATG
Rasgrf1-4-3	ATACCTGGTAAACAACCTGACTGAA
Rasgrf1-5-5	TGCCTTTCTGTGCCTAGCTT
Rasgrf1-5-3	GTTTAAACGCACAGGGAGGT

Rasgrf1-6-5	AAACAAAGTGCCTGGTTCTCA
Rasgrf1-6-3	GGGATGGAGAAATGGGAAAT

Cloning of pit-RNA (fig. S5C)

Rasgrf1-J F	ATATGGGGCTGCTTCACATT
Rasgrf1-J R	AGCACAGCTCTGGCTTTACA

Expression of chr7 piRNA precursor (fig. S5D)

Chr7-piRNA F	TGTTAACAGTTGAGGTATTTATTTTTG
Chr7-piRNA R	TAGTCACCTTAATGGGAGCAAAATC

References

S1. R. Anderson, K. Schaible, J. Heasman, C. Wylie, *J Reprod Fertil* **116**, 379 (Jul, 1999).

S2. T. Yoshimizu *et al.*, *Dev Growth Differ* **41**, 675 (Dec, 1999).

S3. R. Hirasawa *et al.*, *Genes Dev* **22**, 1607 (Jun 15, 2008).

S4. H. Jiang, W. H. Wong, *Bioinformatics* **24**, 2395 (Oct 15, 2008).

S5. T. Watanabe *et al.*, *Nature* **453**, 539 (May 22, 2008).

S6. Y. Kato *et al.*, *Hum Mol Genet* **16**, 2272 (Oct 1, 2007).

S7. B. J. Yoon *et al.*, *Nat Genet* **30**, 92 (Jan, 2002).

S8. S. Kuramochi-Miyagawa *et al.*, *Genes Dev* **22**, 908 (Apr 1, 2008).

S9. A. A. Aravin *et al.*, *Mol Cell* **31**, 785 (Sep 26, 2008).

S10. N. Lane *et al.*, *Genesis* **35**, 88 (Feb, 2003).

S11. S. H. Kim *et al.*, *Biochem Biophys Res Commun* **324**, 58 (Nov 5, 2004).

S12. R. Holmes, Y. Chang, P. D. Soloway, *Mol Cell Biol* **26**, 9564 (Dec, 2006).

S13. S. Tomizawa *et al.*, *Development* **138**, 811 (Mar, 2011).

S14. M. Kaneda *et al.*, *Nature* **429**, 900 (Jun 24, 2004).

figure S1

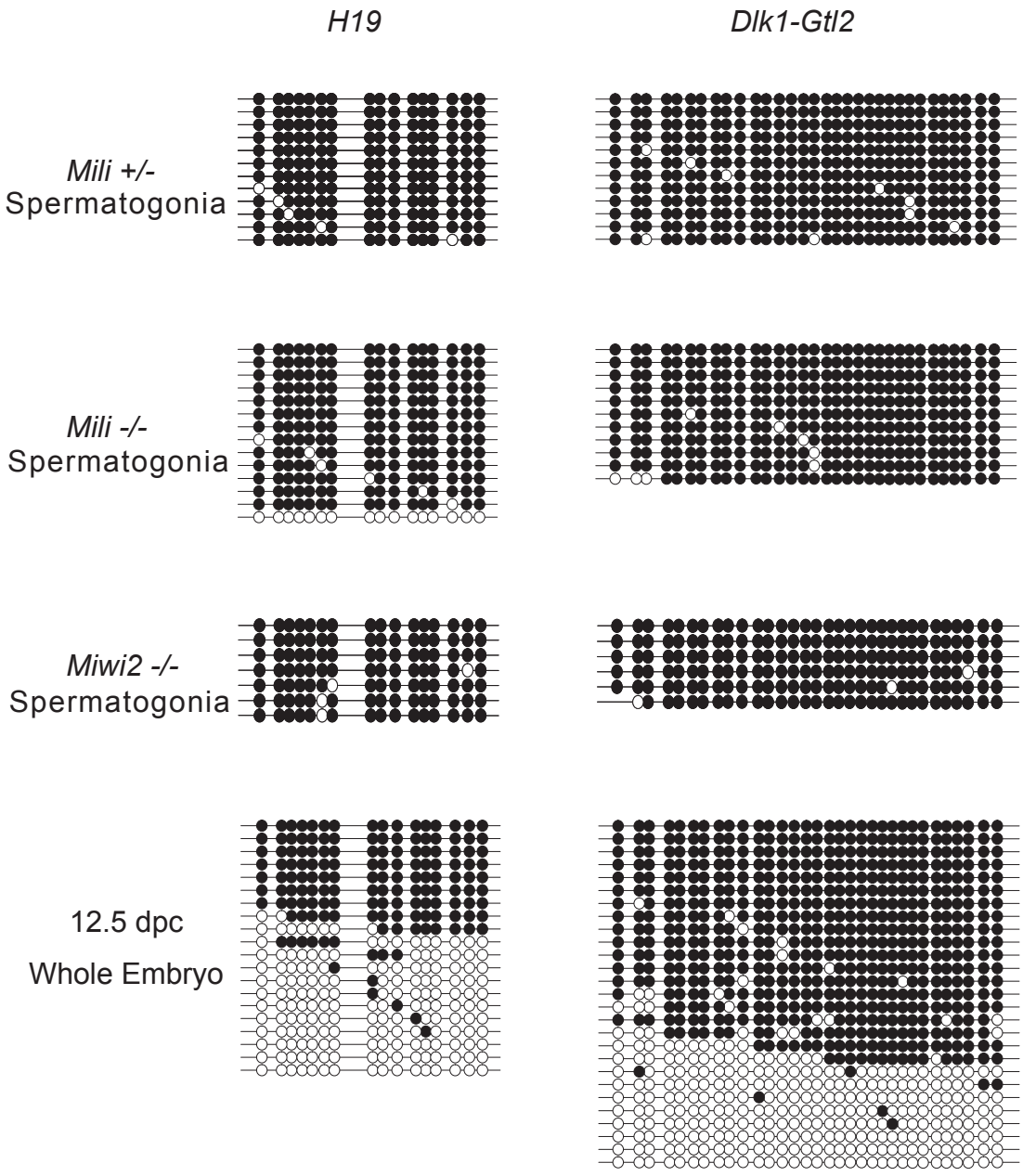

H19
Dlk1-Gtl2
Mili +/-
Spermatogonia
Mili -/-
Spermatogonia
Miwi2 -/-
Spermatogonia
12.5 dpc
Whole Embryo

figure S2

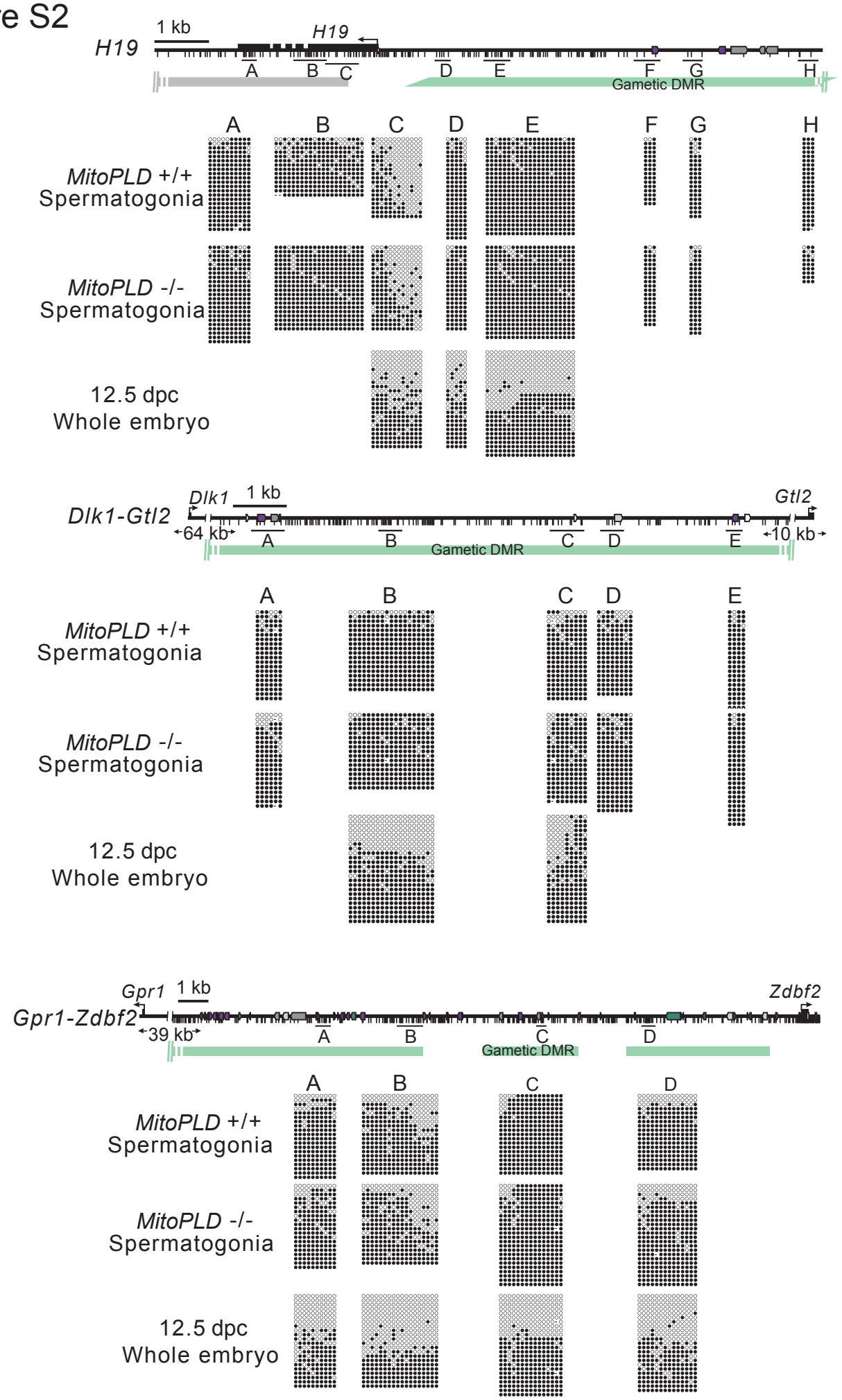
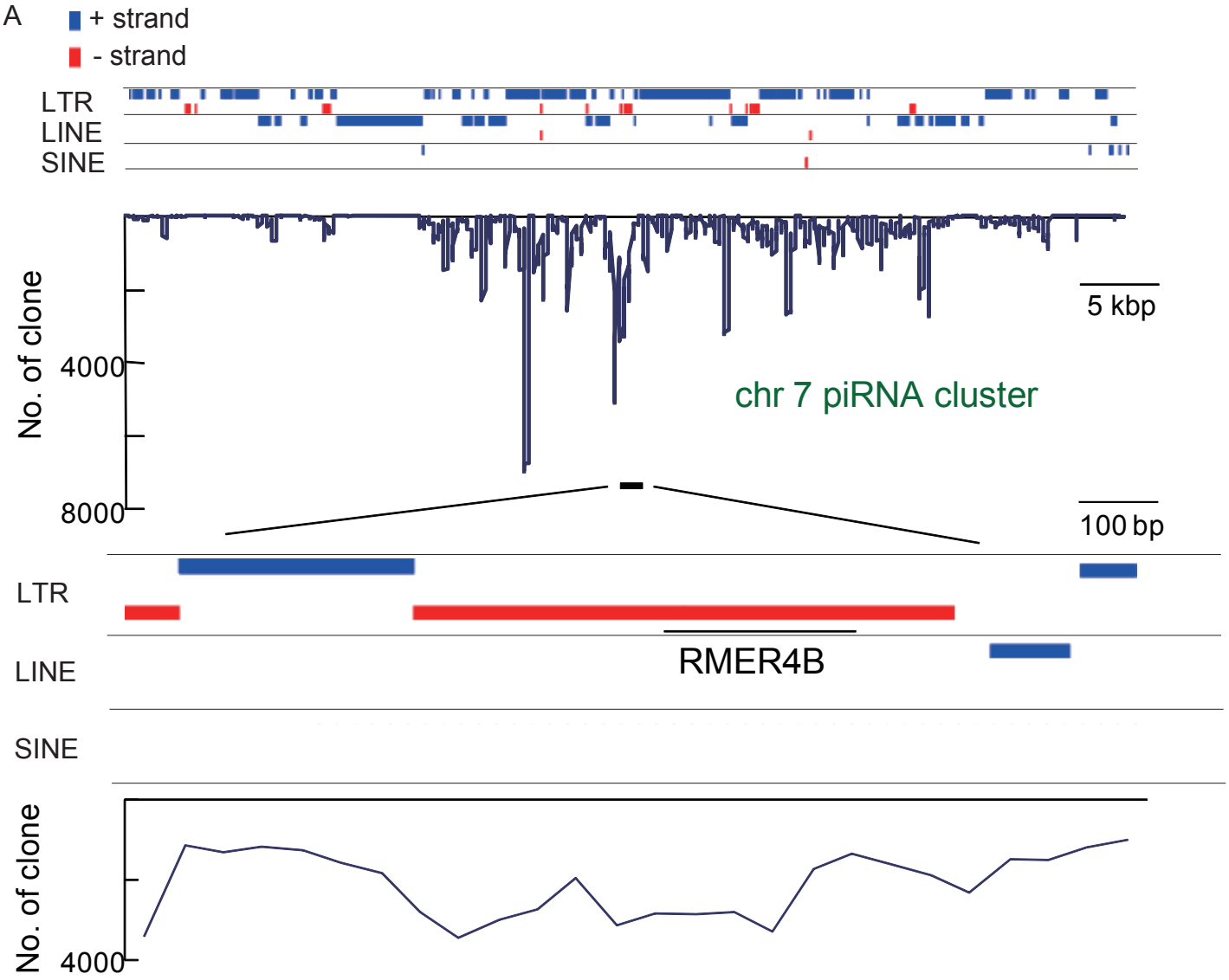

H19
1 kb
H19
A
B
C
D
E
F
G
H
Gametic DMR
MitoPLD +/+
Spermatogonia
MitoPLD -/-
Spermatogonia
12.5 dpc
Whole embryo
Dlk1-Gtl2
Dlk1
1 kb
Gtl2
64 kb
10 kb
Gametic DMR
Gpr1-Zdbf2
Gpr1
1 kb
Zdbf2
39 kb
Gametic DMR

figure S3

A



B

```
Rasgrf1_target_site1      CTGGCTATAATTTTTCTCCATGCTCTAGCAGGCATGTGGTTTTGCCAGCTGAAGATAGTT
Rasgrf1_target_site2      ----------------------------CAGAGCTGTGCTGCTGCCG-CTAAAGATAGTT
chr7_piRcluster           CTGACTATAATTTG-CCTCATGTTCTAGCAGGGGCATGGTTTTGCCAGCTGCAGATAGTT
                                                ***.   .** *  ****. **..********

Rasgrf1_target_site1      TAG---------------ATATGGAATTCTGGGAACTCTTCAGAGAGTATGTAAAG----
Rasgrf1_target_site2      TAG---------------ATATGGAATTCTGGGGACTCTTCAGAGAGTTTATAAAG----
chr7_piRcluster           TCTGTGCAAGCGTGTAACATTTGGAATTCTGGGAACTCTTCAGAGAATATATAACCGGTT
                          *.               **:************.************.*:*.***.

Rasgrf1_target_site1      ----CCAGAGC-------------------
Rasgrf1_target_site2      ----CCAGCGC-------------------
chr7_piRcluster           GGGCCCTGAGAGGCATGGTGAGTTGGTGGT
                              **:*.*.
```

figure S4

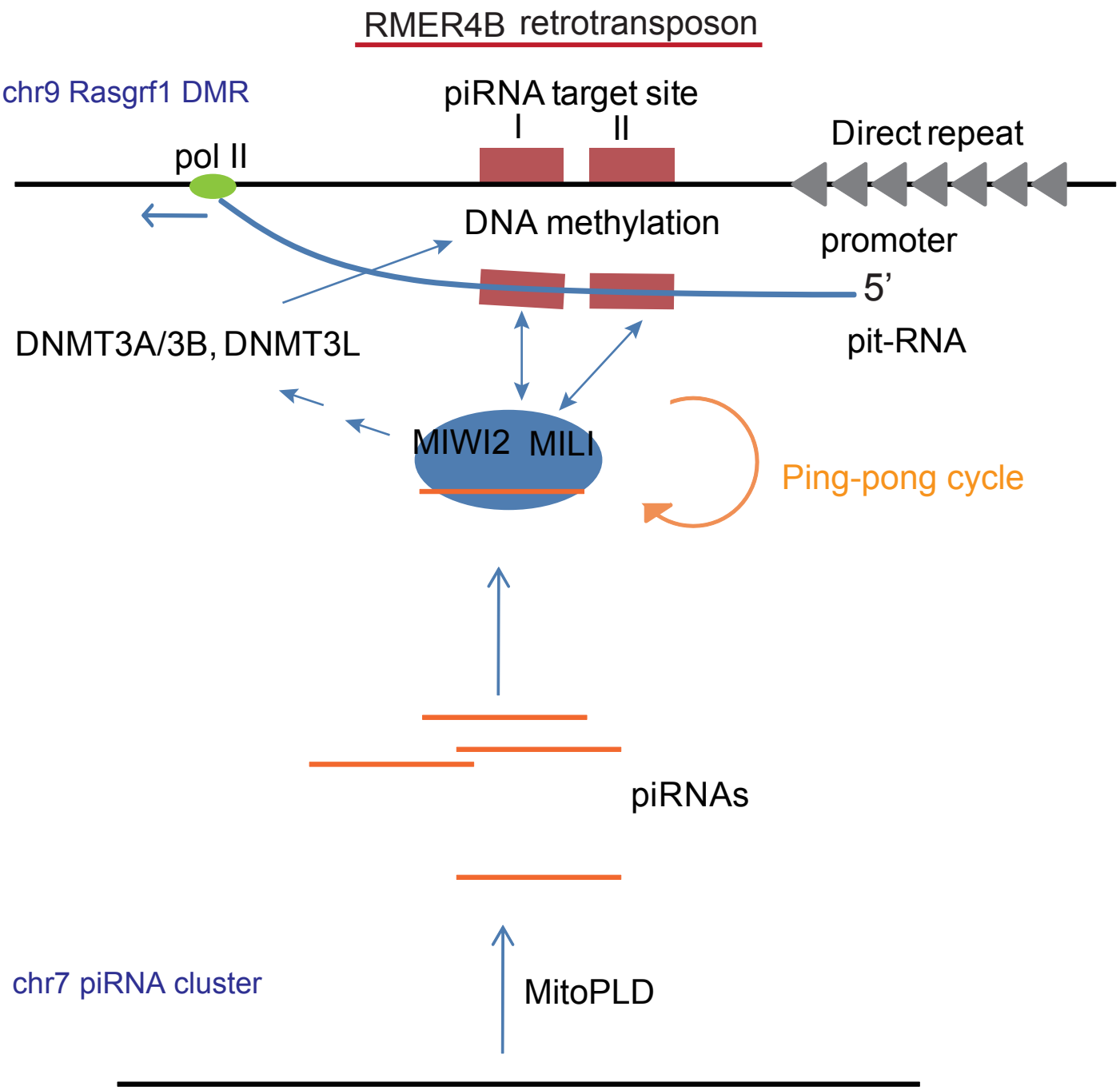
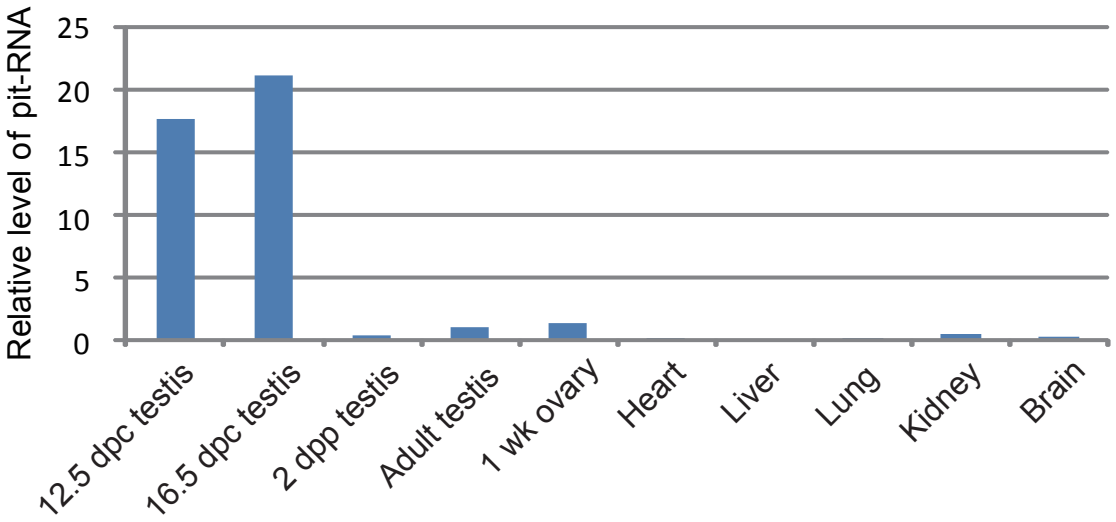
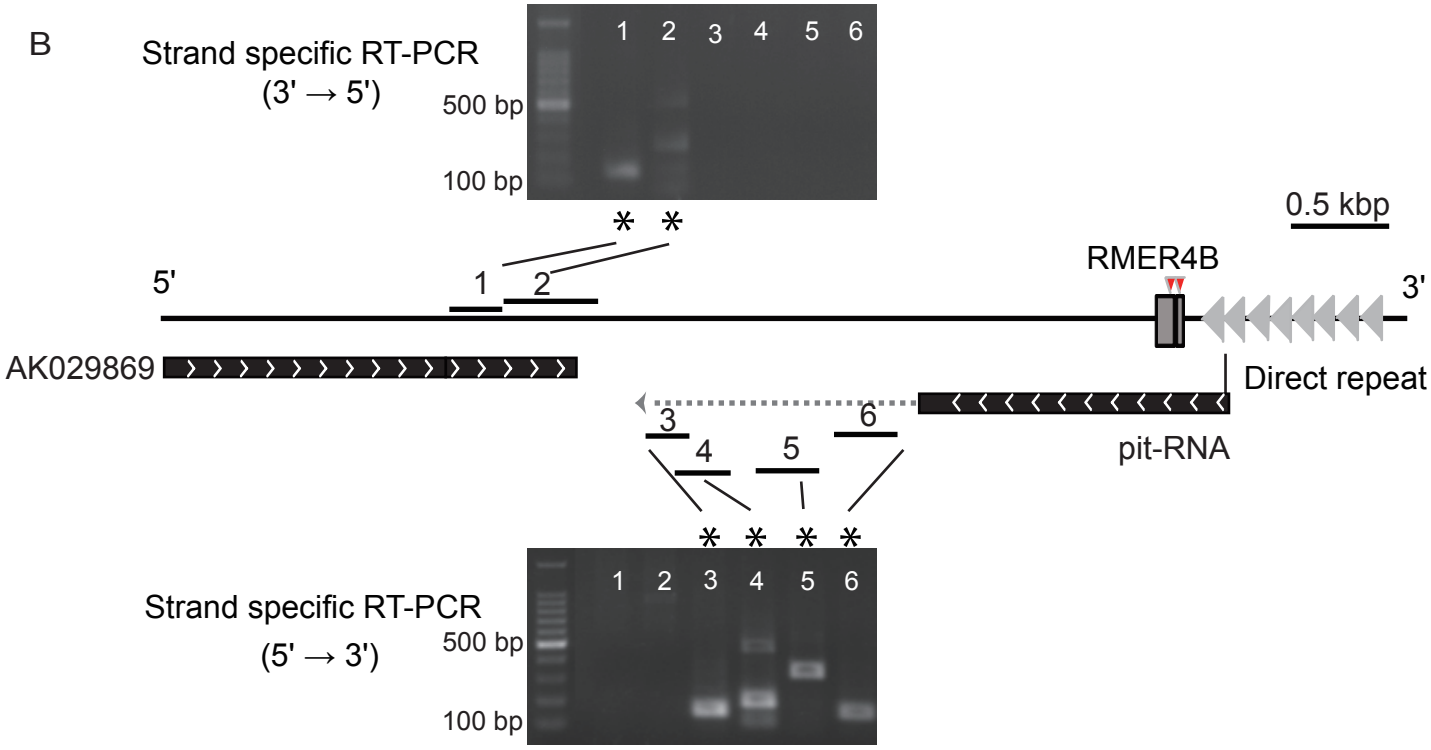

RMER4B retrotransposon
chr9 Rasgrf1 DMR
piRNA target site
I
II
Direct repeat
pol II
DNA methylation
promoter
5'
DNMT3A/3B, DNMT3L
pit-RNA
MIWI2 MILI
Ping-pong cycle
piRNAs
chr7 piRNA cluster
MitoPLD

figure S5

A



B



C

B6 ♀ X JF1 ♂

16.5 dpc testis pit-RNA

	No. of sequences	
Individual	B6	JF1
1	8	7
2	7	8
3	7	5

D

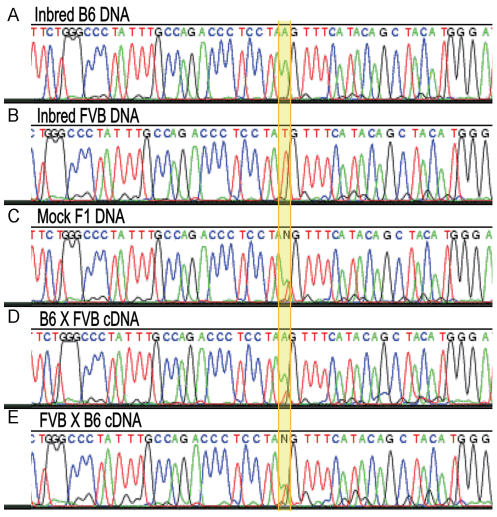
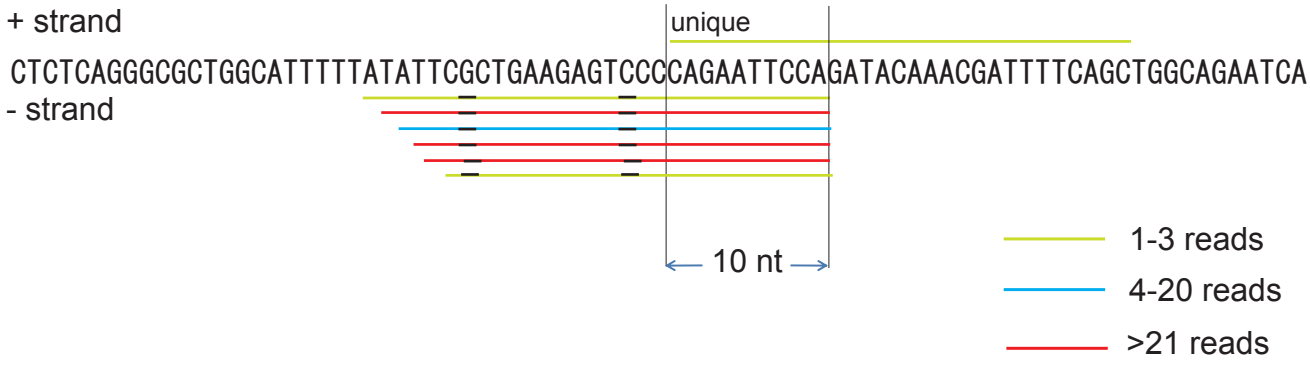


figure S6

chr2:129208680-129208760 (UCSC 2007)



chr11:116358615-116358695 (UCSC 2007)

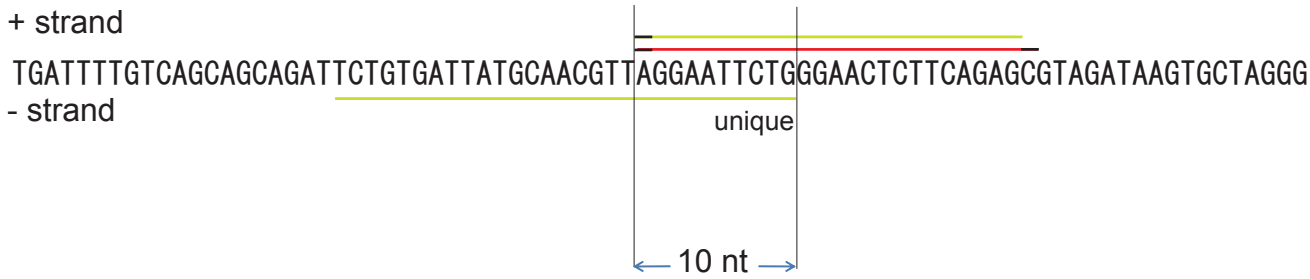
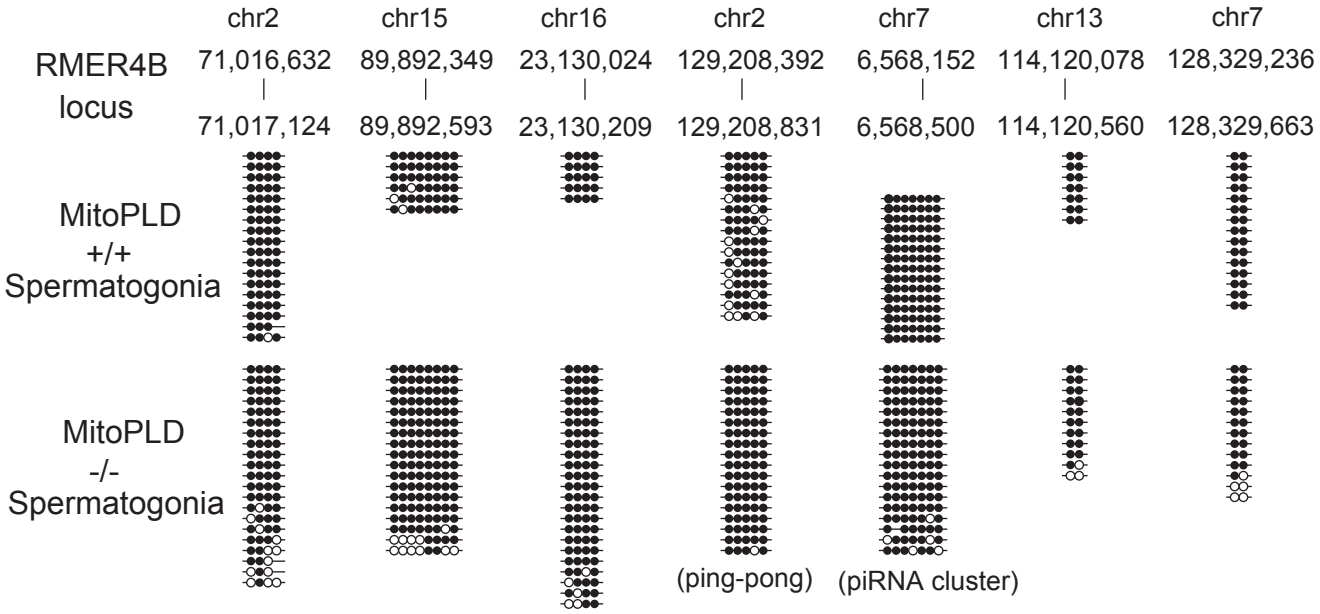


figure S7


chr2
chr15
chr16
chr2
chr7
chr13
chr7
RMER4B locus
71,016,632
71,017,124
89,892,349
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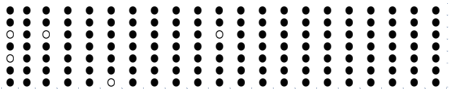
23,130,024
23,130,209
129,208,392
129,208,831
6,568,152
6,568,500
114,120,078
114,120,560
128,329,236
128,329,663
MitoPLD +/+ Spermatogonia
MitoPLD -/- Spermatogonia
(ping-pong)
(piRNA cluster)

figure S8

chr3:133,061,477-133,061,958

IAPEY_LTR



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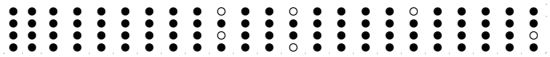

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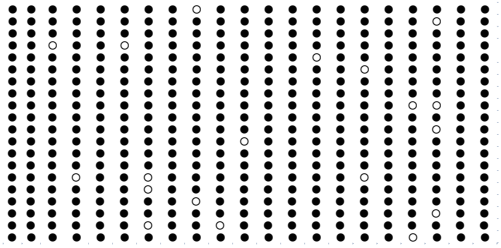

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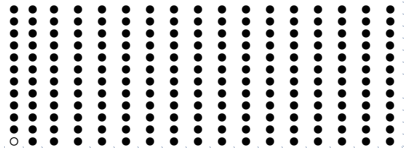

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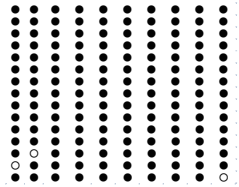

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IAPLTR2b

chr4:155,050,647-155,051,160

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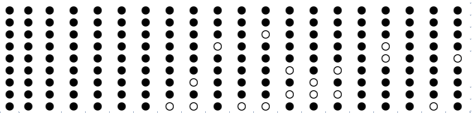

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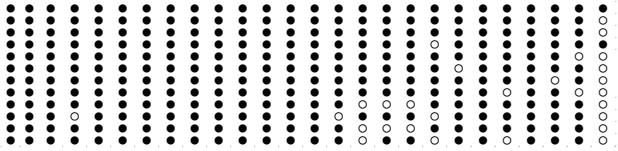

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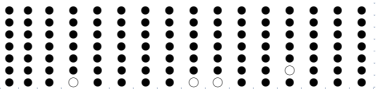
L1Md_A



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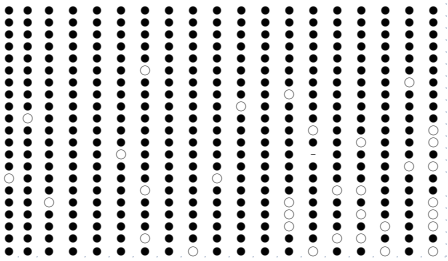
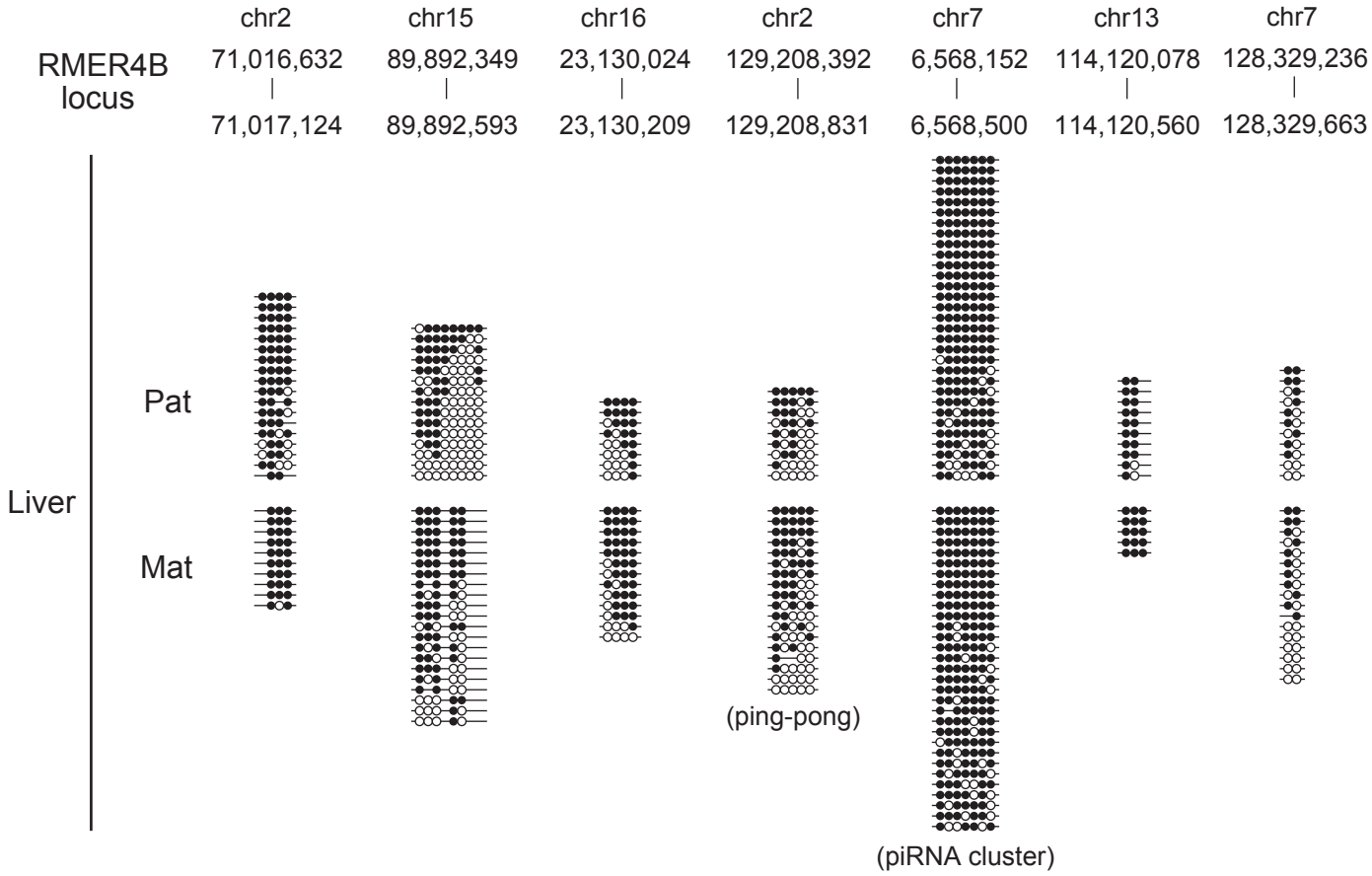


figure S9

A


chr2
chr15
chr16
chr2
chr7
chr13
chr7
RMER4B locus
71,016,632
71,017,124
89,892,349
89,892,593
23,130,024
23,130,209
129,208,392
129,208,831
6,568,152
6,568,500
114,120,078
114,120,560
128,329,236
128,329,663
Liver
Pat
Mat
(ping-pong)
(piRNA cluster)

B

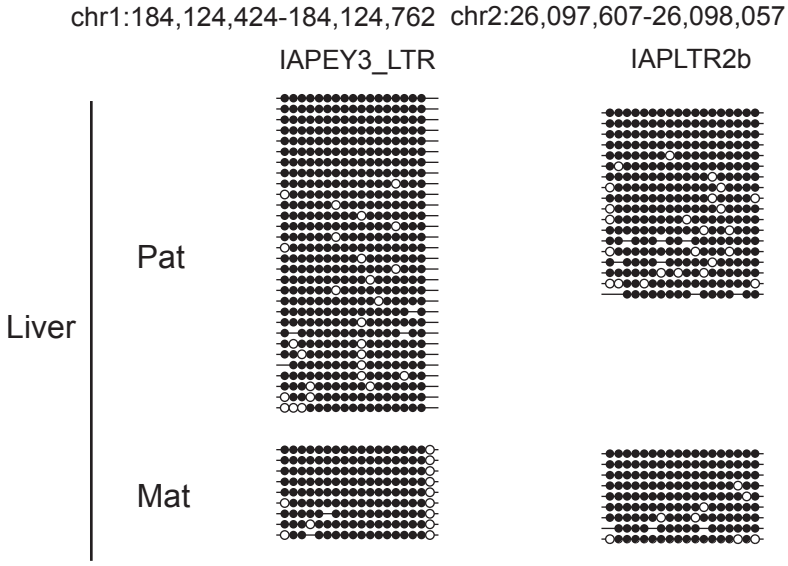
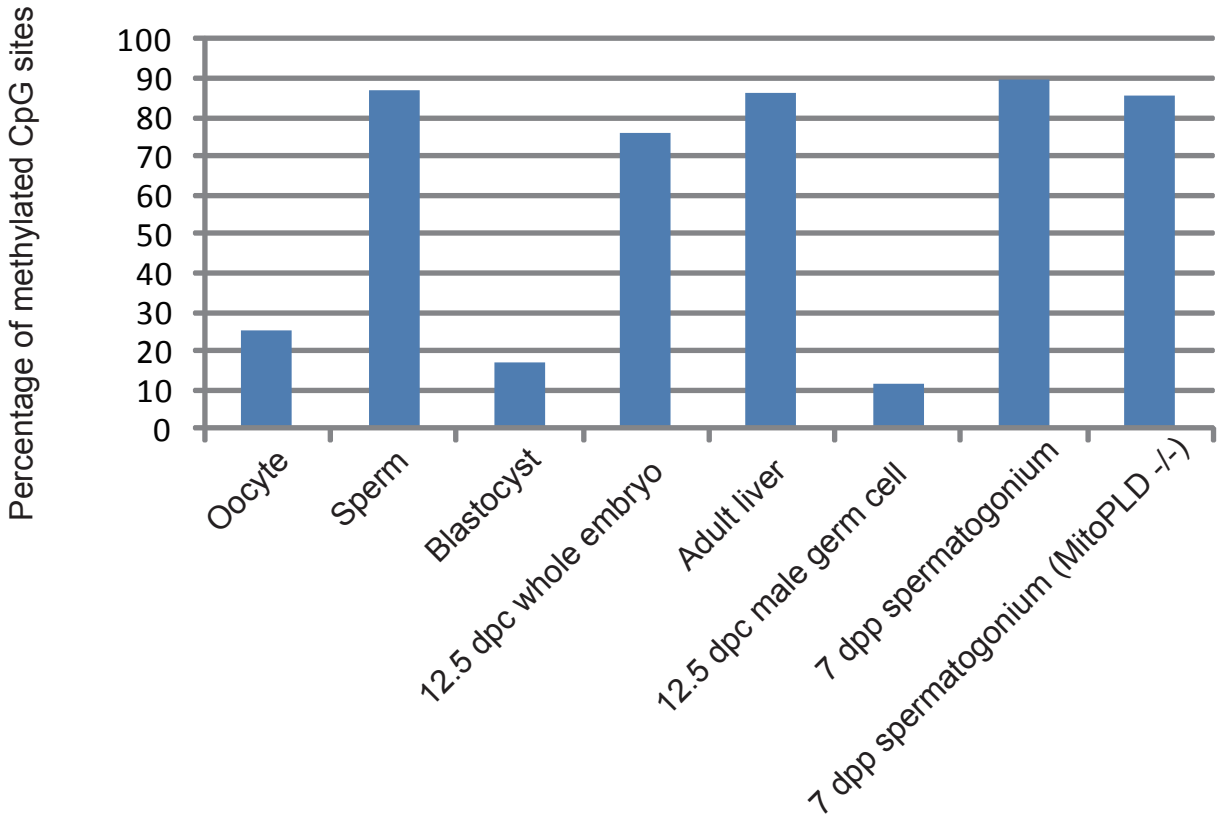

chr1:184,124,424-184,124,762
chr2:26,097,607-26,098,057
IAPEY3_LTR
IAPLTR2b
Liver
Pat
Mat

figure S10



	No. of total CpGs	No. of methyl CpGs	%	No. of clones
Oocyte	79	20	25.3	52
Sperm	112	97	86.6	64
Blastocyst	47	8	17.0	36
12.5 dpc Whole Embryo	59	45	76.2	59
Adult Liver	148	128	86.4	86
12.5 dpc male germcell	176	20	11.3	128
7 dpp Spermatogonium	67	60	89.5	58
7 dpp Spermatogonium (MitoPLD -/-)	63	54	85.7	48

table S1. Number of small RNAs cloned from 16.5 dpc testes

	Wild-type	*Mili* −/−	*MitoPLD* −/−	*Miwi2* −/−
Total	3133698	8734491	2634403	3991758
Genomic match	2083883	5029991	1628406	2474169
Cellular non-coding RNA[1]	604431	3284645	903211	302992
miRNAs	310134	1122346	563819	655686
Repeat[2] (normalized[3])	733161(733161)	142733(67511)	34980(16545)	928212(439036)
mRNA (normalized[3])	92101(92101)	230963(109243)	47027(22243)	146377(69235)
Others (normalized[3])	350208(350208)	246934(116797)	85107(40254)	455074(215246)
Rasgrf1 RMER4B hit allowing mismatches (normalized[3])	258(258)	6(1.657959)	3(1.650179)	381(180.2098)
Rasgrf1 RMER4B unique hit (normalized[3])	6(6)	2(0.552653)	0(0)	6(2.83795)

[1] This class includes rRNA, tRNA, snRNA, snoRNA, scRNA, srpRNA and RNA (RepeatMasker annotation)

[2] A majority of this class is piRNAs

[3] The number of clones is normalized using that of miRNAs

table S2. Unique small RNAs that hit the *Ragrf1* DMR[1]

sequence	length	strand	No. of clone	chr	start	end
TTTAAAAATCACAGACAACAGAAGCTGAA	29	-	1	chr9	89772369	89772397
TTACAACAGGGAAGTCCGGGATTTG	25	-	1	chr9	89772908	89772932
AGCAGAATTAGTGACGATTACAACAGGG	28	-	1	chr9	89772922	89772949
TTCAATGGCAACAAGGACCAGGATATCAATC	31	-	1	chr9	89773600	89773630
TAGCAGAACAGATCAAACAAGCAGGCA	27	-	1	chr9	89774080	89774106
TAAAACCTCTTAGCAGAACAGATCAAACAA	30	-	1	chr9	89774087	89774116
AAGGAGGCGTTTATCTGAATAGTCGCA	27	-	1	chr9	89774163	89774189
CAGAATTCCATATCTAAACTATCTTCAGC	29	-	1	chr9	89774367	89774395
TGGCTTTACATACTCTCTGAAGAGTTCC	28	-	2	chr9	89774396	89774423
AGCAGCACAGCTCTGGCTTTACATACTCTC	30	-	1	chr9	89774407	89774436
CAGAATTCCATATCTAAACTATCTTTA	27	-	2	chr9	89774442	89774468
TCGGCAATGGCTGGGATGATGGCAGAAA	28	-	1	chr9	89776553	89776580
TCGGCAATGGCTGGGATGATGGCAGA	26	-	1	chr9	89776555	89776580
AAACAAATATTTATCATGATCAAACGG	27	-	1	chr9	89776839	89776865
TAGGAAAGTTAAACAAAGCAAAAACGAATA	30	-	1	chr9	89776928	89776957
TAGAAACAGACAGGAGGATTATGAAT	26	-	1	chr9	89777201	89777226
TAATACTGTAGCTTTGAAAACAAACAGA	28	-	1	chr9	89777783	89777810
TACTGTAGCTTTGAAAACAAACA	23	-	1	chr9	89777785	89777807
TGTAGCGCTATAACAATTCTGGCTCCC	27	+	1	chr9	89778319	89778345

[1]Coordinates: chr9: 89769659-89779158 (mm9)

table S3. Origin of small RNAs that hit the *Rasgrf1* RMER4B sequence with up to 2 mismatches

Sequence	length	No. of clone	No. of genomic match	chr7 piRNA cluster	*Rasgrf1* RMER4B
TGGAATTCTGGGAACTCTTCAGAGAATATA	30	18	1	unique hit	
TGGAATTCTGGGAACTCTTCAGAGAA	26	88	1	unique hit	
TGGAATTCTGGGAACTCTTCAGAGAATA	28	50	1	unique hit	
TGGAATTCTGGGAACTCTTCAGAGAATAT	29	1	1	unique hit	
TGGAATTCTGGGAACTCTTCAGAGA	25	35	4	hit[1]	
TTGGAATTCTGGGAACTCTTCAGAGA	26	16	2	hit[1]	
TGGAATTCTGGGAACTCTTCAGAGAAT	27	15	1	unique hit	
TTGGAATTCTGGGAACTCTTCAGAGAAT	28	3	1	unique hit	
TTTGGAATTCTGGGAACTCTTCAGAGAA	28	1	1	unique hit	
TTGGAATTCTGGGAACTCTTCAGAGAA	27	11	1	unique hit	
TGCCAGCTGCAGATAGTTTC	20	1	302		
AGCAGCACAGCTCTGGCTTTACATACTCTC	30	1	1		unique hit
TAGGAATTCTGGGAACTCTTCAGAGCGTA	29	1	1	unique hit	
TGGAATTCTGGGAACTCTTCAGAGT	25	1	2		
TGGAATTCTGGGAACTCTTCAGAGAATATATAA	33	1	1	unique hit	
CAGAATTCCATATCTAAACTATCTTCAGC	29	1	1		unique hit
CAGAATTCCATATCTAAACTATCTTTA	27	2	1		unique hit
TGGAATTCTGGGAACTCTTCAGAG	24	1	18	hit[1]	
TGGAATTCTGGGGACTTTTCAGAGGGT	27	2	74		
CATTTGGAATTCTGGGAACTCTTCAGAGA	29	1	1	unique hit	
TTCTGGGAACTTTTCAGAGGGTATG	25	1	12		
TGGCTTTACATACTCTCTGAAGAGTTCC	28	2	1		unique hit
TTGGAATTCTGGGAACTCTTCAGAGAATA	29	2	1	unique hit	
TCTGGGAACTCTTCAGAGAAT	21	1	1	unique hit	
GAATTCTGGGAACTCTTCAGAGAATATATAA	31	1	1	unique hit	
TGGAATTCTGGGGACTTTTCAGAGG	25	1	122		

[1]This represents a non-unique mapping with a perfect sequence match.