Establishment of Paternal Methylation Imprints in Normal and *Dnmt*-Deficient Male Germ Cells

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Abstract

Genomic imprinting is a germline-specific epigenetic phenomenon that causes monoallelic expression of a small subset of mammalian genes depending on their parental origin. It is known that imprinted gene expression is regulated by cis-regulatory elements called differentially methylated regions (DMRs) that show parental origin-specific DNA methylation. Based on the molecular and genetic studies, it has been shown that DNA methylation is the epigenetic mark for imprinting.

While the allele-specific differential methylation at the DMRs is stably maintained in the somatic lineages, it should be erased and reestablished in the germline of each generation depending on the sex of the individual. In female germ cells, the establishment of the maternal methylation imprints begins after birth and proceeds during oocyte growth. In contrast, how and when the paternal methylation imprints are established is not fully understood, although it is known that the process begins in the gonocyte stage. In addition, although it is known that DNA methyltransferase family genes *Dnmt3a* and *Dnmt3L* are essential for the establishment of the maternal methylation imprints, which *Dnmt3* genes are required for the establishment of the paternal methylation imprints has not been conclusively determined.

To investigate the process of the establishment of the paternal methylation imprints, I performed bisulphite methylation analysis on the fetal and postnatal male germ cells in wildtype testis. The results demonstrated that methylation imprints at all paternally methyted DMRs are established in gonocytes before birth, and that the established methylation imprints are maintained through meiosis in adult testes. I also performed methylation analysis on the male germ cells derived from the germline-specific *Dnmt3a* and *Dnmt3b* mutant mice and from conventional *Dnmt3L* mutant mice. The results demonstrated that *Dnmt3a* and *Dnmt3L* play a major role in the establishment of the paternal methylation imprints in a DMR dependent manner. The results also suggested the contribution of *Dnmt3b* in the establishment

of the paternal methylation imprints at least one DMR. These observations help to understand the precise mechanisms of genomic imprinting in male germline.

Abbreviations

AP : alkaline phosphatase

DMR: differentially methylated region

EDTA : ethylenediamine tetraacetic acid

Gnas1A : guanine nucleotide binding protein, alpha stimulating exon 1A

kb : kilo base

PBS : phosphate-buffered saline

PCR : polymerase chain reaction

PGC : primordial germ cell

SDS : sodium dodecyl sulfate

SNP : single nucleotide polymorphism

Gene symbols

Dlk1 : delta-like 1

Dnmt : DNA methyltransferase

Igf2 : insulin-like growth factor 2

Igf2r : insulin-like growth factor 2 receptor

Kcnq1ot1 : Kcnq1-overlapping transcript 1

Peg3 : paternally expressed gene 3

Rasgrf1 : Ras protein-specific guanine nucleotide-releasing factor 1

TNAP : tissue non-specific alkaline phosphatase

Introduction

Nuclear transfer experiments carried out in early 1980s demonstrated that the maternal and paternal genomes of mouse are not equivalent in function and both are required for normal embryonic development (MacGrath and Solter, 1984; Surani *et al.*, 1984; Barton *et al.*, 1984). The functional difference between the parental genomes is caused by genomic imprinting, which is a germline-specific epigenetic phenomenon resulting in monoallelic expression of a small subset of genes depending on their parental origin in the progeny. So far, about 80 imprinted genes have been identified in mouse, which are involved in diverse biological phenomena such as embryonic development, placental formation, fetal and postnatal growth and maternal behavior (Reik and Walter, 2001). Also, some human diseases such as Prader-Willi syndrome, Angelman syndrome and Beckwith-Wiedemann syndrome are known to occur as a consequence of aberrant expression of imprinted genes (Robertson, 2005).

Methylation of cytosine residues in CpG dinucleotides plays a major role in gene repression, transposon silencing and genome stabilization (Bird, 2002). In human somatic cells, methylated cytosine residues account for ~1% of total DNA bases and affect about 70% of all CpG dinucleotides in the genome (Bird, 2002). The enzymatic activities catalyzing DNA methylation can be classified into two types. One is maintenance methylation, which is an activity to methylate unmethylated cytosine residues of hemimethylated CpGs after DNA replication, and DNA methyltransferase 1 (*Dnmt1*) is responsible for the function (Gruenbaum *et al.*, 1982; Bestor and Ingram, 1983). The other is *de novo* methylation, which is an activity to add methyl groups to cytosine at unmethylated CpGs, and it is known that *Dnmt3a* and *Dnmt3b* catalyze the reaction (Okano *et al.*, 1998). Genetic studies have indicated that DNA methylation is required for normal mouse development, because functional deficiency of *Dnmt1* results in embryonic lethality at around embryonic day 8.5-9.5 (E8.5-9.5) with a severe reduction of genomic DNA methylation (Li *et al.*, 1992). Similarly, animals deficient for

Dnmt3a and *Dnmt3b* show postnatal and embryonic lethality, respectively (Okano *et al.*, 1999).

The relationship between genomic imprinting and DNA methylation was first reported at *H19* and *Igf2r*: the active maternal *H19* allele showed hypomethylation while the inactive paternal *H19* showed hypermethylation in the promoter region (Ferguson-Smith *et al.*, 1993); the active maternal *Igf2r* allele showed hypermethylation while the inactive paternal *Igf2r* showed hypomethylation in the second intron (Stoger *et al.*, 1993). Sequences showing such differential DNA methylation between the parental chromosomes are called differentially methylated regions (DMRs). It has been demonstrated that at least some DMRs act as the cis-regulatory elements that control the allele-specific expression of imprinted genes (Wutz *et al.*, 1997; Thorvaldsen *et al.*, 1998; Fitzpatrick *et al.*, 2002; Lin *et al.*, 2003; Williamson *et al.*, 2004; Liu *et al.*, 2005). More direct evidence for the requirement of DNA methylation in imprinting comes from the study of *Dnmt1*-deficient embryos, in which the differential methylation has been lost at the imprinted loci (Li *et al.*, 1993). In these embryos, both parental alleles of *Igf2* and *Igf2r* were silent, while both alleles of *H19* were active. These observations indicate that DNA methylation is indeed the epigenetic mark for imprinting.

Allele-specific methylation of the DMRs should be established in the parental germline and stably maintained during preimplantation stages, when genomic methylation is dramatically reduced (Reik *et al.*, 2001). It has been reported that the *H19, Dlk1/Gtl2* and *Rasgrf1* DMRs are methylated in sperm but not in oocytes (Bartolomei *et al.*, 1993; Tremblay *et al.*, 1995; Takada *et al.*, 2002; Li *et al.*, 2004), and the *Igf2r, Kcnq1ot1* and *Gnas1A* DMRs are methylated in oocytes but not in sperm (Stoger *et al.*, 1993; Engemann *et al.*, 2000; Liu *et al.*, 2000). In addition, such gametic methylation is stably maintained in preimplantation embryos (Stoger *et al.*, 1993; Tremblay *et al.*, 1995; Shemer *et al.*, 1997). While the differential methylation is stably maintained in somatic cells, it should be erased and reestablished in the germline before being passed onto the next generation. It has been

reported that both alleles of the DMRs are hypomethylated in primordial germ cells (PGCs) around E11.5 to 12.5 (Shemer *et al.*, 1997; Davis *et al.*, 2000; Lee *et al.*, 2002).

Molecular studies have been carried out to understand how the differential methylation is established in the parental germline. In the female germline, the establishment of the maternal methylation imprints begins in primary oocytes after birth, and proceeds during the oocyte growth (Lucifero *et al.*, 2002; Lucifero *et al.*, 2004). This was correlated with the expression analysis of some imprinted genes in parthenogenetic embryos produced by nuclear transfer (Obata *et al.*, 2002). In contrast, the details of the establishment of the paternal methylation imprints are not fully described. Among the three paternally methylated DMRs (*H19*, *Dlk1/Gtl2* and *Rasgrf1*), the *H19* DMR is the best investigated. It was shown that methylation of the *H19* DMR begins during the gonocyte stage in the fetal testis (Davis *et al.*, 2000; Ueda *et al.*, 2000), although there are some discrepancies between the reports. Davis *et al.* argued that methylation of the maternal *H19* DMR is not fully established during the gonocyte stage, while Ueda *et al.* argued that methylation of both parental alleles is completed by the end of the gonocyte stage. For the remaining two DMRs, it is known that methylation also begins in the gonocyte stage (Li *et al.*, 2004), but it is still unclear whether methylation of both parental alleles is completed during this stage.

A defect in the establishment of the methylation imprints was first observed in mice deficient for *Dnmt3*-like protein (*Dnmt3L*), which belongs to the *Dnmt3* family of the *de novo* methyltransferases but lacks methyltransferase activity (Bourc'his *et al.*, 2001; Hata *et al.*, 2002). Hypomethylation of the maternal DMRs was observed in both *Dnmt3L*-deficient female oocytes and heterozygous fetuses derived from the *Dnmt3L*-deficient oocytes, indicating that *Dnmt3L* is essential for the establishment of the maternal methylation imprints in oocytes (Bourc'his *et al.*, 2001; Hata *et al.*, 2002). More recently, taking advantage of the germline-specific *Dnmt3a* and *Dnmt3b* knockout mice, Kaneda *et al.* demonstrated that, when *Dnmt3a* is deleted in female germ cells, all embryos die in utero even though they inherit a

wildtype allele from their father. As similar to *Dnmt3L* mutants, methylation of the maternally methylated DMRs was lost in these fetuses, indicating that *Dnmt3a* is essential for the establishment of the maternal methylation imprints. In contrast, when *Dnmt3b* was deleted in the same manner, embryos were healthy and had appropriate methylation patterns in the maternally methylated DMRs (Kaneda *et al.*, 2004). This suggests that *Dnmt3b* is not required for the establishment of the maternal methylation imprints. Therefore, *Dnmt3a* and *Dnmt3L*, but not *Dnmt3b*, are indispensable for the establishment of the maternal methylation imprints.

The results of the studies on the establishment of the paternal methylation imprints are rather complicated. In our previous study, the germline-specific *Dnmt3a* knockout male germ cells showed azoospermia and reduction in methylation of the *H19* and *Dlk1/Gtl2* DMRs (but not of the *Rasgrf1* DMR), suggesting that *Dnmt3a* is required for the establishment of the paternal methylation imprints in two of the three loci (Kaneda *et al.*, 2004). In contrast, hypomethylation was observed only at the *H19* DMR in the *Dnmt3L*$^{-/-}$ spermatogonia (Bourc'his and Bestor, 2004; Kaneda *et al.*, 2004), which was at variance with the finding by Webster *et al.* that not only the *H19* DMR but also the *Rasgrf1* DMR was hypomethylated (Webster *et al.*, 2005). These results suggested that different enzymes and factors could operate on different paternally methylated DMRs. Involvement of *Dnmt3b* in the paternal methylation imprinting is not likely, because conditional *Dnmt3b* knockout mice did not show any defects in either the knockout mice themselves or their progeny (Kaneda *et al.*, 2004).

To resolve the current confusions about the establishment mechanisms of the paternal methylation imprints, I decided to investigate in detail 1) how and when the methylation imprints are established at the three paternally methylated DMRs during male germ cell development, and 2) which DNA methyltransferase family member is involved in the establishment of the paternal methylation imprints. In the first part of this thesis, I describe the results of bisulphite sequencing analyses of the paternally methylated DMRs at various stages of male germ cell development. I used F1 mice produced by crossing C57/BL6J

females and JF1 males to distinguish the parental alleles. The results demonstrated that, in wildtype male germ cells, the methylation imprints of all paternally methylated DMRs are established in gonocytes during fetal testis development. This is the first complete description of the establishment process of the paternal methylation imprints. In the second part of this thesis, I investigated the roles of *Dnmt3a*, *Dnmt3b* and *Dnmt3L* in the establishment of the paternal methylation imprints in multiple male germ cell preparations obtained from the mutants. The results demonstrated that *Dnmt3a* and *Dnmt3L* play a major role in the establishment of the paternal methylation imprints. However, the effect of the *Dnmt3a* and *Dnmt3L* disruptions was in many cases partial, and the actual degree of demethylation was different from DMR to DMR and also from sample to sample. The results also suggested a possible involvement of *Dnmt3b* in the establishment of the paternal methylation imprints, although the effect of the *Dnmt3b* disruption was not very clear. Thus, the efficiency and role of each *Dnmt3* member seem quite different between the male and female germlines. The identification of the *Dnmt* proteins involved in the paternal methylation imprinting helps to understand the precise mechanisms of genomic imprinting.

Materials and Methods

Mice

To obtain wildtype male germ cells, in which the parental allele can be distinguished, C57BL/6J female mice and JF1 (Koide *et al.*, 1998) male mice were crossed. Germline-specific *Dnmt3a* and *Dnmt3b* and conventional *Dnmt3L* mutant mice used in this study were produced and reported in the previous studies (Hata *et al.*, 2002; Kaneda *et al.*, 2004; Dodge *et al.*, 2005). A schematic representation of knockout constructs of *Dnmt3a* and *Dnmt3b* are shown in Figure 1A. To obtain *Dnmt3a* and *Dnmt3b* deficient postnatal day 0-2 (P0-2) gonocytes, female mice homozygous for the floxed allele of *Dnmt3a* and *Dnmt3b* ($Dnmt3a^{2lox/2lox}$ and $Dnmt3b^{2lox/2lox}$) and male mice heterozygous for the floxed allele of *Dnmt3a* and *Dnmt3b* and possessing TNAP-Cre ([$Dnmt3a^{2lox/+}$, TNAP-Cre] and [$Dnmt3b^{2lox/+}$, TNAP-Cre]) were crossed. Then, gonocytes were obtained from P0-2 male mice having the genotypes [$Dnmt3a^{2lox/1lox}$, TNAP-Cre] and [$Dnmt3b^{2lox/1lox}$, TNAP-Cre] (Fig. 1B). Cre recombinase driven by TNAP promoter was expressed in the PGCs from E9.5-10.5 to the late-gestation (Lomeli *et al.*, 2000). In the case of *Dnmt3L*, heterozygous ($Dnmt3L^{+/-}$) female and male mice were crossed.

Genotyping

Mouse tail DNA was prepared by a standard protocol for genotyping. Briefly, tail biopsies were incubated with lysis buffer (100 mM Tris (pH8.0), 5 mM EDTA, 200 mM NaCl, 0.2% SDS and 200 ug/ml Proteinase K) at 50°C overnight. After phenol/chloroform extraction and ethanol precipitation, DNA pellet was resuspended in 100 ul of TE (10 mM Tris (pH8.0), 1 mM EDTA). The tail DNA was used for genotyping by PCR analysis. Primers used were:

OM142F ($Dnmt3a^{2lox}$ and $Dnmt3a^{+}$, up), 5'-CTG TGG CAT CTC AGG GTG ATG AGC A-3'

3awt/R ($Dnmt3a^{2lox}$ and $Dnmt3a^{+}$, down), 5'-GCA AAC AGA CCC AAC ATG GAA CCC T -3'

OM159F (*Dnmt3b*^{2lox} and *Dnmt3b*$^{+}$, up), 5'-AGA GCA CTG CAC CAC TAC TGC TGG A-3'

OM147R (*Dnmt3b*^{2lox} and *Dnmt3b*$^{+}$, down), 5'-CAG GTC AGA CCT CTC TGG TGA CAA G-3'

TNAP-Cre/F, 5'-TAA GGG CCA GCT CAT TCC TCC-3'

TNAP-Cre/R, 5'-CAC GTC GAT GGC CGC TCT A-3'

KH15 (*Dnmt3L*, wildtype and mutant, up), 5'-CAC TAC TTC GAA TTC CCC CC-3'

KH28 (*Dnmt3L*, wildtype, down), 5'-TGG TTT GTT TAA GTA GAA GAT ATA TTG-3'

IRES (*Dnmt3L*, mutant, down), 5'-CAC ACT CCA ACC TCC GCA GGC TCC TA -3'

PCR was done under the following parameters for *Dnmt3a*, *Dnmt3b* and TNAP-Cre: 30 cycles of 95°C for 30 sec, 65°C for 45 sec, 72°C for 45 sec, with the final extension at 72°C for 5 min.

Parameters for *Dnmt3L* were: 35 cycles of 95°C for 45 sec, 58°C for 30 sec, 72°C for 30 sec, with the final extension at 72°C for 5 min.

Efficiency of recombination by TNAP-Cre was analyzed by PCR. Primers used were:

3a1-2/F (*Dnmt3a*^{2lox} and *Dnmt3a*^{1lox}), 5'-CAC CTG TGC CAG CTG AGA AGA GGA A-3'

3a1-2/R (*Dnmt3a*^{2lox} and *Dnmt3a*^{1lox}), 5'-TGA GTG GTG AGG CCC AGC TTA TCG A-3'

3b1-2/F (*Dnmt3b*^{2lox} and *Dnmt3b*^{1lox}), 5'-GAG TTA AGC TAG CTT ATC GAT ACC G-3'

3b1-2/R (*Dnmt3b*^{2lox} and *Dnmt3b*^{1lox}), 5'-AGA CAC TAT GTG CAG TAC AGC AGC T-3'

PCR was done under the following parameters: 30 cycles of 94°C for 30 sec, 65°C for 45 sec, 72°C for 2 min, with the final extension at 72°C for 5 min (*Dnmt3a*); 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 5 min, with the final extension at 72°C for 5 min (*Dnmt3b*).

Identification of single nucleotide polymorphisms (SNPs) in the *Dlk1/Gtl2* DMR

Fifty ng of DNA (from C57BL/6J and JF1 liver) was used as templates for PCR under the following parameters: 30 cycles of 95°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min, with the final extension at 72°C for 5 min. Primers used were:

IG-F1, 5'- CTG CAA TTC ACG GTA TAT GAG TCC-3'

IG-R1, 5'- CTG CAA GTA CCA GAT TCC ATC AGG-3'

PCR products were cloned using pGEM-T Easy Vectror System I (Promega). Three colonies were picked up from each strain and cultured in LB medium (1% bacto tryptone, 0.5% bacto yeast extract, 1% NaCl, 2 mM NaOH, and 50 ug/ml ampicillin). Plasmid DNA was isolated by Hispeed Plasmid Midi Kit (Qiagen) and DNA sequencing was done using a BigDye Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems) with the standard primers (M13 Forward; 5'-GTAAAACGACGGCCAGT-3' and M13 Reverse; 5'-CAGGAAACAGCTATG-3'). Sequences were analyzed on an ABI Prism 377 Genetic Analyzer (Applied Biosystems).

Preparation of male germ cells

Seminiferous tubules from E14.5, E16.5, E18.5 and P0-2 testes were dissociated by trypsin/EDTA (0.25% trypsin, 10 mM EDTA in PBS) at 37°C for 10 min. Then, cells were cultured in M199 medium (Sigma) supplemented with 10% fetal bovine serum for 1.5 h in a CO_2 incubator. Cells floating in the supernatant were collected and washed by PBS, and resuspended in 2 ml of 25% percoll/M199. The solution was put on 2 ml of 65% percoll/PBS and overlayed by 1 ml of 12% percoll/PBS in a centrifugation tube. Centrifugation was carried out at 2800*g* for 20 min at room temperature. Somatic cells but not germ cells were precipitated in the bottom of the 25% percoll/M199 fraction. Cells enriched in the 25% percoll/M199 phase were isolated by micropipette. Isolated germ cells were incubated with

lysis buffer at 50°C for 1 h. After phenol/chloroform extraction, ethanol precipitation was performed with Ethachinmate (Nippon gene). DNA pellet was resuspended in 30 ul of distilled water. Pachytene spermatocytes and round spermatids were collected from 9-week old testes by elutriation (Beckman J6-MC; Rotor JE5.0). Briefly, seminiferous tubules were dissociated in PBS containing 0.5 mg/ml of collagenase at 32°C for 15 min and then in PBS containing 0.5 mg/ml of trypsin at 32°C for 20 min. Five minutes later from the beginning of the trypsin treatment, 2 ug/ml of DNase I was added. Then cells were treated with trypsin inhibitor (0.5 mg/ml), passed through a nylon mesh (23 um), and added with 2 ug/ml of DNase I. Elutriation was performed according to the previous report (Grabske *et al.*, 1975). Fraction 8 and 4 were regarded as pachytene spermatocytes and round spermatids, respectively. Spermatozoa were collected by a standard protocol. These male germ cells were incubated with lysis buffer at 50°C overnight. After phenol and phenol/chloroform extraction, ethanol precipitation was done. Genomic DNA was resuspended in 100 ul of TE buffer.

Alkaline phosphatase (AP) staining

Male germ cells isolated from E14.5, E16.5, E18.5 and P0 testes were fixed by 3.7% formaldehyde solution for 15 min at room temperature. Then, the cells were washed twice in PBS, and added with the alkaline phosphatase staining solution (0.1 M Tris-HCl (pH8.8), 2 mM $MgCl_2$, 0.5% N, N-dimetylforamide, 0.1 mg/ml nafthol AS-phosphatase, 0.6 mg/ml fast-blue BB salt), and incubated at 37°C for 20 min. After the cells were washed and resuspended in PBS, those showing blue signals were counted under microscopy.

Bisulphite methylation analysis

The principle of bisulphite reaction was shown in Figure 2. Genomic DNA isolated from male germ cells was subjected to bisulphite methylation analysis (Frommer *et al.*, 1992). The

bisulphite treatment was carried out with EZ DNA Methylation Kit (Zymo Research). Sequences investigated in this study were shown in Figure 3. Semi-nested PCR was performed to amplify the *H19*, *Dlk1/Gtl2*, *Rasgrf1* and *Peg3* DMRs. Sequences of the primers were:

BisOF1 (*H19*, first and second, up), 5'-TTG TGA GTG GAA AGA TTA AAT TGT TG-3' (Kaneda *et al.*, 2004)

BisOR1 (*H19*, first, down), 5'-AAT ACA CAC ATC TTA CCA CCC CTA TA-3' (Kaneda *et al.*, 2004)

BisIR1 (*H19*, second, down), 5'-ATC TTA CCA CCC CTA TAA ATC CCT-3' (Kaneda *et al.*, 2004)

BisIGF2 (*Dlk1/Gtl2*, first, up), 5'-GTG TTA AGG TAT ATT ATG TTA GTG TTA GG-3'

BisIGF3 (*Dlk1/Gtl2*, second, up), 5'-ATA TTA TGT TAG TGT TAG GAA GGA TTG TG-3'

BisIGR3 (*Dlk1/Gtl2*, first and second, down), 5'-TAC AAC CCT TCC CTC ACT CCA AAA ATT-3'

BisRGF1 (*Rasgrf1*, first, up), 5'- GAG AGT ATG TAA AGT TAG AGT TGT GTT G-3'

BisRGF2 (*Rasgrf1*, second, up), 5'-TAA AGA TAG TTT AGA TAT GGA ATT TTG GG-3'

BisRGR2 (*Rasgrf1*, first and second, down), 5'-ATA ATA CAA CAA CAA CAA TAA CAA TC-3'

Peg3-F2 (*Peg3*, first, up), 5'-TTG ATA ATA GTA GTT TGA TTG GTA GGG TGT-3' (Hata *et al.*, 2002)

Peg3-F4 (*Peg3*, second, up), 5'-TTT TGT AGA GGA TTT TGA TAA GGA GGT GTT-3'

Peg3-R2 (*Peg3*, first and second, down), 5'-ATC TAC AAC CTT ATC AAT TAC CCT TAA AAA-3' (Hata *et al.*, 2002)

In the nested PCR, first PCR was carried out using 2-3ng of bisulphite treated DNA under the following parameters: 35 cycles of 95°C for 1 min, 61°C for 1 min, and 72°C for 1 min, with the final extension at 72°C for 5 min; Second PCR was carried out using first PCR products under the same parameters for 15-20 cycles. PCR products were cloned using pGEM-T Easy

Vectror System I (Promega). Colonies were picked up and transferred into 96-well plates. DNA was amplified by rolling circle amplification using Templiphi DNA Amplification Kit (GE Healthcare). DNA sequencing was done using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) with a standard primer (M13 Reverse). Sequences were analyzed on an ABI Prism 3700 and 3130xl Genetic Analyzer (Applied Biosystems).

Results

Identification of SNPs in the paternally methylated DMRs between C57BL/6J and JF1 strains

I first performed genomic sequencing of the *Dlk1/Gtl2* DMR and identified SNPs between C57BL/6J (B6) and JF1 mice, because it was necessary to distinguish the parental alleles for methylation analysis. Four SNPs were found (Fig. 3) and two of them involved CpG: one was a substitution from A (B6) to G (JF1) that creates a new CpG and the other was a substitution from G (B6) to A (JF1) that abrogates a CpG. The total number of CpG sites was not changed. SNPs in the other DMRs were identified by K. Kumaki of our laboratory (personal communication). In the *H19* DMR, there were two SNPs between B6 and JF1 (Fig. 3). One of them was a substitution from A (B6) to C (JF1), giving rise to a new CpG site in JF1. In the *Rasgrf1* DMR, there were three SNPs and a 16-bp deletion in JF1 (Fig. 3). Two of the SNPs involved CpG: one was a substitution from A (B6) to G (JF1) and the other was a substitution from G (B6) to C (JF1). Two CpG sites were included in the deletion. Thus, the total number of CpG was 20 in B6 and 18 in JF1 in the *Rasgrf1* DMR.

Assessment of the purity of male germ cell preparations

Male germ cells were isolated from fetal and newborn testes by brief culture in a plastic dish (only somatic cells attach to the dish) and then by percoll sedimentation (see Materials and Methods for the details). The method was originally used to isolate PGCs (Dolci *et al.*, 1993; M. Tamura, personal communication). To test whether the method is applicable to isolate male germ cells at later stages, I performed alkaline phosphatase staining with the germ cells collected at E14.5, E16.5, E18.5 and P0. The results showed that the proportion of alkaline phosphatase positive cell is about 90% in all preparations (Table 1).

Paternal methylation imprints are established in fetal gonocytes

It was previously shown that the establishment of the paternal methylation imprints begins in fetal gonocytes (Davis *et al.*, 2000; Ueda *et al.*, 2000; Li *et al.*, 2004), but there were unsolved questions on the timing of completion of this *de novo* methylation (see Introduction). I therefore prepared DNA from E14.5, E16.5, E18.5 and P0 gonocytes, and carried out bisulphite methylation analysis on the three paternally methylated DMRs (*H19*, *Dlk1/Gtl2* and *Rasgrf1*) and, as a control, one maternally methylated DMR (*Peg3*).

An example of such studies on *H19* is shown in Figure 4A, and methylation changes at all DMRs are summarized in Figure 4C. (The actual data for the *Dlk1/Gtl2*, *Rasgrf1* and *Peg3* DMR are supplied as supplementary information). In E14.5 gonocytes, methylation levels of all paternally methylated DMRs were extremely low on the maternal chromosome, but they were 20-30% methylated on the paternal chromosome. Such a parental difference was previously observed at the *H19* DMR in this stage (Davis *et al.*, 2000; Ueda *et al.*, 2000). Methylation of the maternal allele of the DMRs first appeared in E16.5 gonocytes. In this stage, increased methylation was observed at all paternally methylated DMRs on both parental chromosomes with the parental differences maintained. I also observed that some CpG sites tend to be more methylated than the other CpG sites and that many molecules show mosaic patterns of methylation. Methylation was further increased at these DMRs in E18.5 gonocytes. Particularly, the DMRs on the paternal chromosome showed over 90% methylation at more than half of the CpGs sites in this stage. In contrast, the DMRs on the maternal chromosome were still 70-80% methylated. In the P0 stage, almost all CpG sites showed nearly or over 90% methylation in all DMRs on the maternal chromosome. The unmethylated 10% or so, which were mostly derived from the maternal chromosome could arise from somatic cell contamination. Thus, I concluded that the paternal methylation imprints of all three DMRs have been established almost fully in P0 gonocytes.

Methylation analysis of the paternally methylated DMRs in the meiotic and haploid male germ cells

Mitotically arrested gonocytes start to divide 3-4 days after birth, giving rise to spermatogonia. Then, after a few weeks, the first wave of spermatogenesis is initiated and the germ cells go into meiosis. The first round of spermatogenesis is completed in 5-6 weeks to produce mature spermatozoa. So far, investigation of the methylation status during spermatogenesis has been performed only on the *H19* DMR (Davis *et al.*, 1999). To investigate the methylation status of all paternally methylated DMRs in postnatal testes, I collected pachytene spermatocytes, round spermatids and spermatozoa by elutriation and carried out bisulphite methylation analysis. An example of such studies on the *H19* DMR is shown in Figure 4B, and the methylation status of all DMRs is summarized in Figure 4C. (The actual data for the *Dlk1/Gtl2, Rasgrf1* and *Peg3* DMR are supplied as supplementary information). All paternally methylated DMRs were nearly 90% methylated at most CpG sites on both parental chromosomes in pachytene spermatocytes and round spermatids, as well as in mature spermatozoa. The results suggest that the paternal methylation imprints that have been established in the gonocyte stage are maintained through meiosis to the haploid stage.

***Dnmt3a* plays a major role in the establishment of the paternal methylation imprints at the *H19* and *Dlk1/Gtl2* DMR**

Having established the normal methylation patterns of the paternally methylated DMR in wildtype male germ cells, I next asked which *Dnmt* proteins play a role in the establishment of the paternal methylation imprints. Previously, it was shown that methylation of the *H19* and *Dlk1/Gtl2* DMRs was reduced in the germline-specific *Dnmt3a* knockout spermatogonia. The results suggested that *Dnmt3a* is required for methylation of the DMRs (Kaneda *et al.*, 2004). To investigate the role of *Dnmt3a* in more detail, I first analyzed the methylation status of the paternally methylated DMRs in newborn (P0-2) gonocytes isolated form germline-specific

Dnmt3a mutant males. Efficient deletion of *Dnmt3a* by TNAP-Cre was reported previously (Kaneda *et al.*, 2004), and I confirmed this in my own P0-2 gonocyte preparations (Fig. 5). The results of the bisulphite sequencing studies are shown in Figure 6A, and summarized in Figure 6D. Methylation of the *H19* and *Dlk1/Gtl2* DMR was severely affected by the disruption of *Dnmt3a* in four and three independent gonocyte preparations, respectively. However, some partial methylation was detected in these DMRs and the degree of methylation was different from sample to sample. In contrast, methylation of the *Rasgrf1* DMR was not severely affected by the disruption of *Dnmt3a,* but again variable degree of methylation was observed in different germ cell preparation. This ranged from almost full methylation (which is the normal methylation level in wildtype P0 gonocytes) to intermediate levels of methylation. The results obtained here are basically consistent with our previous observations (Kaneda *et al.*, 2004). Therefore, I concluded that *Dnmt3a* is the major enzyme that methylates the *H19* and *Dlk1/Gtl2* DMRs. The results also suggest that not only *Dnmt3a*, but also other DNA methyltransferases might methylate the *Rasgrf1* DMR. The observed methylation variations between different germ cell preparations are partly caused by somatic cell contamination because the *Peg3* DMR, which is known to be unmethylated in P0 gonocytes, was partially methylated in two of the four preparations. However, the other two preparations with no methylation at all at the *Peg3* DMR also showed a variation at the *Rasgrf1* DMR, suggesting that at least some variation are real.

Possible role of *Dnmt3b* for methylation of the *Rasgf1* DMR

I next examined whether *Dnmt3b* contributes to the methylation of the paternally methylated DMRs in the male germline. Kaneda *et al.* previously investigated the methylation status of the paternally methylated DMRs in germline-specific *Dnmt3b* mutant spermatozoa and found that there are normally methylated. However, the methylation status of the DMRs in newborn gonocytes is unknown. So, I analyzed the methylation status of the paternally methylated

DMRs in P0-2 gonocytes isolated from germline-specific *Dnmt3b* mutant testes. Efficient deletion of *Dnmt3b* by TNAP-Cre was reported previously (Kaneda *et al.*, 2004), and I again confirmed this in my own P0-2 gonocyte preparations (Fig. 5). The results of the bisulphite sequencing studies are shown in Figure 6B, and summarized in Figure 6D. Methylation status of the *H19* and *Dlk1/Gtl2* DMRs was almost normal and nearly identical in all germ cell preparations, indicating that *Dnmt3b* is not required for methylation of these DMRs. In contrast, the methylation status of the *Rasgrf1* DMR was lower and varied from sample to sample as observed in the germline-specific *Dnmt3a* knockout P0-2 gonocytes. Because the methylation status of the *Peg3* DMR was extremely low in all germ cell preparations, it is unlikely that somatic contamination affected the results. Thus, the results suggest that *Dnmt3b* contributes to the methylation of the *Rasgrf1*DMR.

***Dnmt3L* plays a major role in the establishment of all paternally methylated DMRs**

Functional significance of *Dnmt3L* for methylation of the *H19* DMRs has been shown by the previous studies (Bourc'his and Bestor, 2004; Kaneda *et al.*, 2004; Webster *et al.*, 2005). However, the precise role of *Dnmt3L* for methylation of the other paternally methylated DMRs is still unclear. To investigate the role of *Dnmt3L* in more detail, I performed bisulphite methylation analysis on the *Dnmt3L*-deficient P0-2 gonocytes. The results of the bisulphite sequencing studies are shown in Figure 6C, and summarized in Figure 6D. The results showed that the methylation levels of all paternally methylated DMRs are severely decreased by the lack of *Dnmt3L*. Especially, methylation of the *Rasgrf1* DMR was hardly detectable at all four experiments. The results indicate that *Dnmt3L* is essential for the methylation of the *Rasgrf1* DMR. In the *H19* and *Dlk1/Gtl2* DMRs, substantial levels of methylation were still detected in all four germ cell preparations and the actual methylation status was different between the samples. In contrast, the methylation level of the *Peg3* DMR was extremely low in all germ

cell preparations. The results suggest that the partial methylation observed in these DMRs results from *Dnmt3L*-independent methyltransferase activity.

Discussion

In this study, I investigated the methylation status of the paternally methylated DMRs in fetal (E14.5, E16.5, and E18.5) and newborn (P0) gonocytes, and spermatogenic adult germ cells in wildtype male testes. The results demonstrated that the paternal methylation imprints are established in gonocytes and that the established methylation imprints are maintained through meiosis. I also investigated the methylation status of the DMRs in *Dnmt*-deficient P0-2 gonocytes. The results demonstrated that *Dnmt3a* is the major methylase to methylate the *H19* and *Dlk1/Gtl2* DMRs, and *Dnmt3L* plays a critical role in methylation of all paternally methylated DMRs. In addition, the results suggested that *Dnmt3b* also contributes to the methylation of the *Ragrf1* DMR and possibly the other two DMRs.

Establishment of the paternal methylation imprints in male germ cells

A major conclusion derived from my study is that the paternal methylation imprints at the three paternally methylated DMRs are almost fully established by the P0 gonocyte stage without significant differences in timing among the DMRs. A schematic representation of the timing of the establishment of the methylation imprints in the male germline is shown in Figure 7. The fact that the paternal methylation imprints are established in the mitotically arrested fetal gonocytes, contrasts with the process of the maternal methylation imprint establishment, which occurs during the meiotic prophase I in growing oocytes after birth (Obata *et al.*, 2002; Lucifero *et al.*, 2002; Lucifero *et al.*, 2004). In both germline, the establishment of the methylation imprints is correlated with genome wide methylation (Coffigny *et al.*, 1999; Lees-Murdock *et al.*, 2003). I also found that the paternally methylated DMRs are methylated in a nearly identical time course, which contrasts with the previous observation that the maternally methylated DMRs are methylated in different timing (Lucifero *et al.*, 2004).

Interestingly, the DMRs on the paternal chromosomes were methylated more quickly than those on the maternal chromosomes in fetal (E14.5-E18.5) gonocytes. The results are consistent with the previous observations on the *H19* DMR (Davis *et al.*, 2000; Ueda *et al.*, 2000). Because methylation levels of the DMRs are extremely low in E12.5 PGCs (Davis *et al.*, 2000; Lee *et al.*, 2002; Li *et al.*, 2004), it has been suggested that some parental difference other than DNA methylation such as chromatin structure may be retained on the parental chromosomes. As a result, the DMRs on the paternal chromosome may be more accessible to the methyltransferases than those on the maternal chromosome. However, because substantial levels of methylation was detected at the *Dlk1/Gtl2* DMR in E12.5 PGCs (Li *et al.*, 2004), the paternal-specific methylation observed at this DMR in E14.5 gonocytes could be due to preexisted methylation. Although the molecular mechanism causing the parental chromatin difference is unknown, an insulator binding protein CTCF may be involved. It was demonstrated that CTCF binds to the unmethylated *H19* DMR on the maternal chromosome and regulates the imprinting of *H19* and *Igf2* (Bell *et al.*, 2000; Hark *et al.*, 2000) and that abrogation of CTCF in oocytes causes methylation of the normally unmethylated *H19* DMR (Fedoriw *et al.*, 2004). Thus, CTCF can protect unmethylated DMRs from methylation in oocytes. Whether CTCF is expressed in fetal gonocytes and whether it bind to the other DMR are future questions.

Davis *et al.* reported that the *H19* DMR on the maternal chromosome is methylated only slightly in the fetal gonocytes and that hypermethylation is not observed until pachytene spermatocyte stage (Davis *et al.*, 1999; Davis *et al.*, 2000). However, Ueda *et al.* reported that the *H19* DMR on the maternal chromosome is almost fully methylated in P0 gonocytes (Ueda *et al.*, 2000). My study strongly supports the latter finding. Because the CpG sites investigated in Davis *et al.* are almost identical with those investigated in this study, the observed difference did not arise from a difference in the region analyzed. One possible explanation for the difference is the mouse strains used for the analyses. To distinguish the parental alleles,

Davis *et al.* used mice with one *H19* allele derived from *M.m.castaneus* (Tremblay *et al.*, 1995), while Ueda *et al.* and I used *M. m. molossinus* JF1 mice to make (C57BL/6J×JF1) hybrids.

Roles of *Dnmt3a* and *Dnmt3b* in the establishment of the paternal methylation imprints

A previous report showed that *Dnmt3a* is required for methylation of the *H19* and *Dlk1/Gtl2* DMRs in the male germline (Kaneda *et al.*, 2004). I obtained basically the same results regarding this enzyme. Interestingly, the actual degree of methylation at these DMRs was different from sample to sample. It is possible that a residual amount of *Dnmt3a* was present at the early gonocyte stage or that the other methyltransferase such as *Dnmt3b* might contribute to the methylation. Methylation of the *Rasgrf1* DMR in the P0-2 gonocytes from the germline-specific *Dnmt3a* mutant was not severely affected, and again, variations were observed among different samples. The results indicate that the role of *Dnmt3a* is minimal in the methylation of this DMR, suggesting an equally important role for *Dnmt3b*. Indeed, methylation analysis of the paternally methylated DMRs in P0-2 gonocytes from the germline-specific *Dnmt3b* mutant showed that the *Rasgrf1* DMR is significantly demethylated. The results suggest that *Dnmt3b* is involved in the methylation of the *Rasgrf1* DMR, although methylation levels at the *H19* and *Dlk1/Gtl2* DMRs in the *Dnmt3b*-deficient gonocytes were normal.

It was previously shown that methylation level of the genome is dramatically increased in later gonocyte stages from the observations of the methylation status of repetitive elements and of the immunocytochemistry using anti-5-methylcytosine antibody (Coffigny *et al.*, 1999; Lees-Murdock *et al.*, 2003). Although all *Dnmts* are expressed in the fetal testis (La Salle *et al.*, 2004), their cellar localization is different. *Dnmt3a* and *Dnmt3b* are nuclear-localized in E17.5 gonocytes, while they are only slightly detectable in E15.5 gonocytes (Lees-Murdock *et al.*, 2005). In contrast, *Dnmt1* is not localized in the nucleus of

E18.5 gonocytes while it is nuclear-localized in E11.5 and E13.5 PGCs (La Salle *et al.*, 2004). These observations are in accordance with my observation that not only *Dnmt3a* but also *Dnmt3b* may acts on the methylation during gonocyte stage. To clarify the role of *Dnmt3a* and *Dnmt3b* for methylation of the paternally methylated DMRs, it is necessary to investigate methylation status of the DMRs in the *Dnmt3a* and *Dnmt3b* double knockout germ cells.

Role of *Dnmt3L* in the establishment of the paternal methylation imprints

Methylation of all paternally methylated DMRs was severely affected in *Dnmt3L*-deficient P0-2 gonocytes, suggesting a functional significance of *Dnmt3L* in the establishment of the paternal methylation imprints. This may appear to be inconsistent with the previous reports that the *Dlk1/Gtl2* and *Rasgrf1* DMRs in the *Dnmt3L*-deficient spermatogonia (Bourc'his and Bestor, 2004; Kaneda *et al.*, 2004). However, it was recently found that the maternally methylated DMRs sometimes retain the normal methylation imprints in the fetuses derived from the cross of *Dnmt3L*-deficient females and wildtype males (Arnaud *et al.*, in press). Thus, the action of *Dnmt3L* on the DMRs may be stochastic. This could explain the discrepancy.

Dnmt3L is abundantly expressed in the fetal testis but its expression is dramatically reduced after birth (La Salle *et al.*, 2004). This dynamic change in expression is well correlated with the timing of the establishment of the paternal methylation imprints and genome wide methylation. Interestingly, while methylation at the *Rasgrf1* DMR was hardly detectable in P0-2 gonocytes from the *Dnmt3L* mutants, substantial methylation was observed at the *H19* and *Dlk1/Gtl2* DMRs. It was shown that *Dnmt3L* interacts with both *Dnmt3a* and *Dnmt3b in vivo* (Hata *et al.*, 2002), and that *DNMT3L*, a human homologue of mouse *Dnmt3L*, stimulates the *de novo* methylation activity of mouse *Dnmt3a* and *Dnmt3b in vitro* (Chedin *et al.*, 2002; Suetake *et al.*, 2004). Therefore, residual methylation observed at the *H19* and *Dlk1/Gtl2* DMRs in the *Dnmt3L*-deficient P0-2 gonocytes might be explained by the basal activities of the *de novo* methyltransferases in the absence of *Dnmt3L*. The different

methylation levels observed among the DMRs might reflect the difference in the mechanism that methylates the DMRs. Its details are future question.

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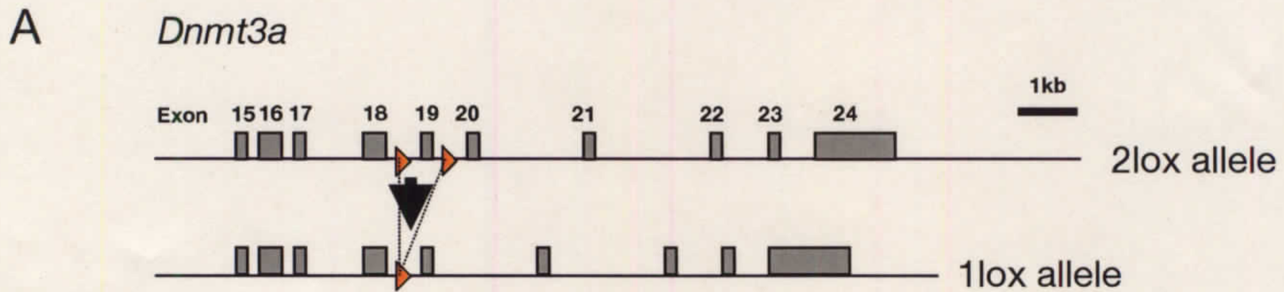
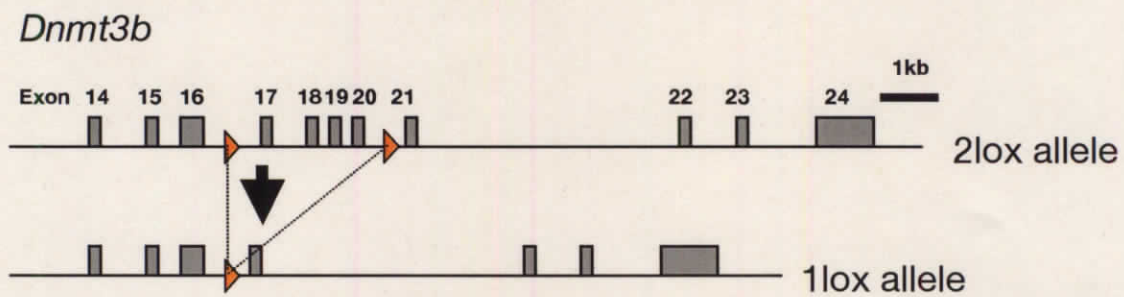
Table 1. Purity of germ cell preparations

Stage	Number of AP* positive cells	Total cell count	% of AP* positive cells
E14.5	192	215	89.3
E16.5	239	250	95.6
E18.5	210	234	89.7
P0	244	274	89.1

*AP, alkaline phosphatase





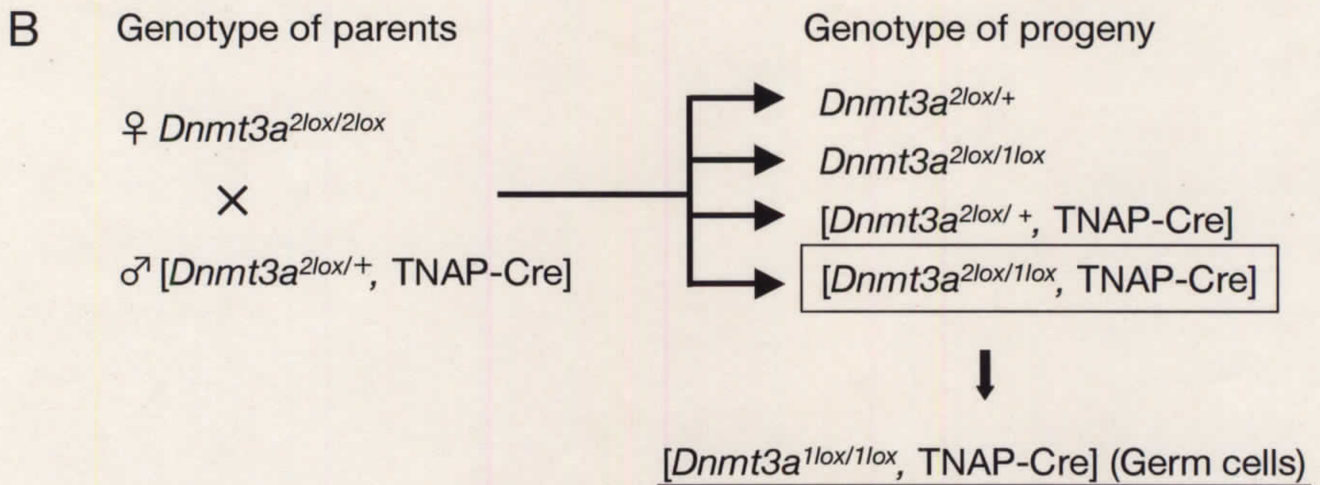


Figure 1. Production of germline-specific *Dnmt3a* and *Dnmt3b* knockout mice

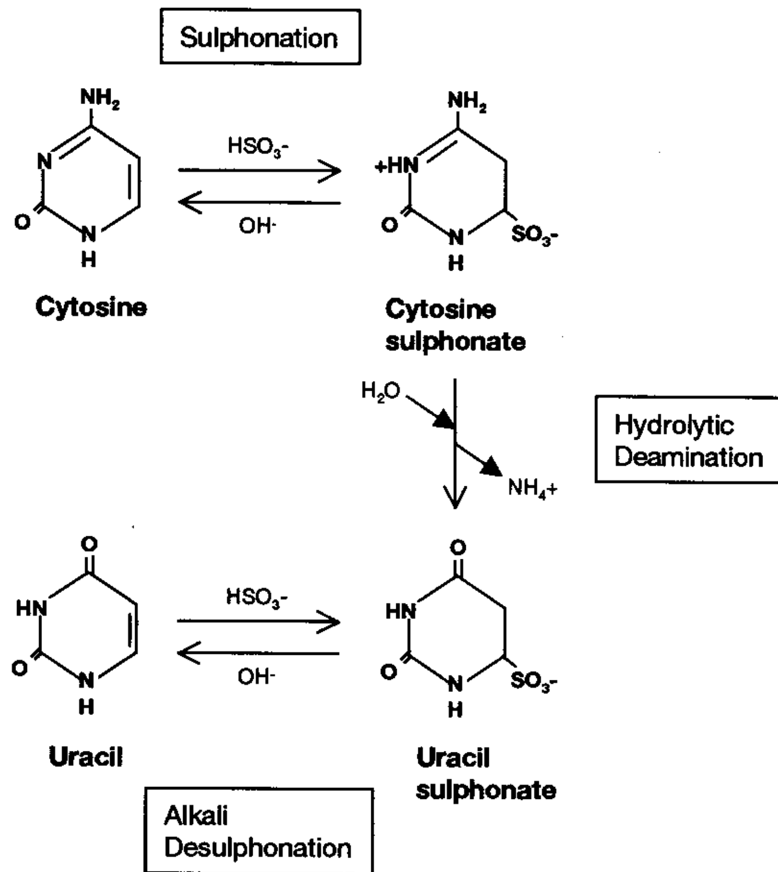
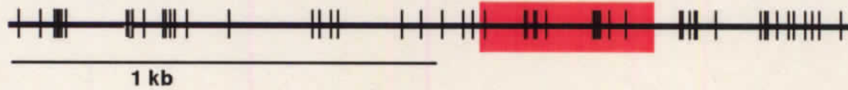


Figure 2. Principle of bisulphite reaction

H19 DMR

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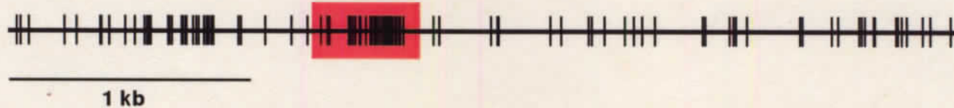
2594     BisOF1
B6   TTGTGAGTGG AAAGACCAAA TTGCTGCGCT GGTGACTGTC ATCTTAAACA TTATGTTCCA GAGACAGCCA AAGTTAAGGT TTGCCCATGA
JF1  TTGTGAGTGG AAAGACCAAA TTGCTGCGCT GGTGACTGTC ATCTTAAACA TTATGTTCCA GAGACAGCCA AAGTTAAGGT TTGCCCGTGA
                               1                                                                 2
B6   CAATGTCCAA GGGCCAAAGT TCGGGTTCGC CCACAGCAAT GTCCGAAGCC GCTATGCCTC AGTGGTCGAT ATGGTTTATA AGAGGTTGGA
JF1  CAATGTCCAA GGGCCAAAGT TCGGGTTCGC CCACAGCAAT GTCCGAAGCC GCTATGCCTC AGTGGTCGAT ATAGTTTATA AGAGGTTGGA
                            3    4                    5      6
B6   ACACTTGTGT TTCTGGAGGG GGTCCCTTTG GTCACTGAAC CCCAAAACCA GCCAGTGTGG CTCACTATAG GAAGGCATAG AAGCTGTTAT
JF1  ACACTTGTGT TTCTGGAGGG GGTCCCTTTG GTCACTGAAC CCCAAAACCA GCCAGTGTGG CTCACTATAG GAAGGCATAG AAGCTGTTAT

B6   GTGCAACAAG GGAACGGATG CTACCGCGCG GTGGCAGCAT ACTCCTATATA TCGTGGCCC AAATGCTGCC AACTTGGGGG GAGCGATTCA
JF1  GTGCAACAAG GGAACGGATG CTACCGCGCG GTGGCAGCAT ACTCCTATATA TCGTGGCCC AAATGCTGCC AACTTGGGGG GAGCGATTCA
                     7         8 9 10                       11                                  12
B6   TTCCCAGCAA TATCCCAGGG TCACCCAAAT AGGGATTCAT AGGGGTGGTAA GATGTGTGC ACC
JF1  TTCCCAGCAA TATCCCAGGG TCACCCAAAT AGGGATTCAT AGGGGTGGTAA GATGTGTGC ACC
                                              BisIR1       BisOR1 3016
```

Dlk1/Gtl2 DMR




```
81038      IG-F1
B6   CTGCAATTCA CGGTATATGA GTCCTATCAT CCTGTATGTG CACAGAGATA TGTCTATATG GCACCATGCA GCCATTTTAT AGTACACGCT
JF1  CTGCAATTCA CGGTATATGA GTCCTATCAT CCTGTATGTG CACAGAGATA TGTCTATATG GCACCATGCA GCCATTTTAT AGTACACGCT
                   BisIGF2
B6   ATATTTGTGC TAAGGTACAT CATGCTAGTG TTAGGAAGGA TTGTGAATCT ATACGGAGAT GTGCTGTGGA CCCAGGCTGC AGTTCACGAT
JF1  ATATTTGTGC TAAGGTACAT CATGCTAGTG TTAGGAAGGA TTGTGAATCT ATACGGAGAT GTGCTGTGGA CCCAGGCTGC AGTTCACGAT
                         BisIGF3                              1                                 2
B6   CGACTAGTAC ACAGGCTGAC CATGTACAAG TGCTGTGGTT TGTCATGGGC AAGTCCCATG GCTTACTGTA CACAATGCTG CCGTTCGCTA
JF1  CGACTAGTAC ACAGGCTGAC CATGCACAAG TGCTGTGGTT TGTCATGGGC AAGTCCCGTG GCTTACTGTA CACAATGCTG CCGTTCGCTA
     3                                                           4                              5   6
B6   TGAACTACCG CTACGGTTCA TAGTGGACAG TCAGTGCCGC AGATCGCTAT GGACTGGTGC CAAGGTTCGC CATGGACTAG TGCCGCGGAC
JF1  TGAACTACCG CTACGGTTCA TAGTGGACAG TCAGTGCCGC AGATCGCTAT GGACTGGTGC CAAGGTTCGC CATGGACTAG TGCCGCGGAC
             7    8                         9       10                       11                 1213
B6   CTCCGTGAAC TAGCGAGGAG GTTCGCCGTG TACTAATGCC GCTTCGCGTA CCGCTGTGTA CGCGTGCCGC GAACCGCCGT GGAATTGTGC
JF1  CTCCGTGAAC TAGCGAGGAG GTTCACCGTG TACTAATGCC GCTTCGCGTA CCGCTGTGTA CGCGTGCCGC GAACCGCCGT GGAATTGTGC
        14        15         16 17            18      1920    21         2223     24 25    26  27
B6   CGCGGTTCGC CGTGGAGTAG CGCTGCAGCC GCTATGCTAT GCTGTTTCTT TCTTTTCCTT AACTCCTGGA GTGAGGGAAG GGCTGCATTA
JF1  CGCGGTTCGC CGTGGAGTAG CGCTGCAGCC GCTATGCTAT GCTGTTTCTT TCTTTTCCTT AACTCCTGGA GTGAGGGAAG GGCTGCATTA
     2829   30 31         32         33                                                  BisIGR2
B6   TTTTGTCAAT GGAGAATGCC TTGAGCACAG GGGATGGCTA AAACATTCTC ACAGATTGGG AATGGGATCA CGCGAGTAAG AGGCTGTCCT
JF1  TTTTGTCAAT GGAGAATGCC TTGAGCACAG GGAATGGCTA AAACATTCTC ACAGATTGGG AATGGGATCA CGCGAGTAAG AGGCTGTCCT

B6   CTCCGGTGCT GTGACCATAC AGACTGTAGT TTAGCTTTGG AATTCCTGAT GGAATCTGGT ACTTGCAG
JF1  CTCCGGTGCT GTGACCATAC AGACTGTAGT TTAGCTTTGG AATTCCTGAT GGAATCTGGT ACTTGCAG
                                                             IG-R1      81735
```

Figure 3. Sequences of the DMRs analyzed in this study

Rasgrf1 DMR

100 bp

```
170941      BisRGF1                                  BisRGF2
B6   GAGAGTATGT AAAGCCAGAG CTGTGCTGCT GCCGCTAAAG ATAGTTTAGA TATGGAATTC TGGGGACTCT TCAGAGAGTT TATAAAGCCA
JF1  GAGAGTATGT AAAGCCAGAG CTGTGCTGCT GCCGCTAAAG ATAGTTTAGA TATGGAATTC TGGGGACTCT TCAGAGAGTT TATAAAGCCA

B6   GCGCTGTGCT GCTGCCGCGC TTCGCGGCTG CACTTCGCTA CCGTTTCGCG GCCGCGCTGC TGCTCCCACA TCCATCCGTG GCTACCGCTA
JF1  GCGCTGTGCT GCTGCCGCGC TTCGCGGCTG CACTTCGCTA CCGTTTCGCG GCCGCGCTGC TGCTCCCACG TCCATCCCTG GCTACCGCTA
      1              2 3     4 5         6         7     8 9    10 11              12        13        14
B6   TTGCTGTTGC TGCACCGCTG CCGCTAAGCT ATGGCTGCCG CACTTCACTG TTGCGCTACC GCTGCGCTAC AACTACCACG ACTGCTACTG
JF1  TTGCTGTTGC TGC------- ---------T GTGGCTGCCG CACTTCACTG TTGCGCTACC GCTGCGCTAC AACTACCACG ACTGCTACTG
                    15        16               17                    18       19     20            21
B6   CTGCTGCTGC ACTAC
JF1  CTGCTGCTGC ACTAC
       BisRGR2     171226
```

Peg3 DMR

1 kb

```
2674              Peg3-F2
B6   TTGACAACAG CAGTCTGATT GGCAGGGTGT GGGAGGCGTG GTGAGGGCCC AAAGCGGGGA ATGGGGTCTT GGATTGGTTA GAGAGGAAGC
JF1  TTGACAACAG CAGTCTGATT GGCAGGGTGT GGGAGGCGTG GTGAGGGCCC AAAGCGGGGA ATGGGGTCTT GGATTGGTTA GAGAGGAAGC
                         Peg3-F4
B6   TCCGCCTCTG CAGAGGACCC TGACAAGGAG GTGTCCCGCA GCCCTTGCTG CAGACGCTGG GGAGTCAGGA GTCGCGGGAG GACGAGCATC
JF1  TCCGCCTCTG CAGAGGACCC TGACAAGGAG GTGTCCCGCA GCCCTTGCTG CAGACGCTGG GGAGTCAGGA GTCGCGGGAG GACGAGCATC
                                              1                    2                    3 4         5       6
B6   GGAGGAGAAG CGGAGAGATG TCCACCCTGG GCTGGTGGCG CCGCCGGGCG CCCGGTTCAG TGTGGGTGCA CTAGACTGCC GACCCTGGTC
JF1  GGAGGAGAAG CGGAGAGATG TCCACCCTGG GCTGGTGGCG CCGCCGGGCG CCCGGTTCAG TGTGGGTGCA CTAGACTGCC GACCCTGGTC
                7                             8    9  10    11    12                            13        14
B6   GGGGTGTGTG CGTAGAGTGC TGTGCTCCGG GAGGTGAGTC AGCCGGCCAC CTGGCTGCTC TGCAGCATGC ACCCTCTTAG ATACCGTCTG
JF1  GGGGTGTGTG CGTAGAGTGC TGTGCTCCGG GAGGTGAGTC AGCCGGCCAC CTGGCTGCTC TGCAGCATGC ACCCTCTTAG ATACCGTCTG
                15                   16             17                                           18
B6   CAGAGTTCAG ATGGTGTTTG GGGTGCGTTG CCGCGGGCCA GGGGCGGCAG ACCATATCAC GGCTCCCAAG GGTAACTGAC AAGGCTGCAG
JF1  CAGAGTTCAG ATGGTGTTTG GGGTGCGTTG CCGCGGACCA GGGGCGGCAG ACCATATCAC GGCTCCCAAG GGTAACTGAC AAGGCTGCAG
                                     19     20 21        22               23
B6   AC                                                                                  Peg3-R2
JF1  AC
3125
```

Figure 3. continued

E14.5 E16.5 E18.5 P0

#1

B6 (Mat)

JF1 (Pat)

#2

B6 (Mat)

JF1 (Pat)

Figure 4A. Methylation changes at the *H19* DMR in fetal gonocytes

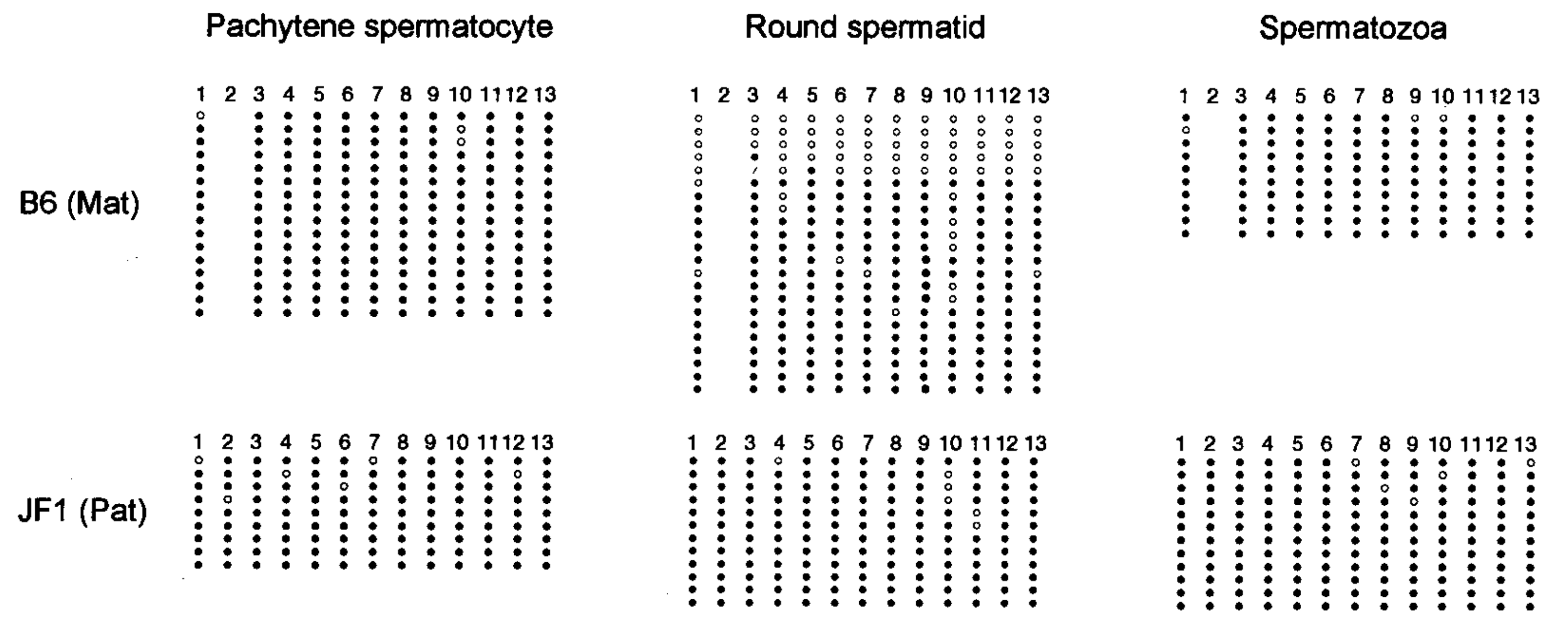


Figure 4B. Methylation changes at the *H19* DMR in meiotic and haploid male germ cells

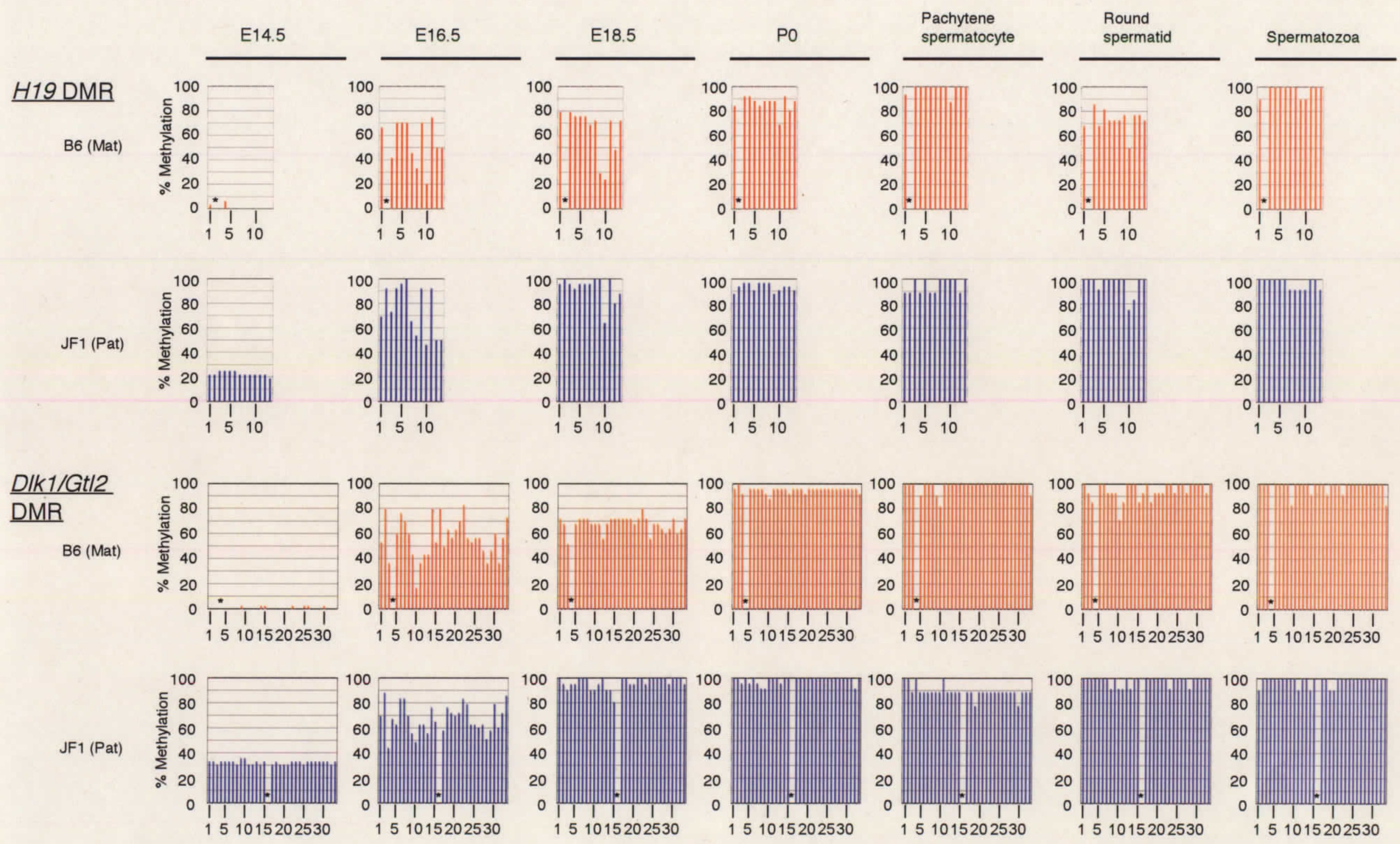


Figure 4C. Methylation changes at the DMRs in wildtype male germ cells

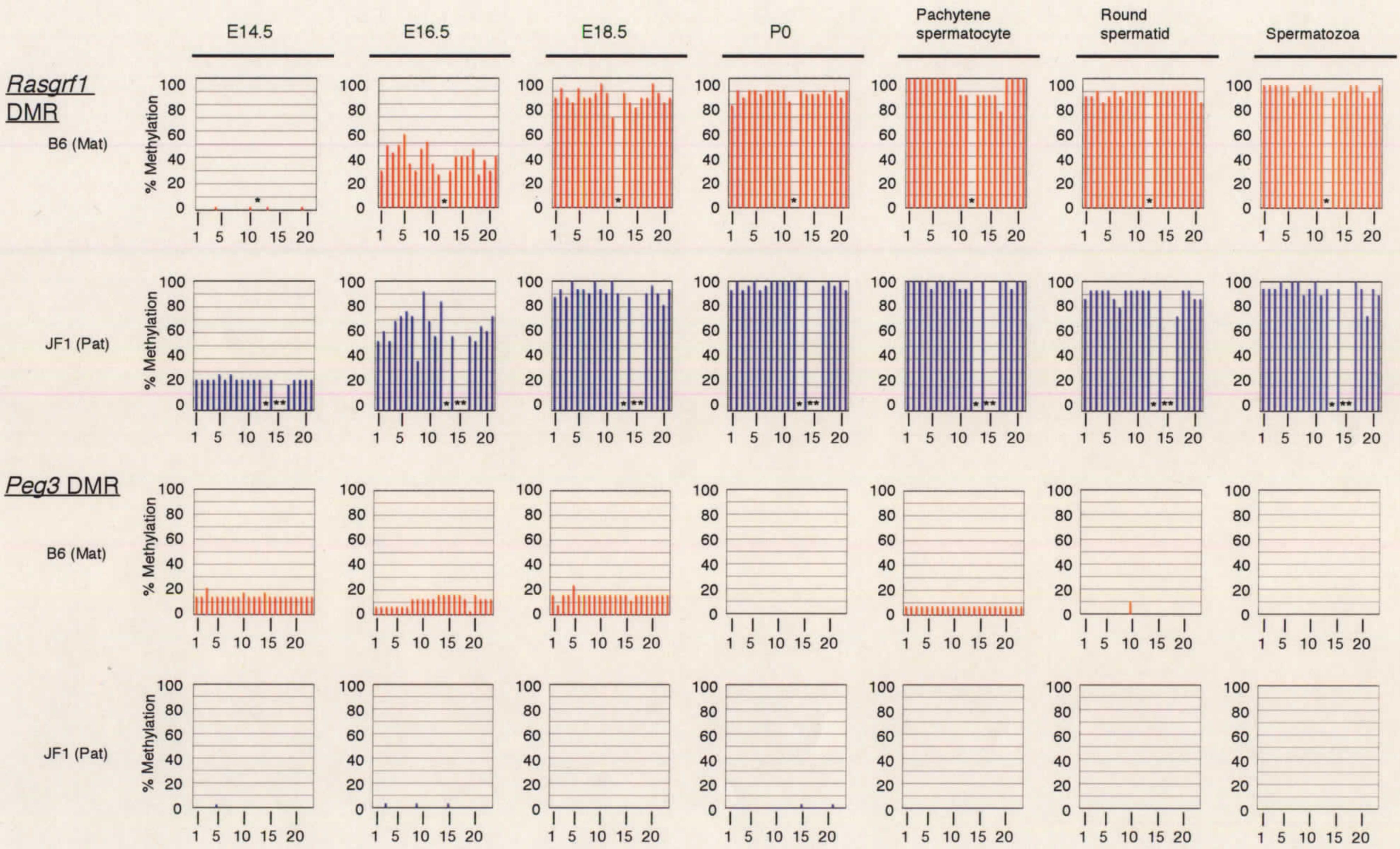
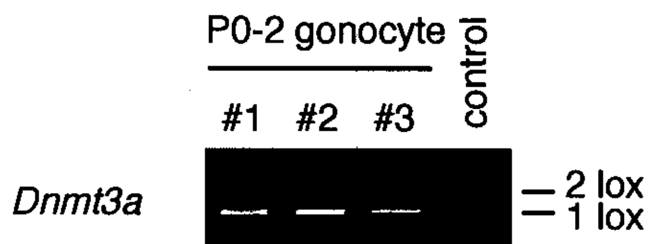


Figure 4C. Continued



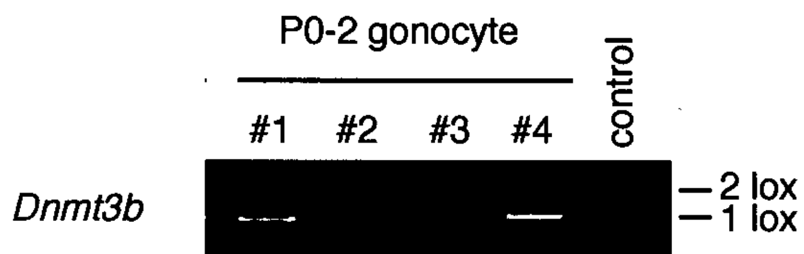


Figure 5. Efficiency of TNAP-Cre

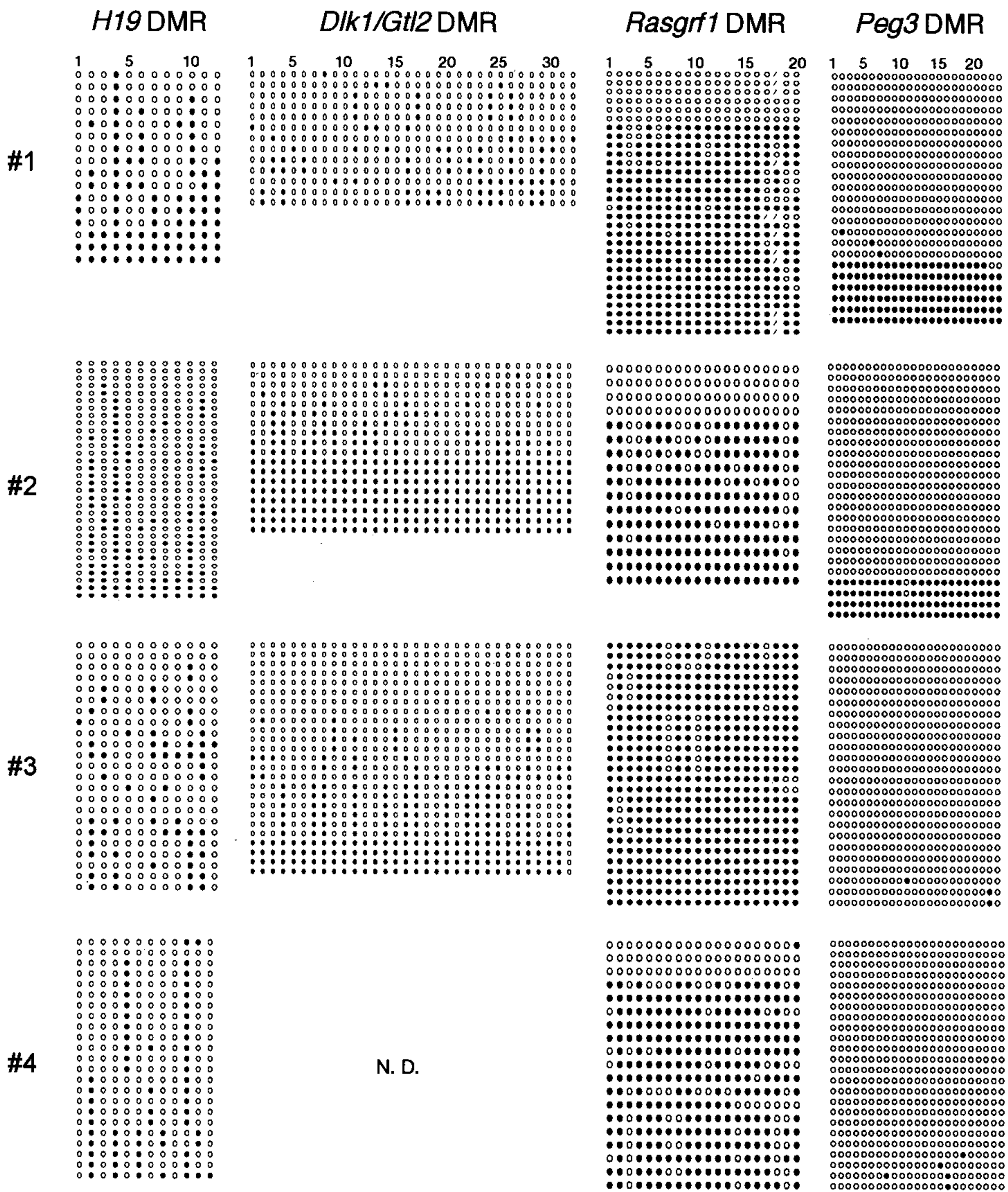


Figure 6A. Methylation analysis of P0-2 gonocytes from [*Dnmt3a*$^{2lox/1lox}$,TNAP-Cre] mice

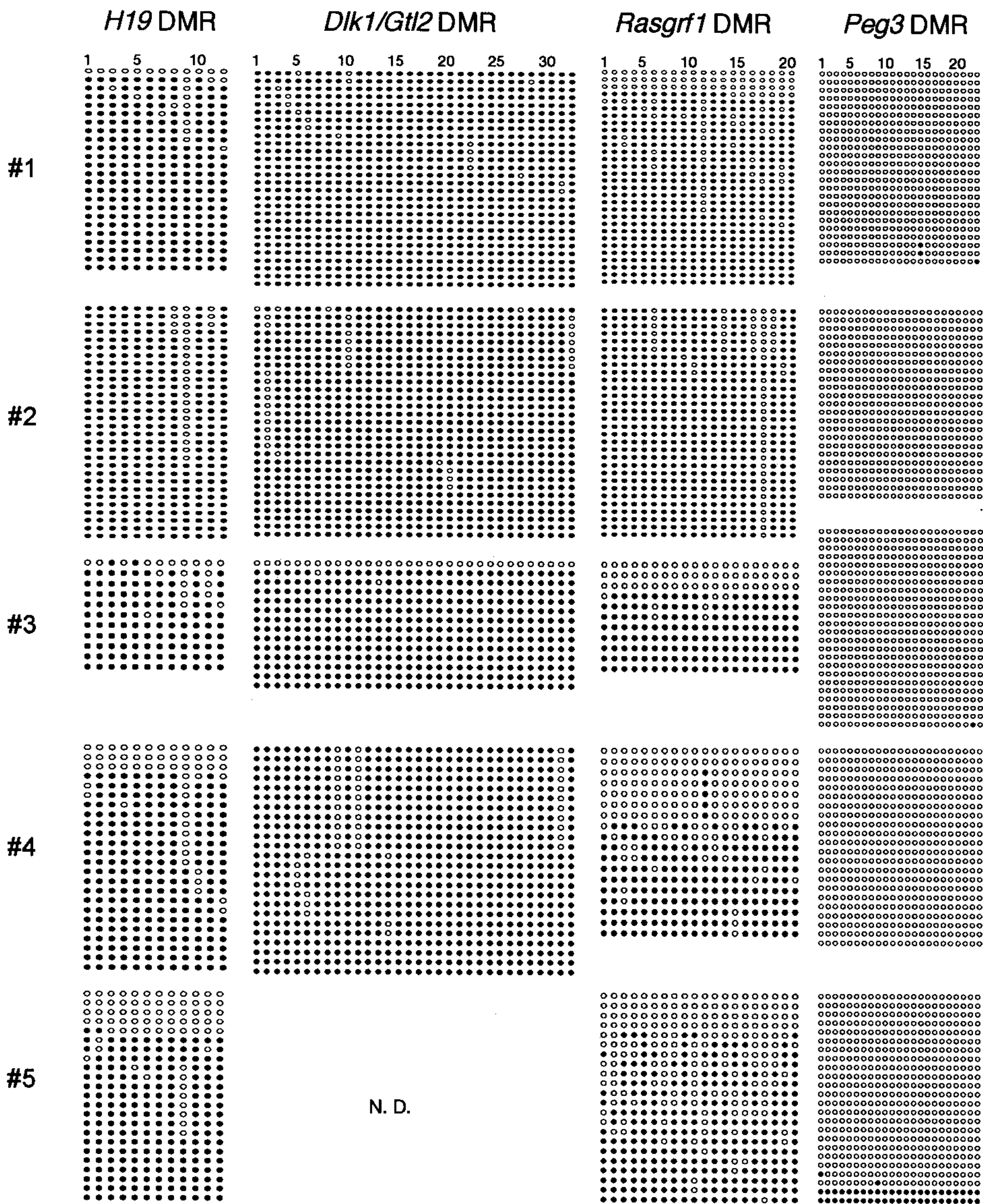


Figure 6B. Methylation analysis of P0-2 gonocytes from [*Dnmt3b*$^{2lox/1lox}$,TNAP-Cre] mice

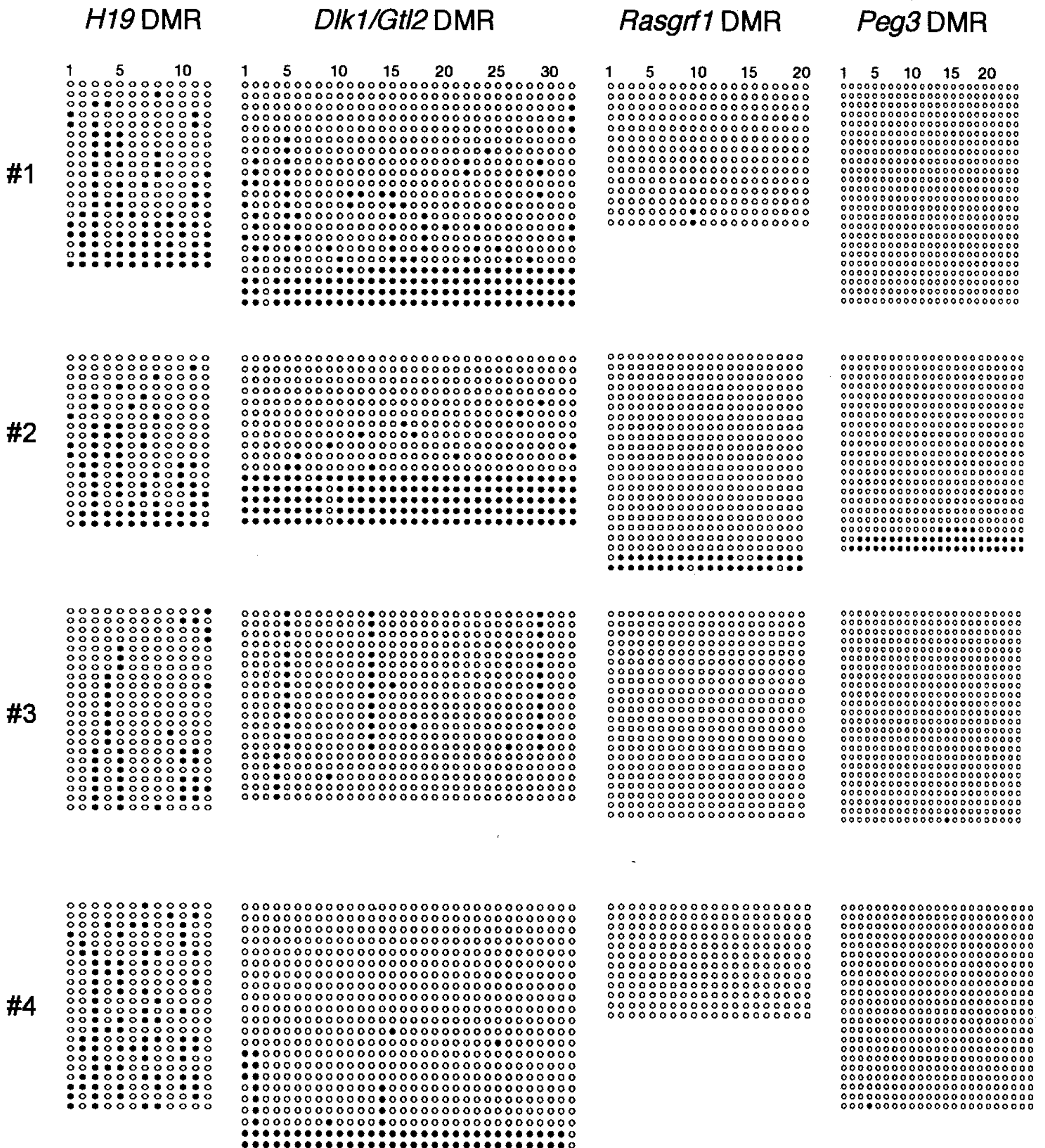


Figure 6C. Methylation analysis of P0-2 gonocytes from *Dnmt3L$^{-/-}$* mice

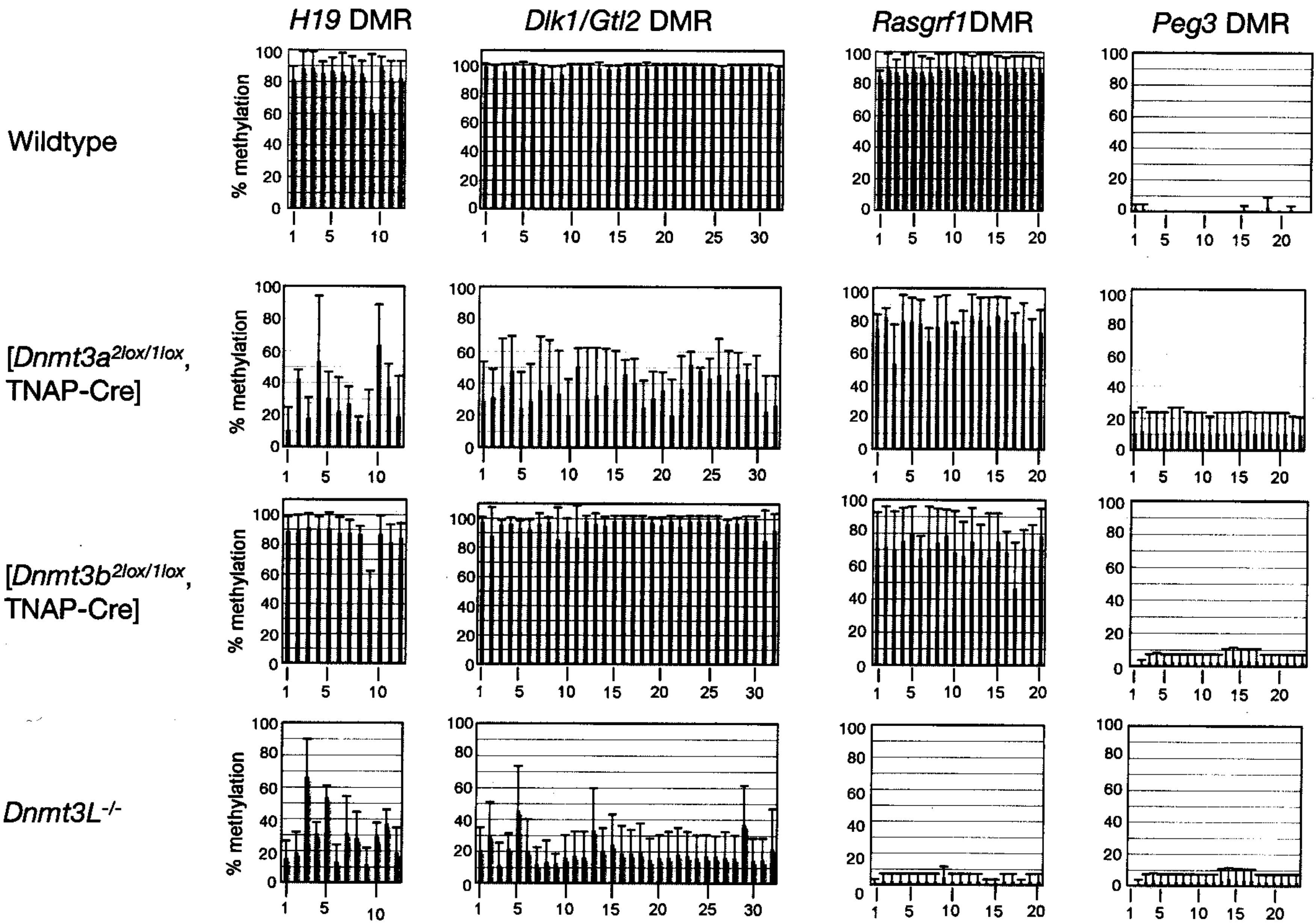


Figure 6D. Summary of the methylation analysis in *Dnmt*-deficient male germ cells

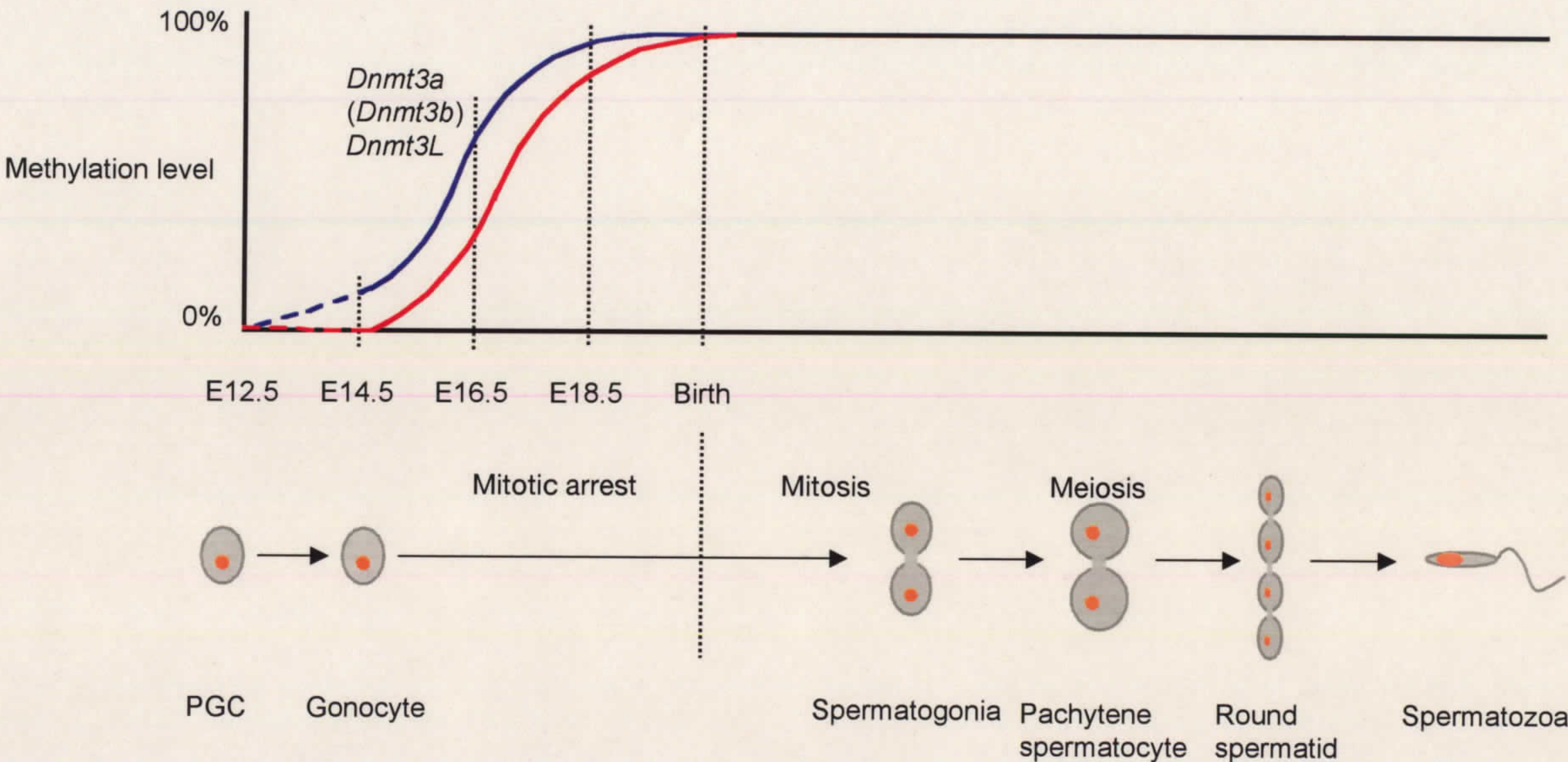
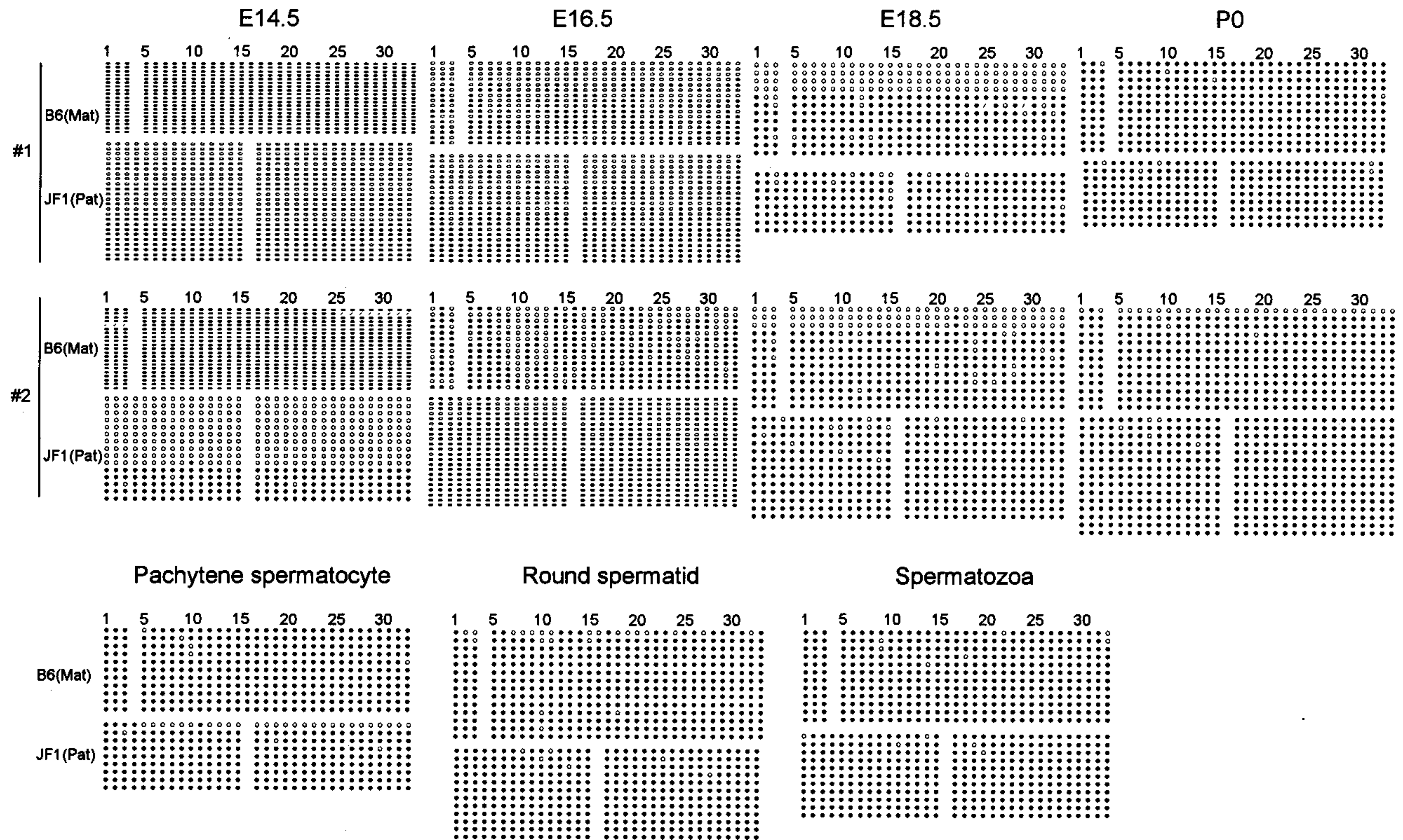
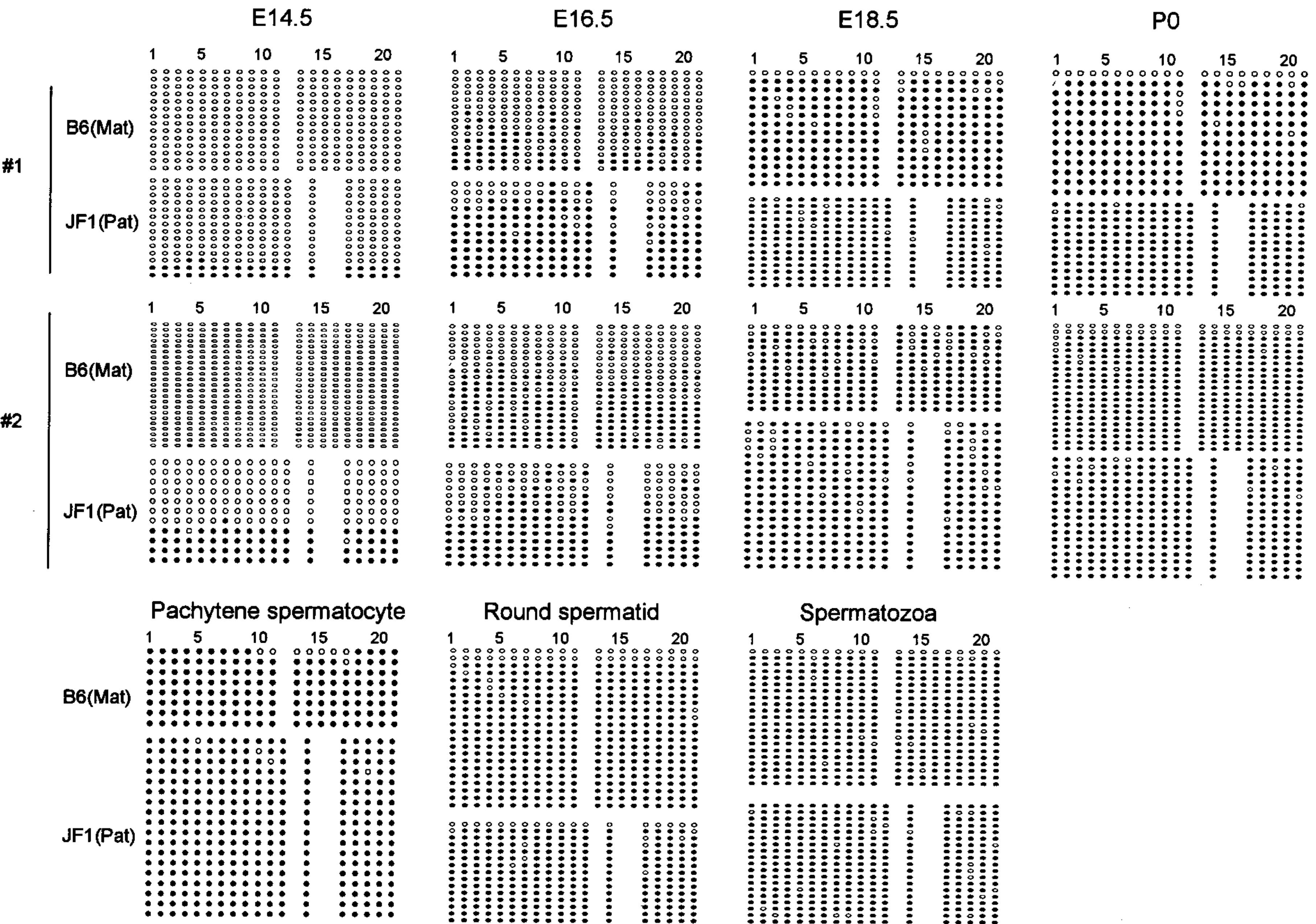


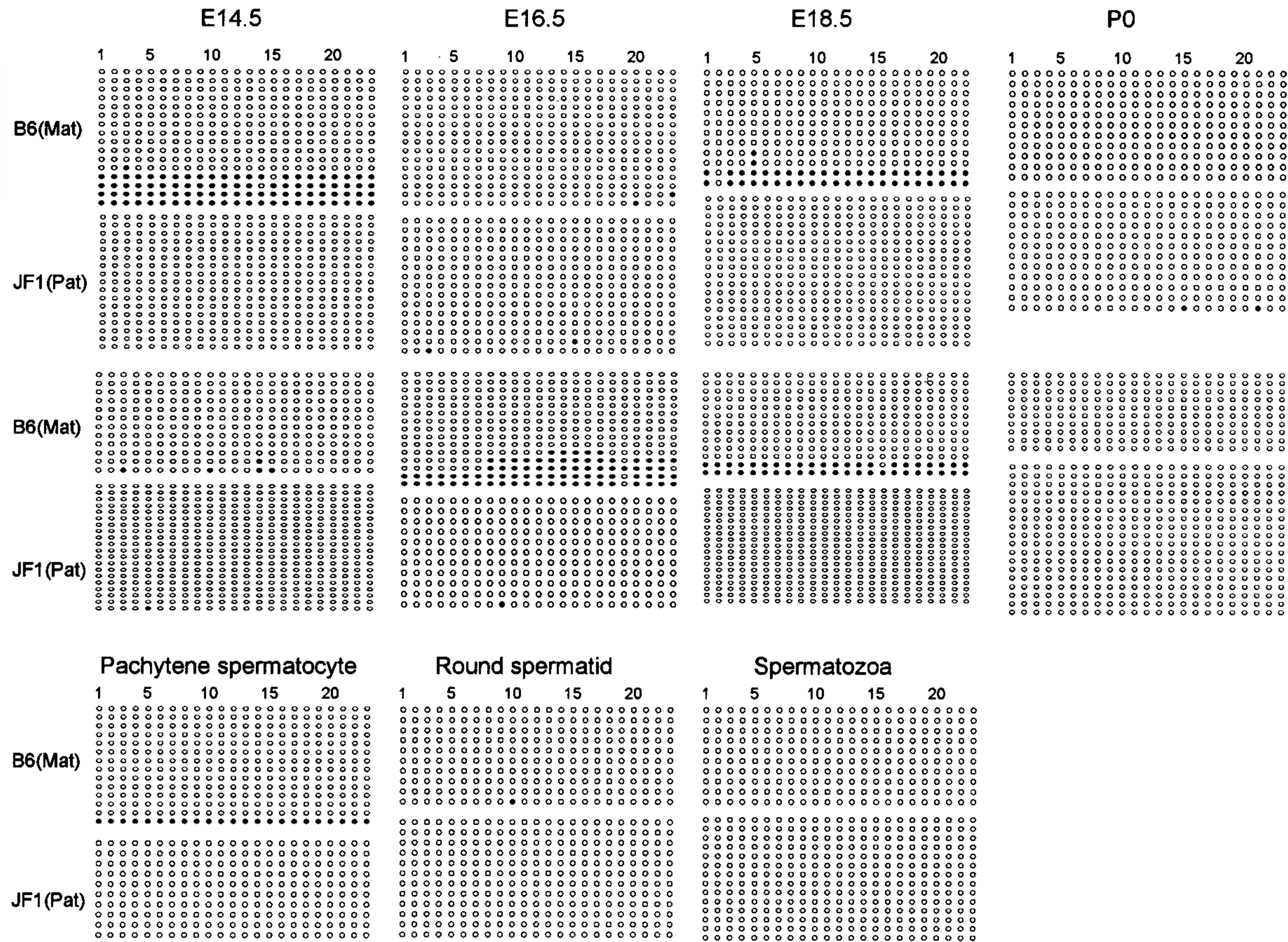
Figure 7. Schematic representation of the establishment of the paternal methylation imprints



Supplementary Figure 1A. Methylation changes at the *Dlk1/Gtl2* DMR in male germ cells



Supplementary Figure 1B. Methylation changes at the *Rasgrf1* DMR in male germ cells



Supplementary Figure 1C. Methylation changes at the *Peg3* DMR in male germ cells