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Role of the Dnmt3 family in *de novo* methylation of imprinted and repetitive sequences during male germ cell development in the mouse

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DNA methylation is an important epigenetic modification regulating various biological phenomena, including genomic imprinting and transposon silencing. It is known that methylation of the differentially methylated regions (DMRs) associated with paternally imprinted genes and of some repetitive elements occurs during male germ cell development in the mouse. We have performed a detailed methylation analysis of the paternally methylated DMRs (*H19*, *Dlk1/Gtl2* and *Rasgrf1*), interspersed repeats [SineB1, intracisternal A particle (IAP) and Line1] and satellite repeats (major and minor) to determine the timing of this *de novo* methylation in male germ cells. Furthermore, we have examined the roles of the *de novo* methyltransferases (Dnmt3a and Dnmt3b) and related protein (Dnmt3L) in this process. We found that methylation of all DMRs and repeats occurred progressively in fetal prospermatogonia and was completed by the newborn stage. Analysis of newborn prospermatogonia from germline-specific *Dnmt3a* and *Dnmt3b* knockout mice revealed that Dnmt3a mainly methylates the *H19* and *Dlk1/Gtl2* DMRs and a short interspersed repeat SineB1. Both Dnmt3a and Dnmt3b were involved in the methylation of *Rasgrf1* DMR and long interspersed repeats IAP and Line1. Only Dnmt3b was required for the methylation of the satellite repeats. These results indicate both common and differential target specificities of Dnmt3a and Dnmt3b *in vivo*. Finally, all these sequences showed moderate to severe hypomethylation in Dnmt3L-deficient prospermatogonia, indicating the critical function and broad specificity of this factor in *de novo* methylation.

INTRODUCTION

Methylation of cytosine residues is the only known epigenetic modification of the mammalian genomic DNA, and this modification is involved in various biological phenomena (1,2). The addition of methyl groups to cytosine residues in DNA is catalyzed by DNA methyltransferases (Dnmts). The methylation activity can be classified into two types: the maintenance activity and *de novo* activity. The maintenance methylation occurs at unmethylated cytosines of hemimethylated CpGs after DNA replication; Dnmt1 is responsible for this activity (3,4). The *de novo* methylation occurs at cytosines of

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unmethylated CpGs; Dnmt3a and Dnmt3b are the enzymes responsible for this activity (5). The biological significance of DNA methylation in mammals has been demonstrated by the embryonic and postnatal lethality of *Dnmt* knockout mice (6,7).

Genomic imprinting is a mammalian-specific epigenetic phenomenon that involves DNA methylation (8,9). Imprinted genes show parental-origin-dependent monoallelic expression, which is controlled by nearby differentially methylated regions (DMRs) (10–15). The allele-specific methylation at the DMRs is established in the parental germline, passed on to the zygote and stably maintained in somatic cells throughout development. However, the methylation imprints are erased in primordial germ cells (PGCs) and re-established during male and female gametogenesis before being passed on to the next generation (16–18). The timing of the establishment of the germline-specific methylation imprints is different between the two sexes. In the female germline, the methylation imprints are established in the postnatal ovary during the oocyte growth phase, corresponding to meiotic prophase I (19–21). The establishment process occurs earlier in the male germline. The *de novo* methylation of the *H19* DMR begins in mitotically arrested fetal prospermatogonia or gonocytes and may or may not continue to the postnatal stages (22–24). Methylation of the other two paternally methylated DMRs (*Dlk1/Gtl2* and *Rasgrf1*) also begins in fetal prospermatogonia (18), but the details of its progression and completion have not been reported.

A defect in the establishment of the germline-specific methylation imprints was first observed in mice deficient for Dnmt3-like protein (Dnmt3L), which belongs to the Dnmt3 family but has no methyltransferase activity (25,26). Hypomethylation of the maternally methylated DMRs was detected in both Dnmt3L-deficient oocytes and heterozygous offspring. More recently, germline-specific gene knockout studies showed that Dnmt3a is essential for the establishment of the maternal methylation imprints (9). However, the results on the establishment of the paternal methylation imprints in Dnmt3a-deficient spermatogonia were rather complicated. They showed extreme hypomethylation at the *H19* and *Dlk1/Gtl2* DMRs but only a slight reduction in methylation at the *Rasgrf1* DMR (9). Also, severe hypomethylation was observed only at the *H19* DMR in the *Dnmt3L*$^{-/-}$ spermatogonia (9,27), which was at variance with another report that described a hypomethylation at the *Rasgrf1* DMR (28). In these studies, germ cells were prepared at different stages by different methods, so it was difficult to correlate these data.

We have now carried out detailed methylation analysis to resolve the current confusion about the establishment of the paternal methylation imprints. It is shown that, in wild-type male germ cells, the methylation imprints of all paternally methylated DMRs are established in prospermatogonia by the newborn stage. We also find that the interspersed repeats and satellite repeats become highly methylated during the fetal prospermatogonia stage. We have then investigated the roles of Dnmt3a, Dnmt3b and Dnmt3L in the *de novo* methylation of the DMRs and repetitive elements in newborn prospermatogonia isolated by our two-step method. We show that sequence-dependent methylation mechanisms involving different Dnmt3 family proteins operate in prospermatogonia.

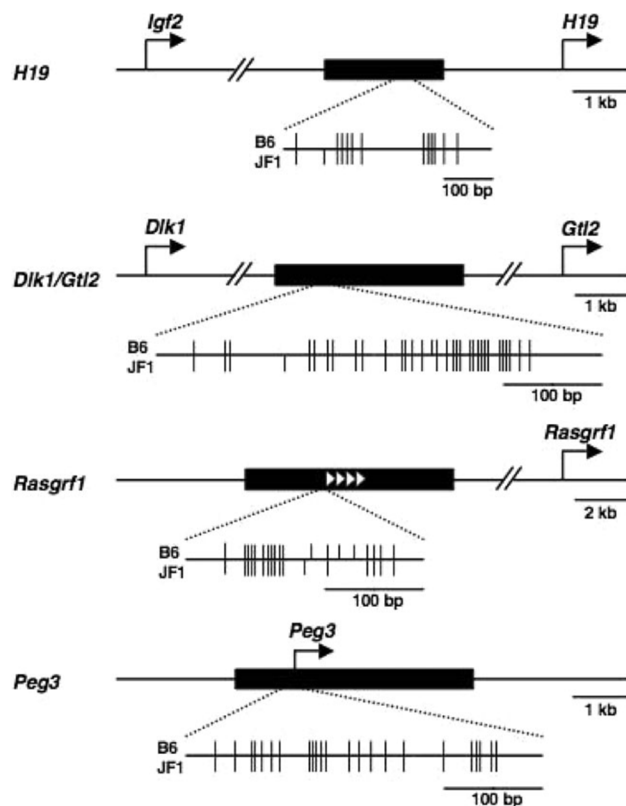

Figure 1. Schematic representation of the three paternally methylated DMRs (*H19*, *Dlk1/Gtl2* and *Rasgrf1*) and one maternally methylated control DMR (*Peg3*) analyzed in this study. The DMRs are shown as black boxes according to the data by Kobayashi *et al*. (47). The tandem repeats at the *Rasgrf1* DMR are indicated by arrowheads. The regions examined by bisulphite sequencing are shown below the boxes as higher-magnification views with the positions of the individual CpG sites. Note that some CpG sites are strain-specific. B6, C57BL/6J.

RESULTS

Establishment of paternal methylation imprints in fetal prospermatogonia

It was previously shown that establishment of the paternal methylation imprints begins in fetal prospermatogonia (or gonocytes) (18,23,24), but the timing of its completion has not been precisely determined. We therefore investigated the developmental changes in methylation at all three paternally methylated DMRs (*H19*, *Dlk1/Gtl2* and *Rasgrf1*) in genomic DNA isolated separately from multiple (at least three) preparations of male germ cells at embryonic day 12.5 (E12.5), E14.5, E16.5, E18.5 and postnatal day 0 (P0). Bisulphite methylation analysis was carried out with the respective preparations for the above three DMRs and, as a control, the maternally methylated *Peg3* DMR. The CpG sites investigated in this study are schematically shown in Figure 1.

Examples of the results (at the *H19* DMR) are shown in Figure 2A, and the methylation changes at all DMRs are summarized in Figure 2B. (The data for the *Dlk1*/*Gtl2*, *Rasgrf1* and *Peg3* DMRs are supplied as Supplementary Material, Fig. S1A–C). In E12.5 PGCs, all paternally methylated

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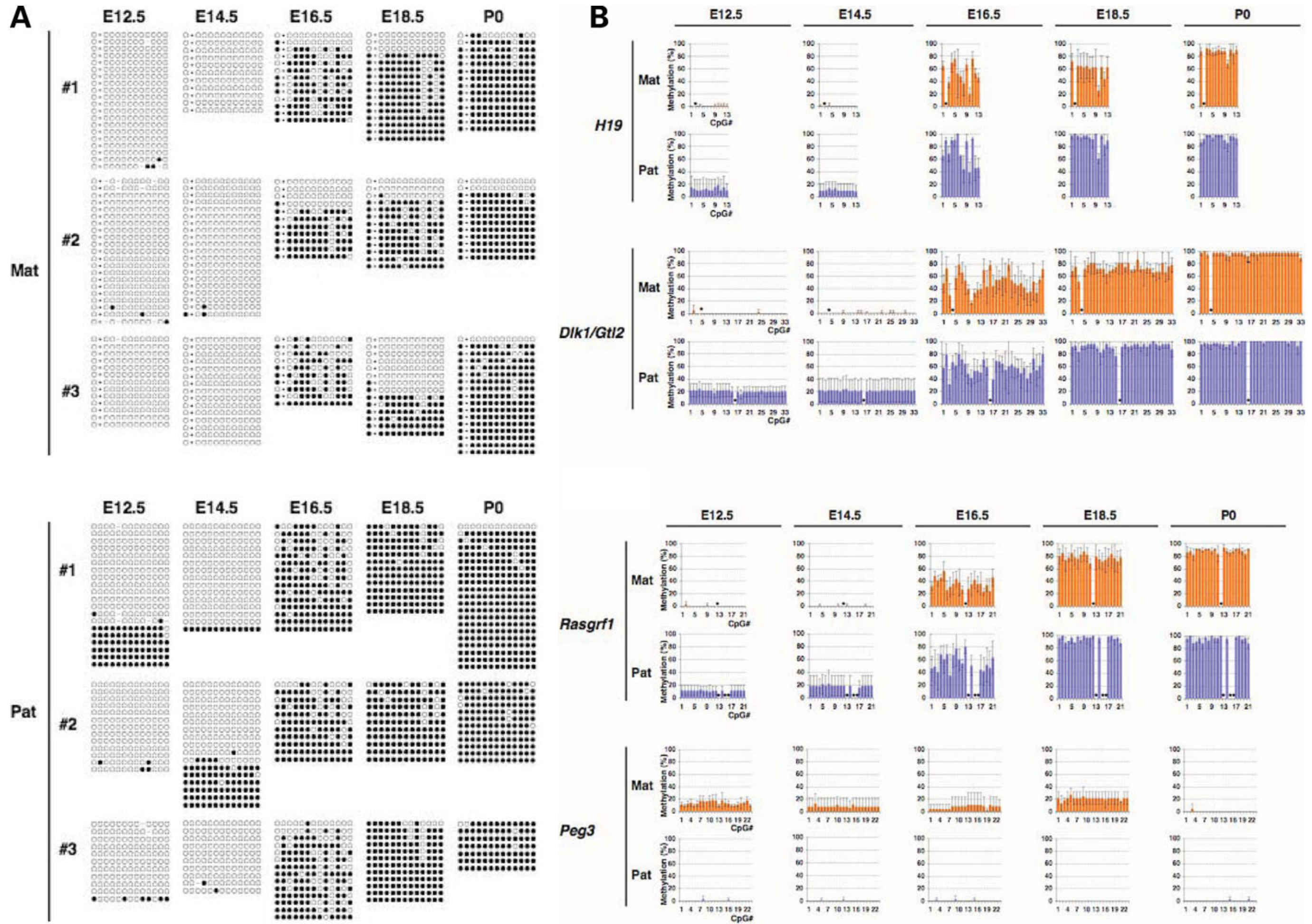

Figure 2. Methylation changes at the DMRs in wild-type male germ cells during fetal testis development. (**A**) The results of the bisulphite methylation studies on the *H19* DMR in three independent germ cell preparations. The maternal and paternal alleles differentially recognized in the (C57BL/6J X JF1) F1 male germ cells are indicated by 'mat' and 'pat', respectively. Horizontally aligned circles represent a single DNA molecule. Open circles indicate unmethylated cytosines and filled circles methylated cytosines. Asterisks show that the CpG sites are not present due to DNA polymorphisms. Hyphens indicate that the positions were unreadable. The sample numbers are indicated on the left. (**B**) Summary of the methylation changes at all DMRs. The vertical axis represents the percentage of methylated cytosines and the horizontal axis the individual CpG sites. Each bar shows the average of the methylation levels obtained from three independent experiments. Standard deviations are shown by the bars. Asterisks are as above.

DMRs were unmethylated on the maternal chromosome, while they were 10–30% methylated on the paternal chromosome. In E14.5 prospermatogonia, the methylation patterns were basically unchanged. The partial methylation observed at the paternal allele of these DMRs and also the partial methylation observed at the maternal allele of the *Peg3* DMR probably reflects incomplete erasure of the pre-existing imprints in germ cells (18,22–24), although we cannot exclude the possible contribution of a small number of contaminating somatic cells. In E16.5 prospermatogonia, all paternally methylated DMRs showed greatly increased levels of methylation on both parental chromosomes. The allelic difference in methylation still existed. At this stage, some CpG sites tended to be more methylated than others and thus many DNA molecules showed mosaic methylation patterns. Methylation was further increased at E18.5, but the allelic difference was still observed. In P0 prospermatogonia, the DMRs showed over 90% methylation on both parental chromosomes at most of the CpG sites. We no more observed the allelic difference. Thus, we concluded that the paternal methylation imprints are fully established at this stage. During the fetal prospermatogonial stages, the *de novo* methylation proceeded in very similar ways at all three DMRs. We also analyzed pachytene spermatocytes and round spermatids from adult testes and mature spermatozoa from adult epididymides and found that these DMRs remained highly methylated through the meiotic and haploid stages (Supplementary Material, Fig. S2).

Roles of Dnmt3a and Dnmt3b in establishment of paternal methylation imprints

We next asked which of the two *de novo* methyltransferases is/are involved in the methylation of these DMRs. We previously showed that both *Dnmt3a* and *Dnmt3b* were efficiently deleted by TNAP-Cre in fetal prospermatogonia. Using these mice, we found that the *H19* and *Dlk1/Gtl2* DMRs were severely

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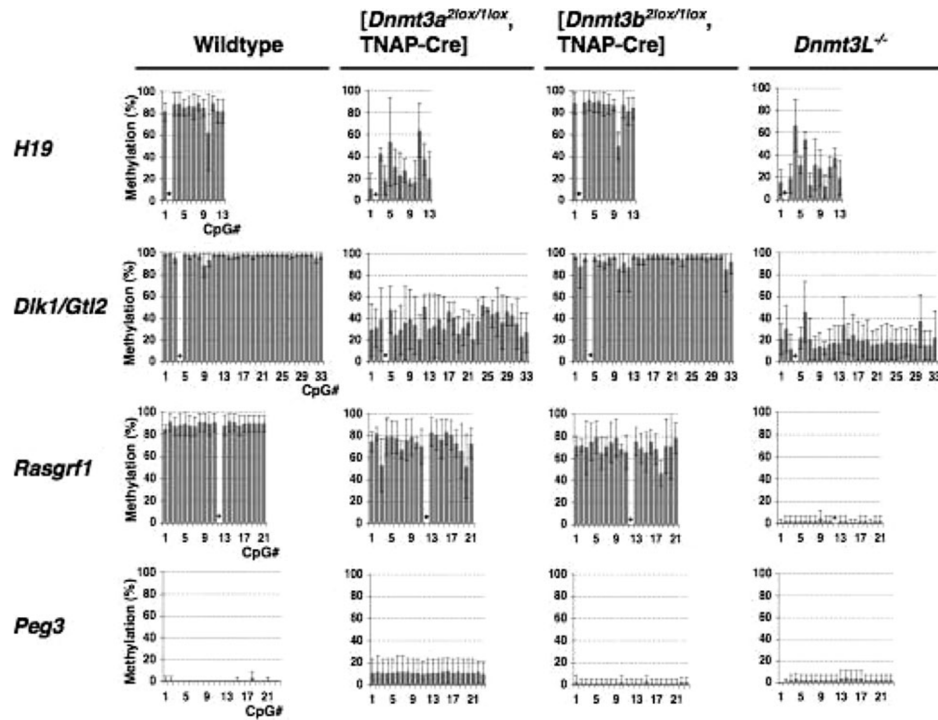

Figure 3. Methylation status of the DMRs in Dnmt3-deficient newborn prospermatogonia. The results of the bisulphite methylation studies of prospermatogonia from (*Dnmt3a*$^{2lox/1lox}$, TNAP-Cre), (*Dnmt3b*$^{2lox/1lox}$, TNAP-Cre) and *Dnmt3L*$^{-/-}$ mice are graphically shown. The methylation analysis was performed with four (Dnmt3a and Dnmt3L) and five (Dnmt3b) independent germ cell preparations. Asterisks are as in Figure 2. The data on the wild-type germ cells were obtained with three independent germ cell preparations from C57BL/6J.

hypomethylated in germline-specific *Dnmt3a* knockout spermatogonia at P11 (9). In contrast, germline-specific Dnmt3b deficiency did not cause any detectable phenotype. To investigate the roles of Dnmt3a and Dnmt3b in further detail, we analyzed the methylation status of the three paternally methylated DMRs in newborn (P0–P2) prospermatogonia isolated from these mutants.

The results of the bisulphite studies on the Dnmt3a-deficient prospermatogonia are shown in Supplementary Material, Figure S3A and summarized in Figure 3. The *H19* and *Dlk1/Gtl2* DMRs were hypomethylated (20–60% methylation) in four and three independent Dnmt3a-deficient prospermatogonium preparations, respectively. In contrast, the *Rasgrf1* DMR was less affected (60–90% methylation). At all DMRs, we observed some variation in overall methylation level between the germ cell preparations from different individuals. The partial but uniform methylation at the *Peg3* DMR in two preparations suggested the presence of some somatic cells, which was confirmed by the presence of a faint PCR band representing the intact *Dnmt3a* allele (data not shown). However, a large proportion of the molecules showed mosaic methylation patterns, which is unlikely to occur in the somatic cell-derived molecules, suggesting that most of the variations were real. These observations indicate that Dnmt3a methylates all paternally methylated DMRs although the contribution of this enzyme is smaller at the *Rasgrf1* DMR.

To know whether Dnmt3b contributes to the *de novo* methylation of any paternally methylated DMR, we next studied the Dnmt3b-deficient newborn prospermatogonia (Fig. 3 and Supplementary Material, Fig. S3B). The *H19* and *Dlk1/Gtl2* DMRs were unaffected, but the *Rasgrf1* DMR was moderately affected. Again, the overall methylation level varied between different preparations but, because the *Peg3* DMR was extremely hypomethylated in all preparations, somatic contamination was unlikely to be the major cause. These findings suggest that the *Rasgrf1* DMR requires both Dnmt3a and Dnmt3b for its full methylation. Unfortunately, we could not analyze prospermatogonia-deficient for both Dnmt3a and Dnmt3b because of the extremely low birth rate of this genotype ($<0.5\%$, whereas the expected rate was 12.5%). The lethality is most likely caused by the leaky TNAP-Cre expression and lack of both *de novo* methyltransferases in somatic cells.

Critical function of Dnmt3L in establishment of paternal methylation imprints

The importance of Dnmt3L in *de novo* methylation of the *H19* DMR was previously demonstrated (9,27,28). However, there have been discrepancies between the reports concerning the other paternally methylated DMRs. To resolve this issue, we analyzed the methylation status of the three DMRs in the Dnmt3L-deficient newborn prospermatogonia (Fig. 3 and Supplementary Material, Fig. S3C). The results unequivocally showed that Dnmt3L plays a critical role in the *de novo* methylation of all DMRs. In particular, methylation was hardly detectable at the *Rasgrf1* DMR in four independent experiments. At the *H19* and *Dlk1/Gtl2* DMRs, a substantial level of methylation was still detected and the actual

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methylation level differed depending on the particular germ cell preparation. Since the methylation level of the *Peg3* DMR was extremely low in all preparations, most of the partial methylation observed at these DMRs probably results from Dnmt3L-independent methyltransferase activities rather than from contaminating somatic cells. Essentially, the same results were obtained in Dnmt3L-deficient germ cells at P17 for all DMRs (data not shown).

De novo methylation of interspersed and satellite repeats in prospermatogonia

A previous study showed that at least three classes of repetitive sequences also undergo *de novo* methylation in fetal prospermatogonia (29). These repeats included intracisternal A particle (IAP) element (an endogenous retrovirus), long interspersed nuclear element 1 (Line1) (a non-retrovirus-type retrotransposon) and minor satellites (centromeric tandem repeats). These repeats are considerably methylated even at E12.5 but become more highly methylated by E17.5 (29). We asked whether two other repeat classes, short interspersed nuclear element B1 (SineB1) and major satellite (a pericentric repeat), are also *de novo*-methylated during this stage. We observed that these two repeat classes also become more methylated between E12.5 and the newborn stage (Fig. 4). The observed methylation level in P0–P2 prospermatogonia was very high (>80%) at the IAP and Line1 elements but only intermediate (30–70%) at SineB1 elements and minor and major satellites.

Roles of Dnmt3a, Dnmt3b and Dnmt3L in *de novo* methylation of interspersed and satellite repeats in prospermatogonia

Previous studies on Dnmt3L-deficient postnatal spermatogonia showed that not only the paternally methylated DMRs but also IAP and Line1 elements are hypomethylated (27,28,30). We therefore asked whether the other repeat classes also require Dnmt3L for *de novo* methylation. Furthermore, we attempted to know which *de novo* methyltransferase (Dnmt3a or Dnmt3b) methylates which repeat class.

The results obtained with the P0–P2 prospermatogonia showed that each class is methylated by a specific *de novo* methyltransferase(s): SineB1 elements are methylated by Dnmt3a; major and minor satellites are methylated by Dnmt3b; IAP and Line1 elements are methylated by both Dnmt3a and Dnmt3b (Fig. 5). For IAP and Line1 elements, the two *de novo* methyltransferases probably function redundantly since the respective single mutants showed only a marginal reduction in methylation. The involvement of Dnmt3b in the methylation of the major and minor satellites is consistent with the previous observations in Dnmt3b-deficient ES cells and embryos (7,31,32). Unexpectedly, however, all repeats required Dnmt3L for their *de novo* methylation. This was surprising because a previous report on Dnmt3L-deficient spermatogonia at P17 detected no demethylation at the minor or major satellites (27). To see whether this discrepancy is due to the difference in developmental time point at which the germ cells were prepared, we compared the

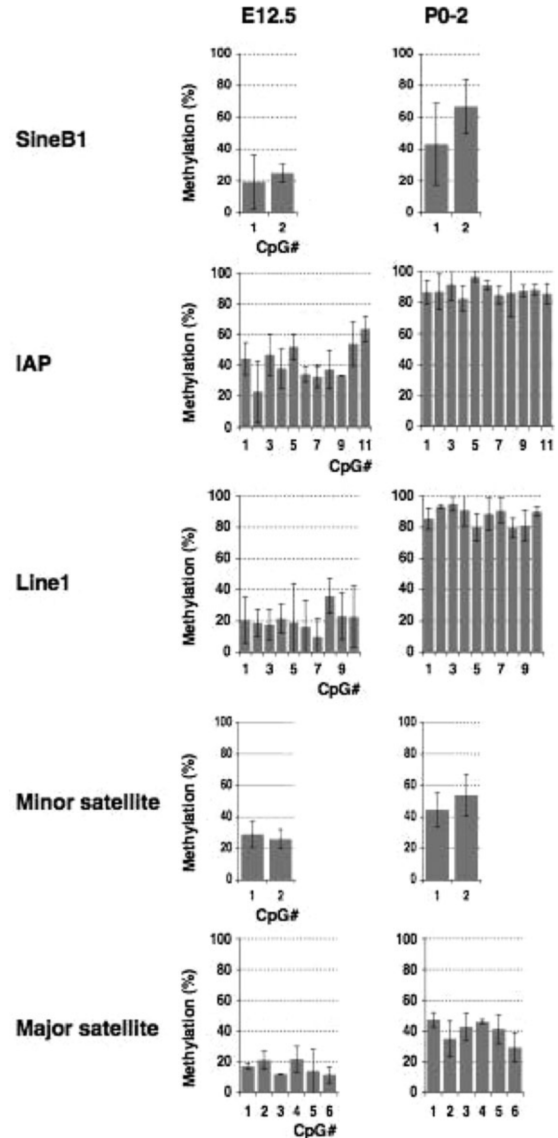

Figure 4. Methylation changes at the repetitive sequences in developing wild-type male germ cells. The results of the bisulphite methylation studies are summarized in the graphs. For SineB1 elements, two of the six CpG sites located in the middle region of the consensus sequence were analyzed (48). Both of the two CpG sites in minor satellites and six of the eight CpG sites in major satellites (49) were analyzed. The regions investigated in IAP and Line1 elements were described previously (50). The experiments were done with at least three independent germ cell preparations.

methylation status of minor and major satellites between P0–4 prospermatogonia and P17–19 germ cells by Southern blotting. Both satellites (especially major satellites) were less methylated at P17–19 than at P0–4 in wild-type germ cells, and thus demethylation in Dnmt3L-deficient cells became less clear at P17–19 (Supplementary Material, Fig. S4). At all repeats, the methylation levels observed in Dnmt3L-deficient prospermatogonia at P0–2 were comparable to those in wild-type PGC at E12.5, suggesting that only *de novo* methylation, but not maintenance methylation, was affected in the mutants.

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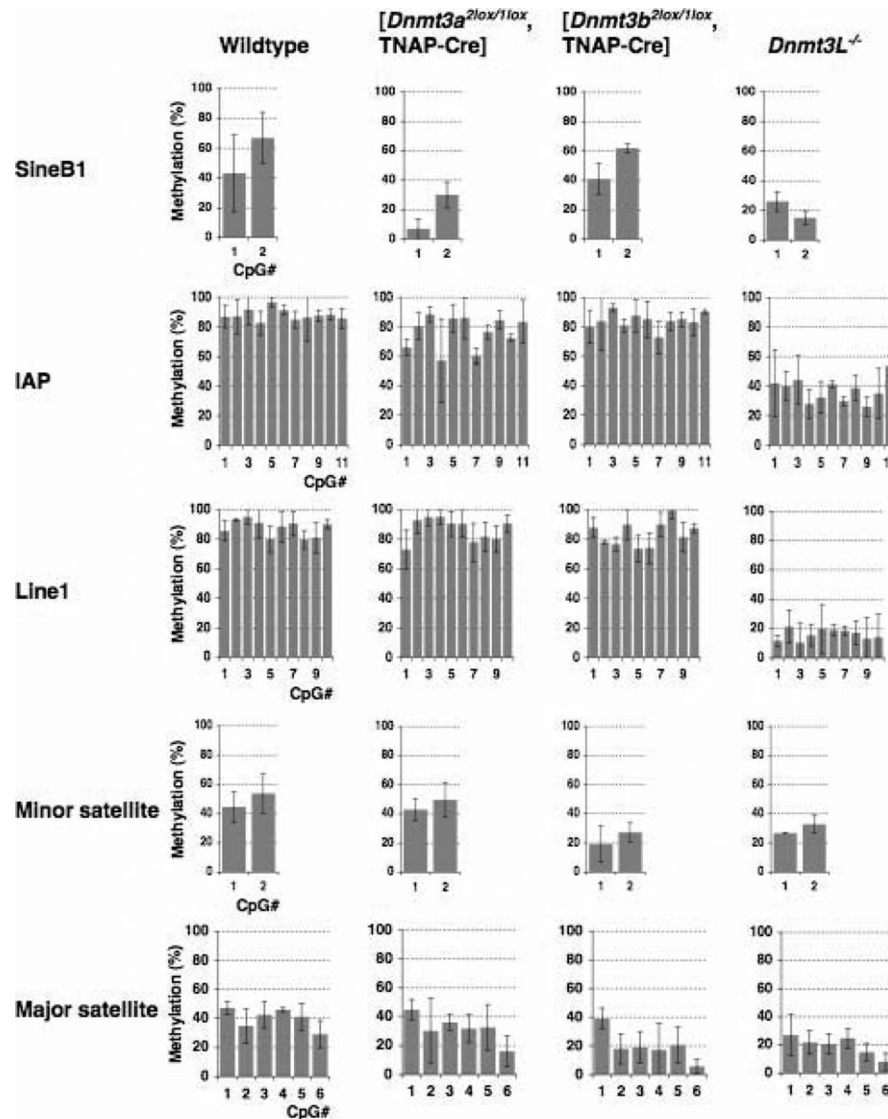

Figure 5. Methylation status of the repetitive sequences in Dnmt3-deficient newborn prospermatogonia. The experiments were done with at least three independent germ cell preparations. The data for the wild-type germ cells are the same as those for P0–2 prospermatogonia in Figure 4.

DISCUSSION

In the present study, we showed that *de novo* methylation of the three paternally methylated DMRs occurs progressively in fetal prospermatogonia after E14.5 and that a full level of methylation is established in newborn prospermatogonia. We also observed that the methylation level of the paternal allele is higher than that of the maternal allele at all these DMRs in fetal prospermatogonia, as described previously (22–24): perhaps, this reflects incomplete erasure of the pre-existing imprints in some germ cells. The establishment of methylation at all these DMRs by the newborn stage is consistent with the previous observation made on the *H19* DMR by Ueda *et al*. (24) but different from those reported by Davis *et al*. (22,23). The latter study described that full methylation of the maternal *H19* DMR is not observed until the pachytene spermatocytes stage. A possible reason for the discrepancy is the difference in mouse strains used for the experiments. Since our observation was reproduced in a commonly used laboratory strain C57BL/6J (data not shown), we can at least say that our result is not restricted to the particular F1 mice that we used. The methylation imprints established in prospermatogonia are then maintained through meiosis and passed to mature spermatozoa.

It was previously reported that both Dnmt3a and Dnmt3b are expressed and nuclear-localized in fetal prospermatogonia (33,34). Our data on the Dnmt3a- and Dnmt3b-deficient newborn prospermatogonia showed that Dnmt3a plays a major role in methylation of the *H19* and *Dlk1/Gtl2* DMRs. Because Dnmt3a2 (encoded by a shorter transcript) is the predominant isoform in prospermatogonia (35), we speculate that this protein catalyzes the *de novo* methylation. For the *Rasgrf1* DMR, Dnmt3a and Dnmt3b appear to operate redundantly: deficiency of either enzyme causes only a small reduction in methylation. The *Rasgrf1* DMR is the first DMR found to be affected significantly by Dnmt3b deficiency. In contrast,

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Dnmt3L, an activator of both Dnmt3a and Dnmt3b (36–39), plays a critical role in the *de novo* methylation of all DMRs. This finding is not in accord with the previous reports that the *Dlk1/Gtl2* and *Rasgrf1* DMRs are only marginally affected in Dnmt3L-deficient spermatogonia (9,27). We observed this variation in the same mouse strain (9 and this study). The discrepancy cannot be explained by the differences in developmental time points at which the germ cells were prepared, as we obtained essentially the same results at P0–4 and P17 (data not shown). Since Arnaud *et al*. recently reported that the maternally methylated DMRs sometimes retain the normal methylation imprints in the fetuses derived from the Dnmt3L-deficient females (40), this kind of stochastic effect in the male germline could account for the discrepancy. Our preliminary study showed that not only the three DMRs, but also the *Xist* promoter and *Igf2* DMR2 are also affected in the Dnmt3L-deficient prospermatogonia (data not shown).

Because previous studies revealed a close link between DMR methylation and transposon silencing in male germ cells (27,28,30), we also studied the methylation status of repetitive sequences. We found that, in addition to IAP elements, Line1 elements and minor satellites (29), both SineB1 elements and major satellites also become more methylated in fetal prospermatogonia. Furthermore, we found that each of these repeat classes is methylated by a specific *de novo* methyltransferase(s): SineB1 by Dnmt3a; the minor and major satellites by Dnmt3b; and IAP and Line1 elements by both Dnmt3a and Dnmt3b. Judging solely on the basis of this target specificity, SineB1 may share some intrinsic features with the *H19* and *Dlk1/Gtl2* DMRs, and IAP and Line1 elements with the *Rasgrf1* DMR. Along this line, it is noteworthy that the *Rasgrf1* DMR contains a sequence derived from an endogenous retrovirus ERVK similar to IAP. How Dnmt3a and Dnmt3b recognize their respective targets is an open question.

Despite the moderate demethylation at the *Rasgrf1* DMR, minor satellites and major satellites, the Dnmt3b-deficient prospermatogonia developed normally and produced functional spermatozoa, which were capable of supporting normal development of the offspring (9). There was no reduction in litter size, and no offspring showed prenatal or postnatal growth retardation. Since the *Rasgrf1* DMR was fully methylated in Dnmt3b-deficient spermatozoa (data not shown), the germ cells harboring a reduced level of methylation may be eliminated, or the normal methylation level can be restored by the action of Dnmt3a in postnatal germ cells.

Finally, an unexpected finding of this study was that the Dnmt3L-deficiency affected all repeat classes, because minor and major satellites were previously reported to be unaffected in the same mutants at P17 (27). This apparent discrepancy could be explained by the difference in developmental time point at which the methylation status was examined: we found that decreased methylation of these satellites in wild-type germ cells at P17–19 makes it more difficult to detect the demethylation in Dnmt3L-deficient cells. Together with the fact that unique sequences, including the three paternally methylated DMRs, are also affected, Dnmt3L is probably not a sequence-specific regulator of the *de novo* methyltransferases but is rather a general activator. This is consistent with the biochemical properties of this protein (36–39).

MATERIALS AND METHODS

Mice

To obtain wild-type male germ cells, B6 females were crossed with JF1 (41) males. This allowed us to distinguish the parental alleles in the germ cell preparations from the offspring based on DNA polymorphisms between the strains. Germline-specific *Dnmt3a* mutants, germline-specific *Dnmt3b* mutants and conventional *Dnmt3L* mutants were described previously (9,26,42). To obtain Dnmt3a-deficient (or Dnmt3b-deficient) prospermatogonia, $Dnmt3a^{2lox/2lox}$ females (or $Dnmt3b^{2lox/2lox}$ females) were crossed with ($Dnmt3a^{2lox/+}$, TNAP-Cre) males [or ($Dnmt3b^{2lox/+}$, TNAP-Cre)]. Prospermatogonia were obtained from [$Dnmt3a^{2lox/1lox}$, TNAP-Cre] [or ($Dnmt3b^{2lox/1lox}$, TNAP-Cre)] males at P0–2. Cre recombinase driven by the TNAP promoter was expressed in the PGCs from E9.5-10.5 to late-gestation (43).

Preparation of male germ cells and extraction of genomic DNA

Isolation of male germ cells was basically done as described (44). Seminiferous tubules from E12.5, E14.5, E16.5, E18.5 and P0–2 testes were dissociated with trypsin/EDTA (0.25% trypsin, 10 mM EDTA in PBS) at 37°C for 10 min. Cells from two to six testes were cultured in M199 medium (Sigma) supplemented with 10% fetal bovine serum in a 90-mm dish for 1.5 h in a CO_2 incubator. Cells floating in the culture medium were collected, washed in PBS and resuspended in 2 ml of 25% Percoll/M199. The cell suspension was put on 2 ml of 65% Percoll/PBS and overlaid with 1 ml of 12% Percoll/PBS in a centrifugation tube. Centrifugation was carried out at 2800*g* for 20 min at room temperature. Cells enriched in the 25% Percoll/M199 phase were collected using a micropipette. The germ cell preparations were checked by morphological observation and by alkaline phosphatase staining and their purity always exceeded 90%. To collect germ cells from P0–4 and P17–19 testes, fluorescence-activated cell sorting (FACS) was also used. Cells dissociated by 0.5 mg/ml of collagenase and trypsin/EDTA were fixed with 4% paraformaldehyde/PBS for 20 min at 4°C and them permeabilized with 0.1% Triton X-100/PBS. Germ cells were reacted with anti-GCNA1 antibody (45) and Alexa fluor 488 goat anti-rat IgG (Molecular Probes) and collected using FACS JSAN (Bay Bioscience). More than 90% of the collected cells were positive for GCNA1, as confirmed by fluorescent microscopy. Isolated germ cells were incubated in a lysis buffer (100 mM Tris pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, 200 μg/ml proteinase K) at 50°C for 1 h. After phenol/chloroform extraction, ethanol precipitation was performed with Ethachinmate (Nippon Gene). The DNA pellet was resuspended in 30 μl of TE buffer.

Pachytene spermatocytes and round spermatids were collected from 9-week-old testes by elutriation (Beckman J6-MC; Rotor JE5.0). Briefly, seminiferous tubules from two testes were dissociated in PBS containing 0.5 mg/ml of col-

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lagenase at 32°C for 15 min and then in PBS containing 0.5 mg/ml of trypsin at 32°C for 20 min. After 5 min of trypsin treatment, 2 μg/ml of DNase I was added. Then, cells were treated with 0.5 mg/ml of Trypsin Inhibitor (Nacalai Tesque), passed through a nylon mesh (23 μm), and treated with 2 μg/ml of DNase I. Elutriation was performed as described (46). Fractions 8 and 5 contained pachytene spermatocytes and round spermatids, respectively. Epididymal spermatozoa were collected according to a standard protocol. The cells were incubated in the lysis buffer at 50°C overnight. After phenol and phenol/chloroform extractions, ethanol precipitation was performed. The genomic DNA was resuspended in 100 μl of TE buffer.

Bisulphite methylation analysis

Bisulphite treatment of the genomic DNA isolated from male germ cells was carried out with an EZ DNA Methylation Kit (Zymo Research). Sequences of the PCR primers are listed in Supplementary Material, Table S1. Semi-nested PCR was performed to amplify the *H19*, *Dlk1/Gtl2*, *Rasgrf1* and *Peg3* DMRs. In the nested PCR, the first-round PCR was carried out using 2–3 ng of bisulphite-treated DNA with the following parameters: 35 cycles of 95°C for 1 min, 61°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min. The second-round PCR was carried out using a portion of the first-round PCR products for 15–20 cycles with the same parameters. PCR amplification of the repetitive sequences was carried out with the following parameters: SineB1, 30 cycles of 95°C for 30 s, 61°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 3 min; IAP, 30 cycles of 95°C for 1 min, 64°C for 3 min and 72°C for 1 min, with a final extension at 72°C for 5 min; Line1, 30 cycles of 95°C for 1 min, 56°C for 2 min and 72°C for 1 min, with a final extension at 72°C for 5 min; minor satellites, 30 cycles of 95°C for 30 s, 59°C for 30 s and 72°C for 2 s; major satellites, 5 cycles of 95°C for 30 s, 65°C for 30 s (with a gradual decrease of 1°C/cycle) and 72°C for 5 s, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 5 s. The PCR products were cloned using pGEM-T Easy Vector System I (Promega). Colonies were picked up and DNA was amplified in 96-well plates by rolling circle amplification using a 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 using an ABI Prism 3700 and 3130xl Genetic Analyzer (Applied Biosystems).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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