Studies on expression of DNA methyltransferases during mouse germ cell development

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CONTENTS

ABSTRACT

Germline-specific differential methylation that persists through fertilization and embryonic development is thought to be the "imprint" distinguishing the parental alleles of imprinted genes. It was previously reported that the paternal-specific methylation of a mouse imprinted gene, *H19*, is established in the gonocyte stage during fetal testis development. This *de novo* methylation is concomitant with the global genomic methylation. In female, it was shown that maternal-specific methylation of *Igf2r*, for example, is established during growing oocyte stage. Three DNA methyltransferases are known in mammals: Dnmt1, Dnmt3a and Dnmt3b. To ask which methyltransferase is responsible for the *de novo* methylation in the male and female germline, I carried out immunohistochemistry with polyclonal anti-Dnmt antibodies. In fetal testes, Dnmt3b was localized in the nuclei of gonocyte. I confirmed that the active forms of the enzyme are present in these cells by RT-PCR using RNA from highly purified gonocytes. By contrast, in the nuclei of growing oocytes, both Dnmt1 and Dnmt3b were detected. However, it is known that Dnmt1 is not required for the establishment of maternal genomic imprints. Additional study using a monoclonal antibody specific to Dnmt3a suggests the presence of a novel isoform(s) of Dnmt3a, which is not detected by the polyclonal anti-Dnmt3a antibodies, in gonocytes and growing oocyte. These results suggest that Dnmt3b and a novel isoform(s) of Dnmt3a are the key methyltransferases that establish the global methylation pattern in the male germline and methylation imprints in the male and female germline.

INTRODUCTION

Methylation at the 5th position of the cytosine residue is the unique physiological modification found in genomic DNA in mammals, which almost exclusively occurs in a CpG dinucleotide-sequence. This DNA methylation is catalyzed by the enzyme activity known as DNA (cytosine-5) methyltransferase (Dnmt). Two distinct families of Dnmts, Dnmt1 and Dnmt3, have been identified in mammals, both of which contain motifs evolutionarily conserved among all known cytosine methyltransferases, although overall structures of the proteins are rather diverged. Dnmt1 prefers hemimathylated CpG sites as substrates rather than unmethylated ones (Bester et al, 1992), which implies that its major role is to maintain the methylation patterns of the genome shortly after DNA replication. The Dnmt3 family, on the other hand, contains two closely related proteins, termed Dnmt3a and Dnmt3b, which are encoded by distinct genes (Okano et al., 1998). They are highly expressed in ES cells, but down-regulated upon their differentiation. Dnmt3a and Dnmt3b do not have strong preference to hemimethylated sites as Dnmt1 does, and methylate both hemimethylated and unmethylated sites with similar kinetics (Okano et al., 1998). Based on studies with knockout mice (Okano et al., 1999) and transgenic flies (Lyko et al., 1999), it is suggested that Dnmt3a and Dnmt3b are *de novo* methyltransferases. There are, at least, three different isoforms for Dnmt3b, which are produced by alternative splicing. Dnmt3b1 and Dnmt3b2 are enzymatically active isoforms, whereas Dnmt3b3 seems to be inactive because it lacks a part of the indispensable catalytic domain.

In mammals, DNA methylation is essential for normal embryonic development (Li et al., 1992; Lei et al., 1996; Okano et al., 1999), and plays important roles in regulation of gene expression, chromosome stability (Xu et al., 1999; Okano et al., 1999; Hansen et al., 1999), silencing of endogenous retroviruses (Walsh et al., 1998), X chromosome inactivation (Panning and Jeanisch, 1996), and genomic imprinting (Li et al., 1993). Genomic imprinting is a process, by which a subset of genes in mammals is differentially marked in the parental germlines so as to

be expressed in a parent-of-origin-specific manner in the embryo and adult. Most of the imprinted genes examined so far show differences in DNA methylation between the parental alleles, suggesting a crucial role for differential methylation in the mechanism of imprinting. The 5' flank of the mouse imprinted gene *H19* is more methylated on the paternal chromosome than on the maternal chromosome in adult somatic tissues (Bartolomei et al., 1993; Ferguoson-Smith et al., 1993). Of the region with differential methylation, however, only a 2-kb region located from –4 kb to –2 kb upstream of the *H19* gene shows clear methylation differences between oocyte and sperm, which persist throughout preimplantation development (Tremblay et al., 1995, 1997; Olek et al., 1997). This suggests that the differential methylation of the 2-kb differentially methylated region (DMR) may be the gametic imprints that differentiate the parental alleles. It has been shown that this DMR is essential for the imprinting of an *H19* transgene (Elson et al., 1997) or of the endogenous *H19* locus (Thorvaldsen et al., 1998). Recently, Ueda et al. (2000) reported that the paternal methylation imprint of *H19* is, for the most part, acquired in gonocyte or prospermatogonia, which are premeiotic germ cells mitotically arrested in the fetal testis. In contrast, the maternal methylation imprints seems to be established during oocyte growth (Chaillet et al., 1991; Ueda et al., 1992; Stöger et al., 1993), corresponding to the diplotene or dictyotene stage of meiotic prophase I, which is consistent with the improved development of diploid parthenogenetic embryos with one genome from a non-growing newborn oocyte and the other from a fully-grown oocyte (Kono et al., 1996; Obata et al., 1998).

To understand the mechanisms of genomic imprinting, it is important to identify which Dnmt(s) is responsible for the gametic methylation imprints laid on the parental chromosomes in the parental germline. In this study, I carried out immunohistochemistry to examine the cellular localization of the three known of Dnmts in developing germ cells.

MATERIALS AND METHODS

Antibodies

Rabbit anti-Dnmt3a and Dnmt3b antisera were the kind gifts from E. Li (Harvard Med. Sch.), which had been raised against bacterially expressed N-terminal regions of the mouse proteins. The monoclonal antibody against Dnmt3a was purchased from IMGENEX, which had been raised against bacterially expressed recombinant mouse Dnmt3a with HS-tag. Rabbit anti-Dnmt1 antibodies were obtained from S. Tajima (Osaka Univ.) (Takagi et al., 1995; Suetake et al., 2001). A germ cell-specific monoclonal antibody TRA98 (Tanaka et al, 1997) and anti-5mC monoclonal antibody were kindly provided by Y. Nishimune (Osaka Univ.) and by Y. Kagawa (Toray Research Center), respectively.

Immunoblot analysis

Full-length mouse Dnmt3a, Dnmt3b1, Dnmt3b2 and Dnmt3b3 cDNAs were subcloned into an expression vector called pcDNA3 (Invitrogen). Mouse 293T cells were transfected with the resulting constructs by lipofection using Lipofectamine Plus (Gibco-BRL). Cells were harvested 24 hours posttransfection and homogenized in 62.5 mM Tris-HCl (pH 6.8), 0.5 x PBS, 2% SDS, 10% Glycerol, and 5% β-mercaptoethanol. Proteins were denatured by heating at 95°C for 2 min, separated by electrophoresis on SDS-7.5% polyacrylamide gels, and transferred onto nitrocellulose membranes (Amersham). Blots were blocked with 5% skimmed milk, incubated with a 1:20000 dilution of anti-Dnmt3a or anti-Dnmt3b antibodies. After several washes, blots were incubated with a 1:300 dilution of biotinylated anti-rabbit Ig antibody (Amersham), soaked in an ABC solution (Vector), and detected by ECL (Amersham).

Preparation of histological specimens and immunostaining

Histological specimens for immunostaining were obtained from the fetuses and animals resulting from crosses between (C57Bl/6 X C3H/HeJ) F1 males and females. Gonads dissected from fetuses and adult animals were placed in O.T.C. compound embedding medium for frozen tissue specimens (TISSUE-TEK, Mile Inc, Elkhart IN) and frozen at –20°C. Sections of 10 μm in thickness were prepared using a freezing microtome (Cryostat CM3000, Leica) and soaked in ethanol prior to RNase A treatment. The specimens were fixed with 4% formaldehyde for 15 min at room temperature. Each section were serially treated with PBD (0.5% Triton X-100, 0.25% Tween 20 in phosphate-buffered saline), with 0.3% hydrogen peroxide in PBD, and blocking buffer (10% fetal bovine serum in PBD), and then reacted with antibodies. To visualize anti-Dnmt antibodies, sections were stained with biotinylated anti-rabbit Ig antibody (Amersham) and a tyramide signal amplification system (TSA Cyanine 3 System, NEN). An anti-Dnmt3a monoclonal antibody from IMGENEX, a germ cell specific monoclonal antibody TRA98 and anti-5mC monoclonal antibody were revealed by a second antibody labeled with Alexa 488 (Molecular Probe). Slides were counterstained with TOTO-3 (Molecular Probes) and mounted with antifade solution containing 2.5% 1,4-diazobicyclo-(2,2,2)-octane (DABCO, Sigma) in 90% glycerol. For double staining using anti-Dnmt3b antibody and anti-5mC antibody, specimens were treated as previously described (Coffigny, 1999) after staining of anti-Dnmt3b. The specimens were examined with an Olympus FV500 confocal microscope or a Zeiss Axioplan fluorescence microscope.

Preparation of germ cells and RT-PCR

Male germ cells were purified from Oct-4/GFP transgenic mice (Yoshimizu et al., 1999) by a fluorescent-activated cell sorting (FACS) method (Abe et al., 1996). Male gonads were

dissected at embryonic day (E) 14.5, E16.5, E18.5, postnatal day (P) 1 and P7, incubated in 0.25% Tripsin/1mM EDTA in PBS for 5 min at 37°C, and triturated to produce single cell suspension. The cells were run on a FACS Vantage cell sorter (Becton Dickinson). Small aliquots of the cells collected as the fluorescence-positive population were histochemically stained for alkaline phosphatase activity to determine the purity. The purity of the germ cell preparations always exceeded 98%. Growing oocytes were isolated from ovaries from P10, P15 and P20 mice as previously described (Bao et al., 2000).

Total RNA was prepared from purified male germ cells by Trizol and then incubated with RQ1 DNase (Promega). cDNA was synthesized using Oligo-dT primers and PCR-amplified using Dnmt3b-specific primers: m3b-8, 5'-CAA CCG TCC ATT CTT CTG GAT GTT-3'; m3b-14, 5'-TCC CCA GTC CTG GGT AGA ACT AT -3'. PCR was carried out using 1 Unit of Ex Taq Hot Start Version and performed for 2 min at 94°C and 30 cycles for 30 sec at 94°C, 30 sec at 72°C and 30 sec at 68°C, and for 5 min at 72°C. A novel PCR product (resulting from a splicing variant) was cloned into a plasmid and sequenced on an ABI PRISM 377 Sequencer (Perkin Elmer) using m3b-8 or m3b-9, 5'-GAG CTA AAA AGG GGA CAG GTG AG-3' as a primer.

RESULTS

Specificities of antibodies against Dnmt3a and Dnmt3b

Specificities of polyclonal antibodies against Dnmt3a and Dnmt3b were respectively confirmed by western blotting using whole cell extracts prepared from mouse 293T cells transiently expressing mouse Dnmt3a, Dnmt3b1, Dnmt3b2, or Dnmt3b3. Figure 1A shows that anti-Dnmt3a antibodies raised against full-length and N-terminus of mouse Dnmt3a reacted with only Dnmt3a but not with any isoforms of Dnmt3b. Similarly, anti-Dnmt3b antibodies raised against N-terminus of mouse Dnmt3b recognized all the isoforms of Dnmt3b regardless of the methylating activity of the proteins (Figure 1C). Therefore, both antibodies were highly specific to the respective Dnmt3 and did not cross-react.

Cellular localization of each Dnmt in the developing testis

To identify which Dnmt(s) is present in male germ cells at the time when methylation imprints are established, immunohistochemistry was carried out. Since the paternal methylation imprint of *H19* is established in gonocyte (also called prospermatogonia) (Ueda et al., 2000), the fetal testes were examined for the cellular localization of Dnmts at E14.5, E16.5, and E18.5 by immunostaining with antibodies against each Dnmt (Figure 2). To distinguish between gonocytes and somatic cells, a germ cell-specific monoclonal antibody, TRA98 (Tanaka et al., 1997), was applied simultaneously.

Figure 2 shows the representative results of immunostaining of each Dnmt (red) and gonocytes (green). In the testes at E14.5, Dnmt1 was distributed in nucleus of both gonocytes and somatic cells. The cytoplasm of these cells also appeared to contain some Dnmt1. At E16.5, however, the staining of Dnmt1 in gonocyte nuclei became less intense than that in somatic cell nuclei, and eventually undetectable at E18.5. In contrast, the distribution of Dnmt1

in somatic cells apparently did not change from E14.5 through E18.5. The absence of the maintenance type methyltransferase Dnmt1 in gonocytes at E18.5 is in good agreement with the fact that they are mitotically arrested and DNA replication does not take place. Dnmt3a was retained in somatic cell nuclei, whereas gonocytes were devoid of a detectable level of Dnmt3a at all stages examined. Although the level of Dnmt3b was moderate in the nucleus of gonocytes at E14.5, most of them showed more intense staining for Dnmt3b at E16.5 and E18.5. My result, therefore, indicates that Dnmt3b was the only Dnmt present in male germ cells at the time when the gametic methylation imprints is settled (see later, however).

Accumulation of Dnmt3b coincides with the elevation of global methylation in male germ cells

To investigate the relationship between Dnmt3b and global methylation of gonocyte genome, immunostaining of the developing testis was performed with a monoclonal antibody against 5-methylcytosine (5mC) in combination with the anti-Dnmt3b antibodies. At E16.5 when the gonocyte had been arrested, the anti-5mC antibody produced uniform staining in most of the germ cell nuclei, although it was weaker than that in somatic cell nuclei (Figure 3). In contrast, almost all nuclei in E18.5 germ cells were more intensely stained with the anti-5mC antibody than in somatic cells (Figure 3). These observations were consistent with the previous report by Coffigny et al. (1999). Double-staining of 5mC and Dnmt3b revealed that accumulation of Dnmt3b in gonocyte nuclei was concomitant with the elevation of their genomic methylation levels in the developing fetal testes.

Isoforms of Dnmt3b expressed in male germ cells

Immunohistochemistry described above suggests that Dnmt3b is involved in methylation of the DMR at the *H19* locus and the whole genome occurring in gonocytes. However, there are at

least three different splicing variants for Dnmt3b, one of which, Dnmt3b3, has been shown to be enzymatically inactive (Aoki et al., 2001). It was, therefore, important to verify that functionally active isoforms of Dnmt3b were expressed in germ cells during testis development.

Germ cells at various developmental stages were purified from the testes of transgenic mice expressing green fluorescent protein (GFP) specifically in the germ cell lineage (Yoshimizu et al., 1999) by means of FACS. This purification procedure gave germ cell preparations with a purity of 98% or more. RNA isolated from the purified germ cells was then subjected to RT-PCR with primers that distinguish between active and inactive isoforms (Figure 4C). Expression of the active isoform, either Dnmt3b1 or Dnmt3b2 or both, was confirmed by the presence of a 518-bp band at all stages examined (Figure 4A). The inactive isoform, Dnmt3b3, which corresponded to a 329-bp band, was also found in E18.5 gonocytes (Figure 4A). It should be noted that, in addition to these expected bands, an extra band with a smaller DNA fragment size was also amplified at P1 (Figure 4A), suggesting the presence of another isoform. When this novel band was cloned and sequenced, it turned out to be a novel splicing variant lacking not only exons 21 and 22 but also exon 20 (Figure 4C). This alternative splicing removes the 29 amino acids encoded by exon 20 from the inactive Dnmt3b3 isoform and further causes a frameshift at the exon 19/23 boundary, with a stop codon emerging 45 amino acids downstream (Figure 4D). This results in a new isoform with an aberrant C-terminal region, which most probably lacks Dnmt activity. It appears that, like Dnmt3b3, this novel isoform does not encode an active enzyme. Both Dnmt3b3 and the novel isoform disappeared by P7 (Figure 4A).

Cellular localization of each Dnmt in the developing ovary

Female germ cells complete the last round of mitosis in the fetal ovaries and became arrested at meiotic prophase I prior to birth. Growth of arrested primary oocytes commences after birth,

and gonadotropin stimulation causes fully-grown oocytes to resume meiosis around 4 weeks later. It has been shown that functioned maternal imprints are established in growing oocyte at the diplotene or dictyotene stage in meiotic prophase I (Kono et al., 1996; Obata et al., 1998, 2001). Furthermore, the studies on some imprinted transgenes and the endogenous *Igf2r/Mpr* demonstrated that the maternal-specific methylation is established during this stage (Chaillet et al., 1991; Ueda et al., 1993; Stöger et al., 1993). I, therefore, examined the distribution of each Dnmt in developing ovaries at E18.5, P7 and P18.

Figure 5A shows immunostaining of the developing ovary at E18.5. Anti-Dnmt1 antibodies showed intense staining in the nucleus of oocytes. Dnmt3a, although present in the nucleus of somatic cells, was not found in TRA98-positive oocytes. Dnmt3b was detected in both oocytes and somatic cells. In oocytes, it was predominantly localized in the cytoplasm although it was also distributed in the nucleus of a subset of cells (Figure 5A). In the ovary at P7, Dnmt1 and Dnmt3b were detected in both the cytoplasm and nucleus of the majority of growing oocytes. Dnmt3a was not detected in oocyte nuclei, but faint signals may be seen in the cytoplasm. As shown in Figure 5C, all the Dnmts were excluded from the nucleus and predominantly found in the cytoplasm of oocytes at P18. Staining of Dnmt1 was extremely intense as reported by Mertineit et al. (1998). While Dnmt3a showed uniform cytoplasmic distribution, Dnmt3b was characterized by peripheral localization in the oocyte cytoplasm.

These results imply that either Dnmt1 or Dnmt3b may be involved in the establishment of maternal imprints during oocyte growth. Howell et al. (2001) recently reported, however, that targeted disruption of an oocyte-specific isoform of Dnmt1, Dnmt1o, did not affect the establishment of methylation imprints in oocytes of homozygous females. Therefore, it appears that Dnmt3b plays an important role in the establishment of the maternal-specific methylation at the imprinted loci (see later, however).

Isoforms of Dnmt3b expressed in growing oocytes

To ask which Dnmt3b isoform is expressed in growing oocytes, RNA isolated from oocytes at E18.5, P10, P15 and P20 was subjected to RT-PCR as described above. As shown in Figure 4B, Dnmt3b1, Dnmt3b2 or both were expressed in oocytes at all stages examined. Dnmt3b3 and the novel isoform were not detected. This result suggests that Dnmt3b found in the nuclei of growing oocytes is functionally active.

Presence of a Dnmt3a isoform in the developing testis and ovary

The results so far suggest that Dnmt3b is the *de novo* methyltransferase that is involved in methylation imprinting in both male and female germline. However, the expression study of the targeted alleles carrying a promoterless IRES-βgeo cassette (Okano et al., 1999) revealed a significant expression of βgeo driven by the endogenous *Dnmt3a* promoter in the developing testis and ovary (Beppu and Li, personal communication). This raised the possibility that, although I did not detect Dnmt3a in the germ cell nuclei with the antibodies against N-terminus of Dnmt3a, there may be some other isoform(s) which could not be recognized by the polyclonal antibodies. Since a monoclonal antibody against Dnmt3a had been commercially available from IMGENEX, I carried out immunostaining with this second anti-Dnmt3a antibody to address this possibility. The specificity of the monoclonal antibody was confirmed by western blotting (figure 1B), just as for the polyclonal antibodies

Figure 6 shows the immunostaining of developing gonads from both sexes, obtained by using this anti-Dnmt3a antibody. To our surprise, the monoclonal antibody yielded uniform staining in the nuclei of gonocytes, which was much more intense than the staining seen in the somatic nuclei (Figure 6A). At E14.5, many gonocytes were stained by the monoclonal antibody, but some others were not. All gonocyte nuclei showed intense uniform staining at E16.5 and E18.5. In the ovary at E18.5, the nuclei of oocytes showed uniform staining at a

level comparable to that of the somatic nuclei (Figure 6B). However, a remarkable accumulation of Dnmt3a was evident in the periphery of the cytoplasm of growing oocytes at P7 and P18. It is worth mentioning that the oocyte nuclei also retained some Dnmt3a, the level of which is comparable to that in the somatic nuclei at this stage. These unexpected results strongly suggest that there is a yet-unidentified isoform(s) of Dnmt3a that is present in the developing testis and ovary.

DISCUSSION

In this study, immunostaining was conducted to examine the expression and distribution of Dnmts in the male and female germ cells. Immunostaining using the polyclonal antibodies raised against the C-terminus of Dnmt1 and the N-terminus of Dnmt3a and Dnmt3b demonstrated that only Dnmt3b was found in the nucleus at the gonocyte stage when gametic methylation is established in the fetal testis. The study also revealed that not only Dnmt3b but also Dnmt1 were present in the nucleus of growing oocytes, in which oocyte-specific methylation pattern is formed. These observations suggest that Dnmt3b may be the only methyltransferase involved in the *de novo* methylation of imprinted loci in both male and female germlines. A monoclonal antibody against Dnmt3a, however, displayed significant staining in the nucleus of germ cells, which were negative with the polyclonal antibodies. The differences between the staining patterns produced by the polyclonal and monoclonal anitbodies suggest the presence of a novel isoform(s) for Dnmt3a that is unrecognizable by the polyclonal anitbodies. Figure 7 summarizes the distribution of each Dnmt in the male and female germlines. Based on the findings, I discuss the role for Dnmts in the establishment of the methylaiton patterns of sperm and oocytes.

Dnmt responsible for the methylaiton imprinting in the male germline

Ueda et al. (2000) recently showed that the DMR at the *H19* locus acquires the paternal-specific methylation pattern between E16.5 and E18.5, suggesting that the methylation imprints are established at the fetal gonocyte stage in the male germline. This implies that Dnmt present in the gonocyte nuclei at this stage plays a crucial role in the establishment of the gametic methylation pattern.

Dnmt1 in the gonocyte nuclei at E14.5, which is detected at the level equivalent to that of

the somatic nuclei, disappeared by E18.5. Male primordial germ cells that have migrated into the genital ridge continue to proliferate before being mitotically arrested around E14.5. Given that the major role of Dnmt1 is to maintain methylation patterns through DNA replication, the gradual loss of this enzyme in mitotically arrested gonocytes is reasonable. Polyclonal antibodies recognizing the N-terminus of Dnmt3a did not detect the protein in gonocytes although its presence was evident in somatic cells. Unlike Dnmt1 and Dnmt3a, Dnmt3b became accumulated in the gonocyte nuclei during this period. This coincides with the establishment of not only the paternal methylation imprints but also the global methylation pattern of the genome. The presence of the active isoforms of Dnmt3b was confirmed by RT-PCR in highly purified male germ cells. It is therefore likely that Dnmt3b is involved in the *de novo* methylation in the male germline.

Dnmt3b, however, did not seem to be the sole enzyme for the *de novo* methylation in gonocytes. A monoclonal antibody (of which epitope is unknown) against Dnmt3a produced intense uniform staining in the gonocyte nuclei at E18.5, which did not necessarily contradict with the results obtained with the polyclonal antibodies against the N-terminus. It is possible that a novel isoform(s) of Dnmt3a is present in the male germ cells, from which the N-terminus of the known Dnmt3a protein is missing. In fact, a preliminary western blot analysis using the extract prepared from the testis at E18.5 demonstrated the presence of a novel Dnmt3a protein with a lower molecular weight as well as the expected one (data not shown). The monoclonal antibody probably recognizes an epitope in the middle or in the C-terminal region of Dnmt3a that is common to all the isoforms. Although further analysis is required, it is tempting to speculate that, in addition to Dnmt3b, this novel isoform(s) of Dnmt3a plays a role in the *de novo* methylation occurring in gonocytes.

Dnmt responsible for the methylation imprinting in the female germline

Kono and colleagues have shown that the maternal functional imprints are established during oocyte growth by generating diploid parthenogenetic embryos with one genome from a non-growing newborn oocyte and the other from a growing oocyte at various stages (Kono et al., 1996; Obata et al., 1998; Obata et al., 2001). Female-specific methylation patterns seem to be formed during this period (Chaillet et al., 1991; Ueda et al., 1993; Stöger et al., 1993), most likely from P5 to P10 (Kono, personal communication). The ovary at P7 was, therefore, analyzed for the distribution of Dnmts, together with the ovaries at E18.5 and P18. Immunostaining with the three polyclonal antibodies revealed that the Dnmts present in the nucleus of growing oocytes were Dnmt1 and Dnmt3b. As was the case for the male germ cells, however, the monoclonal antibody against Dnmt3a disclosed the presence of a presumed new isoform of Dnmt3a in the cytoplasm of growing oocytes at P7. This antibody also stained the oocyte nuclei at P7, though at much reduced levels. It is known that there is a distinct oocyte-specific isofrom of Dnmt1 (Dnmt1o), which is the only isoform found in oocytes (Mertineit et al., 1998). Howell et al. (2001) recently showed that the genomic imprints were appropriately established in oocytes derived from the Dnmt1o-deficient females, indicating that Dnmt1o is not required for the establishment of maternal genomic imprints. It is unlikely that Dnmt1 plays a major role in the *de novo* methyation of the oocyte genome, despite its presence in the nucleus of growing oocytes. Taken together, my results suggest that Dnmt3b and, perhaps, the hypothetical new isoform of Dnmt3a are responsible for the *de novo* methylaiton at the imprinted loci in the female germline.

Role of Dnmts in global demethylation in male germ cells

Based on the analysis of mitotic chromosomes, Coffigny et al. (1999) previously reported that global demethylation occurs in neonatal spermatogonia between P0 and P5. The demethylation is a semiconservative loss of methylation and occurs in a replication-dependent manner. This is

consistent with my immunostaining data on the neonatal testis (data not shown), which is obtained with the anti-5mC antibody. These results argue that the whole genome undergoes a global demethylation despite the presence of Dnmts in the nucleus of germ cells. My RT-PCR analysis revealed that the inactive isoforms for Dnmt3b, i.e. Dnmt3b3 and a novel isoform found in this study, were expressed in male germ cells from E18.5 to P1, prior to and during the global demethylation. These inactive Dnmt3b isoforms disappeared by P7. Although the precise function of these inactive isoforms remains to be determined, they might play an important role in the global demethylation process. For example, these inactive isoforms could inactivate the active enzymes in a dominant-negative manner, perhaps by titrating some regulatory factors.

While the methylation level of the male germ cells declines in the perinatal stage, the *H19* DMR stays methylated during this period (Ueda et al., 2000). It is, therefore, probable that the paternal methylation imprints are protected from the global demethylation. It is of particular interest that Dnmt makes the paternal methylation imprints resistant to the global demethylation in the neonatal testis.

Factors involved in the germline-specific methylation imprinting

Although this study suggests that both the *de novo* methyltransferases Dnmt3a and Dnmt3b are involved in the establishment of the methylation imprints in both sexes, it is still unclear how germline-specific differential methylation is produced. It is possible that some other factors play a role in this process, perhaps in cooperation with the *de novo* methyltransferases. Most recently, Bourc'his et al. (2001) showed that the maternal-specific methylation is disrupted in fetuses derived from the females deficient for Dnmt3l. Dnmt3l belongs to the Dnmt3 family, but does not look like a cytosine-methyltransferase because it lacks a large part of the evolutionarily conserved catalytic domain. This raises the possibility that Dnmt3l may cooperate with the *de novo* methyltransferases to promote the maternal-specific methylation

imprinting. Further study will shed lights on the mechanism how gamete-specific methyaiton imprints are established.

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FIGURES

Figure 1. Immunoblot analysis with polyclonal anti-Dnmt3a (A), monoclonal anti-Dnmt3a (B) and anti-Dnmt3b antibodies (C). Cell lysates prepared from 293T cells expressing exogenous Dnmt3a, Dnmt3b1, Dnmt3b2 or Dnmt3b3, were used to verify the specificity of the antibodies. They did not cross-react with the related proteins.

Figure 1

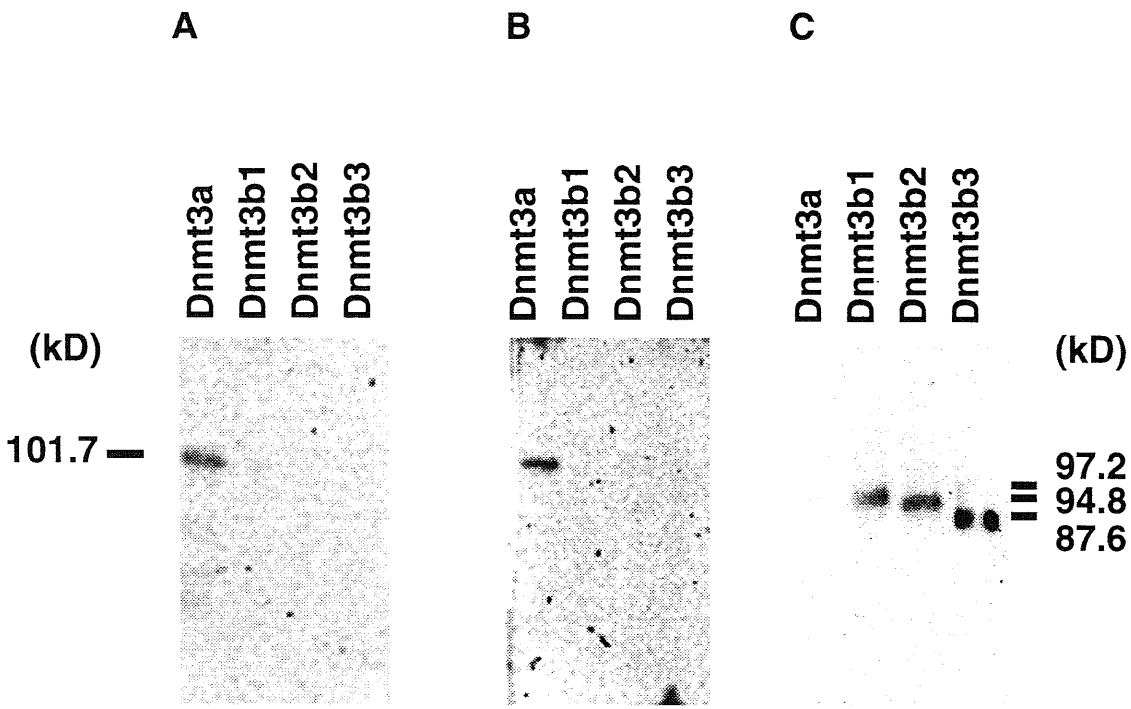


Figure 2. Localization of Dnmts during male germ cell development. Cryosections of the fetal testis at E14.5 (A), E16.5 (B) and E18.5 (C) were stained with the antibodies against each Dnmt (red) in combination with the germ cell-specific antibody TRA98 (green). In the germ cells at this stage (gonocytes), a gradual loss of Dnmt1 was evident. Dnmt3a was not detected in gonocytes at any stages. Dnmt3b was the major Dnmt found in gonocytes, at E16.5 and E18.5.

Figure 2

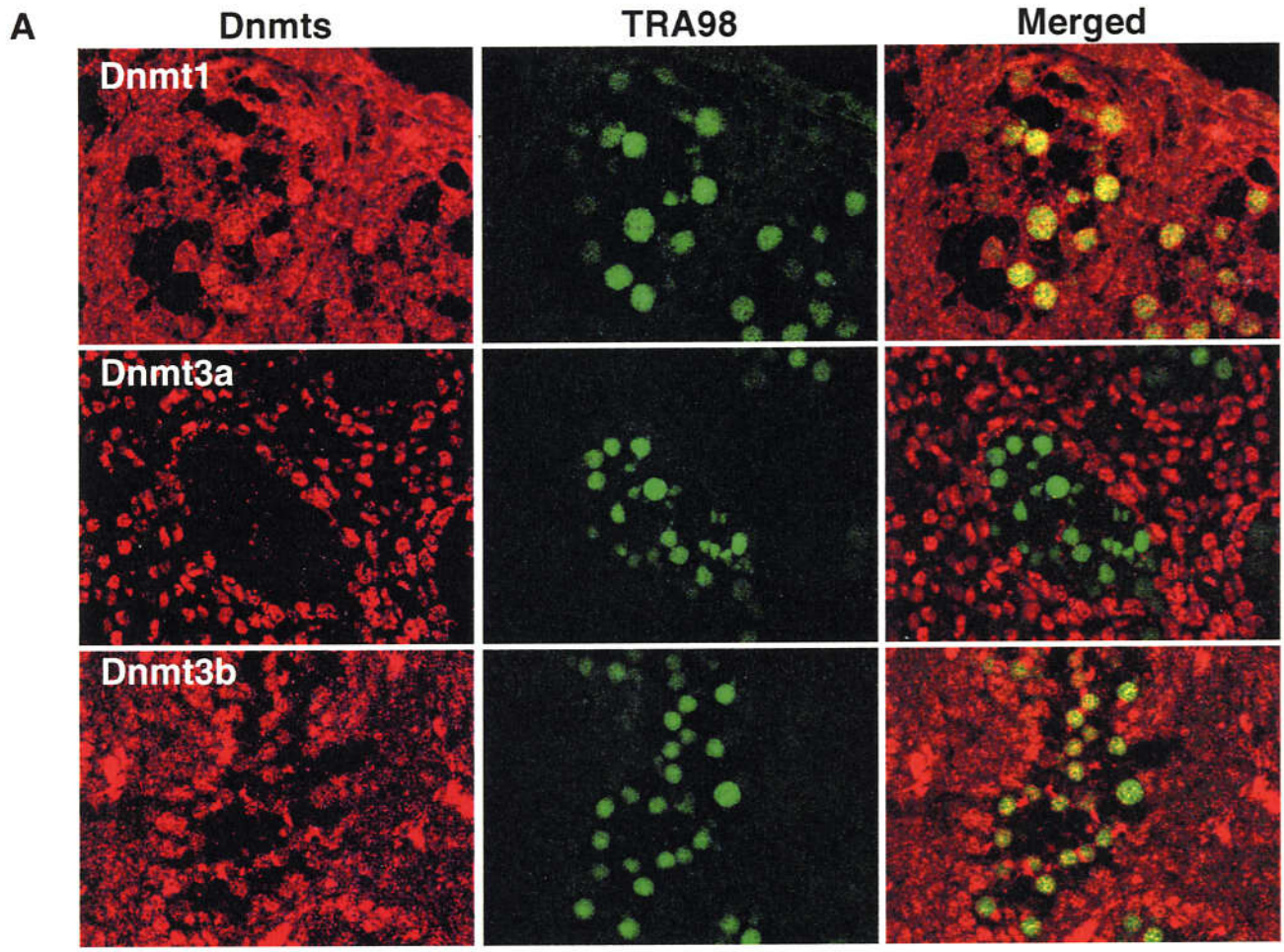


Figure 2

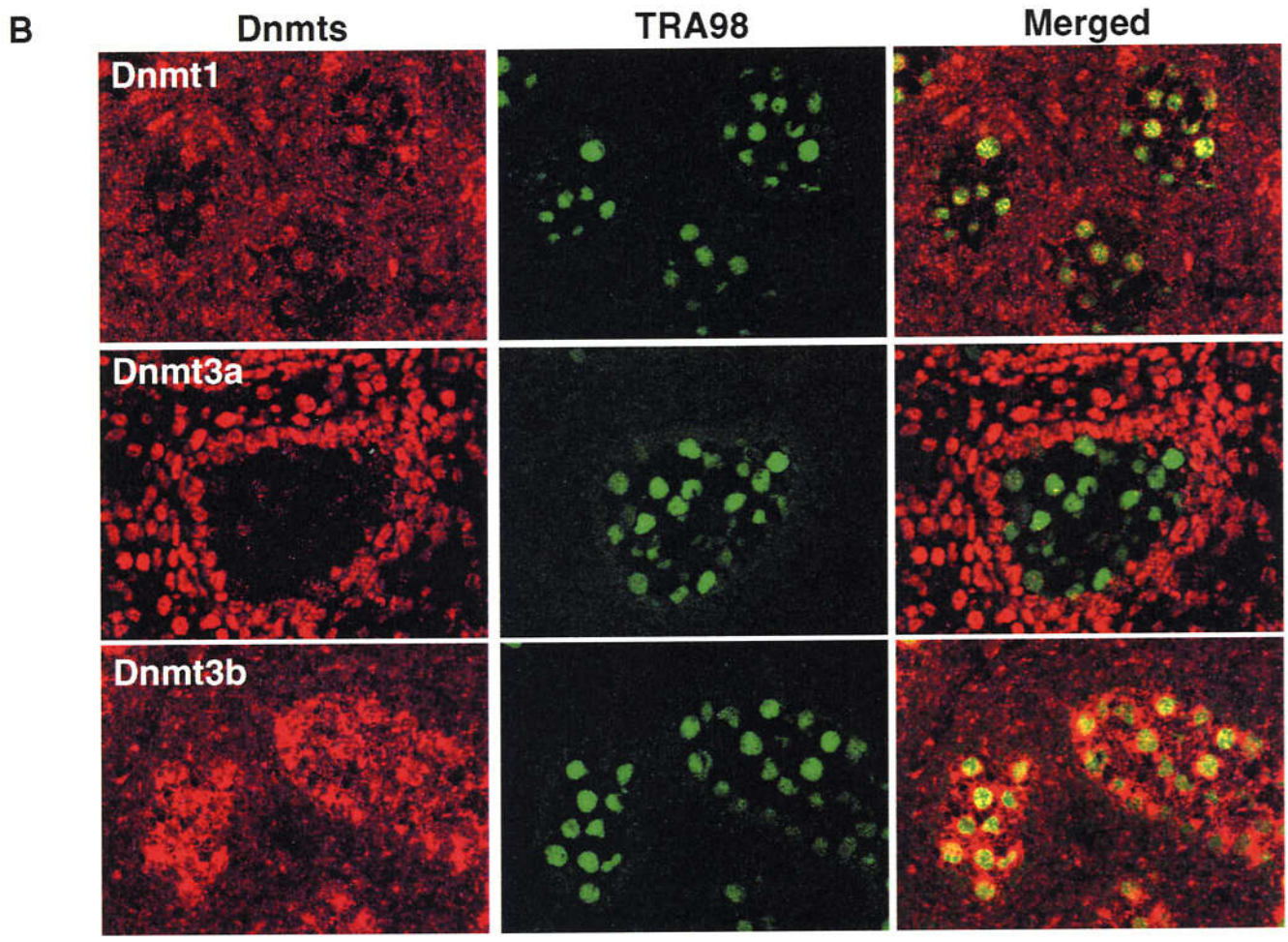


Figure 2

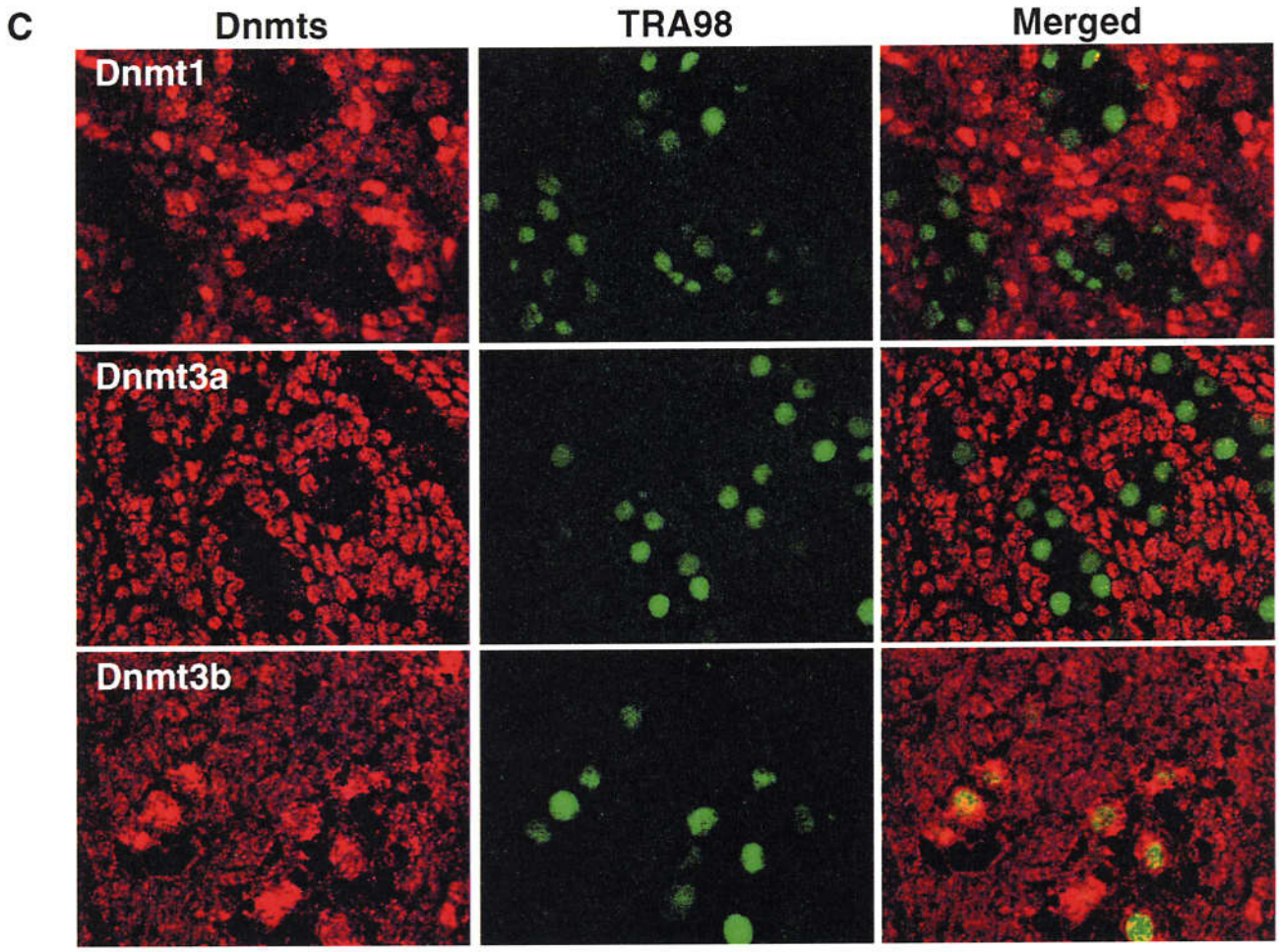


Figure 3. Double immunostaining of the fetal testis at E16.5 (A-C) and E18.5 (D-F) with anti-5mC (A, D) and anti-Dnmt3b antibodies (B, E). The staining with the anti-5mC antibody strikingly increased between E16.5 and E18.5. DNA was counterstained by TOTO-3.

Figure 3

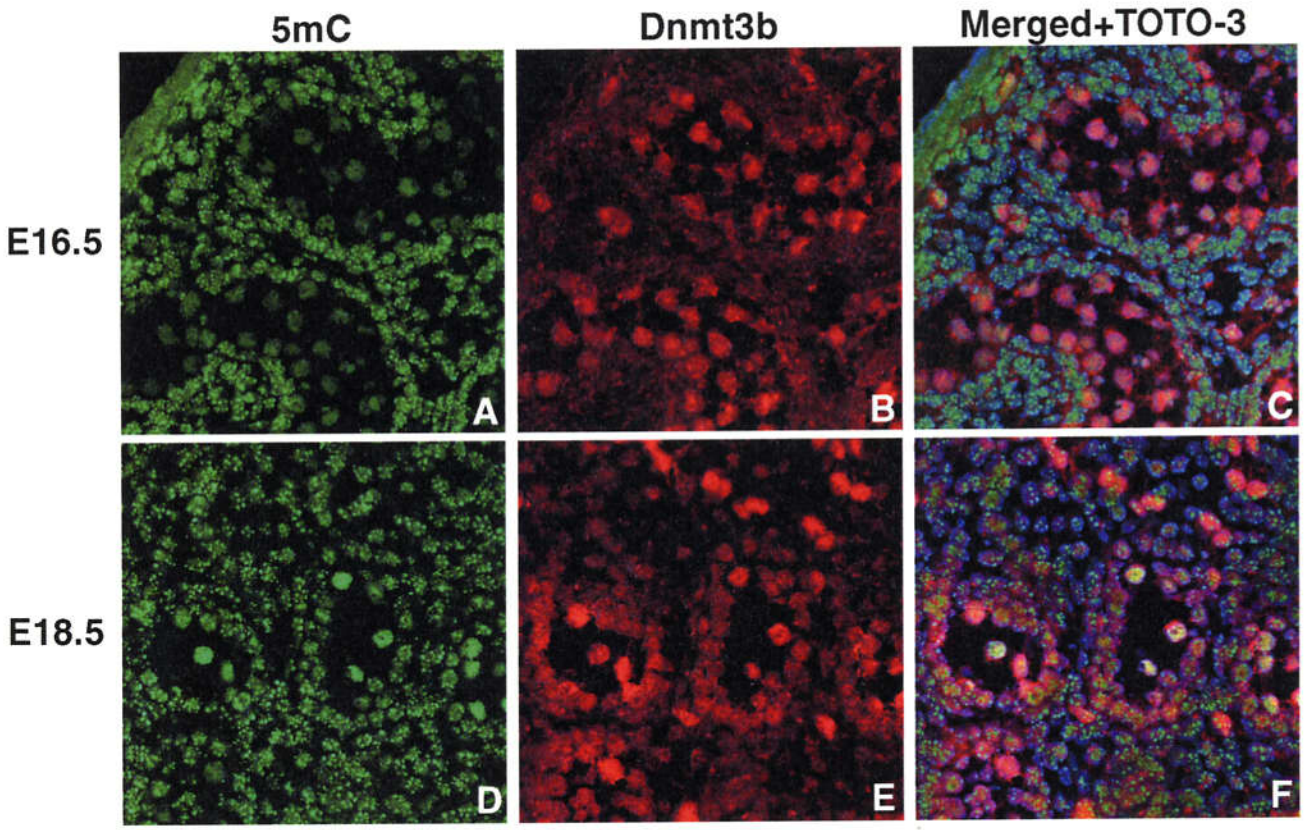
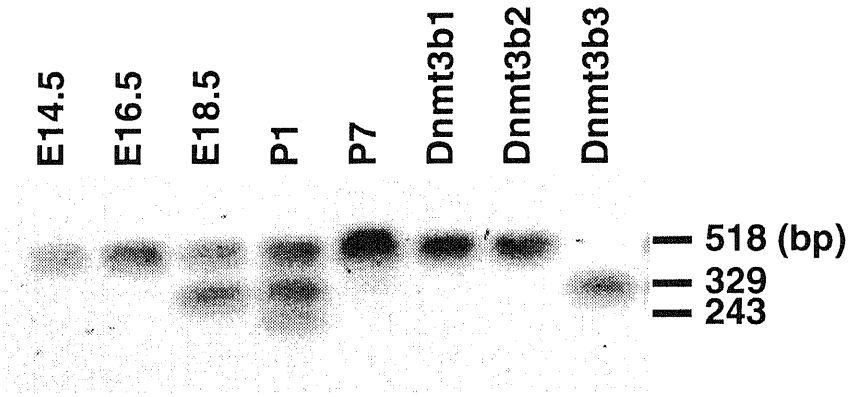


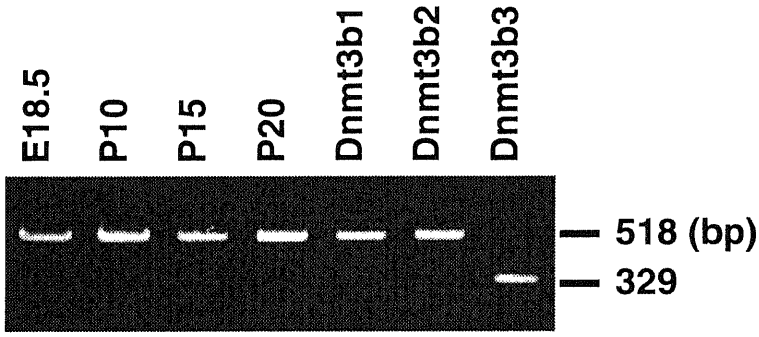
Figure 4. Analysis of Dnmt3b splicing isoforms in germ cells by RT-PCR. (A) Splicing isoforms in male germ cell development. RT-PCR products were run on a 1.5% alkaline agarose gel and subjected to Southern blotting. Active isoforms were expressed at all stages examined, while expression of the inactive isoform was restricted at E18.5 and P1. A novel splicing isoform was found at P1. (B) Splicing isoform in oocyte development. RT-PCR products were run on a 1.5% agarose gel. Only the active isoform was expressed in oocytes at all stages examined. (C) Structure of the novel isoform of Dnmt3b in comparison with the known isoforms. (D) Amino acid sequences of Dnmt3b3 and the novel isofrom. Loss of exon 20 in the novel isoform causes a frame-shift in the C-terminal region. Evolutionarily conserved motifs present in all known cytosine methyltransferase are also shown (V, VI, VII, IX and X).

Figure 4

A


E14.5
E16.5
E18.5
P1
P7
Dnmt3b1
Dnmt3b2
Dnmt3b3
518 (bp)
329
243

B


E18.5
P10
P15
P20
Dnmt3b1
Dnmt3b2
Dnmt3b3
518 (bp)
329

C

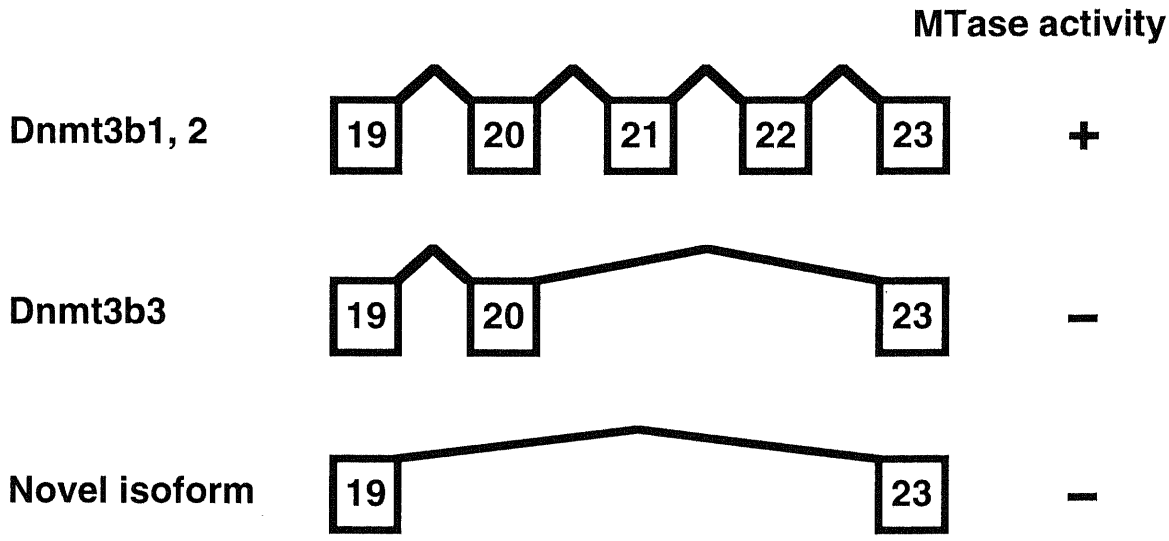

MTase activity
Dnmt3b1, 2
19
20
21
22
23
+
Dnmt3b3
19
20
23
–
Novel isoform
19
23
–

Figure 4

D

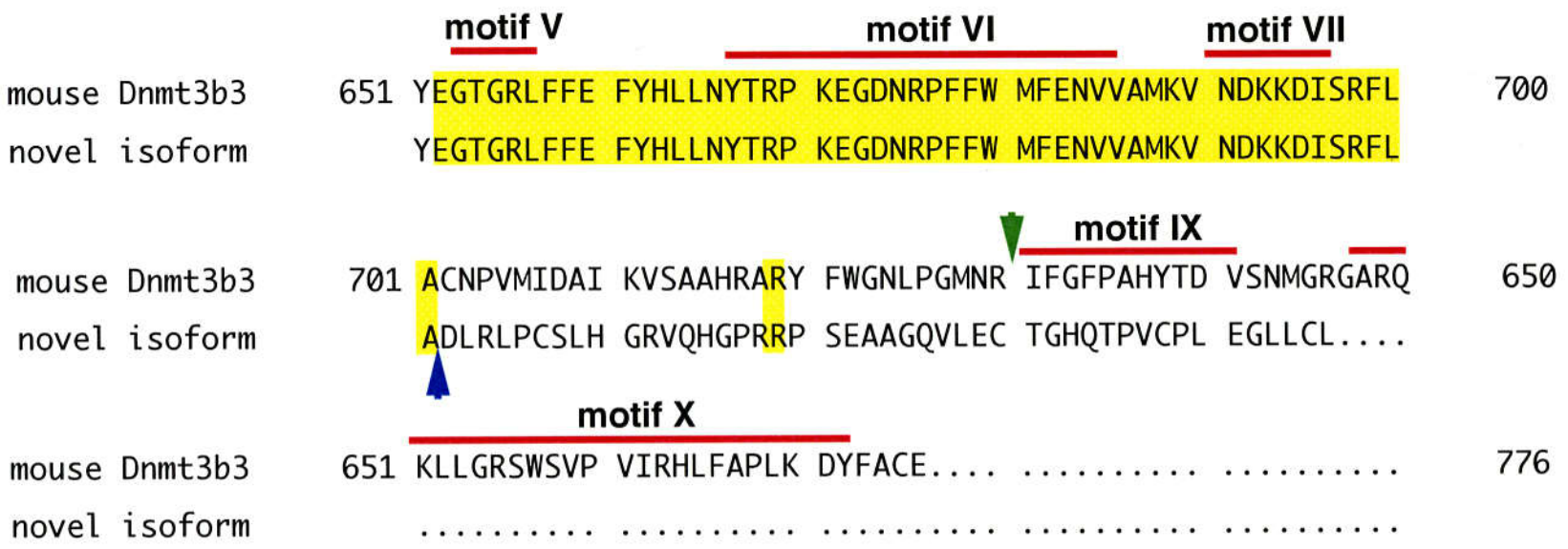

motif V
motif VI
motif VII
mouse Dnmt3b3 651 YEGTGRLFFE FYHLLNYTRP KEGDNRPFFW MFENVVAMKV NDKKDISRFL 700
novel isoform YEGTGRLFFE FYHLLNYTRP KEGDNRPFFW MFENVVAMKV NDKKDISRFL
motif IX
mouse Dnmt3b3 701 ACNPVMIDAI KVSAAHRARY FWGNLPGMNR IFGFPAHYTD VSNMGRGARQ 650
novel isoform ADLRLPCSLH GRVQHGPRRP SEAAGQVLEC TGHQTPVCPL EGLLCL....
motif X
mouse Dnmt3b3 651 KLLGRSWSVP VIRHLFAPLK DYFACE.... 776
novel isoform ....

Figure 5. Localization of Dnmts during oogenesis. Cryosections of the ovary at E18.5 (A), P7 (B), and P18 (C) were stained with antibodies against each Dnmt (red). In panel A, double staining with TRA98 was used to identify oocytes (green). In panels B and C, DNA was counterstained with TOTO-3. Dnmt1 was detected in the nucleus of non-growing oocytes at E18.5 (A) and in both the nucleus and cytoplasm of growing oocytes at P7 (B). The oocyte at P18 (C) showed strong staining for Dnmt1 in the cytoplasm. Dnmt3a was detected in somatic cells, but virtually undetectable in oocytes between E18.5 and P7 (A, B). Dnmt3a was found in the cytoplasm of oocytes at P18 (C). Dnmt3b was predominantly found in cytoplasm of oocytes at E18.5 (A), but in both the nucleus and cytoplasm at P7. Dnmt3b was retained in the periphery of the oocyte cytoplasm at P18 (C).

Figure 5

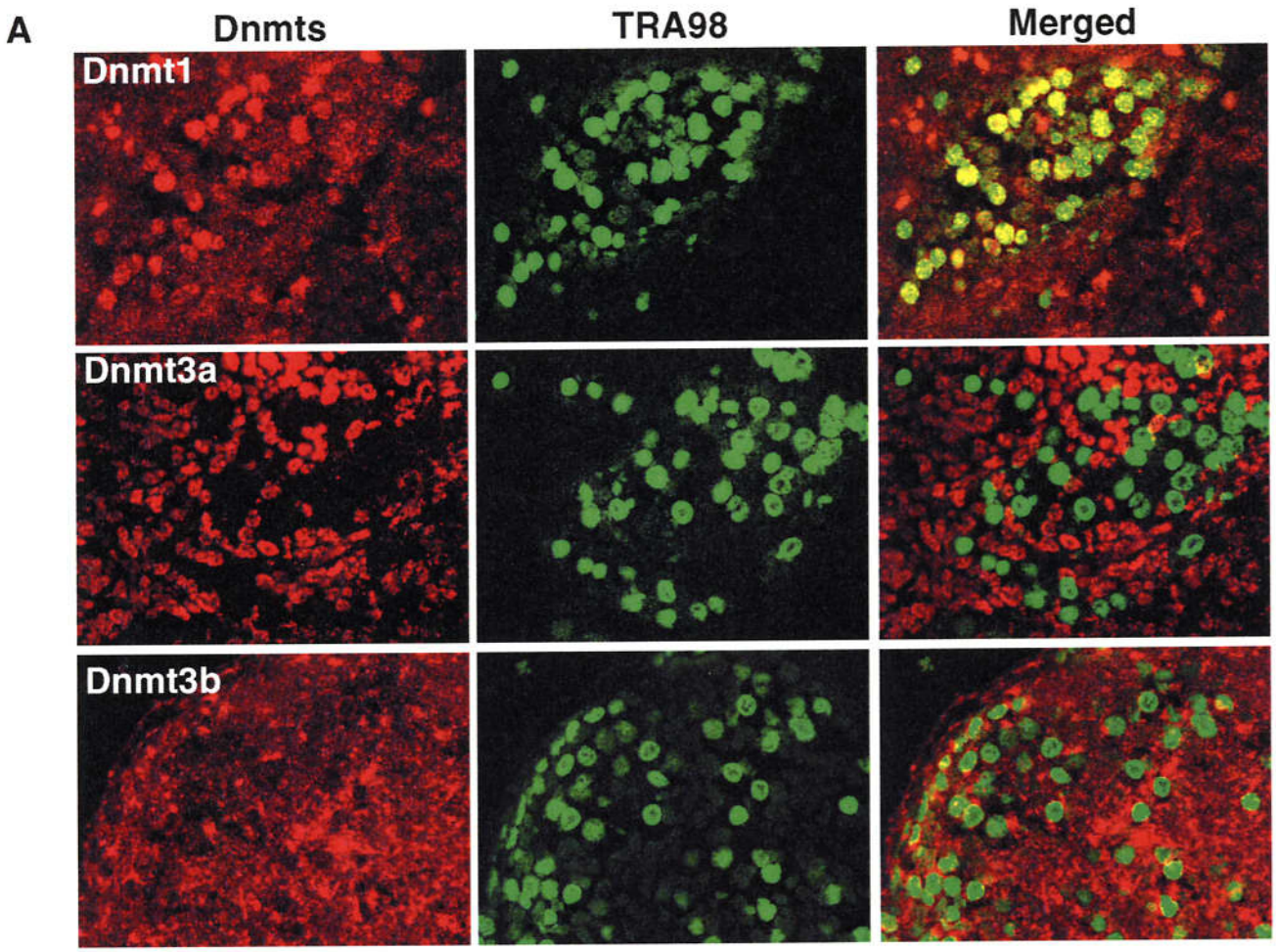


Figure 5

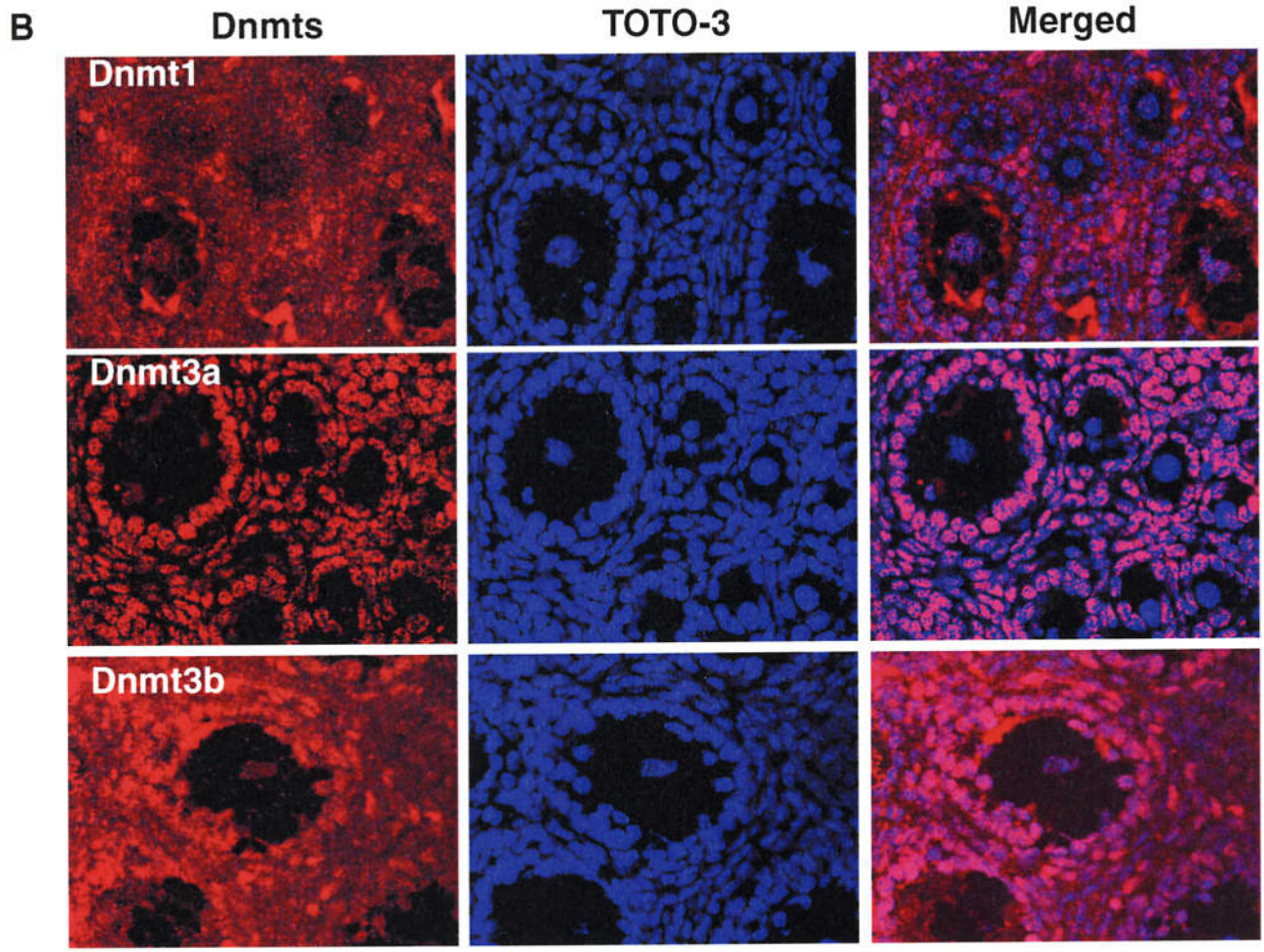


Figure 5

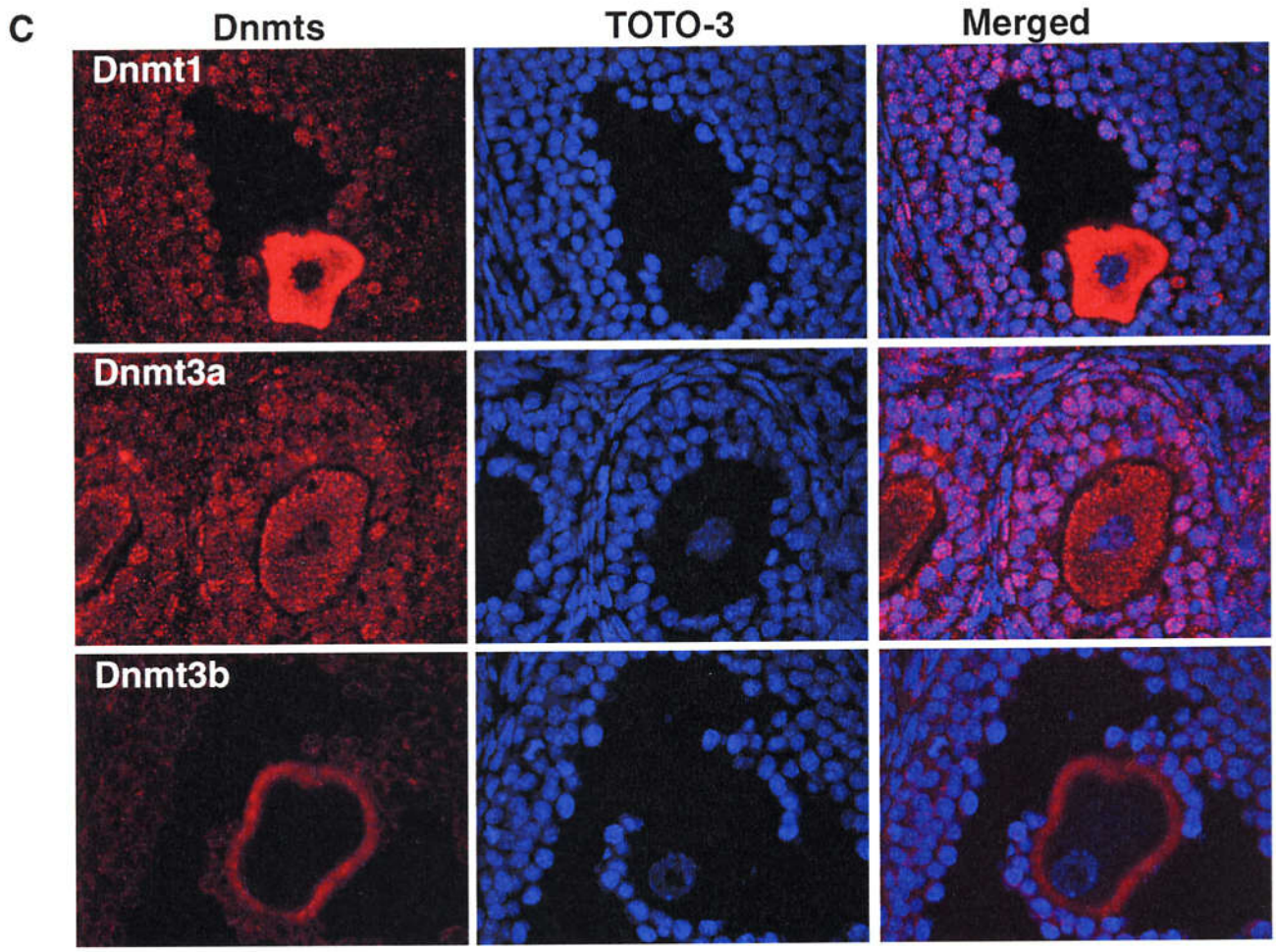


Figure 6. Distribution of Dnmt3a detected by a monoclonal antibody from IMGENEX. Cryosections of male gonads at E14.5, E16.5 or E18.5 (A) and female gonads at E18.5, P7 or P18 were reacted with the monoclonal antibody against Dnmt3a. Unlike the polyclonal antibody against the N-terminus of Dnmt3a, staining was evident in the nucleus of gonocytes at the all time examined and growing oocytes at P7 and P18. Although staining was extremely intense in the oocyte cytoplasm at P7, the nucleus also retain detectable levels of Dnmt3a.

Figure 6

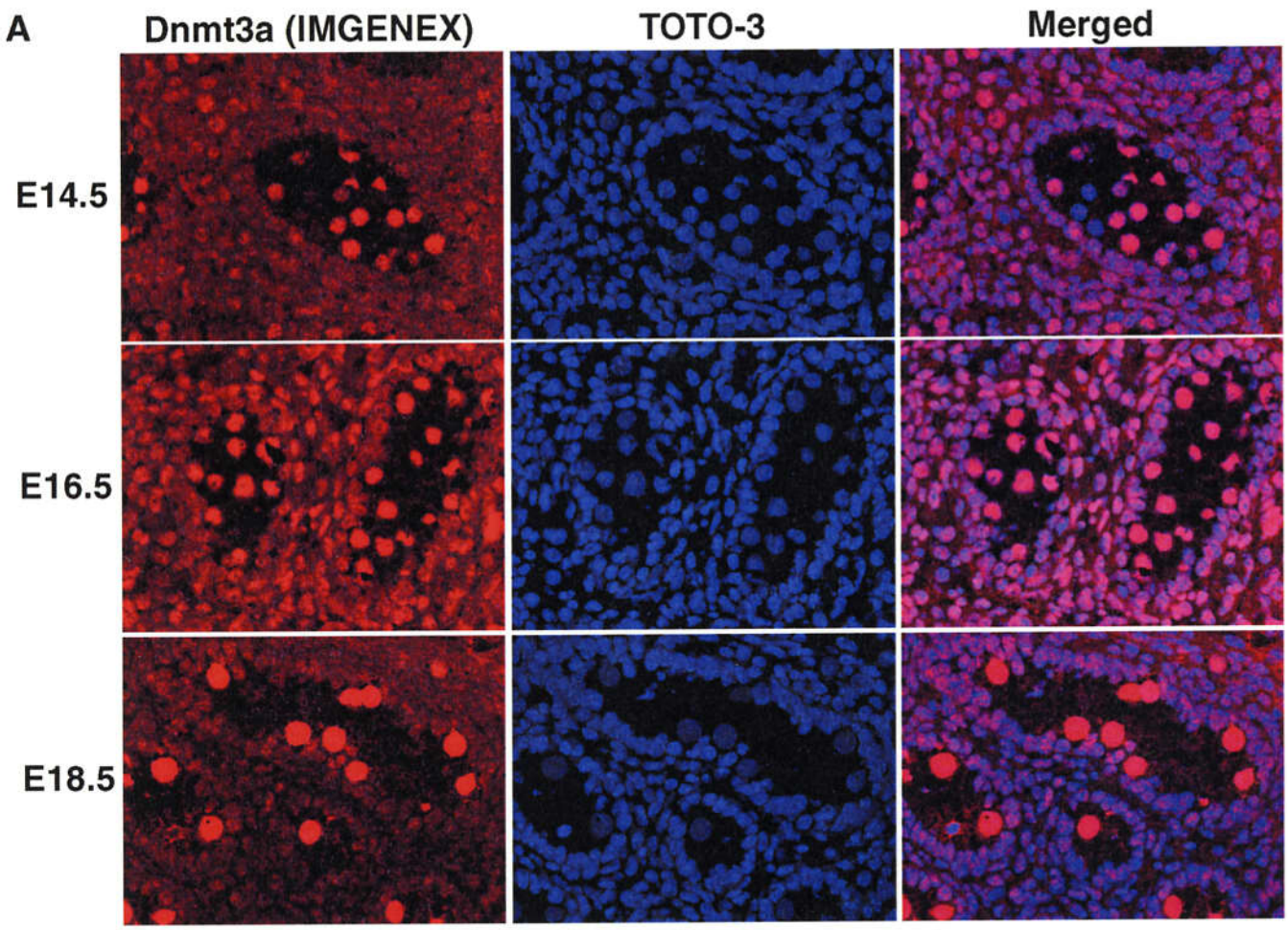


Figure 6

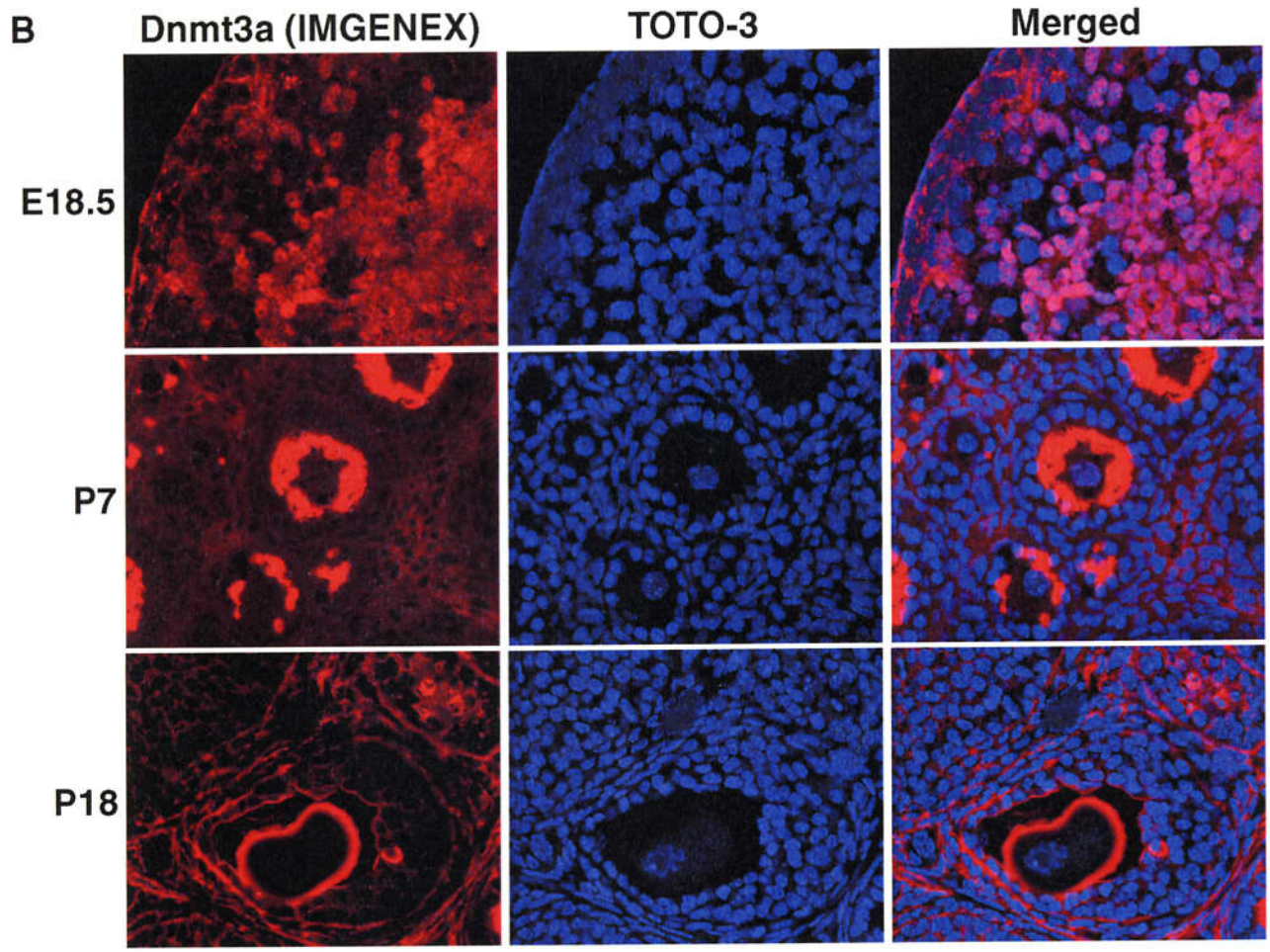


Figure 7. Summary showing the expression and localization of Dnmts in the male (A) and female germline (B). Changes in methylation levels at the *H19* locus (A) and the *Igf2r* locus (B) as well as the whole genome (both A and B) are also shown. The presence or absence of the active isoforms of Dnmt3b is shown by + or -.

Figure 7

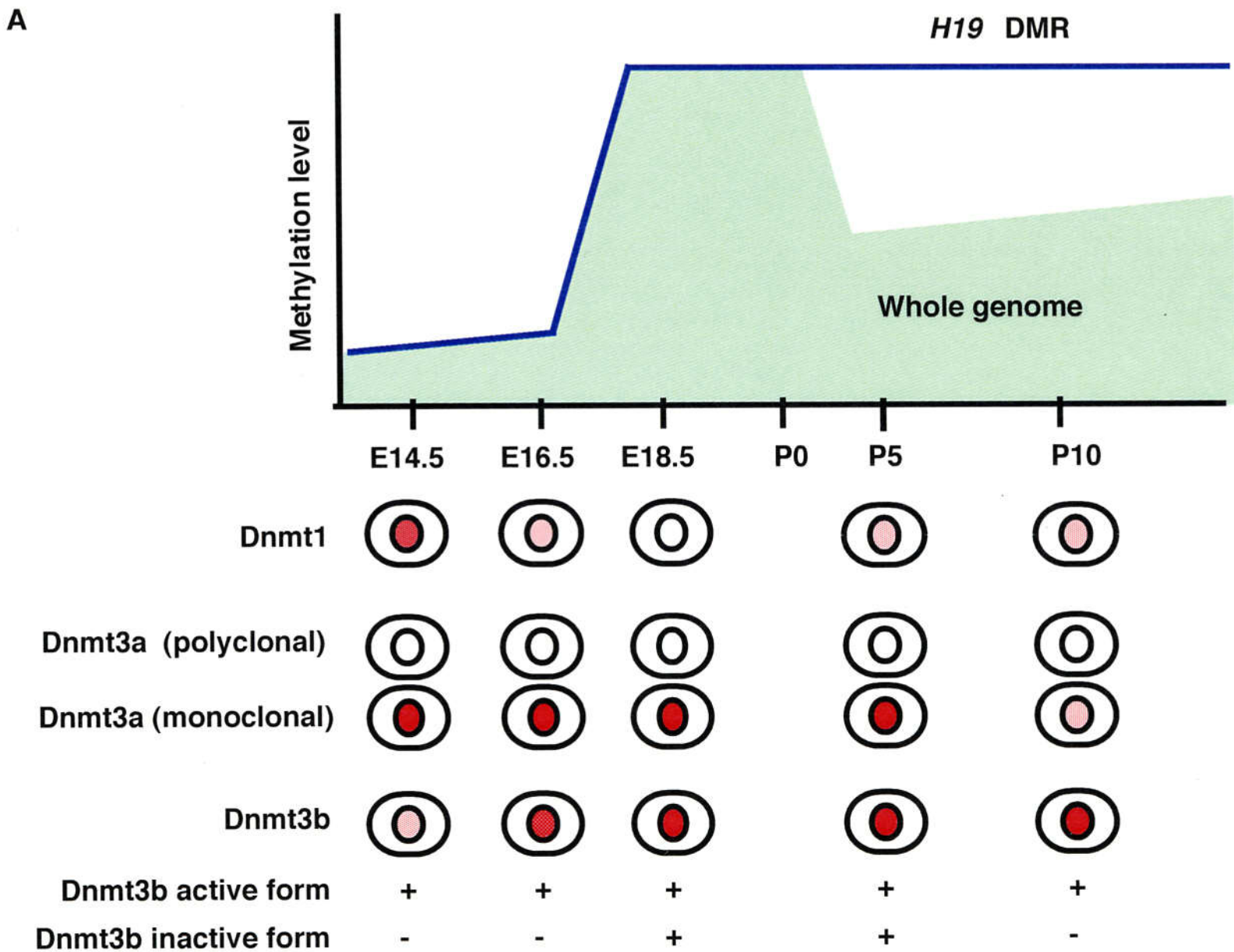
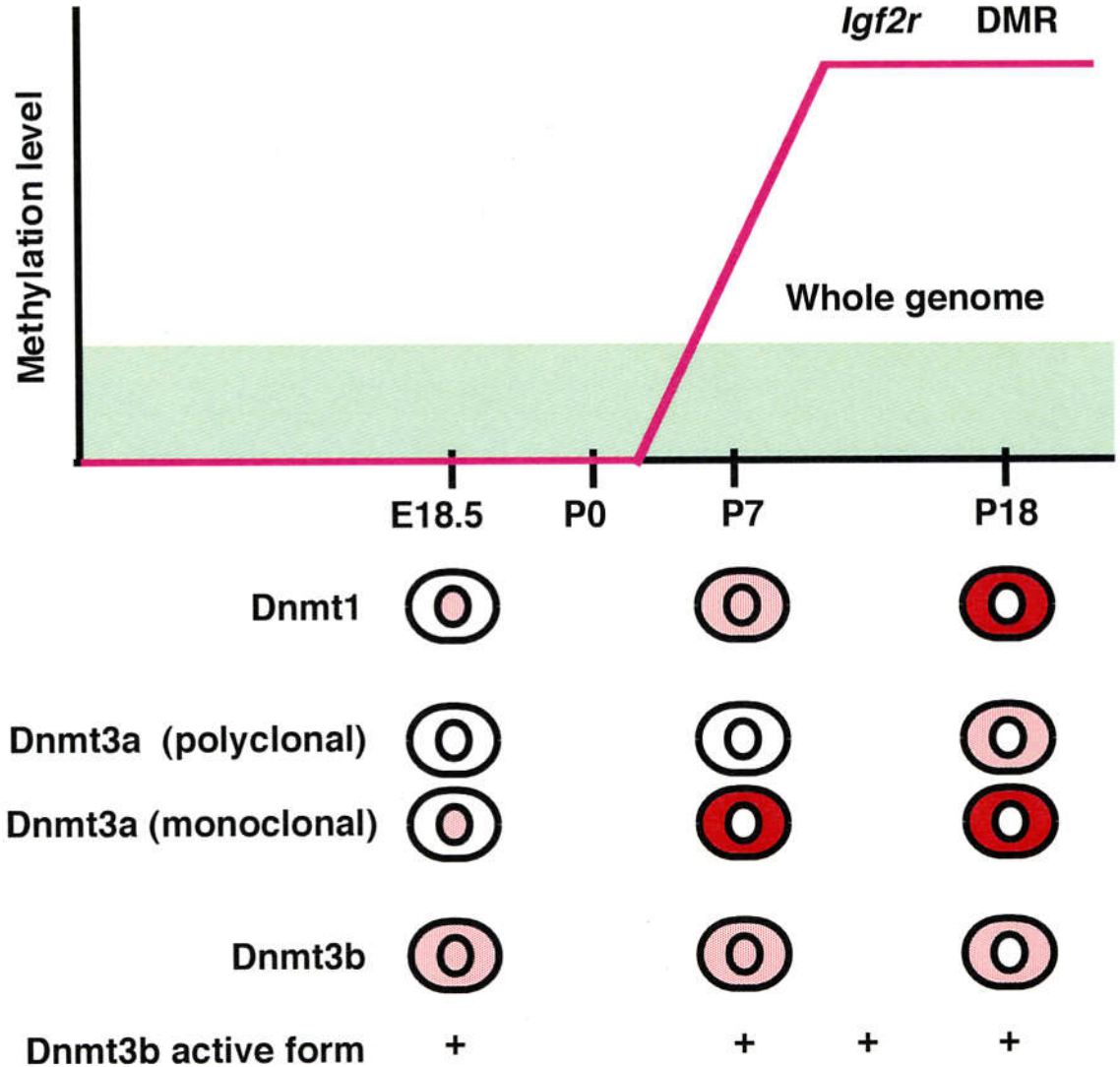

A
H19 DMR
Methylation level
Whole genome
E14.5
E16.5
E18.5
P0
P5
P10
Dnmt1
Dnmt3a (polyclonal)
Dnmt3a (monoclonal)
Dnmt3b
Dnmt3b active form + + + + +
Dnmt3b inactive form - - + + -

Figure 7

B


Igf2r DMR
Methylation level
Whole genome
E18.5
P0
P7
P18
Dnmt1
Dnmt3a (polyclonal)
Dnmt3a (monoclonal)
Dnmt3b
Dnmt3b active form
+
+
+
+