# Role of *de novo* DNA Methyltransferases *Dnmt3a* and *Dnmt3b* in the Establishment of Genomic Imprinting

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**Gene symbols**

*Gapd*: glyceraldehyde-3-phosphate dehydrogenase

*Igf2r*: insulin-like growth-factor 2 receptor

*P57$^{kip2}$*: cyclin-dependent kinase inhibitor p57

*Peg1/Mest*: paternally expressed gene 1/mesoderm specific transcript

*Peg3*: paternally expressed gene 3

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

*Snrpn*: small nuclear ribonucleoprotein N

*TNAP*: tissue non-specific alkaline phosphatase

**Abstract**

Genomic imprinting refers to the parental-origin-specific gene expression of a subset of autosomal genes in mammals. Disruption of imprinting causes embryonic or postnatal lethality, growth retardation, abnormal behavior and many human diseases. It has been proposed that DNA methylation marks the imprinted genes differently during male and female gametogenesis. The epigenetic differences (imprints) between the two gametes lead to parental-origin-specific gene expression in the offspring. These imprints are maintained during development but erased in the fetal germ cells, and then re-established during gametogenesis in a sex-specific manner. Mice deficient for *Dnmt1*, which is a maintenance methyltransferase, show embryonic lethality, genome-wide demethylation and disruption of genomic imprinting. This indicates *Dnmt1* is essential for the maintenance of imprints postfertilization. On the other hand, the primary germline imprints are thought to be established during gametogenesis through the action of a *de novo* DNA methyltransferase(s). However, targeted disruption of the *de novo* DNA methyltransferases (*Dnmt3a* and *Dnmt3b*) in mice results in embryonic or early postnatal lethality. Therefore, the role of DNA methylation in the establishment of the germline imprints cannot be addressed in these mice.

To circumvent this problem, I took advantage of the Cre-loxP system to inactivate the two *de novo* DNA methyltransferases in a germline-specific manner. The floxed *Dnmt3a* and *Dnmt3b* alleles were disrupted in both male and female germlines by introducing Cre recombinase driven by the endogenous *TNAP* (tissue non-specific alkaline phosphatase) promoter. These germline-specific *Dnmt3a* and *Dnmt3b* knockout mice should be viable and expected to grow up to adulthood because the deletion should occur only in germ cells. Analysis

of the embryos and gametes from these germline-specific *Dnmt3a* and *Dnmt3b* knockout mice will determine the function of each enzyme in the establishment of genomic imprints.

The conditional *Dnmt3a* knockout mice that I generated were indeed viable and grew up to adulthood, though somatic tissues had various degrees (56%-80%) of the deletion. Recombination efficiency was determined in fetal germ cells, oocyte and sperm, which suggested that the *Dnmt3a* alleles was mostly inactivated before the onset of methylation imprints. Offsprings from the conditional *Dnmt3a* knockout females crossed with wild-type males died around embryonic day 9.5-10.5 (E9.5-10.5). All embryos that I examined had recombined allele only, suggesting that the recombination efficiency was 100%. Embryos appeared grossly normal at E9.5, but they showed growth retardation, defects in neural tube closure and lack of branchial arches by E10.5. At E11.5, only resorptions were seen. The phenotype was similar to that of the embryos conceived by *Dnmt3L* knockout females, which are defective in establishing the maternal methylation imprints during oogenesis. Indeed, the maternal methylation imprints and the allele-specific expression of several imprinted genes that I examined were lost in the embryos conceived by the conditional *Dnmt3a* knockout mice. These results indicate that Dnmt3a is a key enzyme that establishes maternal methylation imprints during oogenesis. Since Dnmt3L does not have any detectable DNA methyltransferase activity, it is conceivable that Dnmt3a and Dnmt3L cooperate in the process of maternal methylation imprinting. The conditional *Dnmt3a* knockout males were also viable but showed impaired spermatogenesis, again resembling the *Dnmt3L* knockout males. At postnatal day 11 (P11), the testes from these conditional *Dnmt3a* knockout males appeared normal, but, at 11 weeks of age, the size and weight of the testes were significantly reduced. Virtually no spermatids or spermatozoa were observed in the seminiferous tubules of these testes. These results showed that *Dnmt3a,* as well as *Dnmt3L,* is required for spermatogenesis.

Both the conditional *Dnmt3b* knockout males and females were viable and grew up to adulthood, in contrast to the *Dnmt3b*-null mice, which die in late gestation. No deletion was observed in most somatic tissues examined, except for a small proportion of skeletal muscle cells. Most fetal germ cells had recombined *Dnmt3b* allele, suggesting that the floxed *Dnmt3b* allele was mostly deleted before the onset of methylation imprints. A total of 88 pups were born from the conditional *Dnmt3b* knockout females and 87 pups had the recombined allele, suggesting a high rate of recombination by Cre. These pups grew up to adulthood and were fertile. No abnormalities were observed in the embryos. The methylation levels of the imprinted genes and minor satellite DNA, which is the target sequence of *Dnmt3b*, were also normal. These results suggest that *Dnmt3b* is not required for oogenesis or the establishment of maternal methylation imprints. The pups and embryos derived from the conditional *Dnmt3b* knockout males were also normal and showed 100% recombination efficiency (102/102). No abnormal phenotype, imprinting defect, or change in methylation level was observed. Also, histological sections of the testes from these conditional *Dnmt3b* knockout males showed normal spermatogenesis. These results suggest that *Dnmt3b* is not required for spermatogenesis or the establishment of paternal methylation imprints. I also examined whether the *Dnmt3b* allele recombined by Cre was functionally null. Embryos homozygous for the recombined *Dnmt3b* allele were embryonic lethal and the phenotype (growth retardation, rostral neural defects and demethylation of centromeric minor satellite repeats) was very similar to that of *Dnmt3b*-null embryos. These observations confirmed that the floxed *Dnmt3b* allele was successfully inactivated by the Cre recombinase.

This work suggests that *Dnmt3a*, but not *Dnmt3b*, is responsible for the establishment of the maternal imprints. The results from my work also suggest that the primary imprints established in gametogenesis is DNA methylation. *Dnmt3a* is also required for

spermatogenesis, but whether *Dnmt3a* is responsible for the establishment of paternal methylation imprints is yet to be investigated.

## Introduction

Genomic imprinting is a biological phenomenon that causes parental-origin-dependent monoallelic expression of a subset of genes in mammals. So far, approximately fifty imprinted genes have been identified in mouse and human, and it is estimated that a few to several hundred imprinted genes exist in the mammalian genome. Imprinted genes play important roles in fetal growth, development of particular lineages, and maternal behaviors, as well as occurrence of human diseases (Reik *et al.*, 2001). In mice, replacement of male or female pronuclei in fertilized eggs by nuclear transplantation showed that the diploid conceptuses with genomes from only one parent never developed to term (McGrath *et al.*, 1984; Surani *et al.*, 1984). These experiments show that both a paternal and a maternal genome are required for normal development. In human, it is known that parthenogenesis (development with two maternal genomes) causes ovarian teratoma and androgenesis (development with two paternal genomes) causes hydatidiform mole. Uniparental disomies, which inherit a pair of specific chromosome from only one parent, show various disease conditions, including Prader-Willi syndrome, Angelman syndrome and Beckwith-Wiedemann syndrome (Reik *et al.*, 2001).

What is the mechanism of genomic imprinting? The epigenetic memory regarding parental origin should be imposed on the genome during male and female gametogenesis. These memories inherited from the parents are called imprints. Imprints are maintained through fertilization and subsequent rounds of DNA replication and cell divisions during development. In somatic cells, imprints lead to differential gene expression between the parental alleles of imprinted genes. In germ cells, imprints are erased at an early stage. This is followed by re-establishment of sex-specific imprints at a later stage of germ-cell development, thus completing the imprinting cycle. Cytosine methylation at CpG dinucleotides is a major epigenetic regulation

system in mammals and is essential for normal development (Li *et al.*, 1992). DNA methylation can be propagated to daughter cells, thereby acting as an epigenetic mark, and it can be erased by passive demethylation (in the absence of maintenance methyltransferase during or after DNA replication) or active demethylation (by an unknown mechanism). Most of the imprinted genes examined so far show differences in DNA methylation between the parental alleles, suggesting a crucial role for methylation in imprinting. Such differentially methylated regions (DMRs) are often identified in the upstream region or an intron of the imprinted genes. Furthermore, the parental-origin-specific DNA methylation in some DMRs is derived from the gamete DNAs. For example, the paternal-specific methylation of the maternally expressed imprinted gene *H19* is inherited from the sperm DNA, and the maternal-specific methylation of the maternally expressed imprinted gene *Igf2r* is inherited from the oocyte DNA. In each case, the DMR in the gamete of the opposite sex has been shown to be unmethylated. These observations strongly suggest that the differential DNA methylation (also called methylation imprint) is the parental imprint itself.

Direct evidence that DNA methylation is involved in imprinting came from a gene knockout experiment in which the maintenance-type DNA methyltransferase was disrupted. It is known that mammals such as human and mouse have three DNA methyltransferase genes: *Dnmt1* is the maintenance-type DNA methyltransferase, which has a preference for hemimethylated DNA and reproduces the preexisting genomic methylation patterns after DNA replication; *Dnmt3a* and *Dnmt3b* are the *de novo* methyltransferases, which create new methylation patterns on unmethylated DNA (Okano *et al.*, 1998). Mice deficient for Dnmt1 ($Dnmt1^{-/-}$) showed embryonic lethality by E9.5, genome-wide demethylation and disruption of genomic imprinting (Li *et al.*, 1992; Li *et al.*, 1993). In these embryos, normally active paternal allele of *Igf2* and normally active maternal allele of *Igf2r* were silenced while normally silenced

paternal allele of *H19* became activated (Li *et al.*, 1993). These results clearly demonstrate that DNA methylation is essential for the maintenance of imprints in the zygote after fertilization and in subsequent embryonic development. However, this experiment did not address the question of whether DNA methylation is involved in parental imprinting of the gamete genome since the parents of the *Dnmt1*$^{-/-}$ embryos are heterozygous for the mutation and thus carry one intact *Dnmt1* allele.

Although direct evidence for DNA methylation being the gametic imprint is still lacking, changes in methylation at some DMRs during germ cell development were studied in some detail. It has been shown that the methylation imprints are first erased in primordial germ cells (PGCs). Production of cloned embryos by nuclear transfer of donor nuclei from embryonic day 11.5-13.5 (E11.5-13.5) PGCs revealed that the erasure of the imprinting memories occurs between E10.5 and E11.5 (Lee *et al.*, 2002). DNA methylation analysis of PGC genomes also revealed that the erasure of methylation imprints is an active demethylation process and completed within one day (Hajkova *et al.*, 2002). After this erasure process, each DMR acquires its own methylation imprints during male and female gametogenesis. It has been revealed that most imprinted genes, such as *Igf2r*, *Peg1* and *Snrpn*, are methylated in the female germ cells (Stöger *et al.*, 1993; Lefebvre *et al.*, 1997; Shemer *et al.*, 1997), whereas only *H19* and *Rasgrf1* are methylated in the male germ cells (Tremblay *et al.*, 1995; Pearsall *et al.*, 1999). First evidence that the maternal methylation imprints are established during the oocyte growth stage came from the studies on an imprinted transgene (Ueda *et al.* 1992). Then, nuclear transfer experiments from immature oocytes to fully-grown oocytes showed that the maternal imprints of endogenous imprinted genes are also established during oocytes growth (Obata *et al.*, 1998). On the other hand, the studies on the paternally methylated *H19* gene showed that the paternal methylation imprints are established in the gonocyte (prospermatogonia) stage of male germ cell

development (Ueda *et al.*, 2000; Davis *et al.*, 2000).

More recently, efforts were made to know which DNA methyltransferase or DNA methyltransferase-related protein is required for the *de novo* methylation of the imprinted genes in the male and female germ cells. It has been shown that *Dnmt1o*, an oocyte-specific isoform of *Dnmt1*, is highly expressed in oocytes and responsible for the maintenance of methylation imprints at the eight-cell stage (Howell *et al.*, 2001). However, this *Dnmt1* isoform was not required for the establishment of the methylation imprints in oocytes (Howell *et al.*, 2001). Dnmt3L is a protein similar to the *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* but lacks a part of the DNA methyltransferase domain. *Dnmt3L*$^{-/-}$ female mice failed to establish the maternal methylation imprints in oocytes and *Dnmt3L*$^{-/-}$ male mice showed defects in spermatogenesis (Bourc'his *et al.*, 2001; Hata *et al.*, 2002). Since Dnmt3L protein possessed no detectable methyltransferase activity, one or more of the DNA methyltransferases may be involved in the establishment of gametic methylation imprints. Indeed, it has been shown that Dnmt3L interacts with the *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* and colocalizes with these enzymes in the nuclei of transfected cells (Hata *et al.*, 2002). These results suggest that Dnmt3L is a regulator of the *de novo* DNA methyltransferases and that either *Dnmt3a*, *Dnmt3b* or both are involved in the establishment of methylation imprints in the gametes.

Tsujimoto of our laboratory previously studied the expression of Dnmt3a and Dnmt3b in the male and female germ cells by immunostaining and showed that Dnmt3b and a specific isoform of Dnmt3a called Dnmt3a2 are present in growing oocytes and gonocytes (Tsujimoto, 2002 (thesis)). These expression patterns are consistent with the role for the *de novo* DNA methyltransferases in methylation imprinting in germ cells. However, since knockout mice deficient for *Dnmt3a* or *Dnmt3b* die before reaching the reproductive age, direct evidence for the

involvement of these enzymes could not be studied. For example, *Dnmt3a*$^{-/-}$ mice were runted, showed malfunction of the gut, and died at 3-4 weeks of age (Okano *et al.*, 1999). *Dnmt3b*$^{-/-}$ embryos showed mild neural tube defects and demethylation of minor satellite DNA, and died between E14.5-18.5 (Okano *et al.*, 1999). The double mutant [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] showed an even severer phenotype: the embryos failed to initiate *de novo* methylation after implantation and died at E9.5 with growth retardation, turning failure and defects in somite formation (Okano *et al.*, 1999). These embryos were morphologically similar to the *Dnmt1*$^{-/-}$ embryos (Okano *et al.*, 1999). The only information obtained from these mutant embryos on germline imprinting was that [*Dnmt3a*$^{-/-}$,*Dnmt3b*$^{-/+}$] ovaries transplanted to wild-type females could not establish proper maternal methylation imprints in their oocytes (Hata *et al.*, 2002). This result strongly suggests that *Dnmt3a* contributes to the establishment of maternal methylation imprints. However, *Dnmt3a* is mutated not only in the oocytes but also in somatic cells of [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/+}$] ovaries. Also, the genotype [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/+}$] cannot exclude the possibility that *Dnmt3b* participates in this establishment process.

I therefore took advantage of the Cre-loxP system to inactivate each of the *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* in a germline-specific manner. The Cre-loxP recombinase system exploits the ability of Cre recombinase to direct excision of a DNA sequence that has been flanked by a 34-bp sequence called loxP (Sauer *et al.*, 1988). Expression of Cre by an inducible or tissue-specific promoter therefore allows excision of the loxP flanked DNA. I used the TNAP (tissue non-specific alkaline phosphatase)-Cre knock-in allele to inactivate Dnmt3a and Dnmt3b preferentially in germ cells. The germline-specific *Dnmt3a* and *Dnmt3b* knockout mice should be viable because recombination is expected to occur only in germ cells, but rarely in somatic cells. As expected, I could successfully generate such conditional *Dnmt3a* and *Dnmt3b* knockout mice, which were viable and grew up to adulthood.

These phenotypes contrast with the postnatal lethality of the *Dnmt3a*-null mice and embryonic lethality of the *Dnmt3b*-null mice, respectively. As a result of my studies with these conditional knockout mice, it is strongly suggested that *Dnmt3a*, but not *Dnmt3b*, is responsible for the establishment of maternal methylation imprints. This result also shows that DNA methylation is the epigenetic imprints established in gametogenesis and transmitted to the offspring.

## Materials and Methods

### Mice

Mice homozygous for the floxed alleles of *Dnmt3a* and *Dnmt3b* (*Dnmt3a*$^{2lox/2lox}$ and *Dnmt3b*$^{2lox/2lox}$) were generated by Drs. Masaki Okano (Riken CDB) and En Li (Massachusetts General Hospital). In brief, targeting vectors for *Dnmt3a* and *Dnmt3b* were electroporated into J1 ES cells, which were subsequently selected for integration in G418-containing medium as described previously (Li *et al.*, 1992). Correctly targeted ES cells were identified by Southern-blot analysis. The floxed regions contained the exons coding for the conserved catalytic domains (PC motif in exon 19 for *Dnmt3a*, PC and ENV motifs in exons 17-20 for *Dnmt3b*, see Figure 1 and 2). Floxed *Dnmt3b* ES cells (*Dnmt3b*$^{3lox/+}$) were transiently transfected with a Cre expression vector to remove the PGK-neo cassette in ES cells. These ES cells were injected into blastocysts to generate germline chimeras. Floxed *Dnmt3a* mice (*Dnmt3a*$^{3lox/+}$) were crossed with EIIa-Cre mice, which express Cre transgene at a low level, to remove PGK-neo cassette *in vivo* (Xu *et al.*, 2001). To inactivate *Dnmt3a* or *Dnmt3b* specifically in the PGCs, TNAP (tissue non-specific alkaline phosphatase)-Cre knock-in mice, which express Cre recombinase in the PGCs from embryonic day 9.5-10.5 (E9.5-10.5) to the late-gestation (Lomeli *et al.*, 2000 and this study), were crossed with floxed *Dnmt3a* (*Dnmt3a*$^{2lox/2lox}$) or *Dnmt3b* (*Dnmt3b*$^{2lox/2lox}$) mice (Figure 1 and 2). TNAP-Cre mice were a kind gift from Dr. Andras Nagy (Samuel Lunenfeld Research Institute).

### Isolation of DNA and RNA

Mouse tail DNA was prepared by a standard protocol for genotyping. Briefly, tail biopsies were incubated with lysis buffer (100 mM Tris (pH 8), 5 mM EDTA, 200 mM NaCl, 0.2% SDS and

200 μg/ml Proteinase K) at 50°C for overnight. After phenol/chloroform extraction and ethanol precipitation, DNA pellet was resuspended in 100 μl of TE (10 mM Tris (pH 8), 1 mM EDTA). To analyze the tissue specificity and recombination efficiency, I isolated DNA from the cerebrum, cerebellum, thymus, heart, lung, liver, spleen, pancreas, intestine, kidney, skeletal muscle, testis, sperm, ovary and oocytes as described above. Sperm DNA was isolated in lysis buffer containing 10 mM DTT. Genomic DNA was extracted from E10.5 and E11.5 whole embryos as described above for the methylation analysis. Total RNA was isolated from whole embryos using ISOGEN (Nippon Gene) according to the protocol provided by the manufacturer.

**Genotyping**

Mice were genotyped for the 2lox and 1lox (Δ) alleles of *Dnmt3a* and *Dnmt3b* by PCR analysis of tail DNA. Primers used were:

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

OM144R ($Dnmt3a^{2lox}$, down), 5'-AAG CCT CAG GCC CTC TAG GCA AGA T-3';

OM145R ($Dnmt3a^{\Delta}$, down), 5'-TGA GTG GTG AGG CCC AGC TTA TCG A-3';

OM146F ($Dnmt3b^{\Delta}$, up), 5'-GAA CTT GGT CTG CAG GAC GAT CGC T-3';

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

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

Primers used for genotyping of TNAP-Cre mice were:

PgkpA/U1 (TNAP-Cre, up), 5'-TAA GGG CCA GCT CAT TCC TCC-3';

TNAP/EX6L (TNAP-Cre, down), 5'-CAC GTC GAT GGC CGC TCT A-3'.

The tissue specificity and efficiency of site-directed recombination by TNAP-Cre were analyzed

by PCR. Primers used were:

3a1-2/F (both *Dnmt3a*$^{\Delta}$ and *Dnmt3b*$^{2lox}$, up), 5'-CAC CTG TGC CAG CTG AGA AGA GGA A-3';

3a1-2/R (both *Dnmt3a*$^{\Delta}$ and *Dnmt3b*$^{2lox}$, down), 5'-TGA GTG GTG AGG CCC AGC TTA TCG A-3'.

3b1-2/F (both *Dnmt3b*$^{\Delta}$ and *Dnmt3b*$^{2lox}$, up), 5'-GAG TTA AGC TAG CTT ATC GAT ACC G-3';

3b1-2/R (both *Dnmt3b*$^{\Delta}$ and *Dnmt3b*$^{2lox}$, up), 5'-AGA CAC TAT GTG CAG TAC AGC AGC T-3'.

**Histology**

The testes and ovaries from adult mice were fixed with Bouin's solution (Muto Pure Chemicals), dehydrated and embedded in paraffin. Sections of 6$\mu$m in thickness were prepared using a microtome and stained with Hematoxylin and Eosin.

**Preparation of germ cells**

Fetal germ cells were collected from the gonads of E14.5, E16.5 and E18.5 embryos. The tissues were dissociated into single cells by incubation with 0.25% trypsin in PBS for 15 min at 37°C. Dispersed suspensions of PGC-containing tissues were cultured in medium 199 supplemented with 10% fetal bovine serum for 14 hours in a multi-well plate. At the end of the culture period, the solution in the well was gently agitated, and cells floating in the supernatant were removed and collected by centrifugation (Hashimoto *et al.*, 1990). Oocytes from postnatal day 3 (P3) females were obtained according to the protocol by Bao *et al.* (2000). In brief, ovaries of P3 females were immersed in 1 ml of M2 medium (Sigma) containing 1.5 mg/ml crude collagenase (Gibco) and incubated at 37°C for 5-10 minutes. After brief centrifugation and removal of the

supernatant, oocytes-granulosa cell complexes were incubated with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA) at 37°C for 15 minutes. The complexes were washed with M2 medium and the granulosa cells were removed by pipetting. Then oocytes were individually picked up using a microscope and a micromanipulator.

**DNA methylation analysis by Southern blotting**

Genomic DNA isolated from embryos (5 $\mu$g) was digested with methylation-sensitive or methylation-insensitive enzymes, and analyzed by Southern-blot hybridization (Church *et al.*, 1984). The probes used for the methylation studies were: pMO for endogenous C-type retroviruses (Lei *et al.*, 1996); pMR150 for centromeric minor satellite DNA (Chapman *et al.*, 1984); a 2.0-kb fragment from the *H19* upstream DMR (Tremblay *et al.*, 1995); a 702-bp Sp-4 repeat region from the *Rasgrf1* DMR (Pearsall *et al.*, 1999); a 1.1-kb fragment from the *Igf2r* DMR2 (Stöger *et al.*, 1993); a 1.4-kb fragment from intron 1 of *Peg1* (Lefebvre *et al.*, 1997); a 2.2-kb DMR1 fragment from *Snrpn* (Shemer *et al.*, 1997).

**RT-PCR and Northern blotting**

cDNA was synthesized from 1 $\mu$g of total RNA using Superscript II reverse transcriptase (Life Technologies) with random primers. PCR was carried out using specific primers:

#6105 (*Igf2r*, up), 5'-CAG AAG AAG CTC GGG CGT GTC CTA C-3'

#6294 (*Igf2r*, down), 5'-CTC CGC TCC TCG GCC TGA GTG AAC T-3'

kip2/F ($p57^{kip2}$, up), 5'-GCC GGG TA TGA GCT GGG AA-3'

kip2/R ($p57^{kip2}$, down), 5'-AGA GAG GCT GGT CCT TCA GC-3'

Gapd/F (Gapd, up), 5'-ATG GCC TTC CG GTT CCT AC-3'

Gapd/R (Gapd, down), 5'-TGT GAG GGA GAT GCT CAG TG-3'

Total RNA (10 $\mu$g) was fractionated on an agarose-formaldehyde gel, and transferred to Biodyne B nylon membranes (Pall) according to the standard procedures. RNA was UV-crosslinked and hybridized to $^{32}$P-labeled probes. cDNA probes for *Peg1* (a gift from A. Surani), *Snrpn* (amplified by RT-PCR) and Gapd (amplified by RT-PCR) were used.

## Results

Previous studies showed that the imprints of the maternally methylated genes, for example, *Peg1/Mest, Peg3, Igf2r, Snrpn* and $p57^{kip2}$, are established in growing oocytes at the diplotene or dictyotene stage of meiotic prophase I (Obata *et al.*, 2002). This stage corresponds to P5-20 of postnatal development. Also, it has been reported that the paternal methylation imprint of *H19* is established in the gonocyte (prospermatogonia) stage (Ueda *et al.*, 2000; Davis *et al.*, 2000). This stage corresponds to the E14.5-newborn period of fetal development. To inactivate *Dnmt3a* and *Dnmt3b* prior to the establishment of the methylation imprints in both male and female germlines, $Dnmt3a^{2lox/2lox}$ and $Dnmt3b^{2lox/2lox}$ mice were crossed with TNAP-Cre mice, which express Cre recombinase in both male and female PGCs from E9.5 to late gestation (Lomeli *et al.*, 2000 and this study), (Figure 3). The resulting [$Dnmt3a^{2lox/+}$, TNAP-Cre] and [$Dnmt3b^{2lox/+}$, TNAP-Cre] males were crossed with $Dnmt3a^{2lox/2lox}$ and $Dnmt3b^{2lox/2lox}$ females, respectively, since ectopic recombination in somatic tissues was reported upon maternal transmission of the TNAP-Cre locus (Kimura *et al.*, 2003). [$Dnmt3a^{2lox/\Delta}$, TNAP-Cre] and [$Dnmt3b^{2lox/\Delta}$, TNAP-Cre] mice ($\Delta$ represents the 1lox allele) thus obtained should be viable because recombination is expected to occur in germ cells, but rarely in somatic cells (Figure 3).

### Specificity and efficiency of conditional *Dnmt3a* knockout

The specificity and efficiency of conditional deletion of *Dnmt3a* by TNAP-Cre was tested in [$Dnmt3a^{2lox/+}$, TNAP-Cre] mice. DNA samples from various tissues (cerebrum, cerebellum, thymus, heart, lung, liver, pancreas, kidney, intestine, spleen, skeletal muscle, tail, testis, sperm, ovary and oocytes) were analyzed by PCR using the primers that amplify the 2lox (2.3 kb) and $\Delta$ (1.6 kb) alleles but not the wild-type *Dnmt3a* allele (Figure 4). The [$Dnmt3a^{2lox/+}$, TNAP-Cre]

mice showed that the deletion occurred not only in germ cells but also in somatic tissues. While the efficiency of deletion in sperm and oocytes was almost 100%, somatic tissues had various degrees (from 56% in small intestine to 80% in pancreas) of the deletion. Thus the *Dnmt3a* conditional knockout mice [$Dnmt3a^{2lox/\Delta}$, TNAP-Cre] were expected to be mosaic consisting of $Dnmt3a^{2lox/\Delta}$ cells and $Dnmt3a^{\Delta/\Delta}$ cells. However, these mice were viable and grew up to adulthood although some mice showed slightly dwarfish phenotype. This contrasts with the postnatal lethality of the *Dnmt3a*-null mice, which die at 3-4 weeks after birth (Okano *et al.*, 1999).

To assess the recombination efficiency in fetal germ cells of [$Dnmt3a^{2lox/\Delta}$, TNAP-Cre] mice, I purified germ cells from the genital ridges of E14.5, E16.5 and E18.5 male and female embryos according to the method by Hashimoto *et al.* (1990). The purity of the germ cells was determined by alkaline phosphatase staining and was 80-90%. Figure 5 shows the efficiency of deletion in fetal germ cells. In females at E14.5, E16.5 and E18.5, most germ cells had the Δ alleles only. The faint bands representing the 2lox allele could be due to contamination by somatic cells, or to germ cells that were not recombined by Cre. Next, P3 oocytes and ovulated eggs were individually picked up using a microscope to avoid contamination by somatic cells. I found that the floxed *Dnmt3a* alleles were completely deleted in these oocytes (Figure 5). Since the maternal methylation imprints are established in growing oocytes at P5-20 (Obata *et al.*, 2002), this observation suggests that the *Dnmt3a* allele was successfully inactivated before the onset of methylation imprinting. In males at E14.5, E16.5 and E18.5, most germ cells were of $Dnmt3a^{\Delta/\Delta}$ genotype although a faint band representing the 2lox allele was detected. Again, this could be due to either somatic cell contamination or to unrecombined germ cells. As [$Dnmt3a^{2lox/\Delta}$, TNAP-Cre] mice showed azoospermia, I could not obtain germ cells from postnatal testes or mature spermatozoa from the epididymis (see the next section).

**_Dnmt3a_ is essential for spermatogenesis**

The [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] males were crossed with wild-type females (C57BL/6) but no pups were obtained. I therefore examined at day 11 of pregnancy four females plugged by [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] males, but neither embryos nor resorptions were found. These results suggested that the [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] males were sterile. Examination of the testes from [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] males revealed that the testes at P11 looked almost normal but at 11 weeks of age, the size and weight were significantly reduced (19.7±1.6 mg, n=7) as compared to that of control *Dnmt3a*$^{2lox/\Delta}$ testes (96.5±7.9 mg, n=5) (Figure 6). Histological examinations showed that the [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] testes from P11 contained a relatively normal number of spermatogonia in the seminiferous tubules (Figure 6). However, at 11 weeks of age, the testes contained almost no spermatids or spermatozoa, which were present in the lumen of control *Dnmt3a*$^{2lox/\Delta}$ seminiferous tubules (Figure 6). These observations were similar to, or almost identical with, those of *Dnmt3L*$^{-/-}$ males, in which spermatogenesis was arrested at a time when spermatogonia enter meiosis (Bourc'his *et al.*, 2001; Hata *et al.*, 2002). The results indicate that *Dnmt3a* is essential for the differentiation of male germ cells.

**Maternal effect of the _Dnmt3a_ deletion**

When four [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] females were crossed with wild-type males (C56BL/6), no live pups were obtained. To determine when the embryos die, I sacrificed pregnant [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] females and examined the embryos (I designated such embryos as *Dnmt3a*$^{mat\Delta/\Delta}$) at different developmental stages. I found that the embryos were grossly normal at E9.5, but died around E9.5-E10.5 (Table 1). At E9.5, most embryos showed no abnormal phenotype. Some embryos showed heart beating and most embryos turned. At E10.5, embryos showed defects in neural tube closure, lack of branchial arches and pale skin probably due to the impediment of the

circulatory system (Figure 7). Most embryos didn't show hear beating and developmental arrest, but only one out of 17 *Dnmt3a*$^{mat\Delta/\Delta}$ embryos seemed to be almost normal. At E11.5, only resorptions were seen. I found no *Dnmt3a*$^{2lox/+}$ embryos at any of these stages, suggesting that the rate of deletion by Cre during oogenesis was almost 100%. Histological sections of the ovaries from [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] females showed normal process of oogenesis (data not shown). These results demonstrate that the expression of *Dnmt3a* in oogenesis is essential for the embryonic development of the next generation, but not essential for the oocyte development.

**_Dnmt3a_ is essential for the establishment of maternal methylation imprints**

I then investigated whether *Dnmt3a* is required for the establishment of maternal methylation imprints in the oocytes. I analyzed the methylation status of DMRs of several imprinted genes in the *Dnmt3*$^{mat\Delta/\Delta}$ embryos. The regions normally methylated on the maternal alleles, such as the DMR2 of *Igf2r*, the DMR of *Peg1* and the DMR1 of *Snrpn*, were almost completely unmethylated in *Dnmt3a*$^{mat\Delta/\Delta}$ embryos (Figure 8). Wild-type embryos had both a methylated maternal allele and an unmethylated paternal allele, but *Dnmt3a*$^{mat\Delta/\Delta}$ embryos (#1 and #2) had unmethylated alleles only, just as the *Dnmt1*$^{-/-}$ ES cells, which lack almost all methylation (Lei *et al.*, 1996). In contrast, the methylation of the paternally methylated imprinted gene *H19* was unaffected in the *Dnmt3a*$^{mat\Delta/\Delta}$ embryos (Figure 9). Methylation of the repetitive minor satellite DNA was also unaffected in the same embryos (Figure 9). These results indicate that *Dnmt3a* is essential for the establishment of maternal methylation imprints.

**Disruption of the allele-specific expression of maternally methylated genes in *Dnmt3a*$^{mat\Delta/\Delta}$ embryos**

The effect of the lack of maternal methylation imprints on the expression of the imprinted genes,

such as *p57*$^{kip2}$, *Igf2r*, *Peg1* and *Snrpn*, was tested in *Dnmt3a*$^{mat\Delta/\Delta}$ embryos. RT-PCR analysis showed that expression of *p57*$^{kip2}$ and *Igf2r* was almost completely lost in E10.5 *Dnmt3a*$^{mat\Delta/\Delta}$ embryos (Figure 10A, #1 and #2). This is consistent with the loss of the maternal methylation imprints, since it would silence the normally active maternal allele through activation of the antisense transcripts (Stöger *et al.*, 1993; Obata *et al.*, 1998). Northern analysis of *Peg1* and *Snrpn* expression showed an increased expression of *Peg1* (approximately 1.6-fold) and *Snrpn* (approximately 1.1-fold) in E10.5 *Dnmt3a*$^{mat\Delta/\Delta}$ embryos (#1 and #2) compared to wild-type embryos, as determined by a Fuji BAS-2000II Bioimage Analyzer (Figure 10B). This is consistent with the activation of the normally silenced maternal allele due to a loss of the maternal methylation imprints in the DMRs, but the expression levels were lower than those expected from biallelic expression. Perhaps, degradation of RNA from the dying *Dnmt3a*$^{mat\Delta/\Delta}$ embryos hampered accurate quantification of the transcripts.

**Specificity and efficiency of conditional *Dnmt3b* knockout**

The specificity and efficiency of conditional deletion of *Dnmt3b* by TNAP-Cre were tested in [*Dnmt3b*$^{2lox/+}$, TNAP-Cre] mice. DNA samples from various tissues (cerebrum, cerebellum, thymus, heart, lung, liver, pancreas, kidney, intestine, spleen, skeletal muscle, testis, sperm, ovary and oocytes) were analyzed by PCR using the primers that amplify the 2lox (5.0 kb) and Δ (1.9 kb) alleles but not the wild-type *Dnmt3b* allele (Figure 11). The efficiency of deletion was almost 100% in sperm and oocytes. No deletion was observed in most somatic tissues examined, although recombination occurred in a small proportion of the cells in skeletal muscle. This contrasts with the case for the [*Dnmt3a*$^{2lox/+}$, TNAP-Cre] mice, which showed some level of recombination in all somatic tissues. This could be due to either the difference in chromatin structure between the *Dnm3a* locus and the *Dnmt3b* locus, or to the difference in length of the

floxed region between the *Dnmt3a* locus (600 bp) and the *Dnmt3b* locus (3.0 kb).

I then attempted to generate germline-specific *Dnmt3b* knockout mice. The [$Dnmt3b^{2lox/\Delta}$, TNAP-Cre] mice were viable and grew up to adulthood, in contrast to the *Dnmt3b*-null mice, which die in late gestation (Okano *et al.*, 1999). To examine the recombination efficiency in germ cells, I collected fetal germ cells from the [$Dnmt3b^{2lox/\Delta}$, TNAP-Cre] embryos at E14.5, E16.5 and E18.5. In both males and females, the 2lox allele was retained in a small proportion of the germ cells at E14.5, but almost all germ cells had the Δ allele only at E18.5 (Figure 12). The weak 2lox band could be due to contamination by somatic cells, as the purity of germ cells was not 100%, or to germ cells that were not recombined by Cre. Next, P3 oocytes and ovulated eggs were individually picked up using a microscope to avoid contamination by somatic cells. I found that the floxed *Dnmt3b* alleles were completely deleted in these oocytes (Figure 12). This observation suggests that the *Dnmt3b* allele, just as the *Dnmt3a* allele, was successfully inactivated by TNAP-Cre before the onset of methylation imprints in females. In males, adult sperm had the Δ alleles only, but the 2lox allele was still observed in a small proportion of the cells at E14.5-18.5. Again, this band could be due to either somatic cell contamination or to unrecombined germ cells.

**_Dnmt3b_ is not required for spermatogenesis or the establishment of paternal methylation imprints**

The [$Dnmt3b^{2lox/\Delta}$, TNAP-Cre] males were crossed with wild-type females (C57BL/6) and normal pups were obtained. A total of 102 pups were obtained from three [$Dnmt3b^{2lox/\Delta}$, TNAP-Cre] males and all pups had the recombined Δ allele, suggesting that the recombination efficiency was 100% (Table 2). The average litter size was 6.8, which is also slightly lower than the control litter size (7.6). To examine the $Dnmt3b^{pat\Delta/\Delta}$ embryos closely, I dissected them out at

several developmental timepoints but no abnormalities were observed. The *Dnmt3b*$^{pat\Delta/\Delta}$ pups grew up to adulthood and were fertile.

The methylation levels of several imprinted genes were analyzed in E11.5 *Dnmt3b*$^{pat\Delta/\Delta}$ embryos. The methylation levels of *H19* and *rasgrf1*, which are paternally methylated imprinted genes, were normal (Figure 13). Minor satellite DNA was also methylated normally (Figure 13). The methylation levels of *H19* and *rasgrf1* in sperm DNA were also examined by bisulfite sequencing and revealed to be normal (Kumaki *et al.*, personal communication). Histological sections of the testes from [*Dnmt3b*$^{2lox/\Delta}$, TNAP-Cre] males showed normal spermatogenesis (data not shown). These results suggest that *Dnmt3b* is not required for either spermatogenesis or the establishment of paternal methylation imprints.

**_Dnmt3b_ is not required for oogenesis or the establishment of maternal methylation imprints**

The [*Dnmt3b*$^{2lox/\Delta}$, TNAP-Cre] females were crossed with wild-type males (C57BL/6) and normal pups were obtained (Table 2). A total of 88 pups were born from four [*Dnmt3b*$^{2lox/\Delta}$, TNAP-Cre] females and 87 pups had the recombined Δ allele, suggesting a high rate of recombination by Cre. (Note that half of the pups were expected to inherit the Δ allele from the mother even if Cre didn't work.) The average litter size was 6.8, which was only slightly lower than the control litter size (7.6). To examine the *Dnmt3b*$^{mat\Delta/\Delta}$ embryos more closely, I dissected out the *Dnmt3b*$^{mat\Delta/\Delta}$ embryos at several developmental timepoints but no evidence for intrauterine death was obtained. The *Dnmt3b*$^{mat\Delta/\Delta}$ pups looked healthy, grew up to adulthood and were fertile.

Next, the methylation levels of several imprinted genes were analyzed in E11.5 *Dnmt3b*$^{mat\Delta/\Delta}$ embryos. The methylation levels of the maternally methylated imprinted genes,

*Igf2r* and *Peg1*, were normal (Figure 14). Minor satellite DNA, which is the target sequence of Dnmt3b, was also methylated normally (Figure 14). Histological sections of the ovaries from [*Dnmt3b*$^{2lox/\Delta}$, TNAP-Cre] females showed normal process of oogenesis (data not shown). These results showed that *Dnmt3b* is not required for oogenesis or the establishment of maternal methylation imprints.

**_Dnmt3b_$^{\Delta/\Delta}$ mice are phenotypically identical with _Dnmt3b_$^{-/-}$ mice**

As no abnormal phenotype was observed in the embryos and pups of the conditional *Dnmt3b* knockout mice, I wanted to confirm whether the *Dnmt3b*$^{\Delta}$ allele was successfully inactivated. I therefore produced *Dnmt3b*$^{\Delta/\Delta}$ homozygous mice and examined whether the phenotype was the same as the *Dnmt3b*$^{-/-}$ embryos. I dissected the *Dnmt3b*$^{\Delta/\Delta}$ embryos at E14.5 and E16.5 and found the phenotype very similar to that of the *Dnmt3b*$^{-/-}$ embryos (Okano *et al.*, 1999). These embryos showed multiple developmental defects including growth impairment and rostral neural tube defects with variable severity at later stages of development. The minor satellite DNA, which is the target sequence of Dnmt3b, was demethylated in the *Dnmt3b*$^{\Delta/\Delta}$ embryos as in the *Dnmt3b*$^{-/-}$ embryos (data not shown). These observations confirmed that our *Dnmt3b*$^{\Delta}$ allele is functionally null.

**Discussion**

In this study, I used the Cre-loxP system to delete the *de novo* DNA methyltransferase genes *Dnmt3a* and *Dnmt3b* in fetal germ cells and investigated which *de novo* DNA methyltransferase is responsible for the establishment of imprints in the parental germlines. The exons containing the conserved catalytic motifs of *Dnmt3a* and *Dnmt3b* were floxed, and deleted preferentially in both male and female germ cells by the TNAP-Cre knock-in allele. The [$Dnmt3a^{2lox/\Delta}$, TNAP-Cre] and [$Dnmt3b^{2lox/\Delta}$, TNAP-Cre] mice generated in this way were viable and grew up to adulthood. This contrasts with the phenotypes of homozygous *Dnmt3a* and *Dnmt3b* knockout mice, which showed postnatal and late-gestation lethality, respectively (Okano *et al.*, 1999). Thus, using the conditional gene knockout technique, I was able to examine the effect of loss of *Dnmt3a* and *Dnmt3b* on gametogenesis and on development of their offspring (see later).

The [$Dnmt3a^{2lox/\Delta}$, TNAP-Cre] mice showed a nearly 100% deletion rate in both mature oocytes and sperm, while somatic tissues had various degrees (56%-80%) of the deletion. This indicates that the expression of Cre recombinase from the TNAP-Cre locus was not entirely germline-specific. The somatic leak of Cre recombinase and the eventual mosaicism by the $Dnmt3a^{2lox/\Delta}$ and $Dnmt3a^{\Delta/\Delta}$ cells in somatic tissues discounted the specificity, but the only abnormality observed in these mice was the low birth weight. The mice grew normally and, by several weeks of age, their body weight reached the normal level. Detailed studies with purified fetal germ cells showed that the genotype of most germ cells (already at E14.5 in both males and females) was $Dnmt3a^{\Delta/\Delta}$. The [$Dnmt3b^{2lox/\Delta}$, TNAP-Cre] mice showed an almost 100% deletion rate in male and female germ cells and virtually no deletion was observed in most somatic tissues with only one exception of skeletal muscle. A small proportion of skeletal muscle cells of the [$Dnmt3b^{2lox/\Delta}$, TNAP-Cre] mice underwent Cre-mediated deletion. This difference of the

deletion specificity between the *Dnmt3a* and *Dnmt3b* locus could be due to either the difference in chromatin structure or the length between the two loxP sequences at the *Dnmt3a* locus (600 bp) and the *Dnmt3b* locus (3 kb). The fetal germ cells from the [$Dnmt3b^{2lox/\Delta}$, TNAP-Cre] embryos at E14.5 to E18.5 showed that most germ cells had the Δ alleles only in both males and females.

The [$Dnmt3a^{2lox/\Delta}$, TNAP-Cre] males showed an interesting phenotype: they had defects in spermatogenesis and showed azoospermia. These observations are very similar to, or almost identical with, those of *Dnmt3L* knockout males, in which spermatogonia fail to differentiate, giving rise to apoptotic spermatocytes (Bourc'his *et al.*, 2001; Hata *et al.*, 2002). By contrast, [$Dnmt3b^{2lox/\Delta}$, TNAP-Cre] males showed no defect in spermatogenesis. These results suggest that *Dnmt3a* and *Dnmt3L*, but not *Dnmt3b*, cooperate to regulate spermatogenesis. Perhaps *de novo* methylation is required for the differentiation of spermatogonia or for meiosis to occur in spermatocytes. It is still an open question whether *Dnmt3a* is required for the establishment of paternal methylation imprints.

Embryos from [$Dnmt3b^{2lox/\Delta}$, TNAP-Cre] females showed no abnormal phenotype but those from [$Dnmt3a^{2lox/\Delta}$, TNAP-Cre] females showed embryonic lethality between E9.5 and E10.5. These embryos, called $Dnmt3a^{mat\Delta/\Delta}$, showed defects in neural tube closure and somite formation. Furthermore, they showed disruption in methylation and expression of the maternally imprinted genes. The maternally methylated regions such as the DMR2 CpG island of *Igf2r*, the DMR1 of *Snrpn* and the DMR of *Peg3* were almost completely unmethylated in the $Dnmt3a^{mat\Delta/\Delta}$ embryos, in contrast to the approximately 50% methylation of these regions in the wild-type embryos (consistent with the methylation of the maternal allele). By contrast, the methylation patterns of the paternally methylated gene *H19* and minor satellite repetitive sequence were unaffected in the $Dnmt3a^{mat\Delta/\Delta}$ embryos as compared to wild-type embryos. I also examined the

expression of several maternally methylated genes in the *Dnmt3a*$^{mat\Delta/\Delta}$ embryos to see whether the disruption of the methylation imprints in the oocytes leads to abnormal expression of these genes in the offspring. The expression of both *Igf2r* and *p57*$^{kip2}$ was diminished in the *Dnmt3a*$^{mat\Delta/\Delta}$ embryos, consistent with biallelic silencing of these genes due to the lack of their maternal methylation imprints. Northern blot analysis showed that the expression level of both *Peg1* and *Snrpn* was increased in the *Dnmt3a*$^{mat\Delta/\Delta}$ embryos, consistent with their biallelic expression due to the loss of the maternal methylation imprints. These phenotypes were again very similar to those of the *Dnmt3L*$^{mat-/-}$ embryos (Bourc'his *et al.*, 2001; Hata *et al.*, 2002). Since Dnmt3L does not have a DNA methyltransferase activity itself, Dnmt3a is likely to be the key enzyme that establishes the methylation imprints during oogenesis. This provides the formal proof that the primary imprints established in the parental germlines are DNA methylation. My findings are also consistent with the previous experiment in which the embryos derived from a [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/+}$] ovary transplanted to a wild-type female showed defects in maternal methylation imprints (Hata *et al.*, 2002). A more global expression analysis of the *Dnmt3a*$^{mat\Delta/\Delta}$ embryos using, for example, cDNA microarrays will facilitate the identification of genes that are influenced by the methylation status of the oocytes genome.

No abnormal phenotype was observed in the *Dnmt3b*$^{mat\Delta/\Delta}$ and *Dnmt3b*$^{pat\Delta/\Delta}$ embryos, which indicates that *Dnmt3b* is probably not required for gametogenesis and establishment of the methylation imprints. However, there remains a possibility that *Dnmt3b* was not completely inactivated prior to the establishment of methylation imprints. To answer this question, I plan to produce aggregation chimeras between 8-cell *Dnmt3b*$^{+/+}$ embryos and 8-cell *Dnmt3b*$^{\Delta/\Delta}$ embryos, which contain both *Dnmt3b*$^{+/+}$ cells and *Dnmt3b*$^{\Delta/\Delta}$ cells in the germline. If these chimeras give rise to normal embryos with the $\Delta$ allele, it is confirmed that *Dnmt3b* is not required for gametogenesis or the establishment of the parental methylation imprints.

A small form of *Dnmt3a*, denoted *Dnmt3a2*, was recently identified and shown to be expressed in the testis, ovary, spleen and thymus, in which *de novo* methylation is believed to occur during cellular differentiation (Chen *et al.*, 2002). Dnmt3a2 has a methyltransferase activity just as the full-length Dnmt3a form, but it localizes to euchromatin instead of heterochromatin. Moreover, *Dnmt3a2* expression is tightly regulated and correlates with high *de novo* methylation activity, whereas *Dnmt3a* is expressed ubiquitously. Although my conditional knockout inactivated both *Dnmt3a* and *Dnmt3a2* by deleting a common exon, these findings suggest that Dnmt3a2 may be the enzyme form that is responsible for the establishment of the methylation imprints in the germline. Targeted disruption of the *Dnmt3a2*-specific promoter (or exon) (Chen *et al.*, 2002) will give us the answer to this question.

Previous studies revealed that [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] embryos show defects severer than those of *Dnmt3a*$^{-/-}$ or *Dnmt3b*$^{-/-}$ single mutant mice (Okano *et al.*, 1999). The results indicate that *Dnmt3a* and *Dnm3b* have overlapping but some distinct functions during early embryogenesis. Furthermore, [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{+/-}$] mice showed postnatal lethality by 2 weeks of age (Okano *et al.*, personal communication), and [*Dnmt3a*$^{+/-}$, *Dnmt3b*$^{-/-}$] mice showed embryonic lethality at earlier stages than *Dnmt3b*$^{-/-}$ embryos. (Okano *et al.*, personal communication). These observations suggest that the dosage of the *de novo* methyltransferases may be important for embryogenesis. Whether the double knockout of *Dnmt3a* and *Dnmt3b* in germ cells show severer defects is now being investigated.

The methylation level of the paternally methylated imprinted gene *H19* was not affected in *Dnmt3a*$^{mat\Delta/\Delta}$ embryos. It is known that the differential methylation of the DMRs of imprinted genes established in the gametes is maintained during preimplantation development, in spite of the genome-wide demethylation during this period. It is not known, however, which enzyme(s) is involved in this maintenance methylation. The only information obtained thus far is

that Dnmt1o, an oocyte-specific isoform of Dnmt1, maintains the methylation imprints only at the eight-cell stage (Howell *et al.*, 2001). Since this is the only Dnmt1 isoform detected in cleavage-stage embryos, it is possible that either Dnmt3a or Dnmt3b plays a role in this maintenance methylation. Considering that methylation of the *H19* DMR was unaffected, my work showed that the maternal store of Dnmt3a or Dnmt3b alone is enough for the maintenance of the methylation imprints. It will therefore be interesting to ask whether *Dnmt3a* and *Dnmt3b* double knockout in oocytes affect the maintenance methylation during the cleavage stage.

In conclusion, I have produced conditional *Dnmt3a* and *Dnmt3b* knockout mice, in which the genes are inactivated preferentially in germ cells. These mice showed that *Dnmt3a* is definitely involved in the establishment of maternal methylation imprints. They also showed that *de novo* methylation by *Dnmt3a* is required for spermatogenesis. Since the phenotype of the conditional *Dnmt3a* knockout mice was almost identical with that of *Dnmt3L* knockout mice, the products of these genes probably cooperate to create the gametic methylation patterns, including the maternal methylation imprints. The identification of Dnmt3a as the key enzyme involved in gametic methylation should facilitate the clarification of the detailed mechanisms of genomic imprinting and epigenetic reprogramming of the germ cell genome.

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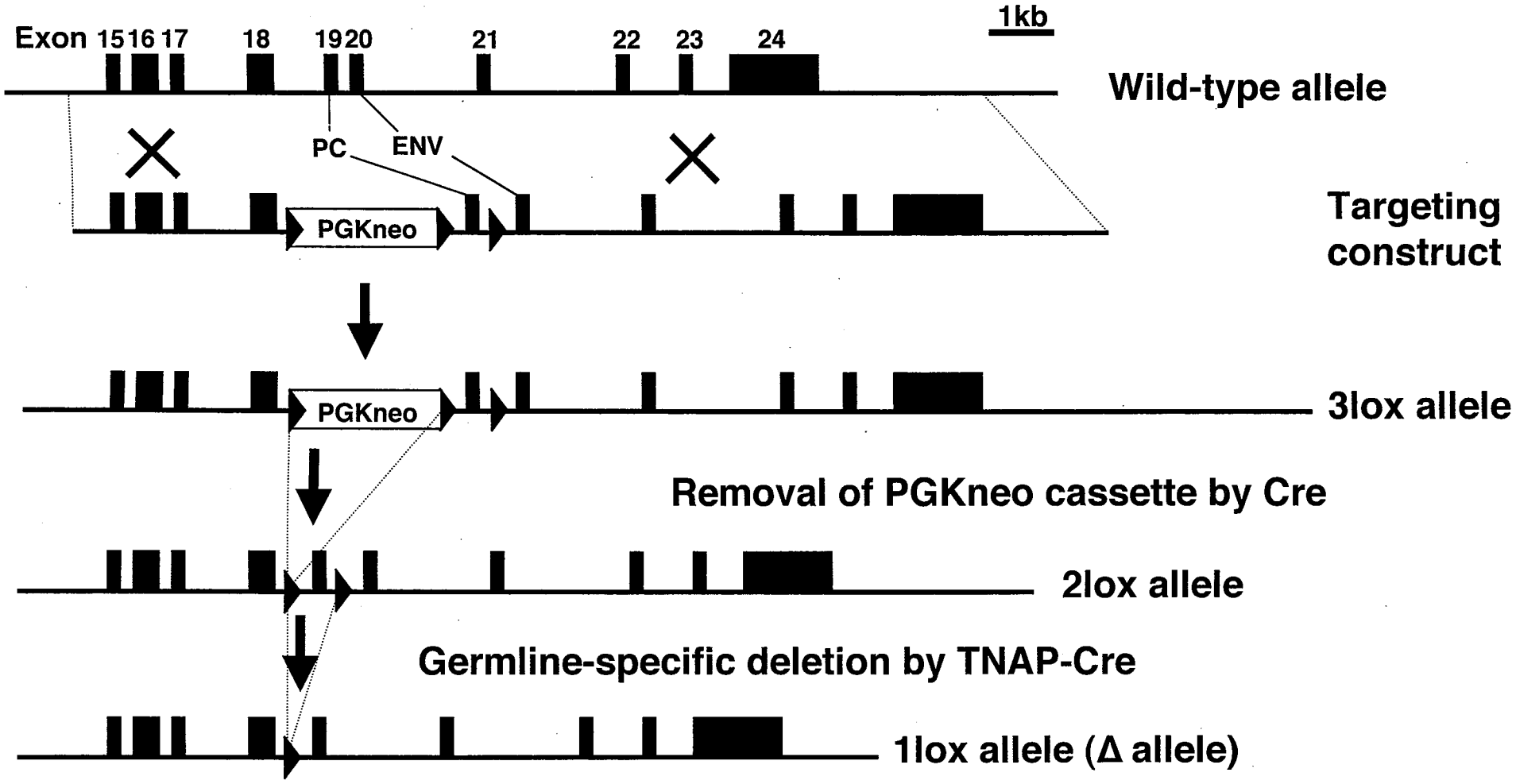



Figure 1. Conditional knockout of the *Dnmt3a* locus. The top line shows the wild-type *Dnmt3a* locus. The black boxes represent the exons. Exon 19, which contains a conserved catalytic motif PC, was flanked by loxP sites (triangles) and deleted by Cre recombinase driven by the *TNAP* promoter.

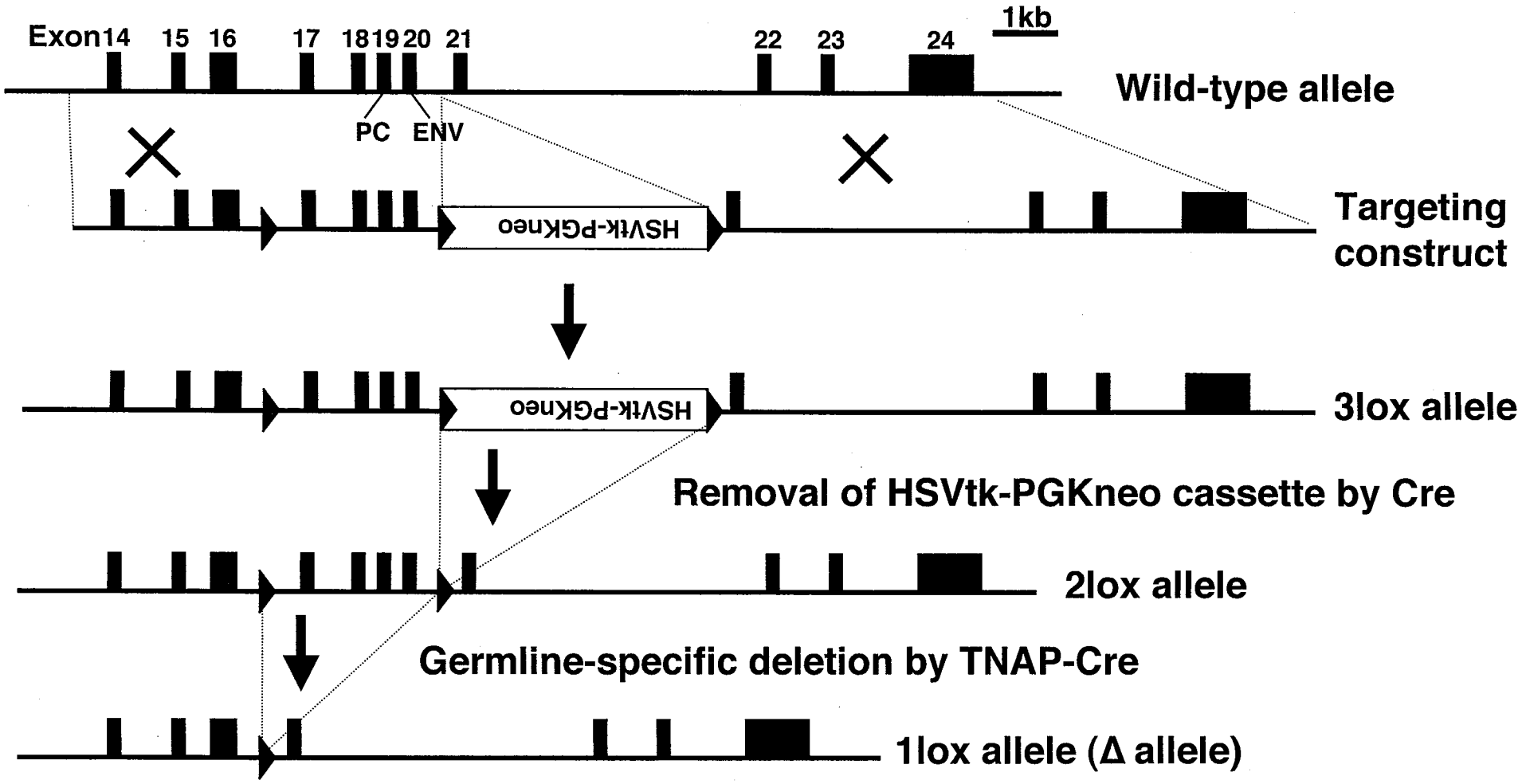



Figure 2. Conditional knockout of the *Dnmt3b* locus. Exons 17-20, which contain the conserved catalytic motifs PC and ENV, were floxed and deleted by the Cre recombinase driven by the *TNAP* locus.

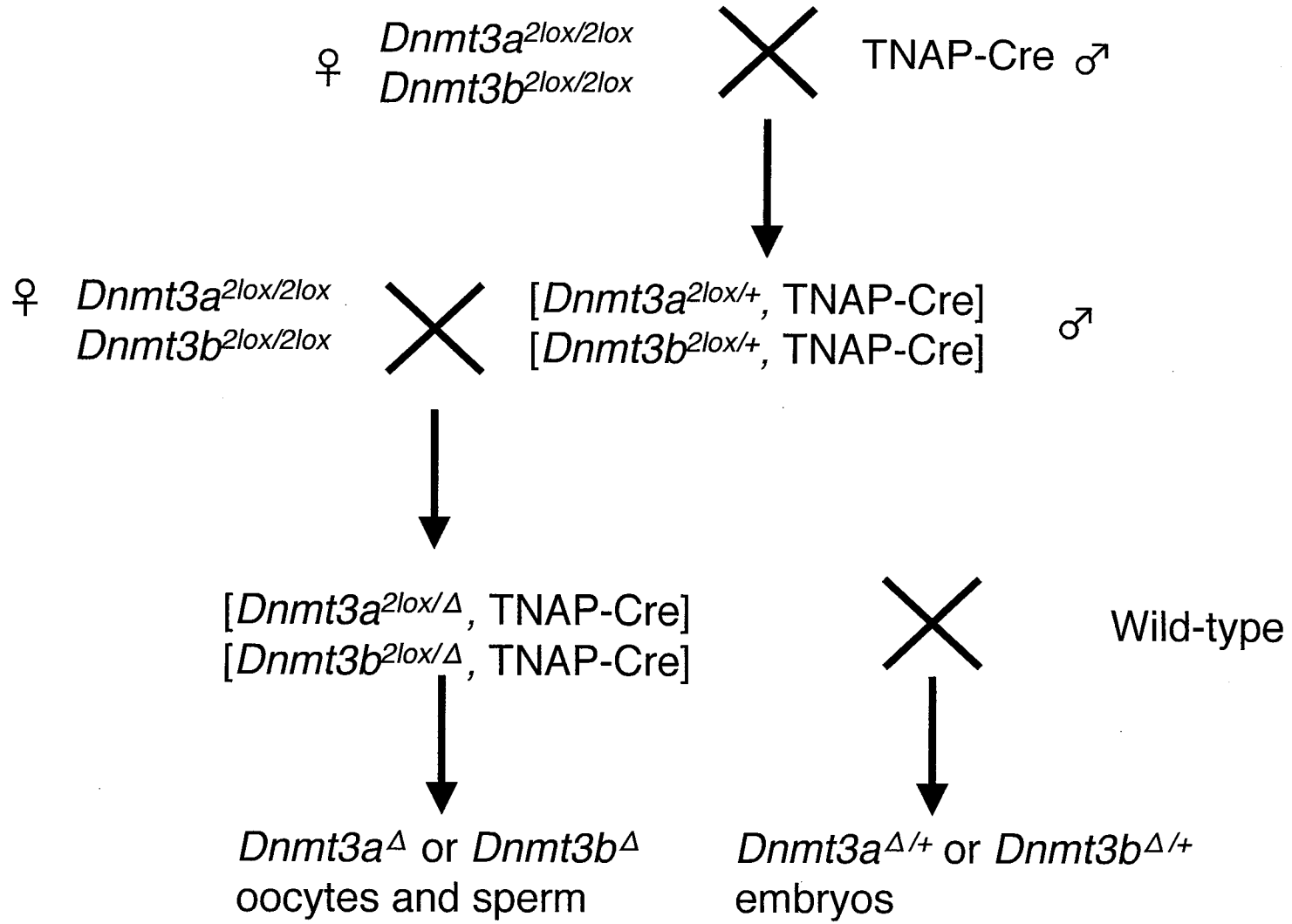



Figure 3. Scheme for the germline-specific *Dnmt3*a and *Dnmt3b* knockout. *Dnmt3a*$^{2lox/2lox}$ and *Dnmt3b*$^{2lox/2lox}$ females were crossed with TNAP-Cre males. Then *Dnmt3a*$^{2lox/2lox}$ and *Dnmt3b*$^{2lox/2lox}$ females were crossed with the resulting [*Dnmt3a*$^{2lox/+}$, TNAP-Cre] and [*Dnmt3b*$^{2lox/+}$, TNAP-Cre] males to avoid the reported ectopic expression of Cre upon maternal transmission. The resulting [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] and [*Dnmt3b*$^{2lox/\Delta}$, TNAP-Cre] mice were selected and crossed with wild-type partners to examine their fertility and effects on embryonic development of the offsprings.

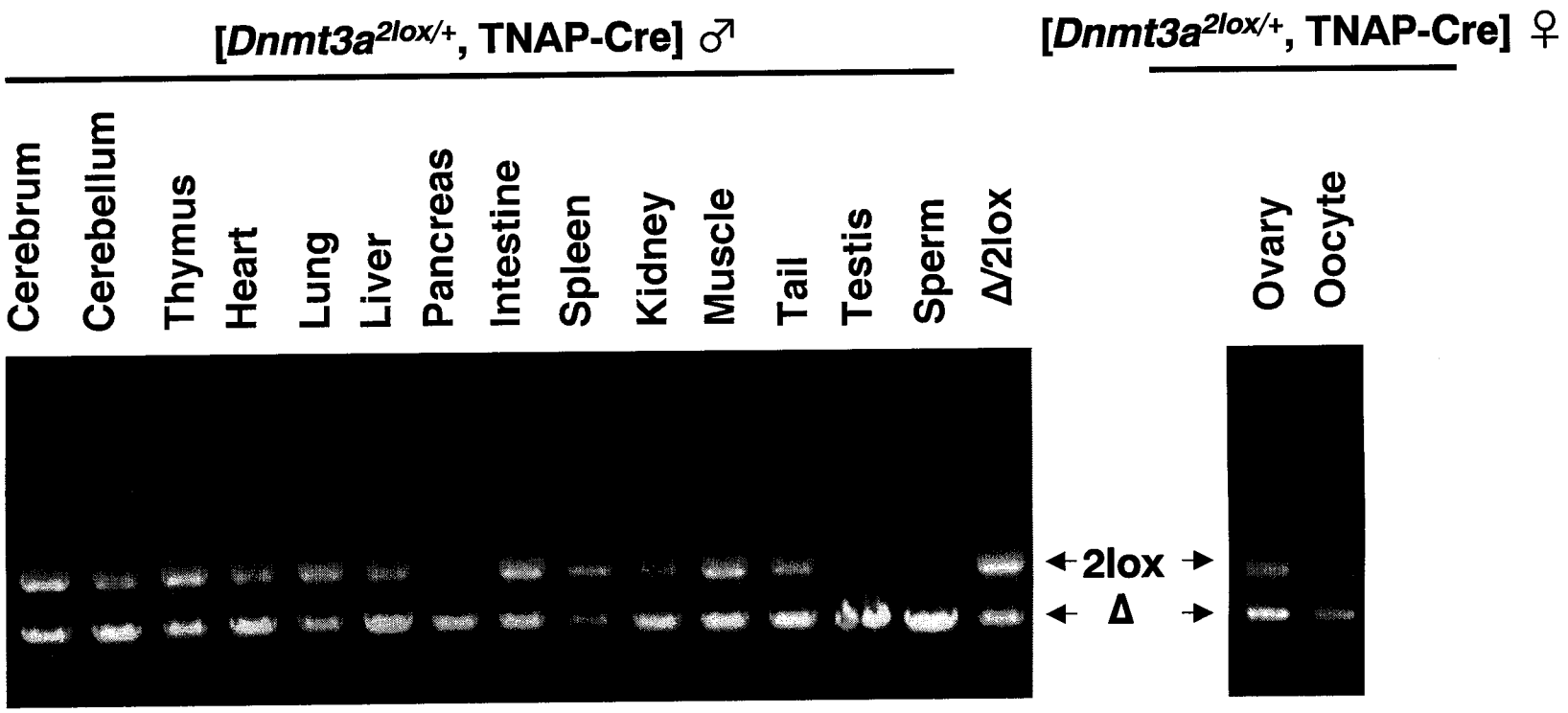



Figure 4. Specificity of conditional knockout of *Dnmt3a* by TNAP-Cre. The efficiency of deletion in mature spermatozoa and oocytes was almost 100%, but somatic tissues showed various degrees of the deletion (from 56% in small intestine to 80% in pancreas).

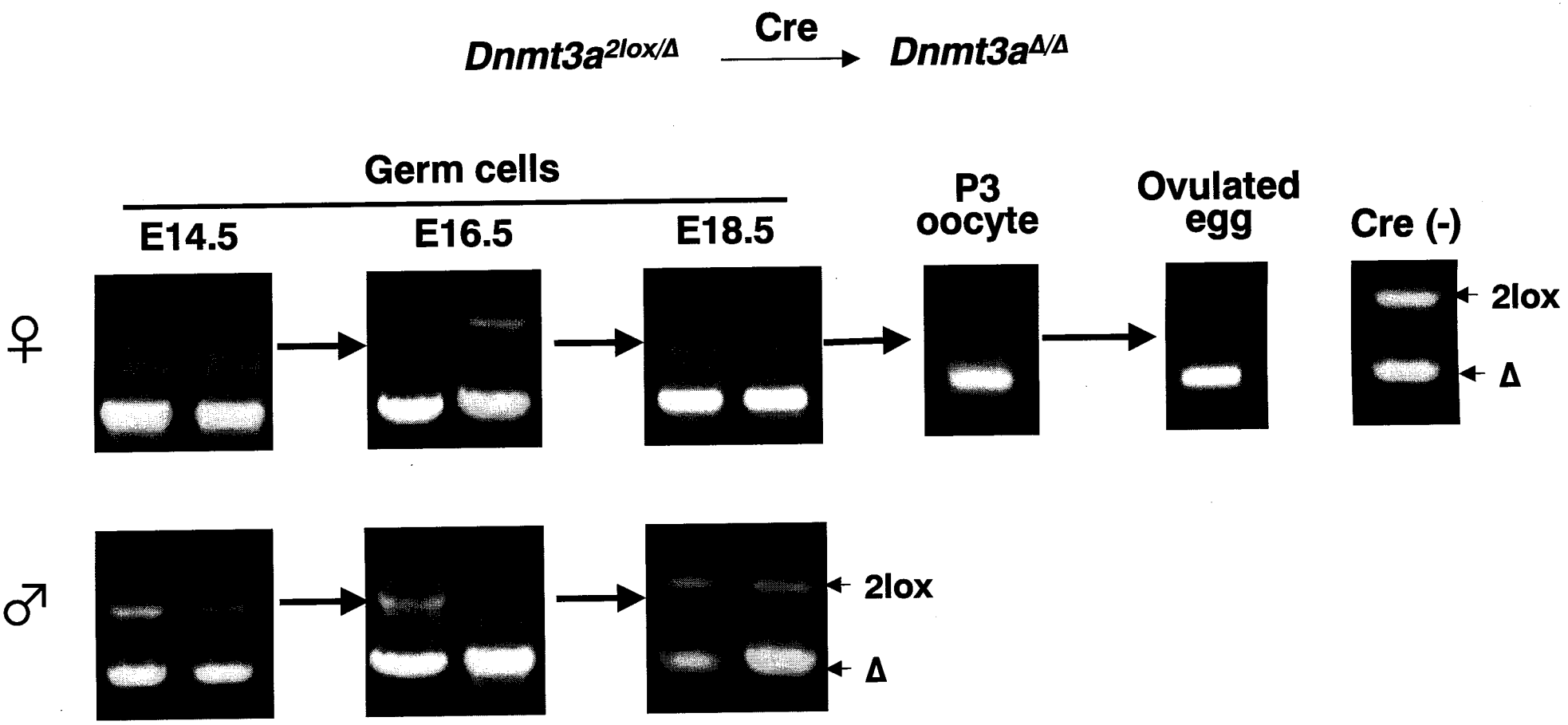



Figure 5. Conditional knockout of *Dnmt3a* by TNAP-Cre during germ cell development. In female [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] embryos at E14.5, E16.5 and E18.5, most germ cells had Δ alleles only. No 2lox allele was detected in P3 oocytes and ovulated eggs by PCR. In male [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] embryos at E14.5, E16.5 and E18.5, some cells still had a 2lox allele. As [*Dnmt3a*$^{2lox/\Delta}$ ,TNAP-Cre] males showed azoospermia, I could not obtain germ cells from postnatal testes or mature spermatozoa from the epididymis.

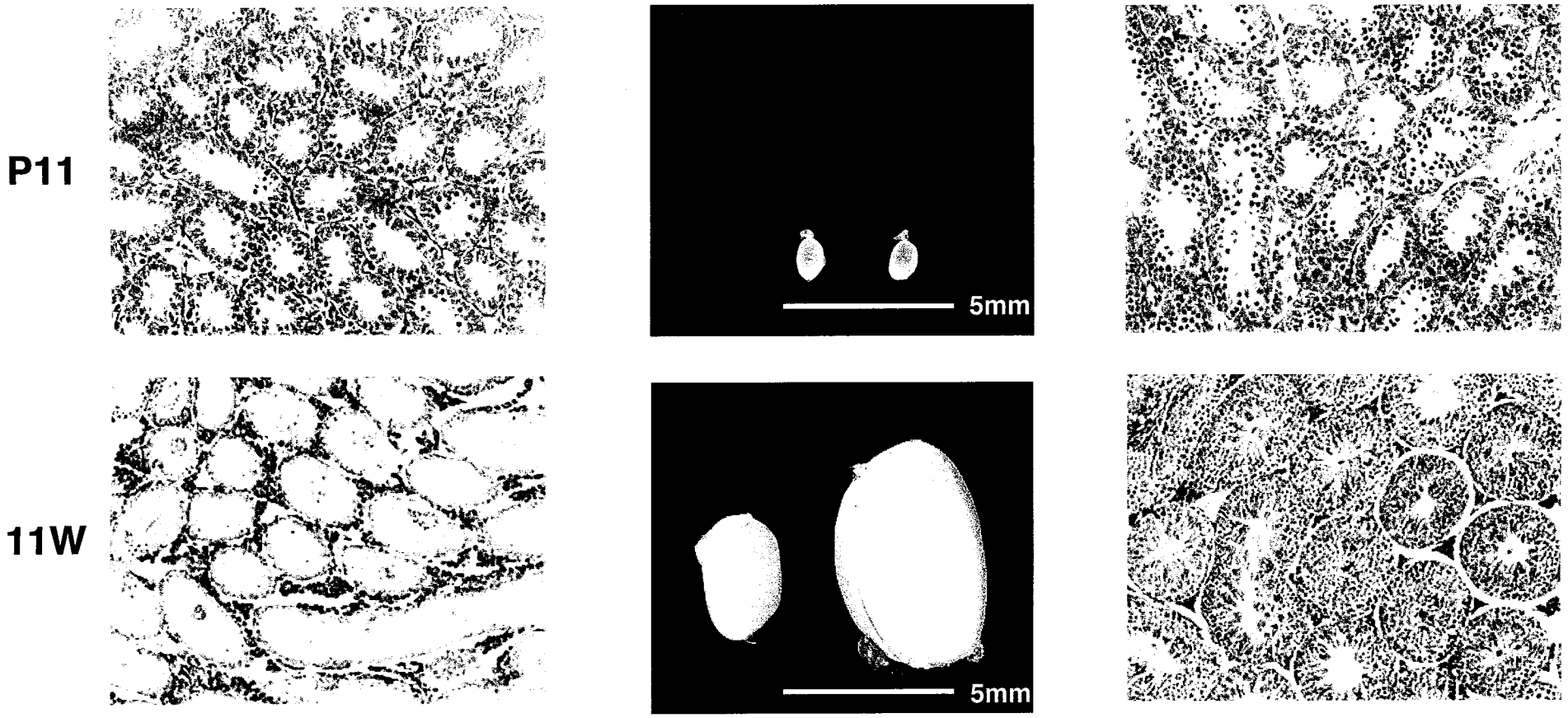



Figure 6. Gross morphology and histology of [*Dnmt3a*$^{2lox/\Delta}$ , TNAP-Cre] (left) and [*Dnmt3a*$^{2lox/\Delta}$ ] (control) testes (right). At P11, the [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] testes looked almost normal. At 11 weeks of age, the [*Dnmt3a*$^{2lox/\Delta}$ , TNAP-Cre] testes contained no spermatids or spermatozoa, which are present in the seminiferous tubules of the control *Dnmt3*$^{2lox/\Delta}$ testes.

| | Genotype of embryo | | |
|---|---|---|---|
| **Embryonic day** | ***Dnmt3a*$^{\Delta/+}$** | ***Dnmt3a*$^{2lox/+}$** | **Resorption** |
| **E9.5** | **16** | **0** | **1** |
| **E10.5** | **17** | **0** | **2** |
| **E11.5** | **0** | **0** | **8** |
| **Total** | **33** | **0** | **11** |

Table 1. Embryonic lethality of the offspring resulting from a cross between [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] females and wild-type males (C57BL/6). At E9.5, all embryos appeared normal. At E10.5, most embryos showed abnormal morphology and some were already dead. At E11.5, only resorptions were seen.

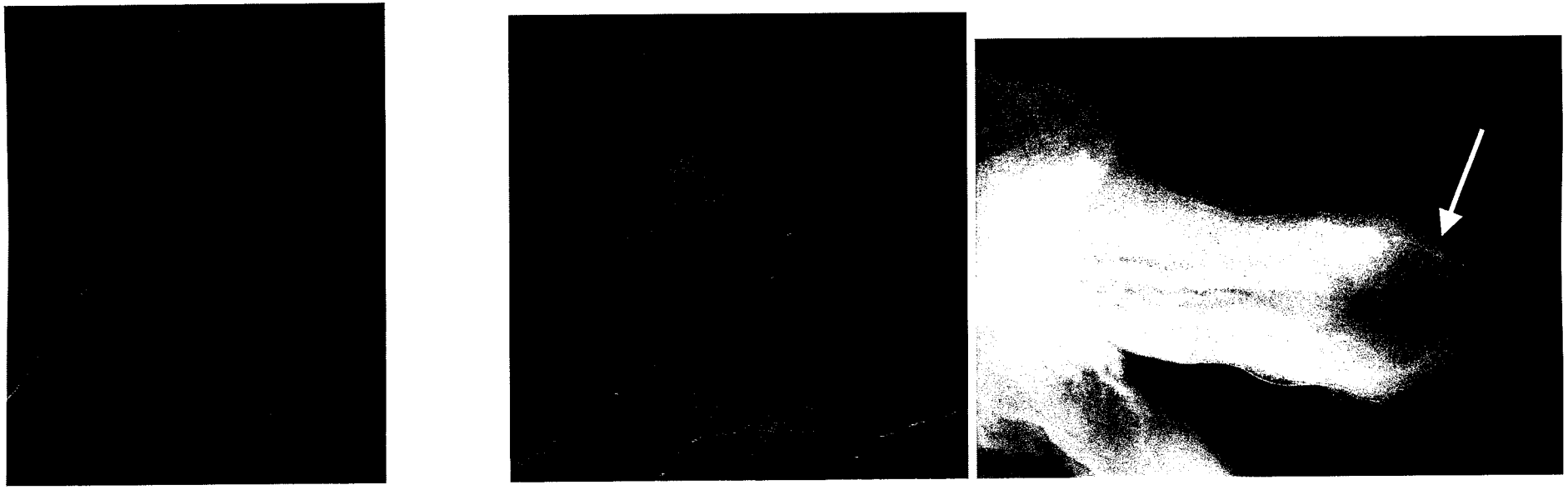



Figure7. Developmental defects of E10.5 embryos (*Dnmt3a*$^{matΔ/Δ}$) from a cross between [*Dnmt3a*$^{2lox/Δ}$, TNAP-Cre] females and wild-type males. Most embryos showed defects such as a smaller brain,an open neural tube in the midbrain region (arrow), and smaller branchial arches.

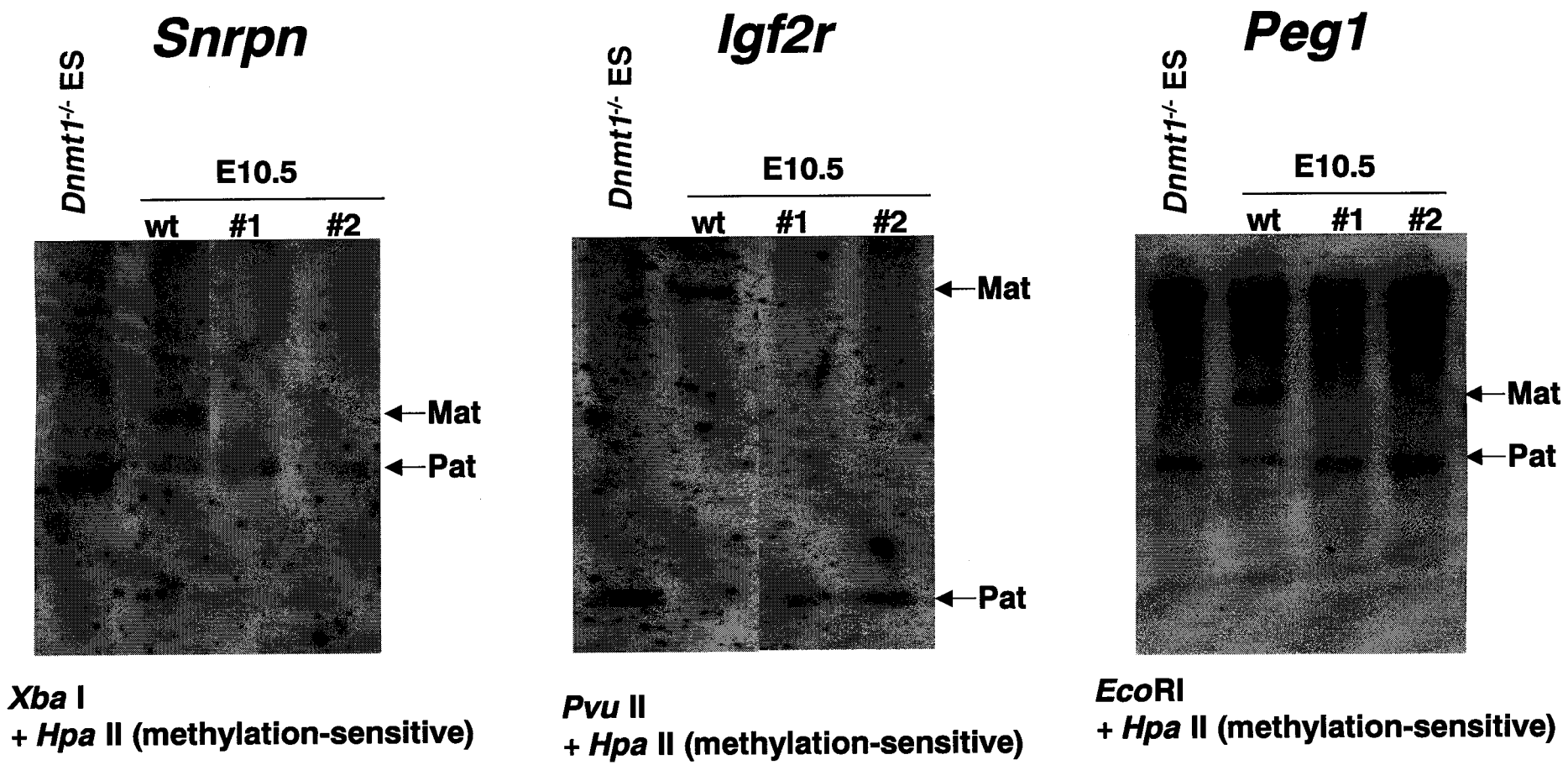
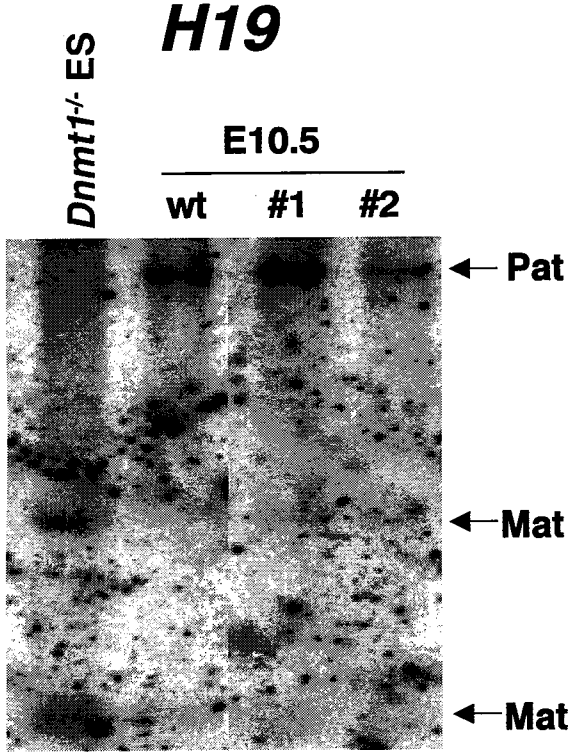



Figure 8. Methylation analysis of maternally methylated imprinted genes *Snrpn*, *Igf2r* and *Peg1* in two *Dnmt3a*$^{matΔ/Δ}$ embryos (#1 and #2) from [*Dnmt3a*$^{2lox/Δ}$, TNAP-Cre] mothers. These embryos lacked the maternal methylation imprints. Mat, Maternal allele; Pat, Paternal allele; wt, wild-type control.



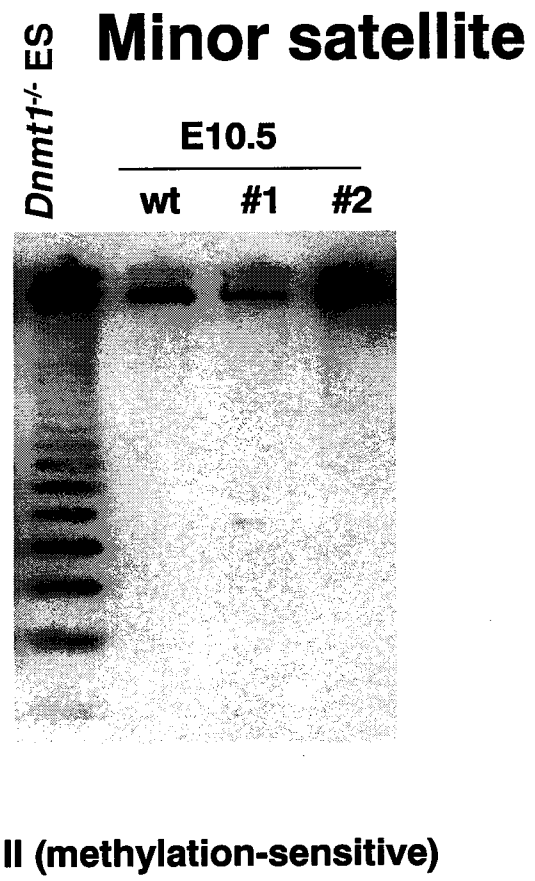




Figure 9. Methylation analysis of the paternally methylated gene *H19* and minor satellites in the *Dnmt3a*$^{mat\Delta/\Delta}$ embryos (#1 and #2) from [*Dnmt3a*$^{2lox/\Delta}$, TNAP-Cre] mothers. No change in methylation was observed. Mat, Maternal allele; Pat, Paternal allele; wt, wild-type control.

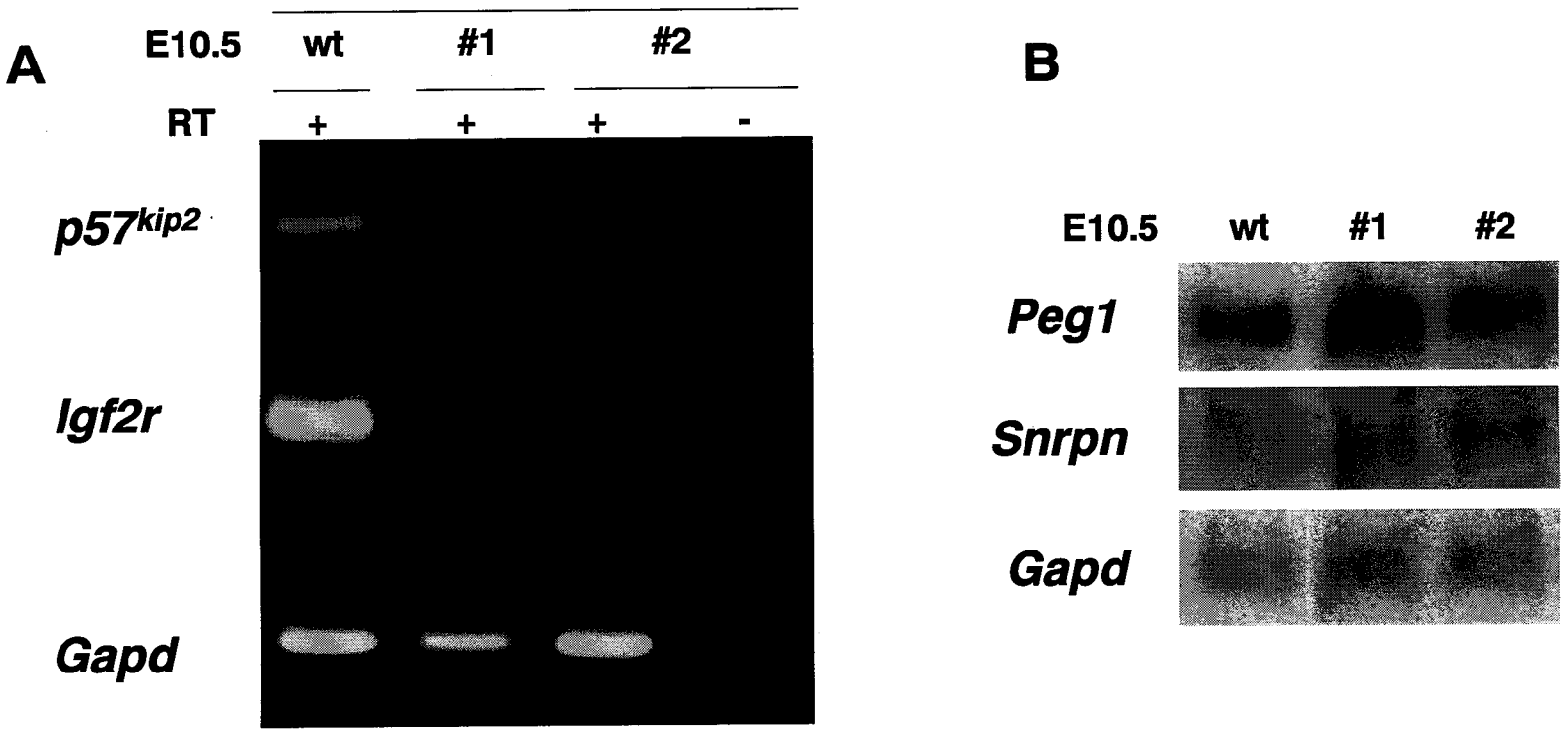



Figure 10. Expression analysis of four maternally methylated genes *p57*$^{kip2}$, *Igf2r, Peg1* and *Snrpn* by either RT-PCR (A) or northern blot hybridization (B). Expression of *p57*$^{kip2}$ and *Igf2r* was lost and expression of *Peg1* and *Snrpn* was increased in E10.5 *Dnmt3a*$^{mat\Delta/\Delta}$ embryos (#1 and #2) compared to wild-type embryos (wt), consistent with the loss of the maternal methylation imprints.

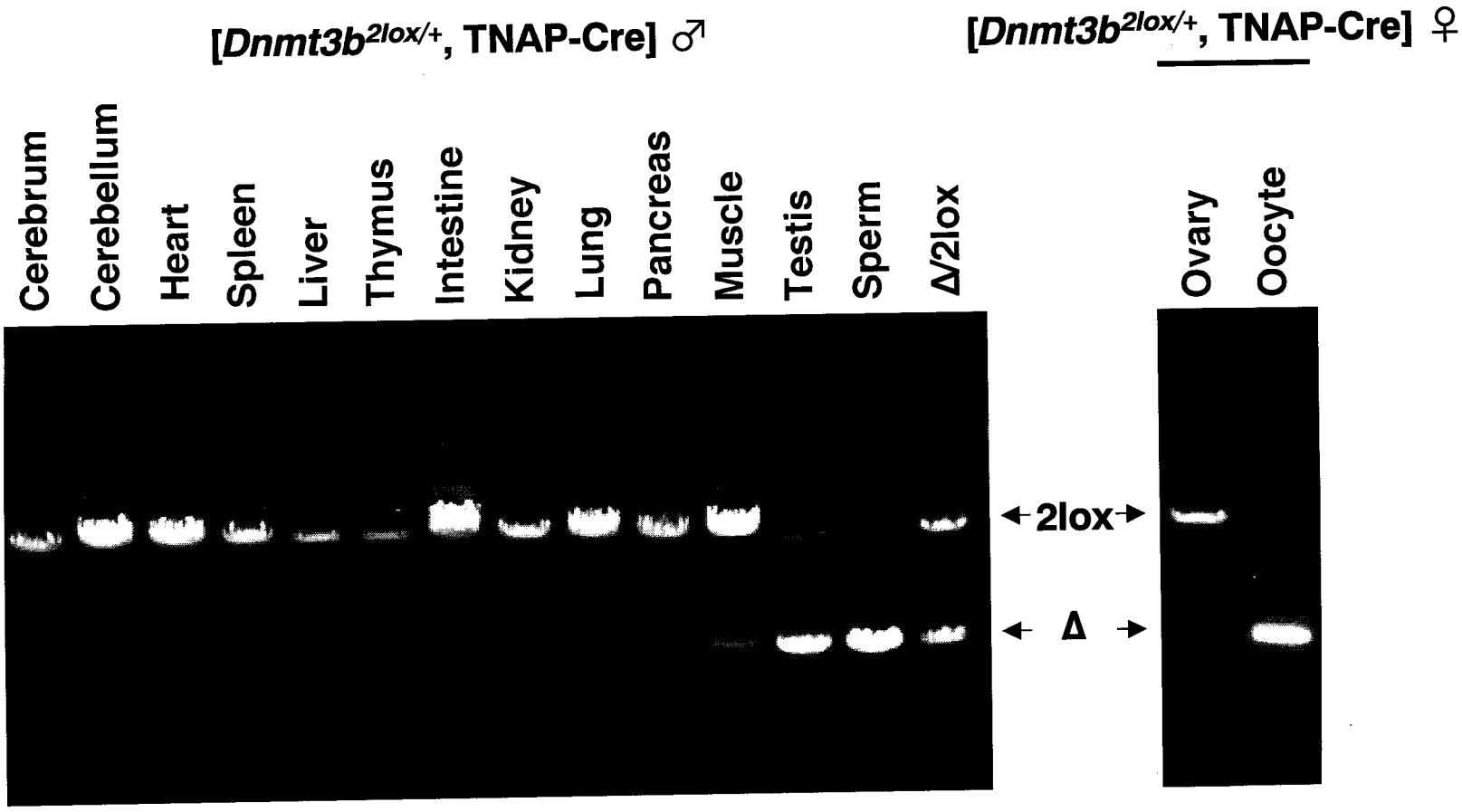



Figure 11. Specificity and efficiency of conditional knockout of *Dnmt3b* by TNAP-Cre. The efficiency of deletion in sperm and oocytes was almost 100%. Cre-mediated deletion also occurred in a small proportion of skeletal muscle cells.

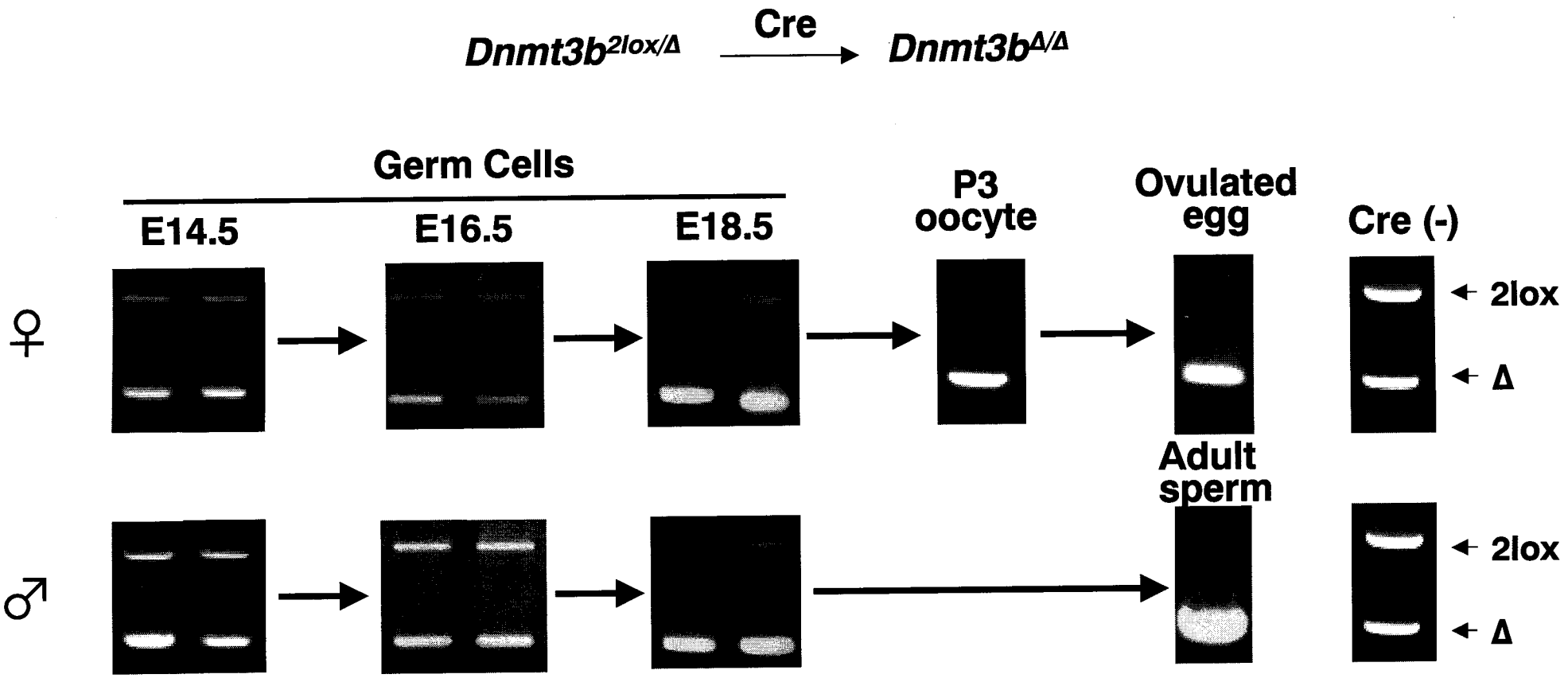



Figure 12. Conditional knockout of *Dnmt3b* by TNAP-Cre during germ cell development. In both females and males, the 2lox allele was progressively converted to the Δ allele. P3 oocytes, ovulated eggs and sperm had the Δ allele only.

| [*Dnmt3b*$^{2lox/\Delta}$, TNAP-Cre] mouse ID | | No. Litters* | No. pups* [*Dnmt3b*$^{\Delta/+}$] | [*Dnmt3b*$^{2lox/+}$] | Efficiency of deletion by Cre | Average litter size* |
|---|---|---|---|---|---|---|
| Female | #787 | 3 | 23 | 0 | | |
| | #796 | 3 | 24 | 0 | | |
| | #797 | 3 | 19 | 1 | 43/44 (97.7%) | 88/13 = 6.8 |
| | #846 | 4 | 21 | 0 | | |
| | Total | 13 | 87 | 1 | | |
| Male | #916 | 6 | 37 | 0 | | |
| | #245 | 6 | 45 | 0 | 51/51 (100.0%) | 102/15 = 6.8 |
| | #883 | 3 | 20 | 0 | | |
| | Total | 15 | 102 | 0 | | |
| Total | | 28 | 189 | 1 | 94/95 (98.9%) | 189/28 = 6.8 |
| Control | | 20 | - | - | - | 152/20 = 7.6 |

*** Germline-specific knockout mice were crossed with wildtype partners.**

Table 2. Efficiency of germline-specific knockout of *Dnmt3b* by TNAP-Cre and its effects on the offspring. Almost all pups had only the recombined Δ allele, suggesting a high rate of recombination. No abnormalities were observed in these pups.

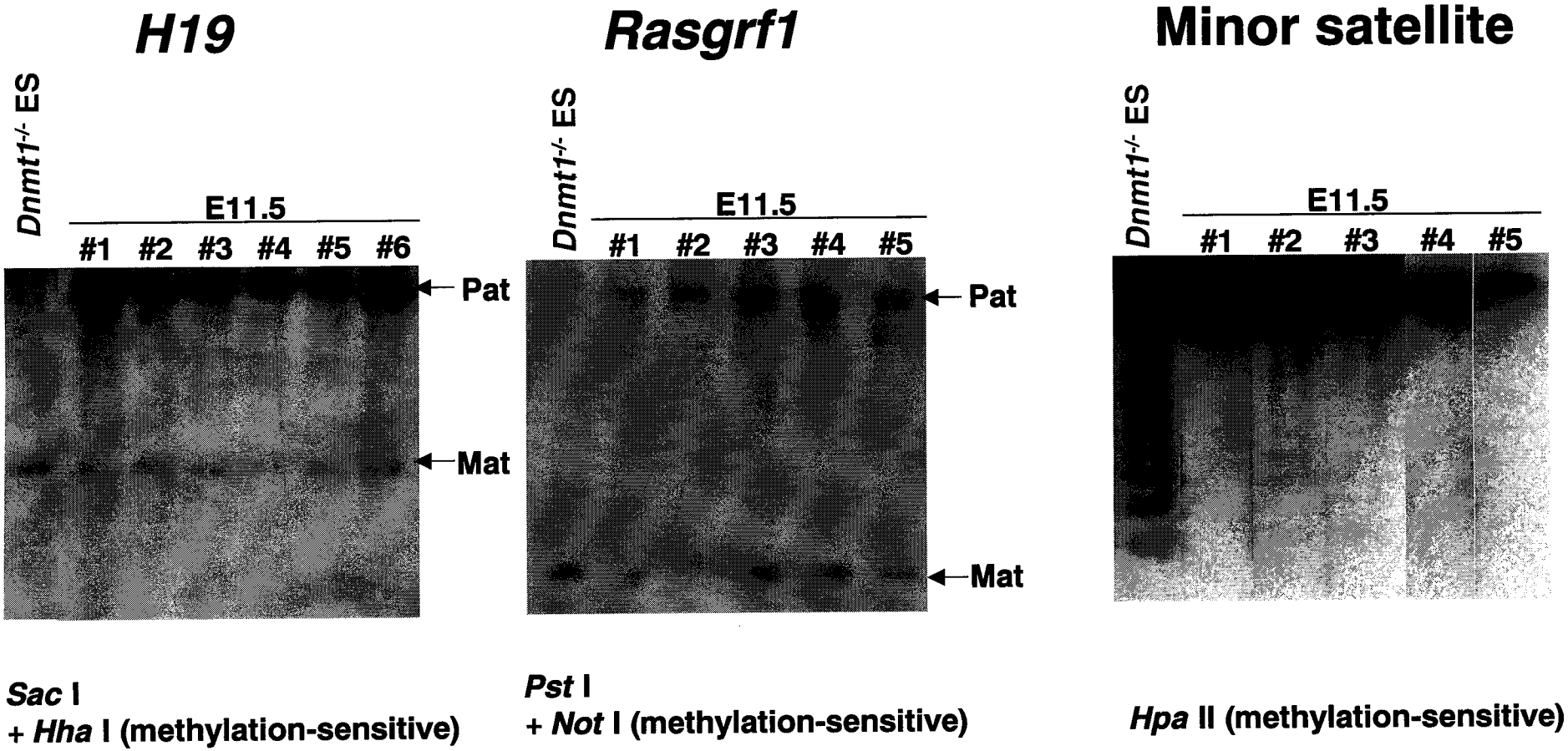



Figure 13. Methylation analysis of imprinted genes and minor satellites in the *Dnmt3b*$^{patΔ/Δ}$ embryos from [*Dnmt3b*$^{2lox/Δ}$ ,TNAP-Cre] fathers. The methylation levels of paternally methylated genes *H19* and *rasgrf1* were normal. Minor satellite DNA, which is the target sequence of Dnmt3b, was completely methylated in all embryos. Mat, Maternal allele; Pat, Paternal allele.

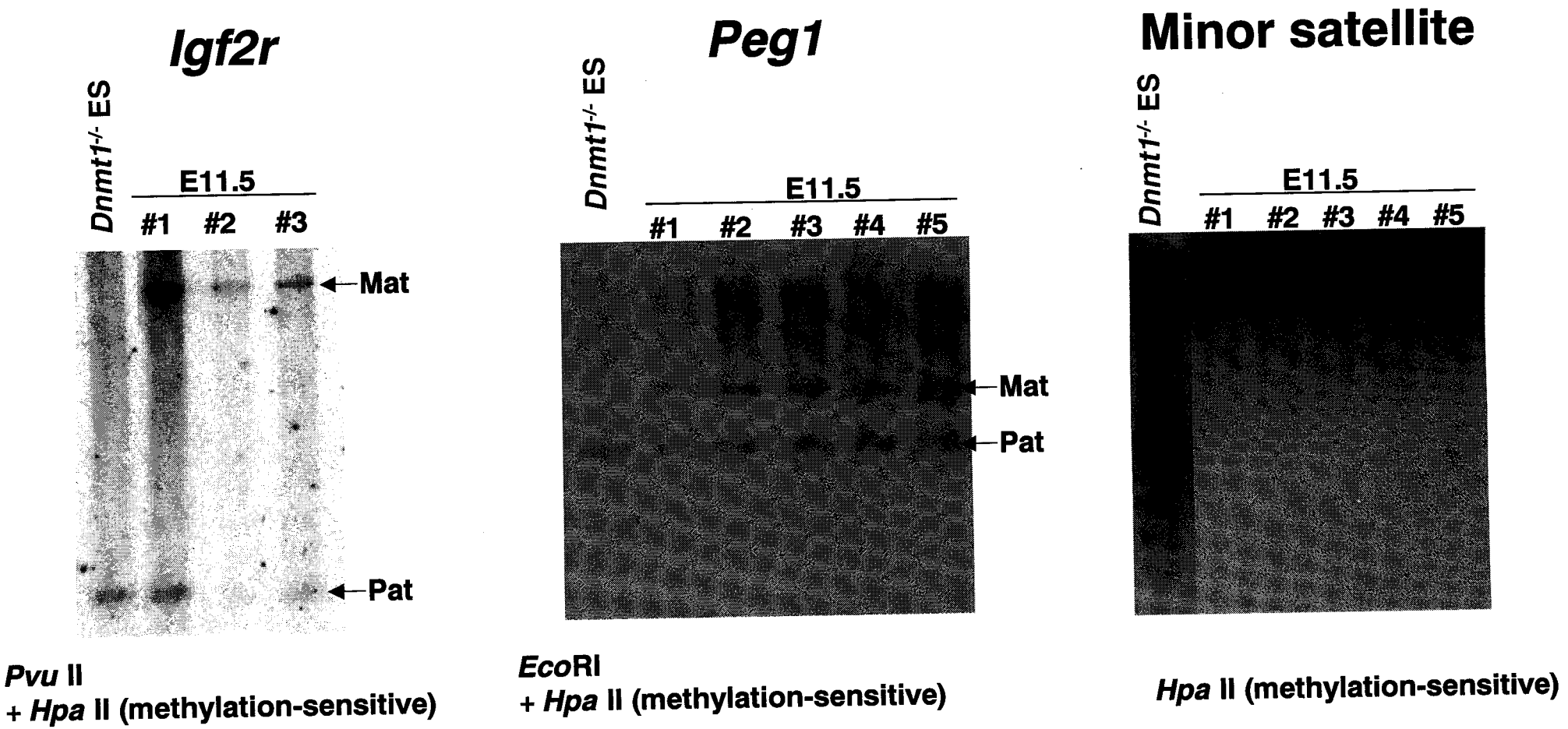



Figure 14. Methylation analysis of imprinted genes and minor satellites in the *Dnmt3b*$^{mat\Delta/\Delta}$ embryos from [*Dnmt3b*$^{2lox/\Delta}$ ,TNAP-Cre] mothers. The methylation levels of maternally imprinted genes *Igf2r* and *Peg1* were normal. Minor satellite DNA, which is the target sequence of Dnmt3b, was methylated normally. Mat, Maternal allele; Pat, Paternal allele.