

## RESEARCH ARTICLE

# *De novo* DNA methylation through the 5'-segment of the *H19* ICR maintains its imprint during early embryogenesis

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## ABSTRACT

Genomic imprinting is a major monoallelic gene expression regulatory mechanism in mammals, and depends on gamete-specific DNA methylation of specialized *cis*-regulatory elements called imprinting control regions (ICRs). Allele-specific DNA methylation of the ICRs is faithfully maintained at the imprinted loci throughout development, even in early embryos where genomes undergo extensive epigenetic reprogramming, including DNA demethylation, to acquire totipotency. We previously found that an ectopically introduced *H19* ICR fragment in transgenic mice acquired paternal allele-specific methylation in the somatic cells of offspring, whereas it was not methylated in sperm, suggesting that its gametic and postfertilization modifications were separable events. We hypothesized that this latter activity might contribute to maintenance of the methylation imprint in early embryos. Here, we demonstrate that methylation of the paternally inherited transgenic *H19* ICR commences soon after fertilization in a maternal DNMT3A- and DNMT3L-dependent manner. When its germline methylation was partially obstructed by insertion of insulator sequences, the endogenous paternal *H19* ICR also exhibited postfertilization methylation. Finally, we refined the responsible sequences for this activity in transgenic mice and found that deletion of the 5' segment of the endogenous paternal *H19* ICR decreased its methylation after fertilization and attenuated *Igf2* gene expression. These results demonstrate that this segment of the *H19* ICR is essential for its *de novo* postfertilization DNA methylation, and that this activity contributes to the maintenance of imprinted methylation at the endogenous *H19* ICR during early embryogenesis.

**KEY WORDS:** DNA methylation, Genomic imprinting, *Igf2/H19* locus, Early embryogenesis

## INTRODUCTION

Genomic imprinting is an epigenetic phenomenon in mammals that causes parental-specific, monoallelic expression of a subset of autosomal genes. The unique expression patterns of imprinted genes are controlled by allele-specific DNA methylation of the *cis*-regulatory sequences, called the imprinting control regions (ICRs).

Because allelic DNA methylation of ICRs is acquired during gametogenesis, ICRs are also called germline differentially methylated regions (gDMRs) (Ferguson-Smith, 2011; Kelsey and Feil, 2013; Tomizawa and Sasaki, 2012). Recent genome-wide profiling has, however, revealed that the number of oocyte- or sperm-specific methylated genomic regions is far more than that of the known ICRs (Kobayashi et al., 2012; Smallwood et al., 2011). Therefore, germline methylation might not be restricted to ICRs, and both the ICRs and the other gDMRs could be methylated by a common mechanism without being strictly discriminated in the germ line (Kelsey and Feil, 2013).

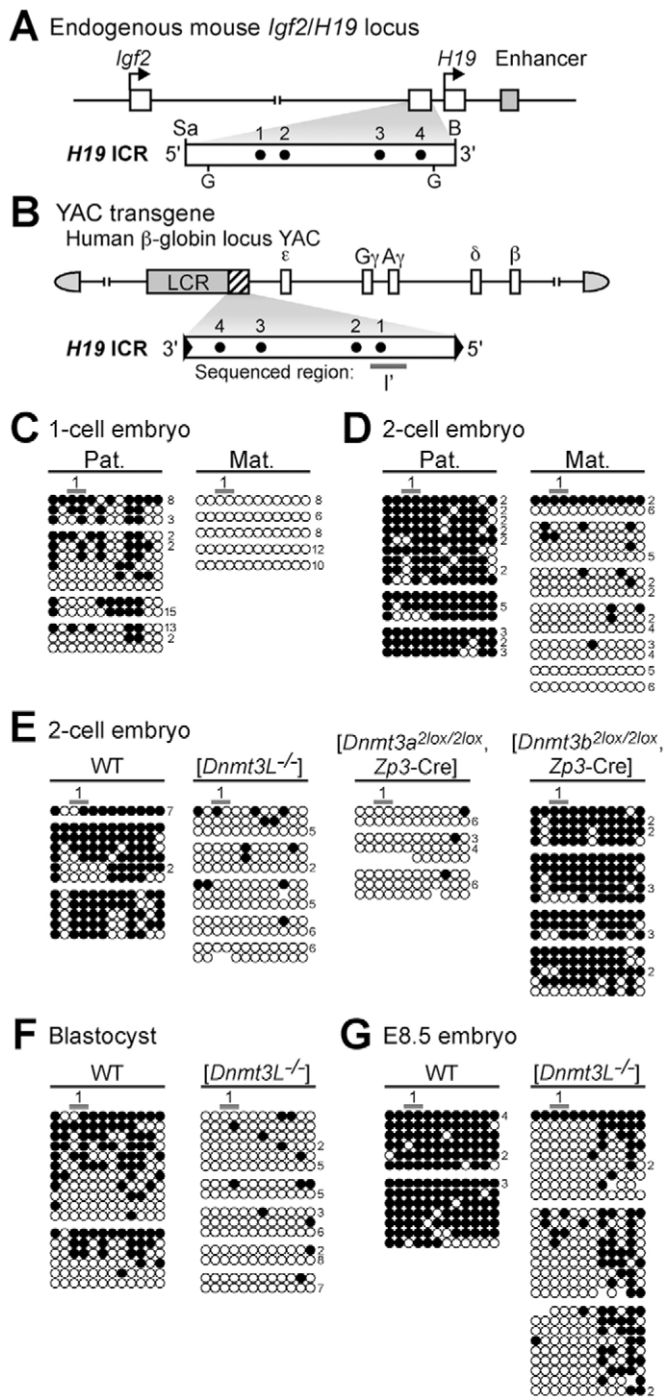
During preimplantation development, although most gDMRs lose their gamete-derived methylation via epigenetic reprogramming activity, allelic methylation of ICRs is faithfully maintained (Kobayashi et al., 2012; Smallwood et al., 2011). Presumably, the methylation maintenance mechanism against genome-wide demethylation activity might operate at restricted genomic loci and selected alleles in preimplantation embryos. We and others have reported that Stella (also known as DPPA3) (Nakamura et al., 2007), KAP1 (also known as TRIM28) (Messerschmidt et al., 2012), and NuRD complex components (Ma et al., 2010; Reese et al., 2007), in addition to the maintenance methyltransferase DNMT1 (Hirasawa et al., 2008), help to maintain ICR methylation in preimplantation embryos. Because these factors have no sequence specificity for DNA binding or intrinsic DNA binding ability themselves, other sequence-specific DNA binding proteins must be required for their recruitment to specific target sites. The Krüppel-associated box (KRAB)-containing zinc-finger protein ZFP57, which was found to interact with KAP1, is a dominant candidate for such a protein (Li et al., 2008; Messerschmidt et al., 2012; Quenneville et al., 2011). However, because the depletion of ZFP57 did not always affect the methylation of ICRs (Li et al., 2008), other factors and their target *cis* elements are apparently engaged in the maintenance mechanism.

The *H19* ICR in the mouse *Igf2/H19* locus (Fig. 1A) is DNA-methylated by the DNMT3A-DNMT3L complex in prospermatogonia, the status of which is maintained on the paternal allele following fertilization (Kaneda et al., 2004; Tremblay et al., 1997), and it is thus classified as a gDMR. Whereas indispensable roles for CTCF (Matsuzaki et al., 2010; Schoenherr et al., 2003) and Sox-Oct binding motifs (Sakaguchi et al., 2013; Zimmerman et al., 2013) in maintaining maternal *H19* ICR hypomethylation during postimplantation periods are well established, little is known about the underlying mechanisms that maintain paternal *H19* ICR hypermethylation during preimplantation periods.

We previously tested the activity of the *H19* ICR in yeast artificial chromosome (YAC) transgenic mice (TgM), in which an *H19* ICR fragment (2.9 kb) was inserted into a YAC bearing the (nonimprinted) human  $\beta$ -globin locus (150 kb, Fig. 1B) to minimize position effects of transgene insertion sites (Tanimoto

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**Fig. 1. DNA methylation status of the transgenic *H19* ICR in early embryos.** (A) Structure of the mouse *Igf2/H19* locus. Mouse *Igf2* and *H19* (open boxes) are ~90 kb apart, and the expression of both genes depends on the shared 3' enhancer (gray box). The *H19* ICR, located approximately at -4 to -2 kb relative to the transcription start site of *H19* is contained within a 2.9-kb *SacI* (*Sa*)-*Bam*HI (*B*) fragment. Dots (1-4) indicate the position of CTCF binding sites. G; *Bgl*II site. (B) Structure of the ICR/ $\beta$ -globin YAC transgene. The 150-kb human  $\beta$ -globin locus YAC carries the LCR (gray box) and the  $\beta$ -like globin genes (open boxes). The 2.9-kb *H19* ICR fragment (inverted orientation) was introduced between the LCR and the  $\epsilon$ -globin gene (Tanimoto et al., 2005). A gray bar below the map indicates sequences (region I') analyzed by bisulfite sequencing. (C,D) Methylation status of the transgenic *H19* ICR in embryos. One- (C) or two-cell (D) embryos that inherited the ICR/ $\beta$ -globin YAC transgene (line 1048) either paternally (Pat.) or maternally (Mat.) were embedded in agarose beads (19-43 embryos per bead in C, 13-37 embryos per bead in D) and treated with sodium bisulfite. The beads were separately and directly used to amplify the region I' of the transgenic *H19* ICR by nested PCR. PCR products were individually subcloned and sequenced. The results from single beads are presented together in a cluster. Each horizontal row represents a single DNA template molecule. The numbers on the right of each row indicate the number of times the pattern was observed in the sequencing. Methylated and unmethylated CpG motifs are shown as filled and open circles, respectively. (E,F) A role of *de novo* DNA methyltransferases in the postfertilization methylation of the paternally inherited transgenic *H19* ICR. Two-cell (E) or blastocyst (F)-stage embryos were obtained from wild-type (WT), [*Dnmt3l*<sup>-/-</sup>], [*Dnmt3a*<sup>2lox/2lox</sup>, *Zp3-Cre*], or [*Dnmt3b*<sup>2lox/2lox</sup>, *Zp3-Cre*] females crossed with the ICR/ $\beta$ -globin male TgM carrying WT Dnmts (5-24 embryos in E, 1-9 embryos in F). The methylation status of the paternally inherited transgenic *H19* ICR (region I') was analyzed by bisulfite sequencing as described previously. (G) E8.5 embryos were obtained from WT or [*Dnmt3l*<sup>-/-</sup>] females crossed with ICR/ $\beta$ -globin male TgM. Genomic DNA was extracted from each embryo and treated with sodium bisulfite, and region I' of the transgenic *H19* ICR was amplified by nested PCR. PCR products were subcloned and sequenced. The results from single embryos are presented together in a cluster. Above each panel in E-G are genotypes of mothers.

et al., 2005). In somatic cells, the *H19* ICR fragment was preferentially methylated when paternally inherited, demonstrating that the 2.9-kb sequence contained sufficient information to recapitulate imprinted methylation. Surprisingly, however, the transgenic *H19* ICR was not methylated in the testes. In addition, randomly integrated *H19* ICR fragments in the mouse genome were hypermethylated in the paternal allele after fertilization, irrespective of their variable methylation levels in the testes of multiple TgM lines (Matsuzaki et al., 2009). It was therefore presumed that the *H19* ICR was marked by an epigenetic modification other than DNA methylation in the germ line, and that paternal allele-specific methylation was acquired after fertilization by referring to this hypothetical mark. Hence, our results and those of others (Gebert

et al., 2010; Park et al., 2004) suggested that two distinct methylation mechanisms operate at the endogenous *H19* ICR: one in the germ line, which is under the control of its surrounding sequences, and the other during the postfertilization period, which is governed by a hypothetical epigenetic mark preset within the *H19* ICR during gametogenesis. We speculated that the latter activity might be actively involved in the region-specific maintenance of allelic methylation at the endogenous *H19* ICR in preimplantation embryos.

In this study, we show that the paternal-allele-specific methylation of the transgenic *H19* ICR commences soon after fertilization in YAC-TgM, and that maternally supplied DNMT3A and DNMT3L are required for this process. By partially obstructing germline methylation of the endogenous *H19* ICR, we discovered that postfertilization methylation activity also exists at the endogenous *H19* ICR. Furthermore, in YAC-TgM, we substantially narrowed the responsible sequences for postfertilization methylation acquisition in the transgenic *H19* ICR. Finally, by deleting the responsible sequences from the endogenous locus, we noted a partial loss of methylation in the paternally inherited *H19* ICR after fertilization, diminished *Igf2* expression, and embryonic growth retardation in the offspring that paternally inherited the mutation. These results demonstrate that the postfertilization methylation imprinting activity of the *H19* ICR is essential for maintaining its imprinted methylation status once established during gametogenesis.

## RESULTS

### Methylation acquisition at the transgenic *H19* ICR in early embryos

The *H19* ICR fragment inserted into the  $\beta$ -globin YAC transgene (Fig. 1B) exhibited preferential DNA methylation in the somatic cells of offspring after paternal transmission, whereas it was not

methylated in sperm (Fig. S1A–C) (Tanimoto et al., 2005). As a first step in elucidating the mechanism of the allele-specific methylation of the transgenic *H19* ICR, we examined the timing of its acquisition in mouse early embryos. Bisulfite sequencing of the transgenic *H19* ICR (region I' including the CTCF site 1, Fig. 1B) revealed that the paternally inherited ICR was moderately and heavily methylated in one- and two-cell stage embryos, respectively (Fig. 1C,D, Fig. S1D), the levels of which were substantially higher than in the maternally inherited alleles. While the DNA methylation level in region I' in two-cell embryos was already high (Fig. 1D, Fig. S1D) and indistinguishable from that in blastocyst-stage embryos (Fig. S1E) (Matsuzaki et al., 2010), DNA methylation around CTCF binding site 4 of the paternally inherited *H19* ICR was low in blastocysts (region II, Fig. S1F), suggesting that DNA methylation acquisition directionally extends from a region near CTCF site 1. These results suggested that the paternally inherited transgenic *H19* ICR is recognized by the DNA methylation machinery soon after fertilization and becomes progressively methylated during embryonic development.

### A role for DNMT3s in the postfertilization methylation of the transgenic *H19* ICR

Methylation acquisition in the endogenous *H19* ICR occurs in fetal prospermatogonia via the actions of DNMT3A and DNMT3L (Kaneda et al., 2004). However, the activity and targets, if any, of the DNMT3 family in early embryos remain obscure. We thus examined which DNMTs were involved in methylation acquisition in the transgenic *H19* ICR. Because the gene products present in early embryos soon after fertilization are mostly derived from oocytes, we assessed the roles of DNMTs on postfertilization methylation of the transgenic *H19* ICR after maternal disruption. To test the function of *Dnmt3l*, *Dnmt3l*-null ( $-/-$ ) (Hata et al., 2002) females were mated with ICR/ $\beta$ -globin YAC transgenic (*Dnmt3l* wild-type) (Fig. 1B) (Tanimoto et al., 2005) males. Because *Dnmt3a* $^{-/-}$  or *Dnmt3b* $^{-/-}$  mice are not viable (Okano et al., 1999), we used Cre-loxP recombination to specifically eliminate these genes in growing oocytes via the *zona pellucida glycoprotein 3* (*Zp3*) promoter-Cre transgene (de Vries et al., 2000; Dodge et al., 2005; Kaneda et al., 2004). After confirming that *Dnmt3* gene products were depleted in both oocytes and early embryos by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) (Fig. S2), we analyzed the methylation status of the transgenic *H19* ICR fragment. In *Dnmt3l*-deficient two-cell embryos, the paternally inherited transgenic *H19* ICR was hypomethylated (Fig. 1E). Depletion of maternally provided *Dnmt3a* gene product also caused hypomethylation of the transgenic *H19* ICR, whereas the loss of the *Dnmt3b* gene product did not affect its methylation (Fig. 1E). These results demonstrated that the postfertilization methylation acquisition of the paternally inherited transgenic *H19* ICR required both DNMT3A and DNMT3L, which were maternally provided to early embryos.

We next examined whether zygotic expression of Dnmts (Fig. S2) (Guenatri et al., 2013; Hu et al., 2008) would compensate for a loss of maternally provided DNMT3L in transgenic *H19* ICR methylation during embryogenesis. The paternally inherited transgenic *H19* ICR remained unmethylated in blastocyst-stage embryos derived from *Dnmt3l* $^{-/-}$  mothers (Fig. 1F). The unmethylated state of the transgenic *H19* ICR did not change even at embryonic day (E) 8.5, despite the fact that allele-nonspecific methylation, probably elicited by postimplantation *de novo* DNA methylation activity, was observed outside of the DMR (Fig. 1G)

(Matsuzaki et al., 2010). These results demonstrated that the paternally inherited transgenic *H19* ICR must be recognized by the DNA methylation machinery, including DNMT3A and DNMT3L, in early embryos to acquire imprinted methylation.

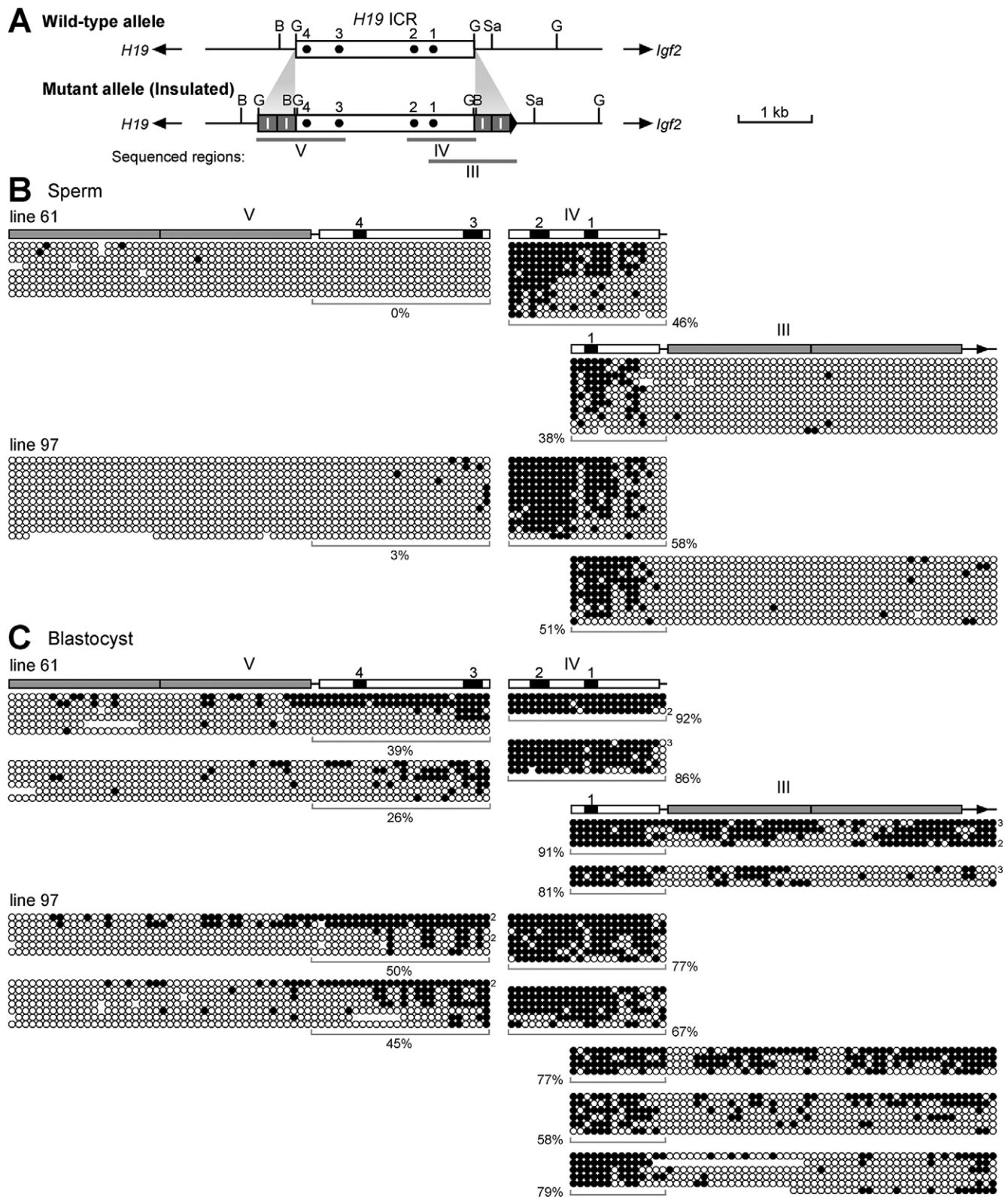
### Evaluation of the postfertilization methylation activity at the endogenous *H19* ICR

Although the transgenic *H19* ICR possesses intrinsic activity to acquire paternal allele-specific methylation in early embryos, it is unclear whether this activity also exists at the endogenous locus. Because the endogenous *H19* ICR is fully methylated in sperm, the postfertilization methylation activity at the endogenous locus, if present, is normally difficult to reveal. Our previous results (Matsuzaki et al., 2009) and those of others (Gebert et al., 2010; Park et al., 2004; Puget et al., 2015) suggested that the gametic methylation of the *H19* ICR was governed by signals from surrounding sequences, i.e. those located outside the 2.9-kb *H19* ICR region. Therefore, by interfering with the transmission of these hypothetical signals and subsequent methylation during spermatogenesis, we sought to verify the postfertilization methylation imprinting activity at the endogenous locus. To this end, we inserted tandemly arrayed chicken HS4 core sequences, (cHS4c)<sub>2</sub>, on both sides of the endogenous *H19* ICR (Fig. 2A), expecting that this would block a presumptive signal to direct DNA methylation of the *H19* ICR in the germ line, as the cHS4 itself was unmethylated in both germ and somatic cells when it was substituted for the endogenous *H19* ICR (Szabo et al., 2002). Importantly, during the postfertilization period, the same manipulation does not prevent methylation imprinting activity in the context of YAC-TgM (Okamura et al., 2013a). Embryonic stem cells (ESCs) were modified by homologous recombination, and accurate targeting events were confirmed by Southern blotting (Fig. S3A,B). Following the establishment of two knock-in mouse lines (Fig. S3B), the neo<sup>r</sup> selectable marker was excised by mating them with Cre-expressing TgM (Fig. S3C).

### Germline methylation of the endogenous *H19* ICR is inhibited by flanking insulator sequences

We examined the methylation status of the insulated *H19* ICR allele in sperm by bisulfite sequencing. The (cHS4c)<sub>2</sub> sequences on both sides of the *H19* ICR were hypomethylated (Fig. 2B). In addition, the *H19* ICR region containing CTCF sites 3 and 4 was also methylated at very low levels. Furthermore, the region around the CTCF sites 1 and 2 was significantly less methylated (Fig. 2B) in comparison to the fully methylated sequences in the wild-type allele (Fig. S3D) ( $P < 0.0001$ , Mann–Whitney U-test; <http://quma.cdb.riken.jp/>). Southern blotting using methylation-sensitive restriction enzymes confirmed these results (Fig. S4A,B). These results indicated that the flanking (cHS4c)<sub>2</sub> fragments at the endogenous *H19* ICR inhibited its methylation acquisition during spermatogenesis. It was reported that USF1 binding to cHS4 sequences induces histone H3/H4 acetylation and H3K4 methylation, thereby interfering with the spread of repressive histone modifications (such as H3K9 methylation) and heterochromatin formation (West et al., 2004). We therefore infer that intrusion of the repressive chromatin state into the *H19* ICR from outside might be a prerequisite for its gametic methylation and that flanking cHS4c might block this process. Alternative hypotheses might be that VEZF1-bound cHS4c sequences or simply its CpG island-like nature somehow interfered with the *de novo* DNA methylation of the neighboring *H19* ICR only during spermatogenesis (Dickson et al., 2010; Kobayashi et al., 2012; Smallwood et al., 2011). It is also possible that cHS4c





**Fig. 2. DNA methylation status of the insulated *H19* ICR in sperm and blastocysts.** (A) Map of the wild-type and mutant *H19* loci. Tandem *CHS4* core fragments (gray rectangles, I for insulator) were inserted at both sides of the *H19* ICR [at *Bg*III (G) sites: mutant allele] (see also Fig. S3). Three regions of the mutant allele (III, IV, and V; gray bars beneath the map) were analyzed by bisulfite sequencing in B and C. B, *Bam*HI; Sa, *Sac*I sites; dots 1-4, CTCF binding sites. (B) Genomic DNA extracted from sperm of mutant mice (lines 61 and 97) was digested with *Xba*I and treated with sodium bisulfite. Three regions of the mutant allele were amplified by PCR, subcloned, and sequenced. The overall percentage of methylated CpGs in region IV or those in the ICR portions of region III or V are indicated next to each panel. (C) Blastocyst-stage embryos that inherited the mutant allele paternally were embedded in agarose beads (6-12 embryos per bead) and treated with sodium bisulfite. The beads were used for amplifying three regions of the mutant allele by PCR, and the resulting fragments were individually subcloned and sequenced.

sequences interfered with transcription read-through at the *H19* ICR, which has been suggested to induce its methylation in male germ cells (Henckel et al., 2012).

### Allele-specific postfertilization DNA methylation at the insulated *H19* ICR

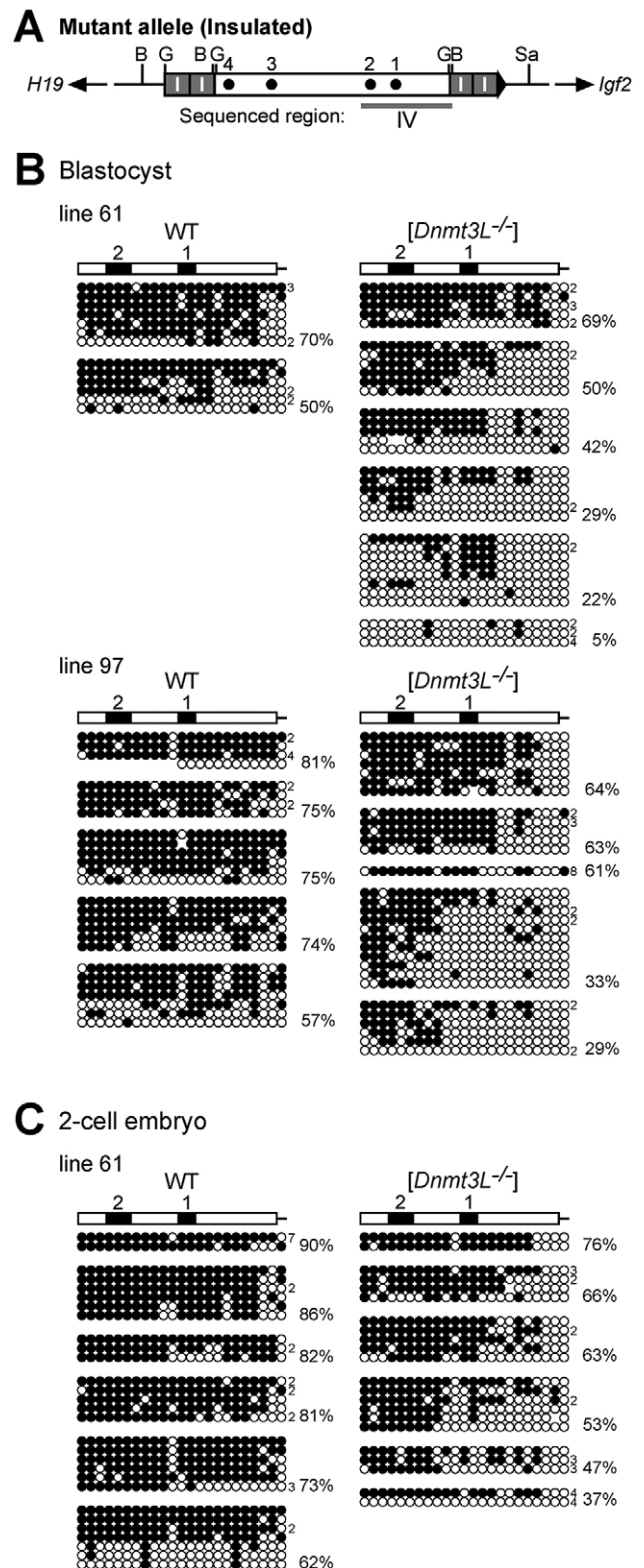
We next examined the methylation status of the insulated *H19* ICR after fertilization. In blastocyst-stage embryos (Fig. 2C), as well as in the somatic cells of neonates (Fig. S4A,C-E), the paternally inherited insulated *H19* ICR exhibited higher methylation levels than those observed in sperm (Fig. 2B). The markedly elevated level of methylation in (cHS4c)<sub>2</sub> sequences on the paternal allele (Fig. S4E) was probably a secondary consequence of imprinted methylation of the adjacent *H19* ICR as the same sequences were unmethylated after maternal transmission. These results demonstrated that the endogenous *H19* ICR was methylated after fertilization in a paternal allele-specific manner, and that the methylation acquisition commenced during the preimplantation period.

In the YAC-TgM, transgenic *H19* ICR methylation in early embryos was dependent on maternally provided DNMT3L and DNMT3A (Fig. 1E,F). We therefore examined whether the same DNA methyltransferases were operating at the endogenous insulated *H19* ICR. When *Dnmt3l*<sup>-/-</sup> female mice were bred, the paternally inherited insulated *H19* ICR region (region IV in Fig. 3A) was less methylated in blastocyst (Fig. 3B) than in control embryos (WT), indicating that DNMT3L is involved in the methylation of the endogenous *H19* locus. In addition, the effect of maternal DNMT3L depletion on the postfertilization methylation at the endogenous insulated *H19* ICR was observed in as early as 2-cell embryos (Fig. 3C), the same timing when postfertilization methylation acquisition took place in the YAC TgM (Fig. 1D).

Taken together, these results suggest that postfertilization paternal allele-specific methylation activity also exists in the endogenous *H19* ICR, which is probably governed by a shared mechanism with the transgenic *H19* ICR.

### Defining the *H19* ICR DNA sequences essential for allele-specific postfertilization DNA methylation in YAC-TgM

To further elucidate the mechanisms underlying the postfertilization methylation of the *H19* ICR, we sought to more precisely define its responsive sequences. To this end, we employed a YAC-TgM system, in which the methylation activity is clearly detectable after fertilization because of its unmethylated state in sperm. We previously demonstrated that the 1.7-kb 'ICR21' fragment covering CTCF sites 1 and 2 (Fig. 4A) was sufficient to recapitulate paternal allele-specific DNA methylation after fertilization in YAC-TgM (Okamura et al., 2013b). We thus generated a series of 5'-truncated *H19* ICR fragments: the ICR4321S fragment, in which the 5'-end of the 2.9-kb *H19* ICR fragment (766 bp) was deleted but all four CTCF sites remained; and the ICR432 fragment, which is 173 bp shorter than the ICR4321S fragment and lacks CTCF site 1 (Fig. 4A). To reduce the time required to obtain mouse lines carrying intact single-copy YAC transgenes, these two fragments were individually floxed using hetero-specific *loxP* sequences, combined to employ a co-placement strategy (Tanimoto et al., 2008), and introduced 3' to the locus control region (LCR) in the human  $\beta$ -globin YAC (Fig. S5A). YAC-TgM were generated by pronuclear injection and intact, single-copy transgene carriers were identified (Fig. S5A,B). Parental YAC-TgM lines (Numbers 443 and 429) were crossed with Cre-TgM to initiate *in vivo* Cre-*loxP* recombination, which generated daughter lines carrying either the ICR4321S or ICR432

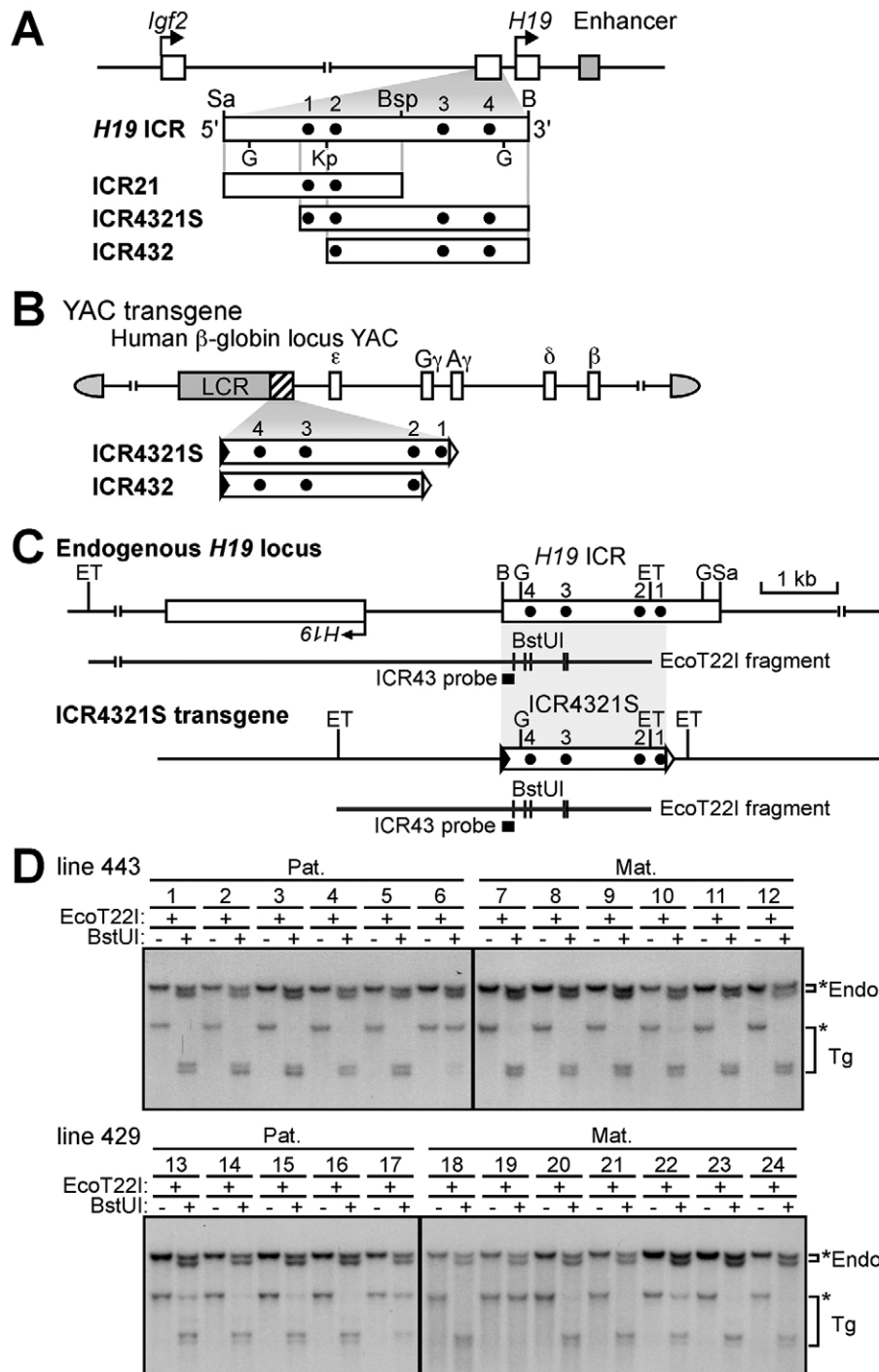


**Fig. 3. Function of DNMT3L in postfertilization methylation of the paternally inherited insulated *H19* ICR.** Blastocyst (lines 61 and 97) and 2-cell (line 61) stage embryos were obtained from wild-type (WT) or [Dnmt3L<sup>-/-</sup>] females crossed with male mice carrying mutant (insulated) *H19* ICR allele. DNA methylation status of region IV (shown in A, map legend as per Fig. 2A) of the paternally inherited mutant allele was analyzed by bisulfite sequencing, as described above, for blastocyst- (B) and 2-cell stage embryos (C). Three to 10 (blastocyst) or 13 to 30 (2-cell) embryos per agarose bead were used. Above each panel in B and C are genotypes of mothers.

transgene at the identical chromosomal integration site (Fig. 4B, Fig. S5C,D).

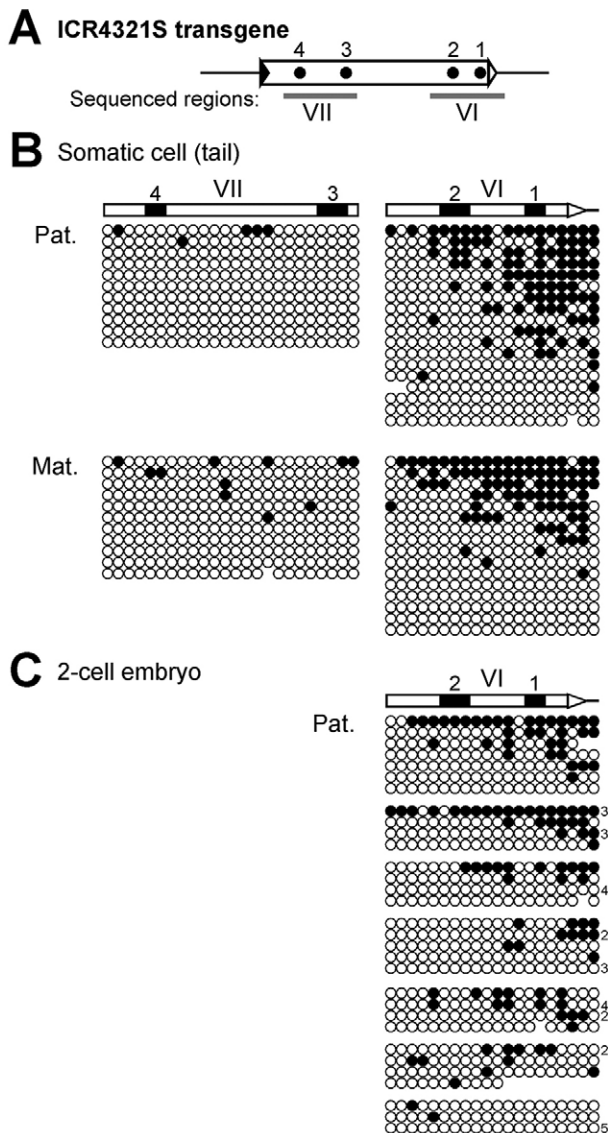
We examined the methylation status of the transgene fragments in somatic cells by Southern blotting (Fig. 4C). The ICR4321S fragment was hypomethylated regardless of its parental origin (Fig. 4D). The methylation status was also determined by bisulfite sequencing. Region VII, covering CTCF sites 3 and 4 (Fig. 5A), was hypomethylated regardless of parental origin (Fig. 5B). Although region VI, containing CTCF sites 1 and 2 (Fig. 5A), was partially methylated, its overall methylation level was low and equally observed on both alleles, implying that this methylation acquisition was allele-nonspecific (Fig. 5B). Because hypomethylation of the

paternally inherited ICR4321S fragment was also observed in two-cell embryos (Fig. 5C), it is suggested that the ICR4321S fragment lost its ability to acquire *de novo* DNA methylation in the early embryo. As was seen in the wild-type 2.9-kb *H19* ICR TgM (Fig. S1B,C) (Tanimoto et al., 2005), the ICR4321S fragment was not methylated in testis (Fig. S6E), indicating that the deleted sequence was not involved in methylation regulation of the *H19* ICR in male germ cells. Essentially the same phenotype, i.e. hypomethylation on both parental alleles in somatic cells (Fig. S6A-C) and in male germ cells (Fig. S6D), was observed in the shorter ICR432 fragment TgM. These results demonstrated that the 5'-region of the *H19* ICR is necessary for postfertilization allele-



**Fig. 4. Generation and DNA methylation analysis of the YAC-TgM carrying the 5'-truncated *H19* ICR fragments.** (A) Schematic representation of the *H19* ICR, ICR21 (Okamura et al., 2013b), ICR4321S, and ICR432 DNA fragments. Sa, *SacI*; B, *Bam*HI; Bsp, *Bsp*EI; G, *Bgl*II; Kp, *Kpn*I sites; dots 1-4, CTCF binding sites. (B) The ICR4321S and ICR432 fragments were inserted 3' to the LCR in the human  $\beta$ -globin YAC, and YAC-TgM were generated (see also Fig. S5). (C) Partial restriction enzyme maps of the endogenous *H19* locus and the  $\beta$ -globin YAC transgene with the inserted ICR4321S fragment. Methylation-sensitive *Bst*UI sites in the *Eco*T22I (ET) fragments are displayed as vertical lines beneath each map. The ICR43 probe used for Southern blotting in D is shown as a filled rectangle. (D) DNA methylation status of the ICR4321S fragment in somatic cells of the YAC-TgM (lines 443 and 429) that inherited the transgenes either paternally (Pat.) or maternally (Mat.). Tail DNA was digested with *Eco*T22I and then *Bst*UI, and the blot was hybridized with the ICR43 probe. Endo., endogenous locus; Tg, transgene. Asterisks indicate the positions of parental or methylated undigested fragments.





**Fig. 5. DNA methylation status of the ICR4321S transgene in tail somatic cells and preimplantation embryos.** (A) Map of the ICR4321S fragment. Two regions (VI and VII) analyzed by bisulfite sequencing in B and C are shown as gray bars beneath the map. (B) DNA methylation status of regions VI and VII in somatic cells of the ICR4321S YAC-TgM (line 443) that inherited the transgenes either paternally (Pat.) or maternally (Mat.). Tail DNA was digested with *Xba*I and treated with sodium bisulfite. Two regions were amplified by nested PCR, subcloned, and sequenced. (C) The DNA methylation status of region VI in 2-cell embryos that inherited the ICR4321S YAC transgene paternally (Pat.) (line 443) was analyzed by bisulfite sequencing as described previously. 16-38 embryos per bead were used.

specific methylation acquisition, which commences during early embryogenesis, in YAC-TgM.

#### The role of the 5'-region of the *H19* ICR in allele-specific methylation at the endogenous locus

To elucidate whether the 5'-end of the *H19* ICR was also essential for its allele-specific methylation at the endogenous locus, we removed a 765-bp sequence, which we deleted in the ICR4321S fragment, from the endogenous *H19* ICR (Fig. 6A, Fig. S7). ESCs were transformed with the targeting vector, in which the 765-bp sequence was flanked by *loxP* sequences (Fig. S7A). Properly targeted ESCs were identified by Southern blotting and used for

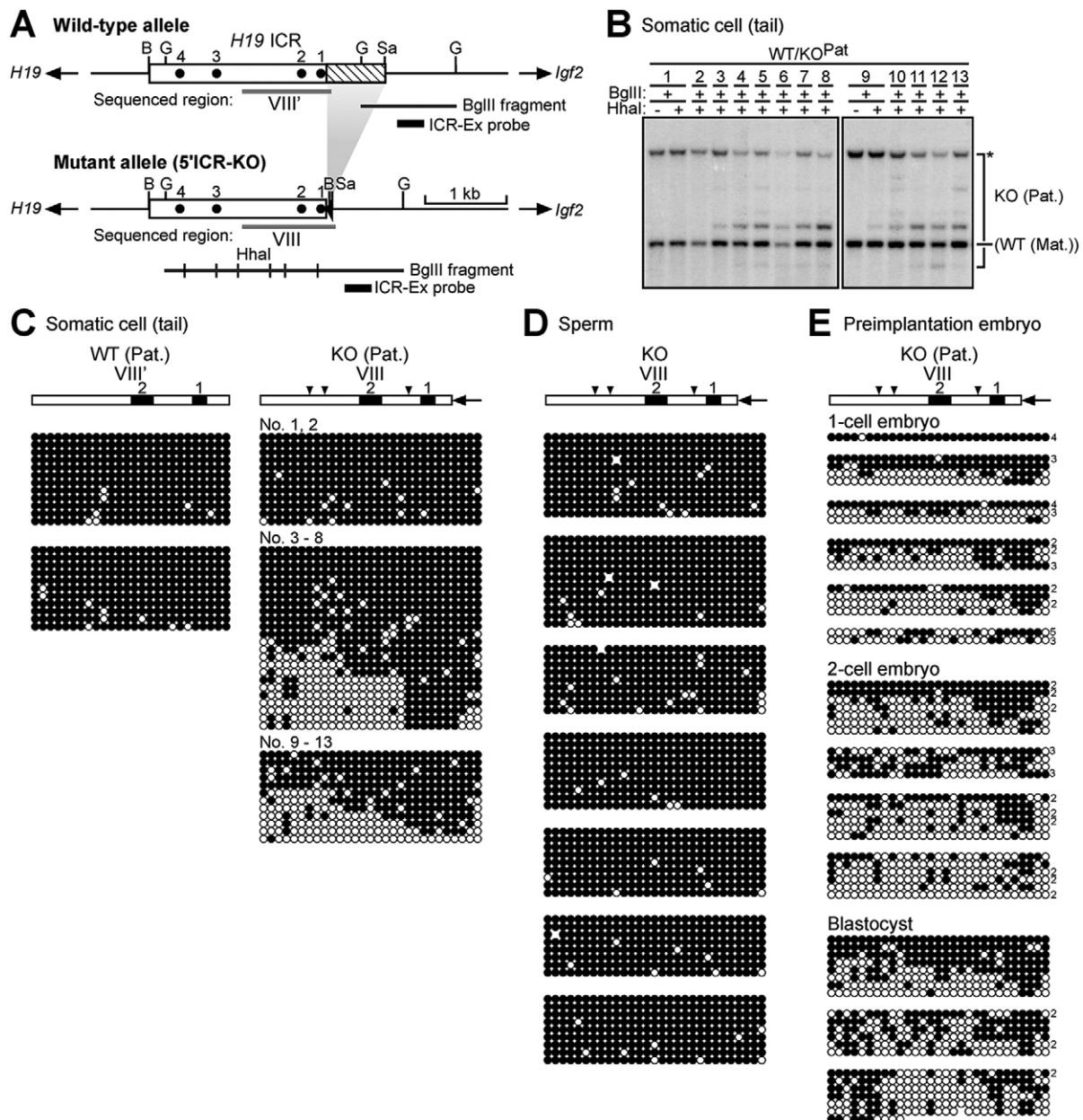
generating a knock-in mouse line (Fig. S7B). The 765-bp sequence and *neo<sup>r</sup>* selectable marker were simultaneously excised from the parental allele by crossing with Cre-expressing TgM, and the precise recombination events were monitored by Southern blotting (5'ICR-KO allele; Fig. S7C).

We first examined the methylation status of the paternally inherited 5'ICR-KO allele in somatic cells (tail tips) of the mutant mice by Southern blotting. The methylation levels of the remaining *H19* ICR sequences were partially reduced in more than half of the offspring (Fig. 6A,B). To further clarify the methylation status, we conducted bisulfite sequencing analysis of the DNA region covering CTCF sites 1 and 2. Based on the Southern blotting results (Fig. 6B), DNA samples from mutant mice carrying highly (numbers 1 and 2) and partially (numbers 3-8 and 9-13) methylated paternally inherited knock out (KO) alleles were individually pooled and subjected to analysis. Consistent with the Southern blotting data, some sequenced clones exhibited partial loss of methylation, particularly around the internal region of the residual *H19* ICR. By contrast, the wild-type paternal allele was fully methylated (Fig. 6C). To explore the reason behind the diminished methylation on the paternal KO allele, we conducted methylation analysis of sperm and preimplantation embryos (1-cell, 2-cell and blastocyst stages). Although the KO allele was fully methylated in sperm (Fig. 6D), the paternally inherited KO allele exhibited a partial loss of methylation even in 1-cell stage embryos (Fig. 6E). These results suggested that the 5'-region of the *H19* ICR was essential for its *de novo* DNA methylation at the endogenous, as well as at the transgene loci, in early embryos, and contributed to ensuring the maintenance of differential methylation during this developmental period.

The partial loss of methylation on the paternal KO allele was also observed in E12.5 embryos (Fig. 7A). Because the *H19* ICR methylation status regulates *Igf2* and *H19* transcription (Bell and Felsenfeld, 2000; Hark et al., 2000; Srivastava et al., 2000), we analyzed the expression of these genes. The expression levels of *Igf2* and *H19* were decreased and increased, respectively, in the livers of E12.5 embryos inheriting the KO allele paternally, when compared with littermate controls (Fig. 7B). Importantly, the degree of methylation loss at the *H19* ICR in each embryo correlated highly with the change in gene expression levels. While the product of *Igf2* promotes fetal growth (DeChiara et al., 1991), that of *H19* [non-coding RNA, carrying a micro RNA (miR-675) sequence] has been proposed to act as a growth repressor (Gabory et al., 2010). In accordance with the expected functions of these genes, the embryos (E12.5) carrying a paternally inherited KO allele tended to be smaller than control embryos from the same litter (Fig. S8). These results suggested that the 5'-region of the *H19* ICR played an essential role in normal *Igf2/H19* expression and fetal growth through the maintenance of paternal allele-specific methylation status after fertilization.

#### DISCUSSION

Using a genetic strategy, we found that maternally supplied DNMT3A and DNMT3L enzymes are essential for the *de novo* DNA methylation of the paternally inherited transgenic *H19* ICR in preimplantation embryos (Fig. 1E). Even in the presence of these enzymes (Fig. S2) (Guenatri et al., 2013; Hirasawa et al., 2008), the maternally inherited transgenic (Fig. 1C,D) and endogenous *H19* ICRs do not acquire methylation. Therefore, it was strongly suggested that an epigenetic mark other than DNA methylation is set within the transgenic *H19* ICR during gametogenesis, and after fertilization, the mark is used for discriminating the parental alleles



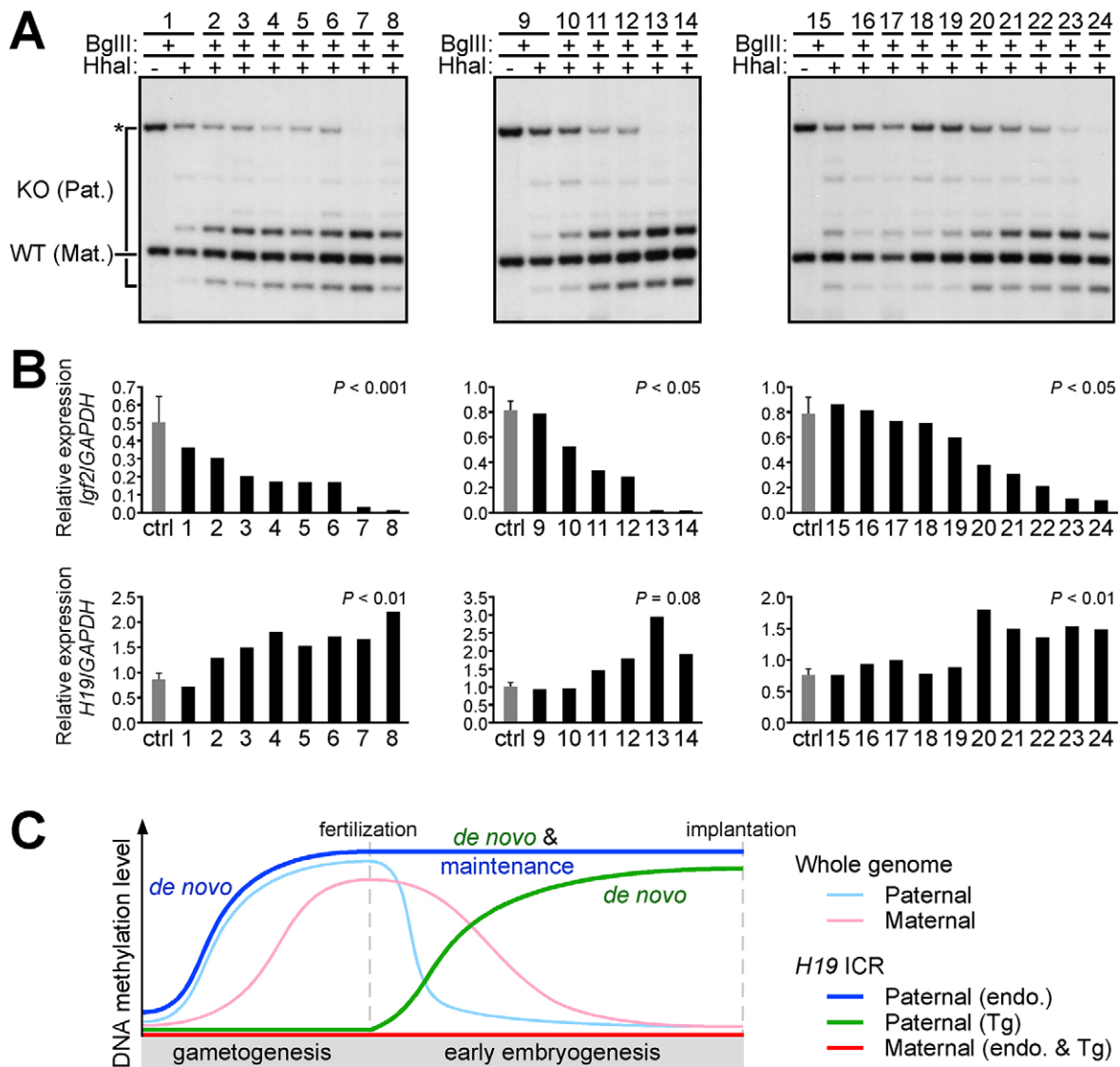
**Fig. 6. DNA methylation status of the 5'-deleted endogenous *H19* ICR.** (A) Map of the wild-type and mutant *H19* gene loci. The *H19* ICR 5'-region (765 bp, shaded area) was excised from the endogenous locus (mutant allele 5'ICR-KO; see also Fig. S7). Methylation-sensitive *HhaI* sites in the *BgIII* (G) fragment are displayed as vertical lines beneath the mutant allele map. The ICR-Ex probe used for Southern blotting in B is shown as a filled rectangle. The *BgIII* fragment detected in the wild-type allele does not have a *HhaI* site. Gray bars below each map (VIII and VIII') indicate regions analyzed by bisulfite sequencing in C, D and E. B, *Bam*HI; Sa, *Sac*I sites; dots 1-4, CTCF binding sites. (B) DNA methylation status of the paternally inherited mutant (5'ICR-KO) allele in tail somatic cells. Tail DNA of mutant mice (WT/KO<sup>pat</sup>) was digested with *BgIII* and then *HhaI*, and the blot was hybridized with the ICR-Ex probe. Asterisks indicate the positions of parental or methylated, undigested fragments. (C) DNA methylation status of the paternally inherited wild-type (region VIII') or 5'ICR-KO (region VIII) *H19* ICR in somatic cells. Tail DNA was pooled, digested with *Xba*I, and treated with sodium bisulfite. Each region was amplified by PCR, subcloned, and sequenced. The numbers above each panel in the 5'ICR-KO results are the ID numbers of pooled samples (in B). The position of the *HhaI* sites in region VIII are shown by arrowheads. (D) DNA methylation status of the 5'ICR-KO allele (region VIII) in sperm from 7 mutant males was analyzed by bisulfite sequencing. (E) DNA methylation status of the paternally inherited 5'ICR-KO allele (region VIII) in 1-cell, 2-cell, and blastocyst stage embryos was analyzed by bisulfite sequencing. 22-33 (1-cell stage), 23-47 (2-cell stage), and 4-12 (blastocyst stage) embryos per agarose bead were used.

by the DNMT complex. In support of this notion, it has been recently reported that several maternal ICRs (*PLAGL1*, *INPP5F\_v2*, and *PEG3*) in the macaque appeared to acquire allele-specific methylation only after fertilization (Cheong et al., 2015).

The paternally inherited hypomethylated transgenic *H19* ICR in two-cell embryos lacking the maternally supplied DNMT3L (Fig. 1E, Fig. S2A) was not capable of acquiring allele-specific

methylation even after the implantation period (Fig. 1G), during which DNMT3L was zygotically expressed (Fig. S2A) (Guenatri et al., 2013; Hu et al., 2008). These results imply that the paternally inherited transgenic *H19* ICR must be recognized by the maternally supplied DNMT complex immediately after fertilization so that it is methylated during early embryogenesis. It is possible that *trans*-acting factors recruiting the DNMT complex to the epigenetically





**Fig. 7. In vivo role of the 5'-region of the endogenous *H19* ICR.** (A) DNA methylation status of the paternally inherited mutant (5'ICR-KO) allele in the fetal liver. E12.5 embryos were obtained by mating wild-type females and hetero-KO males. The liver DNA of hetero-KO embryos (WT/KO<sup>pat</sup>) was extracted and analyzed by Southern blotting as described above. Each panel shows the results of embryos from a single litter. Samples are arranged in order of their *Igf2* gene expression levels determined in B. (B) Expression levels of *Igf2* and *H19* in fetal livers. Total RNA was extracted from the livers of hetero-KO embryos (WT/KO<sup>pat</sup>) and control (ctrl, WT/WT) littermates. The numbers correspond to those in A. The mRNA levels of *Igf2* (top) and *H19* (bottom) were measured by quantitative reverse transcription-PCR. Each value represents the ratio of *Igf2* or *H19* expression to that of *GAPDH*. For control embryos (sample numbers are 5, 8, and 5 in each litter: from left to right), the mean $\pm$ s.d. is shown. The differences in the means between control and KO embryos were tested using an unpaired *t*-test. (C) A model of postfertilization maintenance of methylation imprint at the *H19* ICR. During early embryogenesis, mammalian genomes undergo extensive epigenetic reprogramming, such as genome-wide DNA demethylation. Even in this environment, the allelic DNA methylation of ICRs is faithfully maintained at the imprinted loci, suggesting their states are selectively protected. Based on our observations, we propose that the postfertilization, *de novo* DNA methylation activity of the *H19* ICR contributes to maintain its imprint in early embryos.

marked paternal *H19* ICR might be present only during this short period after fertilization. Alternatively, the hypothetical allele-specific epigenetic mark on the *H19* ICR itself might be lost as embryonic development proceeds.

Possible candidates for the epigenetic signatures marked during spermatogenesis are histone modifications and/or association with specific histone variants. In sperm, 1% and 15% of the genomes are associated with histones rather than protamines in mice and humans, respectively (Miller et al., 2010), and importantly, imprinted loci including the *Igf2/H19* locus are among those regions (Hammoud et al., 2009). We reported that, in sperm, H3K9me2 is preferentially enriched at the paternally methylated endogenous ICRs (e.g. *H19*

and *Rasgrfl* ICRs) (Nakamura et al., 2012). Therefore, histone modification or histone variants within the transgenic *H19* ICR might be transmitted to zygotes to attract the DNMT complex to introduce DNA methylation.

Methylation acquisition at the endogenous *H19* ICR that is flanked by cHS4c sequences was partially obstructed in sperm (Fig. 2B, Fig. S4B). After fertilization, its methylation level was moderately recovered (Fig. 2C, Fig. 3C, Fig. S4C,E), and maternally provided DNMT3L played a significant role in this process (Fig. 3). Therefore, similar to the transgenic *H19* ICR, the endogenous *H19* ICR likely acquired an unknown epigenetic mark during spermatogenesis, and after fertilization, the *de novo*

DNA methylation machinery recognized the mark for allele discrimination. If a common epigenomic state can be identified in the DNA-methylated endogenous *H19* ICR and unmethylated transgenic *H19* ICR sequences in sperm, that would constitute a strong candidate for the primary mark that instructs imprinted DNA methylation in early embryos, because the mark cannot be secondary to DNA methylation.

Although some maternally methylated ICRs (*Snrpn* and *Peg3*) have been suggested to acquire allele-specific methylation after fertilization in mouse, these cases were not maternal DNMT3L dependent (Arnaud et al., 2006; Henckel et al., 2009). Therefore, the mechanistic basis of postfertilization methylation in the *H19* and other ICRs might be distinct. Additionally, as far as we know our data also provide the first example demonstrating a role for DNMT3L during early embryogenesis.

We have previously proposed that, in early embryos, Stella was involved in the maintenance of the hypermethylation status of the endogenous *H19* ICR, by inhibiting Tet3-mediated conversion of 5-methylcytosine to 5-hydroxymethylcytosine (Nakamura et al., 2012). We therefore tested whether the methylation status of the transgenic *H19* ICR, established *de novo* by DNMT3A and DNMT3L was also protected by this molecule. Maternal deletion of the *Stella* gene in oocytes caused a partial loss of methylation at the paternally inherited transgenic *H19* ICR in two-cell embryos (Fig. S1G). It was therefore suggested that Stella plays a shared role in the endogenous and transgenic *H19* ICR.

To demonstrate a role for postfertilization paternal allele-specific methylation activity at the endogenous locus, we narrowed its essential sequences in YAC-TgM (Figs 4 and 5) (Okamura et al., 2013b) and deleted them from the endogenous paternal *H19* ICR. Although gametic methylation of the mutant, endogenous *H19* ICR was not affected, its methylation level was diminished soon after fertilization (Fig. 6), demonstrating that the deleted region contains *cis* elements required for maintaining allele-specific methylation levels during early embryogenesis. A DNA-binding protein complex recognizing this region might interact with the *de novo* DNMT complex or with factors involved in the regulation of the DNA methylation status of the *H19* ICR. Another possibility is that an epigenetic mark to designate its paternal origin might be set in this region of the *H19* ICR during spermatogenesis. Importantly, this is the first example of regulatory sequences that ensure a stable methylation level of the paternal *H19* ICR during preimplantation periods, because all the *cis* regulatory elements identified to date (such as CTCF or Sox-Oct binding motifs) function to maintain the unmethylated state of maternal *H19* ICR following implantation (Matsuzaki et al., 2010; Sakaguchi et al., 2013; Schoenherr et al., 2003; Zimmerman et al., 2013).

Paternal inheritance of the 5'ICR-KO allele resulted in its partial loss of DNA methylation (Fig. 7A), decreased and increased expression of *Igf2* and *H19*, respectively (Fig. 7B), and reduced body weight of the mutant embryos (Fig. S8). Because we observed partial penetrance of these phenotypes, we repeated these experiments after backcrossing the mutant mice onto C57BL/6 for five generations after which essentially the same results were obtained (data not shown). Therefore, any phenotypic variability among embryos is not attributable to genetic background. Some part of maintenance and/or *de novo* methylation machinery in early embryos might have a stochastic nature and contribute to generation of such variations.

In summary, we delineated the postfertilization allele-specific *de novo* DNA methylation activity in the transgenic *H19* ICR and identified its 5'-region as an essential element for this activity. Deletion of this element from the endogenous mouse *H19* ICR

resulted in a loss of methylation on the paternal allele, dysregulation of both *Igf2* and *H19* expression, and growth retardation. We propose that the activity we uncovered plays a role in maintaining allele-specific methylation at the endogenous *H19* ICR in preimplantation embryos, and that the *de novo* methylation imprinting activity at the *H19* ICR and genome-wide demethylation activity are in a state of dynamic equilibrium during this period (Fig. 7C).

## MATERIALS AND METHODS

### Mice

Transgenic mice carrying a human  $\beta$ -globin YAC, in which the 2.9-kb *H19* ICR fragment was inserted between the LCR and the  $\epsilon$ -globin gene were described previously (Tanimoto et al., 2005). *Dnmt3l* KO mice were kindly provided by Dr En Li (Hata et al., 2002). *Dnmt3a-2lox* (RBRC03731) (Kaneda et al., 2004) and *Dnmt3b-2lox* (RBRC03733) (Dodge et al., 2005) mice were generous gifts from Dr Masaki Okano and provided by RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. To conditionally disrupt the floxed alleles in oocytes, these mice were crossed with TgM carrying *Zp3-Cre* gene (Jackson Laboratory; de Vries et al., 2000). Insulated *H19* ICR knock-in mice, ICR4321S TgM, ICR432 TgM, and 5'ICR KO mice were generated as described in the supplementary materials and methods.

Animal experiments were performed in a humane manner and approved by the Institutional Animal Experiment Committee of the University of Tsukuba. Experiments were conducted in accordance with the Regulation of Animal Experiments of the University of Tsukuba and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the MEXT.

### Preparation of oocytes and embryos

Female mice were super-ovulated via injection of pregnant mare serum gonadotropin, followed by human chorionic gonadotropin (hCG) (47–48 h interval). Unfertilized oocytes were collected from oviducts 18 h after hCG injection, and cumulus cells were removed by hyaluronidase treatment. Fertilized one-cell zygotes were collected from mated females 24 h after hCG injection, and cumulus cells were removed. Two- and eight-cell embryos were flushed from oviducts at 44 and 68 h, respectively, after hCG injection. Embryos at E3.5 (blastocysts), E8.5, and E12.5 were obtained by natural mating.

### DNA methylation analysis by bisulfite sequencing

Preimplantation embryos were embedded in agarose beads and treated with sodium bisulfite as described previously (Matsuzaki et al., 2009). Genomic DNA extracted from postimplantation embryos (E8.5), adult male sperm, or the tail tips of ~1-week-old animals was treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research). Sperm and tail tip DNA was digested with *XbaI* prior to the treatment. Subregions of the *H19* ICR in transgenic and knock-in/KO mice were amplified by nested and single-round PCR, respectively, and the fragments were subcloned into the pGEM-T Easy vector (Promega) for sequencing analyses. PCR primers are listed in Tables S1, S2. Nested PCR was employed to distinguish between the endogenous and transgenic *H19* ICR sequences.

### DNA methylation analysis by Southern blotting

Genomic DNA from ICR4321S TgM and 5'ICR-KO mice were first digested by *EcoT22I* and *BglII*, respectively, to liberate the *H19* ICR region and subjected to the methylation-sensitive enzymes *BstUI* and *HhaI*, respectively. Following size separation in agarose gels, Southern blots were hybridized with  $\alpha$ -<sup>32</sup>P-labeled probes and subjected to X-ray film autoradiography.

### RT-qPCR

Total RNA of the liver at E12.5 was extracted by ISOGEN (Nippon Gene) and converted to cDNA using ReverTra Ace qPCR RT Master Mix with

gDNA Remover (TOYOBO). Quantitative amplification of cDNA was performed with the Thermal Cycler Dice (TaKaRa Bio) using SYBR Premix EX TaqII (TaKaRa Bio) and PCR primers listed in Table S3.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

H.M., E.O. and K.T. designed the experiments. H.M., E.O., T.T., A.U. and K.T. performed the experiments. H.M., E.O., T.T. and K.T. analyzed the data. T. Nakamura, T. Nakano, K.H. and A.F. provided scientific advice and materials. H.M., E.O. and K.T. wrote the manuscript.

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#### Supplementary information

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