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Zfp57 mutant ES cell lines directly derived from blastocysts

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Abstract

Zfp57 is a master regulator of genomic imprinting in mouse embryos. To further test its functions, we have derived multiple *Zfp57* mutant ES clones directly from mouse blastocysts. Indeed, we found DNA methylation imprint was lost at most examined imprinting control regions in these *Zfp57* mutant ES clones, similar to what was observed in *Zfp57* mutant embryos in the previous studies. This result indicates that these blastocyst-derived *Zfp57* mutant ES clones can be employed for functional analyses of *Zfp57* in genomic imprinting.

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Resource Table:

Name of Stem Cell construct	Not Applicable
Institution	Icahn School of Medicine at Mount Sinai
Person who created resource	Ho-Tak Lau, Xiajun Li
Contact person and email	Xiajun Li, xiajun.li@mssm.edu
Date archived/stock date	November, 2010
Origin	Mouse blastocysts
Type of resource	Biological reagent: Mouse Embryonic Stem (ES) Cell Lines
Sub-type	*Zfp57* mutant ES cell lines
Key transcription factors	*Zfp57*
Authentication	Undifferentiated ES cell morphology confirmed (Figure 1)
Link to related literature (direct URL links and full references)	http://www.ncbi.nlm.nih.gov/pubmed/18854139
Information in public databases	None

Blastocyst-derived ES clones display typical properties of ES cells

Zfp57 is a maternal-zygotic effect gene and it has both maternal (M) and zygotic (Z) functions (Li et al., 2008; Shamis et al., 2015). Eight *Zfp57*$^{+/-}$ (M^+Z^+) and three *Zfp57*$^{-/-}$ (M^+Z^-) ES clones were derived from the blastocysts generated from the cross between *Zfp57*$^{+/-}$ heterozygous female mice and *Zfp57*$^{-/-}$ homozygous male mice, whereas five *Zfp57*$^{-/-}$ (M^-Z^-) ES clones were derived from the cross between *Zfp57*$^{-/-}$ homozygous female mice and *Zfp57*$^{-/-}$ homozygous male mice. The genotypes for these ES clones were confirmed by PCR-based genotyping (see Fig. 3A below). These ES clones displayed typical ES cell morphology as exemplified by one ES clone for each genotype shown in Fig. 1A, suggesting that undifferentiated ES clones can be properly established without maternal (M^-) or zygotic (Z^-) *Zfp57*. They formed embryoid bodies (EBs) when grown in suspension (Fig. 1A). Based on semi-quantitative RT-PCR analysis, expression of the markers for endoderm (*Foxa2*), mesoderm (*Mlc2a*) and ectoderm (*Ck18*) seems to be increased in the EB samples compared with the ES cell samples in three tested blastocyst-derived ES clones (Fig. 1B). We also counted the metaphase chromosome numbers in five ES clones (Table 1), as exemplified by one metaphase chromosome spread (Fig. 1C). All five ES clones appeared to have relatively normal chromosome numbers, with over 60% or more euploid cells with 40 chromosomes in these ES clones (Table 1). Based on immunostaining, these five ES clones express OCT4 and NANOG, two pluripotency markers (Fig. 2).

Maintenance of maternally inherited DNA methylation imprint

We also analyzed DNA methylation imprint inherited on the maternal chromosomes at four imprinted regions (*Peg1*, *Peg3*, *Snrpn* and *Igf2r*) in these ES clones. Similar to what had been observed in *Zfp57*$^{+/-}$ (M^+Z^+) and *Zfp57*$^{-/-}$ (M^-Z^-) mouse embryos in our previous study (Li et al., 2008), DNA methylation imprint was lost at these four imprinted regions in five *Zfp57*$^{-/-}$ (M^-Z^-) ES clones (M4-M8 of Fig. 3B) in comparison with eight *Zfp57*$^{+/-}$ (M^+Z^+) ES clones (H1-H8 of Fig. 3B). DNA methylation imprint was also lost at these four imprinted regions in three *Zfp57*$^{-/-}$ (M^+Z^-) ES clones (M1-M3 of Fig. 3B), whereas partial loss of DNA methylation was observed at these four imprinted regions in *Zfp57*$^{-/-}$ (M^+Z^-) zygotic mutant embryos lacking just zygotic *Zfp57*. Again these results suggest that maternal *Zfp57* appears to have more lasting effect on the maintenance of DNA methylation imprint inherited on maternal chromosomes in mouse embryos than in blastocyst-derived ES cells.

Maintenance of paternally inherited DNA methylation imprint

We examined paternally inherited DNA methylation imprint at three imprinted regions (*Dlk1-Dio3*, *H19* and *Rasgrf1*) in these ES clones by COBRA. Similar to what was found in *Zfp57*$^{+/-}$ (M^+Z^+) and *Zfp57*$^{-/-}$ (M^-Z^-) mouse embryos in our previous study (Li et al., 2008), DNA methylation imprint was lost at the *Dlk1-Dio3* imprinted region but not at the *H19* imprinted region in five *Zfp57*$^{-/-}$ (M^-Z^-) ES clones (M4-M8 of Fig. 4A) in comparison to eight *Zfp57*$^{+/-}$ (M^+Z^+) ES clones (H1-H8 of Fig. 4A). Unlike partial loss of DNA methylation imprint observed in *Zfp57*$^{-/-}$ (M^+Z^-) zygotic mutant embryos lacking just zygotic *Zfp57* in our previous study (Li et al., 2008), DNA methylation imprint was almost

completely lost at the *Dlk1-Dio3* imprinted region but not at the *H19* imprinted region in three *Zfp57*$^{-/-}$ (M^+Z^-) ES clones (M1-M3 of Fig. 4A), indicating that maternal *Zfp57* has more lasting effect on the maintenance of DNA methylation imprint inherited on paternal chromosomes in mouse embryos than in blastocyst-derived ES cells. We also found that DNA methylation imprint was similarly lost at the *Rasgrf1* imprinted region in three *Zfp57*$^{-/-}$ (M^+Z^-) and five *Zfp57*$^{-/-}$ (M^-Z^-) ES clones in comparison with eight *Zfp57*$^{+/-}$ (M^+Z^+) ES clones (Fig. 4A). This is the first experimental evidence demonstrating that DNA methylation imprint at the *Rasgrf1* imprinted region is lost without *Zfp57*.

DNA methylation at the *IAP* repeats

Previously, we did not observe any loss of DNA methylation at the *IAP* repeat regions in mouse embryos lacking *Zfp57* (Li et al., 2008). Similarly, *IAP* repeat regions were highly methylated in eight *Zfp57*$^{+/-}$ (M^+Z^+) (H1-H8), three *Zfp57*$^{-/-}$ (M^+Z^-) (M1-M3) and five *Zfp57*$^{-/-}$ (M^-Z^-) (M4-M8) ES clones (Fig. 4B). Thus, *Zfp57* is not required for the maintenance of DNA methylation at the *IAP* repeats in blastocyst-derived ES clones.

Materials and Methods

Zfp57 mutant mouse strain

Zfp57 mutant mouse was generated in our previous study (Li et al., 2008). The mouse strain carrying the *Zfp57* mutant allele was maintained in the animal facility of Icahn School of Medicine at Mount Sinai. The animal work was performed in full compliance with the animal care protocol approved by IACUC of the institution.

Derivation of the ES clones from blastocysts

Mouse blastocytsts were isolated from the timed pregnancy mating between *Zfp57*$^{+/-}$ heterozygous female mice and *Zfp57*$^{-/-}$ homozygous male mice or between *Zfp57*$^{-/-}$ homozygous female mice and *Zfp57*$^{-/-}$ homozygous male mice. These blastocysts were used for derivation of the ES clones following the protocol described in this paper (Meissner et al., 2009). Basically, isolated blastocysts were plated on irradiated MEF cells (feeder cells) until undifferentiated ES cell colonies formed on the plate. They were picked individually to a well of 24-well plate to establish the stable ES cell lines. Then the established ES cell lines were expanded in 6-well plates seeded with feeder cells.

ES cell culture

ES cells were cultured in the presence of feeder cells in the ES cell growth medium made of the high-glucose DMEM medium supplemented with 15% fetal bovine serum (FBS). They were passaged once every 3–4 days before the ES cells became too confluent. The ES cell medium was changed daily.

Generation of embryoid bodies (EBs)

Roughly 1–2 million of ES cells grown on a well of 6-well plate with irradiated SNL feeder cells were separated by trypsin digestion before being added to a non-adherent 10-cm petri dish plates pre-treated with poly-hema (Sigma). ES cell growth medium without LIF was

used for the EB culture. The medium was changed once every 2–3 days without removing the aggregated EBs. The floating EBs were harvested for total RNA sample preparation after growing in suspension for 7–8 days. Usually 2 ml of Trizol reagent (Invitrogen) was added to the EBs collected from a 10- cm dish plate after removing the medium. Total RNA samples were prepared according to the manufacturer's manual.

Chromosome number counting of ES clones

Metaphase chromosome spread was prepared for five ES clones. Karyomax (Invitrogen Cat# 15210-040) was added to the ES cells grown in a well of 6-well plates with a final concentration of Colcemid at 1 μg/ml. After incubation for one hour with Karyomax, the ES cells were harvested by trypsin digestion followed by centrifugation. The cell pellets were gently mixed with 5 ml of ice-cold 0.56% KCl solution in sterile distilled water. After incubation for six minutes at room temperature, the ES samples were precipitated by centrifugation and the pellets were mixed with the fixative solution of acetic acid and methanol (1:3). The pellets were resuspended in this fixative solution before being spotted onto the slides for examination under microscope. After photography, the chromosome numbers of about 20 good spreads were counted for each of these five ES clones.

RT-PCR expression analysis of the marker genes for three germ layers

Total RNA samples were prepared from three ES clones and their EBs after growing in suspension culture for 7–8 days. Approximately equal amount of total RNA samples were used for reverse transcription (RT) with Transcriptor First Strand cDNA Synthesis Kit (Roche). RT reaction was initiated with the anchored-oligo$(dT)_{18}$ primer included in the kit, with a negative control without reverse transcriptase (-RT) for one total RNA sample. 1 ul of RT product was used for PCR amplification. The amplification of 25 PCR cycles was applied to *Ck18* and *Rps17* encoding the housekeeping ribosomal protein S17, whereas *Foxa2* and *Mlc2a* were subjected to PCR amplification of 35 cycles. PCR product was separated on agarose gels before photography.

Immunostaining of ES clones

The ES clones grown on a 24-well plate seeded with irradiated SNL feeder cells were directly subjected to immunostaining with antibodies from Santa Cruz Biotechnology against OCT4 (sc-5279) or Nanog (sc-376915). The nuclei were stained with DAPI.

Bisulphite mutagenesis

The genomic DNA samples isolated from these ES clones and the control wild-type mouse tail were subjected to bisulphite treatment with the EZ DNA Methylation-Gold™ Kit (Zymo Research). The bisulphite-treated DNA product was used for COBRA analysis of the imprinting control regions (ICR) and the non-imprinted *IAP* repeats.

Combined Bisulphite Restriction Analysis (COBRA)

COBRA was used for analyzing DNA methylation imprint at the ICRs and *IAP* repeats in this study. After bisulphite mutagenesis, the purified mutagenized genomic DNA was subjected to PCR amplification with the primers covering a portion of the imprinting control

region (ICR) for the imprinted regions or a portion of the non-imprinted *IAP* repeat regions (Zuo et al., 2012). The resultant PCR product was subjected to restriction enzyme digestion targeting the CpG sites located in the amplified ICR or *IAP* repeat regions. The restriction enzyme digested PCR product was separated by gel electrophoresis.

Acknowledgments

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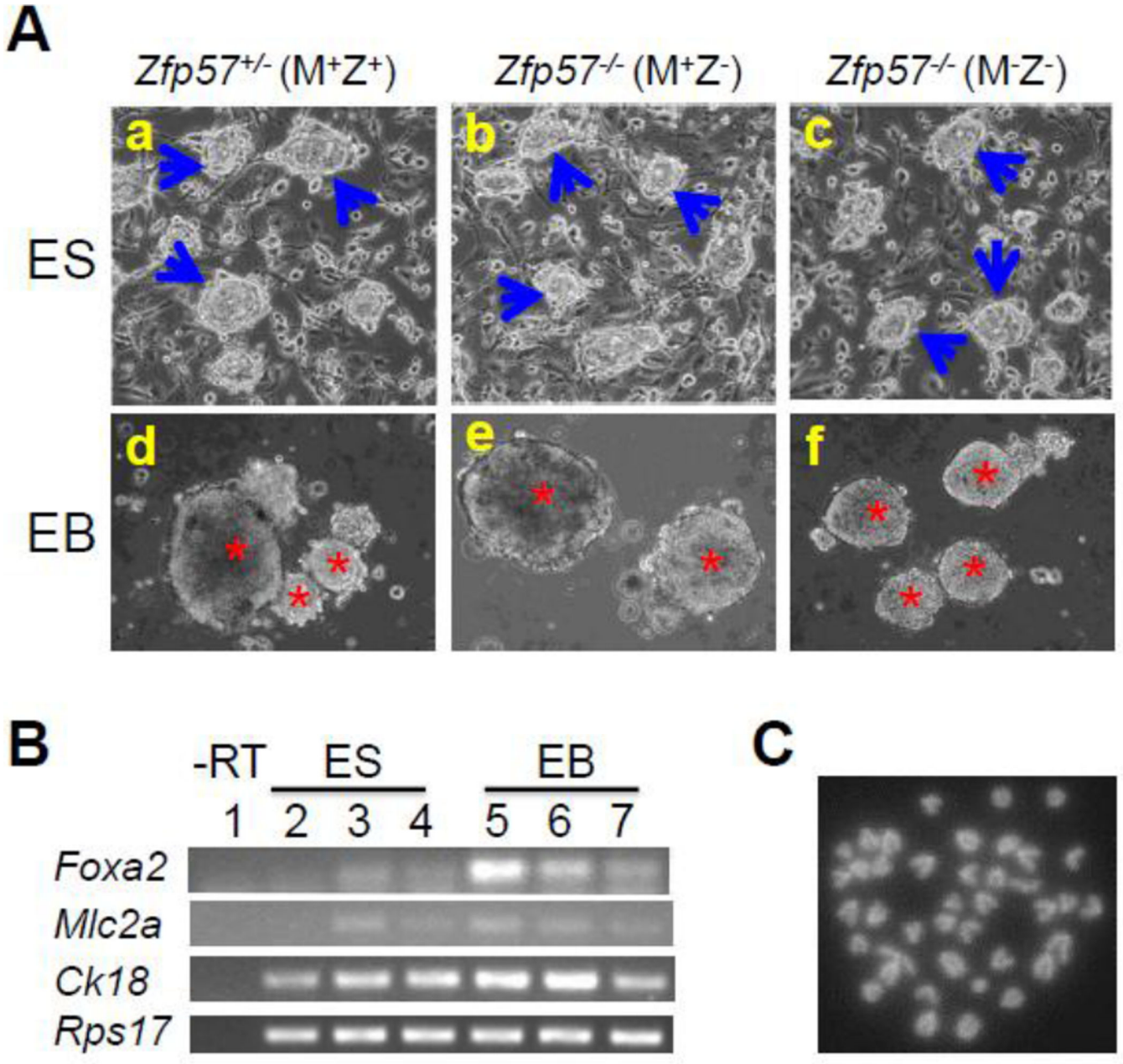

Figure 1. Blastocyst-derived ES clones display normal characters of ES cells

A, ES clones on feeder cells (a-c) or EBs on non-adherent petri dish plates (d-f). One *Zfp57*$^{+/-}$ (M^+Z^+) ES clone (a, d) and one *Zfp57*$^{-/-}$ (M^+Z^-) ES clone (b, e) were shown here as the examples for the ES clones derived from the blastocysts generated from the cross between *Zfp57*$^{+/-}$ heterozygous female mice and *Zfp57*$^{-/-}$ homozygous male mice, whereas one *Zfp57*$^{-/-}$ (M^-Z^-) ES clone (c, f) shown here was an example for the ES clones derived from the cross between *Zfp57*$^{-/-}$ homozygous female mice and *Zfp57*$^{-/-}$ homozygous male mice. Blue arrows in a-c, undifferentiated ES cell colonies grown on feeder cells. Red asterisks in d-f, embryoid bodies (EBs) in suspension formed by ES cells plated on non-adherent petri dish plates. B, RT-PCR expression analysis of the marker genes in three ES

clones and the EBs derived from these three ES clones after growing in suspension culture for 7–8 days. Lane 1, negative control without reverse transcription (-RT) of the same total RNA sample in Lane 4. Lane 2, ES cells of one *Zfp57*$^{-/-}$ (M^-Z^-) ES clone. Lanes 3–4, the ES cells of two *Zfp57*$^{+/-}$ (M^+Z^+) ES clones. Lane 5, EBs of the *Zfp57*$^{-/-}$ (M^-Z^-) ES clone. Lanes 6–7, EBs of two *Zfp57*$^{+/-}$ (M^+Z^+) ES clones. *Foxa2, Mlc2a* and *Ck18* are markers for endoderm, mesoderm and ectoderm, respectively. *Rps17* is a house-keeping gene that was used as the loading control. C, DAPI-stained metaphase chromosome spread derived from a cell of one *Zfp57*$^{-/-}$ (M^-Z^-) ES clone. An example is provided here for the metaphase chromosome spread of five ES clones.

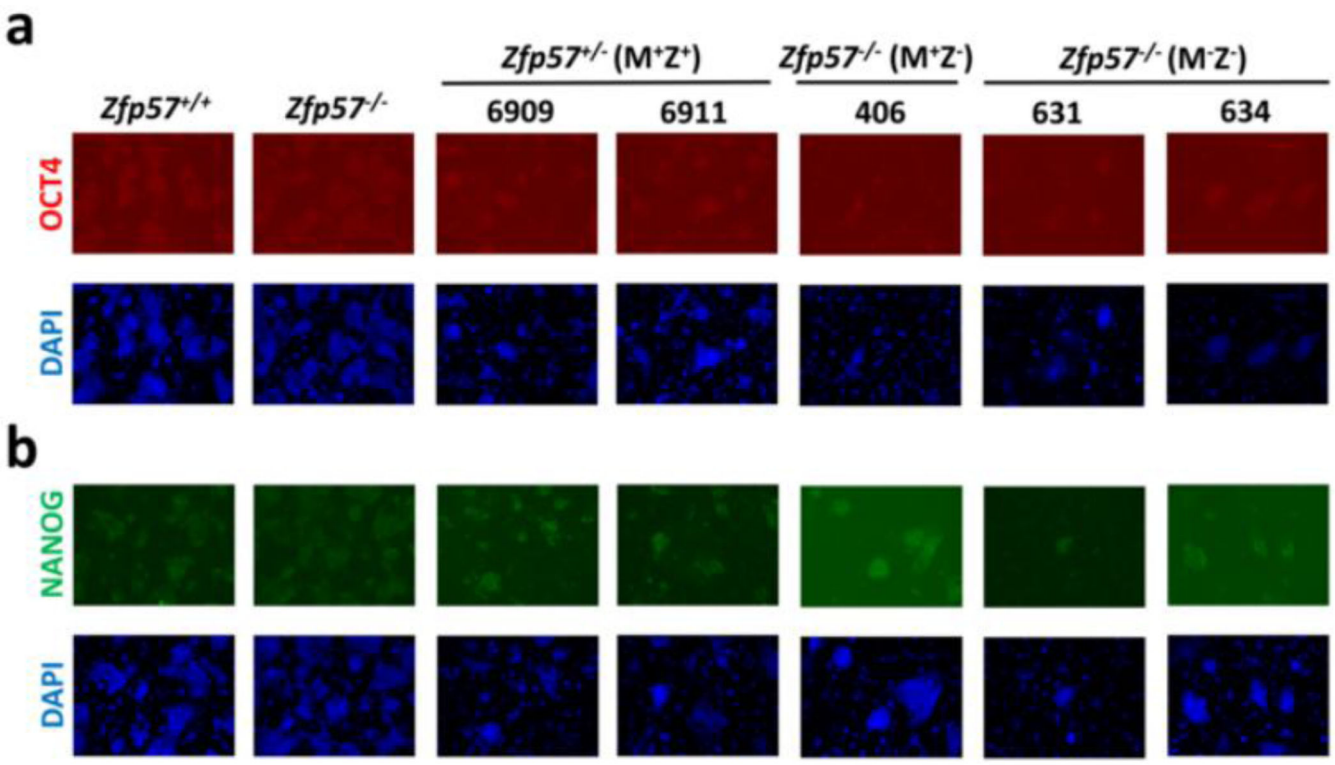

Figure 2. Blastocyst-derived ES clones express pluripotent markers OCT4 and NANOG

Immunostaining was performed to analyze the expression of OCT4 (a) and NANOG (b) in five blastocyst-derived ES clones. Two leftmost columns, control wild-type TC1 (*Zfp57*$^{+/+}$) and a *Zfp57*$^{-/-}$ mutant ES clone generated by homologous recombination in vitro (Li et al., 2008; Zuo et al., 2012). 6909, 6911, 406, 631 and 634 are five blastocyst-derived ES clones generated in this study. Blue signal, DAPI staining. Red signal, OCT4 immunostaining. Green signal, Nanog immunostaining.

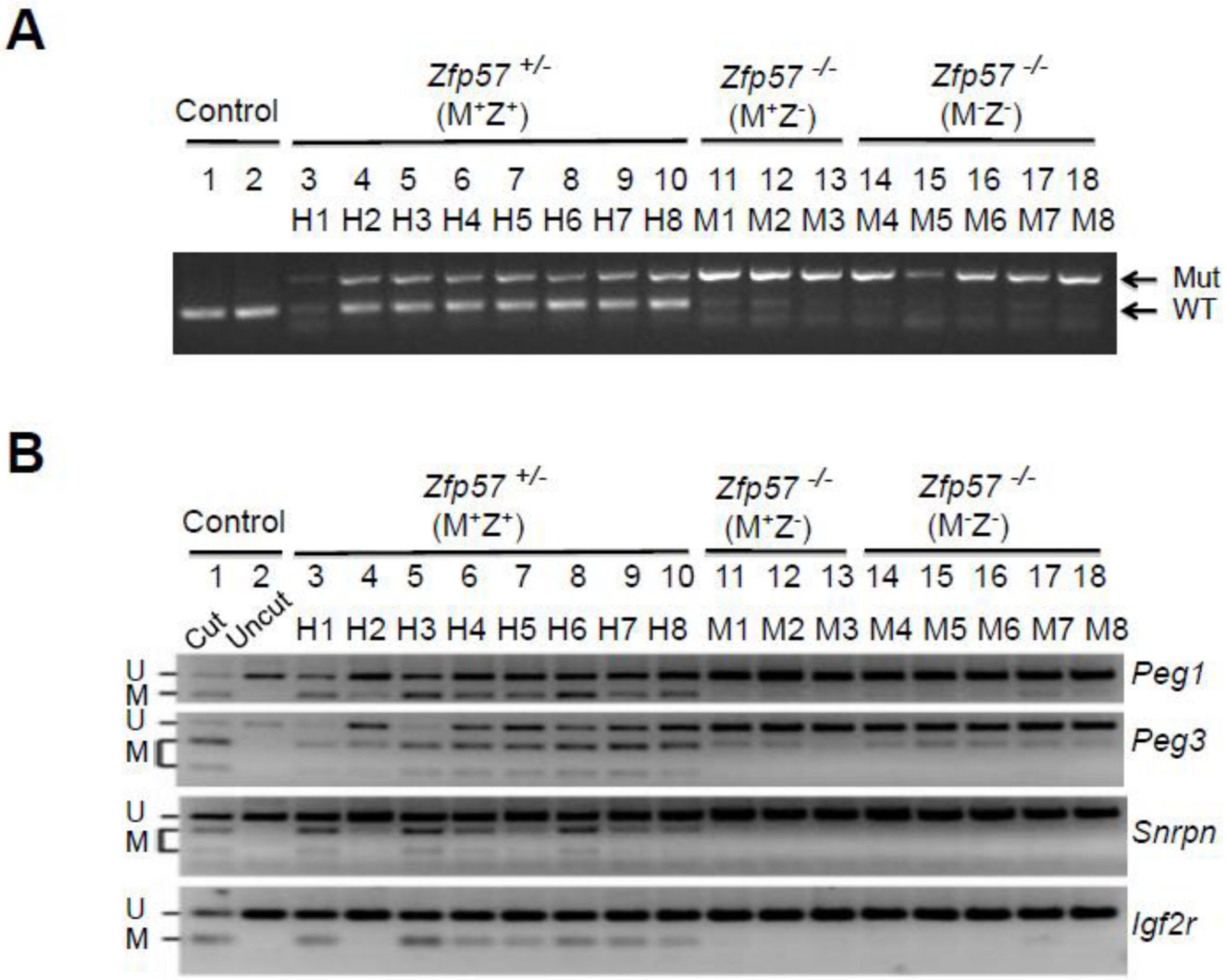

Figure 3. COBRA analysis of maternally inherited DNA methylation imprint

A, PCR genotyping of these ES clones, similar to our previously published study (Li et al., 2008). Mut and WT, the marked gel positions for the PCR product of the mutant (Mut) and wild-type (WT) alleles of *Zfp57*. B, COBRA analysis was carried out for analyzing DNA methylation imprint at the maternally inherited *Peg1*, *Peg3*, *Snrpn* and *Igf2r* imprinted regions. Restriction enzyme (RE) digestion was performed for the bisulphite PCR product of all ES samples, and "Cut" (Lane 1) but not "Uncut" (Lane 2) control wild-type mouse tail DNA sample. U and M, unmethylated (U) and methylated (M) product after RE digestion, respectively. Lanes 3–10, eight $Zfp57^{+/-}$ (M^+Z^+) ES clones derived from the blastocysts generated from the cross between $Zfp57^{+/-}$ heterozygous female mice and $Zfp57^{-/-}$ homozygous male mice. Lanes 11–13, three $Zfp57^{-/-}$ (M^+Z^-) ES clones derived from the blastocysts generated from the cross between $Zfp57^{+/-}$ heterozygous female mice and $Zfp57^{-/-}$ homozygous male mice. Lanes 14–18, five $Zfp57^{-/-}$ (M^-Z^-) ES clones derived from the cross between $Zfp57^{-/-}$ homozygous female mice and $Zfp57^{-/-}$ homozygous male mice.

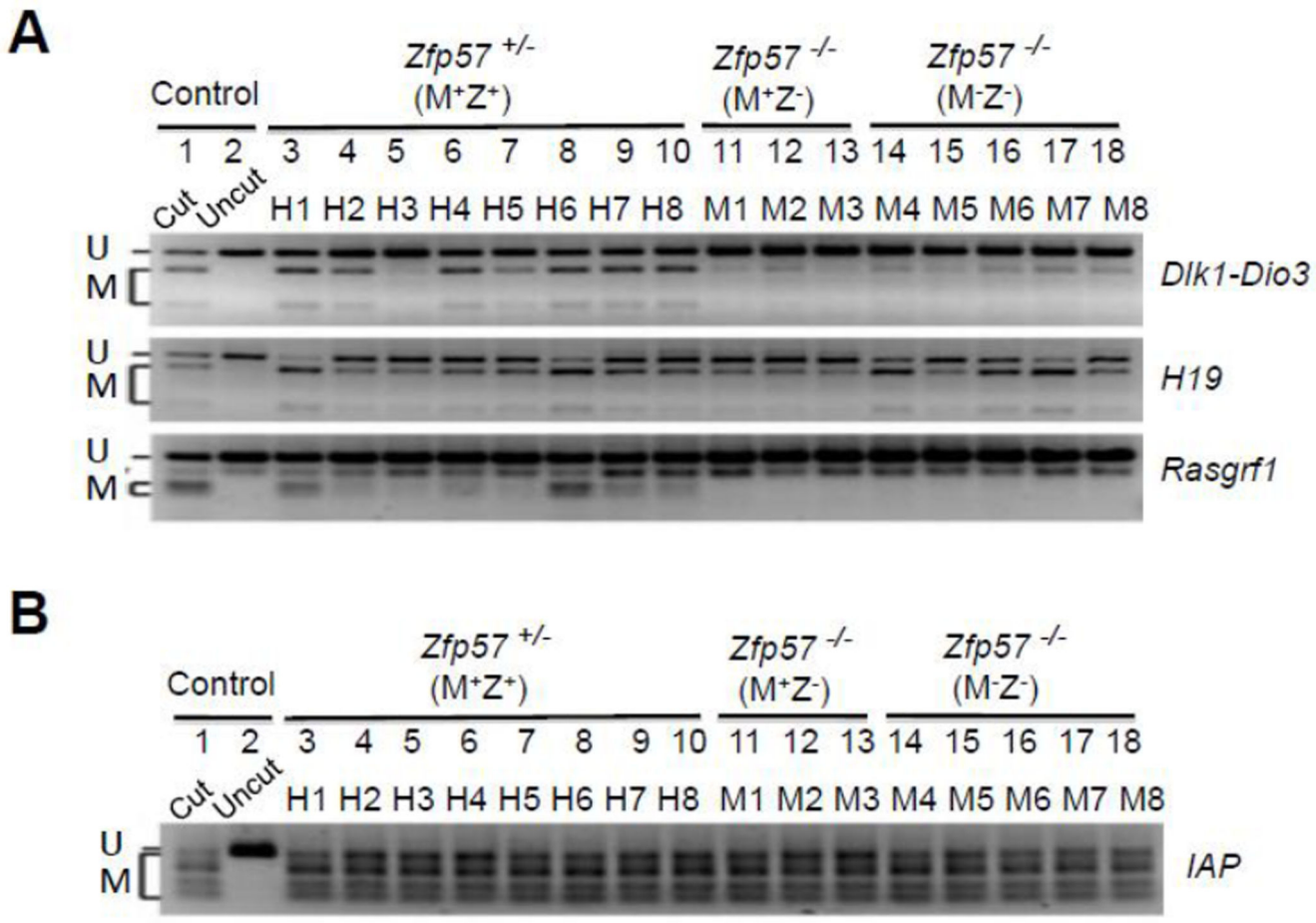

Figure 4. COBRA analysis of paternally inherited DNA methylation imprint

Restriction enzyme (RE) digestion was performed for the bisulphite PCR product of all ES samples, and "Cut" (Lane 1) but not "Uncut" (Lane 2) control wild-type mouse tail DNA sample. U and M, unmethylated (U) and methylated (M) product after RE digestion, respectively. Lanes 3–10, eight *Zfp57*$^{+/-}$ (M^+Z^+) ES clones derived from the blastocysts generated from the cross between *Zfp57*$^{+/-}$ heterozygous female mice and *Zfp57*$^{-/-}$ homozygous male mice. Lanes 11–13, three *Zfp57*$^{-/-}$ (M^+Z^-) ES clones derived from the blastocysts generated from the cross between *Zfp57*$^{+/-}$ heterozygous female mice and *Zfp57*$^{-/-}$ homozygous male mice. Lanes 14–18, five *Zfp57*$^{-/-}$ (M^-Z^-) ES clones derived from the cross between *Zfp57*$^{-/-}$ homozygous female mice and *Zfp57*$^{-/-}$ homozygous male mice.

A, COBRA analysis was carried out at the IG-DMR of the *Dlk1-Dio3* imprinted region, *H19* DMR of the *Igf2-H19* imprinted region and *Rasgrf1* DMR.

B, COBRA analysis of the *IAP* repeats.

Table 1

Counting of metaphase chromosome spreads of five ES clones

ES clone	6909	6911	406	631	634
Genotype	*Zfp57*$^{+/-}$ (M^+Z^+)	*Zfp57*$^{+/-}$ (M^+Z^+)	*Zfp57*$^{-/-}$ (M^+Z^-)	*Zfp57*$^{-/-}$ (M^-Z^-)	*Zfp57*$^{-/-}$ (M^-Z^-)
# of counted metaphase spreads	16	20	20	20	20
# of spreads with 40 chromosomes	10	12	14	14	13
% of euploid cells	62.5%	60%	70%	70%	65%

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