Developmental Cell *15*

Supplemental Data

A Maternal-Zygotic Effect Gene, *Zfp57*,

Maintains Both Maternal and Paternal Imprints

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SUPPLEMENTAL RESULTS

Oocyte-Specific Ablation Is Sufficient to Cause Loss of the Maternal Functions of *Zfp57*

The presence of the maternal function based on the genetic data is consistent with the observed expression of *Zfp57* specifically in the maturing oocytes within the follicles of the ovary (Figure 1E). The oocyte-specific nature of the maternal contribution to the maternal-zygotic embryonic lethality was further confirmed by employing a ZP3-Cre transgene to ablate the maternal function of *Zfp57* specifically in the growing oocytes within the female ovary (de Vries et al., 2000). From the cross between homozygous males and the females containing a ZP3-Cre transgene and two floxed alleles at the *Zfp57* locus, almost half of the embryos were dead by E15.5 and 90% were dead by E17.5 (Supplemental Table S2). In contrast, from the cross between the homozygous males and the females carrying two floxed alleles at the *Zfp57* locus, only 2 out of 45 embryos dissected between E17.5 and E19 were dead and all 25 P0 pups were alive at birth and survived to adulthood. We did not observe much lethality from the cross between the homozygous males and the females carrying a ZP3-Cre transgene and one floxed allele at the *Zfp57* locus. Compared with the midgestation embryonic lethality observed in the cross between homozygous males and homozygous females, the slight delay in embryonic death in this cross involving a ZP3-cre transgene and two floxed alleles may be due to the residual maternal product of *Zfp57* since the ZP3-cre transgene is not expressed until primordial oocytes become activated (de Vries et al., 2000), i.e. about the same period when the transcription of *Zfp57* is turned on in the ovary (Figure 1E).

Dlk1 Transcripts Were Variably Downregulated in *Zfp57* Mutant Embryos

Loss of *Zfp57* resulted in loss of differential methylation at the IG-DMR of the *Dlk1-Gtl2* imprinted region (Figures 3A, S4A and S6A). Since IG-DMR is the controlling element for all the imprinted genes in this region, expression of *Dlk1* and *Gtl2* were both affected in the *Zfp57* mutant embryos (Figure 2). Similar to the methylation studies, we found that *Dlk1* transcripts were almost completely missing in the maternal-zygotic mutant embryos but partially and variably down-regulated in the zygotic mutant embryos (compare Lanes 1 and 5 with Lanes 10, 11 and 15-17 of Supplemental Figure S14). These data are consistent with the genetic data regarding the highly penetrant embryonic lethality of the maternal-zygotic mutants and partial neonatal lethality of the zygotic mutants. Indeed, *Dlk1* is an essential gene (Moon et al., 2002) and variable downregulation of the expression of *Dlk1* may contribute to variable time course of partial lethality observed in the *Zfp57* zygotic mutants.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Targeting Construct

A 9kb genomic fragment of *Zfp57* was subcloned from a BAC clone (BACPAC resources) into the pBluescript vector (Stratagene). A LoxP site was inserted into the third intron of *Zfp57* using the similar recombination cloning system in bacteria (Lee et al., 2001) (Galan-Caridad et al., 2007) (Figure S2A). Oligonucleotides used are ZFP-LOXPFOR 5'-CCATGCAGGTAGTCCAGGCTAGCCTTAAAGTTGCTATGTAGCTGAGAATCGGATCCCCCTCGAGGGACCTA and ZFP-LOXPREV 5'-AGATGACAAGCTAGGCTTGAAAATAAGAGGTAGGAAGACCAGGTTAGGGTTCCGATCATATTCAATAACC, with each oligonucleotide containing 50 base pairs identical to the sequence in the genomic region. Then another cassette containing the Neo drug selection gene and the other LoxP site which has the same orientation as the first LoxP site inserted into the third intron was placed into the first intron using the similar cloning strategy (Figure S2A). Oligonucleotides used are ZFP-NeoFOR 5'-ACATACCTGAATGCGTCACATACCACTCTGAGTTTTGTCTCTTCATCCTCACAAAAGCTGGAGCTCCACC and ZFP-NeoREV 5'-CTGGTCCTGGAAGAGATATAAGCTCTCATCTTTCCAGTGAAATCAAGTCATCCCCTCGAATAACTTCG, with each oligonucleotide containing 50 nucleotides identical to the sequence in the genomic region. This Neo drug selection gene was flanked by two FRT sites and can be removed from the vector or the targeted genomic region by the expression of the Flp recombinase (Figure S2B).

ES Cell Targeting

The targeting vector was linearized with *Apa*I restriction enzyme digestion and purified linearized DNA was electroporated into the TC1 ES cells (129 Sv/Ev) (Deng, C., Wynshaw-Boris, A. and Leder, P., unpublished data). These ES cells were then immediately plated on the 10-cm tissue culture plate seeded with mitomycin c-treated feeder fibroblasts in the DMEM growth medium (Invitrogen/Gibco) containing leukemia inhibitory factor (LIF, Chemicon International). In the following days the transfected ES cells were subjected to 260μg/ml G-418 selection for about 6 days until G-418-resistant colonies appeared. These colonies were picked individually to a well of the 24-well tissue culture plates seeded with mitomycin c-treated feeder fibroblasts. Portions of the ES cells for each clone were frozen. The rest were propagated to prepare genomic DNA for Southern blot screening for the targeted ES clones that have been recombined at the *Zfp57* locus (Figure S2C).

Chimeric Mice and Generation of the Floxed and the Deleted Alleles

Correctly targeted ES clones were transiently transfected with a vector expressing the Flp recombinase. G-418-sensitive ES clones were isolated and loss of the Neo drug resistance gene was confirmed by Southern blot as well as a PCR-based genotyping. These ES clones contain a floxed allele in which one LoxP site was inserted into the third intron and the other LoxP site, together with an FRT site, was placed into the first intron (Figure S2B). Two of these ES clones were used for blastocyst injection into the C57/BL6 recipient and high-grade chimeric 129/BL6 male mice were derived. These chimeric male mice were mated with the female black Swiss mice in a mixed outbred genetic background or the female 129 Sv/Ev mice in the inbred genetic background. In both cases, the floxed allele in the *Zfp57* locus was passed on to the progeny mice. The deleted allele at the *Zfp57* locus was created with the crosses between the floxed allele-containing mice and the EIIa-cre transgenic mice (Lakso et al., 1996)(Figure S2B). The deleted allele in the pure 129 Sv/Ev inbred background was generated by transiently transfecting Cre recombinase into the ES cells containing the targeted allele of *Zfp57* before blastocyst injection. Upon Cre

recombinase-mediated excision, both the second exon with the initiation codon and the third exon encoding the entire conserved KRAB box are deleted with the next available codon encoding an in-frame methionine residue (Met275) located in the region near the carboxyl end of ZFP57 (Figure 1B and Figure S2B). Thus, it is very likely that the deleted allele will be a null allele. Probably due to nonsense-mediated mRNA decay (Chang et al., 2007), there may be an aberrant transcript but no detectable normal *Zfp57* transcript isoforms in the mutant embryos (Figure S2D). Furthermore, no ZFP57 protein was detected in *Zfp57*-null ES cells (Figure 5A).

Generation of *Zfp57*-null ES Clones and the ES Clone with F/T Alleles

For the ES clone containing one floxed allele (F) and one targeted allele (T) at the *Zfp57* locus, ES cells containing one floxed allele mentioned above were electroporated with the targeting construct and subjected to G-418 drug selection. Correctly targeted ES clones with one floxed allele (F) and one targeted allele (T) were screened and verified by Southern blot (data not shown). *Zfp57*-null ES clones were obtained by transiently expressing Cre recombinase in the ES cells with F/T alleles and verified by Southern blot (data not shown).

Genotyping

A three-oligo PCR reaction was used for analyzing the genotype of the mice (Figure S2B). The oligos used are ZLox5'F 5'-GGAAAGGAAACTACACACTGTC, ZLox3'F 5'-CAGCCTGAAAGATCTGAGTCAC and ZLox3'R 5'-GACACTGAACTAAGGTCCTCTAC, which will result in the PCR product of 226 base pairs for the wild-type allele, PCR product of 307 base pairs for the floxed allele and PCR product of 512 base pairs for the deleted allele.

Co-Immunoprecipitation

Two plasmids in which KAP-1 and Myc epitope-tagged ZFP57 are placed under the control of two different constitutive promoters were transfected into COS cells with Fugene (Roche). Two days after the transfection, cells were resuspended in the lysis buffer containing 20mM Tris (pH 7.5), 150mM NaCl, 0.5% NP40, 10% glycerol and protease inhibitors. After brief sonication and centrifuge for 10 minutes at 4^{o}C, the supernatant was collected and used for immunoprecipitation. The lysate was mixed with the antibodies against the myc epitope and protein A/G beads. After 2-hour incubation at 4^{o}C with constant rocking, the protein A/G beads were spun down briefly and washed three times with the lysis buffer. The immunoprecipitate was eluted from the beads and subjected to western blot analysis.

Co-immunoprecipitation was done similarly in ES cells except that purified rabbit polyclonal antibodies against ZFP57 were used to immunoprecipitate the endogenous ZFP57 in ES cells.

Statistical Analysis

To analyze the significance of the observed frequency of homozygous mutant animals, Chi-square test was used for the cross between heterozygous animals (degree of freedom = 2) and the cross between the heterozygous female mice and homozygous male mice (degree of freedom = 1). For the cross between the homozygous female mice and the heterozygous male mice (degree of freedom = 1), the G-test (Chi-square contingency) was used to allow for zero class events (Sokal and Rohlf, 1969)(Table S1).

Genome Accession Numbers of the DMRs

Sequence information for the DMRs of *Snrpn*, IG-DMR, *H19*, *Igf2r*, *Peg1* and *Peg3* can be accessed via the following genome accession numbers: AF081460, AJ320506, U19619, L06446, AF017994, AF105262.1.

Official Locus Names for the Genes Analyzed

Based on the genome database (http://www.ensembl.org/Mus_musculus), the MGI names and IDs for the following genes are: *Zfp57* (99204), *Dlk1* (94900), *Gtl2* (1202886), *Dio3* (1306782), *Rtl1* (2656842), *Igf2* (96434), *H19* (95891), *Peg1* (96968), *Peg3* (104748), *Peg5* (104716) and *Igf2r* (96435).

PCR Primers Used for Bisulphite Experiments

Nested PCR reactions were carried out for bisulphite mutagenized DNA samples. For the *Snrpn* DMR, the outside primers are snrpn OF 5'-TATGTAATATGATATAGTTTAGAAATTAG and snrpn OR 5'-AATAAACCCAAATCTAAAATATTTTAATC. The inside primers are snrpn IF 5'-AATTTGTGTGATGTTTGTAATTATTTGG and snrpn IR 5'-ATAAAATACACTTTCACTACTAAAATCC. For the second biological replicate of oocytes, the following inside primers were used; snrpnF3 5'-TAAGTATTTTTTTTGGTAGTTGTTTT, snrpnF4 5'-TTAAAGGGATATAGATTTTTGTATTG with the reverse inside primer snrpnR4 5'-CACAAACCCAACTAACCTTC.

At the IG-DMR, the same primer IGDMR O/IR 5'-TACAACCCTTCCCTCACTCCAAAAATT was used as both the outside and inside reverse primer. The outside and inside forward primers are IGDMR OF 5'-GTGTTAAGGTATATTATGTTAGTGTTAGG and IGDMR IF 5'-ATATTATGTTAGTGTTAGGAAGGATTGTG, respectively. For the H19 DMR, the outside primers are H19 DMD OF 5'- GAGTATTTAGGAGGTATAAGAATT and H19 DMD OR 5'- ATCAAAAACTAACATAAACCCCT. The inside primers are H19 DMD IF 5'- GTAAGGAGATTATGTTTATTTTTGG and H19 DMD IR 5'-CCTCATTAATCCCATAACTAT. At the *Igf2r* DMR, the same primer Igf2r O/IR 5'-AAATATCCTAAAAATACAAACTACAC was used as both the outside and inside reverse primer. The outside and inside forward primers are Igf2r OF 5'-TTAGTGGGGTATTTTTATTTGTATGG and Igf2r IF 5'-GTGTGGTATTTTTATGTATAGTTAGG, respectively. For the *Peg1* DMR, the outside primers are Peg1 OF 5'- GATTTGGGATATAAAAGGTTAATGAG and Peg1 OR 5'- TCATTAAAAACACAAACCTCCTTTAC. The inside primers are Peg1 IF 5'- TTTTAGATTTTGAGGGTTTTAGGTTG and Peg1 IR 5'-AATCCCTTAAAAATCATCTTTCACAC. For the *Peg3* DMR, the outside primers are Peg3 OF 5'- TTTTTAGATTTTGTTTGGGGGTTT TTAATA and Peg3 OR 5'-AATCCC TATCACCTAAATAACATCCCTAC. The inside primers are Peg3 IF 5'-TTGATAATA GTAGTTTGATTGGTAGGGTGT and Peg3 IR 5'-ATCTACAACCTTATCAAT TACCCTTAAAAA.

For bisulphite sequencing of the *Snrpn* DMR in the 129/DBA/2 hybrid embryos, the outside primers are Sn-F642 5'-GGGTTTTATGTTTGATTGTGTGTG and Sn-R1204 5'- AATCAAATAAAATACACTTTCACTACT. The inside reverse primer is Sn-R1180 5'- ACTAAAATCCACAAACCCAACTAAC. Allele-specific inside forward primers were used for amplifying 129 and DBA/2 derived genomic DNA. For the maternally derived *Snrpn* DMR with the 129 polymorphisms, the inside forward primer is Sn-F780 5'- TGTGTGATGTTTGTAATTATTTGGGAG. For the paternally derived *Snrpn* DMR with the DBA/2 polymorphisms, the inside forward primer is Sn-DF780 5'-TGTGTGATGTTTGTAATTATTTGGGAA.

For bisulphite sequencing of L1 repetitive element *(Line 1)*, the outside primers are Line OF 5'- GTTAGAGAATTTGATAGTTTTTGGAATAGG and Line OR 5'-CCAAAACAAAACCTTTCTCAAACACTATAT. The inside primers are Line IF 5'-

TAGGAAATTAGTTTGAATAGGTGAGAGGGT and Line IR 5'-TCAAACACTATATTACTTTAACAATTCCCA.
For bisulphite sequencing of intracisternal A particle (*IAP*), the outside primers are IAP-OF 5'- TTGATAGTTGTGTTTTAAGTGGTAAATAAA and IAP-OR 5'-CAAAAAAAACACCACAAACCAAAAT. The inside primers are IAP-IF 5'-TTGTGTTTTAAGTGGTAAATAAATAATTTG and IAP-IR 5'-AAAACACCACAAACCAAAATCTTCTAC.

Chromatin Immunoprecipitation
ES cells were grown in gelatin-coated plates for one generation to minimize feeder fibroblast cells and approximately one million ES cells were used for each ChIP experiment. Sonication of ES cells were carried out on ice and ChIP was performed according to the manufacturer's suggested protocol (Upstate, USA). Approximately 0.6 μg of affinity purified rabbit anti-ZFP57 polyclonal antibodies (or equal amount of rabbit nonspecific IgG antibodies) was added to 1ml of the diluted sonicated lysate during immunoprecipitation. Nested PCR reactions were carried out. The first round PCR product was diluted 1250-fold before the second round of PCR amplification. The number of PCR amplification cycles for the first round is 35 and the cycle number for the second round is 25. The outside PCR primers used are SN-DMR-F1 5'- CCGCAGTAGGAATGCTCAAG and SN-DMR-R1 5'- GGCTCCAAAGGATTGCTCAC. The inside PCR primers are SN-DMR-F2 5'- GCTGCCTTTTGGCAGGACA and SN-DMR-R2 5'-GGATGCACTTTCACTACTAGAATCC, respectively.
For the distant upstream region of the *Snrpn* DMR, the outside PCR primers are SNRPN-UF1 5'-GTCTAGCATGATGCTGGCTCTG and SNRPN-UR1 5'-GTACCAGGACTGGGTTGTC. The inside PCR primers are SNRPN-UF2 5'-GACACTTGCTACCCTAGTAG and SNRPN-UR2 5'-ATGCTAGAGGACATGGAG.
For the *H19* DMR, the outside PCR primers are H19-PF3 5'-CACGAGCATCCAGGAGGCAT and H19-PR3 5'-CTGGCCTCATGAAGCCCATGACT. The inside PCR primers are H19-PF1 5'-CAGTTGTGGGGTTTATACGCG and H19-PR4 5'-CTCAATCAGTTGCAATCCGT.

PCR Primers for Southern Blot Probe
For the *Snrpn* DMR, genomic DNA was probed with a 284-bp fragment generated by *Pst*I digestion of the PCR product amplified with the primers of 5'-CCACAGCCTTGGACAAGAGT and 5´-GGGTGTGTTAGTGCGGCACC.

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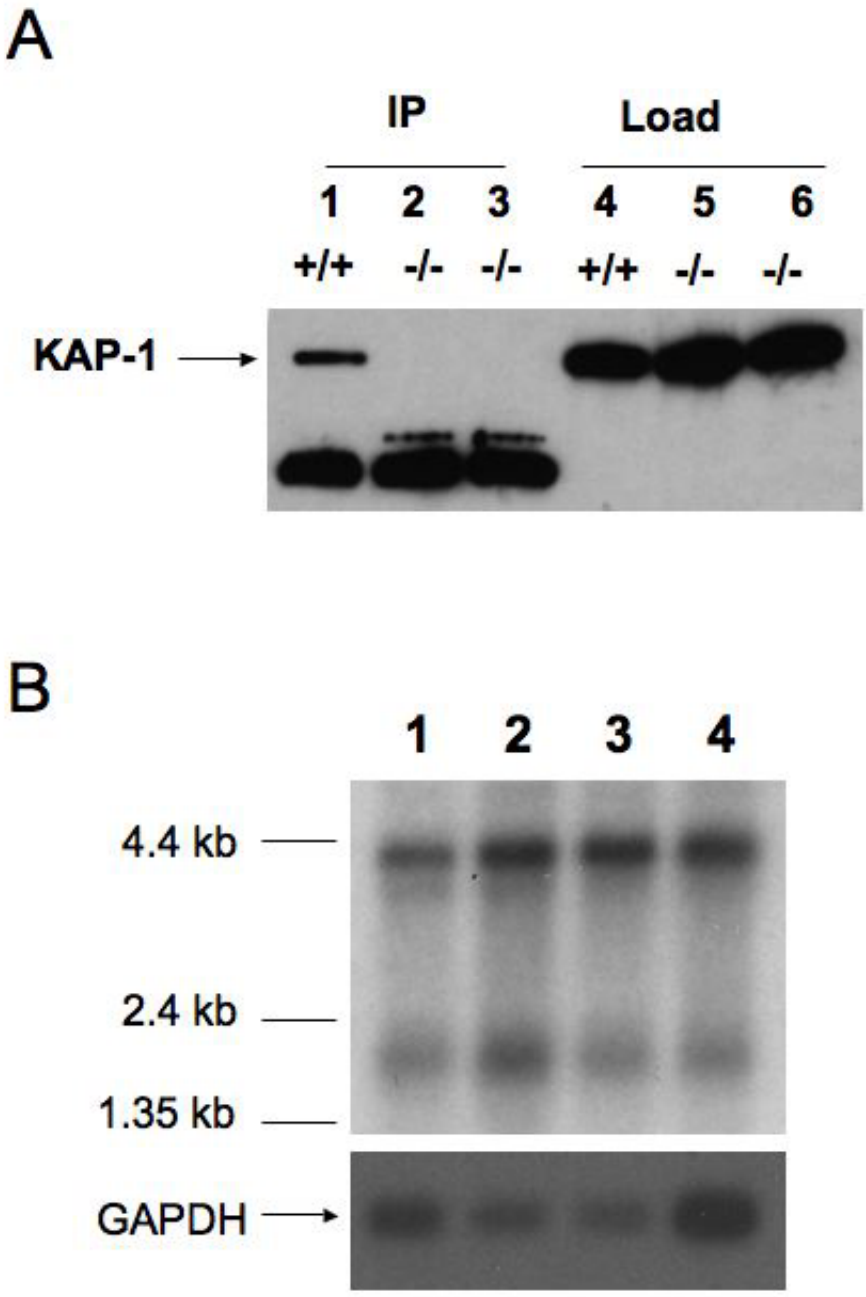


Figure S1. ZFP57 Binds to Endogenous KAP-1 in ES Cells and *Zfp57* Transcripts Are Present in Post-Implantation Embryos

(A) Co-IP in ES cells. Affinity-purified antibodies against ZFP57 were used to immunoprecipitate endogenous ZFP57 expressed in ES cells. Antibodies against KAP-1 were used to probe the western blot. Lanes 1-3 are immunoprecipitated (IP) samples and lanes 4-6 represent the corresponding load control samples. Lane 1, wild-type (+/+) ES cells. Lane 2 and lane 3, two independent *zfp57*-null ES clones.
(B) PolyA RNA Northern blot of *Zfp57* in post-implantation mouse embryos. MTN mouse embryo polyA RNA blot (Clontech cat. No. 636810) was used for examining the expression of *Zfp57* at the following embryonic stages: E7 (lane 1), E11 (lane 2), E15 (lane 3) and E17 (lane 4). The positions of molecular weight markers are indicated. This membrane was also hybridized with a probe for GAPDH as a loading control.

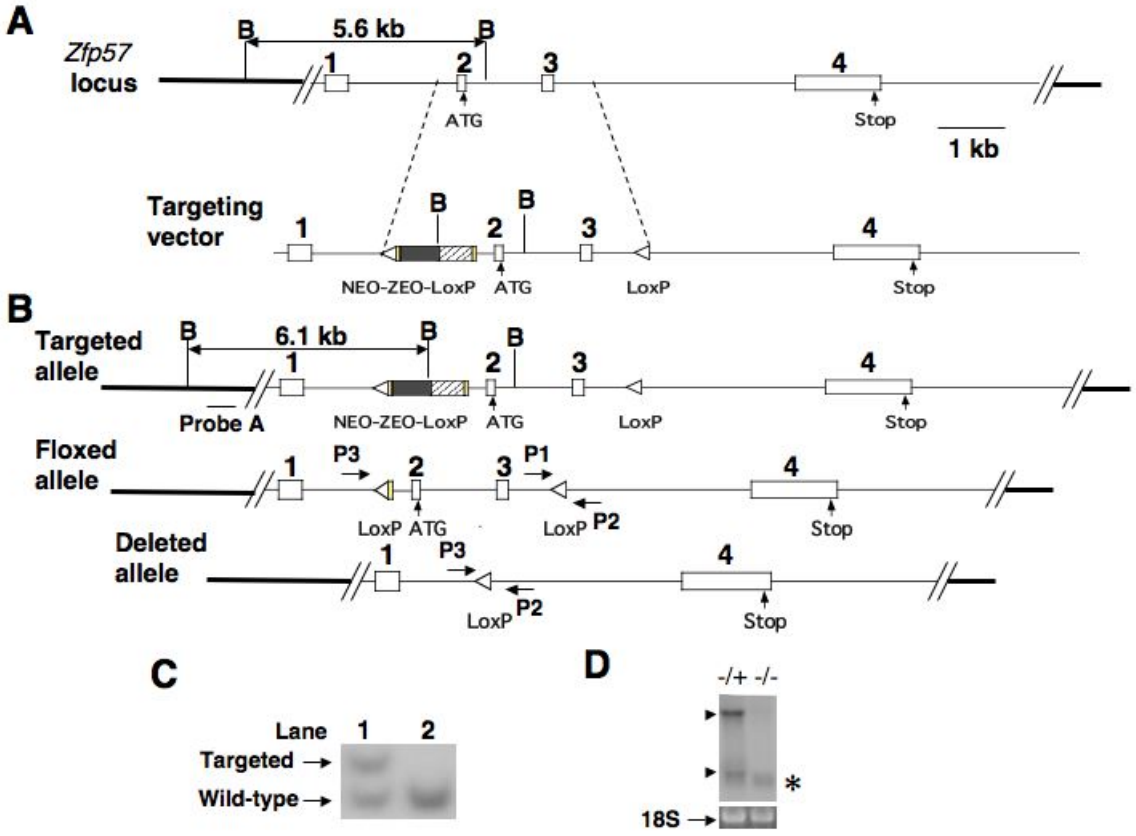


Figure S2. Generation of the Targeted, the Floxed, and the Deleted Alleles at the *Zfp57* Locus
(A) The endogenous locus of *Zfp57* and the targeting vector are illustrated. There are four exons (open boxes) in the *Zfp57* gene (1, 2, 3, 4). Amino acids Met1-Gln6 of Figure 1B are in exon 2. Amino acids Ser7-Val54 are encoded by the entire third exon. Amino acids Gly55-D421 are in exon 4. The start codon is marked with “ATG”. The stop codon is marked with “STOP”. The region in the endogenous *Zfp57* locus that was replaced by the LoxP site and the NEO-ZEO-LoxP cassette in the targeting vector is indicated by the dotted lines. The NEO-ZEO-LoxP cassette harbors a LoxP site as well as the sequences for driving the expression of the Neo resistance gene in ES cells and for driving the expression of the Zeocin resistance gene in bacterial cells, which were flanked by two FRT sites indicated by the yellow bars. “B”, *Bam*HI sites present in the endogenous locus and the targeting vector.
(B) Diagrams are shown for the targeted, the floxed and the deleted alleles of the *Zfp57* gene in ES cells. The targeted allele is the recombined allele between the

endogenous *Zfp57* locus and the targeting vector. Probe A indicates the position of the 5'-external probe (0.32 kb in length) used for confirming the targeted allele by Southern blot as shown in Figure S1C. The floxed allele is the conditional allele in which the NEO-ZEO-LoxP cassette in the targeting locus was removed by transient expression of the Flp recombinase in the targeted ES clones, with one LoxP site and one FRT site remaining. The deleted allele is the putative null allele in which the region encompassing exon 2 and exon 3 of *Zfp57* was deleted from the floxed allele by expression of the Cre recombinase. The positions of the primers used for PCR-based genotyping are indicated as P1 (Zlox3'F), P2 (Zlox3'R) and P3 (Zlox5'F).
(C) Targeted ES clones were confirmed by Southern blot. *Bam*HI-digested genomic DNA samples derived from the control (Lane 2) and a correctly targeted ES clone (Lane 1) were hybridized with a 5' external probe. On the Southern blot, the correctly targeted ES clone has an additional 6.1kb *Bam*HI fragment besides the 5.6kb wild-type *Bam*HI fragment.
(D) No detectable *Zfp57* transcript is found in the homozygous mutant embryo by Northern blot. Total RNA samples of a homozygous (-/-) mutant E 12.5 embryo as well as a littermate heterozygous (-/+) embryo derived from a null female mouse were hybridized with a probe corresponding to the entire open-reading-frame of *Zfp57*. Similar intensity of 18S ribosomal RNA shown in the bottom panel indicates equal loading of the RNA samples. Two *Zfp57* transcript isoforms (arrowheads) are present in the heterozygous embryo. There may be an aberrant transcript indicated by (*) in the homozygous embryo. Both embryos contain the deleted allele.

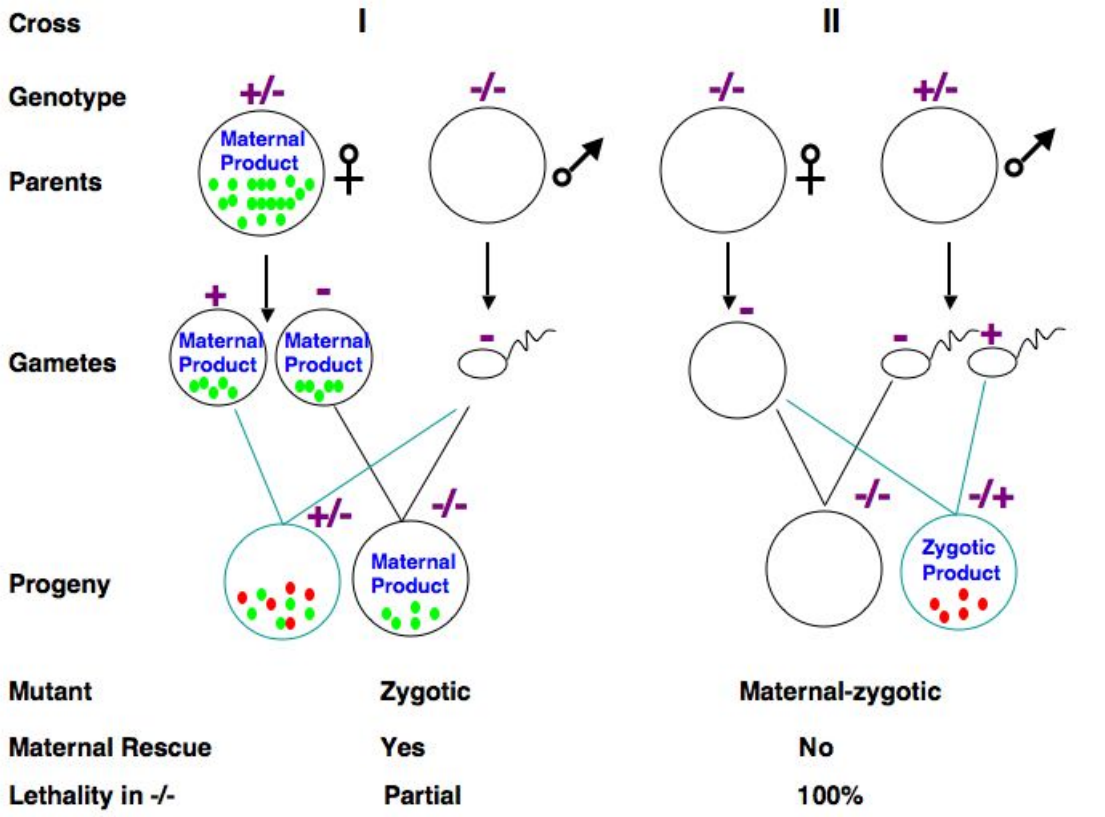


Figure S3. A Diagram Is Shown for the Zygotic Mutant and Maternal-Zygotic Mutant of *Zfp57*

Two reciprocal genetic crosses are shown to illustrate generation of the zygotic mutant and maternal-zygotic mutant. The zygotic mutant is derived from cross I (left) between a heterozygous female mouse and a homozygous male mouse,

whereas the maternal-zygotic mutant is produced by cross II (right) between a homozygous female mouse and a heterozygous male mouse. Maternal and zygotic gene products of *Zfp57* are indicated by green and red dots, respectively. The maternal-zygotic mutant (-/-) of *Zfp57* displays fully penetrant embryonic lethality. By contrast, the zygotic *Zfp57*mutant (-/-) exhibits partial neonatal lethality because of the rescue by the maternal product of *Zfp57*.

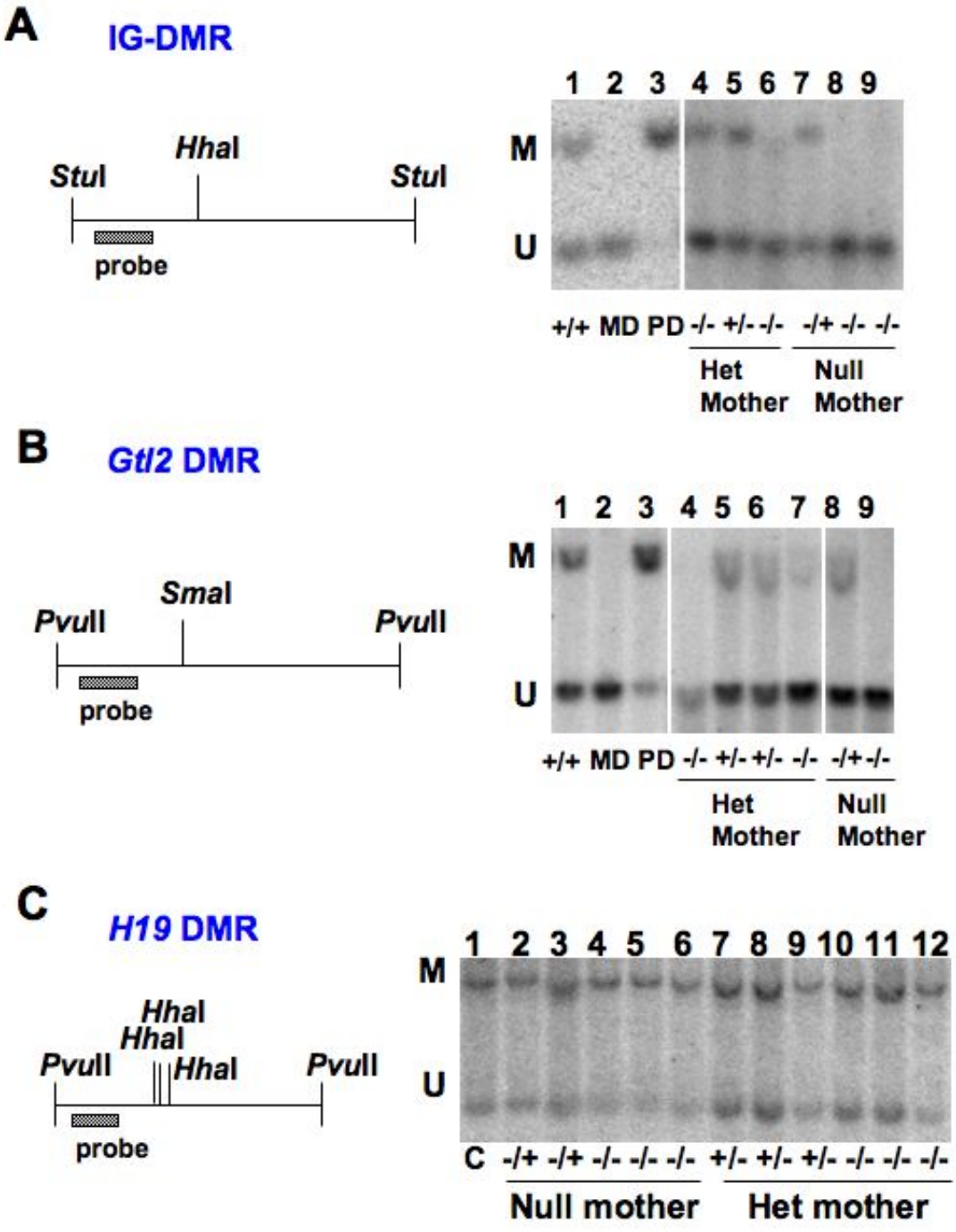


Figure S4. Southern Blot Analysis of Differential Methylation at the *Dlk1-Dio3* and *Igf2-H19* Imprinted Regions

Diagrams (left panels) are shown for the positions of the restriction enzymes as well as the diagnostic probes used for Southern blot analysis (right panels). The DNA samples were derived from the E11.5-E13 mice in the mixed genetic background. M, position of the bands for the methylated DNA. U, position of the bands for the unmethylated DNA. Mouse chromosome 12 harbors the *Dlk1-Gtl2* imprinted domain in which the IG-DMR as well as the *Gtl2* DMR are methylated

on the paternal chromosome but not on the maternal chromosome. All embryos were produced by mice in the mixed genetic background.
(A) Genomic DNA was prepared from heterozygous (+/-, sample 5) or homozygous (-/-, samples 4 and 6) embryos produced by a heterozygous female mouse (Het mother), and from heterozygous (-/+, sample 7) or homozygous (-/-, samples 8 and 9) embryos produced by a homozygous female mouse (Null mother). As controls, genomic DNA was also isolated from the wild-type embryos (+/+, sample 1), embryos carrying a maternal disomy of chromosome 12 (MD, sample 2) and embryos carrying a paternal disomy of chromosome 12 (PD, sample 3). Genomic DNA samples digested with *Stu*I and *Hha*I were hybridized with a probe derived from the IG-DMR. These results were confirmed by COBRA analysis (Figure S6).
(B) Genomic DNA samples digested with *Pvu*II and *Sma*I were hybridized with a probe derived from the *Gtl2* promoter DMR. Heterozygous (+/-, samples 5 and 6) and homozygous (-/-, samples 4 and 7) embryos were from a heterozygous female mouse (Het mother) whereas the heterozygous (-/+, sample 8) and the homozygous (-/-, sample 9) embryos were from a homozygous female mouse (Null mother). MD, maternal disomy of chromosome 12 (sample 2). PD, paternal disomy of chromosome 12 (sample 3).
(C) Differential DNA methylation at the *H19* DMR: Genomic DNA was prepared from heterozygous (-/+, samples 2 and 3) or homozygous (-/-, samples 4, 5 and 6) embryos produced by a homozygous female mouse (Null mother), and from heterozygous (+/-, samples 7, 8 and 9) or homozygous (-/-, samples 10, 11 and 12) embryos produced by a heterozygous female mouse (Het mother). Genomic DNA was also isolated from the wild-type control mice (C, sample 1). Assessment of complete digestion was determined through the absence of partial digestion patterns for the filters in A and C and with a probe for the biallelically unmethylated *Begain* CpG-island promoter located upstream of *Dlk1* which showed complete digestion of *Sma*I fragments (data not shown).

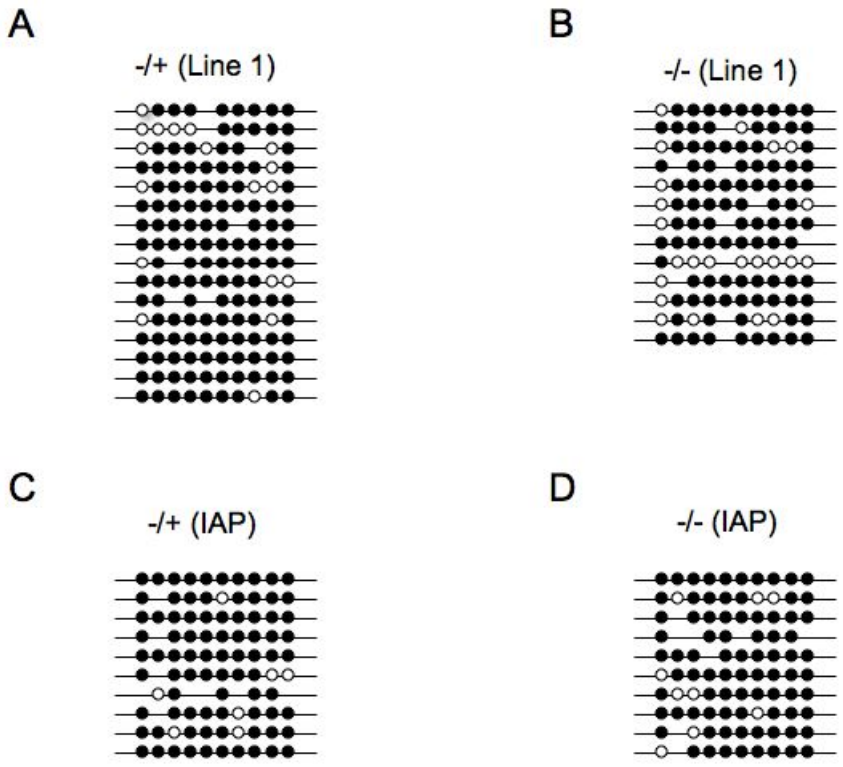


Figure S5. *Zfp57* Does Not Affect Methylation Status of Line1 and IAP Repeats

Bisulphite methylation analysis was performed on intracisternal A particle (IAP) and L1 repetitive elements (Line 1). Genomic DNA samples were prepared from a heterozygous (-/+) and a homozygous (-/-) embryos of the cross between a homozygous female mouse and a heterozygous male mouse in the 129 Sv/Ev background. Similar results were also obtained for Line 1 from the mice in the mixed genetic background (data not shown). Filled oval, methylated CpG site. Open oval, unmethylated CpG sites. Line with ovals, a unique clone.

(A) Line 1 repeats in the heterozygote.

(B) Line 1 repeats in the homzygous mutant.

(C) IAPs in the heterozygote.

(D) IAPs in the homozygous mutant.

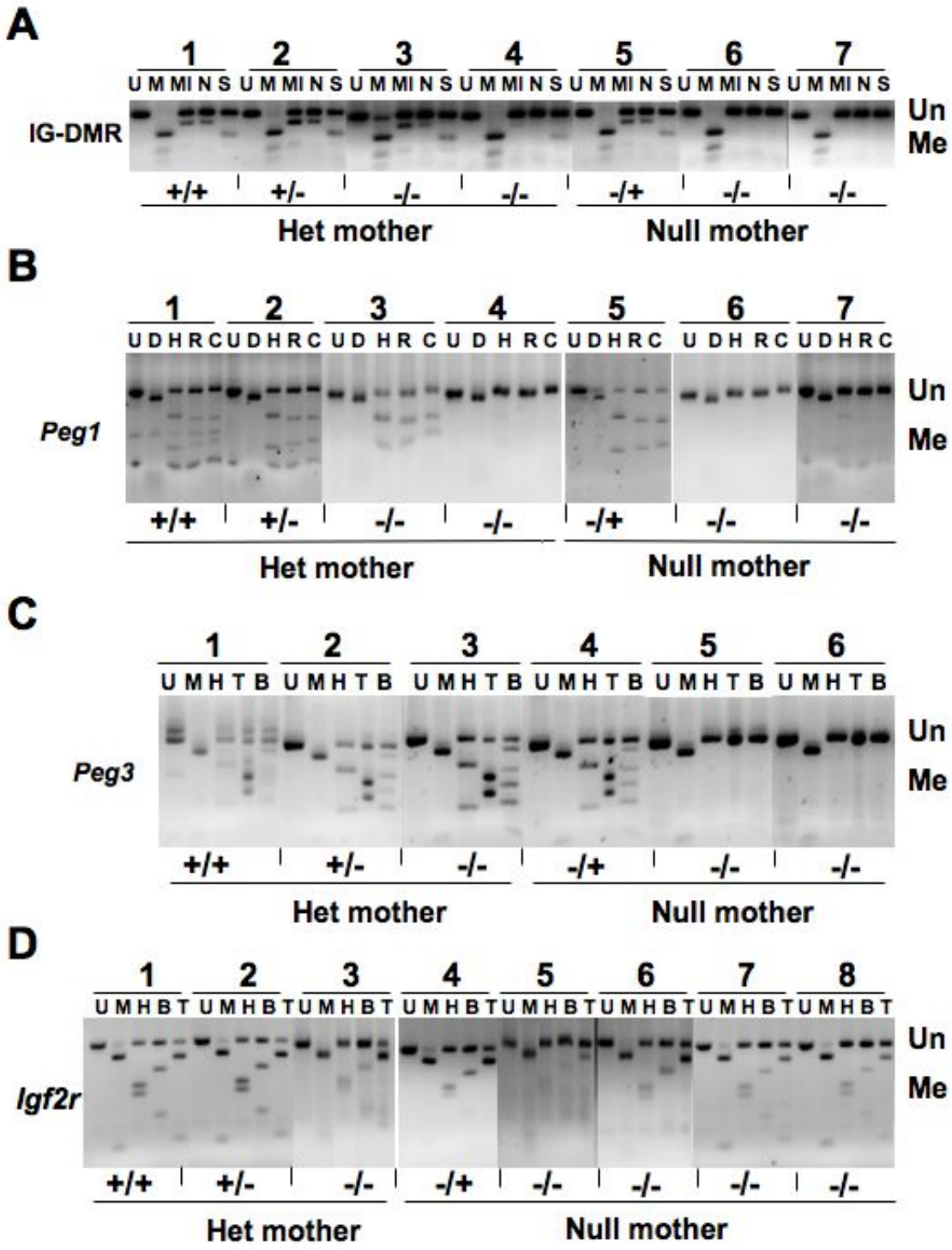


Figure S6. DNA methylation analysis of the imprinting controlling centers in embryos

Combined bisulphite restriction analysis (COBRA) was performed on the IG-DMR and the DMRs for the maternally methylated *Peg1*, *Peg3* and *Igf2r*. DNA samples were prepared from the wild-type (+/+), heterozygous (+/-) and homozygous (-/-) embryos at E11.5-E13.5 in the mixed genetic background produced by a heterozygous female mouse (Het mother) or from the heterozygous (-/+) and homozygous (-/-) embryos produced by a homozygous female mouse (null mother). Bisulphite treated embryonic DNA samples were subjected to restriction enzyme digestions after PCR amplification. U, uncut DNA samples. The restriction enzyme used are as follows: B, *Bst*UI; D, *Dra*I; C, *Cla*I; H, *Hpy*CH4IV; M, *Mse*I; Ml, *Mlu*I; N, *Nru*I; S, *Sau*3AI; R, *Rsa*I; T, *Taq*I. *Dra*I digestion and *Mse*I digestion test the effectiveness of bisulphite mutagenesis. Indeed, the expected

restriction sites were created in all samples in response to bisulphite mutagenesis which changes an unmethylated C nucleotide to a T nucleotide but does not affect a methylated C. The selected restriction enzyme sites for *Mlu*I, *Sau*3AI, *Hpy*CH4IV, *Rsa*I, *Cla*I, *Bst*UI and *Taq*I were protected from bisulphite mutagenesis if the CpG nucleotides within these recognition sites are methylated and were lost after bisulphite treatment if the corresponding CpG sites are unmethylated. Un, position of the bands for the unmethylated DNA. Me, position of the bands for the methylated DNA.
(A) The IG-DMR.
(B) *Peg1* DMR.
(C) *Peg3* DMR.
(D) *Igf2r* DMR
For the IG-DMR (A) and the *Peg1* DMR (B), seven (1-7) DNA samples are shown. Sample 1 was isolated from the cross of a heterozygous female mouse and a heterozygous male mouse. Samples 2-4 were isolated from the embryos generated by the cross of a heterozygous female mouse and a homozygous male mouse. Samples 5-6 were produced between the cross of a homozygous female mouse and a heterozygous male mouse. Sample 7 was generated from the cross of a homozygous female mouse and a homozygous male mouse.
For the *Peg3* DMR (C), six (1-6) DNA samples were shown and they were isolated from the following crosses: Sample 1, a heterozygous mother and a heterozygous father; Samples 2-3, a heterozygous mother and a homozygous father; Samples 4-5, a homozygous mother and a heterozygous father; Sample 6, a homozygous mother and a homozygous father. For the *Igf2r* DMR (D), eight (1-8) DNA samples were shown and they were generated from these crosses: Sample 1, a heterozygous mother and a heterozygous father; Samples 2-3, a heterozygous mother and a homozygous father; Samples 4-6, a homozygous mother and a heterozygous father; Samples 7-8, a homozygous mother and a homozygous father. Similar results were obtained from embryos in the pure 129 Sv/Ev background (data not shown).

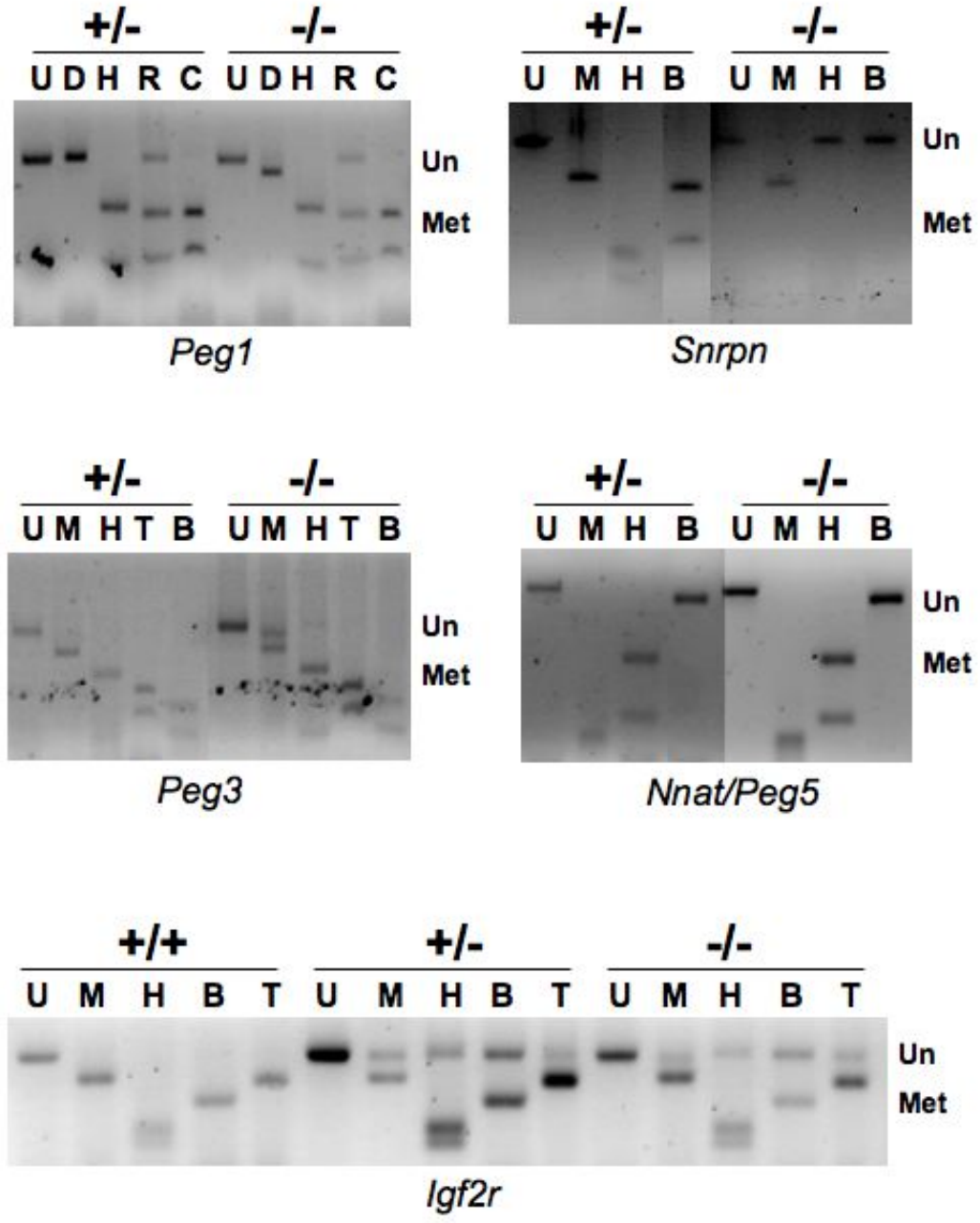


Figure S7. DNA Methylation Analysis of Maternally Established DMRs in the Oocytes

COBRA was performed on the DMRs of the imprinting controlling regions for these maternally imprinted genes (*Peg1*, *Snrpn*, *Peg3*, *Nnat/Peg5* and *Igf2r*). *Dra*I digestion and *Mse*I digestion test the effectiveness of bisulphite mutagenesis. DNA samples isolated from unfertilized oocytes produced by heterozygous female mice (+/-) or homozygous female mice (-/-) in the mixed genetic background were subjected to bisulphite mutagenesis followed by PCR amplification and restriction digestions. U, uncut DNA samples. The restriction enzyme used are as follows: D, *Dra*I; H, *Hpy*CH4IV; R, *Rsa*I; C, *Cla*I; M, *Mse*I; B, *Bst*UI; T, *Taq*I. Un and Met indicate the corresponding restriction enzyme-digested products from unmethylated and methylated DNA samples, respectively. Similar results were obtained for the samples derived in the pure 129 Sv/Ev background (data not shown).

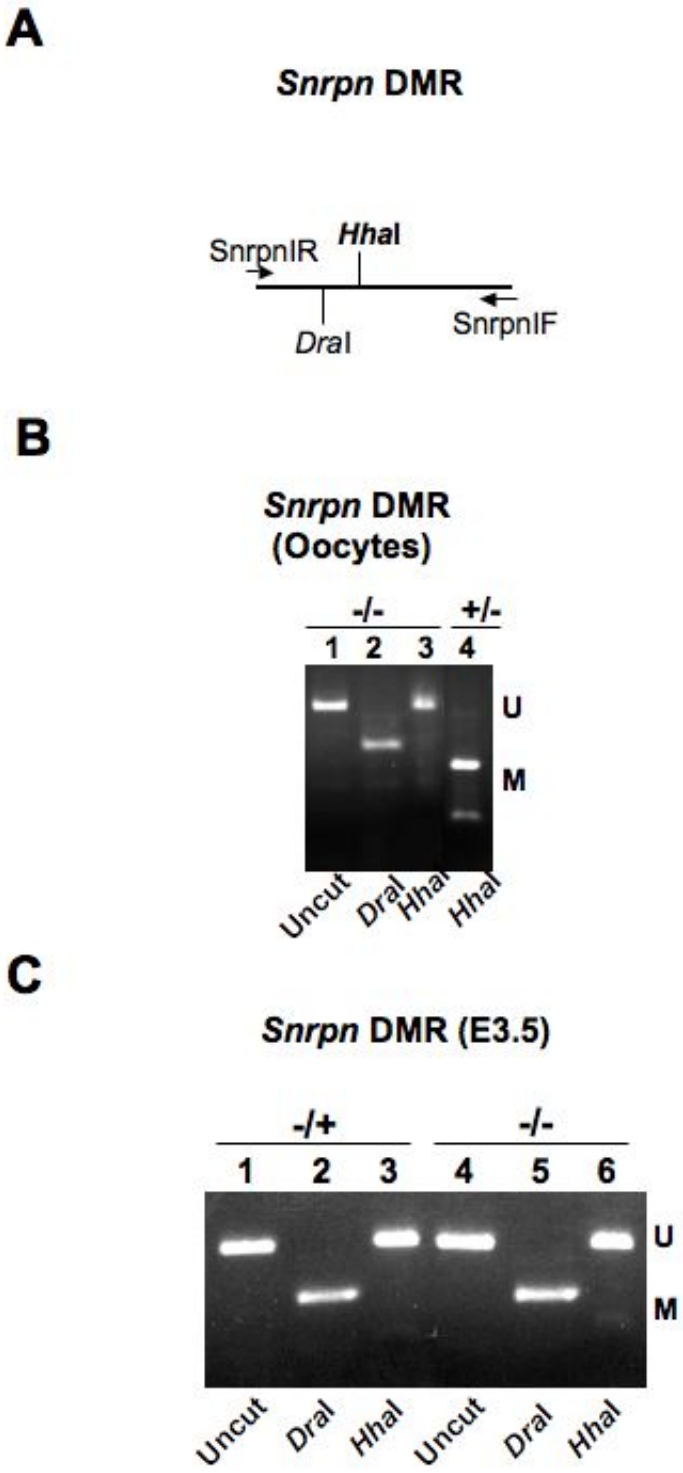


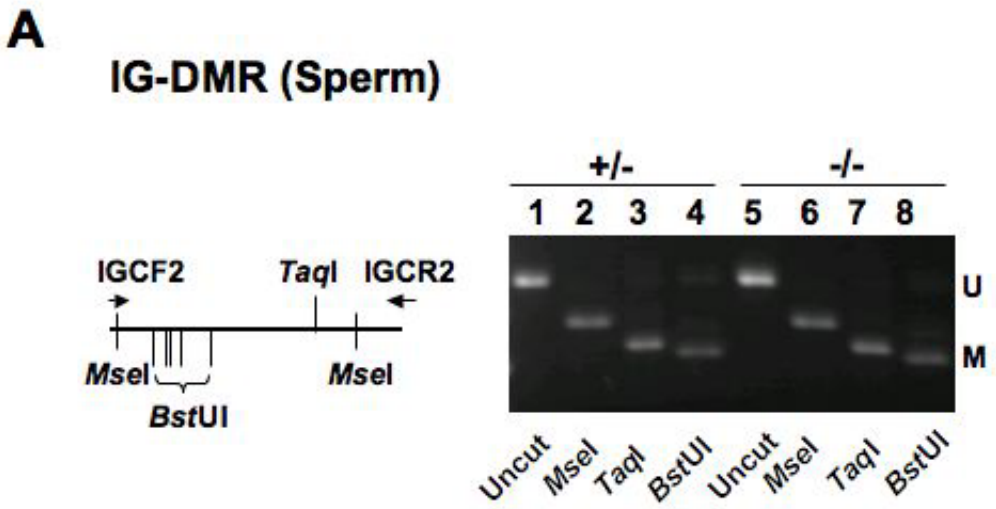
Figure S8. DNA Methylation Analysis of the *Snrpn* DMR in Unfertilized Oocytes and Embryos

*Dra*I digestion and *Mse*I digestion test the effectiveness of bisulphite mutagenesis. U and M indicate the corresponding restriction enzyme-digested products from unmethylated and methylated DNA samples, respectively.

(A) A diagram is shown for the *Snrpn* DMR region which contains a *HhaI* site. A *Dra*I site is created in response to bisulphite mutagenesis. Primers used for PCR are SnrpnIF and SnrpnIR as shown. *DraI* digestion recognizing CTCAAA tests effetiveness of sodium bisulphite mutagenesis which changes an unmethylated C nucleotide to a T nucleotide but does not affect a methylated C. *HhaI* which cuts GCGC tests methylation status of CpG sites.

(B) Restriction digestion of the *Snrpn* DMR PCR products from bisulphite mutagenized oocyte DNA samples derived from the mice in the mixed genetic

background. Lanes 1-3, 185 unfertilized oocytes pooled from 8 homozygous female mice. Lane 4, 174 unfertilized oocytes pooled from 8 heterozygous female mice.
(C) Restriction digestion of the *Snrpn* DMR PCR products from bisulphite mutagenized DNA samples of E3.5 embryos produced by mice in the mixed genetic background. Three pooled samples of 20-25 E3.5 embryos were analyzed. Lanes 1-3, heterozygous E3.5 embryos from a cross between a homozygous female mouse and a wild-type male mouse. Lanes 4-6, homozygous E3.5 embryos from a cross between a homozygous female mouse and a homozygous male mouse.



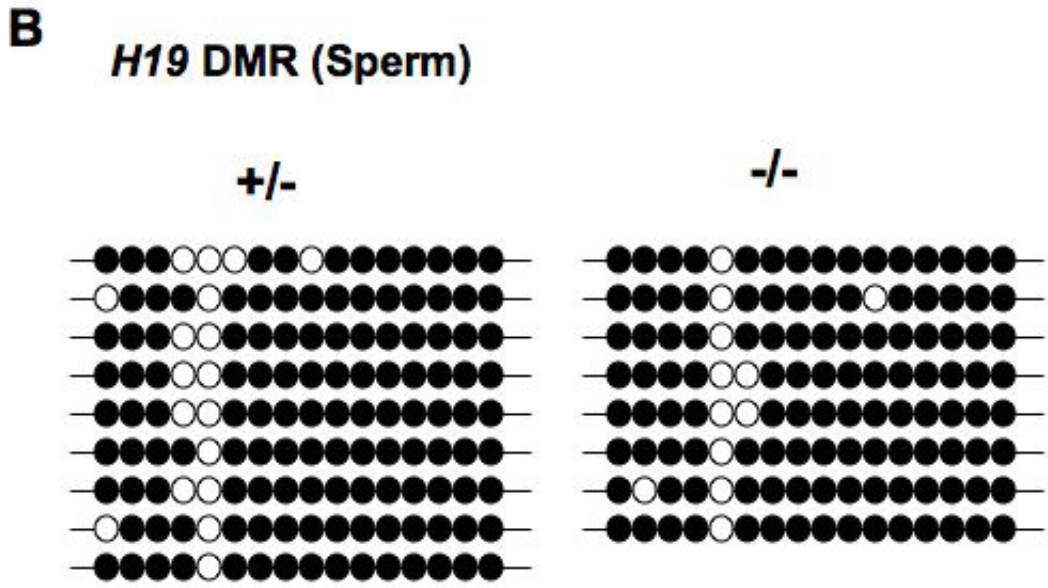


Figure S9. *Zfp57* Is Not Required for the Establishment of Paternal Methylation Imprints at the IG-DMR and *H19* DMR
(A) Combined bisulphite restriction analysis (COBRA) analysis of the IG-DMR. A diagram is shown for the analyzed IG-DMR region. Primers used for PCR amplification of bisulphite mutagenized sperm DNA samples are IGCF2 5'-TTAGGAGTTAAGGAAAAGAAAGAAATAGTATAGT and IGCR2 5'-TATACACAAAAATATATCTATATAACACCATACAA. *Mse*I digestion tests the

effectiveness of bisulphite mutagenesis. The methylation status of the IG-DMR was probed with *Tag*I and *Bstu*I digestions. Lanes 1-4, sperm from heterozygous male mice. Lanes 5-8, sperm from homozygous male mice.
(B) Bisulphite sequencing of the *H19* DMR. Bacterial clones were obtained from the PCR products after bisulphite mutagenesis and sequenced individually. +/-, sperm DNA sample from heterozygous male mice. -/-, sperm DNA sample from homozygous male mice. Filled and unfilled ovals represent methylated and unmethylated CpG sites, respectively.
Sperm samples were derived from the mice in the pure 129 Sv/Ev genetic background.

Figure S10. Sequence Polymorphisms at the *Snrpn* DMR Between 129 Sv/Ev and DBA/2
Sequences for the 129 Sv/Ev and DBA/2 strains were obtained by directly sequencing PCR product amplified from the wild-type genomic DNA samples of these two strains. The reference sequence of the C57/BL6 strain was based on the BLAT search (UC Santa Cruz). Nucleotides that are different between 129 Sv/Ev and DBA/2 are highlighted.

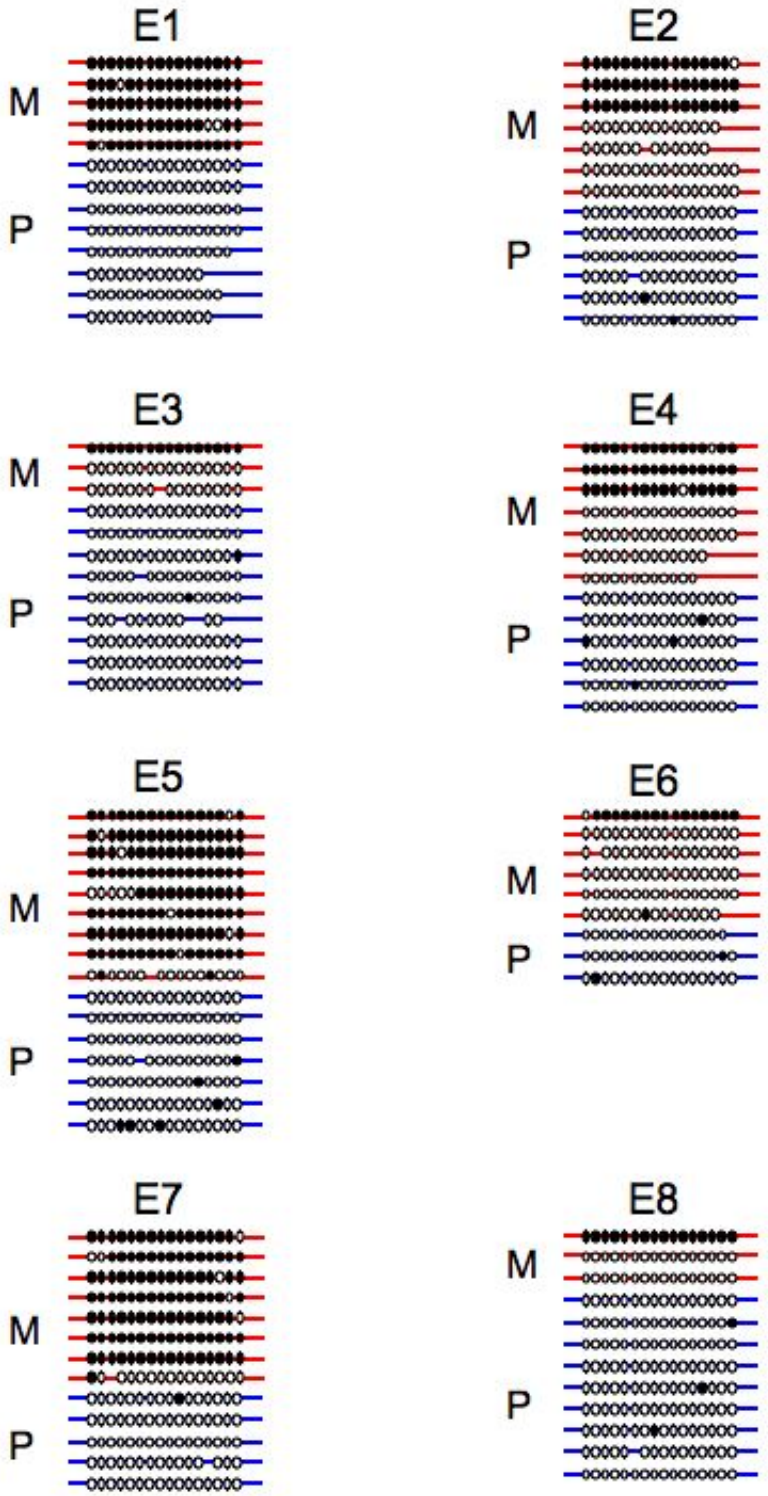


Figure S11. **Methylation Imprint Was Partially Acquired at the Maternally Derived *Snrpn* DMR in the Heterozygous Embryos Derived From Null Female Mice**

Bisulphite sequencing was performed at the *Snrpn* DMR for eight heterozygous (-/+) E12.5 embryos (E1 - E8) derived from the cross between a homozygous female mouse in the 129 Sv/Ev background and a wild-type male mouse in the DBA/2 background. At least three unique clones were obtained for either allele of every embryo. Filled oval, methylated CpG site. Open oval, unmethylated CpG sites. Red line with ovals, a unique clone for the maternally derived 129 allele. Blue line with ovals, a unique clone for the paternally derived DBA/2 allele. M, Maternal. P, Paternal.

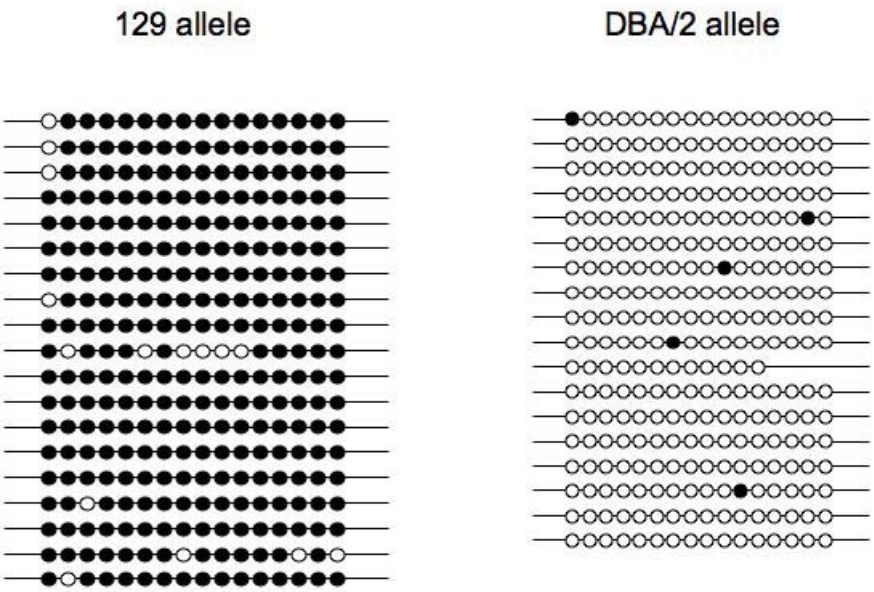


Figure S12. **Differential Methylation Was Retained at the Maternally Derived *Snrpn* DMR in the Embryos Derived from Wild-Type Parents**
Bisulphite sequencing was performed at the *Snrpn* DMR for the seven E12.5 embryos derived from the cross between a wild-type female mouse in the 129 Sv/Ev background and a wild-type male mouse in the DBA/2 background. A total of 19 unique clones with the 129 Sv/Ev polymorphisms (left panel) and 18 unique clones with the DBA/2 polymorphisms (right panel) at the *Snrpn* DMR were obtained. Filled oval, methylated CpG site. Open oval, unmethylated CpG sites. Line with ovals, a unique clone.

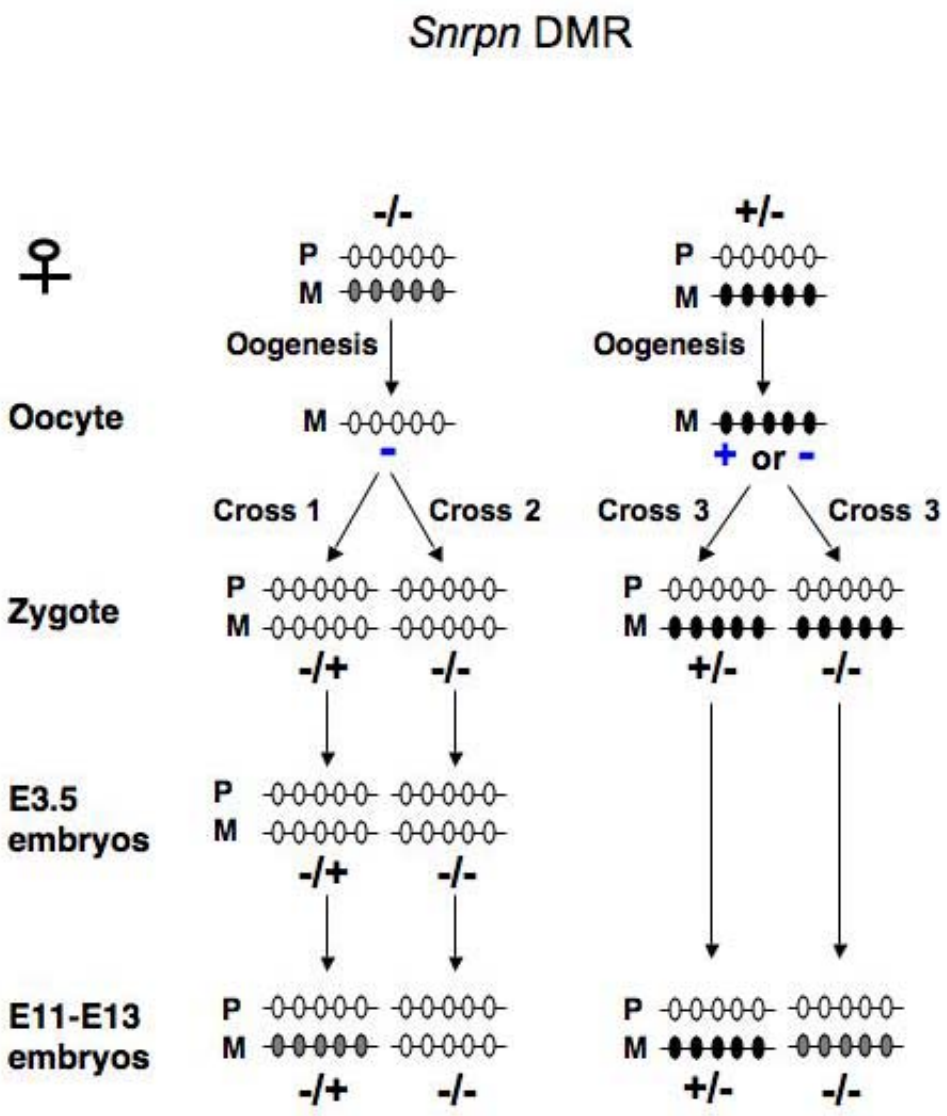


Figure S13. Diagrams Are Shown to Illustrate the Acquisition and Maintenance of DNA Methylation Imprints by the Maternal and Zygotic Functions of *Zfp57*

Cross 1 refers to the mating between homozygous female mice and wild-type male mice (maternal effect) while cross 2 refers to the mating between homozygous female mice and homozygous male mice (maternal-zygotic effect). Cross 3 refers to the mating between heterozygous female mice and homozygous male mice (zygotic effect). The maternal effect of *Zfp57* occurs before fertilization and oocytes derived from null (-/-) female mice fail to establish methylation imprint. The zygotic function provided in cross 1 sometimes results in acquisition of methylation at the maternally derived *Snrpn* DMR region. P, the paternal allele. M, the maternal allele. Filled oval, methylated CpG sites. Open oval, unmethylated CpG sites. Shaded oval, partially methylated CpG sites. +/-, heterozygous female mouse or embryos. -/+, heterozygous embryos from a null mother and a wild-type father.-/-, homozygous female mouse or homozygous embryos. Note: the methylation status of the *Snrpn* DMR at the zygote stage was not determined experimentally. Consistent with Figure 3B, homozygous (-/-) E11-E13 embryos derived from heterozygous female mice were partially methylated whereas heterozygous (+/-) E11-E13 embryos from heterozygous female mice were fully methylated based on bisulphite sequencing (data not shown).

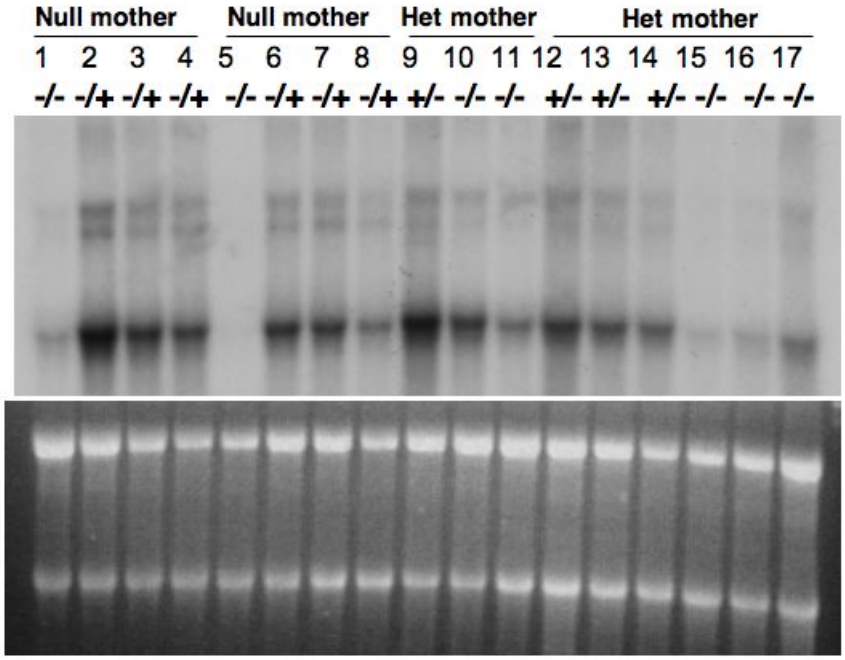


Figure S14. ***Dlk1*** **Transcripts Are Downregulated in** ***Zfp57*** **Mutant Embryos Based on Northern Blotting**

Total RNA samples were made from E13.5 embryos. Samples in lanes 1-8 are derived from the cross between homozygous female mice (Null mother) and heterozygous male mice whereas those in lanes 9-17 are from the cross between heterozygous female mice (Het mother) and homozygous male mice. Top panel, the Northern blot probed with a full-length *Dlk1* cDNA. Bottom panel, ethidium bromomide-stained gel indicates equal loading of total RNA samples.

Table S1. Reduced Numbers of ***Zfp57*** **Mutant Progeny at the Time of Weaning**

Crosses	# of litters	# of -/- mutant/total (%)	Expected % of mutants	Chi-square (P-Value)
+/-(f) X +/-(m)	8	7/65 (10.8%)	25%	7.676 (<0.025)
+/-(f) X -/-(m)	10	19/83 (22.9%)	50%	24.4 (<0.001)
-/-(f) X +/-(m)	6	0/9 (0%)	50%	7.767 (<0.0053) [$]

+/-(f), heterozygous female mouse.
+/-(m), heterozygous male mouse.
-/-(m), homozygous mutant male mouse.
-/-(f), homozygous mutant female mouse.
[$], the G-test (Chi-square contingency) used to allow for zero class events.

Table S2. Ablating Maternal Function of *Zfp57* in Oocytes Is Sufficient to Cause Maternal-Zygotic Embryonic Lethality

Cross	Stage	% of dead progeny/total
Flox/Flox(f) X -/-(m)	**E17.5-E18.5**	**4.4% (n=45)**
	P0	**0% (n=25)**
Flox/+, ZP3-Cre/+(f) X -/-(m)	**E17.5-E18.5**	**5.6% (n=18)**
	P0	**5.8% (n=52)**
Flox/Flox, ZP3-Cre/+(f) X -/-(m)	**E17.5-E18.5**	**91.9% (n=37)**
	E16.5	**75% (n=24)**
	E15.5	**45.5% (n=33)**
	E14.5	**8.3% (n=24)**

Embryos of the different embryonic stages were dissected out from the pregnant females impregnated by homozygous mutant males. Live embryos were confirmed under the dissection microscope by the heartbeat and body movement of the embryos.

-/-(m), homozygous mutant males.

Flox/Flox(f), female mice containing two floxed alleles at the *Zfp57* locus.

Flox/+, ZP3-Cre/+ (f), female mice with a ZP3-Cre transgene and one floxed allele at the *Zfp57* locus.

Flox/Flox, ZP3-Cre/+ (f), female mice with a ZP3-Cre transgene and two floxed alleles at the *Zfp57* locus.