ZFP57 dictates allelic expression switch of target imprinted genes

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ZFP57 is a master regulator of genomic imprinting. It has both maternal and zygotic functions that are partially redundant in maintaining DNA methylation at some imprinting control regions (ICRs). In this study, we found that DNA methylation was lost at most known ICRs in *Zfp57* mutant embryos. Furthermore, loss of ZFP57 caused loss of parent-of-origin–dependent monoallelic expression of the target imprinted genes. The allelic expression switch occurred in the ZFP57 target imprinted genes upon loss of differential DNA methylation at the ICRs in *Zfp57* mutant embryos. Specifically, upon loss of ZFP57, the alleles of the imprinted genes located on the same chromosome with the originally methylated ICR switched their expression to mimic their counterparts on the other chromosome with unmethylated ICR. Consistent with our previous study, ZFP57 could regulate the NOTCH signaling pathway in mouse embryos by impacting allelic expression of a few regulators in the NOTCH pathway. In addition, the imprinted *Dlk1* gene that has been implicated in the NOTCH pathway was significantly down-regulated in *Zfp57* mutant embryos. Our allelic expression switch models apply to the examined target imprinted genes controlled by either maternally or paternally methylated ICRs. Our results support the view that ZFP57 controls imprinted expression of its target imprinted genes primarily through maintaining differential DNA methylation at the ICRs.

genomic imprinting | allelic expression | COBRA | bisulfite sequencing | RNA-seq

Genomic imprinting is characterized by parent-of-origin–dependent expression of a subset of genes in mammals (1–3). It shares some features with other mammalian monoallelic gene expression phenomena (4, 5) and may have coevolved with X chromosome inactivation in mammals (6, 7). To date there are about 150 known imprinted genes, with more than half of them conserved between mice and humans (1, 3). It is well established that genomic imprinting is required for mammalian sexual reproduction and embryonic development (1, 8–10). Loss of genomic imprinting causes many human diseases, such as diabetes, cancer, and neurological disorders (11). Furthermore, genomic imprinting defects are frequently observed in reprogrammed pluripotent stem cells that are promising candidates for cell-based therapies (12–18). Therefore, it is important to understand how genomic imprinting is maintained and what controls parent-of-origin–dependent expression of these imprinted genes (1, 3, 4).

There are some known singleton imprinted genes sparsely populated across the genome (http://www.mousebook.org/mousebook-catalogs/imprinting-resource). However, most of the known imprinted genes in mice are clustered in over 20 imprinted regions that each harbors a few imprinted genes (19, 20). The clustered imprinted genes are coregulated by a *cis*-acting imprinting control region (ICR) (1, 3). Each ICR contains a differentially methylated region (DMR) with germline-derived differential DNA methylation inherited on either maternal or paternal chromosome. There are three known imprinted regions with a paternally methylated DMR located at an intergenic region (21, 22). More than 20 known imprinted regions carry a maternally methylated DMR, which is usually located near the promoter of an imprinted gene (1, 3, 23). Previous studies have demonstrated that differential DNA methylation at the ICR is important for parent-of-origin–dependent expression of the imprinted genes. However, it is still unclear how monoallelic expression of the imprinted genes may be achieved.

DNA methyltransferases are required for the establishment and maintenance of differential DNA methylation at the ICRs (24–26). However, they lack the targeting domains that recognize the ICRs. This gap was bridged by the discovery of ZFP57 as the master regulator of genomic imprinting in both mice and humans (27–29). ZFP57 is a KRAB zinc finger protein with a KRAB domain that can interact with KAP1/TRIM28, the cofactor for all KRAB zinc finger proteins (27). ZFP57 contains a few conserved C2H2-type zinc fingers that can recognize the TGCCGC motifs present in the ICRs (28, 30–32). ZFP57 binds to the methylated ICRs with high affinity (30, 33, 34). It can recruit DNA methyltransferases and other factors via the scaffold protein KAP1/TRIM28 to maintain differential DNA methylation

Significance

This study provides further proof for ZFP57 as a master regulator of genomic imprinting since it maintains DNA methylation at most known imprinted regions and regulates allelic expression of the corresponding imprinted genes. We propose two alternate allelic switch models for ZFP57-dependent regulation of parent-of-origin–dependent expression of the imprinted genes based on allelic and quantitative expression analyses of the imprinted genes. Our results also shed light on the mechanistic link between DNA methylation imprint and monoallelic expression of the imprinted genes. The results indicate that ZFP57 regulates allelic expression of clustered imprinted genes through maintenance of DNA methylation at the imprinting control regions.

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at the ICRs (30, 35). Loss of ZFP57 caused loss of DNA methylation imprint at a few known imprinted regions in both mouse embryos and ES cells (27, 32, 35, 36). It maintains imprinted expression of the target imprinted genes (27, 37). *Zfp57* is a maternal-zygotic effect gene (27). Maternal and zygotic functions of *Zfp57* are partially redundant in the maintenance of DNA methylation imprint and mouse embryonic development (27, 38). Recently, another KRAB zinc finger protein, ZFP445, was found to be partially redundant with ZFP57 in genomic imprinting although loss of ZFP445 alone did not cause significant loss of DNA methylation imprint in mice (39).

In this study, we identified some additional target imprinted regions of ZFP57 and found that loss of ZFP57 caused loss of DNA methylation imprint at most known imprinted regions. In contrast, it did not have much effect on DNA methylation of other putative DMRs or somatic DMRs we examined. Consistent with our previous studies (27, 35), loss of ZFP57 did not affect DNA methylation at the repetitive elements. Next, we took advantage of single nucleotide polymorphisms (SNPs) present in different mouse strains to test the effects of ZFP57 on allelic expression of 23 clustered imprinted genes residing in 18 imprinted regions in mouse embryos. The expression levels of 20 imprinted genes were measured in mouse embryos with or without ZFP57 by qRT-PCR analysis. Interestingly, the maternal alleles of some ZFP57 target imprinted genes exhibited a "maternal to paternal switch" in their allelic expression when differential DNA methylation at the maternally methylated ICRs was lost in *Zfp57* mutant embryos. Similarly, the paternal alleles of some target imprinted genes showed a "paternal to maternal switch" in their allelic expression when the paternally methylated ICRs became unmethylated upon loss of ZFP57. These results were confirmed by RNA sequencing (RNA-seq) analysis of similar mouse embryos. Interestingly, RNA-seq and gene ontology (GO) analyses suggests that allelic expression of some genes in the NOTCH signaling pathway including the imprinted *Dlk1* gene may be perturbed in mouse embryos without ZFP57, which is consistent with our previous study (38).

Results

Zfp57 has both maternal (M) and zygotic (Z) functions in wild-type mouse embryos ($Zfp57^{+/+}$, M^+Z^+). M^- or Z^- indicates loss of maternal or zygotic *Zfp57*, respectively. $Zfp57^{+/+}$ (M^+Z^+), $Zfp57^{+/-}$ (M^+Z^+), and $Zfp57^{-/-}$ zygotic mutant (M^+Z^-) embryos were obtained from the crosses between $Zfp57^{+/-}$ female and $Zfp57^{+/-}$ male mice, whereas $Zfp57^{-/+}$ (M^-Z^+) and $Zfp57^{-/-}$ maternal-zygotic mutant (M^-Z^-) embryos were derived from the crosses between $Zfp57^{-/-}$ female and $Zfp57^{+/-}$ male mice (*SI Appendix*, Figs. S1 and S2).

ZFP57 Maintains DNA Methylation Imprint of Most Imprinted Regions. $Zfp57^{+/-}$ 129S6/SvEvTac (shortened to 129 in this study) female mice were mated with wild-type CAST male mice to generate $Zfp57^{+/-}$ 129/CAST hybrid male mice (*SI Appendix*, Fig. S1*A*). These $Zfp57^{+/-}$ 129/CAST hybrid male mice were used in the timed mating with $Zfp57^{+/-}$ or $Zfp57^{-/-}$ 129 female mice to produce $Zfp57^{+/-}$ (M^+Z^+), $Zfp57^{-/+}$ (M^-Z^+), and $Zfp57^{-/-}$ maternal-zygotic mutant (M^-Z^-) 129/CAST hybrid embryonic day (E)13.5 embryos (*SI Appendix*, Fig. S1 *B* and *C*). Genomic DNA samples prepared from these live embryos were subjected to combined bisulfite restriction analysis (COBRA) to examine differential DNA methylation at the maternally methylated ICRs of 14 known imprinted regions (Fig. 1 *A* and *B*). DNA methylation was mostly intact at the *Snrpn*, *Zac1*, *Peg1*, *Peg3*, *Peg13*, *Nespas*, *Gnas1A*, *Peg5*, and *Igf2r* ICRs in M^+Z^+ or M^-Z^+ embryos, but it was largely lost at these ICRs in M^-Z^- embryos (Fig. 1*A*). Interestingly, DNA methylation was lost at the *Grb10* ICR in M^-Z^+ and M^-Z^- embryos compared with M^+Z^+ embryos, although there was still some DNA methylation left in M^-Z^+ embryos (Fig. 1*A*). No apparent loss of DNA

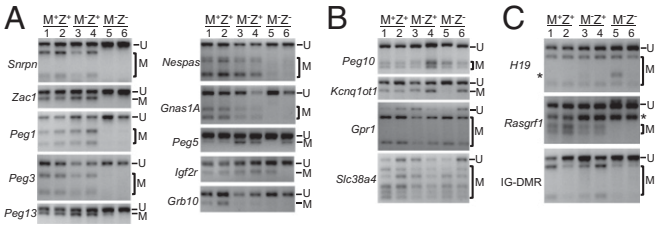

Fig. 1. DNA methylation imprint was lost in the absence of ZFP57 at most imprinted regions examined. Genomic DNA samples were isolated from two $Zfp57^{+/-}$ (M^+Z^+), two $Zfp57^{-/+}$ (M^-Z^+), and two $Zfp57^{-/-}$ (M^-Z^-) hybrid E13.5 embryos derived from the timed mating between $Zfp57^{+/-}$ (or $Zfp57^{-/-}$) 129 female mice and $Zfp57^{+/-}$ 129/CAST hybrid male mice. They were subjected to COBRA for 14 maternally methylated (*A* and *B*) and 3 paternally methylated (*C*) ICRs of the known imprinted regions. Lanes 1–2, two M^+Z^+ embryos. Lanes 3–4, two M^-Z^+ embryos. Lanes 5–6, two M^-Z^- embryos. M, methylated product after COBRA; U, unmethylated product after COBRA. (*A*) DNA methylation imprint was lost at 10 maternally methylated ICRs. These include *Snrpn*, *Zac1*, *Peg1*, *Peg3*, *Peg13*, *Nespas*, *Gnas1A*, *Peg5*, *Igf2r*, and *Grb10* imprinted regions. The restriction enzymes used for COBRA are as follows: *Snrpn*, *Hha*I; *Zac1*, *Taq*$^{\alpha}$I; *Peg1*, *Cla*I; *Peg3*, *Taq*$^{\alpha}$I; *Peg13*, *Taq*$^{\alpha}$I; *Nespas*, *Taq*$^{\alpha}$I; *Gnas1A*, *Cla*I; *Peg5*, *Hha*I; *Igf2r*, *Taq*$^{\alpha}$I; *Grb10*, *Taq*$^{\alpha}$I. (*B*) Loss of ZFP57 did not appear to affect DNA methylation imprint at the maternally methylated ICRs of four known imprinted regions. These include *Peg10*, *Kcnq1ot1*, *Gpr1*, and *Slc38a4* imprinted regions. The restriction enzymes used for COBRA are as follows: *Peg10*, *Bst*UI; *Kcnq1ot1*, *Hha*I; *Gpr1*, *Taq*$^{\alpha}$I; *Slc38a4*, *Taq*$^{\alpha}$I. (*C*) DNA methylation imprint was lost in two of three paternally methylated ICRs. These include *H19* ICR, *Rasgrf1* ICR and IG-DMR of the *Dlk1-Dio3* imprinted region. The restriction enzymes used for COBRA are as follows: *H19*, *Cla*I; *Rasgrf1*, *Bst*UI; IG-DMR, *Taq*$^{\alpha}$I. An asterisk (*) denotes a nonspecific PCR product.

methylation was observed at the *Peg10*, *Kcnq1ot1*, *Gpr1*, and *Slc38a4* ICRs in M^-Z^- embryos compared with M^+Z^+ or M^-Z^+ embryos (Fig. 1*B*). Therefore, loss of ZFP57 led to loss of DNA methylation imprint at most known imprinted regions with maternally methylated ICRs that we examined in mouse embryos.

Currently, there are three known imprinted regions (*Igf2-H19*, *Rasgrf1*, and *Dlk1-Dio3*) with paternally methylated ICRs (Fig. 1*C*). DNA methylation was maintained at the *H19* ICR of the *Igf2-H19* imprinted region in M^+Z^+, M^-Z^+, and M^-Z^- embryos (Fig. 1*C*). In contrast, DNA methylation imprint was largely lost at the *Rasgrf1* ICR and IG-DMR of *Dlk1-Dio3* imprinted region in M^-Z^- embryos compared with M^+Z^+ or M^-Z^+ embryos (Fig. 1*C*). Thus, DNA methylation was lost in two of three paternally methylated ICRs without ZFP57.

We obtained similar COBRA results for 129/DBA hybrid E13.5 embryos (*SI Appendix*, *Supplemental Results* and Fig. S3). Taking these data together, we find that differential DNA methylation was lost at most known imprinted regions we examined in $Zfp57^{-/-}$ (M^-Z^-) embryos. Therefore, ZFP57 is a key factor for the maintenance of differential DNA methylation at the ICRs in mouse embryos.

We also performed whole-genome bisulfite sequencing (WGBS) of four M^+Z^+, four M^-Z^+, and four M^-Z^- 129/DBA hybrid E13.5 embryos (*SI Appendix*, Fig. S2). We found that DNA methylation was indeed completely or partially lost at the maternally methylated ICRs of 17 known imprinted regions of M^-Z^- E13.5 embryos compared with M^+Z^+ embryos (Fig. 2*A* and *SI Appendix*, Tables S1 and S3). It was significantly reduced at the *Snrpn*, *Zrsr1*, *Grb10*, *Mcts2*, and *AK008011* ICRs in M^-Z^+ E13.5 embryos compared with M^+Z^+ embryos (Fig. 2*A* and *SI Appendix*, Tables S1 and S3). It was still retained at the maternally methylated ICRs of four known imprinted regions (Fig. 2*B*). We also found that it was lost at two of three paternally methylated ICRs in M^-Z^- embryos compared with M^+Z^+ or M^-Z^+ embryos (Fig. 2*C* and *SI Appendix*, Tables S1 and S3).

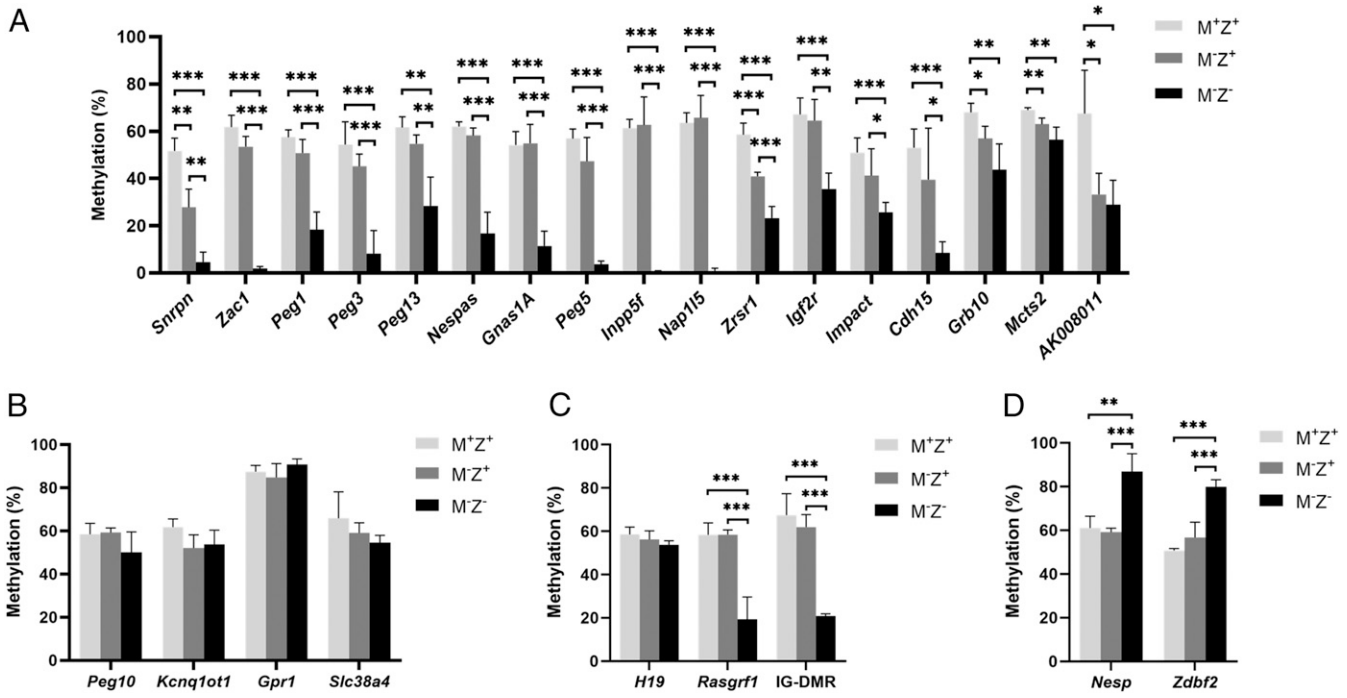

Fig. 2. Loss of ZFP57 caused loss of DNA methylation at most imprinted regions in mouse embryos based on bisulfite sequencing. Genomic DNA samples were isolated from four *Zfp57*$^{+/+}$ (M^+Z^+), four *Zfp57*$^{-/+}$ (M^-Z^+), and four *Zfp57*$^{-/-}$ (M^-Z^-) hybrid E13.5 embryos derived from the timed mating between *Zfp57*$^{+/-}$ (or *Zfp57*$^{-/-}$) 129 female mice and *Zfp57*$^{+/-}$ (DBA*) male mice mainly on the DBA/2J genetic background. These genomic DNA samples were subjected to bisulfite sequencing to examine DNA methylation at 21 maternally methylated (*A* and *B*) and 3 paternally methylated (*C*) ICRs of the known imprinted regions. We also examined DNA methylation at *Nesp* and *Zdbf2* DMRs (*D*). Average percentages of DNA methylation for four M^+Z^+ (light gray bars), four M^-Z^+ (dark gray bars), or four M^-Z^- (black bars) embryos are used for comparison at each ICR or DMR. Standard statistical analysis was performed for the pairwise comparisons and they were marked with one (*$P < 0.05$), two (**$P < 0.01$), or three (***$P < 0.001$) asterisks, or ns (not significant, $P > 0.05$). (*A*) DNA methylation was completely or partially lost at 17 maternally methylated ICRs of the known imprinted regions in M^-Z^- embryos. These include *Snrpn*, *Zac1*, *Peg1*, *Peg3*, *Peg13*, *Nespas*, *Gnas1A*, *Peg5*, *Inpp5f*, *Nap1l5*, *Zrsr1*, *Igf2r*, *Impact*, *Cdh15*, *Grb10*, *Mcts2*, and *AK008011* imprinted regions. (*B*) DNA methylation was retained at four maternally methylated ICRs in M^-Z^- embryos. These include *Peg10*, *Kcnq1ot1*, *Gpr1*, and *Slc38a4* ICRs. (*C*) DNA methylation was lost at two of three paternally methylated ICRs in M^-Z^- embryos. It was reduced at the *Rasgrf1* ICR and IG-DMR of the *Dlk1-Dio3* imprinted region, but not at the *H19* ICR. (*D*) DNA methylation was unexpectedly increased at the *Nesp* and *Zdbf2* DMRs.

Unexpectedly, DNA methylation was significantly increased at the *Nesp* and *Zdbf2* DMRs in M^-Z^- embryos compared with M^+Z^+ or M^-Z^+ embryos (Fig. 2*D* and *SI Appendix*, Tables S1 and S3). Loss of ZFP57 did not significantly impact DNA methylation at 20 other putative or somatic DMRs except for the *Meg3* DMR at the *Dlk1-Dio3* imprinted region (*SI Appendix*, Fig. S4*A* and Tables S1 and S3). Furthermore, DNA methylation was not lost at the long interspersed element (LINE)-1, LINE-2, short interspersed element (SINE), long terminal repeat (LTR), and intracisternal A particle (IAP) repetitive elements in *Zfp57* mutant embryos (*SI Appendix*, Fig. S4*B*). Indeed, no loss of DNA methylation was previously observed at LINE-1 and IAP repeats in *Zfp57* mutant embryos or ES cells either (27, 35). These results suggest that ZFP57 is essential for the maintenance of DNA methylation imprint at most known ICRs; however, it has almost no effect on other DMRs or repetitive elements which may be regulated differently in mouse embryos.

Allelic Expression of the Imprinted Genes Regulated by ZFP57-dependent Maternally Methylated ICRs. Exonic SNPs were identified for many imprinted genes by comparing CAST and 129 mouse genomes. Total RNA samples were isolated from live hybrid E13.5 embryos derived from the timed mating between *Zfp5*$^{+/-}$ or *Zfp57*$^{-/-}$ 129 female mice and *Zfp57*$^{+/-}$ 129/CAST hybrid male mice (*SI Appendix*, Fig. S1 *B* and *C*). Genomic DNA regions harboring exonic SNPs were sequenced to determine if the M^+Z^+, M^-Z^+, and M^-Z^- hybrid embryos contained a maternal 129 allele and a paternal CAST allele for these imprinted genes (Fig. 3 and *SI Appendix*, Fig. S5). The RT-PCR product of each imprinted gene was directly sequenced to gauge the percentages of transcripts derived from both alleles in M^+Z^+, M^-Z^+, and M^-Z^- 129/CAST hybrid embryos (Fig. 3, Table 1, and *SI Appendix*, Fig. S5). Total RNA samples were also isolated from M^+Z^+, M^-Z^+, and M^-Z^- hybrid 129/DBA E13.5 embryos derived from the timed mating between *Zfp5*$^{+/-}$ or *Zfp57*$^{-/-}$ 129 female mice and *Zfp57*$^{+/-}$ DBA* male mice (*SI Appendix*, Fig. S2*B*). RT-PCR analysis was similarly performed for the imprinted genes with exonic SNPs in M^+Z^+, M^-Z^+, and M^-Z^- 129/DBA hybrid embryos (Fig. 3, Table 1, and *SI Appendix*, Fig. S5).

Both *Atp10a* and *Snrpn* at the *Snrpn* imprinted region contain an exonic SNP (Fig. 3). *Snrpn* was expressed from the paternal allele in M^+Z^+ embryos, preferentially paternally expressed in M^-Z^+ embryos, but became biallelic in M^-Z^- embryos, (Fig. 3 and Table 1). This is consistent with loss of differential DNA methylation at the *Snrpn* DMR (Figs. 1*A* and 2*A* and *SI Appendix*, Fig. S3*A*). However, *Atp10a* was close to biallelic in M^+Z^+, M^-Z^+, and M^-Z^- embryos (Fig. 3). Therefore, loss of ZFP57 affected allelic expression of *Snrpn* but not *Atp10a* at the *Snrpn* imprinted region in mouse embryos.

We also determined allelic expression of 15 other imprinted genes controlled by ZFP57-dependent maternally methylated DMRs (Fig. 3, Table 1, and *SI Appendix*, Fig. S5). *Phactr2* is close to *Zac1* and we predict they may reside in the same *Zac1* imprinted region. Consistent with the COBRA and WGBS results (Figs. 1 and 2 and *SI Appendix*, Fig. S3), imprinted expression was lost for *Zac1* and *Phactr2* at *Zac1*, *Peg3* and *Zim1* at *Peg3*, *Trappc9* at *Peg13*, *Nespas* at *Nespas*, *Blcap* at *Peg5*, and

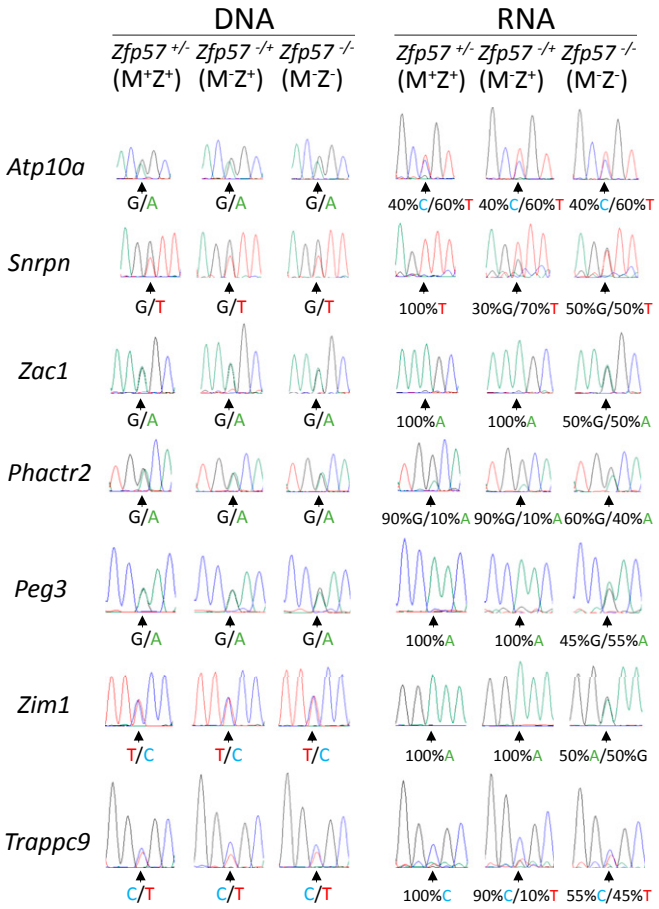

Fig. 3. Allelic expression analysis of the imprinted genes controlled by ZFP57-dependent maternally methylated ICRs in mouse embryos. Total RNA samples and genomic DNA samples were isolated from M^+Z^+, M^-Z^+, and M^-Z^- hybrid E13.5 embryos derived from the timed mating between *Zfp57*$^{+/-}$ (or *Zfp57*$^{-/-}$) 129 female mice and *Zfp57*$^{+/-}$ 129/CAST hybrid male mice or from the timed mating between *Zfp57*$^{+/-}$ (or *Zfp57*$^{-/-}$) 129 female mice and *Zfp57*$^{+/-}$ DBA* male mice (*SI Appendix*, Figs. S1 and S2). The hybrid embryos containing an exonic SNP in a given imprinted gene were confirmed by sequencing the PCR product of genomic DNA samples (*Left*). For example, "G/A" in *Atp10a* means that this embryo has G and A at this SNP of *Atp10a* for the maternal and paternal alleles, respectively. Then the total RNA samples were subjected to RT-PCR analysis followed by sequencing of the RT-PCR product to determine the relative allelic expression levels of this imprinted gene (*Right*). Either the sense or antisense strand was sequenced for *Atp10a* and *Snrpn* at the *Snrpn* imprinted region, *Plagl1(Zac1)* and *Phactr2* (presumably) at the *Zac1* imprinted region, *Peg3* and *Zim1* at the *Peg3* imprinted region, and *Trappc9* at the *Peg13* imprinted region. The estimated percentage is given next to the nucleotide for relative expression levels of two alleles based on the sequencing histograms.

Impact at *Impact* in M^-Z^- embryos compared with M^+Z^+ or M^-Z^+ embryos (Fig. 3 and *SI Appendix*, Fig. S5). However, *Gnas* retained its imprinted expression from the maternal allele in M^+Z^+, M^-Z^+, and M^-Z^- embryos although differential DNA methylation was lost at the *Gnas1A* ICR in M^-Z^- but not in M^+Z^+ or M^-Z^+ embryos (Figs. 1 and 2 and *SI Appendix*, Figs. S3 and S5). In contrast, *Cobl* was mostly biallelic in M^+Z^+, M^-Z^+, and M^-Z^- embryos although DNA methylation was partially lost at the *Grb10* ICR in M^-Z^- and M^-Z^+ embryos compared with M^+Z^+ embryos (Figs. 1 and 2 and *SI Appendix*, Figs. S3 and S5).

Allelic Expression of the Imprinted Genes Regulated by Maternally Methylated ICRs Regardless of ZFP57. We also analyzed the allelic expression of the imprinted genes controlled by maternally methylated ICRs that was not apparently affected in M^-Z^- embryos compared with M^+Z^+ or M^-Z^+ embryos (Table 2). These include *Dlx5* at *Peg10*, *Kcnq1* at *Kcnq1ot1*, *Zdbf2* and *Gpr1* at *Gpr1*, and *Slc38a4* at *Slc38a4*. We found that *Dlx5* was biallelic in M^+Z^+, M^-Z^+, and M^-Z^- embryos (*SI Appendix*, Fig. S6). Thus, *Dlx5* was biallelically expressed in mouse embryos irrespective of ZFP57. In contrast, *Kcnq1* and *Slc38a4* maintained exclusive monoallelic expression in M^+Z^+, M^-Z^+, and M^-Z^- embryos (*SI Appendix*, Fig. S6). *Zdbf2* at *Gpr1* became biallelic in M^-Z^- embryos, whereas it was mostly monoallelic in M^+Z^+ or M^-Z^+ embryos (*SI Appendix*, Fig. S6). However, *Gpr1* was partially biallelic in both M^-Z^+ and M^-Z^- embryos but only expressed from the maternal allele in M^+Z^+ embryos (*SI Appendix*, Fig. S6). Therefore, loss of maternal *Zfp57* caused maternally expressed *Gpr1* to become partially biallelic. Interestingly, there was no loss of DNA methylation at the *Gpr1* ICR but DNA methylation appeared to be increased at the *Zdbf2* DMR in M^-Z^- embryos compared with M^+Z^+ or M^-Z^+ embryos (Figs. 1 and 2 and *SI Appendix*, Fig. S3). These results indicate that allelic expression of *Gpr1* may not be regulated by DNA methylation at the known *Gpr1* ICR and DNA methylation at the *Zdbf2* DMR may be independent of the *Gpr1* ICR in mouse embryos.

In summary, allelic expression of these imprinted genes except *Zdbf2* and *Gpr1* at the *Gpr1* ICR was not affected when ZFP57 was absent in mouse embryos (Table 2). This is consistent with no significant loss of DNA methylation at these maternally methylated ICRs comparing M^-Z^- with M^+Z^+ or M^-Z^+ embryos (Figs. 1 and 2 and *SI Appendix*, Fig. S3). Regulation of allelic expression of *Zdbf2* and *Gpr1* may be independent of DNA methylation at the known *Gpr1* ICR, which will be discussed further in *Discussion*.

Allelic Expression of the Imprinted Genes in the Imprinted Regions with Paternally Methylated ICRs. We examined allelic expression of the imprinted genes in the *Igf2-H19*, *Rasgrf1*, and *Dlk1-Dio3* imprinted regions with paternally methylated ICRs (Fig. 4 and Table 3). *Igf2* was only expressed from the paternal allele in M^+Z^+, M^-Z^+, and M^-Z^- embryos, as there was no observed loss of DNA methylation at the *H19* ICR in M^-Z^- embryos (Figs. 1*C*, 2*C*, and 4 and *SI Appendix*, Fig. S3*C*). Unexpectedly, *Rasgrf1* was biallelically expressed in M^+Z^+ embryos although it was thought to be paternally expressed. It was exclusively expressed from the paternal allele in M^-Z^+ embryos but was only expressed from the maternal allele in M^-Z^- embryos (Fig. 4). Therefore, loss of maternal *Zfp57* in M^-Z^+ embryos caused *Rasgrf1* to be paternally expressed, whereas additional loss of zygotic *Zfp57* in M^-Z^- embryos, together with loss of maternal *Zfp57*, unexpectedly caused *Rasgrf1* to be maternally expressed. Further investigation is needed to explain the observed allelic switch of *Rasgrf1* in these embryos upon loss of maternal and/or zygotic *Zfp57*.

We also analyzed allelic expression of multiple imprinted genes at the *Dlk1-Dio3* imprinted region, including normally paternally expressed *Dlk1* and *Begain*, and maternally expressed *Rian* and *Mirg* (Fig. 4). *Dlk1* was paternally expressed in M^+Z^+ and M^-Z^+ embryos, but it was biallelic in M^-Z^- embryos (Fig. 4). Similarly, both *Rian* and *Mirg* were maternally expressed in M^-Z^+ embryos but were largely biallelic in M^-Z^- embryos (Fig. 4). *Begain* was preferentially expressed from the paternal allele in M^+Z^+ and M^-Z^+ embryos, but was biallelic in M^-Z^- embryos (Fig. 4). Therefore, ZFP57 maintains allelic expression of multiple imprinted genes at the *Dlk1-Dio3* imprinted region.

Quantifying Expression Levels of ICR-Regulated Imprinted Genes by qRT-PCR. We performed qRT-PCR analysis to assess expression levels of the imprinted genes in mouse embryos (Fig. 5). Total RNA samples were prepared from M^+Z^+, M^+Z^-, M^-Z^+, and M^-Z^- 129/DBA hybrid E13.5 embryos (*SI Appendix*, Fig. S2*B*

Table 1. The imprinted genes regulated by ZFP57-dependent maternally methylated ICRs

ICR	Imprinted genes, paternally (P) or maternally (M) expressed	Allelic expression in M^+Z^+ embryos	Allelic expression in M^-Z^+ embryos	Allelic expression in M^-Z^- embryos	Change of expression level in M^-Z^- vs. M^+Z^+ embryos	
Snrpn	*Atp10a* (M)	Almost biallelic	Almost biallelic	Almost biallelic	N/A	
	Snrpn (P)	Paternal	Preferentially Paternal	Biallelic	Up (twofold)	
Zac1	*Zac1* (*Plagl1*) (P)	Paternal	Paternal	Biallelic	Up (twofold)	
	Phactr2 (M)	Maternal (90%)	Maternal (90%)	Almost biallelic	N/A	
Peg1	*Mest* (P)	N/A	N/A	N/A	Up (less than twofold)	
	Klf14 (M)	N/A	N/A	N/A	Down	
Peg3	*Peg3* (P)	Paternal	Paternal	Biallelic	Up (twofold)	
	Zim1 (M)	Maternal	Maternal	Biallelic	Down	
Peg13	*Peg13* (P)	N/A	N/A	N/A	Up (less than twofold)	
	Trappc9 (M)	Maternal	Maternal (90%)	Biallelic	Down	
*Nespas**	(paternally?)	*Nespas* (P)	Paternal	Paternal	Preferentially paternal	Down
Gnas1A	*Gnas* (M)	Maternal	Maternal	Maternal	No change	
Peg5	*Peg5* (*Nnat*) (P)	N/A	N/A	N/A	Up (less than twofold)	
	Blcap (M)	Maternal (95%)	Preferentially maternal	Biallelic	Down	
Impact	*Impact* (P)	Paternal (90%)	Paternal (95%)	Biallelic	Up (less than twofold)	
Igf2r	*Igf2r* (M)	N/A	N/A	N/A	Down	
Grb10	*Cobl* (M)	Biallelic	Almost biallelic	Almost biallelic	N/A	

N/A, not applicable.
**Nespas* may be regulated by a paternally methylated ICR.

and *C*). First, we examined some imprinted genes regulated by maternally methylated ICRs (Fig. 5 and Tables 1 and 2). Expression of the paternally expressed *Snrpn* and *Zac1* (*Plagl1*), located at the respective *Snrpn* and *Zac1* imprinted regions, was increased about twofold in M^-Z^- embryos compared with M^+Z^+ embryos (Fig. 5). A bit less than twofold increase of their expression was observed in M^+Z^- embryos over M^+Z^+ embryos (Fig. 5). There was also significant increased expression of *Snrpn* and *Zac1* in M^-Z^+ compared with M^+Z^+ embryos (Fig. 5). Together with their biallelic expression in M^-Z^- embryos (Fig. 3), we hypothesize that the maternal alleles of these two imprinted genes behave like their paternal counterparts upon loss of ZFP57 (Fig. 6*A*).

Quantitative analyses were also performed for 13 other imprinted genes regulated by 8 maternally methylated ICRs (Fig. 5 and Tables 1 and 2). These include *Mest* and *Klf14* at *Peg1*, *Peg3* and *Zim1* at *Peg3*, *Peg13* and *Trappc9* at *Peg13*, *Nespas*, *Gnas* at *Gnas1A*, *Nnat* and *Blcap* at *Peg5*, *Igf2r*, *Zdbf2* and *Gpr1* at *Gpr1*, and *Impact* (Fig. 5). There was close to twofold increase for the expression of normally paternally expressed *Peg3*, *Zdbf2*, and *Impact* in M^-Z^- embryos compared with M^+Z^+ embryos, similar to what was observed for *Snrpn* and *Zac1*. There was also slightly increased expression of *Peg3*, *Zdbf2*, and *Impact* in M^+Z^- or M^-Z^+ embryos compared with M^+Z^+ embryos. About twofold increased expression of *Gpr1* was observed in M^-Z^+ or M^-Z^- embryos compared with M^+Z^+ embryos. Surprisingly, *Gpr1* expression was even more increased in M^+Z^- embryos (Fig. 5). Although DNA methylation was not lost at the *Gpr1* ICR, it was increased at the *Zdbf2* DMR (Figs. 1 and 2). Expression of *Peg13* and *Nnat* was significantly increased in M^-Z^- embryos compared with M^+Z^+ embryos. Expression of *Nnat* was also increased in M^+Z^- but not M^-Z^+ embryos. There was not much difference in *Peg13* expression in M^+Z^- or M^-Z^+ embryos compared with M^+Z^+ embryos. Together with the allelic expression analysis results (Fig. 3, Tables 1 and 2, and *SI Appendix*, Figs. S5 and S6), the maternal alleles of these imprinted genes controlled by maternally methylated ICRs seem to act like their counterparts on the paternal chromosomes without ZFP57 (Fig. 6*A*).

To our surprise, we observed decreased expression of paternally expressed *Nespas* in M^-Z^- embryos compared with M^+Z^+ or M^+Z^- or M^-Z^+ embryos (Fig. 5). *Nespas* was similarly expressed in M^+Z^-, M^-Z^+, and M^+Z^+ embryos. Interestingly, DNA methylation was lost at the *Nespas* ICR but increased at *Nesp* DMR. We think *Nespas* may harbor a paternally methylated ICR instead (*Discussion*). *Nespas* expression will decrease without ZFP57 if it is regulated by a paternally methylated ICR (*Discussion*).

We observed decreased expression of maternally expressed *Klf14*, *Zim1*, *Trappc9*, *Blcap*, and *Igf2r* in M^-Z^- embryos compared with M^+Z^+ embryos (Fig. 5 and Table 1). However, expression

Table 2. The imprinted genes controlled by maternally methylated ICRs regardless of ZFP57

ICR	Imprinted genes, paternally (P) or maternally (M) expressed	Allelic expression in M^+Z^+ embryos	Allelic expression in M^-Z^+ embryos	Allelic expression in M^-Z^- embryos	Change of expression level in M^-Z^- vs. M^+Z^+ embryos
Peg10	*Dlx5* (M)	Biallelic	Biallelic	Biallelic	N/A
Kcnq1ot1	*Kcnq1* (M)	Maternal	Maternal	Maternal	N/A
Gpr1	*Zdbf2* (P)	Paternal (95%)	Paternal (90%)	Biallelic	Up (twofold)
	Gpr1 (P*)	Maternal	Preferentially maternal	Preferentially maternal	Up (twofold)
Slc38a4	*Slc38a4* (P)	Paternal	Paternal	Paternal	N/A

N/A, not applicable.
**Gpr1* has been reported to be expressed from the paternal allele in wild-type mice.

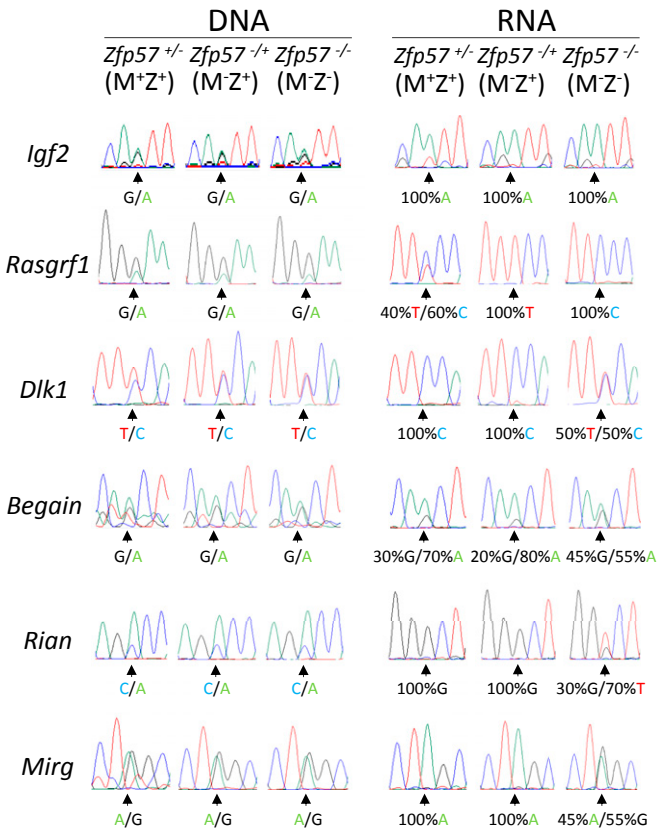

Fig. 4. Allelic expression of the imprinted genes regulated by paternally methylated ICRs in mouse embryos. Total RNA samples and genomic DNA samples were isolated from M^+Z^+, M^-Z^+, and M^-Z^- hybrid E13.5 embryos derived from the timed mating between *Zfp57*$^{+/-}$ (or *Zfp57*$^{-/-}$) 129 female mice and *Zfp57*$^{+/-}$ 129/CAST hybrid male mice or from the timed mating between *Zfp57*$^{+/-}$ (or *Zfp57*$^{-/-}$) 129 female mice and *Zfp57*$^{+/-}$ DBA* male mice (*SI Appendix*, Figs. S1 and S2). The hybrid embryos containing an exonic SNP in a given imprinted gene were confirmed by sequencing the PCR product of genomic DNA samples (*Left*). For example, "G/A" in *Igf2* means that this embryo has G and A at this SNP of *Igf2* for the maternal and paternal alleles, respectively. Then the total RNA samples were subjected to RT-PCR analysis followed by sequencing of the RT-PCR product to determine the relative allelic expression levels of this imprinted gene (*Right*). Either the sense or antisense strand was sequenced for *Igf2* at the *Igf2-H19* imprinted region, *Rasgrf1* at the *Rasgrf1* imprinted region, and *Dlk1*, *Begain*, *Rian*, and *Mirg* at the *Dlk1-Dio3* imprinted region. The estimated percentage is given next to the nucleotide for relative expression levels of two alleles based on the sequencing histograms.

of these maternally expressed imprinted genes was slightly reduced or unchanged or even up-regulated in M^+Z^- and M^-Z^+ embryos compared with M^+Z^+ embryos. *Gnas* was similarly expressed in M^+Z^+ and M^-Z^- embryos although it was slightly up-regulated in M^+Z^- and M^-Z^+ embryos, compared with M^+Z^+ or M^-Z^- embryos (Fig. 5). This is consistent with no difference in allelic expression of *Gnas* in M^-Z^+ and M^-Z^- embryos (*SI Appendix*, Fig. S5). Except for *Nespas* and *Gnas*, loss of ZFP57 caused down-regulation of maternally expressed and up-regulation of paternally expressed imprinted genes controlled by maternally methylated ICRs (Fig. 6*A* and Table 1).

We also performed qRT-PCR analyses of the imprinted genes regulated by paternally methylated ICRs (Fig. 5 and Table 3). The paternally expressed *Igf2* appeared to be slightly reduced in M^-Z^- and M^+Z^- embryos compared with M^+Z^+ or M^-Z^+ embryos (Fig. 5). This slight difference in *Igf2* expression may not mean much because there was no loss of *H19* ICR methylation or loss of monoallelic expression of *Igf2* without ZFP57 (Figs. 1*C* and 4 and *SI Appendix*, Fig. S3*C*). Expression of the paternally expressed *Rasgrf1* was significantly reduced in M^-Z^- embryos compared with M^-Z^+ embryos, but the differences were insignificant in other comparisons (Fig. 5). In contrast, the paternally expressed *Dlk1* was significantly reduced in M^-Z^- and M^+Z^- embryos when compared with M^+Z^+ or M^-Z^+ embryos. This reduction was more dramatic in M^-Z^- embryos (Fig. 5). Expression of the maternally expressed *Rian* at the same *Dlk1-Dio3* imprinted region was significantly increased in M^+Z^- and M^-Z^- embryos compared with M^+Z^+ embryos or M^-Z^+ embryos (Fig. 5). Therefore, loss of ZFP57 caused down-regulation of paternally expressed and up-regulation of maternally expressed imprinted genes regulated by paternally methylated ICRs (Table 3). Together with the allelic expression analysis results (Fig. 4), we propose that the paternal alleles of the imprinted genes regulated by paternally methylated ICRs act like their counterparts on the maternal chromosomes without ZFP57 (Fig. 6*B*).

Allelic Ratio and Gene-Expression Analyses by RNA-Seq. RNA-seq analysis was carried out to examine expression and allelic ratio changes of ZFP57 target genes in mouse embryos. Total RNA samples were isolated from six M^-Z^+ and six M^-Z^- hybrid E13.5 embryos derived from the timed mating between *Zfp57*$^{-/-}$ 129 female mice and *Zfp57*$^{+/-}$ DBA* male mice (*SI Appendix*, Fig. S2*C*), and then were subjected to RNA-seq followed by allelic expression analysis (ASE) (Fig. 7). The RNA transcripts of 498 genes were found to display on average 1.2-fold or more change in the allelic ratios with statistically significant difference when six M^-Z^- embryos were compared with the six M^-Z^+ embryos (Fig. 7*A* and *SI Appendix*, Table S5). Indeed, 14 known imprinted genes with exonic SNPs showed more than 1.2-fold difference in allelic expression ratios (Fig. 7*B*). Among them, *Phactr2*, *Snrpn*, *Impact*, *Peg3*, *Plagl1* (*Zac1*), and *Dlk1* had been found to display allelic ratio changes by above-mentioned allelic RT-PCR sequencing analysis (Figs. 3 and 4 and *SI Appendix*, Fig. S5). Since there are limited exonic SNPs between the mice on the 129 background and the mice on the DBA/2J background, we couldn't detect whether other imprinted genes may display similar allelic expression ratio changes by RNA-seq.

We also identified 114 differentially expressed genes (DEGs) based on RNA-seq analyses that displayed 1.5-fold or more change in RNA transcript levels with statistical significance when 6 M^-Z^+ embryos were compared with 6 M^-Z^- embryos, with 64 up-regulated and 50 down-regulated DEGs (*SI Appendix*, Fig. S7*A* and Table S6). Among them, 21 are known imprinted genes located in the imprinted regions (Figs. 1 and 2 and *SI Appendix*, Figs. S3 and S7*B*).

Effects of ZFP57 on NOTCH Signaling Pathway and Other Biological Processes. To gain further insights into the biological functions of ZFP57, we performed GO analyses for 498 genes with significant allelic ratio differences in M^-Z^- embryos compared with M^-Z^+ embryos (Fig. 7*C*). Interestingly, the NOTCH signaling pathway was 1 of the top 15 identified GO pathways and 2 additional related NOTCH signaling pathways were also found to be significantly perturbed based on GO analysis (Fig. 7 *C* and *D*). In total, eight genes in the NOTCH pathways showed allelic ratio changes in their RNA transcripts which include the *Dlk1* imprinted gene. Intriguingly, some genes in antigen presentation, metabolism, and neural development were also found to display allelic ratio differences in their transcripts upon loss of ZFP57 (Fig. 7*C*). Therefore, ZFP57 appears to regulate food response, nutrient transport, and metabolism based on GO analysis of 114 DEGs (*SI Appendix*, Fig. S7*C*). It may be also involved in a few neurological processes (*SI Appendix*, Fig. S7*C*).

Table 3. The imprinted genes regulated by paternally methylated ICRs

ICR	Imprinted genes, paternally (P) or maternally (M) expressed	Allelic expression in M^+Z^+ embryos	Allelic expression in M^-Z^+ embryos	Allelic expression in M^-Z^- embryos	Change of expression level in M^-Z^- vs. M^+Z^+ embryos
H19	*Igf2* (P)	Paternal	Paternal	Paternal	Down (*)
Rasgrf1[†]	*Rasgrf1* (P)	Almost Biallelic	Paternal	Maternal	Not significant
Dlk1-Dio3	*Dlk1* (P)	Paternal	Paternal	Biallelic	Down
	Begain (P)	Preferentially Paternal	Preferentially Paternal	Biallelic	N/A
	Rian (M)	Maternal	Maternal	Preferentially Paternal	Up (twofold)
	Mirg (M)	Maternal	Maternal	Biallelic	N/A

This slight reduction (*) of *Igf2* expression in M^-Z^- embryos compared with M^+Z^+ embryos may be subtle or indirect because there was no apparent loss of DNA methylation imprint at *H19* ICR or loss of monoallelic expression of *Igf2* comparing M^-Z^- embryos with M^+Z^+ embryos. N/A, not applicable.
[†]*Rasgrf1* has been reported to be expressed from the paternal allele in wild-type mice.

Discussion

One interesting finding of our study is that upon loss of ZFP57, there is an allelic expression switch of many target imprinted genes located on the chromosomes containing the methylated ICRs. The maternal alleles of the imprinted genes will act like their paternal counterparts when differential DNA methylation is lost at the maternally methylated ICRs (e.g., *Peg3* ICR) in *Zfp57* mutant embryos (Fig. 6*A*). We call it a maternal to paternal switch to describe the expression change of the maternal alleles of some target imprinted genes upon loss of ZFP57. In

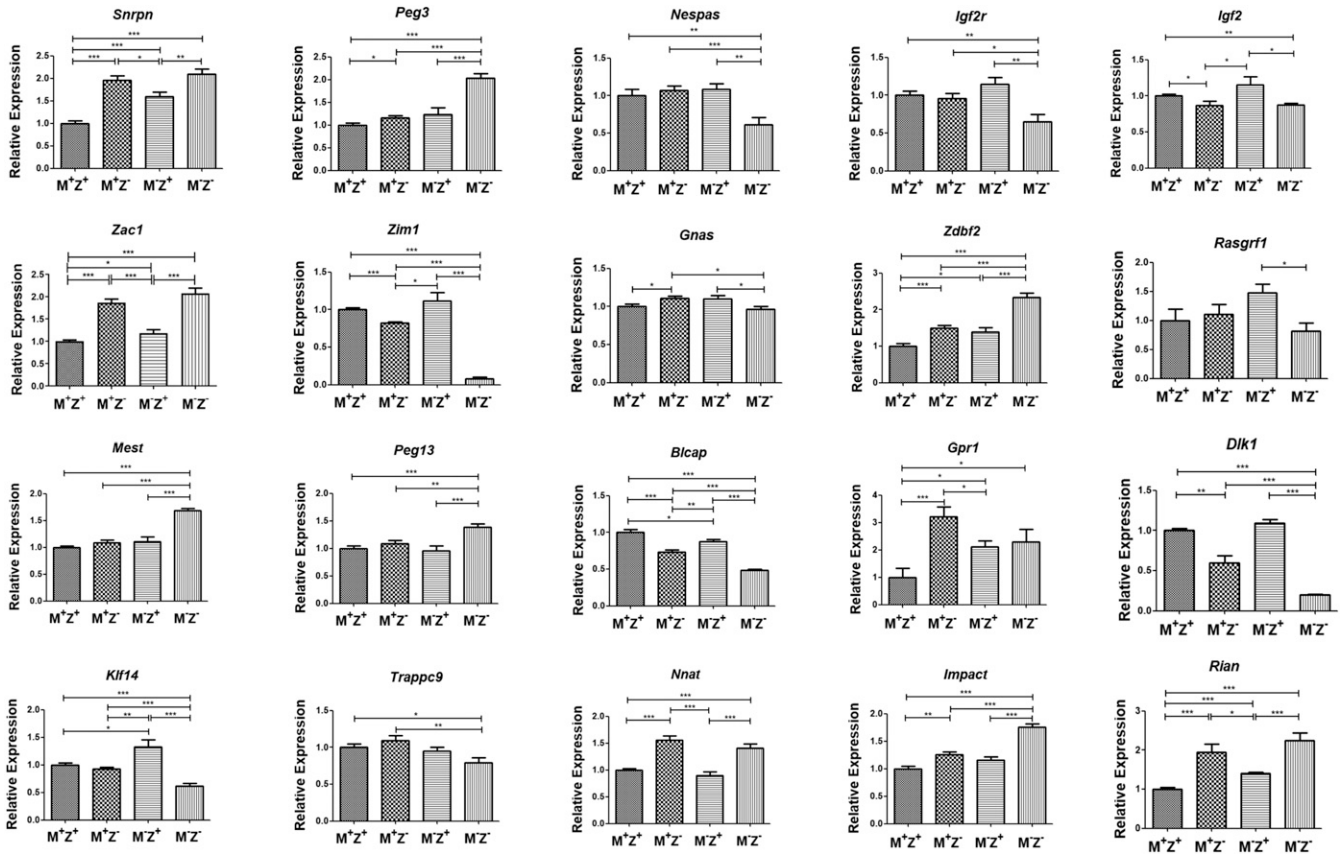

Fig. 5. Expression analysis of the imprinted genes by qRT-PCR. Total RNA samples were isolated from M^-Z^+ and M^-Z^- hybrid E13.5 embryos derived from the timed mating between *Zfp57*$^{-/-}$ 129 female mice and *Zfp57*$^{+/-}$ DBA* male mice (*SI Appendix*, Fig. S2). We also isolated total RNA samples from M^+Z^+ and M^+Z^- hybrid E13.5 embryos derived from the timed mating between *Zfp57*$^{+/-}$ 129 female mice and *Zfp57*$^{+/-}$ DBA* male mice (*SI Appendix*). Three independent embryos for each genotype (M^+Z^+, M^+Z^-, M^-Z^+, and M^-Z^-) were subjected to qRT-PCR analysis of the imprinted genes. We examined expression levels of 15 imprinted genes controlled by 11 maternally methylated ICRs. These include *Snrpn* at *Snrpn*, *Plagl1*(*Zac1*) at *Zac1*, *Mest* (*Peg1*) and *Klf14* at *Peg1*, *Peg3* and *Zim1* at *Peg3*, *Peg13* and *Trappc9* at *Peg13*, *Nespas* at *Nespas*, *Gnas* at *Gnas1A*, *Blcap* and *Nnat* (*Peg5*) at *Peg5*, *Igf2r* at *Igf2r*, *Zdbf2* and *Gpr1* at *Gpr1*, and *Impact* at *Impact*. We also examined expression levels of four imprinted genes controlled by three paternally methylated ICRs. These include *Igf2* at *H19*, *Rasgrf1* at *Rasgrf1*, and *Dlk1* and *Rian* at the *Dlk1-Dio3* imprinted region. Internal housekeeping gene *Gapdh* was used for normalization. Standard statistical analysis was performed for the pairwise comparisons and the statistically significant ones were marked with one (*$P < 0.05$), two (**$P < 0.01$), or three (***$P < 0.001$) asterisks.

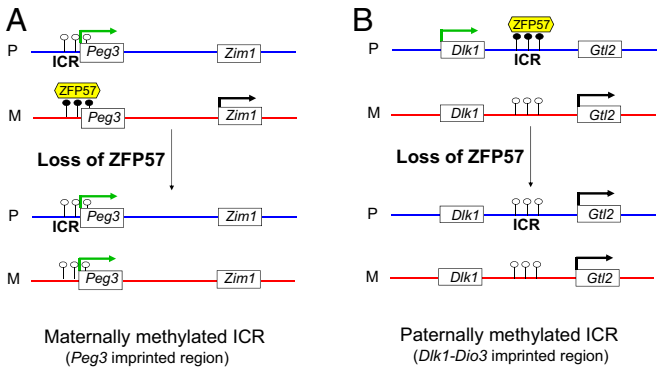

Fig. 6. Diagrams are shown for ZFP57-mediated regulation of DNA methylation imprint and allelic expression of the corresponding imprinted genes. We propose the following two alternative allelic switch models to illustrate the effects of ZFP57 on DNA methylation imprint and allelic expression of the corresponding imprinted genes at some target imprinted regions. The allelic switch may vary at a subset of imprinted regions or for a few imprinted genes. Some of them may not show a complete allelic switch. The *Peg3* and *Dlk1-Dio3* imprinted regions are used as the respective examples, with DNA methylation imprint inherited on either the maternal (M) or paternal (P) chromosome. Maternally methylated ICRs are usually located near the promoters of an imprinted gene (*A*), whereas paternally methylated ICRs usually reside in the intergenic regions of two imprinted genes (*B*). (*A*) ZFP57-dependent allelic switch for the imprinted genes at the *Peg3* imprinted region. *Peg3* and *Zim1* are normally expressed from the paternal or maternal allele, respectively. ZFP57 binds to the maternally methylated *Peg3* ICR and maintains DNA methylation imprint. Upon loss of ZFP57, DNA methylation imprint is lost at this *Peg3* ICR. Accordingly, both maternal and paternal alleles of *Peg3* are expressed whereas expression of *Zim1* is turned off at both alleles. It seems that the maternal alleles of these two imprinted genes act like their paternal alleles without ZFP57. Therefore, we call it maternal to paternal switch to describe the observed switch of allelic expression of the target imprinted genes of ZFP57 regulated by maternally methylated ICRs. (*B*) ZFP57-dependent allelic switch for the imprinted genes at the *Dlk1-Dio3* imprinted region. *Dlk1* and *Gtl2* are normally expressed from the paternal or maternal allele, respectively. ZFP57 binds to the paternally methylated IG-DMR and maintains DNA methylation imprint. Upon loss of ZFP57, DNA methylation imprint is lost at this paternally methylated ICR. As a result, *Gtl2* is biallelically expressed, whereas expression of *Dlk1* is turned off at both alleles. The paternal alleles of the imprinted genes seem to display similar allelic expression patterns to the maternal alleles. Similarly, we name it paternal to maternal switch to describe the observed switch of allelic expression of the target imprinted genes of ZFP57 controlled by paternally methylated ICRs.

contrast, the paternal alleles of some target imprinted genes will behave as if they were the maternal alleles when differential DNA methylation is lost at the paternally methylated ICRs (e.g., the IG-DMR of *Dlk1-Dio3*) in *Zfp57* mutant embryos, which we call a paternal to maternal switch (Fig. 6*B*). These allelic switch models are based on both DNA methylation analysis at the ICRs and expression analyses of the corresponding imprinted genes. Our models fit all ZFP57 target imprinted regions we examined with the exception of the *Nespas* and *Gpr1* imprinted regions, which are discussed below. We notice that the allelic switch may vary at a subset of imprinted regions (e.g., *Peg13*) and it may be incomplete for a few imprinted genes (*Peg13* and *Trappc9*) (Fig. 5). This may be due to incomplete loss of DNA methylation imprint at the *Peg13* ICR in *Zfp57* mutant embryos (Fig. 2*A*). Allelic switch can be further examined for these imprinted genes if DNA methylation imprint at the ICRs can be eliminated in the absence of both ZFP57 and ZFP445 or by any other means. The allelic switch models presented here may not apply to a few imprinted genes, such as *Gnas*, that may be regulated by DNA methylation-independent imprinting mechanisms (see the discussion below).

Nespas was reported to be controlled by a maternally methylated ICR (20). We found DNA methylation was lost at the known *Nespas* ICR in M^-Z^- embryos (Figs. 1 and 2 and *SI Appendix*, Fig. S3). Accordingly, expression of *Nespas* is expected to be increased in M^-Z^- embryos since the maternal allele of *Nespas*, together with the paternal allele, would be expressed upon loss of DNA methylation at the maternally methylated ICR (Fig. 6*A* and *SI Appendix*, Fig. S5). Surprisingly, *Nespas* expression was decreased in M^-Z^- embryos compared with M^+Z^+, M^-Z^+, or M^+Z^-embryos (Fig. 5). Therefore, we hypothesize that *Nespas* is regulated by a paternally methylated ICR instead (Table 1). Interestingly, a paternally methylated *Nespas* ICR may exist based on prior research (40–42). This needs to be further confirmed in future studies. We should also be aware that methylation at the *Nesp* DMR actually increased in M^-Z^- embryos (Fig. 2*D*). How this may affect *Nespas* expression is an interesting subject of future study.

There was no loss of DNA methylation imprint at the known *Gpr1* ICR in M^-Z^- or M^-Z^+ embryos compared with M^+Z^+ embryos (Figs. 1 and 2 and *SI Appendix*, Fig. S3). Surprisingly, *Gpr1* became partially biallelic in M^-Z^- or M^-Z^+ embryos and its expression was increased about twofold in M^-Z^- or M^-Z^+ embryos compared with M^+Z^+ embryos (Fig. 5 and *SI Appendix*, Fig. S6). These results fit the paternal to maternal switch if maternally expressed *Gpr1* is regulated by a paternally methylated ICR that need to be identified (Fig. 6*B*). Interestingly, the allelic expression and total expression levels of *Gpr1* were similarly affected in M^-Z^+ and M^-Z^- embryos compared with M^+Z^+ embryos (Fig. 5 and *SI Appendix*, Fig. S6). These results suggest that loss of just maternal *Zfp57* causes the allelic switch of *Gpr1* in mouse embryos, which is independent of zygotic *Zfp57*. Intriguingly, *Gpr1* expression appeared to be increased even more in M^+Z^- embryos, suggesting that zygotic *Zfp57* may also play a role in *Gpr1* expression (Fig. 5).

There was a twofold increase of *Zdbf2* expression in M^-Z^- embryos compared with M^+Z^+, M^-Z^+, or M^+Z^- embryos (Fig. 5). *Zdbf2* was mostly paternally expressed in M^+Z^+ or M^-Z^+ embryos and became biallelic in M^-Z^- embryos (Table 2 and *SI Appendix*, Fig. S6). There was increased DNA methylation at the *Zdbf2* DMR in M^-Z^- embryos compared with M^+Z^+ or M^-Z^+ embryos (Fig. 2*D*). Therefore, we hypothesize that increased methylation at the *Zdbf2* DMR caused the maternal allele of *Zdbf2* to be equally expressed in M^-Z^- embryos, similar to the paternal allele of *Zdbf2*, so that *Zdbf2* was biallelic with a twofold increase of expression in M^-Z^- embryos. In this case, loss of ZFP57 did not lead to loss of methylation at the *Gpr1* ICR, but still caused an increased methylation at the *Zdbf2* DMR that may be responsible for changed expression of *Zdbf2*.

ZFP57 maintains the DNA methylation imprint at a large subset of imprinted regions that is necessary for imprinted expression of the target imprinted genes (27, 39). However, no apparent loss of DNA methylation imprint was observed at some imprinted regions without ZFP57. It was reported that ZFP445 is partially redundant with ZFP57 in maintaining genomic imprinting in mouse embryos (39). It will be interesting to test our allelic switch models in other imprinted regions when both ZFP57 and ZFP445 are eliminated.

Another interesting finding is that *Gnas* maintained its monoallelic expression in M^+Z^+, M^-Z^+, or M^-Z^- embryos, although DNA methylation imprint was lost at the *Gnas1A* imprinted region in M^-Z^- embryos but not in M^+Z^+ or M^-Z^+ embryos (Figs. 1 and 2 and *SI Appendix*, Figs. S3 and S4). This implies that there might be some kind of DNA methylation-independent imprinting memory that maintains *Gnas* imprinted expression in mouse embryos. Interestingly, we reported similar findings at the *Snrpn* imprinted region in a previous study (27). Some imprinted genes also exhibited imprinted expression without DNA methylation in the placenta (43, 44). It is likely that other epigenetic modifications maintain *Gnas* imprinted expression independent of DNA methylation.

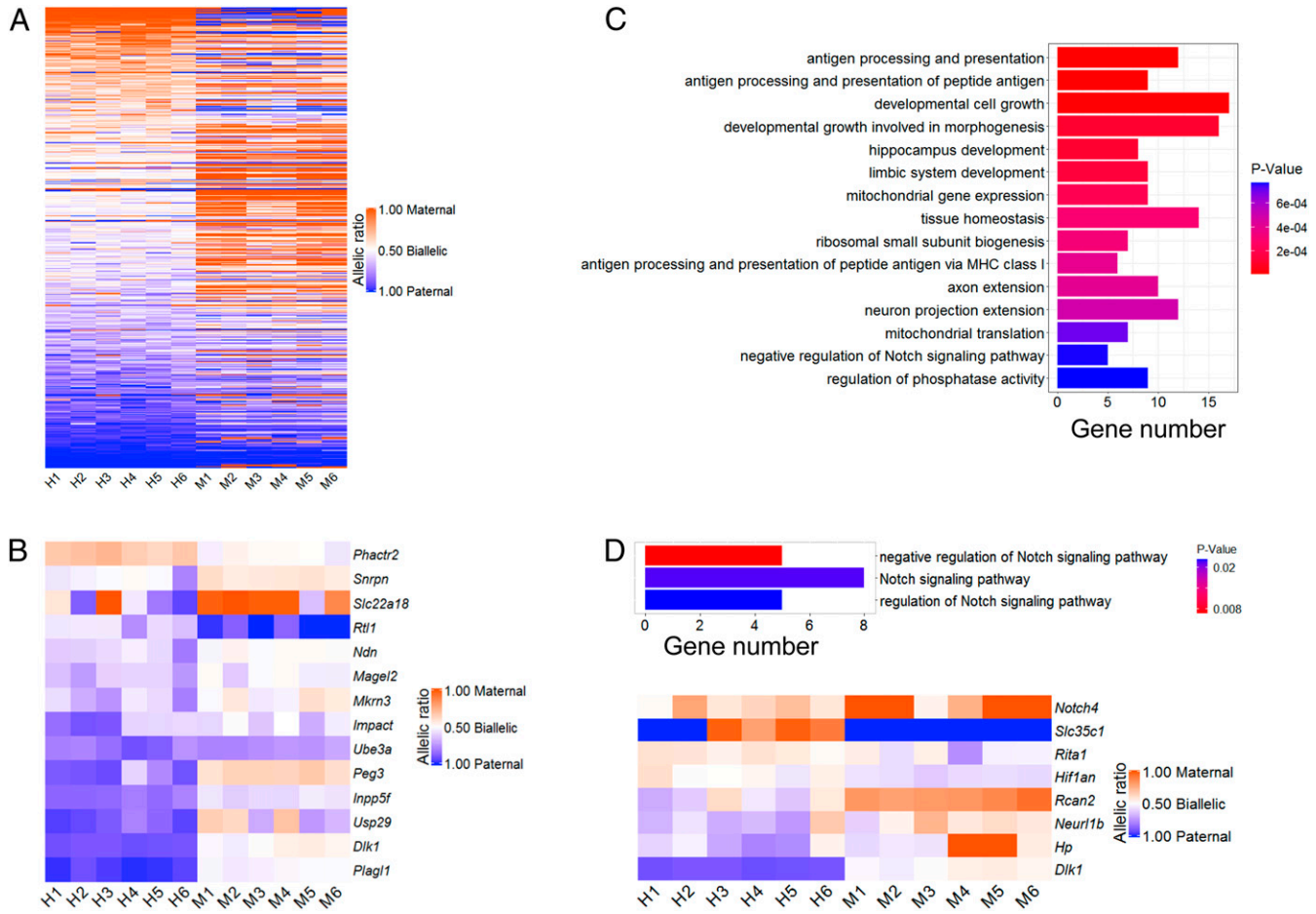

Fig. 7. Loss of ZFP57 caused allelic changes in expression of some known imprinted genes as well as some other genes in mouse embryos. Total RNA samples were isolated from six M^-Z^+ (H1–H6) and six M^-Z^- (M1–M6) hybrid E13.5 embryos derived from the timed mating between *Zfp57*$^{-/-}$ 129 female mice and *Zfp57*$^{+/-}$ DBA* male mice (*SI Appendix*, Fig. S2). RNA-seq analysis was performed for these embryos. The heat maps are used in *A*, *B*, and *D* to show the relative allelic expression ratios of the genes in each embryo, with red color indicating only expressed from the maternal allele (value = 1) and blue color only expressed from the paternal allele (value = 1). The *P* values of the GO analyses increase when the color changes from red to blue in *C* and *D*. (*A*) A total of 498 genes were found to exhibit 1.2-fold or more change on average in the allelic expression ratios comparing 6 M^-Z^- (M1-M6) with 6 M^-Z^+ (H1-H6) embryos. (*B*) Among 498 genes in *A*, 14 are known imprinted genes. (*C*) Top 15 processes or components are listed based on GO analysis of 498 genes with allelic ratio change of 1.2-fold or more. (*D*) Three NOTCH signaling pathway related components or processes, with a total of 8 genes, were found to be significantly different after GO analysis of 498 genes with allelic ratio change of 1.2-fold or more. (*Upper*) Three NOTCH signaling pathways in the GO databases are shown here with a *P* value less than 0.05. (*Lower*) Eight genes in the NOTCH signaling pathway, including the imprinted *Dlk1* gene, exhibited significant changes in the allelic ratios upon loss of ZFP57 in mouse embryos.

Some imprinted genes exhibit tissue-specific imprinted expression (8, 45–47). *Cobl* at the *Grb10* imprinted region was reported to be only imprinted in mouse yolk sac and we found it was biallelic in mouse embryos (*SI Appendix*, Fig. S5) (48). Imprinting may vary for some imprinted genes in different species (8, 45). *Atp10a* is imprinted in human brains but not in mouse brains (49, 50). Indeed, expression of *Atp10a* was similarly biallelic in M^+Z^+, M^-Z^+, and M^-Z^- embryos although *Snrpn* at the same *Snrpn* imprinted region was expressed from the paternal allele in M^+Z^+ embryos, and preferentially paternally expressed in M^-Z^+ embryos, but became biallelic in M^-Z^- embryos (Figs. 3 and 7*B*). *Dlx5* is also imprinted in the human brain but not in the mouse brain (51, 52). It was biallelic in mouse embryos regardless of ZFP57 (*SI Appendix*, Fig. S6). Therefore, it is possible that ZFP57-dependent regulation of some imprinted genes may vary in different tissues or species.

Surprisingly, we found expression of *Rasgrf1* was biallelic in M^+Z^+ embryos. But it became paternally expressed when maternal *Zfp57* was lost in M^-Z^+ embryos. Intriguingly, *Rasgrf1* was switched to become maternally expressed upon complete loss of ZFP57 in M^-Z^- embryos. It was exclusively expressed from the paternal allele in M^-Z^+ embryos, but exclusively expressed from the maternal allele in M^-Z^- embryos (Fig. 4). This is in stark contrast to the biallelic expression observed in other target imprinted genes without ZFP57 (Figs. 3 and 4). It might indicate a unique mechanism for imprinted expression of *Rasgrf1*. Interestingly, the PIWI-interacting RNA (piRNA) pathway is reported to be required for DNA methylation of the *Rasgrf1* DMR but not required for other paternally methylated ICRs (53). Further investigation is needed to determine how ZFP57 regulates *Rasgrf1*.

Interestingly, some genes in the NOTCH signaling pathway showed allelic ratio differences in their RNA transcripts without ZFP57 (Fig. 7 *C* and *D*). This is consistent with our previous study indicating that ZFP57 modulates NOTCH signaling during heart development in mouse embryos (38). Among them, one is the imprinted *Dlk1* gene. DLK1 has been shown to act as a negative or positive regulator in multiple systems (38, 54, 55). It has both membrane-bound and secreted isoforms that may have distinct functions (54). Another is *Neuralized 1b* (*Neurl1b*), which

has been proven to be an important regulator in NOTCH signaling (56). Intriguingly, NOTCH4, one of four mammalian NOTCH receptor proteins, was also shown to change allelic expression ratio without ZFP57. These possibilities can be tested in future studies.

We noticed that three imprinted genes (*Rtl1* at the *Dlk1-Dio3* region, *Ube3a* at the *Snrpn* region, and *Slc22a18* at the *Kcnq1ot1* region) displayed allelic ratio differences in allelic RNA-seq analysis (Fig. 7*B*). They were expected to be expressed from the paternal allele of *Rtl1*, maternal allele of *Ube3a*, and maternal allele of *Slc22a18*, respectively, in M^-Z^+ embryos based on their reported expression in the wild-type mice. However, they were found to be all preferentially expressed from the paternal alleles in M^-Z^+ embryos (Fig. 7*B*). In M^-Z^- embryos, *Rtl1* became more preferentially expressed from the paternal allele and *Slc22a18* switched to become preferentially expressed from the maternal allele, whereas expression from the paternal allele of *Ube3a* decreased (Fig. 7*B*). Their allelic ratio changes were unexpected. Although the reason is presently unknown, it is noteworthy that noncoding RNA (ncRNA) transcripts are widely expressed from these imprinted regions and some are reported to be transcribed from the opposite strands of imprinted genes. Future strand-specific RNA-seq analysis may be needed to distinguish the transcripts of these imprinted genes from other transcripts derived from the opposite strands.

Materials and Methods

Mouse Breeding and Timed Pregnancy Mating. The animal protocol was approved by the Institutional Animal Care and Use Committee of the ShanghaiTech University. The mice were housed in the animal facility of Protein Center of Shanghai Zhangjiang Laboratory. *Zfp57* mutant mice containing the deleted null allele of *Zfp57* on the 129 genetic background that we generated before were used for crosses to obtain the embryos from the timed pregnancy mating (27).

First, $Zfp57^{+/-}$ 129 female mice were mated with the wild-type CAST male mice obtained from the Jackson Laboratories to generate $Zfp57^{+/-}$ 129/CAST hybrid male mice (*SI Appendix*, Fig. S1). Then these $Zfp57^{+/-}$ 129/CAST hybrid male mice were used in the timed pregnancy mating with $Zfp57^{+/-}$ or $Zfp57^{-/-}$ 129 female mice to produce 129/CAST hybrid E13.5 embryos, with about half of the 129/CAST embryos containing one 129 allele and one CAST allele at each locus of interest. These 129/CAST hybrid E13.5 embryos were used for DNA methylation analysis and RT-PCR analysis.

$Zfp57^{+/-}$ mice on the 129 genetic background were backcrossed with the wild-type DBA/2J mice obtained from the Jackson Laboratories 12 times to obtain the $Zfp57^{+/-}$ mice mainly on the DBA/2J genetic background which we named $Zfp57^{+/-}$ (DBA*), meaning that it is almost as if they were on the pure DBA/2J genetic background (*SI Appendix*, Fig. S2). Then the $Zfp57^{+/-}$ (DBA*) male mice were used to set up the timed mating with $Zfp57^{+/-}$ or $Zfp57^{-/-}$ 129 female mice to generate $Zfp57^{+/+}$ (M^+Z^+), $Zfp57^{+/-}$ (M^+Z^+), $Zfp57^{-/+}$ (M^+Z^+), $Zfp57^{-/-}$ (M^+Z^-), $Zfp57^{-/+}$ (M^-Z^+), and $Zfp57^{-/-}$ (M^-Z^-) 129/DBA hybrid E13.5 embryos, containing one 129 allele and one DBA/2J allele at each locus of interest. Genomic DNA and total RNA samples were harvested from these 129/DBA hybrid E13.5 embryos for DNA methylation, RNA-seq, and RT-PCR analyses.

COBRA of the ICRs in the Imprinted Regions. Genomic DNA samples were harvested from the 129/CAST or 129/DBA hybrid E13.5 embryos. Then they were subjected to bisulfite mutagenesis with the EZ DNA methylation-Gold Kit (Zymo Research #D5006). A portion of bisulfite-treated DNA samples was used for PCR amplification with the primers that matched with the sequences located at the DMRs of the imprinted regions after bisulfite mutagenesis. Then a nested PCR was performed to obtain a specific PCR product before it was subjected to restriction enzyme digestion with restriction enzymes recognizing the CpG sites within the amplified PCR product.

Bisulfite Sequencing of the ICRs, DMRs, and Repetitive Elements. The genomic DNA samples isolated from 129/DBA hybrid E13.5 embryos were subjected to WGBS. After sequencing, low-quality reads were removed from the raw reads by Fastp v0.20.0 (https://github.com/OpenGene/fastp). This was followed by the analysis with Trim Galore (v0.4.1, http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to trim the adaptor sequences from the sequence reads in order to obtain the clean reads. Then all clean reads were mapped to the mm10 mouse reference genome by Bismark (v0.15.0; bowtie v2.2.9) (57). The mapped reads were further deduplicated by Bismark and the mapped unique reads were used for CpG methylation calling afterward (57). DNA methylation was examined and quantified for the CpG sites located within 24 ICRs and other DMRs as well as the repetitive elements (Fig. 2 and *SI Appendix*, Fig. S4 and Table S1). Please refer to *SI Appendix*, Table S2 for the information regarding the sequenced reads, mapped reads and bisulfite conversion rates of these samples. Only the CpG sites that were covered at least three times were used for further analysis (*SI Appendix*, Table S3). The total coverages of unique reads are over 72 million for M^+Z^+, over 154 million for M^-Z^+, and over 187 million for M^-Z^- samples (*SI Appendix*, Table S2). The DNA methylation level was calculated for each CpG site first based on total methylated counts divided by total counts for this CpG site, and then average DNA methylation level was calculated for all CpG sites within each ICR or DMR or repetitive element (Fig. 2 and *SI Appendix*, Fig. S4 and Table S3). The group average DNA methylation levels were obtained for each embryo group and used for group comparisons across these ICRs, DMRs, and repetitive elements (Fig. 2 and *SI Appendix*, Fig. S4).

Allelic Expression Analysis of the Imprinted Genes by RT-PCR. Total RNA samples were harvested from the 129/CAST or 129/DBA hybrid E13.5 embryos. Then they were subjected to first-strand cDNA synthesis and RT-PCR analysis. The specific RT-PCR product was directly sequenced to determine the allelic expression of the imprinted genes.

qRT-PCR of the Imprinted Genes. After total RNA samples were harvested from three independent 129/DBA hybrid E13.5 embryos of each genotype, they were subjected to first-strand cDNA synthesis and qRT-PCR analysis. The expression levels of the imprinted genes were normalized to those of the internal housekeeping gene *Gapdh*. Standard statistical analysis was performed for the pairwise comparisons of the relative expression levels of the imprinted genes in these four types of embryos. The statistically significant comparisons were marked with one ($*P < 0.05$), two ($**P < 0.01$), or three ($***P < 0.001$) asterisks in the histograms.

RNA-Seq Analysis. Total RNA samples isolated from 129/DBA hybrid E13.5 embryos were subjected to RNA-seq analysis (*SI Appendix*, Table S4–S6). For allelic expression analysis, transcripts per million was calculated for all genes and only the ones of the mean transcripts per million value over 1 were subjected to further analysis. The percentages of RNA transcripts expressed from the maternal alleles were used to measure allelic ratio changes of the imprinted genes and other genes with exonic SNPs in RNA-seq (*SI Appendix*, Table S5).

For DEGs in RNA-seq, the gene-expression level was calculated based on fragments per kilobase of exon model per million mapped reads (FPKM). Only the genes of mean FPKM value over 1 were used for identification of DEGs (*SI Appendix*, Table S6). Please refer to *SI Appendix*, Table S4 for the information regarding the sequenced reads, mapped reads, and mapping ratios of these RNA-seq samples, as well as *SI Appendix* for more detailed information.

Data Availability. All study data are included in the article and supporting information. The sequences reported in this article have been deposited in the Gene Expression Omnibus (accession no. GSE165079).

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