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# Imprinted DNA methylation reprogramming during early mouse embryogenesis at the *Gpr1-Zdbf2* locus is linked to long *cis*-intergenic transcription

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ABSTRACT

**The paternally-expressed imprinted genes *Gpr1* and *Zdbf2* form a gene cluster wherein the imprinted-methylated regions of these two genes differ. We identified a novel, paternally expressed, long intergenic non-coding *Zdbf2* variant (*Zdbf2linc*) transcribed from maternally methylated *Gpr1* DMR during early embryogenesis in the mouse. While the *Gpr1* DMR displayed biallelic hypermethylation, *Zdbf2linc* expression was rarely observed in the post-gastrulation, despite a positive correlation between the methylation of *Zdbf2* DMRs and the mono-allelic transcription of the original *Zdbf2* coding variant. Furthermore, lack of the maternal methylation imprint resulted in the biallelic expression of both coding and non-coding *Zdbf2* transcripts as well as complete methylation of *Zdbf2* DMRs. Globally, our findings suggest the role of *Zdbf2linc* in the establishment of secondary epigenetic modifications after implantation.**


## 1. Introduction

Functional non-equivalence between parental genomes in mammals is caused by parental origin-specific mono-allelic expression of certain genes, termed "imprinted" genes [1]. To date, over one hundred imprinted genes have been identified in humans and mice (MRC Mammalian Genetics Unit, Harwell, UK, http://www.har.mrc.ac.uk/research/genomic_imprinting), most of which form clusters in specific genomic regions responsible for the abnormal phenotypes of uniparental disomies. Imprinted gene clusters are marked epigenetically and imprinted differentially in the parental germline, depending on the parent. Differentially methylated regions (DMRs), which exhibit parent-of-origin-dependent DNA methylation patterns, have been identified within or near imprinted genes. Two classes of DMRs have been identified: germline DMRs (primary DMRs), which acquire methylation during gametogenesis, and somatic DMRs (secondary DMRs), which establish their allelic methylation patterns after fertilization, most likely through the influence of the germline DMRs at each gene cluster [2–6]. Gene knockout experiments in mice point to the involvement of DNA methylation in this process. De novo methyltransferase *Dnmt3a* and de novo methyltransferase-related protein *Dnmt3L* are required for the establishment of primary methylation imprints in paternal and maternal germlines, as well as the mono-allelic expression patterns of imprinted genes in the embryo proper [7–9]. The targeted deletion of some germline DMRs in mice has also been found to result in the aberrant expression of single or several associated imprinted genes, as well as the loss of the allelic methylation of secondary DMRs [10–19]. Such germline DMRs are called imprinting control regions (ICRs), and act as long-range *cis*-acting regulatory elements.

An imprinted gene cluster containing the *Gpr1* (G protein-coupled receptor 1) and *Zdbf2* (zinc finger, DBF-type containing 2) genes has been identified in mouse chromosome 1; both are specifically expressed from the paternal allele [20–22]. *Zdbf2* was found to have imprinted expression in various embryonic and adult tissues, whereas *Gpr1* showed kidney-specific imprinted expression (Fig. 1A). However, functional roles of these genes in biological processes remain undetermined. Multiple methylome analyses have identified two kinds of imprinted methylated regions in this cluster: maternal allele-specific methylation at a CpG island in *Gpr1* intron 2 (*Gpr1* DMR), and paternal allele-specific

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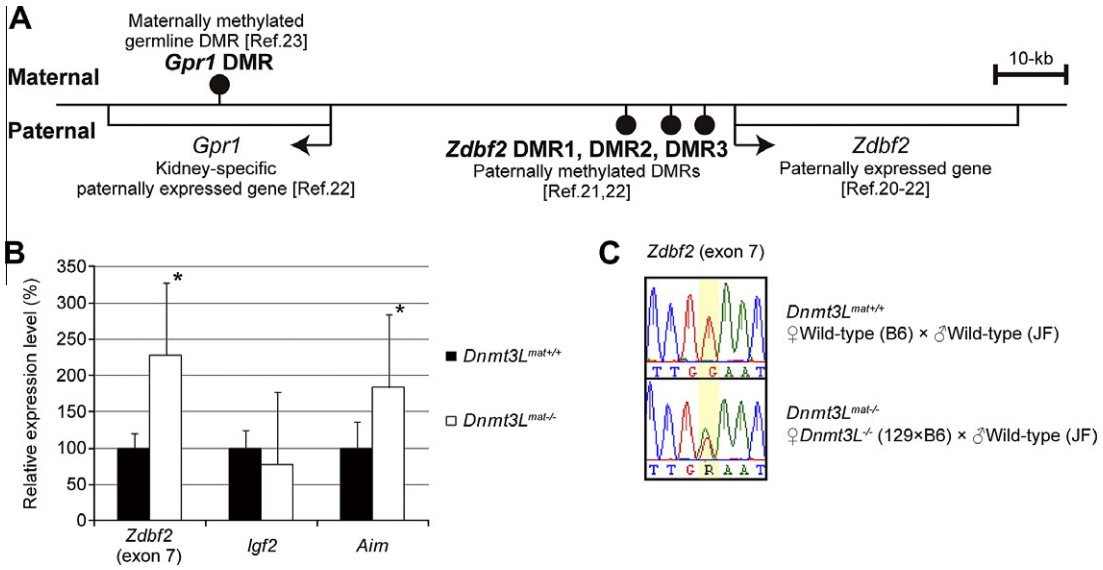


**Fig. 1.** Comparison of *Zdbf2* gene expression in wild-type and $Dnmt3L^{mat-/-}$ embryos. (A) Schematic representation of the imprinted *Gpr1-Zdbf2* domain on mouse chromosome 1C2. Open boxes represent the locations of the two paternally expressed genes; arrows denote the transcriptional direction of these genes. The positions of the differentially methylated regions (DMRs) are indicated by filled pins on the methylated alleles. (B) Quantitative real-time RT-PCR expression analysis of paternally expressed genes *Zdbf2* (exon 7), *Igf2*, and *Airn* in E9.5 mouse embryos from $Dnmt3L^{mat+/+}$ and $Dnmt3L^{mat-/-}$ mice ($n \geqslant 3$). Expression levels of each gene were normalized to those of the housekeeping gene *Gapdh*. Error bars represent SEM ($n = 3$), while asterisks indicate $P < 0.01$ (Student's *t*-test). *Igf2* and *Airn* are regulated by paternal (*H19* DMR) and maternal (*Igf2r* DMR2) methylation imprints, respectively. (C) Allelic expression analysis of *Zdbf2* was performed using $F_1$ hybrid crosses between wild-type C57BL/6 (B6) or *Dnmt3L*-deficient 129SvJae × C57BL/6N (129 × B6) females and JF1/Msf (JF) males. Maternal and paternal alleles were distinguished by an SNP (A/G at chr1. 63,361,080; highlighted in yellow).

methylation at the intergenic regions between the *Gpr1* and *Zdbf2* genes (*Zdbf2* DMR1, DMR2, and DMR3) [21–23]. However, the identity of the DMR that acts as a true ICR on the *Gpr1-Zdbf2* locus remains unknown.

Similar to the *Gpr1-Zdbf2* gene cluster, three other imprinted gene clusters, *Gnas-Nespas*, *Kcnq1ot1*, and *Igf2r-Airn*, have been found to have contradicting imprinted methylation. In these clusters, maternal allele-specific methylations act as ICRs, while the long non-coding RNAs transcribed from them have a role in silencing genes in *cis* through gene- and lineage-specific repressive chromatin modifications [24–26]. Other DMRs may additionally become methylated on the paternal allele after fertilization as somatic DMRs. Furthermore, recent studies have shown that transcription through *Gnas* DMRs is required for their allele-specific methylation to be established during pre- or post-fertilization [26,27]. It is important to elucidate the relationship between imprinted methylation and gene expression profiles on a gene cluster in order to understand the mechanisms by which imprinted expression is regulated.

In this study, we identified a novel, long (>100 kb), imprinted non-coding *Zdbf2* variant transcribed from the *Gpr1* DMR in mice, which showed paternal-allele-specific expression in early embryonic cells. Its expression was found to be associated with the methylation of the *Gpr1* DMR in *cis*. Our results indicate that maternal-allele-specific methylation of the *Gpr1* DMR may directly regulate the imprinted expression of this long non-coding RNA. The transcription of the *Zdbf2* variant may be essential for the establishment of the somatic intergenic differential methylation of *Zdbf2* DMRs that regulates imprinted *Zdbf2* expression after implantation.

## 2. Materials and methods

### 2.1. Sample preparation

Mouse early embryos, embryonic stem (ES) cells, and adult tissues were prepared as described previously [6,21,28]. To use DNA polymorphisms for allele discrimination, wild-type reciprocal $F_1$ hybrids ($Dnmt3L^{mat+/+}$) were obtained by crossing C57BL/6 (B6; Clea Japan, Tokyo, Japan) and JF1/Msf (JF) mice. $Dnmt3L^{mat-/-}$ embryos were obtained by crossing *Dnmt3L*-deficient female (129SvJae × B6 hybrid genetic background) and wild-type JF mice. ES cells were derived from B6 mice (Clea Japan).

### 2.2. Real-time RT-PCR and allelic expression analyses

Total RNA from E3.5 blastocysts, E5.5 whole embryos, E6.5, E7.5 embryonic tissues (extra-embryonic tissues were removed), and E9.5 whole embryos (yolk sacs and amnions were removed) [6], and ES cells was isolated using the Allprep DNA/RNA micro kit (Qiagen, Valencia, CA) and TRIzol reagent (Invitrogen, Carlsbad, CA). Genomic DNA-free total RNA was reverse transcribed to cDNA with SuperScript III (Invitrogen). Quantitative analysis for *Igf2*, *Airn*, and *Zdbf2* gene expression was performed in a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA) with SYBR Green PCR Master Mix (Applied Biosystems). Relative expression levels of each gene were normalized to those of the *Gapdh* or *Actb* housekeeping genes. RT-PCR and direct sequencing for *Zdbf2* variants were performed using an ABI PRISM 3730xl genetic analyzer (Applied Biosystems), as described earlier [21]. Primer sequences and PCR conditions are listed in Table 1.

### 2.3. Rapid amplification of cDNA ends analysis

The 5′-region of the mouse *Zdbf2* gene was obtained using the GeneRacer Kit (Invitrogen). Total RNA was prepared from ES cells, and two rounds of PCR were carried out using TaKaRa EX Taq (TaKaRa) under the following conditions: 25 cycles of 30 s at 94 °C, 30 s at 64 °C, 1 min at 72 °C for the first PCR; and 20 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C for the second PCR. The *Zdbf2* gene-specific primer sets used for the nested PCR were as follows: anti-sense 5RA8: 5′-AGCTGAGGACCCGGAATCCT CACAT-3′ for the first PCR and anti-sense 5RA9: 5′-TGAGGACCCGG AATCCTCACATGGT-3′ for the second PCR. The amplified products were sequenced directly, after purification.