—Original Article—

Deletion of conserved sequences in IG-DMR at *Dlk1-Gtl2* locus suggests their involvement in expression of paternally expressed genes in mice

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Abstract. Expression regulation of the *Dlk1-Dio3* imprinted domain by the intergenic differentially methylated region (IG-DMR) is essential for normal embryonic development in mammals. In this study, we investigated conserved IG-DMR genomic sequences in eutherians to elucidate their role in genomic imprinting of the *Dlk1-Dio3* domain. Using a comparative genomics approach, we identified three highly conserved sequences in IG-DMR. To elucidate the functions of these sequences *in vivo*, we generated mutant mice lacking each of the identified highly conserved sequences using the CRISPR/Cas9 system. Although mutant mice did not exhibit the gross phenotype, deletions of the conserved sequences altered the expression levels of paternally expressed imprinted genes in the mutant embryos without skewing imprinting status. These results suggest that the conserved sequences in IG-DMR are involved in the expression regulation of some of the imprinted genes in the *Dlk1-Dio3* domain.
Key words: CRISPR/Cas9, DNA methylation, Genomic imprinting, Intergenic differentially methylated region (IG-DMR), Mouse

Genomic imprinting is an epigenetic mechanism that results in parental-specific expression of genes inherited from the father and mother. In vertebrates, genomic imprinting is found only in eutherian and marspials. Loss of genomic imprinting leads to embryonic lethality due to abnormal expression of imprinted genes, which are expressed parent-of-origin [1, 2]. Therefore, parent-of-origin-specific expression of imprinted genes is essential for mammalian development. Most imprinted genes form clusters and are regulated by *cis*-acting imprinting control regions (ICRs) [3]. ICRs are differentially methylated regions (DMRs) that exhibit allele-specific DNA methylation patterns [3] established during gametogenesis and maintained throughout embryonic development [4–6]. To date, three paternally methylated DMRs (inherited sperm-specific DNA methylation) and 19 maternally methylated DMRs (inherited oocyte-specific DNA methylation) have been identified in mice [7].

The *Dlk1-Dio3* domain is an imprinted gene cluster located on mouse chromosome 12 (Chr12) and has been identified by producing mouse embryos with uniparental disomy of Chr12 [8]. The *Dlk1-Dio3* domain contains paternally expressed protein-coding genes

(*Dlk1*, *Rtl1*, and *Dio3*), maternally expressed non-coding RNAs, including microRNA clusters (*Gtl2*, *Rtl1as*, *Rian*, and *Mirg*), and the ICR, whose methylation pattern is derived from the sperm (Fig. 1A) [9–11]. It has been reported that the ~ 8-kb intergenic DMR (IG-DMR) located between *Dlk1* and *Gtl2* acts as the ICR of the *Dlk1-Dio3* domain. Analysis of knockout (KO) mice lacking the 4.1-kb IG-DMR showed that this region regulates allele-specific expression of imprinted genes [12]. Maternal inheritance of the deleted allele resulted in embryonic lethality due to biallelic expression of paternally expressed genes and downregulation of maternally expressed genes. On the other hand, paternal inheritance of the deleted allele did not cause embryonic defects [12]. Therefore, hypomethylation of the maternal IG-DMR is functionally essential for embryonic development. However, the precise molecular mechanisms that determine how IG-DMR regulates allele-specific expression of imprinted genes remain largely unclear, given that the IG-DMR is too large for molecular analysis and affects expression of other genes that are located at up to a distance of ~ 1 Mb.

Considering that the *Dlk1-Dio3* domain is imprinted in all eutherian species examined so far [13], the molecular mechanisms underlying IG-DMR function are likely to be conserved among eutherians. To gain insights into IG-DMR function, we searched for conserved sequences in the IG-DMR of eutherians based on the premise that genes crucial for normal development tend to be conserved. In this study, we identified three IG-DMR sequences that are highly conserved among eutherians. Next, to examine the functional roles of these conserved sequences *in vivo*, we generated KO mice lacking each of these regions using the clustered regularly interspaced short
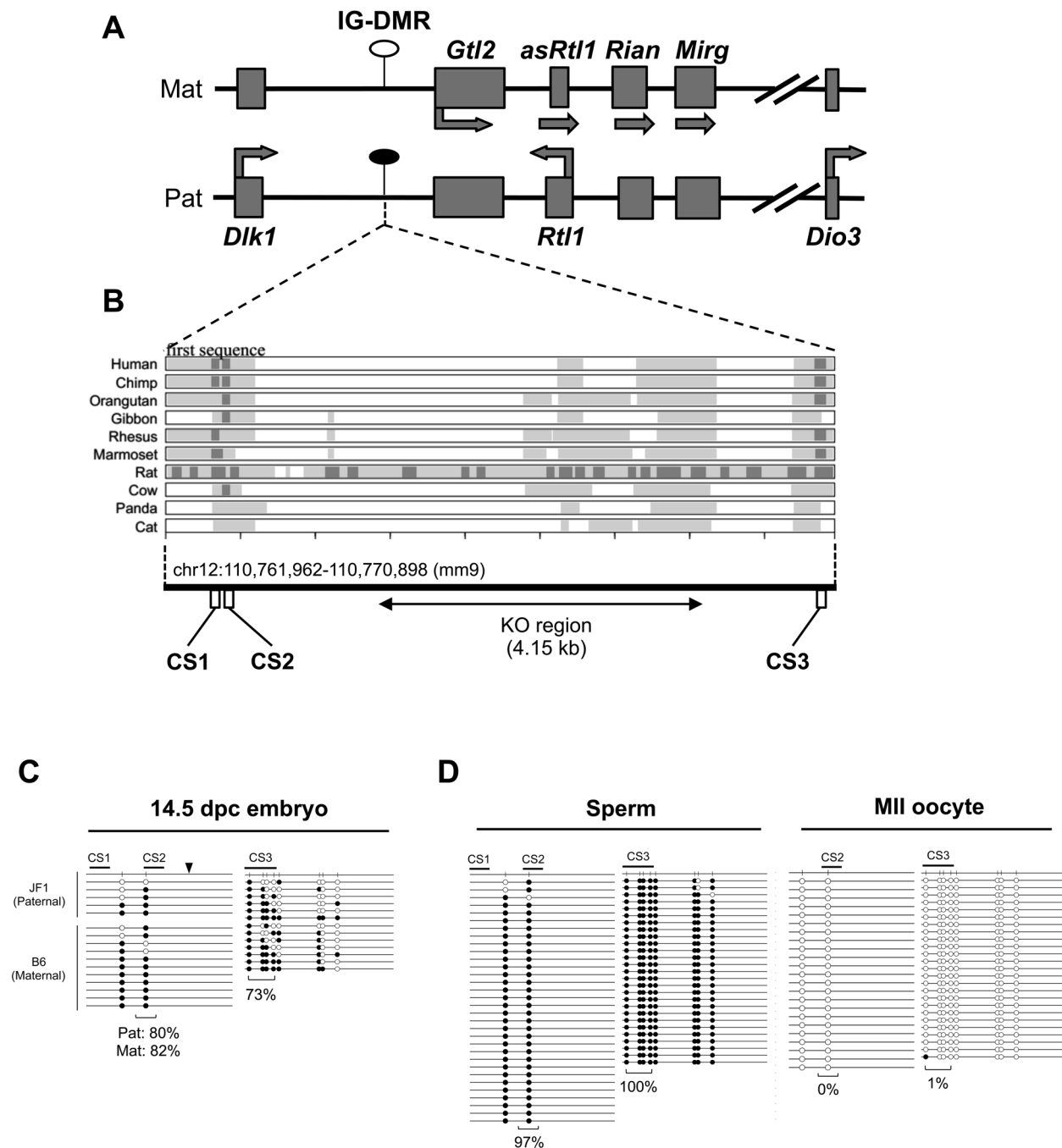
Fig. 1. Identification of conserved sequences in IG-DMR. (A) Schematic representation of the mouse *Dlk1-Dio3* domain. Genomic sequences are indicated with a horizontal black line. Imprinted genes and their expression alleles are indicated with gray boxes and gray arrows, respectively. Names of imprinted genes are shown with their expression alleles. Locations of IG-DMRs are indicated with vertical lines. Black and white circles indicate methylated and unmethylated statuses, respectively. (B) Analysis of sequence conservation of putative IG-DMRs in eutherians. (Top) Results of MultiPipMaker analysis. Alignment of putative IG-DMRs of 10 species is shown. Sequences exhibiting more than 50% and 80% conservation are colored light gray and dark gray, respectively. (Bottom) Identification of three highly conserved sequences in IG-DMR (CS1, CS2, and CS3). Mouse genomic sequence used as a reference is indicated by a black bar. Positions of CS1, CS2, and CS3 are indicated with white boxes. The 4.15-kb region deleted in KO mice [12] is shown by double headed arrows. (C, D) DNA methylation status in CS1, CS2, and CS3 in 14.5 dpc embryos (C) and sperms and MII oocytes (D) analyzed using bisulfite sequencing. The entire amplified sequences and positions of CpG sites are shown by horizontal and vertical lines at the top, respectively. SNP position is indicated with an arrowhead at the top. JF1 (paternal) and B6 (maternal) alleles aligned at the top and bottom, respectively. Each clone is indicated with a vertical line. Methylated and unmethylated CpGs are indicated with black and white circles, respectively. Percent methylation values of CS2 and CS3 are shown at the bottom. Positions of CS1, CS2, and CS3 in the amplified regions are indicated above the methylation status.

Table 1. Genetic conservation of *Dlk1-Gtl2* locus

Species	Location of putative IG-DMR	Length (bp)	Data set
Mouse (Reference)	chr12:110,761,962-110,770,898	8937	NCBI37/mm9
Human	chr14:101,271,066-101,282,625	11560	GRCh37/hg19
Chimp	chr14:100,497,937-100,509,499	11563	CGSC 2.1.3/panTro3
Orangutan	chr14:102,345,370-102,361,570	16201	WUGSC 2.0.2/ponAbe2
Gibbon	GL397390:288,960-299,501	10542	GGSC Nleu1.0/nomLeu1
Rhesus	chr7:164,700,679-164,712,080	11402	BGI CR_1.0/rheMac3
Marmoset	chr10:126,706,354-126,716,922	10569	WUGSC 3.2/calJac3
Rat	chr6:134,099,981-134,108,698	8718	Baylor 3.4/rn4
Cow	chr21:67,352,219-67,364,122	11904	Baylor 4.0/bosTau4
Panda	GL192537.1:392,649-402,737	10089	BGI-Shenzhen 1.0/ailMel1
Cat	chrB3:151,838,799-151,859,155	20357	NHGRI/GTB V17e/felCat4

palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system [14, 15].

Materials and Methods

Comparative genomics analysis

Draft genome sequences were obtained from the University of California, Santa Cruz (UCSC) Genome Browser (http://genome. ucsc.edu/). Genome assemblies of each species are listed in Table 1. Repeat sequences in genomic sequence were identified using RepeatMasker (Smit A.F.A., Hubley R. & Green P., http://www. repeatmasker.org/cgi-bin/WEBRepeatMasker). Sequence homology search and alignment were carried out using MultiPipMaker (http:// pipmaker.bx.psu.edu/pipmaker/) [16].

Generation of mutant mice using the CRISPR/Cas9 system

Single-guide RNA (sgRNA) cloning vectors and human codon-optimized Cas9 (hCas9) plasmids were a generous gift from George Church (Addgene plasmid #41824 and #41815) [14]. sgRNAs were cloned into the sgRNA cloning vector as described previously [17] using the primers listed in Supplementary Table 1 (online only). sgRNA and hCas9 mRNA were prepared via *in vitro* transcription using mMESSAGE/mMACHINE T7 *in vitro* transcription kit (Ambion, Austin, TX, USA). PCR products were amplified using primers containing the T7 RNA promoter sequence and subsequently used as templates for *in vitro* transcription. For microinjection, fertilized eggs were collected from superovulated F_1 hybrids of C57BL/6 × DBA/2 (BDF1) female mice crossed with BDF1 male mice (Sankyo Lab Service, Tokyo, Japan). sgRNAs and hCas9 mRNAs (167 ng/ μl each) were combined and microinjected into zygotes. Embryos were cultured in KSOM medium for one day. Two-cell embryos were transferred to pseudopregnant ICR female mice. For genotyping, genomic DNA was extracted from the tail or fingertips of pups using DirectPCR Lysis Reagents (Viagen Biotech, Los Angeles, CA, USA). To determine individual mouse genotypes, genomic DNA was PCR-amplified using the primers listed in Supplementary Table 1. Sequences of the mutant alleles were determined by Sanger sequencing analysis using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosciences, Foster City, CA, USA). Founder mutant mice were crossed with C57BL/6 mice (Sankyo Lab Service) to obtain the F_1 generation. To produce mice with the deleted allele inherited paternally and maternally, male and female F_1 mutant mice were crossed with JF1 female and male mice, respectively. JF1 mice were obtained from the National Institute of Genetics (NIG, Mishima, Japan). All animal protocols were approved by the Animal Care and Use Committee of the National Research Institute for Child Health and Development, Tokyo, Japan. All experiments were conducted in accordance with the approved animal protocols.

Expression analysis of imprinted genes

Total RNA and genomic DNA were separately isolated from 14.5 days post coitum (dpc) embryos using AllPrep DNA/RNA/Protein Mini Kit (QIAGEN GmbH, Hilden, Germany). Total RNA (1 μg) was treated with TURBO DNase (Thermo Fisher Scientific, Waltham, MA, USA) and used for cDNA synthesis using SuperScript II (Invitrogen, Carlsbad, CA, USA) reverse transcriptase and oligo(dT) primers. Quantitative RT-PCR (qRT-PCR) analyses were carried out using Power SYBR Green PCR Master Mix (Applied Biosciences) using the primers listed in Supplementary Table 1 [18–21]. PCR was performed on a Thermal Cycler Dice Real Time System Single TP850 instrument (TaKaRa Bio, Shiga, Japan) using the following profile: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, and 60°C for 1 min by. For expression analysis of the *Rtl1* gene, cDNA was synthesized from 1 μg of DNase-treated total RNA using the 3′ RACE primer (5′-CTGATCTAGAGGTACCGGATCCGACTCGAGTC-GACATCGTTTTTTTTTTTTTTTTTT-3′) and SuperScript II reverse transcriptase. qRT-PCR analysis was performed using the Forward primer: 5′-ATACCCAGAGTACTGTGCCAAG-3′ and Reverse primer: 5′-AGAGGTACCGGATCCGACTCGAGTCGACATCG-3′.

Allelic expression analysis

To determine allelic expression of *Dlk1*, *Gtl2*, *Rtl1*, and *Dio3*, cDNAs were amplified with GoTaq DNA Polymerase (Promega, Madison, WI, USA) using the primers listed in Supplementary Table 1. Reaction conditions were as follows: 35 cycles of 98°C for 10 sec, 58°C for 30 sec, and 72°C for 1 min. PCR products were treated with ExoSAP-IT (USB; Affymetrix , Santa Clara, CA, USA) and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit.

DNA methylation analysis

Sperms were collected from adult C57BL/6N mice and lysed in extraction buffer (10 mM Tris HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl, 1% SDS, 0.1 mg/ml Proteinase K, and 10 mM DTT) at 50°C overnight. Sperm DNA was purified using phenol/chloroform extraction and EtOH precipitation. MII oocytes were collected from superovulated C57BL/6N adult female mice. Cumulus cells were completely removed by pipetting. DNA was extracted from 100–120 oocytes by incubation in DNA lysis buffer (1 mg/ml Proteinase K, 1% SDS, and 2 μg of yeast tRNA) at 37°C for 1 h. For bisulfite conversion, DNA was treated with EpiTect Bisulfite Kit (QIAGEN). PCR (nested PCR for oocyte DNA) was performed using EpiTaq HS (TaKaRa Bio) and the primers listed in Supplementary Table 1. PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN) and subsequently cloned into the pGEM-T Easy vector (Promega). Colonies were selected and amplified using Illustra TempliPhi DNA amplification Kit (GE Healthcare, Piscataway, NJ, USA). Sequences were analyzed using M13 reverse primer. Obtained sequence data were aligned using the online web tool QUMA (http://quma.cdb. riken.jp/top/index.html) [22]. Statistical analysis of differences in methylation levels was performed using the Mann-Whitney U test.

Results

Identification of conserved sequences in IG-DMR among eutherians

To examine sequence conservation of IG-DMR in mammals, we first collected putative IG-DMR sequences from different mammalian species. The reference IG-DMR sequence used in this study was set between nearest outside HpaII or HhaI recognition sequences from mouse IG-DMR (8937 bp) as defined by Takada *et al.* (2002). Methylation levels of the HpaII and HhaI recognition sequences are similar between paternal and maternal alleles (Fig. 1A) [11]. Draft sequences of the entire *Dlk1-Gtl2* region in 14 species (human, chimp, orangutan, rhesus, marmoset, rat, guinea pig, pig, cow, sheep, dog, cat, panda, and elephant) were obtained from the UCSC Genome Browser. We excluded wallaby (marsupial) and platypus (monotreme) from the analysis, since the gene corresponding to *Gtl2* could not be identified in these two species via homology search using the obtained *Gtl2* sequences of the 14 abovementioned species. Next, putative IG-DMRs were searched using BLAT using the following criteria: SCORE > 100, IDENTITY > 80%, and SPAN > 1000. Results showed that out of the 14 eutherians, ten species contained putative IG-DMRs (human, chimpanzee, orangutan, gibbon, rhesus, marmoset, rat, cow, panda, and cat) (Table 1). We aligned the obtained sequences using MultiPipMaker after masking the repetitive elements in mouse sequence using RepeatMasker. Results showed that the sequence corresponding to the conserved 4.1-kb region (KO region), contained conserved sequences among eutherians at more than 50% homology; however, only mouse and rat sequences showed more than 80% homology. On the other hand, we identified three sequences (referred to as CS1, CS2, and CS3) that exhibit more than 80% conservation in more than half of the 11 species, whereas and 50% in the rest at outside of 4.1 kb region deleted in previous report (Fig. 1B) [12].

To determine whether CS1, CS2, and CS3 are DMRs, we char-

acterized their DNA methylation patterns. Since CS1, CS2, and CS3 have 0, 1, and 4 CpG sites, respectively, DNA methylation statuses of CS2 and CS3 were analyzed using bisulfite sequencing in wild-type C57BL/6 × JF1 hybrid F_1 whole embryos at 14.5 dpc. In CS2, paternal and maternal alleles could be distinguished based on an intersubspecific polymorphism, as shown in Fig. 1C. Results showed that the CpG site at CS2 was biallelically hypermethylated (paternal: 80%; maternal: 82%), and parent-of-origin effect was not observed (Fig. 1C). Because no polymorphisms were identified in CS3, the analysis was performed without discriminating between paternal and maternal alleles. Results showed that CS3 has a mean methylation level of 72% in the CpG sites and did not possess DMR-like methylation patterns (Fig. 1C). In addition, to verify methylation status at the same loci in germ cells, we also determined methylation patterns in the genomes of sperms and MII oocytes. We observed that both CS2 and CS3 were hypermethylated in sperm but hypomethylated in oocytes (Fig. 1D). These results suggest that sperm-specific DNA methylation of CS1, CS2, and CS3 are established in germ cells, whereas the germline-derived DNA methylation patterns are lost during post-fertilization.

Generation of knockout mice lacking IG-DMR conserved sequences using CRISPR/Cas9

To elucidate the function of the conserved sequences of IG-DMR *in vivo*, we generated mice lacking the CS1, CS2, and CS3 sequences using the CRISPR/Cas9 system. A pair of single-guide RNAs (sgRNAs) was designed flanking each of CS1, CS2, and CS3 regions (Fig. 2A). sgRNAs for CS1, CS2, and CS3 were microinjected into zygotes with Cas9 mRNA to generate founder mice. We selected mouse lines with the following deletions: 438-bp deletion of CS1 (ΔCS1), 292-bp deletion of CS2 (ΔCS2), and 303 bp-deletion of CS3 (ΔCS3, Fig. 2B). The founder mice were fertile; pups in the F_1 generation exhibited normal growth and were fertile. Next, we obtained heterozygous offspring with inherited deletion alleles from the father (+/ΔCS1-3) or mother (ΔCS1-3/+) in the next generation by crossing F_1 mice with JF1 mice. Resulting mice also grew normal until adulthood, suggesting that deletion of conserved sequences of IG-DMR does not affect embryonic development and growth after birth (Fig. 3).

Expression levels of paternally expressed genes are altered in CS-deficient mice

Next, to test whether the loss of CS regions affects the expression of imprinted genes in the *Dlk1-Dio3* domain, we performed gene expression analysis of the paternally expressed genes *Dlk1, Rtl1,* and *Dio3* and the maternally expressed gene *Gtl2* in the +/ΔCS1-3 and ΔCS1-3/+ embryos. Quantitative RT-PCR analysis of +/ΔCS1-3, ΔCS1-3/+, and wild-type embryos at 14.5 dpc revealed no significant differences in *Gtl2* expression levels in all genotypes. On the other hand, *Dlk1* expression was 1.5-fold upregulated in ΔCS1/+ embryos compared to wild-type and the other genotypes. In addition, *Dio3* expression levels were significantly lower in +/ΔCS1-3 but not in ΔCS1-3/+ embryos. *Rtl1* expression was also downregulated in ΔCS2/+ embryos compared to the other genotypes (Fig. 4A). To elucidate whether the observed upregulation of *Dlk1* and downregulation of *Rtl1* and *Dio3* were due to loss of imprinting, we
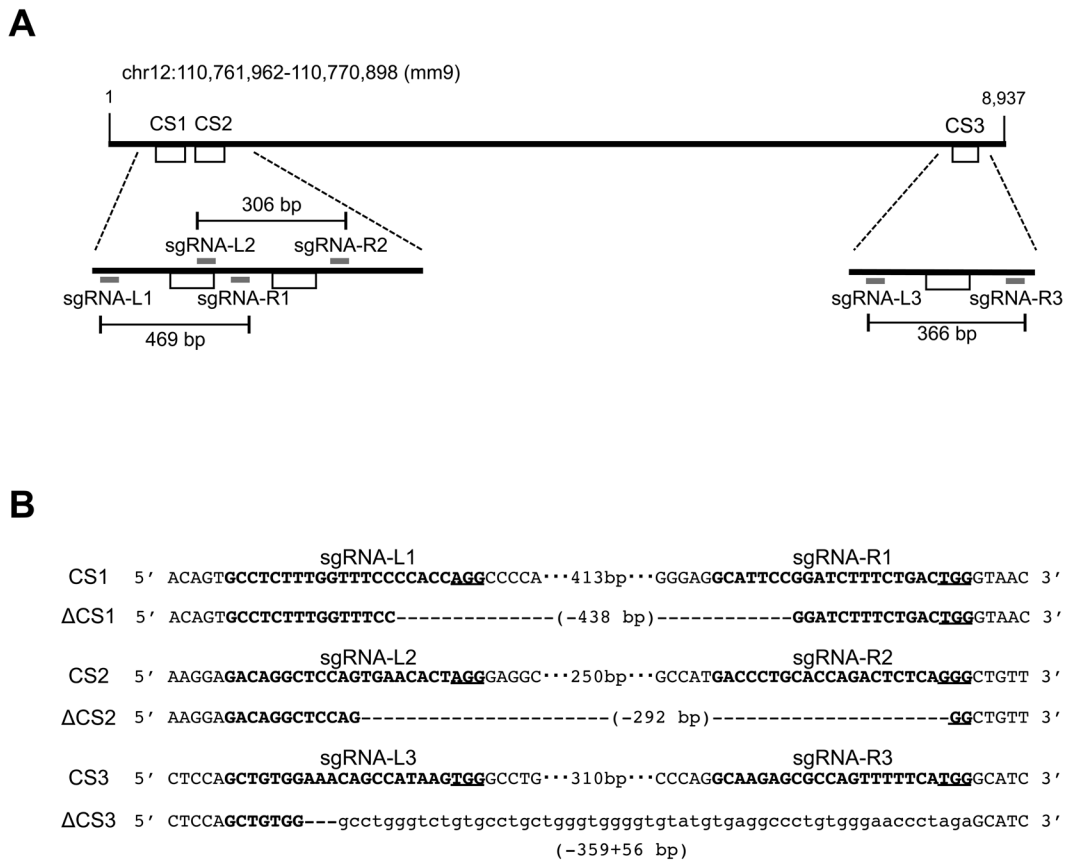
A

chr12:110,761,962-110,770,898 (mm9)

1   8,937

CS1 CS2   CS3

306 bp

sgRNA-L2   sgRNA-R2

sgRNA-L1   sgRNA-R1   sgRNA-L3   sgRNA-R3

469 bp   366 bp

B

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                        sgRNA-L1                                sgRNA-R1
CS1   5' ACAGTGCCTCTTTGGTTTCCCCACCAGGCCCCA···413bp···GGGAGGCATTCCGGATCTTTCTGACTGGGTAAC 3'

ΔCS1  5' ACAGTGCCTCTTTGGTTTCC--------------(-438 bp)-----------GGATCTTTCTGACTGGGTAAC 3'

                        sgRNA-L2                                sgRNA-R2
CS2   5' AAGGAGACAGGCTCCAGTGAACACTAGGGAGGC···250bp···GCCATGACCCTGCACCAGACTCTCAGGGCTGTT 3'

ΔCS2  5' AAGGAGACAGGCTCCAG--------------------(-292 bp)--------------------GGCTGTT 3'

                        sgRNA-L3                                sgRNA-R3
CS3   5' CTCCAGCTGTGGAAACAGCCATAAGTGGGCCTG···310bp···CCCAGGCAAGAGCGCCAGTTTTTCATGGGCATC 3'

ΔCS3  5' CTCCAGCTGTGG---gcctgggtctgtgcctgctgggtggggtgtatgtgaggccctgtgggaaccctagaGCATC 3'
                                    (-359+56 bp)
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Fig. 2. Generation of CS-deficient mice by the CRISPR/Cas9 system. (A) Design of sgRNAs for deletion of CS1, CS2, and CS3. Schematic representation of mouse IG-DMR. Genomic sequence of IG-DMR is shown as a black line. Positions of CS1, CS2, and CS3 are shown with white boxes. Enlarged region around CS1, CS2, and CS3 is also shown at bottom. Target sequences of sgRNA pairs for CS1 (sgRNA-L1/R1), CS2 (sgRNA-L2/R2), and CS3 (sgRNA-L3/R3) are indicated with gray lines. (B) Genotyping of mice carrying ΔCS1-3 alleles. Nucleotide sequences around sgRNA target sequences of CS1-3 and ΔCS1-3 are shown. Numbers in parentheses indicate counts of deleted and inserted nucleotides. Lowercase letters represent inserted sequences.

examined allelic expression of imprinted genes in the +/ΔCS1-3 and ΔCS1-3/+ embryos. Results showed that all genes tested, including *Dlk1, Rtl1,* and *Dio3*, exhibit the same imprinting patterns as the wild-type, suggesting that the observed *Dlk1* upregulation and *Rtl1* and *Dio3* downregulation were not caused by loss of imprinting (Fig. 4B). These results are consistent with that of allelic expression analysis, which showed that DNA methylation status of IG-DMR and promoter CpG island of *Gtl2* gene (also known as *Gtl2*-DMR, Fig. 5A) in +/ΔCS1-3 and ΔCS1-3/+ embryos were similar to those of wild-type embryos (Fig. 5B).

Taken together, these results suggest that conserved regions in IG-DMR enhance the expression of paternally expressed genes without skewing imprinting patterns.

Discussion

Deletion of the 4.1-kb sequence (KO region) in IG-DMR is known to result in similar phenotypes in humans and mice [12, 23]. It was previously assumed that the KO region constitutes the most functionally essential region(s) of IG-DMR and thus represents the most highly conserved sequences among eutherians. However, the three most highly conserved regions (CS1, CS2, and CS3) identified in this study, which exhibit more than 80% sequence conservation, were located outside the KO region. The KO region contains sequences with only 50-80% conservation, which is lower than when 8.9-kb region analyzed in this study is included in the alignment [11]. Thus, the functionally important sequences in IG-DMR that are located in the KO region may not be highly conserved among eutherians and act via different molecular mechanisms. To date, all studies show that the molecular functions of imprinted genes in the *Dlk1-Dio3* domain are associated with the KO region. Kota *et al*. (2014) demonstrated that maternally expressed non-coding RNAs regulate the expression of imprinted genes in the *Dlk1-Dio3* domain. In addition, Luo *et al*. (2016) showed that the AFF3 transcription factor is essential for maternal expression of *Gtl2-Rian-Mirg* genes in mice and acts by binding to allele-specific enhancers in IG-DMR [24, 25]. Some parts of the non-coding RNAs sequences and of the AFF3 binding sites are located more than 50% conserved region, but some are not. In addition, *IPW*, a non-coding RNA located in the Prader-Willi syndrome region on chromosome 15, was reported to
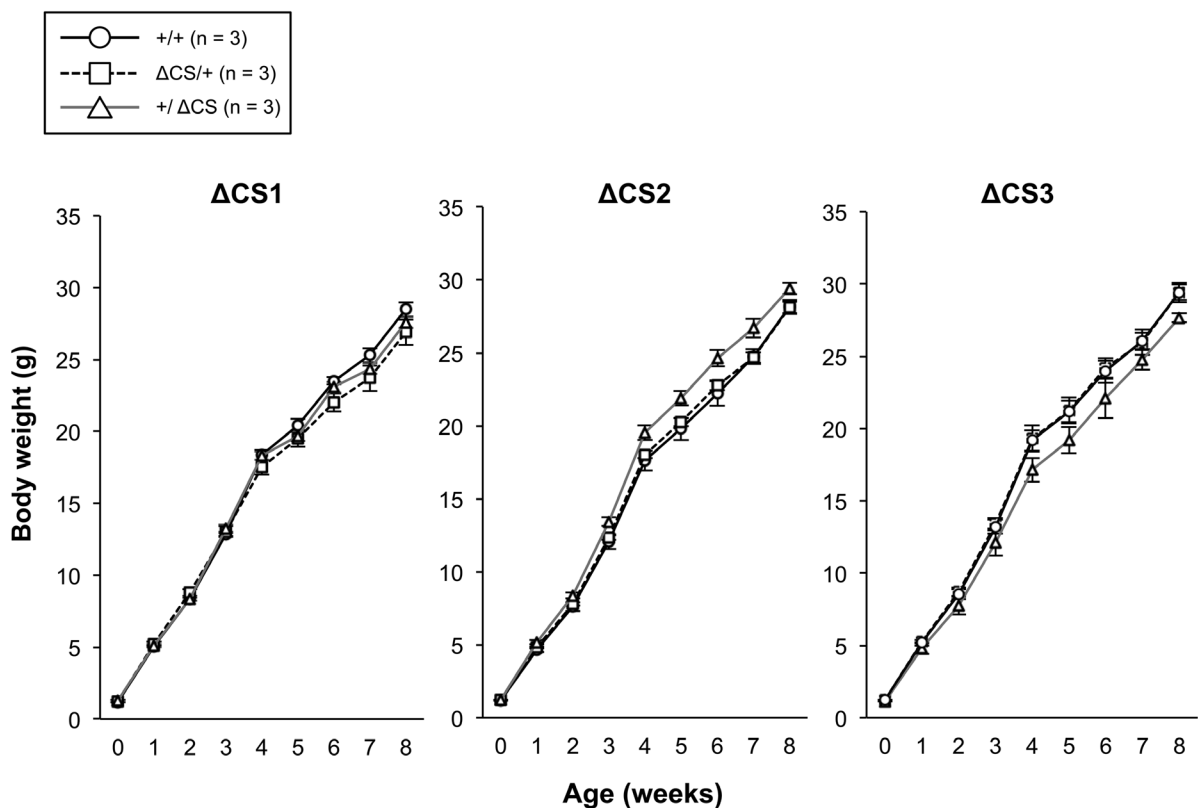
Fig. 3.   Growth of mutant mice carrying ΔCS1-3 alleles. Body weights of mice after birth are shown. Error bars indicate standard error.

regulate the epigenetic status of the *DLK1-DIO3* domain in human induced pluripotent cells. However, the *IPW* gene sequence is not highly conserved between humans and mice [26]. Thus, the results point to species-specific variations in regulatory mechanisms in the *DLK1-DIO3* domain.

In this study, we identified three highly conserved sequences (CS1, CS2, and CS3) in IG-DMR and generated knockout mice lacking each of these regions. Mutant mice lacking CS1, CS2, and CS3 did not exhibit gross phenotypes and uniparental expression in the *Dlk1-Dio3* domain that are usually observed in wild-type mice, suggesting that these regions are not required for maintenance or regulation of imprinted expression in *Dlk1-Dio3*. However, *Dlk1* was found to be upregulated in ΔCS1/+ embryos, whereas *Rtl1* and *Dio3* genes were downregulated in +/ΔCS2 and +/ΔCS1-3 embryos, respectively. These results suggest that the conserved sequences in IG-DMR can directly or indirectly modulate expression levels of paternally expressed imprinted genes in the *Dlk1-Dio3* domain and act in a *cis* manner. Interestingly, maternal deletion of CS1 resulted in *Dlk1* upregulation, suggesting *trans* modulation of paternal *Dlk1* expression by the maternal CS1 in embryos. In addition, *Dlk1* was 1.5-fold upregulated and *Rtl1* and *Dio3* were 0.5-fold downregulated in CS-deficient embryos, but no gross phenotype was observed at postnatal stages. These results suggest that the changes in expression levels occur either in the entire embryo or in a tissue-specific manner at levels that do not impair embryonic development. In either

or mixed cases, results indicate that CSs are involved in expression modulation of the imprinted genes. Further studies are required to elucidate the detailed molecular mechanisms that determine how CSs modulate the expression of paternally expressed genes.

In summary, we identified three highly conserved sequences in the IG-DMR of eutherians. By generating knockout mice lacking these sequences using the CRISPR/Cas9 system, we showed that these conserved sequences in IG-DMR influence the expression of paternally expressed genes. These findings provide new insights into the molecular mechanisms underlying gene expression modulation of imprinted genes in the *Dlk1-Dio3* domain.
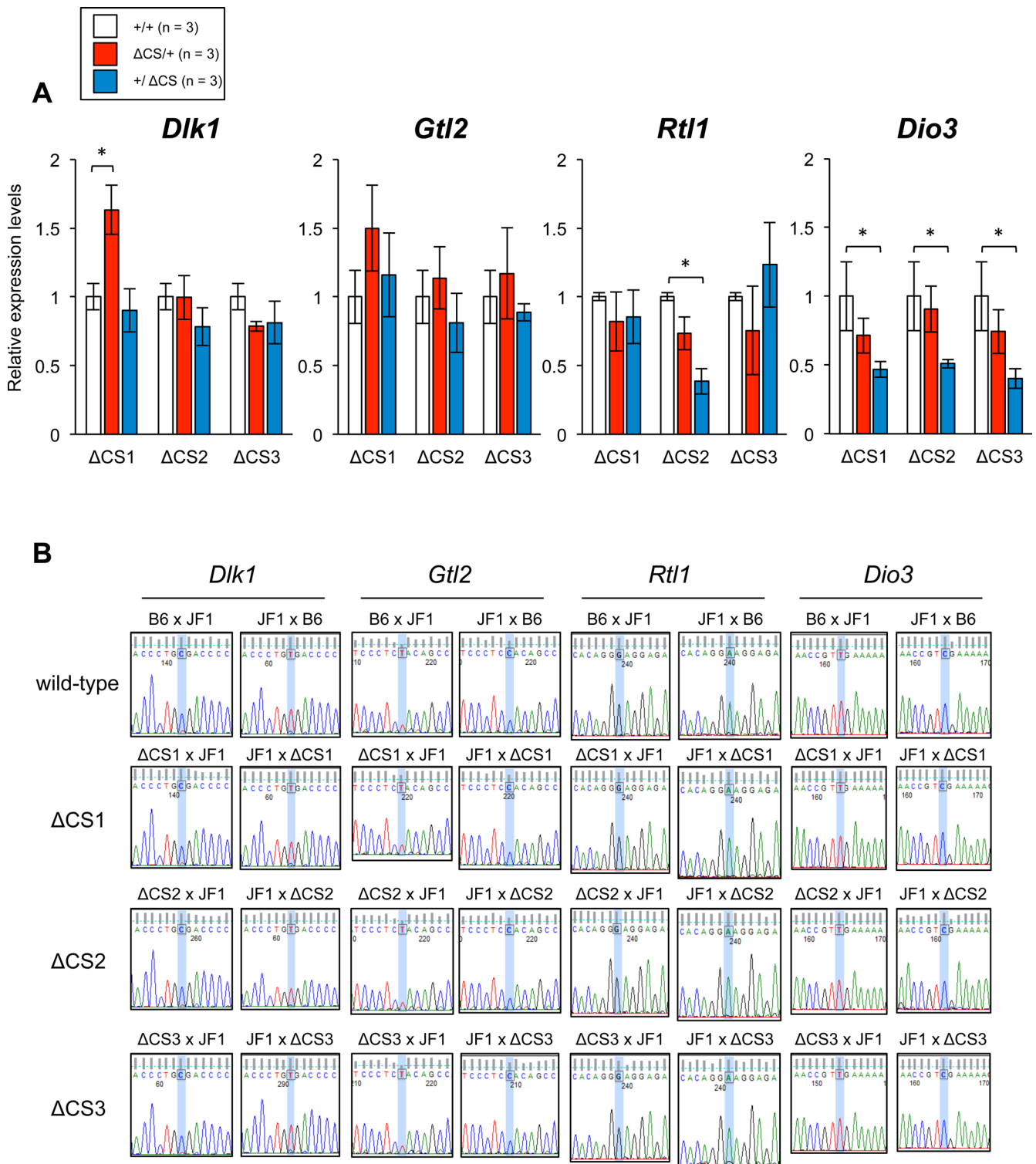
Acknowledgements

Fig. 4. Expression analysis of imprinted genes in CS-deficient embryos. (A) Quantitative RT-PCR analysis of imprinted genes in mutant embryos carrying ΔCS1, ΔCS2, and ΔCS3 alleles. Expression levels of imprinted genes in wild-type (+/+), maternally transmitted (ΔCS/+), and paternally transmitted (+/ΔCS) embryos are indicated with white, red, and blue bars, respectively. Expression levels are normalized by of the mean value of wild-type embryos. Error bars indicate standard error. * $P < 0.01$, t-test. Representative data are shown. Results were obtained from embryos in a single littermate. Similar results were obtained using embryos obtained from independent littermate. (B) Expression alleles of imprinted genes in mutant embryos carrying the ΔCS1, ΔCS2, and ΔCS3 alleles. Electropherograms obtained by direct sequencing of RT-PCR products of *Dlk1*, *Gtl2*, *Rtl1*, and *Dio3* are shown. Genotypes are indicated at the left. Mating combination is indicated at the top of each electropherogram. Polymorphic nucleotides are shaded in blue. Representative data are shown. Similar results were obtained using embryos obtained from independent littermates.
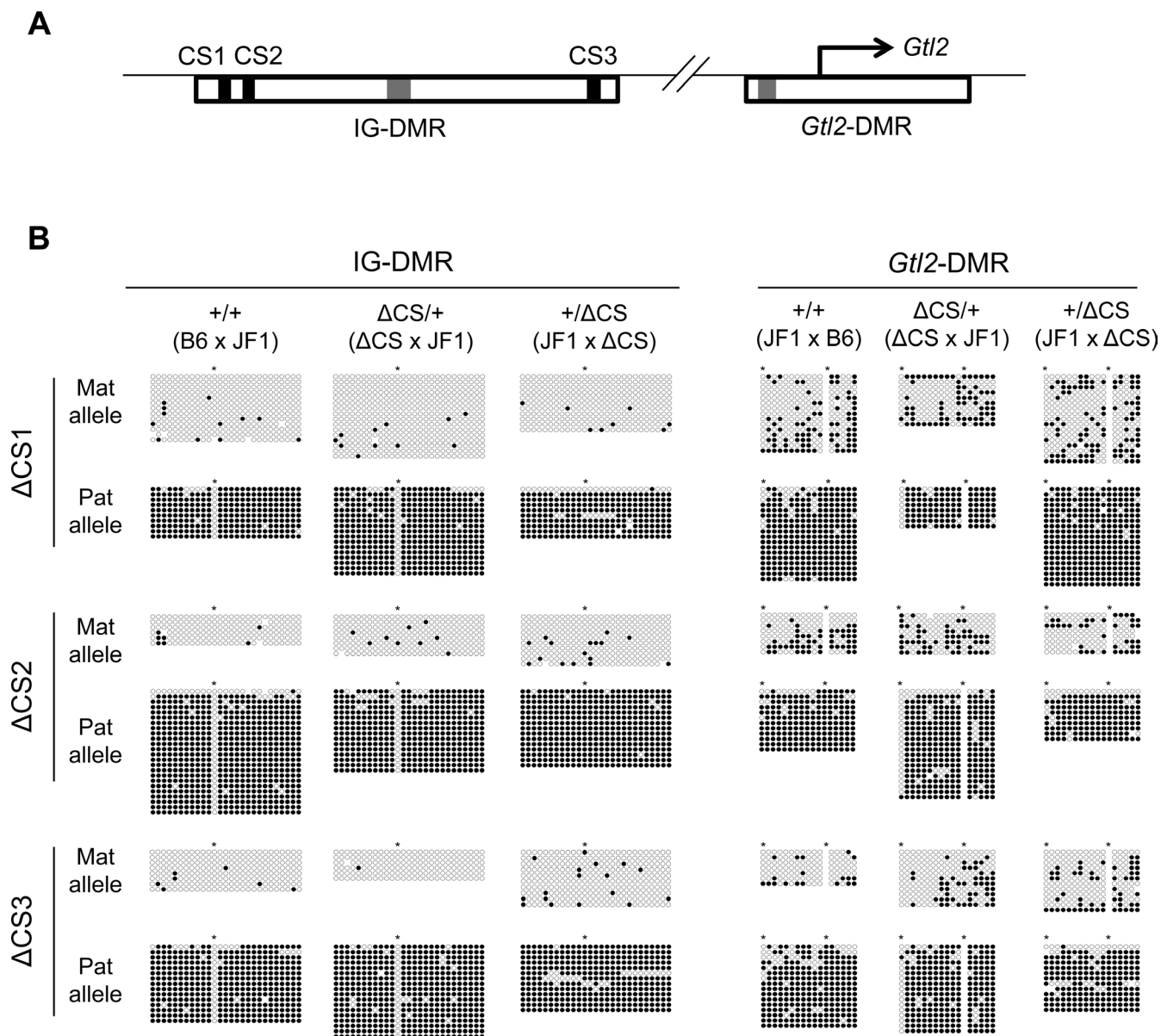
A

B

Fig. 5. DNA methylation analysis of IG-DMR and *Gtl2*-DMR in CS-deficient embryos. (A) Schematic representation of mouse IG-DMR and *Gtl2*-DMR. Genomic sequences are indicated with a black line. Positions of IG-DMR and *Gtl2*-DMR are shown in white boxes. CS1, CS2, and CS3 are indicated with black boxes. Transcription start site of *Gtl2* is shown with an arrow. Regions amplified by bisulfite PCR are indicated with gray boxes. (B) Bisulfite sequencing analysis of IG-DMR and *Gtl2*-DMR in mutant embryos carrying ΔCS1, ΔCS2, and ΔCS3 alleles. White and black circles indicate unmethylated and methylated CpG sites, respectively. Asterisks show the positions of CpG sites harboring polymorphisms. Representative data are shown. Similar results were obtained using embryos obtained from independent littermates.

References

1. **Sotomaru Y, Katsuzawa Y, Hatada I, Obata Y, Sasaki H, Kono T.** Unregulated expression of the imprinted genes H19 and Igf2r in mouse uniparental fetuses. *J Biol Chem* 2002; **277**: 12474–12478. [Medline] [CrossRef]

2. **Wu Q, Kumagai T, Kawahara M, Ogawa H, Hiura H, Obata Y, Takano R, Kono T.** Regulated expression of two sets of paternally imprinted genes is necessary for mouse parthenogenetic development to term. *Reproduction* 2006; **131**: 481–488. [Medline] [CrossRef]

3. **Edwards CA, Ferguson-Smith AC.** Mechanisms regulating imprinted genes in clusters. *Curr Opin Cell Biol* 2007; **19**: 281–289. [Medline] [CrossRef]

4. **Reik W, Walter J.** Genomic imprinting: parental influence on the genome. *Nat Rev Genet* 2001; **2**: 21–32. [Medline] [CrossRef]

5. **Hiura H, Obata Y, Komiyama J, Shirai M, Kono T.** Oocyte growth-dependent progression of maternal imprinting in mice. *Genes Cells* 2006; **11**: 353–361. [Medline] [CrossRef]

6. **Lucifero D, Mann MR, Bartolomei MS, Trasler JM.** Gene-specific timing and epigenetic memory in oocyte imprinting. *Hum Mol Genet* 2004; **13**: 839–849. [Medline] [CrossRef]

7. **Kobayashi H, Sakurai T, Miura F, Imai M, Mochiduki K, Yanagisawa E, Sakashita A, Wakai T, Suzuki Y, Ito T, Matsui Y, Kono T.** High-resolution DNA methylome analysis of primordial germ cells identifies gender-specific reprogramming in mice. *Genome Res* 2013; **23**: 616–627. [Medline] [CrossRef]

8. **Georgiades P, Watkins M, Surani MA, Ferguson-Smith AC.** Parental origin-specific developmental defects in mice with uniparental disomy for chromosome 12. *Development* 2000; **127**: 4719–4728. [Medline]

9. **da Rocha ST, Edwards CA, Ito M, Ogata T, Ferguson-Smith AC.** Genomic imprinting at the mammalian Dlk1-Dio3 domain. *Trends Genet* 2008; **24**: 306–316. [Medline] [CrossRef]

10. **Takada S, Tevendale M, Baker J, Georgiades P, Campbell E, Freeman T, Johnson MH, Paulsen M, Ferguson-Smith AC.** Delta-like and gtl2 are reciprocally expressed, differentially methylated linked imprinted genes on mouse chromosome 12. *Curr Biol* 2000; **10**: 1135–1138. [Medline] [CrossRef]

11. **Takada S, Paulsen M, Tevendale M, Tsai CE, Kelsey G, Cattanach BM, Ferguson-Smith AC.** Epigenetic analysis of the Dlk1-Gtl2 imprinted domain on mouse chromosome 12: implications for imprinting control from comparison with Igf2-H19. *Hum Mol Genet* 2002; **11**: 77–86. [Medline] [CrossRef]

12. **Lin SP, Youngson N, Takada S, Seitz H, Reik W, Paulsen M, Cavaille J, Ferguson-Smith AC.** Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the Dlk1-Gtl2 imprinted cluster on mouse chromosome 12. *Nat Genet* 2003; **35**: 97–102. [Medline] [CrossRef]

13. **Edwards CA, Mungall AJ, Matthews L, Ryder E, Gray DJ, Pask AJ, Shaw G, Graves JA, Rogers J, Dunham I, Renfree MB, Ferguson-Smith AC. SAVOIR consortium** The evolution of the DLK1-DIO3 imprinted domain in mammals. *PLoS Biol* 2008; **6**: e135. [Medline] [CrossRef]

14. **Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM.** RNA-guided human genome engineering via Cas9. *Science* 2013; **339**: 823–826. [Medline] [CrossRef]

15. **Shen B, Zhang J, Wu H, Wang J, Ma K, Li Z, Zhang X, Zhang P, Huang X.** Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Res* 2013; **23**: 720–723. [Medline] [CrossRef]

16. **Schwartz S, Zhang Z, Frazer KA, Smit A, Riemer C, Bouck J, Gibbs R, Hardison R, Miller W.** PipMaker a web server for aligning two genomic DNA sequences. *Genome Res* 2000; **10**: 577–586. [Medline] [CrossRef]

17. **Inui M, Miyado M, Igarashi M, Tamano M, Kubo A, Yamashita S, Asahara H, Fukami M, Takada S.** Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system. *Sci Rep* 2014; **4**: 5396. [Medline] [CrossRef]

18. **Sekita Y, Wagatsuma H, Irie M, Kobayashi S, Kohda T, Matsuda J, Yokoyama M, Ogura A, Schuster-Gossler K, Gossler A, Ishino F, Kaneko-Ishino T.** Aberrant regulation of imprinted gene expression in Gtl2lacZ mice. *Cytogenet Genome Res* 2006; **113**: 223–229. [Medline] [CrossRef]

19. **Takahashi N, Okamoto A, Kobayashi R, Shirai M, Obata Y, Ogawa H, Sotomaru Y, Kono T.** Deletion of Gtl2, imprinted non-coding RNA, with its differentially methylated region induces lethal parent-origin-dependent defects in mice. *Hum Mol Genet* 2009; **18**: 1879–1888. [Medline] [CrossRef]

20. **Sato S, Yoshida W, Soejima H, Nakabayashi K, Hata K.** Methylation dynamics of IG-DMR and Gtl2-DMR during murine embryonic and placental development. *Genomics* 2011; **98**: 120–127. [Medline] [CrossRef]

21. **Tsai CE, Lin SP, Ito M, Takagi N, Takada S, Ferguson-Smith AC.** Genomic imprinting contributes to thyroid hormone metabolism in the mouse embryo. *Curr Biol* 2002; **12**: 1221–1226. [Medline] [CrossRef]

22. **Kumaki Y, Oda M, Okano M.** QUMA: quantification tool for methylation analysis. *Nucleic Acids Res* 2008; **36**: W170-175. [Medline] [CrossRef]

23. **Kagami M, Sekita Y, Nishimura G, Irie M, Kato F, Okada M, Yamamori S, Kishimoto H, Nakayama M, Tanaka Y, Matsuoka K, Takahashi T, Noguchi M, Tanaka Y, Masumoto K, Utsunomiya T, Kouzan H, Komatsu Y, Ohashi H, Kurosawa K, Kosaki K, Ferguson-Smith AC, Ishino F, Ogata T.** Deletions and epimutations affecting the human 14q32.2 imprinted region in individuals with paternal and maternal upd(14)-like phenotypes. *Nat Genet* 2008; **40**: 237–242. [Medline] [CrossRef]

24. **Kota SK, Llères D, Bouschet T, Hirasawa R, Marchand A, Begon-Pescia C, Sanli I, Arnaud P, Journot L, Girardot M, Feil R.** ICR noncoding RNA expression controls imprinting and DNA replication at the Dlk1-Dio3 domain. *Dev Cell* 2014; **31**: 19–33. [Medline] [CrossRef]

25. **Luo Z, Lin C, Woodfin AR, Bartom ET, Gao X, Smith ER, Shilatifard A.** Regulation of the imprinted Dlk1-Dio3 locus by allele-specific enhancer activity. *Genes Dev* 2016; **30**: 92–101. [Medline] [CrossRef]

26. **Stelzer Y, Sagi I, Yanuka O, Eiges R, Benvenisty N.** The noncoding RNA IPW regulates the imprinted DLK1-DIO3 locus in an induced pluripotent stem cell model of Prader-Willi syndrome. *Nat Genet* 2014; **46**: 551–557. [Medline] [CrossRef]