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Restoration of *Dlk1* and *Rtl1* Is Necessary but Insufficient to Rescue Lethality in Intergenic Differentially Methylated Region (IG-DMR)-deficient Mice*

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In the *Dlk1-Dio3* imprinted domain, an intergenic differentially methylated region (IG-DMR) regulates the parental allele-specific expression of imprinted genes. The maternally inherited deletion of IG-DMR (IG-DMR$^{(-/+)}$) results in perinatal lethality because of the overexpression of paternally expressed genes and repression of maternally expressed noncoding RNAs (ncRNAs), including *Gtl2*. To better understand the possible contribution of paternally expressed genes to the lethality, we attempted to rescue the lethality of IG-DMR$^{(-/+)}$ mutants by restoring the paternally expressed genes. Because the paternally inherited *Gtl2* deletion (*Gtl2*$^{(+/-)}$) induced a decrease in the expression of paternally expressed genes, we crossed female IG-DMR heterozygous mice and male *Gtl2* heterozygous mutant mice. The resultant IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutant mice had normal expression levels of paternally expressed genes, and none of them showed perinatal lethality; however, most mice showed postnatal lethality with decreased expression of the maternally expressed ncRNAs. Thus, we inferred that paternally expressed genes are necessary for perinatal survivability and that maternally expressed ncRNAs are involved in postnatal lethality.

Genomic imprinting is an epigenetic mechanism in mammals that results in the functional nonequivalence of parental genomes (1, 2). Imprinted genes are regulated by methylation at differentially methylated regions (DMRs),[2] which are dependent on the parent of origin and mainly show monoallelic expression. The loss of imprinting leads to the biallelic expression or silencing of genes, which can result in various developmental defects and diseases (3, 4). Most imprinted genes are regulated by maternally derived methylation, whereas only three domains are reported to be regulated by paternally methylated imprinted domains: *Dlk1-Dio3*, *Igf2-H19*, and *Rasgrf1* domains (5–7). Studies on mouse embryos containing only the maternal genome have reported that the *Dlk1-Dio3* and *Igf2-H19* domains are essential for fetal survivability (8, 9).

The *Dlk1-Dio3* domain on the distal arm of mouse chromosome 12 contains the paternally expressed genes *Dlk1*, *Rtl1*, and *Dio3* and the maternally expressed noncoding RNAs (ncRNAs) *Gtl2*, *anti-Rtl1*, *Rian*, and *Mirg* (see Fig. 1) (10–15). These imprinted genes have been shown to be essential for perinatal survivability in a previous study reporting that embryos with MatD12 and PatD12 (maternal and paternal uniparental disomy for chromosome 12) died late in gestation with biallelic or no expression of these genes (16).

Imprinting on the *Dlk1-Dio3* domain is regulated primarily by an intergenic DMR (IG-DMR) that is methylated during spermatogenesis (5). In mice, when the IG-DMR deletion is inherited from the paternal allele, the mutants have a normal phenotype and gene expression. In contrast, the maternally inherited IG-DMR deletion (IG-DMR$^{(-/+)}$) results in perinatal lethality as well as biallelic expression of the paternally expressed genes and repression of the maternally expressed genes. The overexpression of *Dlk1* and *Rtl1* induces partial perinatal lethality in mice; therefore, biallelic expression is a possible cause of the lethality (17, 18). However, this possibility has not been confirmed completely because the expression levels of all of the genes in this domain are significantly different between the IG-DMR$^{(-/+)}$ mutant and wild-type (WT) mice. To clarify the relation between the lethality of the IG-DMR$^{(-/+)}$ mutants and overexpression of *Dlk1* and *Rtl1*, we attempted a rescue experiment by mating female IG-DMR heterozygous mutants with male *Gtl2* heterozygous mutants. The paternally inherited *Gtl2*/*Gtl2* DMR deletion (*Gtl2*$^{(+/-)}$) leads to a decrease in the expression levels of *Dlk1* and *Rtl1*; consequently, the double mutant mice (IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$) would have normal expression levels of *Dlk1* and *Rtl1* (Fig. 1) (19). The double mutants showed no lethality at the perinatal stage; however, most of them died at the postnatal stage. These results indicate that the restoration of *Dlk1* and *Rtl1* was necessary but insufficient for normal development in the IG-DMR$^{(-/+)}$ mutants.

EXPERIMENTAL PROCEDURES

Mice—The IG-DMR deletion and *Gtl2* knock-out (KO) mutant mice were produced as described previously (5, 19). These mutant lines only could be maintained when the deletion was inherited from the father, as deletion of maternally inherited IG-DMR results in perinatal lethality and maternally inherited *Gtl2* deletion results in postnatal lethality. The IG-DMR deletion and *Gtl2* KO mutant mice were maintained in a C57BL/6 and B6D2F1 background, respectively. The IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutant mice were obtained by mating female IG-DMR$^{(+/-)}$ with male *Gtl2*$^{(+/-)}$ mutant mice.

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[2] The abbreviations used are: DMR, differentially methylated region; IG-DMR, intergenic DMR; ncRNA, noncoding RNA; WT, wild-type; KO, knock-out; E, embryonic day.

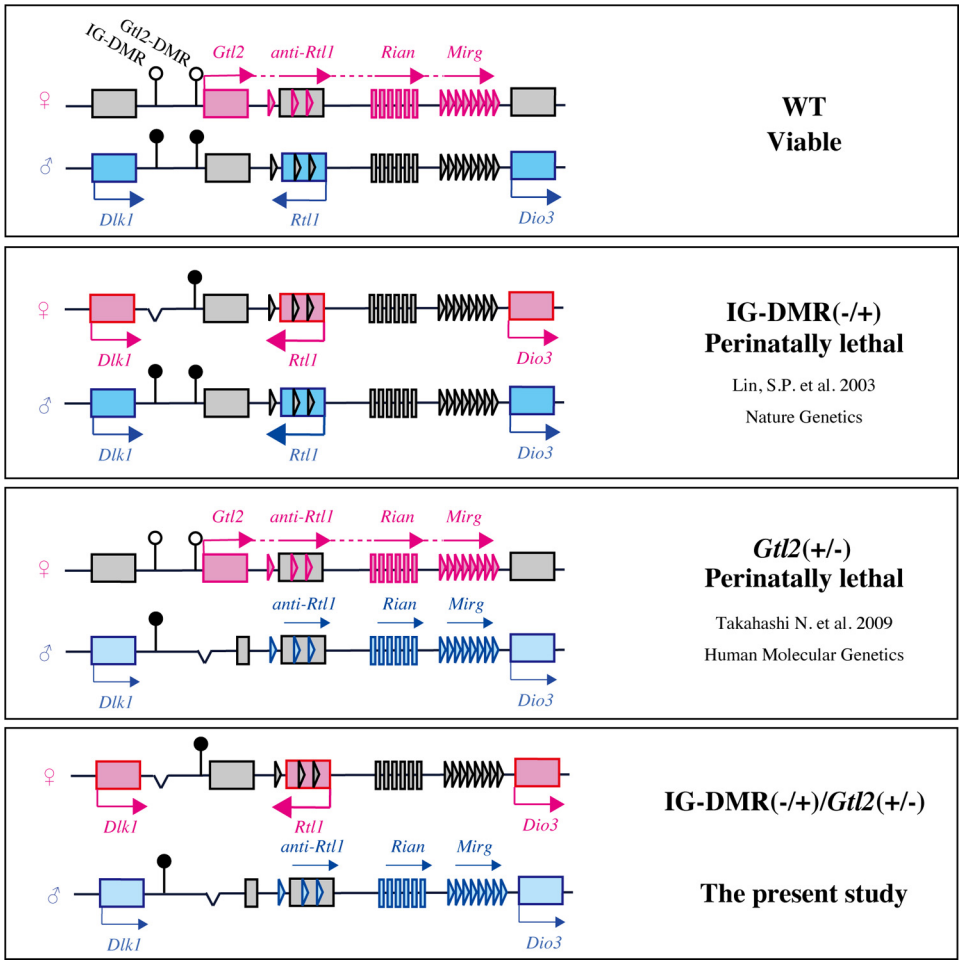

FIGURE 1. **The predicted expression pattern of imprinted genes in the *Dlk1-Dio3* domain.** The position of the imprinted genes is indicated by *squares*, *vertical bars* (small nucleolar RNAs), or *triangles* (microRNAs). The elements are shown in *gray* when inactive, in *pink* when active on the maternal allele, and in *blue* when active on paternal allele. IG-DMR and *Gtl2*-DMR are indicated by circles (*filled circles*, hypermethylated; *open circles*, hypomethylated).

Genotyping of the mutants was conducted by PCR amplification of DNA as described previously. These mutant mice were marked at birth and weighed every 3 days from birth to 7 weeks.

Skeletal Analysis—E18.5 embryos were eviscerated and fixed in 99.5% ethanol for 1 week. For cartilage staining, samples were incubated with 0.015% Alcian blue (Sigma) in 80% ethanol and 20% glacial acetic acid for 1 day. Samples were hydrolyzed overnight in 2% KOH. For bone staining, samples were stained with 0.001% Alizarin red (Sigma) in 1% KOH for 3 h.

Gene Expression Analysis—Total RNA was extracted from the brain, tongue, lungs, and liver of E18.5 embryos using a mirVana miRNA isolation kit (Ambion). The cDNAs were synthesized using the SuperScript III reverse transcriptase (Invitrogen) in a reaction solution (20 μl) containing total RNA (1 μg). Finally, we performed quantitative analysis using real-time PCR (7500 Real-time PCR system; Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems). The primers used for the analysis have been described previously (19).

To conduct the Northern blot hybridization analysis for *Rtl1*, we isolated total and poly(A)$^+$ RNA from the tongue and placenta using TRIzol (Invitrogen) and the PolyATract(R) mRNA isolation system (Promega). We used pooled samples ($n = 3$ for each genotype), and the analysis was conducted three times. Signal intensities were calculated using ImageJ software (http://rsb.info.nih.gov/ij/index.html). The probes and methods used for analysis have been described previously (8).

RESULTS

The Mode of Inheritance and Phenotype—By crossing female IG-DMR heterozygous with male *Gtl2* heterozygous mutants, we generated three genotypes of mice: IG-DMR$^{(-/+)}$, *Gtl2*$^{(+/-)}$, and IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ (Fig. 2*A*). The IG-DMR$^{(-/+)}$ mutants died at the perinatal stage showing morphological phenotypes, including a short body length and broad neck, whereas the *Gtl2*$^{(+/-)}$ mutants showed perinatal lethality with growth retardation of the fetus and placenta (Fig. 2*B*; Table 1); these findings are consistent with those of previous studies (19, 20). In contrast, the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ mutant mice were born in the expected Mendelian ratio and had normal physical characteristics (Fig. 2, *B–E*; Table 1). Although the double mutants survived at the perinatal stage, 70% of them died within 3 weeks of birth. The remaining double mutants showed weight loss at the weaning stage but grew into normal adults with reproductive ability. This suggests that although perinatal lethality disappears, postnatal abnormalities appear in the double mutants.

Recovery of the Skeletal Defects in the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ *Double Mutants*—Over- or underexpression of *Dlk1* leads to skeletal defects. The IG-DMR$^{(-/+)}$ mutants showed the abnormal attachment of the eighth rib to the sternum (two of four embryos), unlike the WT embryos in whom only seven ribs were attached to the sternum. The *Gtl2*$^{(+/-)}$ mutants lacked the fifth ossification center (four of five embryos) (Fig. 3). In contrast, these skeletal defects were absent in all the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutants (four embryos) (Fig. 3). This indicates that the defect induced by abnormal *Dlk1* expression was resolved in the double mutants.

Gene Expression Analysis for the Dlk1-Dio3 Domain in the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ *Double Mutants*—To obtain a better understanding of the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutant phenotype, we performed gene expression analysis of the imprinted genes in this domain (Fig. 4, *A* and *B*). First, we assayed the expression of *Dlk1* and *Rtl1* to determine the reason

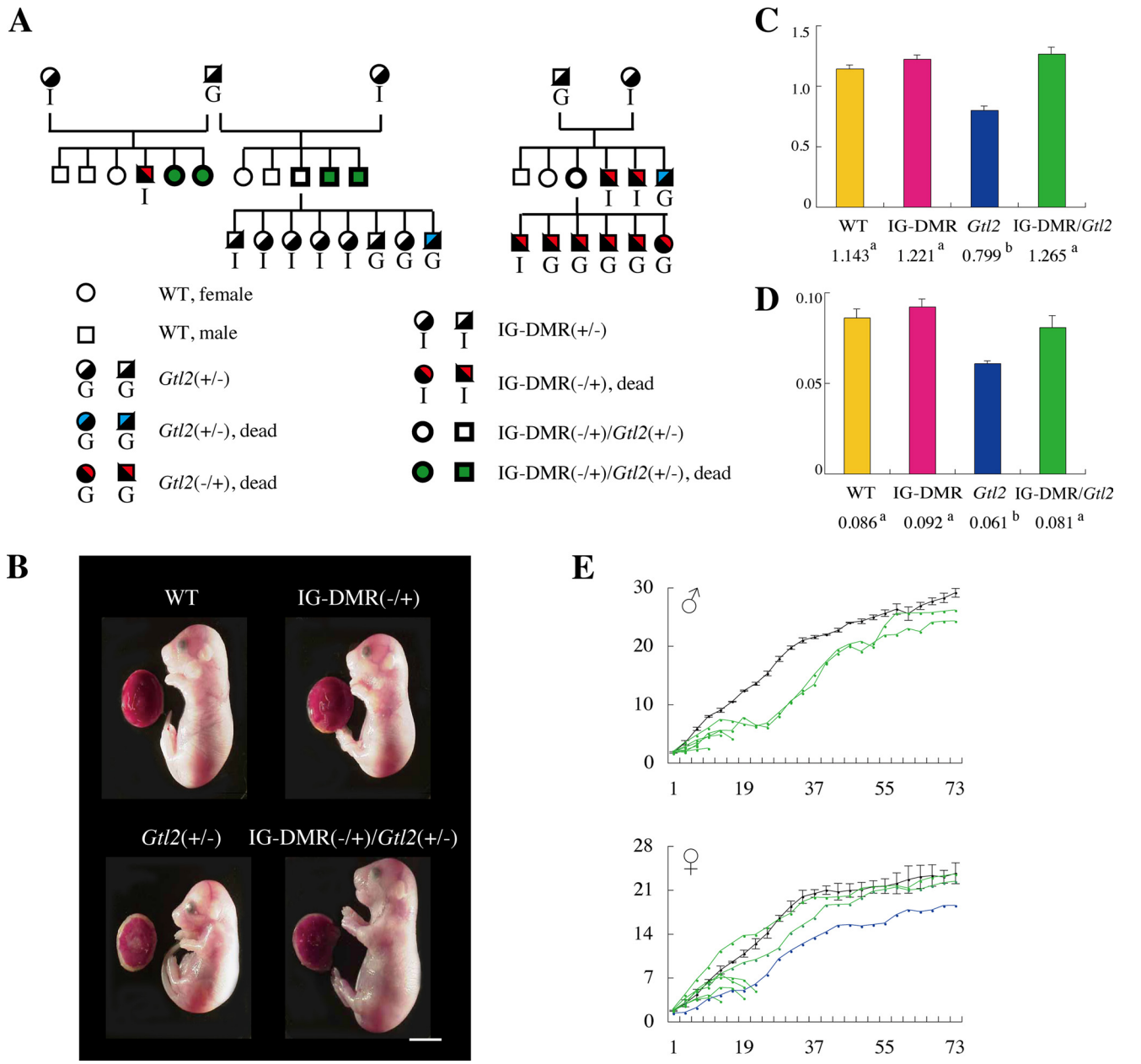

FIGURE 2. **Growth phenotype of the mutant mice.** *A*, pedigree of the mutants. *B*, picture of the fetus and placenta at E18.5. *Scale bar*, 5 mm. *C* and *D*, fetus body weight (*C*) and placenta weight (*D*) are represented by the *bar chart*. Wild-type, $n = 9$; IG-DMR$^{(-/+)}$, $n = 6$; *Gtl2*$^{(+/-)}$, $n = 5$; and IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$, $n = 5$. The values are expressed as the means ± S.E. For comparison of the date, statistical analyses were carried out with one-way analysis of variance. The different superscripts denote the statistically differences ($p < 0.05$) between the genotype. *E*, the growth curve after birth. Wild-type, $n = 11$, *black*; *Gtl2*$^{(+/-)}$, $n = 1$, *blue*; IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$, $n = 14$, *green*.

TABLE 1
The postnatal lethality depending on genotype

Stage	No. of alive pups/total (%)			
	WT	IG-DMR$^{(-/+)}$	*Gtl2*$^{(+/-)}$	IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$
~1 day	14/15 (93)	0/6 (0)	1/11 (9)	13/15 (87)
~1 week	14/14 (100)		1/1 (100)	9/13 (69)
~2 weeks	14/14 (100)		1/1 (100)	5/9 (56)
~3 weeks	14/14 (100)		1/1 (100)	4/5 (80)
Adult	14/14 (100)		1/1 (100)	4/4 (100)
Total	14/15 (93)	0/6 (0)	1/11 (9)	4/15 (27)

for perinatal survivability in the double mutants. The assay results showed that the IG-DMR$^{(-/+)}$ mutants overexpressed *Dlk1* and *Rtl1*, whereas the *Gtl2*$^{(+/-)}$ mutants had decreased expression levels of *Dlk1* and *Rtl1*, as expected. In the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutants, the expression levels of *Dlk1* and *Rtl1* were similar to those of the controls in all the tissues examined. This suggests that the restoration of *Dlk1* and *Rtl1* expression rescued perinatal lethality in the double mutants.

To gain further insight into the mechanism underlying the postnatal lethality of the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutant mice, we performed gene expression analysis of *Rian* and *Mirg*. The expression of *Rian* and *Mirg* was repressed in the IG-DMR$^{(-/+)}$ mutants and enhanced in *Gtl2*$^{(+/-)}$ mutants, as expected. In the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutants, the average expression level of *Rian* and *Mirg* in the brain was reduced to nearly one-fourth of that in the controls, and the

average expression level of these genes in the tongue and lungs were half that in the controls. This demonstrates that postnatal lethality might be caused by the decrease in the expression levels of *Rian* and *Mirg*. The expression levels of these ncRNAs varied among the double mutants. In four of five samples, the expression levels of *Rian* and *Mirg* in the lungs decreased to <54% of that in the control levels; however, one sample showed an expression level identical to that of the controls. Additionally, in one of the five samples, the *Rian* expression level in the brain was two times the average of the other four samples. This might explain why 30% of the double mutants survived. These data suggest that the expression levels of *Rian* and *Mirg* determine the viability of the double mutant at the postnatal stage.

DISCUSSION

The correct dosage of *Dlk1* and *Rtl1* is necessary for normal development, which is clearly evidenced in overexpression and deletion studies on genetically modified mice. One-third of the mutants expressing twice the normal dose of *Dlk1* exhibit skeletal defects and die within the first 3 days of life (17). *Dlk1* KO mice show dwarfism, and half of them die at the perinatal stage (21). In contrast, mutants overexpressing *Rtl1* display partial lethality with placentomegaly, and *Rtl1* KO mice show perinatal lethality with growth retardation (18). These reports suggested that over- and underexpression of *Dlk1* and *Rtl1* induced perinatal lethality.

The IG-DMR regulates the expression of all the imprinted genes in the *Dlk1-Dio3* domain, and all of the IG-DMR$^{(-/+)}$ mutants die perinatally and show biallelic expression of *Dlk1* and *Rtl1* (5). To investigate whether *Dlk1* and *Rtl1* overexpression caused perinatal lethality, we tried to restore the expression levels of *Dlk1* and *Rtl1* in the IG-DMR$^{(-/+)}$ mutants by mating IG-DMR$^{(-/+)}$ females with male *Gtl2* heterozygous mutants. The paternally inherited *Gtl2* deletion led to a lowering of the expression levels of *Dlk1* and *Rtl1*; consequently, the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutants showed normal expression levels of *Dlk1* and *Rtl1*. The double mutants were normal at

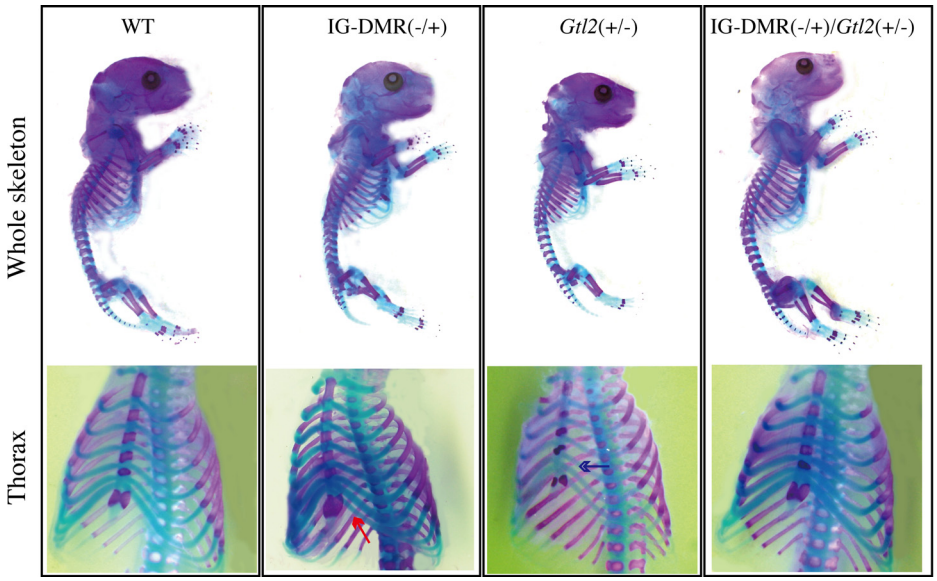

FIGURE 3. **Comparison of skeletal defects among the mutants.** The *red arrow* indicates that the eight ribs were attached the sternum in the IG-DMR$^{(-/+)}$ mutants. The *blue arrow* points the absence of the fifth ossification center in the *Gtl2*$^{(+/-)}$ mutants. The skeletal defect was absent in the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutants.

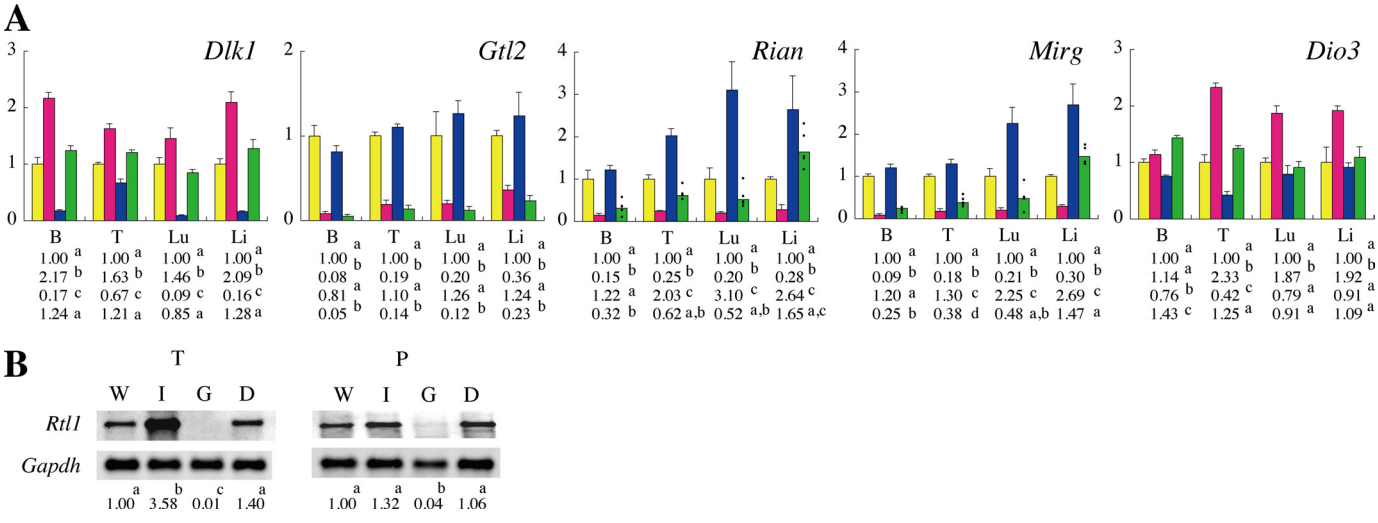

FIGURE 4. **Gene expression in the *Dlk1-Dio3* domain.** *A*, graphical representations of the expression levels of the five imprinted genes *Dlk1*, *Gtl2*, *Rian*, *Mirg*, and *Dio3* at E18.5. The values are expressed as the means ± S.E. Wild-type, $n = 4$, *yellow*; IG-DMR$^{(-/+)}$, $n = 4$, *red*; *Gtl2*$^{(+/-)}$, $n = 3$, *blue*; and IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$, $n = 5$, *green*. It is normalized to 1 in each tissue, which represents the average expression level of the WT. The expression value of *Rian* and *Mirg* in the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutants was individually indicated by *dots* because the expression was varied among samples. *B*, Northern blot analysis of *Rtl1* expression at E18.5. *B*, brain; *T*, tongue; *Lu*, lungs; *Li*, liver; and *P*, placenta. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as control. For comparison of the date, statistical analyses were carried out with one-way analysis of variance. The superscripts denote the differences ($p < 0.05$) between the genotype means. *W*, wild-type; *I*, IG-DMR$^{(-/+)}$; *G*, *Gtl2*$^{(+/-)}$; *D*, IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$.

birth and demonstrated no skeletal defects, indicating that the perinatal abnormalities were rescued by the restoration of the *Dlk1* and *Rtl1* expression dosage. However, all of the double mutants showed growth retardation at the weaning stage, and 70% of them died within 3 weeks of birth. These findings indicate that the restoration of *Dlk1* and *Rtl1* expression was not sufficient to rescue the lethality of the IG-DMR$^{(-/+)}$ mutants.

Gene expression analysis showed that the levels of maternally expressed ncRNA in the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutants did not return to those in the WT. Our previous study showed that the maternal *Gtl2* deletion led to postnatal lethality due to hypoplastic pulmonary alveoli and hepatocellular necrosis, probably due to the decreased expression of small nucleolar RNAs and miRNAs derived from *Rian* and anti-*Rtl1*/*Mirg* (19). Histological abnormalities of the liver and lungs also were observed in the IG-DMR$^{(-/+)}$/*Gtl2*$^{(+/-)}$ double mutants (two of three; data not shown). Thus, it is highly likely that the postnatal abnormalities observed in the double mutants were caused by the decrease in the expression levels of these ncRNAs. We measured the gene expression levels in five double mutant samples and observed that the expression dosage of *Rian* and *Mirg* varied largely among the samples. One sample showed normal expression levels of *Rian* and *Mirg* in the lungs, while another sample had expression levels two times the average of the other double-mutant samples in the brain. Four out of the 15 double mutant mice survived; therefore, it can be considered that the sample-specific recovery of gene expression might induce partial survivability of the double mutants. Our finding would be supported by recently published reports indicating that ncRNAs in the *Dlk1-Dio3* domain have a great influence on the pluripotency level of induced pluripotent stem cells (22, 23).

In conclusion, the present study suggests that the decrease in the ncRNA expression downstream of *Gtl2* might be involved in postnatal development and lethality. Further studies are required to identify the ncRNAs involved in the lethality induced by altered imprinting pattern in the *Dlk1-Dio3* domain and to understand rhe mechanism by which these ncRNAs exert their effects.

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