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Activation of paternally expressed genes and perinatal death caused by deletion of the *Gtl2* gene

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SUMMARY

The *Dlk1-Gtl2* imprinting locus is located on mouse distal chromosome 12 and consists of multiple maternally expressed non-coding RNAs and several paternally expressed protein-coding genes. The imprinting of this locus plays a crucial role in embryonic development and postnatal growth. At least one cis-element, the intergenic differentially methylated region (IG-DMR) is required for expression of maternally expressed genes and repression of silenced paternally expressed genes. The mechanism by which the IG-DMR functions is largely unknown. However, it has been suggested that the unmethylated IG-DMR acts as a positive regulator activating expression of non-coding RNAs. *Gtl2* is the first non-coding RNA gene downstream of the IG-DMR. Although its in vivo function in the mouse is largely unknown, its human ortholog MEG3 has been linked to tumor suppression in human tumor-derived cell lines. We generated a knockout mouse model, in which the first five exons and adjacent promoter region of the *Gtl2* gene were deleted. Maternal deletion of *Gtl2* resulted in perinatal death and skeletal muscle defects, indicating that *Gtl2* plays an important role in embryonic development. The maternal deletion also completely abolished expression of downstream maternally expressed genes, activated expression of silenced paternally expressed genes and resulted in methylation of the IG-DMR. By contrast, the paternal inherited deletion did not have this effect. These data strongly indicate that activation of *Gtl2* and its downstream maternal genes play an essential role in regulating *Dlk1-Gtl2* imprinting, possibly by maintaining active status of the IG-DMR.

KEY WORDS: *Gtl2*, *Meg3*, *Dlk1-Gtl2* locus, Genomic imprinting, Mouse, Methylation, Muscle development

INTRODUCTION

The imprinted *Dlk1-Gtl2* (delta like 1 homolog – gene trap locus 2) locus is approximately 1 Mb long and located on mouse distal chromosome 12 and on human chromosome 14q32 (da Rocha et al., 2008). This locus consists of multiple genes, which are active either on the chromosome inherited from the mother (maternally expressed) or the one inherited from the father (paternally expressed). The maternally expressed genes (MEGs) in this locus are all non-coding genes, including *Gtl2*, also known as maternally expressed gene 3 (*Meg3*) (Miyoshi et al., 2000), RNA imprinted and accumulated in the nucleus (*Rian*) (Hatada et al., 2001), miRNA containing gene (*Mirg*) (Seitz et al., 2003), anti-retrotransposon-like 1 (*anti-Rtl1*) and numerous C/D-box-containing small nucleolar RNAs (snoRNAs) (Cavaille et al., 2002) and micro RNAs (Seitz et al., 2004). Only three paternally expressed genes (PEGs), all protein coding, are found in this locus, including *Dlk1* (Schmidt et al., 2000; Takada et al., 2000), *Rtl1* (Byrne et al., 2010) and deiodinase, iodothyronine type III (*Dio3*) (Hernandez et al., 2002). The imprinting of these genes plays an important role in prenatal development of multiple tissues,

including muscle, bone and brain, and controls postnatal neurological and metabolic functions (Charalambous et al., 2007; Wilkinson et al., 2007). Duplication of maternal distal chromosome 12 along with a deficiency of the corresponding paternal distal portion of the chromosome, MatDp(dist12), causes prenatal growth retardation, hypotrophic immature skeletal muscle and perinatal death (Tevendale et al., 2006); whereas duplication of paternal distal chromosome 12 with deletion of the corresponding maternal region, PatDp(dist12), causes a much more severe phenotype, i.e. death before 16.5 days of gestation (Tevendale et al., 2006). In humans, uniparental disomy of maternal chromosome 14 (mUPD14) or paternal chromosome 14 (pUPD14) is compatible with life. However, mUPD14 patients manifest short stature, small hands, scoliosis, mild developmental delay and early puberty (Kotzot, 2004). By contrast, pUPD14 patients display characteristic facial anomalies and skeletal defects (Cotter et al., 1997; Kagami et al., 2005). Recently, Stadtfeld et al. (Stadtfeld et al., 2010) reported that mouse induced pluripotent stem cells (iPSCs) with repressed expression of MEGs, including *Gtl2*, *Rian*, *Mirg* and numerous miRNAs, contributed poorly to chimeras and failed to generate all-iPSC mice, whereas iPSCs with normal MEG expressions contributed to high-grade chimeras and produced viable all-iPSC mice. These data indicate that overexpression or lack of expression of imprinted genes in the *Dlk1-Gtl2* locus has detrimental consequences to prenatal development and postnatal growth.

Imprinting of the *Dlk1-Gtl2* locus is regulated by at least one cis-element called the intergenic differentially methylated region (IG-DMR) located between *Dlk1* and *Gtl2* (Lin et al., 2003). The IG-DMR on the maternally inherited chromosome is unmethylated,

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whereas the paternally inherited chromosome is methylated (Takada et al., 2002). Deletion of the IG-DMR on the maternal chromosome silences expression of MEGs and activated maternal alleles of the PEGs. However, a deletion on the paternal chromosome does not have this effect (Lin et al., 2003). The mechanism by which the IG-DMR regulates *Dlk1-Gtl2* imprinting is unclear. It is likely that the IG-DMR acts as a positive regulator activating *Gtl2* transcription and its downstream non-coding RNAs. Transcription of these genes in turn suppresses expression of PEGs on the same chromosome (da Rocha et al., 2008). *Gtl2* is the first maternally expressed gene downstream of the IG-DMR. The *Gtl2* gene contains a well-defined TATA-containing promoter. By contrast, no apparent promoters have been found for any other maternally expressed genes downstream of *Gtl2* (Tierling et al., 2006). In addition, all these gene transcripts have the same orientation as *Gtl2*. Therefore, it has been suggested that all maternally expressed genes are transcribed from the *Gtl2* promoter as one long polycistronic RNA, which is processed post-transcriptionally to generate individual intergenic transcripts (da Rocha et al., 2008). The *Gtl2* gene was discovered in dwarf mice carrying paternal transmission of a *lacZ* insertion on distal chromosome 12 (Schuster-Gossler et al., 1998). The insertion location is approximately 1.7 kb upstream of the *Gtl2* gene (Steshina et al., 2006). Paternal transmission of the *lacZ* insertion reduces expression of PEGs including *Dlk1*, *Dio3* and *Rtl1*, which was believed to cause dwarfism (Sekita et al., 2006; Steshina et al., 2006). Interestingly, maternal transmission of this insertion reduces *Gtl2* expression and causes an increase in *Dlk1* expression on a C57BL/6 background (Steshina et al., 2006). The role of the *Gtl2* gene in regulating imprinting remains elusive.

To investigate the *in vivo* function of *Gtl2* and its role in regulating *Dlk1-Gtl2* imprinting, we created a mouse model carrying a 5.9 kb deletion of genomic DNA including ~300 bp of the *Gtl2* promoter and exon 1 through exon 5 of the *Gtl2* gene. We found that the maternally inherited *Gtl2* deletion in embryos resulted in methylation of the IG-DMR, completely abolished expression of downstream MEGs, activated expression of PEGs and caused perinatal death. By contrast, the paternally inherited deletion did not have this effect. Our data indicate that activation of *Gtl2* and its downstream maternal genes plays a crucial role in regulating *Dlk1-Gtl2* imprinting, possibly by maintaining active status of the IG-DMR.

MATERIALS AND METHODS

Mice

Gtl2 knockout mice were created using inGenious Targeting Laboratories (iTL, Stony Brook, NY). A 15.9 kb DNA fragment containing partial *Gtl2* gene sequences from a mouse 129SvJ BAC clone was used to construct the targeting vector (Fig. 1A). An 8.0 kb region upstream of exon 1 and a 2.0 kb region after exon 5 were cloned into a vector to flank the *neo* cassette; designated long arm and short arm, respectively (Fig. 1B). After linearization, the target vector was transfected into iTL 129 Sv/Ev embryonic stem (ES) cells by electroporation. Seven positive clones were injected into mouse blastocysts and implanted into pseudopregnant C57BL/6J females. Male chimeras from two ES clones were mated to C57BL/6J females to achieve germline transmission. Two F1 male *Gtl2* heterozygous mice were backcrossed to C57BL/6J females and bred up to ten generations. Phenotypic analysis was performed using generation F7 mice or later. Similar numbers of mice derived from each of the two F1 male *Gtl2* heterozygous mice were used in the study. Animals were housed in the Center for Comparative Medicine at Massachusetts General Hospital and the protocol was approved by the Subcommittee on Research Animal Care.

Genotyping

The targeted allele was identified by PCR using tail DNA with primers GtlN2F (5'-AAAAGTCGCCTCCCCTACCCGGTAGAATTGAC-3') and GtlwmR1 (5'-TAGAAGGAACCAAGAGCCGTGAAACCAATGGAGC-3'). The wild-type allele was identified with primers GtlwtF1 (5'-AGTGGACATGAACCTGTTCCAGCCTGCATCC-3') and GtlwmR1. Genotyping was confirmed by Southern blotting. Genomic DNA was digested with *Bam*HI, and probed P₁ and P_s (Fig. 1).

Histology

Embryonic tissues were fixed in Bouin's solution, dehydrated and embedded in paraffin. Six-micrometer sections were prepared and stained with Hematoxylin and Eosin (H&E). For glycogen examination, embryo forelimbs were dissected, snap-frozen (cooled isopentane) in liquid nitrogen and embedded in OCT. Ten-micrometer sections were prepared and stained using PAS (Periodic acid-Schiff).

Electron microscopy

Whole embryo forelimbs without skin were fixed overnight at 4°C with a solution of 2.5% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Fixed muscles were washed with cacodylate buffer, post-fixed in osmium tetroxide, dehydrated in graded ethanol and Epon embedded. Semithin sections were stained with Toluidine Blue alkaline. Ultrathin sections were stained with uranyl acetate, lead citrate and viewed with a JEOL 1200EX transmission electron microscope.

RNA analysis

Total embryo and placental RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Gene expression was determined by northern blotting. Ten micrograms of total RNA was resolved on a 1% denaturing agarose gel. Blots were pre-hybridized at 65°C in a pre-hybridization solution (7% SDS and 0.5 M sodium phosphate, pH 7.5) and hybridized with radiolabeled *Gtl2*-specific cDNA probes (accession number: Y13832) (Schuster-Gossler et al., 1998), *Dlk1* (NM_010052), *anti-Rtl1* and *Rtl1* (NM_184109), *Rian* (AK017440) (Cavaille et al., 2002), *Mirg* (AJ517767) (Seitz et al., 2003) or *Dio3* (NM_172119) (Hernandez et al., 1999). The blots were stripped and re-probed with radiolabeled *Gapdh* probe as internal controls. All cDNA probes were labeled with [α -³²P]dCTP using Ready-To-Go DNA Labeling Beads (GE Healthcare, Piscataway, NJ, USA). Expression was quantified using a Storm 860 Phosphorimager and associated ImageQuant program (GE Healthcare). Total RNA from oocytes (approximately 100) was isolated and treated with DNase using a NucleoSpin RNA II Kit following the manufacturer's instructions (Macherey-Nagel, Bethlehem, PA). The first strand cDNA was synthesized using the ProtoScript AMV First Strand cDNA Synthesis Kit using the manufacturer's protocol (New England BioLabs, Ipswich, MA, USA). Expression of *neo* was detected by RT-PCR with the primer set 5'-ACAGTCGATGAATCCAGAAAAGCGG-3' (forward) and 5'-GGTA-GCCAACGCTATGTCCTGATAGC-3' (reverse).

Quantitative RT-PCR

Ten nanograms of RNA were used to synthesize cDNAs for micro RNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed in quadruplicate using *mir-127* or *mir-410* specific TaqMan probes following the manufacturer's instructions on an Applied Biosystems 7500 Fast Real-Time PCR system. *SnoRNA202* was used as the endogenous control. Relative quantities for individual microRNA were calculated using the associated SDS software. Values from wild-type embryos were designated as 100%. Values from *Gtl2* KO/+ and *Gtl2* +/-KO embryos were normalized against those from wild-type embryos.

Biallelic expression of *Dlk1*

Biallelic expression of the *Dlk1* gene in embryos was done as previously described (Lin et al., 2003). Total RNA was isolated from 16.5 days post coitum (dpc) embryos. After reverse transcription, a fragment flanking the polymorphism was amplified by PCR using primers: 5'-TCCTGAA-GGTGTCCATGAAAGAGC-3' (forward) and 5'-AAGCATAGCGTTC-ACTCGATTCCAC-3' (reverse). Multiple PCR products were generated

from several embryos obtained from at least three litters for each genotype and their respective wild-type littermates. The biallelic expression of *Dlk1* was detected by sequencing the PCR products.

Analysis of differentially methylated regions by Southern blotting and bisulfite sequencing

Fifteen micrograms of genomic DNA was used for restriction digestion, as described below. After being resolved on a 2% agarose gel, DNA was transferred onto Nytran SPC membranes using a Whatman TurboBlotter (GE Healthcare). The membrane was pre-hybridized with hybridization buffer (1% bovine serum albumin in 7% SDS and 0.5 M sodium phosphate, pH 7.5) at 65°C and hybridized with ³²P-labelled DNA probes as described below. The image was acquired using a Storm 860 phosphorimager. For the IG-DMR, DNA was digested with *StuI* plus methylation-insensitive *MspI* or methylation-sensitive *HpaII* or *HhaI*. The blot was hybridized with probe M4 as previously described (Takada et al., 2002). For *Gtl2*-DMR, DNA was digested with *BsrI* and enzymes indicated, and hybridized with a 1 kb DNA probe approximately 350 bp upstream the first *Gtl2* exon. For the *Dlk1*-DMR, DNA was digested with *NheI* and enzymes indicated, and hybridized with D2 probe as previously described (Takada et al., 2000). Bisulfite treatment was performed using an EpiTect Bisulfite Kit from Qiagen following the manufacturer's instructions. After purification of the treated DNA, two round PCRs were used to amplify the IG-DMR region using Takara Premix EX Taq. Primers for the first round PCR were 5'-TAAGTGTGTTGGTTTGTATGGGTA-3' (forward) and 5'-CCATCCCCTATACTCAAAACATTCT-3' (reverse). Primers for the second round PCR were 5'-TGGTTTGTATGGGTA-AGTTTTATG-3' (forward) and 5'-CTTCCCTCACTCCAAAATTA-AAA-3' (reverse). PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced by the Massachusetts General Hospital DNA Core facility. For bisulfite sequencing of gamete DNA, approximately 120 oocytes, collected as previously described (Morita et al., 2000), were treated with an Easy DNA Methylation-Direct Kit using the manufacturer's protocol (Zymo Research, Orange, CA, USA).

Statistical analysis

Fisher's exact test was used to compare survival rates between embryos and neonates (Table 1). Student's *t*-test was used to analyze data of embryo body weight, placenta weight, myofiber count (Fig. 2G) and placental expression of *Dlk1* and *Rtl1* (Fig. 6).

RESULTS

Generation of *Gtl2* knockout mice

Analysis of genomic sequences surrounding the transcription initiation site of the *Gtl2* gene revealed that the *Gtl2* core promoter contains a TATA box and a CAAT box. The *Gtl2* gene consists of ten exons (Miyoshi et al., 2000; Schuster-Gossler et al., 1998). To inactivate the *Gtl2* gene, we deleted the first five exons of the gene and the adjacent upstream promoter sequences of ~300 bp by gene targeting (Fig. 1). The targeted region encodes approximately half

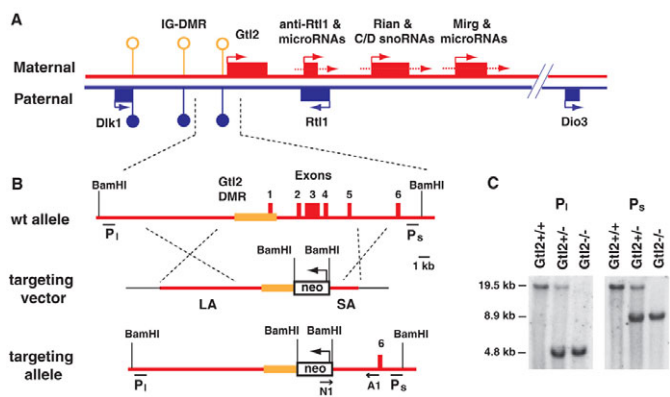


Fig. 1. Targeted deletion of the *Gtl2* gene. (A) Schematic representation of the *Dlk1-Gtl2* locus on mouse chromosome 12. Maternally expressed genes are in red and paternally expressed genes are in blue. Differentially methylated regions are shown as circles. Filled circle, methylated; open circle, unmethylated. (B) The *Gtl2* gene consists of ten exons (only six exons are shown). The gene targeting vector contains an 8.0 kb genomic DNA upstream exon 1 (LA) and a 2.0 kb DNA after exon 5 (SA) flanking the *neo* cassette. After homologous recombination, the targeted region consisting of exons 1-5 of the *Gtl2* gene was deleted. P₁ and P₅ are the probes for detection of long and short arms by Southern blotting, respectively. N1 and A1 are PCR primers for genotyping. (C) Deletion of the targeted region was confirmed by Southern blotting. Genomic DNA was digested with *Bam*HI, and probed with P₁ and P₅ as indicated.

of the mature *Gtl2* transcript – approximately 1900 nucleotides long (Schuster-Gossler et al., 1998). Two lines of *Gtl2* knockout KO mice obtained from two independent ES clones were used. Because it is a maternally expressed gene, we maintained the generated mice by crossing heterozygous males with wild-type C57BL/6J females.

Maternal transmission of *Gtl2* deletion resulted in pre- or perinatal death

Newborn pups carrying maternal or paternal *Gtl2* gene deletions (*Gtl2* KO/+ and *Gtl2* +/KO, respectively) were obtained and examined postnatally (Table 1). All pups carrying the paternal deletion appeared healthy and lived well beyond 1 year. By contrast, none of the pups carrying the maternal *Gtl2* deletion survived. They had similar body weight to their wild-type littermates (data not shown). No milk was visible in the dead pups' stomachs and their lungs sank in water, consistent with pre- or perinatal death. We also examined mice with deletions of both *Gtl2*

Table 1. Maternal deletion of *Gtl2* causes perinatal death

	Embryos (dead)			Neonates (dead)		
	Mating: female × male			Mating: female × male		
	+/+ × +/KO	+/KO × +/+	+/KO × +/KO	+/+ × +/KO	+/KO × +/+	+/KO × +/KO
Total number	70	78	97	162	78	44
Litter number	10	13	15	24	14	9
Av. litter size	7±0.8	6±0.8	6.5±1.2	6.9±1.9	5.6±2.3	4.9±1.5
+/+	32	36	27	80	43	13
+/KO or KO/+	38	42 (5)*	47 (7)	82	35 (35)*	21 (10)
KO/KO	n/a	n/a	23 (2)†	n/a	n/a	10 (10)†

The lethality of neonates carrying a maternal deletion of *Gtl2* (KO/+) or homozygous deletion (KO/KO) is significantly increased compared with their littermates. Numbers in parentheses indicate dead embryos or neonates.
 *, $P=9.70 \times 10^{-17}$; †, $P=7.13 \times 10^{-7}$; Fisher's exact test.
 n/a, not applicable.

alleles and found that half of the heterozygous pups were dead postnatally, while all *Gtl2* KO/KO pups were dead (Table 1). We next examined embryos before birth at 18.5 dpc to determine whether pups carrying the maternal deletion died prenatally. As expected, all *Gtl2* +/KO embryos were alive before birth (Table 1) and weighed approximately 13.3% less than their wild-type littermates (*Gtl2* +/KO 1.02 ± 0.10 g versus *Gtl2*^{+/+} 1.16 ± 0.11 g; $P=2 \times 10^{-7}$). *Gtl2* KO/+ embryos were mostly alive at 18.5 dpc (Table 1). In contrast to the *Gtl2* +/KO embryos, *Gtl2* KO/+ embryos did not significantly differ in weight from their wild-type littermates (*Gtl2* KO/+ 1.09 ± 0.16 g versus *Gtl2*^{+/+} 1.14 ± 0.10 g; $P=0.227$). We examined 23 *Gtl2* KO/KO embryos; 21 of them were alive. They also had similar body weight to their wild-type littermates (*Gtl2* KO/KO 1.23 ± 0.13 g versus *Gtl2*^{+/+} 1.21 ± 0.11 g; $P=0.638$). No apparent physical abnormalities were observed in these embryos. These data indicate that paternal transmission of the *Gtl2* deletion did not cause perinatal death but resulted in embryonic growth retardation. By contrast, maternal deletion of the gene resulted in perinatal death, but did not affect embryonic growth.

Maternal transmission of the *Gtl2* deletion resulted in skeletal muscle defects

Gtl2 KO/+ and *Gtl2* KO/KO embryos of 18.5 dpc were dissected and examined histologically for abnormalities. No apparent anatomic defects were observed in the brain, heart, lung, kidney, liver or spleen. However, they contained obvious defects in all skeletal muscles, including diaphragm, intercostal, paraspinal and limb muscles. As shown in Fig. 2, for example, paraspinal muscles from wild-type embryos as well as *Gtl2* +/KO embryos consist of mainly small, rounded myofibers with peripherally placed nuclei and eosinophilic contractile apparatus throughout the myocyte cytoplasm. An extremely low number of myofibers contain small areas of clearing (Fig. 2A,B). By contrast, the muscles from *Gtl2* KO/+ and *Gtl2* KO/KO embryos consisted of mainly myofibers with centrally localized nuclei or areas of central clearing (Fig. 2C,D). Staining of longitudinally sectioned muscles demonstrated that the area of central clearing is the space between centrally located nuclei along myofibers (Fig. 2D, inset). Central nucleation and central clearing are characteristics of immature muscle fibers called myotubes (Platzer, 1978; Stromer et al., 1974) or can be found postnatally in a severe congenital myopathy called X-linked myotubular myopathy (Pierson et al., 2005). Indeed, H&E skeletal muscle staining from wild-type 15.5 dpc embryos showed that virtually all myotubes contain either centrally localized nuclei or areas of central clearing (Fig. 2E). Similar phenotypes were observed in muscles from *Gtl2* KO/+ embryos at the same age, although the myotubes showed larger areas of clearing (Fig. 2F). The number of myofibers with central nucleation or central clearing decreases with age in wild-type embryos as skeletal muscles mature (Fig. 2G). However, the number remained high in *Gtl2* KO/+ embryos compared with age-matched wild-type littermates (Fig. 2G). Ultrastructural examination revealed appropriate organization of organelles and contractile apparatus in skeletal muscles from 18.5 dpc wild-type embryos (Fig. 3A). By contrast, myofibers from *Gtl2* KO/+ embryos at the same age displayed internally placed nuclei and areas of central clearing, consistent with deposition of a substance that was dissolved during processing for electron microscopy (Fig. 3B,C). The most common muscle constituent with these staining characteristics is glycogen. We stained longitudinal section of forelimb muscle from 18.5 dpc *Gtl2* KO/+ embryos by periodic acid Schiff (PAS) (Fig. 3D). The

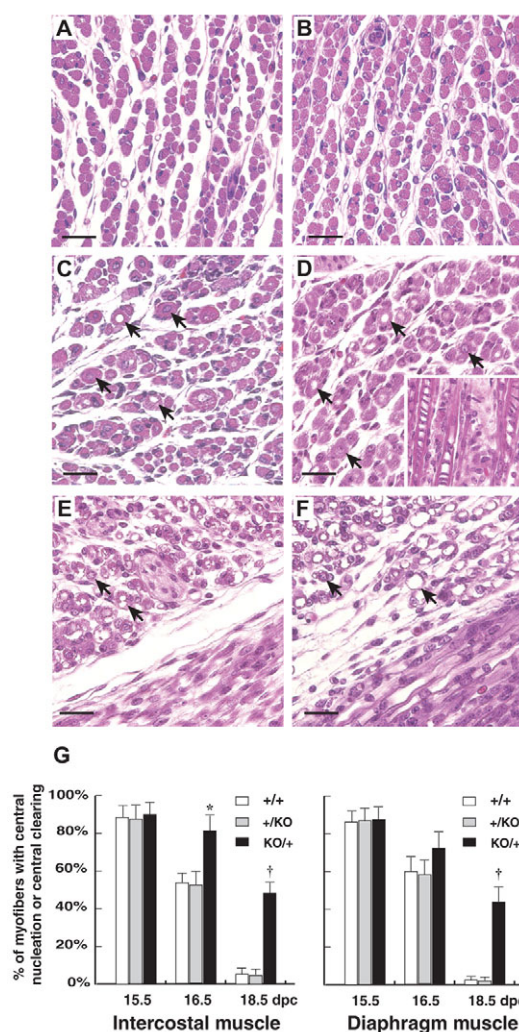


Fig. 2. *Gtl2* KO/+ embryos display delayed skeletal muscle fiber development. (A–F) Hematoxylin and Eosin (H&E) staining of skeletal muscle from (A) 18.5 dpc wild-type and (B) 18.5 dpc *Gtl2* +/KO embryos shows peripherally located nuclei and no central clearing within myofibers. H&E staining of skeletal muscle from (C) 18.5 dpc *Gtl2* KO/+ and (D) 18.5 dpc *Gtl2* KO/KO embryos display centrally located nuclei and central clearing within myofibers. Similar findings are seen in longitudinal sections (D, inset). H&E staining of skeletal muscle from (E) 15.5 dpc *Gtl2*^{+/+} and (F) 15.5 dpc KO/+ embryos show centrally located nuclei and central clearing within myotubes. Arrows denote central nucleation and central clearing within myofibers. (G) Total numbers of myofibers in multiple fields as well as numbers of myofibers with centrally located nuclei or central clearing in the same fields were counted. Data were obtained from at least three embryos for each genotype from three different litters. Percentages of myofibers with central nucleation or central clearing were calculated in intercostal muscles (left) and diaphragm (right) from embryos at ages 15.5, 16.5 and 18.5 dpc, respectively. Data were represented as mean \pm s.d. Student's *t*-test was used to compare values between *Gtl2* KO embryos and their litter-matched *Gtl2*^{+/+} embryos. *, $P < 0.05$; †, $P < 0.01$. Scale bars: 25 μ m.

PAS-positive areas resided in the middle of the myofibers. The staining pattern was very similar to that of the central clearing found in the H&E-stained longitudinal section of *Gtl2* KO/+ paraspinal muscle (Fig. 2D, inset) suggesting that the areas of clearing seen on H&E staining were filled with glycogen (Fig. 3D). The

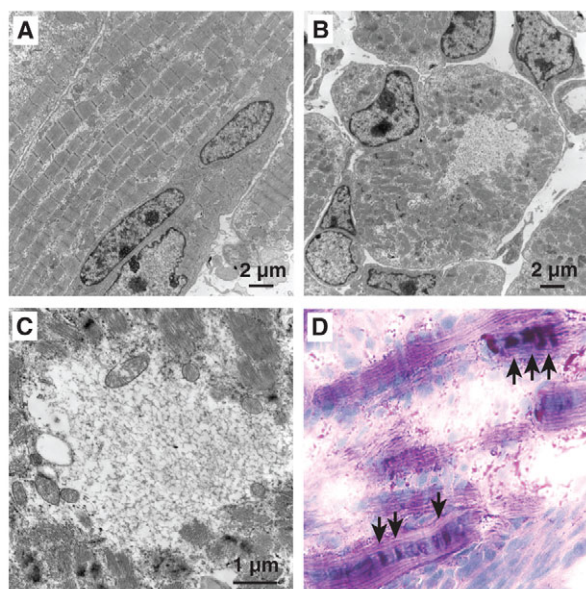


Fig. 3. Skeletal muscles from *Gtl2* KO/+ embryos have central accumulation of glycogen within myofibers. (A) Electron micrographs of limb muscle from 18.5 dpc wild-type embryos show peripherally located nuclei. (B,C) Electron micrographs of limb muscle from 18.5 dpc *Gtl2* KO/+ embryos reveal internal nuclei and central washed out areas, and slightly granular material within myofibers. (D) PAS staining of limb muscles from 18.5 dpc *Gtl2* KO/+ embryos highlights areas between central nuclei (arrows) that had been clear on H&E staining (Fig. 2D, inset).

These findings are consistent with reported ultrastructural studies of myotubes at gestational day 14–15 in mice (Platzer, 1978). These data suggest a delay in muscle maturation beyond the myotube stage in embryos with maternal deletion of the *Gtl2* gene.

Maternal deletion of *Gtl2* resulted in silencing of maternally expressed genes and activation of paternally expressed genes in embryos

Two cDNA probes were used to determine *Gtl2* transcript levels: one overlaps the exon 3 region (P_{ex3}), which had been deleted in the targeted allele; the other spans exons 8, 9 and 10 (P_{ex10}), which had been retained in the targeted allele. As expected, *Gtl2* +/KO embryos expressed *Gtl2* at a level comparable with the wild-type embryos (Fig. 4A,C), whereas no *Gtl2* expression was detected in *Gtl2* KO/+ embryos with both probes (Fig. 4A), suggesting that *Gtl2* gene transcription was completely abolished in embryos with the maternal transmission of the deletion. To investigate how this deletion affects other downstream MEGs, we examined expression of several maternally expressed non-coding RNAs in 16.5 dpc embryos. By northern blotting, RNA levels of *anti-Rtl1*, *Rian* and *Mirg* in *Gtl2* KO/+ embryos were reduced to 9.0, 15.0 and 5.8%, respectively, of those in the wild-type littermates (Fig. 4B,C). By quantitative RT-PCR, levels of *miR-127* and *miR-410* in *Gtl2* KO/+ embryos were reduced to 3.5% and 4.1%, respectively, of those in wild-type littermates (Fig. 4D). By contrast, their expression levels did not change in *Gtl2* +/KO embryos compared with wild-type littermates (Fig. 4D). Paternal deletion of the *Gtl2* gene did not affect expression of PEGs, including *Dlk1*, *Rtl1* and *Dio3* (Fig. 4B,C). However, the transcript levels of *Dlk1*, *Dio3* and *Rtl1* were 210, 180 and 490% in *Gtl2* KO/+ embryos, respectively, compared

with wild-type littermates (Fig. 4C). This phenomenon is similar to that found in embryos carrying maternal transmission of the IG-DMR deletion, where elevated levels of *Dlk1*, *Rtl1* and *Dio3* in IG-DMR KO/+ embryos are due to activation of their maternal alleles (Lin et al., 2003). Increased *Rtl1* expression is also partly due to lack of expression of *anti-Rtl1*, which is believed to contain micro RNAs inhibitory to *Rtl1* (Lin et al., 2003; Seitz et al., 2003). To investigate whether deletion of the *Gtl2* gene activates the maternal allele of PEGs, we crossed DBA/2 mice with heterozygous *Gtl2* mice to generate embryos with paternal or maternal deletion of the *Gtl2* gene. *Dlk1* contains a polymorphism in the coding region (Lin et al., 2003). Transcripts from both alleles of *Dlk1* were detected in embryos carrying maternal transmission of the *Gtl2* deletion, indicating that maternal deletion of the *Gtl2* gene activates the maternal allele of the paternal gene (Fig. 5).

Maternal deletion of *Gtl2* resulted in silencing of maternally expressed genes in placenta but did not affect placental growth

Placentas of 18.5 dpc were stained with H&E. No apparent defects were observed in placentas carrying paternal, maternal or homozygous deletion of the *Gtl2* gene (data not shown). In addition, maternal deletion and homozygous deletion of the *Gtl2* gene did not significantly affect placental growth. The average weight of the *Gtl2* KO/KO placentas was 0.104 ± 0.018 g, while that of the litter-matched *Gtl2*^{+/+} placentas was 0.105 ± 0.015 g ($P=0.8814$). Similarly, the *Gtl2* KO/+ placental weight was 0.121 ± 0.023 g versus the litter-matched *Gtl2*^{+/+} placentas, at 0.113 ± 0.016 g ($P=0.2982$). By contrast, paternal gene deletion resulted in a significant decrease in placental weight (*Gtl2* +/KO 0.086 ± 0.015 g versus *Gtl2*^{+/+} 0.101 ± 0.015 g, $P=0.0241$). The smaller placenta may be responsible for the smaller size of the *Gtl2* +/KO embryos.

To determine the effect of *Gtl2* deletion on placental gene expression, we investigated expression of maternal and paternal genes in 18.5 dpc placentas. Expression of MEGs was completely abolished in placentas carrying maternal deletion of the *Gtl2* gene (Fig. 6), similar to the expression patterns in embryos (Fig. 4). In addition, *Rtl1* expression was increased to approximately 400% of that seen in wild type (Fig. 6). Surprisingly, maternal *Gtl2* deletion did not affect placental *Dlk1* expression, which is in contrast to the increased expression seen in *Gtl2* KO/+ embryos (Figs 4 and 6). We failed to detect any *Dio3* expression in 18.5 dpc placenta. Therefore, we are unable to determine whether *Dio3* expression was changed in *Gtl2* KO/+ placenta. Paternal *Gtl2* deletion did not affect expression of any placental MEGs (Fig. 6). By contrast, the paternal deletion significantly reduced expression of *Dlk1* and *Rtl1* to 50 and 60% of their respective wild types (Fig. 6). Taken together, these data raise the possibility that maternal and paternal alleles are regulated individually by separate mechanisms in placenta. We also investigated the methylation status at the IG-DMR in *Gtl2* KO/+ placentas. We found that the IG-DMR was fully methylated (data not shown), which is consistent with IG-DMR methylation in embryos carrying a maternal *Gtl2* deletion.

Maternal deletion of *Gtl2* resulted in methylation of the maternal allele of the IG-DMR in embryos but not in oocytes

Deletion of the maternal IG-DMR, which is unmethylated, silences maternally expressed genes and activates maternal alleles of the paternally expressed genes in embryos (Lin et al., 2003). By contrast, deletion of the paternal IG-DMR, which is methylated,

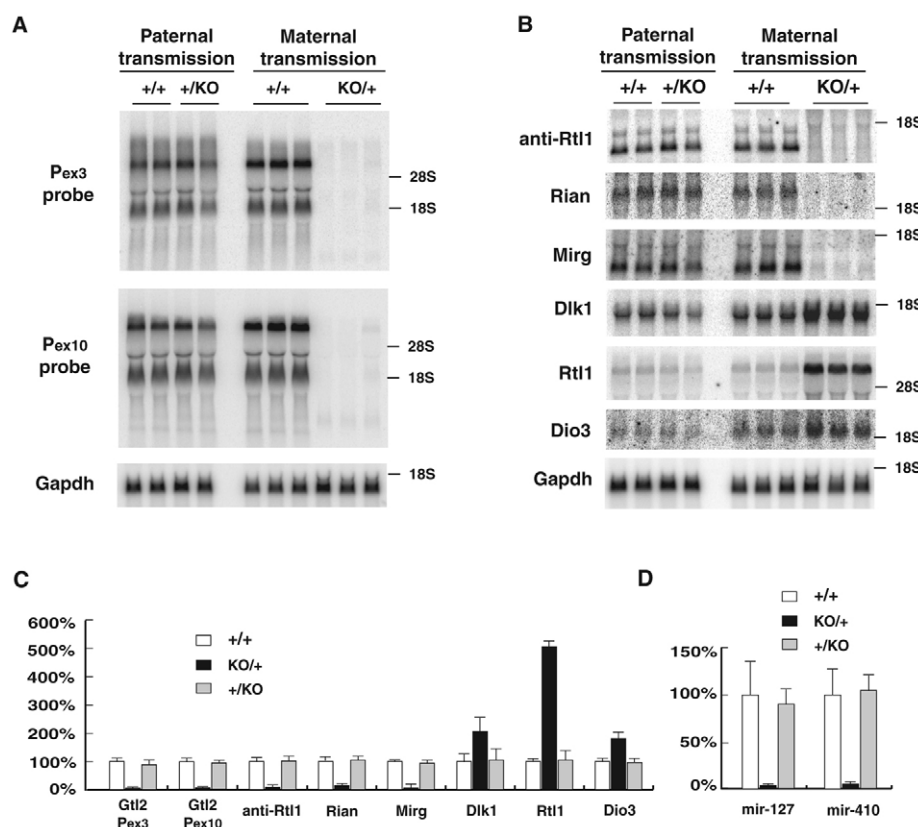


Fig. 4. Expression of imprinted genes in *Gtl2* KO embryos. (A) Northern blot analysis of *Gtl2* expression. Total RNA was isolated from whole 16.5 dpc embryos with paternal (+/KO) or maternal (KO/+) deletion of the *Gtl2* gene and their wild-type littermates (+/+). Each track represents RNA from one embryo. Blots were hybridized with *Gtl2* probe P_{ex3} or P_{ex10}. *Gapdh* was used as a control for equal loading. (B) Northern blot analysis of maternally and paternally expressed genes. (C) Comparison of the imprinted gene expression between embryos carrying maternal (black bar), or paternal (gray bar) *Gtl2* deletion and their wild-type littermates (white bar). Values were calculated using control and mutant embryos from multiple litters. (D) Expression of maternally imprinted microRNAs by qRT-PCR. Values from wild-type embryos were designated as 100%, against which values from *Gtl2* KO/+ and *Gtl2* +/KO embryos were normalized. Data were obtained using at least three embryos for each genotype from two different litters and were represented as mean \pm s.d.

does not (Lin et al., 2003), suggesting that the function of IG-DMR is inactivated by methylation. Using methylation-sensitive Southern blotting, we detected both methylated and unmethylated IG-DMR bands in *Gtl2*^{+/+} and *Gtl2* +/KO embryos (Fig. 7A). However, only methylated IG-DMR was detected in *Gtl2* KO/+ embryos (Fig. 7A). This finding was confirmed by bisulfite sequencing of the IG-DMR core region (Fig. 7D), indicating that the maternal deletion of *Gtl2* results in IG-DMR methylation. The *Gtl2* DMR covers approximately 1.5 kb upstream of the *Gtl2* promoter, the first exon and part of the first intron (Paulsen et al., 2001; Takada et al., 2002). In our *Gtl2* knockout model, the 3' end of *Gtl2*-DMR is deleted. The remaining *Gtl2*-DMR is approximately 1 kb long and we examined its methylation. As shown in Fig. 7B, digestion of this region with *Hpa*II revealed three unmethylated bands in *Gtl2*^{+/+} embryos. However, two of them were not detected in *Gtl2* KO/+ embryos, indicating that these sites were methylated in embryos carrying a maternal deletion of *Gtl2*. Similarly, *Hha*I digestion demonstrated increased methylation in the *Gtl2*-DMR in the *Gtl2* KO/+ embryos compared with that in the *Gtl2*^{+/+} embryos (Fig. 7B). This increase is not observed in *Gtl2* +/KO embryos. The *Dlk1* gene also contains a DMR located near its exon 5 (Takada et al., 2002). Neither paternal nor maternal *Gtl2* gene deletion altered *Dlk1*-DMR methylation (Fig. 7C), indicating that maternal deletion of the *Gtl2* gene leads to a partial epigenotype switch from maternal allele to paternal allele.

The *neo* cassette from the targeting vector was inserted approximately 12.7 kb downstream of the IG-DMR and is transcribed toward the IG-DMR (Fig. 1). Chotalia et al. (Chotalia et al., 2009) reported that transcription from the *Nesp* gene establishes methylation of DMRs in the *Gnas* locus in maternal germ cells. This raises the question as to whether gain of methylation at the IG-DMR in *Gtl2* KO mice is due to transcription

from the *neo* cassette. We examined the methylation status of the IG-DMR in oocytes obtained from mice carrying the *Gtl2* deletion. We found that the IG-DMR remained unmethylated in oocytes carrying the *Gtl2* deletion (Fig. 8A). It is important to mention that oocytes isolated from *Gtl2* KO mice consist of two populations: those carrying the wild-type allele of *Gtl2* and those carrying the targeted allele. Because the IG-DMR is unmethylated in wild-type oocytes, the fact that we did not detect any fully methylated DNA indicates that oocytes carrying the targeted allele do not contain methylated IG-DMR. We also detected *neo* cDNA in the oocytes obtained from *Gtl2* KO mice, indicating that the *neo* is

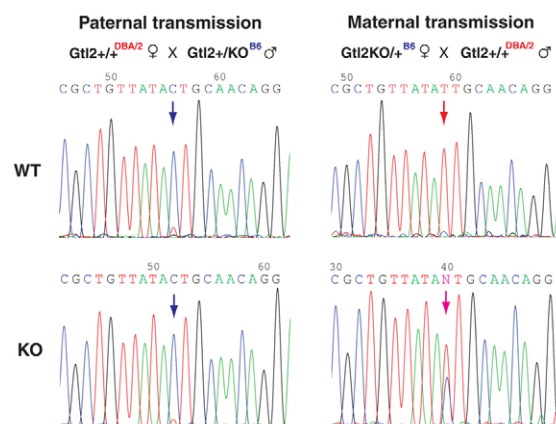


Fig. 5. Biallelic expression of *Dlk1* in *Gtl2* KO/+ embryos. Total RNA was isolated from embryos obtained by crossing DBA/2 with *Gtl2* +/KO C57BL/6 mice. The region containing the polymorphism was amplified by RT-PCR. The allelic expression of *Dlk1* was determined by sequencing PCR products.

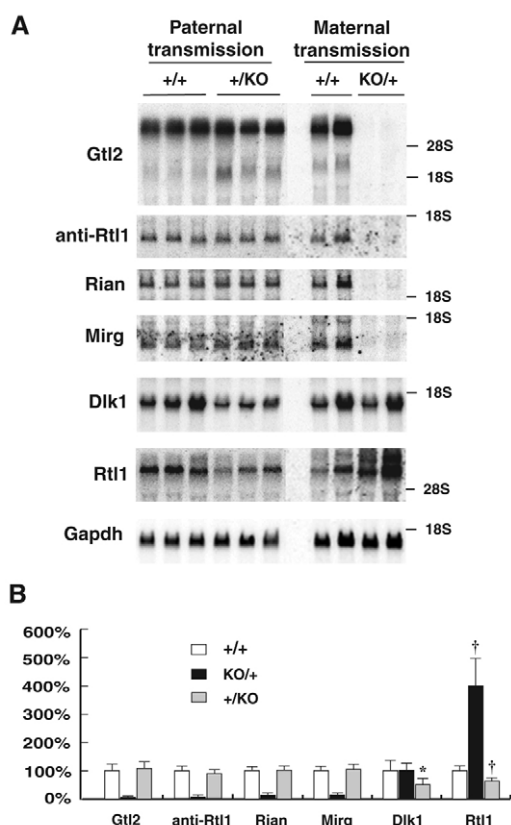


Fig. 6. Expression of imprinted genes in *Gtl2* KO placentas.

(A) Northern blot analysis of maternally and paternally expressed genes in 18.5 dpc placentas. Each track represents RNA from one embryo. *Gapdh* was used as the internal control. (B) Comparison of the imprinted gene expression between 18.5 dpc placentas carrying maternal (black bar), or paternal (gray bar) *Gtl2* deletions and their wild-type littermates (white bar). Values from wild-type embryos were designated as 100%, against which values from *Gtl2* KO/+ and *Gtl2* +/-KO placentas were normalized. Data were obtained using at least five placentas for each genotype from three different litters and were represented as mean \pm s.d. The one-sample *t*-test was used to compare values between *Gtl2* KO embryos and their litter-matched *Gtl2*^{+/+} embryos. *, *P* < 0.02; †, *P* < 0.01.

transcriptionally active in germ cells (Fig. 8B). These data suggest that existence of the *neo* does not influence IG-DMR methylation in germ cells.

DISCUSSION

We demonstrated that deletion of the maternal allele of the *Gtl2* gene caused perinatal death (Table 1) and embryonic skeletal muscle developmental defects (Figs 2 and 3). Skeletal muscles from late gestation embryos carrying the maternal deletion of *Gtl2* display characteristics of immature myotubes found in much younger embryos (Figs 2 and 3). The concept that developmental arrest at the myotube stage could cause severe muscle disease was first proposed by Spiro et al. (Spiro et al., 1966), where patients with severe congenital myopathy and skeletal muscle biopsies displaying small myofibers and markedly increased central nucleation were diagnosed with ‘myotubular myopathy’. Subsequent work has proven this term to be a misnomer; a murine model of myotubular myopathy displays normal histological

development at birth, with central nucleation developing later in the course of the disease (Buj-Bello et al., 2002). Our histological findings in mice with maternal transmission of the *Gtl2* deletion suggest arrest or delay in progression from the myotube stage in our mice, which may contribute to their lack of survival through the perinatal period.

Takahashi et al. (Takahashi et al., 2009) reported that deletion of both *Gtl2* and its DMR abolished *Gtl2* expression, but retained various levels of expression of other MEGs and did not significantly affect expression of PEGs. Interestingly, mice carrying this maternal deletion have a much milder phenotype; they were born alive and lived up to 4 weeks after birth (Takahashi et al., 2009). These data suggest that lack of *Gtl2* expression may not directly cause the perinatal death and skeletal muscle developmental delays found in our *Gtl2* KO/+ embryos. *Dlk1* has been implicated in skeletal muscle development. It is involved in differentiation of skeletal stem cells (Abdallah et al., 2004) and skeletal muscle remodeling (Andersen et al., 2009). Davis et al. (Davis et al., 2004) reported that overexpression of *Dlk1* resulted in skeletal muscle hypertrophy. However, a recent study by the Ferguson-Smith group (da Rocha et al., 2009) showed that high levels (two- to threefold of wild type) of *Dlk1* expression enhances embryo growth, leading to skeletal but not skeletal muscle defects. Therefore, *Dlk1* may not be the gene responsible for the delay in skeletal muscle development in *Gtl2* KO mice. The gene expression pattern of the *Dlk1-Gtl2* locus in *Gtl2* KO/+ mice resembles that found in embryos carrying maternal deletion of the IG-DMR and with paternal uniparental disomy for chromosome 12 (da Rocha et al., 2008; Georgiades et al., 2000; Lin et al., 2007). These mice all had skeletal muscle developmental defects and died at late gestation or perinatally (da Rocha et al., 2008; Georgiades et al., 2000; Lin et al., 2007). Therefore, it is likely that the severe phenotype found in *Gtl2* KO/+ mice is caused by contributions from more than one gene.

Maternal deletion of *Gtl2* abolished expression of all MEGs examined (Figs 4 and 6). Although not yet proven, it has been suggested that all maternally expressed genes are transcribed into one giant polycistronic RNA, from which individual genes are derived by post-transcriptional processing (Tierling et al., 2006). Because a small portion of the *Gtl2* promoter was deleted in the targeted allele, it is possible that lack of downstream MEG expression is due to promoter truncation. It is important to point out that it is not possible to discern whether the effect of the *Gtl2* deletion in our animal model is due to lack of transcription from the *Gtl2* promoter, which leads to silencing of all MEGs, or due to lack of *Gtl2* expression, which in turn causes silencing of the downstream MEGs.

The gene expression pattern, i.e. silencing of MEGs and activation of PEGs, and the skeletal muscle defects in *Gtl2* KO/+ embryos were strikingly similar to those seen in IG-DMR KO/+ embryos (Lin et al., 2003). Interestingly, the maternal allele of the IG-DMR was fully methylated in *Gtl2* KO/+ embryos (Fig. 7). This suggests that IG-DMR methylation may be another mechanism by which *Gtl2* deletion causes silencing of MEGs and activation of PEGs, resulting in the severe skeletal muscle phenotype. The mouse model by Takahashi et al. (Takahashi et al., 2009) carried a 10 kb DNA deletion including the *Gtl2*-DMR and the first five exons of *Gtl2* (to distinguish this animal model from ours, we designated it as *Gtl2*-10kb KO). *Gtl2* expression was abolished completely in embryos carrying the maternal deletion of this region (*Gtl2*-10kb MatKO). However, expression of other MEGs was not completely diminished, but rather modestly

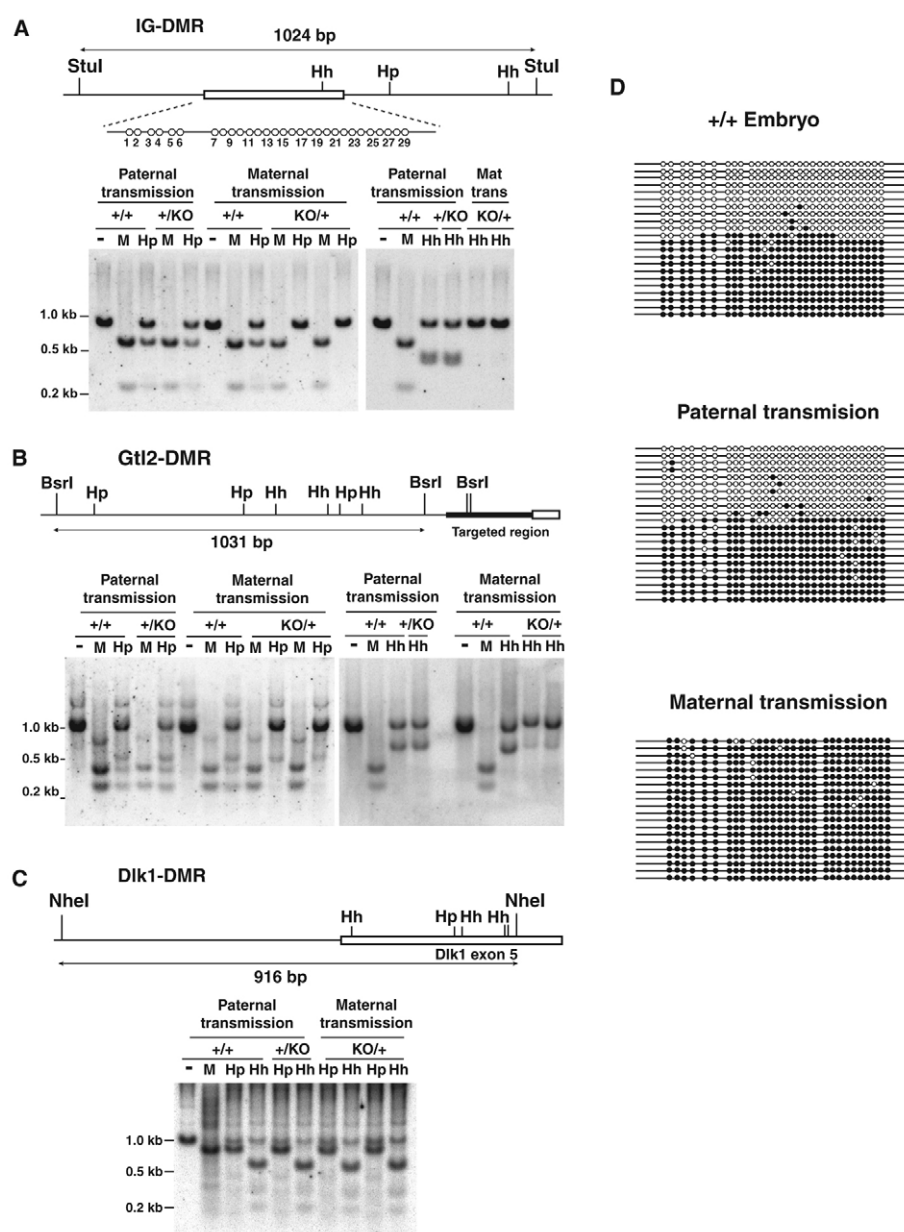


Fig. 7. Methylation analysis of IG-DMR, *Gtl2*-DMR and *Dlk1*-DMR. (A–C) Genomic DNA from 16.5 dpc embryos carrying paternal *Gtl2* deletion (+/KO) and maternal *Gtl2* deletion (KO/+) as well as their wild-type littermates (+/+) were digested with (A) *Stul*, (B) *Bsrl* or (C) *NheI*. The fragmented DNAs were further digested with methylation-insensitive *MspI*, or methylation-sensitive *HpaII* or *HhaI*. The digested DNAs were analyzed by Southern hybridization with probes specific against (A) IG-DMR, (B) *Gtl2*-DMR and (C) *Dlk1*-DMR, respectively. Restriction enzymes: M, *MspI*; Hp, *HpaII*; Hh, *HhaI*. (D) IG-DMR bisulfite sequencing analysis. Each horizontal bar represents a sequence from each clone. Circles indicate CpG sites. White and black circles designate unmethylated and methylated CpGs, respectively.

expressed in *Gtl2*-10kb MatKO embryos. In addition, the PEGs were not activated in the *Gtl2*-10kb MatKO embryos, but rather activated in embryos carrying a paternal deletion or a homozygous deletion (Takahashi et al., 2009). This contrasts significantly with our finding, as well as reports of the maternal IG-DMR deletion (Lin et al., 2003). One of the main differences in the targeted deletion between our *Gtl2* KO mice and *Gtl2*-10kb KO mice is the orientation of the *neo* cassette in the targeted allele. It appears that transcription of the *neo* in the *Gtl2*-10kb KO mice is transcribed away from the IG-DMR and has the same orientation as the MEGs (Takahashi et al., 2009). By contrast, the *neo* cassette in our *Gtl2* KO mice is transcribed towards the IG-DMR and has the opposite orientation to the MEGs. It is very interesting to note that *Rian* and *Mirg* levels in *Gtl2*-10kb KO embryos carrying a paternal or homozygous deletion are significantly higher than those in wild-type embryos. For example, levels of *Rian* in lung are 1.00, 0.66, 2.44 and 1.88 that of wild-type embryos, embryos with maternal, paternal deletion and homozygous deletion of *Gtl2*-10kb,

respectively (Takahashi et al., 2009). One very likely interpretation is that the *neo* promoter, *PGK*, is responsible for the modest expression of MEGs in *Gtl2*-10kb KO embryos with a maternal deletion. Similarly, the *neo* promoter would also be responsible for additional expression of MEGs in embryos with a paternal or homozygous deletion, because the *neo* promoter renders MEGs bi-allelically active in these *Gtl2*-10kb KO mice. Therefore, one possible mechanism responsible for the discrepancy in imprinted gene expression between *Gtl2*-10kb KO mice and our *Gtl2* KO mice is the transcription of the *neo* cassette.

Another major difference between our data and Takahashi's data is that maternal deletion of the *Gtl2* gene in our animal model resulted in methylation of the IG-DMR (Fig. 7), whereas maternal deletion of the *Gtl2* gene and its DMR did not affect IG-DMR methylation in Takahashi's model (Takahashi et al., 2009). The failure of IG-DMR methylation in *Gtl2*-10kb MatKO embryos may be attributed to the additional deletion upstream of the *Gtl2* promoter, which may contain a cis-element necessary for IG-DMR

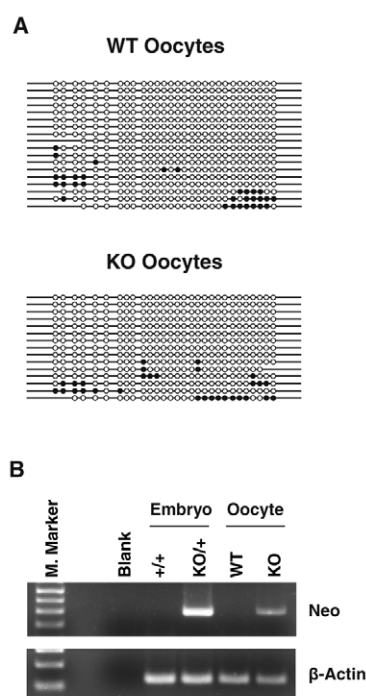


Fig. 8. Methylation analysis of IG-DMR and *neo* expression in female germ cells. (A) Bisulfite sequencing analysis of IG-DMR in oocytes. The region analyzed is the same as the IG-DMR in Fig. 7A. Each horizontal bar represents a sequence from each clone. Circles indicate CpG sites. White and black circles designate unmethylated and methylated CpGs, respectively. (B) The *Neo* expression was detected by RT-PCR in embryos and oocytes as indicated. β -actin was used as the internal control.

methylation. Chotalia et al. (Chotalia et al., 2009) reported that transcription from an upstream gene results in methylation of germline DMRs in the *Gnas* locus. Therefore, another possibility is that the transcription from the *neo* cassette causes IG-DMR methylation in *Gtl2* KO embryos. However, in our study, the IG-DMR was unmethylated in *Gtl2* KO oocytes even though transcription of the *neo* was active in the oocytes (Fig. 8). Therefore, the gain of methylation at the IG-DMR in *Gtl2* KO/+ mice occurs during embryogenesis. Sekita et al. (Sekita et al., 2006) reported that the IG-DMR remained unmethylated in *Gtl2lacZ*Mat mice, in which two to three copies of transgenes carrying *neo* cassettes, whose transcription is towards the IG-DMR, were inserted at 1.7 kb upstream of the *Gtl2* gene. This suggests that the gain of methylation at the IG-DMR in our *Gtl2* KO/+ embryos is not likely to be due to the existence of a *neo* cassette. Mice carrying a maternal transmission of a transgene insertion, such as the *neo* (Steshina et al., 2006) and *neo/lacZ* (Sekita et al., 2006), between the IG-DMR and the *Gtl2* gene have reduced expression of MEGs, but the maternal IG-DMR remained unmethylated. This phenomenon is similar to that observed in Takahashi's *Gtl2*-10kb MatKO mice, in which the expression of MEGs, except *Gtl2*, was modest and the maternal IG-DMR was unmethylated (Takahashi et al., 2009). By contrast, mice carrying a maternal *Gtl2* deletion did not express any MEGs and the IG-DMR was fully methylated. Taken together, we propose that a certain level of MEG expression is required to maintain an unmethylated status at the IG-DMR. MEG non-coding RNAs may act as inhibitors of IG-DMR methylation. In our *Gtl2* KO mouse,

Gtl2 deletion may reduce expression of downstream MEGs to a level unable to prevent IG-DMR methylation. The methylated IG-DMR in turn causes methylation at the rest of the *Gtl2*-DMR and further represses MEG expression.

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Competing interests statement

The authors declare no competing financial interests.

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