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Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the *Dlk1-Gtl2* imprinted cluster on mouse chromosome 12

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Genomic imprinting causes parental origin–specific gene expression[1]. *Cis*-acting regulatory elements that control imprinting are not fully understood but involve regions that become differentially methylated on the two parental chromosomes during male and female gametogenesis[2–4]. Understanding properties of maternally and paternally inherited imprints provides insight into the mechanisms and evolution of genomic imprinting. Previously we identified an intergenic germline-derived differentially methylated region (IG-DMR) that is a candidate control element for an imprinted domain on distal mouse chromosome 12 (ref. 5). The 1-Mb cluster contains the paternally expressed protein-coding genes *Dlk1* (refs. 6,7) and *Dio3* (ref. 8,9) and several maternally expressed non-coding RNAs, including *Gtl2* (refs. 6,7,10) and C/D snoRNAs[11]. A retrotransposon-like gene (*Rtl1*) is expressed from the paternal chromosome and has an antisense transcript expressed from the maternal chromosome containing two microRNAs with full complementarity to *Rtl1* (ref. 12). Here we show that deletion of the IG-DMR from the maternally inherited chromosome causes bidirectional loss of imprinting of all genes in the cluster. When the deletion is transmitted from the father, imprinting is unaltered. These results prove that the IG-DMR is a control element for all imprinted genes on the maternal chromosome only and indicate that the two parental chromosomes control allele-specific gene expression differently.

The organization of the 1-Mb imprinted locus on distal mouse chromosome 12 is illustrated in **Figure 1a**. To determine the mechanism of imprinting at the *Dlk1-Gtl2* domain, we generated a targeted deletion of the IG-DMR between *Dlk1* and *Gtl2*. We deleted 4.15 kb including the tandem repeats[5,13] of the IG-DMR (**Fig. 1b**) in embryonic stem (ES) cells, which we then injected into host blastocysts to generate male chimeras that successfully transmitted the deletion through the germ line (**Fig. 1c–e**). Heterozygous IG-DMR-deleted progeny were generated at the expected frequency at embryonic day (E) 16. At later gestational stages, the percentage of live heterozygous embryos fell when the deletion was maternally inherited but remained the same when the deletion was paternally inherited. We found no adult mice carrying the maternally transmitted deletion (in five litters at 4 weeks of age; $n = 32$). Thirteen neonates in seven litters were heterozygous with respect to the maternally inherited deletion, but they were born dead or died within several hours of birth. Therefore, maternal transmission of the IG-DMR deletion is lethal after E16.

We next quantified the expression of *Dlk1* and *Gtl2* transcripts in embryos after maternal and paternal transmission of the deleted IG-DMR. After maternal transmission, *Gtl2* expression was negligible, indicating repression of *Gtl2* from the usually expressed maternal allele (**Fig. 2**), and *Dlk1* expression was twice that observed in wild-type littermates (**Fig. 2**). We confirmed that the increase in *Dlk1* expression was caused by activation of the usually silent maternal allele using a single-nucleotide polymorphism in DBA/2 and sequencing of RT–PCR products in embryos from females heterozygous with respect to the deletion (129Ola/C57BL6) crossed to DBA/2 males (**Fig. 3a**). Therefore, deletion of the unmethylated copy of the IG-DMR on the maternal allele results in biallelic expression of *Dlk1* and biallelic repression of *Gtl2*.

After paternal inheritance of the IG-DMR deletion, overall expression of *Dlk1* was the same as that observed in wild-type littermates and was from the paternal allele (**Figs. 2** and **3a**). Likewise, expression of *Gtl2* remained from the maternal allele, indicating that the absence of the methylated IG-DMR did not result in loss of imprinting of *Gtl2* on the paternal chromosome. These data show that the methylated IG-DMR on the paternal allele is not involved in *Dlk1* activity and is not necessary for full repression of *Gtl2* on the paternal chromosome.

The *Gtl2* promoter is a DMR that becomes methylated on the paternal allele after fertilization[6]. We carried out methylation analysis to determine whether repression of *Gtl2* after maternal transmission of

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the deletion was associated with a change in promoter methylation. The promoter was hypermethylated relative to the methylation observed in embryos with paternal uniparental disomy for chromosome 12 (pUPD12; refs. 5,14; **Fig. 4**). We also observed slightly less methylation of the *Gtl2* promoter DMR after paternal inheritance of the deletion. This suggests that the maternal allele acquired a paternal epigenotype at *Gtl2* as a consequence of the deletion.

Dlk1 and *Gtl2* are members of a larger cluster of imprinted genes (**Fig. 1a**). Quantitative northern-blot data (**Fig. 2**) show that all the genes normally expressed from the maternally inherited chromosome are repressed after maternal inheritance of the deletion. In contrast, there were no significant alterations in the imprinting of any of the genes in the cluster after paternal transmission of the deleted (methylated) IG-DMR (**Fig. 2**). Therefore, the IG-DMR regulates imprinting of a 1-Mb cluster of genes bidirectionally on the maternal chromosome only.

After maternal inheritance, all the C/D snoRNAs that we tested (MBII-48, MBII-49, MBII-78) as well as miR-127 were expressed at 0–8% of wild-type levels (**Fig. 2**). *Dio3* expression was double that seen in wild-type littermates, owing to activation of the normally repressed maternal allele (**Fig. 3c**). The absence of differential methylation of CpG islands at and around *Dio3* (ref. 8 and data not shown) is consistent with its imprinting being under the control of more distant elements. The usually silent copy of *Rtl1* was activated, and notably, this deregulation resulted in mean expression (450%) that was substantially higher than expected for a double dose (**Fig. 2b**). This suggests that in the mutant, the gene either is transcribed at higher than normal levels or is abnormally stabilized post-transcriptionally. This finding is notable as it suggests a function for the maternally expressed miRNAs that are no longer transcribed in the maternally inherited knockout.

Because of their 100% complementarity with *Rtl1*, we predicted that miR-127 and miR-136 might act as small interfering RNAs (siRNAs) to trigger cleavage of *Rtl1* transcripts[12]. Semi-quantitative, strand-specific, allele-specific analysis indicated that *Rtl1* on the maternal allele was activated to ~80% of the level observed on the paternal allele (**Fig. 3b**). Although a role for miRNAs in *Rtl1* imprinting cannot be ruled out[12], relatively similar upregulation

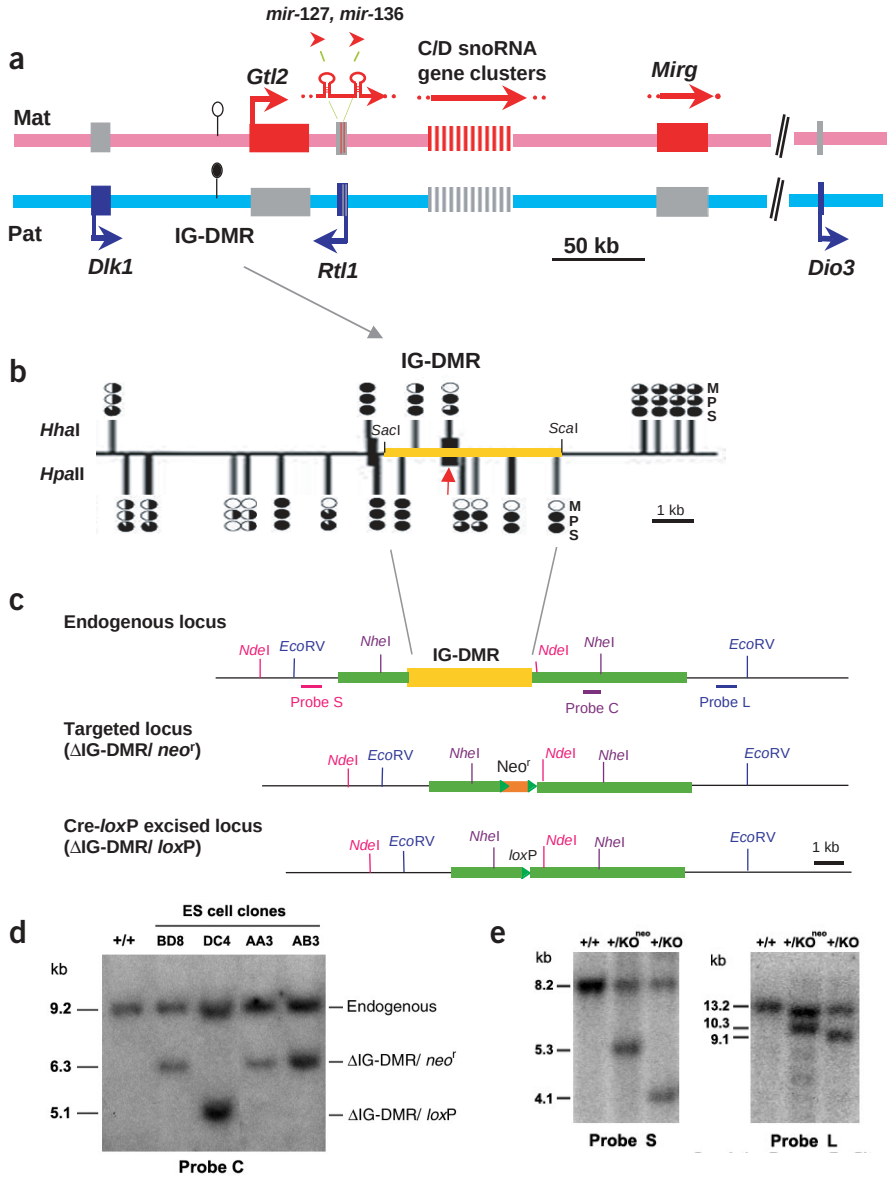

Figure 1 Targeted deletion of the IG-DMR upstream of *Gtl2* on mouse chromosome 12. (**a**) Schematic representation of the 1-Mb imprinted cluster on mouse distal chromosome 12. Differential methylation at the IG-DMR is shown with filled circles representing methylated alleles and open circles representing unmethylated alleles. (**b**) The deleted IG-DMR region is shown in yellow. Vertical bars indicate *Hha*I and *Hpa*II sites. The methylation status (as described in ref. 5) is illustrated with open and filled circles representing lack of methylation and full methylation, respectively. Partially filled circles represent partial methylation. M, P and S represent the methylation status in mUPD12, pUPD12 and sperm DNA, respectively. The location of the conserved tandem repeat array is indicated by a red arrow. The heavy blocks represent CpG islands. (**c**) The targeting construct. Dark green bars represent the extent of short- and long-arm homology, and the yellow bar represents the deleted region. (**d**) Southern-blot hybridization analysis of digested DNA isolated from representative targeted ES cell clones before and after Cre-*lox*P–mediated excision of *neo*^{r}. The restriction enzyme (*Nhe*I) sites and probe C are shown in **c**. (**e**) Southern-blot hybridization using endogenous probes outside the regions of the short-arm (probe S) and long-arm (probe L) homologies indicates correct targeting. DNA isolated from mice with the paternally inherited IG-DMR deletion with (+/KO^{neo}) or without (+/KO) *neo*^{r} and from their wild-type littermates was cut with *Nde*I (for short arm) or *Eco*RV (for long arm).

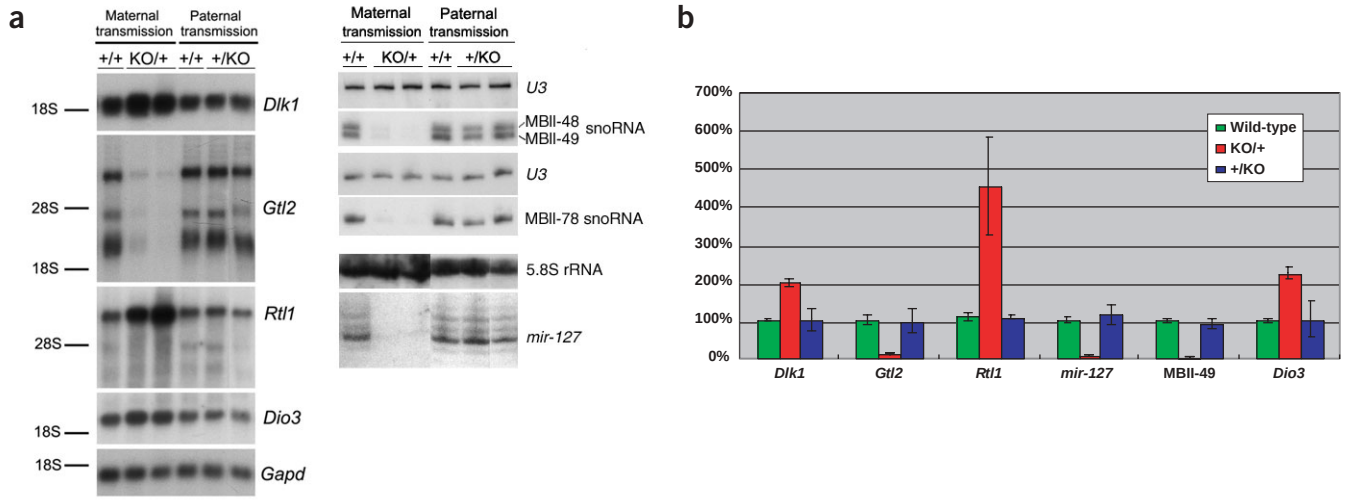

Figure 2 Expression of imprinted genes as a consequence of the IG-DMR deletion. (**a**) Northern-blot analysis of gene expression at the imprinted domain. *Gapd*, *U3* and 5.8S rRNA were used as unlinked control genes for quantification. (**b**) Graphical representation of expression comparing E16 embryos carrying the maternally derived IG-DMR deletion (red; KO/+) with those with the paternally derived deletion (blue; +/KO) and wild-type littermates (green; +/+). Error bars represent s.e.m. Values were calculated from data generated using control and mutant embryos from multiple litters.

from the two chromosomes in the knockout suggests that such post-transcriptional regulation cannot discriminate between *Rtl1* transcripts from either allele. Functional interaction between maternally and paternally expressed transcripts at the orthologous imprinted domain in sheep has been proposed to explain an unusual pattern of inheritance associated with the callipyge (*CLPG*) phenotype[15]. The callipyge defect increases expression on both chromosomes but does not affect imprinting[16]. The mutant phenotype (postnatal muscle hyperplasia) is observed in heterozygotes after paternal transmission only, whereas homozygotes with respect to the *CLPG* allele are normal. The normal phenotype in *CLPG/CLPG* sheep suggests that overexpression of a gene (or genes) on the maternal chromosome abrogates the effect of overexpression of a paternally expressed gene (or genes). Interaction between maternally expressed miRNAs and paternally expressed *Rtl1* transcripts is consistent with this model.

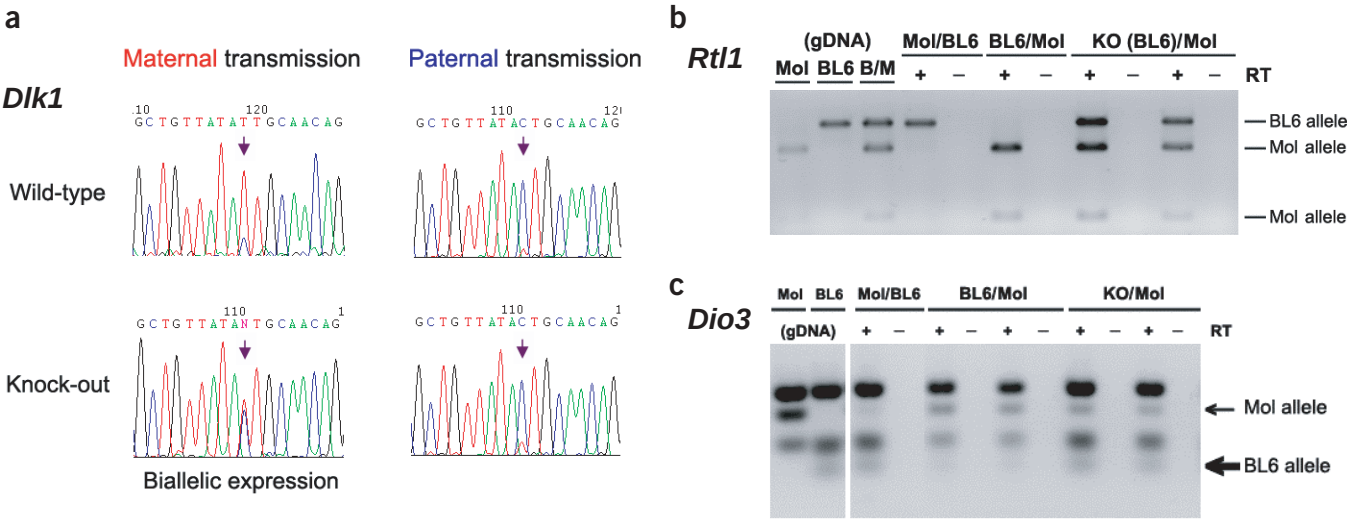

Figure 3 Biallelic expression of *Dlk1*, *Rtl1* and *Dio3* after maternal transmission of IG-DMR deletion. (**a**) *Dlk1*. Sequence analysis of RT–PCR products from control and heterozygous embryos after maternal and paternal transmission of the IG-DMR deletion (knock-out). Biallelic expression of *Dlk1* was detected only when the IG-DMR deletion was maternally transmitted. (**b**) *Rtl1*. Strand-specific RT–PCR followed by digestion distinguished the *Rtl1* transcript in *M. musculus molossinus* (Mol) and C57BL6 (BL6) F_1 hybrid mice. Control hybrids always expressed the paternal allele. Biallelic expression was evident in mice with the maternally inherited deletion (KO (BL/6)/Mol). Semi-quantitative analysis indicated that maternal transcript levels were 77.04 ± 0.63% that of the unmanipulated paternal allele (control BL6/Mol hybrid genomic DNA amplification resulted in maternal allelic amplification of 100.62 ± 0.54% compared to paternal). Control RT–PCR reactions lacking reverse transcriptase (RT) are indicated (–). (**c**) *Dio3*. RT–PCR followed by *Taq*I digestion distinguished *Dio3* transcripts expressed from Mol and BL6 alleles. Control hybrids predominantly expressed the paternal allele, whereas mice with the maternally inherited deletion showed activation of the maternal allele, as evident from the greater intensity of the diagnostic BL6 band (thick arrow) relative to the diagnostic Mol band (thin arrow). Control RT–PCR reactions lacking reverse transcriptase (RT) are indicated (–).

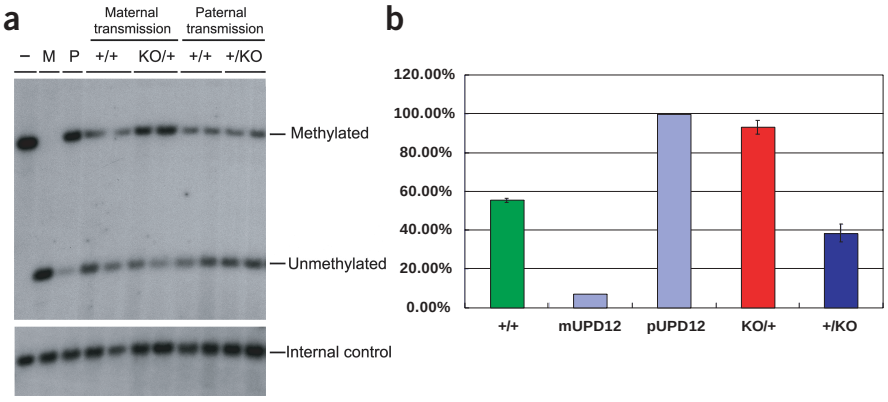

Figure 4 Promoter methylation at *Gtl2* is altered in the absence of the IG-DMR. (**a**) Representative methylation-sensitive Southern-blot hybridization showing the 3-kb methylated band and 1.2-kb unmethylated product in mice that are heterozygous with respect to the IG-DMR deletion after maternal (KO/+) and paternal (+/KO) transmission. Data is shown for *Pvu*II (–) and *Pvu*II with *Sma*I. M, mUPD12; P, pUPD12. (**b**) Graphical representation of quantitative methylation changes associated with the deletion. Data was generated from embryonic DNA digested with *Pvu*II or *Hin*cII and *Hpa*II. The measurement illustrates the percentage of full methylation relative to that observed in pUPD12 mice (normalized at 100% methylation).

Deletion of the IG-DMR on the maternal chromosome results in silencing of the complex array of maternally active non-coding RNAs, including *Gtl2*, which normally expresses multiple alternatively spliced transcripts. *Gtl2* also has little sequence conservation downstream of the first two exons[13]. All maternally expressed imprinted genes are downstream of the IG-DMR, are transcribed in the same orientation and, with the exception of *Gtl2*, have uncharacterized transcriptional start sites[17]. Whether the *Gtl2* promoter has a role in the regulation of these downstream RNAs has not yet been determined.

The IG-DMR is believed to be the only germ line–derived DMR in the mouse 125-kb locus that contains both *Dlk1* and *Gtl2* (ref. 5). Because *DLK1* and *GTL2* are imprinted in humans[10,18] and the region functions in growth control and development, methylation analysis in humans has diagnostic merit, particularly when considering loss of imprinting in human tumors. Using DNA from cells derived from maternal and paternal uniparental disomy for chromosome 14 (UPD14; refs. 19,20), we found that the orthologous IG-DMR region on human chromosome 14 is also differentially methylated on the two parental alleles. The paternally inherited allele is hypermethylated in pUPD14 and hypomethylated in mUPD14 cells relative to wild-type controls (**Fig. 5**).

Three models of postfertilization regulation of imprinted gene clusters have been suggested[1]. First, the insulator model was established from studying the reciprocally imprinted genes *Igf2* and *H19*. In this example, an intergenic germline DMR binds the insulator protein CTCF in a methylation-sensitive manner, blocking access of *Igf2* to shared downstream enhancers on the unmethylated maternal chromosome. The methylated DMR on the paternal chromosome seems to be required for maintaining the reciprocal imprinting of the two genes on that chromosome[21,22]. Second, in the bipartite imprinting center model as described for the PWS-AS (Prader-Willi and Angelman syndromes) locus on human chromosome 15, two distinct regions (imprinting centers; ICs) that function bidirectionally have been mapped, one on each chromosome. On the maternal chromosome, the AS-IC controls methylation and repression of multiple genes, and on the paternal chromosome, the PWS-IC is required for activity and hypomethylation. The PWS-IC is a DMR, and the AS-IC has not yet been characterized at the molecular level *in vivo*[1,23]. Third, the antisense transcript mechanism is exemplified by the *Igf2r* domain, where a large non-coding RNA (*Air*) is required for bidirectional repression of a cluster of paternal alleles (including *Igf2r*) on mouse chromosome 17. *Air* is transcribed from an intronic germline DMR in *Igf2r* on the unmethylated paternally inherited copy[24]. A second imprinted domain on mouse distal chromosome 7, including the genes *Cdkn1c* and *Kcnq1*, is also regulated bidirectionally by a paternally unmethylated germline DMR, and evidence to date suggests regulation by a mechanism also involving antisense RNA[25,26].

Several models could explain the function of the IG-DMR on the maternal chromosome. First, the region might be a transcriptional activator of *Gtl2* and associated transcripts. These non-coding transcripts might then function as *cis*-acting negative regulators of the maternally silent imprinted genes *Dlk1*, *Rtl1* and *Dio3* in a manner reminiscent of the function of *Air* at *Igf2r*[24]. In a second model, the unmethylated maternal IG-DMR might act directly as a long-range regulator of the whole domain, conferring a particular epigenetic state and chromatin conformation associated with activity and repression over at least a 1-Mb domain. The absence of a significant effect of the deletion on the methylated paternal chromosome indicates that repression of the non-coding RNAs and activity of *Dlk1*, *Rtl1* and *Dio3* are regulated differently on that chromosome.

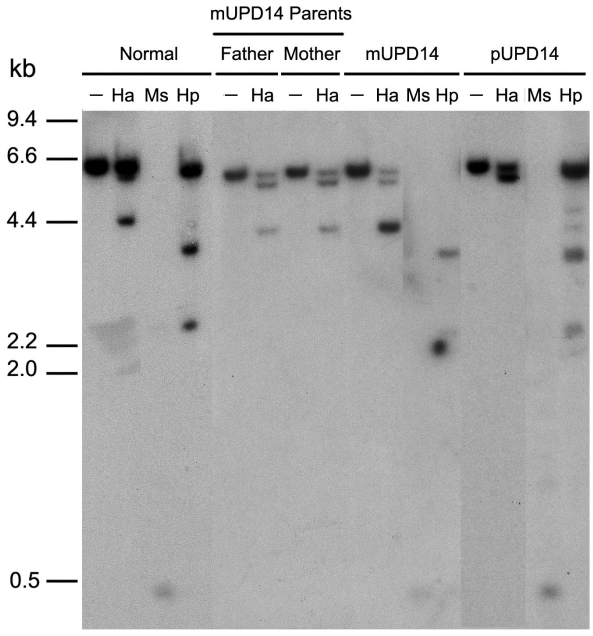

Figure 5 Differential methylation at the IG-DMR is conserved in mouse and human. Comparison of *Hha*I and *Hpa*II tracks between human mUPD14 and pUPD14 shows that the IG-DMR is hypomethylated on the maternal chromosome and hypermethylated on the paternal chromosome. Ha, *Hha*I; Hp, *Hpa*II; Ms, *Msp*I.

Although we cannot rule out an insulator function at the IG-DMR, it seems different from the *Igf2-H19* domain, where an effect is observed after DMR deletion from each of the parental germ lines[22]. Rather, our results suggest a model of imprinting control similar to that described at the PWS-AS locus, in which two different regions regulate imprinting on the two parental chromosomes[1,23]. Alternatively, the methylation and expression status of the paternal chromosome (**Fig. 6**) might represent an 'ancestral' or 'default' state that was present before the evolution of imprinting on the maternal chromosome at this domain. None of these models is mutually exclusive, and regardless of which model (models) best describes it, imprinting of genes in the 1-Mb domain on chromosome 12 is controlled differently on the two parental chromosomes.

Maternal deletion of the IG-DMR switched the epigenotype and transcriptional behavior of the maternal chromosome to resemble that of the paternal chromosome (**Fig. 6**). This causes late-gestational lethality. Because multiple imprinted genes are affected, lethality cannot be attributed to any single gene in the cluster. Neither targeted deletion of maternally expressed transcripts nor overexpression of individual paternally expressed imprinted genes has been described for this locus. But paternal uniparental disomy 12 (pUPD12) embryos also show fetal lethality, and developmental abnormalities have been described[14,27]. Comparative phenotypic analysis of pUPD12 embryos with those carrying maternally inherited deletion of the IG-DMR could determine whether the defects associated with pUPD12 can be attributed to the 1-Mb imprinted domain on distal chromosome 12.

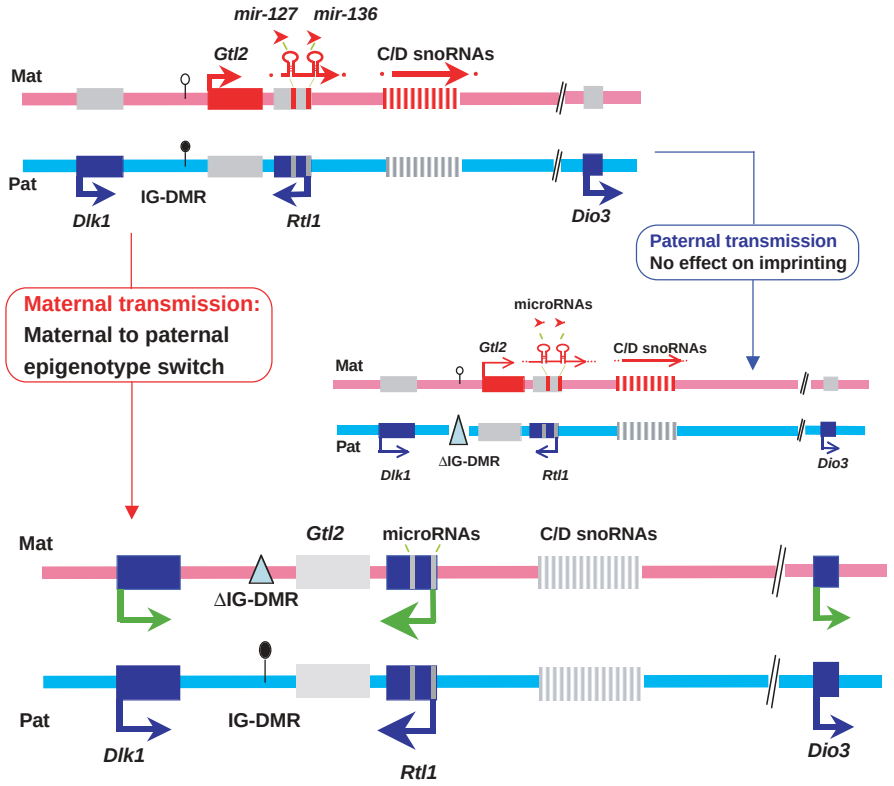

Figure 6 Summary of imprinted gene expression after maternal and paternal transmission of the IG-DMR deletion. When maternally transmitted, deletion of the unmethylated IG-DMR caused bidirectional loss of imprinting of all genes in the domain. No significant differences in imprinting were observed when deletion was paternally transmitted. Schematic is not drawn to scale.

METHODS

Targeted deletion of the IG-DMR. We constructed the targeting vector for deleting the IG-DMR (nucleotides 79,883–84,039) using a 2-kb *Sac*I fragment (nucleotides 77,841–79,883) and a 4.9-kb *Sca*I to *Sph*I fragment (nucleotides 84,039–88,936) ligated into the pNeoFlox8 vector (provided by U. Lichtenberg, Cologne) upstream and downstream, respectively, of the neomycin resistance (*neo*^{r}) gene[28]. We linearized the targeting vector, electroporated it into the male 129/OlaHsd ES cell line E14Ola[29] and selected ES cell clones containing the targeting vector with G418. We confirmed the short-arm and long-arm homologous recombination events of putative targeted ES cell clones by Southern-blot hybridization. We excised *neo*^{r} in the targeted clones *in vitro* by transient expression of an electroporated Cre recombinase gene (pTurboCre, provided by T. Ley, Washington University, St. Louis, USA). Here, we presented only data from mice lacking *neo*^{r}, but mice with *neo*^{r} gave the same results, suggesting that *neo*^{r} had no effect *in vivo*. The Gene Targeting Facility at The Babraham Research Institute carried out ES cell targeting and blastocyst injection. Mice were maintained on a C57BL6 genetic background unless otherwise indicated. Experiments involving mice were carried out in accordance with the UK Government Home Office licensing procedures.

Expression analysis of imprinted genes in the *Dlk1-Gtl2* imprinted cluster. We isolated total and poly(A)$^+$ RNA from embryos with the IG-DMR deleted and control littermates as described previously[13]. We carried out northern-blot hybridization and used probes as described previously for *Dlk1* and *Gtl2* (ref. 6), snoRNAs[11], *Rtl1* and microRNAs[12], and *Dio3* (ref. 8). We quantified expression using a Storm 860 Phosphorimager (Amersham) and associated ImageQuant software according to manufacturer's instructions. We showed biallelic expression of *Dlk1* from E16 embryos heterozygous with respect to a maternally inherited deletion after we identified a single-nucleotide polymorphism between C57BL6 (also 129/Sv, CBA, Balb/C, C3H, SWR, FVB, AKR) and DBA/2 (G→A substitution). We carried out reciprocal crosses between DBA/2 and knockout stock to generate embryos heterozygous with respect to the maternally or paternally inherited deletion and wild-type littermates and then amplified by RT–PCR and sequenced a 223-bp fragment of RNA isolated from these embryos. We sequenced multiple individual PCR products. We evaluated *Dio3* allelic activity using a *Mus musculus molossinus* restriction-site polymorphism as described previously[8] and *Rtl1* allelic expression with strand-specific RT–PCR using a *M. musculus molossinus* restriction-site polymorphism (*Stu*I). We reverse-transcribed RNA using a strand-specific primer and Superscript III (Invitrogen) according to manufacturer's instructions at 55 °C for 1 h. We carried out subsequent PCR reactions in duplicate over multiple cycles to generate a standard curve. We used hybrid genomic DNA as a control to check for equivalence in allelic amplification efficiency and complete digestion. Amplification reactions proceeded in an exponential manner with equivalent slope up to 40–45 cycles of PCR (data not shown). Therefore, we carried out 38 cycles of amplification before digesting samples with *Stu*I and analyzing them by densitometry using Molecular Dynamics ImageQuant software (Amersham). Values were calculated according to their respective molar ratios.

Methylation analysis of the *Gtl2* DMR in IG-DMR knockout embryos. We isolated genomic DNA from embryos using standard techniques[5]. We carried out methylation-sensitive Southern-blot analysis of the *Gtl2* DMR using a 450-bp *Kpn*I-*Sma*I fragment as probe (nucleotides 93,667–94,100) for *Sma*I filters

and probe G1 for *Hpa*II filters[6]. We used a fully methylated region in *Rtl1* as an internal control. To make the filters for quantitative methylation analysis, we digested genomic DNA samples with *Pvu*II and *Sma*I or *Hpa*II or with *Hin*cII with *Hpa*II. A total of ten *Hpa*II sites were analyzed. We quantified the intensity of the undigested product relative to the internal control to provide a 'fully methylated' value and plotted the intensity relative to the level observed in mUPD12 or pUPD12 DNA in which the parental chromosomes are hypo- or hypermethylated, respectively. We quantified intensities of diagnostic and control bands on a Storm 860 phosphorimager using Amersham software. Methylation levels are given relative to pUPD12 mice, represented as 100% methylation. We generated mice with maternal and paternal uniparental disomy for chromosome 12 as described previously[14]. We confirmed complete digestion of the DNA using a probe for exon 1 of *Dlk1* that is fully unmethylated on both alleles[6] (data not shown).

Methylation analysis of the IG-DMR orthologous region on human chromosome 14. We obtained lymphocytes derived from an individual with mUPD14 (DD0217), described in ref. 19, and the parents (DD0216, DD0218) from the European Tissue Culture Collection. We obtained fibroblasts and lymphocytes from individual GR, described in ref. 20, from J. Wang (Alfigen, California). We isolated DNA from primary cultures and digested it with *Bam*HI and methylation-sensitive restriction enzymes. We generated the 654-bp probe corresponding to the human IG-DMR by PCR amplification of human genomic DNA. We carried out Southern-blot hybridization as described previously[6]. Primer sequences and conditions for their use are available on request.

GenBank accession number. Nucleotide numbers refer to sequence accession number AJ320506 for the *Dlk1-Gtl2* cluster.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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