RESEARCH COMMUNICATION

The *Dlk1* and *Gtl2* genes are linked and reciprocally imprinted

Jennifer V. Schmidt,^{1,2} Paul G. Matteson,²
Beverly K. Jones, Xiao-Juan Guan,
and Shirley M. Tilghman³

Howard Hughes Medical Institute and Department
of Molecular Biology, Princeton University, Princeton,
New Jersey 08544 USA

Genes subject to genomic imprinting exist in large chromosomal domains, probably reflecting coordinate regulation of the genes within a cluster. Such regulation has been demonstrated for the *H19*, *Igf2*, and *Ins2* genes that share a bifunctional imprinting control region. We have identified the *Dlk1* gene as a new imprinted gene that is paternally expressed. Furthermore, we show that *Dlk1* is tightly linked to the maternally expressed *Gtl2* gene. *Dlk1* and *Gtl2* are coexpressed and respond in a reciprocal manner to loss of DNA methylation. These genes are likely to represent a new example of coordinated imprinting of linked genes.

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Genomic imprinting refers to the differential expression of the two alleles of a gene that is dependent on their parent of origin. Imprinting was first demonstrated for the insulin-like growth factor 2 receptor (*Igf2r*) and insulin-like growth factor 2 (*Igf2*) genes almost ten years ago (Barlow et al. 1991; DeChiara et al. 1991). Since then, >30 imprinted genes have been described in mammals (<http://www.mgu.har.mrc.ac.uk/imprinting>). Estimates of the total number of imprinted genes, based on the genomic regions of the mouse in which uniparental disomies display deleterious phenotypes, suggest there may be as many as 100 such genes.

The mechanisms that regulate imprinting are only beginning to be elucidated, but several general characteristics have emerged. First, the imprinting of a number of genes has been shown to rely on allele-specific DNA methylation that is established in the gametes and can act to either suppress or activate genes (Li et al. 1993). Second, a significant number of imprinted genes exist in large chromosomal clusters, suggesting coordinate control of linked genes. For example, a direct mechanistic link has been established for the mouse *H19*, *Igf2*, and *Ins2* genes on chromosome 7, which use a single differ-

entially methylated *cis*-acting imprinting control element (Thorvaldson et al. 1998). Third, a majority of imprinted genes for which a function has been identified code for proteins involved in the control of embryonic growth (Tilghman 1999).

Given the role of imprinted genes in fetal growth it is not surprising that they are often expressed in the placenta, the primary tissue regulating nutrient transfer between mother and fetus. To identify new imprinted genes, we designed an allelic differential display strategy (Hagiwara et al. 1997) using placental mRNA derived from two closely related species of North American deer mice, *Peromyscus maniculatus* (BW) and *Peromyscus polionotus* (PO). These mice display a high degree of polymorphism (Vrana et al. 1998), making them useful for screens that depend on allelic differences.

We report here the paternal-specific expression of the *Delta-like* (*Dlk1*) gene in both *Peromyscus* and the laboratory mouse genus *Mus*. We further show that *Dlk1* is tightly linked in *Mus* and humans to *Gtl2*, a gene encoding an untranslated RNA (Schuster-Gossler et al. 1998). Although it had been suggested that *Gtl2* was imprinted and expressed only from the paternal chromosome, we have determined that it is in fact maternally expressed. We demonstrate that the promoter of *Gtl2* but not *Dlk1* is differentially methylated and that these genes respond to loss of CpG methylation in the DNA methyltransferase mouse mutant in a manner identical to that seen for *H19* and *Igf2*. Thus *Dlk1* and *Gtl2* define a new imprinted domain on mouse distal chromosome 12 that has striking structural and regulatory parallels to the well-studied *H19-Igf2* gene pair. This finding raises the possibility that imprinted gene clusters will have regulatory properties in common.

Results

We used multiple sets of differential display RT-PCR primers to amplify RNAs from late gestation placentae of the two *Peromyscus* parental strains and reciprocal F₁ crosses (PO × BW and BW × PO). Given the imprinted expression of the X chromosome in placenta and maternal inheritance of mitochondria, maternal-specific bands could derive from expression of either X-linked or mitochondrial genes, in addition to maternally expressed imprinted genes. Therefore, we focused our initial analysis on paternal-specific bands, which could only be explained by imprinting.

Dlk1 is imprinted in *Peromyscus* and *Mus*

We focused on a differentially displayed band that was amplified from BW placental RNA but not from PO RNA, reflecting the fact that the primers uncovered a polymorphism between the two species (Fig. 1A). In reciprocal hybrids we observed a product in PO × BW RNA, but not BW × PO RNA, suggesting that the transcript is paternally expressed. To confirm this finding we developed a single strand conformational polymorphism

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¹Present address: Laboratory for Molecular Biology, Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois 60607 USA.

²These authors contributed equally to this work.

³Corresponding author.

E-MAIL stilghman@molbio.princeton.edu; FAX (609) 258-3345.

Figure 1. Imprinting of the *Dlk1* gene in *Peromyscus* and *Mus*. (A) Differential display gel showing the polymorphism for *Dlk1*. (BW) *P. maniculatus*; (PO) *P. polionotus*; in the reciprocal crosses the female is listed first. (B) *Dlk1* SSCP analysis for imprinting in *Peromyscus* placenta. RT-PCR was performed using E18.5 placental RNA from the BW and PO parental strains and reciprocal F₁ crosses, as well as a 1:1 mixture of BW and PO RNAs (BW + PO). (C) *Dlk1* SSCP analysis for imprinting in *Peromyscus* embryo. RT-PCR was performed using E18.5 embryo RNA from reciprocal F₁ crosses. The polymorphism is the same as that shown in B, however the gel was run longer to highlight the difference in migration. (D) Imprinting of *Dlk1* in *Mus* embryo. Direct sequencing was performed on RT-PCR products from E12.5 C57BL/6 (B) and Cast/Ei (C) embryos and F₁ offspring (B x C and C x B). The polymorphic base is bold and underlined.

(SSCP) imprinting assay and verified that the gene was paternally expressed in *Peromyscus* placenta and embryo (Fig. 1B,C). We amplified, cloned, and sequenced the band and determined that it was the *Peromyscus* ortholog of the *Dlk1* gene (GenBank accession no. AF272850; also referred to as *Pref-1*, *FA-1*, *Scp-1*, or *pG2*) (Laborda et al. 1993; Smas and Sul 1993). This gene is a member of the *Notch* gene family and encodes a transmembrane protein widely expressed in the developing embryo. The *Dlk1* extracellular domain can be proteolytically cleaved to generate a secreted protein that accumulates in the fetal and maternal circulation, as well as the amniotic fluid (Jensen et al. 1994; Bachmann et al. 1996). *Dlk1* is induced by growth hormone and prolactin in the fetal and maternal pancreas, leading to increased proliferation of β -cells and an increase in insulin production (Carlsson et al. 1997). It has been implicated in the proliferation and differentiation of preadipocytes and stromal cells (Smas and Sul 1993; Moore et al. 1997).

To test whether *Dlk1* was also paternally expressed in *Mus* we cloned and sequenced *Dlk1* RT-PCR products derived from C57BL/6 (B) and Cast/Ei (C) placental RNAs. We identified a single nucleotide polymorphism in exon 5 of the *Dlk1* gene, with the sequence TGAAAGT present in B animals and the sequence TGAAGGT present in C animals. Because this polymor-

phism did not alter a restriction site, we used direct sequencing of RT-PCR products to analyze the allelic expression of the *Dlk1* gene. RT-PCR was performed on embryo and placental RNA from the parental strains and reciprocal F₁ crosses at embryonic day 12.5 (E12.5) and E18.5 (Fig. 1D; data not shown). Exclusive detection of the paternal allele established that *Dlk1* is also paternally expressed in *Mus* embryo and placenta.

Dlk1 maps to *Mus* chromosome 12

The finding that *Dlk1* is paternally expressed in *Mus* was surprising, as the gene had been mapped previously to the X chromosome (Brady et al. 1997). The human *DLK1* gene, on the other hand, maps to 14q32.3, in a region syntenic to distal mouse chromosome 12 (<http://www.ncbi.nlm.nih.gov/genemap99>). To resolve this disparity, we used an *NdeI* polymorphism at *Dlk1* between BTBR and *Mus spretus* to type a (BTBR x *M. spretus*) x BTBR backcross panel (Fig. 2A). The cosegregation of *Dlk1* with Mit markers at the distal end of chromosome 12 confirmed that the *Mus Dlk1* gene maps to chromosome 12 (Fig. 2A).

Dlk1 is linked to the *Gtl2* gene

The localization of *Dlk1* to the distal end of *Mus* chromosome 12 suggested that it might lie near another re-

Figure 2. Linkage of the *Dlk1* and *Gtl2* genes on *Mus* chromosome 12 and maternal expression of *Gtl2*. (A) Genotypes of 20 (BTBR x *M. spretus*) x BTBR backcross animals typed for *D12Mit99* and *Dlk1*. The mapping places *Dlk1* 1 cm distal to *D12Mit99*. (B) Genomic organization of *Dlk1* and *Gtl2* genes compared to *H19* and *Igf2*. Blue boxes denote paternally expressed genes and pink boxes denote maternally expressed genes. The arrows indicate the ICR that regulates imprinting of *H19* and *Igf2* and the insertion site of the *lacZ* gene trap vector in the *Gtl2^{lacZ}* mice (*lacZ*). (C) RT-PCR analysis for *Mus Gtl2* imprinting. RT-PCR products from E12.5 C57BL/6 (B) and Cast/Ei (C) and F₁ embryo (E) and placenta (P) were digested with *SfiI* and analyzed by acrylamide gel electrophoresis.

cently described imprinted gene, *Gtl2*. *Gtl2* was identified in the analysis of a *lacZ* insertional mutation in an embryonic stem cell gene-trap screen (Schuster-Gossler et al. 1996). Paternal inheritance of the *lacZ* transgene gave a proportional dwarfism phenotype, implying that a paternally expressed locus affecting growth was disrupted. The *Gtl2* transcript is widely expressed during development but does not appear to encode a protein, as it is inefficiently spliced and contains no significant open reading frames (Schuster-Gossler et al. 1998). We examined the potential linkage of *Dlk1* and *Gtl2* by screening a mouse bacterial artificial chromosome (BAC) library with probes for each gene. A single BAC was identified that hybridized to both probes (data not shown). Pulsed-field gel analysis of the BAC demonstrated that the two genes are transcribed in the same orientation and their promoters lie ~120 kb apart (Fig. 2B). Sequence analysis of the syntenic region of human chromosome 14 covering the *DLK1* and *GTL2* genes and their intergenic region (GenBank accession nos. AL132711 and AL117190) verified that the linkage and transcriptional orientation of the genes is conserved in humans.

Gtl2 is maternally expressed in *Mus*

Based on the reduced level of *Gtl2* expression in parthenogenetic embryos, it was proposed that the *Gtl2* gene was paternally expressed (Schuster-Gossler et al. 1998). In light of the linkage between *Dlk1* and *Gtl2* we investigated the imprinting status of *Gtl2* more rigorously. The C57BL/6 and Cast/Ei alleles of *Gtl2* were sequenced and a single nucleotide polymorphism was identified in the 3' end of the *Gtl2* transcript. This polymorphism alters an *SfcI* restriction site that is present in the B allele but absent in the C allele. Restriction enzyme digestion of RT-PCR products from embryo and placenta at E12.5 and E18.5 of reciprocal crosses between B and C showed that, contrary to the previous report, *Gtl2* is expressed exclusively from the maternal allele in *Mus* (Fig. 2C; data not shown).

Dlk1 and *Gtl2* are coexpressed during development

The reciprocal imprinting of *Dlk1*, a paternally expressed growth regulator and *Gtl2*, a maternally expressed noncoding RNA, is highly reminiscent of the *H19-Igf2* gene pair on mouse chromosome 7 (Fig. 2B). *H19* and *Igf2* were first suspected to be coordinately regulated based on their linkage and similar patterns of expression during embryogenesis. This coexpression was later demonstrated to be the result of shared enhancers located 3' to the *H19* gene (Leighton et al. 1995b). *Dlk1* has been reported to be widely expressed in the embryo including preadipocytes, placental stromal cells, the adrenal gland, pituitary, and the anlage of the fetal pancreas (Jensen et al. 1993; Laborda et al. 1993; Smas and Sul 1993). *Gtl2* is likewise widely expressed throughout development, with high levels of expression in the paraxial mesoderm, the developing central nervous system, and the epithelia of the kidney, pancreas, and salivary gland (Schuster-Gossler et al. 1998).

To examine in more detail the potential for coordinate regulation of *Dlk1* and *Gtl2*, we performed Northern analysis at various stages of *Mus* development (Fig. 3A). This assay showed that both genes are activated between E7 and E11 in embryos. It has been reported previously, based on in situ hybridization analysis, that *Gtl2* is expressed throughout early embryogenesis, from the one-cell stage onward (Schuster-Gossler et al. 1998). Our Northern analysis, however, was unable to detect expression of *Gtl2* RNA earlier than day E11 even on longer exposure.

In adults, *Dlk1* is expressed primarily in the stromal and β cells of the pancreas, the bone marrow, and adrenal gland (Laborda et al. 1993; Jensen et al. 1994; Moore et al. 1997; Bauer et al. 1998). Based on Northern analysis of adult mouse tissues, we show that *Dlk1* and *Gtl2* are coexpressed, primarily in the pituitary and adrenal gland, but not in brain, where only *Gtl2* RNA is detected (Fig. 3B). In addition, semiquantitative RT-PCR of *Mus* adult pancreas RNA showed both *Dlk1* and *Gtl2* to be highly expressed in this tissue (data not shown).

Expression of *Dlk1* and *Gtl2* is dependent on DNA methylation

The coregulated *H19* and *Igf2* genes have been shown to display reciprocal alterations in expression on the loss of DNA methylation. The DNA methyltransferase mutant mouse (*Dnmt*^{-/-}), which lacks the maintenance DNA methyltransferase, is lethal shortly after day E9.5. At E9.5, on the paternal chromosome *H19* expression is up-regulated in the absence of DNA methylation whereas the *Igf2* gene is silenced (Li et al. 1993). It has been proposed that the loss of DNA methylation at the single imprinting control region (ICR), located between -2 and -4 kb relative to the start of *H19* transcription, activates the adjacent *H19* gene on the paternal chromosome,

Figure 3. Coordinate expression of the *Dlk1* and *Gtl2* genes in *Mus* embryo and adult. (A) Northern analysis for *Dlk1* and *Gtl2* RNA in *Mus* embryos at various stages of development. (B) Northern analysis for *Dlk1* and *Gtl2* RNA in adult *Mus* tissues. The lane designations, left to right, are kidney, pituitary, adrenal, brain, testis, and liver. For both blots the expression of the β -actin gene is included as a loading control. The positions of RNA size markers are indicated.

Figure 4. Altered expression of the *Dlk1* and *Gtl2* genes in *Dnmt*^{-/-} (*Dnmt*^s) embryos. (A) Hybridization of wild-type (WT), *Dnmt*^{-/-}, and *Dnmt*^{-/-}/*H19*^{Δ13} RNA to the Atlas cDNA expression microarray containing the genes indicated. (B) Northern analysis for *Dlk1* and *Gtl2* expression in wild-type and *Dnmt*^{-/-}/*H19*^{Δ13} embryos. Expression of the *rpL32* gene is included as a loading control.

which is normally heavily methylated. The loss of *Igf2* expression, in contrast, has been proposed to be an indirect consequence of the activation of *H19* transcription (Li et al. 1993).

To gain a better understanding of the mechanism regulating the imprinting of *Dlk1* and *Gtl2*, we examined the expression of *Dlk1* and *Gtl2* in *Dnmt*^{-/-} embryo and yolk sac of E9.5 embryos (*Dnmt*^s). Our initial analysis made use of a cDNA microarray containing 588 full-length cDNA clones that included *Dlk1*, as well as the imprinted *Igf2* and *Igf2r* genes (Atlas cDNA microarray, Clontech). Duplicate microarray blots were hybridized with embryo and yolk sac RNA from E9.5 wild-type and *Dnmt*^{-/-} animals. The *Dlk1* gene was highly expressed in yolk sac of the control animals and significantly down regulated in the *Dnmt*^{-/-} animals (Fig. 4A). *Dlk1* expression was also decreased in *Dnmt*^{-/-} embryo RNA. The response of *Dlk1* was very similar to that of *Igf2* and *Igf2r*, both of which are repressed in the absence of DNA methylation (Fig. 4A) (Li et al. 1993). As the amount of RNA obtained from the E9.5 *Dnmt*^{-/-} embryos is very small we took advantage of tissue available from animals deficient in both *Dnmt* and *H19* (*H19*^{Δ13}) to perform Northern analysis. This *H19* mutation results in a loss of imprinting at the adjacent *Igf2* and *Ins2* genes (Leighton et al. 1995a), but it had no effect on the expression of *Dlk1* in either *Dnmt*^{+/-} or mutant animals (Fig. 4A). Northern analysis of RNA from wild-type and *H19*^{Δ13}/*Dnmt*^{-/-} embryos confirmed the microarray results for *Dlk1* and showed that the level of expression was ~30% of wild type (Fig. 4B). Moreover, by reprobing the same blot, *Gtl2* RNA was only detectable in the absence of DNA methylation (Fig. 4B).

These results prompted us to examine *Dlk1* and *Gtl2* for regions of allele-specific methylation. The first exons of both genes have a high CpG content that is conserved in the human *DLK1* and *GTL2* genes. We used Southern

blotting with methylation sensitive restriction enzymes to examine the methylation status of the 5' ends of *Dlk1* and *Gtl2* in the mouse. The promoter and first exon of *Gtl2* showed striking differential methylation, based on cleavage by *Hpa*II, consistent with allele-specific methylation (Fig. 5A). Surprisingly, placental DNA showed far less methylation, consistent with the known global undermethylation of placental DNA. In spite of this lack of methylation, however, *Gtl2* is tightly imprinted in the placenta. In contrast to *Gtl2*, the *Dlk1* promoter and first exon were found to be unmethylated on both alleles (Fig. 5B).

Discussion

Dlk1 and *Gtl2* define a new cluster of imprinted genes on distal mouse chromosome 12, a region predicted to contain imprinted genes from the phenotypes of uniparental chromosomal duplications in mouse and human. Maternal uniparental disomy for distal mouse chromosome 12 results in late embryonic or early neonatal lethality with reduced growth and paternal inheritance of the disomy results in embryonic lethality with enhanced growth (<http://www.mgu.har.mrc.ac.uk/imprinting/imptables.html>). Uniparental disomy involving growth effects has also been reported in humans for the syntenic region of chromosome 14 (Temple et al. 1991; Wang et al. 1991).

The *Gtl2*^{lacZ} gene trap, which integrated just upstream of *Gtl2*, resulted in growth retardation when inherited paternally (Schuster-Gossler et al. 1998). Although it was originally proposed that the phenotype was caused by a decrease in *Gtl2* expression, no decrease was observed in either maternal or paternal heterozygotes, although homozygous mutant embryos expressed reduced levels of RNA. Given our finding of paternal-specific expression of *Dlk1* and its established role in insulin control (Carls-

Figure 5. Methylation analysis of the *Gtl2* and *Dlk1* promoter regions. (A) Methylation analysis of the *Gtl2* promoter and first exon by Southern blotting using *Hinc*II (H), *Hinc*II + *Msp*I (H + M) or *Hinc*II + *Hpa*II (H + Hp). The location of the *Msp*I/*Hpa*II sites are indicated by the arrows and the probe by the dark line. Molecular DNA markers are indicated to the right of the gel. (B) Methylation analysis of the *Dlk1* promoter and first exon. Abbreviations as in A.

son et al. 1997; Efstratiadis 1998], it now seems more likely that deregulation in *Dlk1* expression underlies the *Gtl2^{lacZ}* growth phenotype. If so, then the gene trap may have disrupted an element controlling the expression of *Dlk1*, such as an enhancer or imprinting control element that is required for proper expression of both genes.

The genomic organization, imprinting behavior, methylation status and coexpression of *Dlk1* and *Gtl2* are highly reminiscent of *H19* and *Igf2*. Both clusters encode a maternally expressed untranslated RNA and a paternally expressed fetal growth factor that are coexpressed during embryogenesis. Thus *Dlk1* is added to the list of paternally expressed imprinted genes that are involved in promoting embryonic growth and supports the proposal that imprinting arose to regulate placental growth (Haig 1992). Furthermore *Dlk1* is positively regulated by DNA methylation, like *Igf2*, whereas *H19* and *Gtl2* are negatively regulated, based on our analysis of *Dnmt^{-/-}* embryos. Another similarity extends to the imprinting of *Dlk1* in *Peromyscus* hybrids. In previous work we showed that many paternally expressed genes were deregulated in PO × BW hybrids, which exhibit dramatic growth dysmorphologies of the placenta and embryo (Vrana et al. 1998). One exception to this rule was *Igf2*, whose imprinting was maintained. The maintenance of *Dlk1* imprinting in these hybrids as well further implies that the two genes may be regulated by similar mechanisms.

DNA methylation has been shown to be necessary for the proper expression of all imprinted genes with the exception of *Mash2* (Caspary et al. 1998; Tanaka et al. 1999). Indeed, methylation is believed to represent the primary imprinting mark. Imprinted genes can be divided into two classes based on whether they are activated or repressed by the loss of DNA methylation. It has been proposed that this classification reflects the organization of imprinted genes into clusters in which a single ICR mediates the imprinting of multiple genes (Barlow 1997; Tilghman 1999). In this model, the gene that is adjacent to the ICR will be directly silenced by the methylation of the ICR itself. This applies to *H19*, as well as to the paternally expressed *Snrpn* gene and the maternally expressed antisense transcript at the *Igf2r* locus (Shemer et al. 1997; Wutz et al. 1997). Loss of DNA methylation, then, results in activation of the normally silent allele of these genes, with a concomitant increase in expression levels. *Gtl2* appears to be in this class, based on its differential methylation and activation in *Dnmt^{-/-}* mice.

The silencing of genes such as *Igf2* and *Igf2r* by demethylation is believed to be more indirect. In the case of *Igf2*, experimental evidence points to a chromatin insulator within the unmethylated ICR that is required to block *Igf2* expression on the maternal chromosome (Bell and Felsenfeld 2000; Hark et al. 2000). At this locus, the ICR lies between the *Igf2* gene and the enhancers it shares with *H19* (Fig. 2B). Two groups have recently shown that the unmethylated ICR binds CTCF, a Zn finger-containing protein that binds DNA in a methylation-sensitive manner and has been implicated in insu-

lator function in other vertebrates (Bell et al. 1999; Bell and Felsenfeld 2000; Hark et al. 2000). It is proposed that when the paternal ICR is demethylated in *Dnmt^{-/-}* embryos, an insulator forms inappropriately on the ICR and blocks *Igf2* expression. The inhibition of *Dlk1* expression in *Dnmt^{-/-}* embryos, coupled with its lack of differential methylation, argues that it is in this second class of genes. The similarities we have described between *H19-Igf2* and *Dlk1-Gtl2* suggest that general mechanisms involved in imprinting may be revealed by a comparative analysis of these two loci.

Materials and methods

DNA and RNA analysis

RNA for differential display was isolated by the guanidinium isothiocyanate method followed by CsCl centrifugation; RNA for Northern analysis was prepared by lithium chloride-urea precipitation. The mouse embryo Northern was purchased from Clontech. RNAs for the adult mouse Northern were separated on a formaldehyde-MES agarose gel, transferred to Hybond N+ and hybridized with Express-Hyb (Clontech). Washes were 2 × 15 min in 2× SSC/0.1% SDS at room temperature, followed by 2 × 30 min in 0.1× SSC/0.1% SDS at 50°C. The microarrays were analyzed as detailed by the manufacturer (Clontech). For Southern blotting and methylation analysis, genomic DNAs were separated on 1× TAE agarose gels and transferred to Hybond N+ (Amersham). Hybridization was carried out at 65°C in 5× SSPE, 5× Denhardt's solution, and 0.5% SDS. Washes were as for Northern blots except all were at 65°C. The probe for the *Gtl2* methylation blot was a 532-bp *SacI-XhoI* fragment that recognizes a 4.3-kb *HincII* genomic fragment containing exon 1, and the probe for *Dlk1* methylation was a 1.6-kb *NotI-NcoI* fragment that recognizes a 4.5-kb *HincII* genomic fragment containing exons 1 and 2.

Differential display screen

Placental tissue from the *Peromyscus* parental strains and reciprocal F₁ crosses were isolated between E14.5 and E16.5. The differential display reactions were carried out using primers from the Gene Hunter RNA image kit and a modification of the basic protocol (Liang and Pardee 1992). Cycling parameters were 4 cycles of (94°C for 45 sec, 37°C –42°C over 2.5 min, 72°C for 30 sec) and 35 cycles of (94°C for 45 sec, 42°C for 2.5 min, 72°C for 30 sec) and 72°C for 10 min. Reactions were analyzed on 1× TBE, 5% acrylamide gels and exposed to film overnight. Bands were excised from the gel and reamplification carried out using the cycling parameters 94°C for 45 sec, 42°C for 2.5 min, 72°C for 30 sec for 25–30 cycles, 72°C for 10 min.

Chromosomal mapping of the Dlk1 gene

Dlk1 linkage to *Mus* chromosome 12 was determined by typing 20 (BTBR × *M. spretus*) × BTBR DNAs using PCR with the D12Mit99 primer pair. The same DNAs were also digested with *NdeI* and analyzed by Southern blotting using a 1.5-kb C57BL/6 *Dlk1* cDNA fragment as a probe.

SSCP analysis

Placental tissue from the *Peromyscus* parental strains and reciprocal F₁ crosses were isolated at day E18.5. The RT-PCR reactions were carried out using [³³P]dCTP. Samples were denatured in 95% formamide/10mM NaOH at 94°C for 5 min and the bands resolved on a 0.5× MDE (FMC BioProducts), 0.6× TBE gel. The *Dlk1* primers were 5'-TGAC-CACCTTCAACAAGGAGGC-3' and 5'-GTAGCATGGCACACAG-CAACAC-3', which amplify a 110-bp fragment within exon 5.

Mus imprinting assays

Mus Dlk1 was amplified by RT-PCR using primers (5'-CTGGCGGT-CAATATCATCTTCC-3') and (5'-GAGGAAGGGTCTTAGATA-CCG-3'), which amplify a 288-bp fragment of exon 5. PCR products were sequenced directly and chromatograms analyzed for nucleotide 1258 relative to the transcriptional start. *Mus Gtl2* was amplified by RT-PCR using primers 5'-GCCAAAGCCATCATCTGGAATC-3' and 5'-CACAGATGTAGACTCAACAGTGAAG-3', which amplify a 306-bp fragment spanning exons 8 and 9. PCR products were digested with *SfiI*, which

cuts at nucleotide 1570 relative to the *Gtl2* transcriptional start, and analyzed on a 7.5% acrylamide gel. In all imprinting analyses control reactions in the absence of reverse transcriptase were negative. In addition artificial mixtures of the two parental RNAs were amplified to ensure that there was no bias between the two alleles. In the case of *Dlk1*, these mixtures were also sequenced to verify no bias in the sequencing procedure. Additionally, all imprinting analyses were also verified on genomic DNA from the respective F₁ crosses and showed the equivalent detection of both parental alleles.

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Note added in proof

Miyoshi et al. (2000) have also recently shown that the *Gtl2* gene is maternally expressed in mouse and human.

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Jennifer V. Schmidt, Paul G. Matteson, Beverly K. Jones, et al.

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