Delta-like and gtl2 are reciprocally expressed, differentially methylated linked imprinted genes on mouse chromosome 12

Delta-like and *Gtl2* are reciprocally expressed, differentially methylated linked imprinted genes on mouse chromosome 12

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The distal portion of mouse chromosome 12 is imprinted. To date, however, *Gtl2* is the only imprinted gene identified on chromosome 12. *Gtl2* encodes multiple alternatively spliced transcripts with no apparent open reading frame. Using conceptuses with maternal or paternal uniparental disomy for chromosome 12 (UPD12), we found that *Gtl2* is expressed from the maternal allele and methylated at the 5′ end of the silent paternal allele. A reciprocally imprinted gene, *Delta-like* (*Dlk*), with homology to genes involved in the Notch signalling pathway was identified 80 kb upstream of *Gtl2*. *Dlk* was expressed exclusively from the paternal allele in both the embryo and placenta, but the CpG-island promoter of *Dlk* was completely unmethylated on both parental alleles. Rather, a paternally methylated region was identified in the last exon of the active *Dlk* allele. The proximity, reciprocal imprinting and methylation in this domain are reminiscent of the co-ordinately regulated *Igf2–H19* imprinted domain on mouse chromosome 7. Like *H19* and *Igf2*, *Gtl2* and *Dlk* were found to be co-expressed in the same tissues throughout development, though not after birth. These results have implications for the regulation, function and evolution of imprinted domains.

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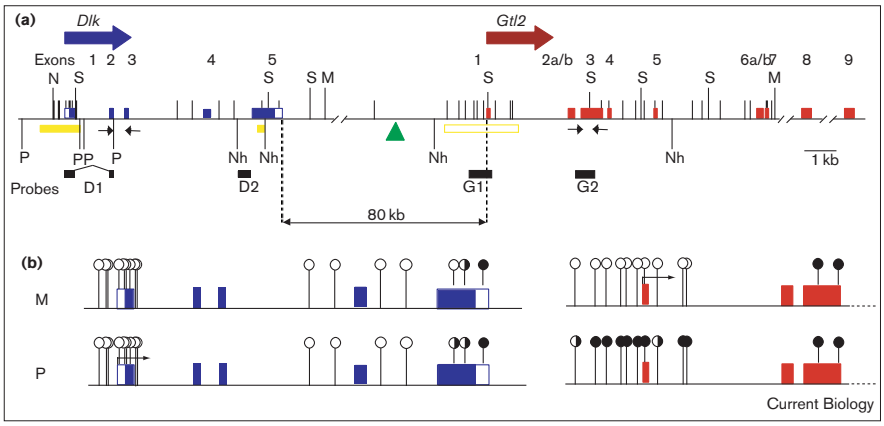
Results and discussion

Many imprinted genes identified in the mouse function in the regulation of prenatal growth and in the development of particular lineages [1,2]. These processes are compromised when the dosage of imprinted genes is perturbed, for example, in androgenetic (bipaternal) and parthenogenetic (bimaternal) embryos [3,4] or in embryos with uniparental duplications and deficiencies that include imprinted domains. Eleven such chromosomal regions have so far been identified in the mouse [5]. One of these is the distal half of mouse chromosome 12 [4]. Paternal UPD12 (pUPD12) causes prenatal lethality after embryonic day 16 (E16) of gestation. These animals have placentomegaly, muscle overgrowth and skeletal defects [6]. Conceptuses with maternal UPD12 (mUPD12) die perinatally and are severely growth retarded [6]. This indicates the presence of imprinted genes on chromosome 12 that are essential for normal growth and development. Consistent with this, a transgene insertion mapping to the distal portion of chromosome 12 results in growth retardation on paternal inheritance [7]. This insertion is located 3 kb upstream of the *Gtl2* gene. *Gtl2* expression is substantially reduced in mutant embryos [8]. Unexpectedly, for a gene proposed to be associated with this paternally inherited mutant phenotype, *Gtl2* is expressed in parthenogenetic embryos [8]. The gene has subsequently been shown to be expressed from the maternal allele and predominantly repressed on the paternal allele in mouse and human [9].

A bacterial artificial chromosome (BAC) clone containing *Gtl2* was isolated from a 129/Sv mouse genomic library (Invitrogen), and a 100 kb stretch encompassing *Gtl2* was mapped (Figure 1). Northern blots using RNA isolated from E15.5 mUPD12, pUPD12 and normal conceptuses were probed with *Gtl2* exon 3 (Figure 2b). *Gtl2* transcripts were expressed in mUPD12 embryos at levels at least twice those seen in normal embryos, but were absent in pUPD12 embryos, indicating that the gene is imprinted. Southern blot analysis of mUPD12 and pUPD12 DNA, cut with methylation-sensitive restriction enzymes, showed that the promoter region of *Gtl2* was differentially methylated (Figure 3c); the inactive paternal promoter was hypermethylated and the promoter of the active maternal allele completely unmethylated. The paternal promoter was not methylated in sperm (Figure 3d, probe G1), suggesting that the promoter methylation on this allele is not the germ-line imprinting signal. The methylation status is summarised in Figure 1b.

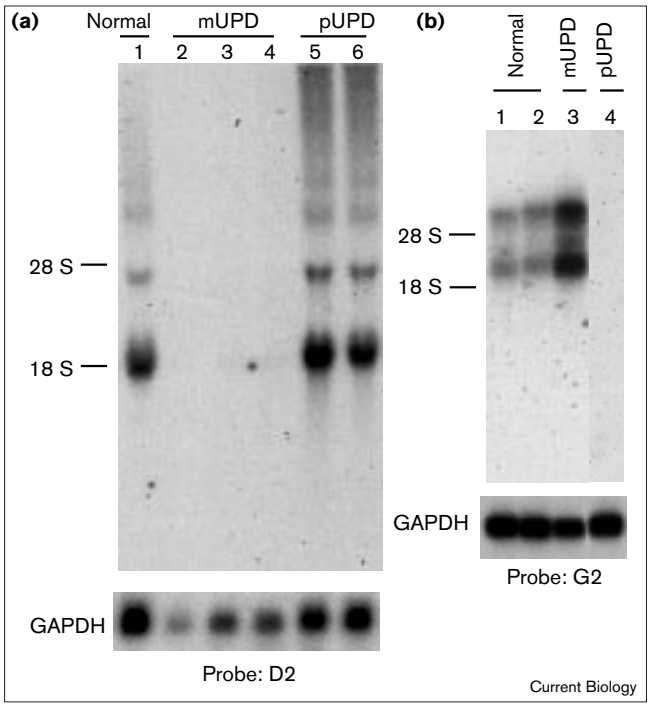
We also identified, from genome databases, candidate genes and expressed sequence tags (ESTs) mapping to distal chromosome 12 or the region of syntenic homology on human chromosome 14q. One of these, *Delta-like* (*Dlk*) was found to map 80 kb upstream of *Gtl2* on the same mouse BAC clone (Figure 1a). *Dlk* encodes an EGF-repeat-containing protein and has been shown to function in several cell types, including pre-adipocytes [10], thymocytes [11], adrenal glomerulosa cells [12], pancreatic

Figure 1



Genomic organisation and differential methylation of the *Dlk* and *Gtl2* genes. **(a)** *Dlk* (blue exons) and *Gtl2* (red exons) gene structures. Yellow boxes, CpG islands; open yellow box, CpG-rich domain at the *Gtl2* promoter; green triangle, *lacZ* insertion site in the *Gtl2*^{lacZ} mouse [7,8]; black bars, probes; arrows, positions of primers used in the reverse transcription (RT)–PCR analysis. A restriction map of the region is shown above, and other relevant sites associated with probes used are shown below. *Hpa*II sites are indicated as unlettered vertical lines. N, *Not*I; S, *Sma*I; M, *Mlu*I; P, *Pst*I; Nh, *Nhe*I. **(b)** Summary of differential methylation and opposite imprinting of the *Dlk* and *Gtl2* genes. M and P, the maternally and paternally inherited chromosomes, respectively; circles, methylation status; white, black and half-filled circles, unmethylated, fully methylated and partially methylated *Hpa*II sites, respectively; arrows, expression of allele and direction of transcription.

β-cells [13], hematopoietic stromal cells and B lymphocytes [14]. A secreted form of the protein has been isolated from amniotic fluid [15]. In mammalian systems, *Dlk* plays a role in differentiation (reviewed in [16]). Although murine *Gtl2* has previously been mapped to chromosome 12, the map position of the *Dlk* gene is ambiguous [17,18]. Using a sequence from intron 2 of the *Dlk* gene, we verified the location of the cloned genomic DNA fragment to mouse chromosome 12 on a mouse × hamster radiation hybrid mapping panel (Research Genetics). The highest anchor LOD (10.6) was obtained for linkage to the marker *D12Mit280*, and the best-fit position for this locus was 9.5 cR proximal to *D12Mit141*, and 1.4 cR distal to *D12Mit280* (data not shown).

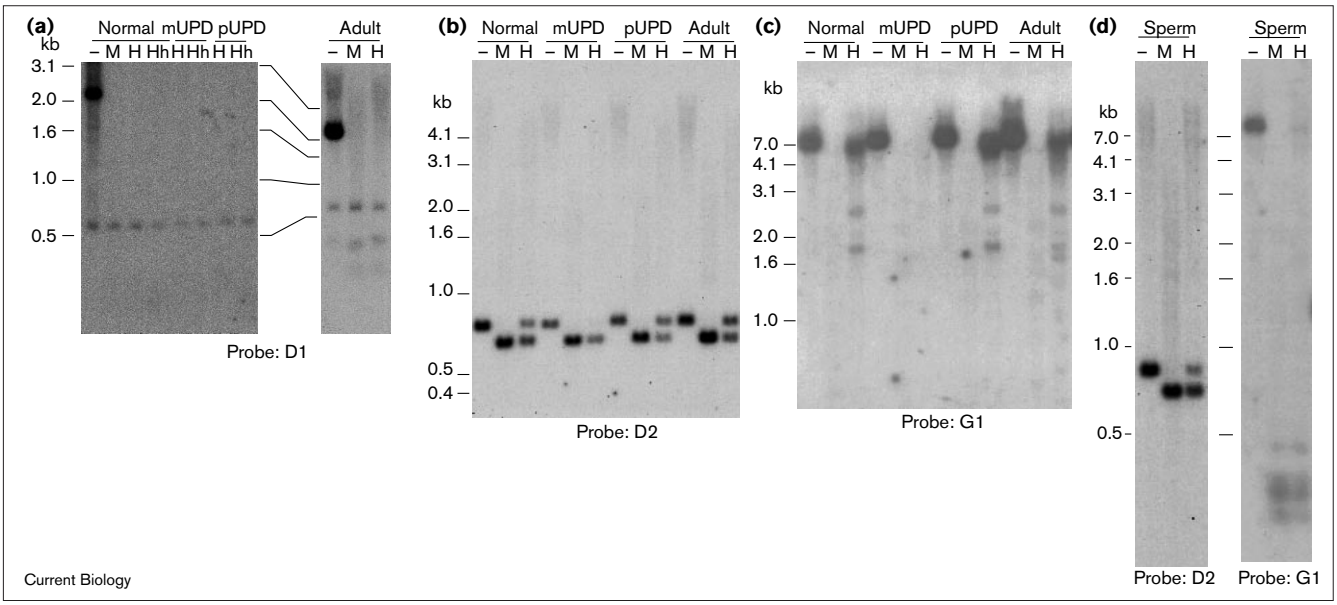


Allele-specific expression of *Dlk* was analysed using RNA isolated from mUPD12 and pUPD12 embryos (and placentae, data not shown). Four different transcripts were evident in both embryos and placentae. *Dlk* was not expressed in mUPD12 material, and was expressed at approximately twice the normal level in pUPD12 conceptuses (Figure 2a), making it the first paternally expressed imprinted gene identified on mouse chromosome 12. Furthermore, *Dlk* and *Gtl2* are imprinted reciprocally. To determine whether the imprinting of *Dlk* is associated with allele-specific methylation, DNA from UPD12 conceptuses was digested with methylation-sensitive restriction enzymes. The promoter of *Dlk* is a CpG island, at which no parental-origin specific methylation differences were observed (Figure 3a, probe D1). Systematic analysis of all the *Hpa*II sites in the gene identified a single differentially methylated site in a smaller CpG island within the last exon. This *Hpa*II site was completely unmethylated

Figure 2

Imprinted expression of *Dlk* and *Gtl2*. **(a)** *Dlk* is exclusively expressed from the paternal allele. Total RNA (10 μg) prepared from normal (lane 1), mUPD12 (lanes 2–4) and pUPD12 mice (lanes 5,6) at E15.5 were analysed by northern blot hybridisation [23] using D2 as a probe. RNA loading was confirmed with a probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Expression of *Dlk* was 1.9 ± 0.3 times stronger in pUPD12 compared with normal embryos, as determined by densitometric analysis on a Storm 860 phosphorimager (Amersham). **(b)** *Gtl2* is expressed exclusively from the maternal allele. PolyA+ RNA (0.5 μg) prepared from normal (lanes 1,2), mUPD12 (lane 3) and pUPD12 mice (lane 4) was analysed as in (a). *Gtl2* exon 3 (G2) was used as a probe. Expression of *Gtl2* was 2.7 ± 0.3 times stronger in mUPD12 compared with normal embryos, as determined by densitometric analysis.

Figure 3



Identification of the differentially methylated regions of *Dlk* and *Gtl2*. **(a)** The CpG island at the start of *Dlk* is completely unmethylated on both parental alleles in embryos and adults. Genomic DNA prepared from normal, mUPD12 and pUPD12 mice at E15.5 and kidney from an adult C57BL/6J mouse was digested with *Pst*I (–), further digested with *Msp*I (M), *Hpa*II (H), or *Hha*I (Hh) and analysed by Southern blot hybridisation [24] using D1 as a probe. **(b)** The CpG island in exon 5 of *Dlk* is differentially methylated on the two parental alleles. Genomic DNA, prepared as in (a), was digested with *Nhe*I (–), further digested with *Msp*I or *Hpa*II and analysed by Southern blot hybridisation using D2 as a probe. **(c)** The CpG-rich 5′ region at the *Gtl2* promoter is hypermethylated on the paternal allele and unmethylated on the maternal allele. Genomic DNA, prepared as in (a), was digested with *Nhe*I (–), further digested with *Msp*I or *Hpa*II and analysed by Southern blot hybridisation using G1 as a probe. **(d)** Analysis of genomic DNA isolated from sperm indicated that the paternal-specific methylation in *Dlk* exon 5 is inherited from sperm (probe D2). In contrast, the paternal methylation at the *Gtl2* promoter was predominantly unmethylated in sperm (probe G1). Thus, this modification was acquired after fertilisation.

on the maternal inactive allele and partially methylated on the active paternal allele (Figure 3b). This partial methylation was present in sperm (Figure 3d, probe D2). To determine whether this differentially methylated region was associated with antisense transcription, as has been described for other imprinted genes with internal differentially methylated sites [19,20], strand-specific RT–PCR was carried out using primers located along the length of the gene. No antisense transcripts from either parental allele were identified 5′ of the last exon (data not shown). Thus, the *Dlk* exon 5 differential methylation is reminiscent, in pattern and parental origin, to that seen in the last exon of the paternally expressed *Igf2* gene. In *Igf2*, the partial methylation pattern represents tissue-specific methylation correlating with organs in which the gene is active and it has been proposed that, when methylated, this region is unable to bind a repressor [21].

Dlk and *Gtl2* represent a new pair of reciprocally imprinted genes. The imprinting characteristics of *Dlk* and *Gtl2* are highly reminiscent of those seen for the *Igf2–H19* locus. Like *Igf2–H19*, *Dlk* and *Gtl2* are 80–100 kb apart, oppositely imprinted, and the 3′ gene, *Gtl2*, like *H19*, encodes an untranslated RNA. The methylation profiles are also very similar: *H19* and *Gtl2* have paternally methylated CpG-rich promoters on the inactive alleles, and *Igf2* and *Dlk* have paternal-specific partial methylation in the last exon of the active allele. *H19* and *Igf2* are expressed in the same tissues in the embryo and the genomic organisation and epigenetic characteristics are important for this co-ordinate regulation (reviewed in [22]). We therefore predicted that *Dlk* and *Gtl2* might also show co-ordinate regulation. Developmental expression of *Gtl2* has been documented by *in situ* hybridisation [8], but *Dlk* expression data are limited. Co-expression of the genes was therefore assessed by PCR amplification of a tissue- and stage-specific panel of cDNAs generated for comprehensive developmental expression assays (E.C., A.C.F.-S., M.H.J. and T.F., unpublished). *Gtl2* and *Dlk* transcripts were detected at most stages in all embryonic and extraembryonic tissues (see Supplementary material). Northern analysis of total RNA from E18.5 foetal tissues confirmed this *Dlk* expression (data not shown). In 88–90% of the prenatal tissues examined, the genes were co-expressed. In adult tissues, no *Dlk* expression was detected but *Gtl2* was expressed in some adult tissues. Thus, at prenatal stages, *Dlk* is expressed in the majority of tissues that also

express *Gtl2*. The relative levels of expression varied between tissues and, because each gene encodes multiple transcripts, may reflect differences in the specific transcripts amplified. The sites of strongest expression were, however, consistent with previous reports [8,10]. Thus, *Dlk* and *Gtl2* share the combination of common methylation imprints, common sites of expression, the same linkage pattern, the same absence of open reading frame in the 3′ gene, and the same reciprocity in imprinting as *Igf2–H19*. Further studies will determine whether the two genes share common regulatory elements, as has been shown for the *Igf2–H19* locus.

Gtl2 was isolated originally as a gene, located 3 kb downstream of a transgene insertion, $Gtl2^{lacZ}$, which caused a foetal and postnatal growth retardation phenotype on paternal transmission [7]. Maternal transmission of the insertion results in normal animals. *Gtl2* expression is reduced though not absent in mutant embryos homozygous for the insertion. Thus *Gtl2* is repressed on the paternal allele, and does not appear to be activated in the transgene homozygotes [8]. The growth retardation phenotype observed on paternal transmission might therefore be explained by the silencing of a paternally expressed imprinted gene, which is in the vicinity of *Gtl2*, with regulatory elements affected by the insertion. Consistent with this is the finding that all mUPD12 embryos are severely growth retarded [6]. Based on the results presented here, absence of *Dlk* expression might be responsible for this growth retardation. Analysis of *Dlk* expression in the $Gtl2^{lacZ}$ mouse, and comparison of the $Gtl2^{lacZ}$ phenotype with that of a *Dlk* knockout mouse, will test this explanation. The lethality and accompanying array of mutant phenotypes identified in mUPD12 and pUPD12 conceptuses indicate that imprinted genes on chromosome 12 play roles in multiple lineages that are not affected in the $Gtl2^{lacZ}$ mutant. It is therefore likely that other imprinted genes exist on mouse chromosome 12, and *Dlk* and *Gtl2* may be two genes within a larger imprinted domain.

Supplementary material

A figure showing co-ordinate expression of *Dlk* and *Gtl2* is available at http://current-biology.com/supmat/supmatin.htm.

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