Deletion of the H19 transcription unit reveals the existence of a putative imprinting control element

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The distal region of mouse chromosome 7 contains a cluster of imprinted genes that includes H19 and Igf2 [insulin-like growth factor 2]. H19 is expressed as an untranslated RNA found at high levels in endodermal and mesodermal embryonic tissues. This gene is imprinted and exclusively expressed from the allele of maternal origin. The Igf2 gene shows a similar pattern of expression but is expressed from the paternal allele. We have generated a targeted deletion of the H19 transcription unit by insertion of a neo replacement cassette. The homozygous mutant animals are viable and fertile and display an overgrowth phenotype of 8% compared with wild-type littermates. This is associated with the disruption of Igf2 imprinting and the consequent biallelic expression of this gene. A striking feature of the recombinant H19 allele is the occurrence of a parental imprint set on the neo replacement cassette. Therefore imprinting of the H19 locus is independent of the H19 gene itself. Taken together with the results of a larger H19 mutation described previously, this indicates that an imprinting control element is located within the region 10 kb upstream of H19.

[Key Words: H19; homologous recombination; Igf2; genomic imprinting]

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The generation of mice carrying a null mutation of this gene can elucidate its role and possibly give further insight into the mechanism of imprinting. Mice carrying a 13-kb deletion encompassing both the H19 gene and its upstream sequences have been described previously [Leighton et al. 1995a]. These mice are viable and fertile and show an increase in weight that is linked to biallelic expression of Igf2. We have created mice harboring a smaller deletion covering only the transcription unit of the gene. We report here that this 3-kb deletion also affects the growth of the mutant animals, although to a lesser extent, and that in contrast with the previous work, the neomycin resistance gene [neo] cassette that has been inserted at the H19 locus is imprinted. This implies that the signals required for the imprint are maintained in these mutant mice and that the H19 gene itself or its RNA product is not involved in the imprint of this locus. Given the difference between the two deletions, we also propose that a putative imprinting control element is located in the region 10 kb upstream of the H19 gene.

Results

Targeted disruption of the H19 gene

The replacement construct that has been used to create a null mutation of the H19 gene by homologous recombination harbors a 3-kb deletion of the entire transcription unit between the 5' EcoRI site and the 3' SalI site of the genomic DNA. All five exons of the gene are deleted, as well as an Sp1 site and the TATA box in the promoter region of H19. The vector contains a 2-kb fragment of 5' homology and a 6.5-kb fragment of 3' homology. The expected homologous recombinant allele results in a 3-kb deletion replaced by the 1.1-kb neo cassette. Both the neo and the herpes simplex virus thymidine kinase [tk] cassettes are under the control of the pMC1 promoter, composed of a Polyoma [Py] enhancer (allowing expression in ES cells) and a tk promoter; contain a poly[A] addition signal; and are inserted in opposite orientation with respect to the H19 transcription direction (Fig. 1B).

After electroporation of the CK35 ES cell line, 4 out of 60 clones displayed a correctly targeted allele as determined by Southern analysis (Fig. 1C). The 5' external probe revealed a 5-kb fragment from the mutant allele and a 4-kb fragment from the wild-type allele in EcoRI-digested genomic DNA from these clones. The 3' external probe revealed 8-kb and 11-kb EcoRI fragments from the mutant and wild-type alleles, respectively [data not shown].

One clone [cl 19] was injected into blastocysts and the resulting male chimeras were mated with MF1 or C57BL/6 females to produce heterozygous F1 animals. These animals were tested for the presence of the neo gene by PCR on rapid-tail DNA preparations [as described in Materials and Methods]. EcoRI-digested DNA was also analyzed by Southern blot using 5' [data not shown], 3', and neo probes (Fig. 1D), showing the expected size fragments. Only one copy of the neo gene was detected in these animals, indicating a single insertion site of the targeting construct. These results confirm the presence of a correctly targeted allele, with the entire 3-kb transcription unit of the H19 gene being replaced by the neo cassette.
Paternal and maternal transmission of the mutation

The H19 gene is imprinted and expressed only from the maternal allele. Therefore heterozygous F1 offspring will display two phenotypes: (1) Mice in which the null allele is paternally inherited will carry a wild-type \( H19 \) allele on the maternal chromosome that is normally expressed: These animals, called paternal heterozygotes (\( \text{pat het} \)), should display a wild-type phenotype; and (2) maternal heterozygotes (\( \text{mat het} \)), in which the null allele is maternally inherited, carry the wild-type \( H19 \) allele and should display the same phenotype as homozygous mutants. This nomenclature was proposed previously for the \( Igf2 \) heterozygous mutants (DeChiara et al. 1990).

The heterozygous offspring from the male chimeras inherit the mutated allele paternally, and these \( \text{pat het} \) mice were identical to wild-type mice. In the F1 offspring from these mice, we found that \( \text{pat het} \), \( \text{mat het} \), and homozygous animals were all viable and fertile. The only observed phenotype was an increase in weight of the \( \text{mat het} \) and homozygous animals compared with their wild-type littersmates. The increase ranged between 4% and 12% with a mean of 8%, estimated on 100 animals (5-day neonates) from 12 litters. Mice from different backgrounds were analyzed and all displayed the same significant increase, as estimated by Student's \( t \)-test (\( P < 0.01 \)). This weight difference was also maintained in adult animals (Table 1; Fig. 2).

Northern analysis on muscle and liver RNA from 5-day neonates showed expression of \( H19 \) in \( \text{pat het} \) mice. No \( H19 \) transcript was detected in \( \text{mat het} \) (Fig. 3A,B) and in homozygous mutants (data not shown). This result confirms that the \( H19 \) targeted allele is correctly mutated and that the imprint is maintained on the normal allele because the paternal \( H19 \) allele is not expressed in \( \text{mat het} \) animals.

Table 1. Weight of mutant animals

| Background       | 5 day neonates (litter no.) | Genotypes | Percent |
|------------------|-----------------------------|-----------|---------|
|                  |                             | \( \text{mat het} \) | wild type |         |
| MF1/129 × C57    | 1                           | 2.16 ± 0.03 (n = 3) | 1.98 ± 0.04 (n = 7) | 8 |
|                  | 2                           | 3.92 ± 0.07 (n = 4) | 3.63 ± 0.07 (n = 6) | 7.4 |
|                  | 3                           | 4.61 ± 0.07 (n = 6) | 4.42 ± 0.09 (n = 4) | 4 |
|                  | 4 and 5                     | 3.97 ± 0.10 (n = 7) | 3.48 ± 0.12 (n = 9) | 12 |
|                  | 6                           | 3.74 ± 0.12 (n = 5) | 3.46 ± 0.04 (n = 5) | 7.4 |
| C57/129 × C57    | 7                           | 3.57 ± 0.11 (n = 4) | 3.15 ± 0.05 (n = 2) | 11.8 |
|                  | 8                           | 3.73 ± 0.08 (n = 3) | 3.46 ± 0.08 (n = 3) | 7 |
| C57/129 × 129    | 9                           | 3.65 ± 0.05 (n = 4) | 3.36 ± 0.08 (n = 3) | 7.7 |
| C57/129          | 10                          | homoygotes     | wild type |         |
|                  |                             | 4.86 ± 0.06 (n = 3) | 4.28 ± 0.15 (n = 5) | 12 |
| 129 × C57/129    | 11 + 12                     | \( \text{pat het} \) | wild type |         |
|                  |                             | 2.90 ± 0.16 (n = 5) | 3.0 ± 0.11 (n = 11) | -3 |
| C57/129 × C57    | females                     | \( \text{mat het} \) | wild type |         |
|                  | males                       | 28.2 ± 1.3 (n = 8) | 25.1 ± 1.2 (n = 6) | 11 |
|                  | males                       | 24.2 ± 2.0 (n = 2) | 22.2 ± 0.9 (n = 6) | 8 |

Expression of the neo gene

The same Northern blots were hybridized with a neo probe: neo transcripts were detected in \( \text{mat het} \) but not in \( \text{pat het} \) animals, which carry the neo gene on the paternally transmitted allele (Fig. 3C,D). These results show that the neo cassette that has been inserted at the \( H19 \) locus is subject to imprinting. The mutation that was introduced into these animals has therefore left intact the signals necessary for this imprint.

RNAs from adult tissues of \( \text{mat het} \) animals (kidney, liver, muscle, testis, and thymus) were hybridized with a neo probe (Fig. 4); expression of neo was detected only in muscle, therefore suggesting that the neo gene is under the same spatio-temporal regulation as the \( H19 \) gene it has replaced.

DNA methylation analysis of the 5' upstream region

It has been shown previously that the 5' upstream region of the \( H19 \) gene is differentially methylated on the maternal and paternal allele [Bartolomei et al. 1993; Tremblay et al. 1995]. To analyze the methylation profile of this region in the \( H19 \) \( \text{mat het} \) and \( \text{pat het} \) mice, genomic DNA from neonatal liver was digested with EcoRI or with EcoRI and \( HpaII \) and hybridized to the 5' external BglII-BamHI probe (Fig. 5A,B). Double digestion of wild-type DNA shows a fully methylated 4-kb fragment (paternal allele) and several \( HpaII \) fragments (maternal allele). In \( \text{mat het} \) and \( \text{pat het} \) DNA samples, 4-kb (wild-type allele) and 5-kb (targeted allele) EcoRI fragments are detected. In \( \text{mat het} \) DNA, the 4-kb paternal fragment was resistant to \( HpaII \) digestion, indicating that the \( HpaII \) sites were methylated on the paternal allele, whereas the 5-kb maternal fragment was sensitive to \( HpaII \). Similarly, in \( \text{pat het} \) DNA samples, the 5-kb paternal fragment was resistant to \( HpaII \) digestion (the ob-
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served 4.2-kb fragment results from the presence of a partially methylated HpaII site in the neo gene) and the 4-kb maternal fragment was sensitive to HpaII. Similar results were obtained with muscle DNA (data not shown). In conclusion, the paternal allele is methylated in both mat het and pat het animals, suggesting that the observed imprint of the paternal neo gene is correlated with allele-specific methylation of the 5' upstream region.

Expression of the Igf2 gene

The H19 gene is located at a distance of 80-kb from the Igf2 gene. To analyze a possible interaction between these two genes, we performed a cross between H19 heterozygous females and Igf2 heterozygous males (kindly provided by A. Efstratiadis, Columbia University, New York, NY). These Igf2 heterozygous mice result from a targeted disruption of the Igf2 gene; it was shown that pat bet animals, which do not express any Igf2, because Igf2 is expressed only from the paternal allele, show a 30% decrease in weight [DeChiara et al. 1990].

This cross gives rise to four genotypes corresponding to different epigenotypes as indicated in Figure 6A. The [H19+/Igf2-] animals, having received a normal H19 allele from their mother and the Igf2 null mutation from their father, should display an Igf2 pat het phenotype. The [H19+/Igf2+] animals, having received the H19 null mutation from their mother and a normal Igf2 allele from their father, should display an H19 mat het phenotype. The [H19-/Igf2-] double heterozygous animals should allow the analysis of Igf2 expression in the absence of H19.

The progeny obtained were genotyped by digestion of tail DNA with EcoRI and Southern analysis using a neo probe. The H19 targeted allele was revealed by a 5-kb diagnostic fragment and the Igf2 targeted allele by an 11-kb diagnostic fragment (data not shown).

Analysis of the weight of 5 day neonates in each group showed the following results [Fig. 6B]: [H19+/Igf2-] animals were 30% lighter than wild-type [H19+/Igf2+] ones, as expected because these animals are Igf2 pat het. The [H19+/Igf2+] animals were 7% lighter than their wild-type littermates (P < 0.1). The [H19+/Igf2+] animals are not shown because of a low sample number in this particular cross; however, in other crosses, as shown in Table 1, H19 mat het animals were on average 8% heavier than wild-type animals.

Figure 3. Expression of H19 and neo genes in neonatal heterozygous mice. (A,B) Northern analysis of 5-day neonate muscle (A) and liver (B) RNA with the H19 probe. Genotypes of the mice are indicated at top; sizes of the transcripts are indicated in middle. (C,D) Northern analysis of 5-day neonate muscle (C) and liver (D) RNA. The same A and B filters were hybridized with the neo probe.

Figure 4. Northern analysis of adult tissues (6 months old) from a mat het animal. The neo probe was hybridized to RNA from the indicated tissues. A GAPDH probe was used as control. Sizes of the transcripts are indicated at right.
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To correlate these observations with the level of Igf2 transcription, Northern analysis was performed on muscle RNA from 5-day neonates [Fig. 6C]. [H19'/Igf2'] animals [Igf2 pat het] showed expression of H19 and no expression of Igf2 mRNA, as reported before [DeChiara et al. 1990]. Unexpectedly, [H19'/Igf2'] animals, which carry only one Igf2 allele of maternal origin, expressed Igf2 transcripts. However, the level of Igf2 was only 25% compared with 100% in wild-type animals [Fig. 6D]. [H19'/Igf2'] animals [H19 mat het] displayed higher amounts of Igf2 transcripts (125%) than wild-type animals and no H19 transcript.

The maternal Igf2 allele, which is normally silent, is therefore expressed in the absence of the maternal H19 gene in cis. However, the maternal Igf2 level of expression is lower than the wild-type paternal Igf2 level. This correlates with the partial rescue of the Igf2 phenotype, as indicated by the 7% weight reduction of these animals compared with wild-type animals.

Discussion

The chromosomal regions subject to imprinting appear to contain clusters of genes displaying parental-specific differential expression. This has been shown not only for the distal region of mouse chromosome 7, containing the H19-Igf2 locus, but also in a more central region of this chromosome containing the Snrpn gene, homologous to the Prader-Willi-Angelman syndrome locus on human chromosome 15 [Nicholls 1994]. X chromosome inactivation is yet another example of long-range control, because the paternal X chromosome is preferentially inactivated in extraembryonic tissues and the Xist gene is
imprinted [Kay et al. 1993, 1994]. The imprinting mechanism must result from a complex gene regulation over large distances, involving chromatin structure, trans-acting factors, and cis-acting sequences. The targeted disruption of the H19 gene has enabled the study of some aspects of the regulatory mechanism involved in imprinting.

We have generated null mutant animals in which only the 3-kb transcription unit of the H19 gene has been deleted. We will refer to this mutation as the H19<sup>a3</sup> allele. Our results have shown that this loss of function mutation does not affect embryogenesis of the mouse: Although H19 is first detected in the trophectoderm cells of the blastocyst and is then expressed at high levels in endodermal and mesodermal tissues of the mid-gestation embryo, it does not appear to play a fundamental role in development. The null mutant mice are viable and fertile, and after one year of breeding, no other obvious phenotype has been detected apart from a weight increase of the maternal heterozygous and homozygous animals compared with wild-type littersmates. This gene might be subject to redundancy and other genes might be accomplishing the same function. There is, however, no indication of a multigene family related to the H19 gene. It is also possible that the sheltered breeding conditions in which the mice are maintained do not allow the detection of a more severe phenotype in these mutant animals. Alternatively, it could be that this gene must be repressed in certain tissues to allow full development of the embryo. To address this question requires experiments involving ectopic expression of H19 in nonexpressing tissues of the embryo.

Several studies have revealed a modification of the imprint in embryonal human tumors related to the Beckwith–Wiedemann syndrome such as Wilm’s tumors and rhabdomyosarcomas [Ohlsson et al. 1993; Moulton et al. 1994; Steenman et al. 1994; Reik et al. 1995; Taniguchi et al. 1995]. In these tumors, expression of H19 is no longer detected and the adjacent Igf2 gene is frequently expressed biallelically. These observations could be related to the tumor suppressor role proposed for H19 [Hao et al. 1993]. It is noteworthy that mutant animals lacking the H19 gene have not shown any incidence of tumors, although they are now over a year of age. The animal model that we have generated suggests that absence of H19 expression is not sufficient to induce tumors in mice.

H19 expression is strongly down-regulated in adult mice and is found only in muscle tissues. Expression analysis of the neo cassette that has replaced the H19 transcription unit has shown that the only adult tissue of mutant mice in which the neo transcripts are detected is muscle. Regulatory elements involved in the correct spatio-temporal expression of the H19 locus have thus been maintained in these mutant animals. Interestingly, this suggests that these regulatory elements can override the tissue-specificity of the Py enhancer, because one of the target organs for polyoma virus is the kidney [Dawe et al. 1987; Rochford et al. 1990].

One striking observation is the imprint of the neo cassette in heterozygous animals. The neo gene is expressed only when inherited from the maternal allele in neonatal muscle and liver, and no expression is detected in pat het animals, which carry the neo cassette on the paternal allele. Signals responsible for inducing a closed chromatin configuration on the paternal allele must have been maintained in the H19<sup>a3</sup> mutation, such that the neo cassette that has replaced the H19 transcription unit is imprinted. A similar regulation was described previously for an X-linked neo transgene that was subject to correct imprinting and X inactivation [Dandolo et al. 1993] and for the neo cassette introduced in the Igf2 mutation [DeChiara et al. 1991]. Recent transgene experiments have suggested that the structural H19 gene or the H19 RNA itself may be involved in its own imprint [Pfeifer et al. 1996; Elson and Bartolomei 1997]. Our present study, however, clearly shows that in the context of the chromatin structure of chromosome 7, neither the H19 DNA sequence nor transcription of the H19 gene itself is required for establishment or maintenance of imprinting at this locus.

In addition, our results are in contrast with the targeted disruption of H19 described previously [Leighton et al. 1995a], in which biallelic expression of neo was observed. In these animals, a 13-kb deletion encompassing not only H19 but also 10-kb of the 5′ upstream region of the transcription unit had been generated. We propose to refer to this latter mutation as the H19<sup>a13</sup> allele. Taken together, these results show that the 10-kb sequence located 5′ to the H19 gene is required for the imprint at the H19 locus [Fig. 7]. This suggests that the H19 5′ upstream region contains an imprinting control element. This 5′ region has been shown to display allele-specific methylation correlated with differential expression of H19 and to include several repeats of a [G]<sub>6</sub>[G]<sub>6</sub> consensus sequence [Bartolomei et al. 1993; Ferguson-Smith et al. 1993; Tremblay et al. 1995]. Analysis of DNA methylation of this region in the H19<sup>a13</sup> mutants showed that only the paternal allele is methylated. Allele-specific methylation has thus been maintained in the mutant animals.

Among known imprinted genes, three have been identified that encode a possible functional RNA: H19, IPW [Wevrick et al. 1994], and Xist. It has been shown that the Xist gene, exclusively expressed from the inactive X chromosome, is involved in the inactivation of adjacent genes [Clemson et al. 1996; Lee et al. 1996; Penny et al. 1996; Herzing et al. 1997; Lee and Jaenisch 1997]. One could suggest a possible similarity in the role of these genes, being involved in the down-regulation of expression of chromosomal domains, and acting through a cis effect of their RNA: Xist on genes of the inactive X chromosome and H19 on the Igf2–Ins2 region. The H19 RNA itself may thus regulate the silencing of the Igf2 gene by a cis effect. A possible example of this effect is the presence of biallelic Igf2 expression in the absence of H19 expression in certain human tumors (as mentioned above).

The results obtained from the cross between H19<sup>a3</sup> and Igf2 heterozygous mutant animals have shown that
a regulatory link can be established between these two genes. Absence of a maternally expressed $H19$ gene is associated with expression of the normally silent adjacent $Igf2$ gene on the same chromosome. This loss of imprint of $Igf2$ resulting in its biallelic expression is correlated with the observed weight increase of the $H19$ mutant animals. Disruption of $Igf2$ imprint has now been observed in two cases (Fig. 7): [1] In these $H19^{\Delta3}$ mutants, the level of maternal $Igf2$ expression corresponds to 25% of the paternal level and is correlated with an 8% weight increase and only partial rescue of mutant animals. Disruption of related with the observed weight increase of the circles. (R) EcoRI; (S) SalI. $H19^{\Delta3}$ animals display imprinted expression of the neo gene, whereas $H19^{\Delta13}$ animals display biallelic expression of this gene.

In conclusion, the $H19^{\Delta3}$ null mutation of the $H19$ gene has shown that this gene is dispensable during embryonic development and leads to a phenotype of larger animals as a result of biallelic expression of the adjacent $Igf2$ gene. Absence of the $H19$ transcription unit modifies the imprint of the $Igf2$ gene. The replacement neo cassette shows the same pattern of parental imprinting and tissue-specific expression as $H19$. Taken together with the $H19^{\Delta13}$ mutants reported previously, our results suggest that the region 10 kb upstream of $H19$ carries a putative imprinting control element. Further experiments using subtle mutations in this upstream region or in the RNA sequence itself of the $H19$ gene should allow a more precise definition of the regulatory events involved in the control of this imprinted region.

Materials and methods
Disruption of the $H19$ gene.

An $H19$ genomic clone was isolated from a $129\ \lambda$FixII mouse genomic library (Stratagene) and the phage insert was subcloned into pBluescript KSII. The targeting vector carries a deletion of the EcoRI–SalI fragment encompassing the $H19$ transcription unit and 50 bp of the promoter (Fig. 1B). It is composed from left to right of pBluescript, a 2-kb BamHI–EcoRI fragment from the 5' end of the gene [-2 to -50 bp], the pMC1–neo cassette (Stratagene) inserted in opposite orientation, a 6.5-kb SalI–BglII fragment from the 3' end of the gene (from +3 to +9.5 kb) and the pMC1–tk cassette. The vector was linearized at a unique NotI site and electroporated into embryonic stem (CK35 ES) cells. The CK35 ES cell line was established according to procedures derived previously using blastocysts from superovulated 129 female mice (Robertson 1987). These cells are maintained on neo mouse embryo fibroblasts in the presence of leukemia inhibitory factor (LIF) (10^3 U/ml). They were tested for efficiency of chimerialism and germ-line transmission after microinjection into C57BL/6 blastocysts prior to transfection procedures.

Embryonic stem (ES) cells (2 x 10^7) in 0.5 ml were electroporated with 15 µg of DNA using a Bio-Rad Gene Pulser apparatus at 220 V and 960 μF in PBS or medium. G418 selection (200 μg/ml effective concentration) (GIBCO) was applied 48 hr later and gancyclovir (2 x 10^-6 M) (Syntex) was added on day 4 or 5 after electroporation. This double selection provided a 5-fold enrichment in positive clones compared with G418 selection.
one being frozen directly in serum with 10% DMSO when con-
fluent and the other being kept for DNA extraction.

Genomic DNA from individual clones was prepared, digested with EcoRI, separated on 1% agarose gels, blotted to Hybond N+ [Amersham] filters, and hybridized to 32P-labeled random primed probes (Sambrook et al. 1989). Hybridization was per-
fomed in Church buffer at 65°C overnight (Church and Gilbert 1984). Blots were washed in 0.4x SSC, 0.5% SDS for 30 min at
65°C.

The 5’ external probe is a BglII-BamHI 200-bp fragment at -2 kb and the 3’ external probe is a BglII-EcoRI 1-kb fragment from +9.5 to +10.5 kb [Fig. 1B]. The neo probe is a 1.1-kb XhoI-BamHI fragment from the pMC1-neo plasmid.

Blastocyst injections and mating of chimeric mice
One of the correctly targeted clones (cl 19) was injected into MF1 or C57BL/6 blastocysts. Germ-line transmission was very low with MF1-derived chimeric males (0.5%–3%) and much higher with C57BL/6-derived chimeras (25%–100%). Heterozy-
gous and homozygous animals were obtained from these differ-
ent crosses.

Rapid testing for heterozygotes
Heterozygous animals were analyzed by a rapid PCR procedure. Tail samples were digested in 200 μl of lysis buffer (50 mM KCl, 10 mM Tris at pH 8.3, 2.5 mM MgCl2, 4.5% NP40, 4.5% Tween 20, 1 mg/ml of proteinase K). A 35-cycle PCR reaction [94°C for 1 min, 55°C for 30 sec, 72°C for 30 sec] using neo-specific oli-
gonucleotides [Dandolo et al. 1993] was directly performed on 2.5 μl of DNA after a preliminary incubation at 95°C for 10 min before adding Taq polymerase [Hanley and Merlie 1991].

Northern blot analysis
RNA was extracted from 5-day neonatal or adult tissues using RNAEXL [Eurobio]. For Northern analysis, 20 μg of total RNA was denatured at 68°C for 10 min in 50% formamide, 2.2 M formaldehyde, 5 mM sodium acetate, 1 mM EDTA, 20 mM MOPS at pH 7.0 and separated in a 1% agarose, 2.2 M formal-
dehyde gel in MOPS buffer (Sambrook et al. 1989). The RNAs were transferred to Hybond N+ filters in 10x SSC. Hybridiza-
tions were performed in Church buffer and filters were washed in 0.4x SSC, 0.5% SDS at 60°C (cDNA probes) or 42°C [oligo-
nucleotide probe]. Ethidium bromide staining, 18S, or Gapdh probes were used as loading controls. Quantification of North-
erns was performed using Image master [Pharmacia].

Igf2 transcripts were detected with a rat Igf2 cDNA probe [Soares et al. 1985] and H19 and Gapdh transcripts were de-
tected with mouse [Fort et al. 1985] and rat [Poirier et al. 1991] cDNA probes, respectively. The 18S probe was an oligonucleo-
tide probe: 5’-AGACGACCGCTCGGGGGTCGCG-3’.

DNA methylation analysis
Liver genomic DNAs from heterozygous (mat het and pat het) and wild-type 5-day neonate animals were digested with EcoRI or with EcoRI and HpaII. DNA samples (20 μg) were separated on 1% agarose gels, transferred to Hybond N+, and hybridized to the BglII-BamHI 200-bp probe as described above.

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