

Identification of an imprinting control region affecting the expression of all transcripts in the *Gnas* cluster

Christine M Williamson^{1,4}, Martin D Turner^{1,4}, Simon T Ball¹, Wade T Nottingham^{1,3}, Peter Glenister¹, Martin Fray¹, Zuzanna Tymowska-Lalanne¹, Antonius Plagge², Nicola Powles-Glover¹, Gavin Kelsey², Mark Macdonochie^{1,3} & Jo Peters¹

Genomic imprinting results in allele-specific silencing according to parental origin¹. Silencing is brought about by imprinting control regions (ICRs) that are differentially marked in gametogenesis². The group of imprinted transcripts in the mouse *Gnas* cluster (*Nesp*, *Nespas*, *Gnasxl*, *Exon 1A* and *Gnas*) provides a model for analyzing the mechanisms of imprint regulation. We previously identified an ICR that specifically regulates the tissue-specific imprinted expression of the *Gnas* gene³. Here we identify a second ICR at the *Gnas* cluster. We show that a paternally derived targeted deletion of the germline differentially methylated region (DMR) associated with the antisense *Nespas* transcript unexpectedly affects both the expression of all transcripts in the cluster and methylation of two DMRs. Our results establish that the *Nespas* DMR is the principal ICR at the *Gnas* cluster and functions bidirectionally as a switch for modulating expression of the antagonistically acting genes *Gnasxl* and *Gnas*. Uniquely, the *Nespas* DMR acts on the downstream ICR at exon 1A to regulate tissue-specific imprinting of the *Gnas* gene.

The *Gnas* cluster is well conserved between human and mouse⁴. It consists of maternally, paternally and biallelically expressed transcripts arising from at least four promoters, such that transcription initiates from alternative first exons and the transcripts splice onto exon 2 of the *Gnas* transcript^{5–7} (Fig. 1a). *Nesp* is maternally expressed⁵ and encodes a neuroendocrine secretory protein⁸. Both *Gnasxl* (ref. 5), encoding the protein XLαs (ref. 9), and *Exon 1A* (ref. 7), corresponding to a noncoding transcript, are paternally expressed. The *Gnas* gene, which encodes the stimulatory G-protein subunit G_sα, is mainly expressed biallelically but shows predominant maternal-specific expression in some tissues¹⁰. *Nespas*, a paternally expressed antisense transcript that is noncoding¹¹, starts about 2 kb upstream of the putative *Gnasxl* initiation site¹² and overlaps the *Nesp* protein coding exon¹³. Three DMRs have been identified in the *Gnas* cluster: first, a paternally methylated region spanning the *Nesp* promoter^{5,6}; second, an extensive germline maternally methylated region at the *Gnasxl* and

Nespas promoters¹⁴; and third, a germline maternally methylated region at the *Exon 1A* promoter⁷. The *Exon 1A* DMR is an ICR that is specifically required for imprinting of the *Gnas* gene^{3,15}. Several ICRs have been associated with the promoters of antisense transcripts^{16,17}. Thus, the DMR that covers the *Nespas* promoter (the *Nespas* DMR) is a candidate ICR for other transcripts in the cluster, and the prominent DNase I-hypersensitive sites associated with the start of *Nespas* are also indicative of regulatory function¹⁴.

To test for an ICR, we deleted 1.6 kb of genomic sequence covering the *Nespas* promoter, its first exon and 631 bp of intronic sequence, leaving the *Gnasxl* minimum promoter region intact (Fig. 1a,b and Supplementary Fig. 1 online). We generated male chimeras that transmitted the deletion, designated ΔNAS-DMR, through the germ line. The resulting mice with the deletion did not feed properly, became thin and lethargic, and died within 2 d of birth. By contrast, when ΔNAS-DMR was propagated through a female germ line by transfer of ovaries from female ΔNAS-DMR carriers to wild-type females, heterozygous ΔNAS-DMR progeny were viable, healthy and fertile. On subsequent paternal transmission, heterozygous progeny were generated at the expected frequency at birth (52.4% of 84 newborns) and died by postnatal day 2.

To understand the molecular changes associated with loss of the *Nespas* DMR, we analyzed the effect of paternal and maternal inheritance of ΔNAS-DMR on expression of transcripts in the *Gnas* cluster. As expected, expression of *Nespas* was not detected when ΔNAS-DMR was paternally inherited (Fig. 1c,d) because the deleted region has promoter activity for *Nespas* (Supplementary Fig. 1). If the *Nespas* DMR is required for silencing *Nesp*, then paternal inheritance of the deletion should give rise to biallelic expression of *Nesp*. Using RNA blot analysis, we showed that *Nesp* transcripts were increased in embryos with the paternally inherited deletion (Fig. 1e). Using mice heterozygous for a *Bst*UI site in exon 11 of the *Gnas* gene^{3,18} to distinguish maternal from paternal RT-PCR products, we showed that the increase in *Nesp* expression was from the usually silent paternal allele (Fig. 1f). On maternal inheritance of ΔNAS-DMR, neither *Nespas* nor *Nesp* expression was altered (Supplementary Fig. 2 online). Thus, deletion

¹MRC Mammalian Genetics Unit, Harwell, Oxfordshire OX11 0RD, UK. ²The Babraham Institute, Cambridge CB2 4AT, UK. ³Present addresses: Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK (W.T.N.); School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK (M.M.). ⁴These authors contributed equally to this work. Correspondence should be addressed to J.P. (j.peters@har.mrc.ac.uk)

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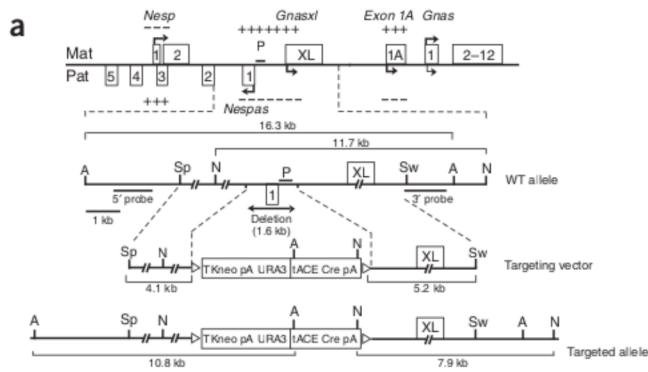


Figure 1 Gene targeting of the *Nespas* DMR region and its effect on transcription and methylation of *Nesp*. (a) Overview of the *Gnas* cluster. For *Gnas*, thick and thin arrows indicate tissue-specific predominant maternal expression and limited paternal expression, respectively. Differential methylation of the DMRs is indicated (—, unmethylated; +++, methylated). P indicates *Nespas* promoter (**Supplementary Fig. 1**). Triangles indicate *loxP* sites. A, *AvrII*; Sp, *Spel*; Sw, *Swal*; N, *Ndel*. (b) Homologous recombination (T) verified by *AvrII* and *Ndel* digestion and hybridization with 5' and 3' probes, respectively. (c) RT-PCR analysis of *Nespas* in neonatal brain after paternal transmission of Δ NAS-DMR (+^{Mat}/KO^{Pat}). Presence (+) and absence (−) of reverse transcriptase is indicated. +^{Mat}/+^{Mat}, MatDp(dist2) mice (maternally uniparental disomic with respect to distal chromosome 2); *Hprt*, amplification control. (d) RNA blot analysis of *Nespas* in poly(A)⁺ RNA (5 μ g) from embryos at 15.5 d post coitum (d.p.c.). Size markers were a 0.24–9.5-kb RNA ladder (Invitrogen). +^{Pat}/+^{Pat}, MatDp(dist2) mice (paternally uniparental disomic with respect to distal chromosome 2). (e) RNA blot analysis (top) and bar chart (bottom) showing expression of *Nesp* in poly(A)⁺ RNA (2.5 μ g) from 15.5-d.p.c. embryos. Means \pm s.e.m. values were calculated for four wild-type (+/+) and four +^{Mat}/KO^{Pat} mice (* t = 4.04; two-tailed P = 6.8×10^{-3}). (f) RT-PCR analysis of brain from neonates carrying a maternal allele of *M. spretus* origin (+^S) and a paternal allele of *M. musculus* origin (+^M or KOM). Digestion with *Bst*UI gives products of 151 bp for transcripts derived from the *M. spretus* allele and 178 bp for transcripts derived from either *M. musculus* allele³ (**Supplementary Fig. 2**). (g) Promoter methylation at the *Nesp* DMR is lost in +^{Mat}/KO^{Pat} mice. Genomic DNA from 15.5-d.p.c. embryos was digested with *Eco*RI (−), *Eco*RI and *Hpa*II (H), or *Eco*RI and *Msp*I (M).

of the unmethylated copy of Δ NAS-DMR on the paternal allele results in derepression in *cis* of *Nesp*, which is normally silent.

We next examined methylation of the *Nesp* promoter. This promoter region is methylated on the paternal allele after fertilization^{7,14}. Loss of silencing of *Nesp* after paternal transmission of Δ NAS-DMR was associated with loss of methylation of the *Nesp*

promoter; the hypomethylation of the *Nesp* promoter was similar to that in mice that were maternally uniparental disomic with respect to distal chromosome 2 (MatDp(dist2)) (**Fig. 1g**). This indicated that in paternal deletion (+/ΔNAS-DMR) carriers, the paternal allele had acquired a maternal epigenotype at *Nesp*. Our findings are consistent with studies in biparental complete hydatidiform moles¹⁹ and in

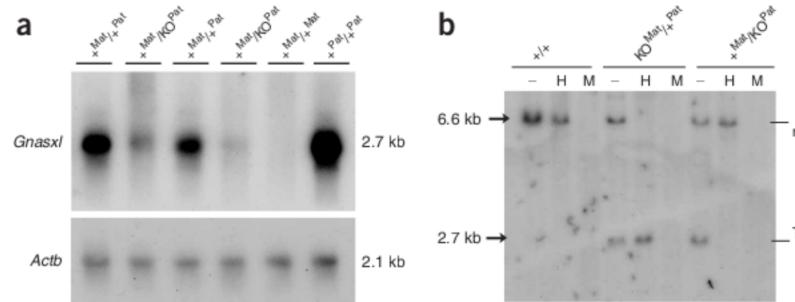
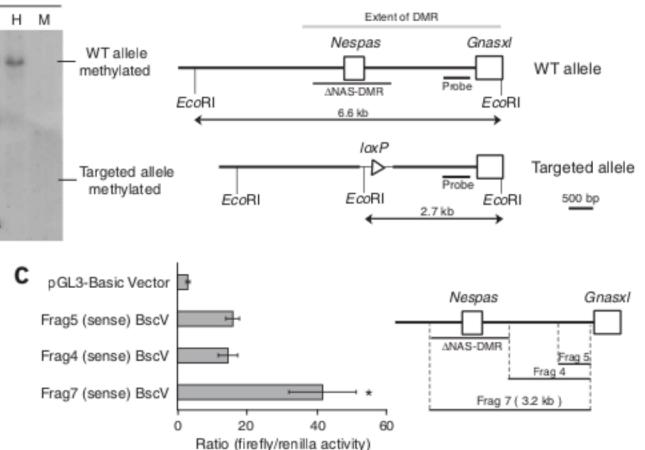


Figure 2 *Gnasxl* expression is reduced and *Gnasxl* promoter methylation is unaltered in mice carrying Δ NAS-DMR (KO). (a) RNA blot analysis of *Gnasxl* in poly(A)⁺ RNA (2.5 μ g) from 15.5-d.p.c. embryos. (b) Differential methylation at the *Gnasxl* minimal promoter region. Left, genomic DNA from neonatal brain was digested with *Eco*RI (−), *Eco*RI and *Hpa*II (H), or *Eco*RI and *Msp*I (M). After maternal and paternal transmission of Δ NAS-DMR, the minimal promoter region of *Gnasxl* on the targeted allele remains methylated and unmethylated, respectively. Right, production of the 6.6-kb and 2.7-kb *Eco*RI fragments in the wild-type and targeted alleles. (c) Location of a *Gnasxl* promoter element in the NAS-DMR by a luciferase reporter assay. Left, mean \pm s.e.m. activities. High activity was detected from fragment 7, which encompasses the NAS-DMR, as compared with fragments 5 (n = 12, t = 3.10, d.f. = 50, one-tailed P = 1.6×10^{-3}) and 4 (n = 6, t = 1.94, d.f. = 50, one-tailed P = 2.9×10^{-2}). Right, position of fragments 4 (nucleotides 152176–153710), 5 (nucleotides 153138–153710) and 7 (nucleotides 150541–153710).



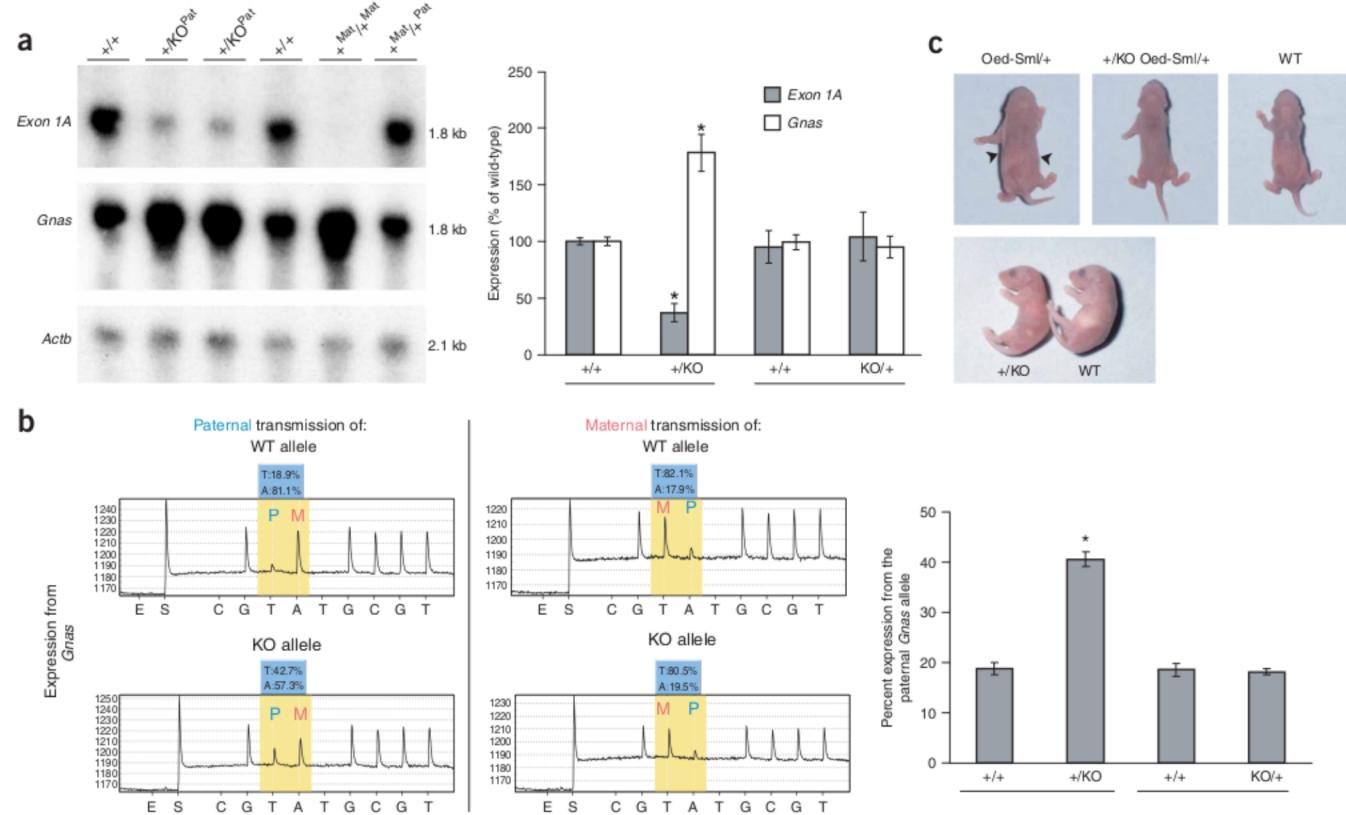


Figure 3 Expression of *Exon 1A* and the *Gnas* gene after paternal and maternal transmission of ΔNAS-DMR (KO). **(a)** RNA blot analysis (left) and bar chart (right) of total RNA in neonatal brown fat. For *Exon 1A*, mean \pm s.e.m. values were calculated for five $+\text{Mat}/KO_{\text{Pat}}$ and six wild-type ($+/+$) sibs, and three $KO_{\text{Mat}}/\text{Pat}$ and three $+/+$ sibs (* $t = 6.90$, two-tailed $P = 7.1 \times 10^{-4}$). For *Gnas*, mean \pm s.e.m. values were calculated for seven $+\text{Mat}/KO_{\text{Pat}}$ and eight $+/+$ sibs, and five $KO_{\text{Mat}}/\text{Pat}$ and four $+/+$ sibs (* $t = 7.40$, two-tailed $P = 5.2 \times 10^{-6}$). **(b)** Left, allele expression analysis of *Gnas* showing that paternal expression is increased in newborn brown fat when ΔNAS-DMR is paternally transmitted ($+/KO$), but is not affected when ΔNAS-DMR is maternally transmitted ($KO/+$). The relative contribution to *Gnas* expression from paternal (P) and maternal (M) alleles is indicated. Right, mean \pm s.e.m. proportion of *Gnas* expression from the paternal allele for five Oed-Sml/+ $+/KO$, three Oed-Sml/+ $+/+$, three $+/Oed-Sml KO/+$, and eight $+/Oed-Sml +/+$ mice (* $P < 1 \times 10^{-6}$ versus each of the other three groups). *Gnas* expression did not differ significantly in the other three groups; thus, there is no significant difference in *Gnas* transcript expressed from Oed-Sml and wild-type alleles. **(c)** Rescue of edematous phenotype of Oed-Sml/+ mice. The maternal genotype is listed first. Edema (arrows) in Oed-Sml/+ mice is absent in compound heterozygous $+/KO$ Oed-Sml/+ and wild-type (WT) mice. The compound heterozygous $+/KO$ Oed-Sml/+ mice do not feed and have a thin body similar to $+/KO$ mice.

individuals with sporadic pseudohypoparathyroidism type Ib (PHP-Ib)²⁰, where loss of maternal-specific methylation at the *NESPAS* and *XLαs* promoters is associated with gain of methylation at *NESP55* on the maternal allele and abnormal silencing.

Because the *Nespas* DMR extends across the *Gnasxl* promoter, we examined the effect of paternal inheritance of ΔNAS-DMR on *Gnasxl* expression and methylation. Expression of *Gnasxl* was diminished (Fig. 2a), but the minimal promoter region remained hypomethylated on the paternal allele (Fig. 2b). Hypomethylation was confirmed by combined bisulfite and restriction analysis and limited sequencing of cloned PCR products (data not shown). Luciferase reporter constructs were generated to test for promoter activity in the *Nespas-Gnasxl* region (Supplementary Fig. 1) and indicated that, although the deletion does not remove the minimal promoter of *Gnasxl*, it does remove a regulatory element required for its expression (Fig. 2c). Thus, the repression of *Gnasxl* when ΔNAS-DMR is paternally derived could be due to the loss of this element. Indeed, the loss of expression of *Gnasxl* probably accounts for the deletion phenotype, because the phenotype is similar to that reported for a null mutant²¹. On maternal inheritance

of ΔNAS-DMR, the maternal *Gnasxl* allele remained repressed and methylated (Supplementary Fig. 2).

We tested whether the deletion had additional effects on regulation of the cluster. We examined the expression of *Exon 1A* and *Gnas* in brown fat, a tissue in which *Exon 1A* is paternally expressed and *Gnas* is predominantly maternally expressed^{3,7,10}. Unexpectedly, on paternal inheritance of ΔNAS-DMR, expression of *Exon 1A* was diminished, and expression of *Gnas* was higher than in wild type (Fig. 3a) owing to increased expression from the paternal allele (Fig. 3b). Expression of *Gnas* was increased from the paternal allele, as shown by quantitative sequencing of RT-PCR products from newborns that had a paternal copy of ΔNAS-DMR and were also heterozygous for a single nucleotide polymorphism in the *Gnas* gene²² (Fig. 3b). These were offspring from female mice heterozygous for the Oed-Sml mutation ($+/Oed-Sml$ females)²³ (see Methods) crossed to male carriers of the *Nespas* deletion. Thus, paternal deletion of the *Nespas* DMR in brown fat leads to decreased expression of *Exon 1A* and increased expression of *Gnas* in *cis* on the paternal allele.

When the nonfunctional Oed-Sml mutation is maternally derived, the mice are grossly edematous at birth^{3,23}, but when we combined it

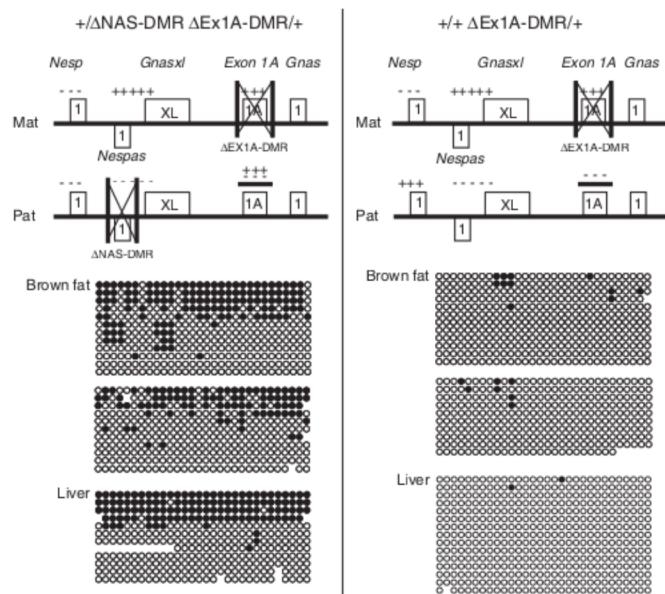


Figure 4 The *Exon 1A* DMR is partially methylated on the paternal allele when ΔNAS-DMR is paternally inherited. Wild-type (+/+) and +/ΔNAS-DMR offspring, with a maternally derived *Exon 1A* DMR deletion (ΔEx1A-DMR/+), were used to enable the methylation status of the paternal *Exon 1A* DMR to be analyzed in the absence of the maternal *Exon 1A* DMR. PCR products (black line) were sequenced after bisulfite modification of DNA from brown fat and liver of compound heterozygous +/ΔNAS-DMR ΔEx1A-DMR/+ and +/- ΔEx1A-DMR/+ newborn mice. Features of the *Gnas* cluster in the two genotypes are shown. The deleted regions, ΔNAS-DMR and ΔEx1A-DMR³, are indicated. The first exon of each sense and antisense transcript are shown above and below the line, respectively. The methylation status of the DMRs is indicated (—, unmethylated; +++, methylated). Each row of circles represents a clone and each circle corresponds to a separate CpG (filled circles, methylated CpGs; open circles, nonmethylated CpGs). Each block of circles represents the data from an individual mouse.

with paternally inherited ΔNAS-DMR, the ΔNAS-DMR allele completely rescued the edema in 26 out of 32 doubly heterozygous mice (Fig. 3c and Table 1). Of the remaining six, two showed partial edema and four showed full edema. Thus, in most double heterozygotes, derepression of the paternal *Gnas* transcript must reach a functionally significant level, at least in tissues relevant to the edema phenotype. On maternal inheritance of ΔNAS-DMR, expression of *Exon 1A* and *Gnas* did not differ significantly from that in wild-type littermates (Fig. 3a), and the maternal *Exon 1A* allele remained repressed (Supplementary Fig. 2).

The *Exon 1A* promoter is located in a germline DMR that is normally unmethylated on the paternal allele⁷. Studies of individuals with PHP-Ib have shown that the methylation status of the *Exon 1A* DMR reflects the imprinting status of GNAS²⁴. We therefore examined methylation of *Exon 1A* and found that the higher expression of *Gnas* in brown fat on paternal inheritance of ΔNAS-DMR was reflected by an increase in methylation at the *Exon 1A* DMR (Fig. 4). In liver, a tissue in which the *Gnas* gene does not show imprinted expression^{10,15},

there was also increased methylation at the *Exon 1A* DMR (Fig. 4), but a modest effect, if any, on *Gnas* expression in six +/ΔNAS-DMR mice (data not shown). As compared with brown fat, *Exon 1A* was very poorly expressed and difficult to detect reliably in liver. It probably has little effect on *Gnas* expression in liver. Increased methylation of the paternal allele was also detected in neonatal brain (data not shown).

Thus, deletion of the paternal *Nespas* germline DMR has bidirectional effects. Loss of the DMR derepresses *Nesp* and *Gnas*, and partially represses *Gnasxl* and *Exon 1A*. It leads to loss of methylation of the *Nesp* DMR and to partial methylation of the *Exon 1A* DMR, but intriguingly it does not affect methylation of the *Gnasxl* promoter (Fig. 5). The derepression of *Nesp* could be due to loss of the antisense transcript *Nespas*, which may function in a similar manner to *Air*²⁵. Increased methylation of *Exon 1A* is associated with increased expression of *Gnas* from the paternal allele in brown fat and no marked increase in liver, but it is unclear how *Exon 1A* regulates the expression of *Gnas*. The methylated DNA at the *Exon 1A* DMR could fail to bind either tissue-specific repressor proteins, thereby facilitating increased *Gnas* transcription, or proteins with insulator function, thereby enabling upstream tissue-specific enhancers to access the *Gnas* promoter^{3,4}. It is possible that the transcript level of *Exon 1A* could be important in modulating that of *Gnas*.

We have shown that the *Nespas* DMR is an ICR and shares similar features with other ICRs². It is the principal ICR for the whole *Gnas* cluster. We previously showed that the *Exon 1A* DMR has the

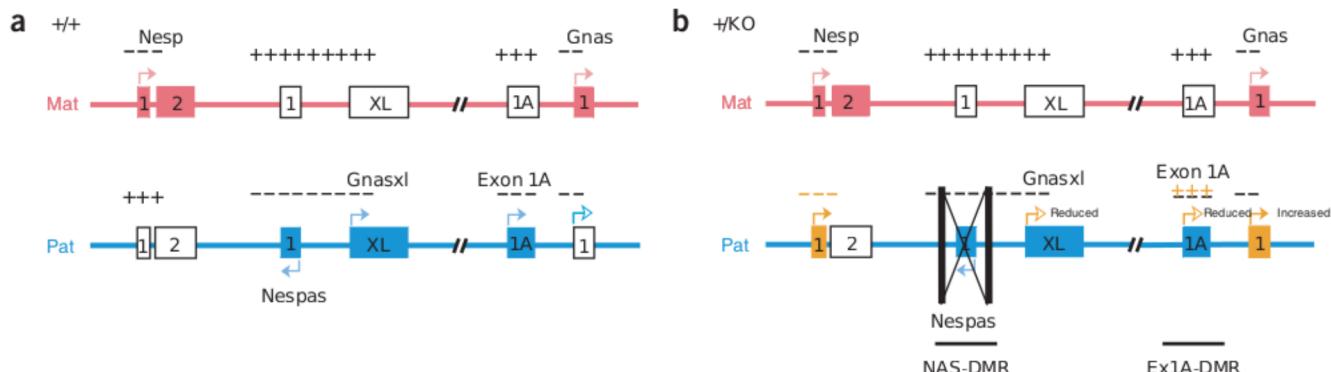


Figure 5 Summary of the transcriptional and methylation status of the *Gnas* cluster. (a) Wild-type. (b) Status after paternal transmission of ΔNAS-DMR. Deletion of the *Nespas* DMR caused, first, loss of *Nespas* transcription, loss of imprinting of *Nesp* and loss of methylation at the *Nesp* promoter; second, a reduction in *Gnasxl* transcripts even though the allele remained unmethylated; and third, a reduction in *Exon 1A* transcripts associated with the allele gaining methylation and thus upregulation of the *Gnas* transcript in brown fat. Changes as a consequence of the deletion are shown in yellow. Paternal transmission of the *Exon 1A* DMR (Ex1A-DMR) deletion caused loss of imprinting of *Gnas* in brown fat but has no effect on *Nesp*, *Gnasxl* and *Nespas*³. The figure is not to scale.

Table 1 Rescue of edematous phenotype of Oed-Sml/+ mice at birth by a nonimprinted Gnas allele

Edema ^a	Genotype			
	Oed-Sml/+	+/ΔNAS-DMR	+/ΔNAS-DMR	Wild type
None	0	50	26	38
Slight	0	0	2	0
Full	20	0	4	0

^aThe number of offspring of each genotype is given. The underrepresentation of the Oed-Sml/+ genotype is consistent with previously detected prenatal loss^{3,23}.

characteristics of an ICR but is unusual in regulating the imprinting of a single closely linked gene, *Gnas*³. All other known imprinting clusters contain a single germline DMR, and the *Gnas* cluster is exceptional in having two. The *Exon 1A* ICR is necessary for controlling imprinted expression of the *Gnas* gene³: on the paternal allele, it is unmethylated and represses *Gnas* expression tissue specifically. Because ΔNAS-DMR leads to increased methylation of the paternally derived *Exon 1A* DMR, however, we can conclude that a normal function of the *Nespas* DMR on the paternal allele is to protect, at least in part, the paternal *Exon 1A* DMR from methylation, thereby facilitating tissue-specific paternal repression of *Gnas*. Thus, two germline DMRs affect transcription of the *Gnas* gene on the paternal allele: the *Nespas* DMR regulates methylation at the *Exon 1A* DMR, which in turn affects *Gnas* tissue-specifically. On the maternal allele, two other *cis*-acting elements, identified from deletions associated with PHP-Ib, are required for methylation of the *Exon 1A* DMR and thus for GNAS expression: the *NESP55* DMR and an element in the neighboring gene *STX16* (refs. 26, 27).

Our results show the *Nespas* DMR acts as a switch to enhance *Gnasxl* expression and to repress *Gnas* expression on the paternal allele. This mechanism seems to be exceptional for an imprinted locus. *Gnasxl*, encoding XLαs, and *Gnas*, encoding Gsα, exert major but opposite effects on postnatal development in the mouse^{10,21}. According to the parental-conflict theory²⁸, it is in the interest of paternal genes in offspring to acquire resources from the mother, whereas it is in the interest of maternal genes in offspring to be sparing in the demand of such resources. As *Gnasxl* is involved in acquisition of maternal resources in the form of milk, it is to the paternal advantage to ensure that *Gnasxl* is expressed²¹. Given the finding that XLαs and Gsα act antagonistically²¹, it is in the paternal interest to enhance *Gnasxl*, but to repress *Gnas*. The *Nespas* DMR facilitates such control of expression on the paternal allele.

METHODS

Construction of the targeting vector. The targeting construct was designed to delete a 1.6-kb region (nucleotides 150599–152175) encompassing the *Nespas* promoter, its first exon and 631 bp of intronic sequence (Fig. 1a). The construct was generated by using the yeast recombinogenic arms system as described^{3,21}. The targeting cassette included a *tACE-Cre* gene for Cre recombinase-mediated excision of the cassette in the male germ line in mice. The backbone of the construct was a 10.9-kb mouse genomic *Spel-Swal* fragment (nucleotides 146517–157430), encompassing *Nespas* exon 1 and the extra large exon of *Gnasxl*, cloned in pRS414 (ref. 21). The 5' and 3' recombinogenic arms that flank the 1.6-kb deletion were amplified by PCR and the primer sequences are available on request. The targeting vector was linearized with *Xba*I and targeting was done in male embryo stem (ES) cells from mouse strain 129/SvEv as described³. Colonies surviving G418 selection were screened by DNA blot analysis of *Ndel*-digested genomic DNA with a 1.4-kb fragment (nucleotides 157431–158817) that lies immediately 3' to the region of vector homology.

Correct targeting at the 5' end was confirmed by probing *Avr*II digests with a 1.2-kb *Scal* fragment (nucleotides 144525–145690; Fig. 1a,b). Karyotype analysis revealed no obvious chromosomal changes in the targeted ES cells. Chimeras were generated by injecting two independently targeted ES clones into C57BL/6 blastocysts.

Breeding studies. To establish breeding lines of ΔNAS-DMR mice, we carried out neonatal ovarian transfers²⁹. To propagate the mutation through the female germline, ovaries were transferred from newborn female +/ΔNAS-DMR donors to 6-week-old recipient 129/SvEv ovariectomized females. The recipients were bred and their offspring were genotyped for ΔNAS-DMR by PCR analysis of DNA from tail tips (genotyping primer sequences are available from the authors on request). Cre recombinase-mediated excision of the cassette was confirmed by PCR and sequencing.

For allelic expression analysis of *Nesp*, *Gnasxl* and *Exon 1A*, offspring of reciprocal crosses between ΔNAS-DMR/+ and SD2 mice³ were produced. SD2 is a stock containing the distal portion of chromosome 2 from *Mus spretus* in a *Mus musculus* background³. For allelic expression analysis of the *Gnas* gene, we generated reciprocal crosses between +/Oed-Sml²³ and ΔNAS-DMR/+ mice. Oed-Sml is a nonfunctional mutation (V159E) in *Gnas* exon 6, resulting in the nucleotide substitution A for T at position 199402 (ref. 22). Offspring were genotyped for the Oed-Sml mutation as described³ and for ΔNAS-DMR as described above. Four classes of offspring were used for allelic expression analysis: Oed-Sml/+ with paternal ΔNAS-DMR; Oed-Sml/+ sibs wild-type for paternal ΔNAS-DMR; Oed-Sml/+ with maternal ΔNAS-DMR and Oed-Sml/+ sibs wild-type for maternal ΔNAS-DMR. For other expression analyses, MatDp(dist2) and PatDp(dist2) mice and embryos were generated and genotyped as described¹¹.

Compound heterozygous +/ΔNAS-DMR ΔEx1A-DMR/+ mice and +/+ ΔEx1A-DMR/+ mice were generated for bisulfite treatment and subsequent methylation analysis by crossing homozygous ΔEx1A-DMR/ΔEx1A-DMR females to heterozygous ΔNAS-DMR/+ males. Offspring were genotyped for ΔEx1A-DMR³ and ΔNAS-DMR.

All mouse studies were done under the guidance issued by the Medical Research Council in 'Responsibility in the Use of Animals for Medical Research' (July 1993) and under the authority of Home Office Project Licence Numbers 30/1518, 30/2065 and 30/1704.

RNA analysis. Total RNA was extracted by the RNA-Bee method (AMS Biotechnology) and poly(A)⁺ RNA was extracted with FastTrack (Invitrogen). Reverse transcription was done with MMLV reverse transcriptase (Invitrogen) as described¹². Expression of the transcripts *Nesp*, *Gnasxl* and *Exon 1A* was analyzed by PCR using transcript-specific forward primers for *Nesp*, *Gnasxl* and *Exon 1A* with a common reverse primer in exon 12 of the *Gnas* gene¹² as described³. *Nespas* was analyzed by RT-PCR using a forward primer specific for exon 1 (BIAR) and a reverse primer specific for exon 3 (NL3) as described¹³.

RNA blots of total RNA (2.5 μg per lane) and poly(A)⁺ RNA were prepared with a NorthernMax-Gly kit (Ambion). Membranes were hybridized with radioactively labeled riboprobes that were made by *in vitro* transcription using a Strip-EZ RNA labeling kit (Ambion) and α-³²P-labeled UTP (Perkin Elmer). Riboprobes to detect *Nespas*, *Nesp*, *Gnas* and *Actb* transcripts have been described^{3,5,11}. *Nespas* was detected as a smear as reported¹¹. Riboprobes for detection of *Gnasxl* and *Exon 1A* mRNA were nucleotides 155917–156264 and 183915–184090, respectively. The *Gapdh* riboprobe was generated from pTRI-GAPDH-Mouse (Ambion). Hybridization was done in ULTRAhyb (Ambion), and blots were washed with Low and High Stringency Wash Buffers (Ambion). Transcript levels were measured by phosphorimager analysis using Molecular Analyst Software (BioRad). The RNA levels were quantified by a regression-based approach in which standard curves were constructed from wild-type total RNA. Transcript levels for *Nesp*, *Exon 1A* and *Gnas* were measured in terms of *Actb* or *Gapdh* and are shown relative to wild type (normalized to 100%).

Methylation analysis. Methylation of the *Nesp* and *Gnasxl* promoter regions was analyzed by digesting genomic DNA (10 μg per track) with *Eco*RI in combination with *Hpa*II and *Msp*I, and probing with radiolabeled PCR products (nucleotides 139675–140530 for *Nesp* and 153138–153710 for

Gnasl). Filters used for DNA blot were incubated with Megaprime-labeled DNA probes in QuikHyb hybridization solution (Stratagene) at 68 °C.

For bisulfite sequence analysis, purified genomic DNA (500 ng) from brown fat and liver was treated and amplified as described¹⁴, except that a nested PCR strategy was not used. The region of the *Exon 1A* DMR analyzed corresponds to nucleotides 183866–184147, and the regions of the *Gnasl* DMR analyzed correspond to PCR products 'e' and 'f' in ref. 15; primer sequences are available from the authors on request. Before cloning, PCR products were tested for full conversion and methylation status by pilot digestion with appropriate restriction enzymes, as well as for any bias in cloning (there was none for the *Exon 1A* DMR). Sequences were analyzed with the BiQ Analyser program³⁰.

Quantification of RT-PCR products by pyrosequencing. Total RNA (0.5 µg) was reverse-transcribed and the PCR product obtained after 30 cycles was analyzed by pyrosequencing on a PSQ8HS96 pyrosequencer (Biotage). Peak heights were determined by the pyrosequencing software. Primer information and conditions are available from the authors on request.

From quantitative sequencing, the relative contribution to *Gnas* expression from the paternal and maternal alleles in compound heterozygous Oed-Sml/+/ΔNAS-DMR mice was 40:60, whereas this ratio was closer to 50:50 from RNA blot analysis of +/ΔNAS-DMR mice. Different genetic backgrounds and/or different methodologies in measuring *Gnas* expression may account for this slight difference.

Luciferase reporter assay. Reporter constructs were generated and the assay was done as described³. The nucleotide positions of the fragments tested are given in the legends of Fig. 2 and Supplementary Fig. 1.

Statistical testing. Expression levels measured on RNA blots and results from the luciferase reporter assays were compared by Student's *t*-test using a pooled sample variance. Analyses were done on the logarithms of the observations to render the sample variances satisfactorily homogeneous. To prevent bias when a sample was run more than once in the same experiment, the mean of the readings was used in the analysis with a statistical weight of *n* (the number of repeat measurements); single measurements with no repeats were given a statistical weight of 1. Pyrosequencing data were analyzed by variance-ratio tests after an analysis of variance on the logarithmically transformed observations.

GenBank accession number. Nucleotide numbers refer to sequence accession number AL593857.10 (the reverse complement of AL593857 cited previously³).

Note: Supplementary information is available on the *Nature Genetics* website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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