Uncoupling Antisense-Mediated Silencing and DNA Methylation in the Imprinted Gnas Cluster

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Abstract

There is increasing evidence that non-coding macroRNAs are major elements for silencing imprinted genes, but their mechanism of action is poorly understood. Within the imprinted Gnas cluster on mouse chromosome 2, Nespas is a paternally expressed macroRNA that arises from an imprinting control region and runs antisense to Nesp, a paternally repressed protein coding transcript. Here we report a knock-in mouse allele that behaves as a Nespa hypomorph. The hypomorph mediates down-regulation of Nesp in cis through chromatin modification at the Nesp promoter but in the absence of somatic DNA methylation. Notably there is reduced demethylation of H3K4me3, sufficient for down-regulation of Nesp, but insufficient for DNA methylation; in addition, there is deletion of the H3K36me3 mark permissive for DNA methylation. We propose an order of events for the regulation of a somatic imprint on the wild-type allele whereby Nespas modulates demethylation of H3K4me3 resulting in repression of Nesp followed by DNA methylation. This study demonstrates that a non-coding antisense transcript or its transcription is associated with silencing an overlapping protein-coding gene by a mechanism independent of DNA methylation. These results have broad implications for understanding the hierarchy of events in epigenetic silencing by macroRNAs.

Introduction

Over recent years it has emerged that most of the mammalian transcriptome is non-coding [1]. Several long non-coding transcripts have been implicated in epigenetic gene regulation and play essential, but incompletely understood, roles in epigenetic gene silencing in X-inactivation and genomic imprinting in mammals. For the latter, more than 100 imprinted genes are known in the mouse and most occur in clusters [2]. Parental specific gene silencing throughout the clusters is brought about by imprinting control regions (ICRs). These are regions that are differentially methylated in gametogenesis and are active when unmethylated. ICRs for three clusters, the Igf2r cluster, the Kcnq1 cluster and the Gnas cluster, contain promoters for macroRNAs that are exclusively expressed from the paternally derived chromosome and run antisense to a protein coding gene that is repressed by the active unmethylated ICR [for reviews, see [3,4]]. Two of these macroRNA genes, Aim in the Igf2r cluster and Kcnq1ot1 in the Kcnq1 cluster, have been shown to be key elements in parental specific silencing of all protein coding genes in their respective clusters [5-7], although their mode of action is incompletely understood. However both are known to be associated with the acquisition of repressive histone marks and DNA methylation marks of some genes. It is not yet known if the third gene for a macroRNA, Nespa in the Gnas cluster has a functional role (Figure 1). It is likely that several other imprinted gene clusters may share this regulatory principle, but their ICRs have not been defined functionally.

The ICR for the Gnas cluster contains the Nespa promoter [8] and lies within an extensive differentially methylated region, the Nespas–Gnasxl DMR, that acquires methylation in the maternal germine [9]. This DMR also contains the promoter for a protein coding paternally expressed transcript Gnasxl. The Gnas cluster, unusually, has a second maternally methylated germline DMR, the Exon1A DMR [10] that specifically controls maternal expression of transcripts arising from the Gnas promoter [11,12]. The ICR regulates the Exon1A DMR that in turn regulates the imprinted expression of Gnas. There is a third DMR in the Gnas cluster and this is a somatic DMR that becomes methylated on the paternal allele post-fertilisation [9,10]. This DMR covers the furthest upstream promoter in the cluster, the Nesp promoter. Nesp is maternally expressed, protein coding and is transcribed for about 80 kb through the whole cluster including the Nespas–Gnasxl DMR and the second germline DMR at Exon1A. Recently it was shown that truncation of this long Nesp transcript upstream of the two germline DMRs disrupted the acquisition of methylation at both DMRs in the oocyte [13]. Thus transcription of the Nesp protein coding transcript in the female germline is required for the

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Author Summary

Genomic imprinting is a process resulting in expression of genes according to parental origin. Some imprinted genes are expressed when paternally derived and others when maternally derived. Thus imprinted genes are monoallelically expressed and one copy has to be silenced. There is evidence that some long non-coding RNAs, acting in cis, have a role in silencing. We investigated the role of Nespas, a gene for a non-coding RNA that is only expressed from the paternally derived chromosome in the Gnas cluster and runs antisense to its sense counterpart, Nes. Expression of Nespas is associated with silencing of Nes and a repressive methylation mark on the Nes DNA. We generated a Nes mutant with reduced levels of activity and showed that it down-regulated its sense counterpart Nes, in the absence of a DNA methylation mark, but in the presence of an altered chromatin mark. We conclude that Nespas can repress Nes by a mechanism independent of DNA methylation, by modulating a chromatin mark.

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Results

Gene targeting generates a Nespas hypomorph (Nesp\textsuperscript{−/−})

In studies to analyse the regulatory function of Nespas, we made use of a knock-in allele that behaved as a Nes hypomorph. We show here that when Nespas is present at a low level in the hypomorph the Nes promoter is unmethylated but Nes expression is considerably down-regulated and the Nes promoter is only partially enriched for an activating histone mark, methylated H3K4. Thus, in the hypomorph, Nespas can mediate the down-regulation of Nes through chromatin modification even in the absence of DNA methylation at Nes. An insufficiency of methylated H3K4 could be a major factor in repression of Nes expression. Furthermore, the results suggest that Nespas has a role in the demethylation of H3K4me3 as a prerequisite for DNA methylation of the Nes promoter on the paternal chromosome. The results provide the first evidence that Nespas has a functional role in regulating imprinted gene expression in the Gnas cluster.

Establishment of maternal methylation across the whole Nespas-Gnasxl DMR including the ICR as well as the Exon1A DMR.

The Nes transcript is the sense counterpart of the paternally expressed antisense Nes. The macroRNA Nes that arises from a promoter within the ICR transcribes through, and is associated with methylation of the Nes DMR [8], but the mechanisms for induction of the post-fertilisation methylation of the Nes DMR on the paternal allele or, for that matter, any somatic DMR, have not been established. De novo establishment of methylation at maternal and some paternal germline DMRs requires the DNA methyltransferase DNMT3A and its non-enzymatic cofactor DNMT3L [14-16]. The process of methylation is mediated by histone modifications; it appears that DNMT3A is recruited to the DNA by complexing with DNMT3L [17-19] which interacts with histone H3 but only when lysine 4 is unmethylated [17]. Interaction of DNMT3A with chromatin is also inhibited by H3K4 methylation [20] but promoted by H3K36 trimethylation [21]. Whether de novo DNA methylation of somatic DMRs depends upon DNMT3A, or the second de novo enzyme DNMT3B, and co-operation with DNMT3L has not been established.

In order to investigate a role for Nespas, we made use of a knock-in allele that behaved as a Nes hypomorph. We show here that when Nespas is present at a low level in the hypomorph the Nes promoter is unmethylated but Nes expression is considerably down-regulated and the Nes promoter is only partially enriched for an activating histone mark, methylated H3K4. Thus, in the hypomorph, Nespas can mediate the down-regulation of Nes through chromatin modification even in the absence of DNA methylation at Nes. An insufficiency of methylated H3K4 could be a major factor in repression of Nes expression. Furthermore, the results suggest that Nespas has a role in the demethylation of H3K4me3 as a prerequisite for DNA methylation of the Nes promoter on the paternal chromosome. The results provide the first evidence that Nespas has a functional role in regulating imprinted gene expression in the Gnas cluster.

Figure 1. Overview of the mouse Gnas locus showing the organisation of the protein-coding transcripts Nes, Gnasxl, and Gnas and the paternally expressed non-coding transcripts Nespas and Exon1A. Transcripts expressed from the maternally inherited allele are shown above the line, those from the paternally inherited allele are below the line and the direction of transcription is shown by arrow heads. Unspliced transcripts are not shown. The first exons of the protein-coding transcripts and the hashed filled boxes are the first exons of the non-coding transcripts. The first exons of Nes, Gnasxl, Exon1A and Gnas all splice into exon 2 of Gnas. A subset of tissues, Gnas itself is tissue-specifically imprinted; the dotted line shows the limited expression of Gnas from the paternal allele. The position of the differentially methylated regions (DMRs) are shown by rows of filled circles on the methylated allele; ICR, shows the position of the Imprinting Control Region [8]. Adapted from Peters and Williamson [49]. doi:10.1371/journal.pgen.1001347.g001
reported elsewhere). The hypomorph resulted from insertion of a polyadenylation cassette from the rabbit β-globin gene, into exon 1 of Nespas, in the reverse orientation (pA) so that truncation of Nespas should not occur (Figure 2A and 2B). Although it was not anticipated that this insertion would influence expression of Nespas, the mutant was shown to be a hypomorph in three ways. Firstly, we used RT-PCR and primers (Figure 2C) that would amplify Nespas but not Nesp. Weak expression of a Nespas splice variant was found in brain from newborn mice with a paternally derived Nesp-Tmun2 allele (+/+Nesp-Tmun2), note that the maternal allele precedes the paternal allele in all genotypes shown here; Figure 2D). Mice carrying a paternal copy of the null Nespas allele, ΔNAS-DMR, in which the Nespas promoter and first exon are deleted [8], were included as a negative control (Figure 2D). Secondly, the hypomorph was verified by RNA blot analysis using a single stranded probe, shown in Figure 2C, that would detect spliced and unspliced Nespas transcripts. The Nespas transcripts, which are detected as a smear [22], were considerably reduced in 15.5 dpc embryos with the paternally inherited Nesp-Tmun2 allele when compared to the wild-type level (Figure 2E). Thirdly, we checked that the primary transcripts of Nespas were reduced in +/+Nesp-Tmun2 newborn brain using a TaqMan real-time qPCR assay. The assay was designed upstream of the Nesp promoter in intron 4 of Nespas [23] and is shown schematically in Figure 2C. We showed that Nespas levels were reduced by 94% in heterozygotes, +/+Nesp-Tmun2, that have a paternally inherited copy of the mutation when compared to wild-type levels (P = 5.91 * 10^{-11}; Figure 2F). The reduced level of Nespas was not due to gain of methylation on the paternal allele at the Nespas-Gnasxl DMR (Figure S1). Thus the position of the cassette close to the Nespas promoter may have affected promoter activity, resulting in a low level of Nespas.

**Paternal inheritance of Nesp-Tmun2 is associated with hypomethylation of the paternal Nesp somatic DMR and partial de-repression of Nesp**

Previous work had shown paternal inheritance of the null ΔNAS-DMR allele was associated with loss of methylation of the Nesp promoter [8]. Therefore we investigated whether a low level of Nespas from the paternal allele in +/+Nesp-Tmun2 was associated with a change in the methylation status of the Nesp somatic DMR. Southern analysis of newborn liver showed complete loss of methylation at the Nesp promoter and first exon on the paternal allele in +/+Nesp-Tmun2 (Figure 3A, Top Right). This result was confirmed by bisulphite analysis of brain DNA from newborn offspring arising from crosses of Nesp-Tmun2 carrier males with SD2 females carrying the Gnas cluster region from Mus spretus. The presence of single nucleotide variants in the parents enabled the distinction of maternal and paternal Nesp alleles. Two wild-type (+SD2/+) and four mutant (+SD2/Nesp-Tmun2) newborns were analysed and Figure 3A (Bottom Right) shows loss of methylation on the paternal allele of +/+Nesp-Tmun2 compared with that of a wild-type. Thus the normally methylated paternal Nesp allele was unmethylated in +/+Nesp-Tmun2, probably due to the low level of Nespas (as summarised in Figure 3A, Left).

As the paternally derived Nesp DMR was unmethylated in +/+Nesp-Tmun2, we expected that Nesp would be expressed from the mutant paternal allele. In addition, as Nesp and Nespas overlap (Figure 1), insertion of the polyadenylation cassette in exon 1 of Nespas on the antisense strand is also an insertion into intron 2 of Nesp on the sense strand and might truncate Nesp. Sequence analysis of RT-PCR products derived from using a Nesp exon 2-specific forward primer and a reverse primer specific to the polyadenylation cassette and therefore specific to the mutant paternal allele revealed a number of splice variants (Figure S2) whereby Nesp splices into the inserted β-globin sequence. Thus Nesp was expressed from the paternal allele and was likely to be truncated. The sizes of the Nesp transcripts in +/+Nesp-Tmun2 embryos were analysed by northern blotting (Figure 3B). Two major transcripts were detected: full length Nesp transcript as expected from the unaltered maternal allele and a smaller weaker band, consistent with Nesp from the targeted paternal allele, being spliced and truncated in the second intron of Nesp.

Quantification of the Nesp level in +/+Nesp-Tmun2 newborn brain by real time RT-qPCR was undertaken using a TaqMan assay in which the probe spanned the junction of exon 1 and 2 of Nesp (Table S1). This showed the level of Nesp transcript was elevated to some extent in +/+Nesp-Tmun2 but was not double the wild-type level expected for full biallelic expression (Figure 3B). The quantification was consistent with full expression from the maternal allele and diminished expression from the mutant paternal allele.

To check whether the insertion of the polyadenylation cassette had disrupted a genomic sequence necessary for full expression of Nesp, we analysed the level of Nesp when the polyadenylation cassette was inserted at the same site in Nespas exon 1 but in the opposite orientation (allele Nespas-Tmun2; Figure 4A and 4B; phenotypic analysis to be reported elsewhere). On paternal inheritance insertion of the cassette caused, as expected, truncation of Nespas (Figure 4C and Figure S3A). Furthermore there was loss of methylation at the Nesp DMR on the paternal allele in +/+Nespas-Tmun2 (Figure 4D). This was consistent with complete loss of silencing of Nesp from the paternal allele (Figure 4E). The Nesp level was increased as shown by northern blotting and confirmed to be double dose when quantified using a TaqMan assay for measuring Nesp exon 1 spliced onto exon 2. The increase in Nesp expression was from the normally silent paternal allele (Figure S3B). As full expression of Nesp was detected from the paternal allele when the genomic sequence was disrupted in +/+Nespas-Tmun2, it was unlikely that the reduced level of Nesp from the paternal allele in +/+Nesp-Tmun2 was due to the disruption of a DNA element. However, it could be due to reduced stability of the Nesp transcripts from the Nesp-Tmun2 allele and/or regulation of Nesp by Nespas.

**Expression of Nespas on the maternal allele in Nesp-Tmun2/+**

It was known that on the wild-type maternal allele, Nesp is expressed and the extensive Nespas-Gnasxl DMR that contains the ICR for the cluster is methylated. Recently the mutant allele NespTmun, which leads to truncation of the Nesp transcript at Nesp exon 2, was shown to be associated with variable, germline-derived loss of methylation of the Nespas-Gnasxl DMR on the maternal allele [13]. Thus some carriers lost methylation of the Nespas-Gnasxl DMR but others did not. It is expected that in those that have lost methylation Nespas is expressed, but in those that retain methylation Nespas is repressed. If a similar variation in the methylation of the Nespas-Gnasxl DMR and Nespas expression occurred on maternal inheritance of Nesp-Tmun2 where Nesp is truncated (Figure 5A) in intron 2, much further 3’ than in NespTmun then we could test whether maternal inheritance of the Nesp-Tmun2 allele has a similar effect to maternal transmission of NespTmun on methylation of the Nespas DMR.

Firstly, Nesp-Tmun2/+129/SvEv heterozygotes in which the wild-type paternal allele was derived from 129/SvEv were used. Variable loss of methylation at the Nespas-Gnasxl DMR region was found by Southern (Figure 5B). Some Nesp-Tmun2/+129 animals had lost methylation on the mutant maternal allele and in other animals within the same litter, the allele remained methylated. Secondly, Nesp-Tmun2/+SD2 heterozygotes, in which the paternal allele was derived from SD2 to enable the parental origin to be distinguished
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A

Mat

Pat

Nesp

Gnasxl

Exon1A

Gnas

16.3 kb

11.7 kb

12.9 kb

8.5 kb

6.2 kb

5.8 kb

pA-neo allele (in ES cells)

Nesp-Tint2 (in mouse)

5' probe

3' probe

B

x+/pA-neo allele

16.3 kb

12.9 kb

11.7 kb

8.5 kb

C

Nesp

Mat

Pat

(primers)

(Neuro probe)

(Taqman probe)

Nespas

D

x+/pA-neo allele

x+/Nesp-Tint2

x+/+/Nesp-Tint2

x+/Nesp-Tint2

Nespas

Hprt

E

Nespas

Actb

F

Nespas expression

+/+(n=6)

+/Nesp-Tint2

(n=6)

*
using single nucleotide variants, were used. Southern analysis of twelve Nesp-Tint2/+ SD2 newborns from two litters showed no loss of methylation in five (42%; designated Nesp-Tint2/+ (methylated)) and loss of methylation in seven (58%). Further investigation of the latter class by bisulphite analysis was carried out. Bisulphite sequence profiles of the seven newborns with loss of methylation confirmed the Nesp promoter region was completely unmethylated on the mutant maternal allele in six (designated Nesp-Tint2/+ (unmethylated)), and partially methylated in one (data not shown).

Thus variable loss of methylation of the Nespas-DMR occurs on maternal inheritance of Nesp-Tint2 just as it does with Nespas.

We predicted that Nespas would be silent in the Nesp-Tint2/+ (methylated) mice, but expressed in the Nesp-Tint2/+ (unmethylated) mice. To check that the detectable level of Nespas was low from the mutant Nesp-Tint2 allele on maternal inheritance as it is on paternal inheritance, we used RT-PCR and double heterozygotes, Nesp-Tint2/+ NAS-DMR (Figure 5C) that had a maternal copy of Nesp-Tint2 and a paternal copy of the Nesp promoter deletion allele, NAS-DMR [9]. As Nespas is not expressed from the NAS-DMR allele (Figure 5D), any expression of Nespas in the double heterozygotes must be from the mutant maternal allele, Nesp-Tint2. As expected, a low level of Nespas was detected in three double heterozygotes, within one litter (Figure 5D), showing that Nespas is expressed from the maternal allele and that the Nesp-Tint2 allele is a hypomorph on maternal inheritance as well as on paternal inheritance. Bisulphite sequence analysis of one of the three double heterozygotes confirmed the active Nespas allele was unmethylated (Figure S4). Nespas expression was not detected in a fourth double heterozygote (Figure 5D) and bisulphite sequencing showed the inactive allele was methylated (Figure S4).

Maternal inheritance of Nesp-Tint2 is associated with down-regulation of Nesp

The finding that maternal inheritance of Nesp-Tint2 resulted in two classes of offspring, one in which Nespas was repressed and one in which Nespas was expressed, enabled us to test whether Nespas expression was associated with down-regulation of Nesp. If the level of Nesp was lower in the Nespas expressing class than in the class in which Nespas was repressed this would provide evidence that Nespas regulates Nesp expression. Both classes carried identical Nesp-Tint2 alleles on the maternal derived chromosome so any effects on the levels of Nesp transcript, such as stability, due to the sequence of the mutant allele should be the same in both classes. Using the TaqMan RT-qPCR assay that measures Nesp exon 1 spliced onto exon 2, a significant difference in Nesp level was detected between four Nesp-Tint2+/SD2 (methylated) and four Nesp-Tint2+/SD2 (unmethylated) littersmates (Figure 5E; \( P = 5.36 \times 10^{-4} \)). The Nesp level was three-fold lower when the Nespas DMR was unmethylated and Nespas is transcribed compared to when the DMR was methylated. Similar results were obtained on a 129/SvEv background between Nesp-Tint2+/129 (methylated) and Nesp-Tint2+/a120 (unmethylated) mice (data not shown).

The reduced level of Nesp in Nesp-Tint2+/SD2 (unmethylated) was not due to a small gain of methylation at the Nesp DMR on the maternal allele (Figure S5). Thus we have shown that expression of Nespas, even at a low level, was sufficient to down-regulate Nesp in the absence of DNA methylation, (summarised in Figure 5E) and provide the first evidence for Nespas-mediated silencing of Nesp.

Altered histone modifications at the Nesp DMR associated with Nespas transcription in Nesp knock-in mutants

Our interpretation from both maternal and paternal inheritance of the hypomorph Nesp-Tint2 is that Nespas transcript/transcription is associated with down-regulation of Nesp expression in the absence of methylation of the Nesp DMR. We next tested whether there were histone modifications at Nesp that would account for its down-regulation on the paternal allele in Nesp-Tint2. We also tested +/-Nesp-Tint1 in which Nesp is fully expressed on the paternal allele, and wild-type in which Nesp is silent. As Nesp and Nespas are expressed in mouse embryonic fibroblast cells (MEFs), we used chromatin prepared from MEFs of wild-type, +/-Nesp-Tint2 and +/-Nesp-Tint1, on a SD2 background, and analysed histone modifications, H3K4me3, H3K4me3, H3K27me3 and H3K36me3 at three regions (designated 1–3; Figure 6A). Region 1 was within a fragment previously shown to have promoter activity (data not shown), region 2 spanned the first intron and part of exon 2, and region 3 was just downstream of Nesp exon 2 in intron 2. Regions 1–3 were chosen as they had been shown to be associated with the activating mark, H3K4me3, and the repressive mark, H3K9me3 on the maternal and paternal allele of Nesp, respectively, in skin fibroblasts (designated Allleic ChIP sites 2, 3 and 4, respectively; [24]). Similarly, H3K4me3 and the repressive mark H3K27me3 were found associated with the 5’ end of Nesp in MEFs but allelic specificity was not analysed [25]. H3K36me3 was included as a marker for transcriptional elongation [26]. Consistent with the reports above, in wild-type MEFs there was depletion of the active mark H3K4me3 on the paternal allele in comparison to the maternal allele at all three regions analysed and enrichment of the repressive mark H3K9me3 at regions 1 and 3 (Figure 6B).

Surprisingly, there was also depletion of H3K27me3 on the paternal allele relative to the maternal allele in wild-type MEFs. These results suggest that H3K9me3 constitutes the repressive mark at the Nesp promoter and DMR in these cells. H3K36me3 showed no parental-allele enrichment, which might
Figure 3. Nesp is expressed and truncated from the paternal allele in +/Nesp-Tint2. (A) Loss of methylation at the Nesp DMR in +/Nesp-Tint2. (A Left) Schematic summary of the transcriptional status of Nespas and the methylation status of Nespas and Nesp on the paternal allele of wild-type and +/Nesp-Tint2. Dashed line indicates low level of transcript. Row of filled circles, methylated allele; row of open circles, unmethylated allele. (A Top Right) Southern analysis showing promoter methylation at the Nesp DMR is lost in +/Nesp-Tint2. Genomic DNA from newborn liver was digested with EcoRI (-), EcoRI and HpaII (H), or EcoRI and MspI (M). (A Bottom Right) Bisulphite profile of the paternal allele of the Nesp DMR in newborn brain from +SD2/+ and +SD2/Nesp-Tint2. Sequence variants allowed the maternal and paternal alleles to be discriminated; see Table S1 for primers. Each row of circles represented a clone derived from the paternal allele and each circle corresponded to a separate CpG (filled circles, methylated CpGs; open circles, unmethylated CpGs). Each block of circles represented the data from an individual mouse. (B) Low level of truncated Nesp from the paternal allele in +/Nesp-Tint2. (B Top Left) RNA blot analysis showing expression of Nesp in poly(A)+ RNA from 15.5 dpc embryos using the single stranded RNA probe shown in Figure 2C. The full length transcript was expressed from the maternal allele. (B Bottom Left) The smaller truncated transcript was expressed from the targeted paternal allele as summarised in the schematic. (B Right) Bar chart showing the relative level of Nesp expression in newborn brain by RT-qPCR, measured using a TaqMan assay that detects exon 1 spliced onto 2. The reference gene was Gapdh. Error bars (RQmin/RQmax) were based on a 95% confidence level.
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Figure 4. Nesp is fully expressed from the paternal allele in +/Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}. (A) The Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} allele was generated by targeting and is identical to Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} except that the polyadenylation cassette was inserted in the opposite orientation (Ap) to truncate Nespa. The selection genes were deleted from the Ap-neo targeted allele upon germline transmission by Cre-recombinase mediated excision to generate Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}. (B) Southern analysis of ES cell DNA from wild-type (+/+4) and targeted (Ap-neo) cells. The Ap-neo targeted clones were identified by the presence of an 8.5 kb Ndel fragment detected with the 3’ external probe. Correct targeting at the 5’ end was confirmed by the detection of a 12.9 kb Avrl fragment with the 5’ external probe. (C) RT-PCR assay in newborn brain showing Nespa is truncated. A primer pair, 3’ of the insertion, detected Nespa whereas a primer pair 5’ of the insertion did not detect Nespa. Symbols + and −, refer to reactions carried out in the presence and absence of reverse transcriptase, respectively. As the Nespa PCR products were weak, it was necessary to blot and probe the Nespa RT-PCR products with appropriate genomic probes. Hprt was included as an amplification control. (D) Southern analysis showing promoter methylation at the Nespa DMR is lost in +/Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}. Genomic DNA from newborn brain was digested with EcoRI (E), EcoRI and HpaII (H) or EcoRI and MspI (M). (E) Biallelic expression of Nespa in +/Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}. (E Top Left) RNA blot analysis showing expression of Nespa in poly(A) RNA from 15.5 dpc embryos. (E Bottom Left) Schematic summary of the transcriptional and methylation status of Nespa and Nespa on the paternal allele of +/Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}. (E Right) Bar chart showing the relative level of Nespa expression in newborn brain by RT-qPCR, measured using a TaqMan assay that detects exon 1 spliced onto 2. The reference gene was Gapdh. Error bars (RQmin/RQmax) were based on a 95% confidence level.

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be consistent with the region being transcribed on both strands, corresponding to Nespa and Nespa, in wild-type MEFs.

The most striking finding in the mutants +/Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} and +/Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} was that the depletion of H3K4me3 on the paternal allele seen in the wild-type MEFs was eliminated (Figure 6B). Furthermore, in the +/+Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} MEFs there was an even greater enrichment for H3K4me3 on the paternal allele at region 1 within the promoter region of Nespa. Thus truncation of Nespa and full expression of Nespa from the paternal allele in the Nespa truncation mutant +/Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} was associated with a high level of H3K4me3 whereas low levels of Nespa and Nespa in the hypomorph +/Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} were associated with a lower level of H3K4me3. Our results suggest that Nespa transcript or transcription mediates the level of enrichment of the activating mark H3K4me3 in the absence of DNA methylation. Furthermore, an insufficiency of H3K4me3 is likely to be a major factor in depressing Nespa expression in the hypomorph.

There were also significant differences between wild-type, +/Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} and +/Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} in the amount of H3K36me3 at region 1, and these correlate with Nespa transcription. Thus, in contrast to the equal allelic enrichment in wild-type MEFs, there was depletion of H3K36me3 on the paternal allele in +/Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} where there is a reduced level of Nespa, and further depletion of H3K36me3 in +/Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} where Nespa is truncated and is not transcribed across the Nespa exons and promoter. The reduction in enrichment of H3K36me3 on the paternal allele is further evidence that transcription downstream of the inverted pA cassette is reduced on the Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} allele.

Differences in the allelic enrichment of the repressive marks H3K9me3 and H3K27me3 were also detected, but were less notable. For H3K9me3 it appeared that the relative enrichment on the paternal allele was reduced in MEFs from both mutants, but this reached significance only for region 3 in +/+Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}, and this accords with full expression of Nespa in +/+Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}. For H3K27me3, the depletion on the paternal allele observed in wild-type MEFs also tended to have been eroded, and this effect was significant at regions 2 and 3. Altered allelic enrichment of H3K27me3 was also detected in newborn brain in +/+Nesp-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} (data not shown). These results suggest that H3K27me3 is not normally a repressive mark on the wild-type paternal allele and that the increased amount in the mutants could be due to absence of DNA methylation, as previously observed at the imprinted Rugg1 locus [27].

Effects on other transcripts in the Gnas cluster

Paternal inheritance of Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} resulted in weak expression; both of Nespa and truncated Nespa so we next investigated whether there were additional effects on the other transcripts and DMRs in the Gnas cluster.

On paternal inheritance of Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}, levels of the normally paternally expressed Gnas transcript were reduced (Figure S6), but the reduction in expression was not due to gain of methylation at the Gnas promoter on the paternal allele (Figure S1). As the cassette was inserted close to the Gnas promoter, it is likely that the position of the insertion had affected the promoter activity of Gnas in some way.

On maternal inheritance of Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} where Nespa was truncated, there was invariable loss of methylation at the Exon1A DMR both when the Nespa DMR was methylated and when it was unmethylated (Figure S7). This result is similar to findings on maternal inheritance of Nespa\textsuperscript{+}\textsuperscript{\textsuperscript{-}} in which the Nespa transcript is truncated at its second exon [13], much further 5’ than in Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}.

As found with paternal inheritance of Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}, paternal inheritance of Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}} also led to reduced levels of Gnas transcript (Figure S6) in the absence of methylation of the Gnas promoter (Figure S8), and was also attributable to the position of the inserted polyadenylation cassette.

In the male allele in Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}/+ mice, full length Nespa remained expressed and Gnas and Exon1A remained repressed (Figure S9). In keeping with this, the Nespa-Gnas and Exon1A DMRs remained methylated on the paternal allele (Figure S10) thus showing that the altered methylation at the two germline DMRs in Nespa-T\textsuperscript{+}\textsuperscript{\textsuperscript{-}}/+ was not due to disruption of a DNA element by insertion of the cassette.

Discussion

Here we provide evidence that Nespa, a gene for a non-coding macroRNA has a role in imprinted gene silencing in the Gnas cluster. Furthermore we have shown that the Nespa transcript or its transcription has a role in setting the histone modifications permissive for DNA methylation of the DMR [17,20,21]. The functional evidence for these findings came from studies of Nespa mutants.

Nespa mediates imprinted gene silencing

Our finding that a low level of maternally expressed Nespa was sufficient to reduce Nespa levels is the first direct evidence for Nespa-mediated silencing of Nespa. This observation is consistent with partial de-repression of paternal Nespa in the Nespa hypomorph and complete loss of silencing of parental Nespa, when paternal Nespa is either truncated or not expressed at all as in the ICR deletion ([8]; summarised in Figure 7).

From the current study, Nespa can be added to the small number of antisense non-coding genes that have been shown to have a functional role in gene silencing. These include Tair, a negative regulator of the non-coding RNA, Xist (for review, see [28]) that is required for X-inactivation [29] as well as two
Figure 5. Maternal transmission of Nesp-Tint2 results in downregulation of Nesp when the Nespas DMR is unmethylated. (A) The maternal Nesp transcript is truncated in Nesp-Tint2/+; RNA blot analysis showing expression of Nesp in poly(A)+ RNA from 15.5 dpc embryos using the single stranded RNA probe shown in Figure 2C. (B) Southern analysis showing the Nespas-Gnasxl DMR promoter can be unmethylated or methylated on the maternal allele in Nesp-Tint2/+; Genomic DNA from newborn brain was digested with EcoRI (-), EcoRI and HpaII (H), or EcoRI and MspI (M) and
paterally expressed non-coding RNAs, *Airn* and *Kcnq1ot1* that are required for imprinted expression in *cis*. *Nespas* does have some similarities to both *Airn* and *Kcnq1ot1* in that it is necessary for imprinted expression, it is transcribed from a promoter contained within the unmethylated ICR on the paternal allele and has an antisense orientation with respect to the coding gene. *Tsix* appears

**Figure 6.** Paternal-specific histone modifications at *Nesp* in MEFs from wild-type, +/Nesp-1int2, and +/Nespas-1ext1. (A) Schematic showing the relative position of regions 1–3, amplified in the ChIP experiments at the 5’end of Nesp. (B) Bar chart showing the ratio of the bound paternal versus bound maternal band intensity for each chromatin modification. Each sample was normalised with the input, mock bound and mock unbound intensities. The “n” number on each bar chart represents the number of MEF lines analysed.

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**P<0.01  * P<0.05**
to silence a single regulatory RNA whereas Airn and Kcnq1ot1 silence multiple protein-coding genes in cis. There is evidence to suggest that Airn and Kcnq1ot1 accumulate at some non-overlapping genes, in placenta, to mediate repressive histone modifications such as H3K9me3 and/or H3K27me3 in a manner similar to the silencing properties of Xist [30]. We have shown that Nespas regulates its sense counterpart Nesp but we do not yet have definitive evidence to show whether or not Nespas regulates other genes in the Gnas cluster.

Relationships among the levels of an antisense Nespas, its sense counterpart Nesp, and epigenetic marks

An inverse relationship exists on the paternal allele between the level of the non-coding RNA Nespas and the level of enrichment of the activating mark H3K4me3 associated with Nesp expression (summarised in Figure 7). As the level of the activating mark was different between the two insertion mutants +/-Nesp-Tm2 and +/-Nespas-Tm1, it was unlikely that the site of insertion of the cassette had disrupted a genomic sequence element controlling H3K4me3. We therefore conclude that the level of expression of the antisense Nespas modulates the level of H3K4me3 at the Nesp promoter, thereby modulating expression of Nesp. Our findings have parallels with the observation that silencing the tumour suppressor gene p15 is modulated by its antisense RNA and expression of the antisense is also associated with a decrease in methylated H3K4 at the p15 promoter [31].

We also identified a striking association between presence of H3K4me3 at the Nesp promoter in both mutants +/-Nesp-Tm2 and +/-Nespas-Tm1, and the absence of somatic DNA methylation. This was consistent with in vitro evidence that H3K4 methylation stops the acquisition of DNA methylation by preventing DNMT3L and DNMT3A from interacting with histone H3 [17,20]. Similar associations between chromatin and DNA methylation have been found at germline DMRs in somatic cells: chromatin on the DNA methylated alleles was devoid of H3K4 methylation whereas chromatin on the parental alleles without DNA methylation had high levels of H3K4 methylation [32,33]. We also noted a direct correlation between Nespas levels (or transcription) and the enrichment of H3K36me3 on the paternal allele. This alteration may also be relevant, since it has been shown recently that the PWWP domain of DNMT3A specifically recognises H3K36 trimethylation [21]. We presume the level of Nespas transcript or transcription was not high enough in the hypomorph to bring about and/or maintain DNA methylation at the Nesp promoter. This is consistent with the finding in an ES cell model where low levels of Airn transcription were associated with a lack of DNA methylation on the paternal Igf2r promoter [34].

Our chromatin analysis showed that demethylation of H3K4 rather than DNA methylation is a prerequisite for silencing Nesp on the paternal allele in the embryo. We propose the DNA methylation of Nesp that occurs post fertilisation is required to stabilise silencing long term. Silencing in the absence of DNA methylation

Figure 7. Summary of the transcriptional, methylation, and chromatin status of the paternal allele from Nesp to Gnasxl in wild-type and mutant mice. (A) wild-type; (B) +/-Nesp-Tm2 and (C) +/-Nespas-Tm1. The H3K4me3 mark, designated mK4 (in red) at the Nesp promoter is shown; bold text and circle, full enrichment of H3K4me3; normal text and dotted circle, partial enrichment of H3K4me3. Full length transcription of Nesp is shown in (C). The pA insertion truncates Nesp and the Ap insertion truncates Nespas. A low level of Nespas is sufficient to downregulate truncated Nesp levels in the absence of DNA methylation and this is associated with a reduction in the level of H3K4me3 at the Nesp promoter. The figure is not to scale.
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methylation has previously been observed in placenta where imprinted genes in the *Kcnq1* cluster on distal chromosome 7 were silenced by repressive histone methylation [35]. The repressive histone marks were not completely effective at silencing the genes and it was suggested that this did not matter as the placenta is a short-lived organ. Evidence has been reported that DNA methylation at two somatic DMRs within the cluster is maintained by interaction of the non-coding *Kcnq1ot1* RNA with Dnmt1 [56].

Model for how the level of an activating mark is controlled at the Nesp promoter

Prior to the de novo methylation of the Nesp DMR post fertilisation [10], we predict the Nesp promoter is enriched for H3K4 methylation. Consistent with this idea, H3K4me3 is present at the 5’ end of Nesp in mouse ES cells [25] and it has recently been shown that most zebrafish genes become marked by H3K4me3 when the genome is activated at the maternal to zygotic transition following fertilisation [37]. We propose that Nespas is required to remove the H3K4me3 mark at Nesp by recruiting histone demethylase(s) to the Nesp promoter region either by a transcript or transcription mediated mechanism. The demethylase(s) are needed to demethylate H3K4 to allow the somatic DMR to be methylated. A histone H3K4 demethylase (KDM1B) has been shown to be required for the establishment of some methylation imprints during oogenesis [38] but it is not known if a non-coding RNA is involved. KDM1B-deficient oocytes showed an increase in H3K4 methylation and failed to acquire DNA methylation marks at several ICs. The recruitment of histone modifying enzymes by non-coding RNAs could be a common mechanism. There is evidence that in the placenta, *Atr* recruits G9A (KMT1C), a histone H3K9-methyltransferase to silence *Slc22a3* [39]. In addition, Nesp transactivation may cause deposition of H3K36me3 across the Nesp promoter region such that, in combination, the two permissive histone marks provide a potent signal for the de novo methylation complex.

Alternatively the reduction in the activating mark H3K4me3 may be the consequence of the down-regulation of Nesp by Nespas by either a transcript or a transcription based mechanism. Given the complementarity between Nesp and Nespas RNAs, Nesp levels could be reduced by an RNA interference mechanism as reported for silencing *Xist* by *Tss* [40]. Alternatively a transcription based model can be envisaged [41]. Thus a cis-acting element may lie within the Nespas transcription unit to silence Nesp completely. In the hypomorph the element will only be partially active so that transcription of Nesp can occur.

A second truncation allele of Nesp; truncation of Nesp in the second intron causes loss of germline methylation

As the transcription units of Nesp and Nespas overlap, the insertion of a polyadenylation cassette to generate the *Nesp-Tint2* allele not only created a Nesp hypomorph on the antisense strand but also created a truncated allele of Nesp, in intron 2, on the sense strand. A truncated allele Nesp*Tint2* had previously been generated at Nesp exon 2 [13]. In *Nesp-Tint2*/*+, as previously observed with Nesp*loc2*/*+, we also found loss of methylation at both the *Nespas-Gnasxl* and *Exon1A* DMRs. In addition we analysed a control, Nespas*Tint2*/*+, whereby the cassette was inserted at the same site but in the opposite orientation. On the maternal allele, Nesp was fully expressed and the two DMRs remained methylated thus showing that the position of the insertion had not disrupted a DNA element that might be required for directing germline methylation. Thus the results from the second truncation allele are consistent with the proposal by Chotalia et al. [13] that transcription of Nesp across the DMRs in oocytes is required for the establishment of germline methylation marks within the *Gnas* cluster. Interestingly, with both truncation alleles, loss of methylation was detected at the *Nespas-Gnasxl* DMR in some mice but not others whereas the *Exon1A* DMR was invariably unmethylated. This occurred even though, in *Nesp-Tint2*/*+, the truncated product of Nesp was longer than in Nesp*loc2*/*+, and truncated at the start of the *Nespas-Gnasxl* DMR, much closer to the *Nespas-Gnasxl* DMR than in Nesp*loc2*. The variation in methylation at the *Nespas-Gnasxl* DMR may be due, at least in part, to variation in termination of Nesp transcription in the oocyte. Thus when the *Nespas-Gnasxl* DMR in *Nesp-Tint2*/*+ is unmethylated, transcription termination must occur close to the polyadenylation site, but when the DMR is methylated there may be ineffective transcription termination. In support of this, full ablation of Nesp transcription by deletion of the *Nesp* DMR on the maternal allele caused almost complete loss of maternal methylation imprints [42].

Both maternal and paternal inheritance of Nesp*Tint2* can result in similar epigenetic and transcriptional outcomes at Nesp and Nespas. Thus with paternal Nesp*Tint2* and also with maternal Nesp*Tint2* (in some animals) both the Nesp and Nespas-Gnasxl DMRs are unmethylated and Nesp and Nespas are weakly expressed, so that parental identity defining the imprinting of the wild-type allele has been lost. Although both maternal and paternal Nesp*Tint2* can lead to similar epigenetic and transcriptional consequences the initiating events on the maternal and paternal alleles are probably different. On the maternal allele the primary event is likely to be the failure of Nesp transcription to induce methylation of the *Nespas-Gnasxl* DMR whereas on the paternal allele the primary event may be the failure of Nesp to methylate and fully suppress Nesp.

Materials and Methods

Targeting to generate Nesp*Tint2* allele

The Nesp*Tint2* targeting construct was designed to insert a polyadenylation cassette from the rabbit β-globin gene [5] into exon 1 of Nespas, in an orientation (designated pA) that would be expected to truncate Nesp (Figure 2A; between nucleotides 151519 and 151520, AL593857.10). The construct was generated by homologous recombination in yeast [43]. Briefly, a 1.2 kb fragment (nucleotides 31392-32553; M18818) from the rabbit β-globin gene, containing part of exon 2, complete intron 2 and exon 3 harbouring the polyadenylation signal was cloned into a XhoI site, 5’ of the loxp site flanking the selection cassette, in pRAY-Cre (AJ627603). The 5’ and 3’ recombinogenic arms (385 bp and 489 bp, respectively), extending upstream and downstream of the site of insertion of the polyadenylation cassette, were amplified by PCR; the 5’ arm was cloned 5’ of the selection cassette, in pRAY-Cre (AJ627603). The 5’ and 3’ recombinogenic arms (385 bp and 489 bp, respectively), extending upstream and downstream of the site of insertion of the polyadenylation cassette, were amplified by PCR; the 5’ arm was cloned 5’ of the polyadenylation cassette and the 3’ arm was cloned downstream of the 3’ loxp site. All primer sequences are available on request. A 10.9 kb mouse genomic *Spel*-SacI fragment, cloned in the yeast–E. coli shuttle vector pRS414 [8,44] was cotransformed into yeast YPH501 with a linear fragment comprising the recombinogenic arms, polyadenylation cassette and selection cassette using the yeast transformation kit (Sigma). The recombined shuttle vector was recovered from yeast colonies and used as the targeting vector (Figure 2A). The targeting vector was linearised with XhoI and electroporated into CJ7 mouse ES cells. Colonies surviving G418 selection were screened for correct targeting (pA-neo allele) by Southern analysis (Figure 2B). Genomic DNA from the clones was digested with *Nde*I and a 3’ external probe (nucleotides 157431-158817, AL593857.10) detected an 11.7 kb fragment in wild-type cells
and an 8.5 kb fragment in correctly targeted cells. Correct targeting at the 5' end was confirmed by probing AsIIdigested DNA with a 5' external probe (nucleotides 14425-145690, AI593857.10; Figure 2B). The probe detected a 16.3 kb fragment in wild-type cells and a 12.9 kb fragment in correctly targeted cells. Two independently targeted clones, with no obvious chromosomal changes checked by karyotype analysis (E.P. Evans, personal communication), were injected into C57BL/6J blastocysts. Excision of the selection cassette occurred in the germline of male chimeras by testes-specific expression of Cre recombinase [45]. Proper excision of the cassette was confirmed by PCR amplification across the remaining loxP site (data not shown).

Targeting to generate Nespas-+/+ allele

The Nespas-+/+ construct was designed to insert the polyadenylation cassette, as described above, in the same site but in the reverse orientation to truncate Nespas. The targeting vector was made as described above except that the polyadenylation cassette was cloned in the opposite orientation in pRAY-Cre (Ap: Figure 4A). Southern blot analysis was performed, as described above, to identify correctly targeted cells (Ap-neo allele; Figure 4B). Two independently targeted clones were injected into C57BL/6J blastocysts.

Mouse breeding

Mice carrying the Nesps-+/+ and Nespas-+/+ alleles were maintained on a 129/SvEv background. As +/+ Nespas-+/+ mice were postnatal lethals, a breeding line had to be established by performing neonatal ovarian transfers as reported previously [8]. Offspring were genotyped for the Nesps-+/+ and Nespas-+/+ alleles by PCR analysis of DNA from tail tips using a forward primer specific for the loxP region (5'-AGTACCCCGGTTCGAAATC-3') and a reverse primer specific to the arm (5'-CGAAATGGGC-GAAACGGTTTG-3'). For some experiments, offspring of reciprocal crosses between Nesps-+/+ or Nespas-+/+ and SD2 mice were produced. SD2 is a stock containing the distal portion of chromosome 2 from Mus musculus background [11]. Compound heterozygous Nesps-+/+ x Nespas-+/+ mice were generated by crossing Nesps-+/+ and Nespas-+/+ males and females to generate Nesps-+/+ and Nespas-+/+ mice. SD2 mice were produced. SD2 is a stock containing the distal portion of chromosome 2 from Mus musculus background [11]. Compound heterozygous Nesps-+/+ x Nespas-+/+ mice were generated by crossing Nesps-+/+ and Nespas-+/+ males and females to generate Nesps-+/+ and Nespas-+/+ mice [8]. 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 1995) and under the authority of Home Office Project Licence Numbers 30/1518, 30/2526 and 30/1704.

Northern and RT-PCR analysis

Total RNA for RT-PCR was extracted from newborn brain using RNA-Be (AMS Biotechnology) and DNA contamination was removed by treating the RNA with DNAsel (Message Clean kit; BioGene Ltd). RT-PCR was performed by reverse transcribing RNA with M-MLV reverse transcriptase (Invitrogen) and oligo(dT)15 primer (Promega). A spliced form of Nespas was analysed using primers F6 and R2 as described previously [23]. Poly(A)+ RNA for blot analysis was extracted using a FastTrack kit (Invitrogen). Northern blots were performed as described previously [8].

Reverse Transcription-Quantitative Real-Time PCR (RT-qPCR)

Frozen tissues were homogenised using a 230 V Ultra-Turrax T25 basic homogeniser. Total RNA, extracted using the Allprep kit (Qiagen), was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). To avoid amplification of contaminating genomic DNA, samples were treated with RNase-free DNase (Qiagen). The FAM dye-labelled TaqMan MGB probe and unlabelled primer sets (Table S1) for Nesps exon 1/exon2 (assay ID nesp0-N0), Nespas intron 4 (assay ID A183XP8) and Gapdh (assay ID Mm99999915_g) were purchased from Applied Biosystems and all amplified with equal efficiency, at 99%, as determined from the slope of calibration curves [46]. The qPCR was performed with TaqMan Fast Universal PCR Master Mix (Applied Biosystems) using the 7500 Fast Real-Time PCR machine. The concentrations of the oligos at 1 × concentration were 900 nM for each primer and 200 nM for the probe. The expression levels of Nespas and Nesps normalised to the reference gene Gapdh, were determined using the comparative threshold cycle method as described previously [47].

Methylation analysis

Methylation-sensitive Southern blot analysis of the Nespas-Gnasxl and Nesps promoter region was performed by digesting genomic DNA with EcoRI in combination with HpaII and MspI [8]. For bisulphite sequence analysis, purified genomic DNA from newborn brain was treated and amplified as described previously [9]. The DNA was treated using the EpiTect Bisulfite Kit (Qiagen). The primer sequences used to amplify each region are available in Table S1.

Chromatin Immunoprecipitation (ChIP) and PCR-SSCP

MEFs, derived from 13.5 dpc embryos from crosses of SD2 homozygous females with Nesps-+/+ and Nespas-+/+ carrier males, were used for ChIP analysis as described previously [48]. Briefly, cells were collected and washed with PBS, the nuclei were purified using a sucrose cushion and incubated with MNase in order to obtain fragments of 1 to 3 nucleosomes in length. Approximately 20 μg of chromatin was incubated with 5 μg of antibody overnight at 4°C. The DNA from the ChIP was amplified by PCR and the parental alleles were distinguished by restriction digest and acrylamide gel electrophoresis. For each amplified region, the relative intensities of the maternal and paternal bands were measured using AIDA image analysis software (v3.27) in the ChIP input, the unbound fraction and antibody-bound fraction. We used antisera directed against trimethylated H3-Lys4 (Active Motif), trimethylated H3-Lys9 (Upstate), trimethylated H3-Lys27 (Abcam) and trimethylated H3-Lys36 (Abcam). The primer sequences for regions 1–3 are available in Table S1.

Supporting Information

Figure S1 The Nespas-Gnasxl region is unmethylated on the targeted allele in +/+ Nesps-+/+. (A) Genomic DNA from neonatal brain was digested with EcoRI (EcoRI), EcoRI and HpaII (H), or EcoRI and MspI (M). (B) Schematic showing the probe and location of the 6.6 kb and 3.3 kb EcoRI fragments in the wild-type and targeted alleles, respectively. Found at: doi:10.1371/journal.pgen.1001347.s001 (0.71 MB TIF)

Figure S2 Schematic representation of the splice variants detected from the splicing of Nesps into the inserted β-globin sequence on the paternal allele in +/+ Nesps-+/+ in newborn brain. Nesps transcription starts at exon 1 and the splice variants were amplified using a Nesps exon 2-specific forward primer and a reverse primer specific to the polyadenylation cassette (arrowheads show the position of the primers). The inserted sequence contains part of exon 2, complete intron 2 and exon 3 harbouring the polyadenylation signal of the β-globin gene. Thick black line labelled AATAAA is
part of the β-globin gene. Exon a (nucleotides 6187-6282, AJ251761); exon b (nucleotides 12997, AJ251761 - 31410, M109819); exon c (nucleotides 12043, AJ251761 - 31410, M109819). Found at: doi:10.1371/journal.pgen.1001347.s002 (0.21 MB TIF)

**Figure S3** Nespas is expressed and truncated in +/Nespas-Te**, (A) Sequence of truncation product from 3′ RACE showing transcription had been terminated in Nespas exon 1 at the expected position. The product was amplified with primers BIBR and linker-poly(dT) (Table S1) and confirms Nespas is polyadenylated. The direction of Nespas transcription is shown by an arrow and the polyadenylation signal is boxed. The 5′ end of Nespas is shown in bold type and the inserted sequence as grey type. The vertical line shows where exon 2 and exon 3 of the inserted β-globin sequence splice together. (B) Schematic representation of the Gnas cluster showing a BanII variant between an allele of 129 (+129) and C57BL/6 (+EcoRI). Arrowheads show the position of the primers; sequences are available in Table S1. (C) The RT-PCR assay uses the BanII variant to distinguish the maternal and paternal transcripts of Nesp. Digestion with BanII gives products of 964 bp for transcripts derived from the C57BL/6 allele and 909 bp for transcripts derived from the 129 allele. Found at: doi:10.1371/journal.pgen.1001347.s003 (0.66 MB TIF)

**Figure S4** Bisulphite sequence profile of the maternal allele of Nasp-DMR in double heterozygotes that had a maternal copy of Nesp T**M** and were also heterozygous for a paternal copy of the Nesp promoter deletion ΔNAS-DMR. (A) Double heterozygote that expressed Nesp, (B) Double heterozygote in which Nesp was not detected. Found at: doi:10.1371/journal.pgen.1001347.s004 (0.07 MB TIF)

**Figure S5** Bisulphite sequence profile of the *Nesp* DMR on the maternal allele showing no gain of DNA methylation in *Nesp-T**W**+/+ (unmethylated). A variant between 129 SvEv and SD2 (nucleotide 140755; AL593857.10) allowed maternal and paternal alleles to be distinguished. Each row of circles represented a clone derived from the maternal allele and each circle corresponded to a separate CpG (filled circles, methylated CpGs; open circles, nonmethylated CpGs). Each block of circles represented the data from an individual mouse. Found at: doi:10.1371/journal.pgen.1001347.s005 (0.51 MB TIF)

**Figure S6** *Gnas* expression is reduced in both +/+Nesp-**T**M** and +/+Nesp-**T**W**. Northern blot analysis of Gnas at poly (A)+ RNA (50 µg) from 15.5 dpc embryos. The level of Gnas in the two mutants is substantially reduced. In +/+Nesp-**T**M**, the polyA cassette will truncate the *Nesp* transcript if transcribed from the paternal allele, and in +/+Nesp-**T**W**, the polyA cassette will truncate the *Nesp* transcript. Gnas transcript is not expressed in +M/+M, mice with maternal uniparental disomy for the distal chromosome 2 imprinting region. Found at: doi:10.1371/journal.pgen.1001347.s006 (0.90 MB TIF)

**Figure S7** Loss of methylation on the maternal allele at the *Eox1A* DMR in both Nesp-**T**M**+/+ (methylated) and (unmethylated) samples. In Nesp-**T**M**+/+ (methylated), the *Nesp* DMR is methylated on the maternal allele whereas in Nesp-**T**W**+/+ (unmethylated), the *Nesp* DMR is unmethylated on the maternal allele. Genomic DNA from newborn lung was digested with BanHI (•), BamHI, and HpaII (H), and BamHI and MspI (M). The probe was a 1.9 kb BamHI - HpaII fragment that encompasses exon 1A. Found at: doi:10.1371/journal.pgen.1001347.s007 (0.56 MB TIF)

**Figure S8** Bisulphite analysis showing the Gnas promoter region is unmethylated on the paternal allele in +/+Nesp-**T**W**. A variant between 129 SvEv and SD2 (nucleotide 153610; AL593857.10) allowed maternal and paternal alleles to be distinguished. Each row of circles represents a clone derived from the paternal allele 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. Found at: doi:10.1371/journal.pgen.1001347.s008 (0.63 MB TIF)

**Figure S9** Maternal inheritance of *Nesp-**Te*** does not alter the imprinted expression of (B) Nesp, (C) Gnasxl, (D) Exon1A. Expression was analysed by RT-PCR in neonates using brain. (A) Schematic representation of the Gnas cluster showing a BstUI variant between an allele of *M. spretus* origin (+SD2) and an allele of *M. musculus* origin (−SD2) to distinguish the maternal and paternal overlapping transcripts. Arrowheads show the position of the forward primers specific for each imprinted sense transcript and a common reverse primer in exon 12 of the Gnas gene [Williamson et al. [8]]. The primer sequences are available in Table S1. RT-PCR products, shown in (B), (C) and (D) were digested with BstUI (Bst), which cuts at sites in the common set of downstream exons 2-12. Products of the same size but arising from alternative first exons, were detected. The additional site (Bst*) in the *M. spretus* derived allele but not the *M. musculus* allele allowed the maternal and paternal products to be distinguished. Neonates arising from reciprocal crosses between SD2 mice, which have a *M. musculus* genetic background but the distal region of chromosome 2 derived from *M. spretus* (+SD2/+SD2), and *M. musculus* carriers of *Nesp-**Te*** were used. Found at: doi:10.1371/journal.pgen.1001347.s009 (0.62 MB TIF)

**Figure S10** The *Nespas-Gnasxl* DMR and the *Eox1A* DMR remain methylated when the *Nespas-**Te** is maternally inherited. (A) Differential methylation at the *Nespas-Gnasxl* promoter region. Genomic DNA was digested with EcoRI (•), EcoRI, and HpaII (H), or EcoRI and MspI (M). The *Nespas-Gnasxl* promoter region remained methylated on the targeted allele when the *Nespas-**Te** allele was maternally inherited. The probe is shown in Figure S1. (B) Methylation analysis at the *Eox1A* DMR. Wild-type (+/+), and *Nespas-**Te**+/− offspring, with a paternally derived *Eox1A* DMR deletion (+/−ΔExox1A-DMR, Williamson et al. [11]) were used to enable the methylation status of the maternal *Eox1A* DMR to be analysed in the absence of the paternal *Eox1A* DMR. Newborn brain genomic DNA was digested with BanHI (•), BanHI, and HpaII (H), or BanHI and MspI (M) and probed with a 1.9 kb BanHI - HpaII fragment that encompasses exon 1A. Found at: doi:10.1371/journal.pgen.1001347.s010 (0.57 MB TIF)

**Table S1** Primer information. Found at: doi:10.1371/journal.pgen.1001347.s011 (0.06 MB DOC)

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**Author Contributions**

Conceived and designed the experiments: CMW GK JP. Performed the experiments: CMW STB CD SM CVB. Analyzed the data: CMW STB CD SM GK JP. Contributed reagents/materials/analysis tools: MF LT TND. Wrote the paper: CMW GK JP.
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