Supporting Information

Fröhlich et al. 10.1073/pnas.0910224107

SI Methods

Construction of the Targeting Vector. The targeting pACN vector contained the diphtheria toxin cassette driven by the thymidine kinase promoter and the neomycin gene driven by the RNA polymerase II promoter for negative and positive selection, respectively, of transfected embryonic stem (ES) cells; this selection cassette was flanked by *lox*P sites (1). The Cre recombinase gene *Cre* was driven by tACE derived from the gene encoding angiotensin-converting enzyme, thus allowing self-excision of the selection cassette upon germline transmission. A 129/SvJ mouse genomic library constructed in the Lambda DASH II vector was screened using a PCR-amplified genomic mouse probe comprising Nesp55 exons 1 through 2. A single phage clone, λ N3, with an insert of approximately 16 kb was isolated. The *Sal*I insert was cloned into the *Sal*I polylinker site of the pBluescript II vector (Stratagene; pBSλN3) and a 3.2-kb *Bam*HI-*Sac*I fragment (nucleotides 74,481–77,956) was then cloned into *Bgl*II and *Bam*HI sites of the pACN targeting vector (1); this provided the 5′ recombinogenic arm of the targeting construct. The 3′ recombinogenic arm that flanks the 4.3-kb deletion (nucleotides 64,934–70,087) was amplified by PCR and cloned using the TA Cloning Kit (pCR4-TOPO; Invitrogen). After removing the 3′ recombinogenic arm from pCR4-TOPO with *Eco*RI, this DNA fragment was inserted into the *Eco*RI site of pACN. In addition, a diphtheria toxin cassette driven by the thymidine kinase promoter was inserted into the *Sal*I site of pACN for negative selection of transfected ES cells. The selection cassette, flanked by *lox*P sites, contained the neomycin gene driven by the RNA polymerase II promoter for positive ES cell selection, and *Cre* driven by tACE derived from the gene encoding angiotensin-converting enzyme. Thus, pACN contains a testis-specific Cre expression cassette, allowing self-excision of the selection cassette upon germline transmission. Primer sequences and PCR conditions are available on request.

Genotyping of Mice with Nesp55 DMR Deletion. Mice carrying the Nesp55 DMR deletion were genotyped by PCR using tail DNA and a forward primer derived from the 5′ recombinogenic arm (Nespas exon 4) and a reverse primer derived from the 3′recombinogenic arm (Nespas intron 1); primers specific for the deleted region (Nesp55 exons 1 and 2) served as an amplification control. The mutant-specific amplification product was cloned and sequenced to confirm that correct loxP-mediated Cre recombination had occurred. Primer sequences and PCR conditions are available upon request.

Generation of Mice with Uniparental Isodisomy for Distal Chromosome 2. Mice with a reciprocal translocation for the distal chromosome 2 imprinting region, T(2;8)26H/J, were obtained from Jackson Laboratories and genotyped as described previously (2). We then generated mice with maternal (mDp2) or paternal (pDp2) uniparental isodisomy for distal chromosome 2 by intercrossing +/T26H males with +/T26H females.

RNA Analyses. Total RNA of different mouse tissues was isolated by the TRIZol method (Gibco/BRL). The prepared RNA was treated with RNase-free DNaseI (Sigma) for 30 min at 37 °C to eliminate residual DNA contaminations. The reaction was phenol-chloroform–extracted once, precipitated, and resuspended in diethylpyrocarbonate water. For Northern blot analysis, 10 μg of total RNA was mixed with an equal volume of glyoxal sample loading dye (Ambion), incubated for 60 min at 50 °C, electrophoresed through a 1% glyoxal gel, and blotted. Membranes (Hybond N+; Amersham) were hybridized with radioactively labeled probes, which were generated using the Prime-It II Random Primed DNA labeling kit (Stratagene), washed stringently, and exposed to films. The probe for the detection of the Nesp55 mRNA was isolated by digesting pBSλN3 with *Bam*HI and *Sac*I (nucleotides 72,526–73,104; Nesp55 exon 2). The 1.15-kb β-actin mRNA probe was isolated from the plasmid pAL41. Two-step RT-PCR was performed on total RNA (1 μg per sample) with DNaseI treatment using the SuperScript III First-Strand Synthesis System (Invitrogen) with oligo-dT priming. Expression of the transcripts encoding Xlαs and 1A was analyzed by PCR using exon-specific forward primers with a common reverse primer in exon 12 of the *Gnas* locus. The PCR products of 1,163 bp and 1,195 bp, respectively, were digested with *Ban*II (NEB), which generates fragments of 960 bp and 992 bp, respectively, for the 129/SvJ-derived mRNA because of a SNP located in *Gnas* exon 10 (3); the digested products were analyzed on 1.5% agarose (Biozyme) gels. Nesp55 mRNA was amplified using primers located in exons 1 and 2, whereas Nespas transcripts were detected by RT-PCR using a forward primer specific for antisense exon 1 and a reverse primer specific for antisense exon 3. A 501-bp β-globin fragment was PCR-amplified from brain cDNA as an amplification control. Primer sequences and PCR conditions are available upon request.

Methylation Analyses. For methylation analyses, we digested 5 μg of genomic DNA from liver with *Bam*HI and *Bgl*II for analysis of the exon 1A DMR; with *Hind*III for the analyses of the Gnasxl and Nesp55 DMRs; or with *Sac*I for the analysis of the Nespas DMR alone or in combination with *Hpa*II or *Msp*I, respectively. Filters used for Southern blot analysis (Hybond N+; Amersham) were hybridized with the following PCR products, which had been subcloned, released from the vector and gel purified, and radiolabeled (Prime-It II Random Primed DNA labeling kit; Stratagene): nucleotides 28,939–30,099 were used for assessing the exon 1A DMR, nucleotides 56,773–60,440 for the Gnasxl DMR, nucleotides 61,009–63,437 for the Nespas DMR, and nucleotides 71,016–74,455 for the Nesp55 DMR. As the 3.4-kb Nesp55 probe is located within the deleted Nesp55 DMR, methylation could only be assessed on the nondeleted allele after *Hpa*II digestion. Primer sequences and PCR conditions are available upon request.

For bisulfite sequence analysis, purified genomic DNA (500 ng) from liver was treated with the EpiTect Bisulfite Kit (Qiagen) and the exon 1A and Nesp55 DMR regions were amplified as described (4), except that a nested PCR strategy was not used. The regions of the Gnasxl and Nespas DMRs that were analyzed correspond to PCR products "d" and "f", as described (5). PCR products were tested for full conversion and methylation status by combined bisulfite restriction analysis. Primer sequences and PCR conditions are available from the authors on request.

Quantification of *Gnas*-Derived Transcripts by qRT-PCR. Quantification of the 1A, Xlαs, and Gsα transcripts was determined by qRT-PCR on the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) by using the QuantiFast SYBR Green PCR Kit (Qiagen). For each sample, we used 50 ng of cDNA per well (see RNA analysis) and intron-spanning primer pairs for amplification of the reference (β-actin) and the target transcripts. For each measurement, duplicate samples using total RNA from three different mice were analyzed. Nontemplate controls and nonamplification controls were included and dis-

sociation curves were generated to rule out nonspecific amplification. The correlation between the C_T and the fold difference in RNA concentration for each primer set was measured by generating serial dilutions (up to eightfold) over the entire detection range. For each probe and primer pair combination used in this study, the correlation coefficient of detection was at least 0.99. The fold difference of relative target gene expression levels between the different mouse genotypes was validated using the Pfaffl method (6). Primer sequences and PCR conditions are available from the authors upon request.

1. Bunting M, Bernstein KE, Greer JM, Capecchi MR, Thomas KR (1999) Targeting genes for self-excision in the germ line. *Genes Dev* 13:1524–1528.
2. Peters J, et al. (1999) A cluster of oppositely imprinted transcripts at the Gnas locus in the distal imprinting region of mouse chromosome 2. *Proc Natl Acad Sci USA* 96: 3830–3835.
3. Fröhlich LF, Bastepe M, Ozturk D, Abu-Zahra H, Jüppner H (2007) Lack of Gnas epigenetic changes and pseudohypoparathyroidism type Ib in mice with targeted disruption of syntaxin-16. *Endocrinology* 148:2925–2935.
4. Liu J, Yu S, Litman D, Chen W, Weinstein LS (2000) Identification of a methylation imprint mark within the mouse Gnas locus. *Mol Cell Biol* 20:5808–5817.
5. Coombes C, et al. (2003) Epigenetic properties and identification of an imprint mark in the Nesp-Gnasxl domain of the mouse Gnas imprinted locus. *Mol Cell Biol* 23: 5475–5488.
6. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.

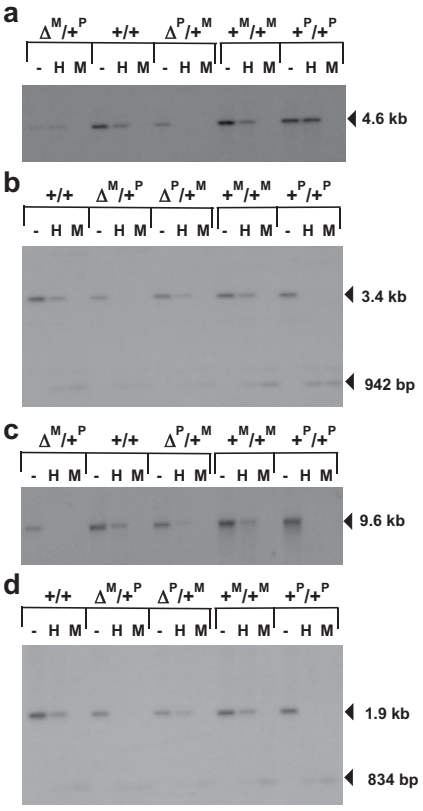

Fig. S1. Methylation analysis of the different *GNAS* DMRs using genomic DNA from liver of 2-d-old WT mice (+/+), from animals with ΔNesp55 on the paternal ($\Delta^P/+^M$) or the maternal ($\Delta^M/+^P$) allele, and from mice with maternal ($+^M/+^M$) or paternal ($+^P/+^P$) uniparental isodisomy with respect to distal chromosome 2. (*A*) Analysis of the Nesp55 DMR; incubation with *Hind*III (–), *Hind*III/*Hpa*II (H), or *Hind*III/*Msp*I (M) before Southern blot analysis. (B) Analysis of the Nespas exon 1 (Nespas/Gnasxl DMR); incubation with *Sac*I (–), *Sac*I/*Hpa*II (H), or *Sac*I/*Msp*I (M) before Southern blot analysis. (C) Analysis of exon Xl (Nespas/Gnasxl DMR); incubation with *Hind*III (–), *Hind*III/*Hpa*II (H), and *Hind*III/*Msp*I (M) before Southern blot analysis. (D) Analysis of exon 1A DMR; incubation with *Bam*HI/*Bgl*II (–), *Bam*HI/*Bgl*II/*Hpa*II (H), or *Bam*HI/*Bgl*II/*Msp*I (M) before Southern blot analysis.

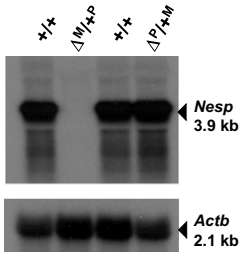

Fig. S2. Northern blot analysis of Nesp55 transcripts in total RNA (10 μg) from brain of WT mice (+/+) or 2-d-old animals after paternal or maternal transmission of ΔNesp55 ($\Delta^P/+^M$ or $\Delta^M/+^P$, respectively); Actb, loading control.