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# A new deletion refines the boundaries of the murine Prader–Willi syndrome imprinting center

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**The human chromosomal 15q11–15q13 region is subject to both maternal and paternal genomic imprinting. Absence of paternal gene expression from this region results in Prader–Willi syndrome (PWS), while absence of maternal gene expression leads to Angelman syndrome. Transcription of paternally expressed genes in the region depends upon an imprinting center termed the PWS-IC. Imprinting defects in PWS can be caused by microdeletions and the smallest commonly deleted region indicates that the PWS-IC lies within a region of 4.3 kb. The function and location of the PWS-IC is evolutionarily conserved, but delineation of the PWS-IC in mouse has proven difficult. The first targeted mutation of the PWS-IC, a deletion of 35 kb spanning *Snrpn* exon 1, exhibited a complete PWS-IC deletion phenotype. Pups inheriting this mutation paternally showed a complete loss of paternal gene expression and died neonatally. A reported deletion of 4.8 kb showed only a reduction in paternal gene expression and incomplete penetrance of neonatal lethality, suggesting that some PWS-IC function had been retained. Here, we report that a 6 kb deletion spanning *Snrpn* exon 1 exhibits a complete PWS-IC deletion phenotype. Pups inheriting this mutation paternally lack detectable expression of all PWS genes and paternal silencing of *Ube3a*, exhibit maternal DNA methylation imprints at *Ndn* and *Mkrn3* and suffer failure to thrive leading to a fully penetrant neonatal lethality.**

## INTRODUCTION

A small percentage of mammalian genes are subject to genomic imprinting, an epigenetic mechanism causing unequal expression of parental alleles. Imprinted genes tend to be organized in clusters regulated by one or more imprinting centers (ICs). The IC controls both gene expression and epigenotype within the domain. An imprinted region located at 15q11–q13 is responsible for both Prader–Willi syndrome (PWS) and Angelman syndrome (AS), two neurobehavioral disorders arising from reciprocal patterns of imprinted gene expression (1). Both gene order and allelic patterns of gene expression are conserved at the syntenic region on mouse chromosome 7.

PWS patients lack the paternal-only expression of a number of genes, including *NDN*, *MAGEL2*, *MKRN3*, *C15ORF2*, *SNRPN* (a bicistronic transcript of *SNURF* and *SNRPN*), *UBE3A-AS* and several small nucleolar RNAs (snoRNAs) (1). In some regions of the brain, *UBE3A* expression is restricted to the maternal allele and its function is disrupted in AS patients (2–4). Although most cases of PWS or AS result from a 5–7 mb deletion that removes the entire imprinted domain, some patients harbor microdeletions which disrupt imprinted gene expression (5). The smallest regions of overlap shared by these microdeletions define a bipartite IC comprised of the AS-IC and the PWS-IC (6). Gene expression patterns in both PWS individuals and mouse mutants support a model in which the PWS-IC functions as a positive regulator of transcription of paternal-only genes at the locus. The AS-IC functions in the maternal germline to epigenetically inactivate the PWS-IC so that paternal-only genes are silenced on the future maternal

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allele. AS-IC mediated silencing of a large transcript encoding *SNURF/SNRPN*, several snoRNAs and the *UBE3A*-antisense transcript (*UBE3A-AS*) on the maternal allele allows for *UBE3A* expression by an unknown mechanism (7).

Conservation of gene order and imprinting patterns suggests that mouse mutants can provide faithful models of imprinting mechanisms at the PWS/AS locus. The smallest region of overlap of microdeletions defining the human PWS-IC currently stands at 4.3 kb including the *SNRPN* promoter and exon 1, and includes a differentially DNA methylated region (DMR) characterized by DNA hypermethylation of the maternal allele (8). A differentially methylated enhancer associated with an evolutionarily conserved sequence located just outside of the minimal PWS-IC in the first intron of *SNRPN*, termed the conserved activator sequence (CAS), may also contribute to PWS-IC function (9).

The murine PWS-IC remains poorly characterized. A 35 kb deletion removing the first six exons of *Snrpn* and 16 kb of 5′ flanking sequence exhibits a complete PWS-IC imprinting defect, indicating that the entire murine PWS-IC is contained within this deletion. Paternal inheritance of this deletion is characterized by a highly penetrant neonatal lethality and absent expression of paternal-only genes (10). To date, smaller deletions within the boundaries of the 35 kb deletion have not yielded a similar complete PWS-IC phenotype. Paternal transmission of a 0.9 kb deletion removing *Snrpn* exon1 led to normal expression of paternal-only genes and appropriate DNA methylation at the remaining portion of the *Snrpn* DMR (11). A 4.8 kb deletion, revealed to be 5.07 kb by complete DNA sequencing of the region, that extended further into the DMR yielded partial neonatal lethality with residual expression of the paternal-only genes *Mkrn*3 and *Ndn* (11). More recently, we reported a mutant in which a 6.9 kb fragment containing the entire human PWS-IC replaced 6.0 kb of mouse sequence with the same 3′ breakpoint as the 4.8 kb PWS-IC deletion. Following paternal transmission of this PWS-IC$^{Hs}$ allele, both *Mkrn*3 and *Ndn* were silenced and acquired a maternal DNA methylation pattern (12). Together, these results suggest that the 6.0 kb region replaced in the mutant contains the entire PWS-IC. We have now tested this idea by creating a targeted deletion of this 6.0 kb interval. Paternal transmission of this deletion leads to undetectable expression of paternal-only genes at the locus and a highly penetrant neonatal lethality. We conclude that all elements of the murine PWS-IC are contained within the boundaries of this deletion.

## RESULTS

### Generation of a 6 kb deletion at the PWS-IC

The imprinting defects characteristic of the PWS-IC$^{Hs}$ allele suggest that the entire PWS-IC is located within a 6 kb region centered around *Snrpn* exon (12). An ES cell clone containing a *lox*P site at −3.7 kb, with reference to the 5′ end of *Snrpn* exon 1, and a floxed PGK-*neo* cassette at +2.3 kb was generated by gene targeting (Fig. 1C). Following transfection of a Cre-expressing plasmid, G418-sensitive clones lacking the PGK-*neo* cassette but retaining the floxed *Snrpn* allele were identified by polymerase chain reaction (PCR) and southern blot (Fig. 1D). A clone with the 6 kb region flanked by *lox*P sites was successfully transmitted to the germline. These mice were mated with the germline Cre-expressing line, 129-*Alpl*$^{tm1(cre)Nagy}$/J (13), to create the 6 kb deletion allele termed PWS-IC$^{\Delta 6kb}$ (Fig. 1E and F).

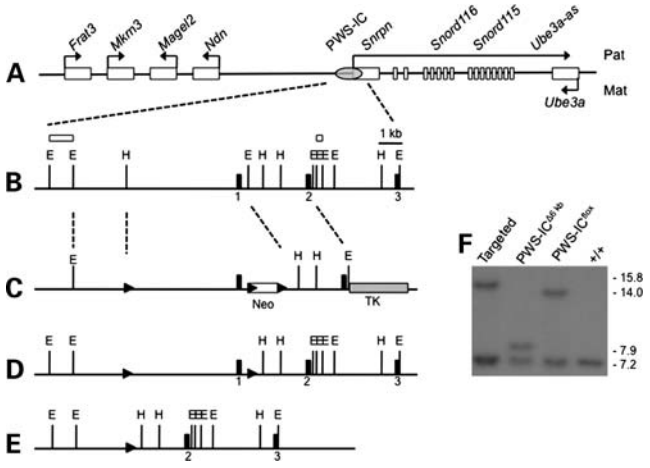


**Figure 1.** Generation of mice with a targeted 6 kb deletion of the PWS-IC. (**A**) A map of the murine PWS/AS locus, not drawn to scale. Genes listed above the line are normally expressed from the paternal allele. *Ube3a* is normally expressed from the maternal allele. The PWS-IC is represented by a grey oval. Arrows indicate the direction of transcription. (**B**) Restriction map of the PWS-IC region. The first three exons of *Snrpn* are shown as black rectangles. Selected *Eco*RI (E) and *Hpa*I (H) sites are indicated. Open rectangles indicate the positions of probes used in determining recombination. Dashed lines illustrate the region of the locus shown in this panel. (**C**) The gene targeting construct. *lox*P sites are shown as black arrowheads. The open box shows the positive PGK-*neo* selection cassette and the grey box indicates the negative HSV-*tk* selection cassette. Dashed lines represent regions for homologous recombination. Open rectangles above the map indicate the position of probes used in southern analysis of recombinants. (**D**) The recombinant allele created by homologous recombination within the arms of homology. After transient transfection with a Cre expressing plasmid, the conditional floxed allele was identified. These ES cells were utilized for blastocyst injection. (**E**) The PWS-IC$^{\Delta 6kb}$ allele was generated by deletion of the PWS-IC in the germline. (**F**) Southern blot analysis of various ES cell and mouse DNAs. Genomic DNA was digested with *Hpa*I and *Eco*RV and hybridized with the 5′ probe shown in (B). The wild-type allele generates a 7.2 kb fragment present in all samples. Correct homologous recombination with the targeting vector yields a 15.8 kb allele arising from the disruption of the *Hpa*I site by the upstream *lox*P site and insertion of the floxed PGK-*neo* cassette. Removal of the PGK-*neo* cassette reduces this band to 14 kb. Deletion of the PWS-IC yields a 7.9 kb band.

### Paternal inheritance of the 6 kb PWS-IC deletion leads to reduced birth weight and survival

Paternal inheritance of a 35 kb deletion removing the entire murine PWS-IC is associated with low birth weight, failure to thrive, fully penetrant neonatal lethality and loss of expression of paternal-only genes from the region. At birth, mice inheriting the PWS-IC$^{\Delta 6kb}$ allele paternally (PWS-IC$^{+/\Delta 6kb}$) are smaller than wild-type littermates and often lack milk spots. PWS-IC$^{+/\Delta 6kb}$ neonates weigh significantly less than their wild-type littermates and rarely survive beyond postnatal day (P) 2, although we did observe one pup surviving to P7 (Fig. 2). Similar to the previously reported PWS-IC$^{\Delta 35kb}$ mutation, we observed no overt phenotypes following maternal inheritance of the PWS-IC$^{\Delta 6kb}$ allele.

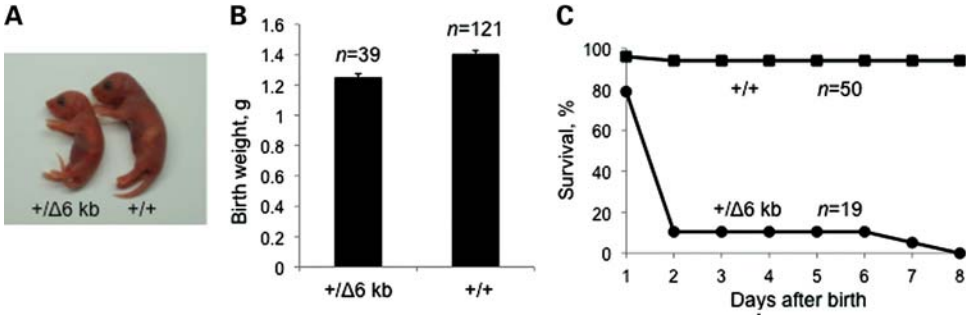


**Figure 2.** Physical characteristics of PWS-IC$^{+/\Delta 6kb}$ pups. (**A**) Newborn PWS-IC$^{+/\Delta 6kb}$ pups are distinguishable from wild-type littermates by their small size and absence of a milk spot. At P2, this PWS-IC$^{+/\Delta 6kb}$ pup weighed 1.4 g and the wild-type littermate weighed 1.9 g. (**B**) At birth, PWS-IC$^{+/\Delta 6kb}$ pups typically weigh less than their littermates. The mean birth weight for PWS-IC$^{+/\Delta 6kb}$ pups is 1.25 g ($N = 39$) and for PWS-IC$^{+/+}$ pups is 1.40 g ($N = 121$) ($P$-value $< 0.0001$). (**C**) PWS-IC$^{+/\Delta 6kb}$ pups have reduced survival compared with wild-type littermates with most PWS-IC$^{+/\Delta 6kb}$ pups dying within 2 days and none surviving past P7 ($N = 19$ PWS-IC$^{+/\Delta 6kb}$ pups, $N = 50$ PWS-IC$^{+/+}$ pups).

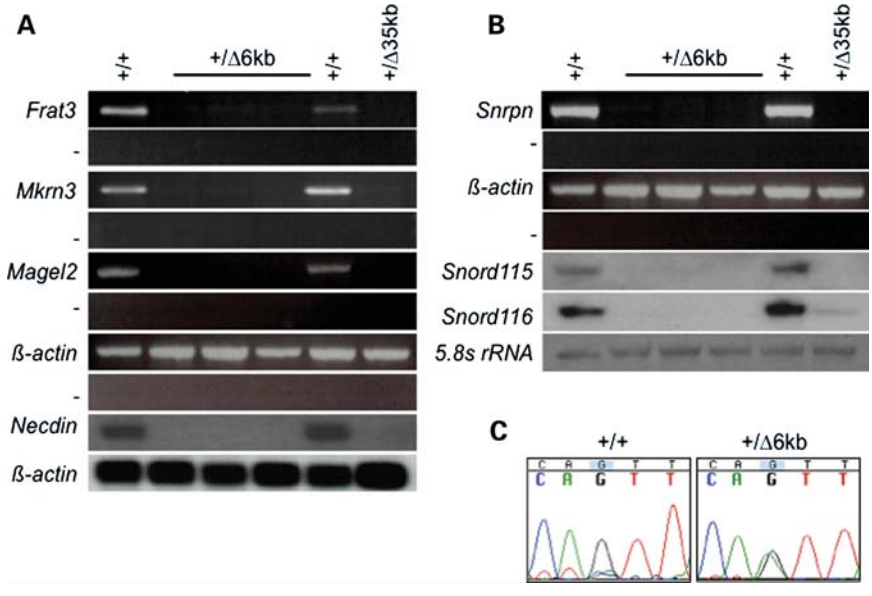


**Figure 3.** Gene expression patterns in PWS-IC$^{+/\Delta 6kb}$ newborn brain. Total brain RNA was prepared from wild-type (+/+), PWS-IC$^{+/\Delta 35kb}$ (+/Δ35 kb) and PWS-IC$^{+/\Delta 6kb}$ (+/Δ6 kb) neonates and analyzed either by RT–PCR or northern blot. (**A**) Analysis of the upstream group of genes. The minus sign in RT–PCR analyses indicates control samples in which reverse transcriptase was omitted during cDNA synthesis. (**B**) Analysis of the downstream cluster of genes. (**C**) Allelic analysis of *Ube3a* expression. P1 progeny from a cross between a B6.CAST.c7 homozygous female and a PWS-IC$^{\Delta 6kb/+}$ male were analyzed for allelic expression of *Ube3a* in the brain. The maternal *castaneus* allele has a guanine residue at the analyzed single nucleotide polymorphism, while the paternal *domesticus* allele has an adenine residue. Wild-type mice exhibit predominantly maternal *Ube3a* expression, while biallelic expression is evident in PWS-IC$^{+/\Delta 6kb}$ mice.

## PWS gene expression is undetectable in PWS-IC$^{+/\Delta 6kb}$ pups

We investigated the molecular consequences of paternal inheritance of the 6 kb PWS-IC deletion by analyzing gene expression in newborn brain. Paternally expressed genes at the PWS/AS locus are arranged in two clusters. The centromeric cluster of genes comprising *Snrpn*, several snoRNAs and *Ube3a-as* are likely to be processed from a common primary transcript (14–18). Snrpn RNA and the snoRNAs Snord115 and Snord116 were not detected in the newborn PWS-IC$^{+/\Delta 6kb}$ brain (Fig. 3B). A low level of Snord116 RNA was detected in the 35 kb PWS-IC deletion control. As previously noted, this low level of expression may be a consequence of continued maintenance of the mouse line or result from the backcross onto the C57BL/6 background (19). We also examined expression of the telomeric cluster of genes composed of *Frat3*, *Magel2*, *Mkrn3* and *Ndn*. Again, expression of these genes was not detectable in brains of pups with a paternal PWS-IC 6 kb deletion allele (Fig. 3A).

## *Ube3a* expression is increased in PWS-IC$^{+/\Delta 6kb}$ mice

*Ube3a* is normally paternally silenced by a mechanism speculated to depend upon *Ube3a-as*, a transcript antisense to *Ube3a*. Consistent with this model, paternal inheritance of the 35 kb deletion of the PWS-IC results in increased paternal expression of *Ube3a* (20). To determine the imprinted status of *Ube3a* in PWS-IC$^{+/\Delta 6kb}$ mice, we took advantage of the B6.*CAST*.c7 strain in which the PWS/AS region is congenic

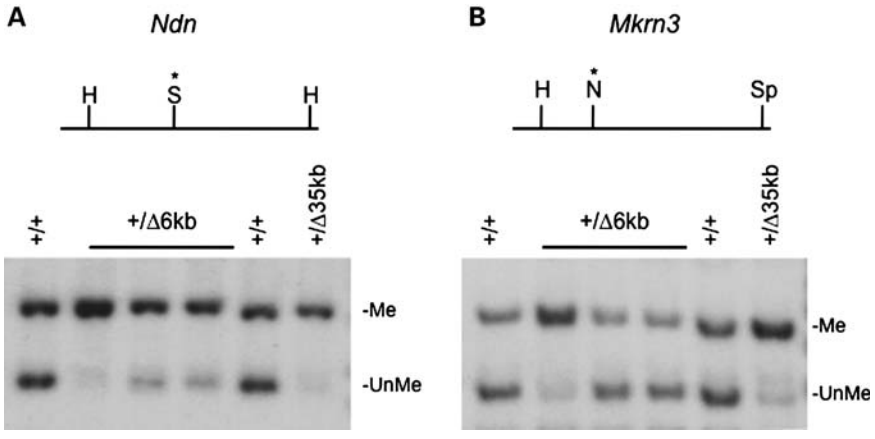


**Figure 4.** DNA methylation analysis of the *Ndn* and *Mkrn3* DMRs. DNA from brains of wild-type (+/+), PWS-IC$^{+/\Delta 35kb}$ (+/Δ35 kb) and PWS-IC$^{+/\Delta 6kb}$ (+/Δ6 kb) newborns was digested with the indicated restriction endonucleases, southern blotted and probed. (**A**) The upper part of the panel shows a restriction map of the *Ndn* locus. The DNA methylation sensitive endonuclease *Sac*II (S) is located between two *Hin*dIII (H) sites.The *Ndn* probe detects a 3.3 kb fragmet (Me) resulting from DNA methylation as the *Sac*II site and a 1.8 kb fragment obtained from unmethylated DNA (UnMe). (**B**) A DNA methylation sensitive *Not*I is located between *Hin*dIII and *Spe*I (Sp) sites at the *Mkrn3* locus. The probe detects methylated and unmethylated fragments of 6.8 and 5.0 kb, respectively. The same DNA preparations were analyzed in both (A) and (B).

for *Mus musculus castaneus* on a C57BL/6 background. DNA sequencing of a region containing a single nucleotide polymorphism demonstrated biparental expression of *Ube3a* in PWS-IC$^{+/\Delta 6kb}$ mice, while the wild-type littermates exhibited predominantly maternal expression (Fig. 3C). Thus, similar to the 35 kb deletion, the 6 kb PWS-IC deletion allele lacks elements necessary for silencing paternal *Ube3a*.

### DNA methylation analysis of *Mkrn3* and *Ndn* in PWS-IC$^{+/\Delta 6kb}$ mice

The paternally expressed genes *Ndn* and *Mkrn3* both contain DMRs characterized by DNA hypermethylation of the normally silent maternal allele. Disruption of the PWS-IC leads to paternal hypermethylation in both individuals with PWS and mice lacking PWS-IC function (21–23). We used DNA methylation sensitive restriction endonucleases to investigate the role of the 6 kb PWS-IC deletion on DNA methylation status of these two loci. Paternal inheritance of the 6 kb PWS-IC deletion resulted in increased DNA methylation at both the *Mkrn3* and *Ndn* loci, although to varying extent (Fig. 4). Increased DNA methylation appeared more extensive and reproducible at *Ndn* than at *Mkrn3*. The same DNA preparations were analyzed at both loci, suggesting that DNA methylation defects in the absence of the PWS-IC may be more penetrant at the *Ndn* locus. Partial hypermethylation of these paternal alleles was evident following paternal inheritance of both the 4.8 and 35 kb PWS-IC deletions (11,22).

## DISCUSSION

ICs are DNA sequences that regulate both epigenotype and allelic gene expression (12). At the PWS/AS locus, overlapping microdeletions define a bipartite IC controlling both paternal- and maternal-only gene expression. A previously described 4.8 kb deletion at the murine PWS-IC exhibited a partial imprinting phenotype. Here, we have extended this deletion 1 kb further upstream of *Snrpn* exon 1. Paternal transmission results in fully penetrant neonatal lethality, undetectable expression of paternal-only genes and increased DNA methylation at *Mkrn3* and *Ndn*. These traits are identical to a 35 kb PWS-IC deletion and indicate that all PWS-IC elements are located within the 6 kb deletion boundaries.

This 6 kb PWS-IC deletes most of the DMR, the *Snrpn* promoter region and the murine CAS. Both the promoter and the CAS are associated with paternal-specific DNase I hypersensitive (DH) sites at the human *SNRPN* gene in lymphoblasts and have been proposed to be involved in PWS-IC function (9). However, the human CAS is located in *SNRPN* intron 1, just outside of the minimally defined human PWS-IC. The inclusion of the transcription activating CAS in the 6 kb murine PWS-IC is consistent with the postulated function of the PWS-IC as a positive regulator of paternally expressed genes in the PWS/AS domain (7). The partial imprinting defect characteristic of the previously described murine 4.8 kb PWS-IC mutation indicates that this deletion contains some but not all elements required for a fully functional PWS-IC. Both the murine *Snrpn* promoter region and the CAS element are included in the 4.8 kb deletion, suggesting DNA sequence elements associated with these regions may contribute to PWS-IC activity. However, the partial imprinting defect of the 4.8 kb deletion indicates that one or more elements outside of this deletion are additionally required for full PWS-IC activity. Because the 4.8 kb deletion and the 6 kb deletion described here share the same 3′ boundary, we postulate that functional elements that confer full PWS-IC activity must be present within this differential 1.0 kb interval. A DH site has been detected within this 1.0 kb interval (S. Rodriguez-Jato, J.R. Khadake, T.P. Yang, unpublished data). We are currently refining its location and determining its parent of origin. If present only on the paternal allele, this would be consistent with the hypothesis that the PWS-IC is comprised of multiple functional elements that contribute to PWS-IC activity by creating a chromatin

holocomplex, similar to an active chromatin hub, specifically on the paternal chromosome (9).

*Snrpn*, the snoRNAs and *Ube3a-as* are all processed from a common transcript. While the majority of transcription initiates within the PWS-IC, some transcripts initiate at several alternative upstream exons and splice into *Snrpn* exon 2, or less commonly, further downstream (24). Although the splice acceptor site at *Snrpn* exon 2 is intact, these transcripts are not detected in mice with a paternal 6 kb PWS-IC deletion, indicating that these upstream transcription initiation sites are themselves subject to regulation by the PWS-IC.

## MATERIALS AND METHODS

### Gene targeting

A targeting vector was constructed from a phage library of fragments generated from BAC 397F16 (Research Genetics). Homology arms consisted of a 3′ 3.5 kb *Eco*RI and a 5′ 8.6 kb *Hin*dIII/*Eco*RI fragment. Oligonucleotides containing a *lox*P site were inserted into the *Hpa*I site of the 8.6 kb *Hin*dIII/*Eco*RI fragment, splitting it into a 2.6 kb 5′ homologous arm and a 6 kb PWS-IC region. A PGK-*neo* gene flanked by *lox*P sites was placed between the 3′ homology arm and the PWS-IC region, and a PGK-*tk* cassette was placed between the 3′ arm and the pBluescript KS + vector backbone.

Homologous recombination was performed in CJ.7 cells (129S1/Sv strain) as previously described (12). Clones with the appropriate homologous recombination were identified by southern blot. Homologous recombination at the 5′ end was identified by *Spe*I digest and at the 3′ end by *Hpa*I/*Eco*RV digest.

The floxed neomycin cassette was removed by transient transfection of the ES cells with a Cre expressing plasmid, pCAGcre (25). Colonies sensitive to G418 were analyzed by PCR and southern blot. An ES cell line with a floxed 6 kb region of the PWS-IC was selected for injection into C57BL/6 blastocysts and chimeric mice identified. The PWS-IC$^{\Delta 6kb}$ mutation was obtained by breeding the PWS-IC$^{flox6kb}$ allele with 129-*Alpl*$^{tm1(cre)Nagy}$/J mice (Jax stock 008569) that express Cre in primordial germ cells and subsequently breeding progeny that inherited both the Cre transgene and the PWS-IC$^{flox6kb}$ allele to wild-type C57BL/6 mice. All genotyping was done by PCR. Primer sequences are available upon request. All animal procedures were previously approved by the University of Florida Institutional Animal Care and Use Committee.

### Southern blot analysis

For southern blot analysis, ES cell or newborn brain DNA was digested with restriction endonucleases as indicated, electrophoresed through 0.8% TAE agarose gels and transferred to a positively charged nylon membrane (Hybond, GE Healthcare). Membranes were hybridized to $^{32}$P labeled probe and exposed to Kodak XAR film. The probes used for identifying ES clones with homologous recombination were outside of the targeting vector's arms of homology. At the 5′ end, a 800 bp *Eco*RI fragment was used while at the 3′ end, a 358 bp *Eco*RI fragment was used, both from a phage library of BAC 397F16 (Research Genetics) fragments.

### Gene expression analysis

Analysis of paternal-only gene expression by either RT–PCR or northern blot was as previously described (12). *Ube3a* allelic expression was determined in newborn brains obtained from matings of B6.*CAST*.c7 females with PWS-IC$^{+/\Delta 6kb}$ males. B6.*CAST*.c7 are C57BL/6 congenic for the *Mus castaneous* PWS/AS region (26). RT–PCR for *Ube3a* was performed across a polymorphism between *Mus castaneus* and *Mus musculus domesticus* using primers Ube3a 5F, 5′-CACATATGA TGAAGCTACGA-3′ and Ube3a 6R, 5′-CACACTCCC TTCATATTCC-3′ (20). The RT–PCR product was sequenced by the UF Center for Epigenetics.

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*Conflict of Interest statement.* None declared.

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