Temporal and developmental requirements for the Prader–Willi imprinting center

Amanda J. DuBose[1,2], Emily Y. Smith[2], Karen A. Johnstone[3], and James L. Resnick[4]

Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, FL 32610

Edited by Arthur L. Beaudet, Baylor College of Medicine, Houston, TX, and approved January 17, 2012 (received for review September 13, 2011)

Imprinted gene expression associated with Prader–Willi syndrome (PWS) and Angelman syndrome (AS) is controlled by two imprinting centers (ICs), the PWS-IC and the AS-IC. The PWS-IC operates in *cis* to activate transcription of genes that are expressed exclusively from the paternal allele. We have created a conditional allele of the PWS-IC to investigate its developmental activity. Deletion of the paternal PWS-IC in the embryo before implantation abolishes expression of the paternal-only genes in the neonatal brain. Surprisingly, deletion of the PWS-IC in early brain progenitors does not affect the subsequent imprinted status of PWS/AS genes in the newborn brain. These results indicate that the PWS-IC functions to protect the paternal epigenotype at the epiblast stage of development but is dispensable thereafter.

Prader–Willi syndrome (PWS) and Angelman syndrome (AS) are genetic disorders resulting from the loss of expression of a cluster of imprinted genes at chromosome 15q11-q13. Individuals with PWS lack paternal gene expression from this imprinted region whereas individuals with AS lack maternal gene expression (1). In contrast to imprinted domains controlled by a single imprinting center, the PWS/AS region is regulated by a bipartite imprinting center composed of the AS imprinting center (AS-IC) and the PWS-IC (2). Imprinting in the region results from interplay between the AS-IC and the PWS-IC. Substantial evidence suggests that the PWS-IC is a positive-acting element that stimulates the expression of genes in the 2-Mb PWS/AS region. The AS-IC is thought to act in the female germline to inactivate the PWS-IC on the future maternal allele (3). Thus, a functional PWS-IC is necessary for the expression of paternal-only genes and an intact AS-IC is necessary to silence these genes on the maternal chromosome. Using a targeted deletion of a 35-kb region spanning *Snrpn* exons 1 to 6, we previously reported that the location and function of the PWS-IC is conserved in mice (4). More recently, we found that a 6-kb deletion surrounding *Snrpn* exon 1 yields an identical phenotype to the 35-kb deletion, demonstrating that all the functional elements of the PWS-IC are within this 6-kb region (5).

The PWS-IC lies within a region of allele-specific DNA methylation. The silent maternal allele is hypermethylated whereas the paternal allele is hypomethylated. These DNA methylation imprints are erased and reestablished in the germline during gametogenesis (6–10). When the PWS-IC acts in somatic tissue to regulate allele-specific gene expression is unknown. Bielinska et al. (11) found that the PWS-IC is required postzygotically in both humans and mice. An individual with minor clinical symptoms of PWS was shown to be mosaic for a PWS-IC deletion, suggesting that the PWS-IC is necessary to maintain paternal gene expression postzygotically. Mice chimeric for a PWS-IC deletion on the paternal chromosome exhibited promoter hypermethylation and loss of expression of *Ndn* and *Mkrn3*, supporting the interpretation that the PWS-IC is required postzygotically (11).

We have created a conditional allele of the PWS-IC to further investigate the temporal and spatial functions of this element. As predicted from previous work, we find that the PWS-IC is necessary before implantation. Unexpectedly, deletion of the PWS-IC early in neurogenesis does not affect subsequent expression of imprinted genes at the locus in newborn brain. Deletion early in neurogenesis does reduce *Snrpn* and snoRNA expression, but this effect is likely a result of the removal of the *Snrpn* major promoter. These pups appear to bypass the early failure to thrive seen in other PWS models but still exhibit a reduction in post-weaning weight gain. These results confirm that the PWS-IC is necessary postzygotically for paternal gene expression but also demonstrate that it is not required later in development for maintenance of the paternal epigenotype.

Results

Generation of PWS-IC Conditional Deletion Allele. We previously demonstrated that the entire murine PWS-IC lies within a 6-kb region located between −3.7 kb and +2.3 kb with reference to *Snrpn* exon 1. Furthermore, we generated a conditional PWS-IC allele by flanking this region with *lox*P sites (5). The structure of this conditional allele is shown in Fig. S1.

Deletion of PWS-IC Before Implantation Results in Reduced Birth Weight and Neonatal Lethality. To investigate the effects of early embryonic deletion of the PWS-IC, we analyzed offspring from a mating of males heterozygous for the PWS-IC^{flox6kb} allele with transgenic (Tg) Cmv-cre (Tg$^{Cmv\text{-}cre}$) hemizygous females. The Cmv-cre transgene expresses Cre ubiquitously before implantation (12). Similar to paternal inheritance of a PWS-IC deletion (4, 5), PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$ pups were visibly smaller and had significantly lower birth weights than littermates (Fig. 1 *A* and *B*). Cmv-cre–mediated deletion of the PWS-IC before implantation caused 100% postnatal lethality with no pups surviving beyond postnatal day (P) 2 (Fig. 1*C*). Southern blot analysis of DNA isolated from several P1 brains suggests that Cmv-cre mediated deletion is extensive (Fig. S2). As expected, mice with a somatic deletion of the PWS-IC on the maternal chromosome were normal, as were mice inheriting a maternal or paternal PWS-IC^{flox6kb} allele in the absence of Cre expression (5).

Paternal Gene Expression Requires Presence of PWS-IC in Preimplantation Embryo. To determine the extent of the imprinting defect resulting from preimplantation deletion of the PWS-IC, we examined gene expression in RNA isolated from the brains of P1 PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$ pups. Deletion of the PWS-IC before implantation affected the expression of all genes in the region. *Snrpn*, *Frat3*, *Ndn*, *Magel2*, *Mkrn3*, *Snord116*, and *Snord115*

Author contributions: A.J.D., E.Y.S., K.A.J., and J.L.R. designed research; A.J.D., E.Y.S., K.A.J., and J.L.R. performed research; A.J.D., E.Y.S., and K.A.J. contributed new reagents/analytic tools; A.J.D., E.Y.S., K.A.J., and J.L.R. analyzed data; and A.J.D., E.Y.S., K.A.J., and J.L.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]Present address: National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892.

[2]A.J.D. and E.Y.S. contributed equally to this work.

[3]Present address: Institute of Biomedical and Clinical Science, Peninsula Medical School, University of Exeter, Exeter EX2 5DW, United Kingdom.

[4]To whom correspondence should be addressed. E-mail: resnick@mgm.ufl.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1115057109/-/DCSupplemental.

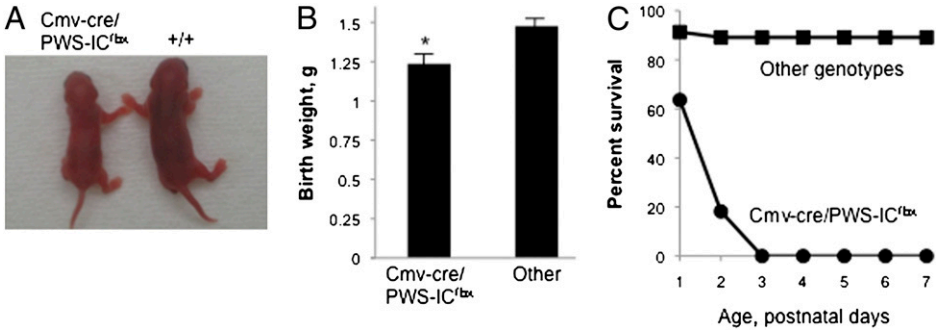

Fig. 1. PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$ pups are small and have reduced survival compared with littermates of other genotypes. (*A*) The PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$ neonate on the left is smaller than the WT littermate on the right (P1). (*B*) PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$ mice weigh significantly less than mice of other genotypes at birth ($P = 0.0007$; $n = 11$ and $n = 59$, respectively). (*C*) PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$ mice (circles, $n = 11$) exhibit postnatal lethality with no survivors beyond P2 compared with other genotypes (squares, $n = 46$).

expression was not detected in PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$ newborn mice (Fig. 2). Gene expression patterns in PWS-IC$^{+/flox6kb}$ pups that did not inherit Tg$^{Cmv\text{-}cre}$ were indistinguishable from WT.

Deletion of PWS-IC in Neuronal Precursors Does Not Result in an Imprinting Defect. Cmv-cre–mediated deletion of the PWS-IC results in a complete imprinting defect and thereby demonstrates that the PWS-IC must be intact in the preimplantation embryo to maintain the paternal pattern of gene expression during subsequent embryonic development. We next sought to establish whether the PWS-IC is required to maintain the imprint at later times in development. PWS-IC^{flox6kb} males were mated to transgenic Nes-cre females to mediate PWS-IC deletion in neuronal precursors and glia by embryonic day (E) 10.5 with widespread deletion by E12.5 (13, 14). The paternally expressed genes *Ndn*, *Frat3*, *Magel2*, and *Mkrn3* were unaltered in the brains of PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ newborns (Fig. 3). Normal expression of these genes indicates that the PWS-IC is not necessary to maintain paternal gene expression in brain precursor cells beyond E12.5.

The downstream cluster of genes, *Snrpn*, several snoRNAs, and *Ube3a-as*, are likely processed from a common 1-Mb transcript (15–17). Deletion of the PWS-IC also removes the major promoter for this transcript. *Snrpn* expression in the brains of P1 PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ mice is reduced by almost 70%, whereas the snoRNAs, *Snord116* and *Snord115*, are reduced by approximately half (Fig. 3). Nes-cre–mediated deletion of the PWS-IC in P1 brain was extensive (Fig. S2), indicating that the observed residual gene expression was not the result of incomplete PWS-IC deletion. We investigated whether deletion of the major promoter for this transcript contributed to the reduction in expression of *Snrpn* and the snoRNAs. Several distant upstream exons (U exons) for *Snrpn* have previously been found to contribute to transcription of the locus (15, 18). We tested whether the residual expression of *Snrpn* and the snoRNAs could originate from the *Snrpn* U exons in PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ animals. Transcripts originating at *Snrpn* upstream exon U1 and extending to *Snrpn* exon 3 were detectable in the brains of PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ mice but not in mice inheriting the same deletion via the germline, nor in PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$ mice (Fig. 4). These results confirm and extend previous findings that the U exons contribute to the *Snrpn* transcription unit and also demonstrate that U exon use requires the presence of the PWS-IC before implantation (18).

Paternal expression of the *Snrpn* transcription unit is widely thought to contribute to silencing of *Ube3a* on the paternal allele via antisense transcription (19–21). Elimination of the major *Snrpn* promoter prompted us to investigate allelic expression of *Ube3a*. Females heterozygous for the Nes-cre transgene and heterozygous for B6.*cast*.c7 (congenic for *Mus musculus castaneus* across a portion of chromosome 7) were crossed with PWS-IC$^{+/flox6kb}$ males, and the progeny were analyzed at 12 wk of age. As shown for other mutations that down-regulate *Snrpn–Ube3a-as* expression, we also found an increase in paternal contribution of *Ube3a* in PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ mice (Fig. S3).

Paternal Epigenotype at Ndn and Mkrn3 Is Stable Postzygotically. The *Ndn* and *Mkrn3* promoters exhibit differential DNA methylation with hypermethylation of the maternal allele (22, 23). Digestion with methyl-sensitive restriction endonucleases was used to analyze the DNA methylation status of *Mkrn3* and *Ndn* in both PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$ and PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ mice. Germline transmission of the 6-kb PWS-IC deletion led to the paternal allele adopting a maternal DNA methylation pattern at both *Mkrn3* and *Ndn* (5), as did deletion with Cmv-cre. Nes-cre–mediated deletion, which does not cause an imprinting defect, resulted in a WT DNA methylation pattern at both loci (Fig. 5). Thus, the DNA methylation imprints at *Ndn* and *Mkrn3* are stable in the brain even in the absence of the PWS-IC.

Deletion of PWS-IC in Neuronal Precursors Results in Postweaning Growth Deficiency. The reduction in *Snrpn–Ube3a-as* expression in the nervous system led us to investigate growth traits of PWS-

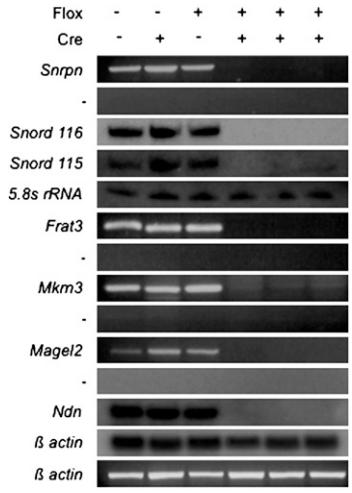

Fig. 2. Expression of PWS genes in PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$ P1 brains. Expression of the indicated genes was determined by either Northern blot or RT-PCR. A minus sign to the left of RT-PCR analyses indicates control samples in which reverse transcriptase was omitted during cDNA synthesis.

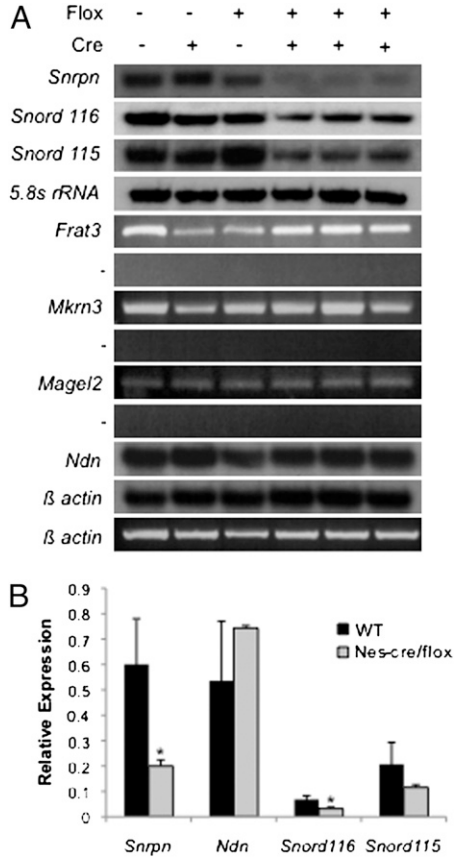

Fig. 3. Expression of upstream PWS genes in P1 brains is preserved whereas expression of the downstream cluster of genes is reduced in PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ mice. (*A*) Gene expression assays were as indicated in Fig. 2. (*B*) Expression levels of *Snrpn*, *Ndn*, *Snord116*, and *Snord115* were quantified by PhosphorImager analysis of the Northern blots. The average of the three control lanes ± SE was compared with the average of the three PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ lanes and normalized to β-actin (*Snrpn* and *Ndn*) or 5.8S RNA (*Snord115* and *116*) exposures on the same blot. *P* values for reduction in *Snrpn*, *Snord116*, and *Snord115* expression levels are 0.018, 0.017, and 0.142, respectively. *Ndn* level was not significantly altered (*P* = 0.2, Student *t* test).

IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ deletion mice. Pups inheriting a PWS-IC^{flox6kb} allele paternally and the Nes-cre transgene maternally did not exhibit a statistically significant difference in birth weight. PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ pups regularly exhibited milk spots and lacked the pallor characteristic of PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$ newborns or pups inheriting a germline deletion of the PWS-IC (5). PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ pups were born with the expected Mendelian frequency (14 of 52; 26.9%) and did not show a reduction in postnatal survival compared with littermates of other genotypes. However, PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ mice did exhibit a growth retardation that became increasingly apparent during the postweaning period (Fig. 6). By 3 wk of age, female PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ mice displayed a significant growth deficiency (8.32 ± 0.48 vs. 10.1 ± 0.32; $P = 0.022$) and, although their growth curve was similar to littermates, they remained growth-restricted (Fig. 6*A*). Males of the same genotype also exhibited a growth deficiency that reached statistical significance by 4 wk of age (15.1 ± 0.52 vs. 18.1 ± 0.36; $P = 0.012$; Fig. 6*B*).

Discussion

We recently demonstrated that paternal transmission of a 6-kb deletion spanning *Snrpn* exon 1 leads to low birth weight, neonatal lethality, and complete loss of PWS gene expression in newborn brain (5). This phenotype is indistinguishable from that

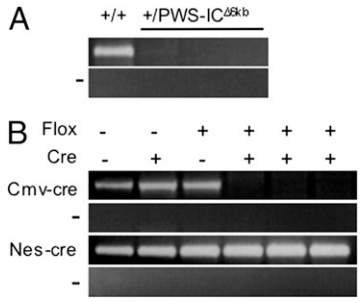

Fig. 4. *Snrpn* upstream exon use in the brain. *Snrpn* upstream exon transcripts were detected by RT-PCR using primers in upstream exon U1 and exon 3. (*A*) Expression from *Snrpn* upstream exon 1 is not detected following germline transmission of the PWS-IC deletion. (*B*) *Snrpn* upstream exon transcripts following Cmv-cre– or Nes-cre–mediated deletion.

of a previously described 35-kb PWS-IC deletion and indicates that the entire murine PWS-IC is included within the boundaries of the 6-kb deletion. Here, we have characterized the consequences of conditional deletion of this minimal PWS-IC.

Evidence from both a mosaic individual with some features of PWS and chimeric mice suggests that the PWS-IC is necessary postzygotically for paternal PWS gene expression and to avoid a maternal methylation imprint (11). Both the mosaic individual and chimeric mice transmitted the deletion, implying that the PWS-IC may not be necessary after separation of the germline from soma. Cmv-cre–mediated deletion of the PWS-IC before implantation results in a gene expression pattern indistinguishable from germline transmission of a PWS-IC deletion, thus indicating that the PWS-IC is necessary before or during implantation. In contrast, Nes-cre–mediated deletion of the PWS-IC in brain progenitors, where extensive deletion occurs by E12.5, does not lead to an imprinting defect. In this case, expression of *Mkrn3*, *Magel2*, *Frat3*, and *Ndn* in newborn brain is unaffected by the absence of the PWS-IC. Together, these results establish that the presence of the PWS-IC is required in the period before implantation through formation of early neural precursors. This period is characterized by dynamic epigenetic changes in somatic tissues. Fertilization is followed by DNA demethylation of the paternal and maternal genomes. The embryonic genome is then remethylated at the epiblast stage early in gastrulation (reviewed in ref. 24). This stage of genomic DNA methylation is likely a critical period for PWS-IC function. Our data are consistent with a model in which the PWS-IC prevents acquisition of a maternal imprint on the paternal allele during this developmental stage.

The finding that the paternal epigenotype can be maintained throughout much of neurogenesis in the absence of the PWS-IC was unexpected. The contribution of epigenetic modifications to neural development is poorly understood, although several reports illuminate the importance of this form of gene regulation. The human brain exhibits region specific DNA methylation patterns, suggesting that DNA methylation contributes to neural development (25). A role for epigenetic regulation in brain development is further suggested by mutations in DNA methyltransferases (reviewed in ref. 26). Our results indicate that the epigenotype of paternally expressed genes at the PWS/AS locus is dependent upon the PWS-IC during early embryonic development. However, by the time Nes-cre becomes active in neural cells at E12.5, maintenance of the paternal epigenotype no longer requires the PWS-IC.

Previous efforts to conditionally inactivate elements regulating imprinted gene expression during embryonic development have focused on the *H19*/*Igf2* and *Rasgrf1* loci. *H19* is normally silenced on the paternal allele whereas *Igf2* is normally silenced on the maternal chromosome. Germline transmission of a paternal IC

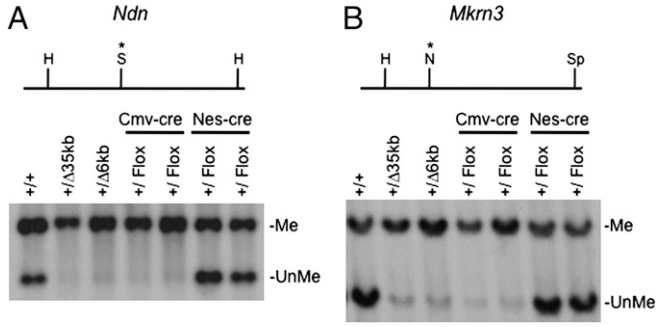

Fig. 5. DNA methylation analysis of the *Ndn* and *Mkrn3* differentially methylated regions. DNA from brains of WT ($^{+/+}$), PWS-IC$^{+/\Delta 35kb}$, PWS-IC$^{+/\Delta 6\ kb}$, PWS-IC$^{+/flox6kb}$ Tg$^{Cmv\text{-}cre}$, and PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ pups was digested with the indicated restriction endonucleases, Southern blotted, and probed. (*A*) The upper part shows a restriction map of the *Ndn* locus. The DNA methylation sensitive endonuclease *Sac*II (S) is located between two *Hind*III (H) sites. The *Ndn* probe detects a 3.3-kb fragment (Me) resulting from DNA methylation at the *Sac*II site and a 1.8-kb fragment obtained from unmethylated DNA (UnMe). (*B*) The upper part shows a restriction map of the *Mkrn3* locus. A site recognized by the DNA methylation sensitive *Not*I (N) endonuclease is located between *Hind*III and *Spe*I (Sp) sites at the *Mkrn3* locus. The probe detects methylated and unmethylated fragments of 6.8 and 5.0 kb, respectively.

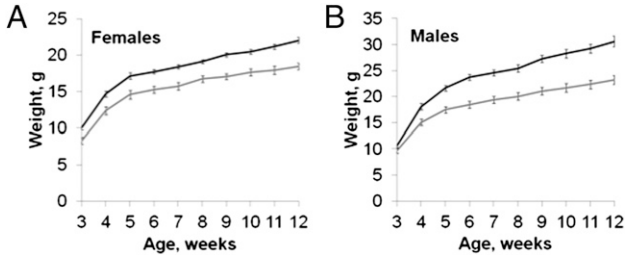

Fig. 6. Postweaning growth of PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ mice and littermates. Mice were weighed weekly after weaning. Weight curves for female (*A*) and male (*B*) PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ mice and their littermates (n = 6 and n = 32 for females and n = 8 and n = 20 for males, PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ and other mice, respectively). The gray line shows weight gain for PWS-IC$^{+/flox6kb}$ Tg$^{Nes\text{-}cre}$ mice and the black line shows the weight gain for the other genotypes. Error bars indicate SE.

deletion at this locus results in expression of the normally silent paternal *H19* allele (27, 28). Conditional deletion of the paternal IC in zygotes also leads to biallelic *H19* expression. However, *H19* expression remains exclusively maternal after paternal IC deletion in terminally differentiated skeletal, cardiac, or liver cells, suggesting that the imprinted status of *H19* becomes IC-independent sometime after implantation. The *Rasgrf1* locus is paternally expressed. Acquisition of DNA methylation at a differentially methylated domain in the male germline is dependent upon a set of repeats located 30 kb upstream of the transcription start site. A conditional deletion strategy targeting these repeats demonstrated that, although the repeats are necessary to maintain differential methylation after fertilization, they are no longer necessary after the epiblast stage (29). Unlike these loci, the DNA methylation imprint is maternally acquired at the PWS/AS locus. The paternal PWS-IC is necessary to establish expression of paternal genes but is dispensable for paternal gene expression by the time neural precursors are formed. The *Rasgrf1* study demonstrates stability of the paternal methylated state of the differentially methylated domain after implantation, whereas the *H19*/*Igf2* studies establish that the paternal IC is dispensable for continued paternal *H19* silencing.

Snrpn, *Snord116*, *Snord115*, and *Ube3a-as* RNAs are generated from primary transcripts with a common initiation site (15–17, 30–32). Expression of these genes is reduced following Nes-cre-mediated deletion, most likely because of the absence of the major promoter at *Snrpn* exon 1 rather than epigenetic modifications of the locus. As previously suggested, residual transcription of these genes is likely to initiate at several upstream promoters that splice into *Snrpn* exon 2 or into acceptor sites located further downstream (18).

Conditional deletion of the PWS-IC with Nes-cre results in adults that are smaller than WT littermates. The only significant alteration in PWS gene expression is a reduction in *Snrpn* and the snoRNAs, and an increase in paternal expression of *Ube3a*. In this regard, these animals resemble other models lacking expression of the *Snrpn* transcription unit as a result of a germline deletion of the PWS-IC or a deletion spanning from *Snrpn* to *Ube3a* (18, 33, 34). In the present PWS-IC^{flox} model, the *Snrpn* U exons drive expression of all of the downstream cluster gene products indicating that the upstream exons are not exclusively dedicated to a particular processing variant. Conditional deletion of the PWS-IC with Nes-cre suggests that even modest changes in the level of the *Snrpn* transcription unit can significantly affect body weight. Previous studies have also shown that components of the large *Snrpn* to *Ube3a-as* transcription unit affect body weight (18, 21, 34–36). Inheritance of a *Snrpn* promoter that is not maternally silenced causes an increase in downstream transcription products and a significant increase in body weight (21). Unlike previous PWS-IC mutants, conditional deletion in brain progenitors of the PWS-IC by Nes-cre does not result in neonatal lethality or overt signs of neonatal failure to thrive. These results suggest that the small size of these mutants is not a result of neonatal failure to thrive but rather a reduction in the postweaning activity of the snoRNAs, as growth retardation is characteristic of other mouse models featuring reduced *Snord116* expression (34–36).

Materials and Methods

Animals. All animal procedures were previously approved by the University of Florida Institutional Animal Care and Use Committee. The PWS-IC^{flox6kb} allele was created as previously described (5). B6.C-Tg (Cmv-cre)1Cgn/J (stock no. 006054) and B6.C-Tg(Nes-cre)1Kln/J (stock no. 006054) were obtained from the Jackson Laboratory. The Nes-cre females were used after 13 to 15 generations of backcross to C57BL/6. The B6.*cast*.c7 line has been previously described (20). Animal weights are expressed as averages ± SE. Statistical significance was determined by unpaired Student *t* test.

Gene Expression Analysis. Northern Blot analysis and RT-PCR were performed as previously described (5). RT-PCR for the *Snrpn* upstream exon use was performed as described previously (18). Northern blot band intensity was digitized on the Storm 860 PhosphorImager (GE Healthcare) and analyzed with ImageQuant TL software.

DNA Methylation Analysis. DNA was isolated from newborn brain, digested with the indicated restriction endonucleases, and analyzed by Southern blot as previously described (5). Probe details are available on request.

ACKNOWLEDGMENTS. The authors thank Theresa Strong, Rob Nicholls, Tom Yang, and Brian Harfe for helpful comments on the manuscript; Deb Morse and Ryan Hallett for technical assistance; Ryan Fiske of the University of Florida Mouse Models Core; and Susan D'Costa, Carlos Sulsona, and Justin Bickford. This work was supported by grants from the Foundation for Prader–Willi Research and National Institute of Child Health and Human Development Grant R01 HD 037872. E.Y.S. was supported by a University of Florida Alumni Fellowship.

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