Mechanisms of activation of the paternally expressed genes by the Prader-Willi imprinting center in the Prader-Willi/Angelman syndromes domains

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Edited* by Arthur D. Riggs, Beckman Research Institute of the City of Hope, Duarte, CA, and approved March 21, 2012 (received for review October 11, 2011)

The Prader-Willi syndrome/Angelman syndrome (PWS/AS) imprinted domain is regulated by a bipartite imprinting control center (IC) composed of a sequence around the *SNRPN* promoter (PWS-IC) and a 880-bp sequence located 35 kb upstream (AS-IC). The AS-IC imprint is established during gametogenesis and confers repression upon PWS-IC on the maternal allele. Mutation at PWS-IC on the paternal allele leads to gene silencing across the entire PWS/AS domain. This silencing implies that PWS-IC functions on the paternal allele as a bidirectional activator. Here we examine the mechanism by which PWS-IC activates the paternally expressed genes (PEGs) using transgenes that include the PWS-IC sequence in the presence or absence of AS-IC and *NDN*, an upstream PEG, as an experimental model. We demonstrate that PWS-IC is in fact an activator of *NDN*. This activation requires an unmethylated PWS-IC in the gametes and during early embryogenesis. PWS-IC is dispensable later in development. Interestingly, a similar activation of a nonimprinted gene (*APOA1*) was observed, implying that PWS-IC is a universal activator. To decipher the mechanism by which PWS-IC confers activation of remote genes, we performed methylated DNA immunoprecipitation (MeDIP) array analysis on lymphoblast cell lines that revealed dispersed, rather than continued differential methylation. However, chromatin conformation capture (3c) experiments revealed a physical interaction between PWS-IC and the PEGs, suggesting that activation of PEGs may require their proximity to PWS-IC.

DNA methylation | epigenetics | genomic imprinting

Genomic imprinting is an epigenetic developmental process by which around 100 imprinted genes are monoallelically expressed in a parent-of-origin–specific manner, so that the paternal and maternal alleles are differentially expressed (1). The chromosomal region 15q11–q13 contains a cluster of imprinted genes within a 2.0-Mb domain. This cluster of genes includes the paternally expressed genes (PEGs) *MKRN3*, *MAGEL2*, *NDN*, *SNURF-SNRPN*, and a large number of downstream genes encoding C/D box snoRNAs (2) and a single maternally expressed gene, the ubiquitin ligase gene (*UBE3A*), located at the 3′ end of the domain (Fig. 1) (3). These same genes are present and imprinted on mouse Chr 7.

Failure to establish a proper imprint of this region in humans results in the neurobehavioral disorders, Prader-Willi syndrome (PWS) and Angelman syndrome (AS) (reviewed in ref. 4). PWS is thought to be a contiguous gene syndrome with several paternally expressed genes as candidates involved in causing the disorder. AS can be caused by the absence of maternal expression of the *UBE3A* gene (3, 5–7). Molecular genetic analyses in individuals with PWS and AS and analysis of the corresponding mouse models provided important insights into regulation of imprinted gene expression in the PWS/AS region.

The coordinated regulation of the imprinted genes within the PWS/AS domain is mediated by a bipartite imprinting center (IC), composed of PWS-IC, a 4.3-kb sequence, which includes the *SNRPN* promoter/exon 1 and AS-IC, a 880-bp sequence, which is located 35 kb upstream of PWS-IC (6, 8). AS-IC, active on the maternal allele, confers imprinting on PWS-IC in the female gametes, which is maintained throughout embryo development, executing differential expression programs for the two parental alleles (4), by methylation of PWS-IC on the maternal allele.

We hypothesized that PWS-IC functions on the paternal allele as a bidirectional activator that controls the expression of the PEGs and indirectly controls the maternally expressed *UBE3A* gene by activating its antisense (9). Deletion or loss of function of PWS-IC on the paternal allele leads to abnormal methylation of the entire 2-Mb domain and inactivation of all PEGs, causing PWS (6). Deletion of a 6.0-kb sequence spanning *SNRPN* exon 1 in the mouse was recently shown to exhibit a complete PWS-IC deletion phenotype, as well (10). Deletion or inactivation of AS-IC on the maternal allele leads to an abnormal methylation pattern of PWS-IC, leading to activation of the paternally repressed genes, including the *UBE3A* antisense gene, thus inactivation of *UBE3A* and causing AS (4).

Using a transgenic experimental system, we demonstrate here that indeed PWS-IC functions as an activator of the PEGs on the paternal allele. Importantly, we found that PWS-IC is required to activate the PEGs in the male gametes and in the very early embryo but is dispensable later in development. This activation is sequence independent because PWS-IC is capable of conferring imprinting on an unimprinted gene such as *APOA1*. The requirement for PWS-IC activating genes appears to be physical proximity, as judged by the chromatin conformation capture (3c) experimental approach used here.

Results

To study how PWS-IC activates the genes on the paternal allele across the PWS/AS domain, we chose *NDN* as a representative gene, which is monoallelically expressed mainly in brain (11), being simple, intronless, and a member of the upstream PEG cluster, which includes *NDN*, *MKRN3*, and *MAGEL2*, and is believed to be a target of the PWS-IC activating function (Fig. 1). First, we analyzed allele-specific DNA methylation patterns of *NDN* in several human somatic tissues and cell lines by Southern blotting (Fig. 2 *A* and *B*). Two bands representing the 2.3-kb and 1.9-kb DNA fragments were observed in fibroblast and brain DNA, indicating that *NDN* is imprinted in these tissues. In human sperm and fibroblasts of an AS patient carrying a paternal duplication of Chr 15, a single 1.9-kb band was observed, whereas in fibroblasts of PWS patients carrying a maternal duplication of Chr 15, only a 2.3-kb band was

Author contributions: A.R. and R.S. designed research; S.R. performed research; Y.K. performed the 3c experiment; G.L. analyzed data; and S.R., A.R., and R.S. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1116661109/-/DCSupplemental.

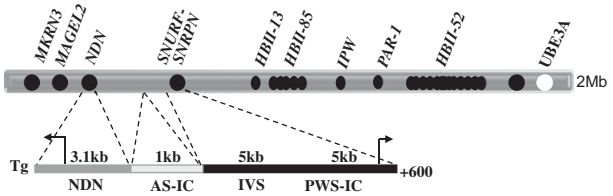

Fig. 1. Schematic presentation of the 2-Mb PWS/AS imprinted domain on human Chr 15q11–13 (not to scale). Paternally expressed imprinted genes are in black. The maternally expressed UBE3A gene is in white. (*Lower*) Schematic presentation of the transgene (Tg) includes 5 kb of the PWS-IC region and 5 kb of intervening sequence (IVS), (in black), 1 kb of AS-IC (in white) and the 3.1-kb sequence of *NDN* (in gray). Arrows represent transcription start sites of *NDN* and *SNRPN*, respectively.

observed, indicating that *NDN* is monoallelically methylated on the maternal allele (Fig. 2*B*).

To understand the process of activation of *NDN*, we made use of a transgenic system. First, we generated a transgene that harbors the *NDN* sequence including its promoter. This transgene was completely methylated in tail and brain DNA upon both paternal and maternal transmissions, as indicated by Southern blot analysis (Fig. 2*C*) and verified by bisulfite analysis (Fig. 2*D*).

These results are in accord with previous observations that activation of *NDN* is controlled by PWS-IC. Microdeletion of PWS-IC on the paternal chromosome leads to loss of monoallelic methylation of *NDN*. In PWS patients with such a microdeletion, *NDN* is completely methylated on both parental copies, indicating that by default *NDN* is methylated (12). It was therefore suggested that *NDN* becomes demethylated and activated on the paternal allele by PWS-IC (9). Therefore, we generated a transgene that carries *NDN* ligated to the PWS-IC sequence, separated by an intervening sequence of 5 kb (presented together in black in Fig. 3*A*). Using this transgene we

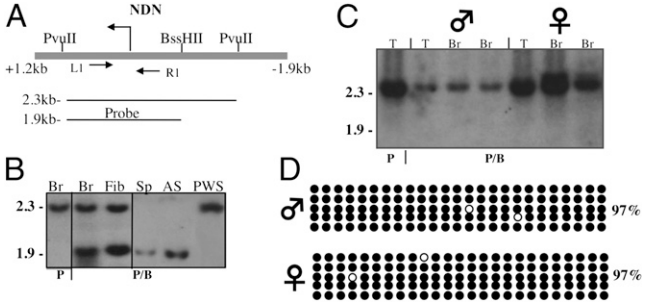

Fig. 2. DNA methylation status of *NDN* in various human tissues and in the *NDN* transgene. (*A*) Schematic presentation of the 3.1=kb human *NDN* sequence that was used to generate the *NDN* transgene (three different lines with 5–20 copy numbers were used). Arrow represents *NDN* transcription start site. PvuII and BssHII designate restriction sites. The 2.3-kb PvuII fragment represents methylated *NDN*. The 1.9-kb PvuII/BssHII fragment represents unmethylated *NDN* (unmethylated BssHII site) and also served to prepare a radiolabeled probe for the Southern blot analysis. Horizontal arrows represent the primers L1 and R1 for the bisulfite assay. (*B*) Southern blot analysis of the methylation status of *NDN* in human cells and tissue DNAs. Br, brain; Fib, fibroblasts; Sp, sperm; AS, AS fibroblasts; PWS, PWS fibroblasts; P, PvuII restriction; P/B, PvuII/BssHII restriction. (*C*) Southern blot analysis of the methylation status of the *NDN* transgene. Lanes 1–3 are three different lines of paternal (♂) transmission; lanes 4–6 are three different lines of maternal (♀) transmission. DNA samples from tail (lanes 1 and 4) or brain (lanes 2, 3, 5, and 6). P, PvuII fragment; P/B, PvuII/BssHII fragment. (*D*) Methylation state of the *NDN* transgene was analyzed in brain by the bisulfite assay upon paternal (♂) and maternal (♀) transmission. Each horizontal line represents one clone; each circle represents one CpG site. Open circle, unmethylated site; solid circle, methylated site.

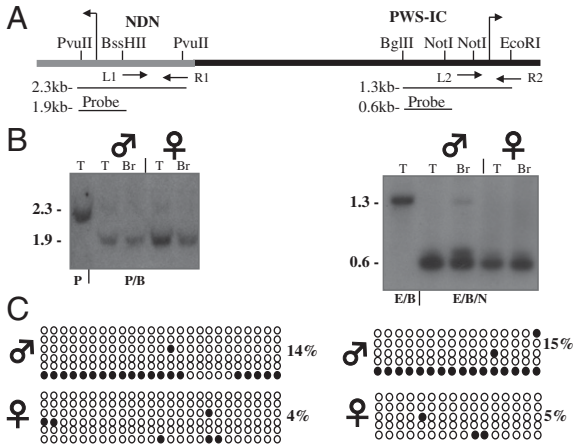

Fig. 3. DNA methylation status of the *NDN*–PWS-IC transgene. (*A*) Schematic presentation of the transgene. 5-kb PWS-IC sequence and 5-kb intervening sequence (IVS) (black) and 3.1-kb *NDN* gene sequence (gray). Arrows represent transcription start sites of *SNRPN* and *NDN* genes, respectively. PvuII, BssHII, BglII, NotI, and EcoRI designate restriction sites. (*Left*) The 2.3-kb PvuII fragment represents methylated *NDN*. The 1.9-kb PvuII/BssHII fragment represents unmethylated *NDN* gene and also served to prepare a radiolabeled probe for the Southern blot. (*Right*) The 1.3-kb EcoRI/BglII fragment represents methylated PWS-IC. The 0.6-kb EcoRI/ BglII/NotI fragment represents unmethylated PWS-IC and also served to prepare a radiolabeled probe for the Southern blot. Horizontal arrows L1, R1 and L2, R2 represent primers for the bisulfite assay for *NDN* gene and PWS-IC, respectively. (*B*) Southern blot analysis of *NDN* (*Left*) and PWS-IC (*Right*). Lanes 1 and 2 represent two different lines of paternal (♂) transmission; lanes 3 and 4 represent two different lines of maternal (♀) transmission. T, tail; Br, brain. (*Left*) *NDN* gene. P, PvuII restriction; P/B, PvuII/BssHII restriction. (*Right*) PWS-IC. E/B, EcoRI/BglII restriction; E/B/*N*, EcoRI/BglII/NotI restriction. (*C*) DNA methylation status of *NDN* (*Left*) and PWS-IC (*Right*) analyzed by the bisulfite assay in brain upon paternal (♂) and maternal (♀) transmission. Details are as in Fig. 2 legend.

observed that the *NDN* and the PWS-IC sequences are unmethylated upon both parental transmissions, as indicated by the 1.9-kb band for *NDN* and the 0.6-kb band for PWS-IC in the corresponding Southern blots (Fig. 3*B*) and by the bisulfite methylation analysis (Fig. 3*C*). These results are in accord with our prediction that PWS-IC confers an active and unmethylated state upon *NDN* on the paternal allele and presumably on the other PEGs.

Because PWS-IC alone did not seem to imprint *NDN* (Fig. 3 *B* and *C*), we made use of a transgene "AS-IC–PWS-IC," which includes both the human AS-IC and PWS-IC sequences and was shown previously to be recognized by the mouse imprinting machinery recapitulating the human imprinting process. In offspring of the transgenic mice, PWS-IC was unmethylated when transmitted paternally and methylated when transmitted maternally (13). Here, we inserted the *NDN* construct into the AS-IC–PWS-IC transgene upstream of AS-IC in an inverted orientation, to recapitulate the situation in vivo (Fig. 4*A*) to examine whether the human AS-IC and PWS-IC are capable of conferring imprinting of the imprinted genes in the region. In this transgene ("*NDN*–AS-IC–PWS-IC"), we found that *NDN* was unmethylated upon paternal transmission (1.9-kb band) and methylated upon maternal transmission (2.3-kb band), as was the PWS-IC sequence (1.3-kb and 0.6-kb bands, respectively) (Fig. 4*B*). These results, verified by the bisulfite assay (Fig. 4*C*), imply that the imprinted state of *NDN* is determined by the imprinting center that includes both AS-IC and PWS-IC.

It has been previously shown that *NDN* promoter is hypomethylated in human oocytes (12), implicating that in human, the maternal imprinted methylation is established postfertilization

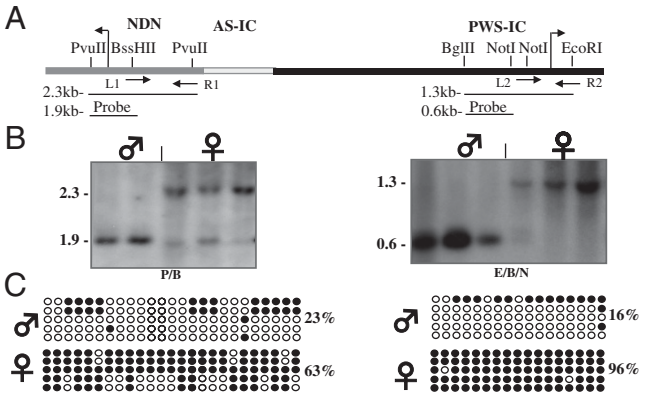

Fig. 4. DNA methylation status of the *NDN–AS-IC–PWS-IC* transgene. (*A*) Schematic presentation of the transgene. The 5-kb PWS-IC and 5-kb intervening sequence (black), 1-kb AS-IC sequence (white) and 3.1-kb *NDN* sequence (gray) (four different lines with 5–30 copy numbers were used). Arrows represent transcription start sites of the *NDN* and *SNRPN* genes, respectively. PvuII, BssHII, BglII, Not1, and EcoRI designate restriction sites. (*Left*) The 2.3-kb PvuII fragment represents methylated *NDN*. The 1.9-kb PvuII/BssHII fragment represents unmethylated *NDN* to prepare a radiolabeled probe for the Southern blot. (*Right*) The 1.3-kb EcoRI/BglII fragment represents methylated PWS-IC. The 0.6-kb EcoRI/BglII/NotI fragment represents unmethylated PWS-IC and also served to prepare a radiolabeled probe for the Southern blot. Horizontal arrows L1, R1 and L2, R2 represent primers for the bisulfite assay for *NDN* gene and PWS-IC, respectively. (*B*) Southern blot analysis of tail tissue DNA of *NDN* (*Left*) and PWS-IC (*Right*). Lanes 1–3 represent three different lines of paternal (♂) transmission and lanes 4–6 represent two different lines of maternal (♀) transmission. Details are as in Fig. 3 legend. (*C*) Methylation status of *NDN* (left) and PWS-IC (right) was analyzed in brain by the bisulfite assay upon paternal (♂) and maternal (♀) transmission. Details are as in Fig. 2 legend.

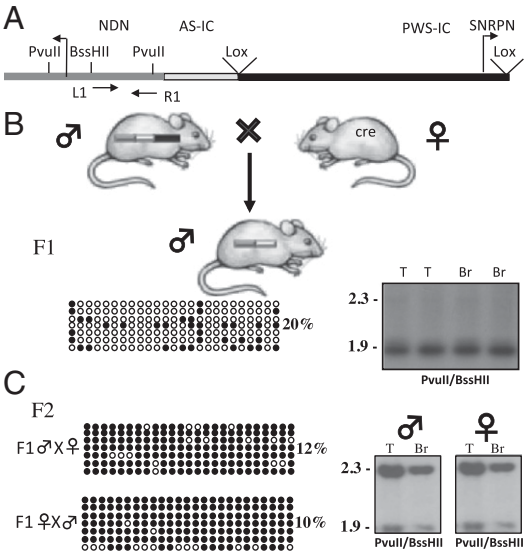

Fig. 5. DNA methylation status of the *NDN–AS-IC–PWS-IC* with floxed PWS-IC transgene. (*A*) Schematic presentation of the transgene. The lox sequences were inserted at the 3′ ends of PWS-IC and AS-IC. Arrows represent transcription start site of *NDN* and *SNRPN*, respectively. (*B*) Male carrying the transgene was mated with female carrying the Cre gene (three different transgenic lines with 20–40 copy numbers were used). Southern blotting analysis was performed on tail (T) and brain (Br) of two different lines of the F_1 progeny of F_1. Brain was also analyzed by the bisulfite method. (*C*) Results of methylation analysis by bisulfite and Southern blot of F_2 brain (Br) and tail (T) obtained by mating of a F_1 male transgene with normal female (F_1 ♂ × ♀) or F_1 female transgene with normal male (F_1 ♀ × ♂). Horizontal arrows L1 and R1 represent primers for the bisulfite assay. Details of results are as described in Fig. 2 legend.

and not in the gametes. The latter interpretation is corroborated by a previous observation that in the mouse the methylation of *NDN*, which prevails in the oocyte, is lost by the blastocyst stage (14), and the monoallelic methylation is probably reestablished postimplantation as is the case for other imprinted genes (15). We assumed that *NDN* acquires its differential methylation under PWS-IC control later in development. Next we asked whether PWS-IC is essential for *NDN* activation only during spermatogenesis and early embryo development, or alternatively, PWS-IC is also required to maintain the active state of *NDN* during late embryogenesis and in adult life. To answer this question we used transgenic mice in which the PWS-IC sequence in the *NDN–AS-IC–PWS-IC* transgene was floxed (Fig. 5*A*). To ensure that the lox sequences do not interfere with the imprinting process, we analyzed the methylation of *NDN* and PWS-IC in progeny of this transgene when crossed with a wild-type mouse, and found that the lox sequences did not interfere with the imprinting process. In the next experiment a male carrying the *NDN–AS-IC–PWS-IC* transgene with floxed PWS-IC was mated with a PGK–Cre-harboring female (Fig. 5*B*). It should be noted that PGK–Cre is expressed ubiquitously in all organs including oocytes (16). An F_1 male progeny of this mating lost the PWS-IC sequence in a very early stage of development, yet *NDN* remained unmethylated as shown by the Southern blot (1.9-kb band) and verified by bisulfite analysis (Fig. 5*B*). PWS-IC was excluded perfectly and no mosaic methylation was observed. As a control the NDN–AS-IC construct established in F_1 progeny was passed through the male and female gametes and found to be methylated as expected (Fig. 5*C*)

These results indicated that, whereas PWS-IC is essential for preventing *NDN* methylation during spermatogenesis or early embryogenesis, it is dispensable later in development.

To examine whether the activation of *NDN* by PWS-IC is sequence specific, we generated a transgenic construct that contains PWS-IC and AS-IC fused to the human nonimprinted apolipoprotein AI gene (*APOA1*) (Fig. 6*A*). *APOA1* is a tissue-specific gene that is unmethylated and expressed in a tissue-specific manner in liver and intestine. The human *APOA1* in transgenic mice was previously shown to be specifically unmethylated in liver but methylated in other tissues including brain and tail (17). Surprisingly, the transgene that includes AS-IC, PWS-IC, and the *APOA1* promoter showed that *APOA1* became imprinted, unmethylated upon paternal transmission (1.4-kb band), and methylated upon maternal transmission (2-kb band) in brain (Fig. 6*B*, *Left*). A similar methylation profile was observed for PWS-IC (0.6-kb and 1.3-kb bands, respectively) (Fig. 6*B*, *Right*). The results of this experiment were reproducible in three transgenic lines of paternal and three lines of maternal transmission and verified by bisulfite analysis (Fig. 6*C*). This experiment suggests that the activation of the genes by PWS-IC on the paternal allele is sequence independent, presumably acting without discrimination on adjacent gene sequences.

Having shown that activation of *NDN* depends on PWS-IC prompted us to ask whether this activation in vivo involves spreading of the epigenetic status of PWS-IC, namely, the differential methylation or, alternatively, is achieved by a physical interaction between PWS-IC and the remote genes. To address this question, we used first immunoprecipitation methylation analysis (MeDIP) followed by genomic tiling arrays (*SI Materials and Methods*). We took advantage of the fact that uniparental disomy (UPD) provides a system that allows the two parental chromosomes to be studied separately. Thus, we used lymphoblast DNA of AS and PWS patients with paternal or maternal disomies, respectively (Mat, maternal allele; Pat, paternal allele in Fig. 7*A*). We found that 133 kb of region A (Chr 15: 21,351,000–21,484,000), which includes the PEGs and the 944 kb of region B, located between the PEG and PWS-IC (Chr 15:

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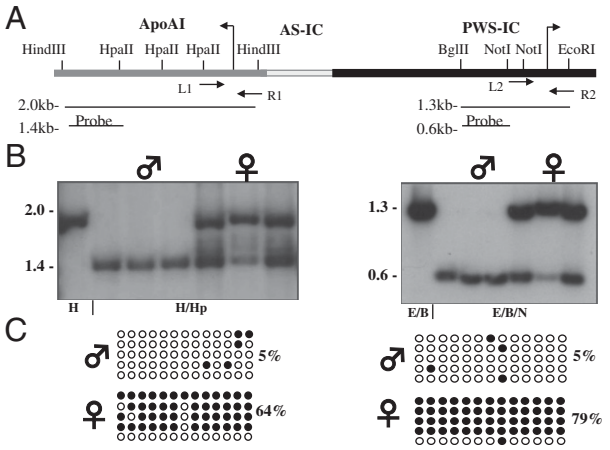

Fig. 6. DNA methylation status of the *APOAI*–AS-IC–PWS-IC transgene. (*A*) Schematic presentation of the transgene. The 5-kb PWS-IC and 5-kb intervening sequence (black), 1-kb AS-IC (white) and 2 kb of the human *APOA1* promoter (light gray) (three different lines with 10–40 copy numbers were used. Arrows represent transcription start sites of *SNRPN* and *APOA1*, respectively. HindIII, HpaII, BglII, NotI, and EcoRI designate restriction sites. (*Left*) The 2-kb HindIII fragment represents methylated *APOAI*. The 1.4-kb HindIII/HpaII fragment represents unmethylated *ApoAI* served to prepare a radiolabeled probe for Southern blot. (*Right*) The 1.3-kb EcoRI/BglII fragment represents methylated PWS-IC. The 0.6-kb EcoRI/BglII/NotI fragment represents unmethylated PWS-IC and to prepare a radiolabeled probe for the Southern blot. Horizontal arrows L1, R1 and L2, R2 represent primers for the bisulfite assay of APOA1 and PWS-IC, respectively. (*B*) Southern blot analysis of brain tissue DNA of the *APOAI* gene and PWS-IC. Lanes 1–3 represent three different lines of paternal (♂) transmission; lanes 4–6 are two different lines of maternal (♀) transmission. (*Left*) *APOAI* gene. H, HindIII restriction; H/Hp, HindIII/HpaII restriction. (*Right*) PWS-IC. E/B, EcoRI/BglII restriction; E/B/N, EcoRI/BglII/NotI restriction. (*C*) DNA methylation of the *APOAI* gene (*Left*) and PWS-IC (*Right*) was analyzed in brain by the bisulfite assay upon paternal (♂) and maternal (♀) transmission. Details of results are as described in Fig. 2 legend.

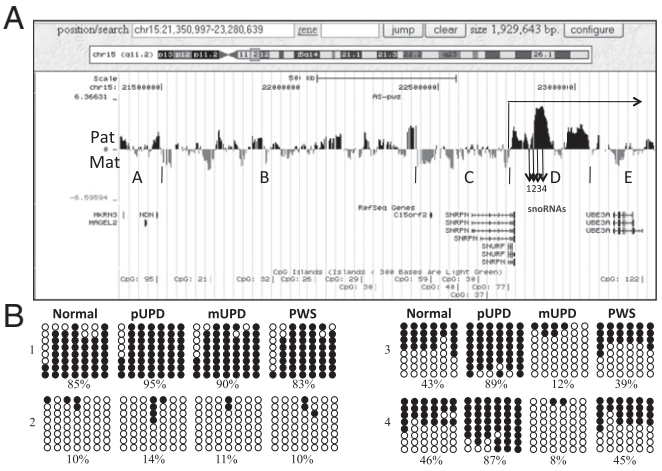

Fig. 7. (*A*) MeDIP analysis of the entire PWS/AS domain. Identification of differentially methylated regions within Chr 15q21.35–23.28. Image shows the smoothed mean $\log_2$ ratios (running average of 10) from paternal UPD15, and from maternal UPD15, the difference obtained by subtracting these two profiles, identified by statistical analysis uploaded as a custom track in the University of California Santa Cruz Genome Browser. Regions of positive differences represent paternally methylated regions (black) and regions of negative differences represent maternally methylated regions (gray). At the *Top* are shown the genomic coordinates (hg18). The horizontal arrow represents the long-range transcript that includes *SNRPN* SnoRNAs and *UBE3A* antisense. Gene names are shown in the *Middle* and CpG islands are shown at the *Bottom*. (*B*) DNA methylation of four CpG clusters 1–4 located at the Snord gene area (designated as vertical arrows in Fig. 7*A*). The corresponding coordinates: (arrow 1) 22,855,026–22,855,363; (arrow 2) 22,865,546–22,865,826; (arrow 3) 22,885,703–22,886,111; (arrow 4) 22,882,902–22,888,351) were analyzed by the bisulfite assay on lymphoblast DNA of normal (normal) and of patients carrying paternal uniparental disomy (pUPD) or maternal uniparental disomy (mUPD) of Chr 15 and of a PWS patient carrying a 45-kb deletion around PWS-IC (PWS) on the paternal allele.

21,484,000–22,428,000), reveal dispersed differential methylation. Although the results cannot ultimately support a spreading mechanism, they do not rule out such a mechanism in the embryo, especially in light of the fact that our results are based on adult cells (Fig. 7*A*). However, the 331 kb of region C (Chr 15: 22,428,000–22,759,000), which includes the PWS-IC sequence, *SNRPN* promoter and upstream sequences, and the regions 3′ to PWS-IC, were methylated on the maternal allele as expected (Fig. 7*A*). Region D, which includes 345 kb (Chr 15: 22,759,000–23,104,000) and contains small nuclear RNAs (snoRNAs) PAR-SN, SNORD107, PAR5, SNORD64, SNORD108, SNORD109, SNORD116, IPW, PAR1, PAR4, SNORD115, HBII-52, and HBII-52, was massively methylated on the paternal allele. This region was previously reported to be transcribed continuously on the paternal allele, starting at the *SNRPN* promoter and running all of the way through the region (2). The function of this unprecedented massive methylation on the transcribed allele remains unexplained. However, this methylation seems to be irrelevant to the imprint of the region because the deletion of PWS-IC did not affect this methylation as judged by the results (Fig. 7*B*) of bisulfite methylation analysis carried out on 10 CpG clusters, 4 of which are designated by arrows 1–4, as presented in Fig. 7*A*. The bisulfite analysis was carried out on lymphoblast DNA of normal individuals and PWS patients carrying a maternal UPD (mUPD) or paternal UPD (pUPD) of Chr 15 or lymphoblasts of a PWS patient carrying a paternal deletion of 45 kb that includes PWS-IC sequences (6). This differential methylation seen in sites 3 and 4 (Fig. 7*B*) was not affected by the deletion of PWS-IC (compare PWS with normal in Fig. 7*B*, sites 3 and 4). The *UBE3A* gene and its flanking sequence, total of 133 kb of region E (Chr 15: 23,104,000–23,237,000) is preferentially methylated on the maternal allele. It should be noted that *UBE3A* promoter is unmethylated on both parental alleles (18) and is regulated by antisense RNA, which is part of the long-range transcript on the paternal allele (horizontal arrow, Fig. 7*A*). In conclusion, the MeDIP analysis reveals a dispersed differential methylation in the region upstream of PWS-IC; therefore, communication of PWS-IC with the remote PEGs by a spreading mechanism is doubtful. However, having used fully differentiated lymphoblast cell lines in these experiments it remains possible that spreading might have occurred earlier and receded by the time of assay. It should be noted that the spreading mechanism was also ruled out in the Igf2r region (19), which showed that the active chromatin is interspersed through modifications localized exclusively on regulatory elements flanking active proteins.

To examine the possibility of physical interaction between PWS-IC and the PEG region, we made use of the 3c method developed by Dekker and colleagues (20) and used successfully by Reik and colleagues (21). We used BglII sites on PWS-IC and the PEG domain as anchor points for genomic interactions. To discriminate between the maternal and paternal alleles, we used lymphoblasts from a Prader-Willi patient and his father, who carried a deletion in their PWS-IC on one chromosome (PWS-U family) and from an Angelman patient and his mother, who were deleted in AS-IC (AS-D family). Although NDN is not expressed in these lymphoblasts, it is differentially methylated (11). Chromatin of these lymphoblast cells was subjected to the 3c procedure (*SI Materials and Methods*). A 180-bp–specific PCR product was observed using primers L1 and L2 in the AS-D patient but not in the PWS-U patient, indicating an interaction on the paternal allele between a BglII site in PWS-IC and a BglII

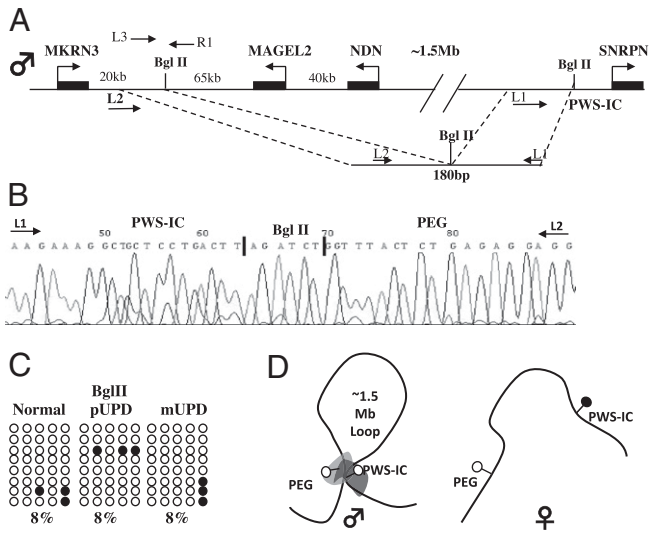

Fig. 8. Physical interaction between PWS-IC and the PEG cluster. (*A*) Schematic presentation of the PWS/AS domain. PWS-IC is at the *Right*; the PEG cluster is at the *Left*. BglII sites used in the 3c experiment are noted. PCR primers flanking the BglII sites are L1 (for PWS-IC) and L2 (for PEG). PCR primers for bisulphite analysis of the BglII site at the PEG region are L3 and R1. The 180-bp hybrid PCR product obtained is magnified at the *Bottom*. (*B*) Sequence of the hybrid PCR product composed of PWS-IC and PEG, with the BglII site at the *Middle*. Primer orientation is noted at the *Top*. The product was obtained from lymphoblast of an Angelman patient (AS-D family) harboring only the paternal PWS-IC. (*C*) Methylation states of the BglII anchor site within the PEG cluster region in normal, paternal UPD (pUPD) and maternal UPD (mUPD) DNAs as analyzed by the bisulfite assay. (*D*) Proposed genomic structure of the paternal domain (♂), which is unmethylated (empty lollipops), with proteins (dark and gray ovals), mediating an interaction between PWS-IC and PEG, looping out the intervening sequence (1.5 Mb). On the maternal chromosome, where PWS-IC is methylated (filled lollipops), no PCR product was detected and presumably no interaction takes place.

site located ~1.5 Mb upstream, between the imprinted *MKRN3* and *MAGEL2* genes (Fig. 8 *A* and *B*). This conclusion was verified by sequence analysis. This BglII site is located 20 kb 3′ to the *MKRN3* gene and 65 kb and 100 kb 5′ to the *MAGEL2* and *NDN* genes, respectively. Numerous BglII sites that lie in the 1.5-Mb intervening region and sites located 3′ to PWS-IC all the way down to the *UBE3A* gene did not reveal interactions with PWS-IC in either cell line. This observation clearly indicates that PWS-IC interacts specifically only with the gene cluster (PEGs) upstream of PWS-IC.

We next asked what controls this interaction. We analyzed methylation of 500 bp surrounding the BglII site in the upstream PEG cluster (Fig. 8*A*) and in DNA samples of normal and PWS and AS patients with mUPD or pUPD of Chr 15. In all DNA samples, the CpGs were unmethylated (Fig. 8*C*), indicating that this region is not differentially methylated. This result implies that the interaction between PWS-IC and the upstream PEGs is controlled only by the unmethylated state of PWS-IC, as only unmethylated PWS-IC on the paternal allele enables this interaction (Fig. 8*D*). This experiment should be interpreted with caution, because it does not prove yet that this interaction is required for the establishment of the imprint.

Discussion

It has previously been suggested that PWS-IC activates bidirectionally the imprinted genes on the paternal chromosome at the PWS/AS domain (9). One gene cluster located at the 5′ end of the domain contains the PEGs (*MKRN3*, *MAGEL2*, and *NDN*). Another gene cluster containing numerous paternally expressed snoRNA genes and the paternally expressed *UBE3A* antisense gene is located at the 3′ half of the domain. As part of our efforts to better understand how PWS-IC confers imprinting and activates the remote PEGs at the 5′ end of the PWS/AS domain, we designed transgenic experiments with the PEG *NDN* as a representative target gene. The results of these experiments allowed us to arrive at several important conclusions. The default state of the PEGs is methylated and silenced, the status when PWS-IC is inactive. PWS-IC in its active unmethylated state is required to establish an active unmethylated state of the PEGs in the gametes and early in development. However, PWS-IC is dispensable later in development, as judged by the experiment where PWS-IC was excised postfertilization (Fig. 5). This result is in accord with a previous observation in a minor PWS patient, which is a mosaic for a deletion of PWS-IC (22). The deletion occurred on his paternal allele in one of the cells at the blastomere stage. The mutated chromosome has acquired a maternal methylation imprint in somatic cells, indicating that the PWS-IC element is required for the establishment of the paternal imprint in the germ line. The deletion of PWS-IC in our transgenic mice had no effect on the imprinting of the domain. The deletion must have occurred in an early embryonic stage because the Cre gene is expressed already in the oocyte (16). Because the Cre gene is expressed constitutively, it rules out mosaic. On the basis of the results obtained by both experiments, it can be suggested that PWS-IC is required in the gametes and is dispensable later in development.

Interestingly, we found that PWS-IC in its active state confers imprinting independent of the target gene sequence. *APOAI*, known to be unimprinted, became unmethylated on the paternal chromosome under the control of the unmethylated active PWS-IC. This surprising result raised the question of how PWS-IC confers imprinting on neighboring sequences. The communication between PWS-IC and the remote PEGs could theoretically be achieved either by spreading of the imprint across the upstream 1.5-Mb intervening sequence or by physical interaction between PWS-IC and the target PEGs, looping out the 1.5-Mb intervening sequence. Performing MeDIP analysis followed by tiling array suggested that the paternal activation of the PEGs after fertilization during somatic development might not involve linear spreading of the unmethylated state from PWS-IC throughout the domain (Fig. 7). However, spreading could have occurred earlier and receded by the time of assay. The 3c assay suggested that the communication of PWS-IC with the remote PEGs is rather through a long-range chromatin interaction between PWS-IC and a site located between the genes *MKRN3* and *MAGEL2* (Fig. 8*A*), 20 kb 3′ to *MKRN3* and 65 kb and 105 kb 5′ to *MAGEL2* and *NDN*, respectively. This site of interaction could play a role as an anchor to establish proximity between PWS-IC and the PEGs. This proximity is required in conferring activation upon the PEGs by PWS-IC. However, this site does not seem to have a role in the gene activation process per se. This long-range chromatin interaction is controlled by the active state of PWS-IC, as the interaction site in the PEG cluster is unmethylated on both alleles (Fig. 8*D*). This conclusion can also be made in light of the fact that in the transgenic experiments *NDN* and *APOA1* were artificially brought to close proximity with PWS-IC, where the interaction site found in vivo was not included. Although this experiment suggested interaction between PWS-IC and the remote genes, the molecular mechanism by which PWS-IC confers imprinting on the remote genes remains elusive, because the site with which PWS-IC interacts is within an intergenic sequence (Fig. 8). As the interaction between PWS-IC and the remote gene cluster is specifically on the paternal allele, it strongly suggests that this interaction can serve to prevent methylation at this region and thereby activation of PEGs.

Unlike the 5′ part of the domain where PWS-IC may confer imprinting by physical interaction, the long-range effect of PWS-

IC on the imprinting of the 3′ part of the PWS/AS domain is very likely explained by the previous observation of a 460-kb continuous transcript that contains at least 148 exons, starting at the *SNRPN* promoter and hosts the *SNURF-SNRPN*, snoRNAs, and *UBE3A* antisense transcription unit that directly controls the maternally exclusive expression of UBE3A by inhibiting the paternal expression of UBE3A (Fig. 7, horizontal arrow) (2). It is likely that the regulation of this long transcript involves a binding protein such as MeCP2, which we have previously shown to bind PWS-IC and control the UBE3A antisense transcription (18). The long transcript serves as a host for the snoRNAs that are encoded within introns of this transcript (23), and most if not all these snoRNAs are indeed expressed by processing the long-range transcript. In conclusion, we suggest two different strategies that PWS-IC uses to activate the paternal genes across the 2-Mb PWS/AS domain. Activation of the remote genes upstream of PWS-IC may be achieved by a physical interaction of PWS-IC with the PEG cluster domain. The genes downstream of PWS-IC are probably expressed through a continuously long transcript starting at the paternally active PWS-IC.

Materials and Methods

Further detailed descriptions of the methods used appear in the figure legends. A detailed description of the preparation of constructs and generation of the transgenic mice appears in *SI Materials and Methods*. The bisulfite methylation analysis, MeDIP-on-ChIP procedure, and the 3c method are also described in detail in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Ben-Zion Tzuberi for help in generating transgenic mice. This study was supported by Israel Science Foundation Grant 104/06 and March of Dimes Foundation Grant 6-FY05-7.

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