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Research Review

Mechanisms of Imprinting of the Prader–Willi/Angelman Region

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Prader–Willi syndrome (PWS) and Angelman syndrome (AS) are two distinct neurodevelopmental disorders, each caused by several genetic and epigenetic mechanisms involving the proximal long arm of chromosome 15. Lack of a functional paternal copy of 15q11–q13 causes PWS; lack of a functional maternal copy of *UBE3A*, a gene within 15q11–q13, causes AS. This region of chromosome 15 contains a number of imprinted genes that are coordinately regulated by an imprinting center (PWS/AS-IC) that contains two functional elements, the PWS-SRO and the AS-SRO. A chromosome lacking the PWS-SRO has the maternal state of gene activity and epigenetic modification after either maternal or paternal transmission; a chromosome lacking the AS-SRO but containing the PWS-SRO has the paternal state of gene activity and epigenetic modification after either maternal or paternal transmission. The maternal state of chromosome 15q11–q13 is associated with methylation of the PWS-SRO, while the paternal state is associated with lack of methylation of the PWS-SRO. Although most models of PWS/AS region imprinting assume that the PWS-SRO is methylated during oogenesis and that this methylation of the maternal PWS-SRO is maintained after fertilization, several lines of evidence suggest that the maternal PWS-SRO is in fact not methylated until after fertilization. Imprinting defects affecting the PWS/AS region can arise from failure to demethylate the PWS-SRO in the male germ line, from failure to methylate the maternal PWS-SRO, or from failure to maintain PWS-SRO methylation after fertilization. © 2008 Wiley-Liss, Inc.

Key words: Prader–Willi syndrome; Angelman syndrome; imprinting; deletion; *UBE3A*; epigenetic; methylation; histone; antisense

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INTRODUCTION

Prader–Willi syndrome (PWS) and Angelman syndrome (AS) are phenotypically distinct disorders involving imprinted genes in 15q11–q13. Molecular genetic analysis of individuals with PWS and AS phenotypes has revealed multiple genetic and epigenetic etiologies for each of these syndromes, and has led to hypotheses regarding the mechanisms of imprinting in the 15q11–q13 region that have been and are being tested using mice with targeted genetic modifications.

Individuals with PWS show neonatal hypotonia and failure to thrive, hyperphagia in early childhood leading to obesity, hypogonadism, short stature, behavior problems, and mild to moderate mental retardation [Goldstone, 2004]. Approximately 70% of individuals with PWS have an ~6 Mb de novo interstitial deletion of 15q11–q13 on the paternal chromosome 15. Most of the remaining PWS individuals (~29%) have maternal uniparental disomy (UPD) of chromosome 15, which, as with paternal 15q11–q13 deletion, causes lack of a paternal 15q11–q13 region. Approximately 1% of individuals with PWS have inherited chromosome 15 homologs from mother and father, but the paternal chromosome carries a maternal imprint, referred to as an imprinting defect.

Individuals with AS exhibit microcephaly, ataxia, severe mental retardation, seizure disorder, absence of speech, and sleep disorder [Williams et al., 2006]. As in PWS, ~70% of AS individuals have large de novo deletions of 15q11–q13, but in AS individuals, these de novo deletions are always of maternal origin. Five to 10% of AS individuals have point

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mutations of the *UBE3A* gene within 15q11–q13, which has led to identification of *UBE3A* as the AS gene. Three-5% of AS individuals have an imprinting defect, with a maternally inherited chromosome 15 that has a paternal imprint, and 1–2% of AS individuals have paternal UPD of chromosome 15.

The 15q11–q13 chromosomal region that is implicated in the pathogenesis of PWS and AS, and the homologous region of mouse chromosome 7, contain a number of genes that show parental-origin-dependent expression or imprinting (Fig. 1). Some of these genes are expressed exclusively from the paternal chromosome, whereas other genes show preferential or tissue-specific expression from either the paternal or maternal chromosome. Other genes within the region show biallelic expression with no evidence of imprinting effects. Molecular genetic analysis in individuals with PWS and AS, and analysis of mouse models, has provided important insights into the complex mechanisms of imprinted gene expression in the PWS/AS region, which involve multiple target imprinted genes, *cis*-acting sites, *trans*-acting factors, and noncoding RNAs.

IMPRINTED GENES IN THE PWS/AS REGION OF 15q11–q13

The PWS/AS region contains a number of genes that are expressed exclusively from the paternal chromosome 15; these genes include *MKRN3*, *MAGEL2*, *NDN*, *SNURF-SNRPN* (referred to hereafter as *SNRPN*), and more than 70 genes encoding C/D box snoRNAs. These genes are also imprinted in the mouse. In both human and mouse, there is evidence for a single large transcriptional unit (>460 kb in human, ~1,000 kb in mouse) that includes the *SNRPN* sense transcript, the transcript from which snoRNAs are processed, and an antisense transcript to the *UBE3A* locus [Runte et al., 2001; Landers et al., 2004].

Another gene, *C15orf2*, encodes an 1156-amino-acid protein of unknown function and is expressed in adult testis and fetal brain [Buiting et al., 2007]. Testis expression is biallelic, whereas fetal brain expression is monoallelic, possibly indicating that it is imprinted. *C15orf2* is present in humans and other primates but is not conserved in mice. The *PWRN1* gene also shows biallelic expression in adult human testis and kidney, but its expression is monoallelic in human fetal brain.

UBE3A, identified as the AS gene [Kishino et al., 1997; Matsuura et al., 1997], shows biallelic expression in most tissues but shows preferential expression of the maternal allele in brains of humans and mice. RNA in situ hybridization studies of mice with paternal UPD for the region of chromosome 7 containing *Ube3a* or with maternal knockout of *Ube3a* showed region-specific imprinting of *Ube3a*,

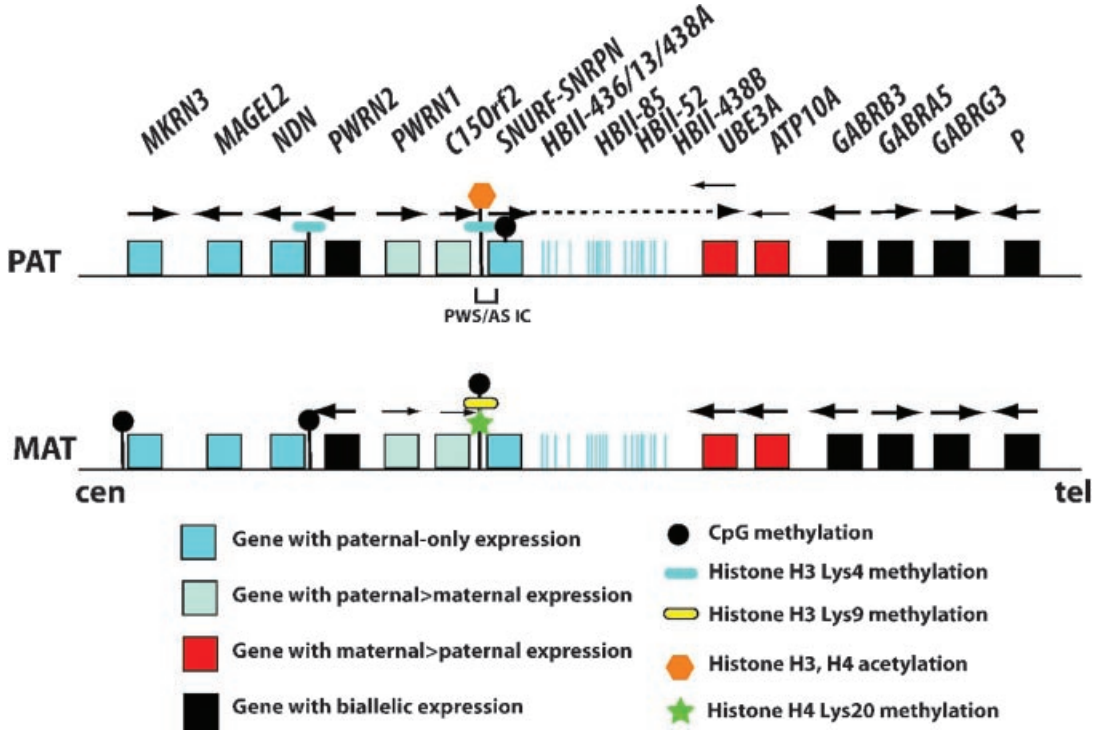

FIG. 1. Imprinted gene expression and epigenetic marks in human chromosome region 15q11–q13. Color coding of boxes indicates whether genes show paternal-only expression, paternal > maternal expression, maternal > paternal expression, or equal expression of paternal and maternal alleles. *PWRN1* and *C15Orf2* show monoallelic expression in human fetal brain, but parental origin of the monoallelic expression has not been determined; because these genes are within a cluster of genes with paternal-only expression, these genes are color coded as paternal > maternal. Parent-specific epigenetic modifications are shown as symbols on vertical black lines. Figure is not drawn to scale.

with no expression of the paternal *Ube3a* allele in hippocampus and cerebellar Purkinje cells, but with equal expression of maternal and paternal *Ube3a* alleles in other brain regions [Albrecht et al., 1997; Jiang et al., 1998]. Yamasaki et al. [2003] used primary neuronal and glial cultures from fetal mouse brains to show that *Ube3a* imprinting is limited to cultured neurons, whereas cultured glial cells show biallelic expression. Dindot et al. [2007] recently used *Ube3a-YFP* (yellow-fluorescent protein) knock-in mice to show that *Ube3a* is imprinted in neurons throughout the brain and is expressed biallelically in glial cells lining the ventricles.

ATP10A is expressed preferentially from the maternal chromosome in human brain and fibroblasts [Herzing et al., 2001; Meguro et al., 2001]. There are conflicting reports as to whether *Atp10a* is imprinted in the mouse [Kashiwagi et al., 2003; Kayashima et al., 2003].

PARENT-SPECIFIC EPIGENETIC MODIFICATIONS IN THE PWS/AS REGION

Silencing of the maternal alleles of *MKRN3*, *NDN*, and *SNRPN* is associated with maternal-specific CpG methylation of the promoter/exon 1 regions of these genes [Driscoll et al., 1992; Glenn et al., 1996; Jay et al., 1997] (Fig. 1). There is also a differentially methylated region in intron 7 of the *SNRPN* locus that is methylated only on the paternal chromosome [Glenn et al., 1996]. In the mouse, the *Snrpn* promoter/exon 1 region is CpG methylated in the oocyte but not in sperm, and this maternal methylation is maintained through early development into adulthood [Shemer et al., 1997], whereas the other regions that show differential methylation in somatic tissues either do not show differential methylation in gametes or their differential methylation inherited from gametes is lost during preimplantation development then reestablished. Therefore, in the mouse, maternal *Snrpn* promoter/exon 1 methylation appears to be a primary gametic imprint for the PWS/AS region, while differential methylation at other sites appears to be a secondary modification that occurs after fertilization. In humans, the *SNRPN* promoter is unmethylated in sperm, but the CpG methylation status of the *SNRPN* promoter in oocytes is unclear. El-Maarri et al. [2001] found that the *SNRPN* promoter/exon 1 region is unmethylated in human oocytes, and they concluded that methylation of the maternal copy of this region must occur after fertilization. By contrast, Geuns et al. [2003] found that the *SNRPN* promoter/exon 1 region is heavily methylated in human oocytes. The reason for the discrepant results from these two studies is probably related to the technical difficulty of performing bisulfite genomic sequencing on the small numbers of human oocytes available for these studies.

In addition to parent-specific DNA methylation, several regions within the PWS/AS region show parent-specific histone modification in somatic cells (Fig. 1). The *SNRPN* promoter region shows paternal-specific histone H3 and H4 acetylation [Saitoh and Wada, 2000; Fulmer-Smentek and Francke, 2001], maternal-specific methylation of histone H3 on Lys9 and of histone H4 on Lys20 [Xin et al., 2001; Fournier et al., 2002; Wu et al., 2006b], and paternal-specific methylation of histone H3 on Lys4 [Xin et al., 2001; Fournier et al., 2002]. The *NDN* promoter region also shows paternal-specific association with histone H3 methylated on Lys4 [Xin et al., 2001; Lau et al., 2004]. The pattern of parental-origin-specific DNA methylation and histone modification of the *SNRPN* promoter region, with maternal-specific DNA methylation, histone H3 Lys9 methylation, and histone H4 Lys20 methylation, and with paternal-specific histone H3 Lys4 methylation, has been observed for a number of imprinting control regions in mouse and human [Mikkelsen et al., 2007].

cis-ACTING SEQUENCES INVOLVED IN PWS/AS IMPRINTING

As described above, approximately 1% of PWS individuals have inherited a paternal copy of chromosome 15 that shows a maternal pattern of imprinted gene expression and epigenetic modification of 15q11–q13, and 3–5% of AS individuals have inherited a maternal copy of chromosome 15 that shows a paternal pattern of imprinted gene expression and epigenetic modification. These individuals are generally recognized by Southern blot or PCR assays for methylation of the *SNRPN* promoter/exon1 region. Somatic cells from normal individuals contain one methylated copy of this region (maternal) and one unmethylated copy of this region (paternal). Individuals with PWS generally have no unmethylated copy of this region, as a result of either large paternal deletion of 15q11–q13 or maternal UPD 15. PWS individuals who have no unmethylated copy of the *SNRPN* promoter/exon 1 region but who do not have either large paternal deletion or maternal UPD are defined as being affected by imprinting defects, with a paternal chromosome 15q11–q13 that shows a maternal pattern of epigenetic modification and gene expression. Most (85%) of PWS imprinting defects are primary epimutations, with no evidence of a DNA sequence change in 15q11–q13 [Buiting et al., 2003]. In these individuals, both copies of the *SNRPN* promoter region are methylated, and the promoter regions of *NDN* and *MKRN3* are also methylated on both the maternal and paternal chromosome 15 homologs. The other 15% have deletions of variable sizes, all of which include the *SNRPN* promoter/exon 1 region. The shortest region of overlap of these deletions is a 4.1 kb region that includes the *SNRPN* promoter [Ohta et al., 1999;

Horsthemke and Buiting, 2006]. This region has been defined as the PWS shortest region of overlap (PWS-SRO) of the PWS/AS imprinting center (PWS/AS-IC), which also includes the AS-SRO, located ~35 kb centromeric to the PWS-SRO (Fig. 2).

Analysis of a male with somatic mosaicism for deletion of the paternal PWS-SRO has shown that the PWS-SRO is required for maintenance of the paternal pattern of epigenetic modification and gene expression [Bielinska et al., 2000]. In this male, somatic cells that have lost the paternal PWS-SRO become methylated on the *NDN* promoter and presumably lose expression of *MKRN3*, *MAGEL2*, *NDN*, and *SNRPN*.

Is the PWS-SRO required for establishment of the paternal pattern of epigenetic modification and gene expression? Sperm from males who have inherited a PWS-SRO deletion from their mothers cannot, of course, be assayed for methylation of the deleted PWS-SRO, but these sperm show normal lack of methylation of the *NDN* promoter [El-Maarri et al., 2001]. After fertilization of a normal oocyte with a PWS-SRO-deletion-bearing sperm, normal paternal expression of *MKRN3*, *MAGEL2*, *NDN*, and *SNRPN* cannot be established and the paternal *MKRN3* and *NDN* promoters become methylated.

A 42-kb targeted deletion of mouse *Snrpn* exons 1–6 plus 23 kb of upstream sequence led to a maternal imprint on paternally transmitted chromosomes carrying the deletion, analogous to human PWS-SRO deletions [Yang et al., 1998] (Fig. 2). By contrast, paternal transmission of a 0.9 kb targeted deletion of the mouse *Snrpn* major promoter and exon 1 region constructed by Bressler et al. [2001] had no effect on imprinting of *Mkrn3*, *Ndn*, *Magel2*, or *Ube3a*. Paternal transmission of a larger, 4.8 kb, deletion led to mosaic imprinting defects and to postnatal lethality in about 50% of mutant mice

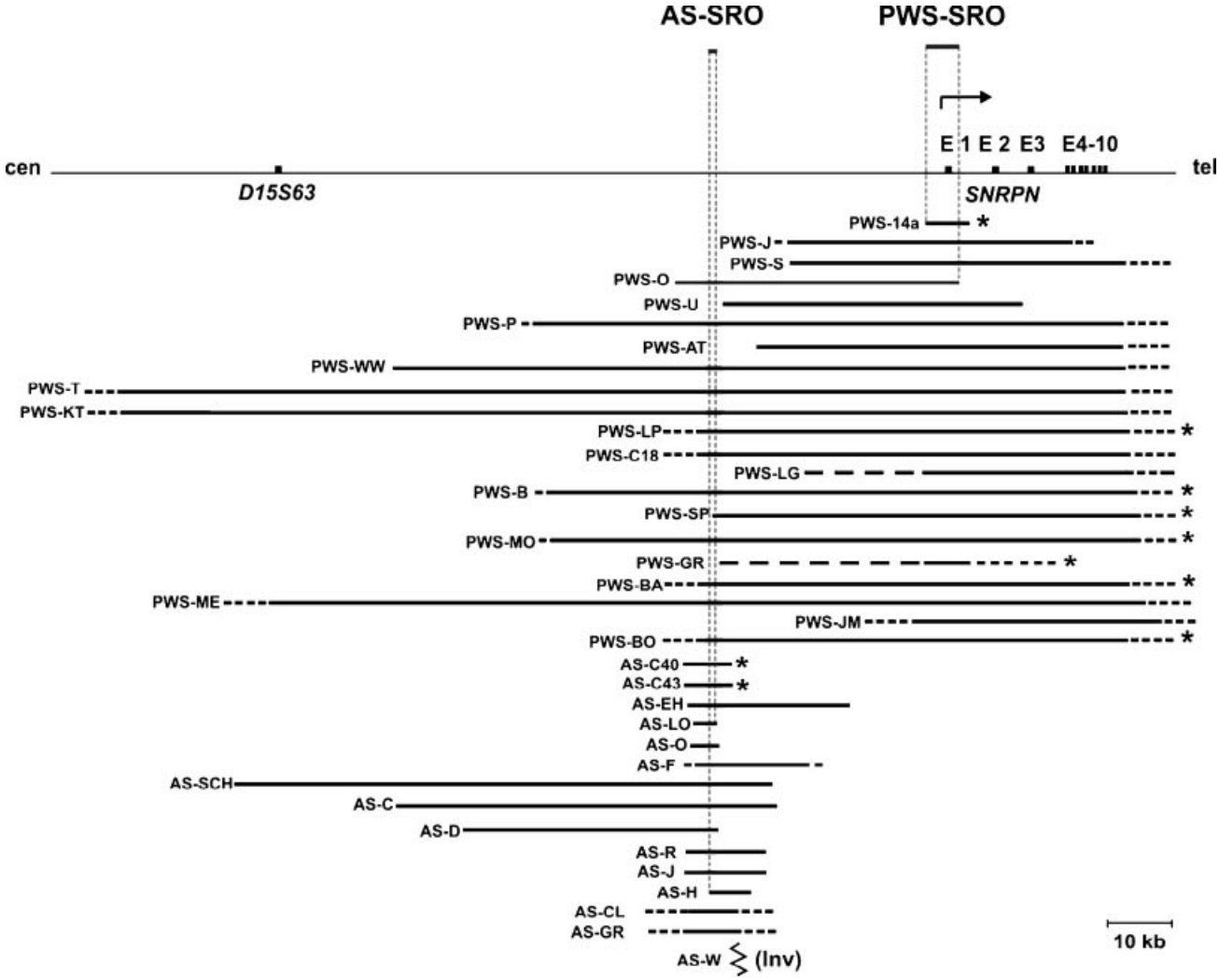

FIG. 2. Microdeletions of the PWS/AS imprinting center that define the PWS-SRO (shortest region of overlap of IC deletions in PWS patients) and the AS-SRO (shortest region of overlap of IC deletions in AS patients). The PWS-SRO overlaps the major promoter and exon 1 of *SNRPN*. The AS-SRO is 35 kb centromeric of the PWS-SRO. Asterisks (*) denote de novo deletions (This figure was published in Advances in Genetics, Vol. 61, Horsthemke B and Buiting K, Genomic imprinting and imprinting defects in humans, p 225–246, Copyright Elsevier Inc. (2008).).

[Bressler et al., 2001]. No targeted deletions intermediate in size between the 4.8 kb deletion, which leads to a partial imprinting defect, and the 42 kb deletion, which leads to a complete imprinting defect, have been reported. The observation that the 0.9 kb deletion leads to almost complete lack of *Snrpn* transcription and has no effect on imprinting of *Mkrn3*, *Ndn*, *Magel2*, or *Ube3a* indicates that transcription from the major *Snrpn* promoter is not required either for paternal activation of *Mkrn3*, *Ndn*, or *Magel2* transcription or for paternal silencing of *Ube3a* transcription in brain. There are, however, upstream alternative *Snrpn* promoters from which a low level of transcription is initiated [Bressler et al., 2001; Landers et al., 2004], and it is possible that transcripts from these alternative promoters may be involved in paternal activation of *Mkrn3*, *Ndn*, and *Magel2* and in paternal silencing of *Ube3a* in brain.

As described above, 3–5% of AS individuals have paternal-only methylation pattern of the *SNRPN* promoter/exon 1 region but do not have large maternal 15q11–q13 deletions or paternal UPD 15. In these individuals, both copies of the *SNRPN* promoter region are unmethylated, and the promoter regions of *NDN* and *MKRN3* are also unmethylated on both the maternal and paternal chromosome 15 homologs. As with PWS imprinting defects, a small fraction (~10%) of these individuals have deletions near *SNRPN*, while the remainder have primary imprinting defects with no evidence of DNA sequence changes in 15q11–q13 [Buiting et al., 2003]. The deletions in AS imprinting defect individuals, in contrast to those in PWS imprinting defect individuals, do not include *SNRPN* promoter/exon 1, but are located centromeric to the PWS-SRO. The smallest region of deletion overlap of these AS imprinting defect deletions, the AS-SRO, is an 880-bp region 35 kb proximal to *SNRPN* exon 1 [Buiting et al., 1999]. This 880-bp region contains upstream exons u5 and u6 of *SNRPN*, which are present in a very small fraction of *SNRPN* transcripts. Transmission of a deletion chromosome through either the female or male germ line leads to a paternal pattern of epigenetic modification and gene expression for the chromosome, so that AS results only when the deleted chromosome is transmitted maternally. There is one familial case of imprinting defect AS in which there is no AS-SRO deletion, but the AS-SRO is separated from the PWS-SRO by a paracentric inversion that spans ~1.5 Mb [Buiting et al., 2001].

Attempts to define a region homologous to the AS-SRO by targeted mutagenesis in the mouse have been unsuccessful. Wu et al. [2006a] constructed mice with a deletion extending from 13 to 93 kb 5′ of *Snrpn* exon 1. When this deletion was transmitted maternally, some offspring showed normal *Snrpn* methylation, some showed reduced methylation, and some showed no methylation of the *Snrpn* 5′ CpG island. Peery et al. [2007] analyzed mice with targeted deletion of the region from 24 to 37 kb 5′ of *Snrpn* exon 1and found normal methylation and imprinted gene expression after maternal transmission of the deletion chromosome. These results indicate that, by contrast to the human situation, there is no sequence between −13 and −93 kb from *Snrpn* exon 1 that is absolutely required for establishment of the maternal pattern of epigenetic modification and gene expression for the mouse PWS/AS region. Peery et al. note, however, that they have analyzed a mouse BAC containing 100 kb of sequence 5′ to *Snrpn* exon 1 that shows normal imprinting when introduced into mice as a single-copy transgene; this result, if confirmed, would indicate the presence of sequence(s) sufficient for establishment of the maternal pattern of epigenetic modification and gene expression for the mouse PWS/AS region within 100 kb 5′ to *Snrpn* exon 1. Wu et al. [2006a] described a duplication/insertion mutation 13 kb 5′ to *Snrpn* exon 1 that causes loss of *Snrpn* methylation, decreased *Ndn* methylation, and decreased *Ube3a* expression when transmitted maternally, and has no effect on methylation or imprinted gene expression when transmitted paternally. It is possible that this duplication/insertion does not remove or disrupt an imprinting control region, but acts as a barrier to the interaction of AS-SRO-like elements 5′ to *Snrpn* with PWS-SRO-like elements in the *Snrpn* promoter/exon 1 region.

Some PWS imprinting defect patients have deletions that include both the PWS-SRO and the AS-SRO. Either maternal or paternal transmission of these deletions leads to a maternal pattern of epigenetic modification and imprinted expression of PWS/AS region genes. The epistasis of the PWS-SRO deletion to the AS-SRO deletion indicates that the PWS-SRO is unconditionally required for a chromosome to have the paternal pattern of epigenetic modification and gene expression, while the AS-SRO is required for a chromosome to have the maternal pattern of epigenetic modification and gene expression only if the chromosome has an intact PWS-SRO. In terms of the PWS/AS-IC, the maternal pattern of gene expression and epigenetic modification, with CpG methylation of the *SNRPN*, *MKRN3*, and *NDN* promoters and silencing of those genes, is the default state of 15q11–q13 and does not require any PWS/AS-IC sequences.

Most patients with PWS or AS imprinting defects (85–90%) do not have IC deletions, and do not have any sequence alteration of the PWS-SRO or AS-SRO. These imprinting defects are therefore described as *primary imprinting defects*, and are thought to represent low-frequency random errors in the imprinting process [Buiting et al., 2003]. These imprinting defects never show familial recurrence, although certain AS-SRO sequence variants may increase the risk of an imprinting defect [Zogel et al., 2006]. Approximately one-third of AS primary

imprinting defects show somatic mosaicism for a methylated maternal *SNRPN* promoter/exon 1 region [Nazlican et al., 2004]; somatic mosaicism for methylation in PWS primary imprinting defects is much rarer, probably occurring in less than 5%. Buiting et al. [2003] have shown that AS primary imprinting defects occur with equal frequency on the grandmaternal and grandpaternal chromosome 15, whereas PWS primary imprinting defects occur only on the chromosome 15 inherited from the paternal grandmother. This observation suggests that PWS primary imprinting defects result from failure to erase the grandmaternal imprint in the paternal germ line, whereas AS primary imprinting defects arise from failure to impose the maternal imprint after imprint erasure in the maternal germ line, or from failure to maintain the maternal imprint after fertilization.

Complementary to studies of PWS-SRO and AS-SRO by targeted deletions in the mouse are studies of these *cis*-acting elements by analysis of transgenic mice. Shemer et al. [2000] showed that a transgene containing 1.0 kb of the human AS-SRO fused to 0.2 kb of the mouse *Snrpn* promoter (homologous to human PWS-SRO) showed correct parent-of-origin-dependent methylation of the PWS-SRO region after maternal transmission and lack of methylation after paternal transmission; this maternal methylation was dependent on presence of the AS-SRO in the transgene. They subsequently found that a transgene containing the human PWS-SRO and the human AS-SRO showed correct methylation, and that Cre-mediated deletion of the AS-SRO in somatic cells did not lead to loss of methylation, indicating that the AS-SRO is required for establishment but not for maintenance of maternal PWS-SRO methylation [Kantor et al., 2004]. They also showed that this multicopy transgene is not methylated in either oocyte or sperm, but that after maternal transmission of the transgene, the PWS-SRO acquires methylation after the blastula stage. This observation of post-fertilization methylation of the human PWS-SRO in transgenic mice provides support for the observation of El-Maarri et al. [2001] that the human PWS-SRO is not methylated in oocytes but is methylated on the maternal allele after fertilization.

trans-ACTING FACTORS INVOLVED IN PWS/AS IMPRINTING

Establishment and maintenance of parent-specific epigenetic modifications and patterns of gene expression presumably involve interaction of proteins or noncoding RNAs with the PWS-SRO, the AS-SRO, and with target imprinted genes in germ cells and in somatic cells. Kantor et al. [2004] have taken initial steps toward identifying proteins that bind to the PWS-SRO and the AS-SRO. However, most of what we know about proteins involved in PWS/AS region imprinting has come from analysis of mice with targeted inactivation of candidate imprinting factors.

Bourc'his et al. [2001] analyzed mice with targeted inactivation of the *Dnmt3L* gene, which encodes a protein with sequence similarity to DNA methyltransferases that does not have enzymatic activity. Males homozygous for an inactivated allele of *Dnmt3L* show azoospermia. Female homozygotes mated to wild-type males produce embryos that die before midgestation. Bisulfite genomic sequencing of DNA from these embryos shows lack of methylation of imprinting control regions, including the *Snrpn* promoter region, that are normally maternally methylated. These embryos also show biallelic expression of genes that are normally expressed only from the paternal allele, indicating that *Dnmt3L* is required in the maternal germ line for methylation of the PWS-SRO region in mouse. Although *Dnmt3L* is highly expressed during mouse oogenesis, it is not expressed during human oogenesis and is only expressed after fertilization [Huntriss et al., 2004].

Kaneda et al. [2004] showed, using a germ-cell-specific conditional knockout, that the de novo DNA methyltransferase *Dnmt3a* is also required in the female germ line for methylation of the PWS-SRO and for silencing of expression of the maternal *Snrpn* allele in early embryos. The other de novo DNA methyltransferase, *Dnmt3b*, is not required for methylation of the maternal PWS-SRO.

Wu et al. [2006b] found that mice homozygous for deletion of the *Arid4a* gene and heterozygous for deletion of the *Arid4b* gene showed reduced DNA methylation, reduced histone H3 Lys9 methylation, and reduced histone H4 Lys20 methylation of the maternal PWS-SRO.

ES cells homozygous for a targeted mutation of the *G9a* histone H3 Lys9 methyltransferase gene were found by Xin et al. [2003] to have reduced H3 Lys9 methylation of the PWS-SRO, complete loss of DNA methylation of the PWS-SRO, and biallelic expression of *Snrpn*. However, day 8.5 pc embryos lacking G9a activity had normal DNA methylation of the PWS-SRO. Xin et al. also showed that ES cells homozygous for an inactivated allele of *Dnmt1*, the major maintenance DNA methyltransferase, retained normal levels of methylated histone H3 Lys 9 and normal monoallelic expression of *Snrpn*. These results suggest that histone methylation is more important than DNA methylation in ES cells for maintenance of silencing of the maternal *Snrpn* allele.

Samaco et al. [2005] reported that *MECP2* deficiency in the brains of individuals with Rett syndrome leads to decreased expression of *UBE3A*, and Makedonski et al. [2005] found that mice with *Mecp2* mutations had biallelic expression of *Ube3a* antisense RNA and decreased expression of *Ube3a* in brain. However, Jordan and Francke [2006] found no decrease in *Ube3a* expression in *Mecp2*-deficient

mouse brains, so that the question of whether *Mecp2* is involved in regulation of *Ube3a* remains unresolved.

Zogel et al. [2006] found that the frequency of the 677C > T variant of the *MTHFR* (5,10-methylene tetrahydrofolate reductase) gene is significantly higher in mothers of children with AS imprinting defects than in their fathers or in the general population. MTHFR is a key regulatory enzyme in one-carbon metabolism and plays an important role in DNA synthesis and DNA methylation. It catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is used by methionine synthase for the methylation of homocysteine to form methionine. Methionine is the precursor of *S*-adenosyl-L-methionine (SAM), which serves as a methyl donor for DNA and histone methyltransferases. The 677C > T transition in the *MTHFR* gene results in a thermolabile protein with reduced enzymatic activity. Changes in MTHFR activity affect the levels of SAM and it is possible that reduced levels of SAM in female germ cells increase the risk that maternal methylation imprints are not or are not completely established.

If loss of function mutations in any of these genes led to imprinting defects in either the male or female germ line, then we might expect to see familial recurrence of PWS or AS imprinting defects with recessive inheritance. The fact that familial recurrence of primary PWS or AS imprinting defects has never been observed in human pedigrees would suggest that sequence variation in the genes encoding these *trans*-acting factors plays only a minor role in predisposition to imprinting defects.

Table I summarizes our knowledge of PWS/AS Imprinting Center.

TABLE I. Synopsis of the PWS/AS Imprinting Center (IC)

The IC is located upstream of the *SNURF-SNRPN* gene, spans ~35 kb of genomic DNA, and contains two distinct functional regions: the PWS-SRO and the AS-SRO
The maternal pattern of epigenetic modification and gene expression is the default state of 15q11–q13
The PWS-SRO is unconditionally required for a chromosome to have the paternal pattern of epigenetic modification and gene expression
The AS-SRO is required for a chromosome to have the maternal pattern of epigenetic modification and gene expression only if the chromosome has an intact PWS-SRO
The AS gene (*UBE3A*) has been identified, but the precise gene(s) for PWS are not yet delineated
Unlike several genes in 15q11–13, *UBE3A* is not associated with a differentially methylated region, but appears to be regulated by coordinated transcription of a relatively long, antisense DNA that overlaps *UBE3A*
Imprinting defects result from errors in imprint erasure in primordial germ cells, errors in imprint establishment in the gametes, or errors in imprint maintenance in postzygotic cells
Most of the imprinting defects are primary epimutations, which are not associated with an increased recurrence risk
Imprinting defects resulting from an inherited IC deletion are associated with a 50% recurrence risk
Abnormalities in *trans*-acting factors (e.g., DNA binding proteins) have not yet been identified as causing either PWS or AS

ANTISENSE RNA AND *UBE3A* IMPRINTING

UBE3A imprinting differs from imprinting of other genes in the PWS/AS region in two important ways: first, *UBE3A* imprinting is brain-specific and appears to be neuron-specific; and second, *UBE3A* does not show parent-specific promoter methylation or other parent-specific epigenetic modifications, even in brain. Rougeulle et al. [1998] identified brain-specific transcription of the paternal allele of *UBE3A* in antisense orientation, and hypothesized that this antisense transcription plays a role in silencing of the paternal *UBE3A* allele in brain. Chamberlain and Brannan [2001] showed that paternal transmission of a 42-kb deletion that includes the *Snrpn* promoter and 35 kb proximal to *Snrpn* causes loss of the *Ube3a* antisense transcript in mouse and leads to equal expression of maternal and paternal *Ube3a* alleles in brain. Runte et al. [2001] provided evidence that the *UBE3A* antisense transcript is the 3′ end of a long transcript that includes *SNRPN*, and more than 70 snoRNAs are processed from this long *SNRPN* sense-*UBE3A* antisense transcript. The major promoter for *SNRPN* transcription is immediately upstream from exon 1, and there are a number of minor promoters further upstream from exon 1, although their use for the *UBE3A* antisense transcript has not yet been analyzed in humans. Other promoters for the *UBE3A* antisense transcript closer to *UBE3A* have not been described.

The hypothesis that brain-specific silencing of the paternal *Ube3a* allele is a consequence of brain-specific paternal antisense transcription of *Ube3a* is supported by the observation of Chamberlain and Brannan [2001] that paternal transmission of the 42-kb deletion that includes the *Snrpn* promoter leads to loss of *Ube3a* imprinting. However, several observations are difficult to reconcile with the antisense hypothesis. First, the 0.9 kb *Snrpn* promoter deletion described by Bressler et al. [2001] causes almost complete loss of *Snrpn* transcription but it apparently causes no disruption of *Ube3a* imprinting when transmitted paternally. Second, Le Meur et al. [2005] examined *Ube3a* sense and antisense transcripts in adult mouse brains by in situ hybridization and found that regions of the brain in which the paternal allele of *Ube3a* is silenced did not contain antisense transcript. Third, Landers et al. [2005] found that *Ube3a* antisense levels were increased in mice with maternal inactivation of *Ube3a*, suggesting that *Ube3a* sense transcripts down-regulate *Ube3a* antisense transcript levels, rather than the reverse.

MODELS FOR PWS/AS REGION IMPRINTING

Although it is clear that the PWS-SRO is CpG-methylated in mouse oocytes and that this methylation

is maintained after fertilization into somatic lineages, the timing of PWS-SRO methylation is unclear in humans. Several lines of evidence support the hypothesis that human PWS-SRO methylation occurs after fertilization: (1) the observation of El-Maarri et al. [2001] that the PWS-SRO is not methylated in human oocytes; (2) the lack of expression of *DNMT3L* in the human oocyte lineage [Huntriss et al., 2004]; and (3) the postfertilization methylation of the PWS-SRO in human AS-SRO/human PWS-SRO transgenic mice [Kantor et al., 2004]. The reason for the discrepancy between results of El-Maarri et al. and those of Geuns et al. [2003] is not clear. In Figure 3, we present a model for interaction of the AS-SRO, the PWS-SRO, and *trans*-acting factors in de novo methylation of the maternal PWS-SRO. In this model, demethylases remove methyl groups from the PWS-SRO during both spermatogenesis and oogenesis. A protein or protein complex that is specific to the oocyte lineage binds to the AS-SRO and leads to an interaction of the AS-SRO and the PWS-SRO. After fertilization, interaction of this protein/protein complex with molecules present in the zygote leads to de novo methylation of the maternal PWS-SRO.

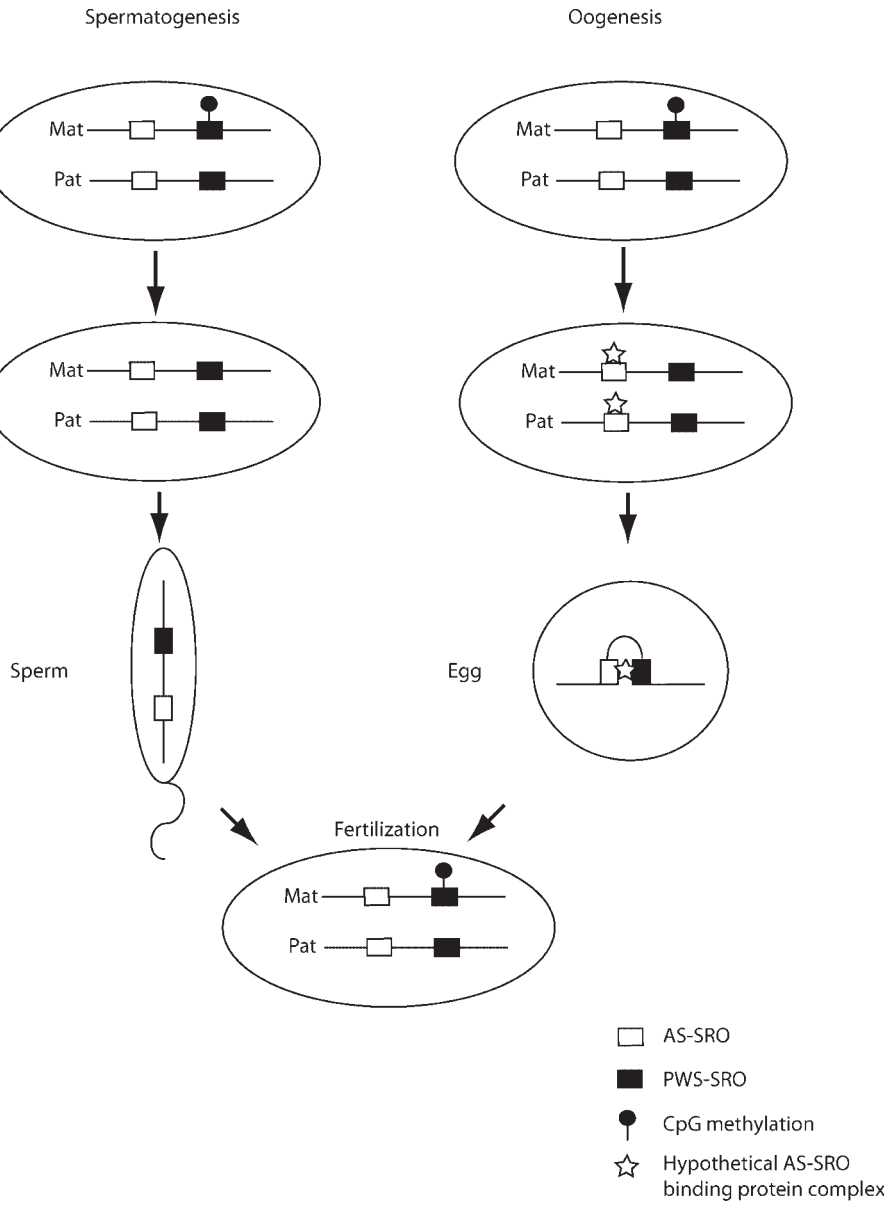

FIG. 3. Model for PWS/AS region imprint erasure and imprint establishment during gametogenesis and early embryogenesis. During spermatogenesis, CpG methylation is removed from the maternal PWS-SRO, which remains unmethylated. During oogenesis, CpG methylation is removed from the PWS-SRO. A protein complex containing at least one protein specific to the oocyte lineage associates with the AS-SRO then with the PWS-SRO. Soon after fertilization, this complex leads to CpG methylation of the maternal PWS-SRO.

After fertilization and establishment of methylation of the maternal PWS-SRO, the events that lead to differential gene expression on the maternal and paternal copies of 15q11–q13 reflect the presence of an active, unmethylated PWS-SRO on the paternal chromosome 15 and the absence of an active PWS-SRO on the maternal chromosome 15. The unmethylated paternal PWS-SRO acts as a promoter for the *SNRPN* transcriptional unit, which includes the snoRNA clusters and the *UBE3A* antisense transcript. The unmethylated paternal PWS-SRO acts at long distances to activate transcription of the *MKRN3*, *MAGEL2*, and *NDN* genes (Fig. 4). The mechanisms by which the PWS-SRO activates transcription of these genes are unclear. These mechanisms may include direct physical interactions of the *MKRN3*, *MAGEL2*, and *NDN* promoters with the PWS-SRO. Alternatively, chromatin structures or chromatin modifications may spread from the PWS-SRO to its target genes. In a formal sense, the unmethylated paternal PWS-SRO acts in cis to repress transcription of *UBE3A* in neurons; whether antisense transcription of *UBE3A* initiated at the PWS-SRO is necessary and sufficient for paternal neuron-specific silencing of *UBE3A* is unclear.

On the maternal chromosome, the AS-SRO and PWS-SRO play no direct role in silencing expression of *MKRN3*, *MAGEL2*, and *NDN*, as evidenced by the fact that chromosomes lacking both AS-SRO and PWS-SRO show no expression of *MKRN3*, *MAGEL2*, and *NDN* and show promoter methylation of *MKRN3* and *NDN*. The silencing of these genes is therefore their default state, at least in terms of AS-SRO and PWS-SRO. Whether there are other *cis*-acting elements required for silencing of these genes is unknown.

Errors in the imprinting process can occur at several different steps (Fig. 5). Failure to demethylate the PWS-SRO in the male germ line can lead to a sperm with PWS-SRO methylation, which will result in an offspring with PWS. Failure to methylate the maternal PWS-SRO after fertilization will result in an offspring with AS. Failure to maintain PWS-SRO methylation after fertilization will lead to a mosaic AS imprinting defect.

UNANSWERED QUESTIONS AND FUTURE RESEARCH DIRECTIONS

Major unanswered questions about the PWS/AS imprinting process include:

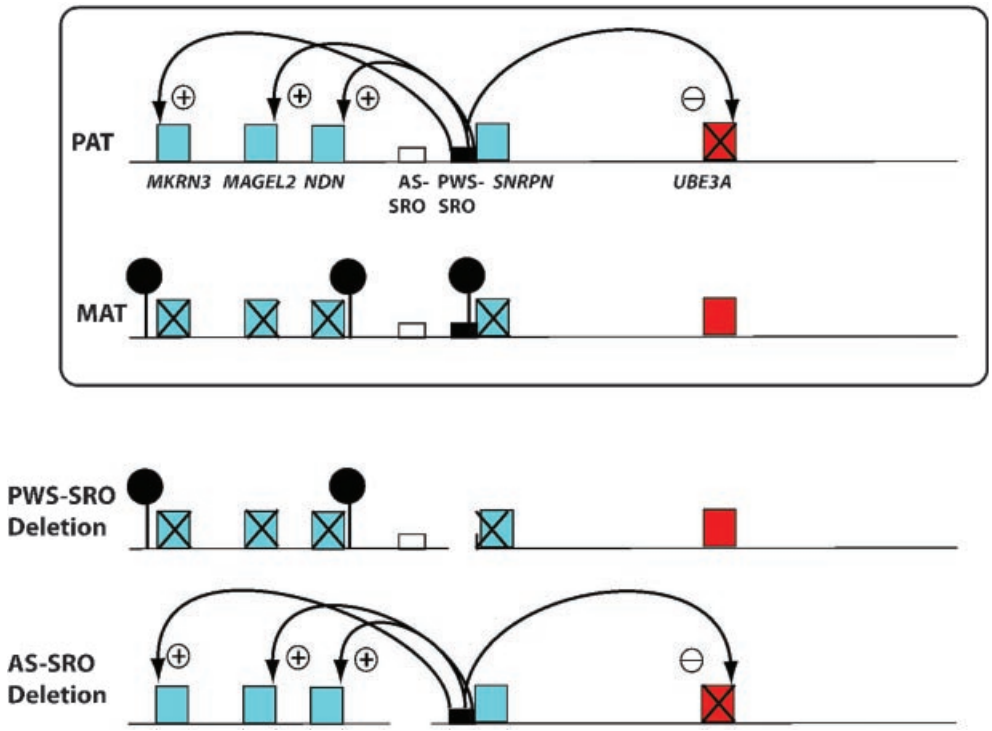

FIG. 4. Model for control of gene expression in 15q11–q13 by the PWS/AS-IC. On the paternal copy of chromosome 15, the PWS-SRO is unmethylated and active. This unmethylated PWS-SRO acts by unknown mechanisms to activate transcription of *MKRN3*, *MAGEL2*, and *NDN*, and it causes brain-specific silencing of *UBE3A* by mechanisms that may include serving as promoter for an antisense transcript. On the maternal copy of chromosome 15, the PWS-SRO is methylated and inactive. In the absence of an active PWS-SRO, *MKRN*, *MAGEL2*, *NDN*, and *SNRPN* are silenced and *UBE3A* is transcriptionally active in brain. In patients with PWS-SRO deletions, the consequences for gene expression are the same as on a maternal chromosome with PWS-SRO methylation. In patients with AS-SRO deletions who have intact PWS-SRO, the PWS-SRO is not methylated after maternal transmission, so that the consequences for gene expression are the same as on a normal paternal chromosome. In patients with deletions encompassing both AS-SRO and PWS-SRO (not shown), the lack of an active PWS-SRO leads to a maternal pattern of gene expression.

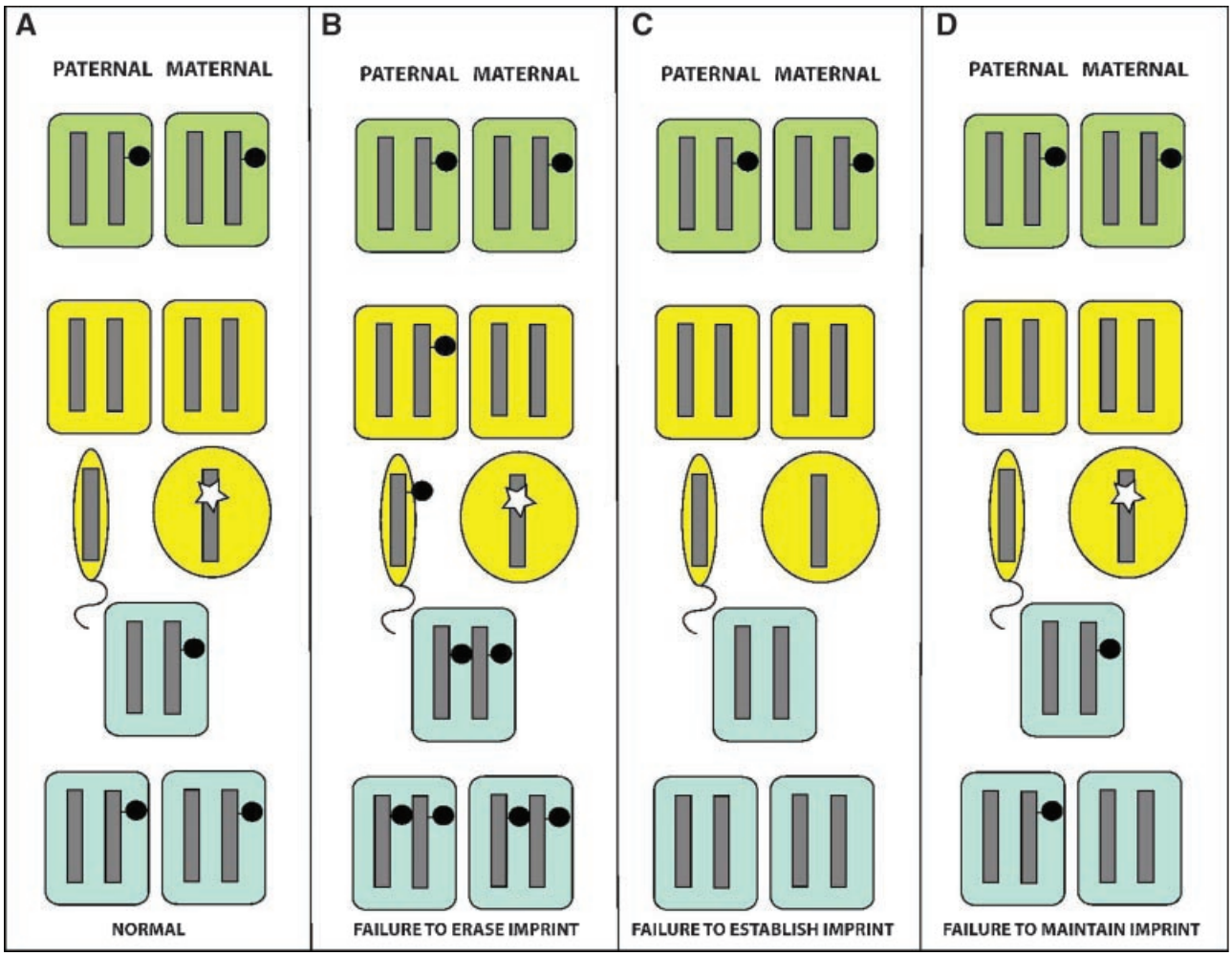

FIG. 5. Model for imprinting errors in the PWS/AS region. In somatic cells (green rectangles) of males and females, the maternal PWS-SRO carries a methylation mark (black circle). In normal imprinting cycle (**A**), methylation mark is erased in primordial germ cells (yellow rectangles). A protein complex containing at least one protein specific to the oocyte lineage (star) associates with the PWS-SRO during oogenesis. Soon after fertilization (blue rectangle), this complex leads to CpG methylation of the maternal PWS-SRO. Imprinting errors can arise from failure to erase the methylation mark in the paternal germ line (**B**), from failure to establish methylation after oogenesis and fertilization (**C**), or from failure to maintain methylation after fertilization (**D**). Failure to maintain methylation leads to somatic mosaicism.

(i) What are the *trans*-acting factors that interact with the AS-SRO in the female germ line, how do these factors lead to methylation of the PWS-SRO in *cis*, and when does this methylation occur?
(ii) How does the unmethylated PWS-SRO act at a distance to promote transcription of *MKRN3*, *MAGEL2*, and *NDN*?
(iii) Is antisense transcription of *UBE3A* necessary and sufficient for neuron-specific silencing of the paternal *UBE3A* allele? If not, how does the unmethylated PWS-SRO silence the paternal *UBE3A* allele in neurons?
(iv) What is the role, if any, of histone H3 Lys9 methylation and histone H4 Lys20 methylation, in establishment or maintenance of CpG methylation of the maternal PWS-SRO?

Investigation of these questions has been hampered by observations suggesting that the timing of PWS-SRO methylation is different in humans and mice, and by lack of success in defining a sequence element in the mouse that functions as the AS-SRO. The observation that multicopy transgenes containing human AS-SRO and human PWS-SRO show correct parent-of-origin-dependent methylation of the PWS-SRO that occurs after fertilization [Kantor et al., 2004] suggests that the molecular mechanisms of PWS/AS imprinting are generally conserved between mouse and human and that the difference in timing of methylation is caused by sequence differences between human and mouse ICs and differences between the temporal expression patterns of murine *Dnmt3L* and human *DNMT3L*

genes. Development of single-site, single-copy mouse transgenic systems containing human AS-SRO and human PWS-SRO would be valuable in allowing more precise definition of sequence requirements for interaction and function of these elements.

Understanding the complex mechanisms of PWS/AS region imprinting should ultimately benefit people with PWS and AS and their families by permitting therapeutic manipulation of the imprinting process. Recently developed animal systems, such as the *Ube3a-YFP* knock-in mouse, should provide important tools to allow screening for drugs that can activate genes that are silenced by the imprinting process.

NOTE ADDED IN PROOF

Dr. Wagstaff was the corresponding author of this article. He died on April 8, 2008. I have the sad privilege of being co-author of his last paper.

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