Cell. Mol. Life Sci. 64 (2007) 947 – 960
1420-682X/07/070947-14
DOI 10.1007/s00018-007-6460-0

Cellular and Molecular Life Sciences

Biomedicine & Diseases: Review

Molecular epigenetics of Angelman syndrome

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Received 28 October 2006; received after revision 11 December 2006; accepted 10 January 2007
Online First 8 March 2007

Abstract. Angelman syndrome (AS) is a neurogenetic disorder characterized by severe mental retardation, ataxia, seizures, EEG abnormalities and bouts of inappropriate laughter. AS individuals fail to inherit a normal active maternal copy of ubiquitin protein ligase E3A (UBE3A). UBE3A is subject to genomic imprinting, with predominant transcription of the maternal allele in brain. The known genetic causes of AS are maternal deletion of chromosome 15q11–q13, paternal chromosome 15 uniparental disomy, UBE3A mutation and an abnormality of the imprinting process, termed imprinting defect. There remain major questions concerning the molecular pathogenesis of AS, including: 1) the mechanisms underlying the imprinting defect class of AS, 2) the identity of proteins targeted by UBE3A, 3) the role of a non-coding antisense transcript in regulating UBE3A imprinting and 4) the contribution of other genes such as methyl-binding CpG-binding protein 2 and γ-aminobutyric acid A receptor, subunit β3 to the AS phenotype.

Keywords. genomic imprinting, ubiquitin ligase E3A (UBE3A), antisense, imprinting center, imprinting defect, DNA methylation, non-coding RNA, autism.

1) Angelman and Prader-Willi syndromes are disorders of imprinting

Genomic imprinting marks the parental origin of chromosomes or chromosomal subregions and results in allele-specific differences in methylation, transcription and replication. There are about 100 transcripts that display allele-specific expression, and these are distributed in twenty different chromosomal regions in mouse (http://www.mgu.har.mrc.ac.uk/research/imprinting/). Although the mechanisms that govern the establishment and maintenance of imprinting are not completely understood, there has been considerable progress in identifying crucial regulatory elements such as regions of differential DNA methylation, boundary elements, allele-specific chromatin modifications and antisense transcripts. This review focuses on the mechanisms that regulate imprinting within human chromosome 15q11–q13 and central mouse chromosome 7B4C, the imprinted regions that encompass the Angelman syndrome (AS) and Prader-Willi syndrome (PWS) loci.

The clinical manifestations of AS include brain growth retardation (microcephaly), severe mental retardation, 'puppet-like' ataxic gait with jerky arm movements, seizures, EEG abnormalities, hyperactivity and bouts of inappropriate laughter [1, 2]. A more detailed description of the clinical abnormalities of AS has been provided in detail elsewhere [1, 3]. PWS is characterized by hypotonia and failure to thrive in infancy, small hands and feet, hypogonadism, variable

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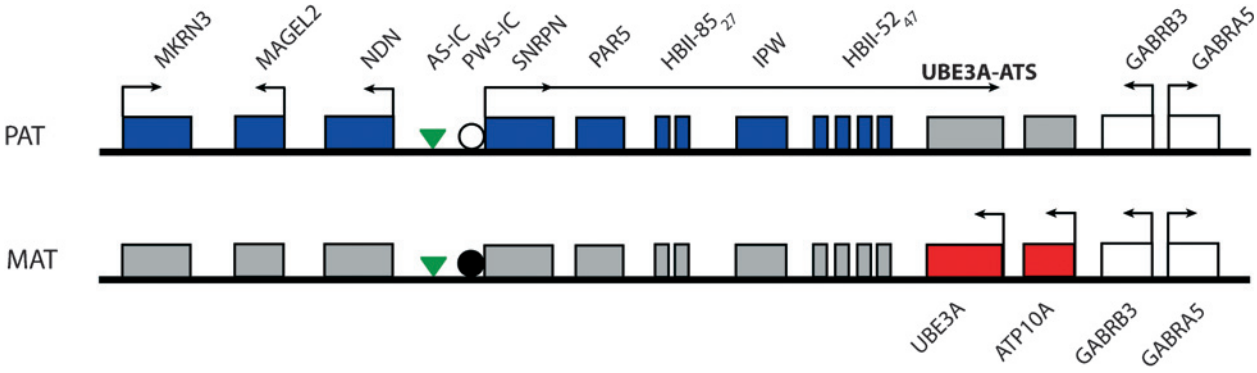

Figure 1. Imprinting map of the human chromosome 15q11–q13 AS/PWS region illustrating the status of allele-specific expression in brain. The paternally expressed genes (blue-filled boxes) that encode proteins are MKRN3 (makorin, ring finger protein 3), NDN (Necdin), MAGEL2 (MAGE-like protein 2) and SNRPN (bicistronic SNURF/small ribonucleoprotein N). Grey-filled boxes indicate silenced alleles. Ubiquitin protein ligase E3A (UBE3A) and the P-type ATPase (ATP10A) are maternally expressed protein-coding genes (red-filled boxes), while the genes encoding the γ-aminobutyric acid receptor β3 (GABRB3) and α5 (GABRA5) subunits display bi-allelic expression (open boxes) in brain. Non-coding paternally expressed transcripts such as PAR5 and IPW are components of a single large (≥460 kb) UBE3A-antisense (UBE3A-ATS) transcript that initiates in SNURF/SNRPN exon 1. UBE3A-ATS is believed to function in silencing the paternal UBE3A allele in brain. UBE3A-ATS serves as a host transcript for the C/D small nucleolar RNAs (snoRNAs) HBII-85 and HBII-52 as well as HBII-13, HBII-436, HBII-437, HBII-438A and HBII-438B (not shown). There are 27 and 47 tandemly repeated copies of HBII-85 and HBII-52, respectively, that are expressed upon processing of the antisense. The PWS imprinting center (PWS-IC) is defined by a cluster of CpG sites that are not methylated (open circles) on the paternal chromosome but are methylated (filled circles) on the maternal chromosome. The AS imprinting center (AS-IC) is denoted by a triangle.

mental retardation, obsessive-compulsive behavior and marked obesity resulting from hyperphagia [4, 5]. The majority of PWS cases (60–70%) are caused by *de novo* deletion of the paternal chromosome 15q11–q13 region. The typical PWS deletions encompass ~5000 kb of the 15q11–q13 region. Non-deletion cases (~25% of all PWS) most often result from maternal uniparental disomy (UPD) of chromosome 15 [6]. There are a small number of cases that result from imprinting defects (ID, see below). With the exception of rare cases with balanced chromosomal translocations [7], PWS can be detected by loss of differential methylation at the PWS-imprinting center (PWS-IC) that is contained in the 5'-region of the bicistronic SNURF/small ribonucleoprotein N (SNRPN) gene (Fig. 1) [8].

2) The PWS-IC

Exon 1 of SNRPN displays allele-specific differential DNA (cytosine) methylation for a cluster of CG dinucleotides. The maternal and paternal copies of the exon 1 differentially methylated region (DMR) are, respectively, methylated and unmethylated. As a result, PWS cases caused by paternal chromosome 15q11–q13 deletion or maternal UPD are characterized by complete methylation rather than differential methylation of the SNRPN exon 1 DMR. Methylation of both alleles of the SNRPN exon 1 DMR is also observed in PWS-ID patients, indicating that in such cases, the paternal chromosome 15 carries a maternal imprint. The identification of a series of small deletions that overlap SNRPN exon 1 in several PWS-ID cases led to the definition of an ~5 kb smallest region of overlap (SRO) for PWS [9, 10]. The term PWS-SRO is used interchangeably with PWS-IC (Fig. 1), the latter term being selected for the purposes of this review. The proposed function of the PWS-IC is to control the switch from maternal to paternal imprint during spermatogenesis. PWS-ID patients not only display the DNA methylation abnormality, they also show loss of expression of the paternal allele-specific genes SNURF/SNRPN (SNRPN upstream reading frame/small nuclear ribonucleoprotein polypeptide N), NDN (Necdin), MKRN3/ZNF127 (makorin/ring finger protein 3), MAGEL2 (MAGE-like 2) as well as the non-coding transcripts PAR5 and IPW (Fig. 1) [9, 10].

A detailed molecular analysis of the PWS-IC in a total of 51 PWS-ID patients revealed deletions in only 7 cases [11]. This low frequency (<15%) of SNURF/SNRPN exon 1 deletions led the authors to speculate that most PWS-ID cases result from epimutations, defined as aberrant epigenetic states that can be either pre- or post-zygotic [11]. Although the nature and mechanism of putative epimutations remain to be demonstrated, there is convincing evidence to suggest that at least some stages of imprint switching occur during or after fertilization [12, 13]. That the IC is essential not only for the germ-line establishment of the paternal imprint but also for its post-zygotic maintenance is suggested by studies of PWS families that are mosaic for a deletion of the PWS-IC [12]. It

Table 1. Molecular classes of AS with methylation status

Class	Chromosome/genetic abnormality	~%	Normal differential methylation
I	15q11–13 deletion	70	No
II	UPD (Uniparental disomy)	5	No
III	ID (Imprinting defect)	5	No
IV	UBE3A mutation	10	Yes
V	Unknown	10	Yes

also appears that chromosome 15 maternal methylation imprints are established during or after fertilization, since there are cases in mouse and human where the incorrect maternal methylation imprint is apparently established *de novo* after fertilization [13]. The SNRPN exon 1 DMR, an element that appears to be essential for the function of PWS-IC, spans 300 nucleotides with extensive maternal allele-specific methylation of 23 CG dinucleotides [13, 14]. Given the role of posttranslational histone modifications in epigenetic regulation, several groups have examined the SNRPN exon 1 DMR for allele-specific differences in histone H3 and H4 acetylation and methylation [15–19]. For the SNRPN exon 1 DMR in both human and mouse, high levels of histone H3 and H4 acetylation are associated with the paternal relative to the maternal allele [15–17]. Moreover, H3 lysine 9 is methylated on the maternal copy of the PWS-IC, while H3 lysine 4 is methylated on the paternal copy [18]. These patterns of histone modification are consistent with the DNA methylation and expression studies indicating that the maternal copy of the PWS-IC is methylated and repressed relative to the paternal copy. In ES cells deficient in the G9a H3 Lys-9/Lys-27 methyltransferase, expression of *Snrpn* appears to be bi-allelic, suggesting loss of imprinting and implying that histone methylation may be involved in the establishment of imprinting at the PWS-IC [19].

3) Differential methylation analysis of the PWS-IC in the molecular diagnosis of AS

Assay of the allele-specific methylation of the SNRPN exon 1 DMR is used for routine molecular diagnostic analysis of AS and is essential for differentiating between the five molecular classes of AS [1, 20] (Table 1). Class I, by far the most frequent (65–70% of all AS cases), is caused by *de novo* deletion of the maternal chromosome 15q11–q13 region. Class II AS individuals (~5%) display paternal UPD for chromosome 15. Class III ($\leq$5%) corresponds to the ID class of AS [21]. Classes I–III of AS are characterized by a loss of methylation rather than differential methylation at the SNRPN exon1 DMR. Differentiation of the three classes following detection of abnormal DNA methylation is made by molecular cytogenetic (FISH) analysis and DNA polymorphism testing [1, 20].

In contrast to the first three classes, patients in AS classes IV and V show normal differential DNA methylation at the PWS-IC (Table 1). The distinction between classes IV and V is made on the mutation status of the AS candidate gene ubiquitin protein ligase E3A (UBE3A) [22, 23]. The great majority of class IV cases result from mutations that are predicted to introduce a stop codon in the UBE3A transcript [20, 24, 25]. These chain-terminating mutations would cause any translation products to be catalytically inactive because of the importance of the extreme C terminus for UBE3A function [26]. The majority of familial cases with normal differential DNA methylation are class IV [20, 24, 25]. Class V includes those patients that are negative for the UBE3A mutation, with the majority of the cases being sporadic rather than familial [20].

Genotype-phenotype correlations for the different molecular classes of AS are emerging, although severe developmental delay, impaired speech, movement and balance disorder, cognitive deficits and unprovoked laughter are shared features [1, 20, 27]. Class I and V cases appear to be the most severe, with the most marked developmental delay, the highest incidence of seizures and the more prominent EEG abnormalities [1, 20, 27, 28]. Classes II and III have a less severe phenotype with a lower incidence of microcephaly and severe seizures and have $\geq$3 words in their vocabulary. The clinical features of class IV patients are intermediate, with seizure severity and microcephaly similar to classes I and V but motor skill and speech performance closer to that of classes II and III. The body mass index is near normal in class I patients and increases somewhat in classes II and III, while class IV patients display a high incidence of early onset obesity [1, 20]. The molecular mechanisms underlying the class-specific phenotypic differences in AS are not understood. It has been suggested that the severity of clinical features in class I patients is due to the haplo-insufficiency of non-imprinted genes such as GABRB3 that are contained

in the chromosome 15q11–q13 deletion. Given that GABRB3 has been implicated in seizure susceptibility [29], the haplo-insufficiency hypothesis would be consistent with the severe seizures observed in class I cases and would suggest that class I AS is a contiguous gene disorder involving UBE3A, ATP10A, GABRB3 and perhaps other genes. The haplo-insufficiency hypothesis would not explain the similar severity of classes I and V of AS, although the nature of the molecular abnormality in class V is completely unknown.

4) The AS-IC

There is further complexity in deciphering the genetics of AS in that a separate regulatory element, the AS-IC, has been identified upstream of SNRPN exon 1 (Fig. 1). The AS-IC was discovered by molecular genetic analysis of class III AS patients (Table 1) that revealed micro-deletions of a region of DNA located 35–40 kb upstream of SNRPN exon 1 [30, 31]. The deleted region contains a number of duplicated and alternative SNURF/SNRPN upstream exons [30, 32]. In class III AS-ID cases, the maternal chromosome 15 has the paternal imprint; the proposed function of the AS-IC is to mediate the switch from the paternal to the maternal imprint during oogenesis [12, 13]. Although the smallest region of overlap (AS-SRO) has refined the delineation of the AS-IC to a single 880bp exon [32], deletion or mutation of this exon is detected in only 20% of AS-ID cases [11]. The observation that the vast majority of AS-ID cases display no abnormalities of the putative AS-IC again suggests the possibility of epimutation in the majority of class III AS [33, 34]. Sporadic imprinting defects account for a sizeable proportion of class III AS, and studies indicate that at least one-third of the sporadic ID cases involve somatic mosaicism [11, 35]. This finding is relevant to the clinical diagnosis of AS, since there appears to be an inverse correlation between the fraction of normally methylated cells and the severity of the clinical manifestations [11, 35].

The discovery of the PWS-IC and AS-IC has led to the model that both these elements regulate imprinting in the PWS/AS region. Evidence for the bipartite regulatory element was first obtained using transgenic mice carrying a 1kb human fragment encompassing the AS-IC and a 200bp mouse PWS-IC segment [36]. It was found that these elements appear to establish the expected patterns of allele-specific DNA methylation, histone modification and expression of the transgene [36, 37]. The transgenic studies have led to a model whereby modification and activation of the AS-IC in the maternal germline results in methylation of the adjacent PWS-IC [36–39]. In this hypothesis, the AS-IC is the primary element in establishing imprinting in the PWS/AS region, since it would function to methylate the PWS-IC and thereby silence the expression of maternal alleles within this region. One concern is that these transgenic studies involved the use of a human/mouse hybrid construct, a strategy that was necessary since a murine equivalent of the human AS-IC had not yet been identified. There is other evidence in support of the bipartite regulatory model, however, based on the identification of AS siblings with a 1–1.5 Mb inversion that separates the AS-IC and PWS-IC [40]. The observation that the inversion was transmitted from the maternal grandfather to the normal mother of the siblings supports a model whereby the AS-IC is required for methylation of the adjacent PWS-IC in the maternal germline.

It is not presently clear whether the AS-IC comprises a single element contained within the region of microdeletion overlap that defines the AS-SRO or whether multiple elements are involved. A transgenic mouse model that shares some features of AS-ID cases has now been reported [41]. This model, generated by an insertion/duplication targeted to 13 kb upstream of *Snrpn* exon 1 [41], may lead to a better definition of the functional elements within the AS-IC. Maternal transmission of this mutation, termed AS-IC^{an}, results in the absence of methylation at *Snrpn* exon 1 DMR, a result that strongly suggests that this insertion/duplication disrupts the AS-IC and is an appropriate mouse model for the class III AS-ID.

The effect of AS-IC^{an} on the function of AS-IC appears to be partial or mosaic in that reduction of rather than complete absence of methylation at the DMR of *Ndn* is observed upon maternal transmission of AS-IC^{an} [41]. On the other hand, maternal transmission of the AS-IC^{an} appears to relieve silencing of the *Snrpn* allele in *cis*, a result that is also consistent with disruption of AS-IC function. Female AS-IC^{an} mice were crossed with males carrying a 4.8kb deletion of the *Snrpn* exon 1 and the putative PWS-IC (PWS$^{\Delta 4.8}$) [42]. PWS$^{\Delta 4.8}$ mice display neonatal lethality and growth retardation, and maternal inheritance of the AS-IC^{an} allele in offspring that carry a paternal PWS$^{\Delta 4.8}$ allele rescues the abnormal phenotype [41]. The normally repressed maternal *Snrpn* allele is expressed in the rescued offspring since the normally methylated PWS-IC carried in *cis* of the maternal AS-IC^{an} allele is unmethylated. The latter results are consistent with the bipartite model discussed above whereby methylation of the PWS-IC in the maternal germline is dependent on a functional AS-IC. Maternal transmission of the AS-IC^{an} results in a marked reduction of *Ube3a* expression in cerebellum and cerebral cortex [41]. The level of

decrease in *Ube3a* expression in AS-IC^{an} brain appears to be comparable to that observed in *Ube3a*-knockout animals [41]. This observation represents the first evidence indicating that the phenotype of AS-ID patients is due to loss of UBE3A expression in the brain.

It is worth noting that significant heterogeneity exists in the phenotype of class III AS-ID cases. In this regard, some individuals classified as AS-ID based on methylation analysis actually display PWS-like features that include obesity, hypotonia and the ability to speak [43]. It has been reported that class IV AS patients have a high incidence of early onset obesity [20]. Obesity is also observed in mice with partial paternal UPD of the mouse chromosome 7 region spanning the AS region [44]. The UPD mouse, which is the first animal model for AS, displays several features of AS including microcephaly and abnormal EEG [44]. UPD mice also show marked changes in body weight during development, with a reduced growth rate in the first few weeks after birth but marked obesity by 6 months. The relationship between obesity and either the loss of maternal-specific methylation at the IC or UPD may involve a second maternal-specific transcript, the P-type ATPase ATP10A, that is located less than 200 kb downstream of *Ube3a* (Fig. 1) [45–47]. Female mice that inherit a maternal deletion encompassing the mouse ortholog, *Atp10a,* have roughly twice the body fat of mice inheriting the deletion paternally [45]. AS-ID patients show loss of expression of ATP10A in peripheral blood, indicating that, like UBE3A, ATP10A is regulated by the AS-IC. The data concerning the maternal allele-specific expression of *Atp10a* have been contradictory [48]. In one study, predominant expression of the maternal *Atp10a* allele was found in hippocampus and olfactory bulb [49], while bi-allelic expression was found in another report [50]. While this might suggest strain-specific differences in *Atp10a* imprinting [48], further studies are needed to determine the imprinting status of *Atp10a* and its potential association with the obesity observed in classes III and IV of AS.

5) *Ube3a*, the gene responsible for AS

UBE3A, first designated as the E6-associated protein (E6-AP), was discovered on the basis of its ability to degrade p53 upon association with the E6 protein of the human papilloma virus [51, 52]. Several studies have established that p53 is not a substrate for UBE3A in E6-negative cells [53–55]. UBE3A is a class of E3 ubiquitin-protein ligase characterized by a 350-aa C-terminal HECT (homologous to the E6-AP carboxyl terminus) domain. UBE3A appears to play a role both in defining target specificity and in catalyzing the transfer of activated ubiquitin to target protein substrates [56]. The HECT domain is conserved among a large group of E3 enzymes, and the terminal 100aa region is essential for transferring the ubiquitin moiety to the protein targeted for degradation by the ubiquitin-proteasome degradation system [26]. The great majority of UBE3A mutations detected thus far in AS are predicted to cause premature protein termination and disruption of the HECT domain [20, 24, 25].

Two mouse models of AS have been generated by targeted inactivation of *Ube3a* [57, 58]. Upon inheritance of the mutation through the maternal but not the paternal germline, both mutant mouse models display several features of AS. These include microcephaly, impaired motor function and long-term potentiation, deficits in context-dependent and spatial learning, inducible seizures, an abnormal hippocampal EEG and disruption of hippocampal calcium/calmodulin-dependent protein kinase II (CamKII) activity [57–59]. The two mouse models of *Ube3a* inactivation were also used to investigate imprinting of *Ube3a* in brain either by examining expression of a reporter gene incorporated into the targeting construct [58] or by *in situ* hybridization [57]. Both studies confirmed the previous report that maternal-specific expression occurs predominantly in hippocampal neurons and cerebellar Purkinje cells [60].

UBE3A also has a distinct function as a member of a large family of co-activators that interact with nuclear receptors and enhance their ability to activate transcription [61]. In transfection experiments with different mutant UBE3A constructs, it was demonstrated that the co-activator function lies in an N-terminal domain of the protein [62], a portion of UBE3A that is intact in the great majority of AS patients [20, 24, 25]. These findings support the current hypothesis that mutation or loss of the maternal UBE3A allele in brain causes the accumulation of a substrate protein(s) that leads to the neurological abnormalities that characterize AS. Several proteins have been identified as potential targets of UBE3A-mediated degradation in non-neuronal cell lines, including HHR23A, the human ortholog of the DNA-repair enzyme RAD23 [63]; the Src family tyrosine kinase Blk [64]; the multicopy maintenance protein Mcm7 [65] and the estrogen receptor [66]. It is not known if the amount or activity of any of these UBE3A targets is disrupted in AS brain.

A search for molecules regulated by UBE3A was also performed by overexpressing human UBE3A in *Drosophila* heads and screening for proteins showing altered levels of expression by two-dimensional gel

electrophoresis [67]. One protein identified in this screen is the Rho/GEF pebble. The mouse ortholog of pebble, epithelial cell transforming sequence 2 (*Ect2*), was observed to be re-distributed in the cerebellum and hippocampus of *Ube3a*-deficient mice [67]. By immunohistochemical staining, increased expression and mis-localization of *Ect2* in the CA3 region of hippocampus is detected in *Ube3a*-null mice, while the levels of *Ect2* are reduced in the cytoplasm of Purkinje cell bodies [67]. While there is a marked re-distribution of *Ect2* upon loss of *Ube3a* in mouse brain [67], the overall levels of *Ect2* appear to be quite low when examined by western blotting, and increased expression cannot be detected in brain and cerebellum of normal or *Ube3a*-mutant mice (Fig. 2).

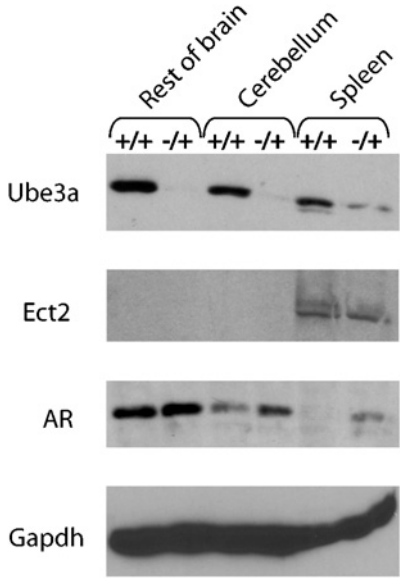

Figure 2. Immunoblot analysis of Ube3a, Ect2 (epithelial cell transforming sequence 2) and AR (androgen receptor) in *Ube3a* maternal-deficient mutant [58] and wild-type littermate brain and spleen. Ect2 expression is undetectable in brain by western blot analysis, whereas AR appears to have elevated levels in rest of brain, cerebellum and spleen of *Ube3a*-deficient relative to control mice. The same blot was successively probed for Ube3a, Ect2, AR and Gapdh (as a loading control). The "rest of brain" sample refers to the remaining brain tissue after the following subregions were excised: cerebellum, olfactory bulb, hippocampus and a small portion of cerebral cortex.

The androgen receptor (AR) has also been identified as a putative target of *Ube3a* [68]. The association between UBE3A and AR was observed in prostate gland and prostate tumors [68]. In particular, it was shown that *Ube3a*-deficient mice display a smaller prostate gland than normal mice. Moreover, it was demonstrated that AR protein levels are increased in heterozygous and homozygous *Ube3a*-knockout mice [68]. An increase in the levels of AR can also be detected in brain, cerebellum and spleen of *Ube3a*-deficient relative to control mice (Fig. 2). AR and *Ect2* are the first proteins identified that appear to be regulated by UBE3A and abnormally expressed in brain as a result of the loss of UBE3A. As additional screening strategies are applied using animal models, it is to be expected that many additional proteins regulated by UBE3A will be identified as potentially contributors to the clinical manifestations of AS.

6) Other functions of UBE3A

There is increasing evidence that alteration of UBE3A (E6-AP) expression is associated with cancer development in a manner that is independent of the E6 papilloma virus protein. Overexpression of the large (~10 kb) *Ube3a* transcript is observed in a mouse model of mammary tumorigenesis [69], while a decrease in UBE3A protein levels has been reported in invasive human breast carcinoma [70]. The link to breast cancer is further supported by the observation that UBE3A regulates estrogen receptor stability [66] as well as the steroid receptor co-activator oncogene amplified in breast cancer 1 (AIB1) [71]. A decrease in the levels of UBE3A protein is also detected in human prostate cancer, a finding that could be directly related to the up-regulation of AR that is observed upon loss or inactivation of UBE3A [70]. The regulation of the ECT2 oncogene [67] is suggestive of a potential role for UBE3A in the development of various human tumors.

Another function of UBE3A is suggested by the finding that the ortholog of UBE3A in *Caenorhabditis elegans* was identified in a screen for oxidative stress-responsive genes [72]. While any role for UBE3A in stress response could be mediated by its function as a ubiqutin ligase, a link between AS and an abnormal stress response has not been previously discussed. For example, reduced expression of UBE3A could sensitize some neurons to oxidative or other stress, resulting in the death of specific neuronal subpopulations. Alternatively, increased resistance to stress could favor the survival of specific neuronal subpopulations during development. There is evidence that UBE3A expression in brain can be affected by stresses such as alcohol exposure. In one study, *Ube3a* was found to be down-regulated by 45% in ethanol-treated compared to untreated cortical mouse neurons [73]. In another study, microarray analysis was performed postmortem in temporal cortex from 11 subjects with a history of alcohol abuse and from 11 control individuals [74]. Average UBE3A expression in samples from alcohol abusers was only 21% of the level of UBE3A expression in controls [74]. These two studies provide evidence

that ethanol exposure significantly decreases UBE3A expression in mammalian systems.

There is also indirect evidence of an interaction between *Ube3a* and ataxin-1, the expanded polyglutamine protein that causes spinocerebellar ataxia type 1 (SCA1) [75]. In these experiments, SCA1 mice were crossed to *Ube3a*-deficient mice, resulting in an acceleration of the polyglutamine-induced phenotype in the SCA1-transgenic mice. There is no evidence to suggest, however, that ataxin-1 plays a role in the development of AS.

7) Non-coding antisense RNA and silencing of the paternal UBE3A allele in brain

UBE3A displays predominant maternal expression in human fetal brain and adult frontal cortex [47, 76, 77]. In mouse, maternal allele-specific expression is detected in specific brain subregions including hippocampus, Purkinje cells of the cerebellum and mitral cells of the olfactory bulb [60]. Although the mechanism of tissue-specific imprinting of *Ube3a* has not been conclusively resolved, the observation that an antisense transcript, UBE3A-ATS, is paternally expressed in human brain (Fig. 1) has fuelled the hypothesis that the ATS functions to silence the paternal UBE3A transcript in *cis* [78, 79]. UBE3A-ATS is a large (~460 kb) noncoding RNA that initiates in the PWS-IC and extends distally through SNURF/SNRPN and IPW and overlaps UBE3A [80]. UBE3A-ATS is alternatively spliced and serves as a host for several types of small nucleolar RNA (snoRNA) of the box C/D class, which are contained within the introns and are expressed upon processing of the paternal copy of the host transcript [80–83]. Several studies have argued for and against a role for the HBII85 and/or the HBII52 snoRNAs in the development of PWS [84–87], and the function of these conserved small RNAs is actively being investigated. The murine *Ube3a-ATS* is also a large (~1000 kb) transcript that encompasses *Ipw* and the MBII85 and MBII52 snoRNAs. Similarly to the human antisense transcript, *Ube3a-ATS* undergoes extensive alternative processing and is observed to be paternal-specific and restricted to brain [88, 89]. The paternal origin and tissue specificity of *Ube3a-ATS* is demonstrated in Fig. 3.

In a PWS mouse model with the 37-kb deletion that encompasses the PWS-IC, there is no expression of the paternal transcripts contained within the PWS region, including *Ube3a-ATS*. In the brains of these mutant mice, the absence of antisense transcription is associated with increased expression of the paternal *Ube3a* allele, suggesting that the silencing process associated with imprinting has been perturbed [88]. While this result is consistent with the proposed role for *Ube3a-ATS* in the imprinting of the *Ube3a* allele in brain, the 37-kb paternal deletion of the PWS-IC could affect the global chromatin structure of the PWS/AS region and cause up-regulation of the paternal *Ube3a* allele by another mechanism. By gene targeting, the mouse PWS-IC has been replaced by the human equivalent [90]. Mice inheriting the human PWS-IC through the maternal germline display a normal methylation imprint in oocytes. These findings suggest that acquisition of the methylation imprint is conserved between human and mouse. The maternal imprint, however, is not maintained in somatic cells. Moreover, this loss of the maternal imprint in the mutant mice results in expression of *Ube3a-ATS* from the maternal allele in brain [90]. The resulting repression of maternal *Ube3a* in *cis* in the brain of mutant mice strongly argues for regulation of *Ube3a* via the antisense mechanism.

The potential involvement of antisense in the control of imprinted gene expression is not unique to UBE3A. Such a mode of regulation was first demonstrated for the maternally expressed insulin growth factor receptor type II (*Igf2r*) [91]. Antisense regulation has also been reported for several other imprinted genes including *Kcnq1* [92, 93], *Xist* [94] and *Gnas* [95]. Several hypotheses have been advanced to explain the epigenetic mechanism of antisense-mediated silencing [96–100]. The proposed silencing mechanisms include degradation of the sense/antisense RNA double-strand, transcriptional interference due to the simultaneous occupancy of the RNA polymerase on the positive and negative strands and an antisense-mediated chromatin alteration. It is not clear which, if any, of the proposed mechanisms are involved in the putative silencing of *Ube3a* by *Ube3a-ATS*. Our laboratory has also demonstrated that the disruption of *Ube3a* causes up-regulation of the *Ube3a-ATS* [101]. The precocious or abnormal expression of UBE3A-ATS could affect the normal processes of neurogenesis and contribute to the phenotypic abnormalities that result from loss or inactivation of UBE3A [101]. This reciprocal modulation of the antisense by the sense is in contrast to the reported cases of epigenetic silencing of a sense transcript by a non-coding antisense RNA.

Other mechanisms of UBE3A imprinting are suggested by RNA-FISH experiments indicating that the maternal UBE3A signals are consistently larger than the paternal-specific signals in fibroblasts and neuronal precursor (NT2) cells [102]. Expression of UBE3A-ATS, however, is not detectable in fibroblasts or NT2 cells (ML, data not shown), suggesting that allele-specific UBE3A expression is regulated, at

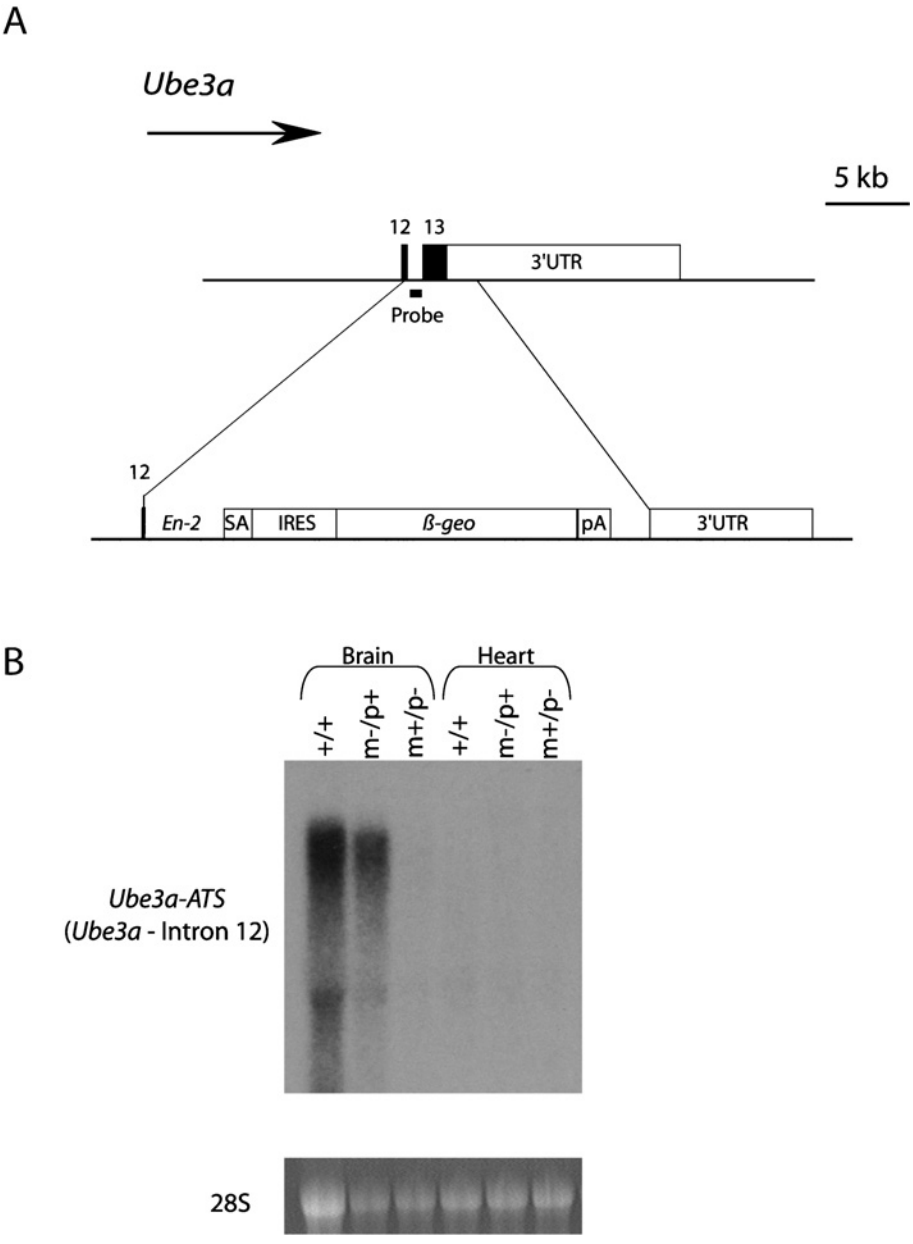

Figure 3. *Ube3a-ATS* displays paternal-specific expression in brain. (*A*) Map of the targeting construct used for inactivating *Ube3a* [58]. (*B*) Northern blot analysis of *Ube3a-ATS* in brain and heart from a wild-type littermate (m+/p+) and mice with maternal (m−/p+) or paternal (m+/p−) inheritance of the mutant *Ube3a* allele. Since *Ube3a-ATS* overlaps the *Ube3a* exons and introns [78, 80, 88, 141], the probe (see *a*) from *Ube3a* intron 12 detects the large (>500 kb) alternatively processed antisense as a high molecular weight smear [101]. The *Ube3a* intron 12 probe does not detect the targeted allele (-) because of the intron 12 deletion. Hybridization is observed in m+/p+ and m-/p+ but not in m+/p− brain, confirming that *Ube3a-ATS* is paternal-specific. No hybridization is detected in heart, consistent with the observation that *Ube3a-ATS* is brain-specific. The 28S ribosomal RNAs serve as a sample loading control.

least in part, by a mechanism not involving UBE3A-ATS. An association between allele-specific UBE3A expression and differential methylation is suggested by an analysis in somatic cell hybrids containing apparently intact chromosomes 15 of paternal or maternal origin [83]. The results of this report are contradicted, however, by reports showing that differential methylation in the 5'-region (exon 1) of UBE3A is not detected in a wide variety of normal human and mouse tissues including brain [20, 103, 104]. Sites of variable and tissue-specific CpG differential methylation are also observed in the UBE3A 3'-UTR [103]. No significant differences in CpG methylation in these UBE3A 3'-UTR sites have been detected in AS or PWS brain samples, however, suggesting that differential methylation of this region is not involved in UBE3A imprinting. Abnormal DNA methylation in UBE3A exon 1 was observed in the brain tissues of 1 of 17 individuals with autism [103]. The association between abnormalities of 15q11–q13 and autism has been the subject of a number of investigations.

8) AS, autism spectrum disorders and Rett syndrome

Duplication of 15q11–q13 is one of the few cytogenetic abnormalities observed in autistic spectrum disorder (ASD) [105–109]. Increased risk of ASD is

associated with maternal but not paternal transmission of interstitial 15q11–q13 duplications. These findings suggest a role for overexpression of one or more maternally expressed genes in ASD, with UBE3A being an obvious candidate gene. The incidence of ASD appears to be increased in PWS relative to AS cases and is significantly higher in PWS UPD compared to deletion cases [110]. The latter observations are consistent with the proposed association between maternal overexpression of genes within 15q11–q13 and ASD.

Several studies have found genetic linkage of markers in the GABRB3 to UBE3A interval to ASD in pedigrees that are negative for chromosome 15q11–q13 rearrangements [111, 112]. There are several reports of markers from this interval being in linkage disequilibrium with ASD [111, 113–118], although there are also studies in which no such association was observed [119, 120]. Overall, the studies support the model that one or more genes in the AS/PWS region contribute to ASD, with a continued interest in GABRB3 [116, 121]. In this regard, there are conflicting reports of whether GABRB3 is subject to genomic imprinting. While it was shown that this gene is expressed from both alleles in both mouse and human brain [79, 122], there is a report that only the paternal allele is expressed in some somatic cell hybrid lines [123]. Mice homozygous for targeted inactivation of *Gabrb3* are characterized by extensive neonatal lethality with associated cleft palate [124]. Surviving mice have a reduced lifespan and display hyperactivity, neurological impairment, movement disorder as well as frequent myoclonus and occasional epileptic seizures [124]. The status of *Gabrb3* imprinting has been examined in heterozygous *Gabrb3*-mutant mice [125]. The parent-of-origin and gender-related differences in *Gabrb3* expression observed in this study [125] were determined by analyzing GABRB3 protein and need to be confirmed by quantitative mRNA analysis in additional animals.

There are also reports of a few AS cases that display mutations of the gene encoding methyl CpG binding protein 2 (MeCP2) [126, 127]. Mutations of MeCP2 result in Rett syndrome (RTT), a neurodevelopmental disorder characterized by cognitive impairment, rhythmic hand movements, seizures and many features of ASD [128]. While AS and RTT are distinct disorders, the overlap in some clinical manifestations could suggest common molecular mechanisms. Given that MeCP2 functions as a transcriptional repressor, two groups have investigated whether MeCP2 might regulate UBE3A by examining UBE3A expression in brain from human RTT cases as well from the MeCP2-knockout mouse model [104, 129]. UBE3A protein and mRNA levels appear to be reduced in MeCP2-mutant relative to control brain samples [104, 129]. GABRB3 levels were also reported to be moderately decreased in the MeCP2-deficient brain [129]. One of the studies suggested that MeCP2 deficiency alters the normal pattern of histone modification at the PWS-IC, resulting in activation of the maternal UBE3A-ATS and a consequent reduction of UBE3A expression [104]. In the second study, however, no change in UBE3A-ATS levels was observed in the MeCP2-deficient brains [129]. Moreover, an additional investigation has reported that no alteration of *Ube3a* expression is observed in MeCP2-mutant mouse brain [130]. Functional studies using the available animal models will be key to resolving whether molecular pathways common to UBE3A, GABRB3 and MeCP2 are associated with the overlapping clinical manifestations of AS, RTT and ASD. In this regard, the *Gabrb3*- and *Ube3a*-deficient mouse models have been studied using ligand autoradiography to measure changes in the amounts of $GABA_A$ receptor. While a functional deficit in $GABA_A$ receptors was observed in hippocampal neurons isolated from *Gabrb3*-deficient mice [131], no such reduction was detected in mice with maternal *Ube3a* deficiency [132]. These studies indicate that alterations in the levels of $GABA_A$ receptor do not contribute to the AS features of the *Ube3a*-knockout mouse model.

9) Does assisted reproductive technology increase the risk of AS?

Over one million births have been achieved through assisted reproductive technology (ART), and there is continued interest in whether there is an associated increase in the risk of birth defects. There are now several reports indicating a link between ART and an increased risk for Beckwith-Weidemann syndrome and AS, both disorders of imprinting [133–136]. Given that AS is relatively rare in the general population and that the number of cases in individuals born through ART is small, caution must be exercised in determining whether there is an increased incidence in this population. Nonetheless, it is striking that the first three such cases were the rare class III of AS rather than the more prevalent class I [137, 138]. Moreover, both individuals were conceived by intracytoplasmic injection, suggesting to the authors that this particular technique might interfere with maternal imprint establishment either in the oocyte or post-*in vitro* fertilization [137]. Additional studies suggest that the increased incidence of AS-ID may result from other factors associated with ART such as superovulation or could also be associated with sub-fertility

[139]. Another recent study of AS incidence in ART reports one class I and two class II cases of AS [140]. More investigations are needed before it can be determined if some ART procedures are associated with an increased risk of AS.

Conclusions

Angelman syndrome is a disorder of genomic imprinting that causes severe neurological and developmental defects. The molecular classes of AS include chromosomal abnormalities, imprinting defects, UBE3A mutations as well as a group of patients with an unknown genetic defect. The identification of UBE3A as a causative gene in AS and the production of several mouse models of AS should allow major progress in addressing a number of big questions that remain, the most significant of which may be determination of the molecules targeted by and/or interacting with UBE3A in brain. The characterization of UBE3A targets may, in the long term, contribute to therapies that will ameliorate the clinical manifestations of AS. Other significant questions include whether: 1) silencing of the paternal UBE3A allele in brain is mediated by the antisense transcript; 2) imprinting defects cause loss of UBE3A expression in brain and 3) abnormal expression of UBE3A contributes to other neurodevelopmental diseases including Rett syndrome and autism spectrum disorder.

Acknowledgements. We wish to thank Dr. V. Sotirova for her encouragement and Dr. J. Wagstaff for kindly providing the Ube3a mutant mice.

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