Maternal disruption of Ube3a leads to increased expression of Ube3a-ATS in trans

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ABSTRACT

Angelman syndrome (AS) is a neurogenetic disorder characterized by severe mental retardation, ‘puppet-like’ ataxic gait with jerky arm movements, seizures, EEG abnormalities, hyperactivity and bouts of inappropriate laughter. Individuals with AS fail to inherit a normal active maternal copy of the gene encoding ubiquitin protein ligase E3A (UBE3A). UBE3A is transcribed predominantly from the maternal allele in brain, but is expressed from both alleles in most other tissues. It has been proposed that brain-specific silencing of the paternal UBE3A allele is mediated by a large (>500 kb) paternal non-coding antisense transcript (UBE3A-ATS). There are several other examples of imprinting regulation involving antisense transcripts that share two main properties: (i) the sense transcript is repressed by antisense and (ii) the interaction between sense and antisense occurs in cis. We show here that, in a mouse model of AS, maternal transmission of Ube3a mutation leads to increased expression of the paternal Ube3a-ATS, suggesting that the antisense is modulated by sense rather than the reciprocal mode of regulation. Our observation that Ube3a regulates expression of Ube3a-ATS in trans is in contrast to the other cases of sense–antisense epigenetic cis-interactions and argues against a major role for Ube3a-ATS in the imprinting of Ube3a.

INTRODUCTION

Epigenetic silencing via an antisense-mediated mechanism has been observed at the X-inactive specific transcript (Xist) and Tsix antisense locus (1) as well as for imprinted genes such as insulin-like growth factor-type-2 receptor (Igf2r) (2) and the potassium voltage-gated channel, KQT-like subfamily, member 1 (Kcnql1) (3,4). Silencing of the paternal alleles of Igf2r and Kcnql1 is mediated by the paternal-specific non-coding antisense transcripts Air and Kcnlqot1, respectively. It has been shown that these antisense transcripts are regulated by differentially methylated regions (DMRs) since paternal deletion of the DMRs ablates expression of Air and Kcnlqot1 (2–4). Truncation of the Air transcript not only results in loss of silencing of the paternal Igf2r allele, but also of two other paternal transcripts that are upstream of Air (5). There is evidence to suggest, however, that Air does not have a direct role in silencing Igf2r (6,7), based on the observation that Igf2r is not imprinted in the brain despite the paternal-specific expression of Air in this tissue (6). Moreover, it has been suggested that the mechanism of Igf2r imprinting involves parental allele-specific histone modifications at the Igf2r promoter rather than the action of Air and/or differential DNA methylation at the Igf2r locus (7). As a result, the concept that non-coding antisense transcripts have a direct role in regulating the expression of neighboring imprinted genes in cis remains controversial.

Like Air and Kcnlqot1, Ube3a-ATS is a paternal transcript that appears to be regulated by a DMR, the Prader–Willi syndrome imprinting center (PWS-IC) (8). It has been proposed that Ube3a-ATS is part of a long transcript (>500 kb) that initiates in the PWS-IC, a region that encompasses the Snurf/Snrpn promoter, and extends downstream (9–11). Ube3a-ATS is expressed in cultured neurons but not in glial cells (12), and its structure and organization is conserved in human, mouse and rat. Inheritance of a paternal deletion of the PWS-IC (ΔPWS-IC) abolishes transcription of all paternal transcripts in the region including small nuclear ribonucleoprotein N (Snrfl/Snrpn) and Ube3a-ATS (8). A putative role for Ube3a-ATS in the cis-regulation of Ube3a imprinting is supported by the observation of increased expression of the paternal Ube3a allele in the ΔPWS-IC mice (8). One mechanism by which the PWS-IC regulates imprinting of
Ube3a-ATS could involve an interaction of the transcriptional repressor, methyl-CpG-binding protein 2 (MeCP2), with the maternal (methylated) PWS-IC locus. In this regard, Ube3a expression is observed to decrease in mutant mice that are deficient in MeCP2, with an associated change in Ube3a-ATS expression observed in one study (13) but not in the other (14). The question of whether Ube3a-ATS plays a role in Ube3a imprinting, therefore, remains unresolved. As an alternative approach to investigating the interaction between Ube3a and Ube3a-ATS, we analyzed the expression of Ube3a-ATS in mice bearing a targeted disruption of Ube3a.

Two mouse models of AS have been generated by targeted disruption of Ube3a (15,16). Upon inheritance of mutation through the maternal but not the paternal germline, both mutant mouse models display several features of AS. These include 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 (15–17). In both mouse models, the inactive allele contained a reporter gene thus allowing the confirmation of a previous report (18) that maternal-specific inactive allele contained a reporter gene thus allowing the confirmation of a previous report (18) that maternal-specific imprinting, therefore, remains unresolved. As an alternative approach to investigating the interaction between Ube3a and Ube3a-ATS, we analyzed the expression of Ube3a-ATS in mice bearing a targeted disruption of Ube3a.

Partial map of Figure 1. In this regard, Ube3a-ATS spans most of the Ube3a gene including intronic regions. Horizontal arrow heads indicate positions of primers (A–G) used for RT–PCR expression studies; oligonucleotide primer G is deleted upon replacement by the targeting construct as denoted by the open arrow head. The asterisk (*) indicates the nucleotide polymorphism used for the allele-specific expression studies.

MATERIALS AND METHODS

RNA preparation, cDNA synthesis and RT–PCR

Total RNA was isolated, using RNAzol (Tel-Test, Inc., Friendswood, TX), from mouse brain of B6, CAST and F1 offspring carrying either a maternally or a paternally transmitted mutant Ube3a allele (16) and from wild-type littersmates. Reverse transcription reactions (+RT) were performed with 2 µg of RNA and Superscript reverse transcriptase (Invitrogen) using random hexamer primers or the Ube3a-ATS strand-specific primer E (5’-AGTCTCGGAAATTGTTCTATCG-3’). A 2 µg aliquot of total RNA was incubated in a similar manner, but without reverse transcriptase (−RT) as a control. One-twentieth of the +RT or −RT reaction was used in PCR using Taq polymerase (Invitrogen) under conditions suggested by the manufacturer. Specific annealing temperatures and extension times are available upon request. Oligonucleotides for PCR (Figure 1) were as follows: C (5’-TTGTCAATCTCTATTACG-3’); D (5’-CAACAGATTCCCTCGTATAGC-3’); E (5’-TTTACAGATGAAAGAGACTG-3’); and G (5’-GTTTACAGCATGCAACACTCC-3’). The oligonucleotide primers and PCR conditions for Gapdh have been described previously (19).

Northern blot analysis

Twelve micrograms of total RNA was size-fractionated on a 1.2% agarose/formaldehyde gel and transferred to a Biotrans nylon membrane (ICN, Irvine, CA). Hybridization was performed in Expresshybe solution according to the manufacturer’s (Clontech, Palo Alto, CA) specifications using the following probes: a 320 bp Ube3a cDNA probe from exons 5 and 6; an RT–PCR product from the distal Ube3a 3’-UTR [1.3 kb, using the M-1F/L-1R primer pair (11)] and a neomycin-specific probe. The Ube3a-ATS-specific probe was generated by PCR using the Ube3a intron 12-specific primers 5’-ACTTTGTACCCACTTGAACT-3’ and 5’-CTTGATACGTCTGACTCTCC-3’. After washing, membranes were exposed to Kodak Biomax film and autoradiographed.

Allele-specific expression analysis

For conventional RT–PCR analysis (Figure 4), the parental alleles of Ube3a-ATS and Atp10a were distinguished using
previously described polymorphisms (8,20). Allele-specific analysis of the Ube3a polymorphism (8) was performed using PCR oligonucleotide primers A (5′-AATCTGGCTCGGCATGTC-3′) and B (5′-AGATCACATCATCGTTGGGTA-3′) (Figure 1).

Real-time PCR amplification (Figure 4B) of Ube3a-ATS in B6 and B6×CAST maternal Ube3a mutation litters was carried out with the iQ™ SYBR® green Super mix using the iCycler iQ real-time detection system (Bio-Rad, Hercules, CA) with the following conditions: 95°C, 1 m; (94°C, 30 s; 60°C, 30 s; 72°C, 20 s) × 45; 72°C, 2 min. The specificity of the reactions was checked by melting curve profiles to monitor the presence of only one duplex DNA species and by agarose gel electrophoresis analysis of certain products to confirm the amplification of a single band of the expected size. The oligonucleotides for PCR were ATS-F (5′-TTGTATACAG-GAAGCTAATGGGG-3′) and ATS-R (5′-CAAAAGTTATCT-AATAATGATTTCC-3′). Amplifications for RT+ and RT− samples were performed at least two times in triplicate with independent cDNA preparations and raw Ct values were obtained using iCycler iQ Optical System Software version 3.0. After normalizing Ube3a-ATS values to Gapdh, the comparative Ct method was used to calculate differences in expression levels between samples (21).

For the allelic discrimination analysis of the Ube3a locus in B6×CAST and CAST×B6 cerebellum P55 RNA samples, a slightly modified TaqMan® assay (PE Applied Biosystems, Foster City, CA) was used. Ube3a maternal and paternal alleles were detected with the same nucleotide polymorphism as described above for allele-specific RFLP expression studies. Sequences of the PCR primers were as follows: Ube3aEx8-F (5′-GCATAGTCTTGGCTTGATTT-3′) and Ube3aEx9-R (5′-AGATCTGCTTGTATCGTAAGGTA-3′). These primers were used together with modified oligonucleotide probes specific for B6 (5′-VIC-ACAAATATTGTATCTGGGATGTCC-MGB-3′) and CAST (5′-FAM-ACAAATATTGTATCGTAGGATGTCC-MGB-3′). Amplifications were carried out with TaqMan® Universal PCR Master Mix (Roche, Branchburg, NJ) on an iCycler iQ real-time detection system (Bio-Rad) with the following conditions: 95°C, 3 m; (95°C, 15 s; 60°C, 1 m) × 60. Amplicons were analyzed at least two times in triplicate with independent cDNA preparations using iCycler iQ Optical System Software version 3.0 (Bio-Rad). The allelic discrimination function was used to plot and automatically call genotypes based on the fluorescence intensities of FAM and VIC at 49 cycles. Allelic discrimination plot values were further corrected with FAM and VIC fluorescence data obtained from B6 and CAST P55 cerebellum RNA included as controls in each plate assay to correct for cross-reactivity of the B6-specific VIC probe. The approach is validated by the consistency of the Ube3a parental expression ratios between wild-type reciprocal crosses (B × C +/+ and C × B +/+) (Figure 5).

RESULTS

Northern blot analysis using a probe from the Ube3a coding region reveals a prominent doublet of ≈10 kb and fainter 4.5–5.5 kb bands in normal m+/p+ brain (Figure 2A), a finding similar to that previously reported for Ube3a expression in normal tissues (22). In the Ube3a mutant mouse model studied here, the disrupted Ube3a transcript is detectable by northern blot hybridization (indicated by the * and ** in Figure 2A), consistent with expression of the lacZ reporter from the targeted allele in brain tissue sections (16). The smaller (* ) re-arranged mRNA (Figure 2A) most probably represents the Ube3a-LacZ-neo transcript since this fragment is not detected by a probe from the distal Ube3a 3′-UTR (Figure 2B), but is detected by a neomycin-specific probe (Figure 2C). The larger (**) re-arranged mRNA, however, appears to use an endogenous termination signal in the Ube3a 3′-UTR since it is detected by both the probe from the Ube3a 3′ non-coding region (Figure 2B) and by the neo probe (Figure 2C).

The re-arranged transcripts (* and **, Figure 2A) are highly prominent in the brain sample from mice with the maternal disruption (m−/p+) of the Ube3a allele, but very faint in the m+/p− sample (Figure 2A). The greater expression of the re-arranged transcripts in m−/p+ compared to m+/p− corresponds to the predominant transcription of the targeted maternal (m−) allele in brain. Similarly, the normal ≈10 kb doublet predominates in m+/p− brain as a result of maternal-specific expression of the normal Ube3a (m+) allele in this sample. Densitometric analysis of the maternal- and paternal-specific hybridization bands detected using the Ube3a and neo probes (Figure 2A and C) reveals a >10-fold maternal:paternal allele expression ratio for Ube3a. A similar ratio is also observed in a replicate northern blot experiment (data not shown). The strong bias (>10:1) towards maternal expression in brain is difficult to reconcile with previous observations of Ube3a imprinting in a relatively limited number of neuronal tissues (15,16,18).

The Ube3a-ATS is a large and complex transcript that initiates upstream of Smpn and extends to overlap most of the Ube3a transcript (9,11). In order to distinguish between Ube3a and Ube3a-ATS by northern blot hybridization, we generated an Ube3a intron 12-specific probe by PCR. Hybridization with the intron 12-specific probe reveals a smear in m+/p+ and m−/p− but not in m+/p− brain samples (Figure 2D) suggesting that this probe is specific for Ube3a-ATS. The hybridization smear is typical of natural antisense transcripts (23) and has previously been observed using the Ube3a-ATS probe, Ipw (24). Because intron 12 is deleted in the targeted allele and since Ube3a-ATS displays paternal-specific expression, hybridization of the intron 12-specific probe is only detected in those samples with a normal paternal (p+) allele (Figure 2D). Examination of the intensity of the Ube3a-ATS hybridization signals in Figure 2D suggested an increase in the m−/p+ relative to the m+/p+ sample. We measured the total hybridization signal in these samples by densitometry and obtained a value of 2.9 for the ratio of the m−/p+ to the m+/p+ signals (normalized to the gel densitometric measurement of 28S bands). A similar analysis of a replicate experiment (data not shown) gave a ratio of 1.8.

These findings suggested that disruption of the maternal Ube3a allele could affect the paternal-specific expression of Ube3a-ATS. In order to investigate the possibility of this trans interaction between sense and antisense, we performed semiquantitative RT–PCR using primers for different regions of overlap between Ube3a and Ube3a-ATS. Total brain RNA from mutant and normal mice analyzed by RT–PCR using primers F and G (Figure 1) in exons 12 and 13, respectively,
is shown in Figure 3A. Two products are detected; one of 244 bp corresponding to the Ube3a exons 12 and 13, and one of 1200 bp that is the unspliced Ube3a-ATS containing intron 12 (Figure 3A). Only the normal (non-targeted) Ube3a and Ube3a-ATS alleles are detected in this analysis since primer G is specific for the part of exon 13 that is deleted upon replacement by the targeting cassette. Ube3a levels are significantly reduced in the m−/p+ relative to the m+/p− brain. (A) Hybridization with an Ube3a cDNA probe. The doublet of hybridization bands at ≥10 kb corresponds to normal (non-targeted) Ube3a transcripts while the re-arranged transcripts are indicated by an asterisk. (B) The blot from A is re-hybridized with a probe from the distal Ube3a 3′-UTR that hybridizes to both Ube3a and Ube3a-ATS. The background smear results, at least in part, from multiple Ube3a-ATS splice forms. The smaller (*) Ube3a re-arranged transcript is not detected using this probe suggesting that the (*) transcript corresponds to the major Ube3a-LacZ-neo fusion transcript that uses the polyadenylation site of the targeting construct (Figure 1). The larger (**) fragment, however, is detected using the Ube3a 3′-UTR probe suggesting that this re-arranged Ube3a transcript terminates in the distal 3′-UTR. (C) Consistent with this suggestion, a neomycin-specific probe detects both (*) and (**) re-arranged transcripts. (D) Hybridization of a probe from Ube3a intron 12 reveals a smear in m+/p+ and m−/p+ but not in m+/p− brain samples. The smear results from hybridization of the intron 12 probe to the paternal Ube3a-ATS allele in the m+/p+ and m−/p+ samples. The absence of a hybridization smear in the m+/p− RNA is because intron 12 is deleted in the targeted paternal allele. The 28S ribosomal RNAs serve as a sample loading control.
size results from the unspliced Ube3a-ATS containing intron 10 (Figure 3C). Ube3a-ATS is observed in all three samples indicating that the targeted paternal deletion of exons 12 and 13 does not eliminate expression of the antisense in upstream regions of Ube3a overlap. Another important feature of the analysis in Figure 3C is the markedly increased levels of expression of Ube3a-ATS that are observed in the m–p+/p+ relative to the m+p+/p+ control littermate and the m+p/+ sample. The increased Ube3a-ATS expression in m–p+/p+ brain is also evident in intron 12 (compare intensity of 1200 bp products in lanes 1 and 3, Figure 3A and B). Replicate RT–PCR experiments indicated that the level of Ube3a-ATS was increased at least 2-fold in the m–p+/p+ relative to m+p+/p+ brain, a ratio that is similar to that obtained by northern blot analysis (Figure 2D). The results of Ube3a–ATS expression analysis (Figures 2 and 3) strongly suggest that disruption of the maternal Ube3a allele affects the levels of Ube3a–ATS expression in trans. To further confirm the putative trans sense/antisense interaction, we performed Ube3a–ATS analysis on a different mouse strain background by crossing C57Bl/6J (B6) mice carrying the targeted Ube3a allele to M. m. castaneus (CAST/Ei) animals.

We exploited a Tsp509I restriction fragment polymorphism in Ube3a exon 8 (*, Figure 1) (8) to analyze the sense and antisense expression in cerebellum of C57Bl/6J (B6), CAST/Ei (CAST) and F1 offspring of CAST × B6 and B6 × CAST crosses (Figure 4A). There are two Tsp509I sites in the B6 Ube3a exon 8 versus a single one in CAST (8). To amplify Ube3a–ATS by RT–PCR, a forward primer upstream of the Tsp509I restriction site in exon 8 is used in combination with a reverse primer from intron 8 (8). The expression of Ube3a–ATS using template RNA from cerebellum of 7.5-week-old mice is summarized in the top panel of Figure 4A. The 840 bp Ube3a–ATS product (lanes 1, 3, 5, 7, 9 and 11) yields sizes of 553 and 640 bp (smaller products not shown), respectively, for the B6 (lane 2) and CAST (lane 4) alleles upon cleavage with Tsp509I. Paternal-specific expression of Ube3a–ATS is evident in both the +/+ hybrid crosses (lanes 6 and 12) and in the mice that derive the Ube3a mutation from either B6 parent (lanes 8 and 10). These findings confirm that inheritance of the Ube3a mutation does not affect the paternal-specific expression pattern of Ube3a–ATS.

Again, increased expression of Ube3a–ATS is evident in B6 × CAST m–p+/p+ relative to B6 × CAST m+p+/p+ cerebellum for the samples either cleaved with Tsp509I (compare lanes 8 and 6) or not treated with the restriction enzyme (compare lanes 7 and 5). These experiments also suggest that the CAST Ube3a–ATS allele is expressed at a higher level than that of the B6 (compare lane 3 to 1, top panel, Figure 4A).

The allele-specific expression of Ube3a in cerebellum is shown in the second panel of Figure 4A. A forward primer (A, Figure 1) upstream of the B6-specific Tsp509I restriction site and a reverse primer (B, Figure 1) in exon 9 (11) are used for allele-specific RT–PCR. The RT–PCR product using the Ube3a primer pair is 300 bp in size (lanes 1, 3, 5, 7, 9 and 11). Upon Tsp509I digestion of the resulting RT–PCR products, the CAST allele (300 bp) is not cleaved (lanes 3 and 4) whereas the B6 allele digests to yield a 200 bp fragment (lanes 1 and 2, smaller product not seen). The predominant expression of the maternal Ube3a allele is evident in all crosses upon analysis of Tsp509I restriction of RT–PCR.

and m+p– samples (compare lane 3 to lanes 1 and 5, Figure 3A), a finding consistent with the observation that the paternal Ube3a allele is repressed in brain (15,16,18).

To confirm that the 1200 bp corresponds to Ube3a–ATS, we performed an RT with the antisense strand-specific primer E, followed by PCR with the F and G oligonucleotides (Figure 3B). Only the 1200 bp product is detected in Figure 3B, confirming that this is the Ube3a-ATS product. The analysis of Ube3a–ATS expression (Figure 3A and B) confirms that the antisense is exclusively paternal in brain since RT–PCR products are detectable in the RNA samples from maternal Ube3a-deficient (m–/p+) mice and control littermates (m+/p+) but not from paternal-deficient (m+/p−) animals.

We also examined Ube3a–ATS expression across exons 10 and 11, a region of Ube3a that is not replaced by gene targeting, by RT–PCR using primers C and D (Figure 3C). In this RT–PCR analysis, the 230 bp band size corresponds to the Ube3a exon 10/11 splice product while the 366 bp band
products (compare intensity of 200 and 300 bp products in lanes 6, 8, 10 and 12, second panel, Figure 4A). We also examined the allele-specific expression of the P-type ATPase Atp10a in these samples (third panel, Figure 4A). Atp10a maps/C24 500 kb downstream of Ube3a on mouse chromosome 7C (25) and displays predominantly maternal expression in human (26). In mouse, Atp10a has been reported to be both bi-allelic (27) and maternal-specific (20), suggesting the possibility of strain-dependent imprinting of Atp10a (28).

Allele-specific expression of Atp10a was performed as described previously (20) using a B6-specific MspI restriction site to distinguish the B6 and CAST alleles. RT–PCR analysis of Atp10a using these primers yields a 288 bp product (lanes 1, 3, 5, 7, 9 and 11) with the B6 allele identified by the 217 bp size band upon MspI digestion (lane 2). The two parental alleles display roughly equal levels (compare the intensity of 288 and 217 bp bands in lanes 6, 8, 10 and 12, Figure 4A, third panel) suggesting bi-allelic expression rather than predominantly maternal expression in cerebellum.

We then used quantitative PCR (qPCR) to measure Ube3a-ATS expression levels in the mutant and normal mice (Figure 4B). We quantified the levels of Ube3a-ATS in total brain of 3.5-week-old m/C0/p+ and m/+p+ B6 mice using primers from exon 8 and intron 8 for RT–qPCR. This analysis reveals a 2-fold increase of Ube3a-ATS levels in the m/C0/p+ relative to m/+p+ (Figure 4B) and confirms the results obtained by northern blot analysis (Figure 2D) and conventional RT–PCR analysis (Figure 3). A ≈2-fold increase in
DISCUSSION

We report here that the maternal disruption of Ube3a causes an up-regulation of the paternal Ube3a-ATS. In contrast to other examples of epigenetic silencing involving the repression of sense by the antisense in cis, this observation indicates that there is trans interaction between Ube3a and Ube3a-ATS. Another important finding is that the trans interaction involves modulation of the antisense by the sense rather than the reciprocal as has been reported in cases of epigenetic silencing of a sense transcript by a non-coding antisense RNA.

A role for trans effects in the regulation of imprinting was first suggested by the observation of a physical association between the homologous 15q11–q13 regions during interphase in human cells (29). Moreover, cells of PWS and AS individuals were deficient in homologous association, suggesting that this process might be involved in the establishment and/or maintenance of imprinting (29). Data supporting the physical association of oppositely imprinted 15q11–q13 chromosomal domains were reported in one study (30) but not in another (31), while homologous association of the oppositely imprinted regions of distal mouse chromosome 7 has also been observed (32). It was suggested that homologous association was not required for normal imprinting in a transgenic insertion mouse model of AS/PWS (33). On the other hand, the study of uniparental fetuses has suggested that both parental genomes are required to establish maternal-specific expression of Igf2r (34). There are also several reports of the transfer of the DNA methylation patterns between alleles in gene targeting experiments at both imprinted and non-imprinted loci (35–39). Strong evidence for the involvement of trans-interactions in the imprinting at the AS/PWS locus was reported upon gene targeting of Snurf/Snrpn (40). In the latter study, inactivation of the paternal Snurf/Snrpn allele resulted in partial or complete demethylation of the maternal allele, indicating that imprint switching can occur in trans upon disruption of the paternal copy of Snurf/Snrpn (40).

In terms of mechanisms involved in the trans interaction between Ube3a and Ube3a-ATS, one possibility is that the Ube3a gene product normally functions to repress Ube3a-ATS expression, perhaps via pathways that involve ubiquitination and chromatin silencing. Brain-specific expression of Ube3a-ATS is controlled by the PWS-IC and additional upstream exons distributed within an ~500 kb region upstream of Snrpn (8,11). Targeted disruption of the maternal Ube3a allele and the resulting diminution of Ube3a protein could impede an Ube3a-mediated chromatin silencing process in the Snrpn upstream region and result in the up-regulation of Ube3a-ATS in trans. In this regard, recruitment of an E3 ligase has been shown to be involved in the regulation of gene silencing in Saccharomyces cerevisiae (41). There is currently no evidence, however, suggesting that Ube3a is involved in histone ubiquitination or in other aspects of chromatin silencing.

Another mechanism could involve a direct interaction between the Ube3a and Ube3a-ATS RNAs. In this regard, the formation of duplexes between complementary sense and antisense transcripts from different chromosomes has recently been demonstrated for the ribosomal S2 and S9 proteins and for Keratin 8 (42). Annealing of the complementary maternal sense to the paternal antisense RNA strands could regulate expression in several ways. One possibility is that a double strand formed between sense and antisense would decrease the stability of Ube3a and Ube3a-ATS. In this scenario, the replacement of Ube3a by the targeting construct would disrupt duplex formation between sense and antisense strands in trans and thereby stabilize the Ube3a-ATS RNA in m−/p+ brain. RNA editing or RNA interference (RNAi)
might be involved in the aforementioned dsRNA degradation, although both processes would be predicted to affect the levels of both sense and antisense. While the hypothesis needs to be tested in individual neurons, our qPCR data (Figure 5) appear to be inconsistent with the involvement of dsRNA degradation by RNAi or RNA editing since the increase in Ube3a-ATS observed in the m→p/p brain does not appear to be associated with a coordinated increase in the levels of the maternal (targeted) Ube3a allele.

The modulation of Ube3a-ATS by Ube3a in trans contrasts with the other reported cases of epigenetic silencing that involve cis-repression of sense by the antisense. The cis and trans modes of sense/antisense coupling could be associated with differences in function. The cis-interaction between Xist and Tsix is characterized by a 10–100 times excess of antisense over sense (43,44), whereas we observe no such increase in steady-state Ube3a-ATS levels relative to Ube3a. It has also been shown that both Xist and Tsix are nuclear transcripts and their cis-interaction is constrained by their tight association with a single X chromosome (1). The putative trans interaction for Ube3a could result from the absence of any local association of sense and antisense with chromosome 7 or be due to local localization outside the nucleus. In this regard, detailed in situ hybridization experiments in mouse brain have revealed a mainly cytoplasmic localization for one Ube3a-ATS splice form that overlaps Ube3a (E. Le Meur, F. Watrin, M. Landers, R. Sturny, M. Lalande and F. Muscatelli, submitted for publication). These findings suggest Ube3a sense and antisense can interact in the cytoplasm and raise the possibility that Ube3a-ATS functions in the post-transcriptional control rather than the epigenetic regulation of Ube3a.

Using a hybridization probe from Ube3a intron 12, we observe a high molecular weight smear in the m→p/p and m→p/p brain samples upon northern blot analysis (Figure 2D). This finding is entirely consistent with previous reports indicating that Ube3a-ATS is a large (>500 kb) alternatively processed paternal-specific transcript that overlaps Ube3a (9,11). In line with the current hypothesis, these observations suggest that the brain-specific Ube3a-ATS could function to silence the paternal Ube3a allele in cis. While our results do not directly address antisense function, it is clear that the increased expression of Ube3a-ATS in m→p/p brain does not affect the steady-state level of the paternal Ube3a allele (Figure 5). Our findings are, therefore, inconsistent with a simple model whereby increased Ube3a-ATS expression results in a corresponding decrease in Ube3a RNA in cis and argue that elements other than Ube3a-ATS control the allele-specific expression of Ube3a. In support of this hypothesis, RNA-FISH analysis suggests a preferential maternal UBE3A expression in non-neuronal human tissues, such as fibroblasts and lymphoblasts, where UBE3A-ATS is not expressed (30).

Finally, our allele-specific expression analysis of Ube3a in brain reveals a very significant bias for maternal expression (Figures 2 and 4). While a rough estimate of 2:1 for the maternal:paternal allele-specific expression ratio was previously obtained by RT–PCR using total brain RNA (8), our northern blot analysis indicates that the Ube3a maternal: paternal expression ratio is ≈10:1 in total brain (Figure 2 and data not shown). Despite the differences in the values obtained by RT–PCR and northern blot analysis, the results seem surprising given that imprinting of Ube3a has been reported to be restricted mainly to hippocampal neurons and the Purkinje cells of the cerebellum (15,18). In this regard, the disrupted Ube3a allele in our mutant mice contains a lacZ reporter gene thereby permitting an analysis of the allele-specific expression of Ube3a in different brain regions (16). Visualisation of the β-galactosidase activity in mice upon maternal and paternal inheritance of the disrupted allele reveals that Ube3a is imprinted in hippocampus and cerebellum (16), a result consistent with previous studies. While these histochemical and in situ hybridization studies suggest a near-complete repression of the paternal Ube3a allele in hippocampus and cerebellum, our experiments strongly suggest that there is a marked bias for maternal expression throughout most regions of brain.

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