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A Mixed Epigenetic/Genetic Model for Oligogenic Inheritance of Autism With a Limited Role for *UBE3A*

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The genetic contribution to autism is often attributed to the combined effects of many loci (ten or more). This conclusion is based in part on the much lower concordance for dizygotic (DZ) than for monozygotic (MZ) twins, and is consistent with the failure to find strong evidence for linkage in genome-wide studies. We propose that the twin data are compatible with oligogenic inheritance combined with a major, genetic or epigenetic, de novo component to the etiology. Based on evidence that maternal but not paternal duplications of chromosome 15q cause autism, we attempted to test the hypothesis that autism involves oligogenic inheritance (two or more loci) and that the Angelman gene (*UBE3A*), which encodes the E6-AP ubiquitin ligase, is one of the contributing genes. A search for epigenetic abnormalities led to the discovery of a tissue-specific differentially methylated region (DMR) downstream of the *UBE3A* coding exons, but the region was not abnormal in autism lymphoblasts or brain samples. Based on evidence for allele sharing in 15q among sib-pairs, abnormal DNA methylation at the 5′-CpG island of *UBE3A* in one of 17 autism brains, and decreased E6-AP protein in some autism brains, we propose a mixed epigenetic and genetic model for autism with both de novo and inherited contributions. The role of *UBE3A* may be quantitatively modest, but interacting proteins such as those ubiquitinated by *UBE3A* may be candidates for a larger role in an oligogenic model. A *m*ixed *e*pigenetic and *g*enetic and mixed *d*e novo and *i*nherited (MEGDI) model could be relevant to other "complex disease traits". © 2004 Wiley-Liss, Inc.

KEY WORDS: Angelman syndrome; autism; complex disease traits; DNA methylation; epigenetics; ubiquitin ligase

INTRODUCTION

Autism is characterized by impairment of social interaction, impairment of communication usually in the form of limitation of spoken language, and repetitive and stereotyped patterns of behavior [American Psyciatric Association, 1994]. Autism (or autistic disorder) is often defined more narrowly, while a broader definition of autistic spectrum disorders includes patients with autism-related findings diagnosed as Asperger syndrome or pervasive developmental disorder, not otherwise specified. Affected children often have a completely normal appearance. Cognitive impairment coexists in about 70% and seizures in about 30% of autistic patients [Tuchman and Rapin, 2002]. By current definitions, autistic spectrum disorders affect about 1 in 200 children [Fombonne, 2003], and both severe and milder phenotypes are approximately four times more frequent in males (~1 in 125) than in females (~1 in 500). Significantly higher male to female ratios have been found for groups with absence of dysmorphic features and/or normal magnetic resonance imaging of the brain [Miles and Hillman, 2000].

The genetic contribution to the etiology of autism is thought to be significant, largely because the concordance in monozygotic (MZ) twins is quite high with 60% of MZ pairs being concordant if autism is defined narrowly, and 92% being concordant if the criteria include a broader spectrum of autism-like phenotypes [Bailey et al., 1995; Folstein and Rosen-Sheidley, 2001; Lauritsen and Ewald, 2001]. In contrast, the concordance in dizygotic (DZ) twins appears to be no higher than for siblings of autism probands, which is generally reported to be about 3% [Jorde et al., 1991; Szatmari et al., 1998; Lauritsen and Ewald, 2001]. Risch et al. [1999] suggest that "The very high (25-fold) MZ:DZ concordance ratio is indicative of at least several interacting loci and, potentially, of many such loci." The twin data and the failure to identify strong evidence for autism-related loci in genome-wide linkage studies using affected sib-pairs have led some investigators to suggest that ten or more loci might contribute to the etiology of autism [Risch et al., 1999; Lamb et al., 2000; Gutknecht, 2001]. Other authors have suggested oligogenic inheritance with many fewer genes [Pickles et al., 1995], and one excellent review suggests that "oligogenicity with epistasis" is the most likely genetic model of autism [Folstein and Rosen-Sheidley, 2001]. Linkage data are not consistent with X-linked inheritance, although a small fraction of patients have fragile X syndrome [Rogers et al., 2001] or mutations in neuroligins encoded on the X chromosome [Jamain et al., 2003]. There is

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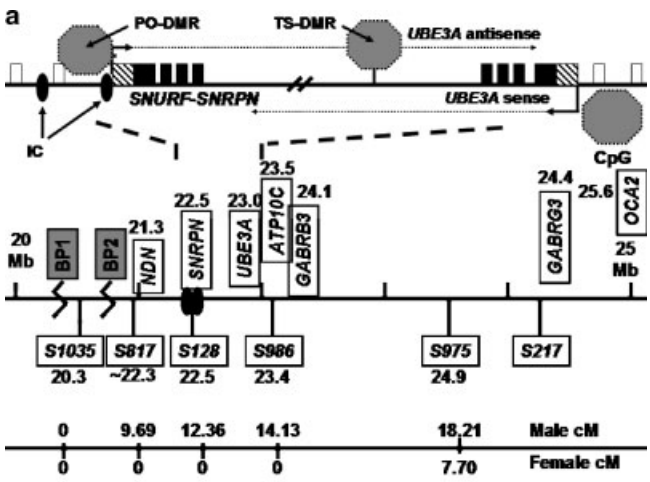
considerable evidence for cytogenetic abnormalities of chromosome 15q11-q13 in autism.

Chromosome 15q11-q13 in Autism and Angelman Syndrome (AS)

There is strong evidence that cytogenetic abnormalities of chromosome 15q11-q13 cause autism with a dependence on parent of origin. Prader–Willi syndrome (PWS) and AS are known to map to 15q11-q13, and the region includes a 3–4 Mb domain subject to genomic imprinting (Fig. 1a). Large paternal deletions of 15q11-q13 cause PWS, and large maternal deletions cause AS; see reviews [Shemer et al., 2000; Nicholls and Knepper, 2001]. There is a bipartite imprinting center (IC)



b

	Paternal sharing at *D15S#*				
	S817	***S128***	***S986***	***S975***	***S217***
29					
18					
9		**Single recombinant**			
2		**Single recombinant**			
	38*/58*				**31/58**

***Shared/total**

Fig. 1. Map of human chromosome 15q11-q13 and linkage analysis of sib-pairs. In **panel a**, the region from 20 to 25 Mb of chromosome 15 on the Marshfield genomic map is depicted with the region encompassing the coding exons of *SNRPN* and *UBE3A* expanded above; additional upstream exons for both genes are not shown. The PWS-IC (**right**) and AS-IC (**left**) are shown. The shaded octagons depict the regions subject to DNA methylation: the parent of origin DMR (PO-DMR), the tissue-specific DMR (TS-DMR), and the 5′-CpG island for *UBE3A*. Gene symbols are standard and DNA markers are all D15 (e.g., *S1035* = *D15S1035*). BP1 and BP2 refer to the centromeric breakpoints typical of PWS and AS deletions. **Panel b** depicts sharing of paternal alleles in the 15q11-q13 region for 58 AGRE sib-pairs. Filled boxes represent sharing of alleles and hatched boxes represent nonsharing.

within 15q11-q13. Deletions of the telomeric portion of the IC—the PWS-IC—cause PWS due to imprinting defects when inherited paternally. The PWS-IC overlaps with the CpG island at the 5′-end of the *SNRPN* gene and is methylated on the maternal allele and unmethylated on the paternal allele. The AS-IC is centromeric to the PWS-IC, and deletions of the AS-IC cause AS due to imprinting defects when inherited maternally. The *UBE3A* ubiquitin ligase gene is within 15q11-q13 and is justifiably regarded as "the Angelman gene," because loss-of-function point mutations cause a complete AS phenotype when inherited maternally but are benign when inherited paternally. *UBE3A* is subject to genomic imprinting with silencing of the paternal allele in selected brain regions and cell types in humans [Rougeulle et al., 1997; Vu and Hoffman, 1997] and mice [Albrecht et al., 1997; Yamasaki et al., 2003].

The evidence for an autism gene within chromosome 15q11-q13 is substantial. There are more than 30 affected individuals in more than 20 reported families with interstitial duplications causing trisomy for the region and many dozen cases of autism with an extra isodicentric chromosome 15 yielding trisomic or tetrasomic dosage for 15q11-q13 [Bolton et al., 2001; Roberts et al., 2002]. The interstitial duplications often cause autism if on the maternal chromosome but are benign when on the paternal chromosome [Cook et al., 1997; Schroer et al., 1998; Roberts et al., 2002], but the consistency of this genotype–phenotype correlation is not complete [Bolton et al., 2001]. When an extra isodicentric chromosome 15 is found in association with autism, the extra chromosome is virtually always of maternal origin [Robinson et al., 1993; Schroer et al., 1998; Borgatti et al., 2001]. These parent-of-origin effects suggest that abnormal expression of an imprinted gene or genes within 15q11-q13 can cause autism. Although not the only candidate for such an effect (see discussion of *GABRB3* and *ATP10A* below), *UBE3A* is a strong candidate, because it is subject to brain-specific paternal silencing, and maternal deficiency causes AS, a syndrome with neurological and behavioral abnormalities similar in many aspects to autism. Linkage analysis for 15q11-q13 in autism has yielded negative results in many cases but positive results in others [Philippe et al., 1999; Risch et al., 1999; Collaborative Linkage Study of Autism, 2001; Gutknecht, 2001]; Nurmi et al. [2001] provide a bibliography and present evidence for linkage disequilibrium at *D15S122* near the 5′-end of *UBE3A*. One report using ordered-subset analysis found a logarithm of the odds (LOD) score of 4.71 at *GABRB3*, a gene that encodes a GABA receptor subunit and lies telomeric to the 5′-end of *UBE3A* [Shao et al., 2003]. A recent very large study found no evidence for linkage to 15q11-q13, despite the use of dense microsatellite markers in this region [Yonan et al., 2003].

There is considerable phenotypic overlap between the AS and autism spectrum disorders, although this has attracted only limited attention [Williams et al., 2001]. Both disorders share prominent impairment of language and a high frequency of seizures and mental retardation. Impairment of social interactions is more widely recognized in autism, but social behavior is often inappropriate in AS. There are significant differences; for example, ataxia is common in AS and rare in autism except perhaps in cases with an isodicentric chromosome [Bundey et al., 1994; Borgatti et al., 2001]. Delayed or impaired walking and microcephaly are typical for AS, but not autism. One study found that four of four AS patients met the full behavioral criteria for autism spectrum disorder [Steffenburg et al., 1996b], but multiple studies have found that a high proportion of children with mental retardation and epilepsy meet the diagnostic criteria for autism [Rossi et al., 1995; Nordin and Gillberg, 1996; Steffenburg et al., 1996a; Swillen et al., 1996]. Our own studies (Peters et al., manuscript in preparation) found that nine of 19 patients with a

molecularly confirmed diagnosis of AS met the criteria for autism disorder.

HYPOTHESIS

An Oligogenic Model for Autism

In this report, we emphasize that changes in gene expression may have a genetic basis (with a change in DNA sequence) or an epigenetic basis (with no change in DNA sequence), and we propose that autism is an oligogenic disorder with *m*ixed *e*pigenetic and *g*enetic and mixed *d*e novo and *i*nherited (MEGDI) contributions to the etiology. First, based on the evidence for parent-of-origin effects, we propose that there is a major epigenetic component in the etiology of autism involving genomic imprinting. Second, we propose that epigenetic and genetic factors (both de novo and inherited) cause autism through dysregulation of two or more principal genes, one of which maps within chromosome 15q11-q13, with the Angelman gene encoding E6-AP ubiquitin-protein ligase (*UBE3A*) being the strongest candidate in this region. Third, we propose that the dysregulation of *UBE3A* involves some combination of overexpression, gene silencing, or misexpression of the three potential isoforms of E6-AP [Yamamoto et al., 1997; Kishino and Wagstaff, 1998]. Fourth, as we have suggested previously [Beaudet, 2002], we believe that the twin data for autism are best explained by de novo defects (epigenetic or genetic) arising in germ cells or in the embryo prior to MZ twinning. Fifth, we speculate that one to a few additional genes functioning upstream (affecting expression of *UBE3A*) or downstream (producing a protein that is ubiquitinated by the *UBE3A* ligase) are likely to participate with the Angelman gene in an oligogenic inheritance model. We propose that the role of *UBE3A* in the oligogenic model is quantitatively modest, but that proteins interacting with or ubiquitinated by *UBE3A* are candidates for involvement in autism.

MATERIALS AND METHODS

Rationale

Interphase fluorescence in situ hybridization (FISH) was used to test whether interstitial duplications of chromosome 15q11-q13 might be represent in a collection of sib pairs with autism; the parental origin of the duplicated chromosome for individuals in the one family identified was determined using short tandem repeat (STR) polymorphisms and Southern blotting using pulsed field gel electrophoresis (PFGE) and restriction enzymes sensitive to DNA methylation to assess a differentially methylated region (DMR) in DNA. Linkage was assessed across the 15q11-q13 region using a dense set of markers and analyzing maternal and paternal linkage (sharing of parental alleles in sib pairs) separately based on the hypothesis that any effect could involve an imprinted gene. Previously unidentified sites of differential DNA methylation were sought using Southern blotting and methylation sensitive restriction enzymes, and a tissue specific DMR (TS-DMR) was discovered. Autism brain samples were analyzed for abnormalities of DNA methylation on the hypothesis that brain specific imprinting defects might be found that could never be discovered by studying blood or cultured cells. Finally, the hypothesis that brain specific dysregulation of expression for *UBE3A* might contribute to autism was examined using Western blotting to assess the levels of the E6-AP ubiquitin ligase product of this gene.

Autism DNAs, Cell Lines, and Brain Tissue

DNA samples and lymphoblast cell lines were collected from 1995 to 1998 as part of the South Carolina Autism Project (SCAP) [Schroer et al., 1998] or were obtained from the Autism Genetic Resource Exchange (AGRE; www.agre.org/) or the National Institute of Mental Health (NIMH) Center for Collaborative Genetic Studies (www.zork.wustl.edu/nimh/). The phenotypic definition for autism is described in at the web sites for the AGRE and NIMH collections; for the 186 probands from the SCAP, 151 were diagnosed by the Autism Diagnostic Interview-Revised (ADI-R), 30 by the Childhood Autism Rating Scale (CARS), and five by physician assessment. For all probands and for affected sib pairs from the AGRE and NIMH collections, we studied only those in which the proband or both sibs were diagnosed with autism excluding those diagnosed as "broad spectrum." For the AGRE probands tested by FISH, 60 overlap completely with 60 of the AGRE sib pairs studied for linkage and 23 were from singleton affected families. The numbers of sib pairs studied for linkage are specified in Table I and Figure 1b. Genotype data will be deposited with the AGRE and NIMH programs upon publication.

Brain tissue was obtained from The Autism Research Foundation (TARF; www.ladders.org/tarf) in conjunction with the Harvard Brain Bank and from the Autism Tissue Program (ATP; www.brainbank.org) in conjunction with the NIH supported brain banks at the University of Maryland and University of Miami (www.medschool.umaryland.edu/BTBank/gflorida.html). Brain samples from the Harvard, Maryland, and Miami banks are indicated as Ha, MD, or Mi, respectively.

Southern Blotting, STR Genotyping, PFGE, and FISH

Southern blotting was performed using standard methods, and genotyping for STRs was performed as previously described [Stockton et al., 1998]. In initial genotyping, we encountered some individuals both from autism and from normal families, who appeared to have three alleles for *D15S817*. We confirmed the report [Longo et al., 2004] of two copies for the *D15S817* marker; we have tabulated data for copy 1, but we found no recombinants between the two copies of *D15S817*. Certain primers including those in the genome database (GDB) amplify copy 1 exclusively in most cases but occasionally amplify an allele of copy 2 leading to the apparent presence of three alleles with one pair of primers. Various primer sequences are available from the authors on request.

The probe for Southern blotting to detect the tissue specific DMR (TS-DMR) and for PFGE was prepared by amplification of a genomic DNA fragment near the last exon of *UBE3A* with the following primers: 5′-GACTTTCCACCTAACTCACTCACA and 5′-GCAGCTGAGTGCCATATAATGTTG. The Southern probe for analysis of the 5′-CpG island of *UBE3A* was amplified from genomic DNA with the following primers: 5′-CACTGGTGAAGCTTGGCAACCTTG and 5′-GGACTGCACAAGTGGGATGAGAAC. PFGE was performed as previously described [Pentao et al., 1992].

FISH was performed on interphase and metaphase cells from 60 affected probands from families with affected sib pairs and from 23 sporadic cases of autism, all from the AGRE collection. FISH was performed according to standard proto-

TABLE I. Sharing of Paternal Alleles for the *D15S817*/*D15S1035* Haplotype for Autism Affected Sib-Pairs

	Shared[a]	NS	Total	χ^2	*P*
AGRE	51	26	77	8.12	0.004
NIMH	30	26	56	0.28	0.60
Total	81	52	133	6.32	0.012

[a]Sharing is scored for *S817* in the case of two recombinants between *S817* and *S1035*.

cols using Nick-translated biotin or digoxigenin-labeled bacterial artificial chromosome (BAC) or P1 artificial chromosome (PAC) probes within the proximal 15q region spanning the PWS/AS critical region. FISH slides were analyzed using a Zeiss AxioplanII microscope with a cooled charged-coupled-device camera and Applied Imaging MacProbe software. Probes were as follows: chromosome 15 centromere Cep (satellite III) probe labeled with spectrum green (Cytocell, Rainbow Scientific, Windsor, CT); PAC 14I12 for *UBE3A* and PAC 5e9 for *SNRPN* [Sutcliffe et al., 1997].

Protein Extraction From Brain Tissue and Western Blotting

Human post-mortem tissue was obtained from three brain banks described above. Mice were sacrificed by cervical dislocation and the cerebrum and cerebellum were dissected. Human frozen brain tissues as provided by the brain banks were cut to small pieces and homogenized in NP40/SDS buffer (1% Nonidet P-40, 0.01% SDS, 0.1 M Tris-HCl, pH 7.2, and complete Protease Inhibitor Cocktail Tablet; Roche Applied Sciences, Indianapolis, IN) on ice with a pellet pestle motor. The lysates were then rotated at 4°C for 15 min before centrifugation at 16,000*g* for 15 min at 4°C. The supernatants were transferred to fresh tubes and put on ice. The pellets were homogenized again in NP40/SDS buffer prior to rotation and centrifugation under the same conditions. The two supernatants were collected, combined, and centrifuged again at 16,000*g* for 15 min at 4°C to remove insoluble protein. The supernatants of soluble protein were used for Western blotting.

The protein concentrations were measured by Bradford assay, and 50 μg of mouse brain protein or 100 μg of human brain protein was used for electrophoresis on 10% Tris-HCl ready gels® (Bio-Rad, Hercules, CA). Proteins were transferred to pure nitrocellulose membrane (Bio-Rad) at 4°C overnight. The membranes were incubated in 5% nonfat dry milk in phosphate-buffered saline with 0.1% Tween-20 (TPBS) for 1 hr at room temperature. Incubations with antibodies were performed in TPBS-milk at 4°C overnight as follows: rabbit anti-human E6-AP diluted 1:1,000 (BL447; Bethyl Labs, Montgomery, TX); goat IgG anti-human calbindin diluted 1:500 (sc-7691 Santa-Cruz Biotechnology, Santa Cruz, CA); or 1:1,000 diluted goat IgG anti-human actin diluted 1:1,000 sc-1616 Santa-Cruz). Antibodies to calbindin and actin were used as loading controls for cerebellum and cerebral cortex, respectively. The membranes were washed three times for 10 min each with TPBS, and then incubated with the appropriate secondary antibody, either donkey anti-goat IgG horseradish peroxidase (HRP) or goat anti-rabbit IgG HRP (sc-2020 and sc-2004, respectively, Santa Cruz). After another three washes in TPBS, the signals were detected by enhanced chemiluminescence (ECL, Amersham Life Science, Newark, NJ).

RESULTS

Duplications of 15q11-q13 but no Coding Mutations in *UBE3A*

We began our studies of autism by sequencing genomic DNA for all known coding and noncoding exons of *UBE3A* in 30 probands from autistic sib-pairs available through the AGRE. Consistent with a previous report [Veenstra-VanderWeele et al., 1999], we found no abnormalities other than benign variants (data not shown). We next performed FISH using a probe overlapping *UBE3A* and interphase cells from cultured lymphoblasts on 60 probands from the AGRE collection. For family AU-0106, we detected an interstitial duplication of 15q11-q13 (Fig. 2a) similar to those described in other families [Cook et al., 1997]. Analysis with STR markers in 15q11-q13 confirmed the duplication with the presence of three alleles in both of the autistic sibs and the mother; each sib inherited two alleles from the mother (Fig. 2b). Analysis was performed using PFGE and Southern blotting with the methylation sensitive enzyme *Not*I and a genomic DNA probe near *UBE3A* (Fig. 2c)

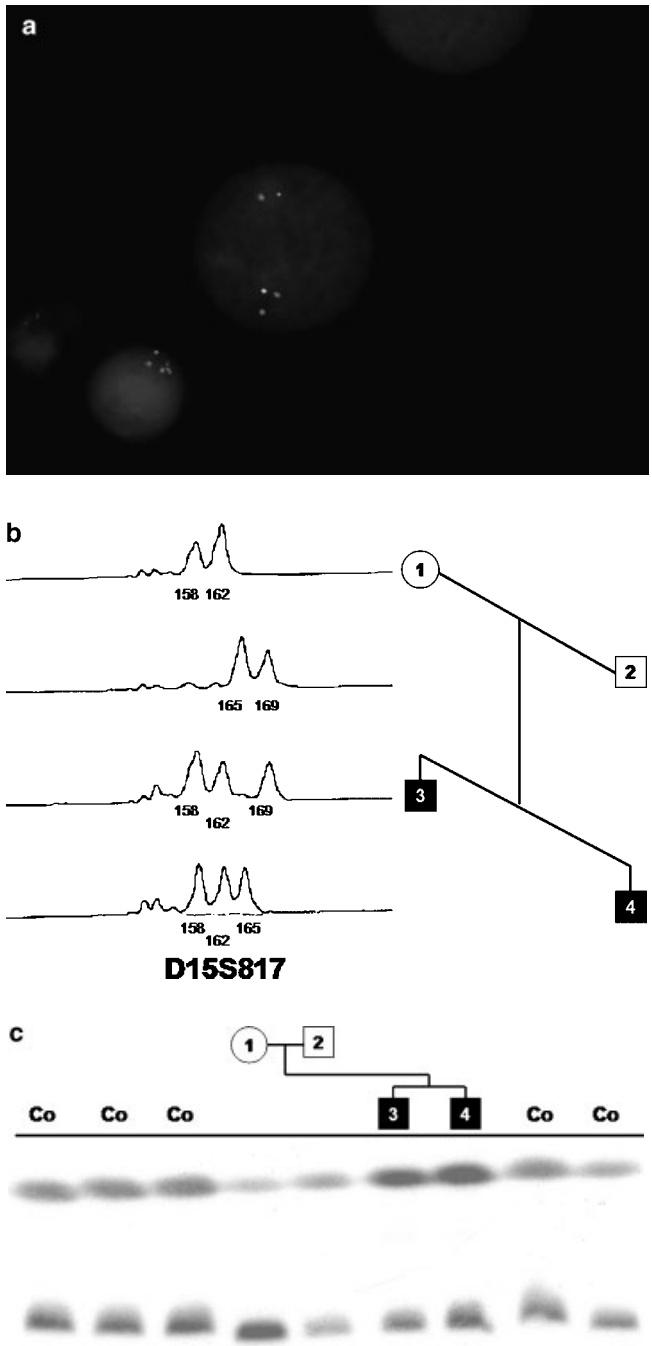


Fig. 2. Analysis of family with interstitial duplication of 15q11-q13. **a**: FISH showing duplication on affected child; green is 15 centromere probe and red is *UBE3A* probe. **b**: Tracing from ABI Gene Scanner for analysis with *D15S817* showing three alleles in two affected children; mother has two copies of 162 allele and one copy of 158. **c**: Pulsed field gel electrophoresis (PFGE) with *UBE3A* probe and *Not*I digestion showing greater intensity (two copies) of the upper methylated maternal allele in the affected sibs (3 and 4), greater intensity (two copies) of the lower unmethylated paternal allele in the mother (1), and approximately equal intensity (one maternal and one paternal allele) in the father (2) and normal controls (Co). The differentially methylated *Not*I site lies in the PWS-IC at the 5′-end of *SNRPN*.

demonstrating that the duplication was on the methylated (maternal) allele in the autistic sibs and on the unmethylated (paternal) allele in the mother. Three instances of maternal duplications were previously identified among 100 families from the South Carolina Autism Project (SCAP) [Schroer et al., 1998]; we did not screen the additional AGRE or the NIMH families studied below using FISH.

Allele Sharing at 15q11-q13

The hypothesis that dysregulation of *UBE3A* causes autism at least in some cases predicts that sequence variation in 15q11-q13 might contribute to susceptibility; if so, affected sibs might show increased sharing of parental alleles to the extent that effects are genetic and inherited rather than epigenetic or de novo. Given the imprinting of *UBE3A* and the evidence that maternal but not paternal duplications of 15q11-q13 cause autism, some families might result from imprinting defects or genetic defects that are of maternal origin and other families might have defects of paternal origin, so it is clear that sharing of alleles should be analyzed according to parent of origin.

Analysis for allelic sharing (i.e., whether affected sibs inherit the same haplotype from a parent) at multiple markers in 15q11-q13 for 58 autistic sib-pairs from the AGRE samples yielded two results of interest (Fig. 1b). First, there was increased sharing for paternal but not for maternal haplotypes, and second, the region of maximum paternal sharing was centromeric to the imprinting center and thus closer to the IC than to *UBE3A*. Among sib-pairs without recombinants, there were 29 with paternal sharing and 18 with paternal nonsharing for all the markers tested (Fig. 1b); there were 22 nonrecombinant pairs with maternal sharing and 28 with maternal nonsharing (data not shown). There were 11 sib-pairs with one paternal recombination between the most centromeric markers and *D15S217* (Fig. 1b), and nine of the 11 showed sharing at the centromeric but not at the telomeric end of the region. The female genetic map in this region shows that recombination is rare between the most centromeric markers and *D15S986*; in contrast, the male recombination rate per megabase of DNA is much higher than average (Fig. 1a). Thus there is a region of below average female recombination and above average male recombination. The map is consistent with the number of paternal recombinants observed over the short physical distance from *D15S1035*/*D15S817* to *D15S975*. The sharing of paternal alleles was greatest at the most centromeric marker (*D15S817*) which lies close to the necdin locus (*NDN*) on the opposite side of the imprinting center relative to *UBE3A* (Fig. 1a).

In order to test further the finding of increased sharing of paternal alleles in the 15q11-q13 region in autistic sib-pairs detected by analysis of *D15S817*, we next studied a dinucleotide repeat adjacent to *D15S817* and a more centromeric marker *D15S1035*, and we expanded the sample size to include 19 additional sib-pairs from the AGRE samples and 56 sib-pairs from an NIMH/Stanford collection (Table I). We found no recombinants between the two copies of *D15S817* and the adjacent dinucleotide repeat, and only two recombinants between *D15S817* and *D15S1035*; both sib pairs showed sharing at *D15S817* but not at *D15S1035*. Since there was extensive redundancy of informativeness for markers *D15S817*, the dinucleotide repeat, and *D15S1035*, sharing is recorded on the basis of haplotypes in Table I. Increased paternal sharing remained substantial (66%) for the expanded AGRE sample (nominal P value = 0.004), but there was no significant increased sharing for the NIMH samples. In the combined data, there was moderately increased sharing (nominal P = 0.012); even if a correction is made for two tests, because we would have considered either increased paternal or maternal to be of importance, the P value is still significant at the 0.02 level. Even for the AGRE samples, 26 of 77 sib-pairs did not share paternal alleles at *D15S817* indicating that a substantial fraction of autism in sib-pairs occurs in the absence of paternal allele sharing at 15q11-q13. Because of the epigenetic and de novo components of our model, we did not calculate a LOD score. Because the evidence of allele sharing was weak, although possibly significant, we chose to explore other strategies to test the oligogenic hypothesis.

Brain-Specific DNA Methylation in 15q11-q13

Sites of differential DNA methylation in 15q11-q13 are indicated in Figure 1a with a region of differential methylation according to parent-of-origin (PO-DMR) at the 5′-end of *SNRPN* and a region of tissue-specific differential methylation (TS-DMR) at the 3′-end of *UBE3A*; in addition, there is an unmethylated CpG island at the 5′-end of *UBE3A*. The PO-DMR is widely used to assess DNA methylation in diagnostic studies of PWS and AS [Sutcliffe et al., 1994]. The TS-DMR is located ~10 kb downstream (centromeric) to the stop codon for *UBE3A* and has not been described previously. The TS-DMR is not part of a CpG island, and we have studied the TS-DMR as a single CpG dinucleotide that is completely methylated in the non-CNS tissues examined (e.g., liver in Fig. 3a,b; other tissues not shown) and in cultured lymphoblasts, but it is variably methylated in cultured fibroblasts and in the brain (Fig. 3). The differential methylation in fibroblasts is not according to parent of origin as shown by analysis of PWS and AS deletion cases (Fig. 3a), but rather there is mosaicism of methylation for both maternal and paternal chromosomes. The TS-DMR is predominantly methylated in the cerebral cortex, but there is considerable variation among Brodmann regions of the cortex (Fig. 3c). The site is predominantly unmethylated in cerebellar cortex and vermis, and the degree of methylation in the hippocampus is intermediate. We have been unable to locate a homologous TS-DMR in mouse to date, although the similarity of tissue-specific silencing in mouse and human suggests that an undiscovered site could exist. The PO-DMR is extremely invariant with methylation being complete for all CpGs in the region on the maternal allele and totally absent on the paternal allele, even when chromosomes are transferred into somatic

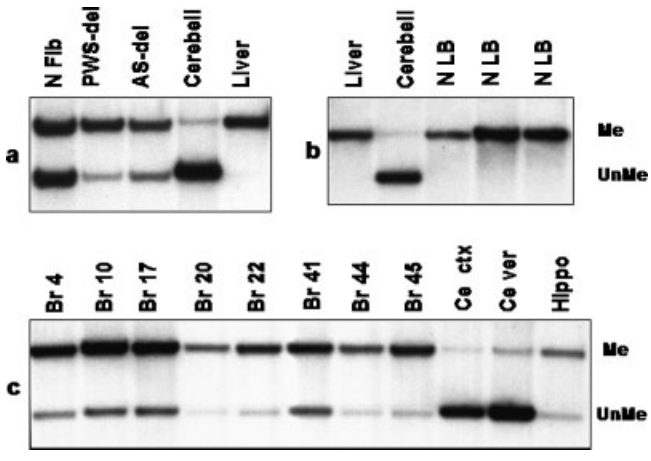


Fig. 3. Southern blot analysis of tissue specific differential methylation. DNA was digested with *Bam*HI and *Hpa*II and probed with a fragment near the 3′-end of *UBE3A*. **a**, **b**: Normal tissues and cultured cells. **c**: Normal human brain. DNA is partially methylated in cultures fibroblasts from a control (N Fib) and PWS and AS deletion cases (PWS-del and AS-del). DNA is highly methylated (Me) in liver and normal control lymphoblasts (N Lb) and is unmethylated (UnMe) in cerebellum (cerebell). Analysis from a single human brain includes Brodmann regions of cerebral cortex (Br 4, etc), cerebellar cortex (Ce ctx), cerebellar vermis (Ce ver), and hippocampus (Hippo).

cell hybrids [Sutcliffe et al., 1994]. In contrast, the TS-DMR is highly variable between tissues.

DNA Methylation in Autism Brain

In an attempt to search for abnormalities of DNA methylation in 15q11-q13 that might be brain-specific, autopsy brain tissue was obtained from 17 cases of autism, available from three different brain banks. Some of the specimens were collected many years ago, and the validity of the diagnosis of autism is documented to varying degrees for these samples; thus it is possible that not all diagnoses are correct. Although, we hypothesized that the TS-DMR was an excellent candidate for tissue-specific abnormalities in autism brain, no abnormalities of methylation for the PO-DMR or the TS-DMR were found when cerebellum and cerebral cortex were studied using Southern blotting in 17 samples (data not shown). The CpG island at the 5′-end of *UBE3A* was examined and definitive abnormalities were found in one sample (Ha-3511). This site was unmethylated in all other human samples examined including a wide range of tissues and cell cultures (Fig. 4a). As shown for a representative subset of samples in Figure 4b, this site was completely unmethylated in 60 autopsy brain samples from controls. For brain Ha-3511 (Fig. 4c,d), this site was approximately 50% methylated in cerebellum and cerebral cortex using two different methylation-sensitive restriction enzymes. In addition, bisulfite treatment followed by polymerase chain reaction (PCR) amplification, cloning, and sequencing confirmed the methylation with almost all of CpGs methylated in some clones and almost all unmethylated in the others. Inquiry regarding the phenotype of this patient indicated that he had received psychiatrist's diagnoses as an adult of mental retardation, hyperactive, autistic, and atypical development. Spoken language was limited and repetitive, mostly three-word phrases and requests. There was a history of aggressive behavior and two seizures. Due to lack of parental samples, we are unable to determine if the differential methylation in Ha-3511 is according to parent-of-origin, although the approximately equal intensity of methylated and unmethylated bands and the two classes of results with bisulfite analysis make this a significant possibility. This CpG island is located at the 5′-end of cDNA clone GenBank U84044 upstream of all known coding and 5′-noncoding exons of *UBE3A* [Yamamoto et al., 1997; Kishino and Wagstaff, 1998]. We suggest that this abnormal DNA methylation likely is associated with some form of dysregulation of *UBE3A* leading to the autism in case Ha-3511, although levels of E6-AP protein in this brain appear normal as shown below.

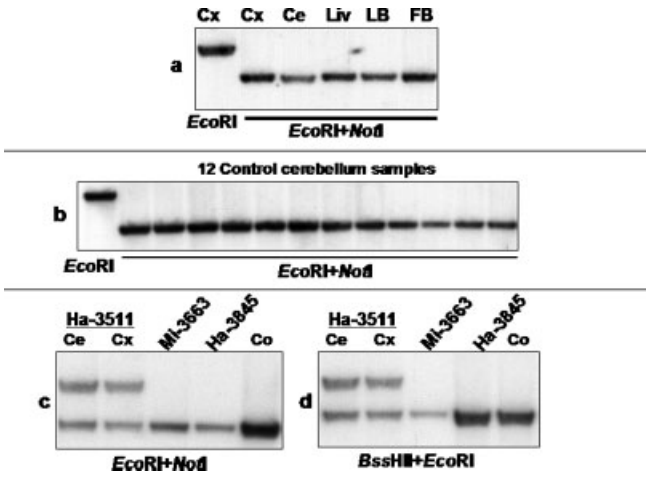


Fig. 4. Southern blot analysis of methylation of CpG island at 5′-*UBE3A*. **a**: Control samples: Cx, cerebral cortex; Ce, cerebellum; Liv, liver; LB, lymphoblasts; FB, fibroblasts. **b**: Control autopsy cerebellum samples. **c**–**d**: Brain from autism case Ha-3511 (cerebellum, Ce and cerebral cortex, Cx) and cerebellum for two other autism cases and from a control (Co) using two different methylation sensitive restriction enzymes *Not*I and *Bss*HII within the CpG island of *UBE3A*.

Western Blot Analysis of Autism Brain

Based on the evidence that maternal but not paternal duplications of 15q cause autism, we considered the hypothesis that over-expression of *UBE3A* might cause autism and that failure of silencing of the paternal allele might occur in some families. In the context of this hypothesis, we performed Western blotting to analyze cerebellum and cerebral cortex samples from the available autism brain specimens (Fig. 5). Analysis of 30 control brain samples showed a relatively constant abundance of antigen as compared to control proteins, calbindin for cerebellar cortex and actin for cerebral cortex. Only one (Ha-4270) of the 30 control samples showed a reduction (severe) of antigen in cerebellum, but results were normal on cerebral cortex for this case. One brain sample from AS showed a severe reduction in E6-AP as expected. In contrast to normal controls, the level of E6-AP antigen in autism cerebellum was severely reduced to at or near the AS level in 3/17, was moderately reduced in 4/17, and was approximately equal to controls in 10/17 (Fig. 5 and Table II). Similar reductions were seen in cerebral cortex (Fig. 5 and Table II). Sample Ha-3511 which had the definitive abnormality of DNA methylation had normal abundance of antigen. Although we hypothesized the possibility of over-expression of *UBE3A*, we in fact found opposite evidence that the abundance of E6-AP was reduced in some autism brains. Interpretation of the Western blot data is hazardous because of the possibilities of secondary effects of autism on abundance of the E6-AP protein, and more importantly because of the potential for premortem

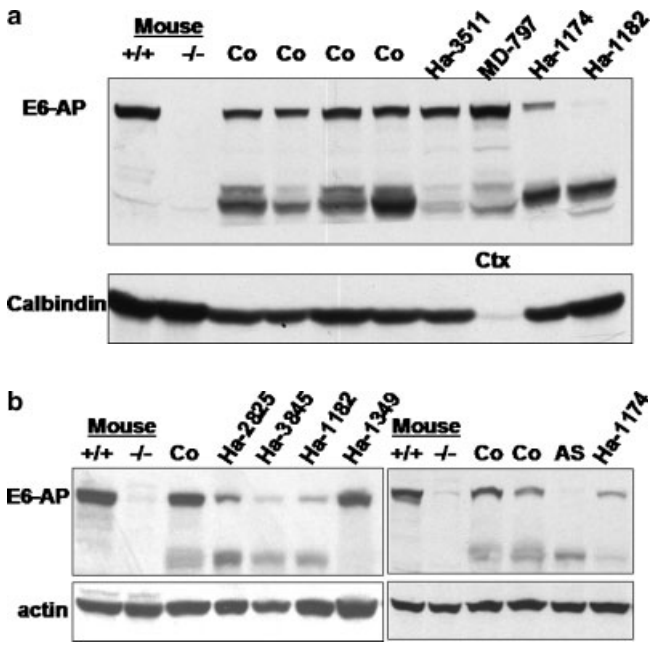


Fig. 5. Western blot analysis of autism brain. Analysis was performed as described in Materials and Methods using an antibody to E6-AP. All lanes in **panel a** are cerebellum except one lane of cerebral cortex (Ctx) from MD-797. Cerebellum (panel a) and cerebral cortex (**panel b**) from wild-type (+/+) and homozygous *Ube3a* null (−/−) mice are shown. Control (Co) and autism (specifically identified) samples are shown. Panel b is samples from cerebral cortex with labeling as in panel a. Control antibodies were to calbindin (panel a) and actin (panel b).

TABLE II. DNA Methylation at the 5′-CpG Island of *UBE3A* and E6-AP Protein in Autism Brain

				Western blot[c]	
Brain[a]	DNA methylation[b]	PMI hours	RNA	Cerebell	Cortex
MD-797	Nl	13	Pass	+	+
MD-1174	Nl	14	Pass	▼	▼▼
MD-1182	Nl	24	Fail	▼▼	▼
MD-1349	Nl	39	Pass	+	+
Ha-1469	Nl	42	na	▼	▼
MD-1638	Nl	50	na	▼/▼▼	na
Ha-1664	Nl	15	na	+	+
Ha-1978	Nl	23	na	+	+
Ha-2825	Nl	9.5	na	+/▼	▼▼
Ha-2830	Nl	24	na	+/▼	▼▼
Ha-2993	Nl	5	na	+	+
Ha-3511	Abn	15	na	+	+
Mi-3663	Nl	30	Pass	+	+
Ha-3845	Nl	28	na	▼▼	▼▼
Ha-4498	Nl	19	Fail	+	▼
Ha-4925	Nl	27	Pass	+	+
Ha-5144	Nl	24	Pass	+	+

[a]Ha, Harvard Brain Bank; MD, Maryland Brain Bank; and Mi, Miami Brain Bank.
[b]Nl, normal; Abn, abnormal.
[c]+, similar to normal controls; ▼, ~50% of controls; ▼▼, near absent as in AS; na, not available.

and postmortem artifacts. Although the postmortem intervals for control and autism brains were matched to the extent possible, artifactual degradation of E6-AP remains a possibility. In particular, other investigators have raised concerns about degradation of RNA for samples MD-1182 and Ha-4498 (Jane Pickett, personal communication, Autism Tissue Program; www.atpportal.org). There could be a bias for brain banks to reject control brains more readily than autism brains, if there is a questionable perimortem or postmortem history. However, the data are more compatible with silencing of expression or temporal or cell-specific dysregulation than with the initial hypothesis of over-expression.

DISCUSSION

Tissue-specific expression of *UBE3A*/*Ube3a* in human and mouse shows paternal silencing in brain but not in somatic tissues. In human, region-specific data are not available, but reports indicate silencing in all samples of "brain" tested [Vu and Hoffman, 1997]; for region-specific data in mouse, paternal silencing is most evident in Purkinje cells and hippocampal neurons [Albrecht et al., 1997]. In the mouse, there is evidence that *Ube3a* is imprinted in neurons but not in glial cells [Yamasaki et al., 2003]. The TS-DMR is methylated in non-CNS tissues, where expression is bialleleic, while the site is unmethylated to varying extents in different regions of the brain where the paternal allele is variably silenced. The absence of methylation is most complete in cerebellum, where the combined mouse and human data suggest that paternal silencing is prominent. There is an antisense transcript for *Ube3a*/*UBE3A* expressed exclusively from the paternal allele in mouse and human; the antisense transcript is expressed preferentially in brain, although it can be detected at low levels in cultured human lymphoblasts [Rougeulle et al., 1998; Chamberlain and Brannan, 2001; Runte et al., 2001; Yamasaki et al., 2003]. Combining information from mouse and humans, the following correlations are suggested for human brain, although causal relationships are not proven: only paternal alleles in certain brain cells (e.g., Purkinje cells) are (1) unmethylated at both the PO-DMR and the TS-DMR, (2) preferentially expressing antisense transcript, and (3) silenced for sense expression of *UBE3A*. Examination of the TS-DMR did not reveal any abnormalities in autism brain.

Our approach to autism is colored by the extensive molecular information available for AS, by the evidence that maternal but not paternal duplications of 15q11-q13 cause autism, and by the phenotypic overlap of autism and AS. AS provides a clear precedent for oligogenic inheritance with MEGDI etiology. We have focused on the hypothesis that dysregulation of *UBE3A* causes autism, at least in a fraction of cases. For the purpose of this discussion, we will define "genetic disease" as a disorder of gene expression caused by an alteration of genomic DNA sequence, and "epigenetic disease" as a disorder of gene expression not accompanied by a change in DNA sequence. As depicted in Figure 6a, the data on AS demonstrate that an epigenetic defect [e.g., the paternal uniparental disomy (UPD) cases of AS] can give an essentially identical phenotype to that caused by a genetic defect (e.g., a common maternal deletion form of AS). The data on AS also demonstrate that despite parent-of-origin effects in all circumstances, there can be extremely heterogeneous etiology for a single phenotype including a complex mixture of de novo and inherited components; for AS this includes large deletion, UPD, imprinting defect with and without IC deletion, and loss-of-function point mutations in *UBE3A*. In addition, there is evidence that nongenetic factors such as intracytoplasmic sperm injection (ICSI) can cause AS via imprinting defects [Cox et al., 2002; Orstavik et al., 2003] that appear to be of de novo epigenetic rather than genetic origin. AS also provides a precedent for very different molecular changes in affected sib-pairs as contrasted to isolated cases. Recurrence risk is very low for large deletion and UPD cases, but high for point mutations in *UBE3A* and imprinting defects caused by deletions of the AS-IC. If the situation for autism is similar to AS, the implication would be that analysis of familial cases like affected sib-pairs might yield very different epigenetic or genetic results compared to analysis of isolated cases.

There is extensive evidence that duplicons are abundant within and adjacent to chromosome 15q11-q13 (see Segmental Duplication Database; http://humanparalogy.gene.cwru.edu/) and that these predispose to a variety of deletions, duplications, and inversions causing disease phenotypes [Christian et al., 1999; Ji et al., 2000; Pujana et al., 2002], including inversions that may themselves be benign but predispose to deletion cases of AS [Gimelli et al., 2003]. The findings in AS also provide a precedent for a mixture of de novo and inherited factors, and there is the potential for semi-heritable epigenetic

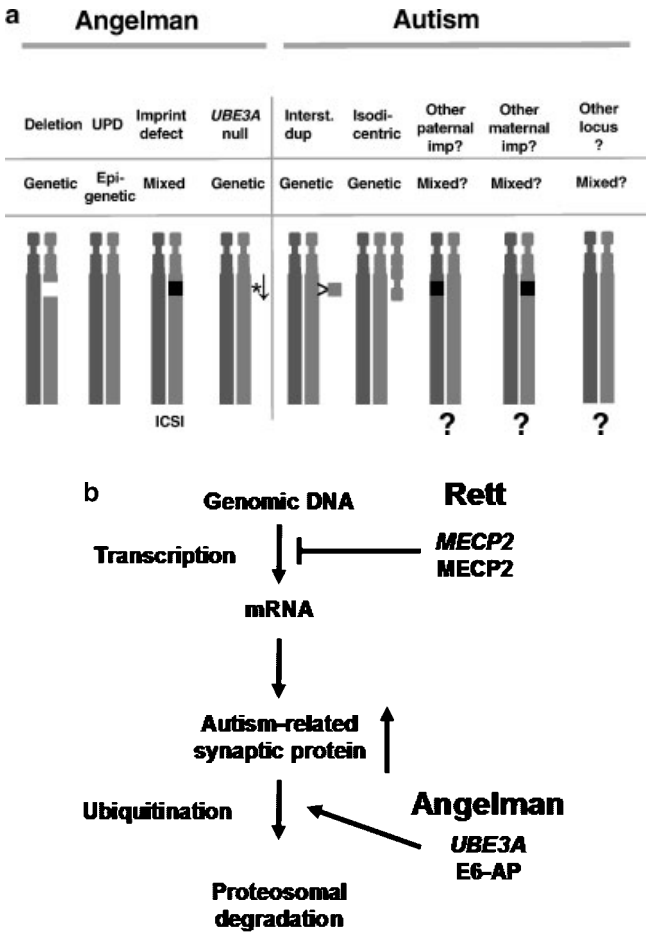


Fig. 6. Hypothetical depiction of molecular heterogeneity and genetic interactions in autism and AS. **Panel a** depicts various molecular classes of AS and autism defects. For autism, the interstitial duplication and isodicentric classes are documented and the other three classes are hypothetical as described in text. Deletion is shown as a white block in the chromosome and imprinting defects as black blocks. Loss-of-function point is shown as an asterisk with downward arrow. **Panel b** depicts potential interactions of the Rett and Angelman syndrome genes with a hypothetical gene encoding an autism-related synaptic protein. MECP2 is hypothesized to repress transcription of the gene and E6-AP is hypothesized to ubiquitinate the protein.

changes. Semi-heritable refers to the fact that differences of genomic imprinting such as differential DNA methylation according to parent of origin may require more than one generation of maternal or paternal transmission to be fully erased and reset. There is unequivocal evidence of incomplete erasure and resetting of imprints in one generation in the mouse [Rakyan et al., 2002]. By analogy to AS, we propose that similar mixtures of epigenetic and genetic and of de novo and inherited factors might contribute to dysregulation of *UBE3A* in the brain as the primary biochemical defect in some fraction of autism cases (Fig. 6a). The maternal interstitial duplications causing autism would be the reciprocal products of unequal cross-overs leading to deletion cases of AS. The cases of maternally derived extra chromosomes (isodicentric 15) represent a variation on the duplication theme with three rather than two maternal alleles, four rather than three alleles in total. Also depicted in Figure 6a is the hypothetical potential for paternal and/or maternal imprinting defects, as well as a hypothetical group of patients with abnormalities of other genes, possibly upstream or downstream in a pathway involving *UBE3A*.

By DSM IV terminology, the autism spectrum disorders include autistic disorder; pervasive developmental disorder, not otherwise specified; and Asperger disorder. We propose that there could be a genotype/epigenotype to phenotype correlation along a continuum with the nature or extent of dysregulation of *UBE3A* in the brain associated with the severity of the phenotype. Consistent with this rationale is the fact that patients with extra isodicentric chromosome 15 have the highest maternal copy number for 15q compared to interstitial duplications, and the former have the more severe phenotypes [Rineer et al., 1998] including ataxia which is otherwise rare in autism [Borgatti et al., 2001].

We propose a MEGDI oligogenic interaction model for the etiology of autism. In this model, some combination of genetic and epigenetic factors exhibiting both de novo and inherited variation could affect the expression of a small number of principal causative genes, one of which is proposed to be *UBE3A*. Although this model is somewhat speculative for autism, there is clear precedent for the MEGDI model in AS where *UBE3A* acts as a single major gene. In the case of autism, the model is compatible with the known data and with the studies reported here. For cases of interstitial duplications and isodicentric abnormalities of 15q11-q13, there is strong evidence that these abnormalities can give a high penetrance for autism, apart from any modifier effects. For example, there are no recorded examples of lack of penetrance when interstitial duplications are maternally inherited in families. On the other hand, the genetic data, such as the lack of either maternal or paternal allele sharing in 15q11-q13 in many sib pairs, suggest that it is unlikely that *UBE3A* or some other gene in 15q11-q13 is the major determining locus in the majority of patients. Rather we propose that *UBE3A* is a major gene in only a fraction of cases and that there is at least a second major gene, perhaps one encoding a protein that is subject to ubiquitination by E6-AP (Fig. 6b). The failure to detect another major gene in linkage studies might be explained by a substantial frequency of de novo events, as we believe is suggested by the twin data. It has been suggested [Zoghbi, 2003] that "both Rett (syndrome) and autism could be disorders of synaptic modulation or maintenance," and this raises the interesting possibility of a candidate autism gene encoding a synaptic protein that is subject to transcriptional regulation by the MECP2 protein and to ubiquitination by E6-AP (Fig. 6b). We do not argue that our data prove each of the various components of the oligogenic hypothesis, but rather that the model (1) is generally compatible with the data presented, (2) represents some fresh perspectives for autism, and (3) can be tested in a number of ways. We believe that this model is highly likely to apply to some small fraction of autism cases, but more importantly and more speculatively, we propose that it will explain the majority of cases of autism. Discussion of models with 10 or more loci and oligogenic models will continue until more definitive data become available. The MEGDI model could be relevant to other "complex disease traits" of unclear etiology.

Although maternal duplications of 15q11-q13 might suggest over-expression of *UBE3A* as a biochemical basis of autism, Western blot data did not suggest increased abundance of E6-AP in brain. Although, it is known that use of alternative promoters and splicing can give rise to three potential isoforms of E6-AP with differing N-terminal amino acid sequences [Yamamoto et al., 1997; Kishino and Wagstaff, 1998], there is minimal information about the relative abundance of each, the cellular or subcellular expression patterns, or any functional differences between the isoforms. Many other forms of dysregulation are possible including increased or decreased transcription, temporal or cell-type specific changes in transcription, long-range position effects between the coding exons and the IC, changes in expression of isoforms, or altered

subcellular localization of isoforms. Epigenetic studies in plants, yeast, fungi, and *Drosophila* provide extensive precedent for the possibilities of transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) of an imprinted gene in 15q11-q13 causing autism. PTGS is most widely studied in plants, and it is known as quelling in fungi and as RNA interference (RNAi) in animals [Matzke et al., 2001; Dykxhoorn et al., 2003; Matzke and Matzke, 2003]. Paramutation, a form of trans-acting TGS that sometimes involves changes in DNA methylation [Wolffe and Matzke, 1999], could contribute to the etiology of autism in the case of invdup15 or intragenetic duplications of 15q11-q13. A paramutation-like effect has been produced artificially at the imprinted *Rasgrf1* locus in mice [Que and Jorgensen, 1998], and a potential effect of the nontransmitted allele of the insulin gene in the causation of type I diabetes suggests the possibility that the "untransmitted chromosome can have functional effects on the biological properties of transmitted chromosomes" [Bennett et al., 1997]. This would be analogous to paramutation in plants [Hollick et al., 1997] or trans-inactivation in yeast [Grewal and Klar, 1996]. The epigenetic data from other species make it clear that both cis-acting and trans-acting effects are possible.

We have attempted to test the role of *UBE3A* in an oligogenic model in a number of ways. In the AGRE but not the NIMH families, linkage analysis of affected sib-pairs was suggestive of increased paternal sharing at *D15S817* in 15q11-q13 nearer to the imprinting center than to *UBE3A*. The difference in the two collections could be real, for example, reflecting diagnostic criteria and time of collection. On the other hand, the AGRE data may reflect a chance statistical finding. Although the cytogenetics evidence for a parent-of-origin effect involving 15q11-q13 is strong, there is evidence for and against linkage to this region in autism. The report of a LOD score of 4.71 at *GABRB3* resulted entirely from sharing of maternal alleles [Shao et al., 2003]. The possibility of maternal sharing at *GABRB3* and paternal sharing near the IC are not necessarily incompatible, since the *GABRB3* data involve a subset analysis.

Against the oligogenic model is the failure to find stronger statistical evidence of genetic loci, but this could be explained by major de novo and epigenetic components in the etiology. The specific focus on *UBE3A* as the relevant gene could prove incorrect, since *ATP10A* is another maternally expressed imprinted gene immediately adjacent to *UBE3A* and *GABRB3* is a neurologically relevant gene in the region. However, we believe that *UBE3A* is the strongest candidate taking into account genomic imprinting, phenotypic overlap with AS, and the altered DNA methylation in one of 17 autism brains. While our data do not prove that the MEGDI model holds for autism, we believe that the hypothesis deserves further testing.

There is an active debate as to whether the incidence of autism is increasing [Fombonne, 2003; Yeargin-Allsopp et al., 2003]. The epigenetic component of the oligogenic model can be considered in the context of possible environmental factors affecting the risk of de novo imprinting defects. Reports that ICSI causes imprinting defects resulting in AS [Cox et al., 2002; Orstavik et al., 2003] and Beckwith–Wiedemann syndrome [Maher et al., 2003] provide an example of how nongenetic factors could affect the risk for an epigenetic form of autism.

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