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Autism and other Neuropsychiatric Symptoms are Prevalent in Individuals with *MECP2* Duplication Syndrome

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Abstract

Objective—There have been no objective assessments to determine whether boys with *MECP2* duplication have autism or whether female carriers manifest phenotypes. This study characterizes the clinical and neuropsychiatric phenotypes of affected boys and carrier females.

Methods—Eight families (9 males and 9 females) with *MECP2* duplication participated. A detailed history, physical examination, electroencephalogram, developmental evaluation, Autism Diagnostic Observation Schedule, and Autism Diagnostic Interview – Revised was performed for each boy. Carrier females completed the Symptom Checklist-90-R, Wechsler Abbreviated Scale of Intelligence, Broad Autism Phenotype Questionnaire, and detailed medical and mental health histories. Size and gene content of each duplication were determined by array-CGH. X-chromosome inactivation patterns were analyzed using leukocyte DNA. *MECP2* and *IRAK1* RNA levels were quantified from lymphoblast cell lines, and Western blots were performed to assess MeCP2 protein levels.

Results—All of the boys demonstrate mental retardation and autism. Poor expressive language, gaze avoidance, repetitive behaviors, anxiety, and atypical socialization were prevalent. Female carriers have psychiatric symptoms including generalized anxiety, depression, and compulsions that preceded the birth of their children. The majority exhibited features of the broad autism phenotype and had higher nonverbal compared to verbal reasoning skills.

Interpretation—Autism is a defining feature of the *MECP2* duplication syndrome in boys. Females manifest phenotypes despite 100% skewing of X-inactivation and normal *MECP2* RNA levels in peripheral blood. Analysis of the duplication size, *MECP2* and *IRAK1* RNA levels, and MeCP2 protein levels revealed that most of the traits in affected boys are likely due to the genomic region spanning *MECP2* and *IRAK1*. The phenotypes observed in carrier females may be secondary to tissue-specific dosage alterations and require further study.

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Introduction

Since the discovery that mutations in the X-linked methyl-CpG-binding protein 2 gene (*MECP2*) cause Rett syndrome (RTT) in 1:10,000 girls¹, *MECP2* mutations were described in females with RTT variants, Angelman-like phenotypes, isolated autism or mental retardation, learning disabilities, attention disorders, as well as in males with classic RTT, fatal infantile encephalopathy, mental retardation with movement disorders and/or epilepsy, and early onset psychosis including schizophrenia²⁻⁷. A *MECP2* duplication syndrome was predicted by the observation that mice engineered to overexpress *MECP2* develop a progressive neurological disorder, stereotyped and repetitive movements, epilepsy, spasticity, hypoactivity, and early death⁸. Human *MECP2* duplications were initially identified in a girl with atypical RTT due to preserved speech, a boy with severe mental retardation and RTT features, males with mental retardation and progressive spasticity, and in members of three families with X-linked mental retardation⁹⁻¹². Additional males with *MECP2* duplications were reported in the past few years with the following common phenotypes: infantile hypotonia, severe to profound mental retardation, poor speech development, recurrent infections, epilepsy, and progressive spasticity¹³⁻¹⁵. While most of the reported cases are inherited, *de novo* cases have been documented^{15, 16}. Autistic features were noted in three boys¹³, however, no objective characterization of neuropsychiatric phenotypes has been performed in males with *MECP2* duplication and no objective clinical or psychiatric characterization has been performed for female carriers of *MECP2* duplication.

There is considerable clinical overlap in patients with classic RTT and autism; in fact some girls with RTT meet formal autism criteria¹. Specifically, stereotypic hand/body movements, anxiety, and social avoidance are examples of overlapping behavioral features². Forepaw stereotypies, anxiety, and abnormalities in social interaction are present in a mouse model of RTT¹⁷ and mice that overexpress *MECP2* also demonstrate forepaw stereotypies and abnormal behaviors⁸. Therefore, we hypothesized that *MECP2* gain of function may also lead to specific autism spectrum behaviors in humans. Furthermore, since many carrier females of X-linked disorders manifest milder disease symptoms than affected males¹⁸, and because data demonstrate that *MECP2* is one of the most dosage-sensitive genes involved in neuronal functional integrity^{8, 19-21}, we also hypothesized that carrier females may manifest mild symptoms. Elucidating the specific behaviors and neuropsychiatric endophenotypes associated with *MECP2* dysfunction may shed light into the pathogenesis of autism and related neuropsychiatric disorders and help define novel research paths.

Methods

SUBJECTS

Eight families including 9 males (subjects 4 and 5 are brothers) and 9 females (7 carrier mothers, 1 carrier maternal aunt who had an affected son who is deceased, and 1 carrier maternal grandmother) with *MECP2* duplication identified through clinical quantitative DNA methods and BAC-based chromosomal microarray analysis were referred to participate in our study between 2006 and 2008. This study was approved by the institutional review board for Baylor College of Medicine and affiliated hospitals. Written and verbal informed consent was obtained from all participants and/or their caregivers. Subjects were admitted to the General Clinical Research Center at Texas Children's Hospital. For each boy, a detailed history, physical examination, electroencephalogram (EEG), cognitive/developmental evaluation (using the Bayley Scales of Infant Development – 3, and the Vineland Adaptive Behavior Scales – 2) 22, Autism Diagnostic Observation Schedule (ADOS)²³, and Autism Diagnostic Interview – Revised (ADI-R)²⁴ were performed. Clinical histories were also obtained for deceased male relatives who presumably inherited a *MECP2* duplication from their carrier mother (documented prior to death in one instance). Carrier females completed the Symptom

Checklist-90-R²⁵, the Wechsler Abbreviated Scale of Intelligence²⁶, the Broad Autism Phenotype Questionnaire²⁷, and detailed medical and mental health histories. Each subject was examined by a board certified neurologist with special qualification in child neurology (H.Y.Z.) and all psychological testing was performed by a psychologist with research reliability in the ADOS and ADI-R (S.P.). EEGs were performed by trained professionals in the Texas Children's Hospital EEG laboratory and interpreted by board certified pediatric neurophysiologists.

Duplication size and genome content

To determine the size, extent, and gene content for each rearrangement, we designed a tiling path oligonucleotide microarray spanning 4 Mb around the *MECP2* region on Xq28. The custom 4×44k Agilent Technologies microarray was designed using the Agilent website (<http://earray.chem.agilent.com/earray/>). We selected 8,323 probes covering ChrX: 150,400,000–154,400,000 (NCBI build 35), including the *MECP2* gene, which represents an average resolution of 1 probe per each 500 bp. Probe labeling and hybridization were performed following the Agilent Oligonucleotide Array-based CGH for Genomic DNA analysis, version 4.0 protocol, plus the 4×44k complementary protocol with modifications unique to the four-pack format. Briefly, 1.5 µg of a single genomic male/female reference and patient DNA were digested with *Alu* I (5 U) and *Rsa* I (5 U) (Promega) for 2 hr at 37°C. Digestions were verified by agarose gel electrophoresis. Labeling reactions with Cy5-dUTP (patient DNA) and Cy3-dUTP (reference DNA) were performed according to the Agilent Genomic DNA Labeling Kit Plus, #5188–5309. Individual dye-labeled reference and patient samples were purified using Microcon YM-30 filters (Millipore Corporation). DNA yield was determined using a NanoDrop ND-1000 UV-VIS spectrophotometer. Each dye-labeled patient and gender-matched reference DNA was combined with 5 µg human Cot-1 DNA (Invitrogen Corporation), Agilent Blocking Agent, and Agilent hybridization buffer (#5188–5220). These mixtures were denatured at 95°C for 3 min, preincubated at 37°C for 30 min, and hybridized to the array in a hybridization chamber for 40 hr at 65°C in a rotating oven (Agilent Technologies). Array slides were washed using Agilent Wash solutions 1 and 2 (#5188–5226), Acetonitrile (Sigma-Aldrich), and Stabilization and Drying Solution (#5185–5979), according to the manufacturer's instructions. Slides were scanned on a GenePix 4000B Microarray Scanner (Axon Instruments). Images were analyzed and data were extracted, background subtracted, and normalized using Agilent Feature Extraction Software A.7.5.1. These data were subsequently imported into array CGH analytics software v3.1.28 (Agilent Technologies). The genomic copy number was defined by analysis of the normalized log₂ (Cy5/Cy3) ratio average of the CGH signal, amid a 5 kb window. Regions that reached a threshold of at least 0.6 were considered as gains consistent with duplication, whereas thresholds of at least 1.2 were considered as triplication.

Quantitative real-time rt-pcr

Total RNA was extracted from immortalized lymphoblast cell lines from affected and control males and carrier and control females using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA), DNaseI treated, and purified using the Rneasy mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA). cDNA was synthesized from 0.5 µg of RNA using the RT2 First Strand Kit (SuperArray Bioscience Corporation, Frederick, MD). Quantitative real-time PCR reactions were performed on 25 ng of cDNA using TaqMan master mix, *MECP2* primers (forward primer 5'AGACCGTACTCCCCATCAAGA, reverse primer 5'CACTTCCTTGACCTCGATGCT, and probe 5'-FAM-ACCGTCTCCCGGGTCTTGCGBHQ), *IRAK1* primers (forward primer 5'GGCCTCAGCGACTGGACAT, reverse primer 5'GAAGGACGTTGGAACCTTTGATG, and probe 5'-FAMCCCGGGCAATTCAGTTTCTACATCAGG-BHQ), and commercially

available human *GAPDH* primers (Applied Biosystems, Foster City, CA). All RNA samples were analyzed in triplicate and normalized relative to *GAPDH* levels.

Production of MeCP2 antibody 0535

The N-terminal MeCP2 antigen used to produce antibody 0535 consisted of a 60 kDa degradation product from a full length GST-mMeCP2 fusion protein expressed in BL-21 cells. The 60 kDa fragment was purified using GSH beads followed by SDS gel purification. The band was cut out and the protein was eluted, dialyzed, freeze dried, and injected into New Zealand White rabbits per standard protocol. The estimated dilution for probing westerns containing 1 µg of acid extract from HEK293T cells transfected with *MECP2* or 40 µg of SDS extract from wild-type or *MECP2* 308 (a mutation that truncates the protein after amino acid 308) mouse cerebellum was 1/25,000 using ECL detection.

Western blot analysis

Proteins were isolated via acid extraction from confluent cultures of each immortalized human lymphoblast cell line. Briefly, 15 ml of confluent culture were harvested by centrifugation, washed with washing buffer [1X PBS, 1 mM PMSF, 10 mM sodium butyrate, complete protease inhibitor cocktail (Roche)], resuspended in 1 ml extraction buffer (0.4 N H₂SO₄, 10 mM sodium butyrate), and incubated on a rotating shelf at 4° C for 60 minutes. After centrifugation, proteins were precipitated by the addition of precipitation buffer (4 mg/ml sodium deoxycholic acid in 100% trichloroacetic acid) for 30 minutes on ice. After centrifugation, the pellet was washed with acidified acetone (0.1% HCl in acetone), then with regular acetone. The pellet was resuspended in water. Determination of the protein concentration was performed using the BCA assay (Thermo Fisher Scientific). 5 µg of total protein from each sample were used for Western blotting. Sample buffer (1X NuPAGE LDS sample buffer, Invitrogen) was added and the samples were boiled for 10 minutes. Proteins were run on NuPAGE Novex 4–12% Bis-Tris Gels (Invitrogen) and then transferred to PVDF membrane [NuPAGE transfer buffer (Invitrogen), 20% methanol]. Immunodetection was performed using the primary antibodies anti-MeCP2 (MeCP2 antibody 0535; 1:10,000) and anti-Histone H3 (Millipore #06–755, 1:10,000) as a loading control. Secondary antibodies were conjugated to HRP and detected by chemiluminescence. MeCP2 and histone H3 protein levels were quantified using ImageJ software (National Institutes of Health), and the total MeCP2 protein was normalized to the level of histone H3 protein detected for each subject.

X-chromosome inactivation studies

X-inactivation studies were performed based on the protocol described by Allen *et al.* with modification²⁸. Briefly, 100 ng of genomic leukocyte DNA from each female (DNA from males was not treated) was digested with and without the methylation-sensitive restriction enzyme *HpaII* (New England Biolabs). PCR primers flanking the androgen receptor CAG_n repeat region were designed as follows:
 5'ACCAGGTAGCCTGTGGGGCCTCTACGATGGGC3' (forward) and
 5'CCAGAGCGTGCGCGAAGTGATCCAGAACCCGG3' (reverse), and 0.5 ng DNA from each sample was subjected to PCR amplification. PCR products were analyzed on an ABI 3100 instrument with GeneMapper software.

Results

Clinical summary of boys

Clinical data were gathered for nine males with a *MECP2* duplication; these males ranged in age from 3–15 years (refer to Table 1). Family pedigrees are shown in Figure 1. All boys manifested abnormal tone best characterized as global hypotonia with 89% exhibiting

progressive lower extremity spasticity (the boy who did not was only 3 years at the time of exam). Choreiform movements characterized by intermittent, spontaneous writhing movements of the arms, hands, and fingers that at times also involved the head and tongue were present in 89%; tremor was notably absent. Frequent respiratory infections and/or recurrent pneumonia occur in 78% and all deceased male relatives with presumed *MECP2* duplication (three maternal uncles and a maternal first cousin of subject 3; and the brother of subject 2) succumbed to respiratory infections. Each of these deceased male relatives had epilepsy and 44% of our male subjects have epilepsy. Clinically, their seizures are partial onset with or without secondary generalization or generalized. Subject 3 also manifests a rare reflex generalized epilepsy triggered by feeding and confirmed by continuous video EEG monitoring in the epilepsy monitoring unit. Other common problems include: cryptorchidism (33%), bruxism (67%), abnormal breathing patterns characterized by grunting, aerophagia, or snoring (56%), sleep difficulties (33%), constipation requiring medical management (56%), and moderate to severe drooling (78%).

All nine boys had one or more abnormal EEGs which were characterized by mild to moderate slowing of the background EEG activity, slowing of the occipital dominant rhythm, and/or absence of the occipital dominant rhythm. Paroxysmal rhythmic slow (theta) activity was recorded in the posterior regions of 4 boys. Typically, these abnormal findings are considered indicative of a diffuse disturbance in brain function. Two of the boys had epileptiform patterns characterized by either multifocal spike discharges or generalized spike and slow wave activity (subjects 1 and 6). Clinical (atonic) and EEG seizure discharges (multifocal) were recorded in subject 1.

Three of nine boys (33%) experienced developmental regression substantiated by using the ADI-R. In subjects 1 and 7, regression correlated with seizure onset and was unaffected by anti-seizure medication. Regression was global in subject 1 and included a marked loss of hand use. Subject 7 experienced loss of ambulation, speech, and hand use with gain of hand wringing. Subject 8 lost hand use and the few words that he had learned.

All of the boys had brain magnetic resonance imaging (MRI) performed in the past. There were no unifying brain MRI findings. Review of images and/or neuroradiology reports revealed normal imaging studies or mild, non-specific abnormalities including white matter changes such as thin corpus callosum, mild cerebral volume loss, and choroid plexus cyst.

Results of psychological evaluation of males

Eight of nine males with the *MECP2* duplication syndrome completed formal evaluation of their cognitive/developmental skills. All of the boys (100%) exhibited cognitive abilities that fell within the severe to profound range of mental retardation. Their abilities did not seem to be dependent upon age, although 2 of the boys (subjects 1 and 7) were much more functional prior to developmental regression. Relative weaknesses were noted in expressive language for all patients. Table 2 provides a comparison of expressive and receptive language delays with respect to developmental abilities.

Six of nine boys (67%) had prior diagnoses of autism and, in two cases (subjects 1 and 7), this diagnosis was made prior to developmental regression. All nine subjects were re-evaluated for the current study for the presence of autism using the ADOS (8/9) and the ADI-R (9/9). Seven of eight boys (88%) exceeded cutoff criteria scores for autism on the ADOS, and the eighth boy exceeded cutoff criteria for autism spectrum disorder (refer to Table 2). All nine boys (100%) exceeded threshold scores for autism on the ADI-R. Specifically, all of the subjects exhibited significant difficulties using eye gaze to modulate social interactions. The majority of the boys exhibited gaze avoidance and at times would actively avoid social interaction. Most of the boys did not initiate social contact and, if they did so, it revolved around their areas of

restricted interest or served the function of directing the examiner's behavior. The majority used another person's hand as a "tool" to communicate "for" them (by directly placing the person's hand on the desired object). Although most of the boys are nonverbal, those who speak use language in a repetitive manner. One boy with significant developmental regression (subject 7) spoke in phrases prior to regressing, but exhibited considerable echolalia and used repetitive phrases (often quoting words/phrases from favorite videos). All of the boys exhibited a limited range of facial expression, as well as limited shared enjoyment in interactions. It is important to note that many of the boys enjoy specific toys and activities, but there was not a sense of true reciprocity in social interactions. Six of nine boys (67%) exhibited significant anxiety in response to certain noises and/or objects and also exhibited sensory interests/aversions. Several of the boys exhibited difficulty with transitions and fixated on objects/activities of interest to them. All of the boys examined exhibited stereotypies and repetitive behaviors including prevalent midline hand movements that varied only slightly among the boys. Rocking, spinning, and self-injurious behavior (typically biting hands or fingers when excited) were also prevalent.

Duplication size, gene content, and *MECP2* rna and protein levels

The genomic regions duplicated in the boys range from 0.32 Mb to 0.71 Mb in size. The smallest region of overlap (SRO) is 231 kb and includes the *MECP2* gene (Figure 2). Similar to previous reports, the SRO also includes the interleukin-1 receptor-associated kinase 1 (*IRAK1*) locus and part of the polymorphic *Opsin* array. The phenotypic severity of the males does not correlate to duplication size, confirming previous reports¹³⁻¹⁵. In fact, the core phenotypes of the disorder (mental retardation, autism, hypotonia with progressive lower extremity spasticity, hand stereotypies, and choreiform movements) are very consistent among this group of males. Slightly more variable phenotypes such as epilepsy and recurrent respiratory infections are highly expressive in the presence of the duplication and may be modulated by genetic background. We quantified *MECP2* and *IRAK1* mRNA levels in all of the affected boys and in three male controls and observed that the cells from boys with *MECP2* duplication expressed two to six-fold more *MECP2* mRNA and two to seven-fold more *IRAK1* mRNA than cells from control males (Figure 3A). The level of mRNA expression observed does not correlate with disease severity in the boys. There was no difference in *MECP2* or *IRAK1* mRNA expression levels between carrier and control females (Figure 3B). We also quantified MeCP2 protein levels in all of the affected boys and in two male controls and observed that the cells from boys with *MECP2* duplication produced approximately 1.5–1.7-fold more MeCP2 protein than cells from control males (Figure 4A and B). These data further support the conclusion that both *MECP2* and *IRAK1* are dosage-responsive genes in the Xq28 duplication syndrome.

Clinical summary of carrier females

Clinical data were gathered for nine female *MECP2* duplication carriers ranging in age from 34–64 (refer to Table 3). Several common features were identified. Five of the women endured abnormal menstrual cycles (56%) with four of five of these women describing a lifelong history of irregular periods. The fifth woman described heavy menses. Three of the four women with irregular menstrual cycles experienced premature menopause (prior to age 40) and the fourth woman (age 34) is experiencing clinical symptoms of impending premature menopause (hot flashes, hair and skin changes). Endocrine and autoimmune disorders are also prevalent in these subjects. Two of the women have adult onset diabetes (subjects 11 and 16) and subject 10 was told by her physician that she is in a "pre-diabetic state." Subject 11 is the mother of subjects 10 and 16 and all three of these women also have hypothyroidism. Interestingly, these women are represented in the group with abnormal menstrual cycles and premature menopause. A fourth woman carries the diagnoses of hypothyroidism, Sjogrens disease, and fibromyalgia.

Results of psychological evaluation of carrier females

Eight of the women completed a psychiatric history and the Symptom Checklist 90-R, seven subjects completed the Broad Autism Phenotype Questionnaire, and seven completed cognitive testing (refer to Table 3). The results revealed that 50% of the women were treated for depression prior to the birth of a child with *MECP2* duplication. All eight women endorsed symptoms of anxiety; for 75%, anxiety was present prior to the birth of the affected child. Five of the women use psychiatric medications and a sixth subject requires a beta blocker. All of the women manifest compulsive behaviors (e.g. a strong need for structure, routine, cleaning, and ordering). Results of the Symptom Checklist 90-R revealed clinically important elevations in the following areas: somatization (63%), interpersonal sensitivity (63%), anxiety (63%), hostility (63%), psychoticism (75%), and global distress (63%). The Broad Autism Phenotype Questionnaire identified elevations in the areas of rigid personality (100%), pragmatic language deficits (57%), and aloof personality (43%). Four of seven subjects (57%) exceeded the overall cutoff score for the broad autism phenotype (BAP). The results of cognitive testing revealed a broad range of scores. Specifically, two subjects scored in the low average range, two scored in the average range, one scored in the high average range, and two scored in the superior range of intelligence. Of note is that 71% achieved significantly higher nonverbal as compared to verbal reasoning skills ($p < 0.05$). The clinical relevance of this finding is that these women also exhibited difficulties with language pragmatics (i.e. the social use of language).

Results of X-chromosome inactivation studies

Leukocyte DNA from nine carrier females was tested for X-chromosome inactivation (XCI) status. Eight of the females had nearly 100% skewing of XCI in peripheral blood and one sample was non-informative (Figure 5). The fact that all of the carrier females expressed wild-type levels of *MECP2* and *IRAK1* mRNA suggests that the abnormal duplication bearing X chromosome was preferentially inactivated in peripheral blood.

Discussion

All nine boys with the *MECP2* duplication syndrome exhibited features consistent with those observed in children with idiopathic autism including difficulties with eye gaze, limited shared enjoyment in interactions, limited range of facial expression, the use of another person's hand as a tool to communicate, several unusual sensory interests and aversions, and repetitive behaviors and interests. Anxiety was also prevalent. The language of the boys who speak consisted largely of repetitive word use. These behaviors cannot be fully accounted for by significant cognitive and language delays since typically developing infants acquire social communication milestones (e.g. a responsive social smile, gaze monitoring) that were not present in these boys. It is also important to note that these behaviors were quite prevalent, regardless of the functional level of the patient. For example, the higher functioning boys (subjects 1, 5, 7, and 8) exhibited significant symptoms of autism and their symptoms were even more prominent (related to anxiety, gaze avoidance, restricted interests, and use of repetitive language). In addition to autism and mental retardation, the majority of boys in our sample also manifest hypotonia with lower extremity spasticity, recurrent respiratory infections, hand stereotypies, choreiform movements, aerophagia, drooling, bruxism, and epilepsy.

All of the female *MECP2* duplication carriers exhibited phenotypic changes despite favorable skewing of XCI. They exhibited psychiatric symptoms (anxiety, depression, compulsions) that preceded the birth of their children. They all exhibited less flexible personality traits, and the majority met formal criteria for the broad autism phenotype. Although cognitive abilities

varied, most of the women exhibited higher nonverbal reasoning as compared to verbal reasoning skills.

In aggregate, phenotypic findings from the boys and the female carriers suggest that gain of MeCP2 function contributes to certain behaviors associated with autism and the BAP. This is evident with regard to repetitive behaviors in the boys and compulsions in the female carriers, anxiety, rigidity, and difficulties with the functional use of language for social purposes. The BAP is the milder expression of underlying genetic liability for autism and associated neuropsychiatric symptoms that are manifest in non-autistic relatives²⁹. It is an intermediate phenotype that refers to personality and language characteristics that reflect the phenotypic expression of this genetic liability²⁹. Utilizing family history and direct observational methods, researchers found that parents of children with autism are more likely to have inflexible personalities, to exhibit difficulty with transitions, to be aloof, blunt, anxious, and hypersensitive to criticism, and to have fewer high quality friendships compared to control parents of children with Down Syndrome^{30, 31}. These characteristics parallel the social communication deficits and the repetitive/stereotypic behaviors observed in children with autism.

Our data also suggest that menstrual irregularities, premature menopause, and endocrine and/or autoimmune disease may be more prevalent in female duplication carriers. It is possible that MeCP2 contributes to these phenotypes via methylation sensitive estrogen or progesterone responsive elements. Alternatively, the IRAK1 protein has been postulated to have a role in the regulation of autoimmunity³² and may contribute to these phenotypes. Others have suggested that increased *IRAK1* dosage may contribute to the recurrent infection phenotype in affected boys¹⁵, a hypothesis that warrants further investigation.

Clinically, the high prevalence of stereotyped and repetitive behaviors (commonly manifested by midline hand movements), breathing abnormalities, epilepsy, hypotonia, autism, mental retardation, and anxiety in our series suggests that Rett syndrome and the *MECP2* duplication syndrome are more similar than previously suspected. Of note, the EEGs of the boys with *MECP2* duplication were less abnormal than girls with RTT and *MECP2* mutations. For example, by age three most girls with RTT have EEGs characterized by epileptiform abnormalities and background activity that is moderately to severely slow in contrast to these boys³³. Longitudinal studies of the EEG in these boys may reveal a characteristic EEG pattern. Longitudinal studies have shown that girls with RTT undergo typical clinical and developmental changes with time; similar longitudinal studies of boys with *MECP2* duplication will provide important prognostic information.

The fact that loss and gain of MeCP2 function lead to very similar and predominantly neurological syndromes indicates that tight regulation of *MECP2* is critical for the appropriate development and maintenance of neuronal function²¹. Furthermore, the fact that female *MECP2* duplication carriers manifest the BAP supports the hypothesis that MeCP2 participates in dosage-sensitive neuronal pathways which directly influence the phenotypic expression of autism spectrum behaviors. Interestingly, hemizygous male mice that express twice the endogenous level of *Mecp2* have significant anxiety and manifest abnormal social interaction, suggesting that this transgenic mouse model reproduces the autism phenotype observed in the boys (Samaco and Zoghbi, unpublished data). The neurobiology of MeCP2 function suggests that the protein has an essential role in fine-tuning gene expression changes in response to neuronal activity. For example, phosphorylation of MeCP2 at S421 occurs in response to neuronal activity and is necessary for MeCP2 to modulate dendritic growth and spine maturation³⁴, and MeCP2 regulates the expression of *Bdnf*, an activity dependent gene³⁴⁻³⁶. The *Mecp2*^{308/y} Rett syndrome mouse model demonstrates exaggerated physiological responses to stress manifested by heightened anxiety and increased corticosterone levels³⁷.

The *Crh* gene is a direct target of MeCP2, and altered *Crh* expression occurs only in brain regions where it is normally expressed³⁷. These data reinforce the hypothesis that MeCP2 is a modulator of neuronal function and suggest that failure of MeCP2 to appropriately modulate internal and external stimuli at the molecular level may underlie the BAP and autism phenotypes observed in our subjects.

The observation that *MECP2* duplication may lead to neuropsychiatric symptoms in women despite nearly 100% skewing of XCI and wild-type expression levels in peripheral blood is interesting. Perhaps duplication of the *IRAK1* gene, an important kinase in the Toll-like receptor signaling pathway³², results in selective survival of cells with wild-type XCI patterns in blood but not in brain. It is also possible that extreme skewing of X-inactivation itself contributes to the observed phenotypes. The women in our study may have some neurons expressing the duplicated allele throughout the brain, in specific brain regions or nuclei, or during specific developmental periods, and one can imagine that even small *MECP2* dosage increases in certain brain regions may lead to mild changes in gene expression or synapse number and the mild neuropsychiatric and cognitive phenotypes observed. We cannot exclude the possibility that other additive environmental or genetic factors also contribute to the phenotypes observed in carrier females. Heterozygous, transgenic, female mice with *MECP2* duplication express lower levels of MeCP2 protein than hemizygous males likely due to XCI, yet they still manifest neurological phenotypes⁸. Conditional knock-out of *MECP2* from Sim1-expressing neurons in the mouse hypothalamus led to mice with abnormal responses to stress, abnormal aggressive behavior, hyperphagia, and obesity providing a precedent for the idea that altered *MECP2* dosage in discrete brain regions can be sufficient to cause significant biological phenotypes³⁸.

The data presented in this study reveal that the full extent of neuropsychiatric phenotypes resulting from MeCP2 dysfunction is not yet appreciated and awaits large-scale studies searching for non-conventional mutations in large cohorts of subjects with neuropsychiatric disorders and appropriate controls. Recent mouse data suggest that even a 50% reduction in MeCP2 dosage is sufficient to cause neurological phenotypes and predict that human patients with mild reductions in MeCP2 protein exist²⁰. Subtle alteration of MeCP2 levels (decrease or increase) is likely to result from non-coding mutations that might alter transcription, mRNA stability, or translation. Some hypomorphic coding region mutations might also affect protein stability or protein-protein interactions. Given the detrimental developmental effects of *MECP2* duplication, we predict that mutations that slightly enhance MeCP2 function will also cause neuropsychiatric phenotypes. The fact that autism is penetrant in 100% of boys in this cohort suggests that *MECP2* duplication should be added to the several dozen other rare causes of syndromic autism, and suggests that *MECP2* gain of function may be another excellent autism model. Males with the appropriate phenotype should be tested for *MECP2* duplication, and since the duplication appears to be inherited frequently from carrier mothers, genetic counseling should be offered to the family.

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Figure 1. Pedigrees for eight of the families included in this study. The pedigree for subject one is not included since he was adopted. Maternal subject numbers are purposefully excluded to preserve anonymity. Standard pedigree symbols are used.

Figure 2.

Genomic region duplicated in subjects with *MECP2* duplications determined by oligonucleotide array CGH. The smallest region of overlap (SRO) includes the *MECP2* and *IRAK1* genes and covers 231 kb. Solid red bars represent oligonucleotide probes for which the mean normalized $\log_2$ (Cy5/Cy3) ratio of the CGH signal, amid a 5 kb window, indicated a gain consistent with duplication. Subject 9 presented $\log_2$ values suggestive of both triplicated (in blue) and duplicated regions; the triplication does not involve the *MECP2* gene. The graphical normalized data for each patient was obtained by inputting the most distal and proximal oligonucleotide genomic probe coordinates into the custom track at UCSC site <http://genome.ucsc.edu/cgi-bin/hgGateway>. Positions are given relative to NCBI build 35 for the X chromosome.

Figure 3.

Quantification of *MECP2* and *IRAK1* mRNA levels as measured by quantitative real-time PCR and normalized to *GAPDH*. A. *MECP2* mRNA levels are 2 to 6 fold higher and *IRAK1* mRNA levels are 2 to 7 fold higher in boys with Xq28 duplication compared to control boys. B. *MECP2* and *IRAK1* mRNA levels are not altered in carrier vs. control females.

Figure 4.

Analysis of MeCP2 protein levels as measured by Western blot and normalized to histone H3. A. Western blot demonstrating MeCP2 protein (upper half of blot, MW ~64 kDa) and histone H3 protein (lower half of blot, MW ~17 kDa) levels in boys with *MECP2* duplication compared with control boys. B. MeCP2 protein levels are ~1.5 to 1.7-fold greater in the lymphoblast cell lines of boys with *MECP2* duplication compared with control boys. MeCP2 protein levels are normalized to total histone H3 protein levels.

Figure 5.

X-chromosome inactivation (XCI) patterns in females with *MECP2* duplication. Panel A demonstrates control DNA with random XCI; Panel B demonstrates control DNA with 100% skewed XCI; The remaining panels demonstrate DNA from *MECP2* duplication carriers with 100% skewed XCI. In each pair, the top panel represents treatment with *HpaII* enzyme and the bottom panel is without enzyme. R = number of repeats. Panel C is subject 10, D is subject 11, E is subject 12, F is subject 13, G is subject 13's mother, H is subject 14, I is subject 15, and J is subject 18.

Table 1

Clinical summary of boys with *MECP2* duplication

Subject	1	2	3	4	5	6	7	8 ^a	9 ^b	Summary
Age at time of examination (years)	5	3	7	15	5	13	12	10	4	
Autism confirmed on ADOS/ ADI-R	x	x	x	x	x	x	x	x	x	9/9
Prior diagnosis of autism	x		x	x	x	x	x			6/9
Severe to profound mental retardation	x	x	x	x	x	x	x	x	x	9/9
Repetitive behaviors	x	x	x	x	x	x	x	x	x	9/9
Hand stereotypies	x	x	x	x	x	x	x	x	x	9/9
Choreiform movements	x	x	x	x		x	x	x	x	8/9
Epilepsy	x		x			x	x			4/9
Poor eye contact	x	x	x	x	x	x	x	x	x	9/9
Rigidity/ difficulty with change in situations		x	x		x	x		x		5/9
Anxiety			x	x	x	x		x	x	6/9
Truncal/axial hypotonia, spasticity in legs	x		x	x	x	x	x	x	x	8/9
Hypotonic throughout		x								1/9
Abnormal breathing patterns, swallowing air	x	x	x			x	x			5/9
Bruxism		x	x		x	x	x		x	6/9
Pain insensitivity		x	x			x				3/9
Developmental regression	x						x	x		3/9
Recurrent respiratory infections/pneumonia	x	x	x		x	x	x	x	x	7/9
Sleep disturbances		x				x	x			3/9
Gastroesophageal reflux			x				x			2/9
Drooling	x	x	x	x		x	x	x	x	8/9
Constipation		x	x			x		x	x	5/9
Cryptorchidism	x					x		x	x	3/9

^aThis subject was previously published¹³.^bThis subject was unable to travel to Texas and we therefore relied on review of his medical records, history obtained via phone interviews, and ADI-R performed via phone interview.

Table 2
Quantification of developmental and autism phenotypes in boys with *MECP2* duplication

Subject	1	2	3	4	5	6	7	8
Age in months	53	39	89	198	80	166	147	125
Bayley Scales of Infant Development, 3rd Edition								
Developmental age (mo)	3	3	11	25	25	25	4	9
Receptive Language (mo)	4	3	9	25	20	25	3	2
Expressive Language (mo)	2	<1	3.5	12	16	9	3	2
Fine Motor (mo)	3	4	12	20	20	22	2	8
Gross Motor (mo)	1	4	13	17	18	NT	5	10
Autism Diagnostic Observation Schedule Scores								
ADOS communication score	4	4	5	4	3	4	5	6
ADOS social interaction score	14	14	12	7	12	8	14	14
Combined communication and social interaction score	18	18	17	11	15	12	19	20

mo = months, NT = not tested
Communication score: autism cutoff ≥ 4 ; autism spectrum cutoff ≥ 2
Reciprocal social interaction score: autism cutoff ≥ 7 ; autism spectrum cutoff ≥ 4
Combined communication and social interaction score: autism cutoff ≥ 12 ; autism spectrum cutoff ≥ 7

Table 3Clinical summary of females with *MECP2* duplication

Subject	10	11	12	13	14	15	16	17	18	Summary
Medical history										
Premature menopause	x	x			x		x			4/9
Abnormal menstrual cycles	x	x			x		x	x		5/9
Hypertension				x						1/9
Diabetes	pre	x					x			2/9
Hypothyroidism	x	x					x			3/9
Sjogren's disease					x					1/9
Fibromyalgia					x					1/9
Migraine									x	1/9
Arrhythmia									x	1/9
Psychiatric history										
History of depression	x	x			x		na		x	4/8
Anxiety	x	x	x	x	x	x	na	x	x	8/8
Uses psychiatric medications	x	x		x	x	x	na		rec	5/8
Compulsions	x	x	x	x	x	x	na	x	x	8/8
Results of SCL-90-R										
Somatization	x	x		x	x		na	x		5/8
Obsessive-Compulsive				x	x		na		x	3/8
Interpersonal Sensitivity	x			x	x		na	x	x	5/8
Depression	x				x		na		x	3/8
Anxiety	x			x	x	x	na	x		5/8
Hostility	x			x	x		na	x	x	5/8
Phobic Anxiety					x		na	x		2/8
Paranoia	x		x				na	x		3/8
Psychoticism	x		x	x	x		na	x	x	6/8
Global Severity	x			x	x		na	x	x	5/8
Results of BAP Questionnaire										

Subject	10	11	12	13	14	15	16	17	18	Summary
Aloof Personality		x	x	x	na		na			3/7
Pragmatic Language Deficits	x	x	x	x	na		na			4/7
Rigid Personality	x	x	x	x	na	x	na	x	x	7/7
BAPQ cutoff	x	x	x	x	na		na			4/7
Results of WASI										
Verbal IQ	106	80	82	109	117	95	na	82	na	
Performance IQ	124	97	93	129	124	97	na	80	na	
Full scale IQ	115	87	86	120	123	96	na	81	na	

An “x,” signifies clinically significant findings (i.e. t-scores beyond the normative range).
WASI = Wechsler Abbreviated Scale of Intelligence; SCL-90-R = Symptom Checklist-90-R; BAP = Broad Autism Phenotype; na = information not available; rec = recommended