Microduplications in an Autism Multiplex Family Narrow the Region of Susceptibility for Developmental Disorders on 15q24 and Implicate 7p21

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Copy number variations (CNVs) play a crucial role in the intricate genetics of autism spectrum disorders. A region on chromosome 15q24 vulnerable to both deletions and duplications has been previously implicated in a range of phenotypes including autism, Asperger's syndrome, delayed development, and mild to severe mental retardation. Prior studies have delineated a minimal critical region of approximately 1.33 Mb. In this study, a multiplex autism family was evaluated for CNVs using genotyping data from the Illumina 1 M BeadChip and analyzed with the PennCNV algorithm. Variants were then identified that co-segregate with autism features in this family. Here, we report autistic first cousins who carry two microduplications concordant with disease. Both duplications were inherited maternally and found to be identical by descent. The first is an approximately 10,000 base pair microduplication within the minimal region on 15q24 that falls across a single gene, *ubiquitin-like 7*. This is the smallest duplication in the region to result in a neuropsychiatric disorder, potentially narrowing the critical region for susceptibility to developmental and autism spectrum disorders. The second is a novel, 352 kb tandem duplication on 7p21 that replicates part of the *neurexophilin 1* and *islet cell autoantigen 1* genes. The breakpoint junction falls within the intronic regions of these genes and demonstrates a microhomology of four base pairs. Each of these microduplications may contribute to the complex etiology of autism spectrum disorders. © 2011 Wiley-Liss, Inc.

Key words: autism spectrum disorder (ASD); copy number variation (CNV); low-copy repeat (LCR); non-allelic homologous recombination

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INTRODUCTION

Genetic discoveries have highlighted the contribution of dosage sensitive genes to human disease [Stankiewicz and Lupski, 2010].

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The availability of dense single nucleotide polymorphism (SNP) arrays and comparative genomic hybridization arrays has further increased the identification of genomic structural aberrations. Within the chromosome 15q24 region, both deletions and duplications have been identified that result in a range of clinical phenotypes including autism, mental retardation, developmental delay, and Asperger's syndrome [Smith et al., 2000; Cushman et al., 2005; Sharp et al., 2006, 2007; Kiholm Lund et al., 2008; Klopocki et al., 2008; Marshall et al., 2008; Andrieux et al., 2009; El-Hattab et al., 2009, 2010; Masurel-Paulet et al., 2009; McInnes et al., 2010; Pinto et al., 2010]. A majority of these copy number variations (CNVs) appear to be recurrent rearrangements, suggesting that they stem from non-allelic homologous recombination events between the five low-copy repeats (LCRs or segmental duplications) in the region, designated 15q24A, 15q24B, 15q24C, 15q24D, and 15q24E (Fig. 1). These five LCRs are complex in nature and share 15 homologous subunits in both forward and inverted orientations [El-Hattab et al., 2009]. Thus far, 20 deletions and 6 duplications have been reported to define a minimal critical region of approximately 1.33 Mb [Smith et al., 2000; Cushman et al., 2005; Sharp et al., 2006, 2007; Kiholm Lund et al., 2008; Klopocki et al., 2008; Marshall et al., 2008; Andrieux et al., 2009; El-Hattab et al., 2009, 2010; Masurel-Paulet et al., 2009; McInnes et al., 2010; Pinto et al., 2010].

In this report, we identify two distinct microduplications in a multiplex autism family that co-segregate with disease. The first is a duplication in the minimal 15q24 region, potentially reducing the critical region to approximately 10,000 nucleotides. To our knowl-

edge, this duplication is the smallest reported in the region, encompassing a single known gene and a hypothetical gene. Data also suggest that, unlike a majority of the previously reported CNVs in the 15q24 region, the duplication described here does not appear to be caused by non-allelic homologous recombination between the currently defined LCRs. The second CNV that we found concordant with disease is a novel 352 kb duplication located on 7p21.3, whose breakpoints were mapped within the *neurexophilin 1* and *islet cell autoantigen 1* genes. From what is known about these genes on chromosome 7 as well as the previously reported patients on chromosome 15, it is possible that each of these microduplications may contribute to this family's autistic features.

MATERIALS AND METHODS

Patient Ascertainment

The family of interest (17122) was ascertained as part of a larger study on autism genetics whose participants were enrolled under protocols previously described [Ma et al., 2009]. All protocols were approved by the Institutional Review Boards at the University of Miami, the University of South Carolina, and Vanderbilt University. Briefly, participants were ascertained on the basis of a previously existing clinical diagnosis of autism spectrum disorder and enrolled if they met the following minimal inclusion criteria: (1) age between 3 and 21 years, (2) expert clinical determination of diagnosis based on the DSM-IV criteria for an autism spectrum disorder, (3) absence of severe sensory or significant motor impairments, and (4) absence of identified metabolic, genetic, or progres-

FIG. 1. Copy number variations (CNVs) identified in the 15q24 region. Green lines denote duplications and red lines signify deletions. Each line represents all individuals carrying a CNV from one family. The purple bars correspond to the low-copy repeats in the region. The microduplication found in family 17122 falls between 15q24B and 15q24C, within the 1.33 Mb critical region.

sive neurological disorders. Research diagnoses were assigned using DSM-IV criteria [American Psychiatric Association, 1994] supported by structured diagnostic instruments including the autism diagnostic interview-revised (ADI-R) and the autism diagnostic observation schedule (ADOS) [Lord et al., 1999; Rutter et al., 2003b]. In addition to diagnostic and psychometric assessments, whole blood was collected from participants by venipuncture.

The control group consisted of 727 apparently healthy Caucasian individuals with no known indicators of behavioral, developmental, or cognitive abnormalities (357 males and 370 females). Three hundred eighteen of the control samples came from Caucasian singleton live births at Centennial Women's Hospital and The Perinatal Research Center, in Nashville, Tennessee. Inclusion criteria consisted of full term births (>37 weeks of gestation) to mothers between ages 18 and 40 years on admission to the hospital with no medical or obstetrical complications during pregnancy or delivery [Velez et al., 2009]. Umbilical cord blood samples were collected after placental delivery. Gender information was recorded when known. The remaining 409 DNA samples were purified from the saliva of children ages 3–21 years recruited via word of mouth, health fairs, daycares, and other child organizations. The social communication questionnaire was collected to rule out developmental problems along with a brief medical history and information on the family's ethnicity [Rutter et al., 2003a].

SNP Array to Identify Copy Number Variations

Family 17122 was genotyped with the Illumina Human 1M Beadchip, assaying over one million SNPs. Samples were processed and quality control checks were performed as previously described [Ma et al., 2009]. The PennCNV algorithm using the Log R ratio and B allele frequency was used to identify CNVs that spanned across multiple SNPs [Wang et al., 2007]. Individual CNV calling was modified according to default parameters to correct for intensity wave artifacts (GC-waves) across the genome. CNVs were then sorted to identify deletions and duplications concordant with autism in all affected members within a family.

Real-Time PCR Copy Number Assays

TaqMan real-time polymerase chain reaction (PCR) copy number assays predesigned by Applied Biosystems (Foster City, CA) were used for validation and delineation of the regions identified by the SNP array. Assays were chosen within each region using the GeneAssist™ Copy Number Assay Workflow Builder. Each sample was tested in quadruplicate using 10 µl reactions according to the manufacturer's protocol. Analysis of the data was performed in the CopyCaller Software v1.0 (Applied Biosystems).

Long Range PCR With Outward Facing Primer Sets

To determine whether the duplications occurred in tandem, we designed outward facing primers for each duplication. Individuals carrying the CNVs were assayed with the Roche Expand Long Range dNTPack. In a 25 µl master mix, reagents were combined as follows: 5 µl of 5× buffer with MgCl₂, 1 µl of 10 mM dNTP mix, 4 ng/µl of each primer, 0.28 µl of the expand enzyme mix, and 50 ng of DNA

and water. Long range PCR was performed under the following conditions: 2 min at 92°C, thirty-five cycles through 10 sec at 92°C, 30 sec at 58°C, and 4 min at 68°C, followed by 10 min at 68°C, and a 4°C hold. PCR products were run on a 1% agarose gel, bands were extracted, purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and sequenced using the Applied Biosystems Big Dye Terminator v3.1 Cycle Sequencing Kit. For the chromosome 7 region, the following primers were used: upstream in the *islet cell autoantigen 1* gene, CTGAAGCCGTGCATTACCT, and downstream in the *neurexophilin 1* gene, GCTCAAGATTCCTGCTTTGC.

In Silico Sequence Analysis

The NCBI BLAST 2 program was used to evaluate the breakpoint regions and determine whether there were shared regions of homology (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

RESULTS

Clinical Findings

This family is a multiplex family of European descent (Fig. 2). Individual 0001 met DSM-IV and ADI-R diagnostic criteria for autism. Pregnancy was unremarkable and the patient was born with a bilateral congenital hip dislocation. Initial parental concerns (~20 months of age) included language delay, social disinterest, and the appearance of odd mannerisms. Individual 0001 first walked at 18 months and acquired first words at 24 months. There is a history of multiple abnormal EEGs, but no evidence of frank seizures. A brain MRI was described as normal and fragile X-testing was negative. Individual 0001 did not display dysmorphic features but has macrocephaly. Additional clinical findings from his medical records include hypotonia, allergies, fine and gross motor delay,

FIG. 2. Pedigree of the multiplex family 17122. Individuals 0001, 0100, and 0101 were clinically evaluated and diagnosed with autism, while individual 1004 was reported to have mental retardation, repetitive behaviors, and be non-verbal.

and minimal language regression. Cognitive assessment reveals moderate intellectual disability (full scale IQ = 46). Adaptive skills fell in the low range (Vineland adaptive behavior composite = 61).

The sole sibling of 0001, individual 0100, also met DSM-IV and ADI-R criteria for autism. During pregnancy, individual 0100 was found to be large for his gestational age. Initial parental concerns (~12 months of age) were because of language problems. Individual 0100 first walked at 18 months and acquired his first word at 40 months; he developed phrase speech at 57 months. His medical records include EEG findings of uncertain significance, but no clinical seizures. In addition, he has a history of allergies, sensory problems, and hyperactivity. No dysmorphic features were observed, but individual 0100 had head circumference measurements in the high normal range. Fragile X testing was negative. Cognitive testing revealed a moderate intellectual disability (full scale IQ = 46) and adaptive skills fell in the low range (Vineland adaptive behavior composite = 55). While no formal evaluation was conducted, the mother of individuals 0001 and 0100, individual 1001, was reported to have migraines, mitral valve prolapse, and allergies.

A third individual in this family, 0101, carries a research diagnosis of an autism spectrum disorder based on both DSM-IV and ADI-R criteria. This individual is the first cousin to individuals 0001 and 0100, related through their mothers (individuals 1001 and 1006, Fig. 2). Initial parental concerns (~96 months of age) were not specific to autism, but included attention problems, language difficulties, and learning impairments. Subsequently, he was diagnosed with ADHD, depressive disorder, and a reading disability. A review of his medical records reveals that he showed significant social problems and no inclination to interact with peers. As part of this research study, a standard diagnostic measure (ADI-R) was completed with his parents. In conjunction with medical record and observational data, a best estimate research diagnosis of autism spectrum disorder was assigned. Individual 0101 first walked at 11 months, acquired first words at 12 months, and phrase speech at 24 months. His medical records reveal that he has dental misalignment, articulation and oral motor problems, an awkward gait, and overly acute hearing. Cognitive assessment placed him in the average range of intellectual functioning (full scale IQ = 99). However, adaptive skills fell below the average range (Vineland adaptive behavior composite = 65). His mother, individual 1006, was reported to have a history of allergies, Raynaud's syndrome, and hearing loss due to high fever with varicella infection as a child. In addition, she reported that she has a "tipped uterus" and that she experienced three first trimester miscarriages. Finally, she described suffering from multiple fears.

Interestingly, there is a fourth individual in the family (1004), the maternal uncle of the other ASD individuals, who has several features of an autism spectrum disorder, although he has never been clinically evaluated or diagnosed. Anecdotally, this individual was reported to be non-verbal, have mental retardation and perform repetitive behaviors such as rocking, humming, and hand flapping. These problems may have been related to birth trauma. While the family has four members who exhibit features along the autism spectrum, individuals 0001 and 1004 were unavailable for genetic testing.

Detection and Validation of the Microduplications

Two duplications were identified as being shared between two autism spectrum disorder individuals (individuals 0100 and 0101) and present in their mothers as well (individuals 1001 and 1006). The first shared duplication was isolated on chromosome 15q24.1 and defined by three SNPs for a size of approximately 5.8 kb (Fig. 1). The second duplication encompassed 162 SNPs on chromosome 7p21.3 for a total size of approximately 348.5 kb. Individual 0101's unaffected sibling 0102 was negative for both microduplications, as were both fathers (individuals 1000 and 1007). Neither of these duplications was identified in 727 pediatric control individuals nor have they been reported in the Database of Genomic Variants (DGV, <http://projects.tcag.ca/variation/>). Only a single inversion in DGV was reported to cross the 15q24.1 region, with breakpoints mapping to approximately the 15q24B and 15q24C LCRs (Kidd et al., 2008).

The 7p21.3 and 15q24.1 regions were each evaluated with six TaqMan real-time PCR copy number assays to confirm the duplications and refine the breakpoint regions (Table I). Both duplications were validated by real-time PCR. These experiments established that the duplication on 7p21.3 is between 350,803 and 353,216 base pairs in length (maximum region chr7:8,144,214–8,497,430, NCBI Build 36) with the breakpoints falling within the *neurexophilin 1* (*NXPH1*, OMIM:604639) and *islet cell autoantigen 1* (*ICA1*, OMIM:147625) genes (Fig. 3). In addition, the duplication on 15q24.1 was narrowed to be between 9,689 and 11,808 base pairs in size (maximum region chr15:72,536,068–72,547,876) and disrupt a single annotated gene, *ubiquitin-like 7* (*UBL7*, OMIM:609748). Both of the duplications were carried through two sisters (1001, 1006) to their affected sons (0100, 0101). An unaffected son and both unaffected spouses were negative for the CNVs.

Identification of the 7p21.3 Breakpoints

We hypothesized that the CNVs may result in tandem duplications and therefore attempted to sequence across these regions by designing outward facing primers from each of the breakpoints and amplifying by long range PCR. Primers were constructed to test whether the duplications might be either in direct or inverted positions to their original location. Using this method, we successfully identified the chromosome 7 CNV as a tandem duplication of the chr7:8,144,894–8,497,305 region, with the breakpoints falling within the intronic regions of the *NXPH1* and *ICA1* genes (Figs. 3C and 4). This generates a new, fused gene composed of the first 2 exons of *NXPH1* and the first 12 exons of *ICA1* oriented to face each other. The breakpoint junction was identified by Sanger sequencing of individual 1006 and confirmed to be identical throughout the family by also sequencing individuals 0100, 0101, and 1001. When evaluating the sequence at the breakpoint junction, a microhomology of the four base pairs "GCAA" was identified (Fig. 4).

Evaluation of Genomic Architectural Elements on 15q24.1

A previous investigation of the large LCRs in the chromosome 15 region identified five complex segmental duplications (Fig. 1)

TABLE I. Flanking SNPs on Illumina 1 M Chip and TaqMan Real-Time PCR Copy Number Assays

Chr.	SNP	CNV assay ID	Location (bp) ^a	Gene	Copy number
7	rs17330124		8144214	ICA1	2
		Hs02960372	8145214	ICA1	3
	rs3807843		8146180	ICA1	3
		Hs01817569	8227474	ICA1	3
		Hs04963570	8443081	NXPH1	3
	rs10486229		8494690	NXPH1	3
		Hs04978506	8496017	NXPH1	3
		Hs04933524	8497430	NXPH1	2
	rs6959225		8499029	NXPH1	2
	15	rs7166701		72536068	UBL7
Hs02844041			72538187	UBL7	3
Hs02194368			72540549	UBL7	3
rs4887152			72543407	LOC440288	3
		Hs04451741	72544757	LOC440288	3
		Hs03910824	72547876	LOC440288	3
rs4077703			72549215	LOC440288	3
		Hs03898004	72550460	LOC440288	2
		Hs03905957	72554610	LOC440288	2
rs8027459			72557387	LOC440288	2

^aNCBI Build 36.

that appear to facilitate the generation of most CNVs in the region through non-allelic homologous recombination (El-Hattab et al., 2009). Since the 15q24 microduplication we describe here does not align with the LCRs in the region, we appraised the area for other genomic elements that may have contributed to the generation of the CNV. We evaluated the proximal (chr15:72,536,068–72,538,187) and distal (chr15:72,549,215–72,550,460) breakpoint regions with the BLAST 2 program for areas of homology. The proximal breakpoint falls within *UBL7*'s fourth intron while the distal breakpoint is located in the second intron of the hypothetical gene *LOC440288*. Four Alu transposable elements were identified in the proximal breakpoint region that had 73–78% homology to a single AluJb segment located in the distal breakpoint region (Table II).

DISCUSSION

This extended family presents an intriguing case in which two potentially significant microduplications are concordant with disease. The region on chromosome 15 is especially interesting due to reported cases of autism, mental retardation, developmental delay, and Asperger's syndrome caused by CNVs in this area [Smith et al., 2000; Cushman et al., 2005; Sharp et al., 2006, 2007; Kiholm Lund et al., 2008; Klopocki et al., 2008; Marshall et al., 2008; Andrieux et al., 2009; El-Hattab et al., 2009; Masurel-Paulet et al., 2009; McInnes et al., 2010; Pinto et al., 2010]. Previous cases of duplications and deletions within 15q24 defined a minimal critical region of approximately 1.33 Mb. This region contains 26 genes: *GOLGA6*, *ISLR2*, *ISLR*, *STRA6*, *CCDC33*, *CYP11A1*, *SEMA7A*, *UBL7*, *ARID3B*, *CLK3*, *EDC3*, *CYP1A1*, *CYP1A2*, *CSK*, *LMAN1L*, *CPLX3*, *ULK3*, *SCAMP2*, *MPI*, *C15orf17*, *COX5A*, *RPP25*, *SCAMP5*,

PPCDC, *C15orf39*, and *COMMD4*. From the microduplication identified in this family, the maximum region of the CNV has now been reduced to approximately 10,000 base pairs (chr15:72,536,068–72,547,876) and only includes one known gene, the first four exons of the *UBL7* gene and two predicted exons of the hypothetical gene *LOC440288* (Fig. 3D). Expression of *UBL7* (also known as *BMSC-Ubp*) is widespread, including in the brain and spinal cord, though its function is unknown [Liu et al., 2003]. It is also possible that the CNV contains a regulatory element and is causing misregulation of a nearby gene or genes. One such candidate is the gene directly upstream of *UBL7*, *semaphorin 7A* (*SEMA7A*, OMIM:607961). Previous data has demonstrated that *SEMA7A* acts to promote axonal growth during embryonic development [Pasterkamp et al., 2003]. Significantly, the closely related *SEMA5A* gene on chromosome 5 was identified in an autism genome wide association study and there is evidence of decreased expression of this gene in patients with autism [Melin et al., 2006; Weiss et al., 2009].

There are also five reported CNVs identified in individuals with either autism or developmental delay that fail to overlap with our newly defined minimal region on 15q24 (Fig. 1) [El-Hattab et al., 2009, 2010; McInnes et al., 2010; Pinto et al., 2010]. Two deletions and a single duplication fall completely outside the previously delineated 1.33 Mb critical region and, therefore, the pathogenic implications are unclear [El-Hattab et al., 2009; McInnes et al., 2010; Pinto et al., 2010]. The fourth CNV is a duplication located at 72.67–73.09 Mb in an individual reported to have delays in development, speech, and motor skills, approximately 117.8 kb away from our newly recognized CNV [El-Hattab et al., 2010]. Perhaps these CNVs are close enough to alter distinct regulatory regions for the same gene. The final, non-overlapping CNV is an 82 kb dupli-

While evidence of duplications and deletions in the chromosome 15 region demonstrate that the 15q24 duplication could contribute

TABLE II. Areas of Homology on Chromosome 15 Across the Breakpoint Regions

Proximal region ^a	Elements in region	Distal region ^a	Elements in region	Homology (%)	Length in nucleotides
72536210–72536507	AluJb	72549217–72549512	AluJb	78	298
72536656–72536945	AluSx	72549219–72549510	AluJb	76	292
72537541–72537820	AluSg	72549217–72549492	AluJb	75	285
72536977–72537141	AluSg	72549347–72549512	AluJb	73	166

^aNCBI Build.

strongly to the clinical phenotypes in this family, the tandem duplication identified on chromosome 7 may also play a role. Two genes fall within the duplicated 7p21.3 region (Fig. 3), *NXPH1* and *ICA1*. *NXPH1* is an appealing candidate due to previous evidence of the protein NXPH1 complexing with neurexin 1 α , which has already been implicated in autism [Kim et al., 2008; Yan et al., 2008]. The neuropsychophilin family of proteins is preferentially expressed in the brain, undergo glycosylation, and act as ligands for α -neuroligins [Missler and Sudhof, 1998]. *ICA1* is also an intriguing candidate due to its role in AMPA regulation [Cao et al., 2007]. It is possible that the 7p21 and 15q24 microduplications are acting in concert within family 17122. This synergistic effect was also suggested for three other families carrying 15q24 CNVs where a second, potentially deleterious CNV was identified [El-Hattab et al., 2010].

It is interesting to note the pattern of inheritance within the family. There are four individuals that appear to fall along the spectrum of autism disorders, all male. Furthermore, both duplications are carried through two sisters, who fail to present with autistic features. For duplications on chromosome 15, this is consistent with earlier studies demonstrating inheritance from individuals that are not autistic and may be the result of incomplete or sex specific penetrance, X-linked inheritance or imprinting [Kiholm Lund et al., 2008; El-Hattab et al., 2009]. In the six previously reported 15q24 duplication families, two duplications were inherited maternally, three duplications were inherited paternally, and the last duplication was undetermined for inheritance status [Kiholm Lund et al., 2008; El-Hattab et al., 2009, 2010; Pinto et al., 2010]. This mirrors our finding of the duplication being inherited from individuals failing to present clinically with autism. In contrast, deletions reported across the 15q24 region all appear to occur *de novo*, with the exception of a deletion inherited paternally to two autistic sons that falls outside of the critical region [Smith et al., 2000; Cushman et al., 2005; Sharp et al., 2006; Kiholm Lund et al., 2008; Klopocki et al., 2008; Marshall et al., 2008; Andrieux et al., 2009; El-Hattab et al., 2009, 2010; Masurel-Paulet et al., 2009; McInnes et al., 2010]. This suggests that the deletions falling across the critical region confer a severe and fully penetrant phenotype, while duplications have a milder effect, may be passed through clinically normal individuals, or may need to act in concert with another genetic aberration.

The mechanisms by which each of the duplications arose in this family are still unclear. Nonetheless, we defined the breakpoints in the 7p21.3 region, demonstrating that the tandem duplication is present and there is microhomology of four base pairs

where the breakpoints join. This microhomology hints at potential mechanisms including fork stalling and template switching (FoSTeS) and microhomology-mediated break-induced replication (MMBIR) [Zhang et al., 2009]. While the chromosome 15 duplication was not delineated to single base pair resolution, an AluJb segment in the distal region was identified that may facilitate non-allelic homologous recombination with one of three Alu elements located in the proximal breakpoint region, a process reviewed by Hedges and Deininger [2007]. It also does not appear that the 15q24 duplication is directly adjacent to the original DNA in either direct or inverted orientations. This leaves open the possibility that some of the clinical phenotypes are not a result of the duplication itself but are perhaps due to a disruption at the insertion site. However, the previous reports of individuals demonstrating autism, mental retardation, developmental delay, and Asperger's syndrome due to larger duplications and deletions supports the idea that the chromosome 15 duplication itself, and not a secondary effect, is causing a majority of the clinical features [Smith et al., 2000; Cushman et al., 2005; Sharp et al., 2006, 2007; Kiholm Lund et al., 2008; Klopocki et al., 2008; Marshall et al., 2008; Andrieux et al., 2009; El-Hattab et al., 2009, 2010; Masurel-Paulet et al., 2009; Pinto et al., 2010]. If this is true, then we have successfully narrowed the critical region from 1.33 Mb and 26 genes to approximately 10,000 base pairs and one known gene, *UBL7*. In conclusion, we have identified two microduplications concordant with disease in a multiplex family which may contribute to the genetics of autism.

NOTE

While this manuscript was in press, Gai and colleagues identified two additional ASD families carrying microduplications on 7p21 [Gai et al., 2011]. Each CNV has breakpoints within the *ICA1* and *NXPH1* genes, similar in size and position to the duplication that we report here. We feel that this demonstrates an independent validation of the significance of this region to autism spectrum disorders.

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