A de novo 1p34.2 microdeletion identifies the synaptic vesicle gene RIMS3 as a novel candidate for autism

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KEYWORDS
Autism, microcephaly, mental retardation, copy number variants, synapse
ABSTRACT

**Background:** We report a child with autism and mild microcephaly who was found to have a *de novo* 3.3 Mb microdeletion on chromosome 1p34.2p34.3. Here, we test the hypothesis that this microdeletion contains one or more genes that underlie the autism phenotype in this child and in other children with autism spectrum disorders.

**Methods:** To search for submicroscopic chromosomal rearrangements in the child, we performed array comparative genomic hybridization (aCGH) using a 19K whole-genome human BAC array and the Illumina 610-Quad BeadChip microarray. Ingenuity Pathway Analysis (IPA) was used to construct functional biological networks to identify candidate autism genes. To identify putative functional variants in candidate genes, we performed mutation screening using PCR-based Sanger sequencing in 512 unrelated autism patients and 462 control subjects.

**Results:** We identified a *de novo* 3.3 Mb deletion containing ~43 genes in chromosome 1p34.2p34.3 that we confirmed using fluorescence *in situ* hybridization (FISH). Literature review and bioinformatics analyses identified *RIMS3* (Regulating Synaptic Membrane Exocytosis 3) as the most promising autism candidate gene. Mutation screening of this gene in autism patients identified five inherited coding variants, including one (p.E177A) that segregated with the autism phenotype in a sibship, was predicted to be deleterious, and was absent in 1,161 controls.

**Conclusions:** Our case report and mutation screening data suggest that *RIMS3* is an autism causative or contributory gene. Functional studies of *RIMS3* variants such as p.E177A should provide additional insight into the role of synaptic proteins in the pathophysiology of autism.
INTRODUCTION

Autism is characterized by deficits in reciprocal social interaction, disrupted verbal and non-verbal communication and restricted interests and repetitive behaviors, and is known to have a strong genetic component [1, 2]. Cytogenetic studies indicate that ~3-4% of autism patients have chromosomal imbalances [3-5] that include maternal 15q11q13 duplications (~1-2% of patients) and deletions of 2q37, 22q11.2 and 22q13.3.[4, 6] Higher resolution approaches using array comparative genomic hybridization (aCGH) and single nucleotide polymorphism (SNP) microarrays have recently identified submicroscopic copy number variants (CNVs) [7-10], including recurrent microdeletions and duplications of 16p11.2 that have been reported in ~1% of autism patients.[8, 11, 12] Importantly, the discovery of chromosomal abnormalities and CNVs has led to the identification of rare intragenic mutations through deep resequencing of autism candidate genes, including NLGN3 and NLGN4[13], NRXN1 [10, 14, 15], SHANK3 [16-18] and CNTNAP2.[19-21] Although mutations in these genes account for only a small proportion of individuals with autism, they nonetheless provide insight into potential biological mechanisms that may underlie autism, such as synaptic dysfunction and abnormal brain connectivity.[22, 23]

Here, we report a boy with autism, microcephaly, and other dysmorphic features in whom aCGH detected a ~3.3 Mb interstitial deletion in chromosome 1p34.2p34.3 that contains ~43 genes including RIMS3 (Regulating synaptic membrane exocytosis protein 3), a gene previously shown to be dysregulated in autism subjects.[24] We examined the role of RIMS3 in 512 unrelated autism subjects by performing mutation analyses to look for putative functional variants.

CLINICAL REPORT

This boy (LP99-105) was born at term to healthy and unrelated parents, and appeared normal except for right hydronephrosis and vesicoureteral reflux, which both resolved. His head circumference was normal at birth, but decelerated after 7 months and subsequently followed a curve at or just below –2 standard deviations (Figure 1A). His early motor development was normal, but he walked late at 16 months. He used his first few words at 15 months and slowly increased to 50-75 words by 2.5 years, but then progressively stopped using almost all speech by 3 years.

On exam, he had a normal facial appearance at 1 year (Figure 1B), but by 10 years he had a long narrow face and deep set or sunken eyes (Figure 1C). His general and neurological exams were normal, including good motor coordination. Serial exams between 2 and 10 years all demonstrated poor social communication including poor eye contact and limited interactive play, striking anxiety, difficulty with transitions, constant chewing, short attention span, poor sleep pattern, and repetitive activities such as bouncing on an exercise ball. Brain MRI at 1 year demonstrated mildly prominent extra-axial spaces, but was otherwise normal. Serial audiograms done during his first several years of life demonstrated normal hearing.

He was diagnosed with Autistic Disorder at age 4 years, 0 months at a university-based assessment clinic for children with developmental disorders. On the Autism Diagnostic Observation Schedule – Generic (ADOS-G) [25], he exceeded the Autism Cutoff score on the Communication, Social, and Communication plus Social Domains, thus meeting the criterion for an ADOS-G classification of Autism (domain scores: Communication 6; Social 12; Stereotyped Behaviors and Restricted Interests 4). The diagnosis was further supported by meeting the cutoff scores of autism on the Autism Diagnostic Interview – Revised (ADI-R) [26] with his mother as the informant. More widespread developmental abnormalities were documented at 4 years based on an overall standard score of 28 on the Scales of Independent Behavior – Revised [27], a parent-report measure of adaptive behavior. This falls in the "limited to very limited" range, equivalent to behaviors at 18 months in typically developing children.

During the subsequent six years, he has not regained any language skills, except for using
several signs inconsistently. He developed a striking fear response when placed in a car at age 9 years that has persisted for more than three years. Chromosome analysis and PCR-based fragile X testing were normal, and no other genetic tests were done. This boy is not part of the Autism Genetics Resource Exchange (AGRE) consortium (http://www.agre.org/).

MATERIALS AND METHODS

Autism and control subjects

We obtained phenotype data and DNA from the 1p34 deletion proband under a protocol approved by the Institutional Review Board at the University of Chicago. The remaining autism probands selected for this study were a subset of the AGRE subjects that were collected with informed consent and institutional review board approval. The AGRE cohort consisted of 512 unrelated autism subjects (151 females and 355 males) from 84 simplex and 428 multiplex families. Subjects who missed criteria for autism on the ADI-R were classified as having “Not Quite Autism” (NQA) if their scores on each ADI-R behavioral domain were within 1 point of the criteria, or if they met criteria for each domain except for age of onset less than 3 years of age. Fragile X testing was performed in all families and a subset of families had karyotyping and CNV analyses performed. Genomic DNA from control subjects (225 females and 237 males) was obtained from the National Institute of Mental Health (NIMH) Human Genetics Initiative control sample set. These subjects were screened for Axis I mental health disorders and none were reported as having autism. A summary of the ethnic origins of the autism patients and control subjects is provided in Supplementary Table 1.

Array comparative genomic hybridization (aCGH)

We performed aCGH on the patient using a 19K whole genome tiling path BAC microarray as previously described.[7] We refined the size of the 1p34.2p34.3 microdeletion using the Illumina 610-Quad BeadChip microarray (Illumina, Inc, http://www.illumina.com/).

Fluorescence in situ hybridization (FISH) and microsatellite analyses

FISH and microsatellite analyses were performed using standard techniques (Supplementary Materials).

DNA amplification and sequencing

PCR primers were designed using MIT Primer3 with M13 forward and reverse tails added to each primer to facilitate high-throughput DNA sequencing (Supplementary Table 2). DNA amplification and purification were performed using standard conditions and protocols (Supplementary Materials).

Pathway Analyses

A RIMS3 network was predicted through the use of Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems®; http://www.ingenuity.com/). To generate this network, a dataset of known autism genes (i.e. focus genes) was uploaded into the application. The Ingenuity Pathways Knowledge Base was used to identify interactions between the focus genes and other gene objects. The network was then algorithmically generated based on their connectivity. The network was comprised of “nodes” i.e. the gene or gene product, connected by “edges” that are biological relationships supported by at least one publication. The network was then trimmed to show a refined network with minimal connections between the autism focus genes.
Protein Predictions

We used several applications to predict the effects of missense substitutions on protein function including SIFT (Sorting Intolerant From Tolerant, http://blocks.fhcrc.org/sift/SIFT) [29], PolyPhen (Polymorphism Phenotyping, http://coot.embl.de/PolyPhen/) [30], and SNAP (http://cubic.bioc.columbia.edu/services/snap/).[31]

RESULTS

Discovery of a 1p34.2p34.3 microdeletion in a child with autism and microcephaly

We performed aCGH using a 19K whole genome tiling path BAC microarray in patient LP99-105, and detected a ~3.3 Mb deletion in chromosome 1p34.2p34.3 that extended from RP11-769L8 to RP11-483I17 and included ~47 genes (Figure 2A). The microdeletion was not detected in 372 control subjects analyzed on the same BAC array platform.[7] FISH studies confirmed the deletion in the proband (Figure 2B), and microsatellite analysis demonstrated that the deletion was de novo (data not shown). We used the Illumina 610-Quad BeadChip microarray to refine the breakpoints to approximately chr1:39,794,296 and chr1:43,058,974 (UCSC Genome Browser, http://genome.ucsc.edu; Build 36.1; accessed November 2008), reducing the size of the microdeletion by ~56 kb and identifying ~43 genes within the deleted region.

We reviewed the Database of Genomic Variants (http://projects.tcag.ca/variation/, Build 36; accessed November 2008) to determine whether any CNVs in the general population were present in the 1p34.2p34.3 microdeletion region. No CNVs encompassed the entire deleted region, but we did find several smaller blocks of CNVs (Figure 2A). The largest of these blocks, independently characterized by four groups (Variations 3289, 4219, 5487 and 8322), was ~435 kb in size and contained 11 genes (PPIE, PPT1, TRIT1, OXCT2, MYCL1, BMP8B, MFSD2, RLF, CAP1, TMCO2, and ZMPSTE24). Both copy number gains and losses of this region were reported in the general population.

Identification and characterization of RIMS3 as a candidate gene for autism

We hypothesized that rare mutations in one or more genes in the 1p34.2p34.3 microdeletion region – excluding the 11 in the ~435 kb polymorphic block – may be responsible for autism in other affected individuals as well. Interrogation of these ~32 genes using published literature and online expression databases identified RIMS3 as the most promising candidate. Network analysis using IPA software demonstrated that RIMS3 directly interacts with the protein-tyrosine phosphatase PPFIA3 that belongs to the liprin-alpha gene family (Supplementary Figure 1 in Supplementary Materials). RIMS3 was also associated with many other autism candidate genes through an indirect interaction with FMR1, mutations of which cause Fragile X syndrome (OMIM 300624).

To test the hypothesis that mutations in RIMS3 underlie the autism phenotype, we sequenced all RIMS3 coding regions, their associated splice-sites, and the 5’ and 3’ untranslated regions (UTR) in 512 unrelated autism patients. We also sequenced these regions in 462 control subjects to capture the natural genetic diversity of RIMS3 in the general population. In the autism samples, we identified five novel coding variants (3 non-synonymous and 2 synonymous) that were absent in control subjects and not reported in public DNA databases (Ensembl, http://www.ensembl.org/ and dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/, both accessed February 2009) (Table 1). Demographic data for these five subjects including sex, race, and ethnicity are provided in Table 1. We also identified a 5’UTR variant as well as one intronic variant located 30 bp upstream of the third exon. We found five different sequence changes in the controls (Table 1).
Sequencing of parental DNA indicated that all five coding variants were inherited. We performed segregation analysis in families by sequencing each variant in affected and/or unaffected siblings. In two multiplex families (AU0247 and AU0125, Figure 3), the variant segregated with the autism phenotype in siblings. In family AU0247, the maternally-inherited p.E177A variant first identified in patient HI0515 was also present in a sibling with NQA (Not Quite Autism), but absent in 1,161 control subjects. We reviewed available phenotype data on the carrier mother, who had depression and symptoms of bipolar disorder and anxiety. She also had a repaired cleft palate and had received speech-language therapy (the father had received speech-language therapy as well). The heterozygous sib had a ventricular septal defect that closed spontaneously by one year and diastasis recti. Copy number variant analysis on this family provided on the AGRE website ruled out known CNVs associated with autism, including microdeletion of 22q11.

The p.E177A substitution, which changes a glutamic acid (acidic and polar) to an alanine (neutral and nonpolar) residue, was predicted to be deleterious according to both the SIFT and SNAP programs. We determined the extent of conservation of the E177 residue by comparing it against the RIMS3 primary protein structure of other species (http://genome.ucsc.edu/, hg 18, accessed February 2009). E177 was conserved among all 18 eutherian mammals with data available, but differs from the presumed ancestral Q177 residue found in six species of fish.

In family AU0125, the p.M260V exon 6 variant identified in patient HI0686 was not predicted to affect protein function by any of the three in silico protein programs. The carrier father had obsessive-compulsive disorder (OCD), depression, anxiety, and attention-deficit hyperactivity disorder. The presence of psychiatric disorders in parents of children with autism has been observed previously in several studies [32-34], consistent with the hypothesis of multiple genes with small additive effects in autism. By parental report, both children had compulsions and rituals, and difficulties with changes in routines and their environment. A summary of phenotype information for the affected siblings in families AU0247 and AU0125 is provided in Table 2, with detailed phenotype data for all members of families AU0247 and AU0125 included in the Supplementary Materials. Phenotypic data for the other three families with coding variants (AU0783, AU0679, and AU0841) were not available.

We also sequenced RIMS3 in the boy with the 3.3 Mb deletion to test the hypothesis that the microdeletion might unmask a recessive allele on the non-deleted chromosome. We did not identify any putative functional coding variants.

In control subjects, we identified four coding variants that were distinct from those identified in autism patients; one of these was predicted to affect protein function (Table 1). Three of the four variants were located in exon 2, a region that did not contain any of the five variants reported in the autism subjects. The fourth variant, p.I162V, was located in the same coding region in exon 4 that harbored the p.E177A substitution. Interestingly, the subject harboring this variant presented with symptoms of OCD. Phenotypic data on the parents of the control subjects were not available.

**DISCUSSION**

We report a boy with postnatal microcephaly, minor dysmorphic features, mental retardation and autism associated with a 3.3 Mb deletion involving ~43 genes in chromosome 1p34.2p34.3. The deletion is large and de novo, leading us to hypothesize that it is responsible for his abnormal phenotype. However, the specific genes responsible for different features of the 1p34.2p34.3 deletion phenotype are unknown.

Only a few patients with interstitial deletions of 1p34.2p34.3 have been reported. The closest match is the recent report of a Dutch boy with a 4.1 Mb deletion that completely overlaps the deletion we report, with loss of an additional 14 genes including the glucose transporter gene SLC2A1.[35] Overall, he has a more severe phenotype than our patient with severe
mental retardation and hypotonia, “profound” microcephaly and epilepsy, as well as heterotopia and ponto-cerebellar atrophy on brain imaging. The more severe phenotype is not surprising given the severe phenotype associated with loss of SLC2A1 alone (OMIM 606777). Three other patients have been reported with deletions involving this general region. One boy with a much larger ~17 Mb visible deletion had severe developmental delay, poor growth, and microcephaly.[36] Two sibs who inherited a juxtaposed inversion (inv 1p22.3p34.1) and deletion (1p34.1p34.3) of this region presented with behavior disorders, although their intelligence and appearance were normal.[37] A detailed genotype-phenotype analysis involving 1p34.2p34.3 in these patients is provided in the Supplementary Materials.

Based on the reported altered regulation of RIMS3 in autism [24] (see below) and our pathway analysis, we hypothesize that haploinsufficiency of the synaptic protein RIMS3 is the primary contributor to the autism phenotype in the 1p34.2p34.3 microdeletion patient. A large number of autism-related genes are involved in synapse function, neuronal cell adhesion or both.[38] Several of these have been implicated in autism based on cytogenetic rearrangements, CNVs or rare mutations, including NLGN3 and NLGN4 [13], NRXN1 [10, 14, 15], SHANK3 [16-18], and CNTNAP2 [19-21]. RIMS3 belongs to the RIM protein family that function as important components of the presynaptic machinery for synaptic vesicle fusion and neurotransmitter release.[39] Expression analysis of RIMS3 in the rat demonstrated that it is exclusively expressed in the brain [40] and protein studies indicated that RIMS3 was detected only in rostral brain regions but not in spinal cord, hindbrain, or midbrain.[40] Overexpression of RIMS3 has been demonstrated to greatly facilitate Ca^{2+}-triggered exocytosis, supporting a role for RIMS3 as a regulator of exocytosis in the synaptic membrane.[40] One recent study demonstrated that expression of RIMS3 is dysregulated in lymphoblastoid cells from autism patients with either maternal duplications of 15q11q13 or Fragile X syndrome.[24] Others have reported increased expression of RIMS3 in schizophrenia [41, 42], a neuropsychiatric disorder hypothesized to have shared genetic etiology with autism.[43] Thus, several lines of evidence converge to support the hypothesis that abnormal RIMS3 function due to chromosomal imbalances or rare mutations may underlie autism. However, we cannot exclude the possibility that other genes within or near the ~3.3 Mb 1p34.2p34.3 deletion may contribute to autism.

To address whether RIMS3 point mutations underlie autism, we sequenced the complete RIMS3 coding region in 512 unrelated autism patients. In total, five coding variants were identified in autism patients that were absent in ~460 controls. None of these variants were de novo and all were inherited with no bias between maternal versus paternal transmission. We used several in silico programs to assess the functional impact of these variants on protein function, and identified one variant (p.E177A) that was predicted to be deleterious. This variant segregated with the autism phenotype in two sibs, was not identified in 1,161 controls, and is highly conserved among eutherian mammals.

The p.E177A substitution resides in a C2B domain that is known to bind to several proteins including α-liprins, SNAP-25, and voltage-gated calcium channels.[44, 45] However, the critical residues for some of these interactions are downstream of p.E177 and this amino acid is not conserved among the RIM family of proteins.[40] α-liprins are known to directly interact with CASK, a calcium/calmodulin-dependent serine protein kinase that belongs to the membrane-associated guanylate kinase (MAGUK) family. CASK regulates the trafficking, targeting and signaling of numerous presynaptic proteins including neurexins, APBA1 (Mint1) and ion channels [46], and is also implicated in brain development.[47] Interestingly, a C2B domain is also found in Doc2-alpha (Double C2-like domains, alpha), a protein that belongs to the same superfamily as RIMS3. DOC2A is one of ~24 genes located within the 16p11.2 microdeletion-duplication region that has recently been detected in ~1% of patients with autism.[8, 11, 12]

Although the p.E177A variant was not found in a large number of controls and was predicted to be deleterious using SIFT and SNAP, further studies are needed to prove whether the p.E177A substitution is pathogenic or not, as protein simulation programs do not substitute for
Only two of the three in silico methods we used predicted a deleterious effect from the p.E177A variant. This is not surprising, given that each program varies in methodology, including differences in classification approaches (e.g. machine-learning versus rule-based systems) and input information (e.g. sequence data, functional annotation, protein structure, solvent accessibility). We also identified one variant in a control subject that was predicted to be deleterious. Only limited phenotype data is available for this subject, so we cannot determine whether it is a benign or weakly pathogenic variant. Detailed phenotypic assessment of this subject would help distinguish between these possibilities.

In summary, our data demonstrate that heterozygous loss of one or more of the 43 genes located within a ~3.3 Mb interval on chromosome 1p34.2p34.3 are associated with abnormal development, including mental retardation, postnatal microcephaly and autism. Further, our data suggest but do not prove conclusively that RIMS3 is an autism causative or contributory gene. To our knowledge, this is the first study to suggest that mutations in a RIM protein may underlie autism or other neurodevelopmental disorders. The identification of putative mutations in RIMS3 is consistent with the identification of CNVs and intragenic mutations of other pre- and post-synaptic proteins in patients with autism, and lends further support to the hypothesis that abnormalities in synaptic development and function underlie autism in some (and perhaps many) patients. Functional studies of RIMS3 variants such as p.E177A should provide additional insight into the role of pre-synaptic proteins in autism. Finally, the rare association of microcephaly and autism suggests that other gene(s) in the region are associated with brain growth.

ACKNOWLEDGMENTS
We thank the parents of the first patient for offering so much of their time and support, and disclose that his mother and the senior author (WBD) are related. We gratefully acknowledge the resources provided by the AGRE Consortium and participating AGRE families. Additional autism families and the control samples were acquired from the NIMH Center for Collaborative Genetic Studies on Mental Disorders. We also thank Elizabeth Berry-Kravis of Rush University Medical Center for performing Fragile X testing in our deletion 1p3 proband. This work was supported in part by grants from the National Institute of Neurological Disorders and Strokes to SLC (R01-NS051812) and WBD (R01-NS050375), and the Roswell Park Cancer Institute Comprehensive Cancer Center Grant (P30 CA01605632) to NN. We are grateful to Autism Speaks for awarding Postdoctoral Fellowships to Ravinesh A. Kumar and Camille W. Brune. AGRE is a program of Autism Speaks and is supported, in part, by grant 1U24MH081810 from the National Institute of Mental Health to Clara M. Lajonchere (PI).

COMPETING INTERESTS
The authors declare no competing financial interests.
FIGURE LEGENDS

Figure 1. (A) The patient’s head circumference (HC) was normal at birth, but decelerated after 7 months and subsequently followed a curve at -3 standard deviations (SD). Y-axis shows HC size in both centimeters (CM) and inches (IN). X-axis indicates age. The top and bottom dashed curves represent HC at +2SD (98%) and -2SD (2%), respectively, in relation to the mean (50%) HC curve (solid). Serial HC measurements for the patient are represented as red dots. (B) The patient’s facial appearance is normal at 1 year of age. (C) At 10 years the boy has subtle dysmorphism with a long narrow face and deep set or sunken eyes.

Figure 2. (A) Array comparative genome hybridization (aCGH) using a BAC microarray demonstrates a deletion in chromosome 1p34.2. The aCGH plot shows the log2 ratio of the patient versus reference DNA on the vertical axis. Each individual BAC is represented as a single blue dot and the horizontal axis shows the position of each BAC along chromosome 1. The deletion of 1p34.2 is indicated by an arrow pointing to a vertical line of dots. Interrogation of the UCSC genome browser for the microdeletion region in this region identifies ~47 RefSeq genes (shown below aCGH plot). Known CNVs (orange blocks) and Indels (green blocks) are reported in the Database of Genomic Variants track. (B) FISH analysis confirms the deletion 1p34.2 aCGH results. The distal breakpoint boundary is indicated where RP11-67o15 (green arrows) shows two normal signals while RP11-120G12 (red arrow) shows a single signal in the interphase nucleus.

Figure 3. Mutation analysis of \textit{RIMS3} identifies two missense variants that segregate with autism and autism-related phenotypes. (A) The pedigree for family AU0247 shows that the p.E177A missense variant is present in a patient with autism (AU0247-03), in a sibling with NQA (Not Quite Autism) (AU0247-04), and in the mother who presents with psychiatric symptoms. Chromatograms indicate the presence of the A/C substitution. The SIFT program predicted the p.E177A substitution to be deleterious. (B) The pedigree for family AU0125 shows that the p.M260V missense variant is present in two siblings with autism (AU0125-03 and AU0125-04) as well as in the father who presents with psychiatric symptoms, including OCD. Chromatograms indicate the presence of the A/G substitution. The variant is not predicted to affect protein function.
REFERENCES


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Table 1. *RIMS3* mutation screening identifies putative mutations associated with autism

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*aBased on A00370-05

*bFour spectra were compared (brain, choroid plexus, serum, and saliva (Pag)). Conservation is identified at the amino acid and nucleotide levels for coding and non-coding variants, respectively. Highly conserved, conserved across all four species; conserved, conserved between human, mouse, and mouse; Not conserved, conserved only between human and mouse; only.
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APPENDICES
None
A de novo 1p34.2 microdeletion identifies the synaptic vesicle gene RIMS3 as a novel candidate for autism

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SUPPLEMENTARY NOTES

Additional phenotype data for families AU0247 and AU0125

Family 1. Individual (AU027403) carries the p.E177A variant. He had delayed first words (48 months) and phrase speech (54 months) with no regression of language or other skills. At 5 years, he had an abnormal gait that was not further described. At 6 years, he met criteria for Autism on the ADI-R with the most severe social and communication scores found among our probands. His parents reported that he had mild aggression and overactivity, and excelled at drawing. On the ADOS (module 1) he also met criteria for Autism. His estimated non-verbal IQ was 110, while a verbal IQ was not available. He had symptoms of obsessive compulsive disorder (OCD) and attention deficit hyperactivity disorder (ADHD), and was being treated with naltrexone. Developmentally, he had delayed eruption of teeth, a ventricular septal defect that closed by 1 year, and diastasis recti. At 7 years, his height was below the 3rd percentile; weight and head circumference were not available.

His brother (AU027404), who also carries the p.E177A variant, was first evaluated with an ADI-R at 3 years. He met criteria for autism on all but the Social domain, which he missed by 1 point and so was classified as meeting “Not Quite Autism” using AGRE criteria. His first words (36 months) and phrases (38 months) were delayed, but no regression was reported. His parents reported he had mild anxiety, aggression and overactivity. He had difficulties with fine and gross motor coordination, and an unusual gait that was not described further. Information on savant skills was not reported. At 5 years, he had symptoms of ADHD but was not taking any medications. His head circumference was normal (27th percentile), while height and weight were not reported. Examination demonstrated downslanting palpebral fissures, epicanthal folds, prominent eyes, and posteriorly-rotated ears. Neither sibling had a history of seizures.

The mother of the children with the p.E177A variant had migraines, as well as a history of rheumatic fever, cholecystectomy and cleft palate that was repaired as child. She had a diagnosis of depression with additional symptoms of bipolar disorder and anxiety, and was treated with paroxetine. Examination demonstrated prominent, cup-shaped ears, and a “pear-shaped” nose. She had a cousin who died of sudden infant death syndrome.

Family 2. Individual (AU012503) carries the p.M260V variant and was evaluated with an ADI-R at 10 years. He met criteria for Autism and had deficits in each of the core domains of ASD. His language development was normal, and he had no history of regression of any language or non-language skills. His parents reported that he had severe aggression, mild overactivity, difficulty with gross and fine motor coordination, and an abnormal gait. His memory skills were good and used functionally. On the ADOS (module 3), he met criteria for Autism Spectrum Disorder, but not Autistic Disorder. At 12 years, his estimated IQ scores were average to above average (Peabody Picture Vocabulary Test standard score 126, Ravens Progressive Matrices estimated non-verbal IQ 94). He had diagnoses of anxiety disorder and ADHD, and showed symptoms of OCD. He was treated with paroxetine and risperidone. He had a congenital bone abnormality in one foot that was not described further, and femoral rotation that was corrected by exercises. On exam, his weight (97th percentile), length (77th percentile) and head circumference (98th percentile) were all above average. Examination demonstrated a small jaw, high-arched palate, abnormal philtrum, flat feet and gynecomastia, the latter possibly a side effect of risperidone treatment.

His sister (AU012504), who also had the p.M260V variant, was evaluated by ADI-R at 7 years; she also met Autism criteria. Her language development was normal with no history of regression. However, she had regression of some other skills including hand play, self-help, and motor skills after 5 years. Her parents described severe aggression, mild anxiety, and mild overactivity. She also had difficulties with fine and gross motor coordination, and an abnormal gait. Her drawing skills were above the population mean but not used functionally. On the ADOS (module 3), she also met criteria for Autism. At 10 years, her estimated non-verbal IQ
was 107. Her receptive and expressive language levels were about 3-years below her age on the Vineland Adaptive Behavior Scales. She had several psychiatric diagnoses including bipolar disorder, OCD and ADHD, and had symptoms of depression. She was treated with mixed dextroamphetamine and racemic DL-amphetamine, valproic acid, and clonidine. Her weight (42nd percentile), height (58th percentile) and head circumference (53rd percentile) were normal. Exam demonstrated prominent lips and several café-au-lait spots, but no other signs of neurofibromatosis. Neither sibling had a history of seizures. Their father had diagnoses of depression and anxiety, and also had symptoms of OCD and ADHD. He had a history of peptic ulcers. The father’s family history was unavailable.

**Additional phenotype data for patients with 1p34.2 deletions**

Only a few patients with overlapping 1p34.2p34.3 deletions have been reported. The closest match is the recent report of a Dutch boy with a slightly larger 4.1 Mb deletion between BACs RP11-445L12 and RP11-125O1.[1] This deletion involves chr1:39,063,443-43,259,372 on the UCSC browser Human March 2006 assembly (http://genome.ucsc.edu/cgi-bin/hgGateway) and completely overlaps the deletion we report, with loss of an additional 5 genes on the distal end and another 9 on the proximal end including the glucose transporter gene (SLC2A1 or GLUT1). Heterozygous mutations of SLC2A1 cause a glucose transport deficiency syndrome in brain (MIM 606777) characterized by a severe developmental encephalopathy with infantile seizures, postnatal microcephaly and spasticity [2], and this boy indeed had low levels of glucose in cerebrospinal fluid.

The boy with the large 4.1 Mb deletion had intrauterine growth deficiency first noted at 36 weeks gestation, and was born at term with severe growth deficiency and microcephaly (weight –3 SD, length –3.5 SD and OFC 29.8 cm, which is –3 SD although reported as only –0.5 SD). His exam demonstrated several dysmorphic features including flat occiput, high forehead, small palpebral fissures, epicanthal folds, broad convex nose with short collumella, flat philtrum, small jaw, and dysplastic ears. Later exams demonstrated severe developmental delay and mental retardation, “profound” microcephaly and severe hypotonia. Brain MRI was reported to show periventricular heterotopia, pontocerebellar atrophy and poor myelinization, and brainstem evoked auditory response was normal at two years. Overall, he has a more severe phenotype than our patient, which is not surprising given the additional deletion of SLC2A1.

Three other patients have been reported with deletions involving this general region. A boy with a much larger ~17 Mb cytogenetic deletion (del 1p34.1p36.1) had severe developmental delay, poor growth, microcephaly, and facial dysmorphism consisting of low-set ears, narrow palpebral fissures and high arched palate. He also had pulmonary atresia, ventricular septal defect, cryptorchidism and hypertrichosis.[3] In another family, two sibs who inherited a complex juxtaposed inversion (inv 1p22.3p34.1) and deletion (del 1p34.1p34.3) of this region had normal intelligence and appearance with behaviour disorders [4], but the deletion size and breakpoints were estimated from cytogenetic inspection only and so cannot easily be compared to either of the above patients.

These other reports support association of mental retardation and postnatal microcephaly with deletion 1p34.2p34.3. None of the other patients were reported to have autism, although the boys with the 4.1 and ~17 Mb overlapping deletions had much more severe developmental handicaps that could easily obscure an autistic disorder. Alternatively, the autism in our patient could be unrelated, although we consider this unlikely given the large size and number of genes deleted. Because microcephaly is rarely associated with autism – macrocephaly is much more common [5]– we hypothesize that different genes in this region are responsible for his autism and microcephaly.

Other genes of interest in the 1p34.2 microdeletion region include YBX1 (nuclease sensitive element binding protein 1), which is associated with growth deficiency and may represent a candidate gene for microcephaly. The Ybx1 knockout mouse has embryonic and perinatal
lethality with severe growth retardation and some mice have craniofacial defects and respiratory failure.[6, 7] KCNQ4 (potassium voltage-gated channel KQT-like protein) is an ion channel gene that regulates neuronal excitability in sensory cells of the cochlea. Missense mutations predicted to have dominant negative effects cause congenital or early-onset non-syndromic deafness type 2 (MIM 603537), while null mutations cause a later onset progressive hearing loss with onset between eight and 50 years of age.[8] Thus, our patient with the 3.3 Mb deletion and the boy with the 4.1 Mb deletion are at high risk for future hearing loss. Polymorphisms of COL9A2 have been associated with lumbar disc disease and specific splice site (but so far not null) mutations with autosomal dominant multiple epiphyseal dysplasia (MIM 120260), but no neurodevelopmental disabilities have been reported with these sequence variants.[9, 10]

SUPPLEMENTARY METHODS

**Fluorescence in situ hybridization (FISH)**

Lymphoblastoid cell lines for the patient and his parents were cultured using standard techniques. RPCI-11 BACs that defined the boundaries of the 1p34.2 deletion were acquired from several sources including the Wellcome Trust Sanger Institute and the Roswell Park Cancer Institute. BAC probes were labeled by nick translation using either Spectrum Green or Spectrum Orange fluorescence dyes (Abbott Molecular Inc, Des Plaines, IL). FISH was performed using standard techniques. Slides were analyzed with a Zeiss Axioplan 2 fluorescence microscope with a cooled CCD camera and Applied Imaging CytoVision v3.7 software.

**Microsatellite Analysis**

Microsatellites were selected from the UCSC Genome Browser microsatellite or simple repeat tracks and primers were designed using MIT Primer3 (http://frodo.wi.mit.edu/). For a single reaction, a master mix of 1μL 10x PCR buffer with 15 mM MgCl2, 1 μL 10mM dNTP, 0.1 μL Ampli Taq Gold enzyme, 0.8 μL 10 μM primers (forward & reverse), and 7.1 μL sterile H2O was prepared. 10 ng of DNA was added to each reaction. The PCR reactions were run in ABI 9700 thermocyclers using the following conditions: hot start at 94°C for 10 min, 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, followed by a final extension step at 72°C for 10 min. Samples were analyzed on an ABI 3730 XL DNA sequencing analyzer and processed using GeneMapper 3.7 software (Applied Biosystems).

**DNA amplification and sequencing**

PCR-amplification primers were designed using MIT Primer3 with M13 Forward and Reverse Tails added to each primer for subsequent use in high-throughput DNA sequencing (Supplementary Table 2). DNA was amplified in a reaction comprised of: 20 ng genomic DNA, 1x buffer I (1.5 mM MgCl2, Applied Biosystems, Foster City, CA), 1 mM dNTPs (Applied Biosystems), 0.4 μM primer (each of forward and reverse, IDT, Coralville, IA), and 0.25 units AmpliTaq Gold (Applied Biosystems) in a total volume of 10 μL. Thermocycling conditions were as follows: 94°C for 10 min; 35 cycles of 94°C for 30 sec, annealing temperature (53-60°C) for 30 sec, and 72°C for 30 sec; and final extension of 72°C for 10 min. Variations in reaction composition and cycling conditions were required for a small number of amplicons. PCR products were purified in a 10 μL reaction comprised of 6.6 units Exonuclease I and 0.66 units shrimp alkaline phosphatase that was incubated at 37°C for 30 min followed by 80°C for 15 minutes. Sequencing reactions were performed using Big Dye terminators on an ABI 3730XL 96-capillary automated 3730XL DNA sequencer (Applied Biosystems) at The University of Chicago DNA Sequencing and Genotyping Core Facility. Sequence data were imported as AB1 files into Mutation Surveyor v3.10 (SoftGenetics, State College, PA). Sequence contigs were
assembled by aligning the AB1 files against GenBank reference sequence files that were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Genbank/). The RIMS3 reference sequence (AL031289) included the complete 5’ untranslated region (UTR), coding sequence and associated splice-sites, intronic sequence, and complete 3’ UTR. The imported GenBank file provides annotated features for each gene that include base count, intron/exon boundaries, amino acid sequence, and previously reported single nucleotide polymorphisms (SNPs) from the SNP database (dbSNP) (http://www.ncbi.nlm.nih.gov/projects/SNP/). To screen for putative mutations, the entire length of the sample trace was manually inspected for quality and variation from the reference trace. All detected variants were visually reviewed by two trained individuals and all variants were confirmed using bi-directional sequencing.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Functional network analysis of RIMS3 identifies biological relationships with at least 14 putative and/or known autism-related genes (red circles). Genes are represented as nodes and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Nodes are displayed using various shapes that represent the functional class of the gene product.
Supplementary Table 1. Ethnicity data for autism patients and control subjects

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## Supplementary Table 2. PCR primers used to amplify RIMS3

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<td>5'-CCTGCCAGTGCTCTGT-3'</td>
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The following sequencing tails were added to each primer to facilitate sequencing:

- **Forward tail**: 5'-TGTAAAACGACGGCCAGT-3'
- **Reverse tail**: 5'-CAGGAAACAGCTATGAC-3'
SUPPLEMENTARY REFERENCES


A de novo 1p34.2 microdeletion identifies the synaptic vesicle gene RIMS3 as a novel candidate for autism

Ravinesh A Kumar, Jyotsna Sudi, Timothy D Babatz, Camille W Brune, Donald Oswald, Mayon Yen, Norma J Nowak, Edwin H Cook, Susan L Christian and William B Dobyns

J Med Genet  published online June 21, 2009

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