Multiple Recurrent De Novo CNVs, Including Duplications of the 7q11.23 Williams Syndrome Region, Are Strongly Associated with Autism

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SUMMARY

We have undertaken a genome-wide analysis of rare copy-number variation (CNV) in 1124 autism spectrum disorder (ASD) families, each comprised of a single proband, unaffected parents, and, in most kindreds, an unaffected sibling. We find significant association of ASD with de novo duplications of 7q11.23, where the reciprocal deletion causes Williams-Beuren syndrome, characterized by a highly social personality. We identify rare recurrent de novo CNVs at five additional regions, including 16p13.2 (encompassing genes *USP7* and *C16orf72*) and *Cadherin 13*, and implement a rigorous approach to evaluating the statistical significance of these observations. Overall, large de novo CNVs, particularly those encompassing multiple genes, confer substantial risks (OR = 5.6; CI = 2.6–12.0, p = 2.4 × 10^{-7}). We estimate there are 130–234 ASD-related CNV regions in the human genome and present compelling evidence, based on cumulative data, for association of rare de novo events at 7q11.23, 15q11.2-13.1, 16p11.2, and *Neurexin 1.*

INTRODUCTION

Autism spectrum disorders (ASD) are defined by impairments in reciprocal social interaction, communication, and the presence of stereotyped repetitive behaviors and/or highly restricted interests. A genetic contribution is well established from twin studies (Bailey et al., 1995; Lichtenstein et al., 2010; Liu et al., 2001). Moreover, the large difference between monozygotic and dizygotic concordance rates is consistent with the contribution of de novo mutation and/or complex inheritance. In addition, the overrepresentation of ASD in monogenic developmental disorders (Klauck et al., 1997; Smalley et al., 1992), gene discovery in families with Mendelian forms of the syndrome (Morrow et al., 2008; Strauss et al., 2006), and long-standing evidence for an increased burden of gross chromosomal abnormalities in affected individuals (Bugge et al., 2000; Veenstra-Vanderweele et al., 2004; Vorstman et al., 2006; Wassink et al., 2001) all point to the importance of genetic risks.

Over the last several years, dramatic advances have emerged from studies of copy-number variation (CNV) characterizing submicroscopic chromosomal deletions and duplications (Iafrate et al., 2004; Sebat et al., 2004). Sebat et al. (2007) first noted that "large" (mean size of 2.3 Mb), rare (<1% frequency in the general population), de novo events were more frequent in ASD probands identified in families with only a single affected child (i.e., simplex families) compared to controls, or versus probands from families with more than one affected individual (i.e., multiplex families).

This overrepresentation of large de novo CNVs in ASD has been replicated in three subsequent studies involving cohorts ranging in size from 60 to 393 simplex trios (Itsara et al., 2010; Marshall et al., 2008; Pinto et al., 2010). Two of these studies (Marshall et al., 2008; Pinto et al., 2010) have also confirmed an excess in simplex versus multiplex ASD families. Across all studies, the burden of rare de novo CNVs in simplex probands (i.e., the percentage of individuals carrying ≥1 rare de novo event) has ranged from 5.0% to 11% (Table S1, available online). Rare structural variants, both transmitted and de novo, have also shown varying degrees of evidence for association with ASD. These include deletions and/or duplications at specific loci, including 1q21.1, 15q11.2-13.1, 15q13.2-13.3, 16p11.2, 17q12, and 22q11.2, as well as recurrent structural variations involving one or a small number of genes, including *Neurexin 1* (*NRXN1*), *Contactin 4* (*CNTN4*), *Neuroligin 1* (*NLGN1*), *Astrotactin 2* (*ASTN2*) and the contiguous genes *Patched Domain Containing 1* (*PTCHD1*) and *DEAD box Protein 53* (*DDX53*) (Bucan et al., 2009; Glessner et al., 2009; Kumar et al., 2008; Marshall et al., 2008; Moreno-De-Luca et al., 2010; Noor et al., 2010; Pinto et al., 2010; Weiss et al., 2008).

To date, the number of definitive replicated findings from these studies has remained relatively small and all evidence has pointed to a highly heterogeneous allelic architecture as no risk variant is present in more than ∼1% of affected individuals. In addition, examples of incomplete penetrance (not all mutation carriers have disease) and affected siblings not sharing the same risk variant have been the rule rather than the exception. Moreover, remarkably diverse outcomes have been identified for apparently identical CNVs. For example, chromosome 16p11.2 deletions or duplications have been found in individuals with ASD and intellectual disability (ID) (Weiss et al., 2008), seizure disorder (Mefford et al., 2009), obesity (Bochukova et al., 2010), macrocephaly, and schizophrenia (McCarthy et al., 2009). These complexities suggest that the use of association strategies to demonstrate an excess of specific de novo CNVs will play an important role in definitively implicating loci in ASD.

We have conducted a genome-wide analysis of rare CNVs in 4457 individuals comprising 1174 simplex ASD families from

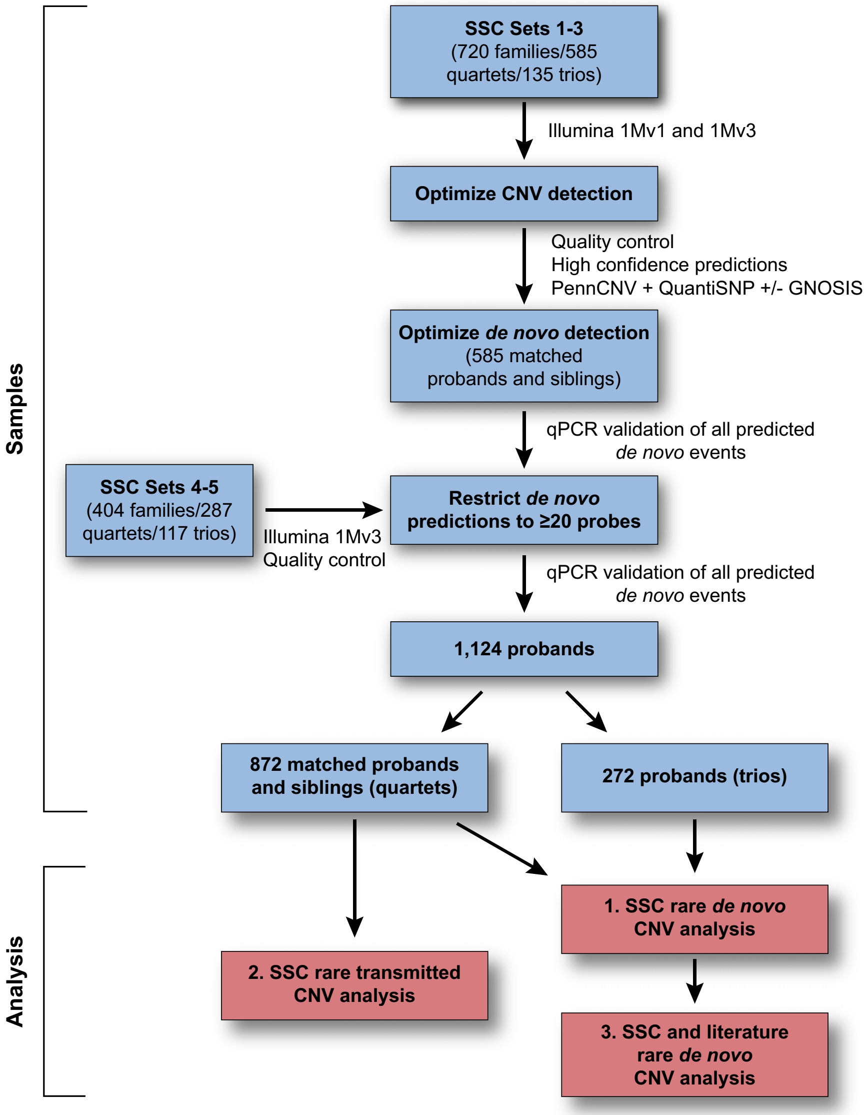

Samples
Analysis
SSC Sets 1-3
(720 families/585 quartets/135 trios)
Illumina 1Mv1 and 1Mv3
Optimize CNV detection
Quality control
High confidence predictions
PennCNV + QuantiSNP +/- GNOSIS
Optimize *de novo* detection
(585 matched probands and siblings)
qPCR validation of all predicted *de novo* events
SSC Sets 4-5
(404 families/287 quartets/117 trios)
Illumina 1Mv3
Quality control
Restrict *de novo* predictions to ≥20 probes
qPCR validation of all predicted *de novo* events
1,124 probands
872 matched probands and siblings (quartets)
272 probands (trios)
1. SSC rare *de novo* CNV analysis
2. SSC rare transmitted CNV analysis
3. SSC and literature rare *de novo* CNV analysis

Figure 1. Flow Chart of CNV Detection and Confirmation in the Simons Simplex Collection

CNV detection was optimized by qPCR analysis of 115 predictions (Table S1 and Figure S1). Quality control was performed to check for identity error and data quality (Supplemental Experimental Procedures). De novo detection was optimized by qPCR analysis of 403 predictions (Figure S1) leading to the threshold of ≥20 probes and refinement of the prediction algorithm. All de novo CNVs reported in the study were confirmed by using qPCR with absolute quantification.

the Simons Simplex Collection (SSC) (Fischbach and Lord, 2010). Each family has been extensively phenotyped, with a single affected offspring, unaffected parents, and, in the majority of cases, at least one unaffected sibling. This ascertainment strategy was designed to enrich for rare de novo risk variants. In addition, the family quartet structure allows for proband versus sibling comparisons that should mitigate a wide range of technical and methodological confounders that have plagued association study designs (Altshuler et al., 2008). We have also developed and apply a rigorous approach to evaluating the genome-wide significance of recurrent rare de novo events. Consequently, both the scale and design of this study provide a valuable opportunity to investigate the contributions of rare de novo and rare transmitted variants in simplex families, to identify ASD risk loci, to evaluate the relationship between rare structural variation and social and intellectual disability (ID), and to place these findings in the context of previous ASD data, particularly with regard to rare de novo CNVs.

RESULTS

Simons Simplex Collection Summary Characteristics

A total of 4457 individuals from 1174 families were included in the study. Data from 1124 families passed all quality control steps; 872 families were quartets that included two unaffected parents, a proband, and one unaffected sibling; 252 families were trios that included two unaffected parents and a proband (Figure 1).

The male-to-female ratio for probands was 6.2:1. All had confirmed ASD diagnoses based on well-accepted research criteria (Risi et al., 2006), including autism, 1006 (89.5%), pervasive developmental disorder-not otherwise specified, 96 (8.5%), and Asperger syndrome, 22 (2%). The mean age at inclusion was 9.1 years for probands (4–18 years) and 10.0 years (3.5–26 years) for siblings. The mean (± 95% CI) full-scale IQ in probands was 85.1 ± 1.5; however, the range was considerable (<20–167, Figure 3): the mean verbal IQ was 81.9 ± 1.7 and the mean nonverbal IQ was 88.4 ± 1.4. Self-reported ancestry was as follows: White non-Hispanic, 74.5%; mixed, 9.3%; Asian, 4.3%; White Hispanic, 4.0%; African-American, 3.8%; other, 4.2%. Additional phenotypic data may be found in recent publications (Fischbach and Lord, 2010) and at www.sfari.org/simons-simplex-collection.

Illumina 1M Arrays Accurately Detect Both Rare De Novo and Transmitted CNVs

DNA samples derived from whole blood (n = 4381), cell lines (n = 68), or saliva (n = 8) were genotyped on the Illumina IMv1 (334 families) or Illumina IMv3 Duo Bead arrays (840 families), which share 1,040,853 probes in common. CNV prediction was performed by PennCNV (PN) (Wang et al., 2007), QuantiSNP (QT) (Colella et al., 2007), and GNOSIS (GN), (www.CNVision.org) (Figure 1). To assess detection accuracy, we evaluated 115 predicted rare CNVs ($\leq$50% of the span of the event found at > 1% in the Database of Genomic Variation [DGV; http://projects.tcag.ca/variation/]) by quantitative polymerase chain reaction (qPCR). A higher positive predictive value was observed for CNVs called by PN and QT, with or without GN (PPV = 97% with GN, PPV = 83% without) than for other combinations of algorithms, irrespective of the number of probes mapping within the structural variation (Table S2 and Figure S1); these "high-confidence" criteria were subsequently used to identify all rare transmitted CNVs.

Given a particular interest in de novo variation and the relative challenge of accurately detecting these CNVs (Lupski, 2007), we sought to optimize our detection strategy further for this class of structural variation by using the first 585 quartets with complete genotyping data (Figure 1). We identified de novo events from among the predicted rare high-confidence CNVs based on the combination of within-family intensity and genotypic data and used a blinded qPCR confirmation process (Figure S1). Fifty-three percent of de novo predictions based on $\geq$20 probes (n = 94) were confirmed compared with 2.6% based on <20 probes (n = 430). Eighty-two percent of failures were false-positive predictions in offspring; 18% were false-negatives in parents. The data from this experiment were then used to further refine de novo prediction thresholds (Supplemental Experimental Procedures). In addition, given the large number of predictions of small CNVs, and the low yield of true positives in the pilot data set (Figure S1), we elected to restrict all further statistical analysis to those rare de novo events that both encompassed $\geq$20 probes and were confirmed by qPCR in whole-blood DNA (Figure S1).

Subsequently, at the conclusion of our study, we were able to evaluate our methods further via a comparison of confirmed de novo CNVs identified in our study versus those detected by Nimblegen 2.1M arrays from among a total of 1340 overlapping subjects (probands or siblings), as described by Levy and colleagues in this issue (Levy et al., 2011). At a threshold of $\geq$20 Illumina probes mapping within a genomic interval a combined total of 58 rare de novo CNVs were identified across the two studies, with each array type identifying 95% (n = 55) of the total. This suggests that the combined results across the two studies are very likely to represent the complete set of large de novo CNVs present in this SSC sample. Though not included in our subsequent statistical analysis, we also compared results for CNVs that mapped to regions encompassing fewer than 20 probes on the Illumina array. A total of 31 small rare de novo CNVs were identified between the two groups with approximately twice as many found by using the 2.1 M Nimblegen array versus the 1 M Illumina array (23 CNVs versus 12 CNVs, respectively). Of these 31 events, only 13% (n = 4) were identified by both groups, suggesting that the sensitivity for small de novo events was low for both arrays and that, as anticipated, there is a pool of small de novo structural events that were not captured in our analyses.

Analysis of Rare De Novo CNVs in the Simons Simplex Collection

Rare De Novo Genic CNVs Are Overrepresented in Simplex Probands

In light of strong prior evidence for an increased burden of de novo CNVS in simplex autism (Itsara et al., 2010; Marshall et al., 2008; Pinto et al., 2010; Sebat et al., 2007), we investigated these events in probands versus their unaffected siblings in all 872 quartets included in this study (Figure 1). A total of 28,610 rare, high-confidence CNVs were identified, 97 were classified as rare and probably de novo, and 83 events were confirmed to be rare de novo CNVs by qPCR in whole-blood DNA (Table S4).

Rare de novo CNVs were significantly more common among probands than siblings. Overall, 5.8% of probands (n = 51 of 872) had at least one rare de novo CNV compared with 1.7% of their unaffected siblings (n = 15 of 872), yielding an odds ratio (OR) of 3.5 (CI = 2.2–7.5, $p = 6.9 \times 10^{-6}$, Fisher's exact test) (Table 1 and Figure 2). When we considered the proportion of individuals carrying at least one rare de novo CNV encompassing more than one gene (multigenic CNVs), the OR increased to 5.6 (43 in probands versus 8 in siblings; CI = 2.6–12.0, $p = 2.4 \times 10^{-7}$). These results remained consistent regardless of whether we analyzed total numbers of CNVs, the proportion of individuals with at least one rare structural variant (Figure 2), or increased the stringency of the definition for rarity (Supplemental Experimental Procedures).

Given the strong male predominance and increased rates of ASD in monogenic X-linked intellectual disability syndromes, we paid particular attention to rare de novo CNVs on the X chromosome but found only two events: one genic deletion present in a male at the gene *DDX53* and a duplication involving six genes in a female sibling (Xq11.1). This small number precluded meaningful group comparisons. Importantly, no statistical results reported in this article were substantively altered by the exclusion of 15 confirmed rare de novo CNVs identified during our detection optimization experiments that did not then meet our minimum probe criteria to be included in our analyses (Table S4). It is of note, however, that one of these was an exonic deletion of *NLGN3* on chromosome X in a male proband (Table S4).

The burden of rare de novo CNVs in these simplex families is remarkably similar to previously published results (Table S1) despite varying CNV discovery approaches and array densities ranging from 85,000 (Sebat et al., 2007) to 1 million probes (Pinto et al., 2010). We reasoned that this was probably due to the particular importance of large de novo events, as their detection would be least sensitive to differences in probe number and distribution. Indeed, we found that rare de novo CNVs in probands tended to be larger than in siblings (mean 1.6 Mb versus 0.7 Mb) (Figure 2 and Figure S2) and to include a greater number of genes (16-fold increase in probands and a 29-fold increase considering only deletions).

In fact, we found that de novo CNVs in probands were both larger and contained a greater number of genes when these measures were considered independently. We fit a series of stepwise linear models that increased in complexity from individual predictors to an analysis of covariance model, with size and affected status as predictors, to a three-term model that included the interaction of size and affected status. We

Table 1. Burden of De Novo CNVs in Probands and Siblings

Category	Analysis	All Probands (n = 1,124)	Matched Probands (n = 872)	Matched Siblings (n = 872)	Ratio (OR)	p Value[a]
De Novo CNVs						
	CNVs	67	54	16		
	Samples[b]	63	51	15		
	Proportion[c]	5.6%	5.8%	1.7%	3.4 (3.5)	3×10^{-6}
	Genes[d]	1417	1153	73	15.8	
De Novo Deletions						
	CNVs	35	31	8		
	Samples	35	31	8		
	Proportion	3.1%	3.6%	0.9%	3.9 (4.0)	1×10^{-4}
	Genes	638	605	21	28.8	
De Novo Duplications						
	CNVs	32	23	8		
	Samples	29	21	7		
	Proportion	2.6%	2.4%	0.8%	3.0 (3.0)	0.006
	Genes	779	548	52	10.5	
De Novo Genic CNVs						
	CNVs	66	53	13		
	Samples	62	50	12		
	Proportion	5.5%	5.7%	1.4%	4.2 (4.4)	4×10^{-7}
	Genes	1417	1153	73	15.8	
De Novo Exonic CNVs						
	CNVs	64	52	11		
	Samples	60	49	10		
	Proportion	5.3%	5.6%	1.1%	4.9 (5.1)	9×10^{-8}
	Genes	1415	1152	71	16.2	
De Novo Multigenic CNVs						
	CNVs	53	44	9		
	Samples	52	43	8		
	Proportion	4.6%	4.9%	0.9%	5.4 (5.6)	2×10^{-7}
	Genes	1404	1144	69	16.6	
De Novo Autosomal CNVs						
	CNVs	66	53	14		
	Samples	62	50	14		
	Proportion	5.5%	5.7%	1.6%	3.6 (3.7)	2×10^{-6}
	Genes	1416	1152	67	17.2	
De Novo chrX CNVs						
	CNVs	1 (male deletion)	1 (male deletion)	2 (female duplications)		
	Samples	1 (male deletion)	1 (male deletion)	1 (female duplication)		
	Proportion	0.1%	0.1%	0.1%	1.0 (1.0)	0.75
	Genes	1	1	6	0.2	
Small De Novo CNVs (<100 kb)						
	CNVs	8	5	3		
	Samples	8	5	3		
	Proportion	0.7%	0.6%	0.3%	1.7 (1.7)	0.36
	Genes	8	5	7	0.7	
Medium De Novo CNVs (100–1000 kb)						
	CNVs	32	26	9		
	Samples	30	25	8		

(*Continued on next page*)

Table 1. *Continued*

Category	Analysis	All Probands (n = 1,124)	Matched Probands (n = 872)	Matched Siblings (n = 872)	Ratio (OR)	p Value[a]
	Proportion	2.7%	2.9%	0.9%	3.1 (3.2)	0.002
	Genes	469	392	34	11.5	
Large De Novo CNVs (≥1,000 kb)						
	CNVs	27	23	4		
	Samples	26	22	4		
	Proportion	2.3%	2.5%	0.5%	5.5 (5.6)	2×10^{-4}
	Genes	940	756	32	23.6	
Single Occurrence De Novo CNVs						
	CNVs	44	37	14		
	Samples	40	34	13		
	Proportion	3.6%	3.9%	1.5%	2.6 (2.7)	0.001
	Genes	862	754	54	14.0	
Double Occurrence De Novo CNVs						
	CNVs	8	8	2		
	Samples	8	8	2		
	Proportion	0.7%	0.9%	0.2%	4.0 (4.0)	0.05
	Genes	89	102	19	5.4	
≥3 Occurrence De Novo CNVs						
	CNVs	15	9	0		
	Samples	15	9	0		
	Proportion	1.3%	1.0%	0.0%	NA (NA)	0.002
	Genes	466	297	0	NA	

[a] Fisher's exact test.
[b] Four individuals have multiple de novo CNVs.
[c] Percent of samples with ≥1 de novo CNV.
[d] RefSeq genes within the CNV.

confirmed a significant difference between probands and siblings with regard to the number of genes within CNVs (estimated $\beta = 11.1$ more genes in a proband's de novo CNV, $p = 0.025$) even after accounting for the strong effect of the size of the event (estimated $\beta = 6.8$ genes per Mb, $p = 1.1 \times 10^{-9}$) (Figure 3A). Considering deletions and duplications separately did not alter these findings. In summary, the burden of rare de novo CNVs was greater in probands than in siblings with regard to total number, size, and gene content.

Strong Association of Rare Recurrent De Novo CNVs

Our interest in identifying specific regions of the genome contributing to ASD led us to investigate next whether multiple overlapping de novo events were present in probands and then to compare these findings to siblings. In total, 23 probands carried recurrent de novo CNVs in six distinct regions of the genome. Each of these intervals contained from 2 to 11 de novo CNVs in unrelated probands; no de novo CNVs overlapping these regions were found in siblings. In contrast, only a single recurrent de novo event was observed in siblings (16p13.11 in two unrelated siblings) and one CNV overlapping the region was also found in a proband (Figure 4).

The six regions found in probands included seven deletions and four duplications at chromosome 16p11.2, four duplications at 7q11.23 (the Williams-Beuren syndrome region), and two CNVs each at 1q21.1 (two duplications), 15q13.2-q13.3 (one deletion, one duplication), 16p13.2 (two duplications), and disrupting the gene *Cadherin 13* (*CDH13*) at 16q23.3 (5 Mb deletion and an overlapping 34 kb exonic deletion).

The presence of multiple regions showing overlapping rare de novo CNVs restricted to probands, and the absence of similar findings in their sibling controls, is striking. However, in contrast to genome-wide association studies (GWAS) of common variants, there is no widely accepted statistical approach or threshold to formally evaluate these results. Consequently, we set out to develop a rigorous method to assess the significance of de novo events (Experimental Procedures). To do so, we determined the null expectation for recurrent rare de novo CNVs based on our data from unaffected siblings and then used this expectation to evaluate the p value for finding multiple recurrences in probands.

With this approach, the probability of finding two rare de novo CNVs at the same position in probands is 0.53. However, the observations of four recurrent de novo duplications at 7q11.23 ($p = 7 \times 10^{-6}$) and 11 recurrent de novo CNVs at 16p11.2 ($p = 6 \times 10^{-23}$) are highly significant. In addition, we found that 16p11.2 deletions ($n = 7$, $p = 2 \times 10^{-14}$) and duplications ($n = 4$, $p = 7 \times 10^{-6}$) are strongly associated with ASD when considered independently (Figure S3).

Prior studies have reported a combination of rare transmitted and de novo CNVs at ASD risk regions. In our data, we observed

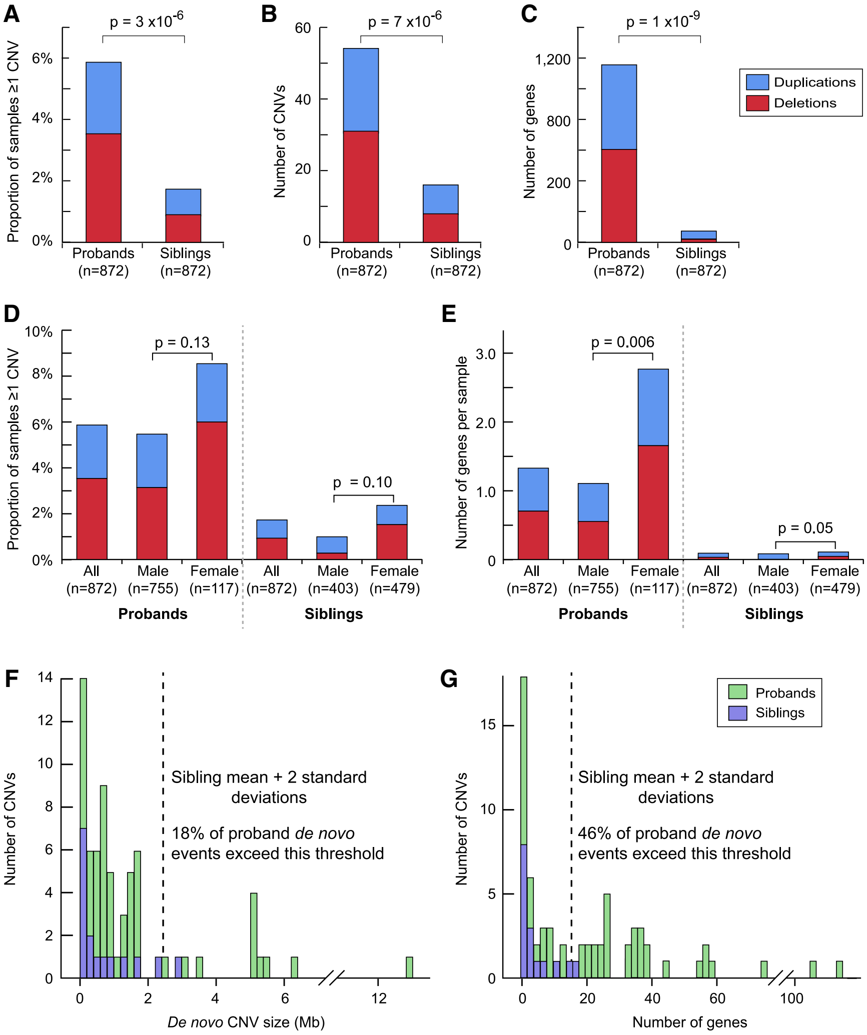


Figure 2. The Burden of Rare De Novo CNVs and Genes Mapping within Them in 872 Probands and 872 Matched Siblings

(A) Percent of individuals with ≥1 rare de novo CNV in probands versus siblings. Red = deletions; blue = duplications for (A) to (E).

(B) Total number of rare de novo CNVs in probands versus siblings (two probands and one sibling have more than one).

(C) Number of RefSeq genes (Pruitt et al., 2007) overlapping rare de novo CNVs in probands versus siblings.

(D) Percent of individuals with ≥1 rare de novo CNV as shown in (A) split by sex. Specific comparisons and associated p values are given.

(E) Number of RefSeq genes overlapping rare de novo CNVs as shown in (C) split by sex.

(F) The distribution of rare de novo CNVs by size in probands (green) and siblings (purple). The dashed vertical line represents the mean plus two standard deviations of the sibling events.

(G) The distribution of rare de novo CNVs by number of RefSeq genes.

Statistical significance was calculated by using Fisher's exact test (A and D), sign test (B), Wilcoxon paired test (C), and Wilcoxon test (E).

eight loci at which rare transmitted CNVs, present only in probands, overlapped one of the 51 regions in probands containing at least one rare de novo CNV. Conversely, in siblings we did not observe any cases in which a rare transmitted CNV, restricted to siblings, overlapped one of the 16 regions showing de novo events. Interestingly, the eight regions in probands showing overlapping rare de novo and rare transmitted CNVs include five of the six intervals characterized by recurrent rare de novo variants, 1q21.1, 15q13.3, 16p13.2, 16p11.2, and 16q23.3 (Figure 4) and three additional genomic segments with one rare de novo event each: 2p15, 6p11.2, and 17q12.

While the use of matched sibling controls should have precluded any confound of population stratification, we explored whether genotype data from the parents of probands with 16p11.2 or 7q11.23 CNVs suggested unusual ancestral clustering (Crossett et al., 2010; Lee et al., 2009) pointing to a particular haplotype that might increase the frequency of de novo events. We found no evidence for this. In addition, given the very large number of 16p11.2 CNVs in this study and the widespread attention afforded previous findings at this locus, we considered the possibility of ascertainment bias. A review of medical histories obtained at the time of recruitment revealed that parents had prior knowledge of a 16p11.2 CNV in two instances (one de novo duplication, one transmitted deletion). With these events removed from the analysis, association of both deletions and duplications remained significant ($p = 3 \times 10^{-19}$, all de novo events [n = 10]; $p = 2 \times 10^{-14}$, deletions [n = 7]; p = 0.002, duplications [n = 3]) (Figure S4).

The Distribution of De Novo CNVs in Probands Supports Marked Locus Heterogeneity

Given the clear risks conferred by large de novo events, we sought to use this class of variation to determine the total number of CNV-mediated de novo ASD risk loci present in the SSC sample. Based on the frequency distribution of 67 de novo events identified in probands, we estimated a total of 130 regions in this SSC cohort (Experimental Procedures).

We then evaluated the implications of this estimate for a second phase of genotyping and CNV analysis, which is currently under way. We used the total predicted number of de novo ASD loci to guide a simulation experiment (Supplemental Experimental Procedures) and found that the most likely outcome of studying a second cohort of similar composition and size would be further confirmation of the 7q11.23 and 16p11.2 findings and the identification of two to three additional regions of significant association. These were most likely to

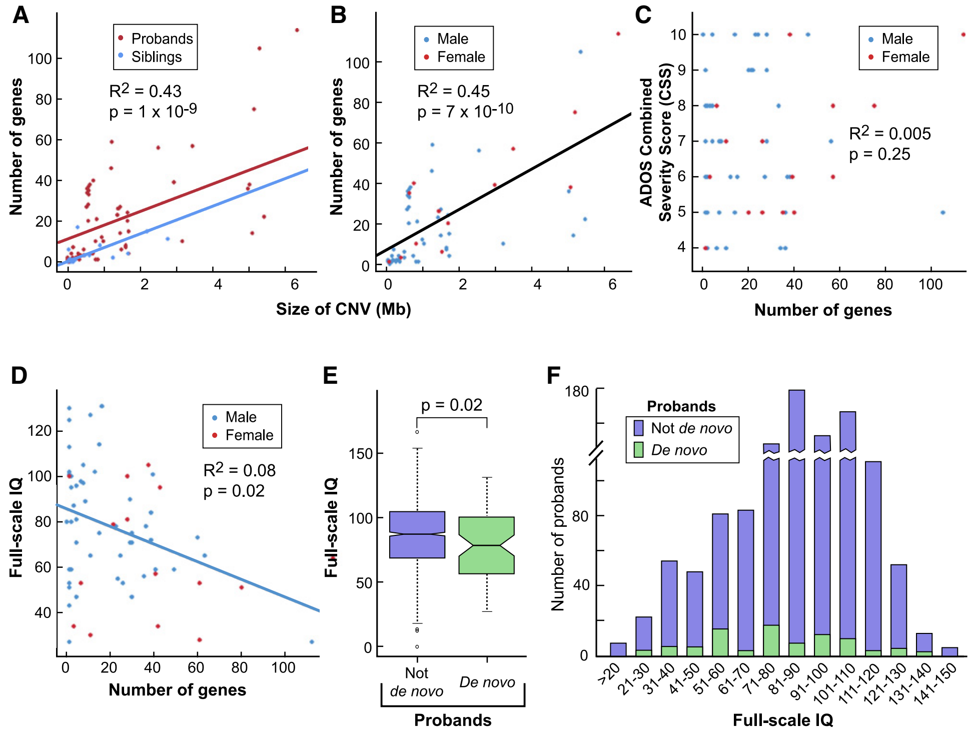


Figure 3. Genotype-Phenotype Analyses of Probands Carrying Rare De Novo CNVs

(A) The number of RefSeq genes within rare de novo CNVs (genes) versus CNV size (size), with probands (red) versus siblings (blue). The slope of the lines shows the fitted significant ($p = 1 \times 10^{-9}$) relationship between genes and size and the difference between the lines shows the fitted difference for probands and siblings ($p = 0.025$). On average, probands have more genes within a rare de novo CNV for any given size.

(B) Genes versus size, with sex of subject encoded by color as noted (sex). The slope of the line shows the fitted significant ($p = 7 \times 10^{-10}$) relationship between genes and size, while the presence of only one line reflects the lack of significant difference by sex ($p = 0.20$).

(C) ADOS combined severity score (CSS), a measure of autism severity, against genes and by sex. The lack of a line indicates the absence of a significant relationship.

(D) Full-scale IQ (IQ) against genes and by sex. The slope shows that IQ declines as a function of genes in males ($p = 0.02$, Wilcoxon test); there is no significant relationship in females.

(E) Boxplot for IQ by presence (green) or absence (purple) of a detected rare de novo event in the probands. The whiskers show the maximal value within a 1.5 multiple of the interquartile range of the upper quartile and the minimal value within a 1.5 multiple of the interquartile range of the lower quartile; the notch shows the 95% confidence intervals of the median.

(F) Distribution of IQ in probands with (green, n = 63) rare de novo CNVs and without (purple, n = 1061).

emerge at the intervals already identified as containing recurrent de novo events, namely 1q21.1, 15q13.2-13.3, 16p13.2, and the *CDH13* locus.

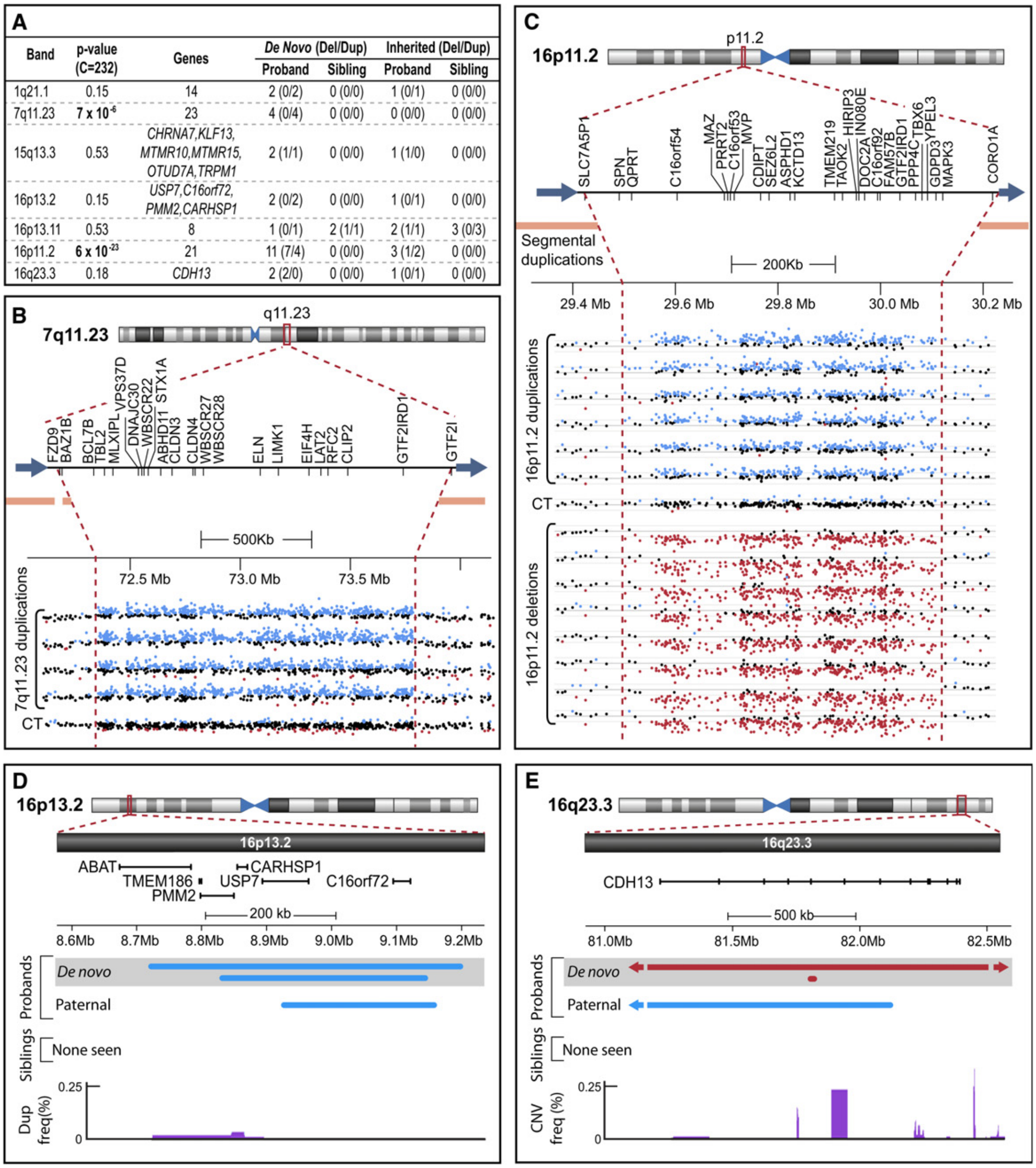
Genotype-Phenotype Analyses of Probands Carrying Any Rare De Novo CNV

Given the availability of highly reliable phenotypic data and longstanding interest in the role of sex in ASD risk and resilience, we investigated whether males or females carried quantitatively different types of rare de novo events and what impact rare de novo CNVs had on intellectual and social functioning.

We found little evidence for larger or more gene-rich de novo CNVs in males versus females. By fitting a series of stepwise linear models, we evaluated whether the number of genes within a de novo CNV tended to differ after accounting for a critical covariate, CNV size. Neither sex ($p = 0.20$) nor the interaction of size and sex ($p = 0.06$) was a significant predictor of gene number. These results should be viewed with some caution, however, given a trend toward significance and a relatively small sample size (Figure 3B).

In contrast, we found that male intellectual functioning was more vulnerable to the effects of rare de novo CNVs. Again, by using a series of stepwise linear models we evaluated the relationship between intellectual functioning, sex, and the number of genes within rare de novo CNVs. For males, there was a significant relationship between IQ and number of genes ($p = 0.02$) with the model predicting a decrease of 0.42 IQ points for each additional gene. In contrast, for females the estimated effect was 10-fold less and did not approach significance (Figure 3D).

To evaluate if low IQ predicted whether a proband carried a de novo CNV, we fit a logistic regression model with de novo CNV status for probands as the outcome and full-scale IQ as the predictor. We found the accuracy of prediction was quite



Band	p-value (C=232)	Genes	*De Novo* (Del/Dup)		Inherited (Del/Dup)	
			Proband	Sibling	Proband	Sibling
1q21.1	0.15	14	2 (0/2)	0 (0/0)	1 (0/1)	0 (0/0)
7q11.23	**7×10^{-6}**	23	4 (0/4)	0 (0/0)	0 (0/0)	0 (0/0)
15q13.3	0.53	*CHRNA7,KLF13, MTMR10,MTMR15, OTUD7A,TRPM1*	2 (1/1)	0 (0/0)	1 (1/0)	0 (0/0)
16p13.2	0.15	*USP7,C16orf72, PMM2,CARHSP1*	2 (0/2)	0 (0/0)	1 (0/1)	0 (0/0)
16p13.11	0.53	8	1 (0/1)	2 (1/1)	2 (1/1)	3 (0/3)
16p11.2	**6×10^{-23}**	21	11 (7/4)	0 (0/0)	3 (1/2)	0 (0/0)
16q23.3	0.18	*CDH13*	2 (2/0)	0 (0/0)	1 (0/1)	0 (0/0)

Figure 4. Confirmed Recurrent Rare De Novo CNVs

(A) All recurrent de novo CNVs identified in 1124 probands and 872 siblings. The gene count is given when >6 RefSeq genes map to an interval; a complete listing of genes is presented in Table S4. The total number of de novo and matching inherited CNVs in probands and siblings is shown for deletions (Del) and duplications (Dup) in parentheses.

(B) LogR data for four de novo duplications and one control with no CNV (CT) in the 7q11.23 interval. RefSeq genes within this region are noted below the ideogram; the orange bars represent flanking segmental duplications. NCBI 36 (hg18) genomic coordinates are shown with the scale indicated. The LogR for all probes within the region is shown; LogR values >0.15 are in blue (suggesting a duplication), while LogR values <−0.15 are in red (suggesting a deletion). B allele frequency data are not shown but support the presence of a corresponding CNV. The approximate boundaries of the CNVs are shown by the vertical dashed red lines and blue arrows.

(C) LogR data for six duplications (four de novo), eight deletions (seven de novo), and one control with no CNV (CT) in the 16p11.2 interval. The ideogram and intensity plots are as in (B).

(D) Overlapping rare de novo and rare inherited CNVs identified in the 16p13.2 interval. The brackets show the boundaries of RefSeq genes; two genes are in common between all three duplications: *USP7* and *C16orf72*. The frequency of duplications in the DGV is shown in purple. The majority of the recurrent de novo region is not present in the DGV.

(E) Overlapping rare de novo and rare inherited CNVs identified in the 16q23.3 interval. A 34 kb deletion overlaps a 5 Mb deletion over a *CDH13* exon (represented by ticks on the gene). The frequency of CNVs observed in the DGV is shown at the bottom in purple.

Table 2. Phenotypic Comparisons between Subjects with 16p11.2 Deletions, 16p11.2 Duplications, and 7q11.23 Duplications and Matched Probands[a]

	16p Deletion (n = 8), Mean (SD)	Deletion Matches (n = 40), Mean (SD)	16p Duplication (n = 6), Mean (SD)	Duplication Matches (n = 30), Mean (SD)	7q Duplications (n = 4), Mean (SD)	Duplication Matches (n = 20), Mean (SD)
Primary						
CPEA Dx-autism	*75%*	*98%*	83%	97%	100%	85%
CPEA Dx-autism spectrum disorder	*13%*	*3%*	0%	0%	0%	10%
CPEA Dx-Asperger syndrome	*13%*	*0%*	17%	3%	0%	5%
ADOS Combined Severity Score	6.5 (1.6)	7.0 (1.7)	7.2 (2.1)	7.4 (1.5)	*7.0 (2.2)*	*7.6 (1.6)*
Full-Scale IQ	76.9 (17.6)	82.5 (27.8)	75.7 (23.2)	81.0 (26)	84.0 (14.9)	81.3 (30.5)
BMI	23.2 (6.4)	20.9 (5.3)	**17.1 (1.4)**	**19.3 (5.2)**	23.1 (5.9)	21.6 (6.4)
Exploratory						
ADI-R social interaction total	*17.9 (6.7)*	*21.3 (5.5)*	21.8 (3.5)	19.1 (5.6)	20.0 (5.5)	19.9 (7.0)
ADOS social affect total	9.9 (4.0)	9.5 (4.2)	*10.8 (5.3)*	*10.7 (3.2)*	11.0 (4.8)	11.7 (3.3)
ADOS social and communication total	12.4 (3.9)	11.5 (4.2)	*14.2 (6.0)*	*12.7 (3.1)*	**11.8 (5.7)**	**13.7 (3.6)**
ADI-R RRB total	5.4 (2.6)	6.8 (2.3)	8.5 (1.9)	6.3 (2.1)	7.3 (1.7)	6.3 (2.6)
ADOS RRB Total	*2.0 (1.3)*	*3.7 (1.9)*	4.3 (2.6)	3.6 (1.7)	*2.0 (1.4)*	*4.1 (2.0)*
ABC total	46.8 (24.3)	42.0 (28.6)	*68.7 (21.5)*	*42.5 (17.9)*	**66.0 (26.7)**	**47.2 (21.0)**
ABC irritability	14.9 (9.3)	9.6 (9.1)	19.5 (6.4)	10.5 (7.8)	**20.0 (13.0)**	**12.0 (7.6)**
ABC hyperactivity	16.5 (9.1)	13.9 (10.3)	**26.7 (6.6)**	**14.0 (7.9)**	20.3 (9.7)	18.3 (9.1)
ABC lethargy/social withdrawal	9.5 (6.6)	10.0 (7.7)	11.5 (8.6)	10.3 (7.2)	*13.8 (8.4)*	*9.8 (6.2)*
Age of first concern	**2.8 (1.5)**	**1.7 (0.9)**	1.7 (0.9)	2.0 (0.9)	1.8 (1.0)	2.1 (1.3)

ABC, Aberrant Behavior Checklist; BMI, body mass index; Dx, diagnosis; RRB, restricted and repetitive behavior.

[a] Significance is shown in bold ($p \leq 0.05$) and italics ($0.05 < p \leq 0.1$).

low (Nagelkerke pseudo $R^2 = 0.014$). Overall, while the odds of carrying a de novo CNV varied 3-fold for those with the lowest versus the highest IQ, the odds were never large (0.111 at IQ = 30, 0.063 at IQ = 80, and 0.036 at IQ = 130). This relationship did not differ significantly by sex (interaction of IQ and sex, $p = 0.12$).

Finally, we investigated the relationship between IQ, sex, and number of genes within rare de novo CNVs to determine whether any of the models significantly predicted ASD severity (measured by the ADOS combined severity score [CSS]). Of these, only full-scale IQ did ($p = 0.02$).

Overall, the data showed a strong effect of large rare genic de novo CNVs on the presence or absence of an ASD diagnosis, but did not support either IQ or ASD severity as useful predictors for probands carrying these risk variants (Figure 3C). We did observe a trend toward more gene-rich de novo CNVs in females (Figure 2) and found females to be less vulnerable to the reduction in IQ associated with rare de novo CNVs.

Genotype-Phenotype Analyses of Probands Carrying 16p11.2 and 7q11.23 CNVs

We next investigated whether individuals with recurrent CNVs at 16p11.2 or 7q11.23 showed distinctive behavioral or cognitive profiles compared with probands who were not carrying rare de novo events. For each proband carrying a de novo CNV at 16p11.2 or 7q11.23, five other probands were selected as controls based on hierarchical matching criteria: first age, then sex, genetic distance, ascertainment site, and whether the sample was from a quartet or trio.

Our primary analysis focused on four variables: full-scale IQ, categorical diagnosis, severity of autism, and body mass index (BMI) (Table 2), with the latter motivated by multiple reports that 16p11.2 deletions contribute to obesity (Bijlsma et al., 2009; Walters et al., 2010). We then pursued a broader exploratory study of additional phenotypic variables, ten of which are presented in Table 2 with the remainder in Table S5.

We found that probands carrying a 16p11.2 or 7q11.23 de novo CNV were indistinguishable from the larger group with regard to IQ, ASD severity, or categorical autism diagnosis (Table 2). However, we did find a relationship between body weight and 16p11.2 deletions and duplications. When we treated copy number as an ordinal variable (one, two, and three copies) and used the matched controls as the diploid sample, BMI diminished as 16p11.2 copy number increased (estimated $\beta = -3.1 \mathrm{kg/m}^2$ for each extra copy, $p = 0.02$).

The extensive phenotypic data available on the SSC sample constitute a great resource for fine-grained analyses of genotype-phenotype relationships. In the current study, the limiting factor with regard to recurrent de novo CNVs was the small sample size, even for 16p11.2 duplications and deletions.

Nonetheless, we undertook an exploratory analysis of a range of phenotypic features and found several that yielded significant p values. While none would survive correction for multiple comparisons, we report them here in the interest of generating hypotheses for future studies (Table 2 and Table S5). For example, individuals with 16p11.2 duplications had higher hyperactivity scores compared to matched control probands, while probands carrying 7q11.23 duplications showed significantly more behavioral problems (Aberrant Behavior Checklist total), but less severe social and communication impairment during ADOS administration.

Analysis of Rare Transmitted CNVs in the SSC

Rare Transmitted Autosomal CNVs Are Equally Represented in Probands and Siblings

Given the very strong association of rare de novo CNVs, we were surprised to find that rare transmitted CNVs were not present in a greater proportion of probands compared to siblings (Figure 5). As prior studies have shown an increased burden of specific subsets of CNVs in neuropsychiatric disorders including autism and schizophrenia, we considered multiple subcategories of rare transmitted events as well, including genic, exonic, brain-expressed, and ASD-related, and did not find a statistically significant result that survived correction for multiple comparisons (Figure 5).

These findings were inconsistent with a recent rigorous, large-scale CNV study undertaken by the Autism Genome Project (AGP) (Pinto et al., 2010). Their sample included both simplex and multiplex families and identified a significantly higher burden of genic and ASD-related CNVs in cases versus unrelated controls. However, there was no differentiation between transmitted and de novo events in this analysis. We reanalyzed our data by using the identical criteria detailed in their article and found nearly identical results (Table S6). However, when we again restricted our evaluation to only rare transmitted CNVs by removing all confirmed de novo events there was no significant difference remaining between probands and siblings, suggesting that the excess burden in the SSC sample was entirely driven by rare de novo events.

We pursued this analysis further because of strong evidence that specific rare transmitted CNVs carry ASD risks as well as recent hypotheses regarding the centrality of maternal transmission of rare CNVs to male probands (Zhao et al., 2007). Consequently, we investigated whether mothers were more likely than fathers to transmit a rare CNV to an affected offspring. We also asked whether there was a greater number of maternally transmitted CNVs in probands versus their unaffected siblings. Neither analysis showed a significant result after correction for multiple comparisons despite considering combinations of the following variables: deletions, duplications, size, exonic, brain-expressed, and ASD-related. In addition, based on the possibility that risk might be confined to only the rarest transmitted events, presumably under the strongest purifying selection, we evaluated "singleton" CNVs, i.e., those observed in only one parent and transmitted to only one proband or sibling. In this case, we found a modest, nonsignificant excess of maternally transmitted CNVs in probands: 344 maternal autosomal singletons were transmitted to probands versus 303 transmissions to siblings (OR = 1.14; p = 0.059, one-sided; p = 0.12, two-sided). For fathers, there was no similar trend (OR = 1.03; p = 0.37 one-sided).

Rare Transmitted X-Linked CNVs Are Equally Represented in Probands and Siblings

We asked similar questions regarding transmission of rare X-linked CNVs from mothers to male probands and obtained similar results. In a group of 353 male probands and 353 matched male siblings we found, contrary to expectation, that more siblings carried maternally transmitted rare X chromosome CNVs than probands (14% probands versus 18% siblings, OR = 0.76; p = 0.11), though this difference was not significant. The result did not change when we evaluated the various subcategories of rare X-linked CNVs including exonic, deletions, duplications, size, brain-expressed, or ASD-associated.

Rare Transmitted CNVs Show Greater Biological Coherence in Probands versus Siblings

We next considered whether the absence of association of rare transmitted CNVs might be a consequence of an inability to differentiate functional from neutral variants. We looked to pathway analyses to help address this question, reasoning that if the specific genic content of CNVs contributed to disease risk, we would find a greater enrichment of biological pathways in probands compared to their unaffected siblings.

We used two gene ontology and pathway analysis tools, MetaCore from GeneGo, Inc. and DAVID (Dennis et al., 2003; Huang et al., 2009), to analyze 1516 genes within CNVs exclusive to probands and 1357 genes exclusive to siblings. The total number and size of rare transmitted CNVs used to determine these gene sets were highly similar in probands and siblings (Figure 5). GeneGo networks identified 22 pathways showing significant enrichment in probands versus only four enriched pathways among siblings. This difference was significant based on 100 permutations of the data set (p = 0.04). DAVID yielded consistent results with 59 pathways enriched in probands and 19 in siblings (p = 0.01, permutation analysis) (Figure 6).

For the present study, we elected to restrict our evaluation of pathways to the general question described here. A manuscript that is in preparation describes a more extensive analysis, focusing on both structural and gene expression data from the SSC.

Transmitted Autosomal and X Chromosome CNVs Overlap with Previously Reported ASD Loci

We next examined all rare CNVs in the SSC in light of previously reported findings, comparing our data to the list of ASD regions included in the recent AGP analysis (Pinto et al., 2010). We also considered genes implicated by recent common variant studies, including *SEMA5A* (Weiss et al., 2009), *MACROD2* (Anney et al., 2010), *CDH9* and *CDH10* (Wang et al., 2009), the *MET* oncogene (Campbell et al., 2006), *EN2* (Gharani et al., 2004), as well as selected schizophrenia loci (International Schizophrenia Consortium, 2008; McCarthy et al., 2009; Millar et al., 2000; Stefansson et al., 2008; Walsh et al., 2008; Xu et al., 2008) (Table 3). We identified multiple regions in which rare transmitted and/or rare de novo events corresponded to previously characterized loci in both ASD and schizophrenia.

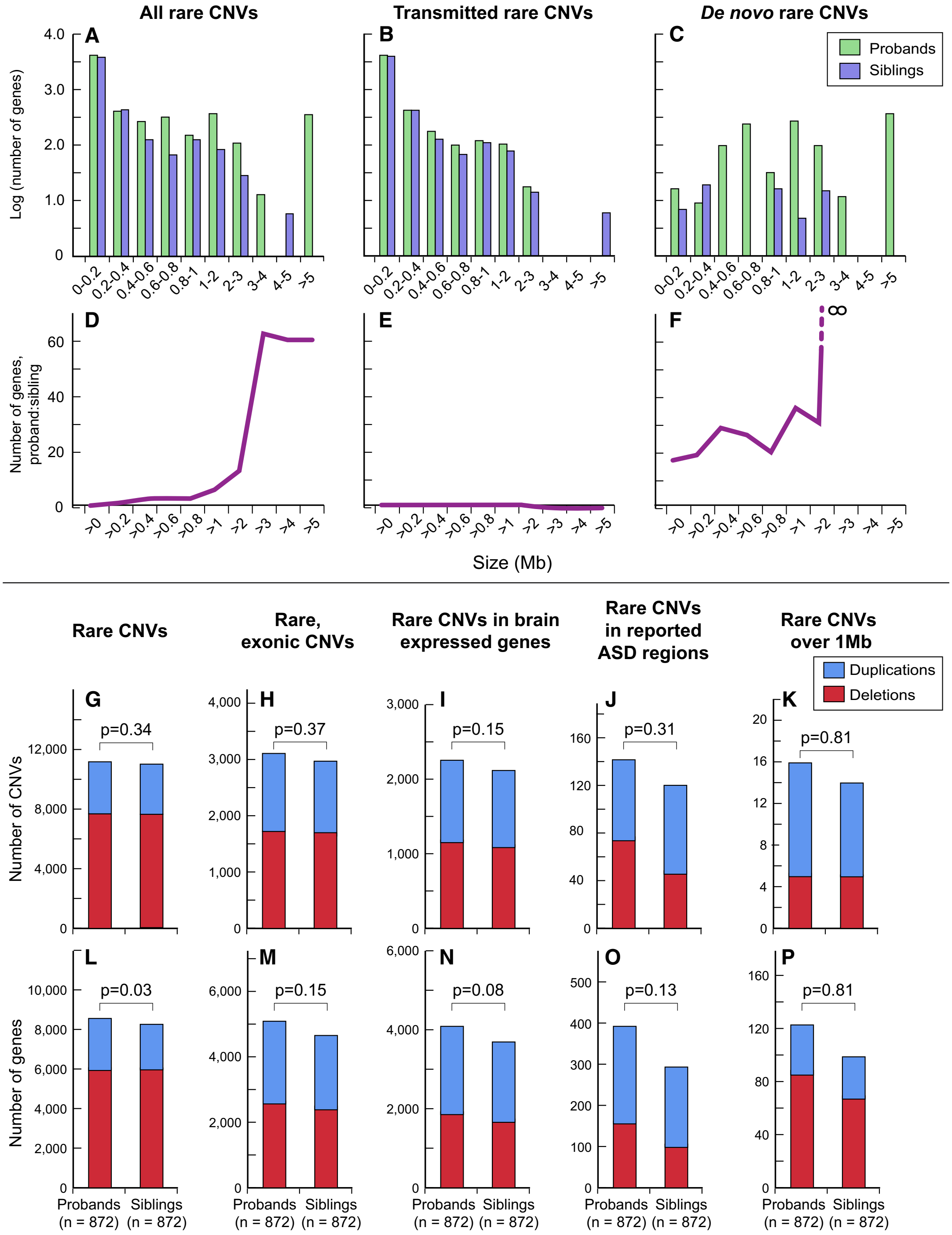


Figure 5. Burden of Rare CNVs in 872 Probands and 872 Matched Siblings

(A) Bar graph showing the log(10) number of genes present in all rare CNVs binned by size (in Mb), with probands shown in green and siblings in purple.

(B) The data from (A) with confirmed de novo events excluded, leaving only CNVs transmitted from a parent to offspring.

(C) Only confirmed de novo events are shown.

(D–F) The ratio (y axis) of number of genes in probands versus siblings for specific size thresholds (x axis). Shown are (D) all rare CNVs (transmitted and de novo); (E) transmitted events; and (F) de novo events only.

(G–K) The total number of transmitted deletions (red) and duplications (blue) for probands and siblings for varying categories of CNV (shown above the graphs). Definitions are in Supplemental Experimental Procedures. p values (noted above the bars) are calculated by using the sign test and are not corrected for multiple comparisons.

(L–P) As in (G–K), with number of RefSeq genes within the CNVs (y axis). p values (noted above the bars) are estimated with a two-tailed paired t test and are not corrected for ∼3000 comparisons.

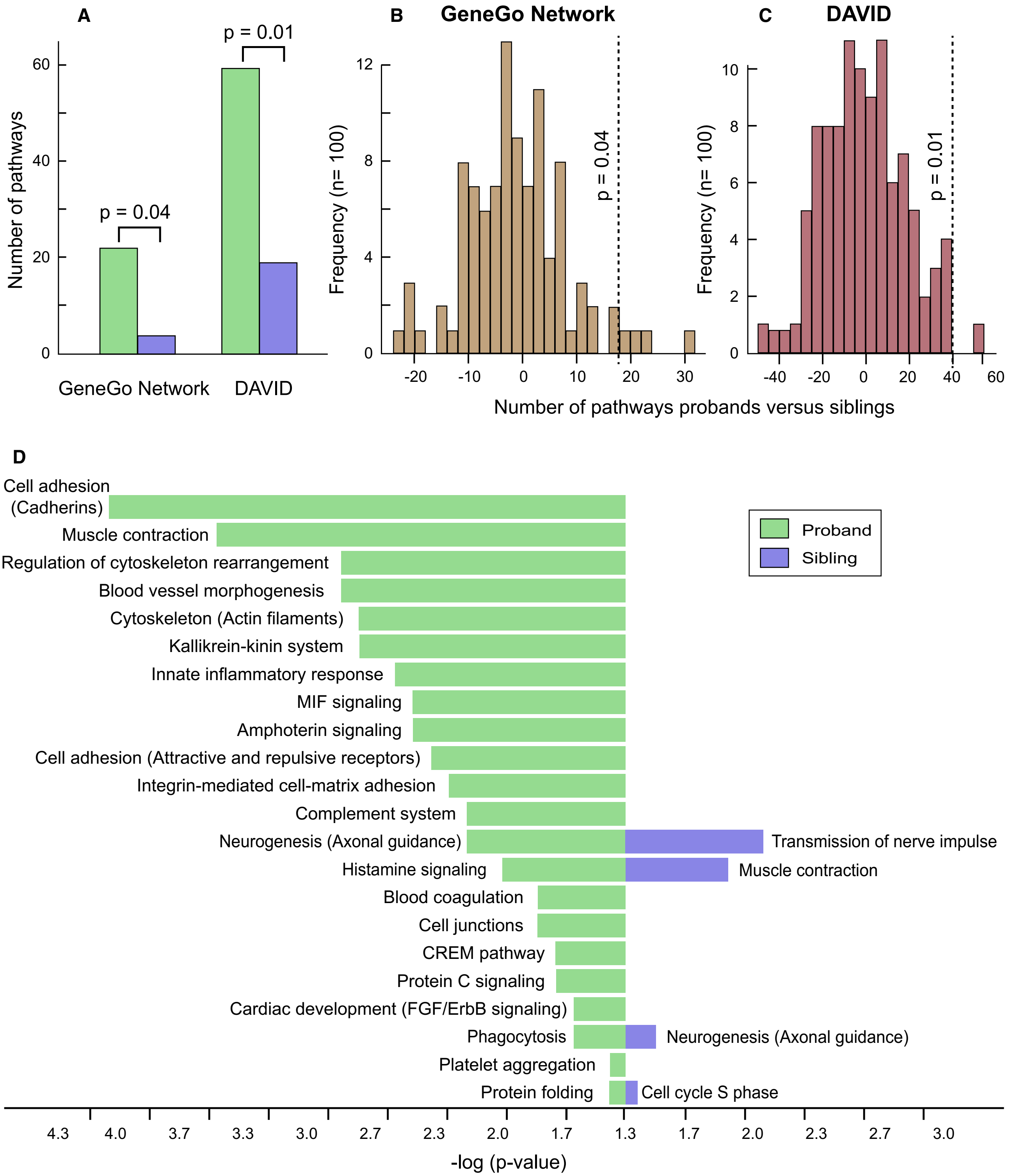


Figure 6. Pathway Analysis of Genes Mapping within Transmitted Rare CNVs

(A) The number of pathways with a corrected p value ≤ 0.05 identified in probands (green) and siblings (purple) by the programs MetaCore (GeneGo networks) and DAVID (level 4 terms). The input consisted of 1516 RefSeq genes found only in transmitted rare CNVs in probands and the 1357 RefSeq genes found only in transmitted rare CNVs in siblings; p values are from (B) and (C).

(B) Permutation analysis to assess significance of the difference between probands and siblings. The 2873 genes identified in probands or siblings were divided randomly between probands and siblings in the same initial proportions. The lists were submitted to GeneGo networks and the difference between the number of

Table 3. CNVs in Genes and Regions Previously Associated with ASD

		All (de novo)[b]		Deletions (de novo)		Duplications (de novo)	
Gene/Region[a]	Location (NCBI 36/hg18)	Proband	Sibling	Proband	Sibling	Proband	Sibling
NRXN1	chr2:50,000,991–51,113,178	3	1	3	1	0	0
CDH10	chr5:24,522,967–24,680,668	0	1	0	1	0	0
MET	chr7:116,099,695–116,225,676	1	0	0	0	1	0
VPS13B	chr8:100,094,669–100,958,984	1	0	1	0	0	0
CACNA1C	chr12:2,032,676–2,677,376	1	1	0	0	1	1
UBE3A	chr15:23,133,488–23,235,221	1 (1)	0	0	0	1 (1)	0
NF1	chr17:26,446,120–26,728,821	1	0	1	0	0	0
MACROD2	chr20:13,924,146–15,981,841	0	1	0	1	0	0
TBX1	chr22:18,124,225–18,151,112	1 (1)	0	1 (1)	0	0	0
ADSL	chr22:39,072,449–39,092,521	2 (1)	2 (0)	1 (1)	1 (0)	1 (0)	1 (0)
NLGN4X	chrX:5,818,082–6,156,706	1 M	0	0	0	1 M	0
DMD	chrX:31,047,265–33,267,647	1 F	1 M, 5F	1 F	1 M, 5 F	0	0
NLGN3	chrX:70,281,435–70,307,776	1 M (1 M)	0	1 M (1 M)	0	0	0
ATRX	chrX:76,647,011–76,928,375	0	1F	0	0	0	1F
FMR1	chrX:146,801,200–146,840,333	1 F	1F	1F	1F	0	0
RPL10	chrX:153,279,911–153,285,232	1 M	0	0	0	1 M	0
1q21.1	chr1:144,022,893–147,496,468	3 (2)	0	0	0	3 (2)	0
3q29	chr3:197,244,288–198,830,238	1 (1)	0	1 (1)	0	0	0
4p16.3	chr4:1–2,043,468	1 (1)	0	0	0	1 (1)	0
7q11.23	chr7:71,970,679–74,254,837	4 (4)	0	0	0	4 (4)	0
15q11.2-13.1	chr15:20,768,955–26,230,781	1 (1)	0	0	0	1 (1)	0
15q13.2-13.3	chr15:28,698,632–30,234,007	3 (2)	0	2 (1)	0	1 (1)	0
16p13.11	chr16:15,421,876–16,200,195	3 (1)	5 (2)	1 (0)	1 (1)	2 (1)	4 (1)
16p11.2	chr16:29,474,810–30,235,818	14 (11)	0	8 (7)	0	6 (4)	0
17q12	chr17:31,893,783–33,277,865	2 (1)	0	2 (1)	0	0	0
22q11.21 typical	chr22:17,412,646–19,797,314	1 (1)	0	1 (1)	0	0	0
22q11.21 distal	chr22:22,028,923–23,368,015	1	0	0	0	1	0

[a] For genes a CNV was included if it overlapped ≥1 exon; for regions CNVs spanning ≥50% of the region and ≥1 exon are included.
[b] De novo CNV count is in parentheses; for chromosome X sex is indicated by M for male and F for female.

Rare Transmitted CNVs Do Not Show Genome-wide Association in the SSC

Finally, we looked for evidence of association for all CNVs in the SSC sample, common or rare, transmitted or de novo, evaluating all high-confidence autosomal CNVs together with all confirmed de novo CNVs. In this instance, we did not use a frequency cutoff to define a set of rare transmitted events. A total of 3667 recurrent regions were identified; 6 showed relative enrichment in probands and 5 showed relative enrichment in siblings. No result reached significance after correction for multiple comparisons (Table S7 and Figure 7C). The region showing the greatest difference in probands compared to siblings was 16p11.2 (p = 0.001).

Expanded Analysis of Rare De Novo CNVs across Multiple ASD Samples

An Analysis of De Novo CNVs in 3816 Probands from Genome-wide Studies of Idiopathic ASD Supports Association of Six Genomic Intervals

Our approach to assessing the genome-wide significance of rare recurrent de novo events provides for a statistical evaluation of CNVs observed in cases without requiring additional matched control samples. Consequently, we were able to conduct a cumulative analysis across multiple studies in search of additional associated ASD loci. We included four other large-scale ASD CNV studies (Itsara et al., 2010; Marshall et al., 2008; Pinto

pathways in probands and siblings was recorded. This process was performed 100 times and the image shows the frequency of the results. Only four events showed a difference ≥18 (the difference seen in [A], vertical dashed line), yielding a p value of 0.04.

(C) Permutation analysis to calculate the significance value with DAVID (level 4 terms) by using the same methods as in (B). A single result was ≥40 (the difference seen in (A), vertical dashed line), giving a p value of 0.01.

(D) All pathways with a corrected p value ≤ 0.05 identified by GeneGo networks for probands (green) and siblings (purple). The length of the bar represents the significance value on a logarithmic scale.

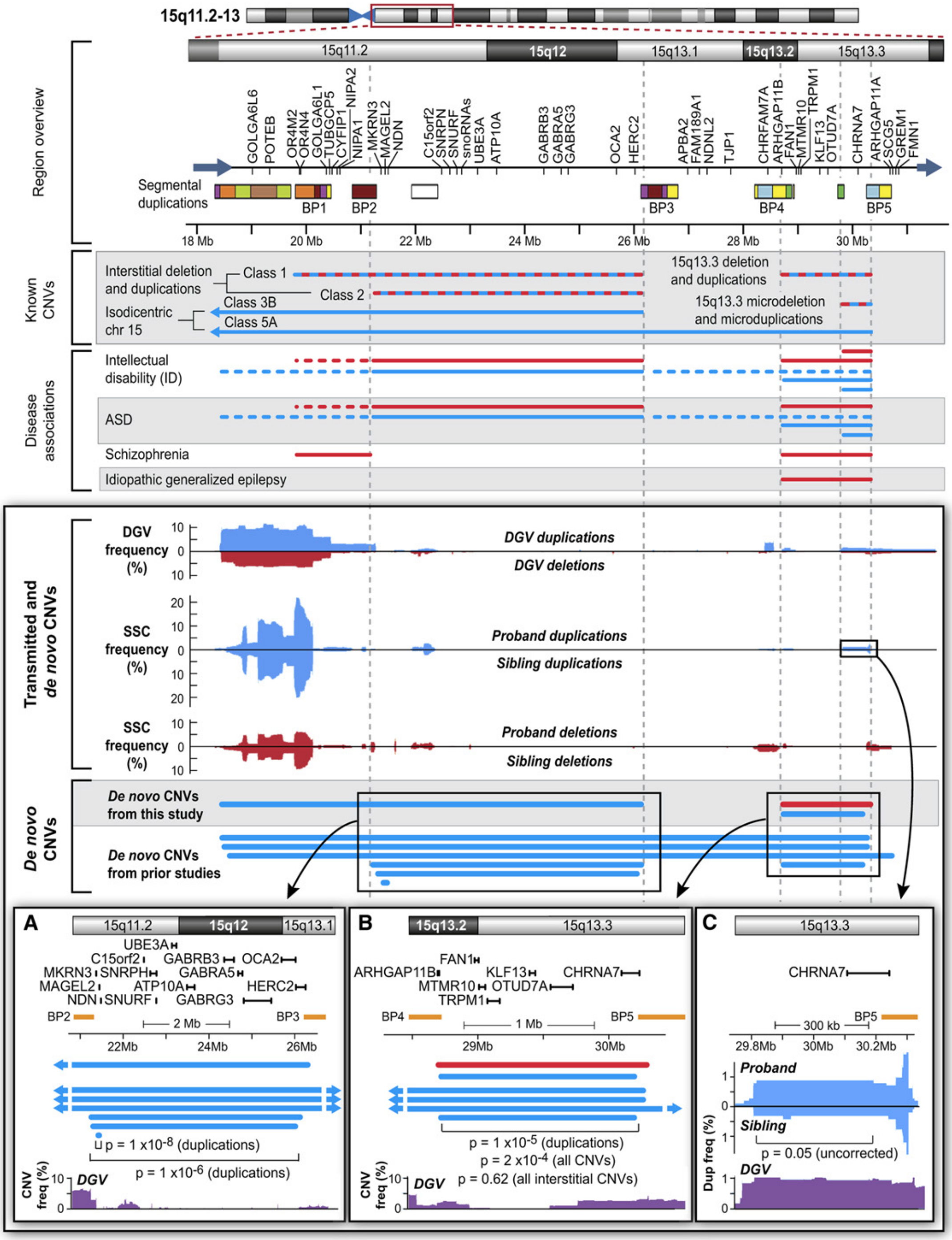


Figure 7. De Novo and Transmitted CNVs in 15q11.2-13

A 13Mb region is identified by the red box on the ideogram at the top.

(Region overview) The RefSeq genes present within the interval and multiple segmental duplications are identified (the colors identify regions of homology; Makoff and Flomen, 2007). Five of these segmental duplications are commonly referred to as BP1-BP5.

(Known CNVs) Duplications (blue) and deletions (red) identified that have been reported in the literature; the alternating red and blue colors denote both deletions and duplications.

(Disease associations) Regions with reported associations to four developmental and neuropsychiatric conditions (Supplemental Experimental Procedures) are identified. Of note, BP2-BP3 deletions lead to Prader-Willi or Angelman syndromes.

(Transmitted and de novo CNVs) The frequency of duplications (blue) and deletions (red) in the DGV and SSC populations is indicated. While CNVs overlying the segmental duplications are common, CNVs between the breakpoints are generally rare.

et al., 2010; Sebat et al., 2007) meeting four criteria: standardized diagnosis, genome-wide detection, confirmed de novo structural variations, and sufficient information to permit the identification of duplicate samples.

These data sets cataloged 219 confirmed rare de novo CNVs from a total of 3816 individuals (Table S1). We found six regions that exceeded the threshold for significance (Experimental Procedures). Given prior evidence and our own data suggesting that reciprocal deletions and duplications at the same locus may both contribute to the ASD phenotype, we evaluated significance for combined events at every interval and calculated probabilities for deletions and duplications separately (Table 4 and Figure S3).

The most frequent recurrent de novo CNV identified across all studies was 16p11.2 with 19 identified probands (14 deletions, 5 duplications) showing extremely strong evidence for association with ASD (2×10^{-55} combined, 5×10^{-29} for deletions, and 2×10^{-5} for duplications). The proximal long arm of chromosome 15 showed two contiguous intervals; the first corresponds to the region 15q11.2-13.1 or BP2-BP3 (seven duplications, 4×10^{-9}) (Figure 7A), long cited as the most common cytogenetic abnormality identified in idiopathic ASD (Cook et al., 1997). We also found evidence of association for the interval mapping to 15q13.2-13.3 or BP4-BP5 (five duplications and one deletion; 1×10^{-4} combined, 2×10^{-5} for duplications) (Figure 7B). Rare deletions and duplications in this region have previously been associated with intellectual disability and ASD and deletions have been associated with schizophrenia and epilepsy (Figure 7). It is important to note, however, that considering only events restricted to 15q13.2-13.3 (i.e., removing three overlapping isodicentric chromosome 15 events) resulted in a loss of statistical significance (0.53 combined, 0.88 for duplications). This suggests either that the result is an incidental finding because of the proximity to a true ASD risk locus or, alternatively, that the smaller 15q13.2-13.3 CNVs might point to a minimum region of overlap mapping to one or more ASD-related genes.

Recurrent de novo CNVs exceeding the significance threshold in the combined sample were also present at 7q11.23 (four duplications, 0.003), in the 22q11.2 region (three deletions and two duplications, 0.002 combined; 0.11 for deletions; 0.88 for duplications), and at the locus coding for the gene *NRXN1*. For *NRXN1* there were five de novo events: one intronic deletion, three exonic deletions, and one exonic duplication (0.002 combined, 0.004 for deletions).

Finally, we used the observed number and distribution of de novo CNVs in the combined proband data set to estimate the likely number of CNV regions contributing to ASD. From the total of 219 confirmed de novo events, we derived an estimate of 234 distinct genomic regions contributing to large ASD-related de novo structural variations (Experimental Procedures).

DISCUSSION

Our results highlight the importance of rare CNVs for simplex ASD. We confirm an overrepresentation of rare de novo events in probands versus siblings with an odds ratio of 3.5 for all variants, 4.0 for rare de novo genic variants, and 5.6 for de novo CNVs encompassing more than one gene. We find very strong evidence for the association of duplications at 7q11.23 by using a rigorous method for assessing genome-wide significance. Moreover, we identify four additional rare recurrent de novo events found only in probands. Two of these, at 1q21 and 15q13.2-13.3, have been previously implicated in neurodevelopmental disorders, including ASD, while, to our knowledge those at 16p13.2 (*USP7* and *C16orf72*) and the *CDH13* locus have not. Each of these four regions also contain rare transmitted CNVs that are restricted to probands. Finally, we find compelling evidence confirming the association of both 16p11.2 duplications and deletions.

It is striking that while we replicate findings of elevated rates of rare de novo CNVs in simplex families (5.8% of probands versus 1.7% in siblings), the percentage of the cohort carrying these events is the same magnitude as that seen previously. This is despite an intensive focus on the ascertainment of simplex quartets and a 10-fold increase in probe density since the earliest CNV studies of ASD. We believe these results are best explained by the particular contribution of large genic de novo variants based on our analysis of gene number, CNV size, and affected status (Figure 3) and by the observation of consistent results across studies despite steadily increasing detection resolution.

While it may not seem surprising that large de novo events carry the greatest risk for developmental disorders, it is interesting to note that we do not find evidence that ASD diagnosis or severity is mediated by intellectual disability (ID). It has been argued that ASD in the presence of ID may reflect an epiphenomenon, in which a nonspecific impairment of brain functioning unmasks and/or exacerbates limitations in an individual's capacity for social reciprocity (Skuse, 2007). It has also been widely held that the detection of large de novo CNVs will be enhanced by the ascertainment of ASD samples with greater intellectual disability. Our data show that large de novo CNVs confer substantial risk for ASD in the SSC, but they are only modestly correlated with lower IQ and largely independent of ASD severity.

These data suggest that our study has identified bona fide high-risk variants for autism spectrum disorders. They also point

(De novo CNVs) Confirmed de novo CNVs in single individuals identified in this study and prior ASD studies are shown (Itsara et al., 2010; Marshall et al., 2008; Pinto et al., 2010; Sebat et al., 2007).

(A) An enlargement of BP2-3 showing the relationship of de novo CNVs, genes, and common regions in the DGV. A small atypical duplication includes the genes MAGEL2, MKRN3, and NDN (Itsara et al., 2010).

(B) An enlargement of BP4-BP5 showing similar data and methods as in (A). Removing the three Class 5A isodicentric chr15 events results in a nonsignificant p value ($p = 0.62$).

(C) An enlargement of the *CHRNA7* region showing enrichment of duplications in probands ($n = 10$) versus siblings ($n = 3$). The p value is $p = 0.05$ (Fisher's exact test), uncorrected for 3,667 comparisons; the rate of duplications in the DGV is similar to that seen in probands (Table S7).

Table 4. Two or More Recurrent De Novo Regions across This and Other Studies

Type	Band	Location (NCBI 36/hg18)	Size (kb)	Recurrence (del/dup)	Frequency (n = 3,816)	p value (C = 232)[a]	Studies[b]	Genes[c] (RefSeq)
Deletions								
	2p16.3	chr2:51,002,576–51,157,742	155	2	0.05%	0.94	3	*NRXN*[d]
	2q24.2	chr2:162,212,720–162,311,972	99	2	0.05%	0.94	5	*SLC4A10* (intronic)
	2q37.3	chr2: 238,217,066–242,701,103	4,484	2	0.05%	0.94	5	41
	3p14.1	chr3:65,674,445–65,725,692	51	2	0.05%	0.94	2,4	*MAGI1* (intronic)
	3p14.1	chr3:67,223,272–70,633,200	3,410	2	0.05%	0.94	1,4	10
	5p15.2	chr5:11,403,359–11,491,117	87	3	0.08%	0.11	1,4	*CTNND2*
	7q31.1-31.31	chr7:113,335,000–119,223,887	5,889	2	0.05%	0.94	4	20
	7q36.2	chr7:153,380,710–154,316,928	936	2	0.05%	0.94	3,4	*DPP6*
	9p24.3	chr9:98,998–334,508	235	2	0.05%	0.94	3	*C9orf66,CBWD1, DOCK8,FOXD4*
	11q13.3	chr11:70,154,458–70,187,872	33	2	0.05%	0.94	3	*SHANK2*
	14q32.12	chr14:92,476,815–92,496,373	19	2	0.05%	0.94	2	*ITPK1*
	15q23-24.1	chr15:69,601,300–71,944,199	2,343	2	0.05%	0.94	1,4	22
	16p11.2	chr16:29,578,715–30,001,681	422	14	0.37%	**5×10^{-29}**	1,2,3,4,5	26
	16q23.3	chr16:81,796,275–81,830,296	34	2	0.05%	0.94	1	*CDH13*
	16q23.3	chr16:82,557,318–82,683,859	126	2	0.05%	0.94	1,2	*MBTPS1,NECAB2, OSGIN1, SLC38A8*
	18q22.1	chr18:64,812,093–6,484,6196	34	2	0.05%	0.94	2,4	*CCDC102B*
	20p12.1	chr20:14,616,243–14,751,454	135	2	0.05%	0.94	1,3	*MACROD2* (intronic)
	22q11.21	chr22:17,257,787–19,786,200	2,528	3	0.08%	0.11	1,3,4	56
	22q13.33	chr22:49,243,247–49,465,883	222	3	0.08%	0.11	4,5	16
Duplications								
	1q21.1	chr1:144,838,175–146,324,832	1,487	2	0.05%	0.88	1	14
	2p25.3	chr2:143,279–196,704	53	2	0.05%	0.88	2	0
	3q21.2	chr3:125,966,642–127,254,388	1,288	2	0.05%	0.88	2	11
	7q11.23	chr7:72,411,506–73,782,113	1,371	4	0.09%	**0.003**	1	22
	8p23.3	chr8:710,491–1,501,580	791	2	0.05%	0.88	3,4	*DLGAP2*
	10q11.23-21.1	chr10:52,699,516–54,408,816	1,709	2	0.05%	0.88	1,2,5	*CSTF2T,DKK1, MBL2,PRKG1*
	12q24.31	chr12:120,628,928–120,862,589	233	2	0.05%	0.88	2,4	7
	15q11.2	chr15:21,343,866–21,505,342	161	7	0.18%	**4×10^{-9}**	1,2,3,4,5	*MAGEL2,MKRN3,NDN*
	15q11.2-13.1	chr15:21,240,037–26,095,621	4,856	6	0.16%	**4×10^{-4}**	1,2,3,4,5	12
	15q13.2-13.3	chr15:28,723,577–30,231,488	1,508	5[e]	0.13%	**2×10^{-5}**	1,2,4,5	*CHRNA7,KLF13,MTMR10, MTMR15,OTUD7A,TRPM1*
	16p13.2	chr16:8,828,382–9,147,487	319	2	0.05%	0.88	1	*PMM2,CARHSP1, USP7,C16orf72*
	16p11.2	chr16:29,563,365–30,085,308	521	5	0.13%	**2×10^{-5}**	1,3,4	26
	20q13.33	chr20:60,949,339–61,220,552	271	2	0.05%	0.88	2,4	7
Combined[f]								
	22q11.21	chr22:17,265,500–19,791,274	2,526	2	0.05%	0.88	2,4	56
	1q21.1	chr1:145,013,719–146,293,282	1,280	3 (1/2)	0.08%	0.53	1,2	14
	2p16.3	chr2:50,539,877–50,677,835	138	2 (1/1)	0.05%	1.00	3	*NRXN*[d]
	7q31.1	chr7:108,242,570–108,393,666	151	2 (1/1)	0.05%	1.00	2,4	*C7orf66*
	7q31.1	chr7:111,065,681–111,454,179	388	2 (1/1)	0.05%	1.00	2,4	*DOCK4*
	9p24.3	chr9:175,632–334,508	159	3 (2/1)	0.08%	0.53	1,3	*C9orf66,DOCK8*
	15q13.2-13.3	chr15:28,723,577–30,231,488	1,508	6 (1/5)[e]	0.16%	**1×10^{-4}**	1,2,4,5	*CHRNA7,KLF13,MTMR10, MTMR15,OTUD7A,TRPM1*
	16p11.2	chr16:29,578,715–30,001,681	521	19 (14/5)	0.50%	**2×10^{-55}**	1,2,3,4,5	26

(*Continued on next page*)

Table 4. *Continued*

Type	Band	Location (NCBI 36/hg18)	Size (kb)	Recurrence (del/dup)	Frequency (n = 3,816)	p value (C = 232)[a]	Studies[b]	Genes[c] (RefSeq)
	16q22.3	chr16:69,987,425–70,647,241	660	2 (1/1)	0.05%	1.00	1,2	13
	20q13.33	chr20:61,056,624–61,076,763	20	3 (1/2)	0.08%	0.53	2,4	*SLC17A9*
	22q11.21	chr22:17,265,500–19,786,200	2,521	5 (3/2)	0.13%	**0.002**	1,2,3,4	56

[a] p values are calculated as described in the Experimental Procedures; values less than p = 0.05 are shown in bold.

[b] 1 = this study; 2 = Itsara et al., 2010; 3 = Pinto et al., 2010; 4 = Marshall et al., 2008; 5 = Sebat et al., 2007.

[c] Counts are given for CNVs with >6 RefSeq genes mapping to the interval. A complete listing of genes is in Table S4. All genes shown represent exonic overlap unless otherwise indicated.

[d] While only two de novo CNVs overlap within *NRXN1*, there are five de novo events overlapping a section of the gene: one intronic deletion, three exonic deletions, and one exonic duplication (p = 0.004 combined, p = 0.007 for deletions).

[e] Three of the duplications contributing to 15q13.2-13.3 are isodicentric chr15 events; because there is a long-standing association with ASD and isodicentric chr15, this region is also considered without these events. For interstitial CNVs alone there are two duplications and one deletion (p = 0.62 combined; p = 0.92 duplications) (Figure 7).

[f] Regions are only listed in the combined category if there is a combination of deletions and duplications resulting in a different p value when the two types of CNVs are considered together.

to a more complex relationship of IQ and large de novo events than has often been supposed: for example, the relatively high rates of 16p11.2 and 7q11.23 CNVs and low rates of 15q11.2-13.1 duplications seen in this study compared to others may reflect the presence of particular subpopulations of rare risk CNVs that are, in fact, more readily ascertained in cohorts with higher mean IQ.

The results further show that the risk associated with large de novo events is related to their greater genic content, even after controlling for larger size. This observation is consistent with two countervailing hypotheses: first, that the greater gene number is a surrogate for the increased chance of disrupting one particular gene or regulatory region because of the involvement of a larger segment of the genome; or second, that it is the contribution of multiple genes and/or regulatory regions simultaneously within these CNVs that increases risk.

Our data do not allow us to resolve this issue. We suspect that if many deletions or duplications encompassing small numbers of genes were as highly penetrant as multigenic events, we would have begun to show more evidence for this either in the form of an overall increased burden for smaller de novo variations and/or an association of specific de novo events. However, it is important to note that despite having higher resolution than some prior studies, we still have a clear ascertainment bias for larger CNVs. It is likely that a combination of high-throughput sequencing, larger patient cohorts, and increasingly sophisticated approaches to evaluating combinations of risk variants will begin to shed light on this issue for both sequence and structural variation.

Our findings with regard to recurrent de novo events in the SSC sample identify six putative ASD loci and two of these, 7q11.23 and 16p11.2, show clear evidence for genome-wide association. Moreover, our simulation analysis suggests that the most likely outcome of the ongoing phase 2 SSC study will be the confirmation of two to three of the remaining four intervals, namely 1q21.1, 15q13.2-13.3, 16p13.2, and 16q23.3 (*CDH13*).

The association of recurrent duplications at 7q11.23 points to particularly promising opportunities to illuminate the molecular mechanisms of social development. Duplications in this interval have previously been described in developmental disorders, including ASD (Berg et al., 2007; Van der Aa et al., 2009), though these have been restricted to case reports or series, with the attendant difficulties in controlling for ascertainment bias. The identification of clear association of duplications in this controlled study of ASD is striking, given that the reciprocal deletion results in a developmental syndrome characterized in part by an empathic, gregarious, and highly social personality (Pober, 2010). Moreover, several lines of evidence, including atypical deletions (Antonell et al., 2010), mouse models (Fujiwara et al., 2006; Hoogenraad et al., 2002; Meng et al., 2002; Sakurai et al., 2011), and gene expression X phenotype studies (Gao et al., 2010; Korenberg et al., 2000) have already identified *CAP-GLY domain containing linker protein 2* (*CLIP2*), *LIM domain kinase 1* (*LIMK1*), *General transcription factor II, i* (*GTF2i*), and *Syntaxin 1A* (*STX1A*) as the leading candidates among the 22 genes within the region for involvement in the cognitive and social phenotypes. The characterization of this single interval in which opposite changes in copy number contribute to contrasting social phenotypes promises to set the stage for a range of intiguing studies of the role gene dosage in this region plays in the genesis and maintenance of social behavior.

The strong replication of findings at 16p11.2 likewise highlights emerging opportunities for translational neuroscience. First, the region is sufficiently circumscribed to investigate by using molecular biological and model systems approaches. Second, though we cannot quantify an odds ratio from our data, given the absence of events in siblings, there is clear evidence from this and prior studies (McCarthy et al., 2009) that 16p11.2 CNVs carry much larger effects than common variants contributing to complex common disorders. Third, the 1% allele frequency observed in ASD cohorts promises an ascertainable cohort of sufficient size to support prospective studies of natural history, neuroimaging, and treatment response as, for example, in the recently launched Simons Variation in Individuals Project (https://sfari.org/simons-vip). Given the reported associations of widely varying outcomes for individuals with either deletions or duplications at 16p11.2, these studies offer an important avenue to address the means by which a single locus may lead to a wide range of psychiatric and developmental outcomes that have previously been conceptualized as distinct.

Multiple lines of evidence suggest that four other recurrent de novo CNVs (1q21.1, 15q13.2-13.3, 16p13.2, and 16q23.3) as well as three intervals in which a single de novo event overlaps with rare transmitted CNVs (2p15, 6p11.2, and 17q12) are likely to be true positives. For example, the 2p15 and 17q12 regions have already been implicated in ASD (Liang et al., 2009; Moreno-De-Luca et al., 2010). Similarly, rare 1q21.1 and 15q13.2-13.3 CNVs have been identified in developmental and neuropsychiatric syndromes, with deletions found in ASD (Miller et al., 2009; Shen et al., 2010), schizophrenia (International Schizophrenia Consortium, 2008; Stefansson et al., 2008), and idiopathic epilepsy (Helbig et al., 2009), and recurrent duplications reported here. To our knowledge, *CDH13* (16q23.3) has not previously been noted to be an ASD risk variant, however the protein family has been implicated in pathogenesis through CNV studies (Glessner et al., 2009), homozygosity mapping (Morrow et al., 2008), and common variant findings (Wang et al., 2009). The 16p13.2 region contains four genes, the most notable of which are *C16orf72*, coding for a protein of unknown function, recently identified in a schizophrenia CNV study (Levinson et al., 2011), and *Ubiquitin Specific Peptidase 7* (*USP7*), which has been shown to have a role in oxidative stress response, histone modification, and regulation of chromatin remodeling (Khoronenkova et al., 2011). Neither gene has been specifically highlighted with regard to ASD, however CNVs involving genes in the ubiquitin pathway have been previously associated with risk (Glessner et al., 2009).

It is somewhat surprising that the family-based design employed here played a central role in the identification and confirmation of rare variant association. The prevailing practice in genome-wide association studies of common variants has been to rely on unrelated case-control designs, given the relative ease of generating very large samples. It is notable that the statistical power afforded by the low probability of observing multiple recurrent rare de novo events by chance more than compensated for the relatively small cohort (compared to those found in contemporary GWAS). The results at 16p11.2 are a striking example: based on a standard case-control comparison, the most statistically significant finding involved 14 events in probands and 0 in siblings ($p = 0.001$, Fisher's exact test) and did not provide evidence sufficient to withstand correction for multiple comparisons. However, the analysis of recurrent de novo events convincingly established association surpassing a genome-wide significance threshold ($p = 6 \times 10^{-23}$).

It is certain that the SSC sample-ascertainment process enhanced certain findings and attenuated others. Restricting the comparison group to siblings limited power to identify association of specific rare recurrent transmitted events; our assessment of significance for de novo CNVs was based on conservative assumptions and may have excluded true risk loci; the filtering for rare de novo CNVs and the small sample size curtailed the assessment of multihit hypotheses; the generally older parental age may have obscured the relationship between age and de novo variation (Figure S3); and, as noted, limited detection accuracy below 20 probes hindered the assessment of small de novo structural variations.

However, despite these limitations, the manner in which the design mitigated important confounds and preserved sufficient power to detect association of recurrent de novo events yielded clear benefits, unambiguously replicating prior findings and identifying additional risk loci. Moreover, this report considers less than half of the SSC: phase 2 of this study is under way, as is high-throughput sequencing of the collection, also focusing on de novo events. Together these endeavors promise to further illuminate the genomic architecture of simplex autism and to provide additional critical points of traction in elaborating the molecular mechanisms and developmental neurobiology underlying ASD.

EXPERIMENTAL PROCEDURES

Genotyping

All members of each family were analyzed on the same array version: either the Illumina IMv1 (334 families) or Illumina IMv3 Duo (840 families) Bead array. These share 1,040,853 probes in common (representing 97% of probes on the IMv1 and 87% of probes on the IMv3). Of the 872 quartet families, 824 (94.5%) had all members hybridized and scanned simultaneously on the Illumina iScan in an effort to minimize batch effects and technical variation.

Identity Quality Control

Genotyped samples were analyzed by using PLINK (Purcell et al., 2007) to identify incorrect sex, Mendelian inconsistencies, and cryptic relatedness by assessing inheritance by descent; 11 families were removed as a result.

CNV Detection

CNV detection was performed by using three algorithms: (1) PennCNV Revision 220, (2) QuantiSNP v1.1, and (3) GNOSIS. PennCNV and QuantiSNP are based on the hidden Markov model. GNOSIS uses a continuous distribution function to fit the intensity values from the HapMap data and determine thresholds for significant points in the tails of the distribution that are used to detect copy-number changes. Analysis and merging of CNV predictions was performed with CNVision (www.CNVision.org), an in-house script.

CNV Quality Control

Specific genotyping and CNV parameters are detailed in the Supplemental Experimental Procedures. Five percent of the samples failed and were rerun; 39 families were removed because of repeated failures.

Criteria for Rare CNVs

A CNV was classified as rare if ≤50% of its length overlapped regions present at >1% frequency in the DGV of March 2010.

CNV Burden

Burden analyses were performed on the matched set of 872 probands and siblings. Typically, three outcomes were assessed: proportion of individuals with ≥1 CNV matching the criteria (p value calculated with Fisher's exact test); number of CNVs matching the criteria (p value calculated with sign test); and number of RefSeq genes within or overlapping CNVs matching the criteria (p value calculated with Wilcoxon paired test). Where burden was assessed for unequal numbers of probands and siblings (e.g., by sex) the sign test and Wilcoxon paired test were replaced with the Wilcoxon test.

Statistical Analysis of De Novo Recurrence

To determine the probability of finding multiple rare de novo CNVs at the same location in probands, we first estimated how many likely positions in the genome were contributing to the observed de novo CNVs in siblings. As there are widely varying mutation rates for structural variation across the genome (Fu et al., 2010), some positions are more likely to result in de novo CNVs observed in our sample than others. Consequently, the likely number of positions is much smaller than the total possible number of positions. We refer to the likely CNV regions as effective copy-number-variable regions (eCNVRs) and calculate their quantity "C" using the so-called "unseen species problem," which uses the frequency and number of observed CNV types (or species) to infer how many species are present in the population. Based on the observed de novo CNVs in the control sibling group, we apply the formula $C = c/u + g^2 d (1 - u)/u$, in which c = the total number of distinct species observed; c_1 = the number of singleton species; d = total number of CNVs observed; g = the coefficient of variation of the fractions of CNVs of each type, and $u = 1 - c_1/d$ (Bunge and Fitzpatrick, 1993). (In this calculation, due to the small number of observations, we assume that g equals 1.) For the de novo events in siblings, $c_1 = 14$, $c = 15$, $d = 16$, and $C = 232$. This calculation is performed in the siblings because the observed rare de novo CNVs in this group are assumed to be predominantly nonrisk variants and consequently represent the null distribution.

Next, we calculate the chance that two de novo events match at any one of C eCNVRs in probands by using methods from the classic "birthday problem" which assesses the likelihood of seeing at least one pair of matching birthdays among a given number of people. Our interest was in seeing >2 matches (m) in probands under the null hypothesis of no association with ASD. This calculation is performed empirically by distributing d events at random among C eCNVRs and then counting the maximum number of CNVs falling in the same location. Repeating this experiment one million times, we obtained an estimate of the probability of finding $\geq$m counts for $\geq$1 eCNVR under the null hypothesis.

Given the importance of the estimate of eCNVRs in unaffected populations for the determination of significance, we recalculated C based on a combined set of confirmed de novo CNVs in controls described in the literature and obtained a highly similar result (C = 242) (Supplemental Experimental Procedures). Moreover, we determined that the results reported here remain significant under the plausible range of estimates for C (Supplemental Experimental Procedures).

Estimate of Number of De Novo CNV Regions Contributing to ASD Risk

The unseen species problem was used to predict the total number of ASD risk loci based on the distribution of de novo CNVs in probands. This required identification of the de novo CNVs that confer risk; to identify such CNVs we estimated that 76% of de novo CNVs in probands confer risk (67 de novo CNVs in probands − 16 de novo CNVs expected in siblings/67 de novo CNVs in probands) and assumed that recurrent de novo CNVs were most likely to be associated with risk and should be included within this 76%. The remainder of the 76% is made up of 27 single occurrence de novo CNVs (though we do not identify which ones), leading to an estimate of the total number of risk-conferring loci as 130 ($c_1 = 27$, $c = 33$, $d = 51$). A similar approach was applied to all de novo CNVs in 3816 probands (count derived from the literature), leading to an estimate of 234 risk-conferring loci ($c_1 = 59$, $c = 88$, $d = 158$).

Stepwise Assessment of Multiple Variables

Predictors were examined in a logical order, e.g., to evaluate the relationship between gene number (G), CNV size (L), and affection status (A, proband versus sibling), we fit a series of increasingly complex linear models in the following steps: (1) regress response G on predictor L, regress G on A; 2) if $\geq$1 term was significant, and assuming L had the best predictive power, we regressed G on L and A; (3) assuming L and A were significant jointly, we regressed G on L, A and L interacting with A. The latter term permits the slope of the relationship between G and L to differ for probands versus siblings. In each step, we determined whether the newest term was significant, given the terms already in the model. We also fit the model by using backward elimination, starting with the full model and simplifying it one term at a time.

Population Structure of Recurrent De Novo CNVs

All parents were projected onto a five-dimensional ancestry map by using eigenvector decomposition (Crossett et al., 2010; Lee et al., 2009). Euclidean distances were measured for the parents of origin. The mean and median distances between these pairs of parents were calculated and were evaluated relative to the remainder of the sample by using a bootstrap procedure (Supplemental Experimental Procedures).

Genotype-Phenotype Analysis

For each sample with a 16p11.2 deletion (eight samples) or duplication (six samples) or 7q11.23 duplication (four samples), five control probands were selected based on a matching hierarchy: age (100% of control probands matched), sex (100%), genetic distance (91%, based on five-dimensional ancestry map), collecting site (46%), and quartet or trio family (34%). Probands with de novo CNVs or CNVs in regions previously associated with ASD were removed prior to matching; each control proband was only included once.

For continuous variables each stratum of a "case" proband matched to five "control" probands was treated as a block and the data were analyzed as a randomized block design by using analysis of covariance. Thus mean values were allowed to vary across blocks and to be altered by case-control status. The difference because of the presence of the CNV of interest was assessed with an F-test with n, M degrees of freedom (n is the number of CNVs of interest and M is the residual degrees of freedom after accounting for model terms). Because IQ is known to affect many behavioral measures associated with ASD, it was treated as a covariate in models for outcomes besides itself and BMI. For diagnostic status, matching was taken into account by using a conditional logit model.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, nine tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.05.002.

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