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Patterns and rates of exonic *de novo* mutations in autism spectrum disorders

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Abstract

Autism spectrum disorders (ASD) are believed to have genetic and environmental origins, yet in only a modest fraction of individuals can specific causes be identified[1,2]. To identify further genetic risk factors, we assess the role of *de novo* mutations in ASD by sequencing the exomes of ASD cases and their parents (n= 175 trios). Fewer than half of the cases (46.3%) carry a missense or nonsense *de novo* variant and the overall rate of mutation is only modestly higher than the expected rate. In contrast, there is significantly enriched connectivity among the proteins encoded by genes harboring *de novo* missense or nonsense mutations, and excess connectivity to prior ASD genes of major effect, suggesting a subset of observed events are relevant to ASD risk. The small increase in rate of *de novo* events, when taken together with the connections among the proteins themselves and to ASD, are consistent with an important but limited role for *de novo* point mutations, similar to that documented for *de novo* copy number variants. Genetic models incorporating these data suggest that the majority of observed *de novo* events are unconnected to ASD, those that do confer risk are distributed across many genes and are incompletely penetrant (i.e., not necessarily causal). Our results support polygenic models in which spontaneous coding mutations in any of a large number of genes increases risk by 5 to 20-fold. Despite the challenge posed by such models, results from *de novo* events and a large parallel case-control study provide strong evidence in favor of *CHD8* and *KATNAL2* as genuine autism risk factors.

In spite of the substantial heritability, few genetic risk factors for ASD have been identified[1,2]. Copy number variants (CNVs), often *de novo* and covering multiple adjacent

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genes, have been identified as conferring risk[3,4]. While these CNVs provide important leads to underlying biology, they rarely implicate single genes, are rarely fully penetrant, and many confer risk to a broad range of conditions including intellectual disability(ID), epilepsy, and schizophrenia[5]. There are also documented instances of rare single nucleotide variants (SNVs) that are highly penetrant for ASD[6].

Large-scale genetic studies make clear that the origins of ASD risk are multifarious, and recent estimates based on CNV data put the number of independent risk loci in the hundreds[4]. Yet knowledge regarding specific risk-determining genes and the overall genetic architecture for ASD remains incomplete. Although new sequencing technologies provide a catalog of most variation in the genome, the profound locus heterogeneity of ASD makes it challenging to distinguish variants that confer risk from the background noise of inconsequential SNVs. *De novo* variation, being less frequent and potentially more deleterious, could offer insights into risk-determining genes. For this reason we sought to carefully evaluate the observed rate and consequence of *de novo* point mutations in the exomes of ASD subjects.

We performed exome sequencing of 175 ASD probands and their parents across five centers with multiple protocols and validation techniques (Supplementary Information). We used a sensitive and specific analytic pipeline based on current best practices[7-9] to analyze all data and observed no heterogeneity of mutation rate among centers.

In the entire sample, we observed 161 coding region point mutations (101 missense, 50 silent and 10 nonsense), with an additional 2 conserved splice site (CSS) SNVs and 6 frameshift indels validated and included in pathway analyses (Supplementary Table 1).

To determine whether the rate of coding region point mutations was elevated, we estimated the mutation rate in light of coverage and base context using two parallel approaches (Supplementary Information). Based on both models, the exome target should have a significantly increased (≈30%) mutation rate compared to the genome. Conservatively, by assuming the low- end of the estimated mutation rate from recent whole-genome data (1.2×10^{-8})[10], we estimate a mutation rate of 1.5×10^{-8} for the exome sequence captured here. The observed point mutation rate of 0.92/exome is slightly but not significantly elevated versus expectation (Table 1) and is insensitive to adjustment for lower coverage regions (Supplementary Information). Indeed our rate is similar to Sanders et al. (in press).

Per-family events were distributed according to the Poisson distribution (Table 1), yielding no evidence for ASD tracing to high rates of *de novo* mutation. The relative rates of ‘functional’ (missense, nonsense, CSS and read-through) versus silent changes did not deviate from expectation (Table 2). We did, however, observe 10 nonsense mutations (6.2%), which exceeded expectation (3.3%) (one-tailed P=0.04; Supplementary Information).

We examined the missense mutations as such variation can cause loss of function[11] using PolyPhen2 scores[12] to measure mutation severity. These also showed no deviation from random expectation. The observed PolyPhen2 scores clearly deviate from standing variation

in the parents (Table 2): Such variation, even the rarest category, has survived selective pressure and is not an appropriate control for *de novo* events.

We observed 3 genes with two *de novo* mutations: *BRCA2* (2 missense), *FAT1* (2 missense) and *KCNMA1* (1 missense, 1 silent). A gene with two or more non-synonymous *de novo* hits across a panel of trios might suggest strong candidacy. However, simulations (Supplementary Information) show that two such hits are inadequate to define a gene as a conclusive risk factor given the number of observed events in the study.

From analyses of secondary phenotypes (Supplementary Tables 2-3), the most striking result is that paternal and maternal age, themselves highly correlated (r^2=0.679, *P*-value<0.0001), each strongly predicts the number of *de novo* events per offspring (paternal age *P*=0.0013, maternal age *P*=0.000365), consistent with aggregating mutations in germ cells in the paternal line[13]. Consistent with genetic theory, there is an increased rate of *de novo* mutation in female versus male cases (1.214 for females vs. 0.914 for males); however, the difference is not significant, perhaps owing to limited sample size. Considering phenotypic correlates, we observed no rate difference between subjects with strict autism versus those with a broader ASD diagnosis, between positive and negative family history, or any significant effect of *de novo* mutation on verbal, nonverbal or full scale IQ (Supplementary Table 3).

While hundreds of loci are apparently involved in autism[4] and *de novo* mutations therein affect ASD risk, modeling of different numbers of risk genes and penetrances (Supplementary Information) shows that a model of hundreds of genes *with high penetrance mutations* is excluded by our data; however, more modest contributions of *de novo* variants are not. For example, 10-20% of cases carrying a *de novo* risk-conferring event and conferring ten to twentyfold increased risk, is consistent with these data (Supplementary Table 4). Thus, our data are consistent with either chance mutation or a modest role for *de novo* mutations on risk.

We therefore posed two questions of the group of genes harboring *de novo* functional mutations: do the protein products of these genes interact with each other more than expected, and are they unusually enriched in, or connected to, prior curated lists of ASD-implicated genes? Using an *in silico* approach (DAPPLE)[14,15] the protein-protein connectivity in the set of 113 genes harboring 'functional' *de novo* mutations was evaluated. These analyses (Figure 1) showed significantly greater connectivity amongst the *de novo* identified proteins than would be expected by chance (*P*<0.001) (Supplementary Information).

Querying previously-defined, manually-curated lists of genes[6] associated with high risk for ASD with and without ID(Supplementary Tables 5), and high risk ID genes (Supplementary Tables 6), we asked whether there was significant enrichment for *de novo* mutations in these genes. Five genes with 'functional' de novo events were previously associated with ASD and/or ID (*STXBP1*, *MEF2C*, *KIRREL3*, *RELN* and *TUBA1A*); for four of these genes (all but *RELN*) the prior evidence indicated autosomal dominant inheritance.

We then assessed the average distance (D_i, Supplementary Figure 2) of the *de novo* coding variants in brain-expressed genes (see supplement) to the ASD/ID list using a Protein-

Protein Interaction background network. To enhance power, data from a companion study (Sanders et al.) were used, including the observed silent *de novo* variants and *de novo* variants in unaffected siblings as comparators. The average distance for non-synonymous variants was significantly smaller for the case set than the comparator set (3.66±0.42 versus 3.78±0.59; permutation *P*=0.033) (Figure 2). Much of this signal comes from 31 synaptic genes identified by three large-scale synaptic proteomic studies (D_i=3.47±0.46 versus 3.57±0.60; permutation P=0.084) (Supplemental Figure 3; see also Supplemental Fig. 4 for the complete data). Taken in total, these independent gene set analyses, along with the modest enrichment of *de novo* variants over background rates in ASD, indicate that a proportion of the *de novo* events observed in this study likely contribute to autism risk.

Using whole-exome sequencing of autism trios, we demonstrate a rate, functional distribution and predicted impact of *de novo* mutation largely consistent with chance mutational processes governed by sequence context. This lack of significant deviation from random mutational processes suggests a more limited role for the contribution of *de novo* mutations to ASD pathogenesis than has previously been suggested[15], and specifically highlights the fact that observing a single *de novo* mutation, even an apparently 'severe' LOF allele, is insufficient to implicate a gene as a risk factor. Yet the pathway analyses presented here assert that the overall set of genes hit with 'functional' *de novo* mutations are not random and are biologically related to each other and to previously identified ASD/ID candidate genes. Modeling the *de novo* mutational process under a range of genetic models reveals that some models are inconsistent with the observed data – e.g., one hundred rare, fully penetrant Mendelian genes similar to Rett syndrome – while others are not such as spontaneous 'functional' mutation in a 1,000 genes that would increase risk by ten or twentyfold (Supplementary Table 4). Models that fit the data are consistent with the relative risks estimated for most *de novo* CNVs[4] and suggest that *de novo* SNVs, like most CNVs, often combine with other risk factors rather than fully cause disease. Furthermore, these models suggest that *de novo* SNVs events will likely explain <5% of the overall variance in autism risk (Supplementary Table 4).

Considering the two companion manuscripts, 18 genes with two functional *de novo* mutations are observed in the complete data. Using simulations, 11.91 genes on average harbor functional mutations by chance (Supplementary Table 7). Thus, a set of 18 genes with two or more hits is not quite significant (p=0.063). Matching loss-of-function variants, however, at *SCN2A*, *KATNAL2 and CHD8* (Supplementary Table 7) are unlikely to occur by chance because the expected very low rate of *de novo* nonsense, splice and frameshift variants. We evaluated these strong candidates further using exome sequencing on 935 cases and 870 controls and at both *KATNAL2* and *CHD8*, three additional LoF mutations were observed in cases with none in controls (no additional LoF mutations were seen at *SCN2A*). Using data from more than 5000 individuals in the NHLBI Exome Variant Server as additional controls, 3 LoF mutations were seen in *KATNAL2* but none again in *CHD8*, making the additional observation of 3 *CHD8* LoF mutations in our cases significant evidence (p<0.01) of this being a genuine autism susceptibility gene. Not all genes with double hits are nearly so promising (Supplementary Information; Supplementary Tables 8-9) supporting the estimate above that most such observations are simply chance events.

Overall, these data underscore the challenge of establishing individual genes as conclusive risk factors for ASD, a challenge that will require larger sample sizes and, likely, deeper analytic integration with inherited variation.

Methods (for online version only)

Phenotype assessment

Affected probands were assessed by research-reliable research personnel using Autism Diagnostic Interview-Revised (ADI-R) and the Autism Diagnostic Observation Schedule-Generic (ADOS) and DSM-IV diagnosis of a pervasive developmental disorder was made by a clinician. All probands met criteria for autism on the ADI-R and either autism or ASD on the ADOS, except for the 3 subjects from AGRE that were not assessed with the ADOS. In all 85% of probands were classified with autism on both the ADI-R and ADOS. All subjects provided informed consent and the research was approved by institutional human subjects board.

Exome sequencing, variant identification, and *de novo* detection

Exome capture and sequencing was performed at each site using similar methods. Exons were captured using the Agilent 38Mb SureSelect v2 (University of Pennsylvania and Broad Institute n=118), the NimbleGen Seq Cap EZ SR v2 (Mt Sinai School of Medicine, Vanderbilt University n=51), or NimbleGen VCRome 2.1 (Baylor n=6). After capture, another round of LM-PCR was performed to increase the quantity of DNA available for sequencing. All libraries were sequenced using an IlluminaHiSeq2000.

Sequence processing and variant calling was performed using a similar computational workflow at all sites. Data was processed with Picard (http://picard.sourceforge.net/), which utilizes base quality-score recalibration and local realignment at known indels[8] and BWA[7] for mapping reads to hg19. SNPs were called using GATK[8,9] for all trios jointly. The variable sites that we have considered in analysis are restricted to those that pass GATK standard filters. From this set of variants, we identified putative *de novo* mutations as sites where both parents were homozygous for the reference sequence and the offspring was heterozygous and each genotype call was made confidently (see Supplementary Information).

Validation of *de novo* events

Putative *de novo* events were validated by sequencing the carrier and both parents using Sanger sequencing methods (University of Pennsylvania, Mt. Sinai School of Medicine, Vanderbilt University, Baylor Medical College) or by Sequenom MALDI-TOF genotyping of trios (Broad).

Gene annotation

All identified mutations were then annotated using Refseq hg19. The functional impact of variants was assessed for all isoforms of each gene, with the most severe annotation taking priority. Splice site variants were identified as occurring within two basepairs of any intron/exon boundary.

Expectation of *de novo* mutation calculation

To calculate the expected *de novo* rate, we assessed the mutability of all possible trinucleotide contexts in the intergenic region of the human genome for variation in two fashions: fixed genomic differences compared to chimpanzee and baboon[12] and variation identified from the 1,000 Genomes project. The overall mutation rate for the exome was then determined by summing the probability of mutation for all bases in the exome that were captured successfully. We also determined the probability of each class functional mutation by summing the annotated variants.

Pathway analyses

We applied DAPPLE[14], which uses the InWeb database[15], to determine whether there is excess protein protein interaction across the genes hit by a functional *de novo* event. We also assessed whether these genes were more closely connected to a list of ASD genes.[6]

Modeling *de novo* events

We modeled a Poisson process consistent with the expected distribution defined by the mutation model and with the observed data. We varied the fraction of genes that influence risk, the probability a variant in a gene would be functional, and the penetrance of functional *de novo* events. We also simulated a random set of *de novo* events to estimate the probability of hitting a gene multiple times.

Association Analysis

We performed association tests using *SKAT*[17], a generalization of C-alpha[18]. Our primary analyses treat case-control data generated at Baylor and Broad separately (23 genes X 2 sites), but we also performed mega and meta-analyses (23 genes X 2 methods).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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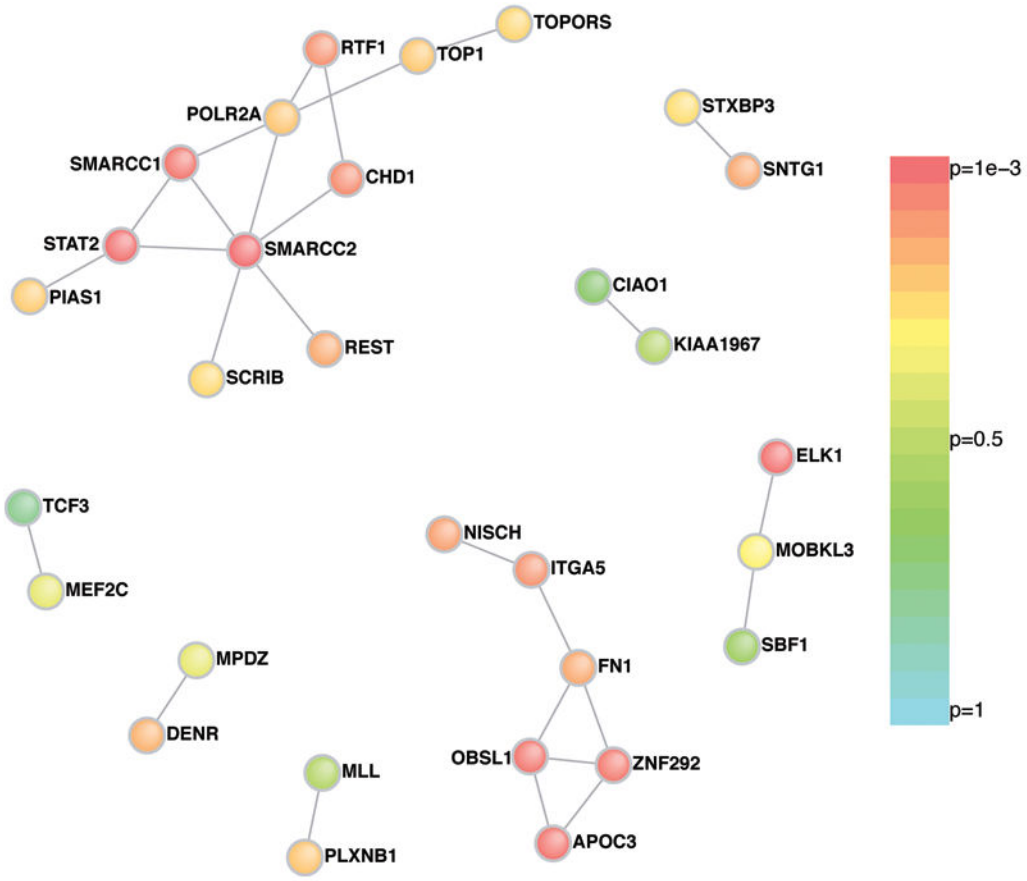

Figure 1. Protein-Protein interaction for genes with an observed functional *de novo* event

Direct protein connections from InWeb, restricting to genes harboring *de novo* mutations for DAPPLE analysis. Two extensive networks are identified, the first centered on SMARCC2 with 12 connections across 11 genes and the second centered on FN1 with 7 connections across 6 genes. The P-value for each gene having as many connections as those observed color the nodes of the network.

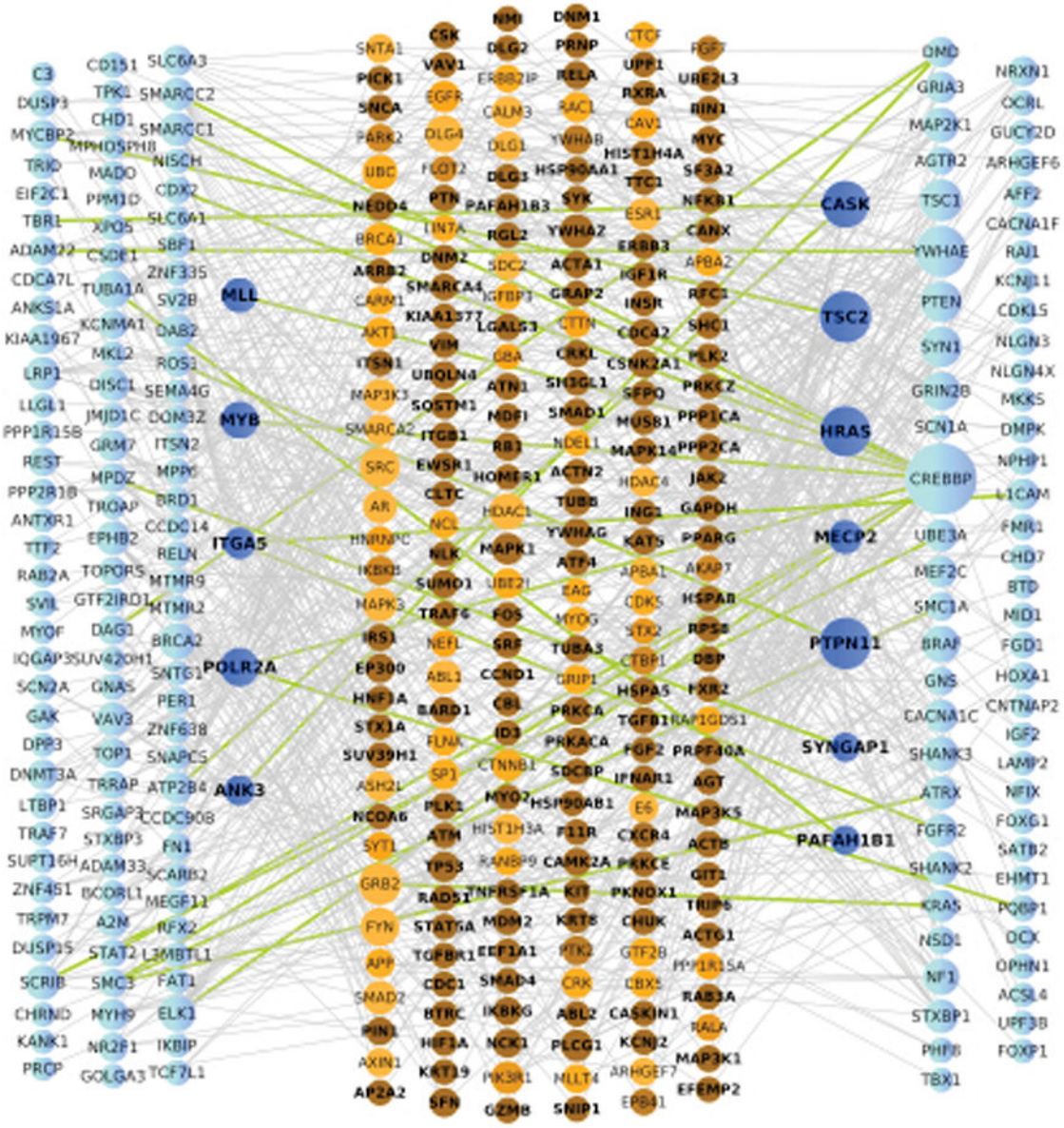

Figure 2. Direct and indirect Protein-Protein interaction for genes with a functional *de novo* event and existing ASD/ID genes

PPI network analysis for de novo variants and prior ASD genes (ASD112). Nodes are sized based on connectivity. Genes harboring de novo variants (left) and prior ASD genes (right) are colored blue with dark blue nodes represent genes that belong to one of these lists and are also intermediate proteins. Intermediate proteins (center) are colored in shades of orange based on a p-value computed using a proportion test where darker color represents a lower p-value. Green edges represent direct connections between genes harboring de novo variants (left) and prior ASD genes. All other edges, connecting to intermediate proteins are shown in grey.

Table 1

Distribution of Events Per Family.

Events per family	All ASD trios		Random Mut-Exp[3]
	exon DN SNVs	Exp	
0	71	69.7	73.2
1	62	64.2	63.8
2	28	29.5	27.8
3	10	9.1	8.1
4	2	2.1	1.8
5	1	0.4	0.3
		0.920	0.871

[1] exon DN-SNVs include all single nucleotide variants in coding sequence but excludes indels and intronic variants

[2] exp is the expected distribution of number of trios with a given event count as determined by the Poisson

[3] Random Mut-Exp is the expectation for 175 trios based on the sequence-context mutation rate model M1 (Supplementary Materials) based on the count of the number of trios that have at least 10× coverage.

Table 2

Rates of mutation annotation given variant type.

Type of *de novo* mutation	*De Novo*[1]	Random *De Novo*	Singletons[2]	Doubletons[2]	≥ 3[2]
Missense	62.7%	66.1%	59.5%	55.4%	48.8%
Nonsense	6.2%	3.3%	1.2%	0.8%	0.4%
Synonymous	31.1%	30.6%	39.3%	43.8%	50.8%
Benign[3]	35.0%	35.9%	46.6%	51.3%	63.4%
Possibly Damaging[3]	21.0%	18.9%	18.8%	17.7%	15.1%
Probably Damaging[3]	44.0%	45.2%	34.7%	31.0%	21.4%

[1] All indels and failing variants were removed.

[2] Singletons, doubletons, and ≥3 (copies) are only those variants called in the parents from Wave 1.

[3] Benign, Possibly Damaging, and Probably Damaging as defined by PolyPhen2