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Recessive gene disruptions in autism spectrum disorder

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AUTHOR CONTRIBUTIONS:

T.W.Y., R.N.D., E.T.L., and M.J.D. designed the study, with important additional contributions by C.B., D.J.C., C.A.W., and J.D.B.; R.N.D., E.T.L., and A.S. performed the data analyses; S.D.R. and S.G. performed Sanger validation; A.C. and C.F. characterized the FEV family; S.G. and T.W.Y. designed the *in situ* expression analyses; R.N.D. and T.W.Y. wrote the manuscript and all authors reviewed and approved the manuscript.

DATA AVAILABILITY

Data included in this manuscript is deposited at dbGAP merged with published data under accession number phs000298.v4.p3.

COMPETING INTERESTS

The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to Timothy.yu@childrens.harvard.edu.

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Abstract

Autism spectrum disorder (ASD) affects up to 1 in 59 individuals¹. Genome-wide association and large-scale sequencing studies strongly implicate both common variants^{2–4} and rare *de novo* variants^{5–10} in ASD. Recessive mutations have also been implicated^{11–14} but their contribution remains less well defined. Here we demonstrate an excess of biallelic loss-of-function and damaging missense mutations in a large ASD cohort, corresponding to ~5% of total cases, including 10% of females, consistent with a female protective effect. We document biallelic disruption of known or emerging recessive neurodevelopmental genes (*CA2*, *DDHD1*, *NSUN2*, *PAH*, *RARB*, *ROGDI*, *SLC1A1*, *USH2A*) as well as other genes not previously implicated in ASD including the transcription factor *FEV*, a key regulator of the serotonergic circuitry. Our data refine estimates of the contribution of recessive mutation to ASD and suggest new paths for illuminating novel biological pathways responsible for this condition.

Editorial Summary:

Analysis of whole exome sequencing data from 2,343 individuals with autism spectrum disorder (ASD) compared to 5,852 unaffected individuals demonstrates an excess of biallelic, autosomal mutations for both loss-of-function and damaging missense variants.

We performed a systematic analysis of exome data from the Autism Sequencing Consortium (ASC)¹⁵, representing 2,343 affected and 5,852 unaffected individuals (Supplementary Table 1, Supplementary Figure 1). We cataloged a total of 696,143 autosomal loss-of-function (LOF) events (representing 28,685 unique variants in 11,745 unique genes) that introduce a stop codon or disrupt a canonical splice site (Table 1). After excluding common variants (allele frequency (AF) >1%), there were 84,645 rare LOF events (27,648 unique alleles) for an average of ~10 LOF mutations per individual. After computational phasing, we found 298 events in the ASC (after filtering to exclude common polymorphisms) consistent with complete gene knockout (homozygous or compound heterozygous LOF mutations), affecting 266 individuals (Table 1). Affected individuals were disproportionately likely to harbor a gene knockout (62% more likely; 0.05 events per affected individual versus 0.031 per unaffected individual, $p=0.0003$ by random permutation testing), with the bulk of the excess arising from the rarest alleles (Table 1; Supplementary Table 2; Figure 1a). To control for possible differences in population and family structure (e.g., founder effects and/or

consanguinity in the Finnish and Middle Eastern cohorts), we also normalized to background burdens of biallelic synonymous variants (see Methods and Supplementary Table 3–6). Individuals with ASD continued to exhibit higher knockout rates after normalization (0.042 biallelic LOF events per affected individual versus 0.031 per unaffected individual, $p=0.008$ by random permutation testing) (Supplementary Figure 2a). Based on the observed ascertainment differentials (AD) between affected and unaffected individuals (0.050 vs. 0.031, or 0.042 vs. 0.031 after normalization), these burdens predict a contribution of biallelic LOF alleles to ~1–2% of ASD cases (see Methods).

We considered whether these findings might be extended to incorporate the impact of missense variation. Missense variation occurs much more frequently than truncating mutations, with a small yet significant subset likely to be damaging. We evaluated potential missense contributions of two different types: all missense/nonsynonymous variants (Nsyn), and damaging missense/nonsynonymous variants (NsynD4, defined as missense changes classified as damaging by 4 or more predictive algorithms, representing 4% of all Nsyn events). Individuals with ASD exhibited an excess of biallelic events specifically involving a LOF allele *in trans* with a NsynD4 allele (Figure 1b), an effect which persisted after normalization to synonymous rates (Supplementary Figure 2b). Similarly, considering biallelic events involving either rare LOF or NsynD4 alleles, individuals with ASD demonstrated an excess of biallelic variation ($p<10^{-4}$) with or without normalization (Supplementary Figure 2c, Figure 1c). Biallelic NsynD4 alleles were also found in excess in cases (Figure 1c) but this did not persist after normalization ($p=0.07$, Supplementary Figure 2c).

Alleles contributing to biallelic LOF knockouts in cases were not in excess in the heterozygous state (Supplementary Figure 3). Genes impacted by biallelic mutation also scored low with respect to haploinsufficiency¹⁶ and gene constraint¹⁷ (Figure 1d–e, Supplementary Table 7), compared to known dominant and *de novo* ASD and ID genes¹⁸. These data support a recessive risk model, as opposed to the additive contributions of alleles acting co-dominantly.

ASD exhibits a striking male bias, and females with ASD exhibit a disproportionate burden of *de novo* and inherited SNVs and CNVs, supporting a female protective/male susceptibility effect^{19,20}. We asked whether this also applies to rare recessive mutations. Unaffected males and females did not differ in burdens of LOF knockout (Figure 2a, Supplementary Figure 4a). Females with ASD, however, exhibited a larger excess of biallelic LOF mutations (Figure 2a, Supplementary Figure 4a). Similar patterns were observed when the analysis was extended to incorporate damaging missense variants, either alone (NsynD4) (Figure 2b, Supplementary Figure 4b) or grouped with LOF mutation (LOF or NsynD4) (Figure 2c, Supplementary Figure 4c). As observed before, LOF alleles were not in excess in the heterozygous state (Supplementary Figure 3). From these patterns we conclude that the female protective/male susceptibility effect extends to recessive mutations as well.

The ascertainment differentials observed led us to estimate the total contribution of biallelic mutation to ASD to be 3–5% (2% from LOF mutations, 1–3% from missense mutations).

Furthermore, based on the ascertainment differentials, identifiable biallelic LOF or damaging missense alleles appeared to contribute to ~10% of female ASD cases in the ASC (Supplementary Figure 4b), suggesting that the category of biallelic mutation could be one of the largest genetic contributors to ASD in females.

To address potential biological implications of these results, we examined the genes in which biallelic mutations were found. We first focused on the category of strict biallelic LOF mutations, estimating from the observed ascertainment differentials that ~25–40% of those found in ASD patients (0.019 out of 0.05; or 0.011 out of 0.042 after normalization to synonymous burdens) contribute to their condition. A total of 109 unique genes were knocked out (biallelic LOF) in affected individuals in our dataset. We reasoned that the most relevant of these genes would be those that are selected against biallelic inactivation in the general population. We therefore developed a filter to remove any gene that is also knocked out in one or more of 60,706 individuals sequenced as a part of ExAC (see Methods)¹⁷. To test its effectiveness, we examined its impact on observed patterns and burdens of gene knockouts in ASD. Overall, 131 of the 227 (58%) gene knockouts observed in the ASC were also observed in ExAC. Application of this filter reduced the overall burdens of observed biallelic knockout by half but left the ascertainment differentials intact (Figure 3a, Supplementary Figure 5). This significantly increased the observed proportional excess of biallelic LOF mutation: after filtering, on average, individuals with ASD were ~2.6 times as likely to have biallelic LOF mutations as individuals without ASD ($p=0.002$) (3.9X in females, $p=0.001$, and 2.6X in males, $p=0.01$) (Figure 3a).

We applied a similar logic to biallelic missense mutations as well. We reasoned that missense alleles encountered in the homozygous state in the general population are not likely to contribute to recessive condition. ~50% of rare, biallelic damaging missense events in the ASC involved an allele encountered in the homozygous state in ExAC (Supplementary Figure 6a, Supplementary Table 8). Filtering these events allowed us to discern for the first time a significant excess of rare biallelic damaging missense variation (*i.e.* NsynD4) in cases compared to controls (Supplementary Figure 6a), with an ascertainment differential of ~4%. Biallelic Nsyn variants were also in slight excess ($p=0.0037$), which arose almost entirely from affected females (Supplementary Figure 6b).

These results indicate that the observed excess in ASD is driven by genes and alleles that are biallelically constrained in the control population, strongly supporting the biological relevance of these findings.

De novo gene disrupting mutations contribute strongly to ASD²¹. To examine possible interactions between *de novo* and biallelic gene disrupting events, we compared the burden of biallelic LOF events in cases with and cases without LOF *de novo* mutations (Supplementary Figure 10)²¹. An excess of rare biallelic LOF events is strongly evident in ASD cases lacking *de novo* LOF variants (Supplementary Figure 10, $p=0.0006$). Their contribution in ASD cases with *de novo* LOF variants is harder to distinguish, as the observed difference in burdens of biallelic LOF events did not achieve statistical significance (Supplementary Figure 7). These may represent orthogonal risk categories, although larger sample sizes will be required to clarify this in the future.

After ExAC filtering, a total of 57 biallelically constrained genes harbored biallelic LOF mutation in at least one ASD case. Of these, we can estimate that ~60% of these (based on the observed burdens, see Methods), and ~80% of the knockouts observed in females, likely confer ASD risk. To further enrich for relevant genes, we also set aside 13 genes knocked out in one or more unaffected individuals in our ASC dataset (Figure 3b, Supplementary Table 7), and three that failed Sanger confirmation, leaving a final set of 41 genes that were specifically knocked out in individuals diagnosed with ASD, but never in 66,557 controls (60,706 from ExAC and 5,852 unaffected individuals from ASC) (Supplementary Table 9,10).

Several of these represent genes already implicated in disease. Eight (*CA2*, *DDHD1*, *NSUN2*, *PAH*, *RARB*, *ROGDI*, *SLC1A1*, *USH2A*) were associated with recessive genetic conditions involving neurodevelopmental delay (Table 2 and Supplemental Information for clinical details). In at least six of these cases (*CA2*, *NSUN2*, *PAH*, *RARB*, *ROGDI*, *USH2A*), available medical and phenotypic records were concordant with the clinical features predicted from the genetic finding (Supplemental Information). Phenotypic data was either unavailable or incomplete, but not discordant, for other cases. In two cases, knockouts were encountered in genes that cause well-characterized autosomal recessive conditions without clear connection to autism (*RFX5* and *DNAI2*, associated with autosomal recessive immunodeficiency and primary ciliary dyskinesia, respectively). In two other cases, knockouts were observed in genes associated with autosomal dominant disorders via recurrent gain-of-function mutations (*IFITM5* and *KIF22*, causing osteogenesis imperfecta type V and spondyloepimetaphyseal dysplasia with joint laxity type 2, respectively). The phenotypic consequence of recessive loss-of-function mutations for these two genes is unknown. Interestingly, *KIF22* is one of 25 genes in the 593kb core interval affected by 16p11.2 deletions.²² *KIF22* was proposed as a one of two genes in the interval that demonstrates dosage sensitivity, with knockdown causing axon tract and behavioral defects in zebrafish,²³ and disruption a *KIF22* ortholog in *Drosophila* causes synapse development defects²⁴.

Several of the remaining 33 genes are of strong neurobiological interest. One affected individual bore a homozygous LOF mutation (p.E48X) in *SLC1A1*, mutations in which have been linked to dicarboxylic aminoaciduria,²⁵ an extremely rare disorder with prior associations with intellectual disability^{26,27} and obsessive compulsive disorder (OCD)²⁵. *SLC1A1* encodes a brain-expressed, activity-regulated glutamate transporter^{28,29} that modulates glutamatergic neurotransmission via a variety of mechanisms^{30,31,32,33,34,35}. *SLC1A1* knockout in mice results in altered locomotor behavior³⁶ and learning and memory defects.³⁷

Two brothers with ASD bore homozygous stop-gain mutations in *FEV*, a transcription factor that is required for both development and function of serotonergic neurons (Supplementary Figure 8a). These neurons send projections that ramify broadly throughout the CNS (Supplementary Figure 8b), and have wide-ranging neuromodulatory effects on many physiological states and behaviors, including mood, aggression, anxiety, sleep, and movement³⁸. Serotonergic dysfunction has been strongly implicated in neuropsychiatric conditions including ASD³⁹, schizophrenia and mood disorders³⁸. In mice, *Fev/Pet-1* has

been shown to be highly expressed in the serotonergic raphe nuclei⁴⁰, and inactivation of *Fev* during development⁴¹ or adulthood⁴² results in depletion of serotonergic neurons and neurobehavioral changes including anxiety and aggression.^{41,42} Human *FEV* is also expressed in the serotonergic raphe in adult brain^{43,44}, and we found similar results via *in situ* hybridization analysis of human fetal brain as well (Supplementary Figure 8c–f). The older affected brother was diagnosed with autism and had a measured IQ of 69. He exhibited severe, stereotyped aggressive and self-injurious behaviors. He had a BMI of 25.6 (99%) and mildly dysmorphic facial features. EEG showed bilateral rolandic focus with generalization. The younger affected brother was diagnosed with PDD-NOS and intellectual disability (IQ not measured), had a BMI of 17.7 (91%), and no facial dysmorphisms (further details in Supplemental Information). We are not aware of any previous report of a human loss-of-function phenotype for *FEV*.

Gene knockouts were also identified in *FCHSD2*, a cytoskeletal adaptor protein that is highly expressed in human fetal brain and is the human homolog of *Drosophila nervous wreck*⁴⁵, *NCOA7*, a brain-specific transcriptional coactivator for steroid and nuclear hormone receptors⁴⁶, and *MOB1B*, a downstream effector in the Hippo signaling pathway, a pathway recently implicated in regulation of brain size⁴⁷. Many additional genes in the candidate set have abundant brain expression (*BRMS1L*, *ELOF1*, *GCN1L1*, *NUPR1*, *SLC22A6*) but less clear neurobiological activity.

We also expanded our scope to incorporate biallelic missense mutation. There were 409 distinct genes that harbored biallelic, damaging missense mutations in cases but not in controls (Supplementary Figure 9, Supplementary Table 11). Only one gene was found that was independently hit in multiple families: *AMT*, an established cause of nonketotic hyperglycinemia (NKH). Hypomorphic mutations in *AMT* have previously been reported to cause ASD without NKH's characteristic metabolic abnormalities. No other gene was hit more than once, so larger sample sizes would be necessary to establish any of these genes as ASD genes on this basis alone (Supplementary Table 11, Supplementary Figures 10 and 11). However, there were thirteen cases in which affected individuals bore biallelic mutations in alleles of established medical significance (in ClinVar as Pathogenic or Likely Pathogenic) (Table 3, Supplementary Table 12, Supplementary Information). All were diagnostic of genetic disorders with known neurodevelopmental consequences including classic metabolic diseases, mitochondrial depletion syndrome, and other syndromic conditions. All together, combined with the previously described eight LOF cases, biallelic mutations in known genes constituted ~1% of our cohort (21 out of 2,343 affected individuals), underscoring the importance of clinical screening for monogenic recessive conditions in this patient population.

Our study extends prior estimates of the contribution of recessive mutations to ASD^{13,14} and proves that the female protective / male susceptibility effect in ASD applies to biallelic mutations as well. Expanding these efforts to larger ASD cohorts should offer a path forward to delineate novel neurobiological mechanisms in autism.

METHODS

Whole exome data analyses

Whole-exome sequencing data were gathered as part of the Autism Sequencing Consortium⁴⁸. We focused our analysis on nine datasets generated using the Illumina sequencing platform, Supplementary Table 1). Alignment and variant calling were performed as previously described. Low-quality variants were filtered with the following criteria: (1) SNPs with GQ<20, (2) SNPs not passing the standard PASS filter, and (3) all indels (due to excessive false positives leading to spurious frameshift calls). Variant annotation was performed using ANNOVAR⁴⁹ to add allele frequencies, functional predictions, conservation, and gene annotations including OMIM and HGMD⁵⁰ disease associations. Allele frequencies were also calculated for each contributing cohort and across the entire collection of ASC samples analyzed. Variants were categorized by their predicted impact into synonymous, nonsynonymous (missense), or loss-of-function (altering a canonical splice site or resulting in a stopgain). Additional classification of damaging missense variants (NsynD4) was performed for variants predicted to be deleterious by at least 4/6 algorithms (SIFT⁵¹, PolyPhen2_HDIV⁵², PolyPhen2_HVAR, MutationTaster⁵³, MutationAssessor⁵⁴, and LRT⁵⁵).

Phasing was performed using Beagle 4⁵⁶ on the ASC dataset. VCFs were subdivided by chromosome and cohort prior to processing with Beagle employing default parameters.

Biallelic burden calculations

Allele frequency filtration was performed as described in the text using available public databases (1000G, EVS, ExAC). For rare variant calculations at maxAF 0.1%, 0.5%, 1%, 5%, and 10%, additional cohort-specific filters (cohort-specific AF of 5% or 10%) were applied to account for allelic variants that may be prevalent in a population but are not well represented in existing control databases. Burdens of homozygous and compound heterozygous variants were then counted for each cohort and for the entire ASC, stratifying by affected/unaffected status, gender, and mutational category (LOF, Nsyn, NsynD4, Syn).

To control for potential differences in underlying family and population structure of some of the constituent cohorts, we also measured rates of synonymous biallelic variation. In two cohorts (from the Middle East and Finland, respectively), rates of biallelic synonymous variation in affected individuals were higher than rates of biallelic synonymous variation in unaffected individuals (Supplementary Table 3). Therefore, for these cohorts, we normalized our LOF, NsynD4, and Nsyn burden calculations to the background rate of biallelic synonymous variation, to isolate the impact of biallelic LOF variation independent of background homozygosity in these cohorts.

The contribution of mutations to ASD risk was estimated using the following approach. First the ascertainment differential (AD) is calculated as the difference between the burden of biallelic mutations in cases and in controls and represents the estimated fraction of mutations that contribute to ASD (for example, an AD of 0.01 suggests that 1% of cases have a biallelic mutation contributing to ASD risk. The fraction of observed biallelic mutations contributing to disease is then calculated as AD divided by the total mutation burden in

cases. For example, given a burden of 0.025 biallelic LOF events in cases and 0.01 biallelic LOF events in controls, the AD is 0.015 and the percentage of mutations identified which contribute to disease risk is 0.015 divided by 0.025, or 60%.

For burden comparisons, significance was assessed by permutation testing using 10,000 random assignments of diagnostic categorization to individuals within the study. A significance threshold of $p < 0.05$ (i.e., without correction for multiple testing) was employed since the allele frequency cutoffs and variant annotation classes (LOF, LOF+NSynD4, LOF or NSynD4, NSynD4, NSyn) analyzed are not discrete. We do note that, all ExAC-filtered biallelic burden results (i.e., excess biallelic LOF, NSynD4, and NSyn mutations, summarized in Figure 3 and Supplementary Figures 5 and 6) were significant even with application of maximally conservative Bonferroni correction (e.g., $p < 0.01$ assuming five fully independent hypotheses).

ExAC filtration

To generate a list of genes commonly inactivated by biallelic LOF mutation, we analyzed whole exome data from 60,706 individuals compiled by the Exome Aggregation Consortium (ExAC)¹⁷. We applied ANNOVAR to systematically re-annotate all variants from ExAC against RefSeq complete protein coding genes. Since phasing was unavailable, we focused on genes bearing putative homozygous LOF variants (any variant in stopgain, frameshift, or canonical splicing change). Homopolymer expansions / contractions in canonical splice regions were ignored, as expansions and contractions in these regions are common and have little impact on splicing. With these parameters, a total of 2986 genes were found to be inactivated by homozygous stopgain, frameshift, or canonical splice site mutation in ExAC. We also used this re-annotated dataset to generate a list of ExAC alleles encountered in the homozygous state, to identify missense and other mutations that are more likely to be clinically benign. For the final LOF candidate genes presented in Supplementary Tables 9 and 10, contributory alleles were also confirmed to be absent in the homozygous state in the gnomAD dataset¹⁷.

Variant confirmation

Sanger sequencing was employed to confirm the accuracy of exome variant calls for a subset of LOF and NSynD4 variants with an empirical validation rate of 96% (107 of 114 alleles tested). These included all LOF alleles reported here for which DNA samples were available. If DNA samples were not available, we note the presence of allelic variants in known databases and/or whether predicted genotypes follow Mendelian segregation patterns in parental exome data (where available) as additional support.

Human tissue collection and *in situ* hybridization for FEV

Human embryonic and fetal tissues were obtained from the MRC-Wellcome Trust Human Developmental Biology Resource (www.hdbbr.org)⁵⁷ following appropriate consent from the women involved and adhering to the relevant HTA guidelines with approval from Research Ethics Committee NRES Committee North East - Newcastle & North Tyneside 1.

RNA probes were produced by amplifying human embryonic cDNA using a forward primer to *FEV* (NM_017521, nt. 1286–1305) tagged with a T7 RNA promoter sequence and a reverse primer (NM_017521, nt. 1862–1843) tagged with a SP6 RNA promoter sequence (Supplementary Table 13). The resulting fragment was *in vitro* transcribed using the DIG RNA Labeling Kit (Roche).

The *in situ* hybridizations were performed as previously described⁴⁸, except that 60ng RNA probe in 100ul ULTRAhyb (Ambion) per slide was used for the hybridization and the post-hybridization washes were modified: slides were washed for 10 minutes in 5 X SSC, twice in 2 X SSC, 50% formamide/1 X SSC, 1 X SSC and finally in 0.1X SSC. The hybridization incubation and all post-hybridization steps were performed at 63.5°C. Three human fetal brain samples were used: a Carnegie Stage (CS) 23 brain (12959), a CS23 head (12011), and a 10 post conception week hindbrain (sample 12721). Findings described were confirmed in at least two specimens. Expression patterns were analyzed relative to spatial models provided by the HuDSeN human gene expression spatial database project (<http://www.hudsen.org>).⁵⁸

Reporting Summary

Further information on research design is available in the Life Sciences Reporting Summary linked to this article.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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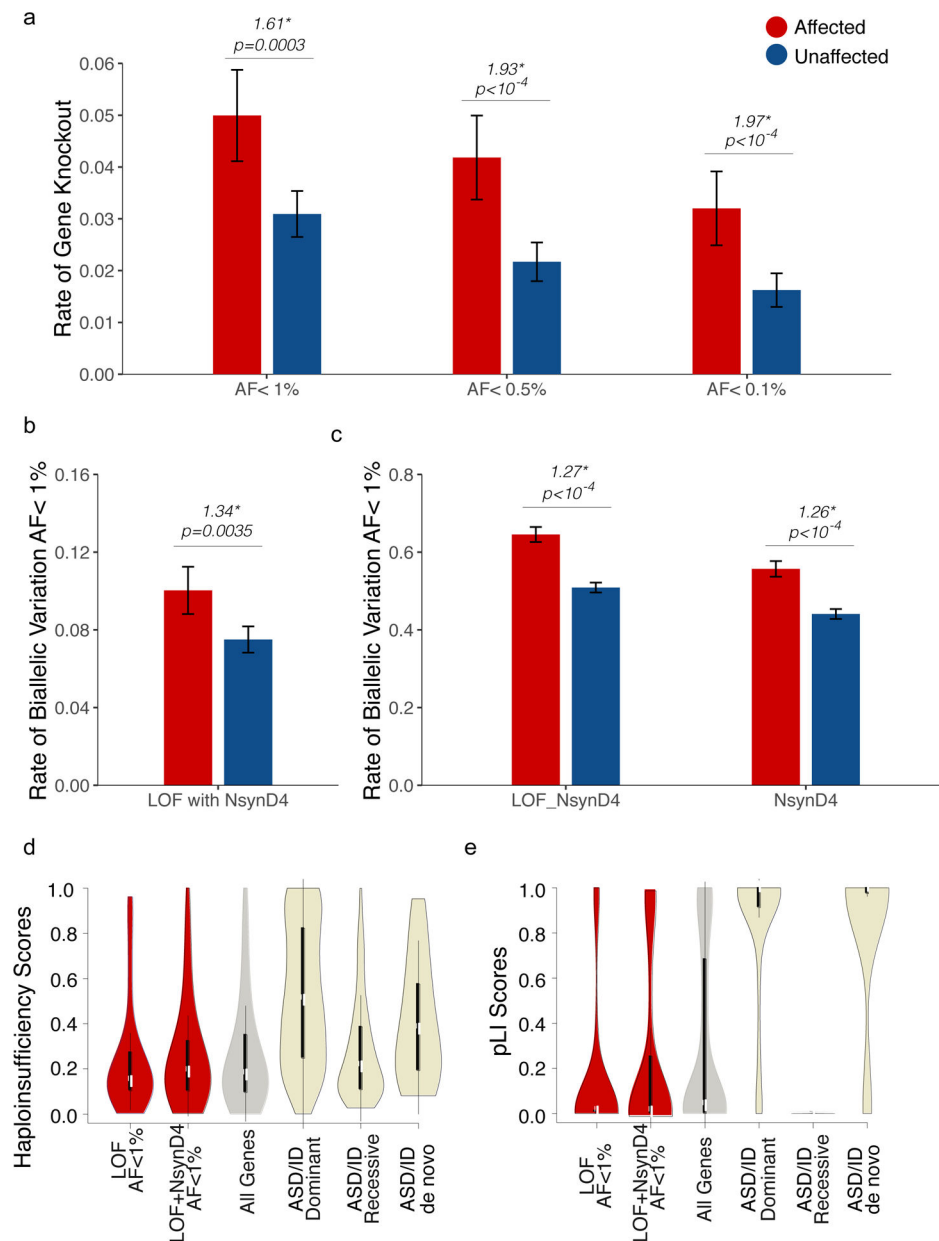
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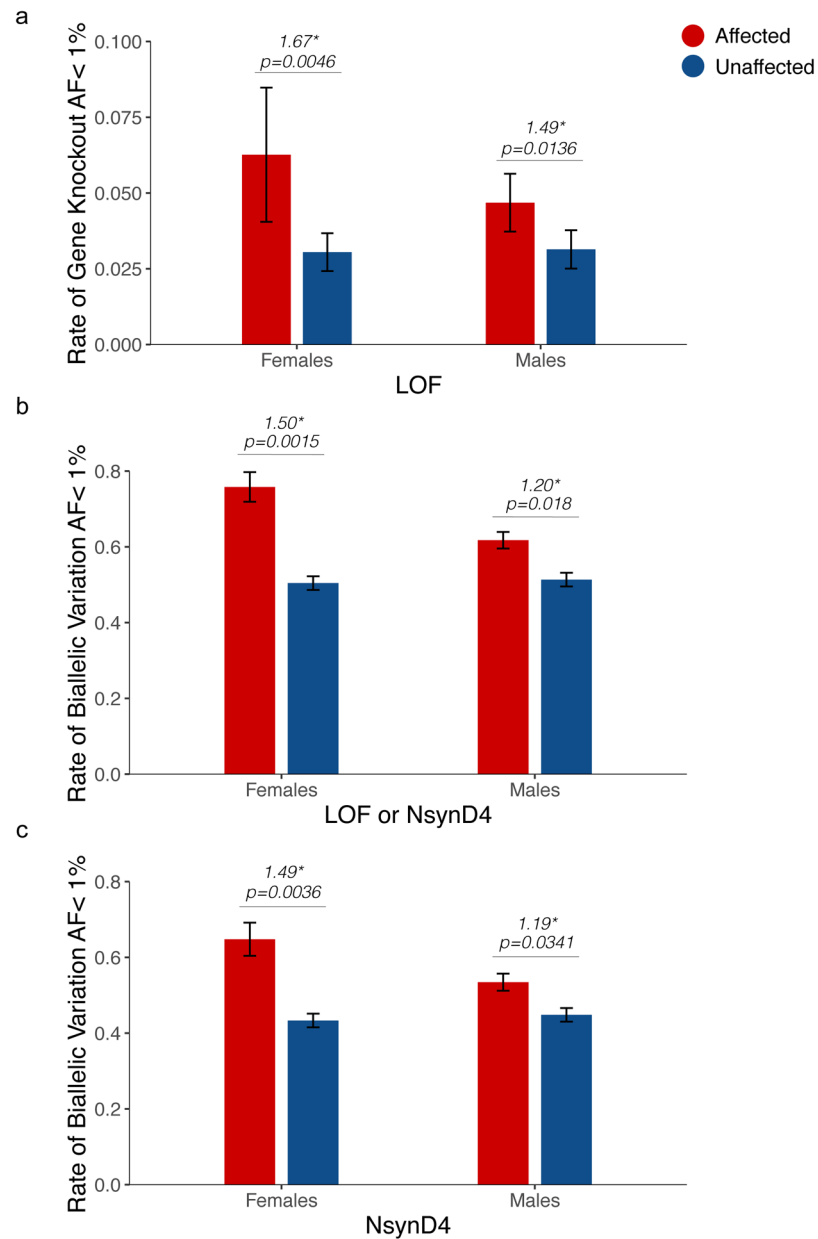
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**Figure 1.**

An excess of rare, damaging, biallelic mutation in ASD. **(a)** Rates of biallelic gene knockout (strict LOF) in the ASD, stratified by diagnosis and allele frequency. Rates of biallelic variation, considering **(b)** LOF variants paired with a damaging missense variant (NsynD4, predicted to be deleterious by at least 4 algorithms), **(c)** LOF or NsynD4 variants, or NsynD4 variants alone. Genes impacted by biallelic variation in cases exhibit **(d)** haploinsufficiency and **(e)** pLI score profiles consistent with recessive genes. Error bars represent the 95% confidence intervals.

**Figure 2.**

Biallelic mutations in ASD: effects of sex. **(a)** Rates of biallelic gene knockout (strict LOF) in the ASC, stratified by diagnosis and sex. **(b, c)** Rates of biallelic variation stratified by sex, considering **(b)** LOF or damaging missense (LOF or NsynD4) variants or **(c)** damaging missense variants alone (NsynD4). Error bars represent the 95% confidence intervals.

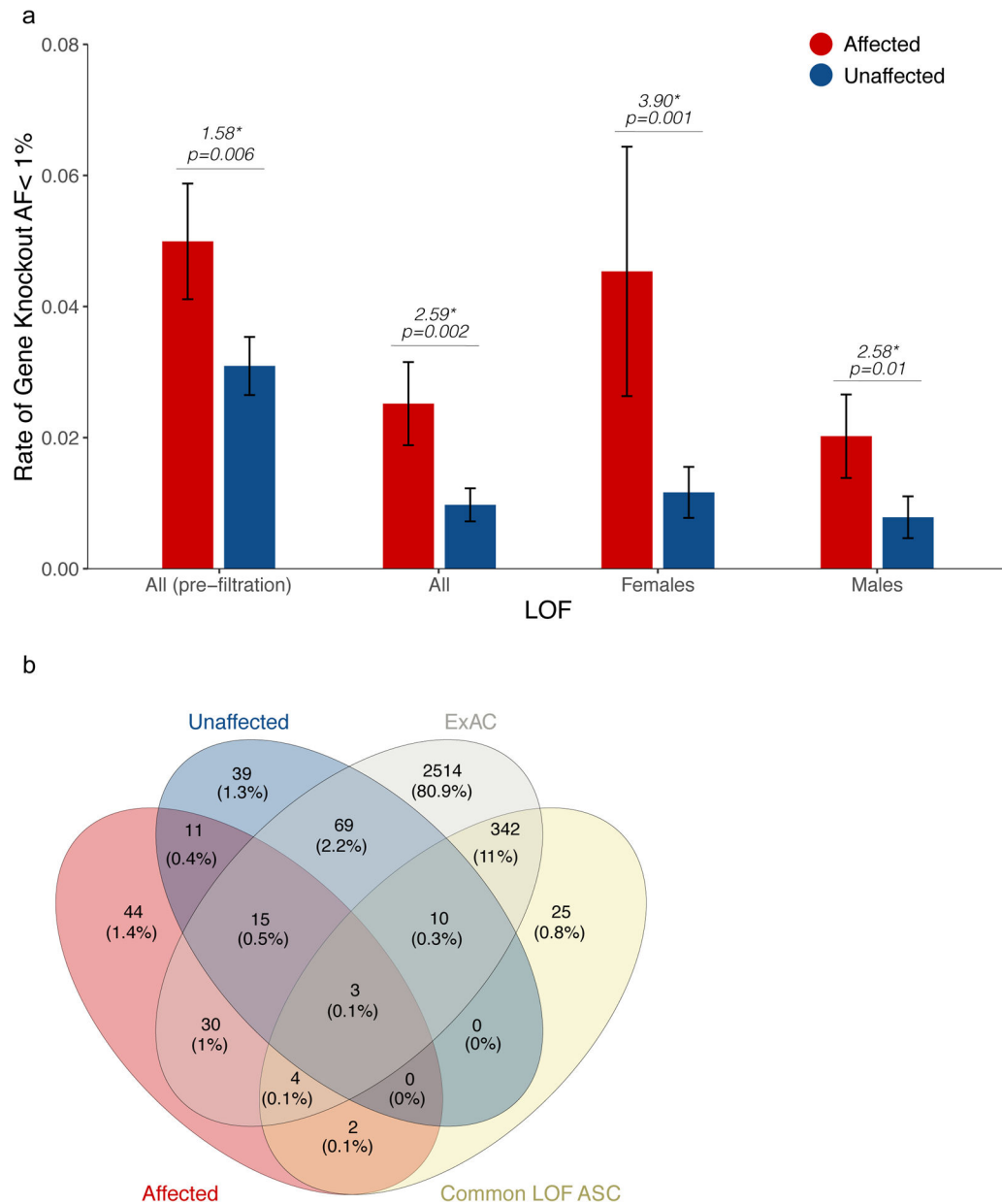


Figure 3. Biallelic mutations in ASD: ExAC filtration. **(a)** Rates of biallelic gene knockout (strict LOF) after filtration of commonly inactivated genes in ExAC. **(b)** Breakdown of candidate ASD genes excluded by presence of knockouts in ExAC, or by knockouts that are common and/or in unaffected individuals of the ASC. Error bars represent the 95% confidence intervals.

Table 1.

Patterns of biallelic LOF mutation in the Autism Sequencing Consortium

Allele frequency	# of biallelic LOF events	# of unique genes involved	# of individuals harboring	Events per individual		p-value
				Unaffected	Affected	
All	237,936	636	8195	28,997	29,128	0.2
10%	1,646	414	1420	0.194	0.219	0.03
5%	621	324	559	0.068	0.096	0.0005
1%	298	237	266	0.031	0.050	0.003
0.5%	225	182	201	0.022	0.042	<0.0001
0.1%	170	138	146	0.016	0.032	<0.0001

Table 2.

Clinically relevant gene knockouts in the ASC

Gene	Mutations	Disease relevance	Notes
<i>CA2</i>	hom c.232+1G>A (NM_000067)	Carbonic anhydrase deficiency	Intellectual disability, osteopetrosis, renal tubular acidosis
<i>DDHD1</i>	comp het c.1311-2A>T/c.2459-2A>T (NM_001160147)	Spastic paraplegia	Spastic paraplegia, mitochondrial abnormalities; only 3 cases reported
<i>NSUN2</i>	hom c.C1708T:p.Q570X (NM_001193455)	Autosomal recessive intellectual disability	Syndromic intellectual disability; only 7 mutations reported
<i>PAH</i>	hom c.C703T:p.Q235X and hom c.592_613del:p.Y198fs (NM_000277) (two separate individuals)	Phenylketonuria	~20% of individuals with PKU have autism (finding previously reported ¹³)
<i>SLCIA1</i>	hom c.G142T:p.E48X (NM_004170)	Dicarboxylic aminoaciduria	Elevated urinary glutamate and aspartate, variable neuropsychiatric symptoms; only 3 cases reported
<i>RARB</i>	hom c.C78A:p.C26X (NM_000965)	PDAC syndrome	Microphthalmia, pulmonary hypoplasia, diaphragmatic hernia, and cardiac defects; milder forms described
<i>ROGDI</i>	hom c.201-1G>T (NM_024589)	Kohlschütter-Tönz syndrome	Global developmental delay, epilepsy, spasticity, amelogenesis imperfecta
<i>USH2A</i>	comp het c.T12714G:p.Y4238X/c.G6224A:p.W2075X (NM_206933)	Usher syndrome	Sensorineural hearing deficiencies, retinitis pigmentosa (finding previously reported ¹⁴)

Table 3.

Biallelic mutations identified in cases and controls previously reported as pathogenic or likely pathogenic in Clin Var.

Gene	Condition	Sample identifier	Sex	Mutation	ClinVar (HGMD)	Variant pathogenicity	Damaging Predictions
<i>Found in 13 cases</i>							
<i>ASS1</i>	Citrullinemia type I	SKUSE5080161	M	hom c.1168G>A:p.G390R (NM_000050.4)	6329 (CM900037)	Pathogenic	10 / 10
<i>ATP7B</i>	Wilson disease	80001103644	M	het c.3207C>A:p.H1069Q (NM_000053.3)	3848 (CM930059)	Pathogenic	11 / 11
				het c.4087T>C:p.S1363P (NM_000053.3)	Same site as p.S1363F (ClinVar 188908, HGMD CM992829)	(p.S1363F=Pathogenic)	12 / 12
<i>B3GALNT2</i>	Muscular dystrophy with brain malformations	80001103921	F	het c.1368+1G>A (NM_152490.4)	na	(Likely Pathogenic)	na
				het c.979G>A:p.D327N (NM_152490.4)	424766 (CM144415)	Likely Pathogenic	10 / 12
<i>CA2</i>	Osteopetrosis, autosomal recessive 3	AU-10501	F	hom c.232+1G>A (NM_000067.2)	288909	Pathogenic	na
<i>COQ8A</i>	Coenzyme Q10 deficiency	AU-13901	M	hom c.1805C>G:p.P602R (NM_020247.4)	214050 (CM141297)	Pathogenic	11 / 12
<i>GBE1</i>	Glycogen storage disease, type IV and Adult polyglucosan body disease	AU-24601	F	hom c.986A>G:p.Y329C (NM_000158.3)	371439 (CM128776)	Pathogenic	11 / 11
<i>GLRA1</i>	Hereditary hyperekplexia	AU-12001	M	hom c.277C>T:p.R93W (NM_000171.3)	225379 (CM104312)	different missense LP in ClinVar (225379)	10 / 11
<i>HADH</i>	Deficiency of 3-hydroxyacyl-CoA dehydrogenase	09C86928	M	hom c.676T>C:p.Y226H (NM_005327.4)	212734 (CM064046)	Likely pathogenic	11 / 11
<i>MYO15A</i>	Deafness, autosomal recessive 3	SAGA-96	F	het c.5515C>T:p.Q1839X (NM_016239.3)	na	(Likely Pathogenic)	na
				het c.8183G>A:p.R2728H (NM_016239.3)	228276 (CM117958)	Pathogenic	10 / 10
<i>NAGLU</i>	Sanfilippo syndrome B	AU-16201	F	homc.934G>A:p.D312N (NM_000263.3)	437446 (CM113463)	Pathogenic	12 / 12
<i>PAH</i>	Phenylketonuria	AU-16201	F	het c.782G>A:p.R261Q (NM_000277.1)	582 (CM910287)	Pathogenic	12 / 12
				het c.842C>T:p.P281L (NM_000277.1)	589 (CM910292)	Pathogenic	11 / 11
<i>POLG</i>	Alpers-Huttenlocher syndrome, Childhood myocerebrohepatopathy spectrum	AU-16201	F	hom c.3151G>A:p.G1051R (NM_002693.2)	13501 (CM040472)	Pathogenic	11 / 11
<i>USH2A</i>	Usher Syndrome, Type 2A	09C96107	M	het c.12714T>G:p.Y4238X (NM_206933.2)	48405 (CM134383)	Pathogenic	na

Gene	Condition	Sample identifier	Sex	Mutation	ClinVar (HGMD)	Variant pathogenicity	Damaging Predictions
<i>Found in 3 controls</i>							
<i>MCCC2</i>	3-methylcrotonyl-CoA carboxylase deficiency	AU-13303	F	het c.6224G>A:p.W2075X (NM_206933.2)	48554 (CM134384)	Pathogenic	
<i>PAH</i>	Phenylketonuria	AU-16503	F	hom c.898G>T:p.A300S(NM_000277.1)	92751 (CM920555)	Pathogenic	10 / 10
		AU-16502	M	hom c.898G>T:p.A300S(NM_000277.1)	92751 (CM920555)	Pathogenic	12 / 12
						Pathogenic	