Systematic evaluation of genome sequencing for the diagnostic assessment of autism spectrum disorder and fetal structural anomalies

Authors

Chelsea Lowther, Elise Valkanas, Jessica L. Giordano, ..., Harrison Brand, Ronald J. Wapner, Michael E. Talkowski

Correspondence

mtalkowski@mgh.harvard.edu

We evaluated genome sequencing (GS) as a single test to displace the sequential application of karyotype, chromosomal microarray, and exome sequencing, three standard-of-care tests used for the assessment of autism and fetal structural anomalies. Our data suggest GS warrants consideration as a first-tier diagnostic approach for these two phenotypes.

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ARTICLE

Systematic evaluation of genome sequencing for the diagnostic assessment of autism spectrum disorder and fetal structural anomalies

Chelsea Lowther,[1,2,3,22] Elise Valkanas,[1,2,4,22] Jessica L. Giordano,[5] Harold Z. Wang,[1,2] Benjamin B. Currall,[1,2,3] Kathryn O'Keefe,[1,2] Emma Pierce-Hoffman,[2] Nehir E. Kurtas,[1,2,3] Christopher W. Whelan,[2] Stephanie P. Hao,[1,2] Ben Weisburd,[1,2] Vahid Jalili,[2] Jack Fu,[1,2,3] Isaac Wong,[1,2] Ryan L. Collins,[1,2,6] Xuefang Zhao,[1,2,3] Christina A. Austin-Tse,[1,7] Emily Evangelista,[2] Gabrielle Lemire,[2] Vimla S. Aggarwal,[8] Diane Lucente,[1] Laura D. Gauthier,[2,9] Charlotte Tolonen,[2,9] Nareh Sahakian,[2,9] Christine Stevens,[2] Joon-Yong An,[10] Shan Dong,[11] Mary E. Norton,[12,20] Tippi C. MacKenzie,[12] Bernie Devlin,[13] Kelly Gilmore,[14] Bradford C. Powell,[15] Alicia Brandt,[15] Francesco Vetrini,[16] Michelle DiVito,[5] Stephan J. Sanders,[11] Daniel G. MacArthur,[1,2,18,19] Jennelle C. Hodge,[16] Anne O'Donnell-Luria,[1,2,17] Heidi L. Rehm,[1,2] Neeta L. Vora,[14] Brynn Levy,[8,21] Harrison Brand,[1,2,3,21] Ronald J. Wapner,[5,21] and Michael E. Talkowski[1,2,3,4,6,21,*]

Summary

Short-read genome sequencing (GS) holds the promise of becoming the primary diagnostic approach for the assessment of autism spectrum disorder (ASD) and fetal structural anomalies (FSAs). However, few studies have comprehensively evaluated its performance against current standard-of-care diagnostic tests: karyotype, chromosomal microarray (CMA), and exome sequencing (ES). To assess the clinical utility of GS, we compared its diagnostic yield against these three tests in 1,612 quartet families including an individual with ASD and in 295 prenatal families. Our GS analytic framework identified a diagnostic variant in 7.8% of ASD probands, almost 2-fold more than CMA (4.3%) and 3-fold more than ES (2.7%). However, when we systematically captured copy-number variants (CNVs) from the exome data, the diagnostic yield of ES (7.4%) was brought much closer to, but did not surpass, GS. Similarly, we estimated that GS could achieve an overall diagnostic yield of 46.1% in unselected FSAs, representing a 17.2% increased yield over karyotype, 14.1% over CMA, and 4.1% over ES with CNV calling or 36.1% increase without CNV discovery. Overall, GS provided an added diagnostic yield of 0.4% and 0.8% beyond the combination of all three standard-of-care tests in ASD and FSAs, respectively. This corresponded to nine GS unique diagnostic variants, including sequence variants in exons not captured by ES, structural variants (SVs) inaccessible to existing standard-of-care tests, and SVs where the resolution of GS changed variant classification. Overall, this large-scale evaluation demonstrated that GS significantly outperforms each individual standard-of-care test while also outperforming the combination of all three tests, thus warranting consideration as the first-tier diagnostic approach for the assessment of ASD and FSAs.

Introduction

Fetal structural anomalies (FSAs) and autism spectrum disorder (ASD) represent developmental defects that share significant overlap in genetic architecture[1–8] and clinical diagnostic recommendations.[9–16] Both are genetically heterogeneous and are associated with many of the same pathogenic variants (e.g., 22q11.2 deletions [MIM: 611867], point mutations in *CHD8* [MIM: 610528])[17,18] that have a wide range of potential clinical outcomes.[19,20] Broad and comprehensive testing strategies are required to maximize diagnostic sensitivity for FSAs and ASD, as it is difficult to predict the genetic basis of these conditions *a priori* due to the diversity of pathogenic variants contributing to these conditions[1–8] and widespread existence of variable expressivity.[21,22] The current standard-of-care testing for

[1]Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA, USA; [2]Program in Medical and Population Genetics, The Broad Institute of MIT and Harvard, Cambridge, MA, USA; [3]Department of Neurology, Harvard Medical School, Boston, MA, USA; [4]Program in Biological and Biomedical Sciences, Division of Medical Sciences, Harvard Medical School, Boston, MA, USA; [5]Department of Obstetrics & Gynecology, Columbia University Medical Center, New York, NY, USA; [6]Program in Bioinformatics and Integrative Genomics, Division of Medical Sciences, Harvard Medical School, Boston, MA, USA; [7]Department of Pathology, Harvard Medical School, Boston, MA, USA; [8]Department of Pathology and Cell Biology, Columbia University Medical Center, New York, NY, USA; [9]Data Science Platform, Broad Institute of MIT and Harvard, Cambridge, MA, USA; [10]School of Biosystem and Biomedical Science, Korea University, Seoul, South Korea; [11]Department of Psychiatry, UCSF Weill Institute for Neurosciences, University of California, San Francisco, San Francisco, CA, USA; [12]Center for Maternal-Fetal Precision Medicine, University of California, San Francisco, San Francisco, CA, USA; [13]Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA; [14]Department of Obstetrics and Gynecology, Division of Maternal-Fetal Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [15]Department of Genetics, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [16]Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN, USA; [17]Division of Genetics and Genomics, Boston Children's Hospital, Boston, MA, USA; [18]Centre for Population Genomics, Garvan Institute of Medical Research, and University of New South Wales Sydney, Sydney, NSW, Australia; [19]Centre for Population Genomics, Murdoch Children's Research Institute, Melbourne, VIC, Australia; [20]Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco, San Francisco, California, USA
[21]Senior authors
[22]These authors contributed equally
*Correspondence: mtalkowski@mgh.harvard.edu
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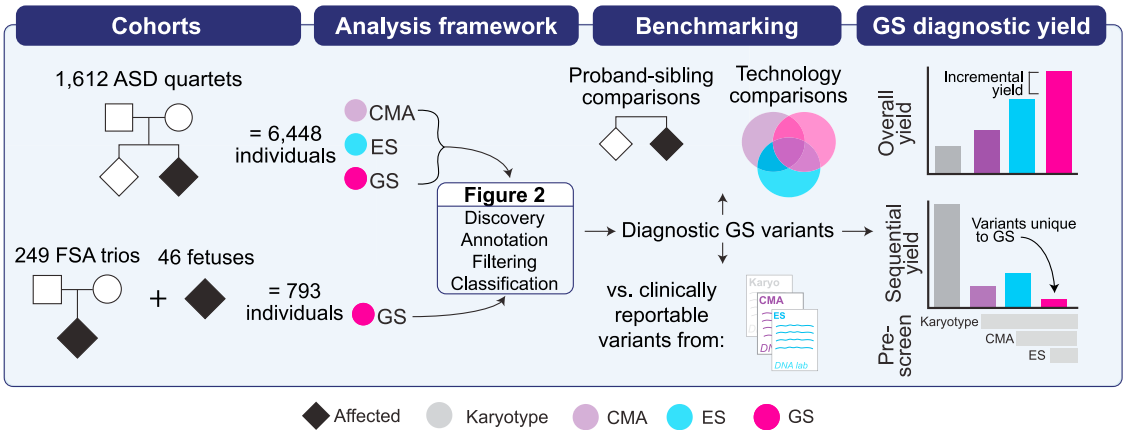

Figure 1. Overall study design

We performed genome sequencing (GS) on 7,241 individuals from two phenotypically ascertained cohorts: autism spectrum disorder (ASD) and fetal structural anomalies (FSAs). The ASD quartet families (n = 6,448 individuals) included one affected proband with ASD, one unaffected sibling, and two unaffected parents. The prenatal cohort included 249 trios (n = 747 individuals) comprising a fetus with an FSA detected by ultrasound and two unaffected parents as well as 46 singleton fetuses ascertained for a diagnostic procedure performed in pregnancy. Fetuses from the 249 trios were pre-screened with one or more standard-of-care diagnostic tests (karyotype, chromosomal microarray [CMA], and/or exome sequencing [ES]) and the 46 singleton fetuses were pre-selected on the basis of having a clinically reportable variant identified by one of the same three standard-of-care tests. For the 1,612 ASD quartet families, we had access to unfiltered data from CMA, ES, and GS available for analysis (see subjects and methods for more details). We performed multiple benchmarking analyses, including comparing the yield of diagnostic variants between ASD probands and their unaffected siblings, direct technology comparisons in the ASD probands, and comparisons against results from clinical diagnostic tests in the fetuses. We assessed the performance of GS by considering the overall, incremental, and sequential diagnostic yields provided by this technology. Plots are demonstrative only and are not drawn to scale nor reflective of real data.

genome-wide genetic surveys involves three orthogonal and largely complementary diagnostic tests: karyotype to discover microscopically visible balanced and unbalanced chromosomal abnormalities, chromosomal microarray (CMA) to capture sub-microscopic copy-number variants (CNVs), and exome sequencing (ES) to identify single-nucleotide variants (SNVs) and small insertions and deletions (indels) within the ~2% of the genome that codes for proteins.[9–16] All three tests are required to capture the full range of genetic variation currently known to be associated with FSAs and ASD. This sequential diagnostic testing strategy is inefficient in the prenatal setting where rapid diagnosis is critical and cumbersome in the pediatric setting where families can be easily lost to follow-up as a result of an unnecessarily long diagnostic odyssey.[23]

Short-read genome sequencing (GS) has the potential to identify almost all pathogenic variation captured by these currently applied technologies in a single test as well as potentially discovering novel diagnostic variants that are cryptic to current approaches.[24–26] To date, studies performing GS for the diagnostic assessment of FSAs and neurodevelopmental disorders (NDDs), of which ASD is a subtype, have only included small cohorts of highly selected individuals with disparate diagnostic pre-screening, resulting in variable GS diagnostic yields ranging from 19.8% to 57.7% for FSAs[27–34] and 30% to 50% for ASD/NDDs.[35–37] These GS studies typically do not provide the opportunity for direct technology comparisons, as multiple standard-of-care tests are rarely available on the same individuals. Given that no single study has quantified the performance of GS against karyotype, CMA, and ES, the added value of GS remains unknown for most phenotypes, including for FSAs and ASD.

The goal of this study was to systematically evaluate the performance of GS against the current standard-of-care diagnostic tests for the assessment of FSAs and ASD. We developed a comprehensive GS analytic framework that characterized nine different classes of genetic variation while maintaining a manageable burden of manual variant review, which currently presents a significant barrier to the widespread implementation of clinical GS.[38,39] We tested our GS analytic framework on 1,612 systematically collected ASD quartet families (n = 6,448 individuals total), which represented an ideal technical benchmarking cohort because each individual in the family had GS and matched CMA and ES data available for re-analysis. To assess the diagnostic yield of GS in FSAs, we applied our analytic framework to 295 prenatal families that had clinical results from karyotype, CMA, and/or ES available for comparison. The diagnostic yields from these large-scale studies suggest that a shift toward recommending GS as a first-tier diagnostic test for the assessment of ASD and FSAs is warranted.

Subjects and methods

Study subjects

We applied our short-read GS analytic framework to 1,612 ASD quartet families from the Simons Foundation for Autism Research Initiative (SFARI) Simons Simplex Collection (SSC; n = 6,448 individuals total; Table S1).[40] Each quartet family comprised one proband diagnosed with ASD, one unaffected sibling, and two

unaffected parents (Figure 1). The ASD cohort was chosen as the primary technical comparison for our GS pipeline because every individual had CMA, ES, and GS data available for re-processing. This facilitated direct technology comparisons that were not impacted by differences in bioinformatic analyses, variant interpretation methods, and/or assessment timepoints.[41] Additionally, given the significant overlap in the types of variants that contribute to ASD and FSAs, particularly SVs,[1,6] the larger size of the ASD cohort enabled the discovery and interpretation of a broader spectrum of diagnostic variants. All participants or their legal guardians provided written informed consent for participation and their data were de-identified by SFARI before sharing with qualified researchers.[40]

We next applied the same analytic framework to 295 fetuses that met criteria for diagnostic testing because of the presence of a structural anomaly (n = 281) or advanced maternal age (AMA) (n = 14; Figure 1). The 295 fetuses included 249 trios (n = 747 individuals) comprising a fetus with a structural anomaly detected by ultrasound and two unaffected parents. Of the 249 FSAs, 85.5% (n = 213) were prescreened (e.g., no diagnostic variant identified) with CMA, the current recommended first-tier diagnostic test for fetuses with structural anomalies,[1] 67.0% (n = 167) with karyotype, and 35.3% (n = 88) with ES. With respect to overlapping tests, 58.6% of the FSA cohort had negative results from both CMA and karyotype and 6.4% had negative results from all three tests (Table S2). We also included 46 singleton fetuses (n = 32 FSAs and n = 14 AMA) that were pre-selected for carrying a clinically reportable variant (n = 53) detected by karyotype, CMA, or ES. We used these samples to benchmark the performance of GS against tests performed in clinical diagnostic laboratories (Table S3). We also wanted to explore the potential for GS to discover variants originally identified by karyotyping. The prenatal cohort includes fetuses recruited from the Carmen and John Thain Center for Prenatal Pediatrics at Columbia University (n = 160), the University of California San Francisco (UCSF; n = 59), and the Prenatal Diagnosis Program at the University of North Carolina Chapel Hill (UNC; n = 30). A subset of the fetuses have had their karyotype, CMA, and ES data previously published.[1,3,42–44] This study was approved by the institutional review boards at Mass General Brigham, Columbia University, UNC, and UCSF. All participants or their legal guardians provided written informed consent prior to participation.

GS and sample level quality control

All 7,241 samples analyzed in this study underwent short-read Illumina GS following standard library protocols to a mean genome coverage of >30× (Tables S1–S3; additional details in the supplemental methods). Whole-blood-derived DNA was sequenced for every individual in the ASD cohort and all the unaffected parents from the fetal structural anomaly trios. Fetal DNA was obtained from chorionic villi, amniocytes, umbilical cord blood, or products of conception. Sample relatedness was confirmed for all individuals via KING[32] and all pregnancies were genetically confirmed to have arisen from non-consanguineous unions (Figure S1). We also used GS data to infer genetic sex by using PLINK[45] and depth-based chromosomal analyses (Figure S2; details in supplemental methods).

GS analytic framework

We developed a framework to identify pathogenic and likely pathogenic (P/LP) variants from GS data with high sensitivity while limiting the number of variants requiring manual review (Figure 2; Table S4). The framework is organized into four components: variant discovery, annotation, filtering, and manual classification. Additional details on the framework can be found in the supplemental methods.

Variant discovery

Variant discovery identified nine different classes of genetic variation, including SNVs, indels, deletions and duplications that ranged from 50 base pairs to full chromosomal aneuploidies, inversions, insertions, translocations, complex rearrangements (16 different sub-classes),[54] and short tandem repeats (STRs), via a suite of algorithms.[55–62] All samples were jointly processed in batches following GATK Best Practices Workflows for SNV and indel discovery with Terra.[63] The SV discovery and genotyping was performed across all samples with GATK-SV, a publicly available cloud-enabled ensemble method that leverages data from multiple SV algorithms to boost sensitivity and filters to improve specificity.[24,54] Here, we ran six individual SV detection algorithms[55–60] on all samples and then ran GATK-SV in cohort mode (a single sample version of GATK-SV is also available as a workflow on Terra). We used GATK-SV for filtering, genotyping, breakpoint refinement, and complex variant resolution to produce a VCF for each cohort. Finally, we ran ExpansionHunter to identify potentially diagnostic STR expansion candidates.[61]

Variant annotation

All variants (SNVs, indels, SVs, and STRs) were annotated for genic overlap and functional consequences against GENCODE v.26 gene boundaries based on the canonical transcript.[64] Sequence variants (SNVs and indels) were annotated with ANNOVAR[65] and any variants predicted to be stop-gain, stop-loss, frameshift insertion, frameshift deletion, or splicing (within 2 bp of a splice junction) according to RefSeq or GENCODE annotations were considered loss of function (LoF). SVs were annotated with GATK-SV and functional consequence was determined for each SV type. LoF SVs were defined as any deletion overlapping coding sequence, an inversion, mobile element insertion, complex SV, or translocation with one or more breakpoints disrupting coding sequence, or an intragenic exonic duplication (a duplication that overlaps coding sequence with both breakpoints contained within the same gene boundary). Full gene copy gains were defined as duplications that fully overlap a gene boundary. Partial gene duplications were defined as duplications with one breakpoint located within the gene boundary and one outside.[46] Additionally, we annotated allele frequency (AF) for all SNVs and indels by using the maximum AF across gnomAD genomes,[66] gnomAD exomes/ExAC,[67] the 1000 Genomes Project,[68] and parental samples from each cohort. The SV and STR allele frequencies were calculated based on the prevalence of each event in gnomAD.[54,69]

Variant filtering

We first filtered variants on the basis of predicted functional impact. For SNVs and indels, we retained all variants annotated as LoF or missense variants that had a CADD score > 15[70] and were not annotated as benign, likely benign, risk factor, association, drug response, or protective in ClinVar.[71] All SNVs and indels that passed our quality control and allele frequency thresholds were retained if they were predicted to functionally alter a gene on our list of disease-associated genes (see Table S4 and supplemental methods for specific thresholds). The only aspect of our

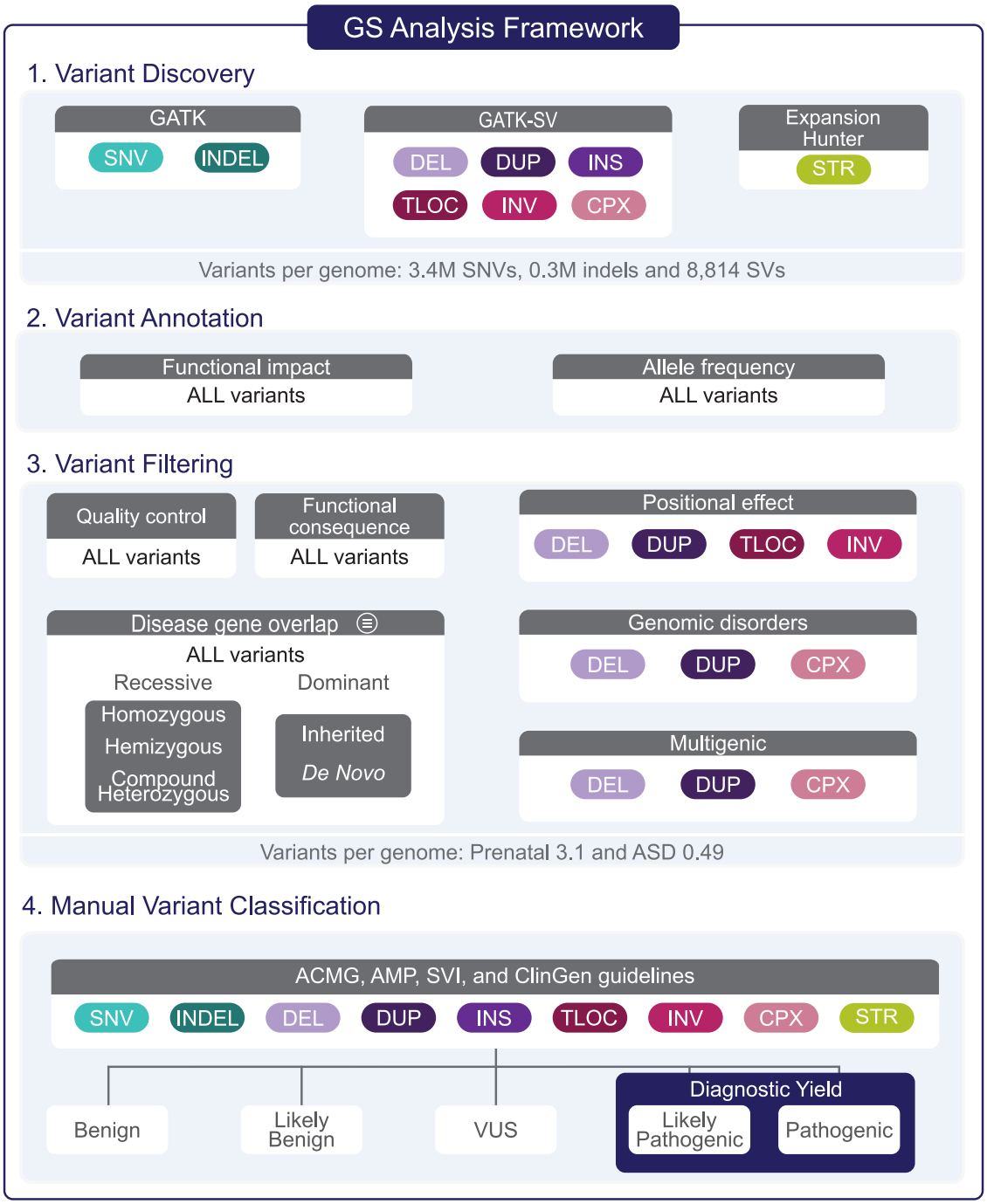

Figure 2. Genome sequencing analytic framework

The comprehensive framework we developed to identify diagnostic variants from GS data, which consists of four components: variant discovery, variant annotation, variant filtering, and manual variant classification. We identified nine different variant classes, including single-nucleotide variants (SNVs), small insertions and deletions (indels; below 50 base pairs), deletions (DELs) and duplications (DUPs) that ranged from over 50 base pairs to full chromosomal aneuploidies, insertions (INSs), translocations (TLOCs), inversions (INVs), complex rearrangements (CPXs), and short tandem repeats (STRs). The filtering strategy was designed to retain P/LP variants while limiting the number of variants requiring manual variant classification. The specific filtering criteria are described in the supplemental methods. All variants output by the filtering pipeline were manually curated by an expert variant review panel following existing clinical guidelines.[46–53] All variants classified as P/LP in genes associated with the indication for testing were considered to represent the diagnostic yield of GS. ASD, autism spectrum disorder; GATK, Genome Analysis Toolkit; SV, structural variant; VUS, variant of uncertain significance; ACMG, American College of Medical Genetics and Genomics; AMP, Association for Molecular Pathology; SVI, Sequence Variant Interpretation Working Group; ClinGen, Clinical Genome Resource.

GS analytic framework that differed between the ASD and prenatal cohorts was the content of the gene lists, which were phenotype-specific and computationally derived to limit the burden of up-front gene curation.[39,72] Briefly, the ASD gene list included 901 genes classified as having a "confirmed" or "probable" association with NDDs in the Developmental Disorders Genotype-Phenotype Database (Table S5).[73] To account for the phenotypic heterogeneity of the structural anomalies observed in our FSA cohort (Tables S2 and S3), we compiled a separate list of 2,535 genes from eight sources that are broadly associated with developmental disorders and congenital anomalies (Table S6 and supplemental methods). All variants were then filtered under four genotype categories (*de novo*, rare inherited, homozygous, and hemizygous) depending on the specific mode(s) of inheritance of the gene-disease association (dominant, recessive, or X-linked). Finally, we applied more stringent filters (described in supplemental methods) to inherited, homozygous, compound heterozygous, and hemizygous missense variants given that they contributed significantly to the number of variants requiring manual review but have not been shown to substantially contribute to the etiology of ASD or FSAs.[2–4,74]

A hierarchical filtering process was applied to all SVs. First, SVs predicted to be LoF or full gene copy gains were retained and partial gene duplications were excluded given their unknown functional impact.[46] Then, following current recommendations, multigenic CNVs (deletions and duplications overlapping $\geq$35 and $\geq$50 protein-coding genes, respectively)[46] were prioritized for manual classification regardless of whether any of the genes have been previously associated with disease. Next, any rare SV overlapping one of the 64 known genomic disorder loci (Table S7) or the 17 noncoding loci associated with pathogenic positional effects (Table S8) were retained. The SVs that did not meet any of the preceding criteria were then filtered on the basis of their overlap with the phenotype-specific disease-associated gene lists following the same inheritance patterns and allele frequency thresholds described above. All STRs that exceeded a pathogenic repeat length based on literature review were retained if they overlapped an STR-mediated locus associated with an early-onset developmental disorder (18 loci described in Table S9). Finally, the identification of candidate compound heterozygous variants comprised three filtering steps: (1) compiling heterozygous SNVs, indels, and LoF SVs located in the same recessive disease-associated gene; (2) annotating each variant with inheritance status; and (3) retaining only the instances where individuals had more than one variant in a recessive disease-associated gene with disparate inheritance patterns (e.g., one maternally inherited, one *de novo*). To retain variants in *trans*, we used inheritance as a proxy for phasing and required that at least one variant per compound heterozygous grouping be inherited from a parent (e.g., not all could occur *de novo*).

Manual variant classification

To ensure all variants were high quality, we visually inspected the read evidence for each candidate diagnostic variant output by our filtering pipeline by using the Integrated Genomics Viewer for SNVs, indels, and SVs;[75] CNView for CNVs;[76] and REViewer for STRs.[77] All variants that passed manual visual inspection were assessed by a variant review panel consisting of board-certified clinical geneticists, cytogeneticists, molecular geneticists, obstetricians, maternal-fetal specialists, pediatricians, and genetic counselors as well as population geneticists and bioinformaticians with expertise in SV identification and interpretation. All variants were first evaluated for a gene-phenotype association on a individual-specific basis.[78] If a reliable match was determined for the individual in question, all variants in that gene were reviewed following guidelines for sequence variant and CNV interpretation from the American College of Medical Genetics and Genomics (ACMG), the Association for Molecular Pathology (AMP), the Clinical Genome (ClinGen),[46,47] and recommendations for adjusting the standard clinical guidelines from the ClinGen Sequence Variant Interpretation (SVI) Working Group.[48–53] Overall, these guidelines provide a systematic and robust method to identify variants with a 90% or greater certainty of causing disease.[47] This method is reliably reproduced across laboratories[79] and rarely results in downgrading P/LP variants over time.[80] All variants classified as P/LP in a gene robustly associated with the individual's phenotype (e.g., the indication for testing) were considered a molecular diagnosis and were counted toward the diagnostic yield of GS.

Benchmarking the performance of the GS analytic framework

ASD proband vs. unaffected sibling comparisons

The quartet family structure of the ASD cohort provided us with a unique opportunity to evaluate our bioinformatic filtering and variant classification methods by comparing the number of variants output at each step between the affected probands with ASD and their unaffected siblings. To confirm that our filtering pipeline was enriching for potentially pathogenic variants as intended and assess the potential false positive rate of the variant interpretation guidelines, we treated each ASD proband and their unaffected sibling as separate trios with both parents. After filtering, we compared the number of variants requiring manual review in the ASD probands to their unaffected siblings then manually reviewed all variants blind to affected status (e.g., all variants were reviewed as if the child was diagnosed with ASD). We then compared the fraction of P/LP variants identified between these two groups.

Cross-technology comparisons

To quantify the sensitivity of GS against CMA and ES, we first leveraged the ASD cohort, which had unfiltered data for each technology available for re-analysis. For the CMA analysis, we obtained CNVs identified from Illumina single-nucleotide polymorphism (SNP) microarrays that were processed as previously described.[6] Briefly, SNP genotyping data were generated via three Illumina CMA platforms and CNVs were identified from these data via PennCNV,[81] QuantiSNPv2.3,[82] and GNOSIS/CNVision.[83] All CNVs identified from CMA were lifted over from GRCh37 to GRCh38 for comparisons against ES and GS. For the ES analysis, we used the SNV, indel, and CNV calls that were generated as part of a larger ASD sequencing initiative.[4,5] To summarize, raw reads from all 6,448 samples were aligned to GRCh38 and SNV and indel discovery was performed with GATK v.4.1.2.0.[62] All samples were jointly genotyped following GATK Best Practices for Variant Calling.[63] We also employed GATK-gCNV for exome CNV detection,[84] a new algorithm that is specifically designed to adjust for known bias factors of exome capture and sequencing (e.g., GC content), while automatically controlling for other technical and systematic differences. The GATK-gCNV workflow is publicly available in a Terra workspace. We applied the same version of our GS analytic pipeline to the CMA and ES data from all 6,448 individuals in the ASD quartet families. The only

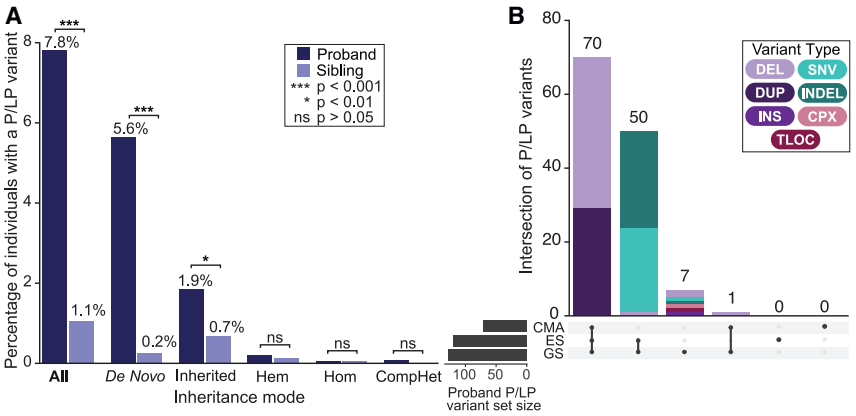

Figure 3. Benchmarking the performance of GS in ASD probands and unaffected siblings

(A) The fraction of ASD probands and unaffected siblings identified to carry a P/LP variant by GS subset by inheritance category. The denominator used for all categories was 1,612 except for hemizygous variants where only males were considered (n = 1,440 male probands and 755 male siblings). p values were calculated by comparing the fraction of probands and siblings with a P/LP variant using Fisher's exact test.

(B) The total number of P/LP variants (n = 128) detected by each technology (GS, CMA, and ES) in n = 126 ASD probands.

modification made was to the allele balance and depth filters to accommodate for the higher coverage of ES compared to GS (Figure S3).

We also analyzed GS of 46 fetuses that were pre-selected for receiving a clinically reportable finding from karyotype, CMA, or ES. Inclusion of these benchmarking fetal samples allowed us to investigate the impact of DNA source (whole blood vs. chorionic villi or amniocytes) on the performance of GS as well as evaluate the ability of GS to identify a range of cytogenetically visible balanced chromosomal rerrangements (BCRs). Each recruitment site provided us with the list of clinically reported variants found in each fetus by using their in-house methods and pipelines (e.g., raw data were not available for re-analysis).[1,3,42–44] We identified STRs across 18 loci (Table S9) in the ASD and FSA cohorts, despite there being no clinical STR test results available for direct comparison. Previous studies using the same computational approach have demonstrated 97.3% and 99.6% sensitivity and specificity against existing PCR tests,[85] respectively. The sensitivity of GS was calculated as the proportion of P/LP variants identified by each diagnostic test (karyotype, CMA, and ES) that were also identified by GS.

Application of GS to a prescreened fetal structural anomaly cohort

After systematically benchmarking the GS analytic framework, we applied it to 249 retrospectively obtained fetal structural anomaly trios (n = 747 individuals) that had been pre-screened with karyotype, CMA, and/or ES (Table S2). The analysis performed on the FSA trios was identical to that applied to the benchmarking samples described above. The added diagnostic yield of GS in this cohort was calculated on the basis of the number of P/LP variants identified by GS.

Results

Assessment of the GS analytic framework

We analyzed short-read GS data from 1,612 ASD quartet families (n = 6,448 individuals) that also had matched CMA and ES data available to directly compare the relative value of each technology. Overall, our GS variant calling methods identified an average of 3.7M short variants (3.4M SNVs, 0.3M indels) and 8,814 SVs per genome that passed filtering criteria as well as 115,821 STR genotypes at 18 targeted disease loci across the cohort. Our filtering strategy reduced the number of variants requiring manual curation to an average of 0.49 variants per child (range = 0–9), totaling 1,743 variants across 901 NDD-associated genes and loci (Table S5) in the ASD probands and unaffected siblings. We observed an enrichment of variants requiring manual review per person in the ASD probands compared to their unaffected siblings (0.58 mean variants per ASD proband and 0.39 per unaffected sibling; $p = 4.12 \times 10^{-14}$; two-sided Wilcoxon test), suggesting that our filtering pipeline was accurately enriching for potentially pathogenic variants. Demonstrating the power of the interpretation guidelines, this proband enrichment further increased following manual variant curation, which identified 128 P/LP variants in 126 ASD probands (7.8% yield; 95% CI 6.5–9.1) compared to 17 P/LP variants in unaffected siblings (1.1% yield; 95% CI 0.6–1.6; odds ratio [OR] = 7.9; 95% CI = 4.7–14.1; $p = 2.2 \times 10^{-16}$; Fisher's exact test; Figure 3; Table S10). Importantly, 71% of the P/LP variants identified in siblings included CNVs associated with reduced penetrance, which are known to be a challenge for genetic counseling and are already encountered by clinicians during routine CMA testing.

Evaluating the diagnostic performance of GS

We benchmarked the diagnostic performance of GS against standard-of-care tests by applying the equivalent GS framework to the CMA and ES data from the ASD cohort, with minor modifications to accommodate each data type (Figure S3; supplemental methods). Overall, GS identified a diagnostic variant in almost 2-fold more probands than CMA (n = 126 vs. n = 71; OR = 1.8; 95% CI 1.3–2.5; $p = 6.5 \times 10^{-5}$) and almost 3-fold more than ES (n = 126 vs. n = 49; OR = 2.7; 95% CI 1.9–3.9; $p = 1.98 \times 10^{-10}$) (Figure 3). When we used a new method to capture CNVs from ES data (GATK-gCNV),[84] the overall diagnostic yield of ES approached that of GS (7.4% vs. 7.8%, respectively), though it still did not capture all

known P/LP variants. For example, a single exon deletion overlapping the first exon of *NRXN1* (MIM: 600565) identified by GS was missed by ES because it did not pass our stringent filtering criteria that required CNVs to overlap >2 exons.[5,84] Manual inspection revealed the deletion was present in the raw ES CNV calls, suggesting strategies for clinical exome CNV calling could consider relaxing filtering for pre-defined disease-associated genes, particularly for those where CNVs are a known mechanism of disease.[86]

Overall, GS captured 100% of the P/LP variants identified by CMA (n = 71) and ES (n = 118) while also uniquely identifying an additional diagnostic variant in seven (0.4%) ASD probands (Figure 3). We reviewed the properties of the variants uniquely identified by GS, which included one SNV and one indel: a *de novo* stop-gain in *ANKRD11* (MIM: 611192) and a 44 bp *de novo* frameshift insertion in *SMARCA4* (MIM: 603254), and five SVs: single exon deletions in *RERE* (MIM: 610226) and *RORA* (MIM: 600825),[87,88] a reciprocal translocation disrupting *GRIN2B* (MIM: 138252), an SVA retrotransposon insertion in *DMD* (MIM: 300377), and a 47.2 Mb complex SV involving chromosome 1 comprised of four deletions, an inversion, and an inverted insertional translocation (Table S11). The *ANKRD11* stop-gain was in an exon with no ES coverage and the *SMARCA4* insertion was within 30 bp of an intron-exon boundary and was not present in the ES read evidence (Figure S4). In contrast to the single exon *NRXN1* deletion described above, the smaller *RERE* (5.6 kb) and *RORA* (0.5 kb) deletions identified by GS were not detectable in the raw ES data, suggesting that ES will not be able to capture all single-exon deletions of clinical relevance. As expected, CMA and ES were unable to detect the balanced translocation. Similarly, while CMA and ES both detected the four *de novo* deletions involved in the complex SV, they were unable to identify the inversions that link the deletions into a single event. Finally, we did not apply a mobile element insertion algorithm to the ES data, as it is not currently implemented in routine clinical diagnostics,[89] but this type of ES analysis could potentially capture variants labeled as GS unique in this study, such as the SVA insertion. Taken together, these data demonstrate that GS outperforms both CMA and ES, capturing all P/LP variants from these two technologies and providing a modest increase in diagnostic yield beyond the combination of both diagnostic tests.

Using DNA obtained from diagnostic procedures performed in pregnancy, we next confirmed the benchmarking results in prenatal samples as well as assessed the performance of GS to detect BCRs routinely identified by karyotype. We chose 46 fetuses that carried 53 reportable variants identified from standard clinical testing due to AMA (n = 14) or ultrasound detection of an FSA (n = 32) (Table S3). These variants included seven aneuploidies, 20 CNVs, and 18 SNVs or indels (including four compound heterozygous variant pairs), all of which are commonly observed in prenatal testing. This benchmarking cohort was also highly enriched for BCRs (n = 8/46 fetuses; 17.4% here vs. 3.0% estimated prevalence across all FSAs).[1] Overall, GS captured 100% of the clinically reportable CNVs and SNVs/indels originally identified by CMA (n = 20) and ES (n = 12) and 62.5% of the BCRs identified by karyotype (n = 5/8). On the basis of the reported karyotype, the three BCRs not captured by GS are localized to highly repetitive telomeric and centromeric regions, which are known to be inaccessible to short-read GS.[26] This class of missed BCRs account for <1% of the total diagnostic yield provided by karyotype in FSAs.[1]

Determining the added diagnostic yield of GS for the assessment of fetal structural anomalies

After systematically benchmarking the performance of our GS analytic framework, we applied it to 249 fetus-parent trios that were pre-screened with karyotype, CMA, and/or ES. The structural anomalies impacted a wide range of organ systems and 36.1% (n = 90/249) of the cohort had multisystem involvement (Figure 4; Table S2). GS identified 816 candidate variants requiring manual review, resulting in an average of 3.1 variants per fetus (median = 3.0, range = 0–21). The increased number of variants output by our GS filtering in fetuses compared to the ASD probands is due to a greater number of SNVs and indels across the larger gene list used, with an average of 2.65 sequence variants across n = 2,535 genes for the fetal cohort compared to an average of 0.31 sequence variants across n = 901 genes for the ASD cohort. Manual variant curation identified 21 P/LP variants in 19 (7.6%) fetuses with a structural anomaly (Table S12). On the basis of our benchmarking analyses, the majority (n = 17/19; 89.5%) of these molecular diagnoses would have also been identified by a combination of contemporary CMA and ES. For example, 78.9% (n = 15/19) of the diagnoses included SNVs and indels identified in fetuses that had not previously undergone ES. Similarly, GS identified a 67 kb deletion in *MED13L* (MIM: 608771) and a maternal uniparental disomy (UPD) event involving chromosome 20 in two fetuses who had previously undergone array comparative genomic hybridization (aCGH). The *MED13L* deletion was missed because the custom aCGH platform did not have probe coverage over the region and the UPD was missed because regions of homozygosity are not identifiable without the analysis of SNP probes, which are absent from aCGH.[1] These data demonstrate the importance of taking previous diagnostic testing, technology platforms, and analysis pipelines into consideration when reporting comparative diagnostic yields.

The most conservative estimate therefore suggests that GS uniquely provided a molecular diagnosis in two FSA probands: a single exon deletion in *MED13L* (1.3 kb in size) and a compound heterozygous variant pair comprising a missense variant in *trans* with a 143 kb intragenic exonic duplication in *DYNC2H1* (MIM: 603297). While the identification of the compound heterozygous variants is technically feasible with the combination of

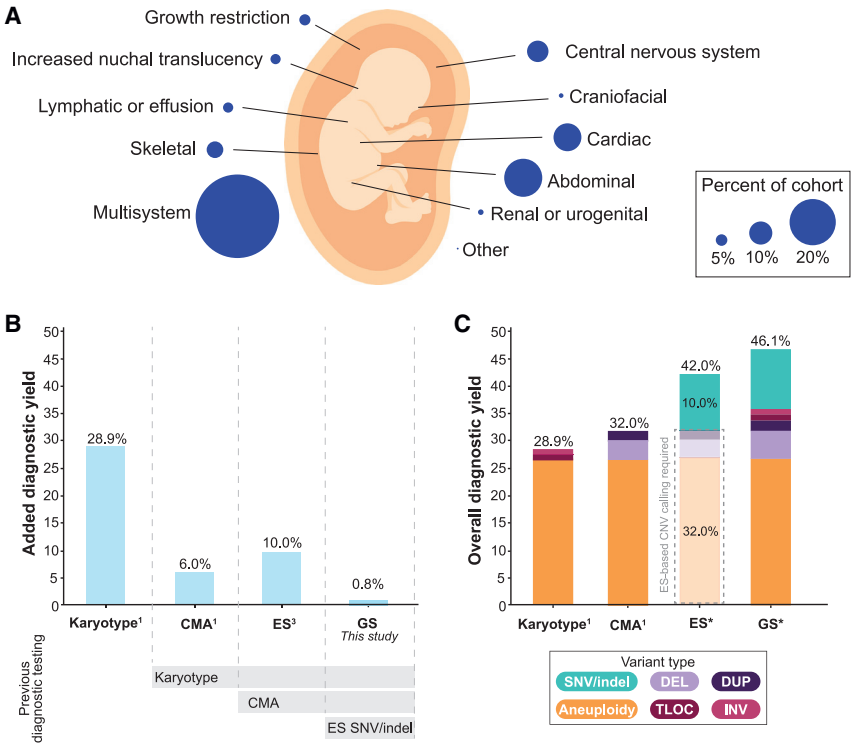

Figure 4. Overview of fetuses with structural anomalies and diagnostic yields across technologies

(A) The phenotypic breakdown of 249 trio fetuses identified to have a structural anomaly detected by ultrasound included in this study. The fetuses were pre-screened with a combination of standard-of-care diagnostic tests (see subjects and methods for details). Fetuses with anomalies impacting more than one body system were counted as having multisystem abnormalities. The remaining categories represent fetuses with isolated structural anomalies.

(B) The added diagnostic yield for each sequencing technology when applied serially to pre-screened fetuses. Each technology is assessed in a cohort that was depleted for diagnostic variants detected by the preceding technology. Yields for karyotype and CMA were taken from Wapner et al.[1] and yields for ES from Petrovski et al.[3]

(C) The estimated overall diagnostic yield provided by each diagnostic test if they were applied to a cohort of unselected fetuses with structural anomalies. *The yields were predicted on the basis of data from this study as well as previously published work.[1,3] The dashed gray box surrounding the ES bar indicates the diagnostic yield that could be captured if ES-based CNV methods are applied.[5,84] Each bar is colored on the basis of the fraction of diagnoses provided by each variant class. CMA, chromosomal microarray; ES, exome sequencing; GS, genome sequencing; SNV, single-nucleotide variant; indel, small insertion and deletion; CNV, copy-number variant; DEL, deletion; DUP, duplication; TLOC, translocation; INV, inversion.

CMA and ES, most clinical analysis pipelines do not systematically integrate variants across technologies. Instead, diagnostic laboratories often manually follow-up on individual genes when there is a strong *a priori* suspicion of a gene-phenotype match, as was true for this fetus in clinic. A pathogenic missense variant in *DYNC2H1* was identified by ES in a fetus with short-rib thoracic dysplasia. Given the specificity of the gene-phenotype association,[90] the diagnostic laboratory manually reviewed the ES read depth profile across this gene, identified the duplication, and confirmed the event with fluorescence *in situ* hybridization.[42] While this ultimately represented a successful approach for this fetus, it is not systematic and the increased burden of these additional steps is unlikely to scale, particularly for phenotypes associated with multiple recessive genes. Overall, these data suggest that GS provided a 0.8% increase in diagnostic yield beyond the combination of karyotype, CMA, and ES in these FSA trios (Figure 4).

Classification of SVs unique to GS

Over 75% (n = 7/9) of the diagnostic variants uniquely identified by GS in the ASD and FSA cohorts were SVs (Figure 5), including SVs below the resolution of and/or inaccessible to existing standard-of-care tests (n = 5) and SVs for which the base pair resolution provided by GS resulted in a medically relevant change in classification from variant of uncertain significance (VUS) to P/LP (n = 2).[80] Notably, while STRs represent a variant class uniquely identifiable from GS, we did not identify any STRs that met P/LP criteria in the ASD or FSA cohorts. As studies examining the contribution of STRs to disease risk increase,[85,91,92] we expect the interpretation of these variants to improve. Indeed, predicting the functional consequences of many GS-unique SVs was challenging, particularly for in-frame single exon deletions like the 5,618 bp *de novo* deletion in *RERE* in an ASD proband. For small rare in-frame CNVs (e.g., that disrupt <10% of the protein),[50] evidence that the altered exon codes for a functional unit of the protein is one way to increase classification of the variant. However, this type of exon-level annotation is unavailable for most genes, suggesting that gene-level metrics quantifying the impact of in-frame CNVs would be of value.

GS also identified SVs that could only be classified as diagnostic using the resolution uniquely provided by this technology, such as the pathogenic balanced translocation disrupting *GRIN2B* in an ASD proband.[24] Reciprocal translocations identified by karyotype are routinely reported back to families, but very little can be said about their contribution to the phenotype because the precise location of the breakpoints, and thus the predicted functional impact, remains unknown.[93–95] Indeed, our previous work has demonstrated that GS revises the location of cytogenetically visible BCRs by one or more cytogenetic bands in over 93% of individuals,[46,93] suggesting that conclusions about pathogenicity for the indication for testing

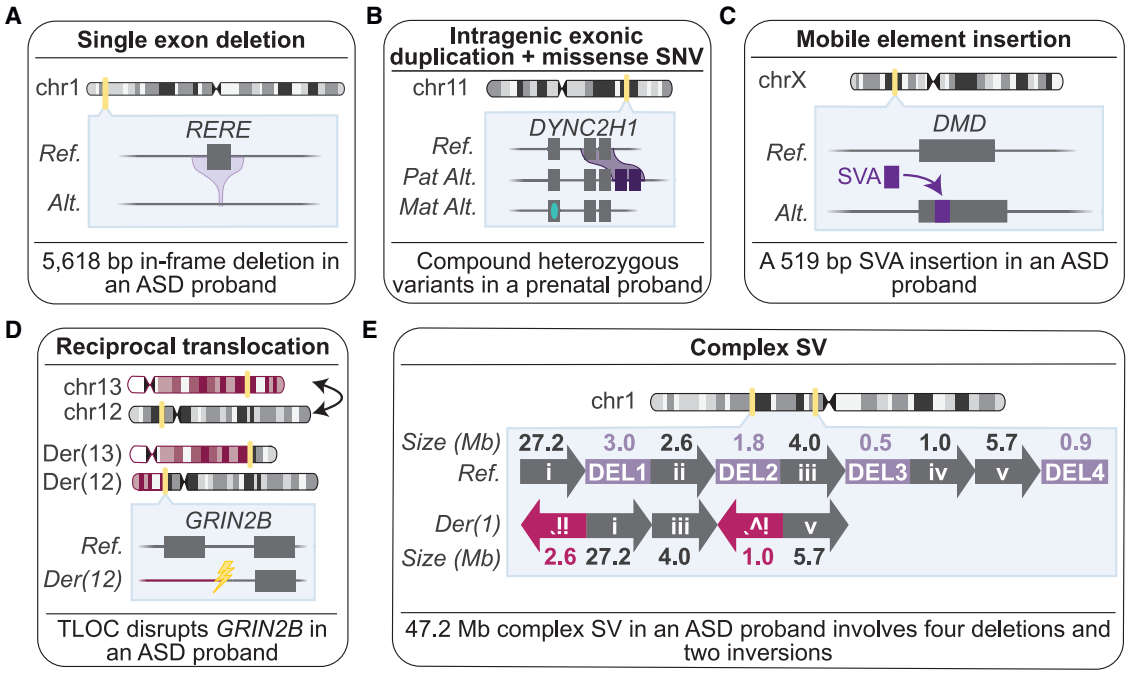

Figure 5. Examples of diagnostic structural variants uniquely identified by genome sequencing
(A) A 5,618 bp single exon in-frame deletion in *RERE* in an ASD proband.
(B) A compound heterozygous missense variant *in trans* with an intragenic exonic duplication in *DYNC2H1* in a fetus with short-rib thoracic dysplasia.
(C) An SVA retrotransposon insertion disrupting *DMD* in an ASD proband.
(D) A balanced reciprocal translocation between chromosomes 12 and 13 in an ASD proband that directly disrupts *GRIN2B*.
(E) Linear representation of a *de novo* complex SV impacting chromosome 1 in an ASD proband. Each rearranged segment of DNA in the derivative chromosome is depicted by a unique roman numeral (i–v), while the four deleted segments of DNA are outlined in purple and sequentially numbered DEL 1–4 (6.3 Mb total deleted). Arrows and chromosomes are not drawn to scale. Inverted segments are denoted by a reverse orientation of arrows. Genomic coordinates for this variant are provided in Table S11.

cannot be drawn on the basis of karyotype results alone. Similarly, we identified a pathogenic *de novo* 47.2 Mb complex SV in an ASD proband that was only resolved by GS. Current guidelines recommend the individual assessment of CNVs involved in a complex SV; however, GS can resolve complex SVs to a single event so there is strong rationale to evaluate the overall rearrangement in diagnostic classification. In this study, we applied the gene-number thresholds to the total number of genes overlapped by all four deletions to classify this complex SV as LP, but we note that these thresholds were derived from very large canonical CNVs and did not include the analysis of complex SV.[46] To improve gene-number thresholds, future studies could consider including complex SVs as well as CNVs below the resolution of CMA, which are now robustly detectable with GS.[96] Taken together, these data provide specific examples of the types of variants, particularly SVs, that will be encountered as comprehensive variant identification from clinical GS becomes more widespread.

Discussion

Since the advent of massively parallel sequencing technologies, the application of clinical short-read GS has represented an enticing approach to ascertain almost all pathogenic variation in a single diagnostic test. Despite this enthusiasm,[97,98] there remains a dearth of unbiased and large-scale studies to systematically assess this technology against conventional tests for any phenotype, and in particular for FSAs. As such, it has been asserted that GS can provide anywhere from no improved diagnostic yield[99] to over 50%.[29,30] Unfortunately, existing studies examining the clinical utility of GS frequently have disparate standard-of-care tests available on individuals for comparison, precluding systematic benchmarking of GS against any individual test as well as the combination of multiple tests. Further, SVs are often not considered[36,100,101] or only identified via a small number of algorithms[102–105] despite evidence demonstrating the need for multiple approaches to maximize sensitivity.[25] This places an unnecessary technical constraint on the diagnostic value of GS and represents a critical limitation for surveying conditions where the contribution of SVs is significant, such as for FSAs and ASD.[1,6] We demonstrate here that these limitations can be circumvented with a comprehensive GS framework to capture, filter, and interpret a broad spectrum of variant classes without significantly increasing the burden of manual variant curation.[39]

The scale of the benchmarking conducted here, namely the 1,612 ASD quartet families that had three technologies

(GS, ES, and CMA) available for re-analysis on all individuals, demonstrated that GS captures all diagnostic variants identified by CMA and ES and provides a molecular diagnosis for almost 2-fold more ASD probands than either technology alone. We also illustrate that the diagnostic yield of ES can approach that of GS if sensitive CNV discovery is performed on the exome data.[106–108] While phenotype, ascertainment, and clinical context are expected to impact comparative diagnostic yields, our study demonstrates the importance of comprehensive variant discovery across technologies to avoid overestimating the added diagnostic yield of a single technology. As exemplified by our FSA cohort, inflated yields of GS (e.g., 7.6% vs. 0.8%) can easily occur when previous testing, technology platforms, assessment timepoints, bioinformatic analyses, and interpretation guidelines are not taken into consideration.

To confirm these results in fetal DNA samples, we applied the GS analytic framework to 46 fetuses preselected to harbor a reportable variant identified by karyotype, CMA, or ES. As expected, GS identified 100% of the CNVs and SNVs/indels identified by CMA and ES, respectively. In contrast, only 62.5% of the BCRs identified by karyotype were recapitulated by GS, largely as a result of the localization of BCRs to highly repetitive acrocentric chromosomes.[109] Previous studies have found that short-read GS may identify upwards of 90.8% of BCR breakpoints when rearrangements involving the acrocentric chromosomes are excluded,[93] suggesting the true performance of GS for detecting all BCRs will likely fall within the 62.5%–90.8% range. However, the impact of these missed BCRs on the total yield of GS will be small (e.g., 0.3%–1.1%), as the fraction of BCRs identified in FSAs is only estimated to be 3%.[1] Indeed, we can extrapolate our benchmarking results to diagnostic yields obtained from unselected FSAs that were ascertained from the same catchment area as the vast majority (64.2%) of our FSA cohort. Using these historical data,[1,3] we estimate that GS can provide an overall diagnostic yield of 46.1% in unselected FSAs, significantly outperforming each individual standard-of-care test by a wide margin: 17.2% increase over karyotype, 14.1% over CMA, and 38.3% over ES when only SNVs and indels are considered, and 4.1% when CNVs are also robustly identified from ES data (Figure 3). Based on diagnostic performance alone, these data strongly argue for GS to displace the serial application of karyotype, CMA, and ES for the assessment of FSAs and ASD, provided analysis and interpretation are sufficiently optimized to identify and interpret all classes of variation.

These studies found that GS uniquely identified nine P/LP variants across ASD probands and fetuses with structural anomalies, representing an added diagnostic yield of 0.4% and 0.8% in each cohort, respectively. Our study revealed that most diagnostic GS-unique variants included SVs that were inaccessible to existing standard-of-care diagnostic tests or were only determined to be pathogenic on the basis of information that was uniquely provided by GS. These included BCRs, complex SVs, single exon in-frame deletions, and mobile element insertions. It may be possible to further increase the yield of ES by improving filtering to recapture single exon CNVs. However, we previously demonstrated that the false positive rate of deletions and duplications detected by GATK-gCNV from ES data can dramatically increase if filtering is relaxed to one exon genome wide without manual curation of individual variants.[5,84] These data should temper enthusiasm regarding immediate significant increases in interpretable pathogenic variation from either ES or GS. Advances in genomics technologies and algorithms will continue to only provide incremental increases in diagnostic yield without improvements in variant annotation (e.g., predicting the functional impact of a variant) and interpretation.

Beyond diagnostic yield, there are additional technical, logistical, and economic considerations when deciding to implement a new diagnostic test such as GS. Among these, technical capacity and timely return-of-results is paramount in the prenatal setting. While assessing turnaround-time and the impact of GS on downstream health care costs was beyond the scope of this study, previous studies have demonstrated that GS results can be delivered within 18–21 days for the assessment of FSAs.[33,34] Additionally, rapid GS (ranging from 26 h to 3.2 days for analysis completion)[100,110] has been demonstrated in the pediatric setting for the assessment of critically ill infants, where, similar to the prenatal diagnostics, time to diagnosis can have a significant impact on medical management and clinical outcomes. Further, clinical GS costs less than existing standard-of-care diagnostic tests for individuals with a developmental disorder and/or congenital anomaly[111] and rapid GS has reduced the cost of hospitalization for children admitted to neonatal or pediatric intensive care units.[112,113] Taken together, these data suggest that the benefits of GS are likely to extend to reductions in health care costs and rapid return-of-results in addition to improved diagnostic yield. Yet, efforts to ensure that GS does not exacerbate health inequities will be critical, as access to testing will be initially isolated to metropolitan areas with major medical centers. Additionally, initiatives to expand diverse population representation in reference databases will be integral to ensuring that individuals from non-European genetic ancestries have an equal opportunity to receive a diagnosis, as population-specific allele frequencies are essential for variant interpretation.

In conclusion, these studies demonstrate the potential for GS to displace a series of standard-of-care diagnostic tests that individually identify only a small portion of the genomic variant spectrum associated with FSAs and ASD. The large-scale benchmarking performed in this study was critical, as these analyses focus on rare variants that span an array of mutational mechanisms but are not frequently observed in the general population or in small cohorts. We demonstrate that GS is unlikely to significantly increase the diagnostic yield in FSAs or ASD without improvements in variant annotation and interpretation,

particularly for noncoding variation, as we were only able to consider a small number of noncoding disease-associated loci. Some discrete phenotypes will also continue to require specialized assays (e.g., methylation tests, microsatellite analysis) for variants not accessible to any short-read GS technology. Overall, these data suggest that GS can effectively displace karyotype, CMA, and ES as a single diagnostic test for the assessment of FSAs and ASD and will provide a marginal, but important, increase in diagnostic yield beyond the combination of all three current standard-of-care diagnostic tests.

Data and code availability

The genomic and phenotype data for the ASD families can be accessed through SFARIbase with permission from the Simons Foundation Autism Research Initiative. The raw sequencing data generated from the fetal structural anomaly cohort is restricted because of consent limitations. However, all diagnostic variants identified in the ASD and FSA cohorts are provided in Tables S10 and S12.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.ajhg.2023.07.010.

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Author contributions

Study design: B.L., H.B., D.G.M., R.W., and M.E.T. Family recruitment and sample collection: J.L.G., V.S.A., D.L., M.E.N., T.M., K.G., B.P., A.B., M.D., N.L.V., B.L., and R.W. Sample library preparation: B.B.C. and K.O'K. Computational analysis: C.L., E.V., H.Z.W., E.P.H., N.K., C.W.W., S.P.H., B.W., V.J., J.F., R.L.C., X.Z., L.D.G., C.T., N.S., J.-Y.A., S.D., B.D., D.B.G., S.J.S., D.G.M., and H.B. Manual variant curation: C.L., E.V., J.L.G., C.A.A.-T., E.E., G.L., K.G., F.V., J.C.H., A.H.O'D-L., H.L.R., N.L.V., B.L., and R.W. Verified the underlying data for these analyses: C.L., E.V., H.B., and M.E.T. Wrote the manuscript and generated the figures: C.L., E.V., H.B., and M.E.T. All authors reviewed the manuscript. C.L. and E.V. contributed equally to this study.

Declaration of interests

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Web resources

ClinGen Sequence Variant Interpretation Resources, https://clinicalgenome.org/working-groups/sequence-variant-interpretation/

GATK Best Practices Workflows, https://gatk.broadinstitute.org/hc/en-us/sections/360007226651-Best-Practices-Workflows

GATK-gCNV, https://app.terra.bio/#workspaces/help-gatk/Germline-CNVs-GATK4

GATK-SV, https://github.com/broadinstitute/gatk-sv

GATK-SV single sample pipeline, https://app.terra.bio/#workspaces/help-gatk/GATK-Structural-Variants-Single-Sample

Terra, https://terra.bio/

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Supplemental information

Systematic evaluation of genome sequencing for the diagnostic assessment of autism spectrum disorder and fetal structural anomalies

Chelsea Lowther, Elise Valkanas, Jessica L. Giordano, Harold Z. Wang, Benjamin B. Currall, Kathryn O'Keefe, Emma Pierce-Hoffman, Nehir E. Kurtas, Christopher W. Whelan, Stephanie P. Hao, Ben Weisburd, Vahid Jalili, Jack Fu, Isaac Wong, Ryan L. Collins, Xuefang Zhao, Christina A. Austin-Tse, Emily Evangelista, Gabrielle Lemire, Vimla S. Aggarwal, Diane Lucente, Laura D. Gauthier, Charlotte Tolonen, Nareh Sahakian, Christine Stevens, Joon-Yong An, Shan Dong, Mary E. Norton, Tippi C. MacKenzie, Bernie Devlin, Kelly Gilmore, Bradford C. Powell, Alicia Brandt, Francesco Vetrini, Michelle DiVito, Stephan J. Sanders, Daniel G. MacArthur, Jennelle C. Hodge, Anne O'Donnell-Luria, Heidi L. Rehm, Neeta L. Vora, Brynn Levy, Harrison Brand, Ronald J. Wapner, and Michael E. Talkowski

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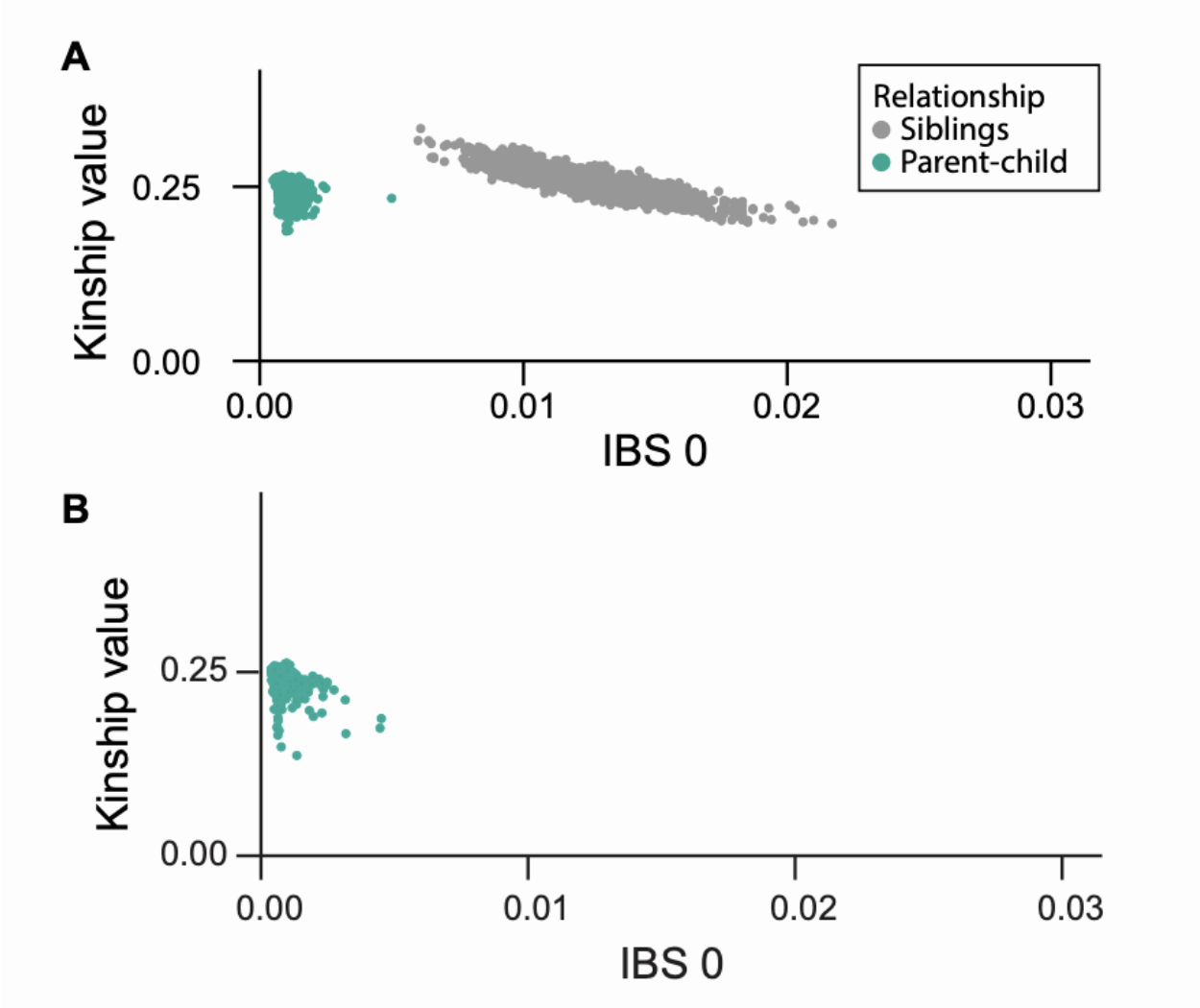

Figure S1. Confirmation of sample relatedness using kinship values
Kinship values for GS were calculated using KING[1] after restricting to SNVs with an alternate allele frequency greater than 5% in gnomAD genomes.[2] Each point on the plot represents a related pair of individuals, colored by relationship status. **(A)** Relatedness metrics for 6,448 individuals from the 1,612 ASD quartet families. **(B)** Relatedness metrics for 747 individuals from the 249 FSA trios.

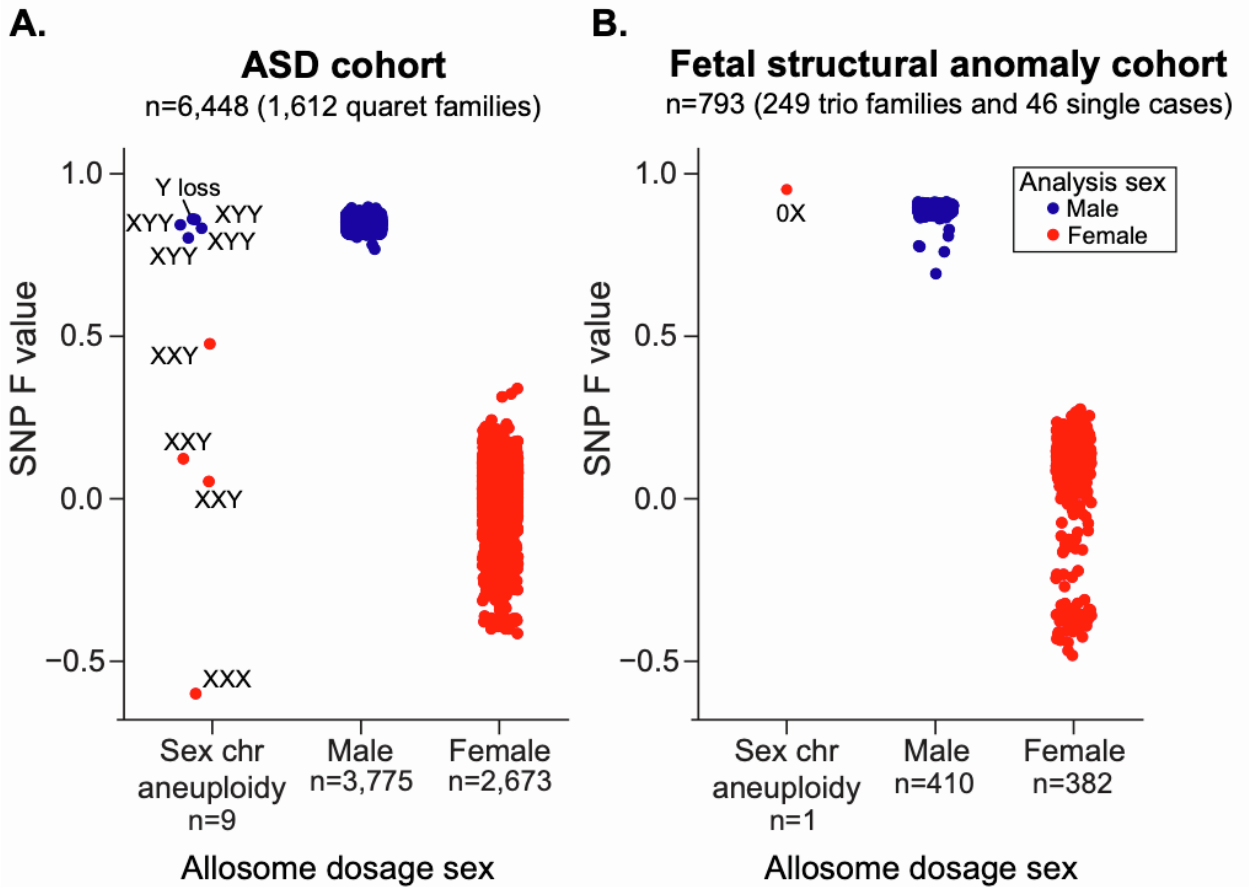

Figure S2. Sample sex QC

Confirmation of sample sex using single nucleotide polymorphism (SNP) and chromosomal read-depth information from GS data. Sex was inferred two ways from GS data: 1) using the F value generated with PLINK[3] based on sex chromosome SNP genotypes, and 2) using read depth (dosage) scores[4] derived from chrX and chrY. Each point represents a sample, colored by final sex used for analysis. **(A)** Sex metrics for the 6,448 individuals from the 1,612 ASD quartet families. Cases with sex chromosomal abnormalities (n=9) have been labeled. **(B)** Sex metrics for the 249 trios individuals from the FSA trios (n=747) that were pre-screened with standard-of-care diagnostic tests and the 46 singleton benchmarking cases.

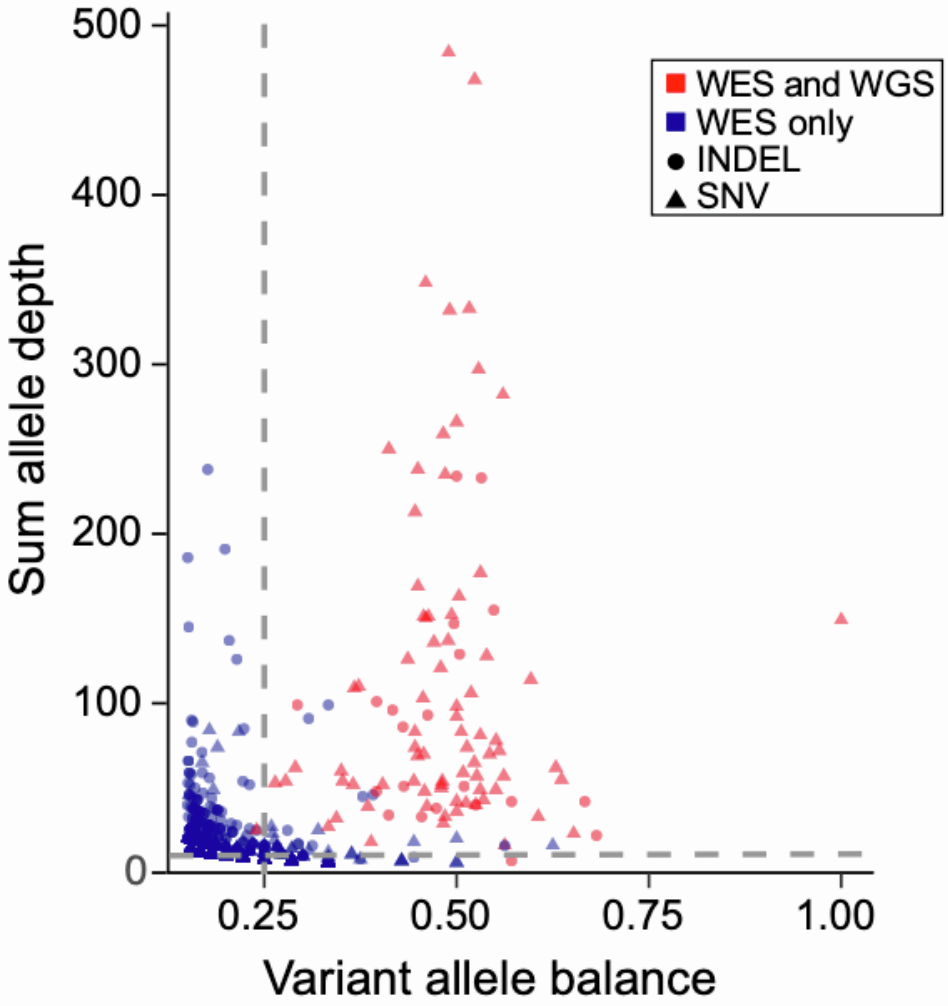

Figure S3. Modified exome sequencing depth and allele balance thresholds
The plot displays the allele balance (AB) and sum allele depth (AD) for all 1,453 *de novo* SNVs and indels detected from ES using the standard GS filters. Color indicates if the variant is unique to ES (blue; n=1,353) or was found in both the ES and GS data (red; n=100). Shape indicates variant type (SNV or indel). The dotted line represents the modified thresholds ultimately used for filtering the ES data before manual review: AB > 0.25 and sum(AD) ≥ 1.

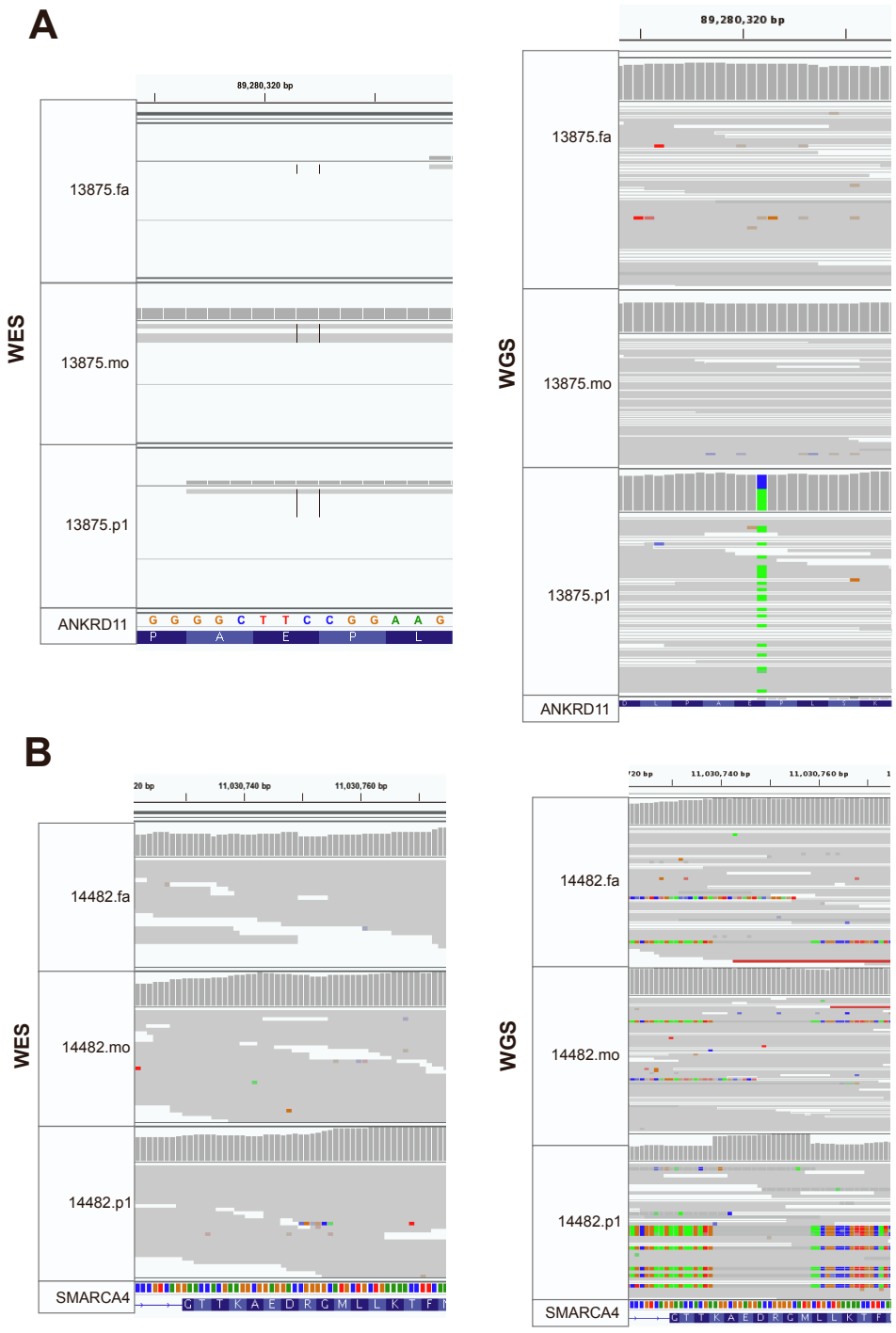

Figure S4. Two pathogenic sequence variants unique to GS in ASD probands
Alignment visualization for two *de novo* variants uniquely identified in GS alongside their raw read evidence from ES. Images were generated using IGV.[5] For each site, the ES and GS screenshots are shown side-by-side. **(A)** A stopgain SNV in *ANKRD11* in proband 13875.p1 that was absent from the ES VCF and has low coverage in the ES CRAM files **(B)** A 44 bp insertion in *SMARCA4* in 14482.p1 that was absent from the ES VCF and shows no supporting evidence in ES CRAM files.

SUPPLEMENTAL METHODS

Participant ascertainment and genome sequencing

We included 1,612 deeply phenotyped quartet families ascertained as part of the Simons Simplex Collection in this study.[6–8] As previously described,[9] each family included two unaffected parents, one unaffected sibling, and an affected proband with autism spectrum disorder (ASD). All affected probands underwent a battery of diagnostic tests, including the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R) to confirm the ASD diagnosis, as well as detailed evaluations of intellectual/cognitive functioning, adaptive behavior, physical/dysmorphic features, developmental milestones, medical comorbidities, and family history. We also included 295 fetuses that met criteria for diagnostic testing due to the presence of a structural anomaly (n=281) or advanced maternal age (n=14) (Figure 1). This included 46 singleton fetuses that were pre-selected for having a clinically reportable variant from karyotype, chromosomal microarray (CMA), and/or exome sequencing (ES), as well as 249 fetal structural anomaly (FSA) trios that had been pre-screened by karyotype, CMA, and/or ES. Recruitment and phenotyping protocols for the fetuses have been previously described.[10–12] All 7,241 individuals underwent paired-end genome sequencing (GS) to a mean target coverage of 30X (see Tables S1-3 for specific sequencing metrics).

Sample-level QC

To confirm sample relatedness, we performed a kinship inference analysis with KING[1] (http://people.virginia.edu/~wc9c/KING) using the GS data after restricting to single nucleotide polymorphisms (SNPs) with an alternate allele frequency (AF) >5% in gnomAD (Figure S1).[2] In parallel, we also predicted genetic sex using two independent approaches: first, we used PLINK to infer sex based on sex chromosome genotypes.[3] Second, we used GATK-SV to calculate copy number estimates for each chromosome per sample, which permitted inference of genetic sex as well as the identification of chromosomal aneuploidies. We compared the predicted sex for each individual between both methods and observed high concordance (Figure S2). Using the relatedness and sex results we resolved sample swaps based on discrepancies in family structures deviating from the expected relatedness metrics for parent-child (IBS0 ≤ 0.005 and kinship coefficient > 0.2) and sibling relationships (IBS0 > 0.005 and kinship coefficient > 0.2; Figure S1).

We also confirmed cross-technology sample relatedness to assure comparisons were performed on the same 6,448 individuals from the 1,612 ASD quartet families. This was accomplished by restricting the ES and GS VCFs to high-quality common SNPs from Purcell et al. 2014[13] that were lifted over to GRCh38/hg38 and limited to 5,862 SNPs common to both ES and GS. Samples were renamed based on their technology of origin and were merged into a single cross-technology ASD VCF for relatedness analysis with KING.[1] ES and GS samples with a kinship coefficient > 0.45 were considered to be identical samples. Finally, we used the confirmed sample metadata from the GS and ES comparisons to identify matching CMA data.[14]

Genome sequencing analysis framework

We developed a GS analytic framework to discover, filter, and interpret nine different classes of variation that are described in detail below and also summarized in Table S4. The aim of this pipeline was to retain as many pathogenic or likely pathogenic (P/LP) variants as possible while reducing the total number of variants requiring manual review. The same GS analysis pipeline was applied to both the ASD and prenatal cohorts with only modifications to the phenotype-specific gene list used.

1.0. Variant discovery

1.1. Sequence variants (GATK)

As previously described,[7,8] the ASD GS data was generated from PCR-free libraries and processed using the Center for Common Disease Genomics functional equivalence pipelines (https://github.com/CCDG/Pipeline-Standardization) and following the Genome Analysis Toolkit (GATK) Best Practices Workflows for sequence variant, single nucleotide variant (SNV) and small insertion/deletion (indel), discovery (https://gatk.broadinstitute.org/hc/en-us/sections/360007226651-Best-Practices-Workflows).[15] Briefly, this included aligning the raw FASTQ reads to the hg38/GRCh38 human reference genome using BWA-mem 0.7.15,[16] sorting and removing duplicate reads with Picard 2.4.1. (http://broadinstitute.github.io/picard/), performing base quality score recalibration, indel realignment, generating single sample gVCFs with GATK HaplotypeCaller 3.5-0,[17] merging single sample gVCFs into batch specific VCFs (ranging in size from 40 to 588 quartets),[8] joint-calling the merged VCFs, and performing Variant Quality Score Recalibration (VQSR). The aligned CRAM and gVCF files were transferred to the Amazon Web Services (AWS) S3 storage system and can be accessed with permission from the Simons Foundation Autism Research Initiative (https://www.sfari.org/resource/sfari-base/).

The GS data from the prenatal samples were generated at the Broad Institute Genomics Platform. After sequencing, individual FASTQ files were transferred to a Google Cloud bucket for storage. All GS data pre-processing and sequence variant discovery was performed using the GATK Best Practices Workflows on the cloud-enabled and freely available Terra platform (https://terra.bio/). Sequence variant calling followed the same steps described above for ASD.

1.2. Structural Variants (GATK-SV)

Structural variant (SV) discovery and genotyping was performed with GATK-SV, which was deployed on the Terra platform (https://terra.bio/). The code for GATK-SV is publicly available at https://github.com/broadinstitute/gatk-sv. All individuals were grouped into batches based on: their dosage bias score (a metric that quantifies the non-uniformity of coverage for a given GS sample),[4] sex, family status, PCR status, and cohort assignment. The ASD cohort included batches comprising 200-400 samples each, and the FSA cohort included one batch of PCR plus (n=186) and two batches of PCR free samples (n=345 and n=346, respectively). All families were kept intact during batching. All 7,241 individuals were analyzed with six SV discovery algorithms, including three paired-end/split-read algorithms (Manta v.1.4.0, Smoove v.0.2.3

[https://github.com/brentp/smoove], and WHAM-GRAPHENING v.1.7.0),[18–20] two read-depth algorithms (GATK-gCNV and cnMOPS v.1.12.0),[21,22] and one mobile element insertion algorithm, MELT v.2.0.5.[23] SV discovery generated six algorithm-specific VCFs per individual that were used as input for GATK-SV, which was run in cohort mode. The GATK-SV pipeline is organized into modules that harmonize predicted SVs across all input algorithms, reduce false positives, resolve overlapping SVs with disparate copy number, identifies complex variants (*e.g.*, inversions flanked by one or more copy number variants [CNVs]),[4,24] and provides cohort-wide SV genotypes and quality metrics available for *post hoc* filtering. We generated a cohort-wide SV VCF for the ASD and FSA cohorts, respectively, that was used as input for all downstream analyses. Further details on the GATK-SV methods can be found in Collins et al. 2020.[4]

1.3. Short tandem repeats (Expansion Hunter)

We identified short tandem repeat (STR) expansions across 18 loci that were selected from the gnomAD disease-associated STR catalog (https://github.com/broadinstitute/str-analysis/tree/main/str_analysis/variant_catalogs) based on conferring an early-onset developmental disorder phenotype (Table S8). STR expansions were genotyped using Expansion Hunter[25] v5.0.0 across 6,435/6,448 individuals from the ASD quartet families (n=9 ASD probands with a sex chromosomal abnormality were removed as well as one quartet family that revoked consent after all other analyses were completed). We also applied Expansion Hunter to all 295 prenatal samples (n=793 individuals in total).

2.0. Variant annotation

Details describing variant annotation are described in the methods of the main text.

3.0 Variant filtering

3.1. Variant QC

After variant discovery, we applied quality control (QC) filters intended to maximize sensitivity for candidate P/LP variants while removing false variant calls. For SVs, this included removing variants with a GATK-SV QUAL score ≤ 1 and multiallelic copy number variants (CNVs). For sequence variants, we removed multiallelic variants, variants with an allele balance (AB) < 0.15 in the case of interest, indels > 50bp, and variants where the sum of the reference and alternate allele depth (AD) was ≤ 5 in any family member. We also removed SNVs that did not pass GATK VQSR. To reduce false positives, we applied additional quality control filters to samples with outlier variant counts, defined as any sample with a variant count (based on raw GATK haplotype caller or individual SV algorithm output) above Q3 + 6*IQR. This definition resulted in relatively few SV outlier samples (n=12 SV in the FSA cohort and none in the ASD cohort) and sequence variant outliers (n=5 in the FSA cohort and n=1 in the ASD cohort). To control the false positive rate in these outlier samples, we removed SVs present in >2 SV outlier individuals and sequence variants with GQ <75.

3.2. Variant functional consequence

All variants were filtered for functional impact. SVs predicted to be loss-of-function (LoF) or full gene copy gain were retained for further filtering.[4] Partial gene duplications, defined as duplications with one breakpoint located outside the gene boundary and one within, were excluded given their unknown functional impact.[26] Any sequence variants predicted to be stop-gain, stop-loss, frameshift insertion, frameshift deletion, splicing (within 2 bp of a splice junction), or missense according to RefSeq or Gencode annotations were retained for additional filtering. We further filtered missense variants based on three tiers (described below) to identify those that are increasingly likely to be functionally damaging and thus classified as P/LP (Tier 1 = most likely to be P/LP, and Tier 3 = least likely to be P/LP). We removed missense variants classified as benign, likely benign, risk factor, association, drug response, or protective in ClinVar from all tiers.

Tier 1 missense:

- Classified as P/LP in ClinVar

Tier 2 missense:

- Classified as P/LP in ClinVar or
- Any missense variant with a CADD score > 30[27]

Tier 3 missense:

- Classified as P/LP in ClinVar or
- Any missense variant with a CADD score > 30 or
- Missense variants with a CADD score between 15 and 30 also located in a missense constrained region[28]

3.3. Disease genes and genomic regions

To facilitate variant filtering, we computationally built a candidate disease gene list for the ASD and FSA cohorts, respectively. The ASD gene list comprised 901 genes (Table S5) broadly associated with neurodevelopmental disorders (NDDs) from the DDG2P database[29] classified as having a 'confirmed' or 'probable' association with developmental disorders that conferred a brain/cognitive phenotype. To account for the variable phenotypes observed in the FSA cohort (Tables S2-3), we compiled 2,535 developmental disorder genes (Table S6) based on the union of eight gene lists, described below:

1) 374 dominant developmental disorder genes from the DDG2P database (accessed July 29, 2019)[29] with a "confirmed" disease association and monoallelic, imprinted, mosaic, x-linked dominant, and x-linked over-dominance modes of inheritance.

2) 800 recessive developmental disorder genes from the DDG2P database[29] with a "confirmed" disease association and biallelic or hemizygous modes of inheritance.

3) 93 genes that were significantly enriched for rare *de novo* variants in the Deciphering Developmental Disorders study.[30]

4) 26 dominant genes significantly enriched for rare *de novo* protein-truncating variants in ASD.[6]

5) 358 genes from the Clinical Genome (ClinGen) Resource Dosage Sensitivity Map with "some evidence for dosage pathogenicity" (haploinsufficiency/triplosensitivity score = 2) or "sufficient evidence for dosage pathogenicity" (haploinsufficiency/triplosensitivity score = 3) (downloaded July 29, 2019; https://www.clinicalgenome.org/curation-activities/dosage-sensitivity/).

6) 708 autosomal dominant and 1,182 recessive disease genes curated from the Online Mendelian Inheritance in Man (OMIM) database.[31,32]

7) 217 recessive and dominant X-linked genes from OMIM (tables were accessed June 12, 2017).

8) 117 genes that have been robustly associated with fetal structural anomalies detectable by ultrasound that were curated by the Prenatal Assessment of Genomes and Exomes study.[33]

Each gene was classified as being associated with a disorder that had a dominant and/or recessive pattern of inheritance based on existing annotations from DDG2P and OMIM. We categorized the inheritance labels provided by DDG2P as recessive: biallelic, and hemizygous or dominant: imprinted, monoallelic, mosaic, x-linked dominant, and x-linked over dominant. When disease inheritance was not available for a gene (n=4 missing from DDG2P), variants in that gene were retained under both dominant and recessive modes of inheritance.

We also compiled a list of 64 known genomic disorder (GD) loci to assess overlap with SVs in both our cohorts. We took all of the known CNV syndromes located on the autosomes and chromosome X from DECIPHER[34] and the haploinsufficient (HI) and triplosensitive (TS) regions from the Clinical Genome (ClinGen) Resource Dosage Sensitivity Map if they had a HI or TS score ≥ 2 ("sufficient evidence for dosage pathogenicity"). We removed any regions that were only associated with late-onset conditions, resulting in 64 candidate regions (Table S7). All SVs that overlapped ≥ 50% of a GD locus were retained for manual review. Following the most recent guidelines for CNV interpretation,[26] we also manually reviewed any rare (<1% frequency in gnomAD-SV)[4] deletion or duplication that overlapped ≥ 25 or ≥ 35 protein-coding genes, respectively, even if it did not overlap a disease gene or GD region from our lists. Finally, we also retained all SVs that overlapped one of 17 non-coding loci known to confer pathogenic long-range position effects (LRPEs; Table S8). To define the non-coding search space, we used topologically-associated domain (TAD) boundaries from the IMR90 fetal fibroblast cell line,[35] which have been previously shown to be associated with pathogenic LRPEs if disrupted,[36,37] that contained each LRPE target gene.

3.4. Inheritance

We filtered variants under the five inheritance modes described below. For the ASD quartets, the unaffected sibling and both parents were treated as independent trios during inheritance filtering. We applied more stringent missense variant filters (tiers described in the variant functional consequence section) to rare inherited and compound heterozygous variants as these two categories resulted in a large number of variants requiring manual review despite there being little evidence supporting their contribution to the etiology of ASD or FSAs.[6,12,33,38,39] The specific functional consequence considered for each inheritance type are as follows:

Dominant disease genes:

- *De novo*
  - All LoF
  - Missense Tier 3

- Rare inherited
  - All LoF
  - Missense Tier 1

Recessive disease genes:

- Homozygous
  - All LoF
  - Missense Tier 3

- X-linked recessive
  - All LoF
  - Missense Tier 3

- Compound heterozygous
  - At least one variant in the pair had to be LoF or Tier 2 missense

The identification of compound heterozygous variants comprised three steps, including: 1) compiling heterozygous SNVs, indels, and LoF SVs located in the same recessive disease gene, 2) annotating each variant with inheritance status, and 3) retaining only the instances where individuals had more than one variant in a recessive disease gene with disparate inheritance patterns (*e.g.*, one maternally inherited, one *de novo*). We required that at least one variant per compound heterozygous grouping be inherited from a parent due to the lack of phasing information from short-read GS.

3.5. Allele frequency

All variants (SNVs, indels, and SVs) meeting the above thresholds were retained if they had an alternate allele frequency (AF) <1% for variants in dominant disease genes or regions and <5% for recessive disease genes. Given that some GDs can occur at an appreciable frequency in disease cohorts,[40] we did not apply any AF cut-off when considering SV that overlapped ≥50% of a known GD locus.

4.0. Variant interpretation

Details describing manual variant curation are described in the methods of the main text.

Benchmarking the performance of GS against conventional tests

Filtering CMA data

As previously described,[7,14] SNP genotyping data was generated for the ASD cases using three microarray platforms, the Illumina 1Mv1, 1Mv3, or Omni2.5. CNV calls for each individual were identified using PennCNV,[41] QuantiSNPv2.3,[42] and GNOSIS/CNVision.[14] CNVs were filtered for rarity based on overlap with CNVs from the Database of Genomic Variants (in GRCh36/hg18) and overlap with CNVs from the ASD parents.[14,43] All CNV coordinates were lifted over from GRCh36/hg18 to GRCh38/hg38 and those classified as high-quality (CNV p-value [pCNV] ≤ 1.0×10^{-9})[14] were filtered following the same steps outlined in the GS SV pipeline (Table S4). There were 14 variants detected by GS that were also detected by CMA but failed filtering because they were not lifted over from hg18 to hg38 (n=6), failed the pCNV high-quality filter (n=5), or were removed due to incorrect CNV coordinates that suggested the variant did not overlap coding sequence (n=3; GS coordinates were used as truth). These variants were recovered and counted towards the overall yield of CMA. We also removed one deletion from CMA manual review that was identified to be rare by CMA but was found in 65 (2.0%) of our 3,224 ASD parents based on GS, which was above our allele frequency threshold.

Filtering exome sequencing data

The ES data for the ASD cases was generated as part of a larger sequencing initiative and has been extensively described.[6,38] We realigned sequencing data from GRCh37/hg19 to GRCh38/hg38[38] and applied the same filtering steps as those outlined in the GS filtering pipeline (Table S4) with minor modifications to account for differences in depth between ES and GS (Figure S3). These included increasing our AB and total allele depth filters for *de novo* variants (both in *de novo* dominant inheritance and as part of a compound heterozygous pair) to account for the higher ES coverage, increased rate of false positives, and potential for somatic variant detection. The new thresholds (AB >0.25 and total AD ≥10) were chosen based on retaining >95% of the variants that were also detected by GS (Figure S3).

To identify CNVs from ES data, we applied GATK-gCNV,[21] a publicly available Bayesian model for germline detection of CNVs. Briefly, this is a read-depth based tool that uses a negative-binomial factor analysis to adjust for known and unknown biases of exome sequencing, while modeling sample and genomic region copy number through a hierarchical hidden Markov model. In this analysis, we jointly processed the 6,448 individuals from 1,612 ASD quartet families described in this study with an additional 66,000 samples.[38] Samples were assigned to batches based on 3D clustering of the first three principal components of coverage depth after normalizing for average depth. The 72,448 samples were processed across 126 batches, with a median batch size of 449 samples (min 136 and maximum 2,259). After raw calling with GATK-gCNV, we applied

our calibrated sample-level quality filters, resulting in 5.49% of the total samples being removed. The GATK-gCNV quality score statistic (QS>400 for homozygous deletions, QS>100 for heterozygous deletions, and QS>50 for duplications) was applied to individual calls to extract rare CNVs with predicted sensitivity and positive predictive value of >90%, which resulted in a resolution of three exons or more. With these filtering metrics, the average ES sample harbored 1-2 rare high-quality CNVs.

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