METHODS

Construction of *Consensus-ASD*

Consensus-ASD consists of aggregated variants from 32 high-quality ASD sequencing studies. Variants were generally curated to only include ASD risk variants that reached study-wide significance; the exact source and curation criteria for each study can be found in **Table S11**. In addition to providing their genomic coordinates, copy-number variants (CNVs) were annotated in two ways: 1) with all genes that they physically overlap ("all genes" approach), and 2) with all genes they physically overlap that are identified in at least one other variant class in *Consensus-ASD* ("known genes" approach). In addition to providing their rsID, non-coding single-nucleotide polymorphisms associated with autism through genome-wide association studies (GWAS SNPs) were assigned to their most likely target gene using the previously published 'Combined SNP-to-Gene' (cS2G) approach (cS2G score > 0.5) (Gazal et al. 2022).

Constraint Analysis

LOEUF scores (oe_lof_upper) were obtained from gnomad v2 (Karczewski et al. 2020) to evaluate genic constraint. For each gene, we calculated a per-gene LOEUF score by averaging the LOEUF values of each gene's associated transcripts. To aid interpretability, we also calculated per-gene LOEUF percentiles, defined as 1 minus the percentile rank of a gene's LOEUF score. In this representation, highly constrained genes have low LOEUF scores and correspondingly high LOEUF percentile values. To evaluate constraint for non-coding GWAS SNPs, we used gnocchi scores from https://pubmed.ncbi.nlm.nih.gov/38057664/. Across all variant classes, a one-sample t-test (mu = 0.5; t.test in base R) was used to assess whether a class's mean constraint percentile was significantly greater than 0.5. While LOEUF is a continuous metric, we utilized LOEUF < 0.6 (approximately 75th LOEUF percentile) when needed to identify a set of constrained genes.

Expression Analysis of *Consensus-ASD*

To assess the longitudinal expression patterns of ASD risk genes, we downloaded normalized RNA-sequencing data from BrainSpan (version: “RNA-Seq Gencode v10 summarized to genes”; https://brainspan.org). Gene expression values were extracted for all available developmental timepoints, and the mean expression of genes within each variant class was computed at each timepoint. Longitudinal trends were visualized by applying cubic spline smoothing (smooth.spline, stats v3.6.2) to these average expression values. To examine cell-type-specific expression patterns, we obtained single-cell RNA-sequencing data from the human fetal cortex (Polioudakis et al. 2019). For each cell type, we calculated and visualized the average normalized expression of genes within each variant class.

Pathway Enrichment Analysis of *Consensus-ASD*

Pathway enrichment analysis of each variant class within *Consensus-ASD* was performed using the fifty Hallmark Molecular Signature Database pathways (Liberzon et al. 2015) (obtained from https://www.gsea-msigdb.org/gsea/msigdb) and a hypergeometric test. P-value correction for multiple hypothesis testing was performed using FDR.

Cross-Enrichment Analysis of *Consensus-ASD*

Cross-enrichment analysis between variant classes within *Consensus-ASD* was performed using hypergeometric tests, and p-value correction for multiple hypothesis testing was performed using FDR. For this analysis, CNVs were annotated using the “known genes” approach (described above) while GWAS SNPs were annotated based on their predicted cS2G target gene (described above).

Cell Culture

All human cell culture work was performed under protocols approved by Boston Children’s

Hospital IRB # S07-02-0087. The control male human iPSC line “280” was previously generated and fully characterized (Schlaeger et al. 2015). Human iPSCs and iPSC-derived NPCs were grown on plates pre-coated with Matrigel (Corning #354277) or Geltrex (ThermoFisher #A1413302). The cells were passaged as needed using Accutase (Millipore Sigma #SCR005) or ReleSR (StemCell Technologies #100-0483). Human iPSCs were cultured in mTeSR™ Plus medium (StemCell Technologies #100-0276). Human NPCs were generated from iPSCs using the STEMdiff™ SMADi Neural Induction Kit (StemCell Technologies #08582) (de Leeuw et al. 2020) by following the monolayer protocol for at least three weeks. NPCs were continuously grown in Neural Induction Medium and were not switched to Neural Progenitor Medium. The medium was supplemented with 10uM Y-27632 (StemCell Technologies #72304) for approximately 1 day following passaging of iPSCs or NPCs. Cerebral organoids were generated from 280 iPSCs using the STEMdiff™ Cerebral Organoid Kit (StemCell Technologies # 08570) with some recommendations from (Giandomenico et al. 2021) as well as additional modifications. In particular, the organoids were embedded in Matrigel (Corning #354277) at day 7 and grown in Expansion Medium until day 14, at which point the organoids were removed from Matrigel and switched to Maturation Medium.

Derivation of Cas13 Knock-in iPSCs

Cas13 (Konermann et al. 2018) was knocked into the *AAVS1* safe harbor locus of 280 iPSCs using constructs that were previously described (Qian et al. 2014). Individual iPSC colonies were picked and expanded. Southern blot analysis was performed by Celplor to confirm correct targeting of the *AAVS1* locus. Karyotype analysis was performed using KaryoStat (ThermoFisher), which revealed a 2.4Mb gain on chromosome 1, which is described in microarray nomenclature as arr[GRCh37] 1q32.1(204108163_206539638)x3. This region did not contain any of the target genes.

Cas13 gRNA design

A deep-learning-based tool (Wei et al. 2023) was used to design Cas13 gRNAs targeting our genes of interest. These gRNAs were filtered to exclude homopolymers and potential off-target interactions, as assessed using BLAST (Camacho et al. 2009). For each target gene, 3 separate gRNAs were selected and combined into a tandem gRNA array, which is processed by Cas13 into individual gRNAs.

Molecular Cloning and Lentivirus Production

For initial NPC KD experiments, the lentiviral pSicoR construct (Addgene #11579) (Ventura et al. 2004) was used as the backbone. The mouse U6 promoter was replaced with the human U6 promoter, which was used to drive expression of the Cas13 gRNA arrays. Lentivirus was generated using standard protocols that have previously been described (Han et al. 2009). These constructs were used in combination with the 280-Cas13 knock-in line. Lentiviruses were applied at a multiplicity of infection (MOI) of 4.0, as this MOI yields >98% transduced cells. RNA was extracted 24 hours later.

For NPC proliferation assays, an all-in-one construct was created that contained Cas13 and EGFP expressed from the CAG promoter element and gRNA arrays expressed from the human U6 promoter. The backbone also contained inverted terminal repeats (ITRs), allowing the construct to be integrated into the genome by piggyBac transposase (Yusa et al. 2011). These constructs were based on the LiOn design (Kumamoto et al. 2020), such that expression of Cas13 and EGFP was dependent upon recombination of the construct by piggyBac transposase, preventing expression from the episomal plasmid.

For organoid experiments, we created the FLEx-based Inducible CRISPR Knockdown (FLICK) construct by engineering several features into a backbone incorporating the FLEx design (Schnütgen et al. 2003). In particular, we included a modified Cre recombinase that includes an intron, which prevents recombination of the plasmid during cloning (Kaczmarczyk

and Green 2001). We also included Cas13 (Konermann et al. 2018) as well as all necessary components of the Tet-On 3G system (Qian et al. 2014) to enable doxycycline-inducible recombination.

Library Preparation for RNA Sequencing (RNA-Seq)

Bulk RNA was extracted using the Direct-zol RNA Microprep Kit (Zymo Research #R2062), and 3' RNA-seq libraries were prepared following a modified version of the TagSeq protocol (Lohman et al. 2016), incorporating recommendations and pooling strategies from the CheapSeq protocol (Rabe 2020). Briefly, 200ng of total RNA from each sample was combined with an indexed oligo(dT) primer and buffer (from NEB #M0466L) as described for CheapSeq. RNA was fragmented in a pre-heated thermocycler at 94C for 2.5 min without the lid, after which the samples were moved immediately to ice. Each sample was then mixed with a template-switching oligo (TSO) that contained an index and a unique molecular identifier (UMI) along with additional reagents required for reverse transcription, as described for CheapSeq. The reverse transcription reaction was performed at 42C for 90 minutes, without heat inactivation. Remaining single-stranded RNA and oligos were then digested by adding 0.5uL of Buffer EB (Qiagen #19086), 0.5uL of thermolabile ExoI (NEB #M0568S), and 0.25uL of RNase A (ThermoFisher #EN0531), and incubating the samples at 37C for 30min, followed by heat inactivation at 85C for 5min and a hold step at 4C. Up to 8 samples with different index sequences were then pooled, and cDNA was purified by performing a 1.4x left-sided SPRI selection (Beckman Coulter #B23317). Q5 UltraII Mastermix (NEB #M0544L) was then used along with partial Read1 and partial Read2 primers to amplify the cDNA for 7 cycles, following the "CheapSeq PCR" parameters described below. Amplified cDNA was purified through a 0.7x left-sided SPRI selection (Beckman Coulter #B23317). Final library construction was performed by using Q5 UltraII Mastermix (NEB #M0544L) along with standard TruSeq indexing primers (NEB #E6440S) and amplifying for 8 cycles following the "CheapSeq PCR" parameters

described below. Libraries were purified by performing a 0.4x-0.65x double-sided SPRI selection (Beckman Coulter #B23317). Purified libraries were assessed using Tapestation HSD1000 (Agilent #5067-5584 and #5067-5585). For oligo sequences, see **Table S9**.

CheapSeq PCR:

1. 98C for 30s
2. X cycles of 98C for 10s, 65C for 75s
3. 65C for 5min
4. 4C forever

Differentially Expressed Gene (DEG) Analysis

Gene expression was quantified using alevin-fry with UMI deduplication (salmon-v1.9). A salmon index and transcript-to-gene map were built using Gencode v45. Counts from distinct transcripts that mapped to the same gene were summed. Only genes with a baseMean expression of >10 in non-targeting control (NTC) samples were considered for transcriptome-wide analyses. Differential expression analysis was performed using DESeq2 (v1.42.0), with the model ~Batch + sgRNA. The p-values were adjusted using false discovery rate (FDR) correction to only perform multiple hypothesis testing for the set of approximately 11,000 analyzed genes (baseMean >10 in NTCs). Enrichment analyses were performed using hypergeometric tests, and p-value correction for multiple hypothesis testing was performed using FDR. To evaluate transcriptome-wide effects of perturbing neighboring lncRNA and PCGs, linear modeling was performed per neighbor pair using the following model: $\log_2$ fold change of gene upon lncRNA KD ~ $\log_2$ fold change of gene upon PCG KD (*lm*, base R).

ddPCR

To confirm KD of targets with low expression, ddPCR was performed. Primers were designed for each target gene (**Table S10**), and specificity was assessed using BLAST (Camacho et al.

2009) and confirmed by Sanger sequencing of PCR products (Azenta). For ddPCR, the same RNA that had been used for RNA-seq (above) was first converted to cDNA using the SuperScript™ IV VILO™ Master Mix (ThermoFisher Scientific # 11756050). Next, ddPCR was performed by combining cDNA at an RNA-equivalent concentration of 0.25ng/uL (5ng per 20uL reaction), primers at a final concentration of 0.1uM, and QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad # 1864033). The ddPCR reactions were performed using the QX200 AutoDG Droplet Digital PCR System (Bio-Rad). Expression levels were normalized using the expression of the housekeeping gene *HPRT1*. The results were analyzed using the model Expr ~ Batch + type, where type can either be NTC or the targeted gene. Results were considered statistically significant if the p-value was <0.05.

MAGIC Analysis

The bioinformatics tool MAGIC (v1.1) (Roopra 2020) was used to identify transcription factors (TFs) whose targets are enriched in the DEGs from individual perturbations. The universe was set as all genes with baseMean > 10 in NTCs. The strategy of “gene body (promoter to the end of the last exon) plus 1Kb flanking sequence either side of the gene body” was used.

Re-Analysis of REST ChIP-Seq Data

Data was extracted from Table S2 from (Rockowitz and Zheng 2015). Mouse genes were mapped to human genes using Orthology.eg.db (v3.18.0), org.Mm.eg.db (v3.18.0), and org.Hs.eg.db (v3.18.0). Enrichment analysis was performed using a hypergeometric test with all BrainSpan (https://www.brainspan.org/) genes with normalized expression >= 1 as the universe. P-value adjustment for multiple hypothesis testing was performed using FDR.

Gene Regulatory Network (GRN) Construction and Benchmarking

A GRN was constructed using the bioinformatic tool ARACNe-AP (Lachmann et al. 2016). The

input was the RNA-seq data from our 3 NTCs and the successful KD samples with at least 50 DEGs. We used curated lists of transcription factors (Lambert et al. 2018) and epigenetic regulators (Boukas et al. 2019) and selected genes expressed in NPCs (baseMean >10 in NTC samples) as the candidate genetic regulators. To create the GRN, we used default parameters and 100 bootstraps that were then aggregated together to form the final network. We then used VIPER (v1.36.0) (Alvarez et al. 2016) to identify differentially active regulons following standard operating procedures. In brief, we identified regulons with significant differential expression between NTCs and KDs of known ASD genes using a t-test, with a null distribution defined by sample permutations. To benchmark the predictive power of the ARACNe-derived GRNs, we generated an additional ARACNe network after holding out perturbation samples for seven target genes that we had previously identified as candidate genetic regulators. For each held-out gene, we assessed overlap using a hypergeometric test between 1) its predicted regulon, and 2) the corresponding set of DEGs identified from perturbation of the same gene. P-values across the seven held-out perturbations were combined using Fisher's method.

PageRank Analysis of GRN

To identify central regulators within our GRN, we converted the GRN to a graph using the R package igraph (v2.0.3) and ran the PageRank algorithm (Page et al. 1999) using the igraph method *page_rank*. We then rank-ordered genes by their PageRank score and ran gene set enrichment analysis (GSEA) using the R package fgsea (v1.28.0), testing the following ontologies in addition to those displayed in **Fig. 3C**: all categories of *Consensus-ASD* and the 50 Molecular Signature Database Hallmark Pathways (Subramanian et al. 2005).

STRING Analysis of GRN

To evaluate known or predicted protein-protein interactions between genes of interest, we utilized the STRING database GUI (Szklarczyk et al. 2023) and set a universe of NPC-

expressed genes (baseMean > 10 in NTCs). All other default parameters were used. Mean normalized degree was calculated using the igraph function (*degree*(graph), normalized = TRUE). To further evaluate the significance of the high mean normalized degree between our 78 candidate genes, we created an empirical null distribution of mean normalized degree by creating 1000 subnetworks of 78 randomly selected GRN regulators, which confirmed that the mean normalized degree of the network consisting of our 78 candidate genes was highly significant ($p = 0$). For clustering of our 78 regulators into 5 modules, we used iterative walktrap clustering using the igraph function *cluster_walktrap* with default parameters. For enrichment analysis of CHD8 targets, we used Supplementary Data 1 from (Sugathan et al. 2014).

Identification of 78 Top Candidate Regulators for Driving Convergence in Autism

VIPER (v1.36.0) (Alvarez et al. 2016) was used to identify regulators whose regulons were significantly differentially active across the KDs of known autism genes compared to controls. From these, we prioritized 78 regulators whose regulons were significantly enriched for both *Consensus-ASD* genes (hypergeometric test with Benjamini-Hochberg correction for multiple hypothesis testing) and high PageRank scores (gene set enrichment analysis with Benjamini-Hochberg correction for multiple hypothesis testing).

Identification of Potential Contributors to the FPE

NPX single-copy Xi-expressed genes were obtained from Table S6 from (San Roman et al. 2023). To identify genes that showed female-biased expression in the neurotypical brain, we combined DEG call sets from the following works:

(1) Table S4 from (Kissel et al. 2024): Identified as significantly female-biased in either the UCLA cohort, BrainVar cohort, or via meta-analysis

(2) (Oliva et al. 2020) (https://storage.cloud.google.com/adult-gtex/bulk-qtl/v8/sb-eqtl/GTEx_Analysis_v8_sbgenes.tar.gz): Identified as significantly female-biased in

either BRNCTXA or BRNCTXB

(3) Table S4 from (Naqvi et al. 2019): Identified as significantly female-biased in the brains of all assessed species (human, mice, rats, dogs, and macaques), suggesting conserved female-biased expression

Re-Analysis of ZFX Targets from Rhie et al., 2018 and San Roman et al., 2023

ZFX ChIP-seq targets were obtained from Table S3E from (Rhie et al. 2018). ZFX targets were also obtained from (San Roman et al. 2023), which performed integrative analysis of ZFX ChIP-seq data (ENCODE) and ZFX KD or KO RNA-seq data from (Rhie et al. 2018). Enrichment was analyzed using hypergeometric tests and p-values were corrected using FDR. For analysis of ZFX targets from (Rhie et al. 2018), the universe was defined as genes expressed across all four cell lines in which ChIP-seq experiments were performed (HEK293T, HCT116, C4-2B, and MCF-7). For analysis of ZFX targets from (San Roman et al. 2023), the universe was defined as genes expressed in the corresponding cell type from (Rhie et al. 2018).

NPC Proliferation Assays

LiOn-based KD constructs were nucleofected into 280 NPCs along with a piggyBac-transposase-expressing plasmid using the Neon Transfection System (ThermoFisher Scientific) with the following parameters: voltage = 1600V, width = 10, pulses = 3. For $2x10^6$ NPCs, the 100uL tips were used to nucleofect 10ug of the KD plasmids and 2ug of the piggyBac-transposase-expressing plasmid. Four days later, the cells were treated for 20 minutes with dye from the CYTOTRACK™ Red 628/643 Cell Proliferation Assay Kit (Bio-Rad #1351205) diluted 1:500 in media. The cells were then rinsed and passaged, with 1/5 of each sample plated into a well of 4 separate plates to be used for the subsequent 4 timepoints. The remaining cells were used for flow cytometry to assess the initial CytoTrack intensity (d0). For the next 4 days, 1 well of each sample was dissociated each day and analyzed by flow cytometry. The results for each

sample were normalized to their initial CytoTrack intensity (d0) and to the average of the NTC samples. Statistical analysis was performed using 2-way ANOVA with Dunnett's multiple comparisons test (GraphPad Prism).

Organoid scRNA-seq

Organoids were dissociated using the Neural Tissue Dissociation Kit – Postnatal Neurons (Miltenyi Biotec # 130-094-802). Cells with recombined FLICK constructs were isolated through FACS for EGFP expression. scRNA-seq libraries were prepared using Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) from 10X Genomics. Libraries containing FLICK barcode sequences were prepared using custom approaches.

Alignment and Quantification of Organoid scRNA-seq Data

scRNA-seq data was aligned to the pre-built CellRanger (v.9.0.0) hg38 reference using *cellranger-count* in feature-barcode mode to perform simultaneous gene expression and guide abundance quantification. Introns were not included in gene expression quantification (--include-introns=false) to avoid quantifying partially degraded transcripts that had been targeted by CRISPR-Cas13. CRISPR guide sequencing library FASTQs for day 60 samples were downsampled to 1.25% of reads using seqtk (v1.4) to match CRISPR library sequencing depth for d30 samples and to minimize background for guide assignment using the cellranger-CRISPR guide quantification pipeline. All other default parameters were used. CellRanger guide assignments were used to filter to only cells with exactly one assigned guide, and these cells and guide assignments were used for all downstream analyses.

Pre-processing and Quality Control of Organoid scRNA-seq Data

Long-term and acute KD samples were processed separately but equivalently. CellBender (v0.3.2) was used to perform detection and filtering of ambient RNA contamination, while

scrublet (v0.2.3) was used to perform automatic doublet detection and filtering. All other downstream analysis was performed with Seurat (v5.0.0). High-quality cells were retained according to the following quality-control metrics: 1) percent mitochondrial (percent.mt) gene expression < 20% and 2) 500 < number of expressed genes (nFeature_RNA) < 6000.

Cell-type Annotation of Organoid scRNA-seq Data

Cell-type annotation was first performed on only non-targeting control (NTC) cells. NTC cells were extracted to create a control-only Seurat object. This object subsequently underwent normalization (*NormalizeData*), variable feature identification (*FindVariableFeatures*), scaling (*ScaleData*; regressed covariates of percent.mt, percent_ribo, nCount_RNA, and nFeature_RNA), and dimensionality reduction (*RunPCA* and *RunUMAP*). Integration was performed using harmony (v1.2.0) across organoid batches and this integrated object was used for unsupervised clustering (*FindNeighbors* and *FindClusters*, resolution = 0.6 and 1.2). A small number of unsupervised clusters (<1000 cells) were filtered due to low cluster-wide QC metrics (i.e. exceptionally high or low nCount_RNA, high percent.mt). All subsequent 2-D visualization of this control-only Seurat object was performed using a UMAP representation of the integrated data. Cell type annotation was performed using canonical brain marker genes. A Seurat object was next constructed for all cells, following the same processing pipeline (e.g., normalization, scaling, dimensionality reduction, and integration) as described above. To perform cell-type annotation, label transfer was performed from the control-only Seurat object (*TransferData*) to the full Seurat object. Expression patterns of canonical brain marker genes were then used to refine and finalize these cell type annotations.

Differential Expression Analysis of Organoid scRNA-seq Data

Differential expression analysis was performed between perturbed cells per guide and cells that received NTCs, stratified by major cell types (RG, IPC, ExN, or IN). Seurat *FindMarkers* was

used to perform differential expression with logfc.threshold = 0, min.pct = 0.10, min.cells.group = 3 and otherwise all default parameters. Enrichment analyses of differentially expressed genes (DEGs) were performed using hypergeometric tests, and p-value correction for multiple hypothesis testing was performed using FDR. The universe was defined as all genes expressed in both comparisons of interest.

Gene Set Enrichment Analysis of Organoid scRNA-seq Data

DEGs were rank-ordered by average log2 fold change. Gene set enrichment analysis (Subramanian et al. 2005) was performed using fgsea (v1.28.0) testing the following ontologies in addition to those displayed in **Fig. S14D** and **Fig. S16F**: all categories of *Consensus-ASD*, the fifty Molecular Signature Database Hallmark Pathways, and the first-degree regulons of each of the five modules of top candidate regulators.

Visualization of Shared DEGs Between Organoid Cell Types

Shared DEGs were visualized between organoid cell types using igraph (v2.0.3). The Fruchterman-Reingold layout algorithm (*layout_with_fr*) was used to determine final node placement.

MAGIC Analysis of Organoid DEGs

The bioinformatics tool MAGIC (v1.1) (Roopra 2020) was used to identify transcription factors (TFs) whose targets are enriched in DEGs from individual perturbations per organoid cell type. The universe was set as all genes expressed in at least 10% of cells of that cell type (Seurat min.pct = 0.10; no log2 fold-change cutoff), as these were the genes assessed for differential expression. The strategy of “gene body (promoter to the end of the last exon) plus 1Kb flanking sequence either side of the gene body” was used.

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