Females with autism spectrum disorders show stronger DNA methylation signatures than males in perinatal tissues

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ABSTRACT

Autism spectrum disorder **(ASD)** comprises a group of neurodevelopmental conditions currently diagnosed by behavioral assessment in childhood, with reported underdiagnosis in females. Though diagnosis in early life is linked to improved outcomes, we currently lack objective screening tools for newborns. To address this gap, we sought to identify a sex-specific DNA methylation signature for ASD using perinatal tissues that reflect dysregulation in the brain. DNA methylation was assayed from ASD and typically developing **(TD)** newborn blood, umbilical cord blood, placenta, and post-mortem cortex samples using whole genome bisulfite sequencing **(WGBS)** in a total of 511 samples. We found that methylation levels of differentially methylated regions **(DMRs)** differentiated samples by ASD diagnosis in females more than males across the perinatal tissues. We tested three theories for ASD sex differences in newborn blood, finding epigenetic support for an X chromosome-related female protective effect, as well as a high replication rate of DMRs (48.1%) in females across two independent cohorts. In our pan-tissue analysis, three genes (X-linked *BCOR*, *GALNT9*, *OPCML*) mapped to ASD DMRs replicated in all four female tissues. ASD DMRs from all tissues were enriched for neuro-related processes (females) and SFARI ASD-risk genes (females and males). Overall, we found a highly replicated methylation signature of ASD in females across perinatal tissues that reflected dysregulation in the brain and involvement of X chromosome epigenetics. This comparative study of perinatal tissues shows the promise of newborn blood DNA methylation biomarkers for early detection of females at risk for ASD and emphasizes the importance of sex-stratification in ASD studies.

Introduction

Autism spectrum disorder **(ASD)** is a neurodevelopmental condition defined by functional impairments in social communication and interactions, restrictive interests, and repetitive behaviors (1). ASD is typically diagnosed in childhood by behavioral observation and clinical assessment (2), although early identification is not always accessible due to long wait lists for diagnostic services (3,4). Treatment options for ASD are limited, though intensive behavioral intervention beginning at an early age and following established guidelines has been associated with better outcomes (5,6).

ASD has a strong sex bias, with males being diagnosed approximately four times more frequently than females. Explanations for the sex bias include biological reasons that may shift the true occurrence of ASD towards males -- such as an increased genetic burden in diagnosed females, epigenetic changes that confer risk to males and/or protection to females, and sex hormone involvement -- as well as social reasons that may lead to the underdiagnosis of true cases in females (7), including compensatory behaviors primarily observed in females and diagnostic definitions that reflect symptoms more common in males (8–11). Indeed, research has found that common diagnostic tools, such as ADOS-2, may be less sensitive to female presentations of ASD (10–12).

The limitations of clinically diagnosing ASD by behavior (13–15), including sex differences in presentation, as well as the positive impact of intensive behavioral intervention beginning at an early age (5), highlight the need for reliable biomarkers that can be used as a screening tool in newborns. A biomarker is an objective measure of a medical state, which can be measured accurately and reproducibly (16). In the case of ASD, the use of biomarkers to identify newborns

at increased likelihood of developing ASD would alert parents and medical practitioners to available resources in their community and encourage behavioral assessments and interventions at an early age. Ideal ASD biomarkers would be clinically accessible and economically viable to allow for widespread use, and measurable at birth to identify children at increased likelihood of developing ASD as early as possible.

One class of potential ASD biomarkers that meet these requirements are epigenetic marks from tissues collected at the time of birth, such as newborn blood, umbilical cord blood, and placenta (17). In contrast to genetic markers, which have not been shown to accurately predict ASD, epigenetic marks such as DNA methylation benefit from their ability to reflect, in a cell-type specific manner, the intersection between the genetic and perinatal environmental factors that increase likelihood for ASD (18–20). Given the neurodevelopmental origins of ASD, the human brain is the most relevant tissue to study but is inaccessible for routine screening in newborns. Studies using the more accessible tissues of placenta and cord blood have shown dysregulated methylation over genes involved in neurodevelopment, demonstrating the promise of these perinatal tissues as a surrogate for the brain (21–28). However, placenta and cord blood are not widely banked and therefore, would be difficult to use for biomarkers in large-scale research studies or screening panels. Newborn blood circumvents these dilemmas since most states collect newborn dried blood spots **(NDBS)** by punching the newborn's heel and absorbing the blood onto Whatman paper, which is typically stored after being used to screen for metabolic diseases. DNA can be easily extracted using commercial kits at a quality and quantity sufficient for DNA methylation profiling and downstream analysis (29,30), making the use of NDBS DNA methylation to screen for ASD a promising method.

In this comparative study, we analyze DNA methylation from ASD and TD individuals using previously published post-mortem cortex (31), placenta (27), and umbilical cord blood (28), as well as unpublished newborn blood from two cohorts (discovery and replication). Our findings build on a study from 2021 that compared DNA methylation across maternal blood, cord blood, and placenta samples from the same individuals and found that DNA CpG methylation sites that were associated with ASD diagnosis were enriched for ASD-associated genes in all tissues (32). In contrast to that study, which assayed DNA methylation using methylation microarrays, all samples in our study were assayed using whole genome bisulfite sequencing **(WGBS)**, which has the benefit of providing insight to the entire genome. Additionally, we performed sex-stratified analyses to improve our understanding of the epigenetic signature of ASD in females in contrast to males given the differences in diagnostic rates and symptom presentation.

We further used our discovery newborn blood dataset to explore three proposed models for sex differences in ASD (33): 1) the multifactorial liability model, including the female protective effect, whereby female-specific protective factors (and/or male-specific vulnerability factors) shift females further away from the liability threshold (34); 2) the extreme male brain theory, whereby all individuals with ASD have a shift towards male phenotypes (35); and 3) the gender incoherence theory, whereby ASD attenuates sex differences present in TD individuals (36). Improved understanding of sex differences in ASD would help us better understand etiologic and clinical variability, increase diagnostic accuracy, and predict treatment needs.

This is the first WGBS study to identify a sex-specific epigenomic signature of ASD in newborn blood, including investigation of ASD sex difference theories, and to compare ASD signatures across tissues (newborn blood, cord blood, placenta, post-mortem cortex) using consistent parameters. This study design allowed us to investigate newborn blood, the most accessible tissue for newborn screening, as a potential source of predictive biomarkers alongside cord blood and placenta, which have previously been used to identify an epigenetic signature of ASD prior to diagnosis, and brain, the most relevant tissue for studying ASD. Overall, we found a stronger epigenetic signature of ASD in females than in males across perinatal tissues, demonstrated epigenetic support for the female protective effect in newborn blood, and identified sex-specific loci that were differentially methylated across tissues and enriched for neuro-related processes and SFARI ASD-risk genes. In particular, the high replication rate of newborn blood DMRs that differentiated TD and ASD females across two independent cohorts shows the promise of newborn blood DNA methylation as a potential biomarker for ASD in female newborns.

Materials and Methods

Study population

We investigated DNA methylation signatures from ASD and TD individuals using samples from newborn blood, umbilical cord blood, placenta, and cortex. The cord blood (28), placenta (27), and cortex (31) samples have been previously published and are available in the Gene Expression Omnibus with accession numbers GSE140730 (cord blood), GSE178206 (placenta), GSE81541 (cortex) and GSE119981 (cortex). We used the “discovery” data set samples from the cord blood and placenta publications (27,28). Newborn blood, cord blood, and placenta samples were all

collected at time of birth, and the post-mortem cortices were collected from individuals aged 4-56 years at the time of death.

All samples were collected from different individuals and cohorts, except for placenta and cord blood, which had considerable overlap. Cord blood and placental samples were obtained through the MARBLES study (37), an enriched-likelihood cohort that enrolls pregnant women in Northern California who already have a biological child with ASD. The TD participants from MARBLES are clinically defined as non-autistic, non-developmentally delayed, having undergone behavioral and cognitive assessments. The human cerebral cortex samples from Broadmann Area 9 were obtained from the National Institute of Child Health and Human Developmental Brain and Tissue Bank for Developmental Disorders at the University of Maryland (31).

Discovery newborn blood samples were obtained through the Child Health and Development Studies **(CHDS)**, a birth cohort that enrolled pregnant women at the Kaiser Foundation Health Plan in Oakland, CA from 1959-1967. Over 98% of eligible women enrolled in the study, therefore reflecting the socio-demographics of the Oakland area at the time. Follow-up studies have collected information and biological samples from the children and grandchildren of the original cohort. We matched the CHDS database with the California Department of Developmental Services **(DDS)** to identify grandchildren of the original CHDS cohort who have been diagnosed with ASD. We sex- and age-matched the ASD individuals with TD individuals, generating 196 deidentified NDBS for the discovery group. Replication newborn blood samples were obtained from participants of the CHARGE case-control study in Northern California, which has been

previously described (38). For both studies, TD participants are those who did not have any neurodevelopmental disorder listed in the DDS.

DNA extraction and whole genome bisulfite sequencing

DNA methylation from all samples included in this study was assayed using whole genome bisulfite sequencing **(WGBS)**. Discovery NDBSs were each punched into six 4 mm punches, which were stored at -80C. The samples were randomized by ASD diagnosis and sex and grouped into 10 batches for DNA extraction. Two DNA extractions were performed per sample, each using three 4mm punches, using the GenTegra Complete DNA Recovery Kit (GenTegra, Pleasanton, CA, USA) and the QIAamp Micro Kit (Qiagen, Hilden, Germany), based on Additional File 2 Protocol GQ from Ghantous et al., 2014 (39). The two isolations from each sample were combined into a single 1.7 mL tube and ethanol precipitation was used to clean the samples. The DNA was re-eluted in 30 ml nuclease-free water and quantified by Qubit. DNA purity was assessed by Nanodrop 260/230 and 260/280 ratios. DNA samples were sonicated to a fragment size of ~350 bp with a peak power of 175, duty of 10%, and 200 cycles/burst for 47 seconds using 15ul DNA and 40ul EB. Sonicated DNA was cleaned and concentrated with gDNA clean and concentrator columns (Zymo Research, Irvine, CA, USA), eluted in 25 ml EB, and re-quantified by Qubit. The maximum mass of DNA for each sample, up to 100 ng, was then bisulfite converted using the Zymo EZ DNA Methylation Lightning Kit. Illumina sequencing libraries were prepared using the ACCEL-NGS MethylSeq DNA library kit (Swift Biosciences, Ann Arbor, MI, USA), with 6 cycles of indexing PCR (7 cycles for lower input samples). The libraries were pooled and sequenced across 13 lanes of NovaSeq S4 flow cells (Illumina, San Diego, CA, USA) for 150 bp paired end reads with a 5% PhiX spike-in to generate ~200 million reads (~8-12x coverage, 60Gb)

per sample. Replication NDBSs were processed and sequenced the same as discovery, except that only one DNA isolation was performed per sample using 2-4 4mm punches and ethanol precipitation was not used to clean the DNA before library preparation.

Cord blood, placenta, and cortex samples were processed as previously described (27,28,31). Briefly, DNA was extracted from cord blood samples with the Qiagen Puregene Blood kit, from placental and cortex samples with the Qiagen Puregene kit. DNA from all three tissues was bisulfite converted with the Zymo EZ DNA Methylation Lightning kit. Illumina sequencing libraries were prepared from cord blood and placental samples using the Illumina TruSeq DNA Methylation kit with indexed PCR primers and a 14-cycle PCR program, and from cortex samples as previously described (31). Cord blood and placental samples were sequenced at 2 per lane with 150 base pair paired-end reads and spiked-in PhiX DNA on the Illumina HiSeq X. Cortex samples were sequenced at one per lane with 100 base pair single-end reads on the Illumina HiSeq 2000.

Sequence alignment and quality control

FASTQ files for each sample were merged across lanes using FASTQ_Me (40) and aligned to the hg38 genome using CpG_Me (41) with the default parameters (42–45). The alignment pipeline includes trimming adapters and correcting for methylation bias, screening for contaminating genomes, aligning to the reference genome, removing PCR duplicates, calculating coverage and insert size, and extracting CpG methylation to generate a cytosine report (CpG count matrix) and a quality control report.

Determination of Sex

Genotypic sex was used in this study to differentiate females and males, whereby those with two X chromosomes are defined as females and those with one X and one Y chromosome are defined as males. Our sample sets did not include any individuals that have other combinations of sex chromosomes. We determined genotypic sex for each sample by calculating the ratio of sex chromosomes from WGBS reads using the SexChecker pipeline (https://github.com/hyeyeon-hwang/SexChecker). The gender identity of study participants is not known to us, and we acknowledge that gender and genotypic sex may not align for some participants. With this manuscript's goal of identifying biomarkers that may be used in newborns, we feel that genotypic sex is an appropriate measure.

Global methylation analyses

Global methylation for each sample was calculated as the total number of methylated CpG counts divided by the total number of CpG counts from all CpGs included in the DMRichR analysis (filtering described below). Differences in global methylation across tissues were tested with one-way ANOVA with Tukey's multiple comparisons while differences across sexes and ASD diagnoses were tested with two-way ANOVA with Fisher's Least Significant Difference using GraphPad Prism 10.0.3. Density plots were made by taking the mean value across samples for each CpG from that tissue's Sex Combined DMR comparison and visualizing them with the "ggplot" function in the ggplot2 R package.

Differentially methylated regions

Sex-combined and sex-stratified ASD vs TD DMRs were identified for each tissue (discovery newborn blood, replication newborn blood, cord blood, placenta, cortex), as well as female vs male

DMRs for discovery newborn blood, using DMRichR (https://github.com/ben-laufer/DMRichR) (29,46,47). We defined regions as sections of the genome that have at minimum 5 CpGs and a maximum gap of 1000 base pairs, with the "universe" for each comparison described as all CpGs included in the analysis. Because each tissue and sex contained a different number of samples, we normalized the analyses by adjusting the percent of samples per group that must have at least 1x coverage over a certain CpG site for its inclusion in the analysis. Datasets with fewer samples tend to produce a greater number of DMRs (many of which may be false positives) and so by requiring datasets with fewer samples to have a greater percent of samples covered over a given CpG, we can normalize the analyses. Because cortex had the fewest number of samples (n = 27), we set perGroup = 1 for all cortex analyses, and for all other tissues, calculated perGroup as 27/n so that each comparison required 27 samples to have coverage over a given CpG for its inclusion in the DMR analysis. Otherwise, we used default DMRichR parameters. ASD vs TD comparisons included autosomal and sex chromosomes while female vs male comparisons included only autosomal chromosomes.

All sex-combined analyses were adjusted for sex, and cortex analyses were additionally corrected for age at time of death. We did not adjust DMRs for cell types because estimations from different tools and reference datasets have shown large inconsistencies when estimating cell type proportions from WGBS data, particularly from NDBS, thereby decreasing our confidence in using cell type as an adjustment variable (29). Additionally, a previous ASD cord blood study showed that when nRBCs were adjusted for, a significant majority of ASD DMRs were maintained (28). To evaluate whether the DMRs distinguished ASD from TD samples, we used heatmaps of

hierarchically clustered nominally significant (p <0.05) DMRs' Z-scores, which are the number of standard deviations from the mean smoothed methylation value.

Principal component analysis

DMRs were also visualized using principal component analysis **(PCA)**. Smoothed methylation values over nominally significant (p <0.05) DMRs from each comparison were obtained using the "getMeth" function in the bsseq R package v1.36.0. PCA was performed using the "prcomp" function in the stats R package with centering to zero and scaling to unit variance. PCA results were plotted using the "autoplot" function in ggfortify v0.4.16 using R v4.3.1 with an ellipse indicating the 95% confidence level for each group, assuming a multivariate normal distribution. If there was zero overlap between the 95% confidence levels for each group, the DMRs were confirmed to differntiate the two groups being compared.

Chromosome enrichment analysis

Enrichr (48–50) was used to test discovery and replication newborn blood ASD vs TD DMRs for enrichment across individual chromosomes. The inputs were DMRs mapped to the nearest gene on hg38 with the background set to the "universe" for each DMR comparison, as described above.

Differentially methylated region overlaps by genomic location and gene name

ASD DMRs from all tissues were overlapped by genomic location using the Genomic Ranges and RegioneR R packages. The permTest function in the RegioneR package, v1.32.0, was used to calculate significance of overlaps by genomic region, using the intersection of all regions included in the two individual analyses as the "genome". For overlap by gene name, ASD DMRs were

mapped to the closest gene and gene names were overlapped pairwise across tissues using the GeneOverlap R package, v1.36.0. The "genome" was defined as the intersection of all regions included in the two analyses, mapped to the nearest gene using the "oneClosest" rule of the Genomic Regions Enrichment of Annotations Tool **(GREAT)** (51).

Discovery newborn blood female vs male DMRs (TD females vs TD males, ASD females vs ASD males, TD females vs ASD males, ASD females vs TD males) were overlapped with discovery newborn blood ASD vs TD autosomal DMRs (ASD females vs TD females, ASD males vs TD males) by gene name, as described above. The number of overlaps across the comparisons were visualized using the UpSetR R package v1.4.0 (52).

Gene ontology term overlaps

Gene ontology **(GO)** enrichment of DMRs from each ASD vs TD comparison were identified with GOfuncR (53). Using the sex-stratified DMRs from each tissue, GO terms related to biological processes, molecular functions, and cellular components that had a p-value < 0.2 were intersected across all tissues. The overlapping biological processes were graphed by their -log(p-value) using GraphPad Prism v 10.0.3, ordered by highest to lowest average -log(p-value) across tissue.

SFARI gene enrichment

DMRs mapped to genes from all comparisons were overlapped with genes listed in the SFARI database, release date 3/28/2024 (54). The significance of the overlaps was calculated with the Fisher's exact test with the background defined as the "universe" for each DMR comparison, as described above.

Results

Study subject characteristics and global methylation signatures from newborn blood, cord blood, placenta, and cortex.

In this study, we investigated WGBS DNA methylation signatures from ASD and TD individuals using samples from newborn blood (discovery and replication; both previously unpublished), cord blood (28), placenta (27), and cortex (31). Three different cohort designs are represented by the perinatal tissues, including a birth cohort (discovery newborn blood), a case-control study (replication newborn blood), and an enriched-likelihood enrollment study (placenta and cord blood). Because ASD is more frequently diagnosed in males, and TD controls were sex-matched, all tissues had more samples from males than females **(Table 1)**.

We first assessed global CpG methylation profiles of our samples and found that all tissues had significantly different average levels of methylation (one-way ANOVA, p <0.0001) **(Supplemental Figure 1A)**, and that partially methylated domains **(PMDs)** were present in placental samples, but no other tissues. When we examined global methylation in ASD vs TD and male vs female samples for each tissue, we found greater differences across sex than across ASD diagnosis **(Supplemental Figure 1C)**, leading us to conduct a sex-stratified (in addition to sex-combined) regional methylation analysis.

Table 1. WGBS samples used in this study.

Tissue	***n***	**Sex**		**Diagnosis**		**Publication**	**Cohort design**
		Females, *n* (%)	**Males, *n* (%)**	**ASD, *n* (%)**	**TD, *n* (%)**		
Discovery Newborn Blood	196	30 (15.3%)	166 (84.7%)	84 (42.9%)	112 (57.1%)	This study	CHDS birth cohort
Replication Newborn Blood	90	11 (12.2%)	79 (87.8%)	49 (54.4%)	41 (45.6%)	This study	CHARGE case-control study
Cord Blood	106	32 (30.2%)	74 (69.8%)	50 (47.2%)	56 (52.8%)	Mordaunt et al., 2020	MARBLES enriched-likelihood study
Placenta	92	30 (32.6%)	62 (67.4%)	46 (50.0%)	46 (50.0%)	Zhu et al., 2022	MARBLES enriched-likelihood study
Cortex	27	10 (37.0%)	17 (63.0%)	16 (59.3%)	11 (40.7%)	Vogel Ciernia et al., 2020	NICHD Brain and Tissue Bank

DMRs distinguish samples by ASD diagnosis better in females than in males across newborn blood, cord blood, and placenta.

To identify potential sex and tissue effects on locus-specific differences in DNA methylation for ASD, we identified differentially methylated regions **(DMRs)** for ASD vs TD samples in sex-combined (corrected for sex), females only, and males only analyses in discovery newborn blood, cord blood, placenta, and cortex **(Supplemental Tables 1-12)**. Given the different number of samples in each tissue, we normalized the analyses by adjusting the percent of samples that must have at least 1x coverage over a given CpG for its inclusion in the analysis ("perGroup" variable).

In females, DMRs differentiated ASD from TD samples by hierarchical clustering **(Figure 1A)** and PCA **(Figure 1B)** among discovery newborn blood, cord blood, and placenta, though the differentiation of male ASD from male TD samples was not revealed among these tissues **(Figure 1C-D)**. DMRs differentiated ASD from TD samples only in cortex from males, but not females, though cortex had the fewest number of samples among tissues. Across all tissues, the sex-combined DMRs did not distinguish ASD from TD samples **(Supplemental Figure 2)** and there were a higher number of DMRs in females only comparisons than in males only or sex-combined comparisons **(Table 2)**.

Table 2. ASD vs TD DMRs by tissue and sex

Tissue	**Comparison**	***n***	**# DMRs**	**# DMR Genes**
Discovery Newborn Blood	Sex-combined	196	59	54
	Females	30	185	181
	Males	166	99	66
Replication Newborn Blood	Sex-combined	90	67	64
	Females	11	5204	4017
	Males	79	189	172
Cord Blood	Sex-combined	106	127	115
	Females	32	430	413
	Males	74	248	235
Placenta	Sex-combined	92	557	384
	Females	30	3012	2147
	Males	62	2168	1493
Cortex	Sex-combined	27	453	435
	Females	10	10405	6599
	Males	17	1067	984

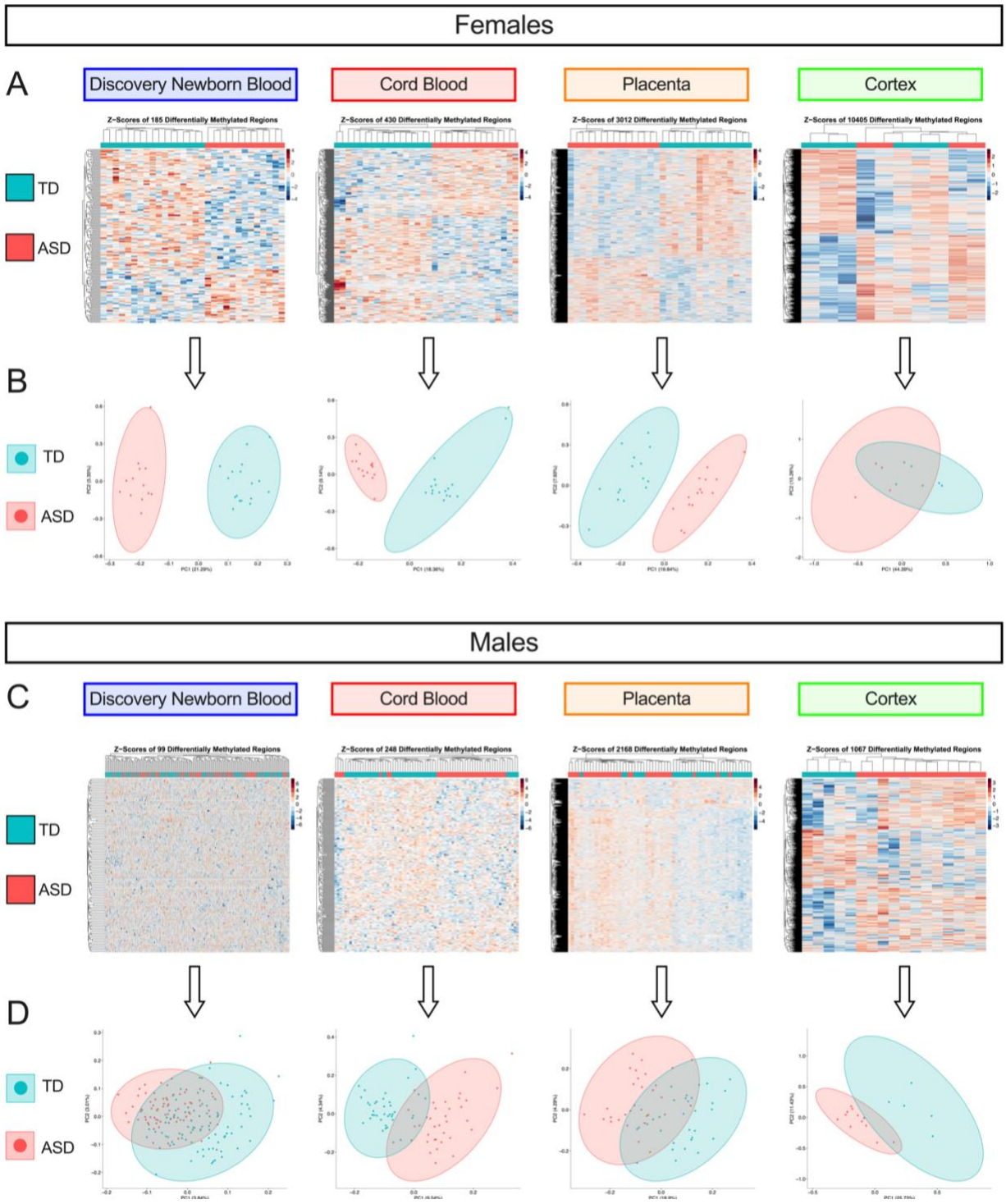

Figure 1. **Differentially methylated regions of ASD vs TD samples in discovery newborn blood, cord blood, placenta, and cortex. A)** Heatmaps of nominally significant ($p < 0.05$) DMRs identified from female samples. DMRs are hierarchically clustered by their Z-scores, which are the number of standard

deviations from the mean smoothed methylation value for each DMR. **B)** PCA plots of smoothed methylation values of the DMRs identified from female samples, colored by ASD diagnosis. The semi-transparent circles on the PCA plots represent 95% confidence intervals. **C)** and **D)** are the same as A) and B) but in male samples.

ASD DMRs identified from females have a signature of hypomethylation and significantly overlap with DMRs identified from males.

ASD females showed a consistent signature of hypomethylation across perinatal tissues, while males varied widely. The percent of DMRs identified from females that were hypomethylated in ASD compared to TD samples ranged from 61% in cord blood to 63% and 69% in placenta and discovery newborn blood, respectively, while males ranged from 19% in placenta, to 55% in discovery newborn blood and 77% in cord blood **(Supplemental Figure 3)**.

Despite these sex differences in the methylation signatures of ASD, we hypothesized that some loci would be consistently differentially methylated in ASD vs TD samples, regardless of sex. To test this sex specificity, we mapped all ASD DMRs to the nearest gene on the hg38 genome and compared the gene lists from DMRs identified in female samples with those identified in male samples from each tissue. We found that DMR genes identified from females significantly overlapped (p <0.05) with DMR genes identified from males in all tissues **(Supplemental Table 13)**. When we compared the genomic location of DMRs from each sex, we found that they were significantly closer together on the genome (even if not overlapping) than expected by random chance in all tissues except discovery newborn blood **(Supplemental Table 13)**.

Newborn blood ASD DMRs replicated in sex-stratified comparisons.

We then evaluated the consistency of newborn blood DMRs across cohorts by using a replication newborn blood dataset from an independent study. Reflecting our findings from the discovery group, there were many more ASD DMRs (p <0.05) identified from the females only comparison (5204 DMRs) compared to the males only (189 DMRs) or sex-combined (67 DMRs) comparisons in the replication newborn blood **(Supplemental Tables 14-16)**, and the DMRs differentiated ASD from TD samples in females but not males or sex-combined analyses **(Supplemental Figure 5)**. The proportion of DMRs that were hypermethylated in ASD vs TD samples also remained consistent across the discovery and replication groups, with 31% and 27% in females, and 45% and 46% in males, respectively **(Supplemental Figure 3)**.

To identify loci that replicated across both newborn blood datasets, we overlapped the sex-stratified DMRs by gene name (less stringent) and by genomic location (more stringent). First, we mapped DMRs to the nearest gene and found that 48.1% of DMR genes identified in discovery newborn blood females were also detected in replication newborn blood females **(Supplemental Tables 17-18)**. In comparison, only 10.6% of DMR genes identified in male comparisons and 1.9% of DMR genes identified in sex-combined comparisons replicated across both cohorts. By genomic location, DMRs from discovery and replication newborn blood overlapped more frequently (p <0.05) than expected by random chance and were physically closer together (p <0.05) in females only, males only, and sex-combined comparisons **(Supplemental Table 17)**. Of the twelve loci that replicated in females across both newborn blood datasets, three were located on the X chromosome **(Supplemental Table 18)**.

Discovery newborn blood DMRs show support for the female protective effect, marked by a shift of ASD females away from TD females.

Because we observed more ASD DMRs in females than in males across all four tissues and the DMRs identified from females better separated the ASD from TD samples, we tested common theories proposed to explain sex differences in ASD. The skew in ASD diagnoses towards males, at about a 4:1 ratio, has led to several theoretical models (33) **(Figure 2A)**, including, 1) the multifactorial liability model, including the female protective effect (34); 2) the extreme male brain theory (35) and; 3) the gender incoherence theory (36). We used the discovery newborn blood dataset to test these theories since we had the greatest number of samples for this tissue and the greatest separation between ASD and TD samples by PCA of DMRs identified in females **(Figure 1B)**.

To understand how sex differences intersect with the methylation signature of ASD, we first identified autosomal DMRs between females and males within TD (TD females vs TD males) **(Supplemental Table 19)** and within ASD (ASD females vs ASD males) **(Supplemental Table 20)**. We found many more DMRs ($p < 0.05$) in the ASD group (682 DMRs) than the TD group (206 DMRs), though both groups had more DMRs than the sex-specific TD vs ASD comparisons in discovery newborn blood. When we performed PCA from smoothed methylation values from all discovery newborn blood samples over the diagnosis-specific female vs male DMRs, we found that ASD males did not separate from TD males in either comparison, while ASD females showed some separation from TD females, though the direction of separation depended on the regions assayed: when smooth methylation was assayed over TD female vs TD male DMRs **(Figure 2B)**,

the ASD females were shifted towards the males, while the opposite occurred when methylation was assayed over ASD female vs ASD male DMRs **(Figure 2C)**.

We next overlapped autosomal DMR gene names from ASD vs TD and female vs male comparisons to better understand the proportion of methylation differences that are common versus unique across sexes and diagnoses. We found that the majority (62.7%; p = 2.3E-106) of TD female vs TD male DMR genes were also found in the ASD female vs ASD male comparison, with many additional DMRs in the latter comparison **(Figure 2D) (Supplemental Table 21)**. In addition, of the 128 ASD female vs TD male DMR genes, 120 (93.8%) were also called as ASD female vs ASD male DMR genes **(Figure 2E)**. This contrasted with the TD female vs ASD male comparison, where only 115/176 (65.3%) of the DMR genes were also called as ASD female vs ASD male DMR genes **(Figure 2E)**. This supports our previous finding that the signature of ASD is more distinct in females than in males, a primary prediction of the multifactorial liability threshold and female protective effect model (33).

Discovery and replication newborn blood DMRs are enriched for X chromosome location. Because we saw evidence of the female protective effect from our analysis of autosomal chromosomes, we wondered if methylation differences on the X chromosome were further contributing to the strong signature of ASD in females. Using both the discovery and replication newborn blood datasets, we tested the ASD vs TD DMRs identified from females only, males only, and sex-combined comparisons for enrichment across all chromosomes. We found that DMRs identified from females in both cohorts were significantly enriched for X chromosome location (q <0.05), while DMRs identified from males were not significantly enriched for any

chromosome **(Figure 2F) (Supplemental Table 22)**. These findings indicate that the strength of the ASD methylation signature in females is being driven by both autosomal and X-linked loci.

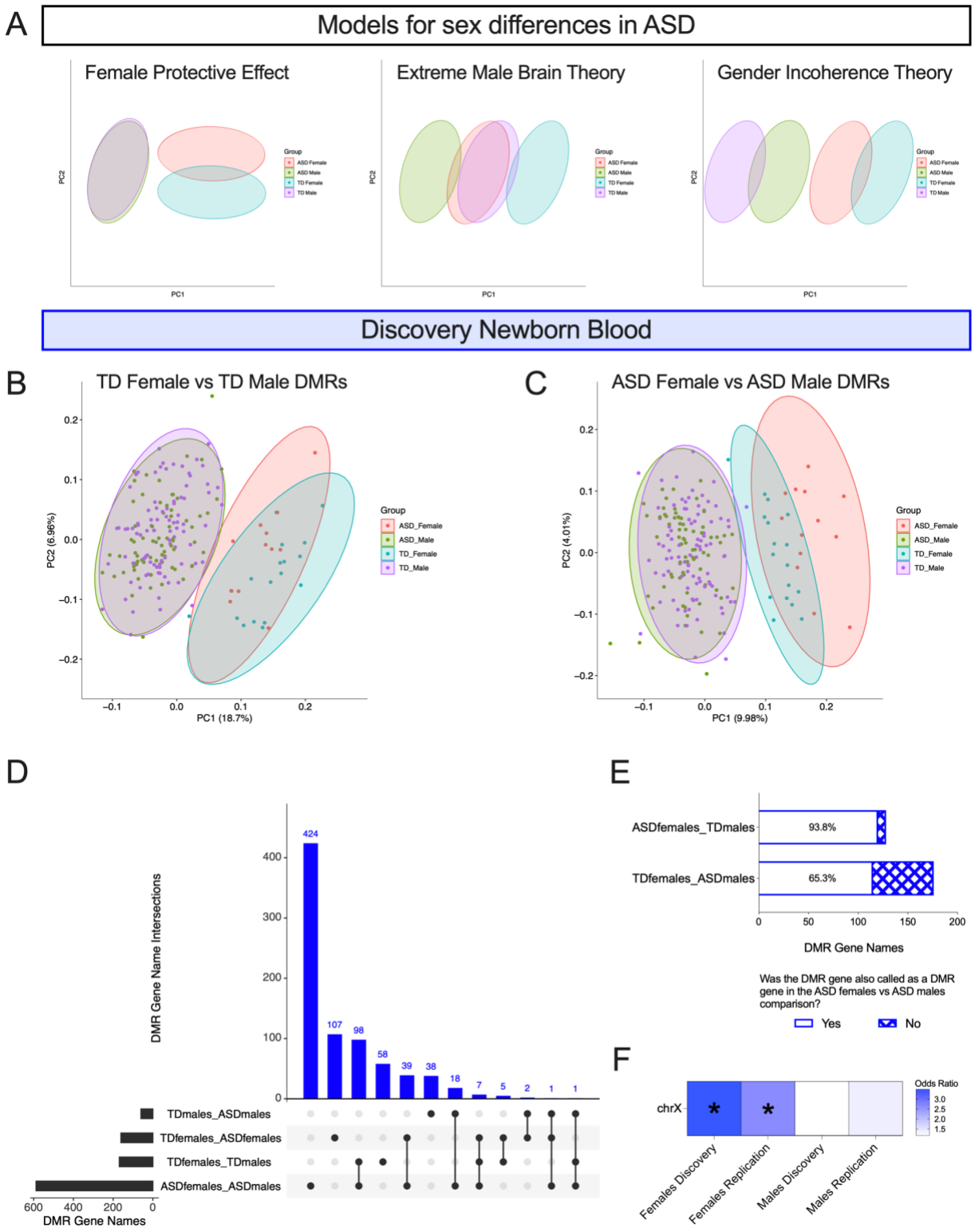

Figure 2. Discovery newborn blood DMRs display female protective effect. A) PCA models reflecting different theories for sex differences in ASD**.** PCA of smooth methylation values in discovery newborn

blood samples over DMRs from **B)** TD female vs TD male comparison and **C)** ASD female vs ASD male comparison. **D)** UpSet Plot of autosomal DMRs intersected by gene name. **E)** Bar plot of ASD female vs TD male and TD female vs ASD male comparisons, showing proportion of autosomal DMR genes that were also called as an autosomal DMR gene in the ASD female vs ASD male comparison. **F)** Heatmap showing enrichment on the X chromosome from ASD vs TD DMRs identified from discovery and replication newborn blood females and males. *q <0.05

ASD DMRs identified from females map to X-linked *BCOR* gene in all tissues.

Once we established the sex-specificity of ASD epigenetic signatures, we wanted to gain insights into potential biomarkers by identifying loci that are differentially methylated in ASD vs TD samples across tissues. First, we mapped DMRs from all analyses to the nearest gene and overlapped gene lists across tissues (discovery newborn blood, cord blood, placenta, and cortex). In all tissue-tissue pairwise comparisons, females had a greater percent overlap of DMRs by gene name than males **(Figure 3A-B)**, except for cord blood vs placenta **(Supplemental Table 23)**. We tested the significance of these tissue-tissue gene overlaps and found that all pairwise comparisons had significantly more overlaps than would be expected by chance, except for discovery newborn blood vs cortex in both sexes and cord blood vs cortex in males. In females, three genes mapped to DMRs identified in all four tissues: *BCOR* (chrX), *GALNT9* (chr12), and *OPCML* (chr11) **(Figure 3A) (Supplemental Figure 6).** *BCOR* and *OPCML* were also replicated in newborn blood from females **(Supplemental Table 15)**. In males, one gene mapped to DMRs in all tissues: *ZNF733P* (chr7) **(Figure 3B)**.

ASD DMRs overlap by genomic location in females better than in males across tissues.

As a more stringent comparison than gene name overlap, we overlapped DMRs from each tissue by genomic location. Very few overlaps would be expected by chance since DMRs cover a very small fraction of the genome and indeed, there were zero loci that had a DMR in all four tissues in either females **(Figure 3C)** or males **(Figure 3D)**. However, in females, there were three overlapping DMRs across three tissues (chr2:130037414-130037709 in newborn blood, cord blood, and cortex; chr20:30899557-30900219 in newborn blood, cord blood, and placenta; and chr20:29741371-29742057 in cord blood, placenta, and cortex) **(Figure 3C) (Supplemental Table 24)**.

We calculated the significance of tissue-tissue pairwise overlaps by genomic location and found that, in females, the overlap of DMRs was significantly higher than expected by chance in newborn blood vs cord blood (p =9.99E-5), newborn blood vs placenta (p =0.048), and placenta vs cortex (p =0.005) **(Supplemental Table 25)**. In males, no overlaps between any two tissues were significant.

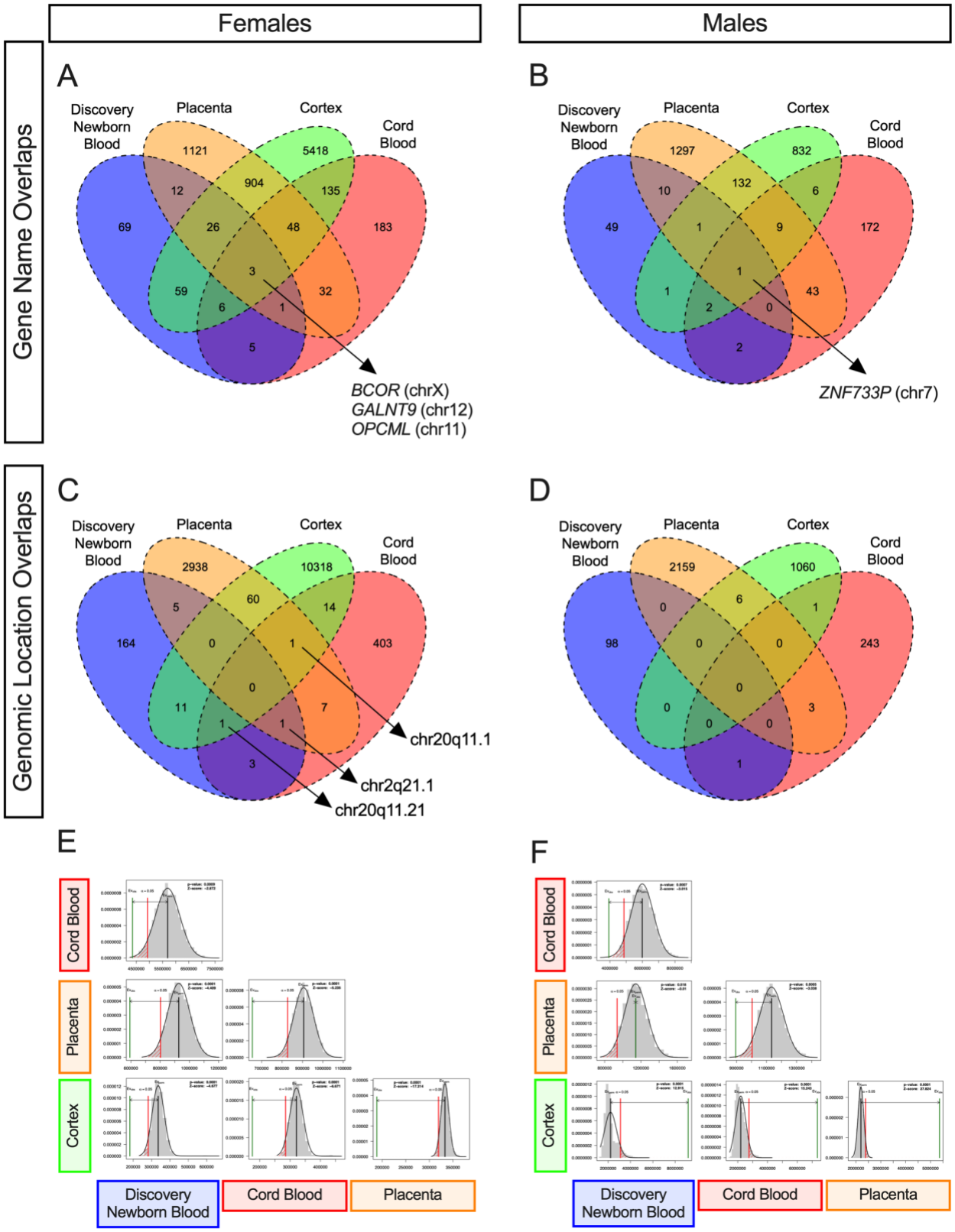
Females
Males
Gene Name Overlaps
Genomic Location Overlaps
A
Discovery Newborn Blood
Placenta
Cortex
Cord Blood
1121
5418
12
904
135
69
26
48
183
3
59
32
6
1
5
BCOR (chrX)
GALNT9 (chr12)
OPCML (chr11)
B
1297
832
10
132
6
49
1
9
172
1
1
43
2
0
2
ZNF733P (chr7)
C
2938
10318
5
60
14
164
0
1
403
0
11
7
1
1
3
chr20q11.1
chr2q21.1
chr20q11.21
D
2159
1060
0
6
1
98
0
0
243
0
0
3
0
0
1
E
F
Cord Blood
Placenta
Cortex
Discovery Newborn Blood
Cord Blood
Placenta

Figure 3. **ASD vs TD DMR overlaps across tissues.** DMRs were overlapped by **A)** gene name in females and **B)** males, and by **C)** genomic location in females and **D)** males. **E)** Pairwise comparisons of the proximity of any given DMR to the closest DMR in another tissue in females and **F)** males using permutation testing. The black line is the expected distance, the red line is alpha = 0.05, and the green line is the observed distance.

ASD DMRs in different tissues are closer together than expected by chance, particularly in females, but are still tissue-specific.

As the likelihood of DMRs overlapping by genomic locations across analyses is very low, we also tested whether DMRs identified in different tissues were closer together on the genome than would be expected by chance. We found that for all pairwise tissue comparisons of DMRs identified from females, the mean distance of a DMR in a given tissue was closer to the nearest DMR in another tissue than would be expected by chance (all *p*-values <0.001) **(Figures 3E) (Supplemental Table 25)**. In males, DMRs from newborn blood vs cord blood and cord blood vs placenta were closer than expected by chance, but DMRs identified from cortex were significantly farther from DMRs identified in all other tissues **(Figures 3F)**.

Despite DMRs being closer together than expected by chance across many tissues, particularly in females, we hypothesized that DMRs are largely tissue specific and would not differentiate ASD from TD samples from other tissues. To test this tissue specificity, we assayed the smoothed CpG methylation values in discovery newborn blood samples over the DMRs identified in cord blood, placenta, and cortex and confirmed by PCA that the samples did not separate by ASD diagnosis in either sex **(Supplemental Figure 7).**

DMRs identified from females are enriched for neurodevelopment-related biological processes in all tissues.

We next performed gene ontology **(GO)** analysis on genes mapped to ASD DMRs from all tissues using GOfuncR and identified terms related to biological processes that had a *p-value* <0.2 in all tissues. There were 15 terms that appeared in all tissues' female DMR analyses, with the most significant GO term being "central nervous system neuron differentiation" **(Figure 4A)**. Other relevant terms in females included "ventral spinal cord interneuron specification" and "regulation of histone H3-K36 methylation". In males, only six terms appeared in all tissues and were related to GTPase activity and muscle cell migration.

DMR genes significantly overlap with SFARI genes, particularly in females.

Lastly, we overlapped DMRs mapped to genes from all analyses with known ASD-associated genes from the SFARI database (54). Across all four tissues, DMR genes were significantly enriched (p <0.05) for SFARI genes in sex-combined, females only, and males only analyses, except for discovery newborn blood sex-combined and males only comparisons **(Figure 4B)** **(Supplemental Table 26)**. DMR genes identified from females were more significantly enriched with SFARI genes than DMR genes identified from males in all four tissues.

To identify loci that replicated across two cohorts of newborn blood and have been genetically linked with ASD, we overlapped discovery and replication newborn blood DMR genes with SFARI genes. We found that, of the 87 newborn blood DMRs that replicated in females across two cohorts, 10 (11.5%) were listed as SFARI genes, two of which are X-linked **(Figure 4C)**:

AGMO (chr7), *ARX* (chrX), *CASZ1* (chr1), *CNTNAP2* (chr7), *GALNT2* (chr1), *KIRREL3* (chr11), *MYT1L* (chr2), *NTRK1* (chr1), *PCDH11X* (chrX), and *SLC4A10* (chr2). No replicated DMRs genes identified from males were also SFARI genes.

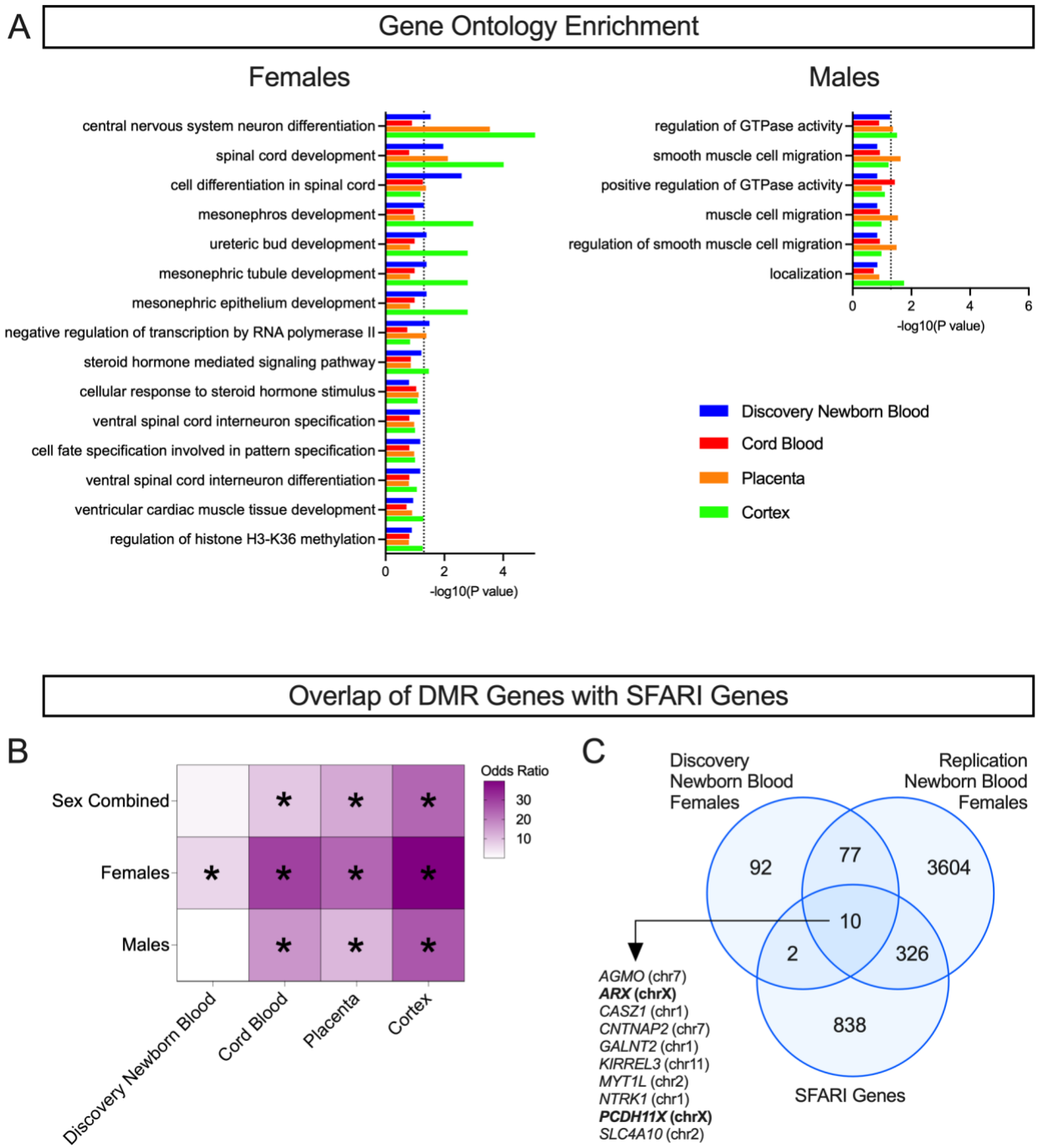

Figure 4. **A)** Gene ontology enrichment. Sex-specific DMRs from each tissue were examined for gene ontology enrichments using GOfuncR. Biological process enrichments with $p < 0.2$ from all tissues were overlapped and graphed by their -log(p-value). The dotted black lines represent $p = 0.05$. **B)** Heatmap of sex-specific DMRs from each tissue overlapped with SFARI genes, with darker purple representing a higher odds ratio (range: 0-40). *$p < 0.05$. **C)** Venn diagram of DMR genes identified in females from discovery newborn blood and replication newborn blood overlapped with SFARI genes.

Discussion

In this study, we investigated a sex-specific epigenomic signature of ASD using previously published umbilical cord blood, placenta, and cortex methylation data as well as two previously unpublished newborn blood datasets. This study was the first to analyze sex-stratified WGBS data from ASD and TD individuals across multiple tissues and cohort designs using consistent analysis parameters, showing the advantages of incorporating published data into new analyses to gain a comprehensive picture. Our findings revealed that the DNA methylation signature of ASD is sex- and tissue-specific, informing future efforts to develop newborn screening tools for ASD.

At the genomic region level, we found a consistently stronger epigenetic signature of ASD in females than in males across multiple tissues and cohorts. In placenta, umbilical cord blood, and two datasets of newborn blood, DMRs differentiated ASD from TD samples in females but not in males, despite the much smaller numbers of females for each of these tissues. While we considered the possibility of false positive results from our female DMR comparisons, the separation of ASD and TD samples across all three perinatal tissues, the high replication of DMRs across two newborn blood cohorts, and the enrichment for gene ontology terms related to neurodevelopment in females

across tissues provided confidence for our interpretation that the ASD epigenetic signature is indeed stronger in females than males. This female-specific ASD signature is characterized by hypomethylation, with the majority of DMRs identified from females being hypomethylated in ASD compared to TD samples across all perinatal tissues, while males varied widely in the proportion of hypo- to hypermethylated DMRs in various tissues. Interestingly, we did find significant overlap across all tissues between ASD DMRs identified in females and those identified in males, indicating that the sexes may share some epigenetic marks of ASD, with females having many more additional methylation differences between ASD and TD individuals.

We also explored proposed models for sex differences in ASD using our discovery newborn blood cohort and found epigenetic support for the female protective effect, but not for either the gender incoherence or the extreme male brain theories. The latter two theories hypothesize that ASD females will look more male compared to TD females, and that ASD males will look either more female (gender incoherence) or more male (extreme male brain) compared to TD males (33). However, we observed in our discovery newborn blood data that ASD and TD males did not differentiate over female vs male DMRs and that ASD females did differentiate some from TD females but the directionality (towards or away from males) depended on the genomic regions assayed. The epigenetic distinction between ASD and TD females, and lack of distinction between ASD and TD males, was also observed when we conducted ASD female vs TD male and TD female vs ASD male DMR comparisons and found a striking overlap (93.8%) of DMR genes between ASD female vs TD male and ASD female vs ASD male comparisons. The much lower overlap (65.3%) between TD female vs ASD male and ASD female vs ASD male comparisons indicate that TD and ASD males have more similar newborn blood methylation profiles than do

TD and ASD females. This may be explained, at least in part, by the enrichment of female-specific ASD vs TD DMRs on the X chromosome. Taken together, these results show that females diagnosed with ASD have a stronger epigenetic signature than do males, aligning with the female protective effect model, which hypothesizes that the protective nature of certain factors in females (such as DNA methylation marks) require more changes to be attenuated to result in ASD, leading to fewer cases in females and stronger biological signatures.

Our results indicating a stronger epigenetic signature in females than in males across perinatal tissues support previous neurological and genetic studies. For example, neuroimaging comparisons of ASD vs TD individuals have found larger differences in females than in males with regard to cortical thickness and development (55), organization of nerve fibers (56,57), gray matter asymmetry (58), amygdala functional connectivity with the cortex (59), local connectivity within brain networks (60). Additionally, many genetic studies have found that females with ASD carry more autism-associated genetic mutations than do males with ASD (61–65). However, statistical genetics models have shed doubt on the female protective effect with respect to the liability threshold model (66), which posits that a greater etiologic load (genetic or environmental) is required for females to cross the ASD threshold than males. As an alternative to the threshold concept, Dougherty et al. (2022) proposed a non-threshold model whereby (the loss of) stochastic developmental processes early in life influence the development of ASD in a sex-specific manner (66). Epigenetic mechanisms, such as DNA methylation, may provide or reflect this stochasticity. It is possible that females retain more stochasticity early in life, potentially due to X chromosome mosaicism or hormonal differences, leading to fewer ASD cases but more severe phenotypes and stronger biological signatures when that stochasticity is lost. Our data support this hypothesis by

showing greater differences between ASD and TD in females than males that are reflected across newborn blood, cord blood, and umbilical cord blood, and enrichment on the X chromosome by newborn blood DMRs identified in females but not males. Because these stochastic sex differences are hypothesized to occur early in life, and because the phenotypes of ASD may influence DNA methylation, our study effectively explores sex differences in ASD by using perinatal tissues that were collected long before the age of diagnosis.

Having established the sex specificity of the epigenetic signature of ASD, we next identified sex-specific loci that were differentially methylated in ASD vs TD samples across all four assayed tissues (newborn blood, cord blood, placenta, cortex). Given the model of sex-specific stochasticity in early life, we hypothesized that DNA methylation differences may be detectable across multiple perinatal tissues if the dysregulation occurred prior to tissue differentiation. In females, one or more DMRs from all four tissues mapped to *BCOR*, *GALNT9*, and *OPCML* while in males, DMRs mapped to *ZNF733P*. X-linked *BCOR* encodes the BCL6 corepressor and plays a critical role in early embryonic development. A *BCOR* homolog, *BCORL1*, has been implicated in ASD in several studies (67–69). *GALNT9* is part of the GALNT gene family that encodes enzymes involved in O-glycosylation; it is expressed specifically in the brain, most highly in the cerebellum, and its deletion has been previously implicated in ASD (70). *OPCML* encodes neural cell adhesion molecules and SNPs in this gene have been linked to schizophrenia (71,72). *ZNF733P* is a pseudogene located nearby many zinc-finger protein-encoding genes close to the centromere of chromosome 7. To our knowledge, this region has not previously been associated with neurodevelopmental or neurodegenerative disorders, though previous genome assemblies have not reliably represented centromeric regions (73). It is known that pericentromeric regions

are often highly methylated and that dysregulated methylation has been associated with genomic instability and disease, suggesting that further investigation into the epigenomic signature of pericentromeric regions is worthwhile (74).

This study demonstrates the value in sex-stratifying samples for ASD studies, despite reduced sample sizes. We found very different epigenetic signatures in females vs males that were not found in sex-combined analyses, with particularly strong signatures identified in females. Given that most ASD studies have vastly more samples from males than from females, it is especially important to study the sexes separately so that the signature in females is not over-shadowed. Additionally, it was useful to directly compare females and males within diagnostic groups, as this led us to learn that sex differences in newborn blood methylation were more pronounced in ASD individuals than TD individuals, though with significant overlap. These results align with a recent neuroimaging study, which found overlap between ASD sex differences and TD sex differences in cortical thickness and the trajectory of cortical thinning across childhood (55). Our study therefore adds to the evidence that the mechanisms governing sex differentiation in neurotypical brains may also contribute to sex differences in ASD and shows that these biological differences are not only measurable in the brain but are reflected by methylation changes in perinatal surrogate tissues.

In addition to furthering our understanding of sex differences in ASD, our results suggest that reproducible DNA methylation differences detectable in newborn blood could be potentially added to molecular screening panels to identify female infants who should be behaviorally screened for ASD. Newborn biomarkers may be particularly useful for cases that are harder to identify

behaviorally, and may help to overcome sex biases and other disparities in ASD diagnosis (75). A positive screening result would inform parents and medical practitioners that diagnostic services will be paramount for the child and, importantly, a negative screening result would not exclude the possibility of future diagnosis. While additional research is needed in this area, our study provides initial results supporting a possible future of ASD screening in newborns, which may help to overcome future sex biases in ASD diagnoses (75).

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Conflict of Interest

The authors declare that they have no conflicting interests.

Availability of Data

Code for this study is available on GitHub (https://github.com/juliamouat/Pan-tissue_ASD). The WGBS data from cord blood, placenta, and cortex are available in the Gene Expression Omnibus

with accession numbers GSE140730 (cord blood), GSE178206 (placenta), GSE81541 (cortex) and GSE119981 (cortex). The WGBS data from the discovery and replication newborn blood are not publicly available: Any uploading of genomic data (including genome-wide DNA methylation data) and/or sharing of California Biobank Program biospecimens or individual data derived from these biospecimens has been determined to violate the statutory scheme of the California Health and Safety Code Sects. 124980(j), 124991(b), (g), (h), and 103850 (a) and (d), which protect the confidential nature of biospecimens and individual data derived from biospecimens. Should we be contacted regarding individual-level data contributing to the findings reported in this study, inquiries will be directed to the California Department of Public Health Institutional Review Board to establish an approved protocol to utilize the data, which cannot otherwise be shared peer-to-peer.

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