Supplementary Files

Placental DNA methylation levels at *CYP2E1* and *IRS2* are associated with child outcome in a prospective autism study

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* Last authors contributed equally to this study

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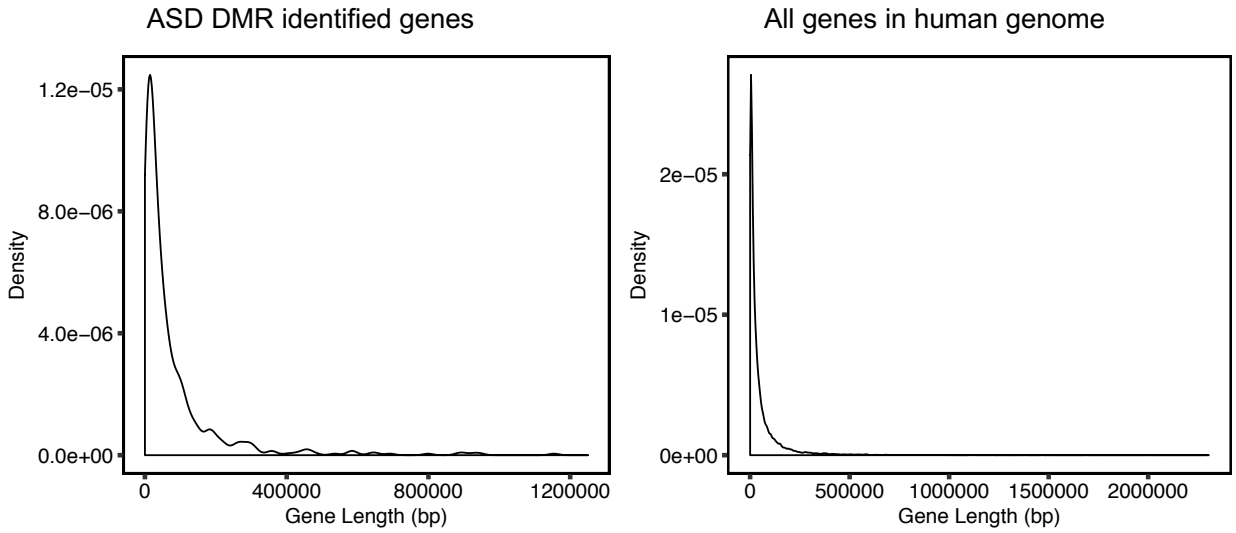
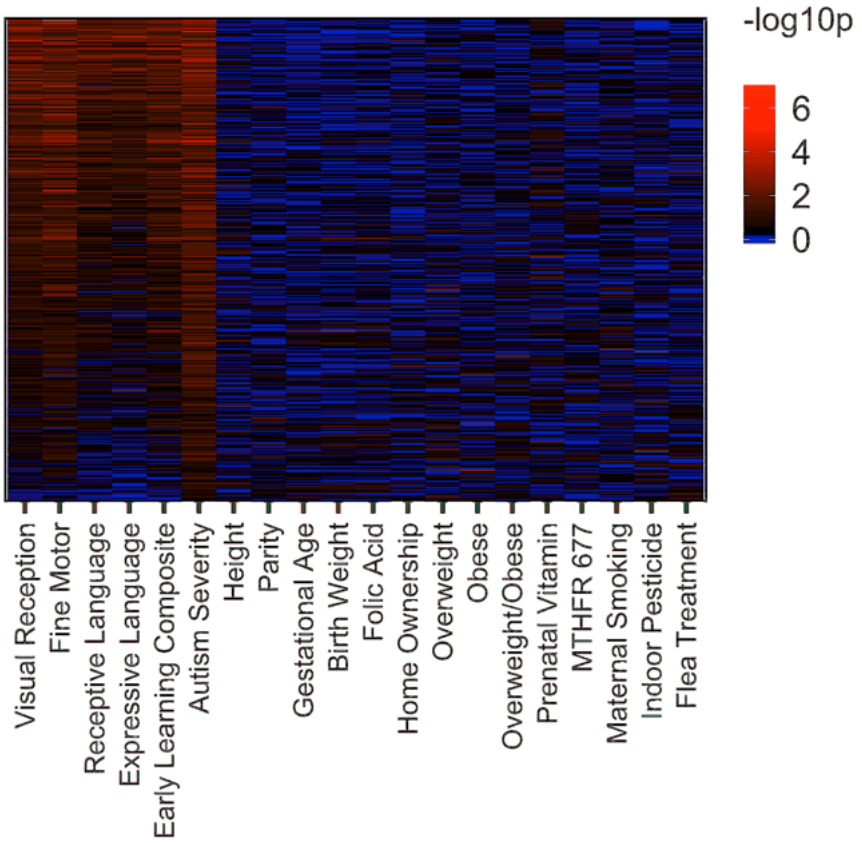


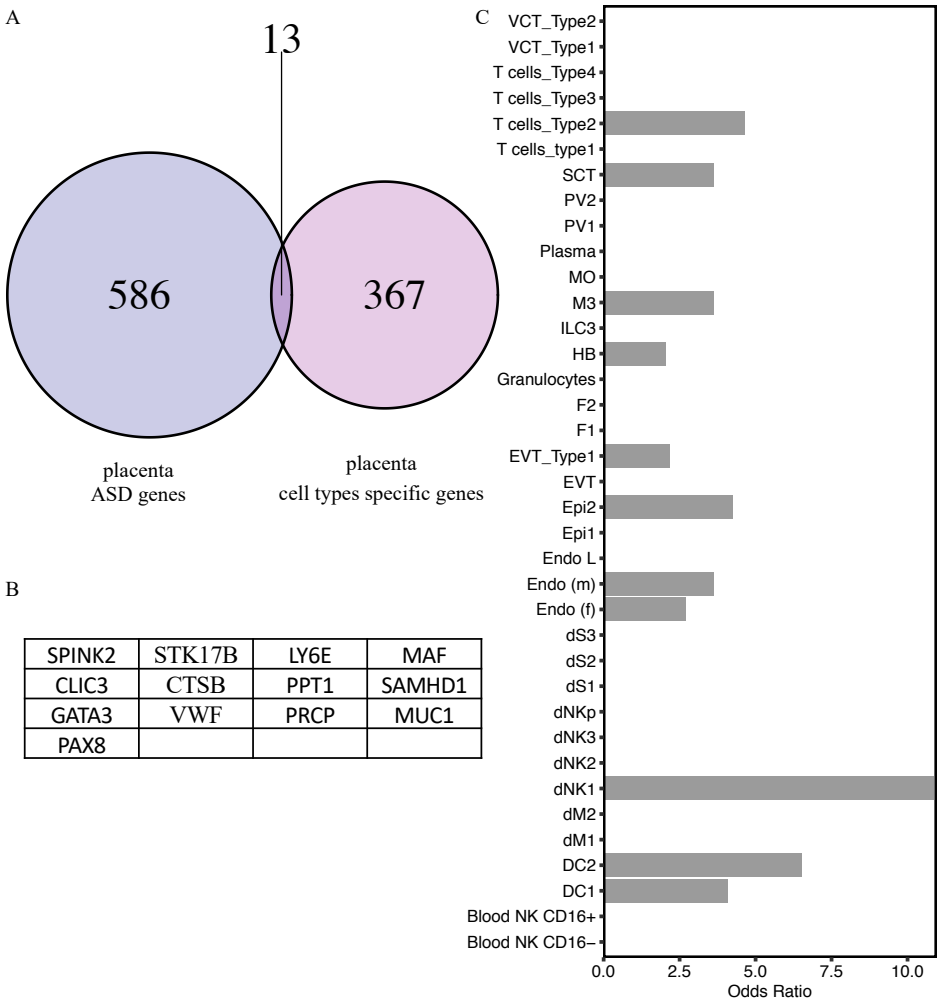
Supplementary Figure 1: The distribution of gene length for ASD DMR genes was similar to all genes in the human genome.

The density plot showed the distribution of gene length of ASD DMR identified genes (left) and all genes (right). The x-axis illustrated the gene length in base pairs. The y-axis was the density for gene length. Person's chi-squared test showed no significant difference between two distributions (p-value = 0.9994).



Supplementary Figure 2: ASD DMRs heatmap by child outcome continuous measurements of cognition and autism severity versus potential cofounding variables.

The plot shows a heatmap of ASD DMRs (y-axis) and the association of % methylation at each DMR with other measured variables. Each row in the heatmap represents one DMR and each column showed one measured variable. The first 5 child outcome variables on the x-axis includes 4 sub-categories of Mullen scores as well as composite score and autism severity score from the ADOS. Significant associations are red ($p < 0.05$). While ASD DMRs were highly associated with autism severity and to a lesser degree with early learning Mullen's scores, other potential confounding variables from MARBLES exhibited only rare associations with individual ASD DMRs.



B

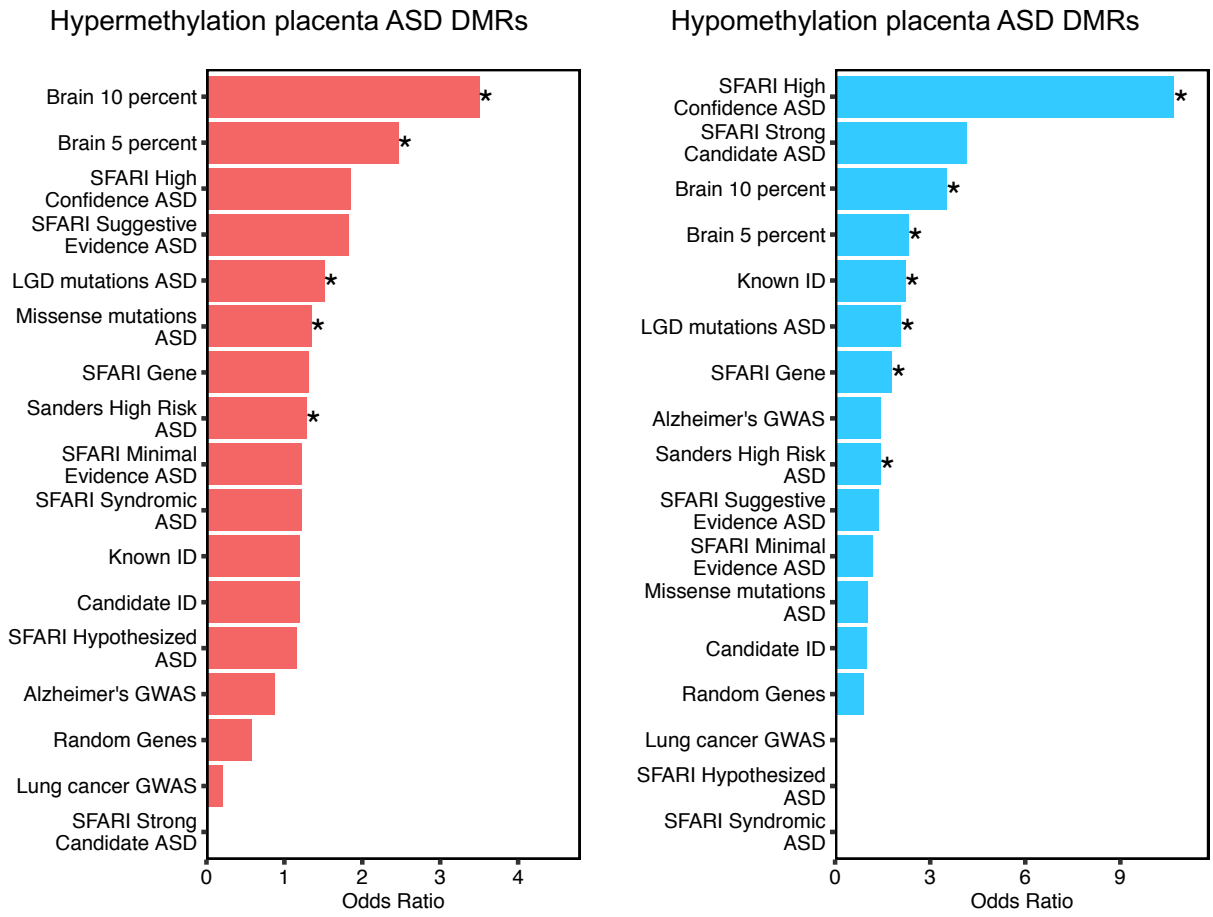
SPINK2	STK17B	LY6E	MAF
CLIC3	CTSB	PPT1	SAMHD1
GATA3	VWF	PRCP	MUC1
PAX8			

Supplementary Figure 3: Placental ASD DMRs genes did not significantly overlap with cell type specific genes in placenta.

A. Venn diagram shows a non-significant overlap of 13 genes associated with placenta ASD DMRs and placental cell type specific genes by two-tailed Fisher's exact test (*p*-value = 0.84).

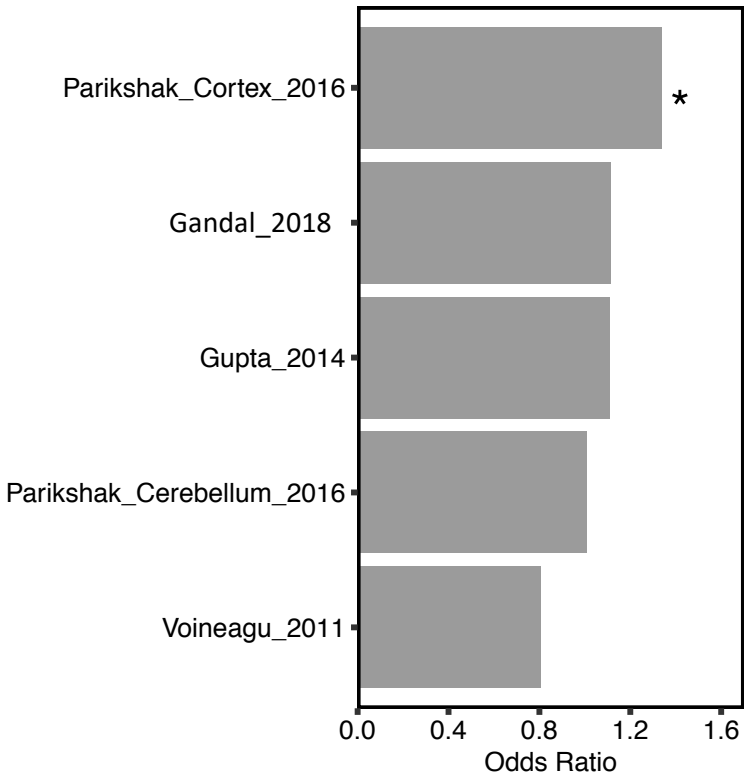
B. Table of those 13 placental ASD DMR genes that were placental cell type markers.

C. Placenta ASD DMR associated genes were compared for overlap by two-tailed Fisher's exact test with each of 38 different identified placental cell types. None of cell type specific genes were significant using two-tailed Fisher's exact test.



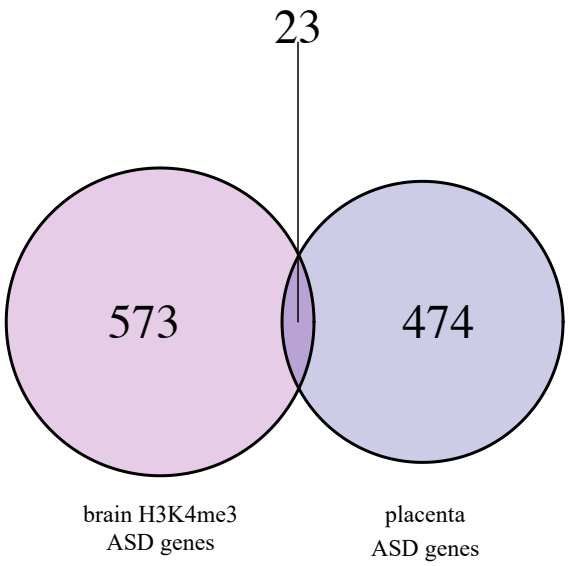
Supplementary Figure 4: Enrichment test on hyper- versus hypomethylated placenta ASD DMR genes for overlap with ASD risk genes and other databases.

Hypermethylated (left) and Hypomethylated (right) placenta ASD DMRs associated genes overlapped with ASD genetics risk database, other intellectual disability and a random gene list ranked by odds ratio. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by two tailed Fisher's exact test after the FDR correction. SFARI: Simons Foundation Autism Research Initiative, LGD: likely gene disrupting mutation, ASD: autism spectrum disorder, Alzheimer: Alzheimer's Disease, ID: intellectual disability.



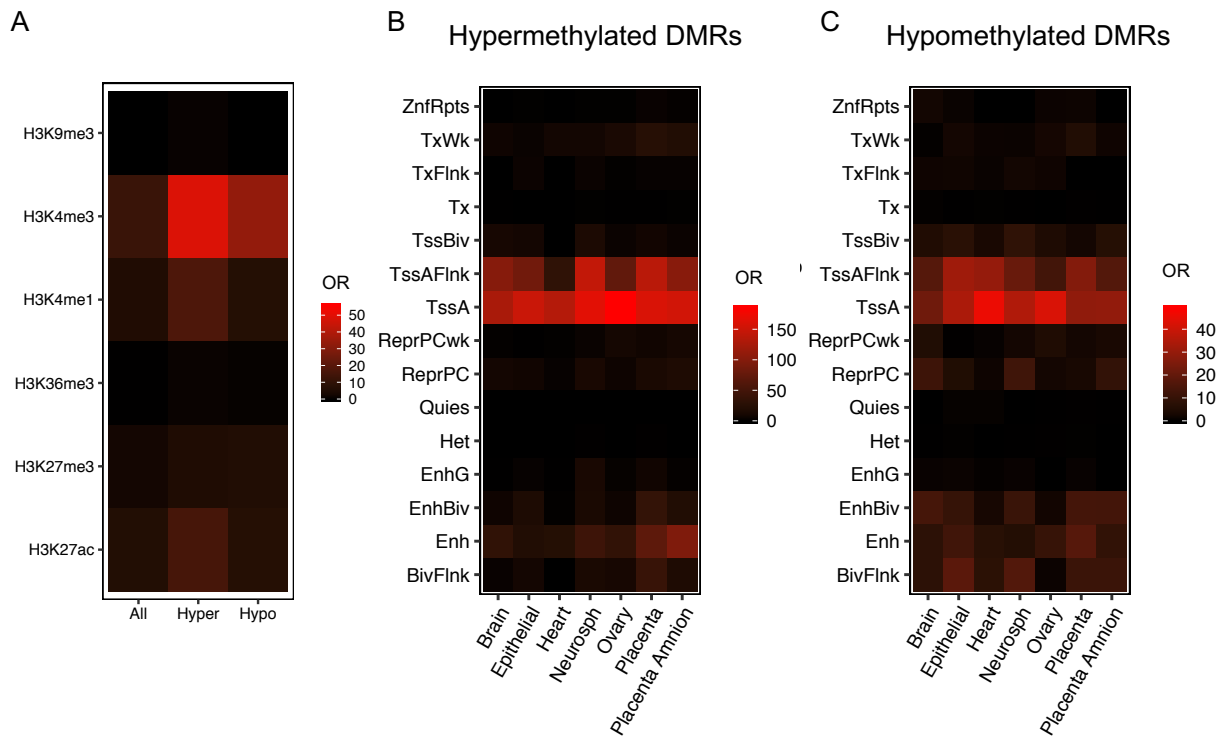
Supplementary Figure 5: Enrichment test on placenta ASD DMR genes and differential expressed genes in ASD postmortem brain.

Placenta ASD DMRs associated genes overlapped with four ASD brain differential expressed genes databases ranked by odds ratio. *$p < 0.05$ by two tailed Fisher's exact test after the FDR correction.



Supplementary Figure 6: Placental ASD DMR genes significantly overlapped with genes associated woth H3K4me3 changes in ASD brain study.

Venn diagram shows the significant overlap between placenta ASD DMRs genes and genes associated with H3K4me3 changes in brain (Shulha et al, 2012) by Fisher's exact test (p-value < 0.05).

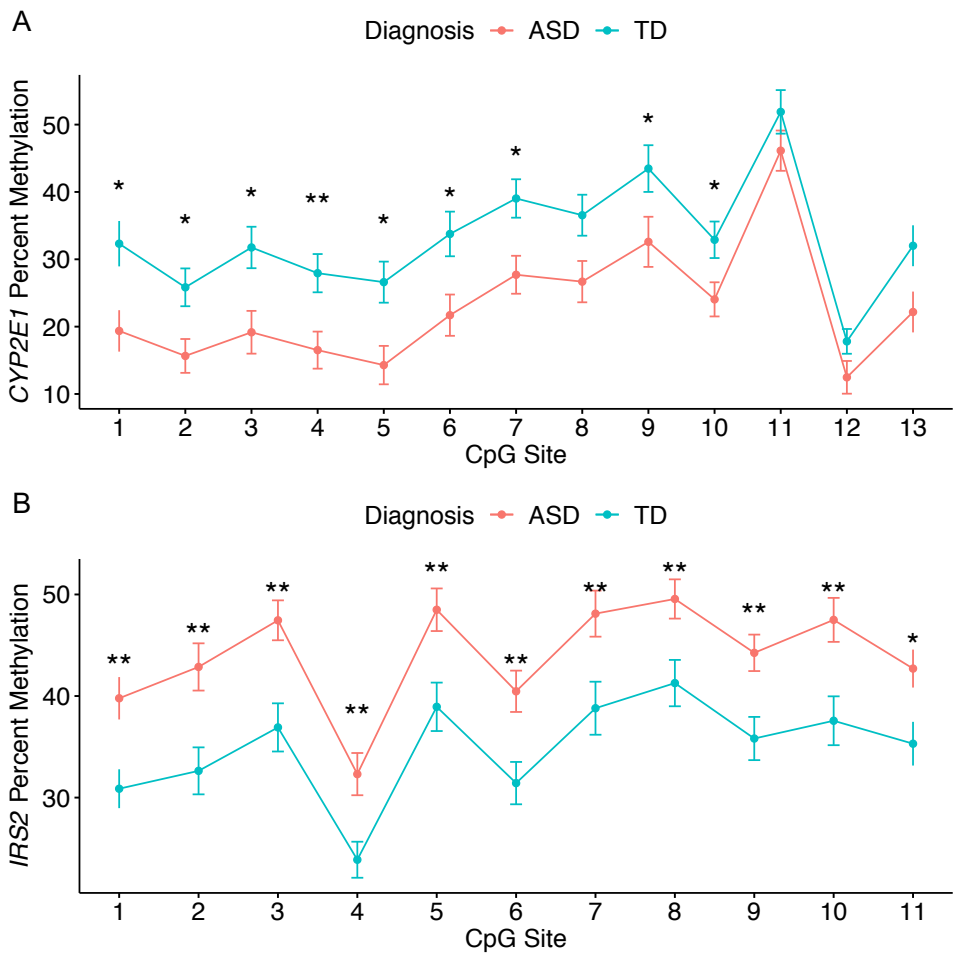


Supplementary Figure 7: Placenta ASD hyper- and hypomethylated DMRs were both enriched at H3K4me3 regions, active promoters and their flanking regions.

A. Hyper- and hypomethylated placenta ASD DMRs were overlapped with histone modification human placenta ChIP-seq peaks from the Epigenome Roadmap with odds ratio plotted

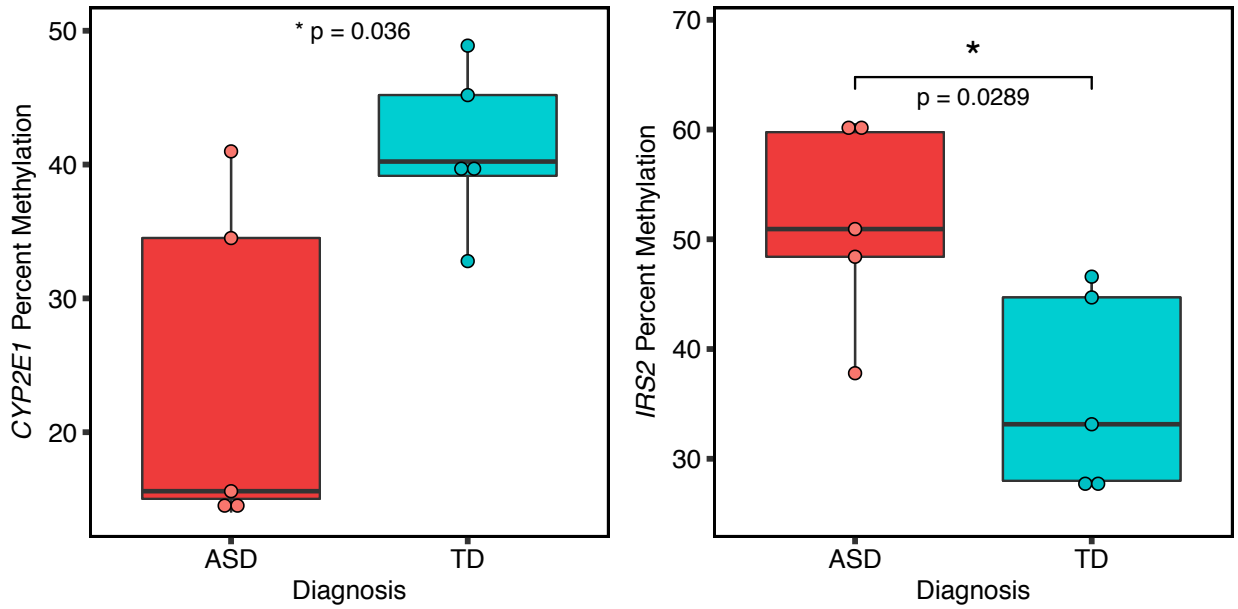
B. Hypermethylated placenta ASD DMRs were tested on chromatin states from the Epigenome Roadmap. The x-axis was different tissue type, the y-axis was represented chromatin states.

C. Hypomethylated placenta ASD DMRs were tested on chromatin states from the Epigenome Roadmap.



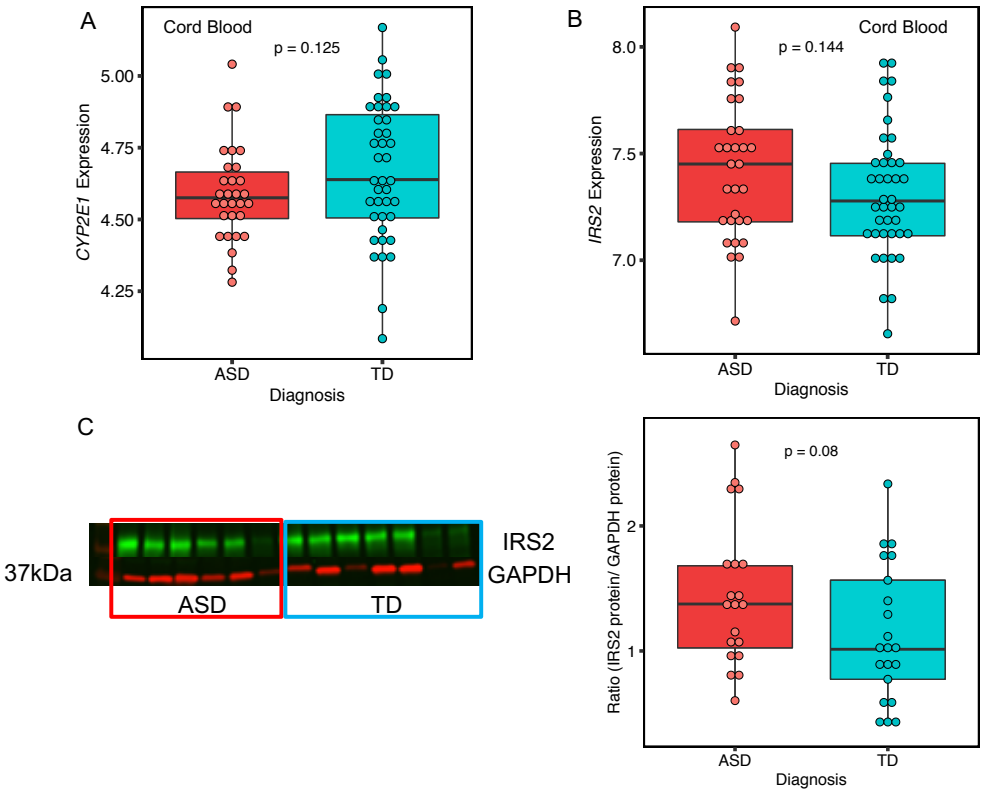
Supplementary Figure 8: Pyrosequencing results from *CYP2E1* and *IRS2* DMRs for each CpG site.

The x-axis represents each CpG sites included in pyrosequencing DMR regions. The y-axis plots the percent methylation at each CpG site. The red line showed the average methylation of ASD samples and the blue line represented the average methylations of TD samples and error bars represent the standard error of the mean. Each CpG site was tested on the significance level with FDR corrected for the numbers of CpGs. *$p < 0.05$, **$p < 0.01$. **A**. 13 CpG sites tested at the *CYP2E1* DMRs with 10 of them showed significant association with diagnosis after FDR correction. **B**. All 11 CpG sites at the *IRS2* DMRs showed significant association with diagnosis after FDR correction.



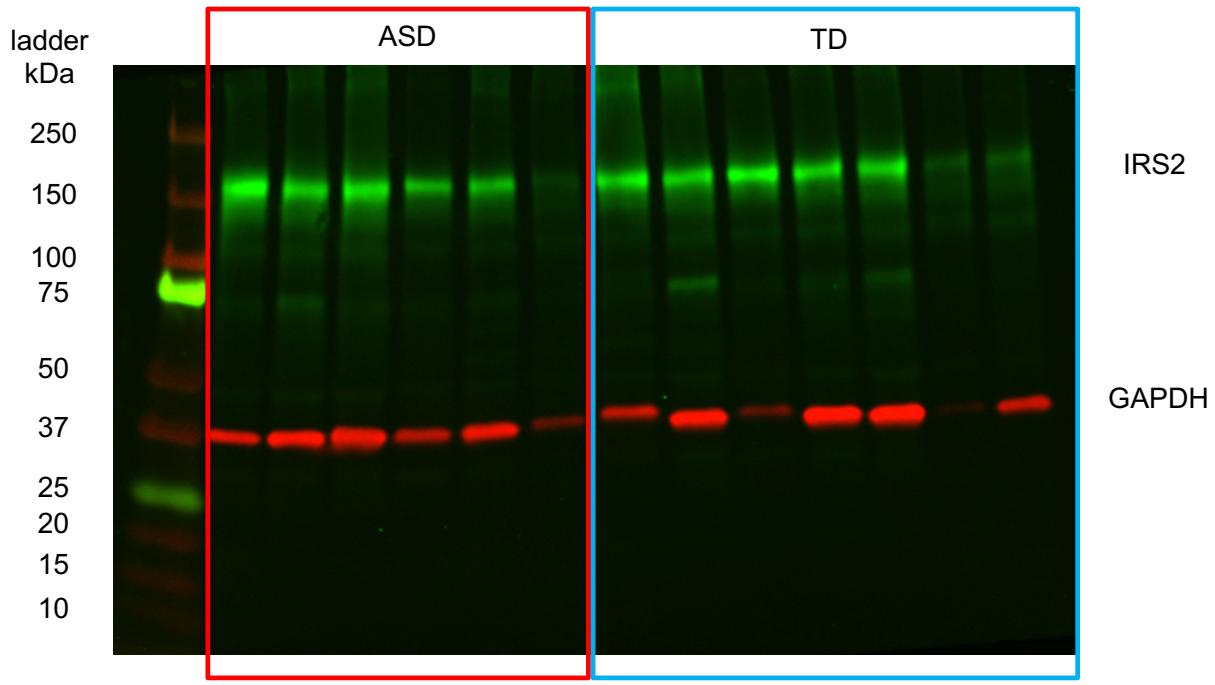
Supplementary Figure 9. Pyrosequencing results from female ASD versus TD placental samples at *CYP2E1* and *IRS2* DMRs.

Five ASD female placental samples and five TD female placental samples were used in the analysis. The left panel shows *CYP2E1* DMR percent methylation was significantly lower in ASD compared with TD (two-tailed t-test, *p*-value = 0.036) in the same direction as that observed in ASD males (Fig. 4C). The right panel shows *IRS2* DMR percent methylation is significantly higher in ASD (two-tailed t-test, *p*-value = 0.029), in the same direction as ASD males (Fig. 4D).



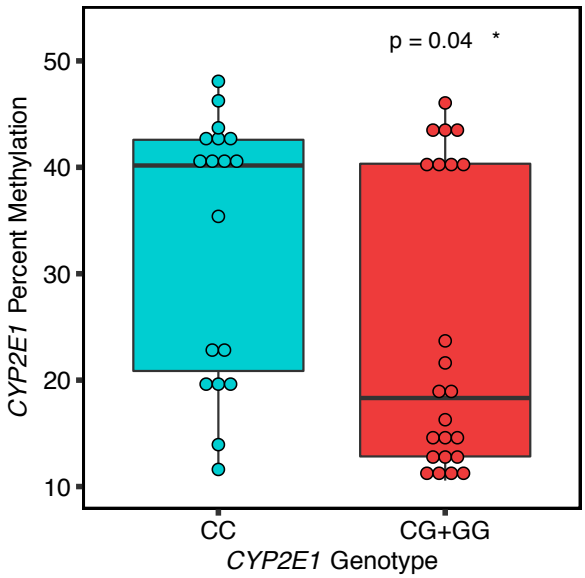
Supplementary Figure 10. For both placental ASD DMRs at *CYP2E1* and *IRS2*, expression trended towards positive correlation with methylation.

A. 30 ASD and 40 TD umbilical cord blood sample in MARBLES were included in this analysis. Affymetric array matrix data on the probe 16711001 was used to represent the expression of *CYP2E1* on the y-axis. Each dot was used to represent one individual (two-tailed t-test, *p*-value = 0.125). **B**. The same umbilical cord blood samples were used for measuring *IRS2* expression at the probe 16780917 (two-tailed t-test, *p*-value = 0.144). **C**. Representative Westerns blots are shown for the ratio of IRS2 to GAPDH (normalization control) in all 41 placenta samples of ASD and TD comparison with each dot representing one sample (two-tailed t-test, *p*-value = 0.08). A Western blot with 6 samples in ASD and 7 samples TD were showed at the left panel. IRS2 protein was labeled with green fluorescence at 185 kDa and GAPDH was marked with red fluorescence at 37 kDa.



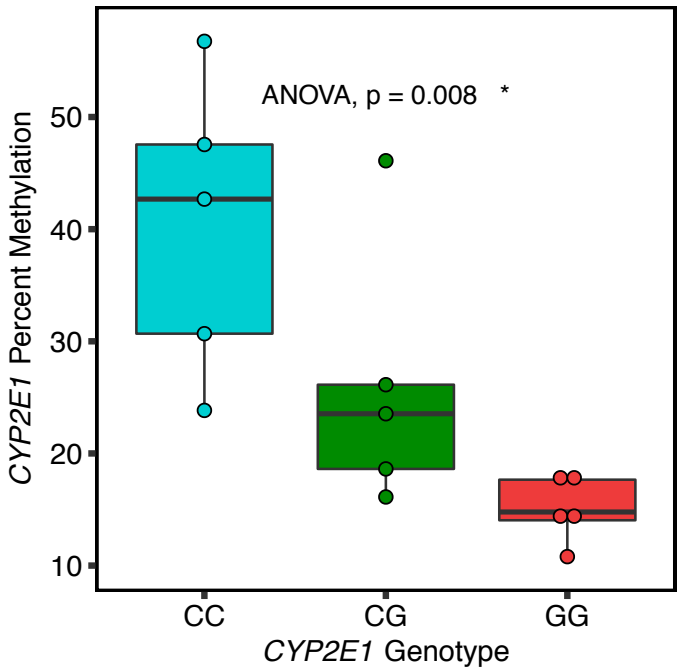
Supplementary Figure 11: Representative IRS2 Western blot image.

A representative Western blot image of placental protein extracts detected by anti-IRS2 (green) or anti-GAPDH (red) antibodies. The left column is the protein size ladder.

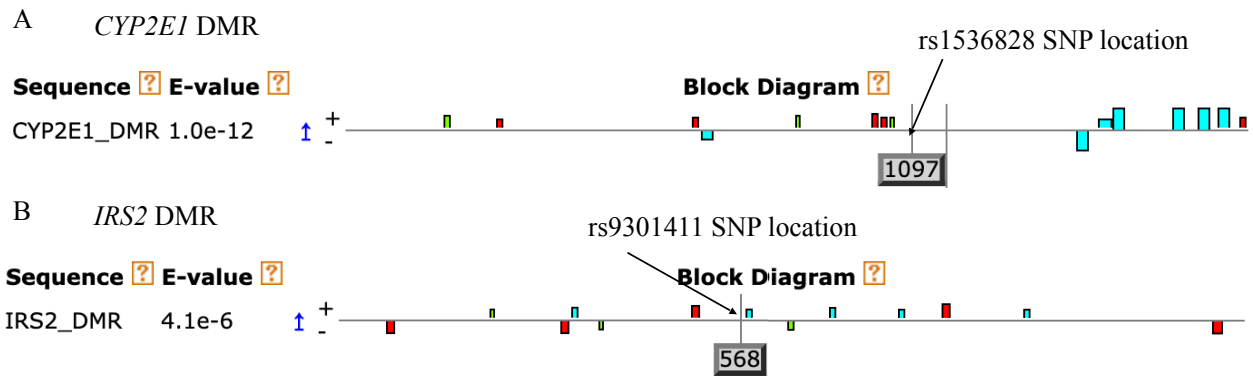


Supplementary Figure 12: *CYP2E1* genotype (rs1536828) was significantly associated with *CYP2E1* DMR methylation levels.

CYP2E1 genotype at rs1536828 combining the minor homozygous genotype (GG) and heterozygous genotype together (CG) together within the ASD DMR was significantly associated with *CYP2E1* DMR average percent methylation tested by two-tailed t-test (*p*-value = 0.04).

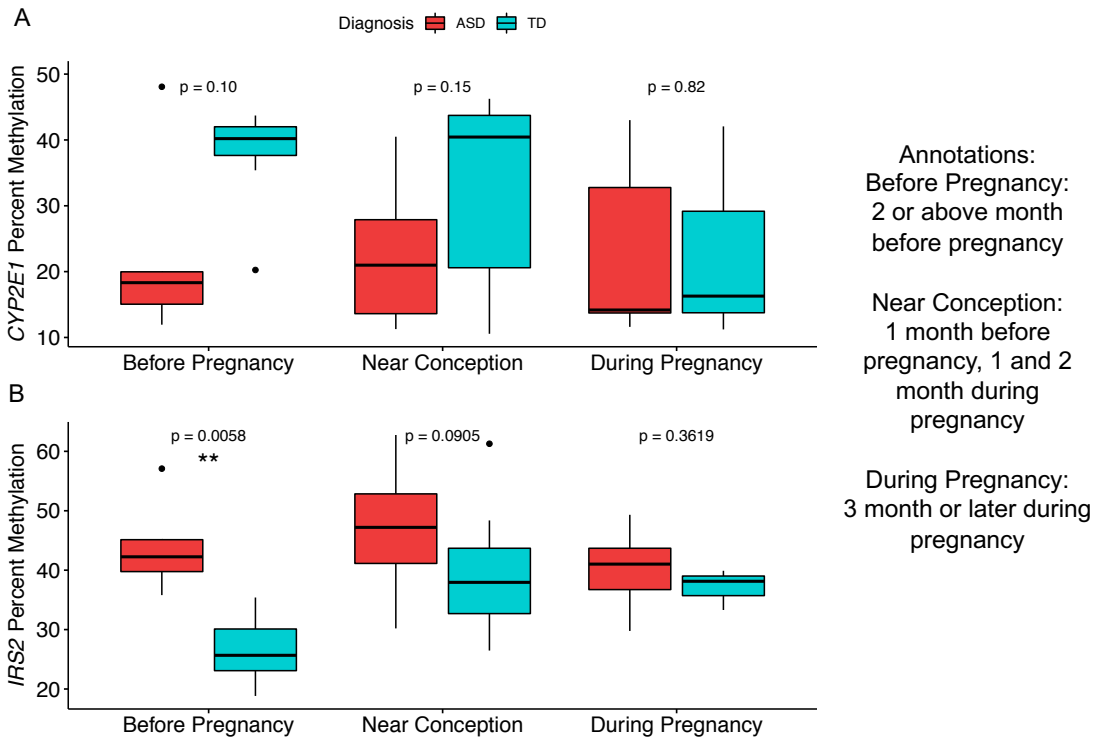


Supplementary Figure 13: *CYP2E1* genotype (rs1536828) was significantly associated with *CYP2E1* DMR methylation levels on an additional 15 MARBLES placental samples. Sanger sequencing and pyrosequencing were performed on an additional 15 MARBLES placental samples, with 5 samples in each genotype groups (CC, CG, and GG). *CYP2E1* genotype at rs1536828 was significantly associated with *CYP2E1* DMR percent methylation using AVOVA (*p*-value = 0.008).



Supplementary Figure 14: *CYP2E1* genotype (rs1536828) and *IRS2* genotype (rs9301411) do not change transcription factor motifs.

Motif structure identified by MEME at *CYP2E1* DMR (**A**) and *IRS2* (**B**) DMR. Horizontal line represents the DMR DNA sequence. Each block shows the location of a transcription factor motif. Arrows point to the SNP location on the sequence. Numbers within grey boxes represent the relative SNP location to the DMR DNA sequence.



Supplementary Figure 7: Prenatal vitamins intake prior to pregnancy was protective for placental DNA methylation patterns at *CYP2E1* and *IRS2* DMRs.

Each sample was labelled at the month the mother started taking prenatal vitamins. The time line was separated into three-time bins. Mothers who started prenatal vitamin use more than two months before pregnancy were grouped as "before pregnancy". Those who started one month before pregnancy, first, or second month of pregnancy were categorized as "near conception", while "during pregnancy" include those who started taking prenatal vitamins 3 months or later into pregnancy. Two-tailed t-test was done within each category between ASD and TD samples. For both *CYP2E1* (**A**) and *IRS2* (**B**) DMRs, the largest protective effect of prenatal vitamin use on methylation levels was observed in placentas from mothers who started prenatal vitamin use before pregnancy.

Supplementary Tables:

Supplementary Table 1:

400 differentially methylated regions (DMRs) in placenta that distinguish ASD and TD samples and their association with 597 genes.

Supplementary Table 2:

Neurodevelopmental outcomes and additional variables for each placenta sample.

Supplementary Table 3:

Demographic and clinical variables of children and their mothers in the MARBLES study, stratified by child diagnosis.

Supplementary Table 4:

Overlapping genes between placenta ASD DMR associated genes and cell type specific genes.

Supplementary Table 5:

597 genes associated with ASD DMRs and the distance (bp) to the transcription start site (TSS).

Supplementary Table 6:

Gene ontology (GO) analysis of ASD DMR genes by Fisher's exact test after FDR (false discovery rate) correction.

Supplementary Table 7:

Overlapping genes between placenta ASD DMR associated genes and other databases, including brain ASD DMR associated genes, ASD genetic risk factors, intellectual disability, Alzheimer's GWAS, and lung cancer GWAS.

Supplementary Table 8:

Overlapping genes between placenta ASD DMR associated genes and differential expressions genes from ASD postmortem brain studies.

Supplementary Table 9:

Methylation data from both *CYP2E1* and *IRS2* DMRs for each sample and CpG site (13 CpG sites for *CYP2E1* and 12 CpG sites for *IRS2*). The average represents all CpG sites for each sample. Methods and primers were described in methods sections.

Supplementary Table 10:

Sanger sequencing results from *CYP2E1* DMR and *IRS2* DMR on rs943975, rs1536828 and rs9301411.

Supplementary Table 11:

376 differentially methylated regions (DMRs) in placenta separated by whether prenatal vitamins were taken or not during the first month of pregnancy. 587 genes were associated with PreVitM1 DMRs. The interaction set of 60 genes with ASD DMRs is also shown.

Supplementary Table 12:

Methylation at the *CYP2E1* DMR and *IRS2* DMR was tested for interaction with diagnosis, genotype, and PreVitM1.