DNA Methylation Signatures of Early Childhood Malnutrition Associated With Impairments in Attention and Cognition

Supplement 1

Contents:

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SUPPLEMENTAL METHODS

Human Subjects and Study Cohort

Written informed consent was obtained from all participants who were compensated for their travel to and from the research center. Approval for this study was granted by the Ministry of Health, Barbados, the Judge Baker Children's Center, Harvard Medical School, Human Research Review Committee (Assurance No. FWA 00001811) and the Massachusetts General IRB (Protocol No.2015P000329/MGH). The research design and sample recruitment for the Barbados Nutrition Study have been described elsewhere (1-3). Briefly, G1 participants were born in Barbados, an English-speaking Caribbean country, between 1967 and 1972. They had been evaluated as children, adolescents and young adults on multiple occasions between 1977 and 1991 (4-9). Those in the previously malnourished group (MAL) had been admitted to the Queen Elizabeth Hospital (Bridgetown, Barbados) in the first year of life with grade II or III protein energy malnutrition (marasmus or kwashiorkor) based on clinical manifestations and the Gomez Scale, which classifies degree of malnutrition on the basis of expected weight for age. Children were eligible for inclusion if they met the following criteria: normal birth weight (>2500 g), absence of pre- or post-natal complications, Apgar score $\geq$ 8, no encephalopathic events during childhood, and no malnutrition after the first year of life. These individuals were subsequently enrolled in a government-supported intervention program that provided health care, growth monitoring, nutrition education, subsidized foods, a pre-school program and regular home visits, and were followed in the program until the age of 12 years. Those in the healthy control group (CON) were recruited in 1977 from the same classrooms and matched to the index children for gender, age (within three months) and handedness. Birth records and growth data were also available for the controls, who met the same inclusion criteria as the index cases, other than having no history of malnutrition or growth faltering. In the current phase of the study we determined that 64 of the original 312 participants in the BNS were not eligible

for follow-up (53 no longer live in Barbados, 10 are deceased and 1 is imprisoned). However, due to limitations in funding, 156/248 were tested in middle adulthood, representing 62.9% of 248 eligible G1 individuals. Prior analyses (2) of this sample indicated that the recruited G1 sample did not differ from the non-participants in terms of gender, age, childhood socioeconomic status, or childhood IQ. Thus, the G1 participants should be considered to be representative of the original participant group.

Epigenetic data were collected in the original cohort and their offspring between 2012 and 2014, when the G1 participants were in their fifth decade of life and their offspring were young adults (Figure 1). Corresponding behavioral data from the same G1 participants were collected at a mean age of 38.1 ± 2.7 (CON) and 38.3 ± 2.0 (MAL). For the current study, there were 94 adult G1 participants (*n* = 50 MAL; *n* = 44 CON) with epigenetic and behavioral data. The study also included epigenetic data from 74 G2 children of G1 participants (*n* = 44 offspring of the MAL group; *n* = 30 offspring of the CON group). The total sample included 168 individuals (Table 1).

Blood Sample Collection

Blood samples (10 ml/EDTA tube) were collected at the Barbados Nutrition Study research center and transported promptly on ice to the Barbados Reference Laboratory (St. Michael, Barbados) for processing and freezing. Blood samples were mixed with RBC Lysis Solution (Qiagen), incubated briefly at room temperature and then spun at 2000 g for 5 min. The plasma was discarded, and the resulting pellets were frozen and shipped in dry ice to New York (Akbarian laboratory, Icahn School of Medicine at Mount Sinai) for processing. At the same time as epigenetic samples were collected, additional blood samples were collected for complete white blood cell differential on 167 of the 168 subjects (Supplemental Table S1).

Genome-wide DNA Methylation Profiling

White blood cell pellets were thawed from -80^{o}C to room temperature, 2.5 ml of cell lysis reagent (Qiagen cat# 1045703) and 100 µg/ml proteinase K (Life Technologies, cat# AM2546) was added and incubated at 55^{o}C for 1 hour on a rotator, followed by an additional incubation overnight at room temperature. Then 15 µl RNAase-A (QIAGEN, cat# 158924) was added, each sample inverted 25 times and incubated at 37^{o}C for 15 minutes. Samples were placed on ice for 10 min, and 833 µl of protein precipitation solution (Qiagen cat# 1045701) was added, followed by vortexing for 20 sec, incubation on ice for 5 min and centrifugation at 2,000xg for 10 min. Supernatant was transferred into a new 14 ml tube, and 2.5 ml of 100% isopropanol added and mixed until a white thread of DNA appeared (~25 inversions). Samples were centrifuged at 3200 rpm for 3 minutes, and supernatant was carefully decanted. The DNA pellet was washed with 2.5 ml of 70% ethanol, centrifuged at 3200g for 3 min, and air dried for 15 min prior to adding 200 µl of DNA hydration solution (Qiagen cat# 1045698). The DNA was rehydrated overnight at 4^{o}C, quantitated using by spectrophotometry (Nanodrop), and stored at -80^{o}C.

Sodium bisulfite-treatment, DNA preparation, and array hybridization were performed according to manufacturer's recommendations. Briefly, white blood cell DNA (approximately 1.25 µg/sample) was bisulfite–treated using the EZ DNA Methylation Kit (Zymo Research), and bisulfite-converted DNA analyzed on the Infinium HumanMethylation450 BeadChip (Illumina) following the manufacturer's guidelines. MAL and CON G1 and G2 samples were distributed evenly across different arrays according to nutrition status, generation and gender, in order to minimize batch effects.

Data Processing and Statistical Analysis of Illumina 450K Methylation Data

Illumina array data were processed using the Methylation Module of GenomeStudio v1.9 software using default parameters. Samples were distributed across the arrays to minimize batch effects, and with each array having an equal proportion of MAL and CON G1 and G2

samples. In each individual, probes with a detection *p*-value > .01 were removed (mean *n* = 348). 482,421 probe sequences (50-mer oligonucleotides) were remapped to the reference human genome hg18 (NCBI36) using BSMAP (10), allowing up to 2 mismatches and 3 gaps; we retained unique sequences for 470,681 autosomal probes and 11,122 probes mapping to the X chromosome. Probes that overlapped SNPs identified by the 1000 Genomes Project (minor allele frequency ≥ 0.05) within 5 bp upstream of the targeted CpG (*n* = 9,409 for autosomes; *n* = 101 for chr. X) were discarded, as such variants can introduce biases in probe performance. After quality control filter, we retained 461,272 autosomal and 11,021 chr. X probe sequences on which we performed a two color channel signal adjustment and quantile normalization on the pooled signals from both channels and recalculation of average β-values as implemented in "lumi" package of R (11). The Illumina Infinium HumanMethylation450 BeadChip contains two assay types (Infinium type I and type II probes) which utilize different probe designs. As the data produced by these two assay types shows distinct profiles, to correct this problem we performed a beta mixture quantile normalization method utilizing BMIQ (12) on the normalized data. These probes were then annotated based on their position relative to RefSeq genes using BEDTools v2.17. We defined promoter regions as ±2 kb from transcriptional start sites, gene body regions as transcription start to transcription end, and intergenic regions not annotated by the preceding categories. We also annotated individual CpG in context of: CpG island based on annotations in the UCSC genome browser (≥50% GC content, length > 200 bp, and > 0.6 ratio of observed /expected CpG dinucleotides), and CpG shore (±2 kb of island), CpG shelf (±2 kb of shore), and CpG sea (regions outside the previous three categories). The positional distribution of autosomal probes within differentially methylated regions (DMRs) with respect to CpG feature and Refseq genes was compared to the overall distribution of all filtered autosomal probes on the array, and enrichment *p* values were determined by Pearson's chi-squared test.

Comparison of Previously Malnourished and Control Groups

We performed the comparison on MAL and CON groups in two different ways: (i) MAL versus CON in Generation G1 and (ii) MAL versus CON in Generation G2 (note that G2 MAL, while the offspring of parent(s) affected by early childhood malnutrition, themselves had adequate nutrition similar to G2 CON). Methylation levels for individual CpG sites in each sample were obtained as β-values, calculated as the ratio of the methylated signal intensity to the sum of both methylated and unmethylated signals after background subtraction. β-values range from 0 to 1, corresponding to completely unmethylated (0) and fully methylated (1) sites, respectively. For each probe, β-values in two groups were compared using two one-sided Student's *t*-tests (e.g. *p*(CON>MAL) and *p*(CON<MAL)). To identify DMRs, we used a 1 kb sliding window based on the start coordinate of each probe, starting at the first probe and moving down consecutively to the last probe on each chromosome, and applied Fisher's method to combine *p*-values for each 1 kb region based on all probes within each window. Significant DMRs were selected at a 1% FDR on autosomes and 5% FDR on the X chromosome. We designed a likelihood ratio model to differentiate the role of malnutrition history on methylation from the potentially confounding of age, gender and cell type composition in peripheral blood (based on relative content of neutrophils, lymphoblasts, monocytes, eosinophils, and basophils) at these DMRs. As a test model, we performed linear regression using age, gender, cell composition and nutrition history as independent variables (Test Model: Methylation ~ Age + Gender + % of major nucleated blood cell types + Nutrition). This test model was tested against the null model in which only age, gender and cell composition were considered as independent variables (Null Model: Methylation ~ Age + Gender + % of major nucleated blood cell types). This test results in more significant *p*-values when postnatal malnutrition in the G1 generation, or parental malnutrition in G2, would play a major role in methylation variation. To combine *p*-values for each 1 kb region based on all probes within each window, we applied Fisher's method as described above. We considered DMRs of this subset with a FDR q-value < 0.05 in favor of the

Test model (as opposed to the Null model, see above), incorporating nutrition status. To further add to stringency of selecting the DMRs, we required at least one probe in the DMR to show more than 2% mean difference between the two groups.

In addition, we compared the nutrition-sensitive DMR identified in G1 individuals with known and putative imprinted loci, overlapping the 1000 probes contained within G1 DMR with a comprehensive set of loci that show parental bias in DNA methylation levels (13-15). Enrichment analysis for co-localization of nutrition associated DMR with imprinted regions was performed using a hypergeometric test to compare the expected overlap of all autosomal probes tested under the null hypothesis.

Behavioral and Cognitive Measures

In earlier studies, we reported that early childhood malnutrition leads to significant impairment in attention and intellectual performance throughout childhood and adolescence (4, 6, 8). These effects were documented as persisting to 40 years of age in the same G1 cohort assessed in the current study (1, 2, 16). At sites that showed significant association between methylation levels and childhood malnutrition, we compared epigenetic profiles with these phenotypes in G1 subjects, using the following measures:

Connors Adult Attention Rating Scale Self-Report Screening Version (17): 93 of the 94 G1 subjects completed this 30-item scale that yielded 4 subscales based on DSM-IV criteria for ADHD symptoms, with each item scored on a four-point Likert scale. The current report used the ADHD Index (CAARS D subscale). The questionnaire was administered by a local interviewer because of the low reading levels of some participants. The CAARS has good internal consistency, test–retest reliability and concurrent validity, ranging from 0.66 to 0.83 for adults (18). The CAARS also has good factor validity (17), and the inter-item correlations are in the expected directions and follow ordinal scaling properties.

Wechsler Abbreviated Scale of Intelligence (WASI) (19): All 94 G1 subjects completed this test. The short-form version (Vocabulary and Matrix Reasoning) of the WASI (19) provided an estimate of full scale IQ. Its internal consistency is excellent, with reliability coefficients ranging from 0.96 to 0.98, as is test-re-test reliabilities, ranging from 0.87 to 0.92 (20). The correlation with the (4-subtest) WASI full scale IQ is reported to be 0.95, and the correlation with the Wechsler Adult Intelligence Scale-III full scale IQ is 0.92.

Methylation/Phenotype Correlations

To test the relationship between DNA methylation and behavioral and cognitive outcomes in the G1 cohort, we performed partial correlations, adjusting for participant age and gender. DNA methylation values (β-values) of the single Illumina CpG probe representing the most significant nutrition group difference within each DMR were used for pairwise correlations with CAARs ADHD Index and WASI full scale IQ.

We have previously reported that malnourished children and adults came from socioeconomically deprived households (21). Childhood standard of living (SES) was assessed using an Ecology Questionnaire whose 50 items queried conditions in the household and the educational and employment history of the parents (21). To derive a household standard of living scale, factor analysis of questionnaire items, using principal components factor-extraction followed by normal varimax rotation, was applied separately at three childhood ages. These data were subsequently combined because the factor structure and content across time points were comparable, although factor scores were calculated separately for each time. The resulting standard of living scale which had good internal consistency (θ = 0.86), and items contributing to the most strongly included the possession of a refrigerator, bath, television, electricity, running water, closet, and gas or electric cooking fuel, as well as number of bedrooms/ rooms, household food expenditure, type of toilet, and weekly household

income. Standard of living scores were standardized at each time point to a mean of 0 and standard deviation of 1.

ZFP57 Genotyping

Human *ZFP57* SNP rs396660 (C/T) was genotyped individually from all 168 DNA samples (G1 and G2 individuals) by PCR amplifying the 629bp fragment spanning the SNP followed by DNA sequencing using an internal primer. Primers are listed in Supplemental Table S16a.

Bisulfite Pyrosequencing

DNA methylation changes detected with the Illumina 450K platform were validated using the bisuflite pyrosequencing analysis for the following genes: *DPPA5*, *PSCORS1C3* and *COMT*. We selected bisulfite-converted DNA samples from 20 individuals in G1 generation (10 G1 MAL and 10 G1 CON). Briefly, 20 ng of bisulfite-converted DNA was PCR amplified using PyroMark PCR kit (Qiagen) and specific primers for each gene region (Supplemental Table S16b) one of which was biotinylated. Biotinylated PCR products were then randomized on a 24-well plate, bound to the streptavidin-coated sepharose beads, and processed on a PyroMark Q24 Vacuum Workstation (Qiagen) according to the standard procedure. Pyrosequencing was performed using a PyroMark Q24 pyrosequencer (Qiagen) with specific pyrosequencing primers (Supplemental Table S16b). The PCR and pyrosequencing primers were designed using PyroMark Assay Design Software 2.0 (Qiagen). Prior to the sample analyses, pyrosequencing assays were validated using unmethylated and hypermethylated human DNA standards (EpiTect PCR Control DNA Set, Qiagen). The average DNA methylation levels of specific CpG sites within each examined region were quantified using PyroMark Q24 2.0.4 software (Qiagen).

Rat Studies

Female Long Evans hooded rats consuming diets containing 6% or 25% casein (Teklad Lab Animal Diets, Harlan Laboratories, Madison, WI) beginning 5 weeks prior to pregnancy were mated with male rats on the same diets. Dams were maintained on the experimental diets throughout pregnancy until parturition. At birth, all litters were fostered to well-nourished, lactating dams from the 25% casein group, thus pups were designated as malnourished (MAL) or controls (CON) , indicating their 6% or 25% casein prenatal diet, respectively. Dams were given ad libitum access to the 25% casein diet during the nursing period and at weaning (PND 21) pups were transferred to a standard laboratory chow diet containing 23% protein (Purina Mills Inc., Richmond, IN; Formula 5001). Starting on postnatal day 90, the prenatally malnourished and control rats underwent tests to examine attention-related behavior (sustained attention task and effects of stress on distractibility) and brain metabolic activity (14C-2-deoxyglucose labeling).

Molecular Studies: Animals used for molecular assays (RT-PCR) were derived from the same breedings used for 2DG and behavioral assays. Primer sequences for gene expression analysis of *Abcf1, Comt, Hprt, Ifng, Inhbb*, *Pbgd, Syngap1, and Vars* are provided in Supplemental Table S16c.

Apparatus and Materials: A system of nine operant chambers (Med Associates, St. Albans, VT) equipped with two retractable levers, a house light (2.8 W), a 45 mg pellet dispenser, a 2900 Hz sonalert tone generator, and three panel lights (2.8 W) were used. The food dispenser, panel lights, and retractable levers were all located on the same wall. The tone generator and house light were located on the opposite wall. Records of signal presentation, lever operation, and food pellet (Noyes Precision Pellets, 45 mg; Research Diets, New Brunswick, NJ) delivery were maintained using a

personal computer with Windows XP (Microsoft, Seattle, WA) and the Med-PC IV software (Med Associates).

Behavioral Training: Training occurred between 9:00 A.M. and 1:00 P.M. 6 d/week. All training occurred with the houselight illuminated. First, rats were initially trained to associate lever pressing with food using a fixed ratio 1 schedule of reinforcement. Response contingencies were set so that lever pressing more than five times on a lever resulted in the lever becoming inactive to prevent the development of a side bias. Once the animals emitted at least 50 responses on both levers for 2 consecutive days, training in the sustained attention task (SAT) was begun.

Sustained Attention Task Shaping: Training sessions consisted of a total of 162 trials. Subjects were placed in the operant chambers and given 1 min to acclimate to the environment. The animals were initially trained to discriminate between signal and nonsignal trials. Signal and nonsignal trials were presented in a pseudorandomized sequence so that each block of 54 trials consisted of an equal number of signal and nonsignal events. Signal trials consisted of illuminating the central and left panel lights for 1 s. The lights were not illuminated for nonsignal trials. Two seconds after the presentation of the signal or the nonsignal event, both levers were extended into the box and remained for 4 s or until a lever press occurred. Animals were reinforced for responding to the presence of the light stimuli by depressing the left lever (hit) and the absence of the lights by pressing the right lever (correct rejection). Incorrect lever presses were defined as misses when they occurred on a signal trial and false alarms when they occurred on a nonsignal trial. If the animal failed to respond or responded incorrectly, the levers were retracted and the intertrial interval (12 ± 3 s) was reinstated. After an incorrect response, the trial was repeated up to three times (correction trials). If the animal failed to respond correctly after three

correction trials, a forced-choice trial was initiated. In forced-choice trials, the event (signal or nonsignal) was repeated but only the correct lever was extended and remained active for 90 s. On forced-choice signal trials, the lights remained illuminated for 90 s. These trials facilitated discriminative conditioning and blocked the development of a side bias. After the animals responded correctly to >70% of both the signal and nonsignal events for at least 2 consecutive testing days, they entered a second shaping task. In this task, only the central panel light was illuminated for 1 s. All other aspects of the task were the same as the previous shaping task. After the animals responded correctly to >75% of both the signal and nonsignal events for at least 2 consecutive testing days in this phase of shaping, they entered the final task.

Sustained Attention Task: Baseline Task: In the final version of the task, the length of the signal duration was changed from 1 s to 25, 100, or 500 ms. Sessions consisted of 27 trials of each of the three signal lengths and 81 trials of the nonsignal trials, yielding a total of 162 trials per session. Because it was planned to analyze performance changes across three blocks of 54 trials each, the sequence of signal and nonsignal trials was pseudorandomized so that one block consisted of 27 signal and 27 nonsignal trials, with each signal length being presented nine times. In addition, both correction and forced-choice trials were discontinued. Animals were trained to a criterion of >75% hits to 500 ms signals and >75% correct rejections to nonsignal trials for at least two consecutive sessions. This task maximizes sustained attentional demands by requiring subjects to discriminate targets from non-targets on successive trials, including a dynamic stimulus range, ensuring that events are temporally unpredictable and testing for a prolonged period (approximately 40 min).

Effects of Stress on Distractibility: Prior work has demonstrated that prenatal malnutrition produces cognitive rigidity in both humans and in a rodent model. This cognitive rigidity impairs performance on tests that require flexible use of attentional resources such as the attentional

set-shifting task while conferring resistance to distraction. The present study extends previous work by assessing the impact of acute restraint stress on distractibility. Rats were placed in plexiglass restraint tubes for 45 minutes. Upon termination of this exposure, rats were immediately placed in a test of distractability. In this session, the houselight was turned off and on in an unpredictable pattern throughout the course of the testing session (0.25, 0.5, 1.5, 1, 2, or 3 s on – off; "unpredictable distractor"). This distractor was chosen as our data (unpublished, McGaughy lab) have shown that it dissociates performance of malnourished and control subjects.

Behavioral Measures: For each test session, the number of hits, misses, correct rejections, false alarms and errors of omission were recorded. The relative number of hits (hits/hits + misses) was computed for each signal length as well as the relative number of correct rejections (correct rejections/correct rejections + false alarms). The relative number of left lever presses (hits + false alarms/all responses) was also calculated in order to detect any side-biases the animal may have developed.

14C-2-deoxyglucose (2DG) Labeling: To evaluate baseline brain activity, rats were injected with the metabolic activity marker 2DG (100 µCi/Kg i.p.; specific activity = 390 mCi/mmol; VWR/GE Healthsource, Radnor, PA) and subjected to the uSAT behavioral assay as described above. Then, 45 min after 2DG injection, animals were deeply anesthetized (pentobarbital; 65 mg/kg) and perfused through the heart with 250 ml of fixative (2% paraformaldehyde and 15% sucrose in 0.1M phosphate buffer, pH 7.4) for 5 minutes. The brains were removed, coated with albumin, frozen at -30°C in 2-methylbutane and stored at −80°C. Brains were later cut into 20 µm thick coronal sections and one out of every five sections was mounted on subbed cover slips and rapidly dried. Sections were batch-processed by affixing them to Bristol board, and apposing them to high-resolution X-ray film (Structurix, Agfa, Belgium) with 14C microscales (Amersham, Piscataway, NJ) and

exposed at −80°C for 10 days. Films were processed and digitized as previously described (22). Uptake of 2DG was assessed densitometrically and values were normalized to white matter (23).

SUPPLEMENTAL TABLES

See Supplement 2 (Excel file)

Table S1. Blood differential cell counts in G1 and G2

Table S2. List of differentially methylated regions (DMRs) G1CON_G1MAL

Table S3. List of differentially methylated regions (DMRs) G2CON_G2MAL

Table S4. X-linked DNA methylation differences. Males only, G1CON_G1MAL

Table S5. X-linked DNA methylation differences. Males only, G2CON_G2MAL

Table S6. X-linked DNA methylation differences. Females only, G1CON_G1MAL

Table S7. Top 100 single probe hits between G1 malnourished subjects and controls

Table S8. Top 100 single probe hits between G2 malnourished subjects and controls

Table S9. Selected differentially methylated genes with relevance to neurobehavioral outcomes and psychiatric disorders

Table S10. Correlations of CpG methylation and ADHD (CAARS) index (G1 generation)

Table S11. Correlations of CpG methylation and IQ index (G1 generation)

Table S12. List of 73 nutrition-sensitive CpGs within imprinted loci (G1)

Table S13. DMR-bound mQTLs

Table S14. Linear regression of ZFP57 DMR with nutrition and genotype as covariates

Table S15. Human-rat conserved DMRs associated with attention and cognition in G1 (human cohort)

Table S16. Primers used in the study

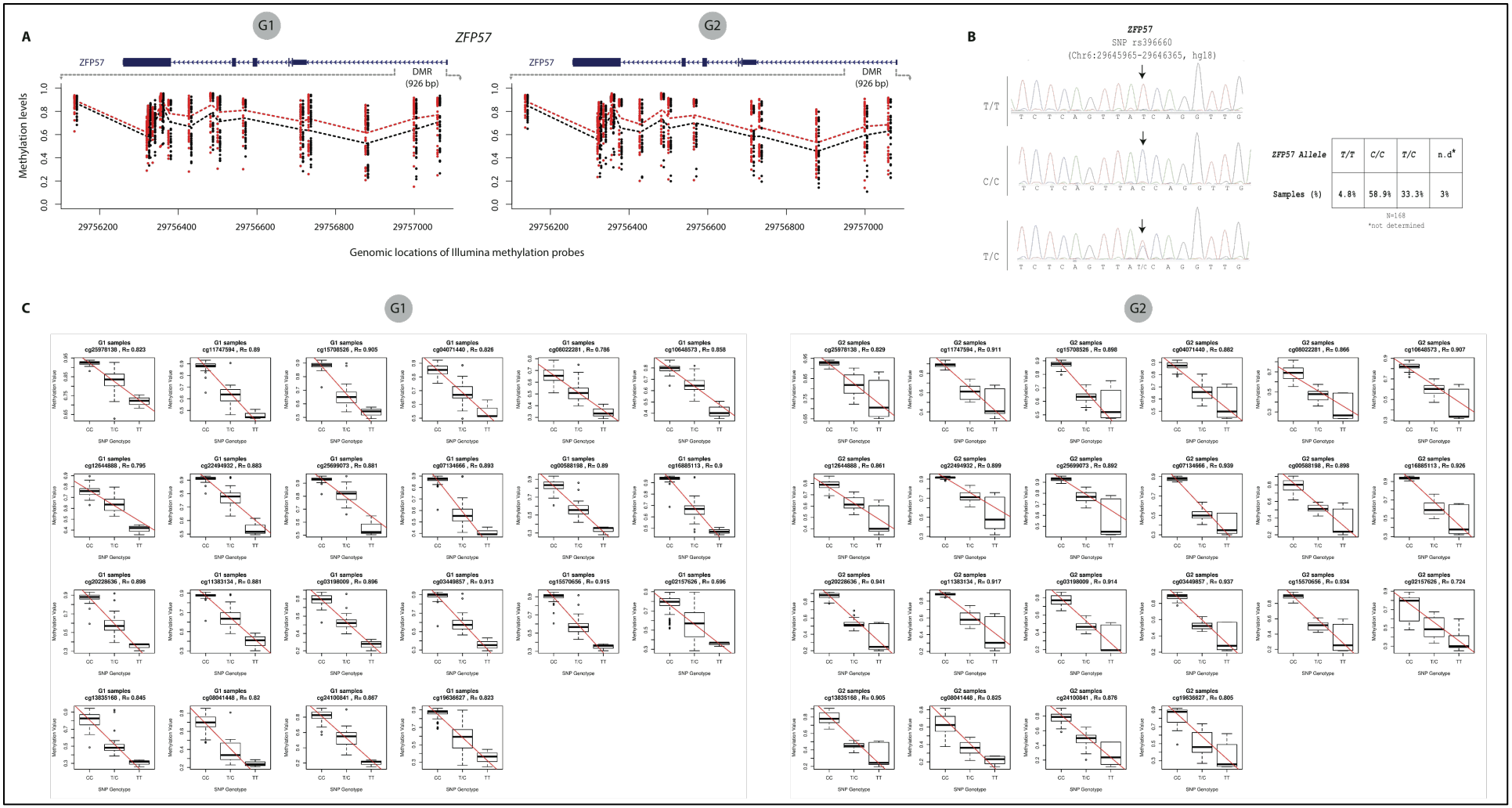

Figure S1. Genotype effects on *ZFP57* CpG methylation. (**A**) Plots of DNA methylation for individual CpGs within *ZFP57* DMR. Data shown separately for (left) G1 and (right) G2 cohorts, as indicated (red, MAL; black, CON). (**B**) (left) Representative electropherograms for *ZFP57* DMR SNP rs396660 for T/T, C/C and C/T subjects from the BNS cohort. Allele frequency table from *n* = 168 BNS subjects tested. (**C**) Box plots showing consistent association between rs396660 allele status and methylation levels of the CpG probes within the *ZFP57* DMR, as indicated, for G1 (left) and G2 (right).

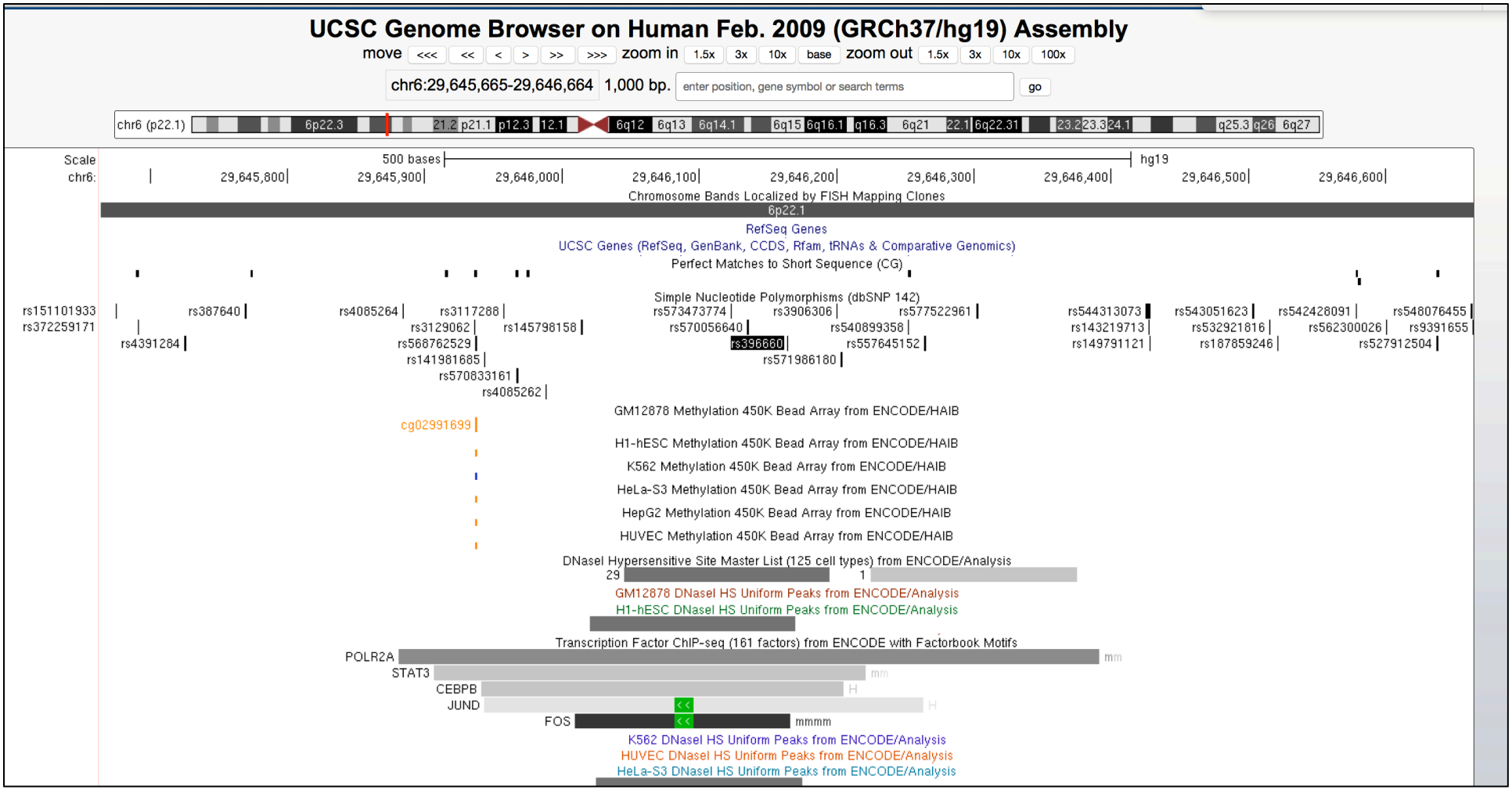

Figure S2. UCSC genome browser shot. ZFP57 mQTL SNP disrupts transcription factor and enhancer protein binding. Figure shows University of California Santa Cruz (UCSC) 1000 basepair browser shot centered on *ZFP57* locus and mQTL rs396660, a SNP that is positioned in POLR2A, STAT3, Jun, FOS, and CEBPB binding sites.

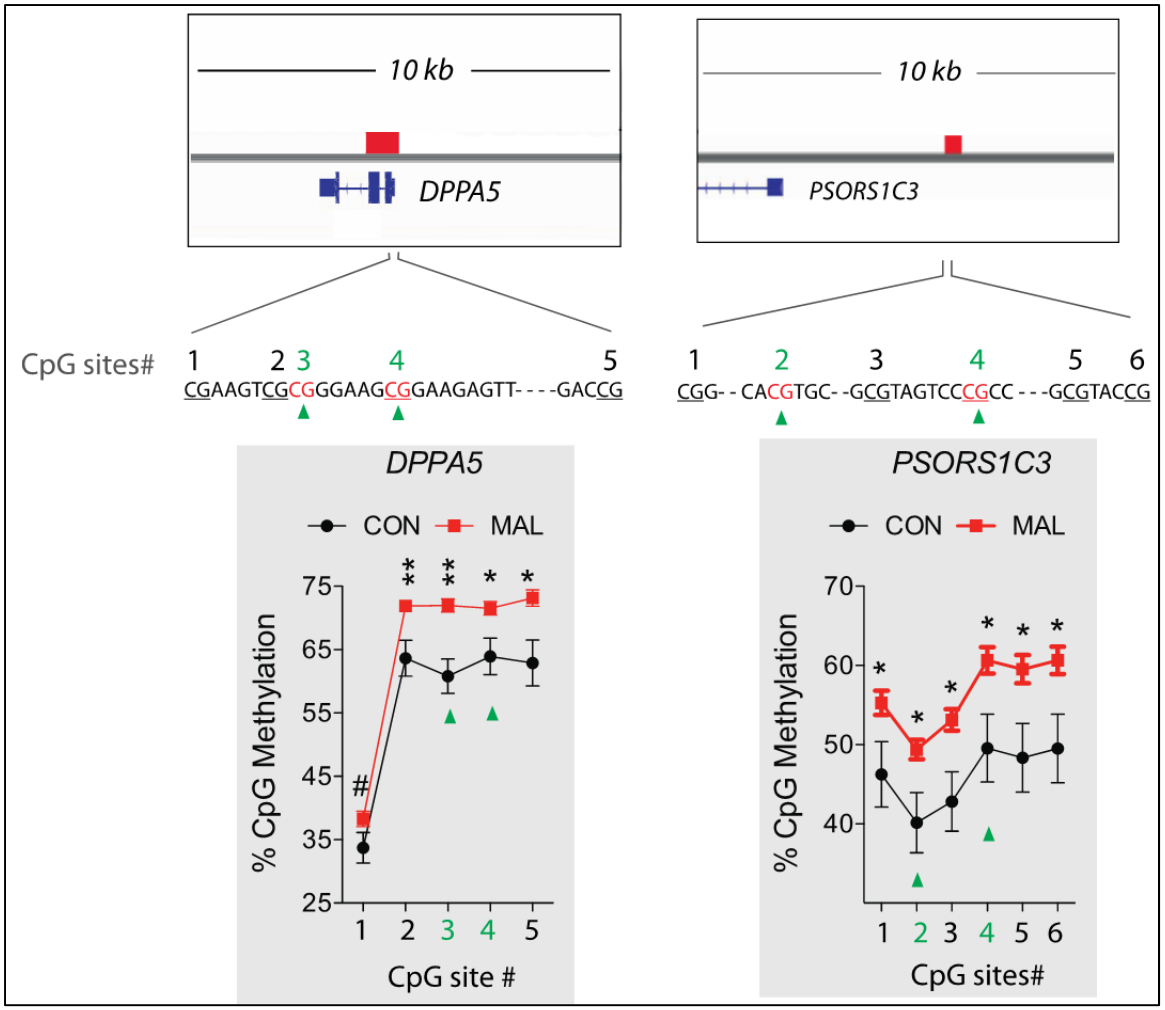

Figure S3. **Validation of CpG methylation changes by pyrosequencing of bisulfite-treated DNA.** (Left) Bisulfite-pyrosequencing within *DPPA5* and *PSORS1C3* DMRs, confirming localized DNA hypermethylation in G1 MAL (red, n = 10) compared to G1 CON subjects (black, n = 10), including (green) CpGs represented on the Illumina array (t-test; $^{\#}p < .1$; $^{*}p < .05$; $^{**}p < .01$). (Right) Summary of Pearson's correlation of CpG sites measured by Illumina array and pyrosequencing assays (n = 10 G1 MAL and 10 G1 CON).

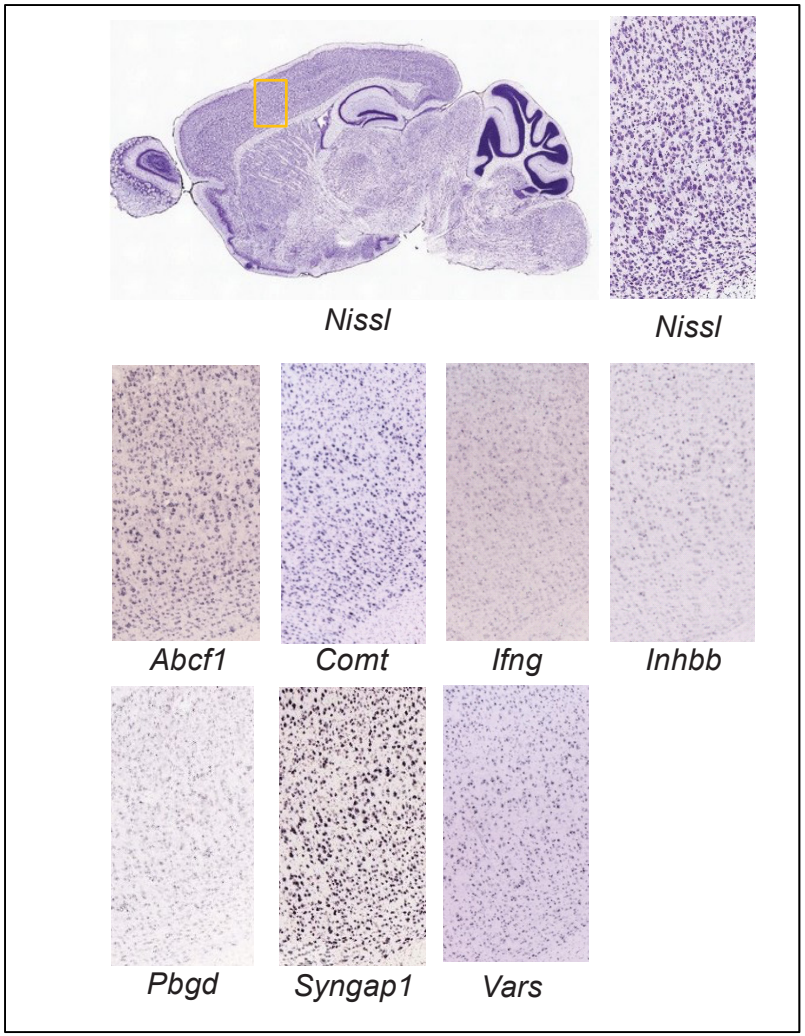

Figure S4. **Gene expression in mouse prefrontal cortex**. Sections through the vertical thickness of rodent (mouse) prefrontal cortex, downloaded from the Allen Brain Atlas, http://mouse.brain-map.org, showing by in situ hybridization moderate or robust prefrontal expression for a subset of genes affected by differential methylation after early childhood malnutrition in the BNS cohort. *Abcf1*, RP_050419_03_G02; *Comt*, RP_041130_01_B06; *Ifng*, RP_050503_02_D06; *Inhbb*, RP_050915_01_D07; *Pbgd*, RP_060608_01_D12; *Syngap1*, RP_050512_03_H06; *Vars*, RP_050203_01_C06.

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