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

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ABSTRACT

BACKGROUND: Early childhood malnutrition affects 113 million children worldwide, impacting health and increasing vulnerability for cognitive and behavioral disorders later in life. Molecular signatures after childhood malnutrition, including the potential for intergenerational transmission, remain unexplored.

METHODS: We surveyed blood DNA methylomes (~483,000 individual CpG sites) in 168 subjects across two generations, including 50 generation 1 individuals hospitalized during the first year of life for moderate to severe protein-energy malnutrition, then followed up to 48 years in the Barbados Nutrition Study. Attention deficits and cognitive performance were evaluated with the Connors Adult Attention Rating Scale and Wechsler Abbreviated Scale of Intelligence. Expression of nutrition-sensitive genes was explored by quantitative reverse transcriptase polymerase chain reaction in rat prefrontal cortex.

RESULTS: We identified 134 nutrition-sensitive, differentially methylated genomic regions, with most (87%) specific for generation 1. Multiple neuropsychiatric risk genes, including *COMT*, *IFNG*, *MIR200B*, *SYNGAP1*, and *VIPR2* showed associations of specific methyl-CpGs with attention and IQ. *IFNG* expression was decreased in prefrontal cortex of rats showing attention deficits after developmental malnutrition.

CONCLUSIONS: Early childhood malnutrition entails long-lasting epigenetic signatures associated with liability for attention and cognition, and limited potential for intergenerational transmission.

Keywords: Attention deficits, Childhood malnutrition, Cognition, DNA methylation, Epigenetics, Prefrontal cortex

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Malnutrition in infancy and early childhood is a major public health challenge, affecting an estimated 113 million children worldwide and causing significant morbidity and mortality (1). This morbidity includes long-lasting vulnerability to psychiatric disease, with increased risk for attention deficits, personality disorders, and impaired cognition for several decades following exposure (2–4). The underlying mechanisms are poorly understood. Nutrition (and malnutrition) during early prenatal periods could affect DNA methylation, a key mechanism for epigenetic regulation affecting genome organization and function without altering underlying DNA sequence (5). However, to date, it remains unclear whether there are long-lasting epigenetic changes in humans exposed to malnutrition in infancy and whether such nutrition-sensitive DNA methylation signatures are associated with changes in brain function and behavior. Furthermore, nutrition-related effects on DNA methylation, particularly effects operating around the time of conception, could potentially be passed on through the germline and affect metabolic function and health of offspring (6,7). However, it is unclear whether epigenetic alterations exist in the offspring of a parent exposed to malnutrition in infancy, similar to the multigenerational transmission of DNA methylation signatures after maternal separation stress in postnatal mice (8,9).

To gain first insights into these questions, we interrogated, on a genome-wide scale, blood DNA methylation in 168 subjects across two generations (generation 1 [G1] and generation 2 [G2]). The subjects included 50 G1 individuals who experienced moderate to severe protein-energy malnutrition during the first year after birth and their G2 offspring (Figure 1). Our population is unique because it has been monitored and examined for 48 years, in the context of the Barbados Nutrition Study (BNS). The study, launched in 1967, provided critical insight into the longitudinal course of malnutrition-related neuropsychological functioning and vulnerability to psychiatric and medical disease (2–4). In this article, we provide multiple lines of evidence that infant malnutrition triggers long-lasting DNA methylation changes associated with liability for defective attention and cognition in adult life.

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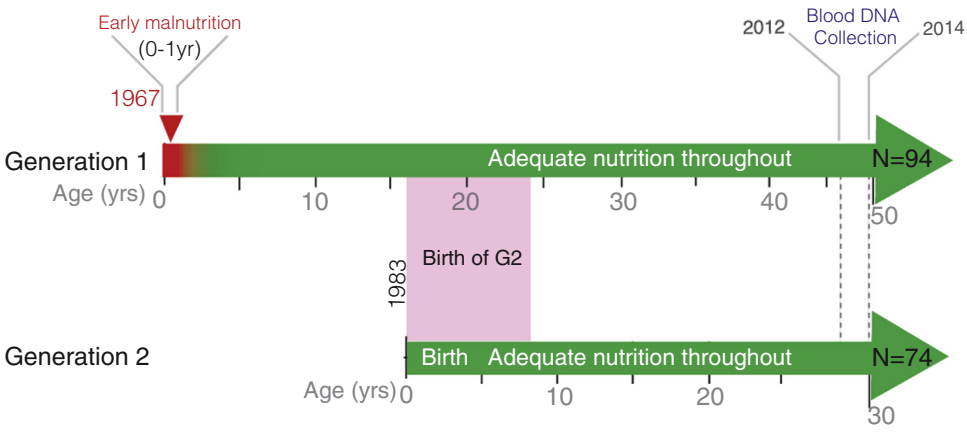


Figure 1. Overview and timeline of the Barbados Nutrition Study. Generation 1 (n = 94) included 44 control subjects and 50 subjects who were malnourished during the first year of life. Subjects with a history of early life malnutrition enrolled in a government-sponsored intervention program that included nutrition education, food subsidies, routine health care, home visits, and a preschool program that extended to 12 years of age. Approximately 44 years after exposure to malnutrition, blood was drawn by venipuncture for the DNA methylation scan. Generation 2 (G2; n = 74), all offspring of members of generation 1 (44 offspring of generation 1 subjects with early malnutrition and 30 offspring of generation 1 control subjects), consistently had adequate nutritional status across the life span; age range when blood was drawn for DNA profiling was 16–30 years.

METHODS AND MATERIALS

The research design and sample recruitment for the BNS have been described elsewhere (2,4,10). 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). The present study included 94 adult G1 participants (n = 50 subjects malnourished in the first year of life [MAL group] and n = 44 healthy control subjects [CON group]) with epigenetic and behavioral data. Also included are epigenetic data from 74 G2 children of G1 participants (n = 44 offspring of the MAL group and n = 30 offspring of the CON group). The total sample included 168 individuals (Table 1). Bisulfite-treated DNA from blood (venipuncture) was assessed with the Infinium HumanMethylation450 BeadChip Kit (Illumina, Inc., San Diego, CA). Attention and cognition were assessed with the Connors Adult Attention Rating Scale Self-Report Screening Version (11) and the Wechsler Abbreviated Scale of Intelligence (12). Childhood standard of living (socioeconomic status) was assessed using a 50-item Ecology Questionnaire that queried conditions in the household and the educational and employment history of the parents (10). Supplemental Methods in Supplement 1 provide details on 1) study cohort, 2) blood sample collection, 3) methylation profiling, 4) data processing and statistical analysis, 5) comparison of MAL and CON groups, 6) cognitive-behavioral measures, 7) methylation/phenotype correlations, 8) *ZFP57* genotyping, 9) bisulfite pyrosequencing, and 10) rat studies.

Table 1. Demographic Characteristics of the Study Population

Characteristic	Generation 1		Generation 2	
	MAL (n = 50)	CON (n = 44)	MAL (n = 44)	CON (n = 30)
Sex, n (%)				
Males	23 (46.0)	24 (54.6)	18 (40.9)	17 (56.7)
Females	27 (54.0)	20 (45.4)	26 (59.1)	13 (43.3)
Mean Age, Years (SD)	44.6 (1.8)	43.9 (2.0)	20.8 (3.5)	21.6 (4.1)

CON, healthy control subjects; MAL, subjects malnourished in the first year of life.

RESULTS

More Than 100 Genomic Loci Show DNA Methylation Changes After Early Childhood Malnutrition

We profiled genome-wide DNA methylation in blood samples of 168 participants enrolled in the BNS (Figure 1), including 50 subjects with moderate to severe protein-energy malnutrition during the first year of life (MAL group) and 44 matched control subjects (CON group) from the G1 generation and 74 of their G2 offspring (44 in the MAL group and 30 in the CON group) (Table 1). Differences in cohort demographics, including age, sex ratios, and blood count/differential, were minimal and without significance (Table 1 and Supplemental Table S1 in Supplement 2). Genome-wide DNA methylation profiles, interrogated via Illumina Infinium HumanMethylation450, queried ~483,000 individual CpG sites genome-wide (covering ~2% of all CpG sites in the genome) (13). We retained 461,272 autosomal and 11,021 chromosome X probes after removing probes with low signal intensity and probes that were within 5 bp vicinity to common single nucleotide polymorphisms (SNPs), as such variants can introduce biases in probe performance. However, larger windows (>5 bp from the CpG tested) tend to have minimal impact (14).

For each sample, methylation values for individual CpG sites were measured as β values ranging from 0 to 1, corresponding to completely unmethylated (β value 0) and fully methylated (β value 1) sites, respectively. Because *cis*-regulatory mechanisms generally encompass multiple CpGs at a given locus, we used a 1-kb sliding window approach (15) to identify methylation differences between cohorts, rather than focusing on isolated changes in single CpGs. Differentially methylated regions (DMR) at 1-kb sliding windows were determined for the G1 (MAL vs. CON) and G2 cohort (MAL offspring vs. CON offspring) separately. The 1-kb sliding window was chosen because of the highly correlative structure of methylation values maintained for probes with separation up to 1 kb (15) and because CpG methylation linkage disequilibrium correlation typically extends for ~500 bp (16), corresponding to the 1-kb window centered on the CpG in question. White blood cell composition, including neutrophil,

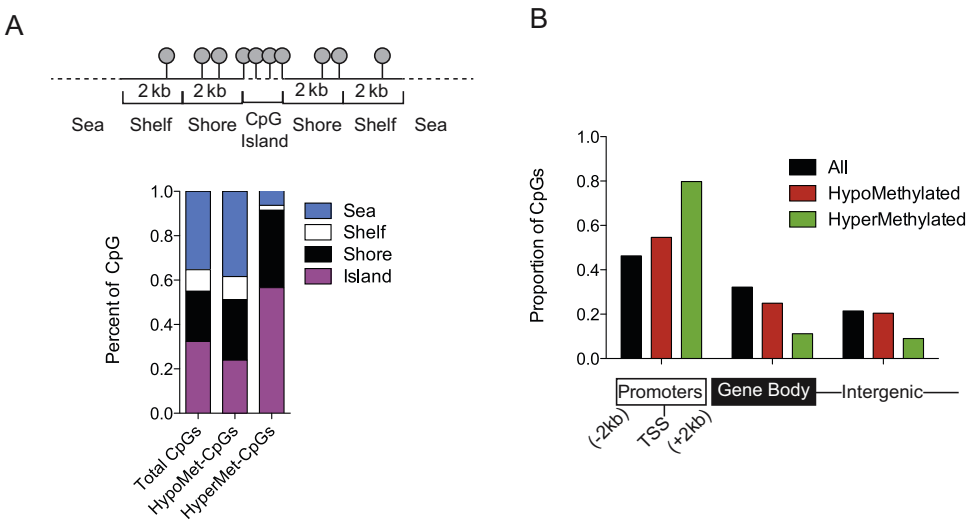


Figure 2. Genome-wide distribution of DNA methylation changes after malnutrition. Distribution of CpG sites **(A)** in CpG islands, shores, shelves, and sea and **(B)** relative to RefSeq gene promoters, gene bodies, and intergenic regions. CpGs in hypomethylated and hypermethylated differentially methylated genomic regions are compared with all autosomal CpGs (total CpGs) on the Illumina array using Pearson's χ^2 test. Note pronounced (approximately twofold) overrepresentation of hypermethylated differentially methylated genomic regions at CpG islands and gene promoters.

lymphocyte, monocyte, eosinophil, and basophil proportions, was indistinguishable between groups (Supplemental Table S1 in Supplement 2). Because a complete blood count was done in each of our samples before performing DNA extraction, we were able to include the potential effect of blood cell heterogeneity using these direct measurements for the five main blood cell types in our statistical analysis and to perform a likelihood ratio test to analyze cell types in our statistical analyses (see Supplemental Methods in Supplement 1). These measures taken together effectively rule out cell type differences as confounds of the DNA methylome analyses. Because direct measurement of blood cell composition was incorporated into our statistical analyses as the most robust approach for accounting for this covariate, indirect modeling (17) was not required.

For G1, we identified 102 autosomal DMRs, comprising 1000 CpGs, of which 609 were hypermethylated and 391 were hypomethylated in MAL subjects. When adjusted for childhood socioeconomic status, 100 of 102 DMRs, or 98%, maintained significance (Supplemental Table S2 in Supplement 2). For G2, we identified 16 DMRs, containing 202 CpGs, of which 104 were hypermethylated and 98 were hypomethylated in offspring of MAL subjects (Supplemental Table S3 in Supplement 2). The aforementioned analyses were limited to autosomal probes, to avoid confounds secondary to X inactivation–related DNA methylation differences between males and females (18). We also analyzed childhood malnutrition–sensitive loci on the X chromosome, identifying 15 X-linked DMRs in G1 males (Supplemental Table S4 in Supplement 2), and three X-linked DMRs in G2 males (Supplemental Table S5 in Supplement 2). Notably, X chromosome methylation at baseline is inherently more variable in females than males (18). There was only a single X-linked DMR in G1 females (Supplemental Table S6 in Supplement 2) and none in G2 females. In addition, we report the top 100 single probe levels (sorted by p value) that are differentially methylated in G1 MAL subjects versus CON subjects (Supplemental Table S7 in Supplement 2) and G2 MAL subjects versus CON subjects (Supplemental Table S8 in Supplement 2).

Enrichment Patterns of Nutrition-Sensitive DNA Methylation Sites

We compared across all G1 and G2 DMRs the distribution of hypomethylated and hypermethylated DMRs with the background CpG distribution in the Illumina 450K array, which has preferential probe placement in gene promoters, CpG islands, and putative regulatory elements. CpG islands showed an approximately twofold significant ($p < 10^{-39}$) overrepresentation among hypermethylated DMRs, together with mild underrepresentation among hypomethylated DMRs (Figure 2A). Consistent with most CpG islands being found in close proximity to 5′ regulatory sequences involved in transcriptional control (19), we also observed a twofold enrichment for gene promoter sequence specifically among hypermethylated DMRs ($p < 10^{-71}$), together with a significant underrepresentation of gene bodies ($p < 10^{-38}$) and intergenic sequences ($p < 10^{-18}$) (Figure 2B). In addition, CpG shores showed preferential enrichment among both hypermethylated ($p < 10^{-12}$) and hypomethylated ($p < .05$) DMRs, which is interesting given that shores often harbor *cis*-regulatory sequences associated with dynamic DNA methylation and modulation of gene expression (20,21). These changes were highly specific because CpG shelves and sea lacked enrichment (Figure 2A). We constructed a genome-wide map of all autosomal DMRs (from Supplemental Tables S2 and S3 in Supplement 2) positioned within 2 kb of an annotated gene transcription start site (Figure 3). In G1 MAL subjects, most promoter-associated DMRs were hypermethylated (compare columns G and H in Supplemental Table S2 in Supplement 2), including genes with a critical role in neurodevelopment as well as multiple regulators of cortical and striatal monoamine signaling (*COMT*, *DCTN1* antisense RNA 1) and connectivity (*MIR200B*, *FOXP2*, *RAB3B*) (Figure 3 and Supplemental Table S9 in Supplement 2).

Nutrition-Sensitive DNA Methylation Sites Correlated With Attention and Cognition

Malnutrition in the first year of life causes long-term impairments in cognition and, independent of lower IQ, attention

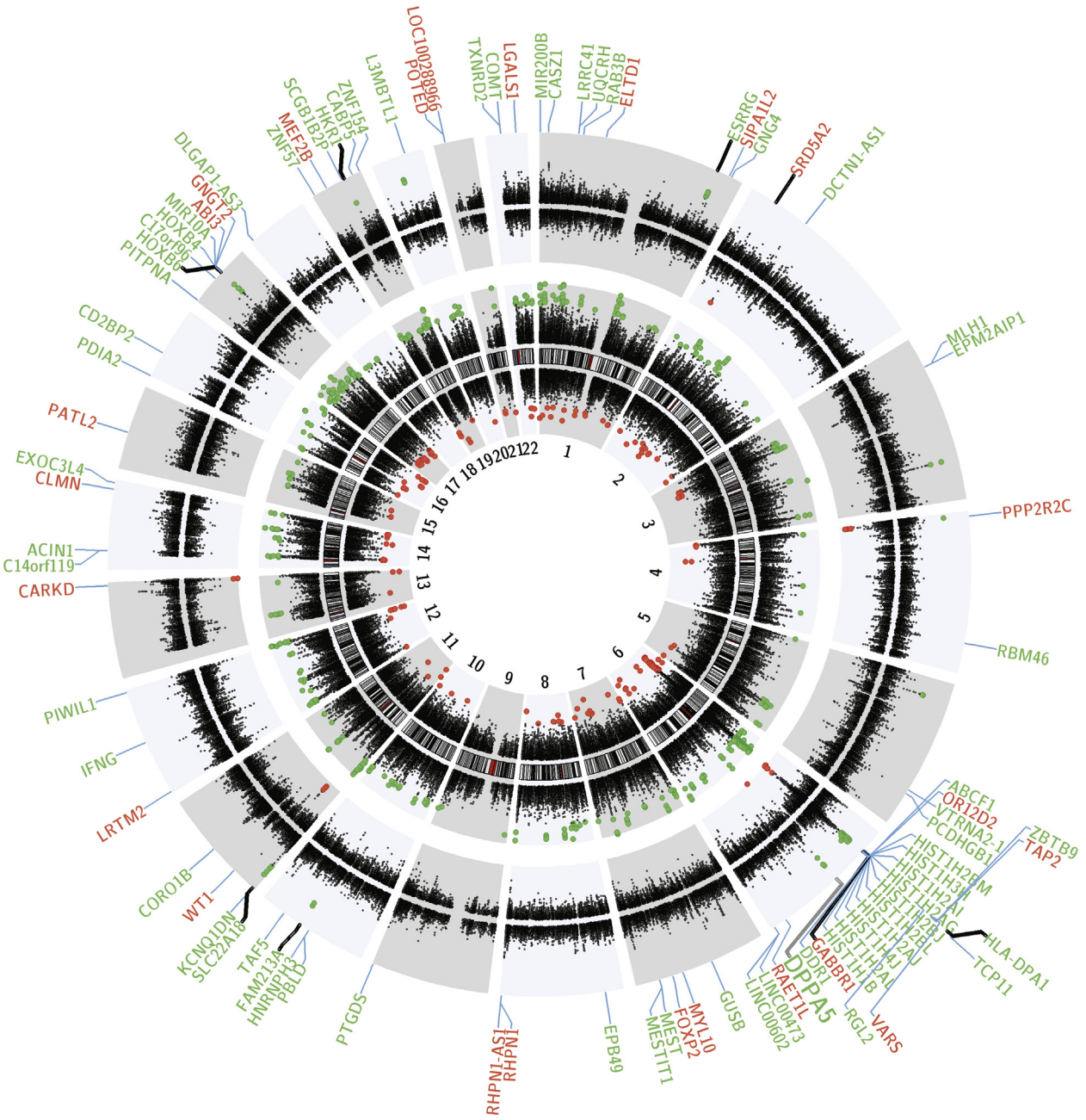


Figure 3. Genome-wide DNA methylation changes in previously malnourished generation 1 subjects and their generation 2 offspring. Circos plot depicting the entire autosomal complement, green (red) genes with transcription start sites within ± 2 kb of significantly hyper-(hypo-)methylated differentially methylated genomic regions. Genes with thin connector (gene abbreviation to plot) line = generation 1; thick connector = generation 2. The black innermost ring (with vertical lines) represents autosome ideograms (annotated is the chromosomal number), with the pter-qter orientation in a clockwise direction. The dots on the inner side (negative) and outer side (positive) of the ideogram represent Fisher's $-\log_{10}$ (*p* value) for each 1-kb window analyzed in generation 1. Each dot marks the location of the Illumina 450K probe along the genome. The next outermost black circle (with dots on the inner/negative and outer/positive side) represents Fisher's $-\log_{10}$ (*p* value) for each 1-kb window analyzed in generation 2. *DPPA5* (green, larger font) was significantly hypermethylated in both generation 1 and generation 2 cohorts. Only genes where the differentially methylated genomic region overlaps its promoter (i.e. ± 2 kb of transcription start site [TSS]) are shown.

deficits (primarily inattention) lasting into adulthood (2,4). Given the strong link between childhood malnutrition and adult attention-deficit/hyperactivity disorder (ADHD) (4), together with cognitive deficits and lower IQ (2), we asked whether such neuropsychiatric sequelae are associated with specific DNA methylation signatures. Here we focus on the G1 cohort, the generation directly exposed to malnutrition. Virtually all (≥95%) of G1 subjects contributed to the psychometric data. Partial correlations after adjusting for age and sex as key demographic factors (22) revealed methylated CpGs in

at least 13 DMRs moderately correlated with the ADHD index on the Connors Adult Attention Rating Scale questionnaire ($R^2 > .04$, false discovery rate <0.1) (Supplemental Table S10A and B in Supplement 2). These included two genes (*IFNG*, *VIPR2*) known to play a role in neuronal function and neuropsychiatric disease (Figure 4A and Supplemental Table S9 in Supplement 2). Furthermore, at least three sites were significantly correlated with IQ scores, including a DMR associated with *ZBTB9* and *SYNGAP1*; *SYNGAP1* is a gene critical for excitatory signaling in cerebral cortex and other brain regions (Figure 4A and Supplemental Tables S9 and S11 in Supplement 2) (23). However, with a more stringent cutoff ($R^2 > .13$, false discovery rate <.05), a single gene, *ABCF1*, was significantly associated with the ADHD index (Figure 4A and Supplemental Tables S9 and S10 in Supplement 2). *ABCF1*, which encodes an adenosine triphosphate–binding cassette, was described as a major "hub" gene in the blood transcriptome of subjects with a diagnosis of schizophrenia, a neurodevelopmental disorder (24). Additional neuropsychiatric risk genes (*COMT*, *DCTN1* antisense RNA 1, *MIR200B*, *WT1*) emerge with less stringent correlational filter criteria (Figure 4A and Supplemental Tables S9–S11 in Supplement 2). Adjustment for potential confounds, including childhood socioeconomic standards of living (see Supplemental Methods in Supplement 1), age, sex, and white blood cell count, overall resulted only in very minor changes in the associations between CpG methylation and ADHD index or IQ (Supplemental Tables S10 and S11 in Supplement 2).

Nutrition-Sensitive DNA Methylation Sites Are Enriched in Imprinted Genes

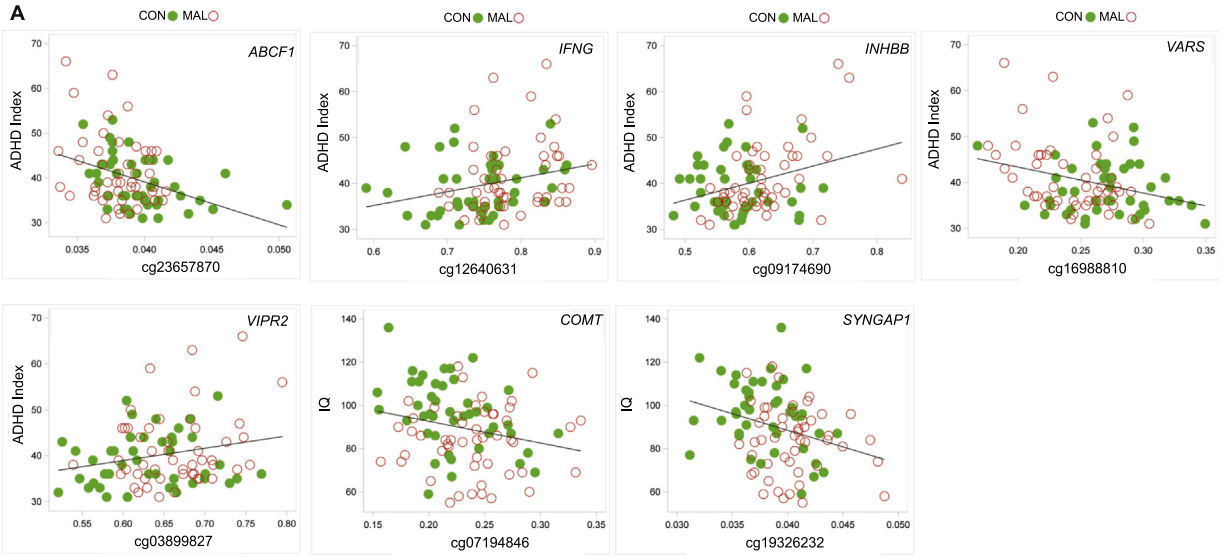
Imprinted genes play an important role in neurodevelopment and remain sensitive to environmentally induced epigenomic disruption (25). We examined whether malnutrition in early life was associated with DNA methylation changes in regions that show parent of origin–specific DNA methylation patterns. We observed that several nutrition-associated DMR loci in our study occurred at known or putative imprinted genes, including *L3MBTL1*, *MEST/MESTIT1*, *JAKMIP1*, and *VTRNA2-1* (26–28). Of the 1000 autosomal probes that showed a significant difference in methylation levels between MAL and CON G1 individuals, 73 correspond to regions that exhibit a parent-of-origin bias in DNA methylation levels, representing a highly significant 40.6-fold enrichment compared with the nonimprinted genomic regions ($p = 3.5 \times 10^{-91}$) (Supplemental Table S12 in Supplement 2). Only 3 of 102 G1 DMRs, or <3%, showed concordant alterations in the G2 cohort, which would suggest that most DNA methylation alterations after early childhood malnutrition, including the aforementioned imprinted genes, are not subject to intergenerational transmission. This could be explained by remethylation of imprinted genes in primordial germ cells—which give rise to the next generation—before birth (at least in males) (29), predating the malnutrition period. All three "intergenerational" DMRs were located on chromosome 6, including an ~700-bp CpG island (*hg18* chromosome 6: 74120243-74121316) next to the *DPPA5* gene promoter and CpG rich sequences surrounding the 5′ ends of *ZFP57* (*hg18* chromosome: 29756140-29757064) and *PSORS1C3* (*hg18* chromosome 6: 31256311-31256592), all three significantly hypermethylated in both MAL1 G1 and MAL G2 subjects (Supplemental Tables S2 and S3 in Supplement 2).

Because CpGs within proximity of common SNPs had been removed from all analyses (see Methods and Materials), 81 of 102 autosomal G1 DMRs—including all neuropsychiatric risk genes listed in Supplemental Table S7 in Supplement 2—did not harbor any CpGs linked to known methylation quantitative trait loci (mQTLs) (a genetic variant, usually an SNP, correlated with methylation levels at a nearby CpG site) (30), and the remaining 22 DMRs mostly harbored a single CpG match (Supplemental Table S13 in Supplement 2). One exception was the DMR of the aforementioned *ZFP57* "intergenerational" gene, with six CpGs matching to an mQTL (Supplemental Table S13 in Supplement 2). We then assessed the genotype of the mQTL driver SNP (rs396660) at the *ZFP57* DMR in MAL and CON subjects and observed strong genotype effects for most of the CpGs, with methylation levels consistently highest in C/C, intermediate in C/T, and lowest in T/T allele carriers (Supplemental Figure S1 in Supplement 1). The mQTL SNP rs396660 is positioned ~2 kb distal to the cluster of probes that form the DMR at *ZFP57*. The SNP does not create or destroy a CpG, suggesting that the methylation state of the SNP position itself is an unlikely determinant for the methylation state of the DMR. Instead, the SNP is located centrally within a putative regulatory element, marked by deoxyribonuclease I hypersensitivity and multiple transcription factor and enhancer protein binding sites (Supplemental Figure S2 in Supplement 1). Therefore, local DNA polymorphisms, affecting transcription factor and enhancer protein binding, are likely to explain at least some of the epigenetic alterations at the *ZFP57* locus reported here and in other nutrition-focused studies (31). To explore this, we reanalyzed the *ZFP57* DMR using linear regression with nutrition and genotype as covariates. In both G1 and G2, genotype consistently showed the strongest *p* values and had the largest impact on the variation of methylation, with a much smaller (but still significant) effect by nutrition (Supplemental Table S14 in Supplement 2).

To technically verify DNA methylation changes detected by the Illumina array, we selected sequences within *DPPA5*, *PSCORS1C*, and *COMT* (encoding catechol-*O*-methyltransferase critical for monoamine signaling at the site of cortical synapses) from the Illumina array and quantified methylation levels of several individual CpG sites within these regions in MAL and CON samples by bisulfite pyrosequencing. Specific CpGs with significant MAL versus CON differences on the array showed similar changes by pyrosequencing. Table 2 shows the summary of Pearson's correlation of CpG sites measured by Illumina array and pyrosequencing assays ($n = 10$ G1 MAL subjects and $n = 10$ G1 CON subjects). Furthermore, there were concordant changes for neighboring CpGs not included in the Illumina array (Supplemental Figure S3 in Supplement 1). These additional studies provide verification and extension of the array data.

Malnutrition-Sensitive Genes Are Dysregulated in Cerebral Cortex of Rats With Attention Deficits

We showed in the present study that long-lasting epigenetic dysregulation in subjects with histories of protein-energy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CON MAL
ABCF1
IFNG
INHBB
VARS
ADHD Index
cg23657870
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VIPR2
COMT
SYNGAP1
IQ
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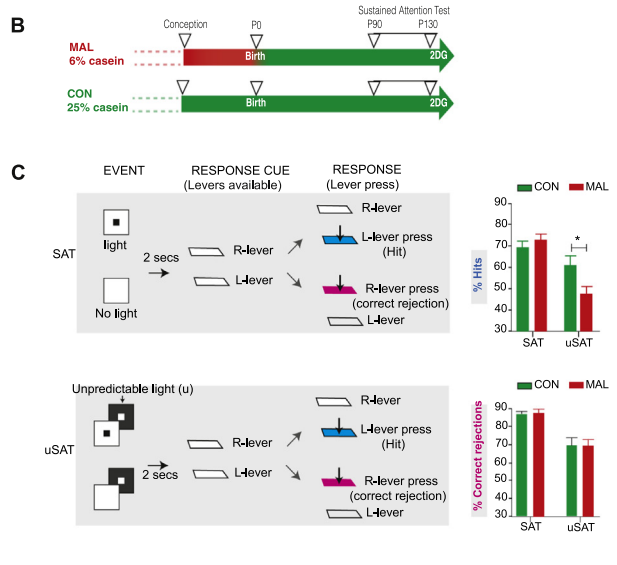
B
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Sustained Attention Test
P90
P130
MAL
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CON
25% casein
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RESPONSE CUE
(Levers available)
RESPONSE
(Lever press)
SAT
light
No light
2 secs
R-lever
L-lever
R-lever press
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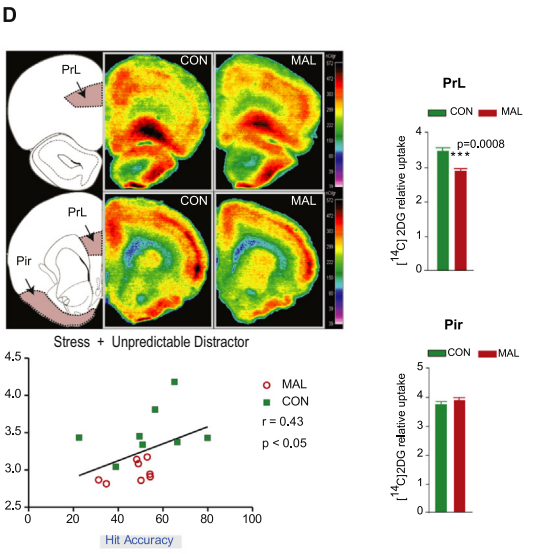
D
PrL
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CON
MAL
Stress + Unpredictable Distractor
2DG Left PrL
Hit Accuracy
r = 0.43
p < 0.05
p=0.0008
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[14C]2DG relative uptake

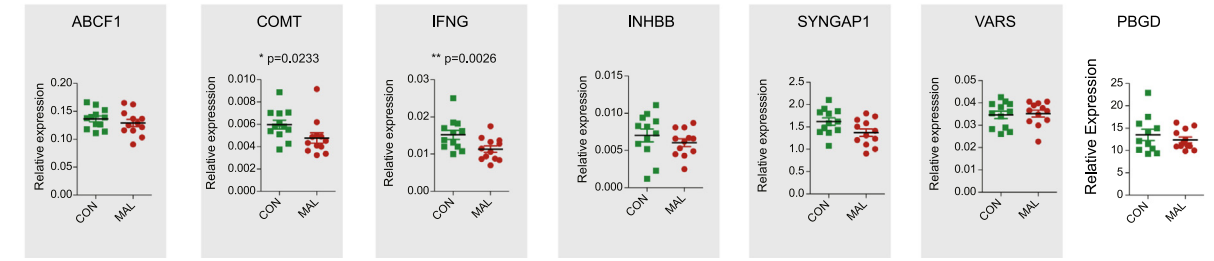
E
ABCF1
COMT
IFNG
INHBB
SYNGAP1
VARS
PBGD
Relative expression
Relative Expression
* p=0.0233
** p=0.0026
CON
MAL

Table 2. Summary of Pearson's Correlation of CpG Sites Measured by Illumina Array and Pyrosequencing Assays

Illumina Probe Identifier	Gene	Methylation Status	Correlation (*r*)	*p* Value
cg09071762	*DPPA5*	↑	.57	.008
cg18052665	*DPPA5*	↑	.54	.015
cg09357589	*PSORS1C3*	↑	.64	.003
cg27547543	*PSORS1C3*	↑	.55	.015
cg06860277	*COMT*	↑	.66	.010
cg07194846	*COMT*	↑	.58	.030

malnutrition limited to the first year of life is correlated with impairments in attention and cognition. This correlation would imply that at least a subset of such genes could impact brain function and behavior. To explore whether animals with defects in attention and cognition show altered gene expression in brain, we exposed rats from (pre)conception to birth to a low-protein diet (Figure 4B) (32). This model is not congruent with the exposure timing in our BNS subjects who were affected by malnutrition after birth and in early childhood. Nonetheless, our prenatally malnourished rats showed attention deficits as adults, which is of interest given that some subjects exposed to early life malnutrition show a similar phenotype (4,22,33). Furthermore, according to our findings presented here, a subset of nutrition-sensitive blood methyl-CpGs shows a significant correlation with attentional scores. Postnatal malnourishment is extremely difficult to model in rodents because of altered maternal behaviors, which may not be dissociated from the nutritional deficit as a cause of later brain and behavioral changes. To confirm that our early life malnourished rats are affected by attentional and cognitive defects as previously reported for this model (4,22,33), we trained prenatally malnourished rats and well-nourished control rats, starting at postnatal day 90, first to make the correct choice between two lever press systems to discriminate brief, temporally asynchronous visual targets from nontargets. Subsequently, the animals performed the same task in the context of an unpredictable distractor (salient light flashing at unpredictable rates) (Figure 4C). There was a significant main effect of nutrition on target discrimination ($F_{1,16} = 5.54$, $p = .032$). Post hoc analyses showed that malnourished rats ($n = 10$ males, 1/litter) performed significantly worse in the sustained attention task in the presence of the unpredictable distractor compared with control rats ($n = 8$ males, 1/litter) ($t_{16} = 2.4$; $p = .03$) (Figure 4C). This attention deficit in malnourished rats, as evidenced by the decrease in the number of correct target choices, was highly specific because nutritional history had no effect on sustained attention in the absence of the distractor. Furthermore, all animals were less able to correctly reject nontargets in the presence of the distractor ($F_{1,16} = 69.59$). None of these effects differed on the basis of prior nutrition (all $p > .10$). All animals maintained approximately neutral side biases for lever pressings (malnourished rats, 0.41 ± 0.01; control rats, 0.42 ± 0.02).

Adult rats exposed to malnourishment during development show region-specific glucose hypometabolism in rostromedial cerebral cortex (34), considered the homologue of frontal association cortex in primates (35). We tested whether these neural circuits are affected by altered expression of differentially methylated genes associated with attention and cognition in our human cohort. We injected malnourished and control animals ($n = 18$; see earlier) with radiolabeled deoxyglucose before the behavioral assay (unpredictable distractor), and brains were harvested 45 minutes later. Densitometry on coronal sections cut through the level of the rostromedial cortex of the left hemisphere showed that radiolabeled deoxyglucose uptake was significantly decreased, specifically in the prelimbic area and surrounding prefrontal cortex in malnourished rats relative to control rats (Figure 4D). Radiolabeled deoxyglucose uptake in the prelimbic area correlated with correct lever press choices in the unpredictable distractor paradigm ($r = .43$, $p < .05$) (Figure 4D). We then quantified RNA in adult prefrontal cortex of malnourished rats and control rats for six genes (*Ifng*, *Inhbb*, *Abcf1*, *Comt*, *Syngap1*, *Vars*, all moderately expressed at baseline) (Supplemental Figure S4 in Supplement 1) associated with nutrition-sensitive DMRs and showing moderate correlation between CpG methylation and attentional/IQ scores in our human cohort (Figure 4A, E). *Ifng* and *Inhbb* genes show a significant degree of conservation of regulatory sequences between human and rat within the nutrition-sensitive DMR (Supplemental Table S15 in Supplement 2). *Ifng* [gamma interferon, implicated in the regulation of cognition and emotion via cytokine signaling pathways (36,37)] transcript was significantly decreased in

Figure 4. Epigenetic and transcriptional dysregulation in adult blood and brain associated with impaired attention and cognition after infant malnutrition. **(A)** Correlation graphs of (y axis) attention-deficit/hyperactivity disorder (ADHD) index/Connors Adult Attention Rating Scale questionnaire or Wechsler Abbreviated Scale of Intelligence IQ and (x axis) β values for top significance scoring Illumina CpG probe showing association between methylation and attention and cognition in generation 1 Barbados Nutrition Study subjects for seven differentially methylated genomic region–associated genes, as indicated (see Supplemental Table S10 and S11 in Supplement 2 for complete list of correlations). **(B)** Timeline for rat malnourishment study, to illustrate exposure to 6% casein/low-protein diet (malnourished subjects group [MAL]) or 25% casein normal diet (control subjects group [CON]) from birth to conception, followed by 25% casein/normal protein diet in both groups onward from birth. Behavioral training (sustained attention task [SAT]) occurred between postnatal day 90 (P90) and postnatal day 130 (P130), followed by SAT testing, radiolabeled deoxyglucose (2DG) injection, and brain harvest. **(C)** Overview of the SAT, including light signal–based event and unpredictable distractor and response scheme. Adult rats previously exposed to prenatal malnutrition (red bars) were more susceptible to the effects on an unpredictable, visual distractor (uSAT) than control rats (green bars) and detected fewer correct targets in the presence of the distractor than control rats (% hits), $^{*}p < .05$. **(D)** Region-specific hypometabolism in adult rat prefrontal cortex after prenatal malnutrition. Coronal sections at level of rostromedial prelimbic cortex (PrL) from CON and MAL animals, showing specific decrease in 2DG signal in rostromedial cortex, including PrL, whereas lateral somatomotor cortex and other corticolimbic structures, including piriform cortex (Pir), show normal glucose metabolism. Bar graphs summarize 2DG uptake (mean ± SEM, normalized to white matter) in PrL and Pir, $n = 8$ MAL animals (red bars) and $n = 8$ CON animals (green bars). $^{***}p = .0008$, two-tailed *t* test. Correlation graph shows strong association between glucose metabolism in left area PrL vs. hit accuracy in (unpredictable distractor) uSAT. **(E)** Left PrL RNA quantification for *Abcf1*, *Comt*, *Ifng*, *Inhbb*, *Syngap1*, and *Vars*, genes associated with nutrition-sensitive differentially methylated genomic regions in the Barbados Nutrition Study cohort, and *Pbdg* control gene. All data normalized to *Hprt* housekeeping gene. $n = 12$/group; $^{*}p < .05$; $^{**}p < .005$, two-tailed *t* test. After Bonferroni correction, only *Ifng* remained significant.

prefrontal cortex of malnourished rats. These changes were specific because expression of the remaining genes (with the possible exception of *Comt*) and *Pbdg* housekeeping gene remained unaltered from control rats (Figure 4E).

DISCUSSION

The BNS is a longitudinal study following, for over 48 years, individuals who had moderate to severe protein-energy malnutrition limited to the first year of life after normal prenatal development. The primary selection criteria, birth weight >2500 g and normal Apgar scores and physical examination at the time of birth, with no evidence of wasting or other clinical signs of malnutrition, essentially exclude the likelihood of fetal malnourishment. In the present study, we provide the first evidence for long-lasting DNA methylation changes in humans exposed to malnutrition in early infancy. Genome-wide DNA methylation scans from 168 subjects revealed, after stringent statistical analyses, 134 nutrition-sensitive genomic loci that showed significant differential methylation in previously malnourished subjects and their offspring after correction for age, sex, and cell type composition. Among the nutrition-sensitive genomic loci, there was a significant overrepresentation of CpG islands and other *cis*-regulatory sequences, including promoters, suggesting long-lasting epigenetic dysregulation of gene expression. The brain is among the organs affected because nutrition-sensitive CpG methylation at various genomic loci showed weak to moderate correlations with measures of attention and cognition in BNS subjects that could not be explained by differences in childhood socioeconomic status or other confounds. In a rat model for attention deficits, prefrontal expression of *Ifng*, a gene associated with a hypermethylated DMR in our human subjects with a history of infant malnutrition, was significantly decreased.

Our studies draw a strong link between protein malnutrition during early childhood and epigenetic dysregulation associated with attentional and cognitive deficits in the adult. These findings significantly extend previous work reporting epigenetic dysregulation after prenatal malnutrition (34,38–40). Of note, DNA and histone methylation landscapes in human prefrontal cortex show highly dynamic developmental regulation of neuronal and to some extent also nonneuronal chromatin during infancy and early childhood (41–45). Therefore, the blood DNA methylation changes as reported here could reflect epigenetic maladaptations in the brain, analogous to epigenetic changes in the brain and behavioral dysregulation in response to early life adversity (46,47).

In the present study, the number of autosomal DMRs in G2 offspring was approximately 10-fold lower compared with G1 parents (16 vs. 102). Of epigenetically altered loci, <3% (3 of 102) showed evidence for intergenerational transmission, or concordant methylation changes in G2 offspring of G1 parents malnourished in their childhood. Therefore, most malnutrition-induced DNA methylation changes after early childhood malnutrition are not transmitted into the next generation. Alternatively, our study may have lacked the power to detect more subtle methylation changes at some of the nutrition-sensitive loci in G2. In the present study, regulatory sequences at *DPPA5* were hypermethylated in MAL G1 subjects and in their G2 offspring. It is not unreasonable to speculate that *DPPA5*, a gene extremely highly expressed in primordial germ cells (48), with its CpG island heavily methylated in blood and other somatic tissues (49) could transduce nutrition-related epigenome remodeling from the G1 parent to the G2 offspring. Finally, as a third generation was not included in this study, it is unclear whether or not epigenomic alterations after childhood malnutrition carry transgenerational potential as reported for nutritional exposures during the fetal period (50).

At least seven of our nutrition-sensitive G1 DMRs are in close spatial proximity to imprinted genes, including *IGF1R* (insulin growth factor 1 receptor) (51); *KCNQ1DN* and *WT1*, important for Wilms' tumor biology (52) and implicated in neurodegenerative disease (*WT1*) (53); the *MEST* and *MEST-TIT1* genes as maternal stress-sensitive genes regulating parental behaviors and body mass in offspring (54); and *SLC22A18AS* and *VTRNA2-1*, which have not yet been explored in the nervous system. Such epigenetic dysregulation—with an impressive >40-fold overrepresentation of methyl-CpG changes for imprinted loci in the present study—could affect not only the risk-exposed G1 subjects but also the earliest stages of development in their offspring, even in the absence of an intergenerational transmission of the G1-specific DNA methylation changes.

In conclusion, our study was the first to assess epigenetic alterations after childhood malnutrition in the first year of postnatal life. However, epigenomic sensitivity to nutritional restriction is likely to exist onward from the earliest phases of the prenatal period. For example, a study reported alterations in six metastable epialleles (CpG sequences with high inter-individual variation presumably as a result of stochastic methylation in early development) in 84 infants conceived during the Gambian rainy season, a period with limitations in the nutritional supply of protein energy and methyl donor molecules, compared to infants conceived during the dry season (55). Strikingly, one of the six nutrition-dependent epialleles in the Gambian cohort (55), *RBM46*, encoding a regulator of early blastocyst and trophoectoderm differentiation (56), completely matched one of the G1 DMRs in our study (Gambia DMR *hg18*, chromosome 4: 155,922,422-155,922,489; Barbados DMR *hg18*, chromosome 4: 155,921,670-155,922,589). Furthermore, DNA methylomics on 24 infants exposed to the war-related 1944–1945 Dutch hunger winter at the time of conception compared with their unaffected siblings identified 181 DMRs, many of which were related to growth and metabolism (57). The Dutch study and our BNS data sets intersect at a single DMR (*GNGT2/ABI3* promoter *hg18*, chromosome 17: 44,641,718-44,642,919). Taken together, these three human studies (including the present study) point to epigenome shaping by nutritional history across a wide age range, whereas DNA methylation signatures differ by exposure timing. For example, malnourishment around the time of conception would affect a set of genes and loci largely different from those epigenetically dysregulated when exposure occurs after birth. Furthermore, DNA methylation at a subset of genes, including the metastable *RBM46* epiallele and *GNGT2/ABI3*, remain sensitive to nutrition from conception onward at least until early childhood. Finally, nutrition-related DNA methylation changes are subtle. For example, in our study, we did not observe all-or-none methylation changes, but rather subtle concordant changes

(either higher or lower β value average) in multiple CpGs distributed at specific gene loci. Therefore, it is likely that the combined effect of genome-wide DNA methylation changes, with a limited contribution from each specific site, amount to an epigenetic risk architecture that could, in conjunction with additional factors, impact long-term physical and mental health after childhood malnutrition.

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Author contributions are as follows: JRG, LKF, CPB, and GM evaluated Barbados Nutrition Study subjects and analyzed neuropsychological data. CJP, MK, CR, MG, EIG, and SA designed and performed molecular assays and designed experiments. ACA, DLR, and JAM designed and performed behavioral and radiolabeled deoxyglucose experiments (animals). AJS, PG, AD, MK, and CJP performed bioinformatics and genomic analyses. SA, JRG, AJS, MK, and CJP wrote the article.

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