Spring 2025 – Epigenetics and Systems Biology
Discussion Session (Environmental Epigenetics)
Michael K. Skinner – Biol 476/576
Week 11 (March 20)

Environmental Epigenetics

Primary Papers

1. Duncan GE, et al. (2022) Sci Rep. 12(1):20166. (PMID: 36424439)
2. McGowan et al., (2009) Nat Neurosci. 12(3):342-8. (PMID: 19234457)
3. Burdge et al., (2009) J Nutr. 139(6):1054-60. (PMID: 19339705)

Discussion

Student 25 – Ref #1 above

- Why are twin studies useful for epigenetic studies?
- Does the data support an environmental impact on the human epigenome and disease?
- What is the application of these epigenetic changes?

Student 26 – Ref #2 above

- What mechanism is proposed for early life effects on brain function?
- Is NGF1 the only gene effected?
- What is the impact of these epigenetic changes?

Student 27 – Ref #3 above

- How does folic acid effect epigenetics?
- Does diet effect epigenetic programming?
- What happens if you have too much folate?

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Epigenome-wide association study of physical activity and physiological parameters in discordant monozygotic twins

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An epigenome-wide association study (EWAS) was performed on buccal cells from monozygotic-twins (MZ) reared together as children, but who live apart as adults. Cohorts of twin pairs were used to investigate associations between neighborhood walkability and objectively measured physical activity (PA) levels. Due to dramatic cellular epigenetic sex differences, male and female MZ twin pairs were analyzed separately to identify differential DNA methylation regions (DMRs). A priori comparisons were made on MZ twin pairs discordant on body mass index (BMI), PA levels, and neighborhood walkability. In addition to direct comparative analysis to identify specific DMRs, a weighted genome coexpression network analysis (WGCNA) was performed to identify DNA methylation sites associated with the physiological traits of interest. The pairs discordant in PA levels had epigenetic alterations that correlated with reduced metabolic parameters (i.e., BMI and waist circumference). The DNA methylation sites are associated with over fifty genes previously found to be specific to vigorous PA, metabolic risk factors, and sex. Combined observations demonstrate that behavioral factors, such as physical activity, appear to promote systemic epigenetic alterations that impact metabolic risk factors. The epigenetic DNA methylation sites and associated genes identified provide insight into PA impacts on metabolic parameters and the etiology of obesity.

Abbreviations

EWAS	Epigenome-wide association study
MZ	Monozygotic twins
PA	Physical activity
DMRs	DNA methylation regions
BMI	Body mass index
WGCNA	Weighted genome complex network analysis
BE	"Built" environment
GWAS	Genome-wide association studies
MeDIP	Methylated DNA immunoprecipitation
MeDIP-Seq	Next generation sequencing
PCA	Principal component analysis
MVPA	Minutes of moderate to vigorous physical activity
WSTR	Washington State Twin Registry

Regular physical activity (PA) and proper nutrition are the foundations of chronic disease prevention and treatment efforts. However, we have failed to motivate adoption and maintenance of these critical health behaviors at the population level. Strategies to increase levels of PA in the population are a critical public health goal[1–4]. The role of the "built" environment in supporting health behaviors has received increased attention over the last decade because of the failure of individual-level approaches (i.e., behavior change) to impact this population-level problem (i.e., obesity and associated metabolic syndrome). "Healthy" or "walkable" built environments provide

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opportunities for PA and better choices for food. These environments potentially lead to lower obesity and associated diseases[5]. Detrimental built environments include sprawling suburbs where automobiles are the only transportation option and fast-food restaurants and strip malls are numerous. Th s "obesogenic" environment minimizes energy expenditure and maximizes energy intake to promote obesity. While it is known that exposure to an obesogenic built environment negatively influences PA and eating behaviors, it is not clear whether exposure to an obesogenic built environment can affect on a molecular level gene activity[6]. These "molecular signatures" of obesity can be investigated through epigenetics.

Our previous work documents "quasi-causal" associations between the built environment and health behaviors in a community-based sample of identical adult twins (monozygotic, MZ) who were reared together as children, but now reside apart[7,8]. This unique sample group permits us to address environmental self-selection by accounting for genetic and shared environmental factors that would otherwise introduce selection biases confounding statistical associations. Because MZ twins have the same genetic background and are matched on numerous childhood exposures, they are an ideal sample to study epigenetics and environmental influences on health behaviors and associated health outcomes.

Genetic mutation and gene expression correlations in with twin studies have demonstrated that most genome-wide association studies (GWAS) have identified genes with a very low frequency correlation often in less than 1% of the patient population examined[9]. Although twin studies help reduce the variation of the GWAS analysis, negligible correlations or associations with genetic mutations have been observed[10]. Therefore, neither PA nor metabolic parameters (e.g., BMI and waist circumference) have been shown to have a high frequency association with genetic mutations[11]. Gene expression analysis has been more useful to correlate PA and metabolic parameters, which supports the hypothesis that gene expression is involved[12]. However, the molecular control of this gene expression and how PA may impact metabolism is unclear. Observations support a lack of genetic DNA sequence alterations in PA and metabolic pathways.

An additional molecular mechanism not involving DNA sequence alterations is epigenetics. Limited associations between PA and metabolic disease have been made using a systems epigenome-wide approach. Since the primary molecular control of gene expression involves epigenetics, environmental factors such as PA may alter epigenetic regulation of gene expression to promote the physiology observed[13]. Classic genetics and genetic mutations do not have the capacity to be environmentally responsive and promote gene expression alterations and physiologies without the inclusion of epigenetics in the process. Therefore, the current study takes a novel systems approach to examine in a genome-wide manner the impacts of PA and metabolic syndrome (e.g., obesity) measures in twin studies with controlled genetics. Epigenetics is defi ed as "molecular factors and processes around DNA that regulate genome activity, independent of DNA sequence, and are mitotically stable"[14]. Epigenetic factors include DNA methylation, histone modifications, chromatin structure and non-coding RNAs[14]. Epigenetics in part evolved to provide a molecular mechanism to be responsive to the environment and impact biology. Examples of environmental and behavioral factors that can regulate epigenetics to impact physiology include environmental toxicants, nutrition, and stress[14,15]. The current study extends these observations to examine the impact of PA[16] on epigenetics and allow associations with metabolic parameters (e.g., BMI and waist circumference)[17,18] measures using identical twins (monozygotic, MZ) to control for genetic background variation.

The initial analysis used is a standard paired comparison between two groups to identify differential DNA methylated regions (DMRs), as previously described[15]. Th s is ideal when the specific groups to compare are known, but not as useful with unknown potential correlations. Therefore, an additional analysis was performed involving weighted genome coexpression network analysis (WGCNA)[19]. The ability to use a WGCNA protocol was established for genetic and physiological parameter correlations[20] with the use of primarily gene expression data[21]. The potential of WGCNA to be used for an epigenetic analysis has also been established[22,23]. The current study uses WGCNA with epigenetic DNA methylation data to correlate PA and measures of metabolic parameters (i.e., BMI and waist circumference). The epigenome gene associations are then used to correlate with PA and metabolic parameters in MZ twin samples. Therefore, positive impacts of PA to reduce measures of obesity can provide insights into the role of epigenetics and physical activity on metabolic measures.

Results

Descriptive parameters for selected characteristics of the study participants are presented in Supplemental Table S1. The participants were on average 50.2 ± 12.6 years, 74.3% female, and most participants identifi d as Non-Hispanic White (94.3%). The majority of the participants were married (70.7%). Most participants had a bachelor's degree or higher (62.6%), and more than half reported an annual income above $100,000 (55.0%). Measured physical activity (minutes of moderate to vigorous physical activity, MVPA) was higher in men (187.8 ± 167.4) compared to women (120.3 ± 131.4).

Within-pair discordance measures were calculated for objective physical activity (PA), walkability, waist size, and body mass index (BMI, kg/m^2). Descriptive statistics for discordant pairs are presented in Supplemental Table S2. Physical activity discordance was defi ed as one twin having at least 150 min of moderate to vigorous PA per week, and their co-twin having less than 150 min. A higher percentage of male pairs (43.8%) were discordant for PA compared to female pairs (36.7%). Neighborhood walkability discordance was determined by one twin living in a car dependent or somewhat walkable neighborhood, and their co-twin living in a very walkable or walker's paradise neighborhood, which involved 27.8% of male pairs and 30.8% of female pairs as discordant for neighborhood walkability. BMI discordance was defi ed as a difference of ≥ 5 kg/m^2 within the twin pair, which involved 11.1% of male pairs and 13.5% of female pairs. Discordant pairs for these parameters were used for the epigenetic analysis.

Buccal cells were used as a marker cell for systemic impacts on the individuals. Buccal cell cheek swabs were obtained under the Washington State University Institutional Review Board (IRB) (#16419). Participants

Discordant Twin Activity and Metabolic Parameter DMR Identification

A Physical Activity Male DMRs

p-value	All Window	Multiple Window
0.001	1974	67
1e-04	**462**	**13**
1e-05	158	4
1e-06	67	2

Significant windows	1	2	3
Number of DMR	449	12	1

D Physical Activity Female DMRs

p-value	All Window	Multiple Window
0.001	689	4
1e-04	**80**	**0**
1e-05	12	0
1e-06	4	0

Significant windows	1
Number of DMR	80

B Walkability Male DMRs

p-value	All Window	Multiple Window
0.001	1041	9
1e-04	**117**	**2**
1e-05	10	0
1e-06	1	0

Significant windows	1	2
Number of DMR	115	2

E Walkability Female DMRs

p-value	All Window	Multiple Window
0.001	632	0
1e-04	**88**	**0**
1e-05	15	0
1e-06	4	0

Significant windows	1
Number of DMR	88

C BMI Male DMRs

p-value	All Window	Multiple Window
0.001	699	3
1e-04	**82**	**1**
1e-05	10	0
1e-06	0	0

Significant windows	1	2
Number of DMR	81	1

F BMI Female DMRs

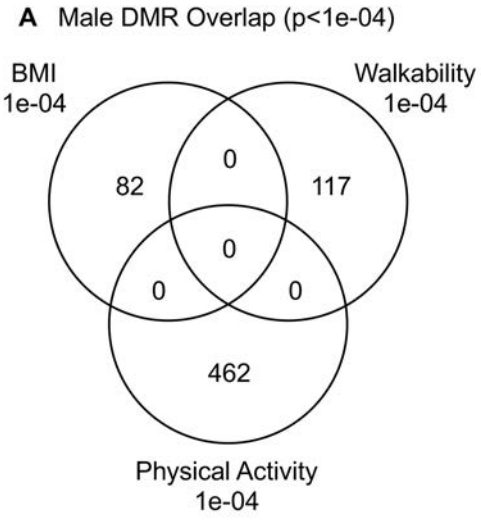
p-value	All Window	Multiple Window
0.001	1802	53
1e-04	**284**	**11**
1e-05	56	6
1e-06	23	4

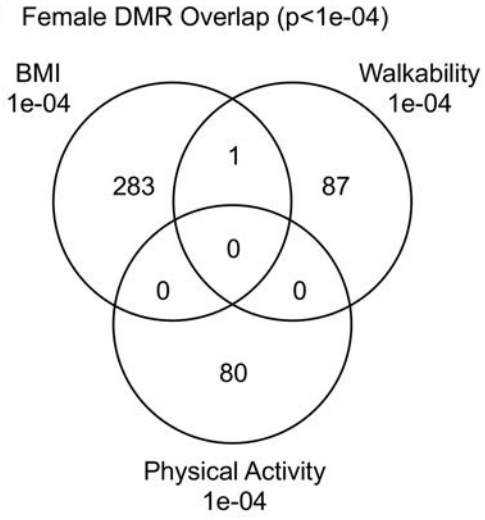
Significant windows	1	2	3	5	7	46
Number of DMR	273	7	1	1	1	1

Figure 1. Discordant twin activity and metabolic parameter DMR identifi ation. The number of DMRs found using different *p* value cutoff thresholds. The All-Window column shows all DMRs. The Multiple Window column shows the number of DMRs containing at least two nearby signifi ant windows (1 kb each). The number of DMRs with the number of signifi ant windows (1 kb per window) at a *p* value threshold of $p < 1e{-}04$ for DMR is bolded. (**A**) Activity male DMRs; (**B**) Walkability male DMRs; (**C**) BMI male DMRs; (**D**) Activity female DMRs; (**E**) Walkability female DMRs; and (**F**) BMI female DMRs.

provided written informed consent prior to sample collection. The buccal cell collection procedure is outlined in the Methods section. The twins were sent kits with a swab brush and following collection the swab brush was sent back to the Washington State Twin Registry (WSTR) laboratory for storage at − 80 °C. At the conclusion of data collection for the full study, collected samples were sent to the Skinner laboratory at the WSU Pullman campus for processing and storage at − 80 °C. Discordant for PA, walkability, and BMI were identifi d and used for the study. Male and female groups were separated for the analysis (Supplemental Tables S1 and S2).

A male and female separation of groups was made due to sex specifi differences in epigenetics observed in previous studies[24,25]. Each individual's buccal cell epigenetic analysis was obtained such that optimal comparisons of parameters could be assessed. The buccal cells were used as a marker cell for alterations in epigenetics for each individual. Similar analyses have been performed for disease specific comparisons, such as female susceptibility for arthritis[24–28]. The DNA was isolated from the buccal cells and used in a methylated DNA immunoprecipitation (MeDIP) protocol followed by next generation sequencing (MeDIP-Seq), as described in the Methods[24]. The analysis and comparisons between the discordant twins for PA, walkability, and BMI were made for each sex (Fig. 1). A variety of edgeR *p* values were used, and the differential DNA methylation regions (DMRs) identifi d for PA (lower 66 min weekly versus higher 266 min weekly, Supplemental Table S2) in the discordant twin comparison identifi d 462 DMRs for males and 80 DMRs for females at $p < 1e{-}04$ (Fig. 1A,D). Walkability (lower 25.7 versus higher 82.0, Supplemental Table S2) in the discordant twin comparison identifi d 117 DMRs for males and 88 DMRs for females at $p < 1e{-}04$ (Fig. 1B,E). BMI (lower 28.4 versus higher 35.2 kg/m^2, Supplemental Table S2) in the discordant twin comparison identified 82 DMRs for males and 284 DMRs for females at $p < 1e{-}04$ (Fig. 1C,F). False discovery rate (FDR) analysis demonstrated with the male PA DMRs an FDR < 0.1, female BMI DMRs an FDR < 0.1 (15%), and with the other DMR comparisons being primarily





C Extended Overlaps (p<1e-04 vs. p<0.05)

p<1e-04 \ p<0.05	BMI Male	Walkability Male	Physical Activity Male	BMI Female	Walkability Female	Physical Activity Female
BMI Male	82 (100.0%)	12 (14.6%)	19 (23.2%)	9 (11.0%)	6 (7.3%)	6 (7.3%)
Walkability Male	10 (8.5%)	117 (100.0%)	35 (29.9%)	20 (17.1%)	19 (16.2%)	16 (13.7%)
Physical Activity Male	50 (10.8%)	163 (35.3%)	462 (100.0%)	97 (21.0%)	192 (41.6%)	137 (29.7%)
BMI Female	50 (17.6%)	53 (18.7%)	58 (20.4%)	284 (100.0%)	67 (23.6%)	51 (18.0%)
Walkability Female	5 (5.7%)	24 (27.3%)	44 (50.0%)	26 (29.5%)	88 (100.0%)	27 (30.7%)
Physical Activity Female	6 (7.5%)	12 (15.0%)	8 (10.0%)	16 (20.0%)	8 (10.0%)	80 (100.0%)

Figure 2. Discordant DMR overlaps. (**A**) Male DMR $p < 1e{-}04$ Venn diagram overlap. (**B**) Female DMR $p < 1e{-}04$ Venn diagram overlap. (**C**) Extended overlaps with $p < 1e{-}04$ and $p < 0.05$ comparisons. DMR number and percent (%) overlap presented within the rows.

FDR > 0.1. The overlap of the DMRs for the three measures was found to be negligible at $p < 1e{-}4$ between the comparison for either male or female (Fig. 2A,B). An extended overlap with the $p < 1e{-}4$ DMR overlap with the other comparison at $p < 0.05$ demonstrated a 5.7–50% overlap (Fig. 2C). The male and female DMR overlaps demonstrated similar percentage overlaps. This extended overlap identified highest overlap at a reduced threshold comparison for PA versus walkability.

The genomic features of the DMRs were then investigated. The chromosomal locations demonstrated that the DMRs were throughout the genome (red arrowheads) and clusters of DMRs were also identifi d (black boxes), but no over-represented sites were observed (Fig. 3). The DMR CpG density was predominantly 1 or 2 CpG/100 bp for PA and walkability (Fig. 4), but the BMI DMRs did have some with higher density (Fig. 4E,K). The DMRs were predominantly 1 kb in length, with some > 2 kb (Fig. 4 and Supplemental Tables S3–S8). A principal component analysis (PCA) for DMR specific genomic features identifi d good separation for principal components 1 and 2 for PA and walkability (Fig. 5A–E). For the discordant twin BMI DMRs, some overlap was observed between the low and high BMI groups (Fig. 5C,F). Lists of all the genomic features for each DMR are presented in Supplemental Table S3 for PA males, Supplemental Table S4 for PA females, Supplemental Table S5 for walkability males, Supplemental Table S6 for walkability females, Supplemental Table S7 for BMI males, and Supplemental Table S8 for BMI females. The chromosomal location, CpG density, length, increase or decrease in DNA methylation (log fold change), and gene annotations and categories are listed in Supplemental Tables S3–S8.

An alternate approach to assess the epigenetic correlations within the discordant twin sets for PA, walkability and BMI used the weighted genome coexpression network analysis (WGCNA). This approach assesses the entire epigenome for variations that correlate with all parameters assessed to provide correlating coeffici ts and associated p values[19,20]. In this approach no specific comparisons are assessed, but all data for individuals are included to identify the complex epigenetic networks involved. Twin pair information is also ignored, since WGCNA does not allow such sample pairs to be considered, so all samples are considered independent. Th s analysis identifies clusters of data that are put in modules that are then correlated with all parameters with correlation coeffici ts and statistics. In this manner, groups of epigenetic sites within the genome can be identifi d that associate with the parameters of interest. The male and female traits and correlations within the methylation data for twins are presented in Supplemental Figs. S1 and S2. The PA sample set associated with moderate to vigorous PA (MVPA) is one of the highly connected parameters for both male and female twins. The walkability scores are also presented, as well as BMI discordance and waist circumference, in Supplemental Figs. S1 and S2.

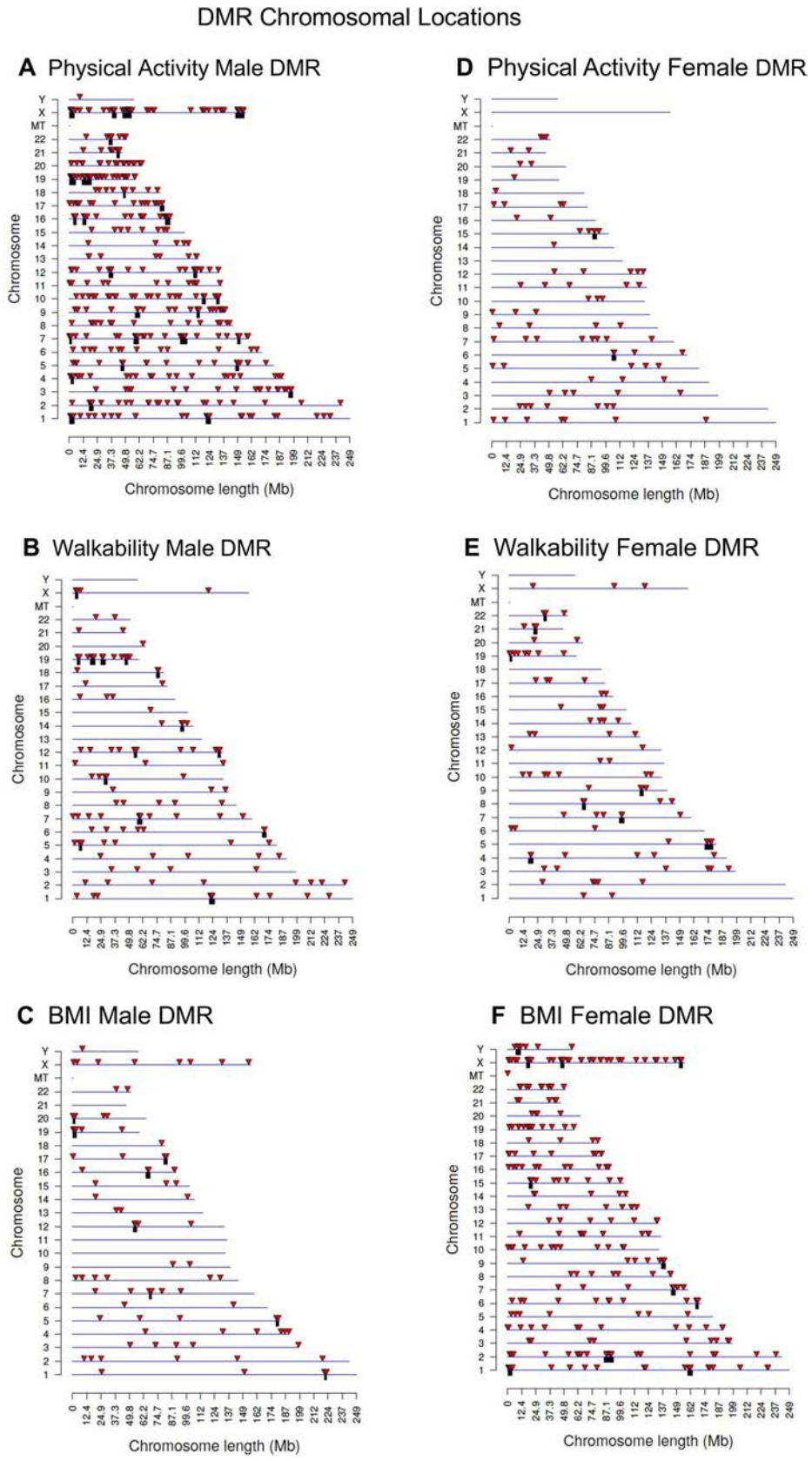


Figure 3. DMR chromosomal locations. The DMR locations on the individual chromosomes is represented with an arrowhead and a cluster of DMRs with a black box. All DMRs containing at least one signifi ant window at a p value threshold of $p < 1e{-}04$ for DMR are shown. (**A**) Activity male DMR; (**B**) Walkability male DMR; (**C**) BMI male DMR; (**D**) Activity female DMR; (**E**) Walkability female DMR; and (**F**) BMI female DMR. The chromosome number versus size (megabase) is presented.

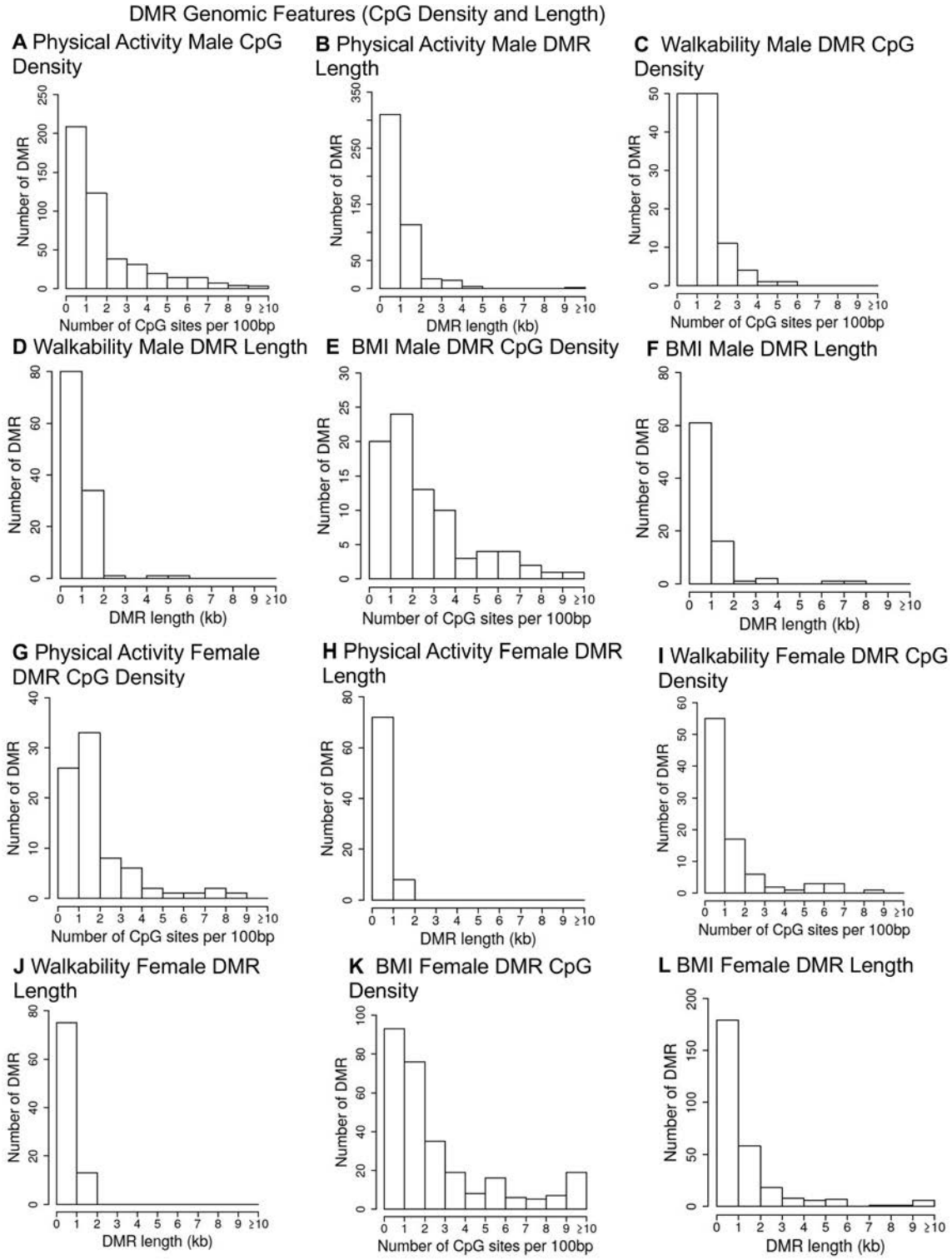


Figure 4. DMR genomic features CpG density and length. (**A**) Activity male CpG density; (**B**) Activity male DMR length; (**C**) Walkability male CpG density; (**D**) Walkability male DMR length; (**E**) BMI male CpG density; (**F**) BMI male DMR length; (**G**) Activity female CpG density; (**H**) Activity female DMR length; (**I**) Walkability female CpG density; (**J**) Walkability female DMR length; (**K**) BMI female CpG density; and (**L**) BMI female DMR length.

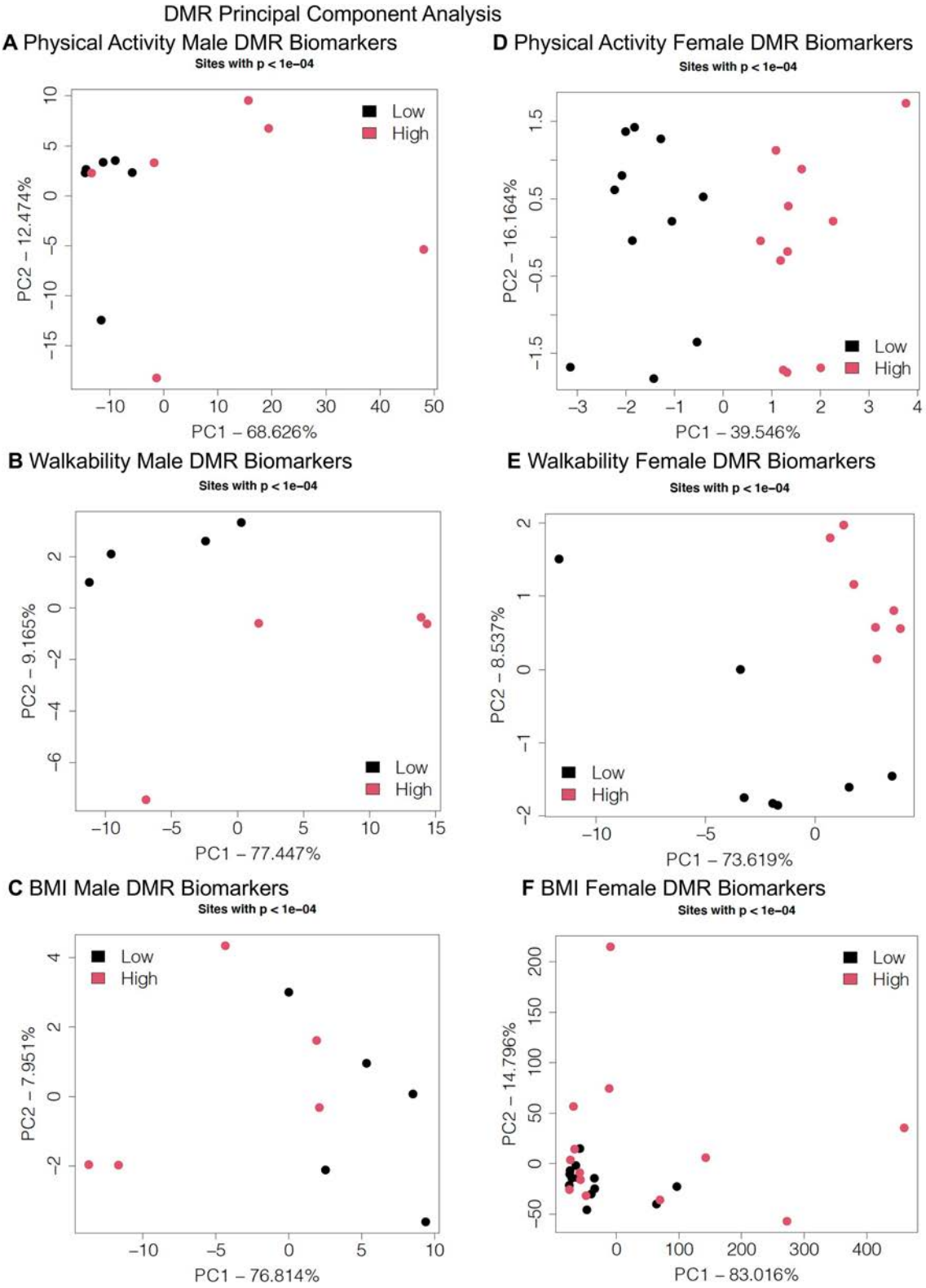


Figure 5. DMR principal component analysis. (**A**) Activity male DMR biomarkers; (**B**) Walkability male DMR biomarkers; (**C**) BMI male DMR biomarkers; (**D**) Activity female DMR biomarkers; (**E**) Walkability female DMR biomarkers; and (**F**) BMI female DMR biomarkers.

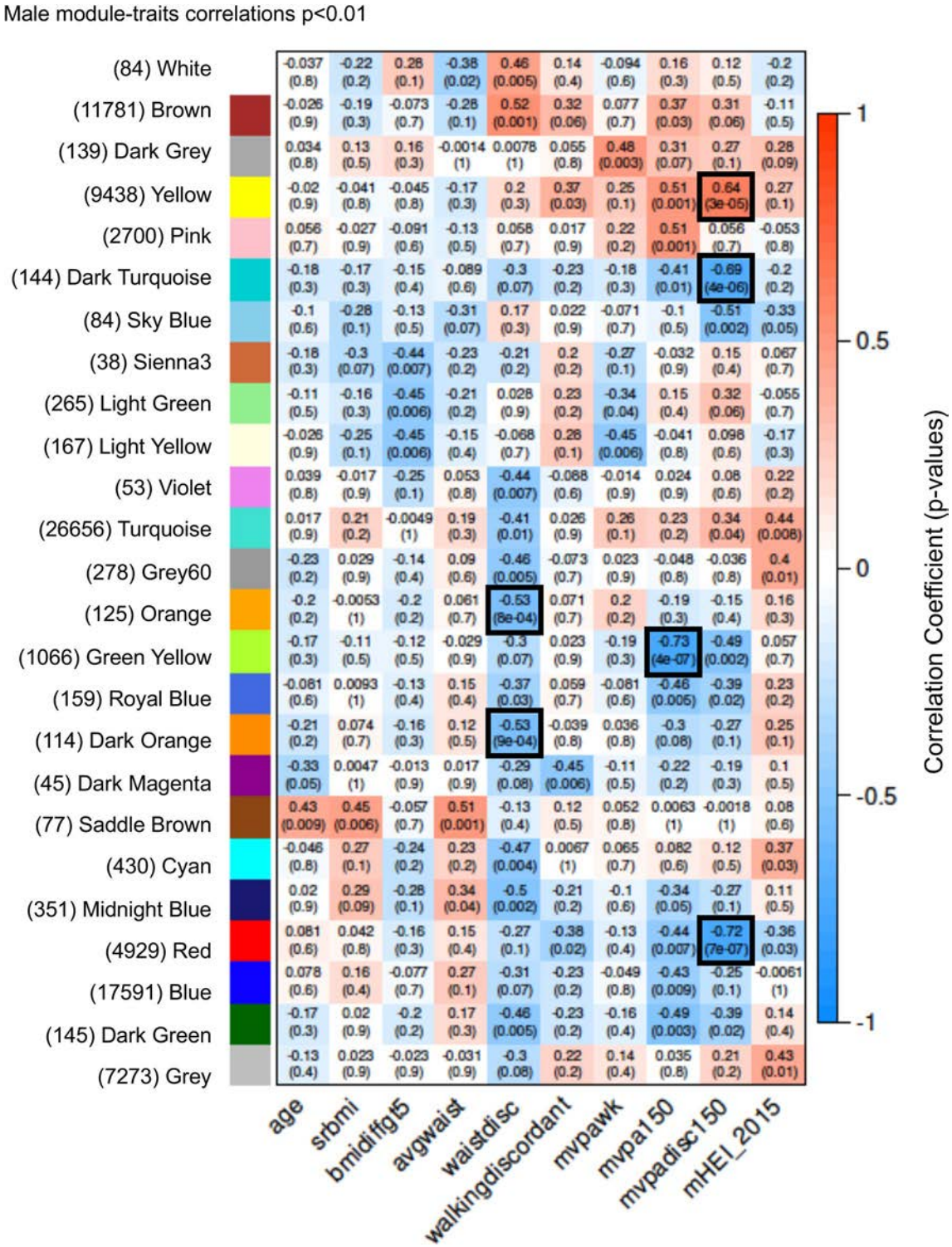


Figure 6. Male module-traits correlations $p < 0.001$. Rows and columns removed if no correlation met threshold.

The trait connectivity information was then used to generate module-trait relationships which provide correlation coeffici ts and p values for all DNA methylation site data (Supplemental Fig. S3 for males and Supplemental Fig. S4 for females). The data was next assessed with a presentation of module-trait relationships with a correlation $p < 0.001$ as shown for males in Fig. 6 and females in Fig. 7. The modules and number of DNA methylation epigenetic sites are presented for all traits with the correlation coefficients and p values listed.

A summary of the module-trait correlations with $p < 0.001$ is presented in Table 1 for both males and females for the PA set, walkability, and waist circumference (waist) parameters indicated. The female set also

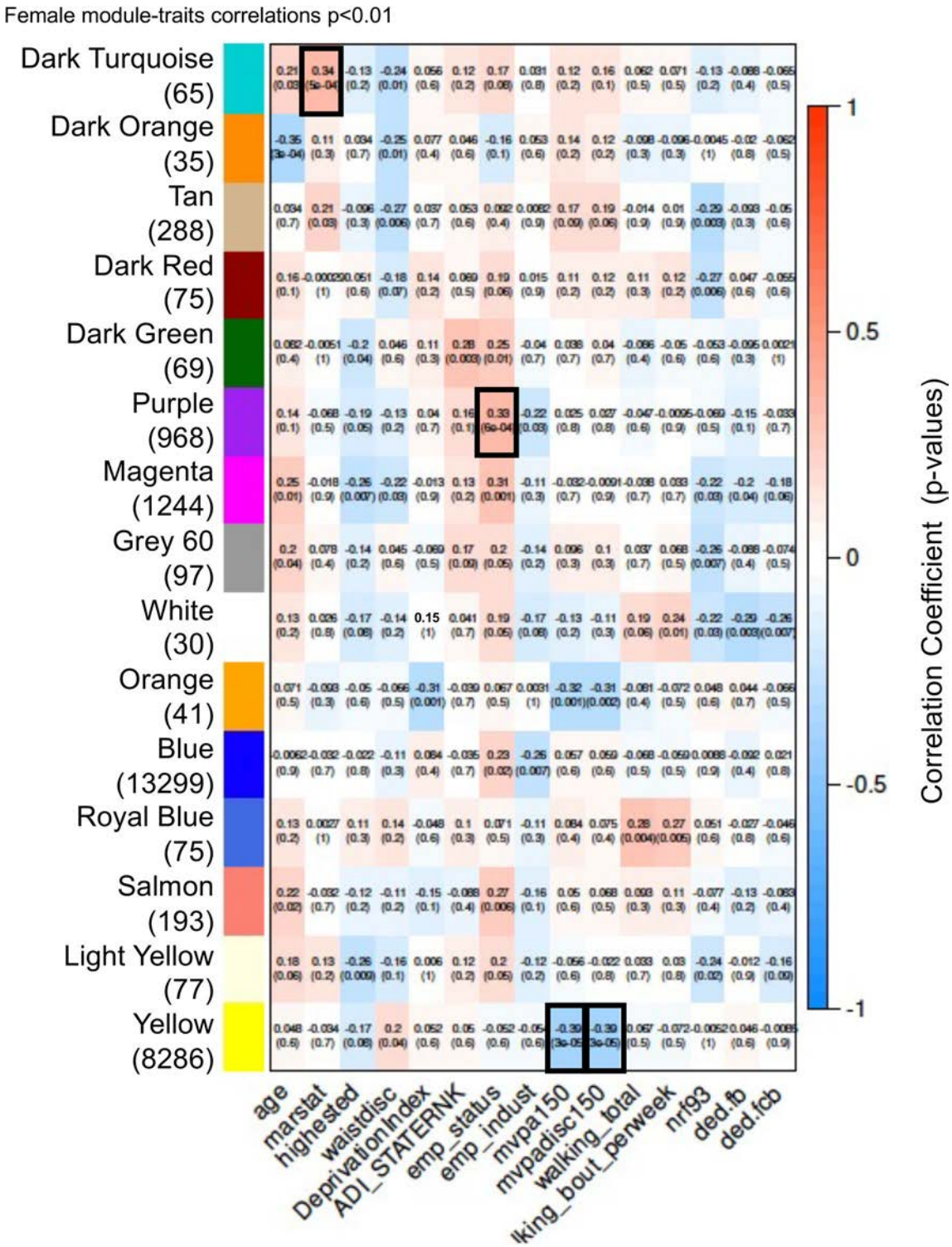


Figure 7. Female module-traits correlations $p < 0.001$. Rows and columns removed if no correlation met threshold.

had module-trait correlations with $p < 0.001$ for marital status (marstat) and employment (EMP) traits (Fig. 7 and Table 1). These traits are not directly relevant or correlated to PA or metabolic traits, so are not considered further. The module epigenetic sites for DNA methylation are presented for those modules with correlated traits ($p < 0.001$), Supplemental Table S9 for male modules and Supplemental Table S10 for female modules. The gene associations were assessed when an epigenetic DNA methylation site was within 10 kb of a gene, so as to include the distal and proximal promoter region. Only the epigenetic sites with gene associations are presented in Supplemental Tables S9 and S10 for each module.

Module	Waistdisc-9	MVPA	MVPA disc	
(A)				
Yellow			M	
Dark turquoise			M	
Orange	M			
Green yellow		M		
Dark orange	M			
Red			M	
Module	**Marstat**	**EMP Status**	**MVPA**	**MVPA disc**
(B)				
Dark turquoise	F			
Purple		F		
Yellow			F	F

Table 1. (A) Male (M) and (B) female (F) summary module-trait correlations $p < 0.001$.

The DMR sites identifi d with the PA, walkability, and metabolic parameters (BMI) were identifi d and associated genes presented in Supplemental Tables S3–S8. The WGCNA used DNA methylation sites and patterns in a genome-wide analysis to identify correlations with traits. The module-trait associations were correlated and statistically identified for each of the major trait correlation coefficients and *p* values involving PA, walkability, and metabolic parameters such as BMI and waist circumference. Th s was correlated to selected modules and the DNA methylation data that associated with genes identifi d, (Supplemental Tables S9 and S10 for males and females separately). The epigenetic gene associations for both data sets were correlated with genes known to be linked to PA and obesity measures in the discordant MZ twins using Pathway Studio software (v 12.5 Elsevier, Inc.)[29]. The DMR associated gene categories related to PA, walkability, and BMI demonstrated that signaling, transport, transcription, and metabolism were all predominant, as expected, since these are the larger gene activity families in the genome, (Fig. 8). The DMR associated gene pathways for each of the traits did not show strong overlap between the traits (Supplemental Fig. S5). The WGCNA epigenetic site analysis identified module-trait relationships with PA, walkability, and BMI (Table 1). The correlated module-trait epigenetic sites identifi d were associated with genes as shown in Supplemental Tables S9 and S10. Gene network analyses were performed with the DMR and module-trait associated genes. The male gene networks for physical activity and obesity measures demonstrated, for the basic DMR analysis, fewer associated genes and smaller networks (Supplemental Figs. S6 and S7) compared to those of the WGCNA module-trait analysis. The male module-trait associated gene networks involved a larger number of genes with common associations to physical activity and obesity (Fig. 9 and Supplemental Fig. S6). The female gene networks for PA and obesity measures also demonstrated a small DMR associated gene network (Supplemental Fig. S7). A large gene network with common PA and obesity gene associations involved the female yellow module (Fig. 10). The male yellow module-trait network also had a large number of associated genes in common between PA and obesity (Supplemental Fig. S6). The smaller module-trait WGCNA associated network genes in common with PA and obesity are also presented in Supplemental Fig. S6. Observations indicate the DMR approach did provide some similar genes in the module-trait network that are indicated with color highlights, however, the majority of associated genes with PA and obesity were identifi d with the WGCNA. Finding genes that have previously been shown to be associated with both PA and obesity helps validate the approach used for both the DMR and WGCNA analyses. As expected, the male and female DMRs, WGCNA modules and gene networks were primarily distinct, due to the sex differences in the epigenome.

Discussion

Epigenetics has a critical role in the regulation of gene expression and maintenance of genome biology[30]. In contrast to genetics, environmental exposures can directly regulate epigenetic processes to subsequently impact gene expression, cell and developmental biology, and the physiology of the organism[31]. These exposures can include nutrition, stress, and toxicants to impact epigenetic processes that in turn impact physiology and associated pathologies[15]. Physical activity (PA) is a common health behavior that is influenced by a number of factors, including environmental exposures, and that in turn impacts biology and disease susceptibility[32]. The current study was designed to minimize genetic variation using monozygotic (MZ) twins to identify DMRs among pairs who are discordant in PA, walkability (an environmental exposure related to PA), and BMI and related metabolic parameters (e.g., waist size). The direct epigenetic associations with PA and metabolic parameters were assessed.

All cell types of an organism have the same DNA sequence, but develop cell specifi ity through cell specific epigenomes[33]. Therefore, mixed cell populations work well for genetic analysis, but are not useful for epigenetic analysis. Th s is why many mixed cell (i.e., blood) analyses have been difficult to interpret[34]. One of the easiest purified cell populations to collect from humans are buccal swab cheek cells. This can be used as a marker cell for exposures and disease susceptibility. Recent studies have demonstrated buccal cells have epigenetic biomarkers for diseases such as arthritis or preterm birth[24,26]. Since ancestral and early life exposures can impact the majority of different cell types development and epigenetics, the current study used buccal cells as marker cells to identify physical activity exposure effects on epigenetics and make associations to metabolic parameters (e.g., BMI).

The initial analysis involved a comparison between MZ twins with discordant PA, where one twin had at least 150 min per week of moderate to vigorous PA while their co-twin had less than 150 min. Th s threshold is

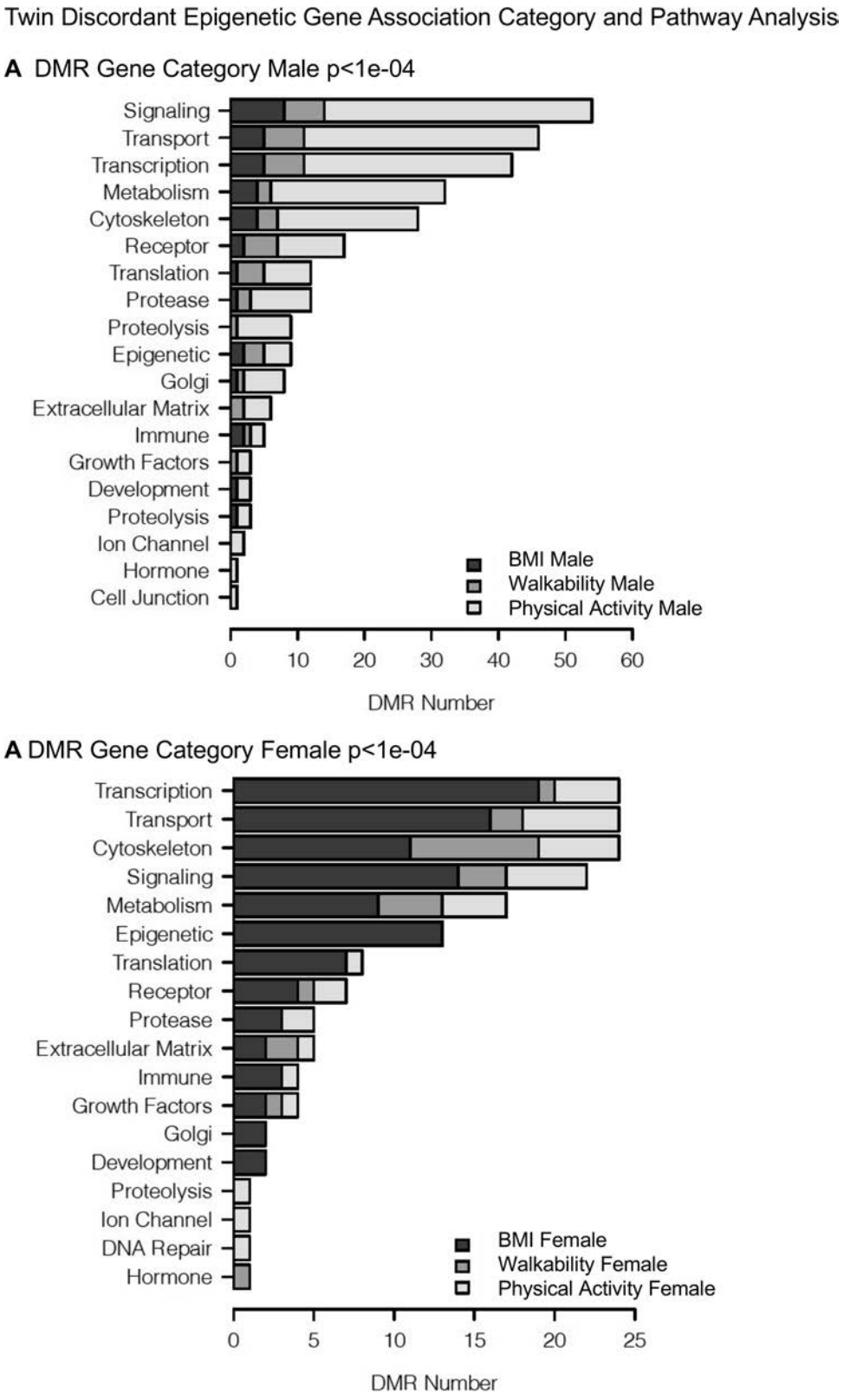


Figure 8. DMR gene categories. (**A**) Male (**B**) Female, with inset legends for the distinct DMR sets.

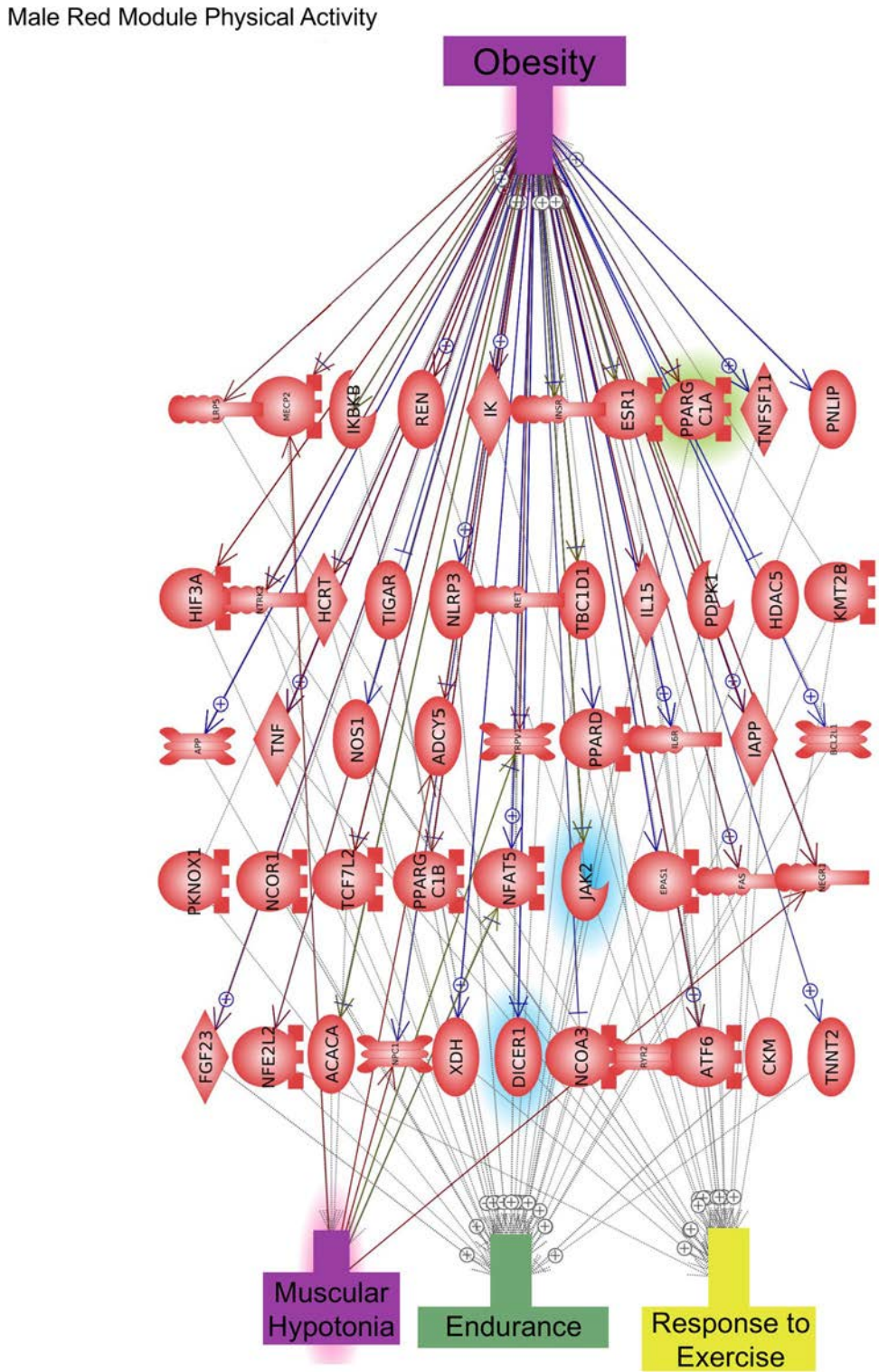


Figure 9. Male Red module-trait physical activity. Twin epigenetic site gene association network analysis. Male red module physical activity. Highlights indicate blue = activity, green = walkability, yellow = BMI, and red = signifi ant over-representation. Gene links in common for obesity and physical activity parameter.

based on guidelines for activity levels in the U.S. population[1]. Using a direct comparison for DMRs, an epigenetic signature for PA was identified. When this was correlated with obesity measures such as body mass index (BMI) and waist size, an epigenetic DMR signature was also observed. Therefore, PA promoted epigenetic

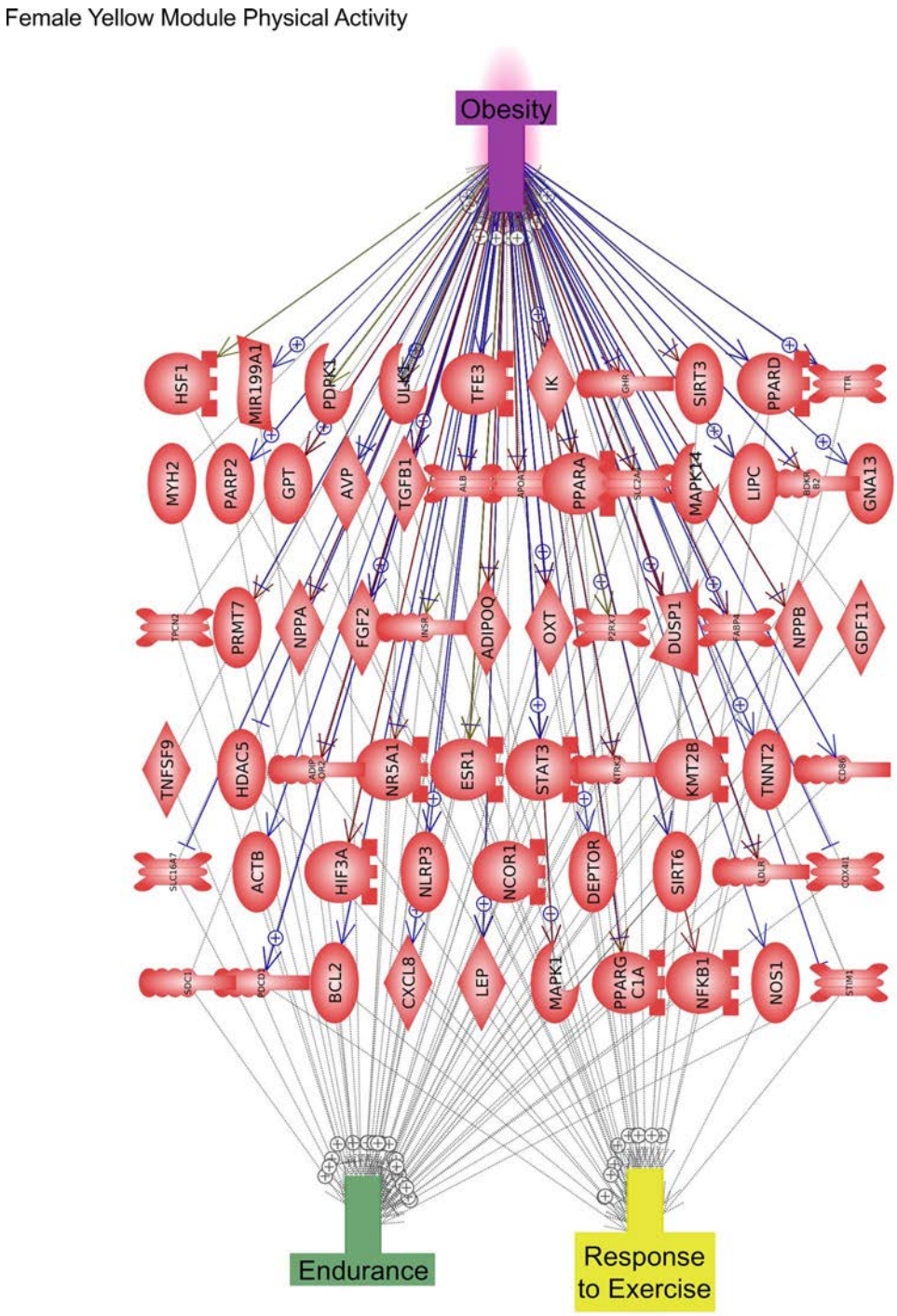


Figure 10. Female Yellow module-trait physical activity. Twin epigenetic site gene association network analysis. Female yellow module physical activity. Gene links in common for obesity and physical activity parameter.

DMR alterations that correlated with a change of metabolic parameters within the sample population. Specific epigenetic DMR signatures were obtained for PA, walkability, and BMI. This analysis identified individuals with a specific discordance and supports the concept that PA and associated metabolic measures have an epigenetic component that allows a mechanistic understanding of the phenomenon.

An alternate approach examined the epigenetics on a genome-wide level for all individuals to identify epigenetic alterations that correlated to the PA, walkability, and metabolic measures. A weighted genome coexpression network analysis (WGCNA) allows such a genome-wide correlation[19,20]. Although primarily used for gene expression analysis, the epigenome can also be assessed to then correlate with genomic actions, gene associations, and physiological parameters[22,23]. Similar observations were made in WGCNA module-trait analyses that PA, walkability, and metabolic parameters (e.g., BMI) did have epigenetic alterations that correlated. The modules

identifi d with statistically signifi ant correlations contained DNA methylation alterations and associated genes that were identifi d.

The DNA methylation WGCNA module and DMR associated genes were identifi d and compared. The DNA methylation alterations had associated genes that have previously been shown to be involved in PA and obesity parameters (Figs. 9 and 10). Although a comparison demonstrated male and female epigenetic changes and associated genes were distinct, similar gene pathways and networks were involved and common PA and obesity associated genes were observed for both male and female networks. The current study demonstrates physical activity (exercise response and duration) through epigenetic (DNA methylation) alterations can impact associated gene expression events that correlate with altered obesity measures (Figs. 9 and 10 and Supplemental Figs. S6–S7). Th s provides a molecular link between PA with the physiology and disease parameters observed in this MZ twin study. The WGCNA analysis also identified other clinical parameters within the twin population investigated. One male parameter that was found to have a correlation was deprivation index, Supplemental Figs. S1 and S2. The females had correlations with marital status (Marstat) and employment (EMP status) that are not directly relevant to the PA and metabolic measures. Future studies will need to further investigate these correlated parameters.

Observations from the current study clearly identified that physical activity (PA) impacts the epigenetics of discordant MZ twins. An increase in PA is correlated with a decrease in metabolic measures such as BMI and waist circumference. Although this is expected, the current study provides direct molecular insights into how this correlation exists. Genetic sequence mutation associations with PA and metabolic disease have not provided high frequency events (i.e., generally less than 1% of study population) that could explain the observations. No previous GWAS correlations could be identifi d that were similar to the EWAS observations. Th s is in part due to GWAS being focused on gene bodies while EWAS is not. Since environmental epigenetics is a high frequency event that is more consistent among individuals, the alterations in DNA methylation that in turn impact gene expression known to be involved in the parameters assessed does reveal how this is correlated on a molecular level. Further research on this topic and correlations with more specific molecular and physiological parameters will help elucidate how environmental epigenetics can mediate on a molecular level how PA reduces pathologies associated with obesity and associated metabolic measures.

Methods

Twin clinical sample collection and information. Participants for this study were MZ twins recruited from the community-based Washington State Twin Registry (WSTR). Recruitment procedures and details about the WSTR have been described[35]. Participants in the current study previously participated in a study of objective measures of physical activity (PA) and locations among individuals residing in the Puget Sound area around Seattle between 2012 and 2015. Follow-up data collection was conducted in 2018 and 2019, 72 pairs completed follow-up collection out of the 144 pairs who completed the baseline study[6,36]. Once participants were enrolled in the study, the study coordinator sent all study materials to the participant for remote data collection. Participants wore a Qstarz BT-Q1000XT (Qstarz International Co. Ltd, Taipei, Taiwan) GPS data logger and Actigraph GT3X+ accelerometer (Actigraph Inc. Pensacola, FL) attached to a belt worn around the waist for one week. They also completed questionnaires and provided a buccal sample, with 70 complete pairs provided buccal samples. The buccal cell collection procedure involved 30 s brushing of each cheek before putting in enclosed vial for shipment by mail and then stored at − 80 °C. Materials were sent to the Skinner lab at WSU Pullman after sample and data collection was completed. Buccal swab brushes were stored at − 80 °C until use. The study protocol was approved by the Washington State University Institutional Review Board (#16419), and informed written consent was obtained from all participants prior to receiving the study materials. All methods were carried out in accordance with relevant guidelines and regulations.

DNA preparation. Frozen human buccal samples were thawed for analysis. Genomic DNA from buccal samples was prepared as follows: The buccal brush was suspended in 750 µl of cell lysis solution and 3.5 µl of Proteinase K (20 mg/ml). Th s suspension was incubated at 55 °C for 3 h, then vortexed and centrifuged briefly. The lysis solution was then transferred to a new 1.5 µl microcentrifuge tube. The microcentrifuge tube with the buccal brush was centrifuged again to retain any remaining solution which was combined with the transferred lysis solution. The buccal brush was discarded and 300 µl of protein precipitation solution (Promega, A795A, Madison, WI) was added to the lysis solution. The sample was incubated on ice for 15 min, then centrifuged at 4 °C for 30 min. The supernatant was transferred to a fresh 2 ml microcentrifuge tube and 1000 µl ice cold isopropanol was added along with 2 µl glycoblue. Th s suspension was mixed thoroughly and incubated at − 20 °C overnight. The suspension was then centrifuged at 4 °C for 20 min, the supernatant was discarded, and the pellet was washed with 75% ethanol, then air-dried and resuspended in 100 µl H2O. DNA concentration was measured using the Nanodrop (Thermo Fisher, Waltham, MA).

Methylated DNA Immunoprecipitation (MeDIP). Methylated DNA Immunoprecipitation (MeDIP) with genomic DNA was performed as follows: individual DNA samples (2–4 µg of total DNA) were diluted to 130 µl with 1 × Tris–EDTA (TE, 10 mM Tris, 1 mM EDTA) and sonicated with the Covaris M220 using the 300 bp setting. Fragment size was verifi d on a 2% E-gel agarose gel. The sonicated DNA was transferred from the Covaris tube to a 1.7 ml microfuge tube, and the volume was measured. The sonicated DNA was then diluted with TE buffer (10 mM Tris HCl, pH7.5; 1 mM EDTA) to 400 µl, heat-denatured for 10 min at 95 °C, then immediately cooled on ice for 10 min. Then 100 µl of 5X IP buffer and 5 µg of antibody (monoclonal mouse anti 5-methyl cytidine; Diagenode #C15200006) were added to the denatured sonicated DNA. The DNA-antibody mixture was incubated overnight on a rotator at 4 °C. The following day magnetic beads (Dynabeads M-280

Sheep anti-Mouse IgG; 11201D) were pre-washed as follows: The beads were resuspended in the vial, then the appropriate volume (50 µl per sample) was transferred to a microfuge tube. The same volume of Washing Buffer (at least 1 mL 1XPBS with 0.1% BSA and 2 mM EDTA) was added and the bead sample was resuspended. The tube was then placed into a magnetic rack for 1–2 min and the supernatant was discarded. The tube was removed from the magnetic rack and the beads were washed once. The washed beads were resuspended in the same volume of 1xIP buffer (50 mM sodium phosphate ph7.0, 700 mM NaCl, 0.25% TritonX-100) as the initial volume of beads. 50 µl of beads were added to the 500 µl of DNA-antibody mixture from the overnight incubation, then incubated for 2 h on a rotator at 4 C. After the incubation, the bead-antibody-DNA complex was washed three times with 1X IP buffer as follows: The tube was placed into a magnetic rack for 1–2 min and the supernatant was discarded, then the magnetic bead antibody pellet was washed with 1xIP buffer 3 times. The washed bead antibody DNA pellet was then resuspended in 250 µl digestion buffer with 3.5 µl Proteinase K (20 mg/ml). The sample was incubated for 2–3 h on a rotator at 55 °C, then 250 µl of buffered Phenol–Chloroform–Isoamylalcohol solution was added to the sample, and the tube was vortexed for 30 s and then centrifuged at 14,000 rpm for 5 min at room temperature. The aqueous supernatant was carefully removed and transferred to a fresh microfuge tube. Then 250 µl chloroform were added to the supernatant from the previous step, vortexed for 30 s and centrifuged at 14,000 rpm for 5 min at room temperature. The aqueous supernatant was removed and transferred to a fresh microfuge tube. To the supernatant 2 µl of glycoblue (20 mg/ml), 20 µl of 5 M NaCl and 500 µl ethanol were added and mixed well, then precipitated in -20 C freezer for 1 h to overnight. The precipitate was centrifuged at 14,000 rpm for 20 min at 4 C and the supernatant was removed, while not disturbing the pellet. The pellet was washed with 500 µl cold 70% ethanol in – 20 °C freezer for 15 min then centrifuged again at 14,000 rpm for 5 min at 4 °C and the supernatant was discarded. The tube was spun again briefly to collect residual ethanol to the bottom of the tube and as much liquid as possible was removed with gel loading tip. The pellet was air-dried at RT until it looked dry (about 5 min) then resuspended in 20 µl TE. DNA concentration was measured in Qubit (Life Technologies) with ssDNA kit (Molecular Probes Q10212).

MeDIP-Seq analysis. The MeDIP DNA samples (50 ng of each) were used to create libraries for next generation sequencing (NGS) using the NEBNext Ultra RNA Library Prep Kit for Illumina (San Diego, CA) starting at step 1.4 of the manufacturer's protocol to generate double stranded DNA. After this step the manufacturer's protocol was followed. Each sample received a separate index primer. NGS was performed at WSU Spokane Genomics Core using the Illumina HiSeq 2500 with a PE50 application, with a read size of approximately 50 bp and approximately 10–35 million reads per sample, and 6–10 sample libraries each were run in one lane.

Molecular bioinformatics and statistics. Basic read quality was verifi d using information produced by the FastQC program[37]. Reads were filtered and trimmed to remove low quality base pairs using Trimmomatic[38]. The reads for each sample were mapped to the GRCh38 human genome using Bowtie2[39] with default parameter options. The mapped read files were then converted to sorted BAM files using SAMtools[40]. Samples with an overall mapping less than 70% were removed from the DMR analysis along the corresponding twin samples. To identify DMR, the reference genome was broken into 1000 bp windows. The MEDIPS R package[41] was used to calculate differential coverage between control and exposure sample groups. The edgeR p value[42] was used to determine the relative difference between the two groups for each genomic window. Windows with an edgeR p value less than 10^{-4} were considered DMRs. The DMR edges were extended until no genomic window with an edgeR p value less than 0.1 remained within 1000 bp of the DMR. False discovery rate (FDR) analysis demonstrated with the male PA 255 DMRs with an FDR < 0.1 (55%), 39 female BMI DMRs an FDR < 0.1 (14%), and with the other DMR comparisons being primarily FDR > 0.1. CpG density and other information was then calculated for the DMR based on the reference genome. DMR were annotated using the NCBI provided annotations. The genes that overlapped with DMR were then input into the KEGG pathway search[43,44] to identify associated pathways. The DMR associated genes were then sorted into functional groups by reducing Panther[45] protein classifi ations into more general categories. All MeDIP-Seq genomic data obtained in the current study have been deposited in the NCBI public GEO database (GEO #: GSE216387).

Weighted genome coexpression network analysis (WGCNA). The weighted genome coexpression network analysis (WGCNA)[46] was performed using the WGCNA R package[47]. All samples were considered independent for the WGCNA analyses, so twin pair correlations were not considered and were ignored. Therefore, the independent twin epigenetic information was considered in the correlations and statistics observed. All MeDIP-Seq genomic windows were ranked by the mean RPKM read depth across all samples. The top 100,000 sites were chosen for inclusion in the analysis. The size of this subset was chosen to allow for a reasonable read depth to be considered and to limit computational time (< 1wk) requirements. WGCNA is a parameter rich analysis and only limited exploration of parameter variations was performed. Modules were calculated using the *blockwiseModules* function with the following parameters: maxBlockSize = 15,000, power = 6 (female), 9 (male), TOMType = "unsigned", minModuleSize = 30, reassignTh eshold = 0, and mergeCutHeight = 0.25. The Pearson correlation was calculated for each development stage and module. The p value for each correlation was calculated using the *corPvalueStudent* function. Sites within each module were annotated using the same methods as the DMRs.

Ethics. The study protocol was approved by the Washington State University Institutional Review Board (#16419), and informed written consent was obtained from all participants prior to receiving the study materials. All methods were carried out in accordance with relevant guidelines and regulations.

Data availability

All molecular data have been deposited into the public database at NCBI https://www.ncbi.nlm.nih.gov/geo/ (GEO # GSE216387), and R code computational tools are available at GitHub (https://github.com/skinnerlab/MeDIP-seq) and www.skinner.wsu.edu.

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Author contributions

G.E.D. conceived, funding acquisition, edited manuscript. A.A. patient sample acquisition, data analysis, edited manuscript. J.L.M.T. molecular analysis, data analysis, edited manuscript. E.N. sample processing, data analysis, edited manuscript. D.B. bioinformatics, data analysis, edited manuscript. M.K.S. conceived, data analysis, funding acquisition, wrote and edited manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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nature neuroscience

Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse

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Maternal care influences hypothalamic-pituitary-adrenal (HPA) function in the rat through epigenetic programming of glucocorticoid receptor expression. In humans, childhood abuse alters HPA stress responses and increases the risk of suicide. We examined epigenetic differences in a neuron-specific glucocorticoid receptor (*NR3C1*) promoter between postmortem hippocampus obtained from suicide victims with a history of childhood abuse and those from either suicide victims with no childhood abuse or controls. We found decreased levels of glucocorticoid receptor mRNA, as well as mRNA transcripts bearing the glucocorticoid receptor 1_F splice variant and increased cytosine methylation of an *NR3C1* promoter. Patch-methylated *NR3C1* promoter constructs that mimicked the methylation state in samples from abused suicide victims showed decreased NGFI-A transcription factor binding and NGFI-A–inducible gene transcription. These findings translate previous results from rat to humans and suggest a common effect of parental care on the epigenetic regulation of hippocampal glucocorticoid receptor expression.

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There are maternal effects on the development of individual differences in behavioral and HPA stress responses in rodents and nonhuman primates[1,2]. Maternal behavior alters the development of HPA responses to stress in the rat through tissue-specific effects on gene transcription[3,4], including forebrain glucocorticoid receptor expression, the activation of which inhibits HPA activity through negative-feedback inhibition[5]. Thus, selective knockdown of glucocorticoid receptor expression in the corticolimbic system in rodents is associated with increased HPA activity under both basal and stressful conditions[6,7]. Conversely, glucocorticoid receptor overexpression is associated with a dampened HPA stress response[8].

Familial function and childhood adversity are linked to altered HPA stress responses in humans, which are associated with an increased risk for multiple forms of psychopathology[9–11]. There is evidence for decreased hippocampal glucocorticoid receptor expression in several psychopathological conditions associated with suicide, including schizophrenia and mood disorders[12–14]. Suicide is also strongly associated with a history of childhood abuse and neglect, and this effect is independent of that associated with psychopathology[15,16]. Thus, environmental events that associate with decreased hippocampal glucocorticoid receptor expression and increased HPA activity enhance the risk of suicide.

The effects of maternal care on hippocampal glucocorticoid receptor expression, and therefore HPA responses to stress, in the adult rodent are associated with an epigenetic modification of a neuron-specific exon 1_7 glucocorticoid receptor (*Nr3c1*) promoter[4,17]. We attempted to translate these findings to humans. We examined glucocorticoid receptor expression and *NR3C1* promoter methylation in hippocampal samples obtained from suicide victims and control subjects who died suddenly of unrelated causes. The focus of our examination was the *NR3C1* (also known as $GR1_F$) promoter, the homolog of the exon 1_7 region in the rat[18], which is highly expressed in human hippocampus[19]. Suicide victims were either positive or negative for history of childhood abuse (sexual contact, severe physical abuse and/or severe neglect), allowing for the separation of the effects associated with childhood abuse from those associated with suicide *per se*. Our controls were all negative for a history of childhood abuse.

RESULTS

Hippocampal glucocorticoid receptor expression

The human glucocorticoid receptor gene *NR3C1* covers a region of more than 80 kb in chromosome 5, containing eight coding exons (exons 2–9) and alternative 5′ noncoding exon 1s[19–21]. The 5′ untranslated region (UTR) of exon 1 of the *NR3C1* gene determines the tissue-specific expression. The 5′ UTR of *NR3C1* contains 11 exon 1 splice variants, all of which bear unique splice donor sites and share a common exon 2 splice acceptor site[19]. Exon 1_F of *NR3C1* is similar to the rat exon 1_7, which reveals a maternal effect on cytosine methylation and expression[8,18,22]. Because individuals with severe forms of major depression show decreased glucocorticoid receptor

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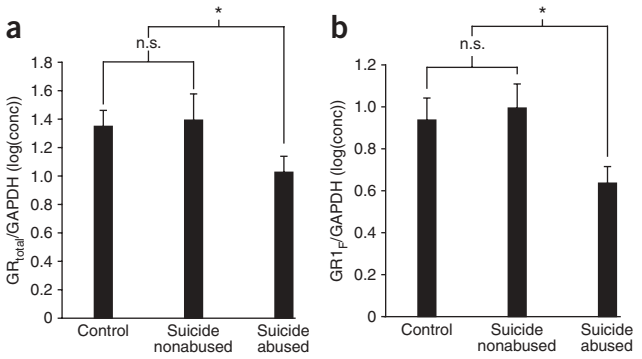

Figure 1 Hippocampal glucocorticoid receptor expression. (**a**,**b**) Mean ± s.e.m. expression levels of total glucocorticoid receptor (GR) mRNA (**a**) and glucocorticoid receptor 1_F (GR1_F) in 12 suicide victims with a history of childhood abuse, 12 nonabused suicide victims and 12 control subjects (**b**). Outliers excluded from analysis included $n = 2$ control subjects, $n = 1$ suicide victims with a history of childhood abuse for glucocorticoid receptor 1_F and an additional $n = 1$ suicide victim with a history of childhood abuse, and $n = 3$ nonabused suicide victims for overall levels of glucocorticoid receptor. * indicates $P < 0.05$; n.s. indicates not statistically significant.

expression and increased HPA activity, we hypothesized that suicide victims would show decreased expression both of glucocorticoid receptor and glucocorticoid receptor 1_F compared with control subjects.

We examined the expression of total glucocorticoid receptor and glucocorticoid receptor 1_F using quantitative reverse transcription PCR (qRT-PCR) with RNA extracted from hippocampal tissue of suicide completers with ($n = 12$) and without ($n = 12$) a history of childhood abuse and from controls ($n = 12$). There was a significant effect on glucocorticoid receptor expression ($F = 3.17$, $P = 0.05$). *Post hoc* tests showed that expression of total glucocorticoid receptor mRNA was significantly reduced in suicide victims with a history of childhood abuse relative to nonabused suicide victims or controls ($P < 0.05$); there was no difference between nonabused suicide victims and controls ($P > 0.05$; **Fig. 1a**). There was also a significant effect on the expression of transcripts containing the exon 1_F *NR3C1* promoter ($F = 3.58$, $P < 0.05$). *Post hoc* tests revealed that glucocorticoid receptor 1_F expression was significantly lower in samples from suicide victims with a history of childhood abuse compared with suicide victims without childhood abuse or controls ($P < 0.05$). Similar to the findings with total glucocorticoid receptor mRNA expression, there was no difference between nonabused suicide victims and controls ($P > 0.05$; **Fig. 1b**).

We examined the relationship between glucocorticoid receptor expression and psychiatric diagnoses (**Table 1**). Mood disorders and substance abuse disorders are risk factors for suicide and have been linked to alterations of glucocorticoid receptor expression[12]. There were no significant effects of psychopathology, even after controlling for childhood abuse status, on overall glucocorticoid receptor or glucocorticoid receptor 1_F expression ($P > 0.05$).

Genotyping and methylation analysis

Because alterations in glucocorticoid receptor 1_F activity could be derived from nucleotide sequence variation and/or epigenetic modifications, we sequenced the *NR3C1* promoter region from each subject. No sequence variation was seen among subjects and all of the sequences were identical to those published previously[19]. Moreover, for each subject, the genomic sequences targeted for binding by the primers used for bisulfite mapping were identical to the published sequence[19], thus eliminating potential primer bias between subjects in sodium bisulfite mapping.

The rat homolog of the exon 1_F *NR3C1* promoter, the exon 1_7 region, is differentially methylated as a function of variations in maternal care[4,17,22]. Cytosine methylation is a highly stable epigenetic mark that regulates gene expression via its effects on transcription factor binding[23,24]. We used sodium bisulfite mapping[25] to examine the methylation status of individual CpG dinucleotides in the *NR3C1* promoter sequence extracted from the hippocampal tissue of the same subjects used for glucocorticoid receptor expression analysis. Sodium bisulfite mapping revealed a significant effect on the percentage of methylated clones (that is, the number of clones with at least one methylated CpG site divided by the total number of clones) between groups ($F = 3.47$, $P < 0.05$). *Post hoc* tests revealed a significant difference between suicide victims with a history of childhood abuse compared with nonabused suicide victims ($P = 0.05$) or controls ($P < 0.05$). There was no difference in the percentage of methylated clones between suicide victims without childhood abuse and controls ($P > 0.05$; **Fig. 2a**). Methylation was limited to specific sites, with no clone showing global methylation (**Fig. 2b**). There were no significant correlations between levels of exon 1_F methylation and age at death ($r = 0.15$, $P > 0.05$), brain pH ($r = 0.08$, $P > 0.05$) or postmortem interval (PMI, $r = 0.24$, $P > 0.05$; **Table 1**).

Patch methylation of the *NR3C1* promoter

DNA methylation of a limited number of sites in the exon 1_F *NR3C1* promoter was associated with decreased expression of the glucocorticoid receptor 1_F variant and of total glucocorticoid receptor mRNA in suicide victims with a history of childhood abuse. Defining a causal relation between the methylation status and transcriptional efficiency of the *NR3C1* promoter is therefore of great importance. We hypothesized that DNA methylation regulates the expression of the *NR3C1* promoter through alterations in transcription factor binding. The transcription factor NGFI-A regulates the expression of *Nr3c1* promoter in the rat, an effect that is inhibited by DNA methylation[17]. To our knowledge, the regulation of NGFI-A (also known as Zif268, EGR1, Krox-24 and ZENK) has not been studied in the human hippocampus, although there is evidence that its expression is downregulated in the prefrontal cortex in schizophrenia[26]. The *NR3C1* promoter contains a number of canonical and noncanonical NGFI-A recognition elements (**Fig. 3a**). We wondered whether, as in the rat[17], NGFI-A could regulate gene transcription through the *NR3C1* promoter and whether this effect might be influenced by the methylation status of the promoter. We used a transient transfection assay in human HEK293 cells to examine transcriptional activity of a *NR3C1* promoter ligated to a promoter-less firefly luciferase expression vector (pGEM-LUC, Promega; **Fig. 3a**) in the presence or absence of ectopic NGFI-A

Table 1 Demographic characteristics and psychiatric diagnoses

	Abused suicide	Nonabused suicide	Control
Male/female	12/0	12/0	12/0
Age (years)	34.2 ± 10	33.8 ± 11	35.8 ± 12
PMI (h)	24.6 ± 5.8	39.0 ± 25.7	23.5 ± 6.0
pH	6.3 ± 0.24	6.5 ± 0.29	6.5 ± 0.22
Childhood abuse/neglect	12/0 (100%)	0/12 (0%)	0/12 (0%)
Mood disorder	8/12 (67%)	8/12 (67%)	0/12 (0%)
Alcohol/drug abuse disorder	9/12 (75%)	6/12 (50%)	5/12 (42%)

Data are presented as mean ± s.d.

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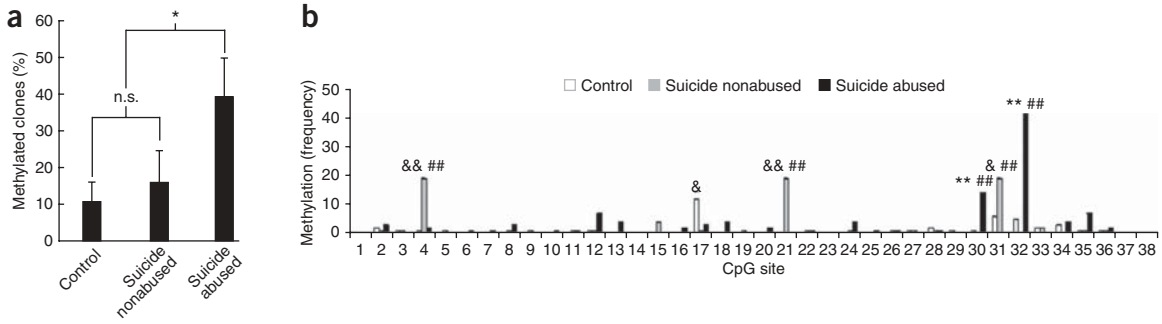

Figure 2 Methylation of the *NR3C1* promoter in the hippocampus. Twenty clones were sequenced for each subject for methylation mapping. (**a**) Mean ± s.e.m. percentage of methylated clones for suicide victims with a history of childhood abuse ($n = 12$), suicide victims without a history of childhood abuse ($n = 12$) and controls ($n = 12$). The methylation percentage was calculated as the number of clones with at least one methylated CpG site divided by the total number of clones (* indicates $P \leq 0.05$; n.s. indicates not statistically significant). (**b**) Methylation of the *NR3C1* promoter region, showing the frequency of methylation observed at each CpG site for suicide victims with a history of childhood abuse, suicide victims with no history of childhood abuse and control subjects ($^{*}P < 0.05$, $^{**}P < 0.001$, abused suicides versus controls; $^{\&}P < 0.05$, $^{\&\&}P < 0.001$, non-abused suicides versus controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, abused suicides versus non-abused suicides; Bonferroni *post hoc* comparisons).

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expression. The use of HEK293 cells allowed us to concurrently transfect a number of expression vectors with high efficiency. The absence of plasmid replication during the transient transfection assay precludes the loss of methylation via passive demethylation[27].

Luciferase expression was measured in the presence or absence of NGFI-A from the unmethylated *NR3C1* promoter plasmid compared with a methylated version. There was a significant effect of testing condition on the transcriptional activity of the exon 1_F *NR3C1* promoter ($F = 110.6$, $P < 0.0001$; **Fig. 3b**). *Post hoc* analysis revealed that the transcriptional activity of the unmethylated *NR3C1* promoter was significantly increased in the presence of the NGFI-A expression vector (*NR3C1*versus *NR3C1* + *EGR1*; $P < 0.0001$). Furthermore, methylation of the *NR3C1* promoter (the entire *NR3C1* construct was methylated *in vitro* and ligated to an unmethylated vector before transfection, *NR3C1-M*) reduced basal transcriptional activity of the *NR3C1* construct (*NR3C1* versus *NR3C1-M*, $P < 0.05$). Methylation of the *NR3C1* construct also blunted NGFI-A induction of transcription (*NR3C1* + *EGR1* versus *NR3C1-M* + *EGR1*, $P < 0.0001$).

These results indicate that methylation attenuates NGFI-A induction of gene expression through the *NR3C1* promoter. However, the decreased glucocorticoid receptor transcription observed in suicide victims with a history of childhood abuse was associated with differences in methylation levels occurring only at specific sites in the exon 1_F *NR3C1* promoter (**Fig. 2b**). An ANOVA examining the methylation of CpG dinucleotides across the exon 1_F *NR3C1* promoter revealed a significant effect of CpG site ($F = 13.86$, $P < 0.0001$), a significant effect of group ($F = 17.12$, $P < 0.0001$) and a significant interaction between CpG site and group ($F = 13.44$, $P < 0.0001$). In NGFI-A recognition elements, methylation was observed at CpG sites 12, 13, 30, 31 and 32 (**Fig. 2b**). We therefore examined whether such selective, site-specific differences in methylation could alter transcriptional activation through the *NR3C1* promoter. Two deletion constructs of the *NR3C1* promoter were generated in which selected CpG dinucleotides were patch-methylated (**Fig. 3a**). CpG sites 12 and 13 were methylated in the 255-bp construct, whereas the 125-bp promoter construct was methylated at CpG sites 30, 31 and 32. Thus, each deletion construct included at least one known or putative NGFI-A binding site[28].

We used patch methylation to examine whether selective methylation at specific sites reduces NGFI-A binding to and transactivation through the exon 1_F *NR3C1* promoter. We found an effect of methylation status on transcription factor–induced gene expression from the *NR3C1* promoter (**Fig. 3c**). For the 125-bp construct, there was a significant effect of methylation status ($F = 57.6$, $P < 0.0001$) and

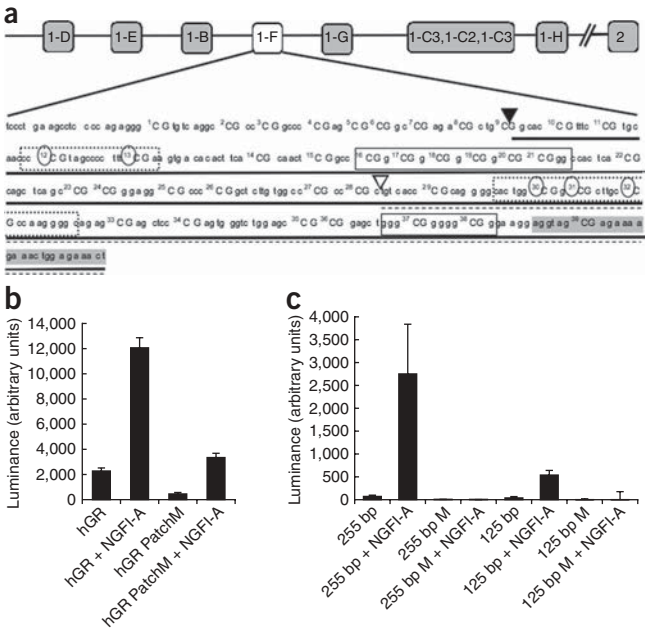

Figure 3 *In vitro* analysis of *NR3C1* promoter methylation. (**a**) The relative position of the *NR3C1* variant and the promoter sequence, showing the location of the CpG dinucleotides. The 255-bp (▼, solid underline) and 125-bp (▽, broken underline) deletion constructs are shown, along with specific CpG dinucleotides that were methylated in each deletion construct, as indicated by circles. Boxes represent known or putative canonical (solid-lined box) and noncanonical (broken-lined box) NGFI-A–binding sites, with the shaded area indicating the beginning of the exon. (**b**,**c**) Mean ± s.e.m. levels of luciferase expression in HEK293 cells. Results are shown after the subtraction of expression of the promoter in the antisense orientation. (**b**) The full *NR3C1* promoter was either unmethylated (hGR) or completely patch methylated (hGR PatchM) and transfected in the presence or absence of NGFI-A. (**c**) The 255-bp and 125-bp *NR3C1* deletion construct were either unmethylated (255 bp or 125 bp) or methylated (255 bp M or 125 bp M), as shown in **a**, and transfected either in the presence or absence of NGFI-A.

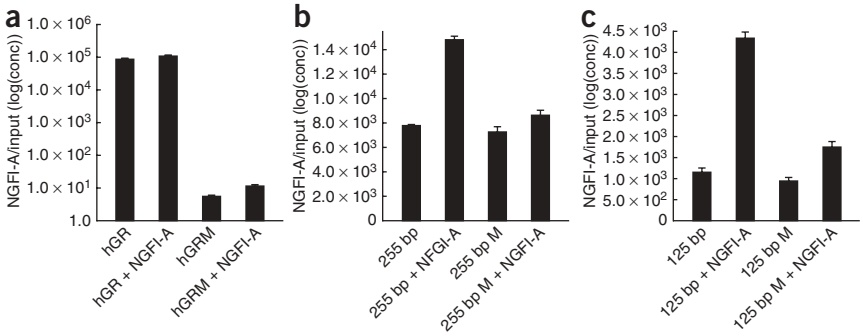

Figure 4 NGFI-A association with exon 1_F *NR3C1* promoter constructs. (**a**–**c**) Quantification of *NR3C1* promoter immunoprecipitated with NGFI-A antibody and normalized to input DNA for the full human *NR3C1* promoter (**a**), the 255-bp deletion construct (**b**) and the 125-bp deletion construct (**c**), each of which was either unmethylated or methylated and transfected in the presence or absence of NGFI-A. Data are presented as means ± s.e.m.

NGFI-A treatment ($F = 6.3$, $P < 0.05$). As predicted, there was also a significant interaction between methylation status and the NGFI-A expression ($F = 48.7$, $P < 0.0001$). *Post hoc* analysis of the 125-bp *NR3C1* promoter construct revealed that the effect of NGFI-A on gene transcription was significantly ($P < 0.001$) greater in the presence of the unmethylated rather than the patch-methylated *NR3C1* promoter construct. The same pattern of results was observed for the 255-bp *NR3C1* promoter construct. Thus, for the 255-bp *NR3C1* promoter construct, there was a significant effect on transcriptional activity of methylation status ($F = 555.4$, $P < 0.0001$) and NGFI-A expression ($F = 387.3$, $P < 0.0001$). There was also a significant interaction between methylation status and the presence of NGFI-A ($F = 489.1$, $P < 0.0001$). *Post hoc* analysis revealed a significantly ($P < 0.001$) greater effect of NGFI-A on gene transcription in the presence of the unmethylated compared with the patch-methylated 255-bp *NR3C1* promoter construct.

NGFI-A association with the *NR3C1* promoter

Site-selective differences in methylation of CpG regions in promoters can alter gene transcription by affecting transcription factor binding[24]. We carried out chromatin immunoprecipitation assays on samples that were obtained using the transfection model described above to examine the association of NGFI-A with the methylated and the nonmethylated *NR3C1* promoter constructs. The results revealed a significant effect of methylation status on NGFI-A association with the exon 1_F *NR3C1* promoter constructs transfected with *EGR1* ($F = 242.92$, $P < 0.0001$; **Fig. 4a**). *Post hoc* tests showed that, in comparison with the nonmethylated construct, patch-methylation inhibited NGFI-A binding to the exon 1_F *NR3C1* promoter ($P < 0.005$; **Fig. 4a**). For the 125-bp promoter construct, there was a significant effect of methylation status ($F = 102.28$, $P < 0.001$) and NGFI-A treatment ($F = 209.99$, $P < 0.0005$) and a significant interaction between methylation status and the presence of NGFI-A ($F = 72.71$, $P < 0.005$). Similarly, for the 255-bp promoter construct, there was a significant effect of methylation status ($F = 95.18$, $P < 0.001$) and NGFI-A treatment ($F = 152.13$, $P < 0.0005$) and a significant interaction between methylation status and the presence of NGFI-A ($F = 67.75$, $P < 0.005$). *Post hoc* testing revealed that, in comparison with the nonmethylated version, patch-methylation inhibited NGFI-A binding to either the 255-bp or 125-bp promoter constructs ($P < 0.01$; **Fig. 4b**,**c**). These findings suggest that the site-selective methylation of the exon 1_F *NR3C1* promoter construct, mimicking the differences observed between samples obtained from suicide victims with a history of childhood abuse, reduces NGFI-A binding and transcriptional activation through the exon 1_F *NR3C1* promoter.

DISCUSSION

Our findings indicate that hippocampal *NR3C1* gene expression is decreased in samples from suicide victims with a history of childhood abuse compared with controls (victims of sudden, accidental death with no history of abuse). In contrast, we found no differences in glucocorticoid receptor expression between suicide victims without a history of childhood abuse and controls. The pattern of results for hippocampal expression of the glucocorticoid receptor 1_F variant was identical to that of total glucocorticoid receptor expression. Our findings suggest that changes in glucocorticoid receptor expression are closely associated with a developmental history of familial adversity, in this case a history of childhood abuse, than with suicide completion. These results are also similar to those of earlier reports in which childhood abuse was associated with an increase in pituitary adrenocorticotropic hormone (ACTH) responses to stress among individuals with or without concurrent major depression[11]. These findings are particularly relevant, as pituitary ACTH directly reflects central activation of the HPA stress response and hippocampal glucocorticoid receptor activation dampens HPA activity.

Our findings are also consistent with those from studies with rodent and nonhuman primates showing that persistent disruptions of mother-infant interactions are associated with increased hypothalamic corticotrophin-releasing hormone expression and increased HPA responses to stress[1,2,29]. Variations in maternal care in the rat influence hippocampal glucocorticoid receptor expression, as well as methylation of the rat fetal calf serum *Nr3c1* promoter, the homolog of the human exon 1_F *NR3C1* promoter[3,4,17,22]. Hippocampal samples from suicide victims showed increased methylation of the exon 1_F *NR3C1* promoter in comparison with samples from controls, but only in cases with a history of childhood abuse. Neither hippocampal glucocorticoid receptor expression nor the methylation status of the exon 1_F *NR3C1* promoter was altered in suicide victims with no history of abuse. These findings suggest that variation in the methylation status of the exon 1_F *NR3C1* promoter, similar to that for glucocorticoid receptor 1_F and total glucocorticoid receptor mRNA expression, associates with childhood adversity and not with suicide *per se*.

A recent study of human cord blood found a correlation between maternal mood and neonatal methylation status of glucocorticoid receptor 1_F[30]. This study reported that increased site-specific methylation of an NGFI-A response element of glucocorticoid receptor 1_F is linked to an enhanced cortisol stress response in infants. Maternal mood disorders are associated with decreased maternal sensitivity and impaired mother-infant interactions[31], as well as with an increased risk for depression in the offspring[32]. Decreased hippocampal glucocorticoid receptor expression associates with depression[12], and psychotic and severe forms of depression are commonly associated with increased HPA activity[13,33]. Thus, our findings suggest that the transmission of vulnerability for depression from parent to offspring could occur, in part, through the epigenetic modification of genomic regions that are implicated in the regulation of stress responses.

One limitation of our design is the absence of samples from control subjects with a history of child abuse. Notably, child abuse predicts ACTH responses to stress[11]. However, the best predictor of HPA

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responses to stress is the interaction between a developmental history of child abuse and stress in adulthood. One interpretation for such findings is that childhood adversity might alter the development of systems that serve to regulate stress responses, such as hippocampal glucocorticoid receptor expression, and thus enhance the effect of stress in adulthood and vulnerability for mood disorders[11]. Rodent models provide evidence for a direct effect of variations in forebrain glucocorticoid receptor expression and the behavioral characteristics of depression. Mice bearing a brain-specific glucocorticoid receptor knockdown show behavioral alterations that mimic some of the features of depression[6,7,34].

The data reported here are consistent with previous reports of alterations in cytosine methylation associated with psychopathology[35–37]. We found increased site-specific methylation of the exon 1_F *NR3C1* promoter in suicide victims with a history of childhood abuse (**Fig. 2**). Our transfection studies with constructs that replicated the *in vivo* methylation profiles indicated that there was a relationship between cytosine methylation, transcription factor binding and gene expression. Variations in maternal care in the rat produce differential methylation of the exon 1_7 *Nr3c1* promoter, the rat homolog of the exon 1_F *NR3C1* promoter, which regulates hippocampal glucocorticoid receptor expression[4,17,22] and HPA responses to stress[3,4]. Increased CpG methylation of the *Nr3c1* promoter decreased NGFI-A binding and reduced hippocampal glucocorticoid receptor expression. Manipulations that reversed the differences in exon 1_7 methylation eliminated the maternal effect on NGFI-A binding, glucocorticoid receptor expression and HPA activity[4,22]. Likewise, our *in vitro* data reveal that differential methylation of the human *NR3C1* promoter altered NGFI-A binding and NGFI-A–induced gene transcription. These findings suggest that selective differences in methylation status at certain sites affect transcription factor binding and gene expression.

Because cytosine methylation is a highly stable, the differences in CpG methylation are unlikely to be a consequence of events immediately preceding death or during the postmortem period. Therefore, changes in brain pH do not affect DNA methylation[38]. The PMI did not differ between the groups and was uncorrelated with the methylation of the *NR3C1* promoter. The intermittent pattern of methylation and the fact that only a portion of the neuronal population was methylated in each subject is consistent with a model in which alterations in methylation occur at later stages in development, after the completion of embryogenesis and neuronal differentiation. Indeed, the maternal effect on the methylation status of the exon 1_7 *Nr3c1* promoter in the rat occurs during early postnatal life[4].

There are precedents for the apparent developmental origins for the observed differences in DNA methylation and glucocorticoid receptor expression. Childhood abuse in humans is associated with altered hippocampal development[39], enhanced HPA activity[9,11] and an increased risk for psychopathology[15,16]. Similarly, children exposed to childhood adversity are more likely to engage in suicidal behavior[40,41]. Variations in the parental care of children are linked with individual differences in HPA and sympathetic and central catecholamine responses to stress[1,11,42,43]. Interventions that target parental care of high-risk children alter HPA activity[44]. Thus, it is tempting to speculate that epigenetic processes might mediate the effects of the social environment during childhood on hippocampal gene expression and that stable epigenetic marks such as DNA methylation might then persist into adulthood and influence the vulnerability for psychopathology through effects on intermediate levels of function, such as HPA activity.

In summary, our data reveal increased site-specific methylation in the exon 1_F *NR3C1* promoter in suicide victims with a history of childhood abuse and suggest that there is a relationship between cytosine methylation, transcription factor binding and gene expression. Our results are consistent with evidence from studies using psychological autopsy methods[40] and epidemiological longitudinal designs[45], which suggest that suicide has a developmental origin. We acknowledge that such conclusions are based on samples that differ along a wide range of experiential and potentially genetic dimensions. Our data certainly do not exclude alternative mechanisms of vulnerability. Indeed, the challenge for the future is to understand how epigenetic variation overlaying that occurring in nucleotide sequence might explain the developmental origins of vulnerability for chronic illness. Our data are merely consistent with observations from animal studies investigating epigenetic regulation of the *Nr3c1* gene and with the hypothesis that early life events can alter the epigenetic state of relevant genomic regions, the expression of which may contribute to individual differences in the risk for psychopathology.

METHODS

Postmortem sample preparation and subject characteristics. We used hippocampal samples from the Quebec Suicide Brain Bank (http://www.douglasrecherche.qc.ca/suicide), which included 12 suicide victims with a history of childhood abuse, 12 suicide victims with a negative history of childhood abuse (matched for psychiatric diagnoses) and 12 controls. All subjects were matched for PMI, gender and age (**Table 1**). Samples were processed as described previously[46] and consisted of hippocampus tissue from male suicides and control subjects of French-Canadian origin, dissected at 4 °C and stored at –80 °C. All subjects died suddenly with no medical or paramedical intervention. Suicides were determined by the Quebec Coroner's Office and the control subjects were individuals who had died suddenly from causes other than suicide. Homogenates of tissue samples were used for genomic DNA (DNeasy, Qiagen) and RNA (Trizol, Invitrogen) extraction. Possible confounds that were examined included PMI, brain pH and the age of the donor at death. Samples were processed and analyzed blindly with respect to demographic and diagnostic variables. Signed informed consent was obtained from next of kin.

Psychological autopsies. Psychiatric diagnoses were obtained using Structured Clinical Interviews for DSM-III-R I (ref. 47) adapted for psychological autopsies, which is a validated method for reconstructing psychiatric history by means of extensive proxy-based interviews, as described elsewhere[48]. History of severe childhood sexual and/or physical abuse or severe neglect was determined by means of structured interviews using the Childhood Experience of Care and Abuse[49] questionnaire adapted for psychological autopsies[45].

Genotyping. Genomic DNA was extracted (DNeasy, Qiagen) according to the manufacturer's protocol. For PCR, we used a 5′-GGG TTC TGC TTT GCA ACT TC-3′ sense primer and a 5′-CCT TTT TCC TGG GGA GTT G-3′ antisense primer that were directed to the *NR3C1* promoter (Genebank accession number AY436590). Primers were selected that covered a 536-bp region that included the region for sodium bisulfite analyses. The resulting PCR products for each subject were sequenced bidirectionally using the forward and the reverse primer on an ABI 3100 genetic analyzer (Applied Biosystems) following the manufacturer's instructions. Genetic variation was assessed throughout the *NR3C1* promoter region used for bisulfite analysis by alignment of genomic DNA with the previously published *NR3C1* promoter sequence[19] using freely available software (CLC Workbench, CLC bio).

Methylation mapping and expression analyses. Sodium bisulfite mapping was performed as previously described[25,50] for 12 suicide victims with a history of childhood abuse, 12 suicide victims with a negative history of childhood abuse and 12 controls. Individual clones were extracted and sequenced (Cequation (8800), Beckman-Coulter). We obtained 20 clones per subject for sequencing from 2–3 independent PCR reactions.

RNA extraction was performed using Trizol (Invitrogen) and was followed by Dnase I treatment, and cDNA conversion was performed using oligo(dT) primers (Invitrogen) according to manufacturers instructions (Roche Molecular Biochemicals). The same subjects used for methylation analysis were studied

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for expression analyses using quantitative RT-PCR. Outliers with expression values that differed more than 1.5 s.d. from the mean were excluded from analysis ($n = 2$ control subjects, $n = 1$ suicide victims with a history of childhood abuse for glucocorticoid receptor 1_F and $n = 2$ control subjects, $n = 2$ suicide victims with a history of childhood abuse and $n = 3$ nonabused suicide victims for overall levels of glucocorticoid receptor; see **Supplementary Methods** online).

HEK293 cell cultures and transient transfection assays. To prepare and transfect (and see ref. 17) the unmethylated and methylated plasmids, we subjected the exon 1_F *NR3C1* promoter to PCR amplification and cloned the resulting PCR product into a PCR2.1 plasmid (Original TA cloning kit, Invitrogen). The *NR3C1* promoter was then ligated into the PCR2.1 plasmid. For patch methylation, the glucocorticoid receptor 1_F plasmid was incubated (2 h, 37 °C) with SssI DNA methyltransferase (20 U, New England Biolabs) in a buffer containing S-adenosylmethionine, and this procedure was repeated until full protection from *Hpa*II digestion was observed. Following digestion with *Hin*dIII and EcoRV restriction enzymes, each fragment was then ligated into a pGEM-luc vector (Promega) at the *Hin*dIII and *Bam*HI or XbaI and *Bam*HI sites in the 5′ to 3′ (sense) or 3′ to 5′ (antisense) orientation, respectively. The concentration of each ligation product was determined by fractionation on a 1.5% agarose gel, by comparing bands of the expected ligation product size against a standard curve of known DNA concentration (ten fivefold serial dilutions of 2 $\mu g/\mu l^{-1}$ micrococal nuclease DNA) to control for possible unequal efficiency of ligation and to ensure that equal amounts of correctly ligated plasmids were used in the transfections. The ligated plasmid was directly transfected into HEK293 cells and was not subcloned to avoid loss of methyl groups from CG dinucleotides during growth in *E. coli*, which do not express CG DNA methyltransferases. For deletion constructs of the exon 1_F *NR3C1* promoter plasmids were prepared and ligations verified in the same manner as described above, except that oligonucleotides for *NR3C1* promoter amplification were designed that incorporated *Hin*dIII and EcoRV restriction sites, obviating the need for ligation into PCR2.1 vector before ligation into the pGEM-luc vector (also see **Supplementary Methods**).

For the NGFI-A plasmid, we subcloned the *EGR1* coding sequence into a TOPO–His expression vector (pEF6/V5-His TOPO TA Expression kit, Invitrogen)[4]. In co-transfection studies, human embryonic kidney HEK293 cells were plated at a density of 6×10^4 in six-well dishes and transiently co-transfected with a total amount of 1.5 μg of plasmid DNA (1.0 μg of *NR3C1* promoter ligated into the pGEM-luc plasmid and 0.5 μg of NGFI-A expression plasmid or 0.5 μg of control pEF6/V5 plasmid) using the calcium phosphate method. HEK293 cells were maintained as a monolayer in DMEM (Invitrogen) containing 10% fetal calf serum (Colorado Serum Company). The cells were harvested 72 h after transfection and lysed, and luciferase activity was assayed using the Luciferase Assay System (Promega) according to the manufacturer's protocol.

Chromatin immunoprecipitation assay for NGFI-A. We carried out chromatin immunoprecipitation assays[30] by postfixing cells in 37% formaldehyde and then pelleted them before lysis and sonication. We reserved one tenth of the sample as 'input' to quantify the amount of DNA before immunoprecipitation. For the remainder of each sample, extracted chomatin was immunoprecipitated using rabbit polyclonal antibody to NGFI-A (antibody) or normal rabbit IgG (nonspecific; both from Santa Cruz Biotechnology). All of the DNA was then uncrosslinked and subjected to qRT-PCR, using primers directed at the luciferase gene immediately downstream of the transfected *NR3C1* promoter (sense, 5′-AGA GAT ACG CCC TGG TTC C-3′; antisense, 5′-CCA ACA CCG GCA TAA AGA A-3′; $T_m = 54$ °C). The signal for each sample was calculated by dividing the value of the antibody by the input. Each resulting value was multiplied by a constant (1×10^6) to plot the values obtained from the experiments on logarithmic axes.

Statistical analyses. Statistical analyses were conducted using Statview or JMP 7 (SAS Institute). Data are presented as mean ± s.e.m. For DNA methylation analysis, the percentage of methylated clones for each subject was tabulated by dividing the number of clones with at least one methylated CpG site by the total number of clones. A factorial ANOVA was used to compare the percentage of methylated clones for each subject as the dependent variable and group (suicide abused, suicide nonabused or control) as the between groups factor. To examine differential methylation across CpG sites methylated *in vitro*, we compared the total number of methylated CpG sites across the *NR3C1* promoter ($n = 33$) for all clones per group (that is, 12 subjects × 20 clones = 240 clones per group) using ANOVA followed by Bonferroni corrected *post hoc* comparisons. For RNA expression analysis, ANOVA followed by PLSD *post hoc* tests were used to examine differences between the suicide victim and control group. Unpaired *t* tests were used to compare groups of subjects with different clinical diagnoses (for example, subjects with mood disorders versus subjects without mood disorders). The relationships between DNA methylation, expression, age at death, PMI and brain pH were analyzed using linear regression analysis. Factorial ANOVA were used for *in vitro* analyses of *NR3C1* promoter, followed by *post hoc* analyses. Statistical significance was determined at $P \leq 0.05$ and analyses included type 1 error correction for multiple comparisons where applicable.

Note: Supplementary information is available on the Nature Neuroscience website.

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Folic Acid Supplementation during the Juvenile-Pubertal Period in Rats Modifies the Phenotype and Epigenotype Induced by Prenatal Nutrition[1–3]

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Abstract

Prenatal nutritional constraint is associated with increased risk of metabolic dysregulation in adulthood contingent on adult diet. In rats, folic acid supplementation of a protein-restricted (PR) diet during pregnancy prevents altered phenotype and epigenotype in the offspring induced by the PR diet. We hypothesized that increasing folic acid intake during the juvenile-pubertal (JP) period would reverse the effects of a maternal PR diet on the offspring. Rats were fed a control (C) or PR diet during pregnancy and AIN93G during lactation. Offspring were weaned on d 28 onto diets containing 1 mg [adequate folate (AF)] or 5 mg [folic acid-supplemented (FS)] folic acid/kg feed. After 28 d, all offspring were fed a high-fat (18% wt:wt) diet and killed on d 84. As expected, offspring of PR dams fed the AF diet had increased fasting plasma triglyceride (TAG) and β-hydroxybutyrate (βHB) concentrations. The FS diet induced increased weight gain, a lower plasma βHB concentration, and increased hepatic and plasma TAG concentration compared with AF offspring irrespective of maternal diet. PPARα and glucocorticoid receptor promoter methylation increased in liver and insulin receptor promoter methylation decreased in liver and adipose tissue in FS compared with AF offspring, with reciprocal changes in mRNA expression irrespective of maternal diet. These findings show that increased folic acid intake during the JP period did not simply reverse the phenotype induced by the maternal diet. This may represent a period of plasticity when specific nutrient intakes may alter the phenotype of the offspring through epigenetic changes in specific genes. J. Nutr. 139: 1054–1060, 2009.

Introduction

Environmental constraint during development, including undernutrition, is causally associated with increased risk of metabolic disease (1). It has been suggested that the developing fetus responds to environmental cues via developmental plasticity in a manner that sets the phenotype in prediction of the future environment, conferring a Darwinian fitness advantage (2). In humans, mismatch between the predicted and future environments has been suggested to underlie the early life origins of metabolic disease (2). Several animal models show that suboptimal nutrition during critical periods in early development induces metabolic dysregulation that resembles human cardiometabolic disease, especially if the offspring are fed a high-fat diet (3,4). In humans and in animal models, the nature of the phenotype induced by maternal dietary constraint differs according the developmental stage of the fetus at the time of exposure (5,6). Thus, the response of individual tissues to nutrient constraint is dependent upon the stage of maturation at the time of exposure.

Moderate maternal dietary protein restriction in rats is a well-established model of induction of an altered phenotype in the offspring (7). The offspring are characterized by persistent hypertension (7,8), dyslipidemia, and impaired glucose metabolism (4). Supplementation of the maternal protein-restricted (PR)[7] diet with folic acid or glycine prevents induction of

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[3] Supplemental Tables 1 and 2, and Supplemental Figure 1 are available with the online posting of this paper at jn.nutrition.org.

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[7] Abbreviations used: AF, folic acid-adequate; AOX, acyl-CoA carboxylase; βHB, β-hydroxybutyrate; C, control group; CPT-1, carnitine palmitoyl transferase-1; C/AF, control/adequate folate; C/FS, control/folate supplemented; Dnmt, DNA methyltransferase; FS, folic acid-supplemented; GR, glucocorticoid receptor; HSL, hormone-sensitive lipase; IR, insulin receptor; JP, juvenile-pubertal; LPL, lipoprotein lipase; NEFA, nonesterified fatty acid; PEPCK, phosphoenolpyruvate carboxykinase; pn, postnatal; PR, protein-restricted, PR/AF, protein-restricted/adequate folate; PR/FS, protein-restricted/folate supplemented; TAG, triglyceride.

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hypertension and endothelial dysfunction in the offspring (9,10), indicating that 1-carbon metabolism is central to the mechanism underlying induction of an altered phenotype. However, increasing the folic acid content of a protein-sufficient maternal diet also induced dyslipidemia and hyperglycemia in the offspring (11). This suggests that the nature of the induced phenotype is contingent upon the nutrient balance of the maternal diet.

Persistent changes in metabolism imply stable alterations to gene transcription. Epigenetic regulation of individual genes during specific periods in the developmental program, primarily by modification of the DNA methylation status of specific cytosines in CpG dinucleotides within their 5′-regulatory regions confers such stable changes in the level of transcription (12). However, stability of the epigenome is reduced during specific periods during the life course that are associated with more intensive changes in tissue function, namely prenatal and neonatal development, puberty, and aging (13). In rodents, manipulation of maternal dietary intakes of folic acid and methyl donors (14), differences in maternal nursing behavior (15), and constricted uterine blood flow (16) induce an altered phenotype in the offspring. We have shown that induction of an altered phenotype by a maternal PR diet during pregnancy involves changes in the epigenetic regulation by DNA methylation (17–20) and by covalent modifications of histones of specific genes, including the glucocorticoid receptor (GR) and PPARα in the liver of juvenile (17) and adult offspring (18). Such epigenetic changes are associated with altered mRNA expression of these genes and of their target genes. Induction of hypomethylation of GR involves downregulation of DNA methyltransferase (Dnmt)-1 mRNA expression and reduced binding to the $GR1_{10}$ promoter (20). This suggests that hypomethylation of the PPARα and GR promoters may involve progressive loss of methyl groups from CpG dinucleotides following mitosis rather than active demethylation (18). These epigenetic changes and altered Dnmt-1 expression were prevented by increasing the folic acid content of the maternal PR diet (17–19).

There is some information that changes in phenotype induced by nutritional constraint during early life can be reversed by subsequent interventions. Vickers et al. (21) have shown that leptin administration to neonatal rats reversed metabolic dysregulation induced by global maternal undernutrition during pregnancy. It is not known whether phenotypes induced by nutritional constraint during pregnancy can be reversed by dietary manipulation after the neonatal period. Folic acid supplementation in pubertal (22) and aging (23) rats altered hepatic whole-genome DNA methylation, although the effect on the epigenetic regulation of individual genes was not reported. Because the stability of the epigenome is decreased during the pubertal period (13), interventions during this time may have the potential to modify phenotypes induced in early life and so change life-long risk of disease. We therefore, tested in rats the hypothesis that increasing folic acid intake of the offspring during their juvenile-pubertal (JP) period could reverse the phenotype and epigenotype induced by the maternal PR diet. Puberty occurs at about postnatal (pn) d 40 in male rats (24) and d 35 in female rats (25). Offspring of control (C) or PR dams were fed a folic acid-adequate (AF) or -supplemented (FS) diet from weaning on d 28 until d 56. Because increased fat intake after weaning exasperates the effect of the maternal PR diet on fat and glucose metabolism (11), offspring were then challenged with a high-fat diet for a further 28 d. We measured the effect of the maternal and JP diets on growth and on markers of lipid and glucose metabolism and on the epigenetic regulation of candidate genes in liver, skeletal muscle, and adipose tissue.

Materials and Methods

Rats and diets. The study was carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986) and approved by internal ethical review. Virgin female Wistar rats (body weight 200–250 g) were mated and fed either C or PR isocaloric diets (**Supplemental Table 1**) (n = 8 per dietary group) from conception to spontaneous delivery on approximately d 21. Dams were fed the AIN93G semipurified diet (Supplemental Table 1) from delivery until the offspring were weaned on pn d 28. Litters were reduced to 8 (approximately equal males and females) within 24 h of delivery. At weaning, offspring were randomly assigned to an AF (1 mg/kg feed) or FS (5 mg/kg feed) diet (Supplemental Table 1) and were maintained on these diets until pn d 56. This produced 4 dietary groups (maternal diet/JP diet); C/AF, PR/AF, C/FS, and PR/FS. The increment in folate intake was comparable to that currently recommended for women in the UK before pregnancy and during the first trimester (26). Offspring were then fed a high-fat diet (Supplemental Table 1) for a further 28 d and were killed on pn d 84. Body weights were recorded at 7-d intervals throughout the postweaning period. Offspring were food deprived for 6 h before they were killed by CO_2 asphyxiation at ~1400 h. Liver, mesenteric adipose tissue, and biceps femoris muscle from the posterior right leg were collected and frozen immediately in liquid nitrogen and stored at −80°C. Blood was collected by cardiac puncture into tubes containing lithium heparin, and plasma was separated from cells by centrifugation and stored at −20°C. Samples from 10 male or female rats in each dietary group were selected for measurements of metabolite concentrations in plasma and for molecular biology analysis.

Measurement of metabolites in plasma. Plasma triglyceride (TAG), nonesterified fatty acid (NEFA), β-hydroxybutyrate (βHB), cholesterol, and glucose concentrations were measured using a Konelab 20 autoanalyzer (27).

Measurement of TAG concentration in liver. Liver TAG concentration was measured by GC (28,29) using triheptadecanoin as internal standard on a 6890 gas chromatograph (Agilent) equipped with a 30-m × 0.25-μm × 0.25-mm BPX-70 fused silica capillary column and flame ionization detection.

Measurement of mRNA expression by real-time RT-PCR. Measurement of the levels of specific mRNA transcripts was carried out using the primers listed (**Supplemental Table 2**). Total RNA was isolated from liver, adipose tissue, and skeletal muscle using Tri Reagent (Sigma) according to the manufacturer's instructions. cDNA was prepared and amplified using real-time RT-PCR (18,30). Samples were analyzed in duplicate and the expression of the individual transcripts were normalized to tissue-specific housekeeping genes, which did not differ in transcript level between groups of offspring (Supplemental Table 2) (18,31).

Measurement of DNA methylation of specific genes by real-time PCR. DNA methylation was conducted using the PCR primers listed (Supplemental Table 2). Genomic DNA was isolated from liver and muscle as described (17) and from adipose tissue using the Wizard SV Genomic DNA Purification system (Promega). Purified DNA was incubated with the methylation-sensitive restriction endonucleases *Aci*I and *Hpa*II according to the manufacturer's instructions (New England Biolabs) (17,18). The resulting DNA was amplified in duplicate using real-time PCR. A region of the PPARγ2 promoter that does not contain *Aci*I or *Hpa*II cleavage sites was used as an internal control (17,18).

Statistical analysis. Data are presented as mean ± 1 SD. Because the PCR measurements of samples from male and female offspring could not be compared directly, all other outcome variables were analyzed separately for males and females. The effect of the different dietary

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regimens on change in body weight over time was assessed using a general linear model with time as a repeated measure and maternal diet and JP diet as fixed factors with weight at weaning as a covariate. Post hoc comparisons were by Bonferroni's method. Comparisons between groups of the effect of different dietary regimens were by a general linear model with maternal diet and JP diet as fixed factors and Bonferroni's post hoc correction for multiple comparisons.

Results

Growth after weaning. Groups of offspring did not differ in body weight at weaning (data not shown). There was an interaction between time and JP diet in male and female offspring (both $P < 0.0001$) but no interaction between time and maternal diet or interaction between time, maternal diet, and JP diet. Weight gain did not differ between C/AF and PR/AF offspring or between C/FS and PR/FS male or female offspring. In males, weight gain was greater in C/FS offspring from pn d 63 and PR/FS offspring from pn d 70 than in C/AF offspring, although body weight tended to be higher ($P < 0.1$) in both C/FS and PR/FS offspring than in C/AF or PR/AF offspring from pn d 49 (**Supplemental Fig. 1**). The mean weight gain at pn d 84 of the C/FS group was 54 g higher than the C/AF group and the PR/FS group was 31 g more than the PR/AF group (Supplemental Fig. 1). In females, weight gain was significantly greater in C/FS than in C/AF offspring from pn d 35 and in PR/FS than in PR/AF offspring from pn d 42 (Supplemental Fig. 1). The mean weight gain at pn d 84 of the C/FS group was 35 g higher than the C/AF group and the PR/FS group was 37 g more than the PR/AF group (Supplemental Fig. 1).

Liver weight and hepatic TAG concentration. The JP diet affected liver weight and liver weight as a proportion of body weight in male offspring but not in females (**Table 1**). There was no effect of maternal diet or interaction between maternal and JP diet in either males or females. The livers of male C/FS and PR/FS offspring were significantly heavier (~25%) and weighed more as a proportion of body weight (~20%) than in C/AF and PR/AF offspring. The JP diet affected liver TAG concentration in males and females, but there was no effect of maternal diet or interaction between maternal diet and JP diet (Table 1). In males and females, liver TAG concentrations were greater in C/FS than C/AF offspring and in the PR/FS than PR/AF offspring.

Concentrations of specific metabolites in plasma. Maternal diet and JP diet induced selective changes in the concentrations of metabolites in plasma. In males, the maternal PR/AF diet increased plasma TAG and βHB concentrations compared with C/AF offspring (Table 1). The NEFA concentration tended to be higher ($P < 0.1$) in PR/AF offspring than in C/AF offspring. The plasma TAG concentration was higher in C/FS and PR/FS offspring than in C/AF offspring (Table 1). In contrast, the βHB concentration was significantly lower in C/FS and PR/FS offspring than in C/AF offspring (Table 1). Maternal diet and JP diet did not affect plasma cholesterol or glucose concentrations.

In females, maternal diet affected plasma TAG and NEFA concentrations such that these metabolites were significantly higher in PR/AF than in C/AF offspring (Table 1). The plasma βHB concentration was significantly higher in PR/AF than in C/AF offspring. Plasma TAG and NEFA concentrations were significantly higher in C/FS and PR/FS offspring than in C/AF and PR/AF offspring, whereas the βHB concentration was lower than in C/AF and PR/AF offspring (Table 1). Maternal diet or JP diet did not affect plasma cholesterol or glucose concentrations.

mRNA expression of specific genes in liver. It was not possible to compare the level of expression or DNA methylation between males and females in the same PCR and so results are presented relative to the C/AF group for each sex. The effects of maternal and JP diet on mRNA expression are summarized (**Tables 2** and **3**). Hepatic PPARα, acyl-CoA oxidase (AOX), carnitine-palmitoyl transferase (CPT-1), GR and phosphoenol-

TABLE 1 Liver weight and TAG and plasma metabolites in rats exposed to diets differing in protein before birth and folic acid in their JP period[1]

	Dietary group				ANOVA		
	C/AF	PR/AF	C/FS	PR/FS	JP diet (*P*)	Maternal diet (*P*)	JP diet × maternal diet (*P*)
Males							
Liver weight							
g	9.4 ± 2.0[a]	9.5 ± 1.6[a]	12.1 ± 1.6[b]	11.9 ± 2.1[b]	<0.0001	—	—
g/100 g body weight	2.5 ± 0.5[a]	2.5 ± 0.6[a]	3.0 ± 0.3[b]	3.0 ± 0.6[b]	<0.0001	—	—
Liver TAG, *μmol/g liver*	12 ± 4[a]	14 ± 3[a]	26 ± 9[b]	21 ± 7[b]	<0.0001	—	—
Plasma TAG, *mmol/L*	1.5 ± 0.7[a]	2.1 ± 0.5[b]	2.7 ± 0.9[b]	2.6 ± 0.8[b]	<0.0001	0.038	—
Plasma βHB, *μmol/L*	193 ± 43[a]	284 ± 79[b]	81 ± 45[c]	58 ± 33[c]	<0.0001	0.003	0.002
Plasma glucose, *mmol/L*	10.7 ± 3.0	9.0 ± 1.6	12.7 ± 4.0	8.8 ± 1.8	—	—	—
Plasma NEFA, *μmol/L*	346 ± 122	606 ± 438	683 ± 587	584 ± 413	—	—	—
Plasma cholesterol, *mmol/L*	2.1 ± 0.3	2.3 ± 0.3	2.8 ± 0.6	2.2 ± 0.3	—	—	—
Females							
Liver weight							
g	7.7 ± 1.3	7.3 ± 1.0	8.3 ± 1.0	8.7 ± 1.2	—	—	—
g/100 g body weight	3.1 ± 0.6	3.0 ± 0.6	3.2 ± 0.3	3.2 ± 0.4	—	—	—
Liver TAG, *μmol/g liver*	14 ± 3[a]	11 ± 6[a]	17 ± 6[b]	17 ± 2[b]	<0.001	—	—
Plasma TAG, *mmol/L*	1.0 ± 0.2[a]	1.4 ± 0.4[b]	1.8 ± 0.7[c]	1.9 ± 0.4[c]	0.001	0.022	—
Plasma βHB, *μmol/L*	229 ± 53[a]	291 ± 64[b]	35 ± 24[c]	49 ± 42[c]	<0.0001	0.019	—
Plasma glucose, *mmol/L*	9.6 ± 2.2	9.3 ± 1.7	7.9 ± 2.6	8.4 ± 2.5	—	—	—
Plasma NEFA, *μmol/L*	362 ± 84[a]	441 ± 115[b]	425 ± 96[b]	484 ± 212[b]	0.011	0.017	—
Plasma cholesterol, *mmol/L*	1.7 ± 0.2	1.6 ± 0.2	2.0 ± 0.3	1.7 ± 0.31	—	—	—

[1] Values are means ± SD, $n = 10$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

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TABLE 2 mRNA expression of genes in liver, adipose tissue, and skeletal muscle in male offspring exposed to diets differing in protein before birth and folic acid in their JP period[1]

	Dietary group				ANOVA		
	C/AF	PR/AF	C/FS	PR/FS	JP diet (*P*)	Maternal diet (*P*)	JP diet × maternal diet (*P*)
Liver			*% of C/AF*				
PPARα	100.0 ± 43.7[a]	176.0 ± 44.3[b]	32.3 ± 14.0[c]	24.4 ± 3.1[c]	<0.0001	0.002	<0.0001
AOX	100.0 ± 16.6[a]	136.0 ± 36.7[b]	63.7 ± 14.0[c]	73.6 ± 13.4[c]	<0.0001	0.03	—
CPT-1	100.0 ± 33.3[a]	139.0 ± 39.8[b]	71.8 ± 24.1[a]	77.3 ± 23.9[a]	<0.0001	0.029	—
GR	100.0 ± 45.6[a]	174.1 ± 43.3[b]	318.5 ± 81.3[c]	297.3 ± 86.2[c]	<0.0001	0.041	—
PEPCK	100.0 ± 27.3[a]	203.6 ± 41.9[b]	256.3 ± 65.5[b]	185.4 ± 45.8[b]	<0.0001	—	—
IR	100.0 ± 23.3[a]	72.8 ± 18.9[a]	229.4 ± 43.2[b]	245.0 ± 58.4[b]	<0.0001	—	—
Adipose tissue							
PPARγ2	100.0 ± 21.0[a]	101.7 ± 37.9[a]	240.5 ± 34.6[b]	218.7 ± 48.3[b]	<0.001	—	—
IR	100.0 ± 38.7[a]	84.3 ± 18.8[a]	281.7 ± 77.9[b]	278.6 ± 82.9[b]			
HSL	100.0 ± 28.8[a]	147.0 ± 44.8[a]	35.0 ± 19.4[b]	41.1 ± 20.4[b]			
LPL	100.0 ± 34.6[a]	107.6 ± 59.6[a]	144.8 ± 56.6[b]	149.5 ± 69.7[b]	0.047	—	—
Skeletal muscle							
PPARα	100.0 ± 34.2[a]	119.7 ± 33.4[a]	224.4 ± 69.4[b]	184.8 ± 46.8[b]	<0.0001	—	—
AOX	100.0 ± 28.9[a]	117.1 ± 29.1[a]	318.7 ± 23.1[b]	317.2 ± 29.0[b]	<0.0001	—	—
CPT-1	100.0 ± 38.2	98.4 ± 29.1	88.2 ± 36.8	87.8 ± 30.2	—	—	—
LPL	100.0 ± 35.6	99.5 ± 66.6	73.4 ± 19.5	76.1 ± 10.1	—	—	—

[1] Values are means ± SD, $n = 10$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

pyruvate carboxykinase (PEPCK) expression were higher in male PR/AF offspring than in C/AF offspring (Table 2). The expression of these genes did not differ between the C/FS and PR/FS offspring. However, PPARα, AOX, and CPT-1 mRNA levels were lower in male C/FS and PR/FS than in C/AF and PR/AF offspring. In contrast, GR and PEPCK expression was higher in C/FS and PR/FS offspring than in C/AF offspring but were expressed at a similar level to PR/AF offspring (Table 2). Insulin receptor (IR) expression was higher in FS offspring than in AF offspring but did not differ between maternal dietary groups (Table 2).

PPARα, AOX, CPT-1, GR, and PEPCK expression was significantly higher in livers of female PR/AF offspring than in C/AF offspring (Table 3). The expression of these genes did not differ between C/FS and PR/FS offspring. PPARα, AOX, and CPT-1 mRNA levels were lower in female C/FS and PR/FS offspring than in C/AF and PR/AF offspring. GR expression was higher in C/FS and PR/FS than in C/AF offspring but at a similar level to PR/AF offspring (Table 3). PEPCK mRNA levels were higher in C/FS and PR/FS offspring than C/AF offspring but were lower than in PR/AF offspring. IR mRNA expression did not differ significantly between PR and C offspring. However, the level of IR mRNA was significantly higher in FS offspring than in C/AF offspring (Table 3).

mRNA expression of specific genes in adipose tissue. Maternal diet did not affect PPARγ2, lipoprotein lipase (LPL), or IR expression in adipose tissue in male or female offspring, although hormone-sensitive lipase (HSL) expression in PR/AF

TABLE 3 mRNA expression of genes in liver, adipose tissue, and skeletal muscle in female offspring exposed to diets differing in protein before birth and folic acid in their JP period[1]

	Dietary group				ANOVA		
	C/AF	PR/AF	C/FS	PR/FS	JP diet (*P*)	Maternal diet (*P*)	JP diet × maternal diet (*P*)
Liver			*% of C/AF*				
PPARα	100.0 ± 45.0[a]	185.6 ± 45.5[b]	29.3 ± 12.7[c]	23.2 ± 8.5[c]	<0.0001	0.001	<0.0001
AOX	100.0 ± 16.7[a]	160.4 ± 45.1[b]	71.1 ± 16.5[a]	75.8 ± 24.3[a]	<0.0001	0.01	0.004
CPT-1	100.0 ± 36.4[a]	168.4 ± 55.3[b]	48.4 ± 26.8[c]	48.6 ± 28.2[c]	<0.0001	0.008	0.008
GR	100.0 ± 39.3[a]	396.4 ± 189.0[b]	394.8 ± 127.5[b]	254.5 ± 83.4[b]	<0.0001	0.032	—
PEPCK	100.0 ± 28.4[a]	305.3 ± 77.6[b]	121.6 ± 56.8[c]	150.1 ± 34.3[c]	<0.0001	<0.0001	<0.001
IR	100.0 ± 26.1[a]	81.4 ± 5.5[a]	169.4 ± 44.6[b]	166.6 ± 46.6[b]	<0.0001	—	—
Adipose tissue							
PPARγ2	100.0 ± 26.5[a]	65.7 ± 18.9[a]	275.4 ± 35.4[b]	373.1 ± 88.1[c]	<0.0001	—	—
IR	100.0 ± 62.5[a]	62.3 ± 52.9[a]	290.1 ± 98.7[b]	202.6 ± 131.4[b]	0.048	<0.0001	—
HSL	100.0 ± 47.2[a]	121.6 ± 41.4[a]	27.8 ± 22.0[b]	13.0 ± 8.4[b]	<0.0001	—	—
LPL	100.0 ± 31.9	91.8 ± 51.1	98.1 ± 45.2	87.0 ± 34.5	—	—	—
Skeletal muscle							
PPARα	100.0 ± 56.8[a]	181.4 ± 72.9[b]	194.8 ± 57.9[b]	233.8 ± 69.4[b]	0.001	0.006	—
AOX	100.0 ± 42.8[a]	123.4 ± 45.4[a]	363.9 ± 49.2[b]	277.6 ± 52.0[b]	<0.0001	—	—
CPT-1	100.0 ± 24.9[a]	86.2 ± 28.7[a]	56.7 ± 13.3[b]	62.0 ± 25.3[b]	<0.0001	—	—
LPL	100.0 ± 46.6	117.4 ± 49.9	78.0 ± 25.7	65.2 ± 25.7	—	—	—

[1] Values are means ± SD, $n = 10$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

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offspring tended be higher ($P < 0.1$) than in C/AF offspring (Tables 2 and 3). PPARγ2 and IR mRNA levels were higher in C/FS and PR/FS male and female offspring than in C/AF and PR/AF offspring (Tables 2 and 3), but HSL expression was lower in C/FS and PR/FS offspring than in C/AF male and female offspring (Tables 2 and 3). LPL mRNA expression was higher in C/FS and PR/FS male offspring than in C/AF and PR/AF offspring but did not differ between C/AF female offspring and FS offspring (Tables 2 and 3).

mRNA expression of specific genes in skeletal muscle. In skeletal muscle, PPARα, AOX, and CPT-1 expression did not differ significantly between male and female PR/AF offspring or between C/FS and PR/FS offspring (Tables 2 and 3). The mRNA levels of PPARα and AOX were significantly higher in FS offspring than in AF offspring, but CPT-1 expression did not differ between these groups (Tables 2 and 3). LPL mRNA expression did not differ between maternal dietary or JP dietary groups in male offspring but was significantly lower in female PR/FS offspring than in C/AF offspring (Tables 2 and 3).

DNA methylation status of specific genes in liver and adipose tissue. In males and females, the maternal diet affected hepatic PPARα and GR, but not IR or methylation and the JP diet affected PPARα, IR, and GR methylation (**Table 4**). In male and female liver, PPARα methylation was significantly lower in PR/AF than C/AF offspring but was greater in FS than C/AF offspring (Table 4). GR promoter methylation was significantly lower in PR/AF and FS offspring than in C/AF offspring (Table 4). IR methylation was lower in FS male and female offspring than in C/AF offspring (Table 4).

Maternal diet did not affect adipose tissue IR or HSL methylation in male or female offspring. JP diet decreased IR methylation in male and female offspring (Table 4) but did not alter HSL methylation (Table 4).

Discussion

Our findings show that in contrast to folic acid supplementation of the maternal PR diet (11,17), supplementation during the JP period did not normalize changes in metabolism and epigenotype induced by the PR diet. Rather, JP folic acid supplementation induced distinct changes in the phenotype and epigenotype of the offspring. Together with our previous observations (11,17), these findings suggest that the effect of increased folic acid intake on phenotype is contingent on the timing of folic acid supplementation relative to the developmental stage of the organism and the nutrient pattern within the diet. One possible mechanism for the difference between the effects of providing the same amount of folic acid to pregnant dams and to JP offspring is that the supply of folic acid to the offspring in utero would have been buffered by maternal metabolism, whereas the JP offspring would have been exposed directly to amount of folic acid in the diet.

Although the maternal dietary groups did not differ in offspring weight gain after weaning, folic acid supplementation increased weight gain in both male and female offspring. Because increased growth started during the pubertal period, this suggests that the JP period is one of plasticity in the regulation of weight gain, which responds positively to increased folic acid intake. Puberty in rats and humans is preceded by a proliferation of preadipocytes (32,33). One possible mechanism by which folic acid supplementation may increase weight gain is by increasing the formation of adipocytes. This is consistent with upregulation in the FS offspring of PPARγ2 expression, which induces adipogenesis (34).

The coordinated activities of metabolic pathways in liver, skeletal muscle, and adipose tissue are critical for maintaining lipid and glucose homeostasis. We therefore measured the epigenetic regulation and mRNA expression of candidate genes in each of these tissues. We did not attempt to provide a comprehensive assessment of the changes in metabolic pathways that underlie the induced phenotypes. The changes in mRNA expression and DNA methylation of hepatic PPARα and GR and in the mRNA levels of their respective targets AOX and CPT-1, and PEPCK in the PR/AF offspring are consistent with our previous findings (20,17,19,18) and, together with higher plasma βHB concentrations, suggests increased capacity for fatty acid β-oxidation. The absence of an effect of maternal diet on glucose concentration suggests that the increased GR and PEPCK expression was a

TABLE 4 Promoter methylation of genes in liver and adipose tissue in the offspring exposed to diets differing in protein before birth and folic acid in their JP period[1]

	Dietary group				ANOVA		
	C/AF	PR/AF	C/FS	PR/FS	JP diet (*P*)	Maternal diet (*P*)	JP diet × maternal diet (*P*)
Male offspring	*% of C/AF*						
Liver							
PPARα	100.0 ± 34.2^a	47.6 ± 32.1^b	172.2 ± 43.3^c	158.7 ± 33.8^c	<0.0001	0.042	—
GR	100.0 ± 44.1^a	45.7 ± 24.0^b	27.7 ± 15.5^b	30.1 ± 29.2^b	<0.0001	0.01	<0.0001
IR	100.0 ± 47.0^a	108.8 ± 24.8^a	55.9 ± 21.9^b	62.3 ± 32.6^b	<0.0001	—	—
Adipose tissue							
IR	100.0 ± 7.9^a	98.7 ± 6.5^a	75.2 ± 8.0^b	73.7 ± 7.1^b	—	<0.0001	—
HSL	100.0 ± 25.8	104.1 ± 32.0	93.4 ± 21.4	91.8 ± 23.9	—	—	—
Female offspring							
Liver							
PPARα	100.0 ± 44.4^a	55.1 ± 45.9^b	141.5 ± 35.7^c	157.5 ± 61.4^c	<0.0001	0.027	0.049
GR	100.0 ± 41.4^a	33.9 ± 20.0^b	11.4 ± 8.5^b	15.2 ± 10.8^b	<0.0001	0.01	<0.0001
IR	100.0 ± 33.5^a	62.8 ± 14.8^a	35.9 ± 20.1^b	38.8 ± 35.3^b	<0.0001	—	—
Adipose tissue							
IR	100.0 ± 11.3^a	99.6 ± 6.8^a	84.8 ± 9.2^b	84.8 ± 14.1^b	—	0.003	—
HSL	100.0 ± 17.1	102.2 ± 29.2	92.0 ± 46.1	93.0 ± 24.6	—	—	—

[1] Values are means ± SD, $n = 10$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

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relatively minor determinant of plasma glucose. PPARγ2 and LPL mRNA expression in adipose tissue and PPARα, CPT-1, and AOX expression in skeletal muscle were not altered in the PR/AF offspring, indicating differential sensitivity of liver, adipose tissue, and skeletal muscle to induction of altered transcription by maternal protein restriction.

JP folic acid supplementation induced differential changes in the methylation status and/or mRNA expression of individual genes that were associated with a shift in lipid metabolism in the food-deprived state. These are summarized in the model presented in **Figure 1**. In adipose tissue, the changes in PPARγ2, IR, LPL, and HSL mRNA expression suggest that JP folic acid supplementation increased capacity for deposition of fatty acids in adipocytes, which is consistent with greater weight gain. Downregulation of HSL and increased LPL expression may reflect increased IR mRNA levels leading to upregulation of the insulin signaling pathway. Although HSL expression was reduced, the plasma NEFA concentration tended to be higher in FS offspring. One possible explanation is that greater fat mass and inefficient entrapment of fatty acids from TAG-rich lipoproteins (35) would tend to increase NEFA flux to the liver.

Lower plasma βHB concentration in FS offspring is consistent with lower expression of PPARα and of the target genes AOX and CPT-1 and suggests constraint in hepatic fatty acid β-oxidation (Fig. 1). Deposition of TAG in liver and increased plasma TAG concentration is consistent with constraint in fatty acid β-oxidation and a shift in fatty acid metabolism toward TAG synthesis, which may be exacerbated by increased NEFA flux. Reduced intakes of folic acid or 1-carbon donors are associated with fatty liver due to reduced phosphatidylcholine synthesis, which is required for VLDL secretion (36,37). Our results suggest that increased folic acid intake after weaning can also induce fatty liver by altering the metabolic partitioning of fatty acids from β-oxidation toward TAG synthesis. Thus, FS supplementation appears to induce opposite changes in equivalent metabolic pathways in different tissues.

PPARα expression is directionally dependent upon the methylation status of its promoter (17,38). Thus, hypermethylation of the PPARα promoter is consistent with its lower expression. In contrast, the methylation status and expression of GR and mRNA expression of PEPCK in FS offspring was similar to PR/AF. Thus, within a single tissue, increased JP folic acid intake induces gene-specific changes in promoter methylation and expression, although the mechanism for such targeting is not known. Furthermore, in skeletal muscle from FS offspring, increased PPARα and AOX expression suggests upregulation of peroxisomal β-oxidation, which suggests the in JP animals, the effects of folic acid supplementation differ between tissues.

Overall, these findings suggest that increased folic acid intake in the JP period induced a change in the partitioning of fatty acids between different metabolic fates. It remains to be determined whether changes in metabolism induced by altered nutrition during the JP period persist throughout the life course and if these observations are influenced by the background diet. Although the effects of increased folic acid intake operate through alterations in the epigenome, the mechanism by which such changes occur cannot be deduced from the present findings. One implication of our findings is that folic acid supplementation as a strategy to reverse the adverse effects of poor nutrition during early life on future health in humans may need to be undertaken with caution, particularly with respect to the timing of the period of supplementation and the composition of the background diet. However, demonstration of plasticity during the JP period supports the possibility of resetting an inappropriate phenotype induced in early life to one better adapted to meet the challenge of the prevailing environment.

Puberty has been identified as a period of relative instability in the epigenome associated with altered epigenetic regulation of genes associated with sexual maturation (13) and variation in nutrition in the prepubertal period in grandparents has been associated with patterns of mortality in the grandchildren (39). Furthermore, exposure during this period to hormones, including estrogens, induces changes in the epigenome in a manner that affects subsequent disease risk, which differs from the effects of hormonal exposure during the neonatal period (40). This may involve changing the DNA demethylation and remethylation cycles that occur in nondividing cells (41), e.g. by altering the balance of Dnmt-1 and demethylase activities (18,41). The JP period is also a time of increased growth and cell proliferation in specific tissues (32,33) that may facilitate epigenetic changes during mitotic cycles (12). Overall, our data support the view that, during the life course, specific periods of plasticity are associated with reduced stability of the epigenome that allow the phenotype of an organism to adapt to environmental cues, such as nutrition. It has been suggested that the phenotype induced in the fetus by environmental cues can promote later reproductive

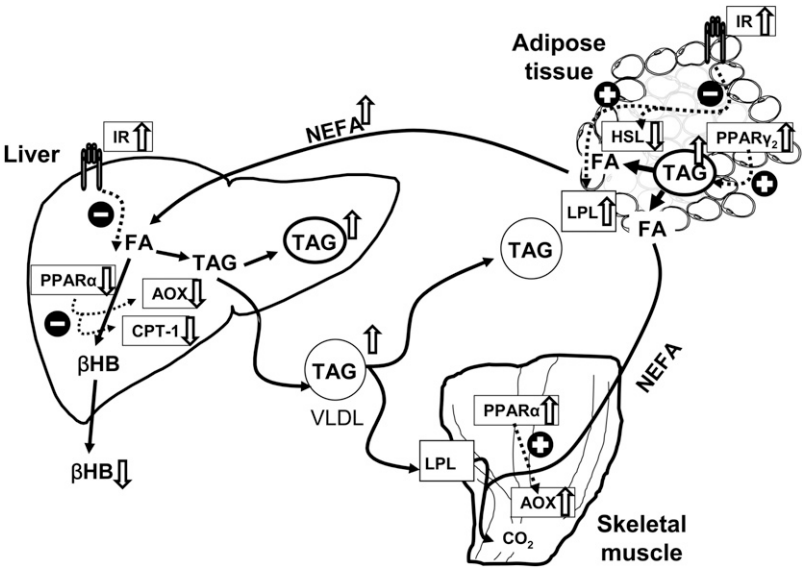

FIGURE 1 A model for the effect of altered gene expression induced in liver, adipose tissue, and skeletal muscle by increased PP folic acid intake. A detailed explanation is provided in the text. Black arrows indicate flux of metabolites. Broken arrows indicate effects of various genes on other genes or on metabolic processes. White arrows indicate the direction of change relative to C/AF offspring. Circle with horizontal bar indicates negative effect on transcription. Circle with cross indicates positive effect on transcription.

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fitness by predicting the future environment and that an incorrect prediction is associated with increased risk of later chronic disease (2). Our findings suggest this hypothesis could be extended to include the phenotypic changes induced during the subsequent period of plasticity in puberty, perhaps enhancing the organism's life course strategy.

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