Received: 10 April 2017 | Revised: 11 August 2017 | Accepted: 1 October 2017

DOI: 10.1002/ajpa.23341

RESEARCH ARTICLE

WILEY American Journal of PHYSICAL ANTHROPOLOGY

DNA methylation of methylation complex genes in relation to stress and genome-wide methylation in mother–newborn dyads

Christopher J. Clukay | David A. Hughes | Nicole C. Rodney | Darlene A. Kertes | Connie J. Mulligan

University of Florida, Gainesville, Florida 32611

Correspondence
Christopher J. Clukay, 2033 Mowry Rd., PO Box 103610, University of Florida, Gainesville, FL 32608.
Email: cjclukay@ufl.edu

Funding information
Grant Sponsorship Supported by NSF grant #BCS 1231264, a UF Research Opportunity Seed Fund award, and a UF College of Liberal Arts and Science award

Abstract

Objectives: Early life stress is known to have enduring biological effects, particularly with respect to health. Epigenetic modifications, such as DNA methylation, are a possible mechanism to mediate the biological effect of stress. We previously found correlations between maternal stress, newborn birthweight, and genome-wide measures of DNA methylation. Here we investigate ten genes related to the methylation/demethylation complex in order to better understand the impact of stress on health.

Materials and methods: DNA methylation and genetic variants at methylation/demethylation genes were assayed. Mean methylation measures were constructed for each gene and tested, in addition to genetic variants, for association with maternal stress measures based on interview and survey data (chronic stress and war trauma), maternal venous, and newborn cord genome-wide mean methylation (GMM), and birthweight.

Results: After cell type correction, we found multiple pairwise associations between war trauma, maternal GMM, maternal methylation at *DNMT1*, *DNMT3A*, *TET3*, and *MBD2*, and birthweight.

Conclusions: The association of maternal GMM and maternal methylation at *DNMT1*, *DNMT3A*, *TET3*, and *MBD2* is consistent with the role of these genes in establishing, maintaining and altering genome-wide methylation patterns, in some cases in response to stress. *DNMT1* produces one of the primary enzymes that reproduces methylation patterns during DNA replication. *DNMT3A* and *TET3* have been implicated in genome-wide hypomethylation in response to glucocorticoid hormones. Although we cannot determine the directionality of the genic and genome-wide changes in methylation, our results suggest that altered methylation of specific methylation genes may be part of the molecular mechanism underlying the human biological response to stress.

KEYWORDS
Democratic Republic of Congo, DNMT3A, methyltransferase, TET3, war trauma

1 | INTRODUCTION

Anthropologists have long recognized the effect of environment on individual biology (Boas, 1910) and the idea that evolutionary forces result in adaptive and maladaptive changes. Early life stressors, particularly those experienced *in utero*, can have substantial biological outcomes. These stressors include psychosocial stress experienced during wartime (Mulligan, D'Errico, Stees, & Hughes, 2012), as well as other types of stress like maternal nutrition, smoking, and depression (Brøns et al., 2010; Jensen Peña, Monk, & Champagne, 2012; Murgatroyd, Quinn, Sharp, Pickles, & Hill, 2015). These early life stressors may be especially important in terms of long-term effects on health and disease risk according to the developmental origins of health and disease hypothesis (DOHaD), which posits that stressors experienced *in utero* affect the postgestational phenotype and range of future possible phenotypic responses.

Recently, epigenetic variation has arisen as one possible molecular mechanism by which environmental stressors result in phenotypic

change (Frisancho, 2009; Mulligan et al., 2012; Nätt, Johansson, Faresjö, Ludvigsson, & Thorsell, 2015; Ponomarev, Wang, Zhang, Harris, & Mayfield, 2012). Epigenetic modifications are changes to the genome that do not alter the DNA sequence, but do change the amount of product, or expression, of encoded genes. Here we test DNA methylation, one type of epigenetic modification, in relationship to maternal stress and newborn health.

Our group has previously explored DNA methylation in relation to stress experienced by 25 new mothers and the health outcomes of their newborns in the Democratic Republic of Congo (Mulligan et al., 2012). We found that maternal stress was correlated with both newborn birthweight and methylation changes in mothers and newborns. When examining genome-wide patterns of methylation, we found a correlation between maternal stress and maternal genome-wide mean methylation (GMM) that was not seen in the newborns (Rodney & Mulligan, 2014); this result suggests that the stress exposures we measured have a broad, genome-wide effect in the individual who directly experiences the stressors, that is, the mother. When studying *NR3C1*, a glucocorticoid receptor gene related to birthweight and the stress response, we found that maternal stress was correlated with newborn methylation, but not maternal methylation, consistent with DOHaD theory that maternal stress modifies offspring biology (Mulligan et al., 2012). Thus, we see a genome-wide effect in the mothers and a gene-specific effect in newborns of maternal stress on DNA methylation.

In the current study, we investigate a possible molecular mechanism underlying the different types of methylation change we have identified in the mother–newborn dyads. The genome-wide effect observed in the mothers prompted us to investigate whether the genes that establish and maintain genome-wide methylation patterns were being affected as well. Therefore, ten genes related to the methylation complex were chosen for epigenetic and genetic analysis; four methyltransferase genes involved in the transfer of methyl groups (*DNMT1*, *DNMT3A*, *DNMT3B*, *DNMT3L*), three genes whose products modify methylated CpG sites to make other methylation products (*TET1*, *TET2*, *TET3*), and three genes that code for methyl-binding proteins (*MECP2*, *MBD2*, *MBD4*). Together, the proteins produced by these genes each contribute to establishing, maintaining, and altering the DNA methylation pattern of a cell.

Regarding the methyltransferase genes, *DNMT1* produces one of the primary enzymes, DNMT1, that reproduces methylation patterns during DNA replication in adult cells, that is, differentiated cells *in utero* and in adults. DNMT1 does this by preferentially methylating hemimethylated CpG sites, where one strand of the DNA helix is already methylated. *DNMT3A* and *DNMT3B* produce the main *de novo* methyltransferases that establish the methylation profile prior to implantation (Fatemi, Hermann, Gowher, & Jeltsch, 2002; Li et al., 2015). These methyltransferase enzymes are also thought to be involved in the maintenance of methylation patterns in adult cells, although their exact role is unclear (Fatemi et al., 2002; Feng et al., 2010; Sharma, De Carvalho, Jeong, Jones, & Liang, 2011). There is also evidence that DNMT1, DNMT3A, DNMT3B and DNMT3L cooperate to establish and maintain methylation patterns (Fatemi et al., 2002; Hu et al., 2008).

The second category of studied genes encodes the TET proteins; TET1, TET2, and TET3. These enzymes act as part of a single pathway to modify methylated CpG sites to more oxidized states, such as 5-hmC, 5-formylC and 5-carboxyC (Kohli & Zhang, 2013). The full consequences of these changes have not been fully elucidated, but are believed to play a role in demethylation. In addition, TET3 has been shown to regulate *DNMT3A* activity, altering genome-wide methylation levels (Bose et al., 2015). Thus, the TET genes may play a role in removing or altering methylation, in contrast to the DNMT genes that generate and maintain DNA methylation.

Lastly, methyl-binding proteins bind to methylated CpG sites and influence gene regulation at those locations. MECP2 binds to different methylated forms of DNA (e.g., mCH and hmC), appears to have a role in both transcriptional silencing and transcriptional activation, and is particularly important in the brain (Pohodich & Zoghbi, 2015). MBD2 and MBD4 proteins contain methyl-binding domains and are involved in DNA demethylation, DNA repair and/or gene expression regulation (Laget et al., 2014; Weaver et al., 2014). In addition, some reports have indicated that the MBD genes may be able to directly demethylate CpG sites as well (Detich, Theberge, & Szyf, 2002). Thus, methyl-binding proteins may affect demethylation activity and gene regulation in the cell.

In sum, we investigate the role that methylation complex genes may play in the associations we found previously between maternal stress, newborn birthweight, genome-wide changes in maternal methylation, and gene-specific changes in cord methylation. We analyze DNA methylation of ten methylation complex genes, and genetic variants in six of these genes. Subsequently, we test measures of methylation and the genetic variants for association with maternal stress, birthweight, and GMM in both maternal venous and newborn cord blood.

2 | MATERIALS AND METHODS

2.1 | Study participants and sample collection

Participants for the study were enrolled as described previously (Rodney & Mulligan, 2014). In brief, 25 mothers who delivered babies in the summer of 2010 at HEAL Africa hospital in Goma, DRC were enrolled in the study. Mothers were interviewed and blood samples from the mother and umbilical cord were collected shortly after delivery. QIAamp DNA Mini Kits (Qiagen, Cat No. 51304) were used to extract genomic DNA. The ten genes chosen for study were based on a literature review that identified all major genes involved in DNA methylation and demethylation. Twenty-four maternal venous and 24 newborn umbilical cord samples were assayed for methylation; two cord blood samples were found to be contaminated and were removed from our analyses. Maternal stress and birthweight data were available for all 25 dyads. The study was approved by the Western Institutional Review Board, Olympia, WA (www.wirb.com, WIRB Project #20100993).

2.2 | Sociocultural data

Sociocultural data focused on maternal stress and trauma were collected from each mother as previously described (Rodney & Mulligan,

2014). In brief, lengthy semi-structured interviews and a standardized survey of perinatal stress derived from an established measure (Brunet et al., 2001) were administered to each participant one day after giving birth. Both interviews and surveys were conducted in the Congolese dialect of the Swahili language. These instruments allowed culturally-relevant stress data to be obtained regarding socio-economic, war-related, and other stressors pertinent to this population. All women discussed their war-related traumatic experiences without being explicitly asked, attesting to the salience of these experiences.

Using data from the survey and interviews, continuous composite measures of stress were constructed based on factor analysis as described previously (Kertes et al., 2016) [these stress measures differ from those described in Rodney & Mulligan (2014)]. Two factors were identified that explained the majority of variance in the stress questions (55% in total). Each factor was confirmed using internal consistency statistics and found to be adequate based on Cronbach's α (all >0.80). All questions contributed to internal consistency with the exception of two questions regarding in-law stress and co-wives, both of which were removed. The remaining questions were retained in the two factor-based summary variables that we use in this study. We refer to the two factors as chronic stress and war trauma since the majority of questions in each factor relate to chronic and war trauma stress, respectively.

2.3 | Genetic data

A custom Affymetrix microarray was designed in our lab with ~30,000 SNPs covering over 3500 genes, including six genes investigated in this study: *DNMT1*, *DNMT3A*, *DNMT3B*, *DNMT3L*, *MBD2*, and *MBD4*. Only the maternal venous blood samples were analyzed. Samples were processed according to the manufacturer's specifications using 200 ng of DNA. The microarray was processed at the University of North Carolina—Chapel Hill Genomics Core. Quality control was performed using the Affymetrix Genotyping Console and the SNPolisher package in R as described by Affymetrix. Probes corresponding to the genes of interest were isolated based on the associated UCSC gene name in the annotation file resulting in 14 SNPs for *DNMT1*, 56 for *DNMT3A*, 39 for *DNMT3B*, 36 for *DNMT3L*, 52 for *MBD2*, and 4 for *MBD4*.

2.4 | Epigenetic data

Methylation of CpG sites was assayed using the Human-Methylation 450 Bead Chip (Illumina). Five-hundred nanograms of each sample was prepared as recommended by Illumina and the chip was processed at the University of Miami Hussman Institute for Human Genetics. Approximately 485,512 CpG sites spanning all chromosomes were assayed. These sites were then filtered as recommended by the manufacturer based on the following criteria: sites were removed if they were not detected with a p value of .01 in every sample, had missing data for one or more samples, exhibited no variance, or were known to cross-hybridize to nonspecific sites (Chen et al., 2013). Lastly, using the 1000 Genomes Project African super population (Consortium, 2012), all probes were removed that contained a SNP at the assayed CpG site, a SNP at a frequency greater than or equal to 5% anywhere in the probe sequence, or three or more SNPs at any frequency.

After filtering, ~366,000 CpG sites remained. Types I and II probes were normalized using the Beta Mixture Quantile Method (BMIQ) in R (Teschendorff et al., 2013). Beta values were converted to M values as recommended by Du et al. (2010). Methylation at each gene of interest was assessed using the subset of CpG sites associated with the gene based on the UCSC gene name in the annotation file. This resulted in 16 CpG sites for *DNMT1*, 70 for *DNMT3A*, 16 for *DNMT3B*, 10 for *DNMT3L*, 15 for *MECP2*, 13 for *MBD2*, 14 for *MBD4*, 27 for *TET1*, 16 for *TET2*, and 14 for *TET3*.

2.5 | Statistical analyses

Genome-wide mean methylation (GMM) was calculated for each individual based on the average methylation of all autosomal CpG sites ($n = 348{,}252$) in that sample; CpG sites on X and Y chromosomes were excluded to avoid the influence of sex-based effects such as X-inactivation on measures of GMM.

Principal component analysis (PCA) was used to summarize methylation at each gene using JMP Genomics (SAS Institute Inc., 2013). PCA is a means to empirically capture the shared methylation signal of intercorrelated CpG sites at each gene and has been used for various purposes in other methylation studies such as ancestry estimation (Barfield et al., 2014; Mulligan et al., 2012; Rahmani et al., 2017). Additionally, methylation principal components (PCs) reduce the multiple testing burden as fewer PCs are tested compared to the number of individual CpG sites. Samples were separated by tissue type prior to analysis, creating two sets of samples; mothers' blood and newborns' cord blood. Even though the first PCs account for the greatest variation in methylation, we chose the inflection point as an unbiased and accepted way to determine the number of retained PCs per gene. An average of five to six methylation PCs were retained per gene accounting for, in total, greater than 50% of the variation in methylation at each gene, with some gene's PCs capturing over 95% of variation in methylation.

Promoter and enhancer methylation PCs were also created to investigate methylation at these genomic regulatory elements. For each gene, a PCA was conducted using CpG sites that fell within regions designated as promoters (within 200 base pairs of the transcription start site) and enhancers (based on the Illumina annotation file). A promoter or enhancer PCA was only performed on genes that had at least three assayed CpGs in that category. This requirement resulted in promoter methylation PCs being created for *DNMT1* (5 CpGs), *DNMT3A* (10 CpGs), *MBD2* (3 CpGs), *MBD4* (5 CpGs), and *TET1* (5 CpGs) and enhancer methylation PCs for *DNMT3A* (26 CpGs), *TET1* (4 CpGs), *TET2* (5 CpGs), and *TET3* (3 CpGs).

Hierarchical linear regressions were used to test both genetic and epigenetic effects. To determine possible genetic effects, SNPs at the genes of interest were coded based on the number of copies of the minor allele. Each SNP was tested separately for association with maternal GMM, cord GMM, methylation PCs at the particular gene of interest, or birthweight. Epigenetic relationships were tested using

both the methylation PCs and the *M* values of individual CpG sites at each gene as methylation measures. These methylation measures were tested individually for association with the stress measures (chronic stress and war trauma), maternal GMM, cord GMM, or birthweight. All calculations other than PCA were performed using R with the code included in the Supporting Information (R Core Team, 2015).

All measures of methylation in maternal venous or newborn cord blood were corrected for cell composition using the Houseman method for the Illumina 450K chip, as implemented in the minfi package for R (Aryee et al., 2014; Houseman et al., 2012). Reference datasets were based on Houseman et al. (2012) for venous blood and Bakulski et al. (2016) for cord blood. Quality control plots were within acceptable ranges for both tissue types. Once cell proportions were calculated, PCA was performed on the cell proportions in order to reduce collinearity, creating cell composition PCs to be included as control variables in all linear regression analyses involving gene-specific methylation. Thus, maternal cell composition PCs were included when testing maternal venous blood methylation and cord cell composition PCs were included when testing newborn cord blood methylation. Hierarchical linear regression was performed with the cell composition PCs included in the first step followed by the variable of interest in the second step.

Bonferroni correction was used for all linear regressions to correct for multiple testing. The Bonferroni threshold for significance was set according to the number of methylation PCs (56 maternal PCs or 53 cord PCs) or individual CpG sites (211 CpG sites) tested, for example, when testing for association of maternal methylation PCs and maternal GMM, the Bonferroni threshold was set to 0.05/(56 maternal methylation PCs) = 8.92 E−04. Individual thresholds are given with each set of results (see "Section 3").

In order to determine which CpG sites were associated with each PC, the mseapca package for the PCA of metabolites was adapted for use in this study (Yamamoto et al., 2014). The pca_scaled function was used to detect which CpG sites were significantly represented by the methylation PC of interest using the hypothesis test published by Yamamoto et al. (2014). Significant CpG sites were selected based on *q* value, a *p* value corrected for multiple testing, using false discovery rate correction at a level of 5% (Benjamini & Hochberg, 1995).

3 | RESULTS

3.1 | Measures of methylation

Three different measures of methylation were used in this study; genome-wide methylation (GMM) to investigate methylation across the genome as well as methylation principal components (PCs) and individual CpG sites to investigate methylation at each gene of interest. Genome-wide mean methylation represents the general level of methylation across the genome for that individual. GMM was calculated for each individual as the average methylation of all autosomal CpG sites

TABLE 1 Number of CpG sites and methylation PCs for each gene

Gene	Function	Total CpG sites[a]	Maternal methylation PCs	Cord methylation PCs
DNMT1	Methyltransferase -Maintenance of methylation	15 (5, 0)	6	6
DNMT3A	Methyltransferase -Maintenance of methylation and *de novo* methylation	70 (9, 26)	5	4
DNMT3B	Methyltransferase -Maintenance of methylation and *de novo* methylation	16 (1, 2)	6	5
DNMT3L	Methyltransferase -Regulates DNMT3A and DNMT3B	10 (0, 0)	6	6
TET1	Methylated CpG modification - Demethylation pathway	27 (5, 4)	4	6
TET2	Methylated CpG modification - Demethylation pathway	16 (2, 5)	6	5
TET3	Methylated CpG modification -Demethylation pathway -Regulates DNMT3A	14 (0, 3)	4	6
MECP2	Methyl-binding protein -Binds various forms of methylated DNA -Promotes transcriptional silencing/activation	15 (2, 2)	6	2
MBD2	Methyl-binding protein -Involved in demethylation/DNA repair -Regulates gene expression	13 (3, 0)	7	7
MBD4	Methyl-binding protein -Regulates gene expression	14 (5, 0)	6	6

[a]Numbers in parenthesis denote the number of CpG sites located in promoters and enhancers, respectively.

TABLE 2 Associations between war trauma, maternal GMM, and newborn birthweight.

Association	β	R^2	*p* value
War trauma and birthweight	−0.22	0.40	0.0009
War trauma and maternal GMM	−0.013	0.34	0.0029
Maternal GMM and birthweight	7.2	0.20	0.0259
Chronic stress and birthweight	0.082	0.46	0.0003

(n = 348,252) in that sample. Methylation PCs were created to empirically capture the shared methylation signal of inter-correlated CpG sites at each gene (Table 1). These PCs allow for trends in each individual gene to be captured, such as groups of CpG sites in a particular region of the gene changing together. Methylation at each CpG site was also tested to determine if individual sites were important and to compare with the results using methylation PCs.

3.2 | Pairwise associations of maternal stress, GMM, and birthweight

Significant associations were detected between war trauma, maternal GMM, and newborn birthweight, with each pairwise comparison being associated (Table 2). As war trauma increases, maternal GMM, and birthweight both decrease. This result confirms our earlier findings using a simpler measure of maternal stress based on an additive model of the stress-related questions (Rodney & Mulligan, 2014). Chronic stress was also found to be associated with birthweight, but not maternal GMM (Table 2). No significant associations were found with cord

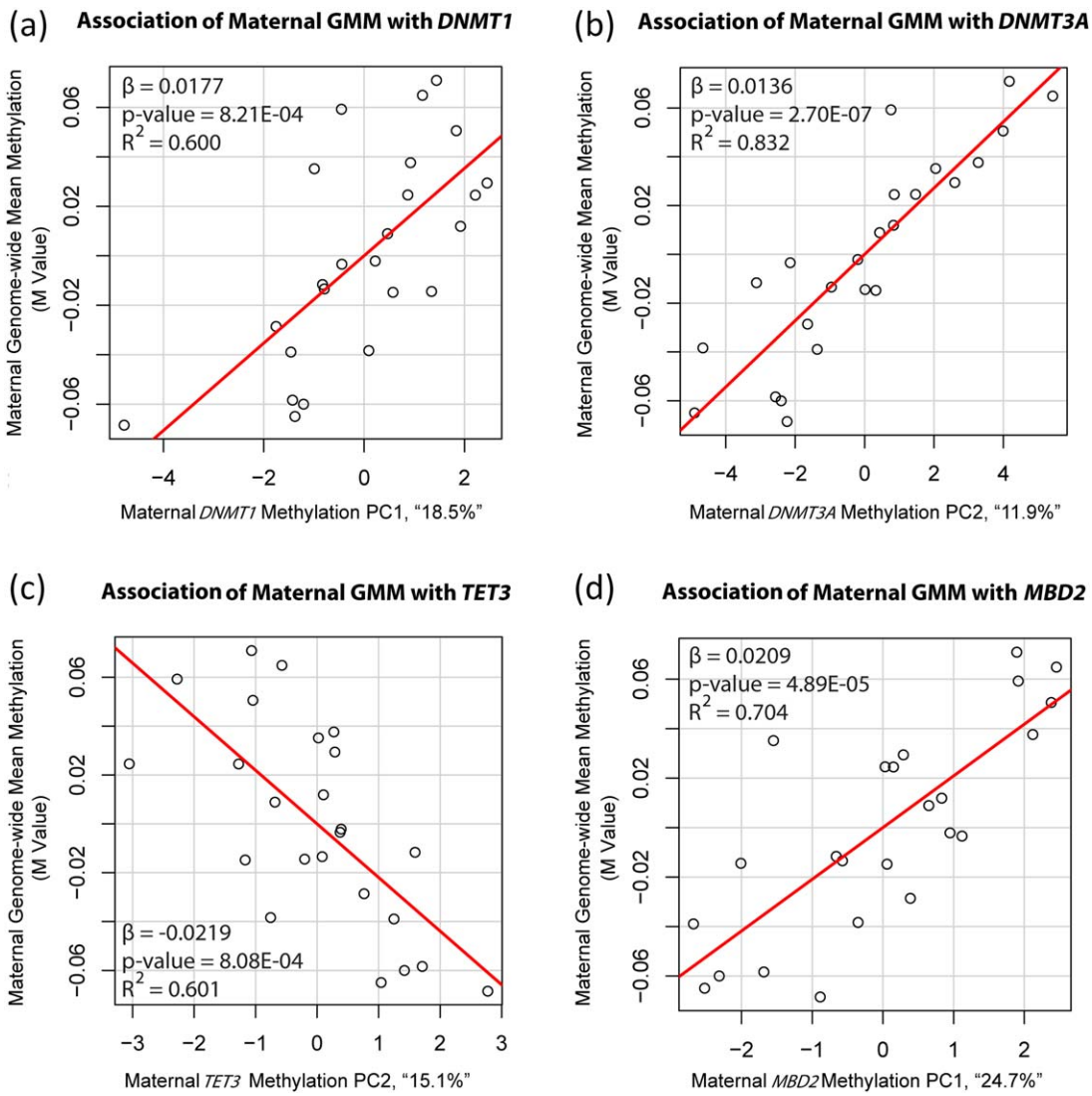

FIGURE 1 Partial regression plots of significant associations between gene-based methylation principal components (PCs; *X* axis) and genome-wide mean methylation (GMM; *Y* axis) in maternal venous blood. The percent variance in methylation explained by the graphed PC is indicated in quotes on the *X* axis. Beta and *p* values represent a model that includes cell composition PCs while R^2 is the variance explained in a simple association. The Bonferroni threshold for significance is based on the number of maternal methylation PCs tested, that is, 0.05/52 maternal methylation PCs = 9.62E−04. PC, principle component; GMM, genome-wide mean methylation

GMM and either maternal stress measure or birthweight, consistent with our previously published results (Rodney & Mulligan, 2014).

3.3 | Association of genetic variants, DNA methylation, and birthweight

Genetic data on the mothers were generated for six of the genes of interest, specifically 14 single nucleotide polymorphisms (SNPs) for *DNMT1*, 56 for *DNMT3A*, 39 for *DNMT3B*, 36 for *DNMT3L*, 52 for *MBD2*, and 4 for *MBD4*. Each SNP was tested individually for association with maternal GMM, cord GMM, or birthweight using hierarchical linear regression. After multiple testing correction, one significant association was seen in relation to cord GMM (rs12462004 in an intergenic region), suggesting that the majority of tested genetic variants do not affect genome-wide methylation or birthweight in our dataset.

The possibility of allele-specific methylation was explored by testing the SNPs against methylation PCs and methylation at individual CpG sites. After multiple-testing correction, no significant associations were seen between individual SNPs at *DNMT1*, *DNMT3A*, *DNMT3B*, *DNMT3L*, *MBD2*, or *MBD4* and either methylation measure, suggesting that the tested genetic variants are not affecting DNA methylation at these genes in our dataset.

3.4 | Association of methylation at methylation genes with maternal stress, GMM, and birthweight

The relationship between methylation at methylation genes and GMM was assessed by testing maternal methylation PCs and individual CpG sites at each gene for association with maternal GMM as well as testing cord methylation PCs and individual CpG sites at each gene for association with cord GMM.

In relation to the methyltransferase genes, maternal GMM was positively associated with the first maternal methylation PC of *DNMT1* and the second maternal methylation PC of *DNMT3A* (Figure 1A,B). When testing individual CpG sites rather than methylation PCs, maternal GMM associated with two maternal CpG sites at *DNMT3A* (Table 3).

Regarding the TET genes, maternal GMM was negatively associated with the second maternal methylation PC of *TET3* (Figure 1C). When testing individual CpG sites, maternal GMM associated with methylation at one CpG site in *TET3* (Table 3).

Regarding the genes that code for methyl-binding proteins, maternal GMM was positively associated with the first methylation PC of *MBD2* (Figure 1D). No association between maternal GMM and methylation at individual CpG sites were found.

TABLE 3 Individual CpG sites associated with maternal GMM

Gene	CpG site	β	*F* value	*p* value[a]	Adj. R^2
DNMT3A	cg25096282	0.185	48.8272	1.59E−06	0.739
DNMT3A	cg03314052	0.242	22.1062	1.78E−04	0.566
TET3	cg02237855	0.209	30.7598	2.89E−05	0.643

[a]Bonferroni threshold = 0.05/211 CpG sites = 2.37E−04.

After multiple testing correction, no significant associations were seen with cord GMM. Promoter and enhancer methylation PCs were created for the genes with at least three CpGs in those categories and tested for association with GMM, but no significant results were found in maternal venous or newborn cord blood. Furthermore, all methylation PCs and individual CpG sites were tested individually for association with chronic stress, war trauma, infant sex, or birthweight using hierarchical linear regression, but no significant associations remained after multiple testing correction.

3.5 | Methylation PC characterization

In order to identify the CpG sites captured in the methylation PCs, the factor loadings of each methylation PC that showed a significant association with maternal GMM (see above) were used to determine which CpG sites were associated with that PC using statistical hypothesis testing.

Regarding the methyltransferase genes, six CpG sites were associated with maternal methylation PC1 of *DNMT1* and 11 CpG sites were associated with maternal methylation PC2 of *DNMT3A* (Supporting Information Table S1). As expected, the three individual CpG sites that associated with maternal GMM (Table 3) were included in the CpG sites associated with the significant methylation PCs (Supporting Information Table S1). All CpG sites associated with methyltransferase genes loaded positively onto the associated methylation PCs with one exception (Supporting Information Table S1). A positive loading means that maternal GMM and methylation at the PC-associated CpG sites increase or decrease together.

In relation to the TET genes, four CpG sites were associated with maternal methylation PC2 of *TET3*; two factors loaded in a positive direction and two factors loaded in a negative direction (Supporting Information Table S1). Lastly, six CpG sites associated with maternal methylation PC1 of *MBD2* in a positive direction.

4 | DISCUSSION

As part of an ongoing study in the Democratic Republic of Congo, our group is investigating the effects of maternal stress on newborn health outcomes and possible associations with maternal or newborn DNA methylation. Previously, we found genome-wide changes in maternal methylation and gene-specific changes in newborn cord methylation that correlated with maternal stress (Rodney & Mulligan, 2014). We hypothesize that genome-wide changes in DNA methylation may be related to changes in the genes responsible for establishing, maintaining, and altering DNA methylation in cells. Thus, in this study, we assay DNA methylation and genetic variation in the methylation complex genes, reasoning that changes in methylation or genetic variants might alter the expression of the methylation complex genes.

We first assayed genetic variants to ensure that no variants in our dataset were associated with methylation of the tested genes or genome-wide methylation or were independently associated with maternal stress or newborn birthweight. Only one SNP showed a significant association, with cord GMM, but cord GMM showed no

subsequent associations and we report no further results regarding cord methylation.

With respect to the methylation data, we found associations between maternal GMM and methylation at *DNMT1*, *DNMT3A*, *TET3*, and *MBD2* in maternal venous blood. The new associations are consistent with the known biological role of these gene products in establishing, maintaining and altering DNA methylation patterns.

Decreased maternal GMM was found to associate with decreased maternal methylation PC1 of *DNMT1* and PC2 of *DNMT3A* (Figure 1A, B). *DNMT1* produces one of the primary enzymes that reproduces methylation patterns during DNA replication in adult cells, thus *DNMT1* is a strong candidate gene to be involved in a genome-wide change in methylation. DNMT3A has also been shown to be necessary in adults for maintaining methylation patterns during cell division as well as other cellular functions (Feng et al., 2010; Sharma et al., 2011). Our results suggest an association between increased maternal stress, decreased maternal GMM, and decreased maternal methylation at *DNMT3A*. This finding is interesting in light of the fact that, in mouse models, exposure to stress hormones has been previously linked to decreased *DNMT3A* expression and genome-wide hypomethylation compared to unexposed mice (Bose et al., 2015). We do not have expression data on *DNMT3A* in our study. However, enrichment analysis of the CpG sites in PC2 does not show an over-representation of promoters (data not shown), meaning that the traditional association between decreased promoter methylation and increased expression cannot be assumed. Thus, based on the results of Bose et al. (2015), we can speculate that the observed changes in *DNMT3A* methylation may be related to the genome-wide signal of reduced methylation we see in association with maternal war stress.

Decreased maternal GMM also associated with increased maternal methylation of PC2 for *TET3* (Figure 1C). TET3 has been shown to regulate DNMT3A activity and has been implicated as a mediator for the effects of stress hormones on genome-wide methylation in the brains of mice (Bose et al., 2015). Maternal GMM also associated positively with maternal methylation PC1 of *MBD2* (Figure 1D). As a methyl-binding protein, MBD2 is believed to affect gene regulation (Laget et al., 2014; Weaver et al., 2014) and, according to some reports, may be able to directly demethylate CpG sites as well (Detich et al., 2002). Thus, all four genes whose methylation was found to associate with maternal GMM have roles in maintaining and altering DNA methylation and two genes have been linked to genome-wide hypomethylation due to stress hormones in mouse models.

We cannot determine the causality or directionality of our observed associations between genome-wide methylation and methylation at the four genes identified in our study (*DNMT1*, *DNMT3A*, *TET3*, and *MBD2*). In other words, we do not know if altered methylation and expression of the four genes directly impacts genome-wide methylation, or if genome-wide methylation is reduced by other genes or processes and the methylation of our four genes is caught up in that larger effect. The genes we tested are those most strongly implicated in DNA methylation and most expected to impact genome-wide methylation. Specifically, we would predict that *DNMT1* would be involved in any genome-wide methylation changes as DNMT1 has long been identified as the primary methyltransferase in maintaining genome-wide methylation (Cai et al., 2017; Majumder et al., 2006). However, it is difficult to know how the changes in methylation we detected impact gene expression since no studies have investigated the relationship between methylation and expression at these genes. Recent studies have shown that methylation at nonpromoter regions can lead to either increased or decreased gene expression depending on the gene and region (Jones, 2012; Plongthongkum, Diep, & Zhang, 2014; Wagner et al., 2014; Yang et al., 2014). Thus, additional studies are needed to determine how gene expression is affected by the methylation changes we detect in *DNMT1*, *DNMT3A*, *TET3*, and *MBD2*.

Association between maternal GMM and gene methylation was only seen in *DNMT1* and *MDB2* when using PCA, that is, no individual CpG sites in *DNMT1* or *MDB2* were associated with maternal GMM. Methylation PCs were created to empirically summarize methylation at and near each gene of interest. The data-driven nature of the technique is particularly useful when studying DNA methylation because, while some CpG sites have been found to be individually significant (Hashimoto, Oreffo, Gibson, Goldring, & Roach, 2009), often multiple CpG sites seem to work in concert (Amabile et al., 2016; Hata & Sakaki, 1997). As such, it can be hypothesized that the cell may process methylation signals in a variety of ways, which may be reflected in different methylation PCs. We used statistical hypothesis testing to determine which CpG sites were significantly associated with each methylation PC (Supporting Information Table S1). As expected, the two CpG sites in *DNMT3A* and one in *TET3* whose methylation associated individually with maternal GMM were also identified as significantly associated with the corresponding PCs associated with maternal GMM. Individual CpG sites explained a lower proportion of GMM variance than their corresponding methylation PCs (data not shown). Thus, methylation PCs enabled more significant genes to be detected and explained a higher proportion of GMM variance relative to individual CpG sites.

Some limitations should be noted when considering our findings. First, our sample size of 25 mother–newborn dyads (with methylation data on 24 maternal and 22 cord blood samples) is small. However, the fact that we find significant p values after strict Bonferroni correction, as well as other methylation studies with sample sizes between 20 and 45 (Cao-Lei et al., 2014; Kinnally, 2014; McGowan et al., 2009; van Dongen et al., 2014), suggests that our sample size may be sufficient, at least to detect the strongest biological relationships. Second, the Illumina 450 K chip used in our study cannot differentiate between DNA methylation and DNA hydroxymethylation, which means the two types of methylation are confounded. However, levels of hydroxymethylation outside the central nervous system are very low, suggesting that hydroxymethylation may not play a significant role in gene regulation in venous blood (Globisch et al., 2010).

In this study, we report associations between genome-wide mean methylation (GMM) and methylation at four methylation complex genes in maternal venous blood. Similar associations were not seen in newborn cord blood. Previously, we reported associations between maternal stress and GMM in maternal venous blood that were also not seen in newborn cord blood (Rodney & Mulligan, 2014). It is interesting to speculate on the implications, and possible biological effects, of this

specific set of results. Our finding of a change in methylation across the maternal genome, that is, GMM, represents a substantial biological effect as it comprises methylation changes at >600 genes out of the 21,000 genes in the human genome (Mulligan, 2012). Furthermore, >99% of those genes showed a decrease in methylation thus revealing a uniform reduction in genome methylation. Such a widespread effect on the maternal genome could reflect a generalized response to stress in mothers, who are directly experiencing the stressors, compared to their newborns. A net reduction in methylation could result in dysregulation of multiple regions across the genome. Such dysregulation, in response to maternal stress, could have both adaptive and maladaptive effects. An adaptive response might enable secondary promoters or cryptic splice sites, which would normally be suppressed by methylation (Yang et al., 2014), to be used, thus enabling a wider variety of proteins to be made available in order to respond to the stressor. Our finding of an association between maternal GMM and methylation at four methylation complex genes suggests that those four genes might be involved in an intentional decrease in methylation in response to stress or an attempt to reestablish regulation after the GMM change. Alternatively, the dysregulation might simply result in wastage and higher energy usage as unwanted proteins are produced. Regardless, GMM represents an important phenotype for future studies of the biological response to stress.

It is also interesting to speculate on why we do not find the same maternal stress–GMM–methylation of methylation complex genes associations in newborn cord blood. Although maternal stress hormones can pass through the placental barrier, the fetus' exposure to maternal stress hormones, and general exposure to maternal stressors, will be muted in comparison to the mother's exposure. Thus, we might predict that cord blood would show a diminished genome-wide response to maternal stress in comparison to maternal blood, as we have found. Furthermore, the lack of an association between maternal war trauma and cord GMM is consistent with DOHaD theory insofar as a change in GMM represents a generalized response across the entire genome. In contrast, DOHaD would predict that the newborn would make more focused changes, most likely through specific genes, to influence optimal phenotypes later in life, such as the changes to *NR3C1* methylation reported in our previous study (Mulligan et al., 2012).

In sum, our results suggest an association between methylation at four methylation complex genes, GMM, and maternal stress that is present in the mothers directly experiencing the stressor, but not their newborns. We propose that further study of GMM, as well as contrasting genome-wide versus gene-specific changes, will help illuminate the molecular and biological responses to stress. Our study provides a short list of genes to be included in future studies of the stress response as well as offers possible insight into the underlying mechanism(s) involved in that response.

ACKNOWLEDGMENTS

This project was supported by NSF Grant # BCS 1231264, a UF Research Opportunity Seed Fund award and a UF College of Liberal Arts and Science award. We gratefully acknowledge the participation of the women of the Democratic Republic of the Congo and our excellent collaborators, staff and research facilities at HEAL Africa, Goma, DRC. C.J.C. designed the study, conducted all data analyses, interpreted results, and co-wrote the manuscript, D.A.H. processed the Illumina 450K data, N.C.R. collected the samples and ethnographic/survey stress data, D.A.K. constructed the stress measures, and C.J.M. oversaw the project, interpreted the results, and co-wrote the manuscript. All Illumina Methylation450K array data have been deposited in Gene Expression Omnibus (GEO) under accession number GSE54399.

ORCID

Christopher J. Clukay http://orcid.org/0000-0002-4310-6484

REFERENCES

Amabile, A., Migliara, A., Capasso, P., Biffi, M., Cittaro, D., Naldini, L., & Lombardo, A. (2016). Inheritable silencing of endogenous genes by hit-and-run targeted epigenetic editing. *Cell*, *167*, 219–232. https://doi.org/10.1016/j.cell.2016.09.006

Aryee, M. J., Jaffe, A. E., Corrada-Bravo, H., Ladd-Acosta, C., Feinberg, A. P., Hansen, K. D., & Irizarry, R. A. (2014). Minfi: A flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics*, *30*, 1363–1369. https://doi.org/10.1093/bioinformatics/btu049

Bakulski, K. M., Feinberg, J. I., Andrews, S. V., Yang, J., Brown, S., McKenney, S. L., … Fallin, M. D. (2016). DNA methylation of cord blood cell types: Applications for mixed cell birth studies. *Epigenetics*, *11*, 1–9. https://doi.org/10.1080/15592294.2016.1161875

Barfield, R. T., Almli, L. M., Kilaru, V., Smith, A. K., Mercer, K. B., Duncan, R., … Conneely, K. N. (2014). Accounting for population stratification in DNA methylation studies. *Genetic Epidemiology*, *38*, 231–241. https://doi.org/10.1002/gepi.21789

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, *57*, 289–300. https://doi.org/10.2307/2346101

Boas, F. (1910). *Changes in bodily form of descendants of immigrants*. Washington: Government Print Office.

Bose, R., Spulber, S., Kilian, P., Heldring, N., Lonnerberg, P., Johnsson, A., … Ceccatelli, S. (2015). Tet3 mediates stable glucocorticoid-induced alterations in DNA methylation and Dnmt3a/Dkk1 expression in neural progenitors. *Cell Death & Disease*, *6*, e1793. https://doi.org/10.1038/cddis.2015.159

Brøns, C., Jacobsen, S., Nilsson, E., Rönn, T., Jensen, C. B., Storgaard, H., … Vaag, A. (2010). Deoxyribonucleic acid methylation and gene expression of PPARGC1A in human muscle is influenced by high-fat overfeeding in a birth-weight-dependent manner. *The Journal of Clinical Endocrinology & Metabolism*, *95*, 3048–3056. https://doi.org/10.1210/jc.2009-2413

Brunet, A., Weiss, D. S., Metzler, T. J., Best, S. R., Neylan, T. C., Rogers, C., … Marmar, C. R. (2001). The peritraumatic distress inventory: A proposed measure of PTSD criterion A2. *American Journal of Psychiatry*, *158*, 1480–1485. https://doi.org/10.1176/appi.ajp.158.9.1480

Cai, Y., Tsai, H.-C., Yen, R.-W. C., Zhang, Y. W., Kong, X., Wang, W., … Baylin, S. B. (2017). Critical threshold levels of DNA methyltransferase 1 are required to maintain DNA methylation across the genome

in human cancer cells. *Genome Research*. *27*, https://doi.org/10.1101/gr.208108.116

Cao-Lei, L., Massart, R., Suderman, M. J., Machnes, Z., Elgbeili, G., Laplante, D. P., … King, S. (2014). DNA methylation signatures triggered by prenatal maternal stress exposure to a natural disaster: Project ice storm. *PLoS ONE*, *9*, e107653. https://doi.org/10.1371/journal.pone.0107653

Chen, Y-A., Lemire, M., Choufani, S., Butcher, D. T., Grafodatskaya, D., Zanke, B. W., … Weksberg, R. (2013). Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. *Epigenetics*, *8*, 203–209. https://doi.org/10.4161/epi.23470

Consortium T. G. P. (2012). An integrated map of genetic variation from 1,092 human genomes. *Nature*, *491*, 56–65. https://doi.org/10.1038/nature11632

Detich, N., Theberge, J., & Szyf, M. (2002). Promoter-specific activation and demethylation by MBD2/demethylase. *Journal of Biological Chemistry*, *277*, 35791–35794. https://doi.org/10.1074/jbc.C200408200

Du, P., Zhang, X., Huang, C.-C., Jafari, N., Kibbe, W., Hou, L., & Lin, S. (2010). Comparison of Beta-value and *M*-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinformatics*, *11*, 587–595. https://doi.org/10.1186/1471-2105-11-587

Fatemi, M., Hermann, A., Gowher, H., & Jeltsch, A. (2002). Dnmt3a and Dnmt1 functionally cooperate during *de novo* methylation of DNA. *European Journal of Biochemistry*, *269*, 4981–4984. https://doi.org/10.1046/j.1432-1033.2002.03198.x

Feng, J., Zhou, Y., Campbell, S. L., Le, T., Li, E., Sweatt, J. D., … Fan, G. (2010). Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nature Neuroscience*, *13*, 423–430. https://doi.org/10.1038/nn.2514

Frisancho, A. R. (2009). Developmental adaptation: Where we go from here. *American Journal of Human Biology*, *21*, 694–703. https://doi.org/10.1002/ajhb.20891

Globisch, D., Münzel, M., Müller, M., Michalakis, S., Wagner, M., Koch, S., … Carell, T. (2010). Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. *PLoS ONE*, *5*, e15367. https://doi.org/10.1371/journal.pone.0015367

Hashimoto, K., Oreffo, R. O. C., Gibson, M. B., Goldring, M. B., & Roach, H. I. (2009). DNA de-methylation at specific CpG sites in the IL1B promoter in response to inflammatory cytokines in human articular chondrocytes. *Arthritis & Rheumatism*, *60*, 3303–3313. https://doi.org/10.1002/art.24882

Hata, K., & Sakaki, Y. (1997). Identification of critical CpG sites for repression of L1 transcription by DNA methylation. *Gene*, *189*, 227–234. https://doi.org/10.1016/S0378-1119(96)00856-6

Houseman, E. A., Accomando, W. P., Koestler, D. C., Christensen, B. C., Marsit, C. J., Nelson, H. H., … Kelsey, K. T. (2012). DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics*, *13*, 1–16. https://doi.org/10.1186/1471-2105-13-86

Hu, Y.-G., Hirasawa, R., Hu, J.-L., Hata, K., Li, C.-L., Jin, Y., … Xu, G.-L. (2008). Regulation of DNA methylation activity through Dnmt3L promoter methylation by Dnmt3 enzymes in embryonic development. *Human Molecular Genetics*, *17*, 2654–2664. https://doi.org/10.1093/hmg/ddn165

Jensen Peña, C., Monk, C., & Champagne, F. A. (2012). Epigenetic effects of prenatal stress on 11β-hydroxysteroid dehydrogenase-2 in the placenta and fetal brain. *PLoS ONE*, *7*, e39791. https://doi.org/10.1371/journal.pone.0039791

Jones, P. A. (2012). Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, *13*, 484–492. https://doi.org/10.1038/nrg3230

Kertes, D. A., Kamin, H. S., Hughes, D. A., Rodney, N. C., Bhatt, S., & Mulligan, C. J. (2016). Prenatal maternal stress predicts methylation of genes regulating the hypothalamic–pituitary–adrenocortical system in mothers and newborns in the Democratic Republic of Congo. *Child Development*, *87*, 61–72. https://doi.org/10.1111/cdev.12487

Kinnally, E. L. (2014). Epigenetic plasticity following early stress predicts long-term health outcomes in rhesus macaques. *American Journal of Physical Anthropology*, *155*, 192–199. https://doi.org/10.1002/ajpa.22565

Kohli, R. M., & Zhang, Y. (2013). TET enzymes, TDG and the dynamics of DNA demethylation. *Nature*, *502*, 472–479. https://doi.org/10.1038/nature12750

Laget, S., Miotto, B., Chin, H. G., Estève, P.-O., Roberts, R. J., Pradhan, S., & Defossez, P.-A. (2014). MBD4 cooperates with DNMT1 to mediate methyl-DNA repression and protects mammalian cells from oxidative stress. *Epigenetics*, *9*, 546–556. https://doi.org/10.4161/epi.27695

Li, Z., Dai, H., Martos, S. N., Xu, B., Gao, Y., Li, T., … Wang, Z. (2015). Distinct roles of DNMT1-dependent and DNMT1-independent methylation patterns in the genome of mouse embryonic stem cells. *Genome Biology*, *16*, 1–15. https://doi.org/10.1186/s13059-015-0685-2

Majumder, S., Ghoshal, K., Datta, J., Smith, D. S., Bai, S., & Jacob, S. T. (2006). Role of DNA methyltransferases in regulation of human ribosomal RNA gene transcription. *Journal of Biological Chemistry*, *281*, 22062–22072. https://doi.org/10.1074/jbc.M601155200

McGowan, P. O., Sasaki, A., D'alessio, A. C., Dymov, S., Labonte, B., Szyf, M., … Meaney, M. J. (2009). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nature Neuroscience*, *12*, 342–348. https://doi.org/10.1038/nn.2270

Mulligan, C. J. (2012). *Epigenetic alterations and stress among new mothers and newborns in the Democratic Republic of Congo*. Paper presented at the Florida Genetics Symposium, Gainesville, FL, USA.

Mulligan, C. J., D'errico, N. C., Stees, J., & Hughes, D. A. (2012). Methylation changes at NR3C1 in newborns associate with maternal prenatal stress exposure and newborn birth weight. *Epigenetics*, *7*, 853–857. https://doi.org/10.4161/epi.21180

Murgatroyd, C., Quinn, J. P., Sharp, H. M., Pickles, A., & Hill, J. (2015). Effects of prenatal and postnatal depression, and maternal stroking, at the glucocorticoid receptor gene. *Translational Psychiatry*, *5*, e560. https://doi.org/10.1038/tp.2014.140

Nätt, D., Johansson, I., Faresjö, T., Ludvigsson, J., & Thorsell, A. (2015). High cortisol in 5-year-old children causes loss of DNA methylation in SINE retrotransposons: A possible role for ZNF263 in stress-related diseases. *Clinical Epigenetics*, *7*, 91. https://doi.org/10.1186/s13148-015-0123-z

Plongthongkum, N., Diep, D. H., & Zhang, K. (2014). Advances in the profiling of DNA modifications: Cytosine methylation and beyond. *Nature Review of Genetics*, *15*, 647–661. https://doi.org/10.1038/nrg3772

Pohodich, A. E., & Zoghbi, H. Y. (2015). Rett syndrome: Disruption of epigenetic control of postnatal neurological functions. *Human Molecular Genetics*, *24*, R10–R16. https://doi.org/10.1093/hmg/ddv217

Ponomarev, I., Wang, S., Zhang, L. L., Harris, R. A., & Mayfield, R. D. (2012). Gene coexpression networks in human brain identify epigenetic modifications in alcohol dependence. *Journal of Neuroscience*, *32*, 1884–1897. https://doi.org/10.1523/JNEUROSCI.3136-11.2012

R Core Team. (2015). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org/

Rahmani, E., Shenhav, L., Schweiger, R., Yousefi, P., Huen, K., Eskenazi, B., … Halperin, E. (2017). Genome-wide methylation data mirror

ancestry information. *Epigenetics & Chromatin*, *10*, 1. https://doi.org/10.1186/s13072-016-0108-y

Rodney, N. C., & Mulligan, C. J. (2014). A biocultural study of the effects of maternal stress on mother and newborn health in the Democratic Republic of Congo. *American Journal of Physical Anthropology*, *155*, 200–209. https://doi.org/10.1002/ajpa.22568

SAS Institute Inc. (2013). JMP® Genomics (Version 7.0). Cary, NC.

Sharma, S., De Carvalho, D. D., Jeong, S., Jones, P. A., & Liang, G. (2011). Nucleosomes Containing methylated DNA stabilize DNA methyltransferases 3A/3B and ensure faithful epigenetic inheritance. *PLoS Genetics*, *7*, e1001286. https://doi.org/10.1371/journal.pgen.1001286

Teschendorff, A. E., Marabita, F., Lechner, M., Bartlett, T., Tegner, J., Gomez-Cabrero, D., & Beck, S. (2013). A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. *Bioinformatics*, *29*, 189–196. https://doi.org/10.1093/bioinformatics/bts680

van Dongen, J., Ehli, E. A., Slieker, R. C., Bartels, M., Weber, Z. M., Davies, G. E., … Boomsma, D. I. (2014). Epigenetic variation in monozygotic twins: A genome-wide analysis of DNA methylation in buccal cells. *Genes*, *5*, 347–365. https://doi.org/10.3390/genes5020347

Wagner, J., Busche, S., Ge, B., Kwan, T., Pastinen, T., & Blanchette, M. (2014). The relationship between DNA methylation, genetic and expression inter-individual variation in untransformed human fibroblasts. *Genome Biology*, *15*, R37.

Weaver, I. C. G., Hellstrom, I. C., Brown, S. E., Andrews, S. D., Dymov, S., Diorio, J., … Meaney, M. J. (2014). The methylated-DNA binding protein MBD2 enhances NGFI-A (egr-1)-mediated transcriptional activation of the glucocorticoid receptor. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *369*, 20130513. https://doi.org/10.1098/rstb.2013.0513

Yamamoto, H., Fujimori, T., Sato, H., Ishikawa, G., Kami, K., & Ohashi, Y. (2014). Statistical hypothesis testing of factor loading in principal component analysis and its application to metabolite set enrichment analysis. *BMC Bioinformatics*, *15*, 51–60. https://doi.org/10.1186/1471-2105-15-51

Yang, X., Han, H., De Carvalho Daniel, D., Lay Fides, D., Jones Peter, A., & Liang, G. (2014). Gene body methylation can alter gene expression and is a therapeutic target in cancer. *Cancer Cell*, *26*, 577–590. https://doi.org/10.1016/j.ccr.2014.07.028

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Clukay CJ, Hughes DA, Rodney NC, Kertes DA, Mulligan CJ. DNA methylation of methylation complex genes in relation to stress and genome-wide methylation in mother–newborn dyads. *Am J Phys Anthropol*. 2018;165:173–182. https://doi.org/10.1002/ajpa.23341