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Aberrant signature methylome by DNMT1 hot spot mutation in hereditary sensory and autonomic neuropathy 1E

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DNA methyltransferase 1 (DNMT1) is essential for DNA methylation, gene regulation and chromatin stability. We previously discovered DNMT1 mutations cause hereditary sensory and autonomic neuropathy type 1 with dementia and hearing loss (HSAN1E; OMIM 614116). HSAN1E is the first adult-onset neurodegenerative disorder caused by a defect in a methyltransferase gene. HSAN1E patients appear clinically normal until young adulthood, then begin developing the characteristic symptoms involving central and peripheral nervous systems. Some HSAN1E patients also develop narcolepsy and it has recently been suggested that HSAN1E is allelic to autosomal dominant cerebellar ataxia, deafness, with narcolepsy (ADCA-DN; OMIM 604121), which is also caused by mutations in DNMT1. A hotspot mutation Y495C within the targeting sequence domain of DNMT1 has been identified among HSAN1E patients. The mutant DNMT1 protein shows premature degradation and reduced DNA methyltransferase activity. Herein, we investigate genome-wide DNA methylation at single-base resolution through whole-genome bisulfite sequencing of germline DNA in 3 pairs of HSAN1E patients and their gender- and age-matched siblings. Over 1 billion 75-bp single-end reads were generated for each sample. In the 3 affected siblings, overall methylation loss was consistently found in all chromosomes with X and 18 being most affected. Paired sample analysis identified 564,218 differentially methylated CpG sites (DMCs; \( p < 0.05 \)), of which 300,134 were intergenic and 264,084 genic CpGs. Hypomethylation was predominant in both genic and intergenic regions, including promoters, exons, most CpG islands, L1, L2, Alu, and satellite repeats and simple repeat sequences. In some CpG islands, hypermethylated CpGs outnumbered hypomethylated CpGs. In 201 imprinted genes, there were more DMCs than in non-imprinted genes and most were hypomethylated. Differentially methylated region (DMR) analysis identified 5649 hypomethylated and 1872 hypermethylated regions. Importantly, pathway analysis revealed 1693 genes associated with the identified DMRs were highly associated in diverse neurological disorders and NAD+/NADH metabolism pathways is implicated in the pathogenesis. Our results provide novel insights into the epigenetic mechanism of neurodegeneration arising from a hotspot DNMT1 mutation and reveal pathways potentially important in a broad category of neurological and psychological disorders.

Introduction

DNA methylation affects many cellular functions including transcription regulation, cell differentiation, gene imprinting and genome stability.1,2 Mammalian DNA methylation is catalyzed by DNMT1, DNMT3A, and DNMT3B. DNA methyltransferase 1 (DNMT1), locates at chromosome 19p13.3-p13.2, is the maintenance methyltransferase and essential for maintaining proper epigenetic inheritance. DNMT3A and DNMT3B establish new methylation patterns and are considered de novo methyltransferases. The line between maintenance and de novo methyltransferases has become increasingly blurred as studies have linked DNMT1 to de novo methylation3 and DNMT3A/B to maintenance methylation.4 Aberrant DNA methylation

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and other epigenetic defects by either Mendelian or polygenic pathogenesis are increasingly associated with diverse forms of neurological diseases.\textsuperscript{1,5} DNMT3B mutation leading to the partial inactivation of its catalytic domain has been linked to a human developmental disorder, ICF1 (immunodeficiency, centromere instability, and facial anomalies) syndrome.\textsuperscript{6} DNMT3B mutations cause DNA hypomethylation in satellites 2 and 3 and \(\alpha\) repetitive sequences, a major part of constitutive heterochromatin. Recently, we identified heterozygous mutations in DNMT1 targeting sequence (TS) domain causing hereditary sensory and autonomic neuropathy type 1 with dementia and hearing loss (HSAN1E; OMIM 614116).\textsuperscript{8} Subsequently, additional DNMT1 TS domain mutations were found underlying autosomal dominant cerebellar ataxia, deafness and narcolepsy (ADCA-DN, OMIM 604121).\textsuperscript{9} It has been suggested that these two adult-onset neurodegenerative disorders can be considered in the differential of each other as HSAN1E and ADCA-DN patients may share a similar set of phenotypes with varied severity and onset age.\textsuperscript{9} More HSAN1E families have been recognized and a hot spot mutation at amino acid 495 was identified as Y495C mutation is the most common causal mutation found among HSAN1E patients.\textsuperscript{8,11} The DNMT1 Y495C mutation leads to DNMT1 protein misfolding and decreased enzymatic activity. DNMT1 is dispensable for development, cellular functions and cell cycle regulation. Genomic deletion of DNMT1 in mice leads to embryonic death,\textsuperscript{12} and complete deletion of DNMT1 in human cancer cells results in significant loss of global methylation, pronounced chromosomal defects and apoptosis.\textsuperscript{13} Dynamic changes in DNA methylation are also linked to neuronal synaptic stimulation in the brain.\textsuperscript{14} DNMT1 mutations in HSAN1E do not lead to malignancy; instead, they result in the neurodegeneration of peripheral and central nervous systems.\textsuperscript{15}

The aim of the present study was to investigate the genome-wide DNA methylation changes in HSAN1E patients arising from the hotspot DNMT1 mutation Y495C. There are \(-30\) million cytosines preceding guanine nucleotides (CpGs)\textsuperscript{16} in the human genome and the majority of them (\(-80\%) are methylated,\textsuperscript{2} especially at repetitive elements and centromeric satellite repeats, which comprise approximately half of human genome. A small percentage of CpG dinucleotides are clustered within gene promoters as CpG islands (CGI), but normally only \(3\%) of CpG islands are methylated.\textsuperscript{17} The pathogenic role of promoter methylation has been extensively studied especially in cancer, but there is limited understanding on how methylation changes in intergenic regions relate to human disease.

We analyzed CpG methylation at single-base resolution in 3 affected individuals and their age-, gender-matched (\(<5\) y difference) unaffected siblings by whole genome bisulfite sequencing (WGBS). This paired-sample study design allowed us to perform statistical analysis of differential methylation while controlling for extraneous factors, minimizing inter-familial and environmental influences. Furthermore, we investigated the pathways downstream of consequent aberrant DNA methylation that are involved in HSAN1E pathogenesis in relation to other neurological and psychological disorders.

**Results**

Genomic DNA was extracted from peripheral blood B cells of three affected patients carrying the heterozygous Y495C DNMT1 mutation and their unaffected siblings and analyzed by WGBS and bioinformatics approaches as illustrated in Figure 1. Our sequencing results generated approximately one billion single-end reads of 75-bp length for each individual sample. Alignment efficiency was high with 94–95\% of these reads mapped to the human reference genome (hg19; Table 1). Our sequencing results covered \(\sim24\) of \(-30\) million CpGs present in the human genome. The bisulfite conversion rate was assessed using all non-CpG sites with \(>10\)X coverage in the genome, and found to be close to 1 (\(\sim0.999\)), indicating almost complete conversion. On average, 18–19 million of CpG cytosines were obtained with \(>10\)X coverage for each individual genome and 12,035,253 CpG sites had \(>10\)X coverage across all 6 samples (Fig. 2A and B), of which 6,051,917 were in genic (5kb upstream or inside gene body) regions and 5,983,334 were in intergenic (outside of genic regions) regions. The genic CpGs had higher overall methylation (Fig. 2C) than the intergenic CpGs (Fig. 2D). Notably, the affected patients consistently had lower methylation than their paired siblings, particularly in intergenic regions. The methylation of CpG islands (CGI) was low (\(-30\%) across all samples, whereas methylation in repeat regions (Alu, L1, L2, Satellite repeat, and simple repeat) was high (\(\geq70\%\)) (Fig. 2E). All the repeat regions had lower methylation in the affected patients than their unaffected siblings except for CGIs (Fig. 2F).
Genome-wide CpG methylation patterns reflect DNMT1 mutation status and kinship

Previous studies have shown that methylation is associated with gender, age, and individual genetic background, as well as environmental influences. Therefore, we designed our study to compare age- and gender-matched sibling pairs to minimize the impact of potentially confounding variables. To validate this approach, we performed unsupervised clustering using the top 126,000 CpGs in which methylation was most varied across the 6 samples (CpGs with the highest standard deviations). We found that the affected and the unaffected samples formed two distinct clusters (Fig. 3A), suggesting a strong effect of DNMT1 mutation on DNA methylation. Interestingly, when we used the top 10,000 most varied CpGs across the 6 samples for unsupervised clustering, the samples clustered by relationship (Fig. 3B), demonstrating an effect of the overall genetic background. Our results from cluster analysis confirm that it is important to compare the methylation change between gender and age-matched individuals within a family, ideally under similar environmental influence. Also, it indicates that our paired-samples design will likely increase the accuracy in interrogating the impact of DNMT1 mutation on methylation changes of different genomic regions.
Differentially methylated CpGs (DMCs) are mostly hypomethylated

We performed paired t-statistics to identify differentially methylated CpGs or DMCs between 3 affected and 3 unaffected siblings after removing 190,349 CpGs without any variance across samples (constant methylation, non-informative). The mean methylation difference was used to measure the scale of a change along with the differential P value. At P < 0.05, 564,218 DMCs were identified, of which 308,962 had mean ratio difference greater than 0.1 (>10% methylation difference). Noted is that even with paired analysis for increased power, the small sample size would only detect the CpGs with a large difference and low variability. Therefore, we compared the P value distribution from the permutation test with randomly assigned sibling pairs and found that the P values from the true pairs were non-uniform with lower P values shifted to the left side of significant difference (Fig. S1A), whereas the P values from the permutation deviated toward the non-significant side (Fig. S1B). These results indicate that the significant CpGs were not likely random. The majority of the DMCs were hypomethylated in all chromosomes (Fig. 4A). Interestingly, chromosome X (3145 hyper- and 10104 hypo-methylated CpGs) and chromosome 18 (4342 hyper- and 11346 hypo-methylated CpGs) had the largest methylation reduction (Fig. 4B and C), suggesting the impact of DNMT1 mutation is chromosome- and region-specific.

Of the 308,962 DMCs with mean methylation change greater than 10%, there were significantly more in intergenic than in genic regions (182,766 vs. 126,196; chi square test P < 2.2e-16). In genic regions, there were 2-fold more hypomethylated CpGs than hypermethylated CpGs; in intergenic regions, there were 3-fold more hypomethylated CpGs than hypermethylated CpGs (Fig. 5A). These results demonstrate higher prevalence of hypomethylation in the intergenic regions. Although the overall, genome-wide reduction in DNA methylation was moderate between affected and unaffected siblings, a greater reduction (5–10%) was observed around transcription start sites (TSS) (Fig. 5B).

When we assessed the DMC distribution in the genome according to the genomic features such as exons, CGIs, Alu elements, L1, L2, satellite, and simple repeats, we found all but the CGIs to be significantly hypomethylated in both genic (Fig. 5C) and intergenic regions (Fig. 5D).

Differentially methylated regions (DMRs) are mostly intergenic and hypomethylated

DNA methylation often occurs in clusters and it is important to evaluate how the differentially methylated regions are scattered across the genome. For DMR analysis, we included CpGs captured in all 6 samples and used a smoothing approach\(^{10}\) to stabilize the CpG sites with lower than 10X coverage. We designated a DMR as the minimum of 5 CpGs with the mean difference ≥10% between the 3 pairs of affected and unaffected siblings. Across the genome, 7521 significantly changed DMRs were found, of which 5649 were hypomethylated and 1872 were hypermethylated in the affected siblings. The majority of these DMRs (4890, 65%) were intergenic and 2631 (35%) were within genic regions. Additionally, over 82% of the 4890 intergenic DMRs and 62% of the 2631 genic DMRs were hypomethylated, indicating most DMRs showed loss of methylation in the affected siblings (Fig. 6A). The DMR analysis further confirmed more prevalent hypomethylation in the intergenic regions in the affected siblings.

DMR-associated genes are enriched in neurological disorders

To investigate whether the methylation changes resulted from DNMT1 mutation are directly associated with genes enriched in a particular pathway, functional class or disease, we conducted a pathway enrichment analysis for the genes that have identified DMRs within 5kb of their TSS using Ingenuity Pathway Analysis (IPA; see Methods for details). IPA constructs the networks that optimize for both interconnectivity and number of focus genes under the constraint of maximal network size.\(^{10}\) The top three significantly enriched gene interaction networks were “hereditary disorder, metabolic disease, cancer,” “hereditary disorder, neurological disease, cellular assembly, and organization,” and “cancer, cellular development, tumor morphology.” Next, we applied canonical pathway analysis using the IPA library of

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Figure 3. (A) Unsupervised clustering using the top 126,000 varied CpGs shows the affected and unaffected samples form different clusters, suggesting the similar methylation patterns by mutation status. (B) Unsupervised clustering using top 10,000 highly varied CpGs shows samples are clustered by family, indicating the strong genetic influence on DNA methylation.
Eleven canonical pathways were significantly enriched ($P < 0.05$; Table S1). Of particular interest, several of the significant pathways are related to NAD+/NADH metabolism which has been implicated in neurodegeneration.20,21 Finally, we applied disease association functional analysis, which identifies the diseases that are most significant to the gene set. We found that the top three significantly enriched diseases were "neurological diseases" with enrichment p-value between 1.51E-04 to 5E-02 (Table S2), "psychological disorders" ($P = 1.8E$ to 04$-4.98E$-02; Table S3), and "skeletal and muscular disorders" ($P = 1.8E$-04 to 4.77E-02; Table S4) Some of the top neurological diseases were "progressive neuropathy," "Parkinson’s disease," "attention deficit disorders," "attention deficit hyperactivity disorder," "narcolepsy," and "dementia." All these findings are consistent with the typical HSAN1E phenotypes which include progressive neuropathy, dementia, sleep disorder, and personality disorders, indicating that the gene set we identified is associated with pathogenesis of central and peripheral neurodegeneration, and may have important implication in other neurodegenerative disorders.

**CpG methylation in imprinted genes**

Genomic imprinting is an epigenetic regulatory mechanism that turns off expression from either the paternal or the maternal
Figure 5. DMC distribution in different genomic regions. (A) DMCs occur across genomic regions; however, intergenic regions have higher percentage of hypomethylated CpGs than genic regions. (B) Hypomethylation around transcription start site (TSS). All genes are aligned around TSS and average methylation is displayed separately for affected (red) and unaffected (green) subjects. (C) DMCs in different genomic features of genic regions. All regions except CpG islands have more hypo-methylated DMCs in the affected individuals. (D) DMCs in different genomic features of intergenic regions. All have more hypo-methylated DMCs in the affected individuals. X-axis, genomic region; y-axis, percentage of hypermethylated (purple bars) and hypomethylated DMCs (green bars).
CpG methylation in histone modification mark sites

To examine whether differential DNA methylation preferentially occurred in regions normally occupied by specific histone marks and variants, we compared the overall CpG methylation between the affected and unaffected siblings in the regions of 11 histone modifications and variants (H2A.Z, H3K4me1, H3K4me2, H3K4me3, H3K9ac, H3K9me3, H3K27ac, H3K27me3, H3K36me3, H3K79me3, H4K20me1) reported in a lymphoblastoid cell line of a healthy donor (GM12878) by the ENCODE project (https://genome.ucsc.edu/ENCODE/). In addition, we also compared the CTCF (CCCTC-Binding factor) binding sites defined in two replicates of the same cell line (CTCF.rep1 and CTCF.rep2). We found that CpG methylation was reduced in the affected individuals in the regions normally occupied by the repressive histone marks H3K27me3 (4.1% median reduction) and H3K9me3 (1.2% median reduction), as well as CTCF (2.3% median reduction) (Fig. 6B). Increasing evidence indicates that DNA methylation and histone modification pathways work synergistically to regulate chromatin structure and gene expression. DNA methylation may serve as a template for histone modifications, and decreased methylation in the repressive histone marks may lead to interruption of proper heterochromatin state.

CpG methylation in genes associated with mitochondrial function

Mitochondrial genes perform the important function in energy generation for cells. The fact that HSAN1E patients manifest some clinical presentations similar to mitochondrial diseases or the mutation leads to the loss of high energy demanding neurons prompted us to investigate the methylation profile of mitochondrial genes. We identified 666 mitochondrial function-related genes encoded either in the nuclear or the mitochondrial genome (Table S5), and 654 were captured in the data set with 10× coverage. Comparing the mean methylation of these genes between the affected and unaffected samples, we did not see noticeable difference (0.83 vs. 0.84). About 4.1% of CpGs in genes related to mitochondrial function were differentially methylated vs. 4.5% methylated CpGs found in genes unrelated to mitochondrial function. Of the differentially methylated genes annotated to mitochondrial functions, 54.8% was hypomethylated.

Discussion

Accumulating evidence suggests DNMT1 plays multiple critical functions in the nervous system, but the exact molecular mechanism(s) remain unclear. HSAN1E is the first identified adult-onset, monogenic neurodegenerative disease caused by DNMT1 mutations. The disease-causal DNMT1 mutation provides a unique window through which we can investigate specific methylation defects underlying an adult-onset neurodegenerative disorder at the molecular level. The role of DNMT1 is also of fundamental biological interest, as it is the only essential methyltransferase to ensure the fidelity of epigenetic inheritance. Through whole genome methylation evaluation with single base resolution, our study reveals a signature pattern of methylation alteration in this adult onset neurodegenerative disease, characterized by a prevalent hypomethylation in intergenic regions, a significant methylation decrease at surrounding regions of transcription start sites (TSS),
and marked methylation reduction on chromosomes X and 18 (5–10%). Additionally, DNMT1 mutation leads to more hypermethylated DMC in some CpG islands.

Our results provide an interesting contrast yet unique resemblance to a recent study that also used whole genome bisulfite sequencing to assess the methylation profile change in a ICF syndrome patient with DNMT3B mutation. This study showed DNMT3B mutations cause severe hypomethylation across the whole genome (~25% overall reduction) and profound losses in chromosome X (63%). ICF syndrome patients have autosomal recessive inheritance and an average life expectancy of only 8 y. In contrast, our whole genome bisulfite sequencing results showed that the impact of DNMT1 heterozygous mutation on global genome methylation is only modestly changed in 3 affected HSAN1E patients relative to their age- and gender-matched siblings. However, the strong impact of DNMT1 heterozygous mutation is rather exerted in intergenic regions, TSS regions and chromosomes 18 and X. These regions have between 5–10% reductions in methylation, suggesting a targeted and insidious effect of DNMT1 methylation defect. HSAN1E patients have autosomal dominant inheritance; they are normal from birth to early teen years, but then start to develop progressive sensory neuropathy, sensorineural hearing loss and memory loss in young adulthood. The life expectancy of HSAN1E is approximately 50 y and the cause of death is usually related to dementia complications. The ICF syndrome’s early-onset and severe phenotypes are consistent with the drastic methylation decrease while HSAN1E’s adult-onset feature, unique phenotypes and progressive course are linked with region-specific methylation reduction. Our findings suggest DNMT1 heterozygous mutation has minimal effect during development, but has deleterious accumulating impact on the nervous system over a long period of time and specifically on memory formation and sensory neurons.

Because patients with DNMT1 mutations have a syndromic appearance similar to many mitochondrial disorders, we investigated the DMC pattern of mitochondrial function-related genes encoded either in the nuclear or the mitochondrial genome, but did not find significant difference. However, our IPA analysis demonstrated that the 1693 genes associated with DMRs are highly enriched in many neurological and psychological disorders. Alzheimer disease, narcolepsy, and dementia were among the top significantly associated genes. The canonical pathway analysis revealed NAD+/NADH metabolism pathways are implicated in the pathogenesis. NAD+ is a coenzyme involved in redox reactions, expressed ubiquitously and has critical functions in the neural cells. NAD+ has been shown not only important in energy metabolism and mitochondrial functions, but also in calcium homeostasis, aging, and cell death. In animal models, increased nuclear NAD+ has neuroprotective function against the negative effects of both mechanical and chemical insult. Emerging evidence shows NAD+ as an important therapeutic target for the treatment of age-related degenerative diseases, such as Alzheimer disease. Our result indicates that DNMT1 likely impacts a specific set of pathway involving NAD+/NADH metabolism that are particularly imperative in degenerative neurological disorders.

Increasingly epigenetic mechanisms are also being recognized important in sporadic neurological diseases without apparent genetic causes. The high fidelity DNMT1 enzyme activity is critical in epigenetic regulation. Our DNMT1 Mendelian disease model provides a unique and valuable avenue for future research in common varieties of human neurodegenerative disorders. Utilizing a unique paired-sample design, our study showed that even modest reductions in DNMT1 enzyme activity and preferential site-specific intergenic changes of DNA methylation can lead to devastating neurological consequences. Understanding how DNMT1 mutation exerts its deleterious impact in a region- and chromosome- specific manner and how the methylation changes specifically lead to sensory nerve axonal loss and brain atrophy with cognitive decline will require a significant amount of future study. Our work provided first insights into a specific neurodegenerative pathway by identifying a signature genome methylation pattern potentially having broader implications beyond HSAN1E.

Materials and Methods

Study subjects
Since methylation is associated with age and gender, we identified three same-gender, similar aged (<5 y) sibling pairs from the large kindred of European origins (Caucasian) we longitudinally studied in our original publication for whole genome methylation analysis. Three HSAN1 patients with DNMT1 heterozygous Y995C mutation were matched with their normal siblings without the mutation. Their genomic DNA samples were collected from blood and whole genome bisulfite sequencing was performed. The clinical features are very similar between the affected persons in this kindred. They all started to lose their sensory nerve action potentials during young adulthood. By the 3rd-4th decade, prominent sensorineural hearing loss with cognitive and behavioral changes was noted with progressive declines, leading to the requirement of total care, and death often occurred in the 4th-5th decades. Brain imaging and limited autopsy of the affected persons showed global atrophy and a reduced weight of the autopsied brains, spinal cord posterior column loss of axons, and ponsensorial fiber loss in distal nerves. Histopathologic analysis did not reveal any inclusion bodies such as β-amyloid, tau, TDP-43, and α-synuclein. The study protocols were approved by Mayo Clinic Institute Review Boards.

DNA extraction
DNA samples were extracted from patients’ blood. Briefly, the anticoagulated blood samples were centrifuged at 2500 g for 10 min, and the separated buffy coat layers were collected. Then Genome DNA was extract from buffy coat using Qiamp DNA Blood Kit (Qiagen).

Whole genome bisulfite methylation sequencing
10μg genomic DNA was used for bisulfite sequencing library construction according to the standard Illumina Paired-End
protocol. Briefly, genomic DNA was fragmented and adaptors were ligated. DNA samples were then subjected to the bisulfite conversion using EZ DNA Methylation-Gold kit (Zymo), purified using AMPure beads (Agencourt) and amplified by 10 cycles PCR. Sequencing was performed on HiSeq2000 (Illumina) with single-end reads on HiSeq2000 using TruSeq SBS sequencing kit version 3 and HCS v2.0.12 data collection software.

WGBS data analysis
The raw sequence fastq data were processed using our bisulfite sequence analytical pipeline, SAAP-BS (v1) with alignment to human reference genome version 19 (hg19) to obtain CpG site methylation including both methylated and unmethylated cytosine counts and methylation ratio. All CpG sites were extracted. To ensure data quality and reliability, we only included CpG sites with ≥10X coverage in the downstream differential methylation CpG analyses. Overall methylation was measured by mean and median genome wide, by chromosome, or special regions of the genome for each individual or comparison group. DMC detection between affected and unaffected siblings was performed by paired t statistics. The constant CpGs across samples (the methylation ratio is either 1 or 0 in all six samples) were first removed. CpGs with P < 0.05 in paired t test were considered differentially methylated. The distribution of these DMCs in the genome was summarized against various genomic regions including intergenic, genic regions, promoters, repetitive sequences, histone modification, and imprinted genes.

Differential methylation region analysis
Differentially methylated regions (DMRs) between affected and unaffected siblings were identified using the R package “bsseq” as previously described.18 For this detection analysis, we retained all CpG sites that were sequenced in all six samples regardless of coverage. A smoothing approach was used to stabilize the sites with a lower coverage since nearby CpGs tend to have similar methylation patterns.19 The smoothing procedures regresses these CpGs to reduce potential outliers at low-covered CpG sites. T statistics was then performed for each CpG independently and similarly changed CpGs were grouped into DMRs according to specified minimum CpG number and methylation difference between affected and unaffected individuals (minimum of 5 CpGs and at least 10% methylation difference in the DMR region were used).

Pathway/gene enrichment analyses by Ingenuity pathway analysis
For the genes with DMRs within 5kb of TSS, we conducted a pathway, gene network and functional enrichment analysis using IPA (http://www.ingenuity.com/, performed on September 5, 2013), a widely used pathway and system biology interpretation tool20 in order to identify relevant biological and molecular networks. IPA uses a proprietary database created from continuously updated, published, peer-reviewed scientific publications stored in the Ingenuity Pathways Knowledge Base (IPKB). It offers several function modules including Core, Tox, Metabolomics, Comparison, Biomarker Filter, and microRNA Target Analysis. The Core Analysis was used for this data, which includes the enrichment for canonical pathway, disease/biological function, toxicological function and gene-gene interaction networks. The enrichment P value is calculated using the right-tailed Fisher Exact Test, representing the likelihood of the association between a set of focus genes and a given process or pathway is due to random chance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/29767

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