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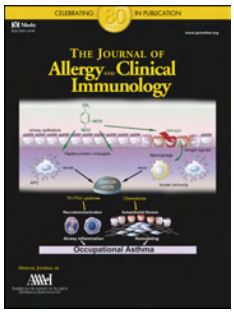

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Inhalation of diesel exhaust and allergen alters human bronchial epithelium DNA methylation

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Abstract

Background: Allergic disease affects 30-40% of the world's population and its development is determined by interplay between environmental and inherited factors. Air pollution, primarily consisting of diesel exhaust emissions, has increased at a similar rate to allergic disease. Exposure to diesel exhaust may play a role in the development and progression of allergic disease, in particular allergic respiratory disease. One potential mechanism underlying the connection between air pollution and increased allergic disease incidence is DNA methylation, an epigenetic process with the capacity to integrate gene/environment interactions. **Objective**: To investigate the effect of allergen and diesel exhaust exposure on bronchial epithelial DNA methylation. **Methods**: We performed a randomized crossover-controlled exposure study to allergen and diesel exhaust in humans, and measured single site (CpG) resolution global DNA methylation in bronchial epithelial cells. **Results:** Exposure to allergen alone, diesel exhaust alone, or allergen and diesel exhaust together (co-exposure) led to significant changes in 7 CpG sites at 48 hours. However, when the same lung was exposed to allergen and diesel exhaust but separated by approximately four weeks, significant changes in over 500 sites were observed. Furthermore, sites of differential methylation differed depending on which exposure was experienced first. Functional analysis of differentially methylated CpG sites found genes involved in transcription factor activity, protein metabolism, cell adhesion and vascular development, among others. **Conclusion:** These findings suggest that specific exposures can prime the lung for changes in DNA methylation induced by a subsequent insult.

Key Messages

- Appreciation for the role of DNA methylation in mediating immunologic effects of environmental exposures is growing.
- We used a controlled human exposure to diesel exhaust and allergen, each alone or in combination, followed by airway (conducting airway epithelium) sampling and global assessment of DNA methylation, to interrogate epigenetic changes due to these acute exposures.
- Exposure to diesel exhaust and allergen each incur risk for DNA methylation changes upon subsequent exposure, and the order of such exposures determines both unique and overlapping epigenetic signals.

Capsule summary

Combined diesel exhaust and allergen is known to intensify the airway immune response, relative to either exposure alone. We demonstrated that DNA methylation of epithelial cells might be a mechanism underpinning such synergy.

Keywords: Allergen, particulate matter, diesel exhaust, air pollution, epigenetics, DNA methylation

Abbreviations:

DEP, diesel exhaust particles; Th2, T helper type 2; Th17, T helper type 17; IgE, Immunoglobulin E; DE, diesel exhaust.

Introduction

Allergic disease is increasing worldwide with approximately 30-40% of the world's population now affected[1]. The development of allergic disease is determined by an interaction between genetic and environmental factors[2]. Air pollution is one of the major environmental factors impacting the development and exacerbation of allergic diseases, in particular respiratory diseases including asthma and allergic rhinitis[3].

Diesel exhaust (DE) contributes the majority of particulate matter present in urban air pollution[4], and diesel exhaust particles (DEP) reach the alveolar/gas exchange regions[5]. DE increases bronchial inflammation and airways resistance in healthy subjects, while short-term exposure to diesel traffic reduces airway function in asthmatic patients[6]. Exposure to DE plays a major role in the development and progression of allergies[4, 5]. For example, high DEP exposure is associated with more frequent asthma symptoms in children with allergic asthma[7]. Interestingly, co-exposure to allergen and DE(P) increases allergen specific IgE, asthma severity, airway inflammation, and airway responsiveness in humans or in mouse models[7-9].

The molecular mechanisms responsible for the synergy between DE and allergen remain unclear. As mutations occur too slowly to explain the recent rapid increase in allergy associated disease, epigenetics is a potential mechanism by which gene-environment interactions may rapidly influence disease incidence and progression[10]. DNA methylation was among the first epigenetic mechanisms to be identified[11] and is the most extensively studied in relationship to disease[12] and human populations[13]. It is the potentially reversible addition of a methyl group to DNA cytosine residues, primarily where a cytosine is followed directly by a guanine (CpG sites)[14]. Consistent with epigenetics serving as a potential mediator between environment and genome, differential DNA methylation of a number of genes involved in multiple cellular processes including immune responses[15-17], nitric oxide synthesis[18, 19], and DNA binding[16], has been associated with air pollution exposure. We have previously shown controlled exposure to DE alters the methylation of CpG sites in circulating mononuclear cells[20]. Similarly, exposure to ambient pollution is associated with increased DNA methylation and decreased expression of the forkhead box protein 3 (Foxp3) and interferon gamma (IFNγ) genes in regulatory and effector T cells respectively, leading to impaired regulatory T cell function and associated increased asthma morbidity[15, 21].

Studies of mechanisms underlying the interaction between allergy and DE exposure have been limited to animals or performed as observational studies (rather than controlled exposure studies), investigation of candidate targets rather than global alterations, or of easily accessible tissues such as blood[20], rather than sampling the primary site of exposure, the lung. Here we tested the hypothesis that exposure to allergen and/or diesel exhaust would alter DNA methylation in bronchial epithelial cells by performing a randomized crossover controlled exposure study to allergen and DE in humans. We collected bronchial epithelial cells, the primary cell type exposed to inhalants, and assessed changes in global DNA methylation in response to exposure. We report that exposure to allergen, DE, or allergen and DE as a co-exposure had modest effects on DNA methylation when assessed at 48 hours post-exposure. However, exposure to both allergen and DE, with the exposures separated by four weeks, was strikingly different, significantly altering bronchial epithelial cell global DNA methylation. These results support the hypothesis that DNA methylation can serve as a molecular mechanism underlying the interaction between allergens and particulate air pollution on respiratory health.

Methods

Study Demographics

Seventeen Caucasian participants (See Table 1 and Table 2) were recruited to the Air Pollution Exposure Laboratory (APEL) in Vancouver, British Columbia, Canada. Written consent was obtained from all subjects, and the protocols were approved by the institutional review board for human studies at the University of British Columbia. Participants were 20 to 46 years old (median = 27, SD = 7.8), all non-smokers and 47% were asthmatic (Table 1). We excluded individuals with any of the following: (1) pregnancy/breastfeeding, (2) use of inhaled corticosteroids, (3) regular use of bronchodilator medication (*i.e.*, use of bronchodilators more than 3 times per week), (4) unstable asthma symptoms, (5) any use of vitamins A, C, E or other antioxidant supplements, (6) co-morbid conditions judged by the investigators to increase risk of dropout, or (7) work in an industrial setting or other setting of significant inhaled exposures. Males (n=7) and females (n=10) were included. Sensitization to birch, Timothy/Pacific grass and house dust mite (Dermatophagoides pteronyssinus group 1) (Table 2) was tested by skin prick, using standardized extracts. Medical grade allergen extracts (Hollister-Stier, Spokane, WA) in solutions were used. A wheal ≥3 mm to at least one of those allergens was required for inclusion in the study. Subjects withheld long-acting β_2-agonists for 48 hours, short-acting β_2-agonists for 6 hours, long-acting anti-histamines for 14 days, non-steroidal anti-inflammatories and aspirin for 7 days, and short-acting anti-histamines for 3 days prior to skin test. A series of 10-fold dilutions of the test allergen were used to determine the lowest skin prick dose needed to elicit a 3-mm wheal, based on the strong correlation between the concentration of allergen leading to skin test positivity and that prompting airway responsiveness[22]. In those whose test allergen was birch, we required that they avoid apple, pear, sweet cherry, peach, plum, apricot, almond, celery, carrot, potato, hazelnut, mango, and chili pepper to minimize concerns for oral allergy syndrome.

Exposure design and procedures

Subjects entered a crossover experiment [23-25] (Figure 1) using two conditions (diesel exhaust [DE] (300μg $PM_{2.5}/m^3$) or filtered air [FA]), the order of which was randomized and counter-balanced. DE exposure used a previously-described system that excludes potential contamination with lipopolysaccharide[26], except that in the present study we utilized a 2.5kW constant load. The Air Pollution Exposure Laboratory (APEL) was designed for the controlled inhalation of human subjects to aged and diluted DE to mimic "real-world" occupational and environmental conditions. Notably, the protocol allows for effective blinding to both the subject[27] and those analyzing all material and data.

One hour following each exposure to DE or FA, bronchoscopy was performed to deliver a saline-controlled segmental allergen challenge (SAC). A 5 mL solution of allergen extract in a concentration 10-fold lower than that minimal dose producing a positive wheal was instilled in a lower lobe bronchial segment, and 5 mL control (normal saline) was instilled in a contralateral lower lobe bronchial segment. Photographs were taken of the segments instilled, to guide subsequent bronchoscopy as needed. DE exposure preceded allergen instillation to avoid the possibility that starting with segmental allergen would lead to acute segmental bronchoconstriction and thus decreased deposition of diesel particulate matter within that segment. 48 hours after allergen challenge, bronchial brushes of airway epithelial cells (AECs) were obtained in the same segments. Side (right versus left) of lower lobe segmental challenge and sampling were reversed in the post-4 week period bronchoscopies (yielding samples 3,4,7 and 8 in Figure 1B) and the exact segment utilized for the pre-4 week period challenge and sampling was avoided in the post-4 week phase (to avoid artefact). Four samples did not generate sufficient DNA for analysis leaving 64 samples in total. All four samples were from the same individual (samples 5-8 of Figure 1A). 8 full sample sets remained for both groups and methylation analysis was not affected.

DNA isolation and DNA methylation arrays

Genomic DNA was isolated from bronchial brush samples, bisufite converted and evaluated by Illumina Infinium HumanMethylation450 BeadChip array as described previously[28]. All procedures were conducted using commercially available kits and done following the manufacturers' protocols. Bronchial brush samples were confirmed as >90% bronchial epithelial cells by light microscopy following cytospin and hematoxylin/eosin staining. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valancia, CA, USA). 750ng of the purified DNA was bisulfite-converted using the EZ-DNA methylation kit (Zymo Research, Orange, CA, USA), changing epigenetic data into sequence-based data by selective conversion of unmethylated cytosines to uracils. Bisulfite-converted DNA was assessed for concentration and quality using the NanoDrop, and 160ng of the conversion product was used for genome-wide DNA methylation evaluation at over 485,000 CpG sites using the Illumina Infinium HumanMethylation450 BeadChip array, as described previously[28].

Data quality control and normalization

Illumina GenomeStudio software was used to obtain raw signal intensities that were imported into R for quality control, normalisation, and statistical analysis. Any probes for which detection p-values were greater than 0.01, probes with missing beta values, and probes for which less than three beads contributed to the signal in 1 or more samples were excluded (29,587 probes removed). 11,648 probes residing on the X or Y chromosome were removed to control for gender-derived differences on the array. Finally, probes known to be polymorphic at the CpG or to cross-hybridize to somatic sites and sites on the X or Y chromosome[29], and those examining single nucleotide polymorphisms were removed (additional 28,211 probes removed). This left 416,066 probes for 64 samples from the 16 subjects for analysis. Colour correction, background adjustment and quantile normalisation were performed using the lumi R package and the data were normalised using Subset-quantile Within Array Normalization (SWAN) [30]. ComBat[31, 32] was used to remove any effects of batch (samples were run across two batches), chip (10 chips) and array location (12 arrays per chip). Average replicate correlations (mean ± SD of 6 replicates) increased from 0.9967 ± 0.0011 to 0.9976 ± 0.003 following ComBat. Two values of DNA methylation were calculated, beta-values (β-values) and M-values. β-values are the ratio of all methylated probe intensities over total signal intensities (methylated and unmethylated) and have a range from 0 to 1. They approximately represent percent methylation. M-values are log transformation of β-values and more statistically robust[33]. All statistical analyses were performed using M-values. Visualisation and discussion are presented as β-values.

Differential methylation analysis

All statistical analysis was performed using R statistical software. Probes were filtered to include only variable probes (probes which have a β-value standard deviation of greater than 0.05 across all samples; 43,485 probes). Probes with DNA methylation levels significantly different between the samples stated in the results section were identified using the R lm linear modelling function (paired where appropriate) followed by q-value calculation to control for false discovery rate (FDR) at 0.05. We did not control for participant demographics (for example age, sex, asthmatic status) as these were balanced between study group (Table 1 and 2) and our initial analysis ensured there was no difference in DNA methylation due solely to study group. A filtering step for potential biological effects was then performed to leave only probes that also had a β-value difference of greater than 0.1 (equivalent to a 10% change in methylation between groups).

DAVID Functional Enrichment Analysis

UCSC refgene accession-IDs corresponding to the 43,485 variable CpG probes were used as background for DAVID GO analysis[34]. UCSC refgene accession-IDs corresponding to our significant hits were input for DAVID GO analysis. Clusters with enrichment scores greater than 1.3 were considered significant.

Bisulfite PCR-pyrosequencing

Bisulfite PCR-pyrosequencing was used to validate differences in DNA methylation at select CpG sites. Bisulfite PCR-pyrosequencing assays were designed with PyroMark Assay Design 2.0 (Qiagen). The regions of interest were amplified by PCR using the HotstarTaq DNA polymerase kit (Qiagen) as

follows: 15 minutes at 95°C (to activate the Taq polymerase), 45 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 30s, and a 5 minute 72°C extension step. For pyrosequencing, a single-stranded DNA was prepared from the PCR product with the Pyromark™ Vacuum Prep Workstation (Qiagen) and sequencing was performed using sequencing primers on a Pyromark™ Q96 MD pyrosequencer (Qiagen). The quantitative levels of methylation for each CpG dinucleotide were calculated with Pyro Q-CpG software (Qiagen). Primer sequences are listed in Supplementary Table 4.

Results

Study cohort and samples

To evaluate the effects of allergen and diesel exhaust exposure on DNA methylation in the human lung, we performed a rigorous randomized crossover controlled exposure study. Specifically, 17 individuals were randomized into two study groups (Group I and Group II), defined by whether filtered air (FA) or diesel exhaust (DE) was inhaled first (Figure 1A). Following inhalation of FA or DE, allergen was instilled into one lung and saline into the other. The process was repeated approximately 4 weeks later with opposite exposures. This resulted in 4 exposure conditions per subject: FAS (filtered air and saline), DES (diesel exhaust and saline), FAA (filtered air and allergen) and DEA (diesel exhaust and allergen). The resulting samples were numbered and are defined in Figure 1B. Importantly, in contrast to previous studies[12], we collected bronchial epithelial cells (BEC), the primary cell type exposed to inhalants. The average percentage of bronchial epithelial cells in the brushings was high and consistent across all conditions (97.3% for FAS, 96.9% for DES, 95.4% for FAA, 98.7% for DEA). We measured DNA methylation in genomic DNA extracted from BECs obtained from the four conditions at each time point, and in the majority of cases unpaired analyses were performed. However, where possible a paired comparison was performed and little difference identified (Figure S1 A and B and supplementary results section). Initial analysis revealed that no CpG sites were significantly differently methylated between Group I and Group II with an q-value< 0.05 (See Supplementary Results and Figure S2 for further detail).

Initial exposure had minimal effect on DNA methylation

After determining that there were no DNA methylation differences between Group I and Group II (defined by whether filtered air (FA) or diesel exhaust (DE) was inhaled first (Figure 1A)), we tested the effect of isolated exposures and co-exposures. To do this, we investigated the effect of allergen exposure only (2.FAA vs 1.FAS), DE only (5.DES vs 1.FAS) and allergen and DE co-exposure (6.DEA vs 1.FAS) (Figure 1B). In these tests, no sample had received any prior study exposure. We did not identify any CpGs that had a significant change in methylation associated with allergen. Six CpGs were significantly altered by DE and seven CpGs by co-exposure. Of these, five CpGs were common between DE and co-exposure (Supplemental Table 5). These data suggested that exposure of BECs to either allergen, DE, or both (co-exposure) had minimal effects on DNA methylation within the 48-hour time period that elapsed from exposure to sample collection.

DNA methylation was significantly affected by allergen in lung previously exposed to DE

Given the limited effects of single exposures, we subsequently tested whether allergen and DE exposure altered DNA methylation when administered individually to the same lung but separated by 4-weeks. Specifically, we assessed differences in DNA methylation in BECs exposed to allergen following exposure to DE 4 weeks earlier (7.FAA) versus those completely unexposed (1.FAS), using linear modelling. The deviation of the raw p-value distributions from random suggested an association of allergen administration and DNA methylation in BECs previously exposed to DE (Figure 2A). This was statistically significant as we identified 150 differentially methylated CpGs at a q-value <0.05 (red points Figure 2B). To assess the likelihood of statistically significant CpGs having biological impact the absolute difference between the means of the β values of the exposures were calculated and referred to as the delta beta (Δβ). We considered a Δβ of 0.1, corresponding to a 10% difference in CpG methylation, to be of biological interest. 75 probes had a q-value <0.05 and a minimum Δβ of 0.1 (green points Figure 2B and Figure 2C). Of these CpGs, 70 showed a decrease in DNA

methylation in response to allergen after prior DE exposure, relative to the control (1.FAS) condition (Figure S3A). In contrast, only 5 probes displayed an increase in DNA methylation (Figure S3B).

Upon further investigation we found that, while not reaching statistical significance, the initial DE exposure (5.DES) caused an intermediate change in DNA methylation, in the same direction (Figure 2D, decrease and Figure 2E, increase) as that caused by sequential DE and allergen exposure. These data suggested that an initial DE exposure might prime DNA methylation of certain CpG sites within BECs and that upon subsequent allergen exposure these sites underwent further DNA methylation modification, resulting in a significant difference in DNA methylation relative to unexposed cells.

The 75 CpG sites differentially methylated in response to allergen following prior DE exposure were primarily in regions of low or intermediate CpG density (Figure 3A), significantly different from the distribution of the full analysis probe set (chi-squared p-value <0.05). Significant sites were within 0.5Mb of a transcription start site (TSS; red points Figure 3B), while the full analysis probe set fell in DNA regions up to 2Mb of a known TSS (black points Figure 3B), however this enrichment did not reach statistical significance (chi-squared p-value 0.3037). 40 probes were downstream of a TSS while 35 probes were upstream. The closest TSS to the 75 differentially methylated CpG sites were associated with 58 different genes (Supplementary Table 1). Several of these had more than one differentially methylated CpG associated with them, including 7 genes that had 2 or more (bold text Supplemental Table 1). Of particular note, we found a significant decrease in DNA methylation in response to allergen following prior DE exposure in 8 CpGs located upstream of the TSS of the TBX3 gene, a member of T-box family of transcription factors (Figure 3C). We confirmed these data using pyrosequencing of cg02824888 (Figure S4), which also showed significant (Mann-Whitney p<0.005) decrease in DNA methylation in response to allergen with prior DE exposure, with the difference in DNA methylation being comparable between the two technologies (10.7% by array versus 16.3% by pyrosequencing, Figure S5). Moving from single gene analysis to broader biological functions, we performed functional analysis using the DAVID Gene Ontology tool and identified two clusters above the recommended 1.3 enrichment score cutoff (1.58 and 1.55), which highlighted genes involved in protein metabolism and hormone/steroid stimulation among the 58 genes differentially methylated after subsequent exposure to DE and allergen (Figure S6).

DNA methylation was significantly affected by DE exposure in lung previously exposed to allergen

Having found significant changes in DNA methylation in lungs in response to DE exposure followed by allergen, we next asked whether the reverse order of exposures would result in similar effects. Specifically, we again used linear modelling to assess changes in DNA methylation in BECs exposed to DE following exposure to allergen 4 weeks earlier (4.DES) versus those completely unexposed (1.FAS). The deviation of the raw p-value distributions from random suggested an effect of DE on DNA methylation in BECs exposed to allergen (Figure 4A). Further, 22,904 CpGs showed a significant difference in methylation at a q-value <0.05 (red points Figure 4B), with 548 of those having a minimum $\Delta\beta$ of 0.1 (green points Figure 4B and Figure 4C). 10 CpGs were in common between the 75 differentially methylated in response to allergen after prior DE exposure (Supplemental Table 3). Of the CpGs with a minimum of 10% change in DNA methylation, 528 probes had a decrease in DNA methylation in response to DE after prior allergen exposure (Figure S6), relative to the control (1.FAS) condition whereas 20 probes had an increase in DNA methylation.

Again, while not sufficient to reach statistical significance, the initial allergen exposure (2.FAA) caused an intermediate change in DNA methylation, in the same direction as that caused by sequential allergen and DE exposure (Figure 4D, decrease and Figure 4E, increase). These data suggest that an initial allergen exposure primes DNA methylation of certain CpG sites (that differ from those altered by an initial DE exposure, as noted above) within BECs and that, upon subsequent DE exposure, these sites undergo further modification to DNA methylation, resulting in a statistical difference in DNA methylation compared to unexposed cells.

Of the 548 probes differentially methylated in response to DE after prior allergen exposure, close to 50% were in regions of low CpG density (Figure 5A), which differed from the distribution of the full analysis probe set (chi-squared p-value < 0.001). Furthermore, the differentially methylated CpG sites were enriched within 0.5Mb of a TSS compared to the full data set (Chi-squared p-value 0.0002) (red points Figure 5A). 260 probes were downstream of a TSS and 288 probes were upstream. The closest TSS to the 548 differentially methylated sites were associated with 450 different genes (Supplementary Table 2). 63 of these had 2 or more differentially methylated CpGs associated with them (bold text Supplemental Table 2). In particular, we found a significant decrease in DNA methylation in response to DE following prior allergen exposure in 19 CpGs associated with genes of the Hox family. Five CpGs associated with HOXA3 (Figure 5C), three CpGs with HOXA4 (Figure 5D), 7 CpGs with HOXB1 (Figure 5E) and three CpGs with HOXB3 (Figure 5F). We confirmed these data using pyrosequencing of cg18680977 of the HOXA3 gene, cg11532431 of the HOXA4 gene, cg26634219 of the HOXB1 gene and cg11060532 of the HOXB3 gene (Figure S4). All showed significant decreases in DNA methylation in response to DE with prior allergen exposure, with the difference in DNA methylation being comparable between the two technologies (Figure S5). Functional analysis using the DAVID Gene Ontology tool identified 5 clusters (enrichment scores; 1.96, 1.43, 1.36, 1.35 and 1.34) and highlighted genes involved in biological/cell adhesion, protein localisation/transport, vascular development/angiogenesis, transcription factor activity/DNA binding and cell motion/migration among the 450 genes associated with the 548 CpGs differently methylated after subsequent exposure to allergen and DE (Figure S6B).

Discussion

The molecular mechanisms regulating the interaction between air pollution and allergic disease are poorly understood. Defining these mechanisms is critical for creating strategies to reduce susceptibility and severity of airways disease[8], especially given dynamics associated with both air pollution and allergens associated with global climate change[35]. DNA methylation may be one of the mechanisms by which environmental exposures, including DE, can exert lasting effects on gene expression, cell function, and human health at a rate consistent with the rapid increase in allergic disease in recent decades[8]. However, current evidence linking DNA methylation to the response to inhaled allergens and traffic pollution comes primarily from animal studies and observational human studies and has focused on repetitive DNA elements[36 37]. While we have previously shown controlled diesel exhaust exposure to alter CpG methylation in peripheral blood mononuclear cells[20], we are not aware of any previous controlled *in vivo* human study investigating global DNA methylation responses to DE in conjunction with allergen, and we pursued this given the commonality of these particular exposures and our desire to embrace the complexity beyond traditional single exposure models.

Here, we used a sequential exposure paradigm in combination with DNA methylation profiling of intact (*in vivo*) human lung cells to test whether multiple carefully characterized exposures over a period of weeks had compounding effects. Our major finding was that prior exposure to allergen or DE significantly altered global DNA methylation in the human epithelium upon subsequent exposure, approximately one month later, to the alternative exposure. Furthermore, the order of these exposures resulted in different patterns of altered DNA methylation. This finding suggests that DNA methylation was highly sensitive to differences in short-term exposure, which has implications for understanding epigenetic dynamics and moving towards preventive measures. Notably, while the methylation of thousands of CpG sites changed when DE and allergen were given sequentially and separated by 4 weeks, only a few had altered methylation 48 hours after initial exposure, suggesting a sequential insult on the lung was *required* to significantly alter DNA methylation. In agreement with this, samples collected from a lung exposed to allergen and diesel exhaust co-exposure 4 weeks previously but without additional exposure through a four week period (8.FAS, compared to 1.FAS) demonstrated no CpGs with significantly altered DNA methylation (Figure S7 vs Supplemental Table 5) other than those already demonstrated after initial DE or DEA exposure (Supplemental Table 5). We suggest that the initial exposure in the sequence did alter methylation of CpG sites to an intermediate (non-significant) level, and that the second exposure then caused further modification of

methylation that reached our statistical thresholds. Of interest, in our study the sample used to assess the effect of DE in a lung previously exposed to allergen was taken from a location distinct to that where the allergen was earlier instilled. This suggests that local allergen instillation can prime the wider lung for future exposure. In support of our observation, published studies instilled allergen in the right middle lung lobe and observed significant effects (eosinophil infiltration) in a separate saline-instilled (control) lobe[38].

Probing in detail, within the broader insights relating to the potential additive effects of DE and allergen, we identified particular genes whose DNA methylation pattern was associated with these agents in our sequential exposure paradigm. The gene associated with the greatest number of differentially methylated CpG sites in response to allergen following prior exposure to DE was TBX3. TBX3 and closely related TBX2 act as transcriptional repressors with roles in vertebrate development, cell fate, cell differentiation and cell cycle progression. Both are up-regulated in tumours and can cause bypass of cell senescence. *Tbx-2*-deficient mice display hypoplastic lungs with reduced branching morphogenesis and decreased mesenchymal and epithelial cell proliferation suggesting a critical role for these proteins in lung development[39]. Four of the Hox family of genes were associated with differentially methylated CpG sites in response to DE following prior exposure to allergen, HOXA3, HOXA4, HOXB1 and HOXB3. Hox genes encode homeodomain transcription factors that determine cell and tissue identities in the developing embryo and patterning of the developing mouse lung[40].

The majority of the changes in DNA methylation we observed were reductions after exposure. Air pollution induces oxidative stress which is thought to favour the demethylation process and cause lesions to DNA which prevent binding of DNA methyltransferases, thus potentially resulting in decreased methylation[10]. The detailed molecular mechanisms regulating the DNA methylation changes we observed should be investigated further via *in vitro* exposure of BECs to diesel exhaust and allergen.

Limitations of our study include the decision to recruit both asthmatic and non-asthmatic individuals knowing that we would not have statistical power to test the modifying effect of asthma status on changes in DNA methylation after allergen or DE exposure. Here we targeted those with atopy, but not necessarily asthma, as we were interested in the immune response in those sensitive to common aeroallergens. We knew that if we were to limit by asthmatic status then recruiting to a sample size with sufficient statistical power would be difficult given the demanding nature of the protocol. At inception we intended to restrict analysis to intra-subject comparison, given the crossover design, but when this became impossible we used other methods to check for demographic confounding. First we confirmed no statistical difference in methylation between the two groups of individuals in "unexposed" conditions. Second, we performed additional paired analyses (each subject serving as his/her own control) where possible (Figure S1). Importantly, the initial allergen alone exposure maintained a non-significant effect, and the DE with prior allergen exposure (4.DES vs 1.FAS) altered minimally, increasing statistically significant hits from 22904 to 23379; however the CpGs with a statistically significant change and a delta beta of >0.1 was maintained at 547 versus 548 in the unpaired analysis. This gives us confidence that individual characteristics, including asthmatic status, age and sex do not confound the data. This also applies to the fact we included subjects reactive to different allergens, primarily to increase our overall sample size. Furthermore, a recent examination of allergen effect on human airways showed no such effect modification on outcomes related to ours (sputum inflammatory cells or methacholine responsiveness)[41]. Furthermore, we recognize that the changes in DNA methylation after 4 weeks could be partially reflective of inflammation related to the stress of the prior bronchoscopy (independent of the exposures), but this concern is attenuated by the expectation that bronchoscopy-related inflammation would have resolved within the 4-week interval. Furthermore, the very high percentage of bronchial epithelial cells in the bronchial brushings is important in ensuring that observed changes in DNA methylation are not simply reflective of a changing distribution of cell types. Finally, it is important to consider the potential for previous exposure to external environmental inhalants to confound the results of our study. While the potential for such an effect cannot be eliminated entirely, all subjects were non-smokers and living in Metro

Vancouver. Thus, it is unlikely that they are exposed to significant levels of air pollution (typical local exposures would be an order of magnitude below the level we use in our study). Also any undetected incidental extra-experimental exposures would be non-differential and thus bias to null results, such that we might miss weaker effects but the significant findings would be then the stronger effects.

Since air pollution contributes to 6% of total mortality and 50% of mortality caused by air pollution is attributed to motorised traffic[42], the global relevance of these findings is already considerable. A recent study reporting related effect of sequential ultrafine particle and allergen exposure on bronchoalveolar lavage cell composition[43], adds further strength to the observations and warrants the initiation of larger studies to investigate this phenomenon in greater detail. There may be even more impact if this opens inquiries into whether similar additive effects occur with other exposures beyond air pollution. In spite of the complexity of epigenomics, we embraced the fact that epigenetic responses to environmental exposures are likely to depend on the timing, length and potency of the exposure and aim to understand these complexities so that we can leverage this knowledge towards preventive measures[44]. We enhanced the biological relevance of the current study by a) using a controlled exposure rather than an observational setting, which is prone to residual confounding; b) using inhalation of whole DE rather than DEP, providing a more realistic inhalant and route of exposure; c) using DNA from bronchial epithelial cells, the first line of defence for the lung in response to inhaled exposures rather than a surrogate tissue (i.e. blood).

In summary, we show that while a single short term exposure to either allergen, DE, or both as a co-exposure causes only minor changes in DNA methylation, sequential insults with allergen and DE separated by a 4-week period mediates significant changes in CpG site methylation. Although requiring further investigation, we suggest the initial insult acts to prime the methylome for the second resulting in an additive methylation change with potential biological relevance. Our data adds biological plausibility to evidence that air pollution and allergen have significant effects on cell biology and are relevant to policy makers who want evidence from models that embrace the complexity of the environment we inhale.

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Figure Legends

Figure 1) Study design (A) and explanation of sample naming according to group status (B). Individuals in Group I were first exposed to filtered air (FA); those in Group II were first exposed to diesel exhaust (DE). Post-exposure, allergen and saline were instilled 48 hours later bronchial brushings were taken for epithelial cell DNA isolation. 4 weeks later the process was repeated with opposing exposures. (filtered air/saline (FAS), filtered air/allergen (FAA), diesel exhaust/saline (DES) and diesel exhaust/allergen (DEA)). Samples 1-4 associated with Group I and samples 5-8 associated with Group II.

Figure 2) CpG methylation was significantly affected by allergen in lung previously exposed to diesel exhaust. A) p-value distribution for the effect of allergen in lung with prior DE exposure (7.FAA vs 1.FAS). Dashed line indicates distribution expected by chance; B) Plot of 43,485 variable CpG probes. Red= q-values < 0.05: green= q-value<0.05 *and* Δβ >0.1 C) Beta values of probes significantly different between control (1.FAS) and 7.FAA with a Δβ >0.1 (75 total probes). Each vertical band is a study subject. Blue: high methylation; yellow: low methylation; D/E) Reduction/increase in DNA methylation (Δβ), relative to 1.FAS, amongst 70/5 hits following the intermediate diesel exhaust exposure. Points are individual CpG sites and lines indicate mean ± SEM.

Figure 3) Characteristics of the CpGs affected by allergen in lung previously exposed to diesel exhaust. A) CpG island type for all analysis probes and the 75 top hits. LC = low CpG density, IC = intermediate CpG density, ICshore = intermediate CpG density overlapping with a high-density region, HC = high CpG density[29]; B) Relationship of Δβ and distance to the transcriptional start site. Red=q-value<0.05 and Δβ of >0.1; B) C) Individual CpG site plots of the 8 CpG hit sites associated with the TBX3 gene. Whiskers represent the minimum and maximum of all the data.

Figure 4) CpG methylation was significantly affected by diesel exhaust in lung previously exposed to allergen. A) p-value distribution for the effect of DE in lung with prior allergen exposure (4.DES Vs 1.FAS). Dashed line indicates distribution expected by chance; B) Plot of 43,485 variable CpG probes. Red=q-value < 0.05; green=q-value<0.05 *and* Δβ >0.1 : C) Heatmap of beta values of probes that were significantly different between control (1.FAS) and 7.DES (DE with prior allergen) with a Δβ >0.1 (548 probes total). Each vertical band is a study subject. Blue: high methylation; yellow: low methylation; D/E) Reduction/increase in DNA methylation (Δβ), relative to 1.FAS,

amongst 528/20 hits following the intermediate allergen exposure. Points are individual CpG sites and lines are mean ± SEM.

Figure 5) Characteristics of the CpGs affected by diesel exhaust in lung previously exposed to allergen. A) CpG island type for all analysis probes and the 548 top hits. LC = low CpG density, IC = intermediate CpG density, ICshore = intermediate CpG density overlapping with a high density region, HC = high CpG[29]; B) Relationship of Δβ and distance to the transcriptional start site. Red=q-value<0.05 and Δβ of >0.1; C-F) Individual CpG site plots of the CpG sites associated with 4 HOX genes (C) HOXA3, D) HOXA4, E) HOXB1, F) HOXB3). Whiskers represent the minimum and maximum of all the data.

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Table 1: Grouped Participant Demographics

	Group I (n=8)	Group II (n=9)	p-value
Age (years)	29.50 ± 2.771	27.33 ± 2.718	0.5859 (t-test)
Sex	Male:3; Female:5	Male:4; Female:5	1 (fishers exact)
Asthmatic Status	Asthmatic:3 ; Nonasthmatic:6	Asthmatic:5; Nonasthmatic:4	0.6372 (fishers exact)

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Table 2: Individual Participant Demographics

Subject	Age	Gender	Asthmatic?	Allergen
DE-53	20	Female	Yes	D. pterinyssinus
DE-58	31	Female	No	D. pterinyssinus
DE-70	24	Female	Yes	D. pterinyssinus
D2E-81	32	Male	No	Timothy/Pacific Grass
DE2-90	34	Female	Yes	Timothy/Pacific Grass
DE2-94	27	Male	No	D. pterinyssinus
DE2-96	25	Female	No	D. pterinyssinus
DE2-103	46	Male	No	Birch
DE2-105	27	Male	Yes	Timothy/Pacific Grass
DE2-106	46	Female	Yes	D. pterinyssinus
DE2-107	20	Female	No	Timothy/Pacific Grass
DE2-109	31	Female	No	Birch
DE2-112	28	Male	Yes	Timothy/Pacific Grass
DE2-114	23	Male	Yes	Timothy/Pacific Grass
DE2-115	23	Female	Yes	D. pterinyssinus
DE2-116	23	Male	No	D. pterinyssinus

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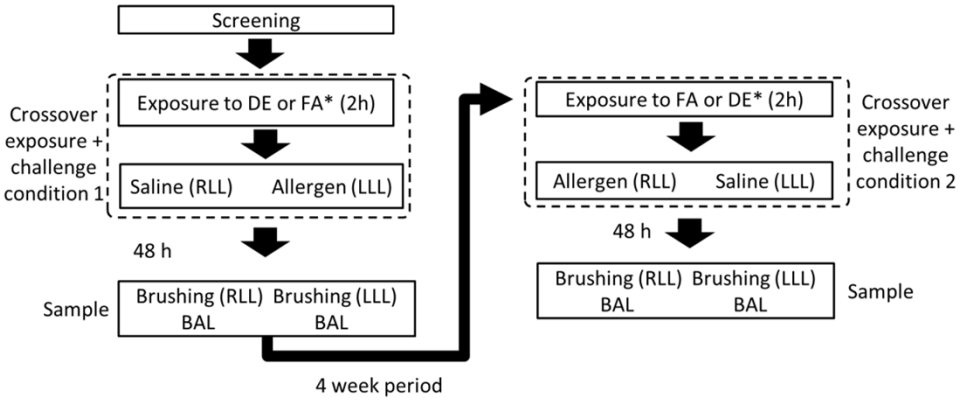
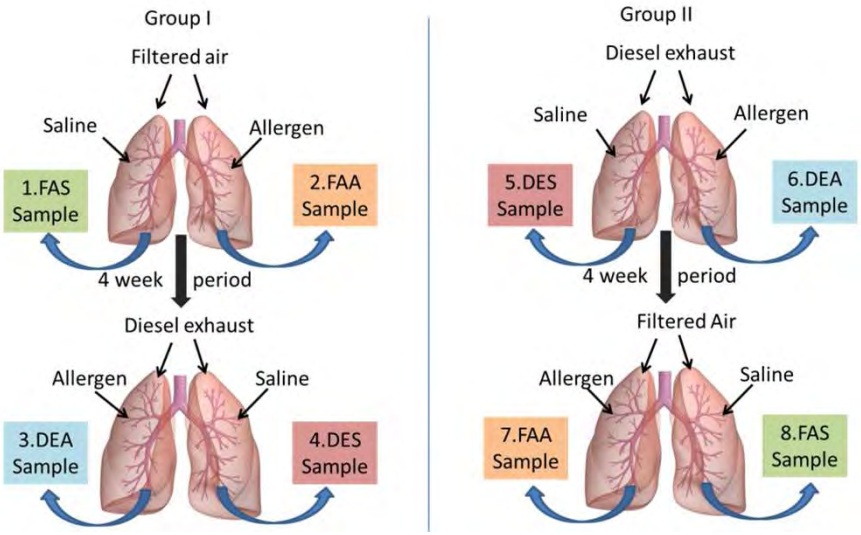
A)


Screening
Crossover exposure + challenge condition 1
Exposure to DE or FA* (2h)
Saline (RLL)
Allergen (LLL)
48 h
Sample
Brushing (RLL)
BAL
Brushing (LLL)
BAL
4 week period
Exposure to FA or DE* (2h)
Allergen (RLL)
Saline (LLL)
Crossover exposure + challenge condition 2
48 h
Brushing (RLL)
BAL
Brushing (LLL)
BAL
Sample

B)


Group I
Filtered air
Saline
Allergen
1.FAS Sample
2.FAA Sample
4 week
period
Diesel exhaust
Allergen
Saline
3.DEA Sample
4.DES Sample
Group II
Diesel exhaust
Saline
Allergen
5.DES Sample
6.DEA Sample
4 week
period
Filtered Air
Allergen
Saline
7.FAA Sample
8.FAS Sample

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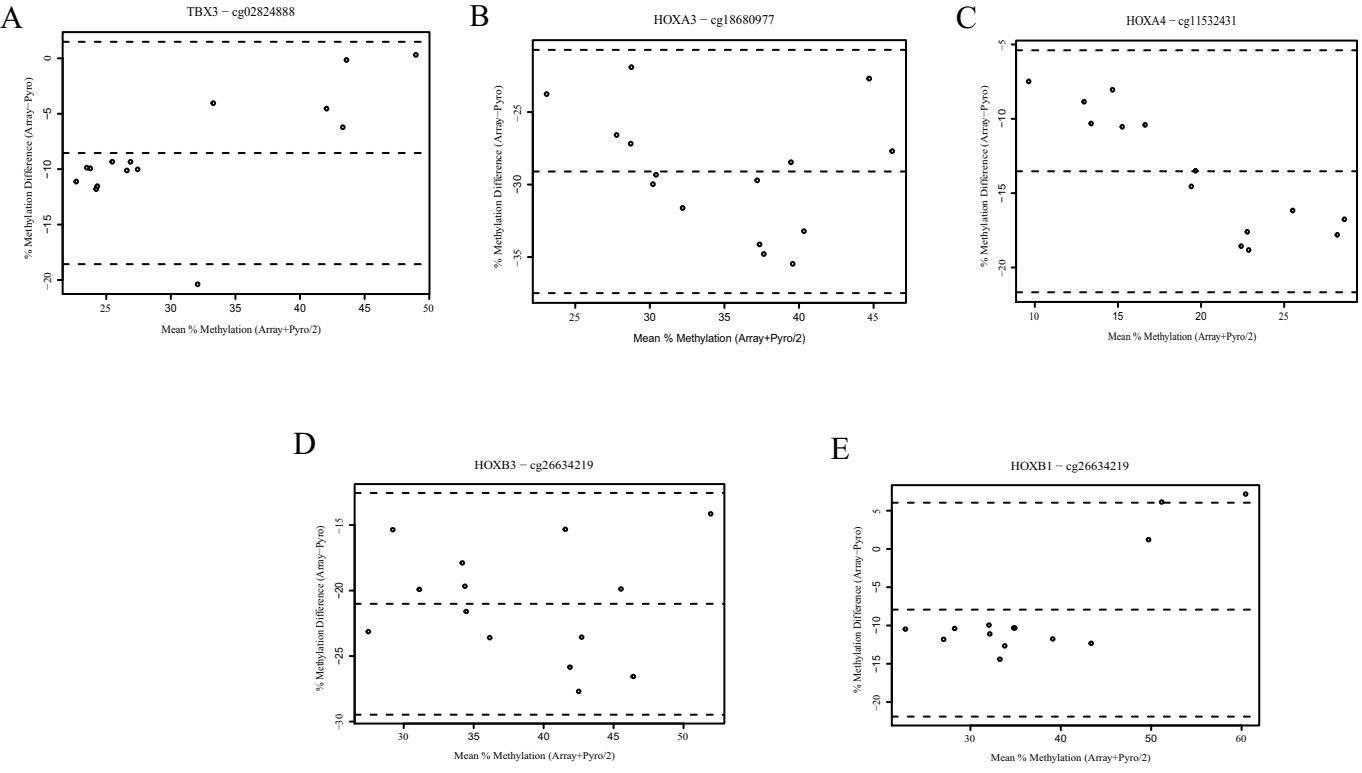

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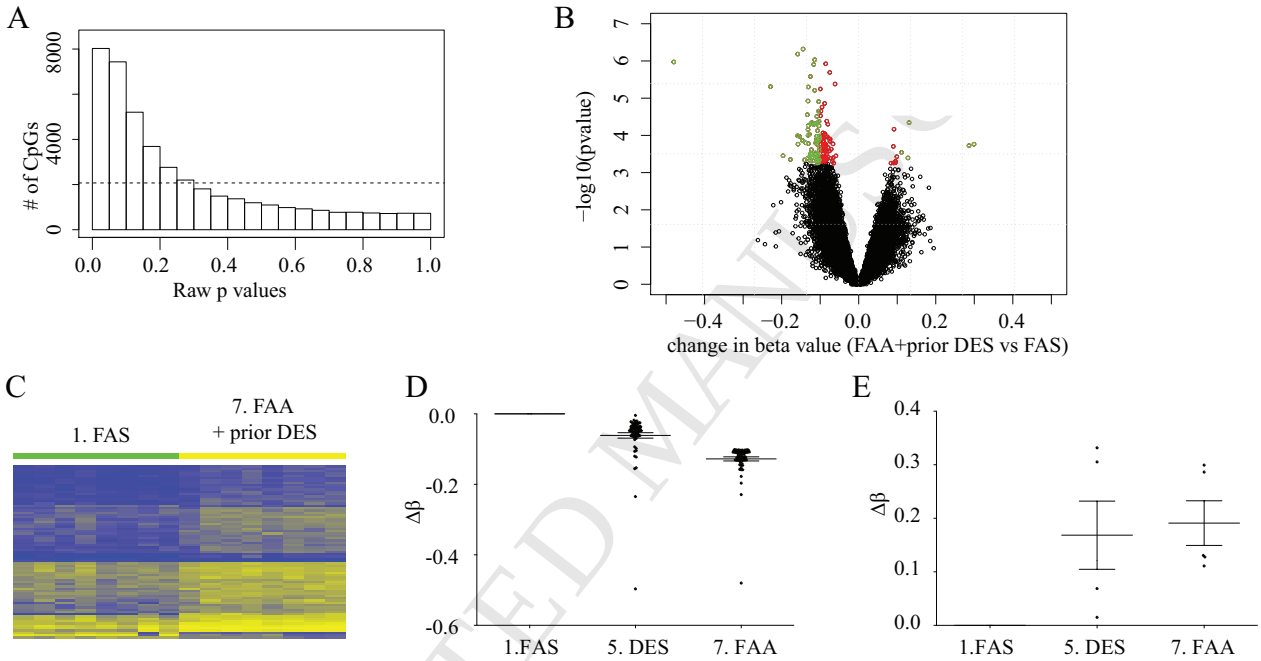

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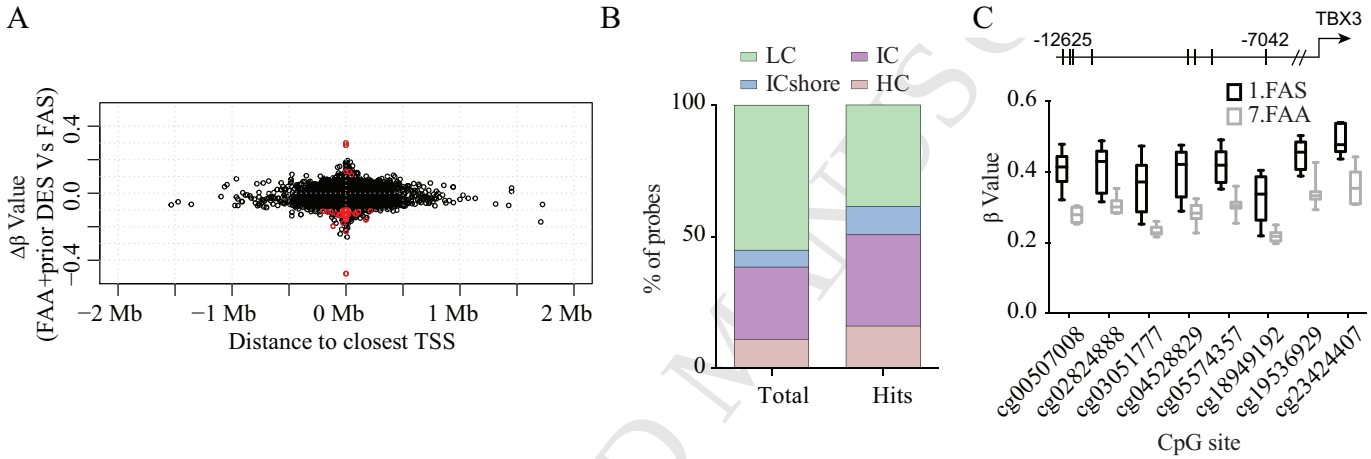

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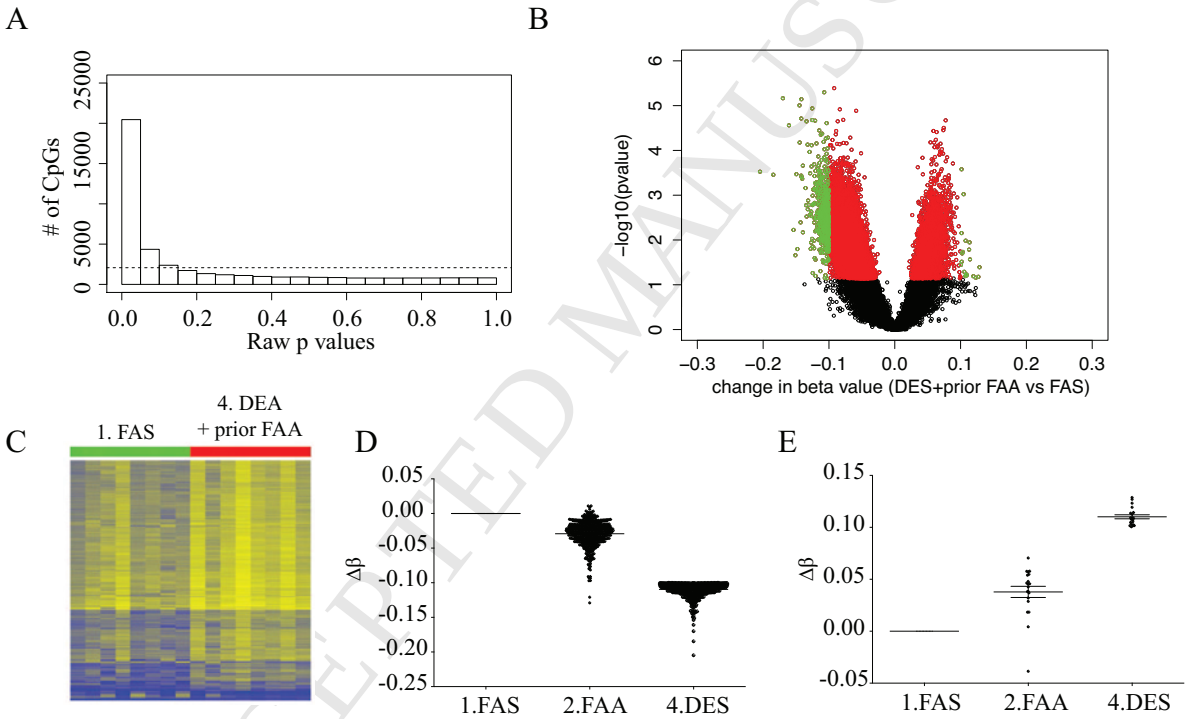

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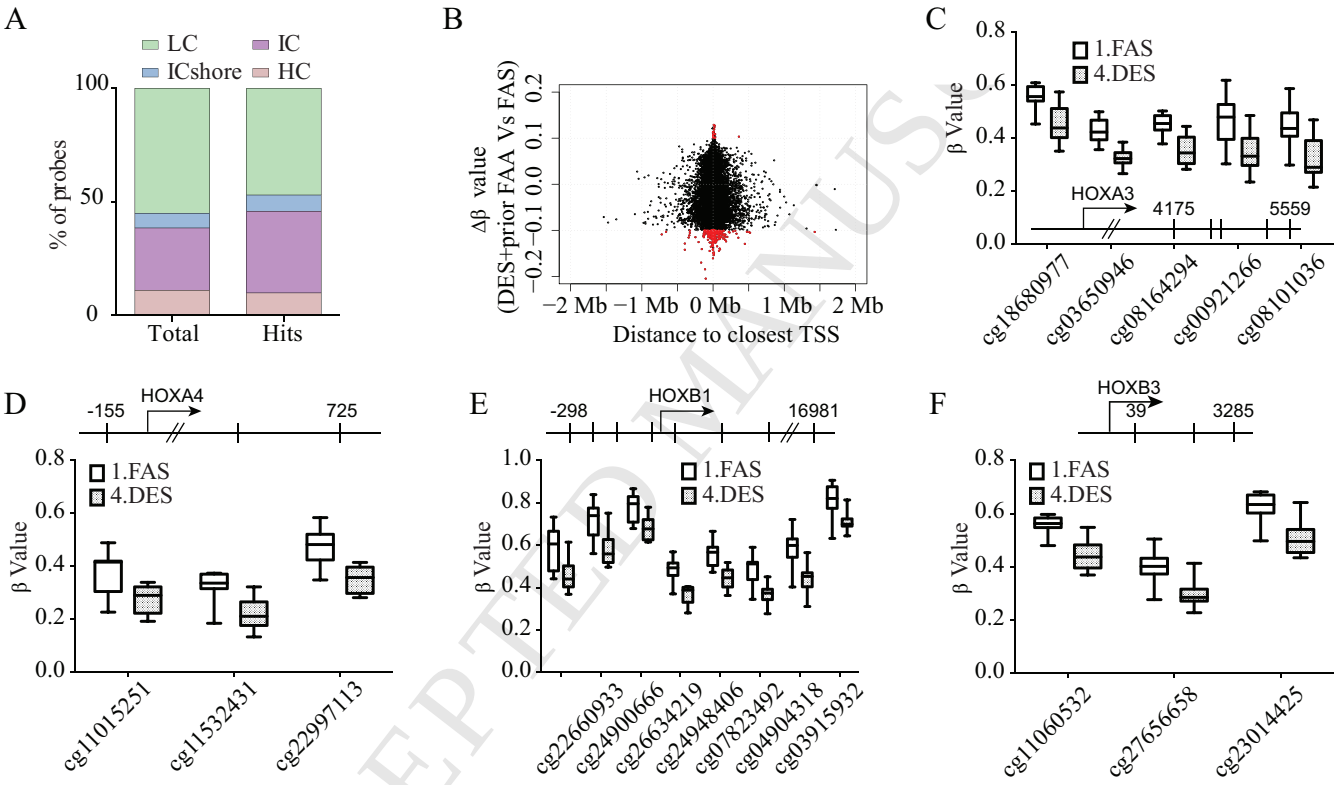

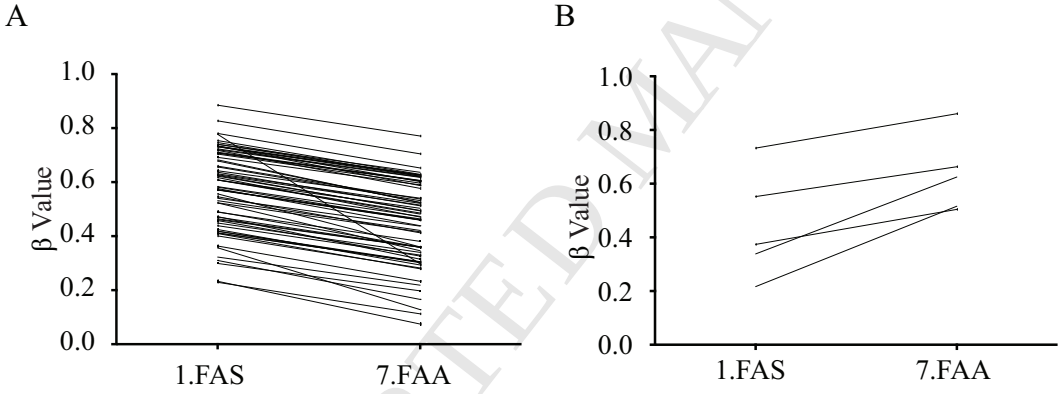

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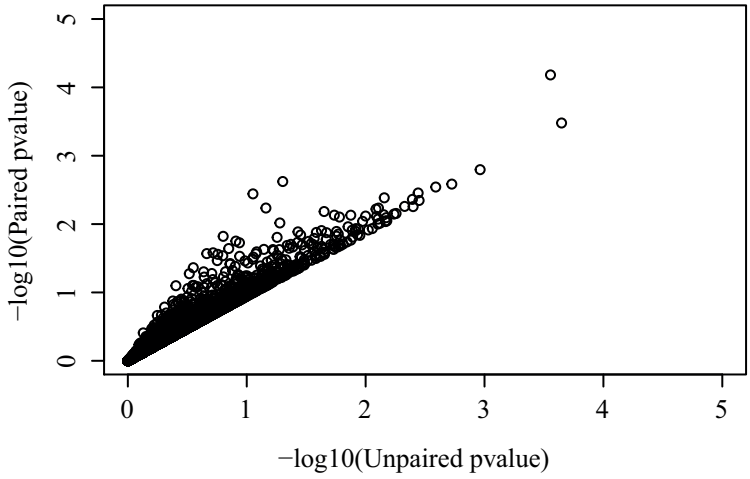
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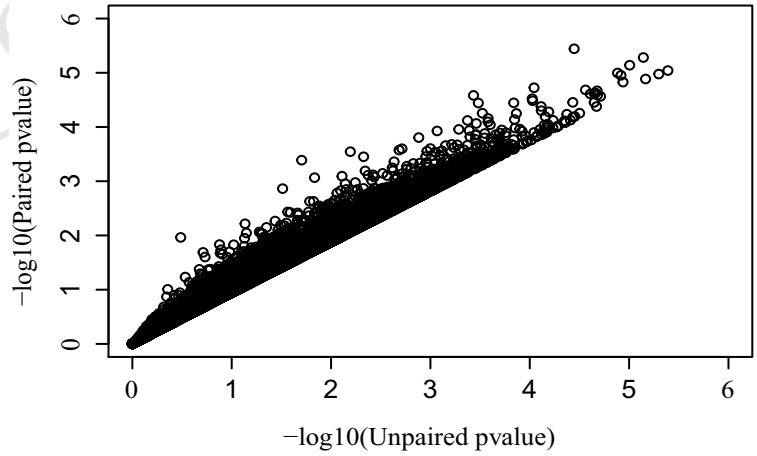
2
3
4
5
0
1
2
3
4
5
−log10(Unpaired pvalue)

B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−log10(Unpaired pvalue)

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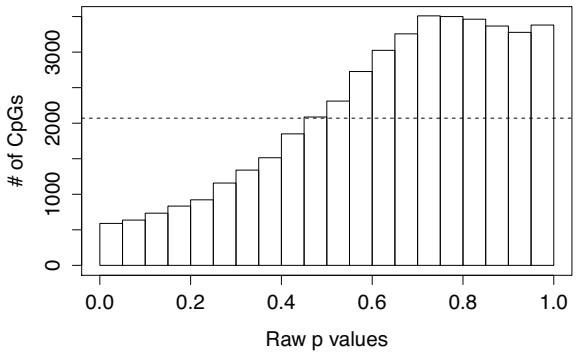

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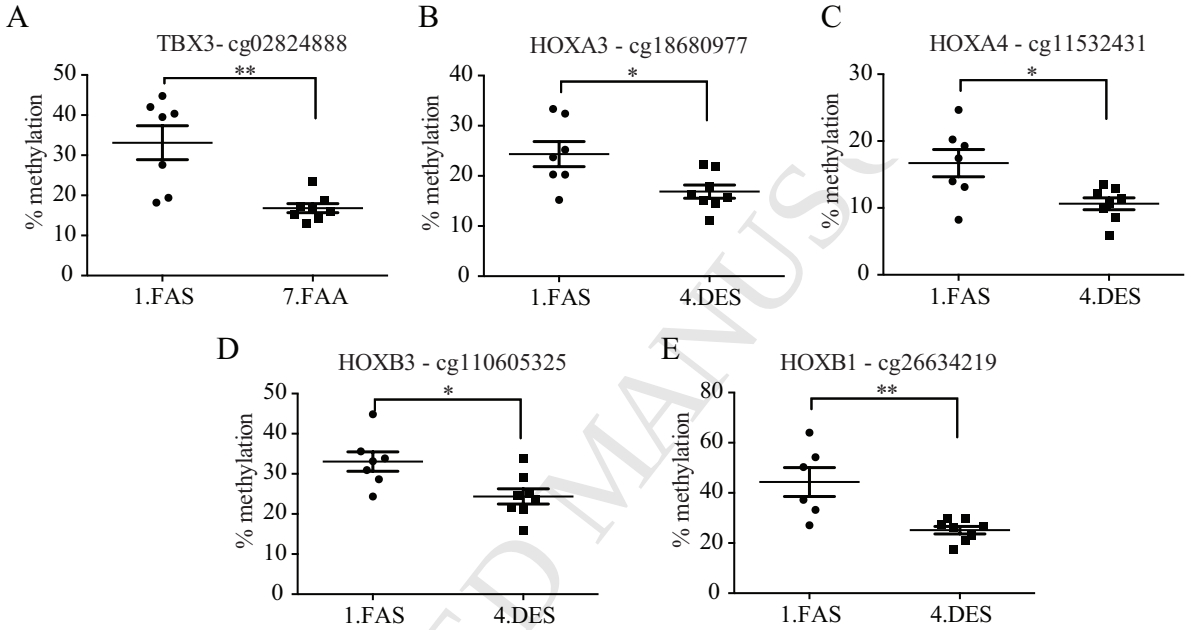

F

CpG	Δβ Array	Δβ Pyrosequencing	Pyrosequencing Mann - Whit ney p value
cg18680977	10.2	7.5	<0.05
cg11532431	10.4	6.1	<0.05
cg26634219	11.8	19.1	<0.005
cg11060532	11.8	8.7	<0.05

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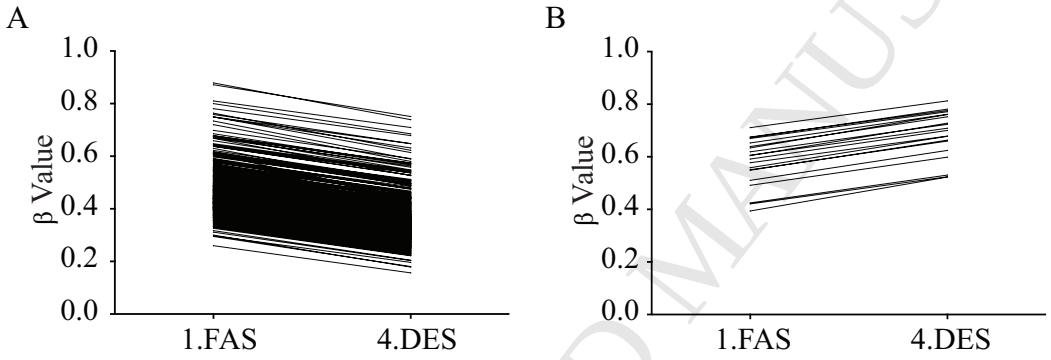
A

Cluster	Enrichment Score	Term	Count	Benjamini P-value
1	1.58	GO:0032269 (negative regulation of cellular protein metabolic process)	4	0.84
		GO:0051248 (negative regulation of protein metabolic process)	4	0.64
		GO:0010605 (negative regulation of macromolecule metabolic process)	6	0.83
		GO:0032268 (regulation of cellular protein metabolic process)	4	0.95
		GO:0005829 (cytosol)	3	0.99
2	1.55	GO:0010033 (response to organic substance)	7	0.61
		GO:0051384 (response to glucocorticoid stimulus)	3	0.77
		GO:0031960 (response to corticosteroid stimulus)	3	0.77
		GO:0009725 (response to hormone stimulus)	4	0.96
		GO:0009719 (response to endogenous stimulus)	4	0.96
		GO:0048545 (response to steroid hormone stimulus)	3	0.96

B

Cluster	Enrichment Score	Term	Count	Benjamini P-value
1	1.96	GO:0022610 (biological adhesion)	28	1
		GO:0007155 (cell adhesion)	28	1
		GO:0016337 (cell-cell adhesion)	11	0.95
2	1.43	GO:0008104 (protein localization)	23	0.93
		GO:0034613 (cellular protein localization)	13	0.93
		GO:0070727 (cellular macromolecule localization)	13	0.91
		GO:0015031 (protein transport)	20	0.91
		GO:0006886 (intracellular protein transport)	12	0.9
		GO:0045184 (establishment of protein localization)	20	0.9
		GO:0046907 (intracellular transport)	15	0.96
3	1.36	GO:0001568 (blood vessel development)	11	0.96
		GO:0001944 (vasculature development)	11	0.97
		GO:0048514 (blood vessel morphogenesis)	8	0.92
		GO:0001525 (angiogenesis)	5	0.98
4	1.35	GO:0043565 (sequence-specific DNA binding)	23	0.13
		GO:0003700 (transcription factor activity)	26	0.72
		GO:0051252 (regulation of RNA metabolic process)	37	0.91
		GO:0006355 (regulation of transcription, DNA-dependent)	36	0.91
		GO:0030528 (transcription regulator activity)	34	0.95
		GO:0006357 (regulation of transcription from RNA	20	0.92
		GO:0006350 (transcription)	35	0.97
		GO:0045449 (regulation of transcription)	43	0.97
		GO:0003677 (DNA binding)	37	0.97
5	1.34	GO:0040012 (regulation of locomotion)	10	0.96
		GO:0051270 (regulation of cell motion)	10	0.94
		GO:0030334 (regulation of cell migration)	9	0.95
		GO:0051272 (positive regulation of cell motion)	6	0.92
		GO:0030335 (positive regulation of cell migration)	5	0.94
		GO:0040017 (positive regulation of locomotion)	5	0.96



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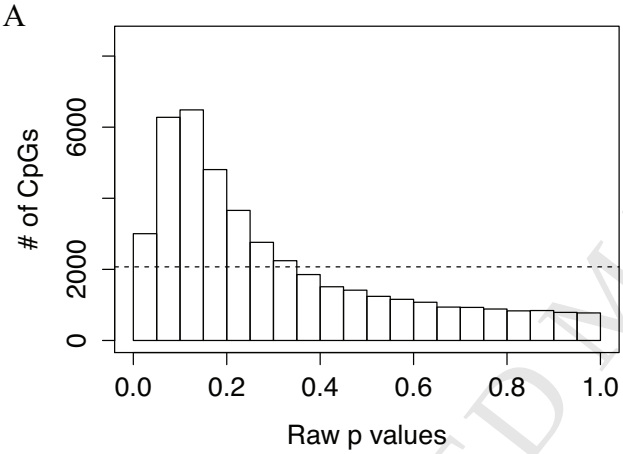
A



B

CpG	Q Value
cg00458681	0.0436
cg04234412	0.0436
cg6437703	0.0436
cg12419862	0.0459
cg20007245	0.0436
cg25703541	0.0436

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Supplementary Table 1: A list of the 58 genes represented by the closest TSS of the 75 differentially methylated sites between 7.FAA and 1.FAS. Bold text highlights genes with 2 or more differentially methylated CpGs associated with them.

CpG	P-Value	Q-Value	Delta Beta (7.FAA-1.FAS)	Gene name associated with closest transcription start site
cg06437703	9.28E-07	0.00278	-0.114	EIF4EBP1
cg04234412	**1.07E-06**	**0.00278**	**-0.480**	**LOC391322**
cg12419862	**6.53E-07**	**0.00278**	**-0.158**	**LOC391322**
cg20007245	**1.24E-06**	**0.00278**	**-0.117**	**LOC391322**
cg25703541	**4.79E-07**	**0.00278**	**-0.144**	**LOC391322**
cg04458567	2.62E-06	0.00439	-0.125	POLR1B
cg09033563	**4.93E-06**	**0.00610**	**-0.229**	**LOC391322**
cg00507008	**5.00E-06**	**0.00610**	**-0.131**	**TBX3**
cg17130982	6.15E-06	0.00633	-0.114	AK054708
cg17745291	1.24E-05	0.01107	-0.104	ALS2CR8
cg11912202	1.19E-05	0.01107	-0.132	OLIG2
cg24864275	2.23E-05	0.01571	-0.104	FCGBP
cg26767081	2.78E-05	0.01863	-0.133	BC061632
cg10156846	3.10E-05	0.01888	-0.109	DLX5
cg22996308	4.93E-05	0.02125	-0.110	5S_rRNA
cg04129308	4.53E-05	0.02125	0.131	AX747860
cg18049328	4.21E-05	0.02125	-0.103	BRSK2
cg25861692	4.52E-05	0.02125	-0.122	CADM1
cg02888990	4.59E-05	0.02125	-0.107	FLJ43860
cg11542165	**4.91E-05**	**0.02125**	**-0.124**	**HNF4A**
cg24626554	5.08E-05	0.02125	-0.117	LOC389458
cg05574357	**4.84E-05**	**0.02125**	**-0.113**	**TBX3**
cg21041701	5.55E-05	0.02186	-0.103	H3F3C
cg23424407	**5.52E-05**	**0.02186**	**-0.131**	**TBX3**
cg23615741	**6.98E-05**	**0.02596**	**-0.132**	**NKX2-3**
cg11676636	7.50E-05	0.02715	-0.107	ARHGAP22
cg25455724	1.04E-04	0.03016	-0.102	APOM
cg05415871	1.03E-04	0.03016	-0.158	BC038783
cg18877361	1.16E-04	0.03016	-0.103	C14orf80
cg07064544	**1.06E-04**	**0.03016**	**-0.154**	**HNF4A**
cg17504394	9.25E-05	0.03016	-0.128	Mir_584
cg16597737	1.22E-04	0.03016	-0.101	SLC9A11
cg24820672	**9.96E-05**	**0.03016**	**-0.131**	**SPACA7**
cg19536929	**1.06E-04**	**0.03016**	**-0.109**	**TBX3**
cg16415457	1.26E-04	0.03016	-0.109	UBE2E1
cg02460314	1.38E-04	0.03139	-0.146	CPN1

cg21058973	**1.37E-04**	**0.03139**	**-0.146**	**HNF4A**
cg00622988	1.39E-04	0.03139	-0.105	IRX2
cg06703856	1.47E-04	0.03173	-0.109	LEAP2
cg15242686	**1.72E-04**	**0.03179**	**0.299**	**GSTTP1**
cg22389375	1.72E-04	0.03179	-0.158	KCNQ1DN
cg04215055	1.60E-04	0.03179	-0.112	MGAT5B
cg11480029	1.59E-04	0.03179	-0.137	OR9A2
cg03051777	**1.67E-04**	**0.03179**	**-0.131**	**TBX3**
cg08261841	1.76E-04	0.03179	-0.108	TMEM139
cg11141652	**1.88E-04**	**0.03318**	**0.286**	**GSTTP1**
cg07803284	2.16E-04	0.03530	-0.102	ETV5
cg07664999	2.42E-04	0.03774	-0.123	TM7SF4
cg08251704	2.60E-04	0.03852	-0.102	DGKD
cg14962049	2.86E-04	0.04078	0.111	PRELID2
cg16738929	2.96E-04	0.04133	-0.112	CUL2
cg23172828	3.08E-04	0.04191	-0.122	SMAD6
cg00005112	3.09E-04	0.04191	-0.125	WWP2
cg07091758	3.26E-04	0.04367	-0.110	PKD1L2
cg08076955	3.40E-04	0.04457	-0.125	HOXB1
cg04033390	3.43E-04	0.04457	-0.110	MIR3612
cg15671083	**3.39E-04**	**0.04457**	**-0.105**	**UCN3**
cg16611278	3.72E-04	0.04476	-0.127	DTX1
cg17479716	3.59E-04	0.04476	-0.112	IGSF21
cg11273702	3.52E-04	0.04476	-0.196	KBTBD8
cg00549574	3.58E-04	0.04476	-0.112	PRPSAP1
cg27349333	**3.77E-04**	**0.04476**	**-0.121**	**UCN3**
cg07659053	4.04E-04	0.04498	0.128	BC036251
cg13424029	**3.92E-04**	**0.04498**	**-0.105**	**NKX2-3**
cg14972210	4.13E-04	0.04498	-0.132	SORCS3
cg26004771	4.45E-04	0.04673	-0.177	C1orf49
cg19103219	4.56E-04	0.04673	-0.107	chromosome 10 open reading frame 139
cg01463540	4.56E-04	0.04673	-0.102	DLGAP4
cg10528482	4.56E-04	0.04673	-0.141	SLC9A3
cg13609544	4.64E-04	0.04674	-0.103	HOXB3
cg22948337	**4.76E-04**	**0.04692**	**-0.126**	**SPACA7**
cg04528829	**4.78E-04**	**0.04692**	**-0.119**	**TBX3**
cg02824888	**4.89E-04**	**0.04751**	**-0.108**	**TBX3**
cg00190355	5.56E-04	0.04976	-0.108	BC013821
cg18949192	**5.44E-04**	**0.04976**	**-0.104**	**TBX3**

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Supplemental Table 2: A list of the 450 genes represented by the closest TSS of the 548 differentially methylated sites between 4.DES vs 1.FAS. Bold text highlights genes with 2 or more differentially methylated CpGs associated with them.

CpG	P-Value	Q-Value	Delta Beta (4.DES-1.FAS)	Gene name assocated with closest transcription start site
cg08960448	0.0040	0.0206	-0.1066	41164
cg26944526	0.0003	0.0206	-0.1125	5S_rRNA
cg02258482	0.0009	0.0206	-0.1070	7SK
cg16350608	0.0004	0.0206	-0.1413	7SK
cg04661040	0.0008	0.0206	-0.1139	AAK1
cg12130225	0.0022	0.0206	-0.1102	ABCB10
cg02328793	0.0010	0.0206	-0.1019	ACOT7
cg20587996	0.0002	0.0206	-0.1177	ACTR3C
cg12505085	0.0069	0.0206	-0.1000	ADK
cg14043752	0.0014	0.0206	-0.1152	AFTPH
cg26927232	0.0007	0.0206	-0.1043	AJ276246
cg18016148	0.0013	0.0206	-0.1046	AK025288
cg19307180	0.0008	0.0206	-0.1159	AK054970
cg27263068	0.0059	0.0206	-0.1009	AK055055
cg03985360	0.0010	0.0206	-0.1070	AK055368
cg01358966	0.0014	0.0206	-0.1032	AK075019
cg19510206	0.0017	0.0206	-0.1042	AK096230
cg23755884	0.0005	0.0206	-0.1090	AK128161
cg26049390	0.0030	0.0206	-0.1003	AK128216
cg08796240	0.0037	0.0206	-0.1049	AK128439
cg03991300	0.0021	0.0206	-0.1022	AK128777
cg26036018	0.0066	0.0206	-0.1055	AK4
cg10600964	0.0026	0.0206	-0.1012	AL050000
cg19765196	**0.0022**	**0.0206**	**-0.1070**	**ALCAM**
cg21832978	**0.0062**	**0.0206**	**-0.1044**	**ALCAM**
cg17665652	0.0005	0.0206	-0.1133	ANK2
cg19660531	0.0003	0.0206	-0.1146	ANO2
cg22222261	0.0016	0.0206	-0.1030	ANO5
cg07813214	0.0010	0.0206	-0.1002	AP1S3
cg16362173	0.0020	0.0206	-0.1002	ARF6
cg05816000	0.0036	0.0206	-0.1008	ARHGAP10
cg11310863	0.0065	0.0206	-0.1010	ARHGAP15
cg20892287	0.0012	0.0206	-0.1082	ARHGAP32
cg25422226	0.0014	0.0206	-0.1073	ARL4A
cg27308445	0.0029	0.0206	-0.1004	ARSI
cg03164112	**0.0000**	**0.0206**	**-0.1419**	**ASB18**
cg06473675	**0.0000**	**0.0206**	**-0.1608**	**ASB18**
cg02647835	**0.0035**	**0.0206**	**-0.1023**	**ASPSCR1**

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cg14419393	**0.0067**	**0.0206**	**-0.1130**	**ASPSCR1**
cg04819081	0.0003	0.0206	-0.1010	ATAD2B
cg12194594	0.0027	0.0206	-0.1183	ATP11A
cg18882091	0.0031	0.0206	-0.1079	ATP5J2
cg22795345	0.0019	0.0206	-0.1083	AX748212
cg11523350	0.0027	0.0206	-0.1286	BACE1
cg20446334	0.0005	0.0206	-0.1148	BAIAP2
cg05471602	0.0047	0.0206	-0.1039	BARX2
cg09831875	0.0040	0.0206	-0.1090	BC017209
cg04778178	0.0008	0.0206	-0.1011	BC035889
cg09576488	0.0004	0.0206	-0.1148	BC039545
cg23094620	0.0017	0.0206	-0.1005	BC040634
cg21594328	0.0003	0.0206	-0.1095	BC047484
cg12893852	0.0009	0.0206	-0.1100	BC047651
cg24361265	0.0057	0.0206	-0.1183	BC062349
cg06422039	0.0057	0.0206	-0.1086	BC084558
cg20477160	**0.0004**	**0.0206**	**-0.1110**	**BC148242**
cg25976672	**0.0050**	**0.0206**	**-0.1044**	**BC148242**
cg00498816	**0.0068**	**0.0206**	**-0.1112**	**BC152380**
cg18183817	**0.0032**	**0.0206**	**-0.1137**	**BC152380**
cg00953399	0.0001	0.0206	-0.1080	BET3L
cg21510704	0.0008	0.0206	-0.1043	BX247991
cg08148252	0.0008	0.0206	-0.1108	BX647900
cg25134818	0.0021	0.0206	-0.1058	BZW2
cg01472043	0.0060	0.0206	-0.1063	C10orf47
cg22871947	0.0051	0.0206	-0.1068	C10orf71
cg01168833	0.0040	0.0206	-0.1048	C12orf75
cg18356785	0.0034	0.0206	-0.1042	C1QTNF4
cg01881444	0.0024	0.0206	-0.1032	C3orf64
cg07579997	0.0039	0.0206	-0.1109	C4orf49
cg06871919	0.0052	0.0206	-0.1023	C5orf22
cg17580613	0.0050	0.0206	-0.1032	C7orf30
cg05632631	0.0000	0.0206	-0.1699	CACNG3
cg27235315	0.0000	0.0206	-0.1211	CAPN8
cg00876266	**0.0036**	**0.0206**	**-0.1003**	**CAPS**
cg13545089	**0.0002**	**0.0206**	**-0.1066**	**CAPS**
cg16465280	0.0011	0.0206	-0.1032	CARD11
cg09118017	0.0060	0.0206	-0.1008	CASZ1
cg07790870	0.0020	0.0206	-0.1049	CBFA2T2
cg09405227	0.0039	0.0206	-0.1044	CCBP2
cg02901065	0.0022	0.0206	-0.1072	CCDC146
cg10370305	0.0035	0.0206	-0.1025	CCDC34
cg05233289	0.0023	0.0206	-0.1271	CCDC88B

cg17489451	0.0018	0.0206	-0.1009	CCDC91
cg17224401	0.0010	0.0206	-0.1118	CCR3
cg00606396	0.0008	0.0206	-0.1160	CDH18
cg26673975	0.0007	0.0206	-0.1219	CDH5
cg12864235	0.0013	0.0206	-0.1036	CDH9
cg08371050	0.0059	0.0206	-0.1023	CDK14
cg23235622	0.0053	0.0206	-0.1080	CEP250
cg01987516	**0.0025**	**0.0206**	**-0.1067**	**chromosome 10 open reading frame 139**
cg19103219	**0.0004**	**0.0206**	**-0.1090**	**chromosome 10 open reading frame 139**
cg14496909	**0.0013**	**0.0206**	**-0.1317**	**CHSY1**
cg18536607	**0.0036**	**0.0206**	**-0.1180**	**CHSY1**
cg10667102	0.0001	0.0206	-0.1324	CIRBP
cg03339668	**0.0075**	**0.0206**	**-0.1005**	**CIT**
cg26097051	**0.0030**	**0.0206**	**-0.1005**	**CIT**
cg00804587	0.0032	0.0206	-0.1017	CLDN1
cg26442107	0.0017	0.0206	-0.1347	CLEC16A
cg15016233	0.0065	0.0206	-0.1053	CMAS
cg02366931	**0.0045**	**0.0206**	**-0.1052**	**CMYA5**
cg21928760	0.0016	0.0206	-0.1032	CNTN4
cg13879411	**0.0010**	**0.0206**	**-0.1038**	**COL28A1**
cg21450738	0.0000	0.0206	-0.1421	COX6C
cg03951662	0.0043	0.0206	-0.1010	CPD
cg02460314	0.0001	0.0206	-0.1162	CPN1
cg02483735	0.0003	0.0206	-0.2047	CSF3R
cg05885484	0.0013	0.0206	-0.1087	CSNK1G3
cg00814909	0.0012	0.0206	-0.1155	CTBP1
cg09916651	**0.0001**	**0.0206**	**-0.1444**	**CUX2**
cg10535597	**0.0066**	**0.0206**	**-0.1015**	**CUX2**
cg11551879	**0.0010**	**0.0206**	**-0.1023**	**CUX2**
cg02971328	0.0044	0.0206	-0.1013	DAGLB
cg12655375	**0.0040**	**0.0206**	**-0.1044**	**DEF6**
cg18486815	**0.0019**	**0.0206**	**-0.1116**	**DEF6**
cg10280963	0.0032	0.0206	-0.1036	DEK
cg08379738	0.0078	0.0206	-0.1008	DENND1C
cg10113526	0.0078	0.0206	-0.1068	DENND3
cg08251704	0.0002	0.0206	-0.1046	DGKD
cg01379412	0.0048	0.0206	-0.1050	DIXDC1
cg11681321	0.0037	0.0206	-0.1039	DJ439531
cg11714341	0.0001	0.0206	-0.1046	DKFZp686M11215
cg03668763	0.0004	0.0206	-0.1114	DM376719
cg06776201	0.0010	0.0206	-0.1247	DPF3
cg05617002	0.0040	0.0206	-0.1009	DPP10

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cg08030922	0.0004	0.0206	-0.1105	DQ571524
cg01502353	**0.0008**	**0.0206**	**-0.1086**	**DST**
cg15750696	**0.0029**	**0.0206**	**-0.1092**	**DST**
cg01192077	**0.0003**	**0.0206**	**-0.1495**	**EBF1**
cg22586996	**0.0040**	**0.0206**	**-0.1007**	**EBF1**
cg24889827	0.0015	0.0206	-0.1005	EEA1
cg18454133	0.0041	0.0206	-0.1225	EFS
cg00813343	0.0011	0.0206	-0.1140	EIF3IP1
cg24935217	0.0062	0.0206	-0.1022	ELF5
cg23208271	0.0069	0.0206	-0.1003	ELMO1
cg16058975	0.0004	0.0206	-0.1088	ELTD1
cg07899060	0.0057	0.0206	-0.1011	EMID2
cg14195925	0.0080	0.0206	-0.1007	ENGASE
cg09313482	0.0002	0.0206	-0.1017	ENPP2
cg07331478	0.0003	0.0206	-0.1032	EPHA4
cg10231182	0.0042	0.0206	-0.1011	EPS8
cg02408480	0.0026	0.0206	-0.1019	ERCC8
cg00768179	0.0031	0.0206	-0.1053	ERRFI1
cg00944421	0.0034	0.0206	-0.1034	ESRP2
cg06429194	**0.0025**	**0.0206**	**-0.1010**	**ETS1**
cg21121082	**0.0048**	**0.0206**	**-0.1138**	**ETS1**
cg27078890	**0.0041**	**0.0206**	**-0.1057**	**ETS1**
cg01423643	0.0013	0.0206	-0.1032	F11R
cg13563867	0.0007	0.0206	-0.1087	F3
cg01089602	0.0030	0.0206	-0.1013	F7
cg07213482	0.0021	0.0206	-0.1040	FAM13A
cg11059712	0.0000	0.0206	-0.1072	FAM171A1
cg22973459	0.0010	0.0206	-0.1033	FAM178A
cg19775206	0.0057	0.0206	-0.1122	FAM181A
cg26815617	0.0034	0.0206	-0.1010	FAM183B
cg13680876	0.0009	0.0206	-0.1024	FAM189A2
cg03003256	0.0020	0.0206	-0.1116	FAM26D
cg00094158	0.0012	0.0206	-0.1006	FAM53B
cg03501539	0.0007	0.0206	-0.1240	FAM55B
cg12883629	0.0009	0.0206	0.1047	FAM65B
cg24052851	0.0016	0.0206	-0.1054	FAM75C2
cg18610569	0.0003	0.0206	-0.1004	FAT4
cg19674178	0.0013	0.0206	-0.1024	FGF7
cg02888990	0.0000	0.0206	-0.1254	FLJ43860
cg11223933	**0.0044**	**0.0206**	**-0.1031**	**FMNL1**
cg19735250	**0.0071**	**0.0206**	**-0.1147**	**FMNL1**
cg06980460	0.0044	0.0206	-0.1082	FOLH1
cg07929259	0.0068	0.0206	-0.1029	FOXA1

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cg19509778	0.0015	0.0206	-0.1064	FOXI2
cg24541672	0.0009	0.0206	-0.1162	FOXK2
cg25481160	0.0052	0.0206	-0.1067	FOXP1
cg14330460	0.0033	0.0206	-0.1009	FRMD1
cg22262233	0.0008	0.0206	-0.1052	GABRG3
cg25556008	**0.0049**	**0.0206**	**-0.1025**	**GADD45B**
cg17679980	**0.0054**	**0.0206**	**-0.1015**	**GAS2**
cg25982561	**0.0052**	**0.0206**	**-0.1107**	**GAS2**
cg21377950	0.0047	0.0206	-0.1025	GJD4
cg06007850	0.0040	0.0206	-0.1005	GMDS
cg02479744	0.0021	0.0206	-0.1068	GMPR
cg04206484	0.0061	0.0206	-0.1161	GPER
cg21986821	0.0056	0.0206	-0.1003	GPR108
cg15720112	0.0012	0.0206	-0.1032	GRAMD3
cg08920233	0.0013	0.0206	-0.1082	GRIP1
cg09805403	0.0030	0.0206	-0.1092	GSPT1
cg03234702	0.0035	0.0206	-0.1116	HIST1H3E
cg09515921	0.0057	0.0206	-0.1028	HLX
cg25494605	0.0017	0.0206	-0.1018	HMP19
cg20848979	0.0031	0.0206	-0.1129	HNF4A
cg03650946	**0.0002**	**0.0206**	**-0.1034**	**HOXA3**
cg08164294	**0.0010**	**0.0206**	**-0.1004**	**HOXA3**
cg18680977	**0.0049**	**0.0206**	**-0.1021**	**HOXA3**
cg11532431	**0.0061**	**0.0206**	**-0.1044**	**HOXA4**
cg22997113	**0.0015**	**0.0206**	**-0.1218**	**HOXA4**
cg04904318	**0.0040**	**0.0206**	**-0.1431**	**HOXB1**
cg07823492	**0.0024**	**0.0206**	**-0.1182**	**HOXB1**
cg22660933	**0.0067**	**0.0206**	**-0.1395**	**HOXB1**
cg24900666	**0.0058**	**0.0206**	**-0.1021**	**HOXB1**
cg24948406	**0.0008**	**0.0206**	**-0.1201**	**HOXB1**
cg26634219	**0.0005**	**0.0206**	**-0.1188**	**HOXB1**
cg11060532	**0.0003**	**0.0206**	**-0.1149**	**HOXB3**
cg23014425	**0.0026**	**0.0206**	**-0.1143**	**HOXB3**
cg27656658	**0.0045**	**0.0206**	**-0.1006**	**HOXB3**
cg14047130	0.0001	0.0206	-0.1034	HS3ST1
cg02332936	0.0011	0.0206	-0.1175	HSDL2
cg06127256	0.0032	0.0206	-0.1003	HTA
cg22713469	0.0021	0.0206	-0.1037	HTR1A
cg08269485	0.0014	0.0206	-0.1035	IFITM10
cg17479716	0.0004	0.0206	-0.1407	IGSF21
cg00796611	0.0006	0.0206	-0.1022	IKBKE
cg09131332	0.0007	0.0206	-0.1355	IL17REL
cg06658391	0.0055	0.0206	-0.1069	IL1RN

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cg18145937	0.0004	0.0206	-0.1029	IL4
cg25861066	0.0001	0.0206	-0.1021	INHBA
cg20783970	0.0006	0.0206	-0.1150	IRF2BP2
cg13327384	0.0039	0.0206	-0.1036	IRX1
cg06742021	**0.0001**	**0.0206**	**-0.1189**	**IRX2**
cg22986569	**0.0002**	**0.0206**	**-0.1396**	**IRX2**
cg04115185	**0.0003**	**0.0206**	**-0.1286**	**IRX4**
cg12924936	**0.0004**	**0.0206**	**-0.1421**	**IRX4**
cg15714227	**0.0002**	**0.0206**	**-0.1156**	**IRX4**
cg17006413	**0.0022**	**0.0206**	**-0.1068**	**ISG15**
cg21472517	**0.0007**	**0.0206**	**-0.1206**	**ISG15**
cg24655428	0.0065	0.0206	-0.1073	ISLR
cg15638366	**0.0006**	**0.0206**	**-0.1090**	**ITGA6**
cg11273702	0.0003	0.0206	-0.1846	KBTBD8
cg15731035	0.0007	0.0206	-0.1207	KCNAB2
cg01323777	**0.0035**	**0.0206**	**-0.1014**	**KCNAB3**
cg13407335	**0.0022**	**0.0206**	**-0.1168**	**KCNAB3**
cg13675753	**0.0016**	**0.0206**	**-0.1046**	**KCNJ1**
cg25194720	**0.0026**	**0.0206**	**-0.1069**	**KCNJ1**
cg27056194	**0.0002**	**0.0206**	**-0.1225**	**KCNJ1**
cg23735602	0.0024	0.0206	-0.1145	KCNMB2
cg07148458	0.0023	0.0206	-0.1119	KCTD11
cg27221338	**0.0026**	**0.0206**	**-0.1125**	**KDELR2**
cg02479497	**0.0047**	**0.0206**	**-0.1009**	**KIAA0182**
cg27048684	0.0024	0.0206	-0.1270	KIAA0556
cg19593009	0.0069	0.0206	-0.1017	KIAA0825
cg07628631	0.0019	0.0206	-0.1044	KIAA1033
cg24814707	0.0000	0.0206	-0.1256	KIAA1211
cg24775327	**0.0059**	**0.0206**	**-0.1161**	**KIAA1274**
cg11035730	0.0049	0.0206	-0.1057	KIF26A
cg26531076	0.0044	0.0206	-0.1140	KLF5
cg10982443	0.0034	0.0206	-0.1085	KLHL33
cg17667972	0.0046	0.0206	-0.1034	KRT4
cg20102877	0.0019	0.0206	-0.1061	KRTCAP3
cg05364691	**0.0062**	**0.0206**	**-0.1006**	**LAMB4**
cg16190209	**0.0037**	**0.0206**	**-0.1009**	**LAMB4**
cg26914392	0.0004	0.0206	-0.1218	LBR
cg20926939	0.0046	0.0206	-0.1067	LDB2
cg25202503	0.0009	0.0206	-0.1014	LINC00346
cg16754346	0.0040	0.0206	-0.1050	LINC00426
cg03548062	**0.0064**	**0.0206**	**-0.1208**	**LINC00523**
cg09942166	**0.0040**	**0.0206**	**-0.1048**	**LINC00523**
cg11806439	**0.0032**	**0.0206**	**-0.1179**	**LINC00523**

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cg21869609	0.0058	0.0206	-0.1002	LINGO3
cg02346342	0.0015	0.0206	-0.1023	LOC100128126
cg01644640	0.0005	0.0206	-0.1025	LOC100128590
cg02473847	**0.0012**	**0.0206**	**-0.1019**	**LOC100129534**
cg09510202	0.0055	0.0206	-0.1022	LOC100130357
cg14845962	0.0032	0.0206	-0.1199	LOC100130776
cg17826530	0.0072	0.0206	-0.1097	LOC100131060
cg26402660	0.0024	0.0206	-0.1025	LOC100133311
cg27322699	0.0063	0.0206	-0.1186	LOC100507043
cg17014914	0.0012	0.0206	-0.1054	LOC100507466
cg21462633	0.0017	0.0206	-0.1087	LOC151171
cg14188346	0.0056	0.0206	-0.1062	LOC158435
cg03826463	0.0050	0.0206	-0.1044	LOC253573
cg16800228	0.0012	0.0206	-0.1032	LOC400456
cg24996154	0.0023	0.0206	-0.1035	LOC503519
cg09556515	0.0029	0.0206	-0.1130	LOC727710
cg09226986	0.0019	0.0206	-0.1094	LPAL2
cg01482958	0.0048	0.0206	-0.1035	LPAR3
cg25078649	0.0006	0.0206	-0.1021	LRRC73
cg08617354	0.0026	0.0206	-0.1092	LRRTM3
cg07395436	0.0001	0.0206	-0.1071	LYPLAL1
cg05258935	0.0072	0.0206	-0.1139	MAP4K1
cg16100355	0.0030	0.0206	-0.1101	MCF2L
cg11602041	0.0047	0.0206	-0.1017	ME3
cg16558822	**0.0011**	**0.0206**	**-0.1111**	**MECOM**
cg18675617	**0.0070**	**0.0206**	**0.1016**	**MEIS1**
cg03260530	0.0016	0.0206	-0.1011	METRNL
cg01218903	0.0002	0.0206	-0.1265	MGAT5B
cg20731875	0.0032	0.0206	-0.1223	MGC12916
cg06553513	**0.0003**	**0.0206**	**-0.1204**	**MGMT**
cg17451493	**0.0000**	**0.0206**	**-0.1104**	**MGMT**
cg14576951	0.0022	0.0206	-0.1020	MICALL2
cg20288000	0.0054	0.0206	-0.1032	Mir_584
cg21935393	0.0001	0.0206	-0.1293	Mir_720
cg09574009	0.0006	0.0206	-0.1056	MIR1297
cg18792131	0.0016	0.0206	-0.1237	MIR200B
cg11616411	0.0033	0.0206	-0.1017	MIR371A
cg10418812	0.0029	0.0206	-0.1270	MIR4655
cg15077193	0.0032	0.0206	-0.1014	MIR4745
cg05778494	0.0019	0.0206	-0.1043	MIR92B
cg08061524	**0.0021**	**0.0206**	**-0.1321**	**MIRLET7BHG**
cg13523819	0.0014	0.0206	-0.1156	MITF
cg06308084	0.0049	0.0206	-0.1070	MLC1

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cg02016467	0.0067	0.0206	-0.1064	MSLN
cg24404533	0.0055	0.0206	-0.1007	MUC6
cg04869379	0.0024	0.0206	-0.1059	MYO16
cg13432945	0.0021	0.0206	-0.1014	MYSM1
cg04784618	0.0003	0.0206	-0.1141	NAALADL2
cg19827875	0.0017	0.0206	-0.1081	NAV1
cg07613333	0.0013	0.0206	-0.1003	NAV2
cg13774505	0.0022	0.0206	-0.1120	NCS1
cg17147885	0.0057	0.0206	-0.1075	NEK10
cg03000585	0.0007	0.0206	-0.1003	NEXN
cg00593773	**0.0039**	**0.0206**	**-0.1044**	**NFATC1**
cg25951288	**0.0030**	**0.0206**	**-0.1023**	**NFATC1**
cg09113483	**0.0070**	**0.0206**	**-0.1007**	**NFIA**
cg06807696	0.0062	0.0206	-0.1149	NFKBIA
cg18980036	0.0004	0.0206	-0.1018	NKX6-2
cg14307471	0.0005	0.0206	-0.1209	NOL4
cg04969340	0.0000	0.0206	-0.1340	NOM1
cg22249735	0.0037	0.0206	-0.1086	NRP2
cg21660452	0.0030	0.0206	-0.1338	NRXN2
cg07657463	0.0059	0.0206	-0.1009	NSUN7
cg00540540	0.0075	0.0206	-0.1055	ODZ2
cg00376979	**0.0001**	**0.0206**	**-0.1263**	**OLIG2**
cg05183668	**0.0000**	**0.0206**	**-0.1034**	**OLIG2**
cg11912202	**0.0000**	**0.0206**	**-0.1447**	**OLIG2**
cg03895540	0.0027	0.0206	-0.1070	OR52N2
cg11480029	0.0000	0.0206	-0.1123	OR9A2
cg12927617	0.0037	0.0206	-0.1119	ORM1
cg00874605	**0.0025**	**0.0206**	**-0.1175**	**PARN**
cg04388123	**0.0059**	**0.0206**	**-0.1054**	**PARN**
cg14397402	0.0001	0.0206	-0.1068	PAX9
cg19637591	0.0009	0.0206	-0.1046	PCDH17
cg00817501	0.0020	0.0206	-0.1009	PCDH18
cg08548659	0.0008	0.0206	-0.1035	PCDH7
cg05148373	0.0003	0.0206	0.1015	PDE1A
cg18425032	0.0069	0.0206	-0.1039	PDE4D
cg19350115	0.0026	0.0206	-0.1148	PDE7B
cg05556923	**0.0060**	**0.0206**	**-0.1537**	**PDGFA**
cg09187695	**0.0022**	**0.0206**	**-0.1024**	**PDGFA**
cg02055988	0.0041	0.0206	-0.1041	PIK3IP1
cg00120783	0.0040	0.0206	-0.1130	PITPNA
cg07091758	0.0001	0.0206	-0.1074	PKD1L2
cg12635662	0.0006	0.0206	-0.1035	PLAC1L
cg24578857	0.0054	0.0206	-0.1123	PLD6

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cg22594071	0.0007	0.0206	-0.1158	PLSCR5
cg18180501	0.0011	0.0206	-0.1014	PP12613
cg00501542	0.0014	0.0206	-0.1067	PPFIA4
cg12069151	0.0036	0.0206	-0.1015	PPP1R10
cg20067575	**0.0048**	**0.0206**	**-0.1040**	**PPP1R14C**
cg26954245	**0.0031**	**0.0206**	**-0.1077**	**PPP1R14C**
cg07056967	0.0058	0.0206	-0.1076	PPP6R3
cg12466022	0.0074	0.0206	-0.1014	PPPDE1
cg11150222	**0.0019**	**0.0206**	**-0.1006**	**PRDM1**
cg21376090	**0.0002**	**0.0206**	**-0.1109**	**PRDM1**
cg23591302	0.0007	0.0206	-0.1095	PRICKLE1
cg27303421	0.0023	0.0206	-0.1145	PRL
cg18235088	**0.0030**	**0.0206**	**-0.1121**	**PRRT1**
cg20151476	0.0005	0.0206	-0.1008	PSMG3
cg00908766	0.0013	0.0206	-0.1086	PSRC1
cg13049961	0.0024	0.0206	-0.1090	PURA
cg15323253	0.0027	0.0206	-0.1011	PXDC1
cg11298446	0.0029	0.0206	-0.1069	RAB11FIP2
cg09772333	0.0035	0.0206	-0.1054	RABAC1
cg11526630	0.0016	0.0206	-0.1177	RADIL
cg10846682	0.0017	0.0206	-0.1250	RAMP1
cg00730887	0.0022	0.0206	-0.1046	RAPGEF2
cg22906709	0.0016	0.0206	-0.1134	RASA3
cg01062942	**0.0017**	**0.0206**	**-0.1304**	**RASAL3**
cg21002957	**0.0019**	**0.0206**	**-0.1096**	**RASAL3**
cg22216196	**0.0009**	**0.0206**	**-0.1063**	**RASAL3**
cg01927373	0.0072	0.0206	-0.1059	RASGEF1B
cg06389444	0.0019	0.0206	-0.1062	RBFOX3
cg08425628	0.0021	0.0206	-0.1035	RBM43
cg14864148	0.0052	0.0206	-0.1047	RELT
cg15934776	0.0011	0.0206	-0.1143	REV1
cg04013650	**0.0030**	**0.0206**	**-0.1042**	**RP1**
cg13833632	0.0004	0.0206	-0.1136	RTKN2
cg00773370	0.0015	0.0206	-0.1048	SCNN1A
cg09245302	0.0056	0.0206	-0.1065	SECTM1
cg19262563	0.0008	0.0206	-0.1049	SEMA3C
cg06653026	0.0013	0.0206	-0.1071	SEMA3D
cg21442419	0.0002	0.0206	-0.1030	SKI
cg00655147	0.0058	0.0206	-0.1042	SLC16A12
cg18158015	0.0017	0.0206	-0.1058	SLC22A25
cg20127102	0.0022	0.0206	-0.1006	SLC43A3
cg27109043	0.0013	0.0206	-0.1040	SLC45A4
cg02344497	0.0023	0.0206	-0.1026	SLC4A7

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cg02102889	0.0006	0.0206	-0.1106	SLC7A11
cg22689016	0.0006	0.0206	-0.1178	SLC9A3
cg05906024	**0.0044**	**0.0206**	**-0.1155**	**SLIT3**
cg13908833	**0.0064**	**0.0206**	**-0.1009**	**SLIT3**
cg25984601	**0.0013**	**0.0206**	**-0.1102**	**SLIT3**
cg21718735	0.0025	0.0206	-0.1093	SMAD7
cg17648080	0.0015	0.0206	-0.1058	SMG6
cg25669593	0.0048	0.0206	-0.1133	SMOC2
cg05584602	0.0031	0.0206	-0.1142	SNX29
cg00572966	0.0020	0.0206	-0.1083	SP4
cg20290367	0.0023	0.0206	-0.1008	SPECC1
cg05694089	0.0022	0.0206	-0.1013	SPEN
cg02813710	0.0049	0.0206	-0.1174	SRPK2
cg18180230	0.0001	0.0206	-0.1017	SRSF8
cg20018563	0.0000	0.0206	-0.1148	ST8SIA2
cg18481642	0.0034	0.0206	-0.1098	STAT1
cg18628646	0.0049	0.0206	-0.1002	STOX2
cg19847038	**0.0005**	**0.0206**	**-0.1124**	**STX19**
cg27200466	**0.0023**	**0.0206**	**-0.1121**	**STX19**
cg15173428	0.0054	0.0206	-0.1056	SYN3
cg04703974	0.0011	0.0206	-0.1051	SYNRG
cg11171466	0.0008	0.0206	-0.1126	SYT8
cg07213771	0.0028	0.0206	-0.1109	TBX18
cg04370442	0.0016	0.0206	-0.1229	TEPP
cg06784602	0.0045	0.0206	-0.1037	TGFBR2
cg01704739	**0.0007**	**0.0206**	**-0.1054**	**TM4SF1**
cg07664999	0.0017	0.0206	-0.1047	TM7SF4
cg10501093	**0.0049**	**0.0206**	**-0.1120**	**TNFAIP2**
cg18620571	**0.0047**	**0.0206**	**-0.1173**	**TNFAIP2**
cg01089914	**0.0031**	**0.0206**	**-0.1070**	**TNS1**
cg20225999	**0.0035**	**0.0206**	**-0.1013**	**TNS1**
cg08040824	0.0003	0.0206	-0.1138	TP63
cg23234149	**0.0016**	**0.0206**	**-0.1096**	**TP73**
cg18062333	0.0002	0.0206	-0.1142	TRNA
cg22731383	**0.0003**	**0.0206**	**-0.1049**	**TRNA_Pseudo**
cg08638929	0.0054	0.0206	-0.1028	TSPAN1
cg25588348	0.0009	0.0206	-0.1182	TTLL5
cg07532576	0.0012	0.0206	-0.1041	TTN
cg16689724	0.0006	0.0206	-0.1166	TUBE1
cg13974313	0.0012	0.0206	-0.1039	TXK
cg16969274	0.0017	0.0206	-0.1155	U6
cg03668982	0.0008	0.0206	-0.1002	UBE2E1
cg06513139	0.0040	0.0206	-0.1004	UBXN6

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cg06634441	**0.0003**	**0.0206**	**-0.1047**	**UGT2A1**
cg23270582	**0.0074**	**0.0206**	**-0.1006**	**UGT2A1**
cg16807101	0.0008	0.0206	-0.1001	UHRF1BP1L
cg01449677	0.0028	0.0206	-0.1185	USP36
cg14855367	0.0020	0.0206	-0.1030	UTS2D
cg06795523	0.0032	0.0206	-0.1036	VRK2
cg24726965	0.0009	0.0206	-0.1047	WDPCP
cg10090326	0.0021	0.0206	-0.1028	WDR25
cg00415665	0.0054	0.0206	-0.1121	ZHX2
cg17970299	0.0064	0.0206	-0.1047	ZNF385A
cg22987448	0.0022	0.0206	-0.1014	ZNF414
cg06022562	0.0060	0.0206	-0.1012	ZNF750
cg05995260	0.0081	0.0206	-0.1105	AF075112
cg18820060	0.0082	0.0206	-0.1014	LOC285577
cg05881698	0.0081	0.0206	-0.1085	NRTN
cg08485527	0.0083	0.0206	-0.1022	SEC14L1
cg14279856	0.0085	0.0206	-0.1021	DDR1
cg14582917	0.0084	0.0206	-0.1027	LOC100506190
cg13568106	0.0087	0.0207	-0.1049	FOXN1
cg01571001	0.0087	0.0207	-0.1093	GATA2
cg26150922	0.0087	0.0207	-0.1017	LONRF2
cg03346415	0.0089	0.0207	-0.1122	WWP2
cg17229698	**0.0089**	**0.0207**	**-0.1116**	**SLIT3**
cg02289739	0.0091	0.0207	-0.1062	AK057978
cg09378456	0.0092	0.0208	-0.1042	CFTR
cg08018572	0.0092	0.0208	-0.1033	LMF1
cg14835484	0.0091	0.0208	-0.1024	TPM3
cg10628201	0.0092	0.0208	-0.1116	YDJC
cg20344388	**0.0093**	**0.0208**	**-0.1019**	**COL18A1**
cg11585071	**0.0094**	**0.0208**	**-0.1093**	**TP73**
cg14316944	0.0099	0.0208	-0.1109	ACTG1
cg01446627	0.0096	0.0208	-0.1090	ADARB2-AS1
cg22641350	0.0095	0.0208	-0.1135	BC038783
cg06607764	0.0098	0.0208	-0.1056	CYTH1
cg07678266	0.0098	0.0208	-0.1162	GLT1D1
cg03915932	**0.0097**	**0.0208**	**-0.1012**	**HOXB1**
cg18812904	0.0094	0.0208	-0.1071	KCNG2
cg14496282	**0.0095**	**0.0208**	**-0.1257**	**PDGFA**
cg04757389	0.0094	0.0208	-0.1030	PTPRS
cg24714094	0.0098	0.0208	-0.1001	RTDR1
cg15087376	**0.0099**	**0.0208**	**-0.1004**	**TM4SF1**
cg26427109	0.0104	0.0208	0.1077	CD6
cg03044452	0.0107	0.0208	-0.1020	GGT6

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cg08101036	**0.0104**	**0.0208**	**-0.1226**	**HOXA3**
cg08622675	**0.0106**	**0.0208**	**-0.1096**	**KDELR2**
cg07474477	0.0103	0.0208	-0.1020	PKMYT1
cg03039843	0.0104	0.0208	-0.1066	SULT2B1
cg21665744	0.0108	0.0208	-0.1017	AK023033
cg05843596	0.0109	0.0208	-0.1124	AK123181
cg26313247	0.0110	0.0209	-0.1076	GPX5
cg08616951	0.0111	0.0209	-0.1030	PKP3
cg12937817	0.0112	0.0209	-0.1023	MAD1L1
cg04276301	**0.0113**	**0.0209**	**0.1137**	**OTX1**
cg09951047	0.0113	0.0209	-0.1004	MIR3074
cg03661817	0.0114	0.0210	-0.1053	KIFC3
cg25844471	0.0116	0.0210	-0.1004	C10orf67
cg23602690	0.0115	0.0210	-0.1271	DM119500
cg21478902	0.0118	0.0210	-0.1015	COL4A1
cg16397032	0.0118	0.0210	-0.1118	BC007399
cg03526459	**0.0119**	**0.0210**	**-0.1114**	**TRNA_Pseudo**
cg04148089	**0.0123**	**0.0211**	**-0.1007**	**KIAA0182**
cg10002850	0.0124	0.0211	-0.1031	EGFR
cg09941601	0.0126	0.0212	-0.1036	FOXL1
cg24591913	0.0130	0.0213	-0.1003	PPYR1
cg12752420	0.0135	0.0215	-0.1059	C1orf210
cg04892182	0.0137	0.0216	-0.1058	KIAA1522
cg14052044	0.0137	0.0216	-0.1000	C19orf21
cg09655403	**0.0140**	**0.0217**	**-0.1058**	**CMYA5**
cg22009751	0.0140	0.0217	-0.1247	MIR2467
cg15744108	0.0143	0.0218	-0.1015	TRNA_Leu
cg22615730	0.0147	0.0219	-0.1027	ANXA4
cg17342469	**0.0152**	**0.0221**	**-0.1098**	**MIRLET7BHG**
cg08407014	**0.0153**	**0.0221**	**-0.1021**	**GADD45B**
cg00921266	**0.0153**	**0.0221**	**-0.1225**	**HOXA3**
cg11015251	**0.0156**	**0.0222**	**-0.1052**	**HOXA4**
cg02944007	0.0157	0.0222	-0.1002	TSPAN10
cg02497558	**0.0158**	**0.0223**	**-0.1276**	**HOXB1**
cg11737879	0.0158	0.0223	-0.1012	PEX3
cg17239876	**0.0159**	**0.0223**	**-0.1480**	**KIAA1274**
cg27331292	0.0161	0.0224	-0.1038	SH2D3C
cg18155888	**0.0162**	**0.0224**	**-0.1002**	**LOC100129534**
cg18451016	0.0163	0.0224	-0.1120	SF3A3
cg16595607	**0.0164**	**0.0224**	**0.1129**	**OTX1**
cg19825437	**0.0166**	**0.0225**	**-0.1145**	**MECOM**
cg12748890	0.0167	0.0225	-0.1026	SYTL1
cg11254532	**0.0174**	**0.0228**	**-0.1028**	**NFIA**

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cg14297340	0.0176	0.0229	-0.1002	LOC100507299
cg03903278	0.0185	0.0232	0.1033	OTOL1
cg06463424	**0.0186**	**0.0233**	**-0.1008**	**RP1**
cg26209990	**0.0188**	**0.0234**	**-0.1111**	**LEP**
cg23792485	0.0190	0.0234	-0.1077	HNF4A
cg13471599	0.0192	0.0235	-0.1064	MSI1
cg03003335	0.0194	0.0236	-0.1123	ACAP3
cg10271192	**0.0199**	**0.0238**	**0.1025**	**OTX1**
cg24812143	**0.0219**	**0.0247**	**-0.1510**	**KIAA1274**
cg09282336	0.0233	0.0254	-0.1030	CMIP
cg22635491	0.0253	0.0263	-0.1216	TBX3
cg25979543	0.0258	0.0266	-0.1012	ARHGEF10L
cg18290233	**0.0271**	**0.0272**	**0.1143**	**NR2F1**
cg12422539	0.0275	0.0274	-0.1204	AK091028
cg14721213	0.0281	0.0278	-0.1003	FMO2
cg08234689	**0.0282**	**0.0278**	**-0.1093**	**LEP**
cg08045906	**0.0290**	**0.0282**	**-0.1013**	**PRRT1**
cg01513157	0.0297	0.0285	-0.1075	SDR42E1
cg22022580	0.0297	0.0286	-0.1024	BC024306
cg00221709	**0.0307**	**0.0290**	**0.1018**	**NR2F2**
cg18524788	0.0316	0.0294	-0.1087	MIR148A
cg02670123	**0.0341**	**0.0306**	**-0.1044**	**ITGA6**
cg01953658	**0.0360**	**0.0316**	**0.1006**	**NR2F2**
cg02081019	**0.0405**	**0.0339**	**0.1288**	**NR2F1**
cg01082798	0.0496	0.0386	-0.1130	AK024243
cg14457782	**0.0515**	**0.0396**	**0.1055**	**WNK4**
cg06528626	**0.0547**	**0.0413**	**-0.1245**	**LMO3**
cg11157253	**0.0590**	**0.0435**	**0.1089**	**NR2F2**
cg01223086	**0.0601**	**0.0440**	**0.1089**	**NR2F2**
cg27624826	**0.0612**	**0.0446**	**-0.1053**	**TRNA_Pseudo**
cg13851508	**0.0619**	**0.0449**	**0.1091**	**MEIS1**
cg16930572	**0.0636**	**0.0458**	**-0.1203**	**LMO3**
cg08409113	**0.0639**	**0.0459**	**0.1072**	**WNK4**
cg20933946	**0.0644**	**0.0461**	**0.1193**	**NR2F2**
cg21293562	**0.0664**	**0.0472**	**0.1270**	**NR2F2**
cg26267430	**0.0699**	**0.0489**	**0.1233**	**NR2F2**
cg01364755	0.0706	0.0493	-0.1062	ADARB2
cg04120686	**0.0719**	**0.0499**	**-0.1031**	**MECOM**

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Supplemental Table 3: CpGs differentially methylated in response to allergen after prior diesel exhaust exposure and in response to diesel exhaust after prior allergen exposure

CpG	1.FAS Vs 7.FAA		1.FAS Vs 4.DES		Gene name of nearest TSS
	Δβ	Q value	Δβ	Q value	
cg02460314	-0.146	0.0314	-0.116	0.0206	CPN1
cg02888990	-0.107	0.0213	-0.125	0.0206	FLJ43860
cg07091758	-0.110	0.0437	-0.107	0.0206	PKD1L2
cg07664999	-0.123	0.0377	-0.105	0.0206	TM7SF4
cg08251704	-0.102	0.0385	-0.105	0.0206	DGKD
cg11273702	-0.196	0.0448	-0.185	0.0206	KBTBD8
cg11480029	-0.137	0.0318	-0.112	0.0206	OR9A2
cg11912202	-0.132	0.0111	-0.145	0.0206	OLIG2
cg17479716	-0.112	0.0448	-0.141	0.0206	IGSF21
cg19103219	-0.107	0.0467	-0.109	0.0206	chromosome 10 open reading frame 139

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Supplemental Table 4: Pyrosequencing Primers

Primer Name	Primer Sequence
3_TBX3 F1	TTG TGT GGT TAT GGT ATA AAG TTA GT
3_TBX3 R1*	/5BiodT/CA AAT ACA ATC CCT CCT ACT ATA ACA TAC
3_TBX3 S1	ATT TTG ATT TTG GTA GTA GAT
4_HOXA3 F1	GGA GAA AGT GAG GGT TGA TTA TTG AT
4_HOXA3 R1*	/5BiodT/AA CCT CAT ACA TAC CAA TAA TTT TTA TCA
4_HOXA3 S.2	GGG TTT GAA GAG AAA ATG
8_HOXA4 F1*	/5BiodT/GT TAG TGG TGT ATT TTT GGA TGA AGA AG
8_HOXA4 R1	CAC ATA CCC ACA TCT CAC C
8_HOXA4 S1	TCC CCT AAA CCT CTC
9_HOXB1 F1	TAG AGT GGG TAT TTT AAG AAG GAG TT
9_HOXB1 R1*	/5BiodT/TA ACC CAT TAA CCT AAA AAA AAT CAC ATA T
9_HOXB1 S1	AAG AAG GAG TTT ATT TTA TTA
12_HOXB3 F1*	/5BiodT/GA GAA ATG TTG GAT GTG GGA AGA TAG
12_HOXB3 R1	ACC CCA ACC CTA ACA ATA AAT CTC CCT A
12_HOXB3 S1	CAA TCT CCC CTT TCT CAC AAT AT

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Supplemental Table 5: CpGs differentially methylated in response to diesel exhaust exposure or diesel exhaust and allergen co-exposure

Exposure	CpG	P value	Q value
Diesel Exhaust	cg04234412	5.66E-07	0.009
	cg06437703	2.75E-06	0.017
	cg09033563	1.97E-06	0.015
	cg12419862	7.25E-07	0.009
	cg20007245	9.43E-07	0.009
	cg25703541	1.82E-07	0.007
Co-exposure	cg00458681	2.09E-06	0.019
	cg04234412	1.87E-06	0.019
	cg09033563	2.73E-06	0.019
	cg12419862	3.03E-06	0.019
	cg20007245	6.04E-06	0.032
	cg25703541	1.92E-06	0.019

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Supplementary Results

Study group status had no effect on DNA methylation

As our analysis plan involved comparisons of samples that spanned our two study groups, our first analysis assessed significant differences in CpG-specific methylation due specifically to group assignment. We used linear modelling to compare two samples, one from each group, with the exact same study exposure; 3.DEA and 6.DEA. Both samples were collected from lungs 48 hours after co-exposure to DE and allergen with no prior allergen or DE exposure. The resulting unadjusted p-value distribution (Figure S1A) showed skewing towards high p-values, suggesting a low likelihood of differential CpG methylation by group assignment beyond than that expected by chance, thus allowing the subsequent between group comparisons of study exposure. Furthermore no sites were significantly different at an FDR of 0.05.

Initial exposure had minimal effect on DNA methylation

Analyses inclusive of DE were unpaired to allow the same model to be run for all comparisons regardless of whether they were within or between groups. For allergen-only analysis, a paired model was possible and performed as both samples were taken from the same group of individuals, but we observed little such difference (Figure S1A).

DNA methylation was significantly affected by DE in lung previously exposed to allergen.

As the samples used in the analysis of DE in lung previously exposed to allergen were all taken from Group I, i.e. from the same individuals, we also performed a paired analysis. This analysis found 23,379 CpGs that were significantly differentially methylated after BH correction between the control samples and the DE after prior allergen exposure samples, compared to the 22,904 identified by non-paired analysis (Figure S1B). When we applied the filter of a $\Delta\beta > 0.1$, 547 probes remained in comparison to 548 probes identified by non-paired analysis.

Supplemental Figure Legends

Supplemental Figure 1) Comparison of paired Vs non-paired analysis. Paired vs non-paired p values for A) 2.FAA vs 1.FAS and B) 4.DES vs 1.FAS.

Supplemental Figure 2) CpGs methylation did not significantly differ between Group I and Group II. A) p-value distribution for the effect of group population differences (3.DEA vs 6.DEA). The uniform distribution expected by chance is indicated with a horizontal dashed line

Supplemental Figure 3) Individual CpG site methylation changes after exposure to allergen with prior diesel exhaust exposure. A) 70 CpG sites that decrease in methylation; B) 5 CpG sites that increase in methylation

Supplemental Figure 4) Bisulfite PCR-pyrosequencing of selected CpG site methylation. A single CpG site per gene was selected for pyrosequencing analysis. Data shown are mean ± SEM. *$p < 0.05$, **$p < 0.01$ for comparison of 1.FAS with 7.FAA/3.DES by unpaired Mann-Whitney test.

Supplemental Figure 5) Assessment of the agreement between array and pyrosequencing analysis. A single CpG site per gene was selected for pyrosequencing validation of methylation changes identified by array analysis.

Supplemental Figure 6) David functional analysis. A) DAVID functional annotation clusters for the 75 top hit probes after exposure to allergen with prior diesel exhaust exposure; B) DAVID functional annotation clusters for the 548 top hit probes after exposure to diesel exhaust with prior allergen exposure.

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Supplemental Figure 7) Individual CpG site methylation changes after exposure to allergen with prior diesel exhaust exposure. A) 528 CpG sites that decrease in methylation; B) 20 CpG sites that increase in methylation.

Supplemental Figure 8) Delayed effect of co-exposure on DNA methylation. p-value distribution for the effect of delayed DEA exposure (1.FAS Vs 8.FAS). The uniform distribution expected by chance is indicated with a horizontal dashed line. Table shows the names and q-values of the CpGs that were significantly different following delayedco-exposure.

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