

RESEARCH ARTICLE

In Utero Cigarette Smoke Affects Allergic Airway Disease But Does Not Alter the Lung Methylome

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Abstract

Prenatal and postnatal cigarette smoke exposure enhances the risk of developing asthma. Despite this as well as other smoking related risks, 11% of women still smoke during pregnancy. We hypothesized that cigarette smoke exposure during prenatal development generates long lasting differential methylation altering transcriptional activity that correlates with disease. In a house dust mite (HDM) model of allergic airway disease, we measured airway hyperresponsiveness (AHR) and airway inflammation between mice exposed prenatally to cigarette smoke (CS) or filtered air (FA). DNA methylation and gene expression were then measured in lung tissue. We demonstrate that HDM-treated CS mice develop a more severe allergic airway disease compared to HDM-treated FA mice including increased AHR and airway inflammation. While DNA methylation changes between the two HDM-treated groups failed to reach genome-wide significance, 99 DMRs had an uncorrected p-value < 0.001. 6 of these 99 DMRs were selected for validation, based on the immune function of adjacent genes, and only 2 of the 6 DMRs confirmed the bisulfite sequencing data. Additionally, genes near these 6 DMRs (*Lif*, *Il27ra*, *Tle4*, *Ptk7*, *Nfatc2*, and *Runx3*) are differentially expressed between HDM-treated CS mice and HDM-treated FA mice. Our findings confirm that prenatal exposure to cigarette smoke is sufficient to modify allergic airway disease; however, it is unlikely that specific methylation changes account for the exposure-response relationship. These findings highlight the important role in utero cigarette smoke exposure plays in the development of allergic airway disease.

Introduction

Smoking during pregnancy has long been identified as an independent risk factor for short term maternal and fetal outcomes, such as intrauterine growth restriction, ectopic pregnancy, premature birth, and developmental deficiencies. Furthermore, this exposure can lead to long-lasting changes in disease susceptibility, including asthma, behavioral disorders, obesity, and respiratory illness [1]. In fact, pre- and postnatal cigarette smoke exposure is one of the

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strongest environmental risk factors of asthma [2], and the risk of developing asthma symptoms is doubled when exposed early in life [3]. However, the mechanisms that result in childhood asthma following maternal smoking during pregnancy are largely unknown.

Despite the well known risks of smoking during pregnancy, an estimated 23% of women reported they smoked 3 months prior to pregnancy while 11% of pregnant women continue to smoke throughout pregnancy in the United States [4]. In the same report, 16% of women smoked 4 months after pregnancy. However, this may underestimate those affected by cigarette smoke as it does not include second hand or environmental cigarette smoke.

Cigarette smoke is known to influence epigenetic mechanisms, including epigenetic machinery [5, 6], post-translational histone modifications [7, 8], and DNA methylation [9, 10]. DNA methylation changes due to cigarette smoke can occur quite rapidly and persist for extended periods of time [11, 12]. One known association is the methylation of RUNX3 with a history of smoking in bladder tumors [13]. We have previously reported that Runx3 is differentially methylated in allergic airway disease in mice [14]; this is supported by the report that Runx3 deficient mouse spontaneously develop a phenotype resembling allergic airway disease [15].

Based on these observations, we hypothesized that cigarette smoke exposure during prenatal development generates long lasting differential methylation altering transcriptional activity that corresponds with altered disease.

Materials and Methods

Mice

C57BL/6J mice were purchased from Jackson Laboratories. Animals were housed under standard conditions and protocols were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver.

Cigarette Smoke Exposure

12 week old C57BL/6J females were exposed to cigarette smoke at ~50 mg/m³ TSP (equivalent to about a pack a day or a heavy smoker) or filtered air for 5 hours/day, 5 days/week for 4 weeks (1 week acclimation included) prior to mating with C57BL/6J males. Cigarette smoke was generated by the TE-10 smoking machine (Teague Enterprises) from 2R4F research cigarettes (University of Kentucky). Cigarette smoke contained a mixture of both side and mainstream smoke. Exposure was continued until birth of pups at which time all exposures were stopped and mothers and pups were placed under normal housing conditions.

Phenotyping

Allergic airway disease was induced using an adapted house dust mite model [16]. Briefly, mice were sensitized to 10 µg of filtered house dust mite extract (HDM, GREER Labs) or saline through intraperitoneal (i.p.) injection on days 0 and 7 followed by sensitization on days 14 and 15 with 5 µg HDM or saline administered intratracheally using a microsprayer (Penn Century). On day 17, mice were anesthetized by an i.p. injection of pentobarbital sodium (60 mg/kg). Following tracheostomy, pancuronium bromide (0.25 mg/kg) was administered, and mice were ventilated on a small animal ventilator (flexiVent FV-FXM1; SCIREQ). Airway resistance was measured through forced oscillation techniques (flexiVent FV-FXM1; SCIREQ) over increasing doses of methacholine. Following procedure, a cardiac stick was performed to euthanize the mouse and collect blood, then whole lung lavage (WLL) was collected. Lung tissue was perfused with phosphate buffered saline then snap frozen in liquid nitrogen. Cytokines in the lung lavage

and IgE in the serum were measured using ELISA MAX Standard Sets and protocols from Bio-Legend. Additional information is provided in the supplemental methods ([S1 Appendix](#)).

Bisulfite Sequencing and Data Analysis

To measure DNA methylation in whole lung tissue, bisulfite sequencing was performed utilizing Agilent's SureSelect Methyl-Seq Target Enrichment System for Illumina Multiplexed Sequencing. Experimental procedures followed SureSelect Human Methyl-Seq Protocol Version B using SureSelect Methyl-Seq Reagent Kit and Mouse Methyl-Seq Capture Library. Additional information is provided in the supplemental methods ([S1 Appendix](#)).

Bisulfite-sequencing reads were handled using *bwa-meth* [17] which also tabulated percent methylation at each CpG motif. Correlating sets of adjacent CpG sites were clustered together using the Adjacent Site Clustering algorithm [18]. Each cluster was required to have a minimum of three CpG sites to constitute a cluster. Methylation clusters were analyzed using a beta regression weighted on sequence read depth, and multiple testing correction was performed using the Benjamini-Hochberg method [19].

Pyrosequencing

DMRs identified through bisulfite sequencing were confirmed through pyrosequencing PCR on Qiagen's Pyromark MD. Additional information is provided in the supplemental methods ([S1 Appendix](#)).

RT-PCR

Differential expression was tested through qRT-PCR on the Viia7 Real-Time PCR system (Applied Biosystems) using Taqman assays (Applied Biosystems). Additional information is provided in the supplemental methods ([S1 Appendix](#)).

Statistics

Data were expressed as mean $\pm$ SEM. Individual comparisons between groups were confirmed by a 1-tailed Mann-Whitney U test because we were testing only 1 outcome, that *in utero* cigarette smoke caused increased allergic airway inflammation. Significant differences between groups were identified by analysis of variance. For validation, pyrosequencing data was analyzed using a 1-tailed Mann-Whitney U test for HDM-treated CS mice versus HDM-treated FA mice. A 2-tailed Mann-Whitney U test was applied to qRT-PCR data to compare HDM-treated CS mice versus HDM-treated FA mice. GraphPad Prism version 5.04 (GraphPad Software, La Jolla, CA) was used to perform statistical calculations. Pathway analysis was performed using Ingenuity Pathway Analysis (IPA) software.

Results

In utero cigarette smoke exposure alters HDM-induced allergic airway disease

These results support previously published data that mice exposed during gestation to cigarette smoke (CS) develop a more severe allergic airway disease phenotype [20–24]. HMD-treated mice exposed to CS during gestation demonstrate increased AHR compared to HDM-treated mice exposed to filtered air (FA) during gestation. Following challenge with HDM, enhanced airway inflammation is observed in CS mice compared to FA mice with an increase in total cells and eosinophils in WLL ([Fig 1A–1D](#)). Both HDM-treated CS and FA mice differ from their respective saline-treated mice counterparts in terms of AHR and cellular infiltration.

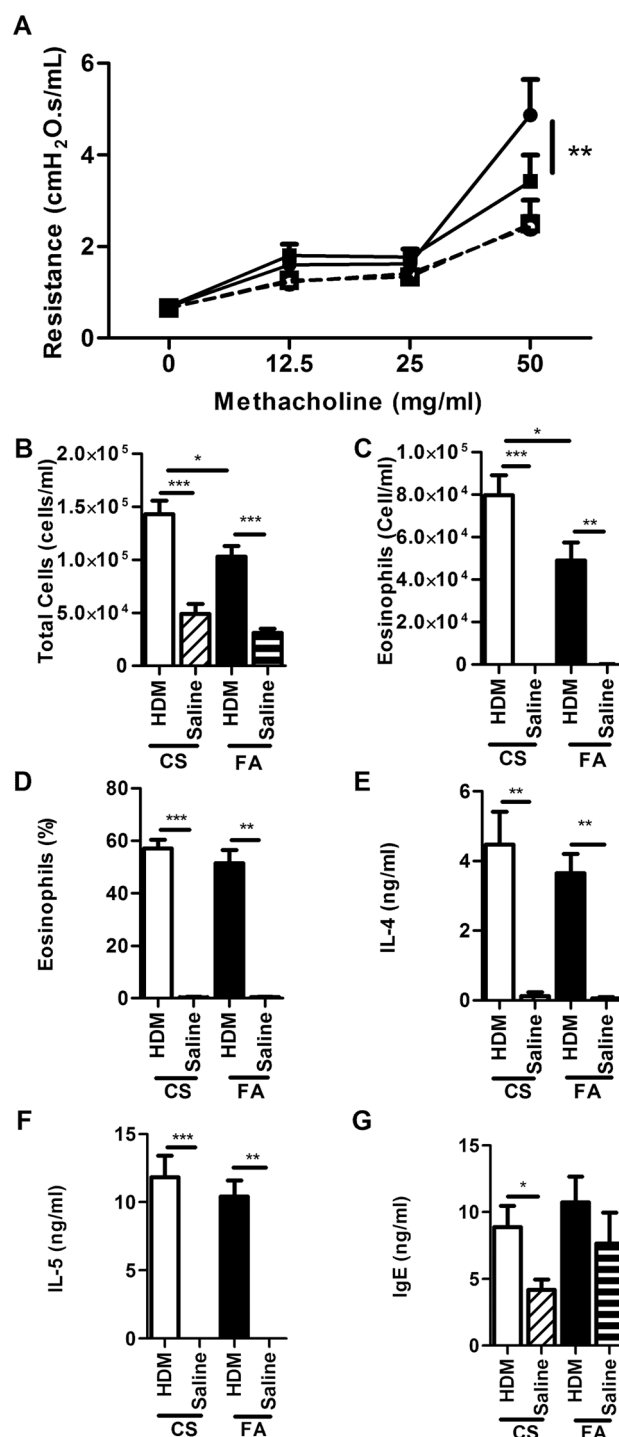


Fig 1. In utero cigarette smoke exposure enhanced the severity of HDM-induced allergic airway disease. (A) airway hyperresponsiveness (HDM-treated FA solid line closed square, HDM-treated CS solid line closed circle, saline-treated FA dashed line open square, and saline-treated CS dashed line open circle), (B) total cell in WLL, and (C) concentration of eosinophils, (D) percentage of eosinophils in the WLL, (E) WLL IL-4 cytokine levels, (F) WLL IL-5 cytokine levels, and (G) total IgE levels in the serum (HDM-treated CS white bar, HDM-treated FA black bar, saline-treated CS white bar with black dots, and saline-treated FA black bar with white dots, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001).

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There is no statistical difference observed for saline-treated mice treated with cigarette smoke (Fig 1A–1D).

We observed no differences in IL-4 and IL-5 concentrations in WLL between HDM-treated CS and FA mice (Fig 1E–1G). HDM-treatment resulted in increased concentrations of IL-4 and IL-5 in WLL compared to saline-treatment in both CS and FA treated mice. However, only HDM-treated CS mice demonstrated a significant increase in total IgE in the serum compared to saline-treated CS mice, and the lack of differences between HDM-treated and saline-treated mice is limited by the measurement of total IgE and not antigen specific IgE (Fig 1E–1G).

DNA methylation changes due to prenatal smoke exposure in allergic airway disease

Agilent's SureSelect targeted methyl-sequencing was performed in whole lung tissue to determine methylation patterns at specific sites throughout the genome. On average, each sample approximated 64 million reads with 88% of the reads falling within the targeted regions with greater than 50% of the regions with 20x coverage (S1 Table). Comparing DNA methylation between the HDM-treated groups failed to identify significant DMRs after multiple testing correction. Due to the lack of association and the increased noise in the data due to the admixture of cell types in whole lung tissue, we therefore reduced the statistical threshold to investigate suggestive DMRs. There are 99 suggestive DMRs with an uncorrected p-value < 0.001 (Fig 2A and S2 Table). These DMRs have an average length of 93 base pairs, are primarily found in gene bodies (intron, exon, 3' untranslated region, or 5' untranslated region; n = 56 or 57%; Fig 2C), and areas outside of CpG islands and shores (>3000 bases from the island; n = 58 or 59%; Fig 2D). 45 DMRs are hypomethylated.

DMRs are annotated based on nearest gene, and these genes were uploaded into IPA. Despite the small number of genes entered, 65 canonical pathways are enriched of which many involved immune function (S3 Table). Ingenuity network analysis on the 99 DMRs identified 6 networks with a minimum score > 20 (Fig 2B).

DMRs selected for validation from the 99 DMRs (uncorrected p-value < 0.001) were chosen based on known immune function of genes within 25kb of the DMR (Fig 3A and S4 Table). Specific CpGs within each selected DMR were chosen for pyrosequencing based on percent difference between experimental groups, a significant t-test on individual CpGs, and ability to design pyrosequencing primers. DMRs designated *Runx3*, *Tle4*, *Nfatc2*, *Lif*, *Ptk7*, and *Il27ra* were tested. *Lif*, and *Ptk7* validated methyl-sequencing data in addition *Nfatc2* had a near significant p-value (0.069) (Fig 3A and S4 Table). It is not unexpected that some of the DMRs did not validate as methyl-sequencing analysis failed to identify any significant targets at genome-wide significance and the reduced threshold increases our type I error.

Transcriptional expression of *Runx3*, *Tle4*, *Nfatc2*, *Lif*, *Ptk7*, and *Il27ra* were measured (Fig 3B). All genes tested demonstrated a significant change in expression with fold-changes ranging from -2.33 to -1.76. Interestingly, the relationship between expression and methylation does not always follow the canonical anti-correlated relationships. The association of gene expression is variable with nearby DMRs suggesting the use of alternative mechanisms in gene regulation.

We also performed subset analyses on methylation clusters within 25kb of asthma related genes as defined by genetic association [25], Ingenuity Pathway Analysis, or overlap of the 2 lists (S5 Table). In aggregate, 329 genes were represented in this analysis. These more targeted analyses focusing on asthma-associated genes did not identify methylation changes associated with *in utero* cigarette smoke exposure. In summary, our results suggest that *in utero* cigarette smoke exposure does not significantly alter the methylome in lung tissue of diseased mice.

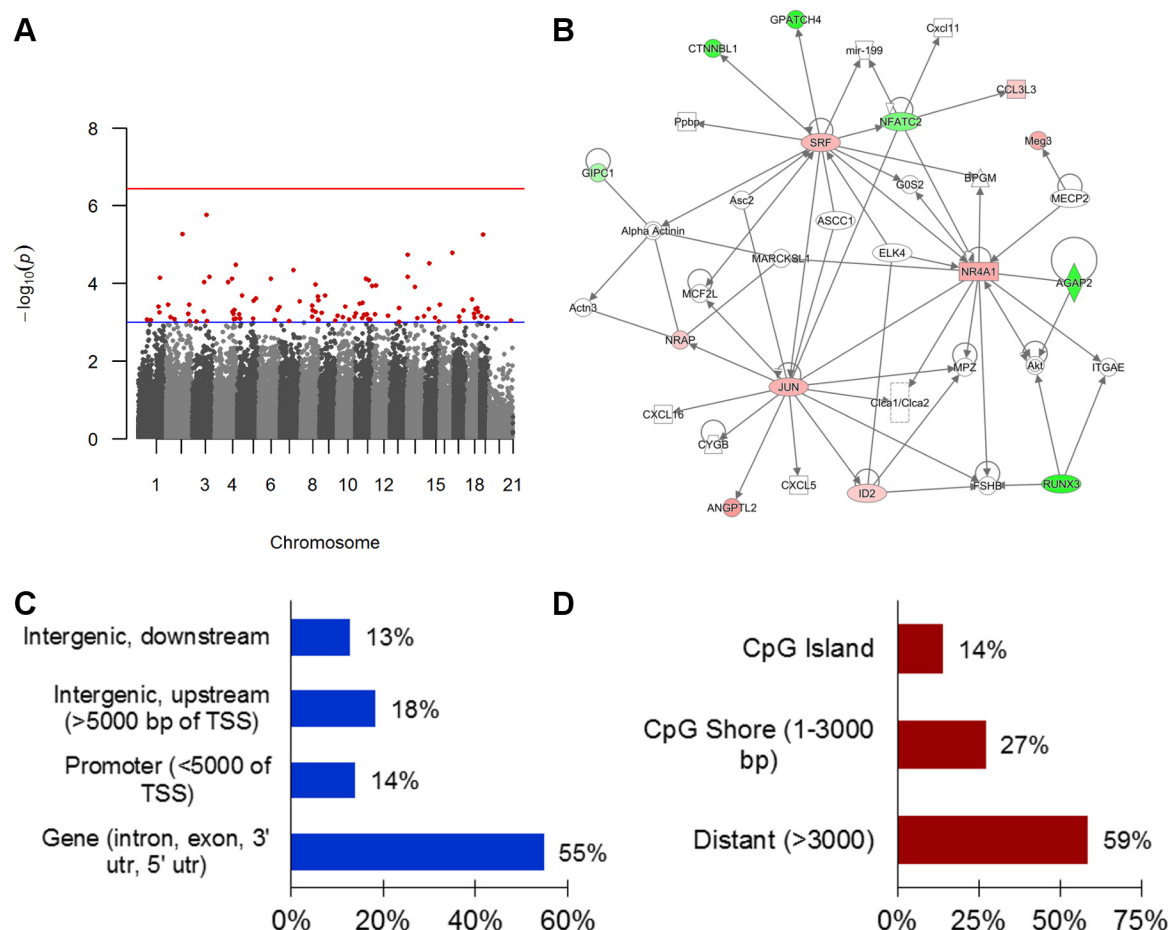


Fig 2. DMRs lack genome-wide association with *in utero* cigarette smoke exposure in allergic airway disease. (A) Manhattan plot of the p-values from a weighted beta regression for HDM-treated CS vs. HDM-treated FA mice. Each dot represents a p-value for correlating CpG clusters as identified through A-clustering with a minimum of 3 adjacent CpGs within a cluster. The red horizontal line is the estimated genome-wide significance threshold of $p = 3.6 \times 10^{-7}$; the blue line is the threshold for suggestive association ($p = 0.001$). Red dots denote suggestive DMRs with an unadjusted p-value < 0.001 ($n = 99$). (B) A molecular network identified by Ingenuity Pathway Analysis of the genes nearest to the 99 DMRs. This network demonstrates that these genes have a number of direct and shared interactions with each other. Network analysis was performed using only direct interactions and a minimum network score of 20. Legend: genes are colored red (hypermethylated) or green (hypomethylated), horizontal ellipse = transcriptional regulator, square = cytokine, double circle = group/complex, vertical diamond = enzyme, vertical rectangle = G-protein coupled receptor, circle = other, and triangle = phosphatase. Genomic distribution of the top 99 DMRs by relationship to (C) gene and (D) CpG Island.

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Discussion

Prenatal cigarette exposure is sufficient to alter the severity of HDM-induced allergic airway disease, and these phenotypic changes are associated with specific molecular changes in the lung. However, the association of methylation changes in lung tissue with *in utero* cigarette smoke modified allergic airway disease remains unconvincing. Although methylation changes in the genome-wide and subset analyses were not significant after multiple testing correction, it remains uncertain if smaller shifts in methylation occur as some of the suggestive DMRs were confirmed through pyrosequencing and nearby genes are enriched for immune pathways. In addition, the variable association between methylation and expression suggests that alternative mechanisms may regulate transcriptional activity, and future studies into the epigenetic mechanisms of prenatal cigarette smoke exposure on allergic airway disease should focus on specific cell types and/or alternative mechanisms of transcriptional regulation.

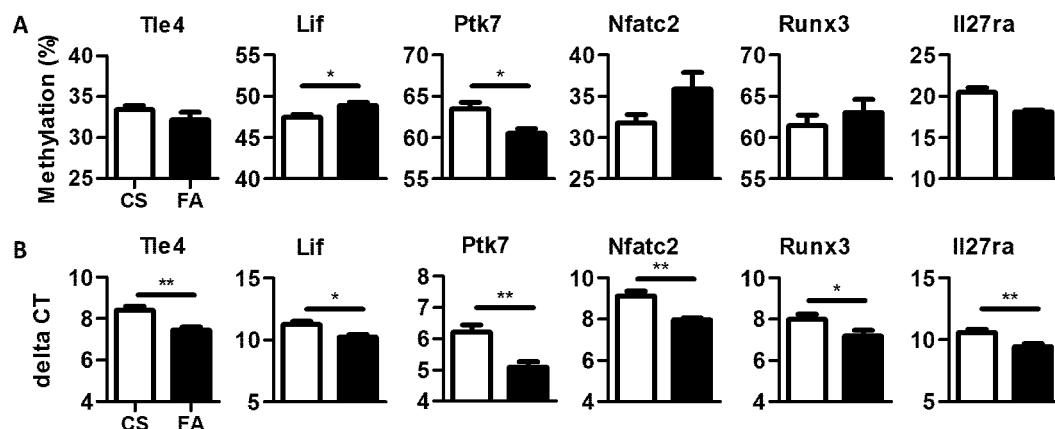


Fig 3. DMR validation is inconsistent despite consistent gene expression differences. (A) Mean methylation levels of 6 selected DMRs for internal validation through pyrosequencing. y-axis = methylation percent (B) Mean transcription levels of 6 selected genes near the 6 selected DMRs. y-axis = delta CT (HDM-treated CS white bar, HDM-treated FA black bar, * p-value < 0.05, ** p-value < 0.01)

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During gestation, the maternal immune system reduces Th1 IFN- γ cell-mediated response to fetal antigens by developing a subtle Th2 state [26]. Fetal immunity reflects that of the mothers, which is dominated by Th2 activity with reduced Th1 function, with underlying epigenetic changes that control gene expression patterns [27]. At birth, the Th1/Th2 cell ratios shift to a proper state; however, continuation of the Th2 state could affect an individual's risk of developing allergic diseases [28]. Smoking during pregnancy is capable of altering proper immune development leading to reduced innate TLR-mediated response [29], a higher Th2 response and proliferation in cord blood mononuclear cells upon challenge [30, 31], and reduced IFN γ production [32]. The suggestive DMRs in this study highlight immune dysfunction through the enrichment of not just immune pathways in general, but to those important to allergic disease, including IL-4 signaling, NFAT regulation of immune response, CD28 signaling in Th cells, and so on.

A limitation in this study is that the methylation experiment was completed on whole lung tissue with an admixture of cells. Cell type specific expression and methylation patterns increases variance in the analysis creating a higher threshold for discovery. The small phenotypic differences likely could not overcome the noise in the system, and this could explain why DMRs did not reach genome-wide significance. Increased sample size and/or read depth would increase our ability to detect changes and compensate for the small phenotypic changes. The measurement of epigenetic changes in environmental exposure and disease is a powerful tool in studying the etiology of asthma which creates a mechanistic link between environmental exposure and disease phenotype providing additional avenues of research into disease development and severity.

Supporting Information

S1 Appendix. Supplemental Materials and Methods.
(DOCX)

S1 Table. Agilent SureSelect Methyl Sequencing Summary.
(DOCX)

S2 Table. Suggestive DMRs in HDM-treated CS vs FA mice.
(DOCX)

S3 Table. Pathway analysis of Suggestive DMRs in HDM-treated CS and FA mice.
(DOCX)

S4 Table. DMR Validation and Transcript Levels.
(DOCX)

S5 Table. DMR Subset Analysis Gene Lists.
(DOCX)

Author Contributions

Conceived and designed the experiments: KE BP IY DS. Performed the experiments: KE. Analyzed the data: KE BP. Contributed reagents/materials/analysis tools: BP IY DS. Wrote the paper: KE DS.

References

1. Murin S, Rafii R, Bilello K. Smoking and smoking cessation in pregnancy. *Clin Chest Med*. 2011; 32(1):75–91, viii. Epub 2011/02/01. doi: [10.1016/j.ccm.2010.11.004](https://doi.org/10.1016/j.ccm.2010.11.004) PMID: [21277451](https://pubmed.ncbi.nlm.nih.gov/21277451/).
2. Jaakkola JJ, Gissler M. Maternal smoking in pregnancy, fetal development, and childhood asthma. *Am J Public Health*. 2004; 94(1):136–40. Epub 2004/01/10. PMID: [14713711](https://pubmed.ncbi.nlm.nih.gov/14713711/); PubMed Central PMCID: PMC1449839.
3. Hu FB, Persky V, Flay BR, Zelli A, Cooksey J, Richardson J. Prevalence of asthma and wheezing in public schoolchildren: association with maternal smoking during pregnancy. *Ann Allergy Asthma Immunol*. 1997; 79(1):80–4. Epub 1997/07/01. doi: [10.1016/S1081-1206\(10\)63090-6](https://doi.org/10.1016/S1081-1206(10)63090-6) PMID: [9236506](https://pubmed.ncbi.nlm.nih.gov/9236506/).
4. Tong VT, Dietz PM, Morrow B, D'Angelo DV, Farr SL, Rockhill KM, et al. Trends in smoking before, during, and after pregnancy—Pregnancy Risk Assessment Monitoring System, United States, 40 sites, 2000–2010. *MMWR Surveill Summ*. 2013; 62(6):1–19. Epub 2013/12/19. PMID: [24196750](https://pubmed.ncbi.nlm.nih.gov/24196750/).
5. Yao H, Rahman I. Current concepts on oxidative/carbonyl stress, inflammation and epigenetics in pathogenesis of chronic obstructive pulmonary disease. *Toxicol Appl Pharmacol*. 2011; 254(2):72–85. Epub 2011/02/08. doi: [10.1016/j.taap.2009.10.022](https://doi.org/10.1016/j.taap.2009.10.022) PMID: [21296096](https://pubmed.ncbi.nlm.nih.gov/21296096/); PubMed Central PMCID: PMC3107364.
6. Akinbami LJ, Moorman JE, Liu X. Asthma prevalence, health care use, and mortality: United States, 2005–2009. *Natl Health Stat Report*. 2011;(32):1–14. Epub 2011/03/02. PMID: [21355352](https://pubmed.ncbi.nlm.nih.gov/21355352/).
7. Akbarian S, Rios M, Liu RJ, Gold SJ, Fong HF, Zeiler S, et al. Brain-derived neurotrophic factor is essential for opiate-induced plasticity of noradrenergic neurons. *J Neurosci*. 2002; 22(10):4153–62. Epub 2002/05/23. PMID: [12019333](https://pubmed.ncbi.nlm.nih.gov/12019333/).
8. Marwick JA, Kirkham PA, Stevenson CS, Danahay H, Giddings J, Butler K, et al. Cigarette smoke alters chromatin remodeling and induces proinflammatory genes in rat lungs. *American journal of respiratory cell and molecular biology*. 2004; 31(6):633–42. Epub 2004/08/31. doi: [10.1165/rcmb.2004-0006OC](https://doi.org/10.1165/rcmb.2004-0006OC) PMID: [15333327](https://pubmed.ncbi.nlm.nih.gov/15333327/).
9. Kim DH, Nelson HH, Wiencke JK, Zheng S, Christiani DC, Wain JC, et al. p16(INK4a) and histology-specific methylation of CpG islands by exposure to tobacco smoke in non-small cell lung cancer. *Cancer research*. 2001; 61(8):3419–24. Epub 2001/04/20. PMID: [11309302](https://pubmed.ncbi.nlm.nih.gov/11309302/).
10. Rosas SL, Koch W, da Costa Carvalho MG, Wu L, Califano J, Westra W, et al. Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. *Cancer research*. 2001; 61(3):939–42. Epub 2001/02/28. PMID: [11221887](https://pubmed.ncbi.nlm.nih.gov/11221887/).
11. Phillips JM, Goodman JL. Inhalation of cigarette smoke induces regions of altered DNA methylation (RAMs) in SENCAR mouse lung. *Toxicology*. 2009; 260(1–3):7–15. Epub 2009/05/26. PMID: [19464564](https://pubmed.ncbi.nlm.nih.gov/19464564/).
12. Launay JM, Del Pino M, Chironi G, Callebort J, Peoc'h K, Megnier JL, et al. Smoking induces long-lasting effects through a monoamine-oxidase epigenetic regulation. *PloS one*. 2009; 4(11):e7959. Epub 2009/12/04. doi: [10.1371/journal.pone.0007959](https://doi.org/10.1371/journal.pone.0007959) PMID: [19956754](https://pubmed.ncbi.nlm.nih.gov/19956754/); PubMed Central PMCID: PMC2775922.
13. Wolff EM, Liang G, Cortez CC, Tsai YC, Castela JE, Cortessis VK, et al. RUNX3 methylation reveals that bladder tumors are older in patients with a history of smoking. *Cancer research*. 2008; 68(15):6208–14. Epub 2008/08/05. doi: [10.1158/0008-5472.CAN-07-6616](https://doi.org/10.1158/0008-5472.CAN-07-6616) PMID: [18676844](https://pubmed.ncbi.nlm.nih.gov/18676844/); PubMed Central PMCID: PMC2536768.

14. Hollingsworth JW, Maruoka S, Boon K, Garantzotis S, Li Z, Tomfohr J, et al. In utero supplementation with methyl donors enhances allergic airway disease in mice. *J Clin Invest*. 2008; 118(10):3462–9. Epub 2008/09/20. doi: [10.1172/JCI34378](https://doi.org/10.1172/JCI34378) PMID: [18802477](https://pubmed.ncbi.nlm.nih.gov/18802477/); PubMed Central PMCID: PMC2542847.
15. Fainaru O, Shseyov D, Hantisteanu S, Groner Y. Accelerated chemokine receptor 7-mediated dendritic cell migration in Runx3 knockout mice and the spontaneous development of asthma-like disease. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102(30):10598–603. Epub 2005/07/20. doi: [10.1073/pnas.0504787102](https://doi.org/10.1073/pnas.0504787102) PMID: [16027362](https://pubmed.ncbi.nlm.nih.gov/16027362/); PubMed Central PMCID: PMC1180803.
16. Kelada SN, Carpenter DE, Aylor DL, Chines P, Rutledge H, Chesler EJ, et al. Integrative genetic analysis of allergic inflammation in the murine lung. *American journal of respiratory cell and molecular biology*. 2014; 51(3):436–45. doi: [10.1165/rcmb.2013-0501OC](https://doi.org/10.1165/rcmb.2013-0501OC) PMID: [24693920](https://pubmed.ncbi.nlm.nih.gov/24693920/); PubMed Central PMCID: PMC4189492.
17. Pedersen BS, Eyring K, De S, Yang IV, Schwartz DA. Fast and accurate alignment of long bisulfite-seq reads. *ArXiv e-prints [Internet]*. 2014 January 1, 2014; 1401:[129 p.]. Available: <http://adsabs.harvard.edu/abs/2014arXiv1401.1129P>.
18. Sofer T, Schifano ED, Hoppin JA, Hou L, Baccarelli AA. A-clustering: a novel method for the detection of co-regulated methylation regions, and regions associated with exposure. *Bioinformatics*. 2013; 29(22):2884–91. Epub 2013/08/31. doi: [10.1093/bioinformatics/btt498](https://doi.org/10.1093/bioinformatics/btt498) PMID: [23990415](https://pubmed.ncbi.nlm.nih.gov/23990415/); PubMed Central PMCID: PMC3810849.
19. Klipper-Aurbach Y, Wasserman M, Braunsiegel-Weintrob N, Borstein D, Peleg S, Assa S, et al. Mathematical formulae for the prediction of the residual beta cell function during the first two years of disease in children and adolescents with insulin-dependent diabetes mellitus. *Medical hypotheses*. 1995; 45(5):486–90. PMID: [8748093](https://pubmed.ncbi.nlm.nih.gov/8748093/).
20. Blacquiére MJ, Timens W, Melgert BN, Geerlings M, Postma DS, Hylkema MN. Maternal smoking during pregnancy induces airway remodelling in mice offspring. *The European respiratory journal*. 2009; 33(5):1133–40. Epub 2009/01/09. 09031936.00129608 [pii] doi: [10.1183/09031936.00129608](https://doi.org/10.1183/09031936.00129608) PMID: [19129273](https://pubmed.ncbi.nlm.nih.gov/19129273/).
21. Rouse RL, Boudreaux MJ, Penn AL. In utero environmental tobacco smoke exposure alters gene expression in lungs of adult BALB/c mice. *Environmental health perspectives*. 2007; 115(12):1757–66. Epub 2007/12/19. PMID: [18087596](https://pubmed.ncbi.nlm.nih.gov/18087596/); PubMed Central PMCID: PMC2137099.
22. Singh SP, Barrett EG, Kalra R, Razani-Boroujerdi S, Langley RJ, Kurup V, et al. Prenatal cigarette smoke decreases lung cAMP and increases airway hyperresponsiveness. *American journal of respiratory and critical care medicine*. 2003; 168(3):342–7. Epub 2003/06/07. [pii]. PMID: [12791581](https://pubmed.ncbi.nlm.nih.gov/12791581/).
23. Singh SP, Mishra NC, Rir-Sima-Ah J, Campen M, Kurup V, Razani-Boroujerdi S, et al. Maternal exposure to secondhand cigarette smoke primes the lung for induction of phosphodiesterase-4D5 isozyme and exacerbated Th2 responses: rolapram attenuates the airway hyperreactivity and muscarinic receptor expression but not lung inflammation and atopy. *J Immunol*. 2009; 183(3):2115–21. Epub 2009/07/15. doi: [10.4049/jimmunol.0900826](https://doi.org/10.4049/jimmunol.0900826) PMID: [19596983](https://pubmed.ncbi.nlm.nih.gov/19596983/); PubMed Central PMCID: PMC3191864.
24. Wu ZX, Hunter DD, Kish VL, Benders KM, Batchelor TP, Dey RD. Prenatal and early, but not late, postnatal exposure of mice to sidestream tobacco smoke increases airway hyperresponsiveness later in life. *Environmental health perspectives*. 2009; 117(9):1434–40. Epub 2009/09/15. doi: [10.1289/ehp.0800511](https://doi.org/10.1289/ehp.0800511) PMID: [19750110](https://pubmed.ncbi.nlm.nih.gov/19750110/); PubMed Central PMCID: PMC2737022.
25. DeWan AT, Egan KB, Hellenbrand K, Sorrentino K, Pizzoferrato N, Walsh KM, et al. Whole-exome sequencing of a pedigree segregating asthma. *BMC medical genetics*. 2012; 13:95. doi: [10.1186/1471-2350-13-95](https://doi.org/10.1186/1471-2350-13-95) PMID: [23046476](https://pubmed.ncbi.nlm.nih.gov/23046476/); PubMed Central PMCID: PMC3563469.
26. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunol Today*. 1993; 14(7):353–6. Epub 1993/07/01. PMID: [8363725](https://pubmed.ncbi.nlm.nih.gov/8363725/).
27. Zaghoulani H, Hoeman CM, Adkins B. Neonatal immunity: faulty T-helpers and the shortcomings of dendritic cells. *Trends Immunol*. 2009; 30(12):585–91. Epub 2009/10/23. doi: [10.1016/j.it.2009.09.002](https://doi.org/10.1016/j.it.2009.09.002) PMID: [19846341](https://pubmed.ncbi.nlm.nih.gov/19846341/); PubMed Central PMCID: PMC2787701.
28. Bousquet J, Jacot W, Yssel H, Vignola AM, Humbert M. Epigenetic inheritance of fetal genes in allergic asthma. *Allergy*. 2004; 59(2):138–47. Epub 2004/02/07. PMID: [14763925](https://pubmed.ncbi.nlm.nih.gov/14763925/).
29. Noakes PS, Hale J, Thomas R, Lane C, Devadason SG, Prescott SL. Maternal smoking is associated with impaired neonatal toll-like-receptor-mediated immune responses. *The European respiratory journal*. 2006; 28(4):721–9. Epub 2006/07/28. doi: [10.1183/09031936.06.00050206](https://doi.org/10.1183/09031936.06.00050206) PMID: [16870663](https://pubmed.ncbi.nlm.nih.gov/16870663/).
30. Devereux G, Barker RN, Seaton A. Antenatal determinants of neonatal immune responses to allergens. *Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology*. 2002; 32(1):43–50. Epub 2002/05/11. PMID: [12002736](https://pubmed.ncbi.nlm.nih.gov/12002736/).

31. Noakes PS, Holt PG, Prescott SL. Maternal smoking in pregnancy alters neonatal cytokine responses. *Allergy*. 2003; 58(10):1053–8. Epub 2003/09/27. PMID: [14510725](#).
32. Tebow G, Sherrill DL, Lohman IC, Stern DA, Wright AL, Martinez FD, et al. Effects of parental smoking on interferon gamma production in children. *Pediatrics*. 2008; 121(6):e1563–9. Epub 2008/06/04. doi: [10.1542/peds.2007-2795](#) PMID: [18519461](#).

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S1 Appendix

Supplemental Materials and Methods

Mice

C57BL/6J mice were purchased from Jackson Laboratories. Animals were housed under standard conditions and protocols were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver.

Cigarette Smoke Exposure

12 week old C57BL/6J females were exposed to cigarette smoke at $\sim 50 \text{ mg/m}^3$ TSP (equivalent to about a pack a day or a heavy smoker) or filtered air for 5 hours/day, 5 days/week for 4 weeks (1 week acclimation included) prior to mating with C57BL/6J males. Cigarette smoke was generated by the TE-10 smoking machine (Teague Enterprises) from 2R4F research cigarettes (University of Kentucky). Cigarette smoke contained a mixture of both side and mainstream smoke. Exposure was continued until birth of pups at which time all exposures were stopped and mothers and pups were placed under normal housing conditions.

House Dust Mite Challenge and Sensitization

Eight- to twelve-week-old C57BL/6^{MTHFR^{-/-}} and C57BL/6 (Charles River) male mice were sensitized to 10 μg house dust mite extract (HDM, GREER Labs) or saline through i.p. injection on days 0 and 7 followed by sensitization on days 14 and 15 with 5 μg HDM or saline administered intratracheally (i.t.) using a microsprayer (Penn Century). Airway function and sample collection occurred on day 17. Prior to administration, HDM was reconstituted in saline to 1 $\mu\text{g/ml}$ and passed through a 0.45 μm filter then a 0.02 μm filter to facilitate microsprayer use.

Airway physiology

Mice were anesthetized by an i.p. injection of pentobarbital sodium (60 mg/kg). Following tracheostomy, pancuronium bromide (0.25 mg/kg) was administered, and mice were ventilated on a small animal ventilator (flexiVent FV-FXM1; SCIREQ). Forced oscillation techniques were used to measure respiratory mechanics in response to an aerosolized methacholine challenge (0, 12.5, 25, and 50 mg/ml).

Whole Lung Lavage, Cell Count, and Cell Differential

Whole lung lavage fluid (WLLF) was collected after airway physiology measurement. The lavage fluid was a preparation of 50 mL of 1x PBS with 60 μ L of 0.5M EDTA added. Mice were lavaged with an initial 1 mL of lavage fluid followed by two consecutive lavages of 0.5 mL of fresh lavage fluid. WLLF was kept on ice throughout processing.

WLLF was then spun down at 3000 x g for 5 minutes. The supernatant was collected into a separate tube and stored at -80°C. The cell pellet was then treated with 200 μ L red blood cell lysing buffer (Sigma) for 1 minute with gentle mixing. 1 mL of cold 1x PBS was immediately added after 1 minute incubation. The cells were then spun down again at 3000 x g for 5 minutes. The supernatant was then discarded and 1 mL of fresh 1x PBS was added to resuspend cells. 20 μ L of cell pellet solution was placed in a separate tube and combined with 20 μ L Turks solution (10mg Crystal Violet, 3 mL glacial acetic acid, in 100 mL deionized water). 10 μ L of solution was placed on a hemacytometer. Cells were counted and total cells were calculated.

Approximately 12,500 cells in 200 μ L was placed in a slide funnel assembled with a glass slide and cartridge. The assembly was then placed in cytopsin centrifuge (Thermo Scientific

Cytospin 4) and run at 700 rpm for 3 minutes. Slide was then removed, placed in a slide rack, and dried overnight. Cells were then stained with Protocol Hema 3 stain set (Fisher), 1 minute solution 1, 3 minutes 45 seconds solution 2, and 1 minute solution 3. After drying, the cover slip was added, and 200 cells were identified and counted based on staining and morphology.

Sample Processing

Blood was withdrawn through cardiac puncture. It was collected in lithium heparin tubes (BD Inc.). Tubes were spun down at 1000 x g for 10 minutes. Serum was then removed and snap frozen in liquid nitrogen and stored at -80°C.

Tissues (spleen, thymus, mediastinal lymph node) collected were snap frozen and stored at -80°C. Lung tissue prior to collection was perfused with 1x PBS. Then, the right lobes were tied off while the left lobe was inflated with a mixture of 50% optimal cutting medium (OCT) and 50% 1x PBS. The inflated left lobe was then quickly removed and embedded in OCT and snap frozen in liquid nitrogen for histology and stored at -80°C. The right lobes were also snap frozen for use in DNA and RNA experiments and stored at -80°C.

ELISA

Sample Preparation

WLLF was concentrated using Amicon Ultra 3K device. 1 mL of WLLF was added to each filter and centrifuged at 4,000 x g for 25 minutes. Then, the filtering device was flipped around and spun for 2 minutes at 1,000 x g. Total volume of the WLLF was concentrated by a factor of 3 on average.

ELISA

IL-4 and IL-5 were measured in the concentrated WLLF and IgE in the serum using ELISA MAX Standard Sets and protocols from BioLegend. First, Costar Assay High Binding 96 well plates were coated with experiment specific capture antibody and incubated at 4°C overnight. Plates were then washed and assay diluent (1% BSA in PBS) was added to each well and incubated at room temperature for 1 hour followed by plate washing. Sample and standard were then added to each well, incubated for 2 hours at room temperature then washed. Detection antibody was added to each well, incubated at room temperature for 1 hour, washed, and then treated with Avidin-HRP at room temperature for 30 minutes. Next, the plate underwent two wash cycles prior to the addition of TMB substrate solution. Plates were allowed to develop until desired color was reached, then the reaction was stopped with stop solution. A plate reader was used to measure absorbance at 450 and 570 nm.

Agilent Expression Array

Sample Preparation and Array Processing

A minimum of 25 ng of high quality total RNA from whole lung tissue, as determined by Bioanalyzer RNA assays, was used for SurePrint G3 8x60K arrays (Agilent). One-Color Microarray-Based Gene Expression Analysis: Low Input Quick Amp Labeling protocol version 6.6 and kits were used for this expression experiment. First, RNA was incubated with Spike-in mix, T7 primer, and a cDNA master mix to generate labeled cRNA and purified using RNeasy columns (Qiagen). Then, cRNA was quantified on NanoDrop Spectrometer to determine total concentration and Cyanine 3 dye concentration. 600 ng of labeled sample was then fragmented then hybridized to the arrays for 17 hours followed by washing. Scanning was modified to meet the specifications of the NimbleGen MS200 scanner. A custom parameter on

the NimbleGen MS200 scanner was developed in order to scan the Agilent arrays. Images were uploaded to Feature Extraction 11.5.11 software to extract data.

Analysis

Extracted features were up-loaded into Partek Genomics Suite (Partek Inc.), then underwent quantile normalization and log-2 transformation. Analysis of variance (ANOVA) was applied to determine differences between all experimental groups. Adjusted p-values were generated using the FDR q-value selection through Partek.

Bisulfite Sequencing

Sample and Library Preparation

To measure DNA methylation in whole lung tissue, I performed bisulfite sequencing utilizing Agilent's SureSelect Methyl-Seq Target Enrichment System for Illumina Multiplexed Sequencing. Experimental procedures followed SureSelect Human Methyl-Seq Protocol Version B using SureSelect Methyl-Seq Reagent Kit and SureSelect Mouse Methyl-Seq Capture Library. I started with 4 µg of DNA instead of the recommended 3 µg due to previous experience with similar techniques. Sample DNA was sheared using the Covaris S2 system to fragment the DNA to a 140-180 bp size. Fragmented DNA, then, underwent end repair and purification with AMPure XP beads (Beckman Coulter Genomics). Bioanalyzer DNA 1000 chips were used to confirm the size of DNA fragments. An adenosine was added to the 3' end of each DNA fragment followed by methylated adaptor ligation. Adaptor-ligated DNA was then purified again using AMPure XP beads then size and quantity was measured through the Bioanalyzer, approximate size of 170-230 bp and minimum of 500 ng. Samples were then dehydrated and reconstituted in 3.4 µL of nuclease-free water to concentrate the sample. Then, samples were

hybridized with SureSelect Capture library for approximately 40 hours at 65°C. Immediately after incubation, captured DNA fragments were isolated using Dynabeads MyOne Streptavidin T1 magnetic beads. Captured DNA was then bisulfite converted using Zymo Research's EZ DNA Methylation-Gold Kit following the SureSelect's Methyl-Seq modified protocol. Bisulfite-treated libraries were then PCR amplified followed again by AMPure XP bead purification. Libraries were then indexed through PCR amplification. Indexed libraries were purified with AMPure XP beads then assayed for quality and quantity with the 2100 Bioanalyzer High Sensitivity DNA assay, size 220-280 bp and a concentration of 2-15 nM. Libraries were pooled to contain either 11 or 12 libraries each at a final DNA concentration of 2 nM per library which was confirmed by a final measurement with the Bioanalyzer. Pooled libraries were then submitted for sequencing on the Illumina HiSeq. Each pool was run twice in separate lanes of a flow cell.

Analysis

Bisulfite-sequencing reads were trimmed and aligned to the mouse genome *mm10* using *bwa-meth* (16) which also tabulated percent methylation at each CpG motif. Correlating sets of neighboring CpG sites were clustered together using the Adjacent Site Clustering (A-clustering) algorithm (17). Each cluster was required to have a minimum of three CpG sites to constitute a cluster. Clusters were then analyzed using a beta regression weighted based on sequence read depth. Multiple testing correction was performed using the *Benjamini-Hochberg* method.

Pyrosequencing

DMRs were validated using pyrosequencing. Extracted DNA samples were bisulfite converted using Zymo EZ DNA Methylation-Gold kits and protocol. After conversion and clean-

up, converted DNA was measured on a Nanodrop Spectrometer as specified by protocol. 35 ng of converted DNA was then used for the pyrosequencing PCR reaction using Qiagen's Pyromark PCR kit with each sample run in duplicate. Unmethylated and methylated controls are included for each primer set. Primers for the PCR reaction were designed using the Pyromark Assay Design software. After amplification, the biotinylated-target strand was purified using streptavidin sepharose beads in binding buffer while shaking at room temperature at 1400 rpm for at least 10 minutes. 96 well pyrosequencing plates were then loaded with sequencing primer in annealing buffer and purified target DNA followed by annealing by heating to 80°C for 2 minutes then the plate was allowed to cool to room temperature for 10 minutes. The Pyromark MD was loaded with appropriate volumes of dNTPs, enzyme solution, and substrate solution from the Pyromark Gold Reagents Q96 kit. The plate was then run on the Pyromark MD. Methylation measurements were averaged across duplicates then across experimental group. A one-tailed Mann-Whitney test was used to determine statistical difference between experimental groups.

RT-PCR

Gene expression was measured through quantitative real-time PCR (qRT-PCR) using TaqMan assays (Life Technologies). 80 ng of RNA was converted to cDNA using Superscript III Reverse Transcriptase (Life Technologies) kit and protocol. cDNA was then mixed with TaqMan Fast Advanced Master Mix and best coverage TaqMan assays then run in triplicate on the ViiA 7 Real-Time. Delta CT values were generated from the reference gene, beta-actin. Triplicates were averaged for each sample, and statistical differences were determined through a one-tailed Mann-Whitney test.

S1 Table: Agilent SureSelect Methyl Sequencing Summary

	n	Average Total Reads	Mean Coverage	2X Coverage (%)	20X Coverage (%)	Off Target (%)
FA HDM	9	7.2E+07	42	0.79	0.55	0.11
FA Saline	6	4.4E+07	28	0.78	0.45	0.11
CS HDM	8	6.6E+07	40	0.79	0.52	0.11
CS Saline	6	7.2E+07	43	0.79	0.56	0.12

S2 Table: Suggestive DMRs in HDM-treated CS vs FA mice

Chrm	Start	End	CpGs	coef	p-value	q-value	Gene	Distance To Gene
chr4	150263029	150263062	5	-1.66	3.38E-05	0.65	Eno1;Eno1b	14156;14156
chr4	135033681	135033743	3	-1.59	8.39E-04	0.92	Runx3	-86901
chr3	88042645	88042681	5	-1.58	9.20E-05	0.74	Gpatch4	-424
chr11	94138540	94138670	7	-1.50	8.32E-04	0.92	Spag9	12458
chr10	127092188	127092356	7	-1.49	7.24E-04	0.92	Agap2	0
chr7	97325919	97325943	3	-1.49	4.51E-05	0.72	Usp35	0
chr2	157901452	157901547	14	-1.45	6.07E-04	0.92	Ctnnbl1	9549
chr5	118063299	118063307	3	-1.42	2.85E-04	0.92	Tesc	1429
chr18	52527547	52527593	4	-1.41	6.07E-04	0.92	Lox	0
chr11	4247416	4247596	5	-1.30	5.99E-04	0.92	Osm	6390
chr16	91617026	91617046	3	-1.21	1.64E-05	0.49	Dnajc28	0
chr8	105327507	105327534	3	-1.21	5.48E-04	0.92	Tmem208	0
chr4	124279006	124279019	3	-1.20	7.56E-05	0.72	1700057H15Rik	170606
chr18	61665597	61665721	5	-1.11	4.63E-04	0.92	Mir143hg	-59
chr5	113287153	113287386	26	-1.08	8.13E-04	0.92	Sgsm1	0
chr8	85076222	85076270	4	-1.05	5.00E-04	0.92	Wdr83	0
chr19	47314049	47314161	7	-1.05	7.88E-04	0.92	Sh3pxd2a	0
chr6	146852634	146852972	9	-1.02	4.04E-04	0.92	Smco2	0
chr15	76542887	76542953	3	-1.01	7.64E-04	0.92	Slc52a2	757
chr2	168525888	168526023	3	-1.00	9.25E-04	0.92	Nfatc2;Nfatc2	0;0
chr10	63160851	63160887	4	-0.97	4.01E-04	0.92	Mypn	0
chr4	148734844	148734916	5	-0.97	8.16E-04	0.92	Gm572	63272
chr14	7027162	7027242	9	-0.94	1.85E-05	0.49	Gm10406	0
chr18	80976446	80976498	4	-0.93	5.38E-04	0.92	Sall3	0
chrX	159414179	159414237	4	-0.93	9.09E-04	0.92	Map7d2	-340
chr11	77514877	77514912	3	-0.88	7.57E-05	0.72	Abhd15	-204
chr8	126476270	126476333	4	-0.86	2.78E-04	0.92	Gm17296	-1205
chr10	89708838	89708877	3	-0.85	8.61E-04	0.92	Actr6	3095
chr3	32396052	32396232	6	-0.85	9.50E-04	0.92	4930429B21Rik	28614
chr5	24325380	24325485	6	-0.82	8.18E-04	0.92	Kcnh2	0
chr3	123117227	123117284	3	-0.81	6.87E-05	0.72	Synpo2	0
chr2	18675994	18676001	3	-0.78	3.54E-04	0.92	Commd3	0
chr13	65781856	65781921	5	-0.77	9.86E-04	0.92	Gm10324	-331531
chr17	43358036	43358086	5	-0.72	7.21E-04	0.92	Gpr116	-31379
chr5	15936199	15936445	4	-0.70	6.30E-04	0.92	Cacna2d1	0
chr14	76109914	76110065	5	-0.67	7.95E-04	0.92	Gpalpp1	0
chr7	140955935	140955981	3	-0.64	2.92E-04	0.92	Ifitm2	0
chr8	83653791	83653980	7	-0.63	7.15E-04	0.92	Gipc1	0
chr15	31368524	31368727	4	-0.59	4.62E-04	0.92	Ankrd33b	-765
chr16	4420943	4421145	4	-0.57	3.55E-04	0.92	Adcy9	-445

chr19	10773386	10773469	3	-0.55	7.00E-04	0.92	A430093F15Rik	0
chr12	18515182	18515297	12	-0.52	6.21E-04	0.92	5730507C01Rik	0
chr1	89932528	89932754	12	-0.50	8.83E-04	0.92	Gbx2	-1352
chr1	151087800	151087913	6	-0.48	5.61E-04	0.92	C730036E19Rik	-50120
chr1	64119373	64119478	5	-0.38	8.48E-04	0.92	Klf7	0
chr14	8216137	8216267	4	0.62	6.85E-05	0.72	Kctd6	0
chr6	135314332	135314366	3	0.66	4.91E-04	0.92	Pbp2	-3948
chr8	127190699	127190758	3	0.68	8.70E-04	0.92	Pard3	0
chr4	127668189	127668383	4	0.75	5.40E-04	0.92	CK137956	259208
chr3	83589324	83589379	3	0.77	5.32E-04	0.92	1700028M03Rik	-15206
chr8	122695929	122696052	4	0.80	8.53E-04	0.92	Cbfa2t3	0
chr14	56669126	56669214	3	0.80	1.25E-04	0.80	Mphosph8	0
chr8	25087920	25088008	4	0.82	1.01E-03	0.92	Plekha2	0
chr9	114330005	114330112	3	0.82	8.94E-04	0.92	Bcl2a1c	-22
chr12	25093649	25093707	3	0.83	1.15E-04	0.78	Id2	91
chr13	65726906	65726983	5	0.85	4.27E-04	0.92	Gm10324	-386469
chr11	83648484	83648664	7	0.86	6.18E-04	0.92	Ccl3	0
chr18	38636664	38636675	3	0.87	2.63E-04	0.92	Spry4	-35396
chr18	53255464	53255528	3	0.88	7.60E-04	0.92	Snx24	0
chr4	132783608	132783640	3	0.89	6.16E-04	0.92	Themis2	0
chr3	108098501	108098798	3	0.89	9.20E-04	0.92	Gnat2	0
chr19	56344247	56344259	3	0.89	7.68E-04	0.92	Nrap	0
chr6	86496987	86497069	3	1.00	7.55E-05	0.72	2310040G24Rik	-8760
chr11	119937292	119937414	5	1.00	1.17E-04	0.78	Baiap2	-5677
chr18	73835755	73835925	3	1.02	4.28E-04	0.92	Me2	-20363
chr4	139974780	139974826	3	1.02	4.81E-04	0.92	Klhdc7a	-6754
chr11	62996643	62996682	3	1.09	6.35E-04	0.92	Mir6354	0
chr2	61811483	61811544	3	1.09	8.28E-04	0.92	Tbr1	0
chr11	84522053	84522086	3	1.10	6.33E-04	0.92	Lhx1	0
chr8	84063944	84064070	4	1.10	3.71E-04	0.92	Rfx1	-2765
chr7	66387811	66387889	8	1.10	9.38E-04	0.92	Lrrk1	0
chr5	134729315	134729388	3	1.12	2.47E-04	0.92	Eln	0
chr15	34016791	34016973	4	1.13	3.11E-05	0.65	Mtdh	-65745
chr17	46552275	46552434	5	1.18	9.65E-04	0.92	Srf	0
chr5	36868773	36868787	4	1.23	2.04E-04	0.92	Ppp2r2c	0
chr17	73037161	73037270	3	1.25	5.01E-04	0.92	Lclat1	-70714
chr4	95106226	95106315	3	1.25	9.40E-05	0.74	Jun	-54004
chr11	49719835	49719888	3	1.29	7.83E-04	0.92	Cnot6	-7113
chr1	156615798	156616051	4	1.29	7.18E-05	0.72	Abl2	0
chr11	95046878	95047005	3	1.31	8.27E-05	0.74	Itga3	0
chr6	113379994	113380143	3	1.36	8.68E-04	0.92	Arpc4;Arpc4	0;0
chr12	109542822	109542870	3	1.38	6.78E-04	0.92	Meg3	0
chr9	21125858	21125923	3	1.39	5.76E-04	0.92	Tyk2	0

chr14	122373730	122373779	4	1.42	7.07E-04	0.92	Clybl;Gm5089	0;0
chr15	101277572	101277651	5	1.42	9.48E-04	0.92	Nr4a1	2778
chr2	118728223	118728285	3	1.46	5.46E-06	0.25	Plcb2	0
chr8	105835357	105835381	3	1.48	1.09E-04	0.78	Tsnaxip1	0
chr11	32552724	32552917	3	1.49	3.35E-04	0.92	Stk10	0
chr10	46235841	46236012	3	1.55	7.39E-04	0.92	Snord14d	-8735
chr10	7867202	7867307	4	1.56	6.85E-04	0.92	Zc3h12d	0
chr2	165332955	165333024	5	1.59	3.52E-04	0.92	Elmo2	-6476
chr11	51644368	51644441	7	1.60	3.20E-04	0.92	N4bp3	0
chr8	121951636	121951670	3	1.62	2.17E-04	0.92	Banp	0
chr2	33219624	33219647	3	1.66	7.43E-04	0.92	Angptl2;Ralgps1	0;0
chr3	103737837	103737888	3	1.69	1.75E-06	0.23	Olfml3;Mir3473f	0;0
chr11	77498744	77498775	3	1.80	6.40E-04	0.92	Git1	0
chr11	105181678	105181764	8	2.08	8.66E-04	0.92	Tlk2	0
chr1	143777067	143777101	3	2.38	4.03E-04	0.92	Trove2	-16
chr9	46012706	46012730	3	2.62	2.05E-04	0.92	Sik3	-89
chr19	24280501	24280538	5	3.35	5.60E-06	0.25	Fxn	0

S3 Table: Pathway analysis of Suggestive DMRs in HDM-treated CS and FA mice

Ingenuity Canonical Pathways	-log(p-value)	Ratio	Molecules
Rac Signaling	4.08	0.05	ITGA3,JUN,BAIAP2,PARD3,ARPC4
PDGF Signaling	3.48	0.05	JUN,ABL2,TYK2,SRF
CXCR4 Signaling	3.31	0.03	ADCY9,PLCB2,JUN,GNAT2,ELMO2
Signaling by Rho Family GTPases	3.30	0.03	ITGA3,JUN,BAIAP2,GNAT2,PARD3,ARPC4
Cdc42 Signaling	3.12	0.03	ITGA3,JUN,BAIAP2,PARD3,ARPC4
Actin Nucleation by ARP-WASP Complex	2.75	0.05	ITGA3,BAIAP2,ARPC4
PI3K Signaling in B Lymphocytes	2.65	0.03	PLCB2,JUN,NFATC2,PLEKHA2
Axonal Guidance Signaling	2.60	0.02	PLCB2,ITGA3,BAIAP2,GNAT2,NFATC2,GIT1,ARPC4
Cardiac Hypertrophy Signaling	2.57	0.02	ADCY9,PLCB2,JUN,SRF,GNAT2
Molecular Mechanisms of Cancer	2.32	0.02	ADCY9,PLCB2,ITGA3,JUN,TYK2,GNAT2
Role of JAK family kinases in IL-6-type Cytokine Signaling	2.29	0.08	TYK2,OSM
Gluconeogenesis I	2.29	0.08	ENO1,ME2
Role of NFAT in Regulation of the Immune Response	2.20	0.02	PLCB2,JUN,GNAT2,NFATC2
Endothelin-1 Signaling	2.20	0.02	ADCY9,PLCB2,JUN,GNAT2
Regulation of Actin-based Motility by Rho	2.15	0.03	ITGA3,BAIAP2,ARPC4
IL-1 Signaling	2.15	0.03	ADCY9,JUN,GNAT2
CDK5 Signaling	2.05	0.03	ADCY9,ITGA3,PPP2R2C
Oncostatin M Signaling	2.03	0.06	TYK2,OSM
Cholecystokinin/Gastrin-mediated Signaling	2.03	0.03	PLCB2,JUN,SRF
Interferon Signaling	1.98	0.06	TYK2,IFITM2
fMLP Signaling in Neutrophils	1.95	0.03	PLCB2,NFATC2,ARPC4
April Mediated Signaling	1.94	0.05	JUN,NFATC2
Corticotropin Releasing Hormone Signaling	1.91	0.03	ADCY9,JUN,NR4A1
B Cell Activating Factor Signaling	1.90	0.05	JUN,NFATC2
Actin Cytoskeleton Signaling	1.85	0.02	ITGA3,BAIAP2,GIT1,ARPC4
CD28 Signaling in T Helper Cells	1.85	0.03	JUN,NFATC2,ARPC4
P2Y Purigenic Receptor Signaling Pathway	1.84	0.03	ADCY9,PLCB2,JUN
iNOS Signaling	1.82	0.05	JUN,TYK2
RhoA Signaling	1.81	0.02	ABL2,BAIAP2,ARPC4
PI3K/AKT Signaling	1.80	0.02	ITGA3,TYK2,PPP2R2C
Cellular Effects of Sildenafil (Viagra)	1.75	0.02	ADCY9,PLCB2,KCNH2
GNRH Signaling	1.75	0.02	ADCY9,PLCB2,JUN
Phospholipase C Signaling	1.71	0.02	ADCY9,PLCB2,ITGA3,NFATC2
Relaxin Signaling	1.69	0.02	ADCY9,JUN,GNAT2
Synaptic Long Term Depression	1.64	0.02	PLCB2,GNAT2,PPP2R2C
Role of CHK Proteins in Cell Cycle Checkpoint	1.63	0.04	PPP2R2C,TLK2

Control			
EGF Signaling	1.62	0.04	JUN,SRF
Epithelial Adherens Junction Signaling	1.61	0.02	BAIAP2,PARD3,ARPC4
ErbB2-ErbB3 Signaling	1.61	0.04	JUN,TYK2
ATM Signaling	1.58	0.03	JUN,TLK2
Tec Kinase Signaling	1.52	0.02	ITGA3,TYK2,GNAT2
Calcium-induced T Lymphocyte Apoptosis	1.51	0.03	NR4A1,NFATC2
Dopamine-DARPP32 Feedback in cAMP Signaling	1.50	0.02	ADCY9,PLCB2,PPP2R2C
Hepatic Cholestasis	1.49	0.02	ADCY9,JUN,OSM
GABA Receptor Signaling	1.48	0.03	ADCY9,KCNH2
IL-10 Signaling	1.46	0.03	JUN,TYK2
Agrin Interactions at Neuromuscular Junction	1.45	0.03	ITGA3,JUN
CREB Signaling in Neurons	1.43	0.02	ADCY9,PLCB2,GNAT2
Chemokine Signaling	1.43	0.03	PLCB2,JUN
JAK/Stat Signaling	1.42	0.03	JUN,TYK2
RhoGDI Signaling	1.42	0.02	ITGA3,GNAT2,ARPC4
Ephrin Receptor Signaling	1.41	0.02	ITGA3,GNAT2,ARPC4
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	1.41	0.01	PLCB2,JUN,NFATC2,OSM
LPS-stimulated MAPK Signaling	1.41	0.03	JUN,SRF
Leptin Signaling in Obesity	1.40	0.03	ADCY9,PLCB2
PPAR α /RXR α Activation	1.38	0.02	ADCY9,PLCB2,JUN
IL-4 Signaling	1.38	0.03	TYK2,NFATC2
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.38	0.02	JUN,TYK2,PPP2R2C
Dopamine Receptor Signaling	1.36	0.03	ADCY9,PPP2R2C
Regulation of the Epithelial-Mesenchymal Transition Pathway	1.35	0.02	LOX,ID2,TYK2
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	1.35	0.03	JUN,NFATC2
Ceramide Signaling	1.34	0.03	JUN,PPP2R2C
ERK/MAPK Signaling	1.34	0.02	ITGA3,SRF,PPP2R2C
Thrombin Signaling	1.31	0.02	ADCY9,PLCB2,GNAT2
Breast Cancer Regulation by Stathmin1	1.31	0.02	ADCY9,PLCB2,PPP2R2C

S4 Table: DMR Validation and Transcript Levels

		Methyl-Sequencing Data				Pyrosequencing Data			
Chrm	postion	HDM-FA	HDM-CS	Difference	DMR p-value	HDM-FA	HDM-CS	Difference	p-value
chr11	4247417	0.75	0.47	-0.28	6.0E-04	0.49	0.47	-0.02	0.014
chr17	46552350	0.48	0.82	0.34	9.6E-04	0.61	0.63	0.02	0.006
chr19	14890412	0.15	0.25	0.10	1.1E-03	0.22	0.23	0.01	0.139
chr2	168525953	0.61	0.42	-0.19	9.2E-04	0.36	0.32	-0.04	0.069
chr4	135033743	0.83	0.48	-0.35	8.4E-04	0.62	0.60	-0.02	0.228
chr8	84064048	0.11	0.25	0.14	3.7E-04	0.19	0.20	0.01	0.109

S5 Table: DMR Subset Analysis Gene Lists

Genetic Association	IPA Asthma Associated
1700012D01Rik	1700012D01Rik
1700119H24Rik	1700119H24Rik
4930455D15Rik	4930455D15Rik
4930488B22Rik	
9130409I23Rik	
9830166K06Rik	9830166K06Rik
Abcb8	
Ace	
Ace3	
Actl7b	
Actr8	Actr8
Adam18	
Adam33	
Adamts12	
Adh4	Adh4
Adh5	Adh5
Adora1	Adora1
Adora2a	Adora2a
Adra1b	Adra1b
Adrb2	Adrb2
Agt	
Ahi1	
Ahnak	
Aicda	
Alas1	Alas1
Alox5ap	
Aoah	
Ap1s1	
Apobec1	
Apof	
Apon	
Arg1	Arg1
Arg2	Arg2
Arrdc2	
Atg9b	
B9d2	B9d2
BC021614	
Bcl2	Bcl2
Bco2	Bco2

Genetic Association	IPA Asthma Associated
Kcnh2	
Kcnh6	
Kcnn1	
Kdsr	Kdsr
Kif3a	Kif3a
Kmo	
Lep	Lep
Lepr	
Lgals9	Lgals9
Lifr	
Llph	Llph
Lrp1	Lrp1
Lst1	Lst1
Lta	Lta
Lta4h	
Ltb	Ltb
Ltc4s	Ltc4s
Ly86	
Maml1	Maml1
Mast3	
Mbl2	
Med23	Med23
Med7	Med7
Mefv	
Mfap5	
Mfsd12	Mfsd12
Mgat4b	Mgat4b
Mif	
Mir3098	Mir3098
Mir3470b	
Mir5098	
Mir6989	Mir6989
Mir7240	
Mir760	
Mir7671	
Mir7688	
Mmp9	Mmp9
Mpdu1	Mpdu1
Mpo	Mpo

Bdnf	
Blmh	
Bzrap1	Bzrap1
C3	C3
Cabin1	
Cactin	Cactin
Cadm4	
Calm3	
Capn9	
Cat	
Ccdc97	Ccdc97
Ccl24	Ccl24
Ccl7	Ccl7
Ccr3	Ccr3
Cd68	Cd68
Cd86	
Cflar	Cflar
Cftr	
Chdh	Chdh
Chil1	Chil1
Ckap2l	
Clec16a	
Csf2	Csf2
Ctla4	
Ctnna3	Ctnna3
Ctnn	
Cxcl12	
Cyb561	
Cyba	
Cyfp2	
Cyp1b1	
Cyp24a1	
Cyp2r1	Cyp2r1
Cysltr1	Cysltr1
Dact3	
Dap3	
Ddt	
Defb1	
Dennd1b	
Derl3	
Dnajc12	Dnajc12

Mrc1	
Mrpl4	Mrpl4
Ms4a2	Ms4a2
Mtmr12	
Muc2	
Muc5ac	
Mvd	
Myb	
Mybph	Mybph
Myl4	
Mylk	Mylk
Nab2	Nab2
Nat1	
Nat2	
Nat3	
Ndufa2	
Ndufv1	
Necap1	Necap1
Nfkbil1	Nfkbil1
Nob1	
Nod1	Nod1
Nos1	
Nos2	Nos2
Nos3	
Npsr1	Npsr1
Nqo1	
Opn3	
Ormdl3	Ormdl3
Osbpl7	Osbpl7
Pan2	
Parp1	
Pde4d	Pde4d
Pdgfra	
Pfdn4	
Pip5k1c	Pip5k1c
Plau	
Plaur	
Pon1	
Pparg	Pparg
Ppm1m	Ppm1m
Ppp1r9a	

Dnttip2	
Dpp10	
E130310I04Rik	E130310I04Rik
Edn1	
Egflam	
Eif4a1	Eif4a1
Elf5	
Ephx1	
Ezh2	Ezh2
F3	
Fam114a1	
Fam206a	
Fbxo21	
Foxj2	Foxj2
Fyn	
Gclm	
Gfra4	
Ggct	Ggct
Gipc3	Gipc3
Gjd3	
Gm11651	
Gm12	Gm12
Gm12216	
Gm14023	
Gm16894	
Gm19583	
Gng8	
Gpr108	Gpr108
Gpx1	
Grb7	Grb7
Gstp2	
Gstt1	
Gstt2	
Gstt3	
Gstt4	
Hacd2	Hacd2
Hal	
Hdac7	Hdac7
Hip1	
Hlx	
Hmg20b	Hmg20b

Prlr	
Prm1	
Prn	
Prnd	
Prnp	
Psemb8	
Psemb9	
Ptgdr	Ptgdr
Ptger2	Ptger2
Ptger3	
Ptger4	
Ptgir	
Ptgs2	Ptgs2
Ptprd	
Ptpre	
Rad50	Rad50
Rara	
Rdh11	Rdh11
Rhbdf1	Rhbdf1
Rnu6	
Rrp15	
Scgb1a1	
Sdhd	Sdhd
Sell	Sell
Serpine1	
Slc12a5	Slc12a5
Slc25a48	Slc25a48
Slc3a2	Slc3a2
Slc6a4	
Socs1	
Sod2	
Specc1l	Specc1l
Sqstm1	Sqstm1
Stard5	Stard5
Stat1	
Stat2	
Stat4	
Stat6	Stat6
Syn1	
Tap1	
Tap2	

Hmox1	Hmox1
Hnmt	Hnmt
Icam1	Icam1
Icam4	Icam4
Icam5	Icam5
Ido1	
Ido2	
Ifna1	
Ifnar1	
Ifne	
Ifng	Ifng
Ifngr2	
Igfbp4	
Ikbkap	
Ikzf3	Ikzf3
Il10	Il10
Il12a	
Il12b	Il12b
Il12rb1	
Il13	Il13
Il16	Il16
Il17c	
Il17rb	Il17rb
Il18r1	
Il1a	
Il1r2	
Il1rl1	Il1rl1
Il1rn	Il1rn
Il21	
Il23a	
Il3	Il3
Il33	Il33
Il4	Il4
Il5	Il5
Il5ra	
Il6	Il6
Il9r	Il9r
Inpp4a	
Irak1	
Irak3	Irak3
Irf1	

Tbkbp1	Tbkbp1
Tbx21	Tbx21
Tbxa2r	Tbxa2r
Tex12	Tex12
Tgfb1	Tgfb1
Tgfb2	
Timd4	
Timeless	
Tle4	
Tlr2	Tlr2
Tlr6	
Tlr8	
Tlr9	Tlr9
Tmbim4	Tmbim4
Tmco6	
Tmem194	Tmem194
Tmem50b	
Tmem63a	
Tmem91	Tmem91
Tnc	
Tnf	Tnf
Tnfsf13	Tnfsf13
Tnfsf14	Tnfsf14
Tnfsfm13	Tnfsfm13
Tnp2	
Tom1	Tom1
Top2a	
Traf3ip2	
Trip10	Trip10
Trnt1	
Trpd52l3	Trpd52l3
Ttc1	Ttc1
Twf2	Twf2
Tyms	Tyms
Vcam1	Vcam1
Vdr	Vdr
Vti1b	Vti1b
Wdr36	Wdr36
Wnt5a	Wnt5a
Wtap	
Zfp263	

lrgc1	
Itga4	Itga4
Itgb3	

bold = overlap

Zfr	
Zpbp2	Zpbp2