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TET1 methylation is associated with childhood asthma and traffic-related air pollution

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Abstract

Background—Asthma is a complex disorder influenced by genetics and the environment. Recent findings have linked abnormal DNA methylation in T cells with asthma; however, the potential dysregulation of methylation in airway epithelial cells is unknown. Studies of mouse models of asthma have observed greater levels of 5-hydoxymethylcytosine (5-hmC) and *TET1* expression in lungs. TET proteins are known to catalyze methylation through modification of 5-mC to 5-hydroxymethylcytosine (5-hmC).

Objective—Associations between *TET1* methylation and asthma and traffic-related air pollution were examined.

Methods—*TET1* methylation levels from DNA derived from nasal airway epithelial cells collected from 12 African-American children with physician-diagnosed asthma and their non-

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Online Repository Information
Figures E1 and Tables E1-3 can be found in the Online Repository.

Author contributions
HJ conceived and designed the experiments in discussion with XZ, JBM and MBK; AU recruited children with the assistance of NJ and performed sample processing; HKS processed samples and performed locus-specific bisulfite pyrosequencing and data analysis; XZ performed 450K array analysis and statistical analysis of the methylation data; HJ wrote the paper with the assistance of HKS, XZ, JBM, MBK, AU and GKKH.

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asthmatic siblings were measured using Illumina 450K arrays. Regions of interest were verified by locus-specific pyrosequencing in 35 additional sibling pairs and replicated in an independent population (N=186). Exposure to traffic-related air pollution (TRAP) at participants' early life and current home addresses was estimated using a land-use regression model. Methylation studies in saliva, PBMCs, and human bronchial epithelial cells (HBEC) were done to support our findings.

Results—Loss of methylation at a single CpG site in the *TET1* promoter (cg23602092) and increased global 5hmC was significantly associated with asthma. In contrast, TRAP exposure at participants' current homes significantly increased methylation at the same site. Patterns were consistent across tissue sample types. 5-aza-2'-deoxycytidine and diesel exhaust particle exposure in HBEC was associated with altered *TET1* methylation, expression and global 5-hmC.

Conclusions—Our findings suggest a possible role of *TET1* methylation in asthma and response to TRAP.

Capsule summary—*TET1* DNA methylation might serve as a biomarker for asthma and higher risk of exposure-related asthma exacerbations.

Keywords

DNA methylation; TET1; 5hmC; nasal epithelial cells; cross-tissue marker; traffic related air pollution; asthma

INTRODUCTION

Asthma is a complex, heterogeneous (1), and inheritable disorder. The prevalence of asthma has increased dramatically worldwide, making it a primary clinical and economic burden. Asthma is known to be influenced by gene-environment interactions (2). In children, asthma exacerbations are commonly triggered by environmental exposures including traffic-related air pollution (TRAP). A ubiquitous environmental exposure in urban areas, TRAP has been shown to induce inflammatory and immunologic responses leading to the exacerbation of asthma (3-8). Both early childhood and longitudinal TRAP exposure have been associated with asthma incidence in epidemiologic studies (9-13). Despite detailed phenotypic characterization of asthma (14), the molecular mechanisms of the initiation and progression of asthma, and the impact of the environmental exposure on these processes are not fully understood.

Epigenetic regulation in asthma has recently become an intensely studied area due to its impact on transgenerational disease susceptibility, phenotypic variability and association with environmental exposures (15, 16). DNA methylation is a heritable epigenetic modification that can be altered by environmental exposures. Its essential role in the regulation of gene expression makes it a potential mechanism in the etiology of complex diseases like asthma. Previous studies have indicated that pathways involved in asthma, such as T-cell differentiation and cytokine production, can be regulated by DNA methylation (17-22). Pharmacological demethylation has been reported to alleviate asthma symptoms in an experimental mouse model of asthma, possibly by altering the methylation on cytokine genes (23). Moreover, exposure to TRAP has been associated with DNA methylation variation in asthma-related genes (22, 24-29), and DNA methylation levels at specific loci

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have also been suggested to be used as a biomarker for asthma severity and exposure related asthma exacerbations (30). Therefore, the epigenome has been suggested to be a mechanistic bridge between exposures in the environment and the development of asthma, possibly via mediating gene-environment interactions. A better understanding of the epigenome will offer new pathophysiological insights into the relationship between genetics and the environment, and may provide therapeutic targets to prevent the onset and delay the progression of asthma.

The Ten-Eleven Translocation 1 (*TET1*) enzyme is a candidate that may play an essential role in asthma development. A known epigenetic modulator, TET1 promotes DNA demethylation by catalyzing the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), 5-formylmethylcytosine (5-fmC), and ultimately 5-carboxylcytosine (5-caC) (31). It is well characterized in cellular differentiation, cancer development and response to hypoxia (32-41). Recently, using a mouse model of house dust mite-induced asthma, a study reported altered methylome, increased levels of 5-hmC, and *TET1* expression in lungs (42), implicating a possible role of *TET1* in asthma. In this paper, we evaluated the associations of *TET1* methylation and expression levels with childhood asthma, and determined whether these associations were modified by TRAP exposures.

MATERIALS AND METHODS

Study Population

The Exposure Sibling Study (ESS) and the Pediatric Environmental Exposure Study (PEES) were used as the discovery and the replication cohorts, respectively. Both studies were approved by the Institutional Review Board at Cincinnati Children's Hospital Medical Center. Written informed consent was obtained from each participant or the participant's parent or legal guardian before study participation.

ESS is a cross-sectional study consisting of African-American siblings between age 5 and 18 years. Participants were recruited from the Greater Cincinnati Pediatric Clinical Repository (GCPCR) (43) and from the general public in response to flyers posted around the medical center and online. Eligible siblings (non-twins) discordant for asthma were born and raised in the same household in the Cincinnati Metropolitan area. Asthma diagnosis was obtained from the parental report, and confirmed via electronic medical record. Children on medication for any heart or lung condition besides asthma were excluded. Nasal, saliva, and blood samples were collected as described below and height and weight were recorded. The parent or guardian was given an electronic survey (Research electronic data capture (REDCap) software) (44) that characterized asthma onset, diagnosis, symptoms, severity, quality of life, medication, environmental exposures, social histories and residential address for the first year of life and for the past 5 years. For the discovery phase of the analysis, nasal epithelium cells from 12 of the 35 sibling pairs participating in ESS were assayed for DNA methylation using the Infinium HumanMethylation450 BeadChip (detailed study design shown in Figure 1).

To replicate the finding from the discovery population (ESS), we assayed saliva DNA from 158 asthmatic and 28 non-asthmatic African American PEES participants. PEES is a case-

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control study of nearly 400 asthmatic and non-asthmatic children aged 5-18 years living in the Cincinnati Metropolitan area (45). Children with asthma in PEES were diagnosed according to American Thoracic Society criteria (46).

Elemental carbon, or soot, is produced by incomplete combustion and the dominant contributors of outdoor elemental carbon are traffic (diesel combustion) and woodburning (47). However, in the Cincinnati metropolitan area outdoor woodburning is not a major source of particulate matter. In both studies, elemental carbon attributable to traffic (ECAT) (a proxy of diesel exhaust particles (DEP), herein referred to as TRAP) was estimated using a land-use regression model from the latitude and longitude coordinates from participants' home addresses using previously established methods (48). Two estimates were generated; birth or early exposure to TRAP was defined as exposure during the first year of life; and current exposure was derived using the current address. Children with 85th body mass index (BMI) percentiles (considering age and sex based on the Centers for Disease Control and Prevention curves) were considered overweight (49).

Sample collection and DNA/RNA extraction

Nasal epithelial cell or nasal mucosa sampling was performed on each participant with a CytoSoft Brush (Medical Packaging Corp, Camarillo, CA, USA), and the samples were immediately taken to the laboratory for processing. Information regarding DNA/RNA extraction, cell-culture studies, 450k array processing, bisulfite pyrosequencing and quantitative PCR can be found in the Online Repository.

Statistical Analysis

The demographics and characteristics were compared between ESS and PEES cohorts using Wilcoxon rank sum or Chi-squared tests according to the distribution of the data. In the discovery phase by 450K methylation array, 24 ESS nasal epithelium samples were assayed. To remove the effects of age and sex on the methylation level, the beta values of each CpG site were first modeled with age and sex in all 24 samples using linear regression; residuals were calculated and used in subsequent association tests with asthma status. As the siblings were paired by family, we compared the residuals between asthmatics and controls using paired t tests for each CpG sites. Sites with p value of the paired t test 0.05 and absolute beta difference between asthma and control 0.1 were selected as differentially methylated sites. As the sample size is small, non-parametric Wilcoxon signed-rank test was also performed for the significant findings to ensure the robustness of the conclusion. All analyses on the microarray data were conducted in R.

To examine whether the methylation levels of the *TET1* site from the 450K array agreed with those from pyrosequencing, we correlated the data from the 24 ESS samples used in the discovery phase using Pearson and Spearman correlation. The agreement was also visually judged by a Bland-Altman plot. Correlation was also examined among PBMC, saliva and NEC from the ESS cohort to evaluate tissue specificity of the methylation of *TET1*.

To verify the *TET1* association with asthma identified by the 450K methylation array, we analyzed the methylation level measured by pyrosequencing in all the 70 ESS samples. As

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ESS samples were paired by family, to account for the inter-family variability, we used a mixed model approach to test the association of methylation% with asthma status, and with ECAT. Age and sex were tested in the same model as potential co-variables. To replicate the *TET1* association with asthma and ECAT, 186 saliva samples from the PEES cohort were examined. The association of *TET1* methylation with asthma and ECAT was tested using a linear regression with age and sex adjusted.

To elucidate the role of methylation on *TET1* expression, we first correlated the methylation levels with the expression levels using Pearson correlation. The methylation levels were also dichotomized into two groups (high and low methylation groups) using hierarchical and k-means clustering. A two-sided t test was then performed to test whether the expression level was different in the low methylation group compared with the high methylation group. *TET1* expression, methylation and global 5hmC levels between controls and 5aza or DEP treated groups in HBEC were compared by two sided t-test. The analyses were performed using Statistical Analysis Software (SAS), version 9.3 unless otherwise specified. A p value cutoff of 0.05 was used to indicate the statistical significance.

RESULTS

Population characteristics

The demographics and characteristics of the ESS and PEES subjects included in the analyses are shown in Table 1. No statistically significant differences were detected between the ESS and PEES populations in age, sex, exposure to secondhand smoke, BMI percentile, obesity current ECAT or ECAT during infancy. Since the ESS study by design utilized siblings, it had 50% asthmatic participants; whereas PEES targeted and therefore had more asthmatics (85%). We also compared the demographics of the participants used in the discovery phase with the rest of the ESS cohort; no significant differences were detected (Table E3).

cg23602092 methylation at *TET1* promoter is associated with asthma

Out of the 312516 CpG sites that passed the filtering procedure described in Materials and Methods, 237 showed p 0.05 and absolute difference in beta values between asthmatics and controls 0.1, including a CpG in the promoter region of the *TET1* gene (cg23602092, p=0.014, paired t test, Figure 2A). Since *TET1* is a candidate gene that might be involved in asthma, we assayed this CpG site in all 70 ESS samples using pyrosequencing. We observed a good agreement in methylation levels between pyrosequencing and the 450K array (Pearson's r=0.96, Spearman's r=0.86, Figure 2B and 2C). Using the 70 ESS samples, we then verified the association of *TET1* methylation percentage with asthma in a mixed model. Age and sex were tested as potential co-variables. As no significant effects were detected, age and sex were excluded from the final model. While age and sex did not show effects on the methylation at cg23602092, a statistically significant association was detected between asthma status and cg23602092 methylation (p=0.040), with the mean methylation level being 22% (±17%) and 15% (±15%) in controls and asthmatics, respectively (Figure 2D).

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Impact of TRAP exposure on cg23602092 methylation differs in asthmatics versus controls

To assess the influence of TRAP exposure on the methylation level of cg23602092 at *TET1* promoter and the *TET1* association with asthma, we added TRAP exposure (current or birth estimates) to the mixed model. As shown in Figure 3, the correlation between current TRAP exposure (estimated as current ECAT) and cg23602092 methylation significantly differed in asthmatics and controls (p=0.033). In controls, higher cg23602092 methylation was associated with higher current ECAT (p<0.001); in asthmatics, a similar trend was observed, but the association was not statistically significant (p=0.23). Interestingly, besides an overall significant cg23602092-asthma association when current ECAT was not considered, our results suggested that the difference in cg23602092 methylation between asthma and control was larger in children who were exposed to high level of TRAP. To ensure the statistical robustness, M values were modeled similarly. The results were consistent with those when methylation percentages were used. The effect of birth TRAP was tested similarly, but no significant association was detected with *TET1* methylation.

Correlation of DNA methylation between tissue types

Epigenetic modifications have been reported to be tissue-specific, but both saliva and peripheral blood mononuclear cells (PBMCs) have been used to profile DNA methylation changes associated with environmental exposures and respiratory diseases due to their accessibility, especially for younger kids. Using bisulfite pyrosequencing, we measured the methylation levels of *TET1* cg23602092 in PBMCs and saliva and compared these values to those measured in nasal cells from the ESS cohort. As shown in Figure 4, methylation levels of cg23602092 were highly correlated in all three tissues (Pearson's r   0.75). These consistent results suggest that cg23602092 methylation changes are robust and that methylation at cg2360292 might be a useful cross-tissue biomarker for asthma.

Hypomethylation of *TET1* promoter was associated with asthma in PEES cohort

In order to substantiate our findings and replicate our results, we assessed the same CG site in saliva DNA from an independent cohort (PEES) containing asthmatic (N=158) and non-allergic, non-asthmatic (N=28) children. Using linear regression, we detected a statistically significant association between the *TET1* cg23602092 and asthma after adjusting for age (p<0.001, Figure 5). Potential effects of sex and current or birth ECAT were also tested; no significant associations were detected. M values were similarly evaluated, and our results support the findings when we modeled methylation percentages.

TET1 expression was negatively associated with the promoter CG site methylation

DNA methylation is an important epigenetic mechanism involved in gene expression regulation. It was recently shown that *TET1* promoter is hypermethylated and transcriptionally silenced in human non-Hodgkin B cell lymphoma (50) and in murine cell lines (51). To investigate the role of methylation modification at cg23602092 on transcription in nasal cells, we evaluated expression profiles of *TET1* using qPCR analysis. Interestingly, while the expression levels were not significantly correlated with the pyrosequencing methylation values, we observed a bimodal distribution of the DNA methylation data (Figure 6A). Hierarchical and k-means clustering further support that high

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and low methylation groups may exist. Therefore, we dichotomized the methylation data and found that the expression level of *TET1* was marginally lower in samples with higher cg23602092 methylation (p=0.107, two-sided t test, Figure 6B). Upon treating HBEC cells with 5-aza-2'-deoxycytidine, a DNA methylation inhibitor, we found that the *TET1* expression is significantly up-regulated after 24 hours, and cg23602092 significantly demethylated ($p<0.0001$, Figure 6C and 6D), which supports the negative correlation between cg23602092 methylation and *TET1* expression.

Up-regulation of global 5-hmC levels in asthmatics

TET enzymes regulate DNA methylation by catalyzing the conversion of 5-mC to 5-hmC in an Fe(II)- and α-KG-dependent manner (52). *TET1* in particular, has been shown to play a pivotal role in DNA demethylation (53). Knockdown of *TET1* resulted in reduced global 5-hmC levels (53, 54), while increased expression of *TET1* was associated with increased 5-hmC in murine lung tissues (42). Consistently, we observed a significant global increase in 5hmC level when *TET1* expression was induced by 5-aza (p<0.0001, Figure 6E). We then measured global 5-hmC in the saliva of the ESS sibling pairs and again observed higher levels of 5-hmC in asthmatics compared to their non-asthmatic siblings (Figure 6F), suggesting *TET1* expression in asthmatic children is increased.

Reduced *TET1* mRNA and global 5-hmC levels in HBECs challenged with DEP

DEP constitute a significant portion of particulate matter in TRAP and is recognized as a key component not only in TRAP-related asthma exacerbations (55, 56), but also were implicated in asthma pathogenesis (57, 58) and asthma severity (45). To determine the effect of controlled DEP exposure on *TET1* expression levels, we exposed HBECs to DEP (5ug/cm^2) and measured *TET1* expression and methylation overtime. At this dose of DEP, the expression of *CYP1A1*, *HMOX*-1, *IL1-β*, *IL-6* and *TSLP* were induced (Figure E1). *TET1* expression was slightly up-regulated in HBECs exposed to DEP at 1 hour after exposure to DEP, and significantly down-regulated 4 hours post-exposure and restored to normal level 24 hours after exposure (p=0.003, Figure 7A). Associated with this down-regulation, *TET1* cg23602092 showed an increase in methylation at 24 hours after exposure (p<0.0001), supporting a negative association between methylation and expression (Figure 7B). Also associated with the less *TET1* expression, we observed significantly lower levels of 5hmC in HBECs exposed to DEP 24 hours post-exposure (Figure 7C).

DISCUSSION

Our study consistently found that the loss of nasal cell-derived DNA methylation at a particular CpG (cg23602092) site in the promoter region of *TET1* was associated with childhood asthma. The methylation level of this CpG site was also significantly associated with levels of TRAP and this was most evident in non-asthmatics. *TET1* expression levels and 5hmC, a product of TET1, were altered in asthmatic children as well as in human bronchial epithelial cells treated with DEP, a major component of TRAP. In addition, inhibition of methylation promoted the expression of *TET1* in human bronchial epithelial cells. Collectively, our results demonstrate the function of methylation in the regulation of *TET1*, which may play a role in asthma etiology and response to traffic pollution. Moreover,

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the methylation level of this CpG site highly correlated across nasal cells, PBMCs and saliva, making it a potential cross-tissue biomarker for childhood asthma.

TET1 encodes a dioxygenase that consecutively converts 5mC into 5hmC, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), thus playing a key role in active DNA demethylation. Redox status, such as oxidative stress, has been shown to increase *TET*-mediated demethylation (40, 41, 59). Oxidative stress has been demonstrated in all forms of asthma by affecting airway epithelial cell damage, airway hyperresponsiveness, airway obstruction, and immune responses (60). Exposure to TRAP alone or in combination with allergens induces oxidative stress, which contributes to increased asthma risk (60). Because of the aforementioned associations among *TET1*, oxidative stress, asthma and TRAP exposure, associations of *TET1* with asthma status and TRAP described in this paper are not unexpected. Though the exact role of *TET1* in such cascade is yet to be elucidated, it is evident that DEP exposure upregulates proinflammatory cytokines in airway epithelial cells with well-established roles in asthma (61-67). Therefore, it is plausible that DEP-associated oxidative stress and inflammation in airways regulate *TET1*, which in turn modulates the 5-hmC levels, resulting in transcriptional activation of downstream genes such as VEGFA, which is known to be associated with lung function (68), particulate matter exposure and asthma (69-71). Interestingly, our study showed methylation of *TET1* promoter may be a mechanism for regulation of *TET1*.

Methylation signatures are tissue specific, therefore selection of relevant tissues and cell types to identify disease-specific modifications and disease etiology is crucial. To date, a majority of the studies have used either blood or saliva to profile DNA methylation patterns associated with complex diseases and environmental exposures. Although there are well-founded reasons why these surrogate tissues are used, the validity of the associations identified using surrogate tissues is questionable. In asthma, the airway epithelium is a major driver of allergic responses through its interaction with immune cells, which plays an essential role in the origin and persistence of the disease (72, 73). Therefore, it would be optimal to use lung epithelial cells for methylation profiling, but obtaining such samples in a clinical setting is impractical, particularly in children. Therefore, we sampled nasal epithelial cells as a proxy of bronchial epithelial cells to study biologically relevant changes occurring in the lung (74). Strikingly, when we quantified *TET1* methylation level at the site of interest in PBMCs, saliva, and nasal cell DNA from our discovery cohort, a strong correlation was observed across all three disease-relevant tissues and cell types, indicating that *TET1* dysregulation could be used as a systemic biomarker for childhood asthma.

We observed a bimodal distribution of *TET1* methylation in our cohort. To further investigate this phenomenon, we dichotomized the ESS cohort into high and low *TET1* methylation levels (cutoff=0.2) and TRAP exposure (cutoff=median of 0.3445). In the high methylation group, 83% had been exposed to high TRAP compared to 36% of the low methylation group (p=0.001, chi-square test). Therefore, TRAP exposure may contribute to the bimodal distribution and it is possible that *TET1* methylation changes markedly once TRAP exposure reaches a certain level. Another contributing factor may be a family effect. Out of 35 families, siblings in 28 had concordant methylation grouping. In our final mixed models, both TRAP exposure and a random family effect were included. Nevertheless, after

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adjusting for both TRAP exposure and family effect, we still observed a significant difference in *TET1* methylation level between asthmatics and controls.

The incidence of asthma is more prevalent among low-income African-American children who are more likely to reside near high TRAP and industrial areas (75-77). Accumulating evidence suggest that TRAP mediates asthma susceptibility and aggravate asthmatic symptoms (9, 10, 13). Recently it was suggested that the association between TRAP and asthma is mediated by epigenetic mechanisms (28). Given the shared mechanisms and co-occurrence of asthma and TRAP exposure, it is expected that the correlation of *TET1* with asthma and TRAP to be in the same direction. However, in our study, children with current exposure to higher levels of TRAP demonstrated increased methylation at *TET1* promoter, while children with asthma exhibited lower *TET1* methylation level. As the current study used a cross-sectional design, subjects were only examined at one particular time point. However, the biological processes are often dynamic and interrelated, thus data from one time point cannot capture all dynamic changes. It is likely that the response of *TET1* to TRAP is time-specific, as suggested by its response to acute DEP exposure in HBECs (Figure 7A). Nevertheless, our findings clearly showed a role of *TET1* in asthma and response to TRAP and future longitudinal studies are needed to further dissect the relationship between TRAP, DNA methylation and asthma in conjunction of a cell culture model of real life TRAP exposure.

One of the strengths of our research design is the recruitment of African-American sibling pairs discordant for asthma who were born and have been living in the same household. Asthma is a complex disease with many influencing risk factors such as race, family history, socio-economic status, and various environmental exposures, most of which have been shown to modify epigenetic landscapes (15, 78). The paired siblings allowed us to better control for genetic factors as well as other unknown environmental factors, thus increasing the power to identify asthma-specific variation.

Conclusions and Implication for Future Studies

Our study demonstrated for the first time that DNA hypomethylation at *TET1* promoter was associated with childhood asthma in African-Americans. We also showed that methylation at the same CpG site was significantly associated with current exposure to TRAP in an opposite direction of the asthma association. Further studies are warranted to understand the underlying mechanism(s) of the opposite directionality in these associations and to determine if they are present in children of various races at crucial developmental windows. Our *in vitro* human cell line experiments support the involvement of *TET1* in response to DEP exposure. Further experiments will be needed to address the role of *TET1* in asthma development and response to TRAP exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ESS	Exposure Sibling Study
PEES	Pediatric Environmental Exposure Study
5-mC	5-methylcytosine
GCPCR	Greater Cincinnati Pediatric Clinical Repository
5-hmC	5-hydroxymethylcytosine
5-fmC	5-formylmethylcytosine
5-caC	5-carboxylcytosine
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
DEP	diesel exhaust particles
TRAP	traffic-related air pollution
HBECs	human bronchial epithelial cells
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
SST	Serum Separator Tube
CPT	Cell Preparation Tube
TET1	Ten-Eleven Translocation 1
REDCap	Research Electronic Data Capture
ELISA	enzyme-linked immunosorbent assay
BMI	body mass index
IQR	Interquartile Range
PBMC	peripheral blood mononuclear cells

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Key messages

- Increased *TET1* promoter methylation and global 5hmC levels are associated with childhood asthma.
- Increased traffic related air pollution is associated with decreased *TET1* promoter methylation.
- *TET1* promoter methylation is conserved between different tissue types.
- There is a negative correlation between *TET1* methylation and its expression.

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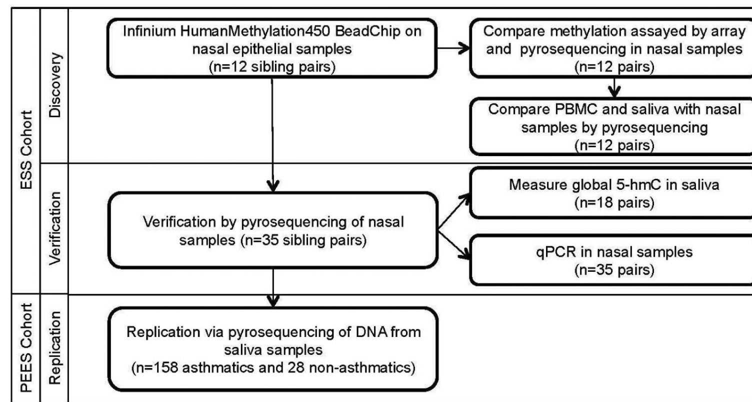

Figure 1. Study schematic

There were 35 sibling pairs in the discovery ESS population. Of these, nasal samples from 12 pairs were selected to run on the Infinium HumanMethylation450 BeadChips. We then verified the results via pyrosequencing on PBMCs and saliva in these same 12 pairs and in nasal epithelial samples from all 35 sibling pairs. We also measured 5-hmC levels in saliva from 18 pairs. To replicate our findings, we performed pyrosequencing on 186 saliva samples from children participating in the PEES cohort.

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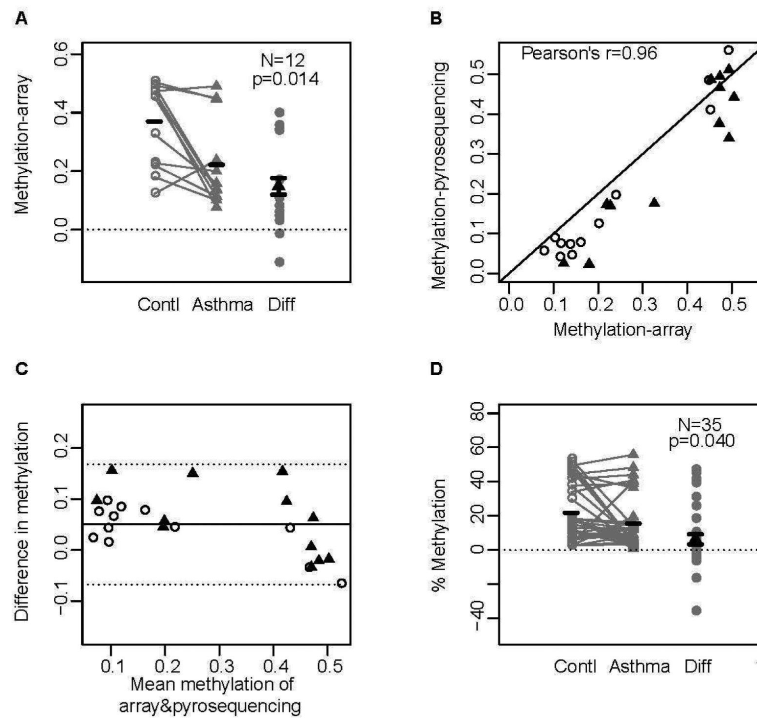

Figure 2. Hypomethylation of cg23602092 located at *TET1* promoter is associated with childhood asthma in ESS cohort

(A) Differentially methylated cg23602092 on the Infinium HumanMethylation450 BeadChip. Beta values and the differences of each of the asthma-control pairs were shown as gray dots; the mean beta for controls or asthmatics was shown as the black bar; the mean difference was shown as a black triangle together with the SEM. **(B)** The correlation between bisulfite pyrosequencing and bead-array measurements. Controls and asthmatics were represented by solid triangles and open dots, respectively. **(C)** Bland-Altman plot showing the limits of agreement. Solid line: mean difference, dotted lines: 95% confidence interval. **(D)** DNA methylation in all ESS participants. Figure legends are the same as (A).

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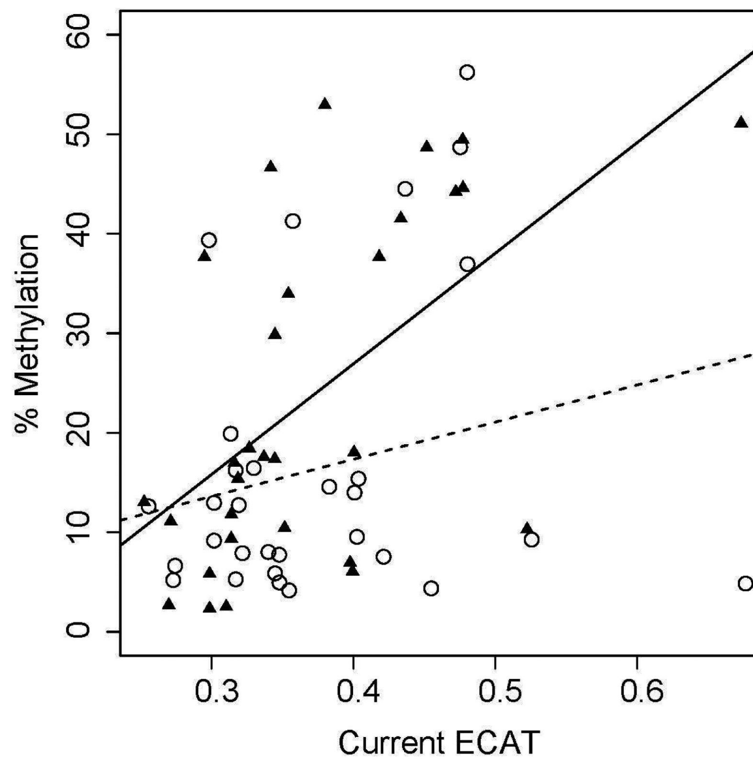

Figure 3. Influences of TRAP on cg23602092 methylation and on the association between cg23602092 methylation and asthma

Percent methylation was shown for controls (solid triangles) and asthmatics (open circles). Model predicted relationship between TRAP exposure (current ECAT) and methylation was shown in controls (solid line) and in asthmatics (dashed line). The distance between the dashed and solid lines represents the differences in methylation between controls and asthmatics at different levels of current ECAT exposure.

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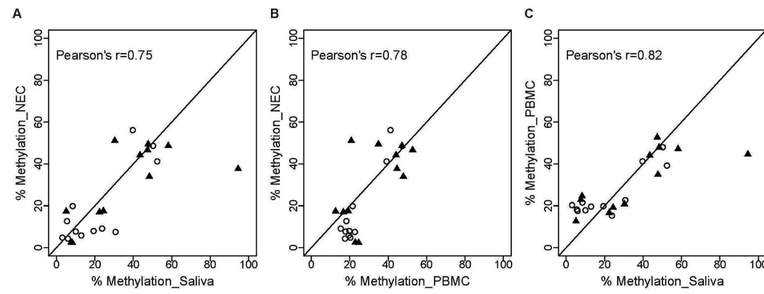

Figure 4. Methylation of cg23602092 is correlated between three tissues

Shown are comparisons of cg23602092 methylation between **(A)** nasal cells and saliva, **(B)** nasal cells and PBMCs and **(C)** Saliva and PBMCs in ESS cohort. Controls and asthmatics were represented by solid triangles and open dots, respectively.

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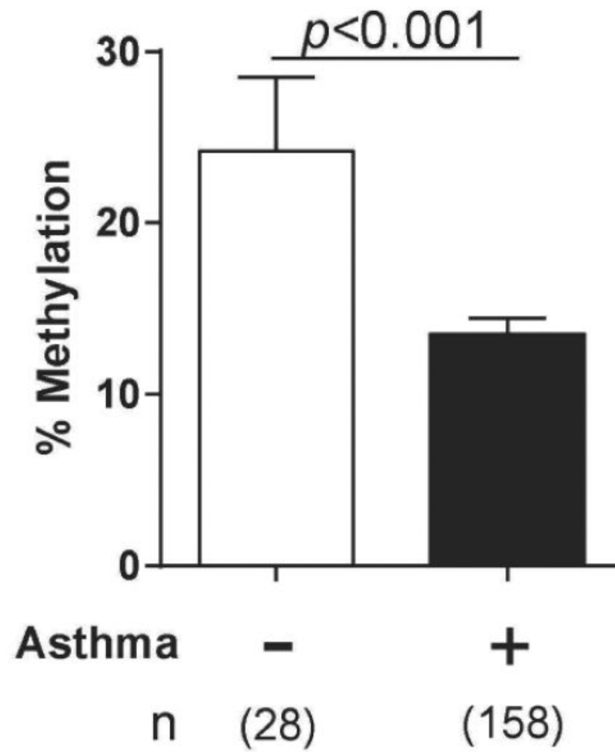

Figure 5. Hypomethylation of cg23602092 is associated with childhood asthma in PEES cohort

Methylation levels measured by locus-specific bisulfite pyrosequencing in the saliva of asthmatic (n=158) and non-asthmatic (n=28) kids enrolled in the PEES cohort. A statistically significant association between TET1 hypomethylation and asthma was detected, thus replicating our finding from the ESS cohort. Bars represent mean ± SEM.

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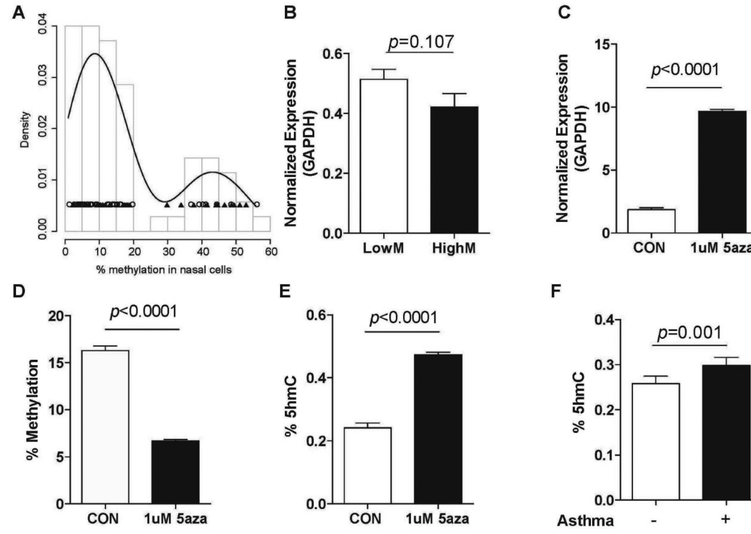

Figure 6. cg23602092 methylation is negatively correlated with *TET1* expression in nasal mucosa samples

(A) Bimodal distribution of cg23602092 methylation shown in histogram (grey bars) and density (solid curve). Solid triangles: controls; open circles: asthmatics**.** **(B)** *TET1* expression levels in nasal epithelial cells. HighM group: methylation%>20%; LowM: methylation %<20%. Values are represented as the mean±SEM. **(C-E)** *TET1* expression (**C**), cg23602092 methylation (**D**), and global 5hmC (**E**) in HBEC treated with 1uM 5-aza. Data represent two technical duplicates of three biological replicates and are shown as mean±SD. (**F**) 5hmC in saliva DNA from 18 pairs of siblings from ESS cohort. Values are represented as the mean±SEM.

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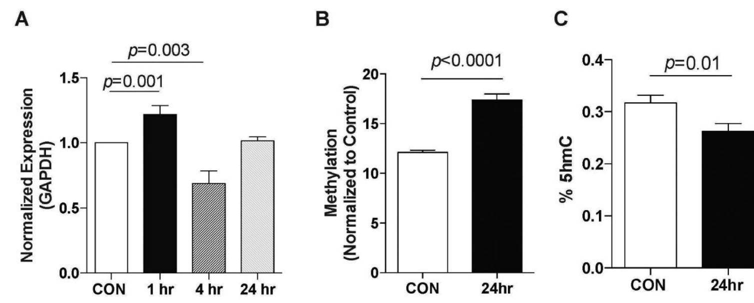

Figure 7. Exposure to DEP induced changes in cg23602092 methylation, *TET1* expression and 5hmC

(A) *TET1* expression in HBECs cultured with DEP (5ug/cm^2) was measured and normalized to untreated controls at indicated time points. **(B)** Percent methylation values were measured and normalized to controls. **(C)** 5hmC ELISA. Data represents three technical replicates of biological duplicates and is shown as mean±SD.

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Table 1

Population characteristics

	ESS N=70	**PEES N=186**	**p value**
Age (mean ± SD)	11.0 (9.0-14.0)	12.0 (8.0-15.0)	0.62
Male Sex (%)	38 (54%)	97 (52%)	0.76
Asthmatic (%)	35 (50%)	158 (85%)	<0.001
Secondhand smoke exposure (%)	33 (47%)	97 (52%)	0.48
BMI percentile	72.9 (46.3-96.8)	76.5 (49.1-96.9)	0.49
OverWT/Obese (%)	29 (41%)	83 (45%)	0.65
ECAT during infancy	0.42 (0.34-0.51)	0.41 (0.34-0.53)	0.61
Current ECAT	0.34 (0.31-0.42)	0.37 (0.30-0.49)	0.18

Note: age, BMI percentile, ECAT during infancy and current ECAT were shown as median (IQR) and compared using Wilcoxon rank sum test; other variables were shown as N (%) and compared using Chi-squared tests.