ORIGINAL RESEARCH

DNA Methylation Is Globally Disrupted and Associated with Expression Changes in Chronic Obstructive Pulmonary Disease Small Airways

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Abstract

DNA methylation is an epigenetic modification that is highly disrupted in response to cigarette smoke and involved in a wide spectrum of malignant and nonmalignant diseases, but surprisingly not previously assessed in small airways of patients with chronic obstructive pulmonary disease (COPD). Small airways are the primary sites of airflow obstruction in COPD. We sought to determine whether DNA methylation patterns are disrupted in small airway epithelia of patients with COPD, and evaluate whether changes in gene expression are associated with these disruptions. Genome-wide methylation and gene expression analysis were performed on small airway epithelial DNA and RNA obtained from the same patient during bronchoscopy, using Illumina's Infinium HM27 and Affymetrix's Genechip Human Gene 1.0 ST arrays. To control for known effects of cigarette smoking on DNA methylation, methylation and gene expression profiles were compared between former smokers with and without COPD matched for age, pack-years, and years of smoking cessation. Our results indicate that aberrant DNA methylation is (*1*) a genome-wide phenomenon in small airways of patients with COPD, and (*2*) associated with altered expression of genes and pathways important to COPD, such as the NF-E2–related factor 2 oxidative response pathway. DNA methylation is likely an important mechanism contributing to modulation of genes important to COPD pathology. Because these methylation events may underlie disease-specific gene expression changes, their characterization is a critical first step toward the development of epigenetic markers and an opportunity for developing novel epigenetic therapeutic interventions for COPD.

Keywords: chronic obstructive pulmonary disease; small airways; epigenetic regulation; DNA methylation; integrative omics

Clinical Relevance

We show, for the first time, that aberrations to DNA methylation in chronic obstructive pulmonary disease (COPD) small airways affects hundreds of genes, and corresponds to gene expression disruptions affecting pathways of known importance to COPD pathology, such as the NF-E2–related factor 2 oxidative response signaling pathway. Our findings lead us to conclude that DNA methylation is likely an important mechanism involved in COPD biology. Because DNA methylation is a reversible gene regulatory modification, and small airways are the primary sites of airflow obstruction in COPD, further work in this area may contribute to the development of much-needed treatment or prevention strategies.

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Chronic obstructive pulmonary disease (COPD) is a highly prevalent lung condition affecting 300 million people worldwide (1). The burden of this disease is expected to increase, and, by 2030, COPD will become the third leading cause of mortality (currently fifth) worldwide (2). Unfortunately, there is a dearth of therapies that can effectively modify disease activity or progression (3). Small airways have been identified as the primary site of airflow limitation, and undergo extensive remodeling that precedes emphysematous destruction of lung parenchyma (4). A better understanding of the molecular mechanisms underlying small airway remodeling is thus relevant to the design of therapeutic and prevention regimes targeting COPD pathogenesis.

To date, efforts to molecularly characterize COPD, or identify biomarkers of COPD predisposition and progression, have largely focused on genome-wide association studies (GWAS) (5–8), transcriptome profiling of parenchymal lung tissues (9–13), or large and small airways (14–17), and, recently, studies that integrate genotype (GWAS) and gene expression data for the purpose of identifying expression quantitative trait loci (18–21). GWAS have revealed a significant interindividual genetic variance underlying lung function and response to major COPD risk factors. Transcriptome studies support the hypothesis that impaired protective mechanisms in response to reactive oxidative species from cigarette smoke and inflammatory cells result in damage to small airway epithelia (SAE) and promote inflammation in COPD airways that persist years after smoking cessation. Expression quantitative trait loci studies are beginning to unravel the functional impact of many disease-related variants in COPD biology, lung function, and smoking response.

Epigenetic changes, which yield somatically heritable changes in gene expression patterns, are important mediators of environmental exposures related to chronic disease. DNA methylation is a heritable, tissue-specific, and reversible gene regulatory mark that is highly modified in response to cigarette smoke and involved in the development and progression of a wide spectrum of diseases (recently reviewed in Ref. 22). Because DNA methylation is a reversible gene regulatory modification, the exploration of epigenetic drugs to treat inflammatory and malignant disease is an enormous field of study (23, 24). Although epigenetic biomarkers associated with COPD have been explored in other clinically relevant tissues, such as sputum and blood (25–27), whole-genome assessment of these markers in small airways of patients with COPD has not been previously performed. Given (*1*) the importance of small airways to COPD pathology, (*2*) the knowledge that epigenetic mechanisms mediate cellular responses to systemic and environmental stimuli, such as inflammation and smoking (24), and (*3*) the highly tissue-specific nature of DNA methylation patterns, we hypothesized assessment of these markers in SAE from individuals with COPD would provide insight into DNA-level disruptions associated with small airway remodeling. In this study, we used an integrative multi-'omics approach on patient-matched small airway DNA and RNA to evaluate the potential impact of aberrant DNA methylation on the biology of COPD. Because disruption of epigenetic events may underlie disease-specific gene expression changes, characterization of DNA methylation is a critical first step toward the development of epigenetic markers and novel epigenetic therapeutic interventions for COPD.

Materials and Methods

For detailed methods please see the Materials and Methods in the online supplement.

Subjects and Sample Collection

Bronchial SAE specimens were obtained by bronchial brushing of small airways (defined as < 2 mm in diameter) using a 1.5-mm brush from former smokers (FS) with ($n = 15$) and without ($n = 23$) COPD (Table 1 and Figure E1 in the online supplement). FS are defined as those who have stopped smoking for 1 year or longer. Two-tailed Student's *t* tests found no significant difference in age, pack-years, or years since quitting smoking between COPD and non-COPD groups (Table 1). All subjects with COPD were GOLD stage II ($n = 9$) or III ($n = 6$).

Molecular Profiling

DNA methylation profiles were obtained using the Illumina Infinium Methylation chip (HM27; Illumina Infinium 27K Methylation Arrays, San Diego, CA), assaying 27,578 CpG sites of 14,475 genes (Gene Expression Omnibus number pending). Gene expression profiles for 22 patient-matched samples were generated using Affymetrix Human Gene 1.0 ST arrays (Gene Expression Omnibus number pending; Affymetrix, Santa Clara, CA). Quantification of percent cytosine methylation for select genes was performed by pyrosequencing on a subset of samples for which adequate material was available from Table 1 and on select differentially methylated (DM) genes of interest for which pyrosequencing probe design was feasible (Table E1) (28).

DNA Methylation Analysis

Sequence-dependent color bias correction and simple scaling normalization normalization algorithms designed for Illumina Infinium HM27 methylation platform were applied (29). Because commonly used β values are heteroscedastic, M values were used for all statistical tests where equal variance is assumed (29, 30). β values were used for dimensional reduction by unsupervised principal component analysis (PCA), as recommended (30). The Illumina Infinium assay was highly reproducible, although less methylated

Table 1: Summary Demographics and Clinical Information

	COPD	Normal	*P* Value
n	15	23	
Age	65 ± 5.76	64 ± 4.8	0.44
Female:male	5:10	8:15	1
Pack years	54.77 ± 30.43	46.64 ± 20.53	0.37
Years quit	10 ± 9.55	14 ± 5.44	0.2
FEV_1 Actual	1.79 ± 0.63	3.06 ± 0.68	5.59E-06
FEV_1 % Predicted	58 ± 15.59	98 ± 9.84	2.10E-08
FEV_1/ FVC%	58 ± 9.57	75 ± 5.38	4.92E-06

Definition of abbreviations: COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume at 1 second; FVC, forced vital capacity.

probes were more variable (Figures E2 and E3). A multivariate ANOVA was used to assess variance in methylation due to disease, age, sex, pack-years, and years quit. To identify DM genes in COPD small airways, we applied a nonparametric permutation test, using 10,000 permutations and corrected for multiple testing using the Benjamini and Hochberg (B-H) method (B-H $P < 0.05$ was considered significant). This test is highly powerful for small sample sizes. We further applied SD less than or equal to 2, and average fold change (FC) cutoffs of greater than 1.25 or less than 0.75 for probes to be considered differentially hyper- or hypomethylated in COPD airways, respectively. A PCA was performed in MatLab (Natick, MA). Genes DM between top and bottom pack-year tertiles of our cohort, regardless of disease status, were deemed "smoking-related."

DNA Methylation and Gene Expression Integration

Nonparametric Spearman tests were applied to identify genes likely regulated epigenetically (Spearman's $\rho \leq -0.4$ and $P < 0.05$) using patient-matched methylation and gene expression profiles. A gene was considered significantly negatively correlated if at least one Illumina and corresponding Affymetrix probe on either array passed the criteria stated. DM genes, the expression levels of which in COPD airways had (*1*) a permutation *P* less than 0.05, and (*2*) an average FC of greater than 1.2 or less than 0.8 compared with non-COPD profiles, were considered differentially expressed (DE). Here, we focused on genes that sustained concomitant inverse methylation and expression alterations (DM and DE). Recent expression studies report subtle differences (i.e., small effect size) induced by cigarette smoke in nonmalignant tissues; thus, we employed the same FC criteria to ensure that we did not overlook subtle changes (31, 32). DM and inversely DE genes were selected for Ingenuity Pathway Analysis (http://www.ingenuity.com), which uses a Fisher's exact test to calculate *P* values corresponding to the probability that pathway enrichment is due to chance alone.

Results

Aberrant DNA Methylation Patterns Affect Hundreds of Genes in COPD Small Airways

We hypothesized that patterns of DNA methylation in COPD small airways would be distinct from those in subjects with normal lung function and similar smoking history. We first evaluated the extent to which DNA methylation was differentially altered in small airways between patients with COPD compared with control subjects. We detected 1,120 unique genes (1,260 CpG probes) as DM in COPD SAE, of which 97% were hypermethylated (Table E2). Increased variance in lowly methylated probes in combination of our SD cut off threshold may also have contributed to the increased proportion of hypermethylated probes (Figures E2 and E3). A subset of these genes was validated by pyrosequencing analysis (Table E1, Figure E4). Of the 1,120 DM genes, 79 were previously associated with COPD in gene expression studies or GWAS (Table E3). These included, for example, hypermethylation of three glutathione S-transferase (GST) genes (*GSTP1*, *GSTM1* and *GSTT1*), three cholinergic receptors (*CHRNB1*, *CHRNB2*, and *CHRND*), as well as *GPR126*, *HTR4*, and *EPHX1*. Hypomethylated COPD-associated genes included *KSR1*, the overexpression of which is indicative of increased bacterial colonization frequently associated with COPD phenotypes in humans and in mice (33).

DNA Methylation Is Correlated with Lung Function Variables

We were next interested in assessing whether methylation may be associated with lung function variables, as opposed to disease status. Although we did not detect any significant methylation differences between nine patients with GOLD II (moderate) COPD and six patients with GOLD III (severe) COPD, a PCA using only 100 of the most DM genes between six subjects with severe COPD and six control subjects separated 15 subjects with COPD and 23 without COPD in COPD and non-COPD overall, although there was substantial overlap between subjects with severe COPD and those with moderate COPD (Figure 1). When we considered methylation and lung function as continuous variables, we found that methylation levels of 62 genes were significantly (B-H *P* value < 0.05) correlated with lung function overall, 48% of which overlapped with our DM COPD genes (Table E4). All significantly correlated lung function probes were negatively correlated with methylation (i.e., higher methylation was associated with lower lung function).

COPD-Related DNA Methylation Alterations Possibly Induced by Smoking

Although two-tailed Student's *t* tests found a significant difference in packyears between our high ($n = 11$ cases) and low ($n = 10$ cases) pack-year groups ($P = 0.000126$), FEV % predicted ($P = 0.042852$), years quit ($P = 0.01543$), and age ($P = 0.017821$) were also significant, therefore "smoking-related" genes also had to be significantly associated with pack years ($P < 0.05$) by a multivariate analysis of variance. We detected 158 unique genes that passed our criteria for "smoking associated" (i.e., DM between high and low pack-year patients in our cohort). Of these, 45 overlapped with our DM COPD genes from Table E2, 11 of which were also significantly associated with pack years and disease status ($P < 0.05$) by a multivariate analysis of variance in the high– and low–pack-year groups. These are: *BAI2*, *C10orf35*, *CD248*, *CDKN2B*, *CHRNB1*, *LIPC*, *PTK9*, *SOX17*, *SUV420H2*, *TREM2*, and *ZNF323*, all of which were DM in the same direction in COPD and high–pack-year groups; nine genes were hypermethylated and two (*TREM2* and *ZNF323*) were hypomethylated (Table E5).

We also compared our findings to a recent study by Buro-Auriemma and colleagues (37) who describe the effects of active smoking on the SAE methylome in current-smoker (CS) and never-smoker subjects without COPD. Of the top 50 hypomethylated and hypermethylated smoking-associated DM genes discovered by Buro-Auriemma and colleagues, none of our smoking-associated genes overlapped, but five of our DM COPD genes did, including: *ALDH1A3* and *SH3TC2*, which were hypermethylated in CS and COPD FS SAE (except one of the two DM *ALDH1A3* probes, which was hypomethylated in COPD), and *CYP1A1*, *GSTM1*, and *KCNJ15*, which were hypomethylated in smokers, but hypermethylated in COPD SAE.

Pathways Affected by DNA Methylation

We next examined what molecular pathways were associated with DM genes in COPD airways. Overall, three pathways were significantly enriched in the 1,120 DM

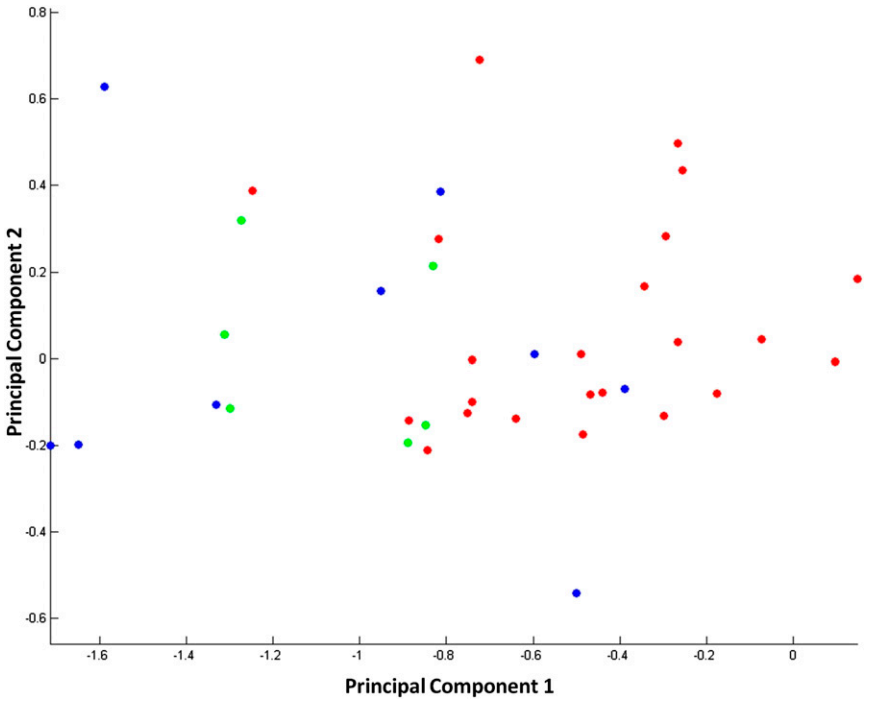

Figure 1. Principal component analysis. Nine GOLD stage II (*blue dots*), six GOLD stage III (*green dots*) and 23 control subjects (*red dots*), were clustered based on 100 of the most differentially methylated (DM) genes between six stage III and six control subjects. While COPD and normal methylation profiles generally clustered separately, severe and moderate COPD subjects did not distinctly separate.

gene set (B-H–corrected P value < 0.05); these included: G protein–coupled receptor signaling (31 genes), Aryl hydrocarbon receptor signaling (20 genes), and cAMP-mediated signaling (26 genes) (Figure 2 and Table E6). These pathways are known to play a role in small airway biology, including COPD small airway remodeling, wound healing, and in mediating cellular response to polycyclic aromatic hydrocarbon (a component of cigarette smoke) exposure.

Integration of DNA Methylation and Gene Expression Changes to Reveal Candidate Genes and Pathways Potentially Involved in COPD Pathogenesis

Epigenetic regulation of gene expression by DNA methylation is dependent on location and CpG content of regulatory elements. For example, hypermethylation of gene promoter elements with high CpG content is associated with repression of gene expression, whereas hypermethylation within the first exon of gene bodies is associated with activation of gene expression. Because Illumina HM27 CpG probes reside primarily within promoters, we focused our analysis on genes the methylation and gene expression values of which were negatively correlated across samples (where matched DNA methylation and gene expression profiles were available) from subjects with COPD and those without. We identified 141 such genes; however, we note that methylation levels of 335 genes were positively associated with gene expression, 99% of which were hypermethylated and overexpressed. Of the inversely correlated genes, 130 were hypermethylated and underexpressed, and 11 were hypomethylated and overexpressed relative to non-COPD airways (Figure 3A, Table E7). A total of 15 of these 141 genes (11%) has been previously associated with COPD, at either the DNA or mRNA level, but none has been previously associated with differential DNA methylation in COPD (Table 2). For example, *TFF3*, the trefoil factor that regulates repair of injured human respiratory epithelium, was hypermethylated and underexpressed in COPD SAE (34). Similarly, the creatine kinase gene, *CKB*, was hypermethylated and underexpressed in COPD airways. Underexpression of *CKB* has been previously detected in COPD bronchial epithelial cells in association with smoke-induced bronchial epithelial cell senescence (35).

Because this is the first study assessing DNA methylation patterns in small airways of patients with COPD, validation of methylation findings in external datasets was not possible. Therefore, to validate our DM and DE gene set, small airway gene expression profiles were downloaded from GSE37147 (14). After matching for smoking status, this cohort included 39 FS with COPD ($n = 32$ GOLD II; $n = 7$ GOLD III) and 63 FS without COPD. We found that 46 out of 141 of our DM and DE genes had similarly altered expression patterns in COPD compared with non-COPD airways in this external dataset (indicated in Table E7), five of which (*EPHX1*, *IGF1R*, *LRIG3*, *MUC13*, and *SDCBP2*) showed statistically significant differential expression between the 39 COPD and 63 non-COPD profiles ($P < 0.05$ by a nonparametric Mann-Whitney U test). Our observation of highly methylated genes exhibiting reduced gene expression levels suggests that aberrant DNA methylation has a concordant effect on gene expression in COPD SAE cells.

To identify the aberrantly methylated gene candidates most likely to be controlled by DNA methylation in COPD SAE, we applied a Spearman correlation cut off ($\rho < -0.4$; $P < 0.05$) to the 141 DM and DE gene set. The most negatively correlated DM and DE gene was *CYP4F11* ($\rho = -0.866742$, Spearman $P = 0.000001$) (Table 3). For these genes, the presence of COPD was the only significant factor ($P < 0.05$) responsible for the observed variance in methylation based on a multivariate ANOVA assessing variance due to COPD, age, sex, pack-years, and years quit, except one of the two methylated probes for *CYP4F11* (cg24655310), which was also affected by sex ($P = 0.01733$). *MUC13* was the gene most significantly associated with hypomethylation in subjects with COPD ($P = 0.0006442$).

We next applied pathway enrichment analysis to the set of 141 DM and DE genes. This 141-gene set was significantly enriched ($P < 0.05$) for three pathways: phosphatase and tensin homolog (PTEN) signaling, the NF-E2–related factor 2 (Nrf2) oxidative stress response pathway, and the IL-17F in allergic inflammatory airway diseases

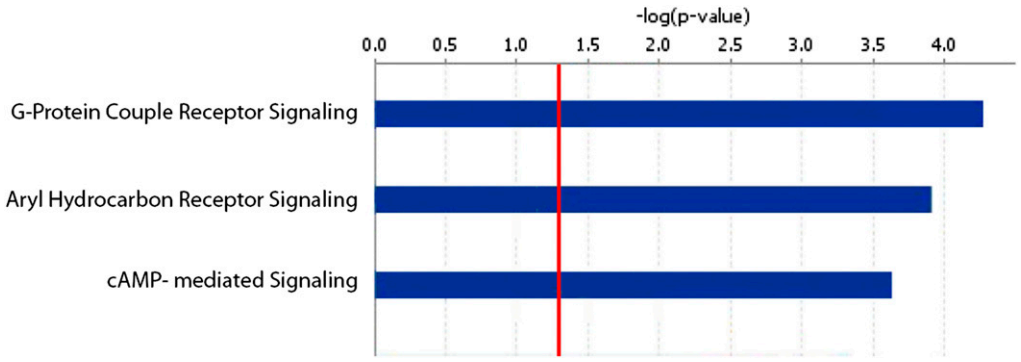

Figure 2. DM genes in COPD small airways correspond to three significantly enriched pathways. We detected 1,120 DM genes in small airways of patients with COPD compared with methylation profiles from individuals without COPD. These genes corresponded to three significantly enriched pathways: G protein–coupled receptor signaling (31 genes; Benjamini and Hochberg [B-H] $P = 0.024$), Aryl hydrocarbon receptor signaling (20 genes; B-H $P = 0.0276$), and cAMP-mediated signaling (26 genes; B-H $P = 0.0345$). The *horizontal axis* displays −log of the B-H P value, calculated by Fisher's exact test right tailed, representing the probability that pathways are enriched in a given gene set by random chance. A B-H P value of 0.05 is indicated by the *vertical red line*.

(Figure 3B). Two modulators of the PTEN signaling pathway, *PTEN* and *CSNK2A2*, were hypermethylated and underexpressed in COPD airways. Multiple upstream Nrf2 regulators (*PKC*, *MEK1*, *Actin*, *PTEN*, *NRF2*) and downstream effector molecules (*MAF*, *MRP4*, *GST*, *HIP2*, *HSP6*, *EPHX1*, *FTH1*) were found to be differentially affected at the level of DNA methylation and/or gene expression. Two of the most strongly negatively correlated genes in our entire study included *EPHX1* and *CYP4F11* (Table 3). In the IL-17F pathway, the upstream receptor, *IL17RC*, together with downstream *CXCL1*, were found to be hypermethylated and underexpressed, whereas the proinflammatory *CSF2* was hypomethylated and overexpressed.

In our external validation data set, the Nrf2 signaling pathway was the most significantly enriched pathway ($P = 0.00614$) based on the 46 genes that were altered in the same direction, and was also the only significantly enriched pathway that overlapped between the DM and the concomitant DM and DE gene sets (Figure 4).

Collectively, our integrative analyses indicate that: (*1*) expression levels of COPD-associated genes are epigenetically deregulated in small airways of patients with COPD; and (*2*) DNA methylation is a likely mechanism through which key pathways of importance to COPD pathology are disrupted.

Discussion

DNA methylation is highly modified by inflammation and cigarette smoke in cells of exposed airways and lung tissues, and is directly involved in the development and progression of a wide spectrum of disease. In the context of COPD, DNA methylation has been explored in sputum and blood (25, 26, 36), but not on a genome-wide level in SAE. Because DNA methylation is highly tissue specific, and small airways are the primary sites of airflow obstruction in COPD, assessment of these markers in SAE from patients with COPD is of significant biological and clinical interest. We provide the first genome-wide methylation and integrative 'omics study applied to the analysis of SAE from individuals with COPD. To avoid confounding effects of active cigarette smoking, which is known to affect both DNA methylation and gene expression in small airways (37), analyses were restricted to FS.

We found that DNA methylation is widely disrupted in SAE of patients with COPD, affecting hundreds of genes, which we found predominately hypermethylated relative to SAE of individuals without COPD (Table E2). Because roughly 90% of gene promoters associated with CpG islands are normally unmethylated, many of these DM events are likely abnormal. Overall, our DM COPD gene set was enriched for three pathways: G protein–coupled receptor signaling, Aryl hydrocarbon receptor signaling, and cAMP-mediated signaling. In the context of COPD, deregulation of these pathways has been implicated at the single-nucleotide polymorphism (SNP), mRNA, and protein levels (38–40), but not previously at the level of DNA methylation as described here.

Although we did not detect any significant differences between cases of moderate and severe COPD, we did detect genes, the methylation status of which was significantly correlated with lung function overall, all in a negative direction, and almost half of which overlapped with our DM COPD genes (Table E4). We found methylation of *GATA4* negatively associated with lung function and DM in COPD; hypermethylation of *GATA4* has been previously associated with lower percent predicted forced expiratory volume at 1 second in wood smoke–associated COPD (27). Methylation levels of genes not detected as DM in COPD, but significantly correlated with lung function and of potential interest to COPD, include *CRABP1*, a cellular retinoic acid–binding protein (41), and *ITPK1*, which has been associated with murine tracheal cell model of cystic fibrosis (42).

Given the overwhelming proportion of DM genes that were hypermethylated in COPD SAE, our results contrast with those of a study that discovered that DNA methylation patterns in blood DNA of large family-based cohorts of patients with COPD were predominantly hypomethylated (26). However, DNA methylation (and gene expression) patterns are tissue specific; therefore, discordance between these studies is expected. COPD is a systemic disease, and results from large-scale epigenomic investigations using peripheral blood DNA are indeed of clinical importance given the accessibility of blood and the potential utility of blood-based biomarkers.

DNA methylation has been explored in lung and airway cells in the context of other

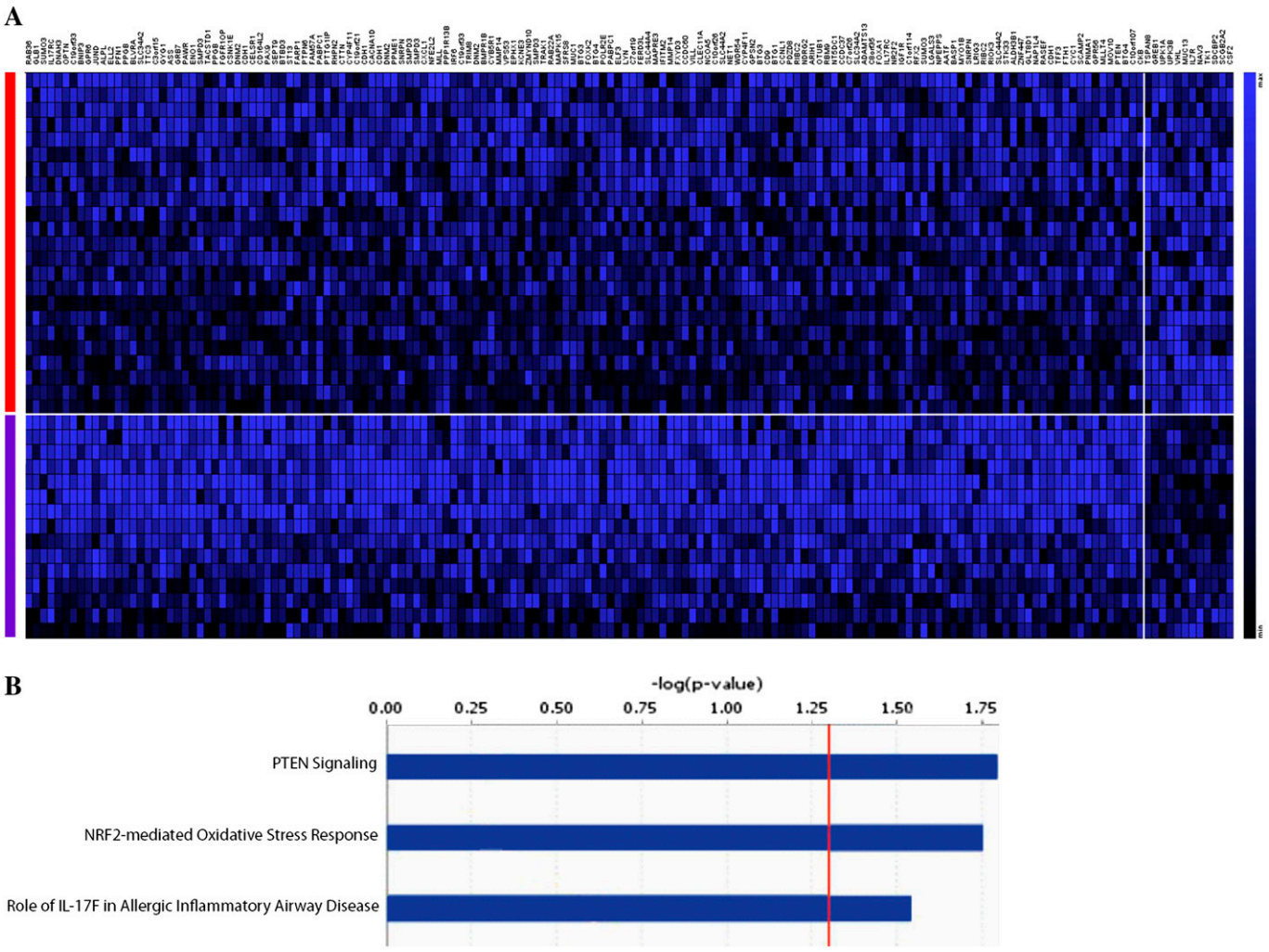

Figure 3. DM and inversely differentially expressed (DE) genes in COPD airways. (*A*) Heat map: 141 DM and inversely DE genes in COPD small airways, corresponding to 130 hypermethylated and underexpressed genes and 11 hypomethylated and overexpressed genes are depicted for 38 samples (COPD = 15, *purple bar*; non-COPD = 23, *red bar*). M values are plotted. Positive M values correspond to more (*bright blue*) and less (*black*) methylation. Genes correspond to Table E3. (*B*) Pathways enriched in DM and DE COPD airway gene set. Three pathways were significantly ($P < 0.05$) enriched in the 141 DM and DE genes. These included: phosphatase and tensin homolog (PTEN) signaling ($P = 0.016$); the NF-E2–related factor (Nrf) 2–mediated oxidative stress response pathway ($P = 0.0178$); and the IL-17F in allergic inflammatory airway diseases ($P = 0.0288$). The *horizontal axis* displays −log of the *P* value which was calculated by Fisher's exact test right tailed, representing the probability that pathways are enriched in a given gene set by random chance. A *P* value of 0.05 is indicated by the *vertical red line*.

chronic lung and airways diseases, including idiopathic pulmonary fibrosis (IPF) (43) and asthma (44). Two hypermethylated COPD genes (*GRASP* and *ABCA8*) overlapped with the 16 genes discovered by Sanders and colleagues (43) as DM and DE in IPF lung tissues, although only *ABCA8* was in the same direction. Of interest, two of our hypermethylated DM COPD genes (*CAV1* and *PTEN*) have been described elsewhere as IPF suppressor genes (45–47), as well as in COPD (48, 49), highlighting the potential importance of these genes to chronic lung disease, particularly in the context of cigarette smoke (48, 50). Our COPD SAE results did not have any overlap with six genes described by Stefanowicz and colleagues (44) as DM between atopic- and asthmatic-derived SAE, possibly reflecting the distinct biology of these diseases.

In attempting to enrich identification of COPD-specific DNA methylation alterations by only assessing FS, we were not able to assess whether our DM COPD genes may be induced by smoking. Therefore, we compared our results to those of a recent study by Buro-Auriemma and colleagues (37), who assessed methylation and gene expression differences between SAE from CS and never smokers without COPD, and found the majority of DM genes hypomethylated in smoker SAE. When we directly compared our results, five DM COPD genes overlapped with the most DM in CS SAE, although primarily in opposite directions. Interestingly, although 20% of Buro-Auriemma and colleagues' top hypomethylated genes are involved in aryl hydrocarbon receptor signaling, we found this pathway significantly and almost entirely hypermethylated in COPD SAE (Table E6). Given the importance of these genes and

Table 2: Differentially Methylated and Differentially Expressed Genes in Chronic Obstructive Pulmonary Disease Small Airways Previously Associated with Chronic Obstructive Pulmonary Disease

Symbol	Meth	Exp	Meth B-H *P* Value	Meth FC	Exp *P* Value	Exp FC
BNIP3	HYPER	UNDER	7.45E-05	1.76	4.06E-03	0.5
TTC3	HYPER	UNDER	6.73E-07	1.57	3.14E-04	0.35
SMPD3	HYPER	UNDER	1.19E-05	1.42	6.43E-09	0.61
CXCL1	HYPER	UNDER	4.80E-05	1.4	2.87E-06	0.15
EPHX1	HYPER	UNDER	3.10E-05	1.37	1.67E-03	0.21
MUC1	HYPER	UNDER	3.08E-05	1.36	5.62E-11	0.55
NFE2L2	HYPER	UNDER	1.29E-04	1.36	7.61E-07	0.26
MMP14	HYPER	UNDER	3.00E-05	1.35	2.44E-02	0.27
CD9	HYPER	UNDER	2.84E-07	1.31	3.79E-04	0.11
LGALS3	HYPER	UNDER	2.25E-04	1.29	7.64E-04	0.26
TFF3	HYPER	UNDER	9.88E-04	1.26	6.07E-03	0.24
PTEN	HYPER	UNDER	2.38E-05	1.26	2.25E-04	0.34
CKB	HYPER	UNDER	1.04E-07	1.25	4.80E-04	0.55
VHL	HYPO	OVER	4.20E-02	0.7	3.55E-03	2.63
MUC13	HYPO	OVER	3.98E-04	0.71	9.07E-10	1.18

Definition of abbreviations: B-H, Benjamini and Hochberg method; Exp, expressed; FC, fold change; Meth, methylated.

pathways to COPD and smoking response (51), these results could suggest that, in individuals without disease, hypomethylation and up-regulation of smoking-induced genes and pathways in CS SAE, such as *CYP1A1*, *GSTM1*, and genes involved in Aryl hydrocarbon receptor signaling, occurs, but abnormally. Hypermethylation of these genes may be related to smoking-induced damage associated with COPD.

We attempted to further assess the contribution of smoking to our COPD results by assessing methylation differences between the individuals with high and low pack-years from our cohort, regardless of disease status. Of our "smoking-related" genes, none overlapped with the top smoking-associated methylated genes described by Buro-Auriemma and colleagues, but 28% overlapped with our DM COPD genes, including the cholinergic receptor, *CHRNB1*; interestingly, SNPs in *CHRNB1* are associated with nicotine dependence and lung cancer (52). Genes that were not detected as DM in COPD, but detected as smoking related in our study, included *CRYGD*, a member of six gene products required for expression of two important smoking-response genes, *AHR* and *CYP1A1* (53), and *CCL26*, a negative regulator for neutrophils in COPD and the expression of which is positively associated with lung function in COPD (but negatively in asthma) (54).

In addition to assessing genome-wide DNA methylation patterns, we further sought to identify genes likely disrupted at the transcriptional level due to aberrant DNA methylation by integrating DNA methylation with gene expression changes using patient-matched DNA and RNA profiles (Table E7). We identified three pathways disrupted at both the DNA methylation and gene expression levels, which we believe are potentially important in COPD pathogenesis, namely: PTEN signaling, the Nrf2-mediated oxidative stress response, and the IL-17F inflammatory response pathways.

PTEN is the master inhibitor of the phosphoinositide 3-kinase–AKT–mammalian target of rapamycin pathway. Acquired mutations of *PTEN* are evident in airway epithelium of smokers, and *PTEN* variants have previously been associated with COPD (49, 55). Activation of the phosphoinositide 3-kinase pathway is an important therapeutic target in a wide spectrum of cancers, and is increasingly implicated in COPD (56, 57). In our study, two modulators of this pathway, *PTEN* and *CSNK2A2*, were hypermethylated and underexpressed in COPD airways, suggesting that DNA methylation may be an additional mechanism regulating this pathway in COPD airways.

The IL-17F inflammatory response pathway is also interesting in the context of COPD. Cytokines are important mediators in allergic and nonallergic inflammatory airway disease. Although overexpression of IL-17 is associated with many inflammatory diseases, its role in COPD is ambiguous, likely due to differences in biological tissue or cell type assayed (e.g., serum, lymphocytes, airway epithelial cells), because IL-17 and IL-17 receptor expression and function varies widely based on cellular context (58, 59). In COPD SAE, we found the upstream receptor in this pathway, *IL17RC*, together with the downstream *CXCL1*, hypermethylated

Table 3: Differentially Methylated and Expressed Genes Most Likely Under Epigenetic Control in Chronic Obstructive Pulmonary Disease Small Airways

Probe	Ref Seq	Rho	*P* value	Meth	Exp	Meth B-H *P* Value	Exp *P* Value	FC Meth	FC Exp	Chr	Map Info
cg03190825	CYP4F11	−0.87	2.00E-06	HYPER	UNDER	4.75E-04	9.69E-03	1.32	0.50	19	15906788
cg09081544	MUC13	−0.63	1.94E-03	HYPO	OVER	3.98E-04	9.07E-10	0.71	1.18	3	126135480
cg27644292	SNRPN	−0.50	1.88E-02	HYPER	UNDER	1.09E-07	4.37E-02	1.42	0.58	15	22674380
cg24655310	CYP4F11	−0.49	2.20E-02	HYPER	UNDER	2.53E-04	9.69E-03	1.43	0.50	19	15906119
cg24928687	EPHX1	−0.49	2.31E-02	HYPER	UNDER	3.10E-05	1.67E-03	1.37	0.21	1	224079536
cg17571291	BLVRA	−0.48	2.61E-02	HYPER	UNDER	1.77E-06	7.94E-03	1.60	0.49	7	43764312
cg16173067	SDCBP2	−0.45	3.59E-02	HYPO	OVER	1.64E-03	7.27E-05	0.72	6.72	20	1257722
cg03242880	BTG4	−0.45	3.80E-02	HYPER	UNDER	2.04E-06	4.39E-02	1.25	0.72	11	110888125

Definition of abbreviations: B-H, Benjamini and Hochberg method; Chr, chromosome; Exp, expressed; FC, fold change; Meth, methylated; Ref Seq, NCBI Reference Sequence Database.

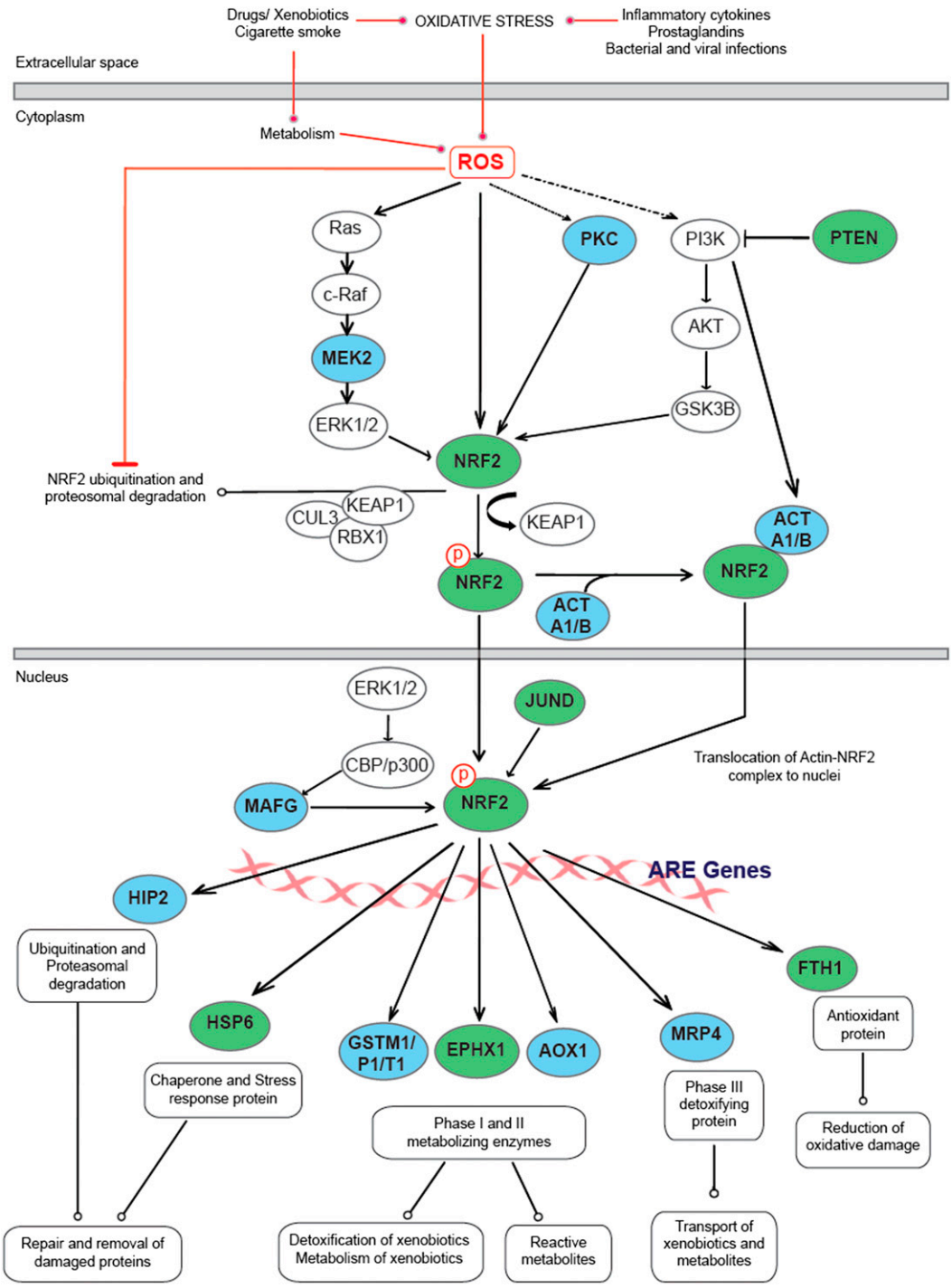

Figure 4. The Nrf2-mediated oxidative stress response pathway is altered at multiple levels by DNA methylation in COPD airways. Increased cellular levels of reactive oxidative species (ROS), which are produced from multiple sources, inhibit Kelch-like ECH-associated protein 1 (KEAP1)/Cullin 3 (CUL3)/ring-box 1, E3 ubiquitin protein ligase (RBX1)-mediated NRF2 ubiquitination and proteosomal degradation, allowing NRF2 nuclear translocation. Antioxidant response element (ARE) genes, which are transcriptionally activated by NRF2, mediate a multitude of processes involved in cellular protection from ROS damage. Compared with small airways from individuals with normal lung function, genes in the Nrf2 pathway are differentially altered at the level of DNA methylation and gene expression at multiple up- and downstream points in COPD small airways. Genes hypermethylated in COPD airways are colored *light blue*; genes hypermethylated and concomitantly underexpressed in COPD airways are colored *green*. An impaired Nrf2 response can result in increased damage from ROS.

and underexpressed, whereas the proinflammatory *CSF2* was hypomethylated and overexpressed.

Increased oxidative stress and generation of free radicals, such as that which occurs in response to cigarette smoke exposure, affect nearly all aspects of COPD pathology. The Nrf2 pathway is the major cellular defense system against oxidative

stress, mediated through NRF2 nuclear translocation and activation of antioxidant response element (ARE) genes. The Nrf2 pathway is normally up-regulated in airways of healthy smokers, but in smokers with severe COPD, expression of key modulators and downstream ARE genes are underexpressed, resulting in impaired cellular defense mechanisms and increased oxidative damage in airway and lung tissues (15, 17, 60, 61). At the DNA level, multiple GWAS have identified functional SNPs in promoters of key genes within this pathway, including downstream ARE and genes associated with xenobiotic metabolism, to be associated with increased COPD and lung cancer risk (62).

Our data strongly suggest that the Nrf2 pathway sustains multiple levels of epigenetic disruption. We detected multiple upstream Nrf2 regulators (*PKC*, *MEK1*, *Actin*, *PTEN*, *NRF2*) and downstream effector molecules (*MAF*, *MRP4*, *GST*, *HIP2*, *HSP6*, *EPHX1*, *FTH1*) differentially affected at the level of DNA methylation alone, or by both DNA methylation and gene expression in COPD small airways (Figure 4). Two genes in this pathway, *EPHX1* and *CYP4F11*, were among the most negatively correlated DM and DE genes overall, strongly suggesting that, in COPD airways, reduced expression of *EPHX1* and *CYP4F11* is likely modulated epigenetically by DNA methylation. *EPHX1* functions in the biotransformation of epoxides resulting from degradation of aromatic compounds, such as those found in cigarette smoke. Underexpression of *EPHX1* is frequently described in COPD airway and lung tissues, and "slow" *EPHX1* SNPs are associated with impaired enzyme activity and increased COPD risk, and "fast" SNPs potentially confer a protective effect (40). Cytochrome P450 4F enzymes, such as *CYP4F11*, are involved in cellular protection, xenobiotic metabolism, detoxification, lipid synthesis, and metabolic activation of drugs, including those used to treat chronic inflammatory disease (63, 64). They also have a direct role in inhibiting inflammation through suppression of leukotriene and prostaglandin signals (64). *CYP4F11* contains both c-Jun N-terminal kinase (JNK)/activator protein 1 and hormone response element binding domains; it is positively regulated by retinoid X receptors and JNK (through TNF-α activation), and is negatively regulated by retinoic acid receptors. However, regulation of CYP4F11 in an environment of chronic inflammation is complex. In human epidermal keratinocytes, although TNF-α leads to immediate activation of *CYP4F11* through JNK, subsequent activation of NF-κβ results in direct inhibition of *CYP4F11* (63, 64). *CYP4F11* regulators are clearly important to COPD biology, although little is known about the function of this enzyme in respiratory tissues. Given the known functions of CYP4F11, it is possible that epigenetic silencing of this enzyme in small airways of patients with COPD may lead to impaired cellular protective responses, increased inflammation, or altered activation of inhaled steroids.

The study and application of antioxidant inflammation modulators to target the Nrf2 pathway is a growing field of study, particularly relevant for COPD therapeutics (65). Interestingly, overactivation of this pathway through mutation of its key inhibitor, *KEAP1*, is a frequent event in lung squamous cell carcinoma (66). Given that squamous cell carcinoma is more frequent in individuals with COPD (67), elucidating the role this pathway plays in promoting inflammation and tumorigenesis may be critical to the rational application of antioxidant inflammation modulator therapy to patients with COPD.

Given that our study is limited by sample size, further investigation of aberrant methylation in small airways across a larger cohort of subjects for which COPD phenotypes are defined by both computed tomography and symptoms, in addition to lung function, is warranted. Moreover, it is possible that populations of cells obtained from small airways of patients with COPD may contain more inflammatory cells than those from individuals without COPD, and should therefore be considered in the interpretation of gene expression and methylation comparisons. Our results provide rationale for further assessment of the involvement of DNA methylation to COPD biology that ideally considers functional elements beyond gene promoters (as is now feasible with newer platforms). Because DNA sequence variants can affect normal methylation patterns affecting gene expression (68), the integration of information from COPD GWAS results with methylation and expression data may help elucidate those genes and mechanisms contributing to COPD biology.

Our findings suggest that, in small airways of patients with COPD, aberrant DNA methylation is a genome-wide phenomenon affecting hundreds of genes and several pathways important to smoking response and COPD biology. As DNA methylation is a reversible gene regulatory modification, further work in this area may contribute to the development of novel treatment strategies or the reappropriation of existing epigenetic-based drugs to the treatment or prevention of COPD. ■

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References

1. Murray CJ, Lopez AD. Global mortality, disability, and the contribution of risk factors: global burden of disease study. *Lancet* 1997;349: 1436–1442.
2. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med* 2006;3:e442.
3. Sin DD, McAlister FA, Man SF, Anthonisen NR. Contemporary management of chronic obstructive pulmonary disease: scientific review. *JAMA* 2003;290:2301–2312.
4. McDonough JE, Yuan R, Suzuki M, Seyednejad N, Elliott WM, Sanchez PG, Wright AC, Gefter WB, Litzky L, Coxson HO, *et al*. Small-airway obstruction and emphysema in chronic obstructive pulmonary disease. *N Engl J Med* 2011;365: 1567–1575.
5. Cho MH, Castaldi PJ, Wan ES, Siedlinski M, Hersh CP, Demeo DL, Himes BE, Sylvia JS, Klanderman BJ, Ziniti JP, *et al*. A genome-wide association study of COPD identifies a susceptibility locus on chromosome 19q13. *Hum Mol Genet* 2012;21:947–957.

6. Artigas MS, Loth DW, Wain LV, Gharib SA, Obeidat M, Tang W, Zhai G, Zhao JH, Smith AV, Huffman JE, *et al*. Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function. *Nat Genet* 2011;43:1082–1090.
7. Kong X, Cho MH, Anderson W, Coxson HO, Muller N, Washko G, Hoffman EA, Bakke P, Gulsvik A, Lomas DA, *et al*. Genome-wide association study identifies BICD1 as a susceptibility gene for emphysema. *Am J Respir Crit Care Med* 2011;183:43–49.
8. Repapi E, Sayers I, Wain LV, Burton PR, Johnson T, Obeidat M, Zhao JH, Ramasamy A, Zhai G, Vitart V, *et al*. Genome-wide association study identifies five loci associated with lung function. *Nat Genet* 2010;42:36–44.
9. Campbell JD, McDonough JE, Zeskind JE, Hackett TL, Pechkovsky DV, Brandsma CA, Suzuki M, Gosselink JV, Liu G, Alekseyev YO, *et al*. A gene expression signature of emphysema-related lung destruction and its reversal by the tripeptide GHK. *Genome Med* 2012;4:67.
10. Wang IM, Stepaniants S, Boie Y, Mortimer JR, Kennedy B, Elliott M, Hayashi S, Loy L, Coulter S, Cervino S, *et al*. Gene expression profiling in patients with chronic obstructive pulmonary disease and lung cancer. *Am J Respir Crit Care Med* 2008;177:402–411.
11. Ning W, Li CJ, Kaminski N, Feghali-Bostwick CA, Alber SM, Di YP, Otterbein SL, Song R, Hayashi S, Zhou Z, *et al*. Comprehensive gene expression profiles reveal pathways related to the pathogenesis of chronic obstructive pulmonary disease. *Proc Natl Acad Sci USA* 2004;101:14895–14900.
12. Golpon HA, Coldren CD, Zamora MR, Cosgrove GP, Moore MD, Tuder RM, Geraci MW, Voelkel NF. Emphysema lung tissue gene expression profiling. *Am J Respir Cell Mol Biol* 2004;31: 595–600.
13. Spira A, Beane J, Pinto-Plata V, Kadar A, Liu G, Shah V, Celli B, Brody JS. Gene expression profiling of human lung tissue from smokers with severe emphysema. *Am J Respir Cell Mol Biol* 2004;31: 601–610.
14. Steiling K, van den Berge M, Hijazi K, Florido R, Campbell J, Liu G, Xiao J, Zhang X, Duclos G, Drizik E, *et al*. A dynamic bronchial airway gene expression signature of COPD and lung function impairment. *Am J Respir Crit Care Med* 2013;187:933–942.
15. Tilley AE, O'Connor TP, Hackett NR, Strulovici-Barel Y, Salit J, Amoroso N, Zhou XK, Raman T, Omberg L, Clark A, *et al*. Biologic phenotyping of the human small airway epithelial response to cigarette smoking. *PLoS ONE* 2011;6:e22798.
16. Ammous Z, Hackett NR, Butler MW, Raman T, Dolgalev I, O'Connor TP, Harvey BG, Crystal RG. Variability in small airway epithelial gene expression among normal smokers. *Chest* 2008;133:1344–1353.
17. Pierrou S, Broberg P, O'Donnell RA, Pawlowski K, Virtala R, Lindqvist E, Richter A, Wilson SJ, Angco G, Moller S, *et al*. Expression of genes involved in oxidative stress responses in airway epithelial cells of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2007;175:577–586.
18. Qiu W, Cho MH, Riley JH, Anderson WH, Singh D, Bakke P, Gulsvik A, Litonjua AA, Lomas DA, Crapo JD, *et al*. Genetics of sputum gene expression in chronic obstructive pulmonary disease. *PLoS One* 2011;6:e24395.
19. Bowler RP, Bahr TM, Hughes G, Lutz S, Kim YI, Coldren CD, Reisdorph N, Kechris KJ. Integrative omics approach identifies interleukin-16 as a biomarker of emphysema. *OMICS* 2013;17:619–626.
20. Obeidat M, Miller S, Probert K, Billington CK, Henry AP, Hodge E, Nelson CP, Stewart CE, Swan C, Wain LV, *et al*. GSTCD and INTS12 regulation and expression in the human lung. *PLoS ONE* 2013;8: e74630.
21. Lamontagne M, Couture C, Postma DS, Timens W, Sin DD, Pare PD, Hogg JC, Nickle D, Laviolette M, Bosse Y. Refining susceptibility loci of chronic obstructive pulmonary disease with lung eQTLs. *PLoS ONE* 2013;8:e70220.
22. Teperino R, Lempradl A, Pospisilik JA. Bridging epigenomics and complex disease: the basics. *Cell Mol Life Sci* 2013;70:1609–1621.
23. Nebbioso A, Carafa V, Benedetti R, Altucci L. Trials with 'epigenetic' drugs: an update. *Mol Oncol* 2012;6:657–682.
24. Kabesch M, Adcock IM. Epigenetics in asthma and COPD. *Biochimie* 2012;94:2231–2241.
25. Wan ES, Qiu W, Baccarelli A, Carey VJ, Bacherman H, Rennard SI, Agusti A, Anderson WH, Lomas DA, Demeo DL. Systemic steroid exposure is associated with differential methylation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2012;186:1248–1255.
26. Qiu W, Baccarelli A, Carey VJ, Boutaoui N, Bacherman H, Klanderman B, Rennard S, Agusti A, Anderson W, Lomas DA, *et al*. Variable DNA methylation is associated with chronic obstructive pulmonary disease and lung function. *Am J Respir Crit Care Med* 2012;185: 373–381.
27. Sood A, Petersen H, Blanchette CM, Meek P, Picchi MA, Belinsky SA, Tesfaigzi Y. Wood smoke exposure and gene promoter methylation are associated with increased risk for COPD in smokers. *Am J Respir Crit Care Med* 2010;182:1098–1104.
28. Cotton AM, Avila L, Penaherrera MS, Affleck JG, Robinson WP, Brown CJ. Inactive X chromosome–specific reduction in placental DNA methylation. *Hum Mol Genet* 2009;18:3544–3552.
29. Du P, Zhang X, Huang CC, Jafari N, Kibbe WA, Hou L, Lin SM. Comparison of beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinformatics* 2010;11:587.
30. Zhuang J, Widschwendter M, Teschendorff AE. A comparison of feature selection and classification methods in DNA methylation studies using the Illumina Infinium platform. *BMC Bioinformatics* 2012;13:59.
31. Hackett NR, Butler MW, Shaykhiev R, Salit J, Omberg L, Rodriguez-Flores JL, Mezey JG, Strulovici-Barel Y, Wang G, Didon L, *et al*. RNA-Seq quantification of the human small airway epithelium transcriptome. *BMC Genomics* 2012;13:82.
32. Bosse Y, Postma DS, Sin DD, Lamontagne M, Couture C, Gaudreault N, Joubert P, Wong V, Elliott M, van den Berge M, *et al*. Molecular signature of smoking in human lung tissues. *Cancer Res* 2012;72: 3753–3763.
33. Zhang Y, Li X, Carpinteiro A, Goettel JA, Soddemann M, Gulbins E. Kinase suppressor of Ras-1 protects against pulmonary pseudomonas aeruginosa infections. *Nat Med* 2011;17:341–346.
34. Chwieralski CE, Schnurra I, Thim L, Hoffmann W. Epidermal growth factor and trefoil factor family 2 synergistically trigger chemotaxis on BEAS-2B cells via different signaling cascades. *Am J Respir Cell Mol Biol* 2004;31:528–537.
35. Hara H, Araya J, Takasaka N, Fujii S, Kojima J, Yumino Y, Shimizu K, Ishikawa T, Numata T, Kawaishi M, *et al*. Involvement of creatine kinase b in cigarette smoke–induced bronchial epithelial cell senescence. *Am J Respir Cell Mol Biol* 2012;46:306–312.
36. Sood A, Petersen H, Blanchette CM, Meek P, Picchi MA, Belinsky SA, Tesfaigzi Y. Methylated genes in sputum among older smokers with asthma. *Chest* 2012;2:425–431.
37. Buro-Auriemma LJ, Salit J, Hackett NR, Walters MS, Strulovici-Barel Y, Staudt MR, Fuller J, Stevenson CS, Hilton H, Ho MW, *et al*. Cigarette smoking induces small airway epithelial epigenetic changes with corresponding modulation of gene expression. *Hum Mol Genet* 2013;22:4726–4738.
38. Allen-Gipson DS, Zimmerman MC, Zhang H, Castellanos G, O'Malley JK, Alvarez-Ramirez H, Kharbanda K, Sisson JH, Wyatt TA. Smoke extract impairs adenosine wound healing: implications of smoke-generated reactive oxygen species. *Am J Respir Cell Mol Biol* 2013; 48:665–673.
39. Fujimoto J, Kadara H, Garcia MM, Kabbout M, Behrens C, Liu DD, Lee JJ, Solis LM, Kim ES, Kalhor N, Moran C, Sharafkhaneh A, Lotan R, Wistuba, II. G-protein coupled receptor family C, group 5, member A (GPRC5A) expression is decreased in the adjacent field and normal bronchial epithelia of patients with chronic obstructive pulmonary disease and non–small-cell lung cancer. *J Thorac Oncol* 2012;7: 1747–1754.
40. Smolonska J, Wijmenga C, Postma DS, Boezen HM. Meta-analyses on suspected chronic obstructive pulmonary disease genes: a summary of 20 years' research. *Am J Respir Crit Care Med* 2009;180:618–631.
41. Plantier L, Rochette-Egly C, Goven D, Boutten A, Bonay M, Leseche G, Fournier M, Crestani B, Boczkowski J. Dysregulation of elastin expression by fibroblasts in pulmonary emphysema: role of cellular retinoic acid binding protein 2. *Thorax* 2008;63:1012–1017.
42. Yang L, Reece J, Gabriel SE, Shears SB. Apical localization of ITPK1 enhances its ability to be a modifier gene product in a murine tracheal cell model of cystic fibrosis. *J Cell Sci* 2006;119:1320–1328.

43. Sanders YY, Ambalavanan N, Halloran B, Zhang X, Liu H, Crossman DK, Bray M, Zhang K, Thannickal VJ, Hagood JS. Altered DNA methylation profile in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2012;186:525–535.
44. Stefanowicz D, Hackett TL, Garmaroudi FS, Gunther OP, Neumann S, Sutanto EN, Ling KM, Kobor MS, Kicic A, Stick SM, *et al*. DNA methylation profiles of airway epithelial cells and PBMCs from healthy, atopic and asthmatic children. *PLoS One* 2012;7:e44213.
45. Wang XM, Zhang Y, Kim HP, Zhou Z, Feghali-Bostwick CA, Liu F, Ifedigbo E, Xu X, Oury TD, Kaminski N, *et al*. Caveolin-1: a critical regulator of lung fibrosis in idiopathic pulmonary fibrosis. *J Exp Med* 2006;203:2895–2906.
46. Miyoshi K, Yanagi S, Kawahara K, Nishio M, Tsubouchi H, Imazu Y, Koshida R, Matsumoto N, Taguchi A, Yamashita S, *et al*. Epithelial PTEN controls acute lung injury and fibrosis by regulating alveolar epithelial cell integrity. *Am J Respir Crit Care Med* 2013;187: 262–275.
47. Xia H, Khalil W, Kahm J, Jessurun J, Kleidon J, Henke CA. Pathologic caveolin-1 regulation of PTEN in idiopathic pulmonary fibrosis. *Am J Pathol* 2010;176:2626–2637.
48. Ryter SW, Lam HC, Chen ZH, Choi AM. Deadly triplex: smoke, autophagy and apoptosis. *Autophagy* 2011;7:436–437.
49. Hosgood HD III, Menashe I, He X, Chanock S, Lan Q. PTEN identified as important risk factor of chronic obstructive pulmonary disease. *Respir Med* 2009;103:1866–1870.
50. Shaykhiev R, Otaki F, Bonsu P, Dang DT, Teater M, Strulovici-Barel Y, Salit J, Harvey BG, Crystal RG. Cigarette smoking reprograms apical junctional complex molecular architecture in the human airway epithelium *in vivo*. *Cell Mol Life Sci* 2011;68:877–892.
51. Lakhdar R, Denden S, Knani J, Leban N, Daimi H, Hassine M, Lefranc G, Chibani JB, Khelil AH. Combined analysis of EPHX1, GSTP1, GSTM1 and GSTT1 gene polymorphisms in relation to chronic obstructive pulmonary disease risk and lung function impairment. *Dis Markers* 2011;30:253–263.
52. Saccone NL, Saccone SF, Hinrichs AL, Stitzel JA, Duan W, Pergadia ML, Agrawal A, Breslau N, Grucza RA, Hatsukami D, *et al*. Multiple distinct risk loci for nicotine dependence identified by dense coverage of the complete family of nicotinic receptor subunit (CHRN) genes. *Am J Med Genet B Neuropsychiatr Genet* 2009;150B: 453–466.
53. Solaimani P, Damoiseaux R, Hankinson O. Genome wide RNAi high throughput screen identifies proteins necessary for the AHR-dependent induction of CYP1a1 by 2,3,7,8-tetrachlorodibenzo-Rho-dioxin. *Toxicol Sci* 2013;136:107–119.
54. Paplinska M, Hermanowicz-Salamon J, Nejman-Gryz P, Bialek-Gosk K, Rubinsztajn R, Arcimowicz M, Placha G, Gora J, Chazan R, Grubek-Jaworska H. Expression of eotaxins in the material from nasal brushing in asthma, allergic rhinitis and COPD patients. *Cytokine* 2012;60:393–399.
55. Anderson GP, Bozinovski S. Acquired somatic mutations in the molecular pathogenesis of copd. *Trends Pharmacol Sci* 2003;24:71–76.
56. Wong KK, Engelman JA, Cantley LC. Targeting the PI3K signaling pathway in cancer. *Curr Opin Genet Dev* 2010;20:87–90.
57. Bozinovski S, Vlahos R, Hansen M, Liu K, Anderson GP. Akt in the pathogenesis of COPD. *Int J Chron Obstruct Pulmon Dis* 2006;1:31–38.
58. Zhang X, Zheng H, Zhang H, Ma W, Wang F, Liu C, He S. Increased interleukin (IL)-8 and decreased IL-17 production in chronic obstructive pulmonary disease (COPD) provoked by cigarette smoke. *Cytokine* 2011;56:717–725.
59. Kramer JM, Gaffen SL. Interleukin-17: a new paradigm in inflammation, autoimmunity, and therapy. *J Periodontol* 2007;78:1083–1093.
60. Hubner RH, Schwartz JD, De Bishnu P, Ferris B, Omberg L, Mezey JG, Hackett NR, Crystal RG. Coordinate control of expression of Nrf2-modulated genes in the human small airway epithelium is highly responsive to cigarette smoking. *Mol Med* 2009;15: 203–219.
61. Tomaki M, Sugiura H, Koarai A, Komaki Y, Akita T, Matsumoto T, Nakanishi A, Ogawa H, Hattori T, Ichinose M. Decreased expression of antioxidant enzymes and increased expression of chemokines in COPD lung. *Pulm Pharmacol Ther* 2007;20:596–605.
62. Cho HY, Kleeberger SR. Nrf2 protects against airway disorders. *Toxicol Appl Pharmacol* 2010;244:43–56.
63. Bell JC, Strobel HW. Regulation of cytochrome p450 4f11 by nuclear transcription factor-kappaB. *Drug Metab Dispos* 2012;40:205–211.
64. Wang Y, Bell JC, Keeney DS, Strobel HW. Gene regulation of CYP4f11 in human keratinocyte hacat cells. *Drug Metab Dispos* 2010;38: 100–107.
65. Rahman I. Pharmacological antioxidant strategies as therapeutic interventions for COPD. *Biochim Biophys Acta* 2011;1822:714–728.
66. Hammerman PS, Hayes DN, Wilkerson MD, Schultz N, Bose R, Chu A, Collisson EA, Cope L, Creighton CJ, Getz G, *et al*. Comprehensive genomic characterization of squamous cell lung cancers. *Nature* 2012;489:519–525.
67. Lau A, Villeneuve NF, Sun Z, Wong PK, Zhang DD. Dual roles of Nrf2 in cancer. *Pharmacol Res* 2008;58:262–270.
68. Aran D, Hellman A. DNA methylation of transcriptional enhancers and cancer predisposition. *Cell* 2013;154:11–13.