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Particulate matter Air Pollution induces hypermethylation of the p16 promoter Via a mitochondrial ROS-JNK-DNMT1 pathway

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Exposure of human populations to chronically elevated levels of ambient particulate matter air pollution < 2.5 µm in diameter (PM_{2.5}) has been associated with an increase in lung cancer incidence. Over 70% of lung cancer cell lines exhibit promoter methylation of the tumor suppressor p16, an epigenetic modification that reduces its expression. We exposed mice to concentrated ambient PM_{2.5} via inhalation, 8 hours daily for 3 weeks and exposed primary murine alveolar epithelial cells to daily doses of fine urban PM (5 µg/cm³). In both mice and alveolar epithelial cells, PM exposure increased ROS production, expression of the DNA methyltransferase 1 (DNMT1), and methylation of the p16 promoter. In alveolar epithelial cells, increased transcription of DNMT1 and methylation of the p16 promoter were inhibited by a mitochondrially targeted antioxidant and a JNK inhibitor. These findings provide a potential mechanism by which PM exposure increases the risk of lung cancer.

Each year, more than 160,000 people in the US and 1.4 million people worldwide die from lung cancer, which is the leading cause of cancer related death¹. Exposure to cigarette smoke remains the most important cause of lung cancer, however, approximately 15% of lung cancers occur in never-smokers and lung cancer in non-smokers as a separate entity remains a leading cause of cancer mortality^{2,3}. Epidemiologists studying the link between exposure to particulate matter air pollution (PM) and lung cancer have consistently observed a positive association³⁻⁵. In one study, Pope et al. reported that for every 10 µg/cm³ elevation in PM_{2.5} concentration there was an approximately 8% increased risk of lung cancer related mortality⁵.

Lung cancer is associated with several characteristic epigenetic changes; one of the most common is the methylation of the promoter for the tumor suppressor p16, which has been reported in >70% cell lines derived human non-small cell lung cancers^{6,7}. Methylation of the p16 promoter is thought to play a critical role in lung cancer development by allowing the uncontrolled clonal expansion of premalignant lesions to cancer^{8,9}. In sputum or cellular samples from smokers without lung cancer, smokers without malignancy, never smokers and lung cancer survivors, Belinsky and colleagues have identified hypermethylation of CpG islands in the promoter of p16 as an early event in the development of lung cancer, particularly in patients with a history of exposure to cigarette smoke⁸. Methylation of the p16 promoter is frequently associated with widespread changes in the methylation of other genes suggesting that promoter methylation is regulated by a common upstream pathway¹⁰.

DNA methylation in mammalian cells is catalyzed by members of the (cytosine-5)-DNA methyltransferase (DNMT) family. DNMT1 is thought to play a major role in the changes in DNA methylation observed in human cancer cells¹¹ and an increase in DNMT1 abundance has been linked to cigarette smoke exposure induced lung carcinogenesis in mice and humans¹². The c-jun-n-terminal protein kinase (JNK), a member of the mitogen activated protein kinase family is induced by oncogenes frequently observed in human lung cancers and up-regulates the transcription of DNMT¹³⁻¹⁵. As we have previously found that exposure to PM induces apoptosis in alveolar epithelial cells through the mitochondrial oxidant-dependent activation of JNK^{16,17}, we hypothesized that

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the PM induced activation of JNK might enhance DNMT1 transcription and p16 promoter methylation via a similar pathway.

RESULTS

Exposure to concentrated ambient PM_{2.5} results in methylation of the p16 promoter in the lungs of mice. We exposed mice to concentrated ambient PM_{2.5} or filtered air 8 hours daily, 5 days per week 3, 6 or 9 weeks (Supplementary Figure S1) after which we harvested the lungs for isolation of whole lung genomic DNA and measured methylation of the promoter for p16. Mean particle concentrations in the PM_{2.5} and filtered air chambers (measured daily at the beginning of the exposure) were 5.5×10^5 and 6.47×10^2 particles/cm³ respectively (Figure 1A). During the exposure, daily PM_{2.5} concentrations reported from a nearby Environmental Protection Agency Monitor averaged $11.55 \mu\text{g}/\text{m}^3$. We observed a similar increase in methylation of the p16 promoter in mice exposed to concentrated ambient PM_{2.5} at all three time points (Figure 1B, combined data in Figure 1C). We observed a similar increase in methylation of the promoter for the matrix metalloproteinase-2 (MMP2) gene (Figure 1C). Promoter methylation of the p16 and MMP promoters, along with those of 4 other genes in the sputum of a high risk smoking cohort was shown to increase the risk for developing lung cancer¹⁸.

Exposure to PM causes a dose-dependent increase in mitochondrial Reactive Oxygen Species generation and cell death in primary alveolar epithelial cells from mice. We and others have reported an increase on cellular ROS production after PM exposure in alveolar epithelial cells including primary human alveolar type II cells^{16,17}. We isolated primary alveolar type II cells from mice and cultured them at air-liquid interfaces. Because these cells differentiate in culture, we refer to them hereafter as alveolar epithelial cells. After 48 hours in culture, we infected them with an adenovirus encoding a mitochondrially localized oxidant sensitive probe (mito-Ro-GFP). Twenty-four hours after infection, we pretreated the cells with a mitochondrially targeted antioxidant (Mito-CP, 50 μM) 30 minutes prior to treatment with increasing doses of PM and measured oxidation of the probe 4 hours later using flow cytometry (Figure 2A). To confirm our previous finding that mitochondrially generated ROS are required for PM induced cell death in alveolar epithelial cells^{16,17}, we treated uninfected cells with increasing doses of PM in the presence or absence of Mito-CP (50 μM) and measured cell death 24 hours later. Cell death in response to high dose PM (50 $\mu\text{g}/\text{cm}^2$) was prevented by treatment with Mito-CP, however, at a dose of 5 $\mu\text{g}/\text{cm}^2$, PM did not cause significant cell death even in untreated cells (Figure 2B). To determine the lowest dose of Mito-CP that would scavenge mitochondrial ROS generated in response to this nonlethal dose of PM, we measured mito-Ro-GFP oxidation in cells treated with PM (5 $\mu\text{g}/\text{cm}^2$) in the presence of increasing doses of Mito-CP. Mito-CP at a dose of 5 μM prevented oxidation of the mitochondrially localized probe, while the control cation used to deliver Mito-CP to the mitochondria, TPP, had no effect even at a dose of 50 μM (Figure 2C).

Long-term exposure to low dose PM induces methylation of the p16 promoter in primary murine alveolar epithelial cells. We treated primary murine alveolar epithelial cells daily with Mito-CP (5 μM) followed 30 minutes later by PM (5 $\mu\text{g}/\text{cm}^2$). After 10 days of treatment, we isolated the DNA from the cells for measurement p16 promoter methylation. Long-term treatment with PM was associated with methylation of the p16 promoter, which was prevented by daily treatment with Mito-CP but not the control cation TPP (Figure 3). PM-induced methylation of the p16 promoter was also prevented by daily treatment with the DNA-methyltransferase inhibitor 5-azacytidine (5-aza) (1 μM) 30 minutes before PM administration (Figure 3).

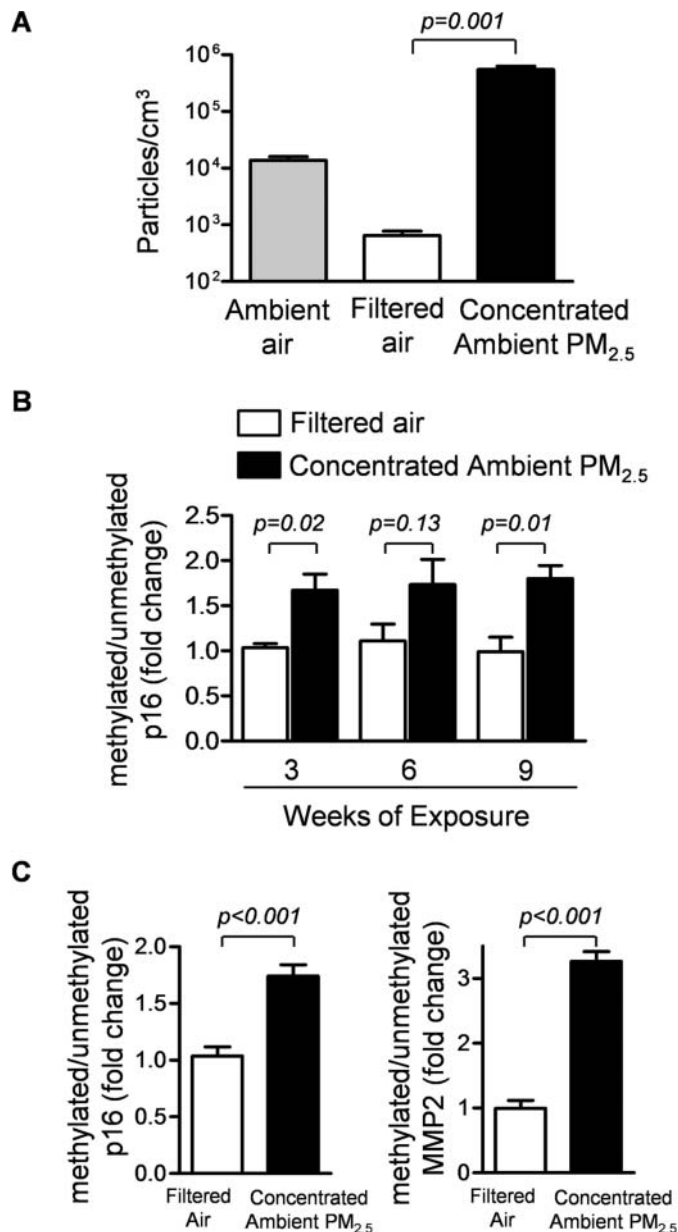


Figure 1 | Inhalation of concentrated ambient PM_{2.5} results in hypermethylation of the p16 promoter in the lungs of mice. A VACES system was used to generate concentrated ambient PM_{2.5}, which was delivered to two identical murine exposure chambers, one of which was equipped with a teflon filter at the chamber inlet, the other of which was not. (A) Mean particle concentrations over the duration of the exposure in the ambient air (inlet to the VACES), the inlet to the concentrated ambient PM_{2.5} chamber and the inlet to the filtered air chamber distal to the filter. (B) Mice were placed in either the concentrated ambient PM_{2.5} or filtered air chamber 8 hours daily, five days per week. At the indicated times the ratio of methylated/unmethylated p16 was measured in DNA isolated from whole methylated lung homogenates using methylation-specific PCR. Each bar represents 3–4 animals exposed to concentrated ambient PM_{2.5} or filtered air. (C) Fold change ratios in the methylated/unmethylated p16 and MMP2 genes of the 10 animals exposed to concentrated ambient PM_{2.5} or filtered air. P values (unpaired two tailed t-tests) are indicated in italics above the bars.

Exposure to PM is associated with mitochondrial-oxidant and JNK-dependent transcription and expression of DNMT1. Promoter methylation in mice and humans is catalyzed by three DNA methyltransferases, DNMT1, 3a and 3b. We measured the levels of mRNA encoding these three proteins in alveolar epithelial cells treated

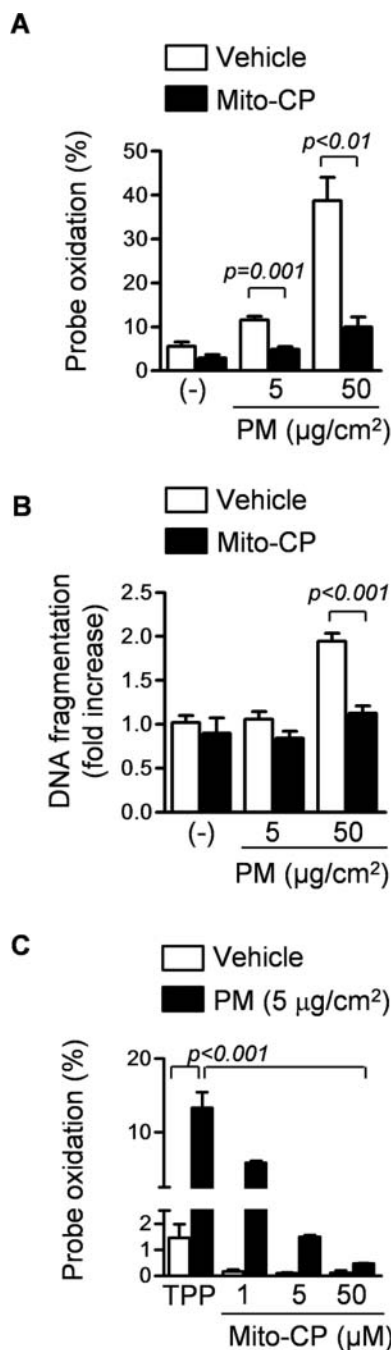


Figure 2 | PM exposure induces mitochondrial ROS production and apoptosis in cultured mouse lung epithelial cells. (A) Primary murine alveolar epithelial cells were infected with an adenovirus (5 pfu/cell) encoding a mitochondrially-localized-oxidant-sensitive GFP probe (mito-Ro-GFP) and 48 hours later were pretreated Mito-CP (50 μM) or vehicle (DMSO) followed 30 minutes later by PBS or PM (5 or 50 $\mu\text{g}/\text{cm}^2$) and oxidation of the probe was measured using flow cytometry 4 hours later. (B) Primary alveolar epithelial cells were pretreated with Mito-CP (5 μM) or the control cation 30 minutes before treatment with PM (5 or 50 $\mu\text{g}/\text{cm}^2$) and cell death was measured 24 hours later using an ELISA that detects DNA fragmentation. (C) Cells were infected with the Mito-Ro-GFP probe as in (A) and 48 hours later were pretreated with increasing doses of Mito-CP or the control cation TPP 30 minutes before exposure to PM (5 $\mu\text{g}/\text{cm}^2$) and oxidation of the probe was measured 4 hours later. $N \geq 3$, P values (unpaired two tailed t-tests) are indicated in italics above the bars.

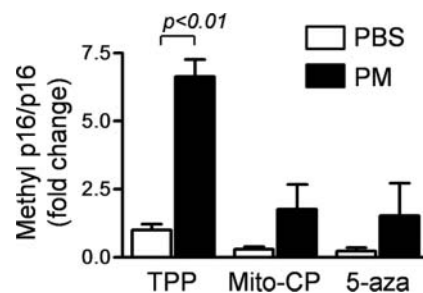


Figure 3 | Exposure of primary murine alveolar epithelial cells to repeated doses of PM results in mitochondrial-oxidant-dependent methylation of the p16 promoter. Primary mouse alveolar epithelial cells were cultured at an air-liquid interface. After 2 days in culture, the cells were treated daily with Mito-CP (5 μM), 5-azacytidine (5-aza) (1 μM) or the cation control TPP (5 μM) followed 30 minutes later by PM (5 $\mu\text{g}/\text{cm}^2$ /day). After 10 days of treatment, DNA was isolated from the cells and methylation of the p16 promoter was measured using methylation specific PCR. $N=4$ for all measures, P values (ANOVA with Bonferroni correction for multiple comparisons) are indicated in italics above the bars.

daily for 10 days with PM (5 $\mu\text{g}/\text{cm}^2$). Treatment with PM increased the level of mRNA encoding DNMT1 but had no effect on the levels of DNMT3a and DNMT3b (Figure 4A). Treatment with PM also increased the levels of DNMT1 protein in whole cell lysates (Figure 4B). The daily administration of Mito-CP (5 μM) 30 minutes before PM treatment prevented the PM-induced increase in DNMT1 transcription and abundance (Figure 4A,B, TPP (5 μM) as control).

Exposure to PM is associated with mitochondrial oxidant-mediated activation of JNK, which is required for methylation of the p16 promoter. We exposed primary murine alveolar epithelial cells to PM and measured JNK activation 30 minutes after exposure using an antibody that recognizes phosphorylated and total JNK. Treatment with PM increased the phosphorylation and kinase activity of JNK (Figure 5A,B). Pretreatment of the cells with the JNK inhibitor SP600125 (20 μM) or Mito-CP (5 μM) prevented the PM-induced activation of JNK (Figure 5A,B). Treatment with the control cation (TPP) had no effect on JNK activation. We then measured DNMT1 mRNA expression and p16 methylation in primary murine alveolar epithelial cells treated daily with SP600125 (20 μM) followed 30 minutes later by PM. Inhibition of JNK with SP600125 prevented the PM-induced increase in expression of DNMT1 protein (figure 5C) and p16 methylation in these cells (Figure 5D). Consistent with our findings *in vivo*, inhibition of either mitochondrial ROS with mito-CP or JNK with SP600125 also prevented the PM-induced methylation of the MPM2 promoter (Figure 5E, TPP (5 μM) as control for Mito-CP). Treatment with an inhibitor of the extracellular-signal related kinase pathway U0126 did not affect the increase in DNMT1 protein abundance or p16 promoter methylation (Supplementary Figure S2).

Exposure of mice to concentrated ambient PM_{2.5} increases lung oxidant stress and the levels of DNMT1. We exposed mice to concentrated ambient PM_{2.5} or filtered air 8 hours daily for 3 days and looked for evidence of lung oxidant stress by immunostaining lung sections for nuclear 7,8-Dihydro-8-Oxo-2'-deoxyguanosine (8-oxo-DG). Exposure to concentrated ambient PM_{2.5} was associated with an increase in the number of nuclei staining positively for 8-oxo-DG (Figure 6A). In identically treated mice, we measured mRNA encoding DNMT1, DNMT3a and DNMT3b (Figure 6B). Similar to our observations in alveolar epithelial cells, we found an increase in DNMT1 mRNA and protein (Figure 6B and 6C) but no change in DNMT3a or DNMT3b in the lungs of mice exposed to CAPS.

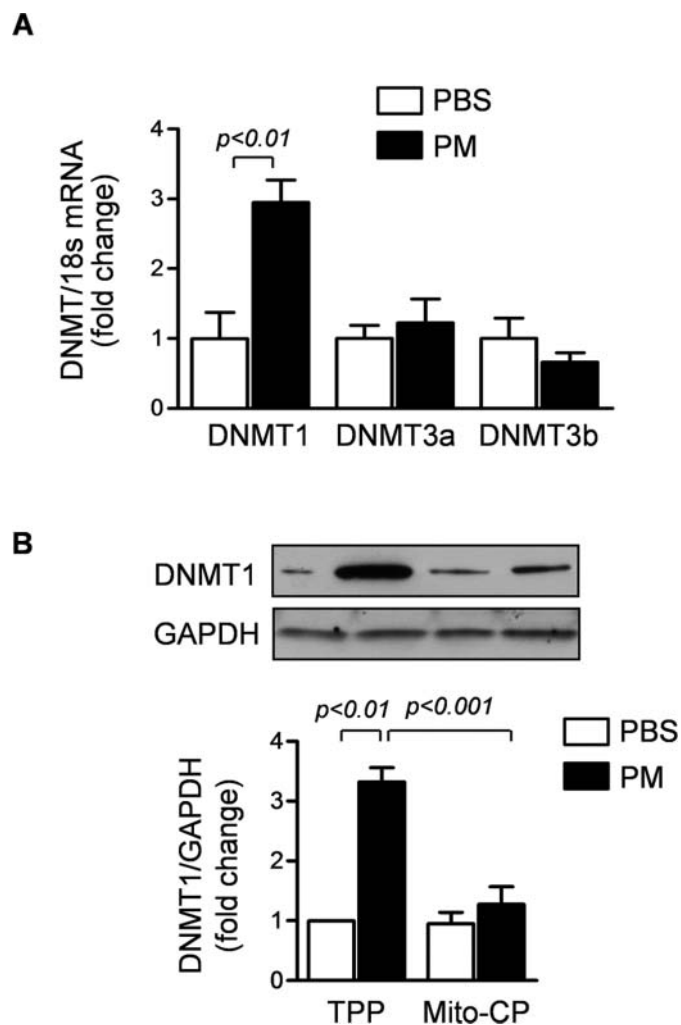


Figure 4 | Exposure of primary murine alveolar epithelial cells to repeated doses of PM increases DNMT1 mRNA and protein. Primary mouse alveolar epithelial cells were cultured at an air-liquid interface. After 2 days in culture, the cells were treated daily with PM (5 $\mu\text{g}/\text{cm}^2/\text{day}$). After 10 days of treatment the cells were lysed and (A) mRNA encoding DNMT1, DNMT3a and DNMT3b (RT-qPCR) and (B) DNMT1 protein (immunoblot) were measured. $N \geq 4$ for all measures, P values (ANOVA with Dunnett correction for multiple comparisons) are indicated in italics above the bars.

Discussion

We found that exposure of mice to concentrated ambient $\text{PM}_{2.5}$ resulted in lung oxidant stress, increased transcription of DNMT1 and caused hypermethylation of the p16 promoter in the lungs of mice. In primary murine alveolar epithelial cells, we found that enhanced transcription of DNMT1 and methylation of the p16 promoter in response to PM was inhibited by a mitochondrially targeted antioxidant and an inhibitor of the JNK pathway. Our data suggest that PM-induced mitochondrial oxidant stress activates JNK to transcriptionally upregulate the expression of DNMT1. These findings provide a potential mechanism explaining the observed link between exposure to PM and the development of lung cancer.

Lung cancer is associated with characteristic epigenetic changes, which include global hypomethylation accompanied by selective hypermethylation of CpG islands in tumor suppressor genes¹⁹. Methylation of the p16 promoter can be detected in $>70\%$ cell lines derived from human NSCLCs and in the sputum of smokers before the development of lung cancer. In the cell, p16 sequesters the cyclin D-dependent kinases CDK4 and CDK6 causing a G1-phase cell-cycle arrest and senescence^{6,7,9}. Therefore, methylation-induced

suppression of p16 allows for the clonal expansion of malignant cells⁹. In mice, genetic loss of p16Ink4a results in spontaneous carcinogenesis and an increased incidence of carcinogen induced cancers²¹. However, the partial loss of function induced by promoter methylation we observed in mice exposed to concentrated ambient $\text{PM}_{2.5}$ is likely insufficient to induce lung cancer in the absence of an additional genetic or environmental stimulus⁹. In support of this hypothesis, Belinsky and colleagues have identified the detection of hypermethylation of CpG islands in the promoter of p16 in the sputum as a risk factor for the development of lung cancer, particularly in patients with a history of cigarette smoke exposure^{8,10,18,20}.

Mammalian DNA is modified by methylation of 60–80% of the cytosine residing in the dinucleotide sequence CpG through a reaction catalyzed by the DNMTs^{11,22}. Mammalian cells express three DNMTs, DNMT1, DNMT3a, and DNMT3b¹¹. DNMT1 is widely expressed in human and murine cells and is required for the maintenance of methylation patterns during cellular replication^{23,24}. In contrast, DNMT3a and DNMT3b participate in *de novo* methylation during early development^{22,25,26}. Studies in transgenic and knockout mice suggest that DNMT1 plays an essential role in tumorigenesis^{11,27}. Blunted expression of DNMT1 using hemizygous knockout mice is associated with delayed and less severe tumor formation in murine models of cancer driven by the loss of tumor suppressor genes^{27,28}. DNMT1 expression has been reported to be increased in patients with lung cancer, with particularly high levels observed in patients who were smoking at the time of their resection¹². We found a relative increase in DNMT1 expression in mice exposed to concentrated ambient $\text{PM}_{2.5}$ and in primary alveolar epithelial cells from mice following prolonged exposure to PM. If PM exposure increases p16 promoter methylation by inducing the expression of DNMT1, then methylation of CpG islands should occur synchronously and early in the development of lung cancer. In support of this hypothesis, Belinsky and colleagues discovered that CpG island hypermethylation of three genes: p16, MMP2 and Basic Helix-loop-helix Family Member e23 (BHLHB4) in lung cancer cell lines was associated with a pattern of widespread CpG island hypermethylation¹⁰. We found evidence for promoter methylation in the MMP2 gene in mice and alveolar epithelial cells exposed to PM.

The c-Jun NH(2)-terminal kinase (JNK) is a member of an evolutionarily conserved subfamily of mitogen-activated protein (MAP) kinases^{29,30}. In lung epithelial cells, we reported that exposure to $\text{PM}_{2.5}$ resulted in the generation of ROS from site III in the mitochondrial electron transport chain^{16,17}. These ROS activated apoptosis signaling kinase 1 (ASK1), a MAP kinase kinase kinase (MAPKKK) that is activated when freed from its normal binding partner thioredoxin upon its oxidation to activate the JNK and p38 MAPKs³¹. The ASK1-mediated activation of JNK, resulted in the phosphorylation and activation of p53, which increased the transcription of the proapoptotic protein NOXA to activate BAX/BAK and induce cell death^{16,17,32}. Here we found that exposure to a lower dose of PM acted via a similar pathway to induce the transcription of DNMT1. These observations are consistent with the known regulation of the DNMT1 gene, which contains several conserved Activator Protein 1 (AP1) binding sites in its promoter^{14,33,34}.

We and others have reported that mitochondrially-derived ROS play diverse and critical roles in the metabolic adaptation of cells to environmental stress. Sometimes these signaling events are maladaptive. For example, we recently reported that oncogene induced tumors require mitochondrial ROS to drive biosynthetic pathways involved in cellular proliferation³⁵ and in this report we show that the inappropriate generation of mitochondrial ROS in response to particulate matter air pollution activates stress kinase pathways to suppress the transcription of tumor suppressors. In contrast, we have also found that mitochondrial ROS initiate adaptive signaling pathways that may prevent cancer, for example the terminal differentiation of human mesenchymal stem cells³⁶. Collectively, these data

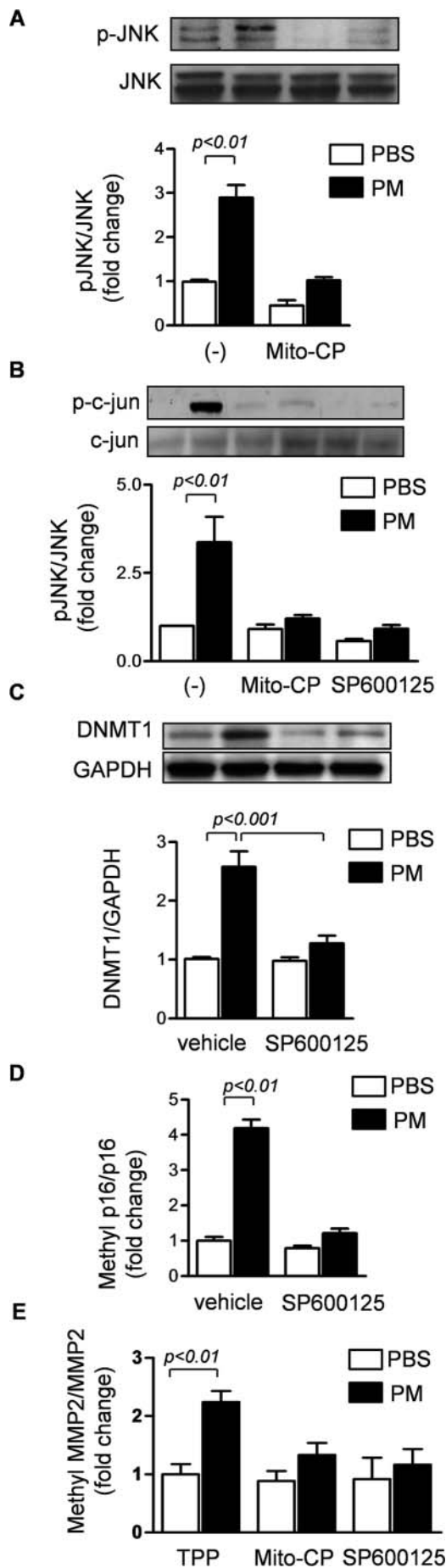


Figure 5 | JNK is required for the PM-induced hypermethylation of the p16 promoter in primary murine alveolar epithelial cells. (A) Primary mouse alveolar epithelial cells were cultured for 2 days at an air-liquid interface and then treated with Mito-CP (5 μ M) or vehicle followed 30 minutes later by PM (5 μ g/cm²) and 30 minutes later the levels of phosphorylated and total JNK were evaluated by immunoblot (densitometry below). (B) Primary mouse alveolar epithelial cells were cultured for 2 days at an air-liquid interface and then treated with Mito-CP (5 μ M), SP600125 (20 μ M) or the appropriate vehicle controls followed 30 minutes later by PM (5 μ g/cm²) and 2 hours later JNK kinase activity in cell lysates was measured. (C,D) Cells were treated daily for 10 days with SP600125 (20 μ M) or vehicle followed 30 minutes later by PM (5 μ g/cm²/day). After 10 days, the levels of DNMT1 were measured in cell lysates by immunoblotting and methylation of the p16 and MMP2 promoters was measured in cellular DNA using methylation-specific PCR. $N \geq 4$ for all measures, *P* values (ANOVA with Dunnett correction for multiple comparisons) are indicated in italics above the bars.

suggest that while mitochondrially targeted antioxidants might be useful as therapeutics for cancer they may not be effective for cancer prevention.

A major strength of our study is our observation that mice exposed to concentrated ambient PM_{2.5} show increased p16 promoter methylation in the lung and increased transcription of DNMT1. The concentrations of PM in the concentrated ambient PM_{2.5} exposure chamber are about 10 fold higher than ambient levels outside our laboratories which are located in an urban area near several major roadways. Based on data reported from EPA monitors near our laboratories during the period of exposure, we estimate chamber PM concentrations between 100–120 μ g/m³. Even these elevated levels are substantially less than those that would be encountered in many world cities¹. Furthermore, the mice in this study were exposed to PM for 8 hours daily, 5 days per week, a schedule that would mimic a typical exposure for an outdoor worker.

There are several important limitations of our study, which we hope will prompt further investigation. Firstly, while we were able to validate some of the key mechanistic findings of our cell culture model in the lungs of mice, loss of function studies in components of the mito-ROS-JNK-DNMT1 pathway will be required to confirm the importance of this pathway *in vivo*. Secondly, we are unable to definitively localize the DNMT1 and p16 promoter methylation signal to the alveolar epithelium in the intact lung. Thirdly, we observed that exposure to PM activated the JNK MAPK pathway within minutes of exposure and that increased levels of DNMT1 were present in cells within a day of exposure. However, we were only able to detect methylation of the p16 promoter after 10 consecutive days of exposure to PM. We speculate that additional inhibitory mechanisms prevent promoter methylation, which are only overcome after repeated and prolonged exposure. Finally, individuals in the population vary widely in terms of their genetic risk for cancer and previous exposure to environmental carcinogens. In our murine model, we examined a relatively small number of animals in a single inbred strain of mice and we limited our analysis to male mice as female sex has been shown to be protective in murine models of gastrointestinal and lung cancer where inflammation plays an important role³⁷.

We conclude that exposure to PM results in a mitochondrial-oxidant and JNK-mediated increase in the transcription and abundance of DNMT1 and increased methylation of the p16 promoter in the lung epithelium. Aberrant upregulation of DNMT1 could result in early and coordinated hypermethylation of key tumor suppressor genes in PM exposed patients, increasing the risk for the development of lung cancer. As these epigenetic changes are reversible and precede the development of lung cancer, they offer a novel target to prevent its development in high risk individuals.

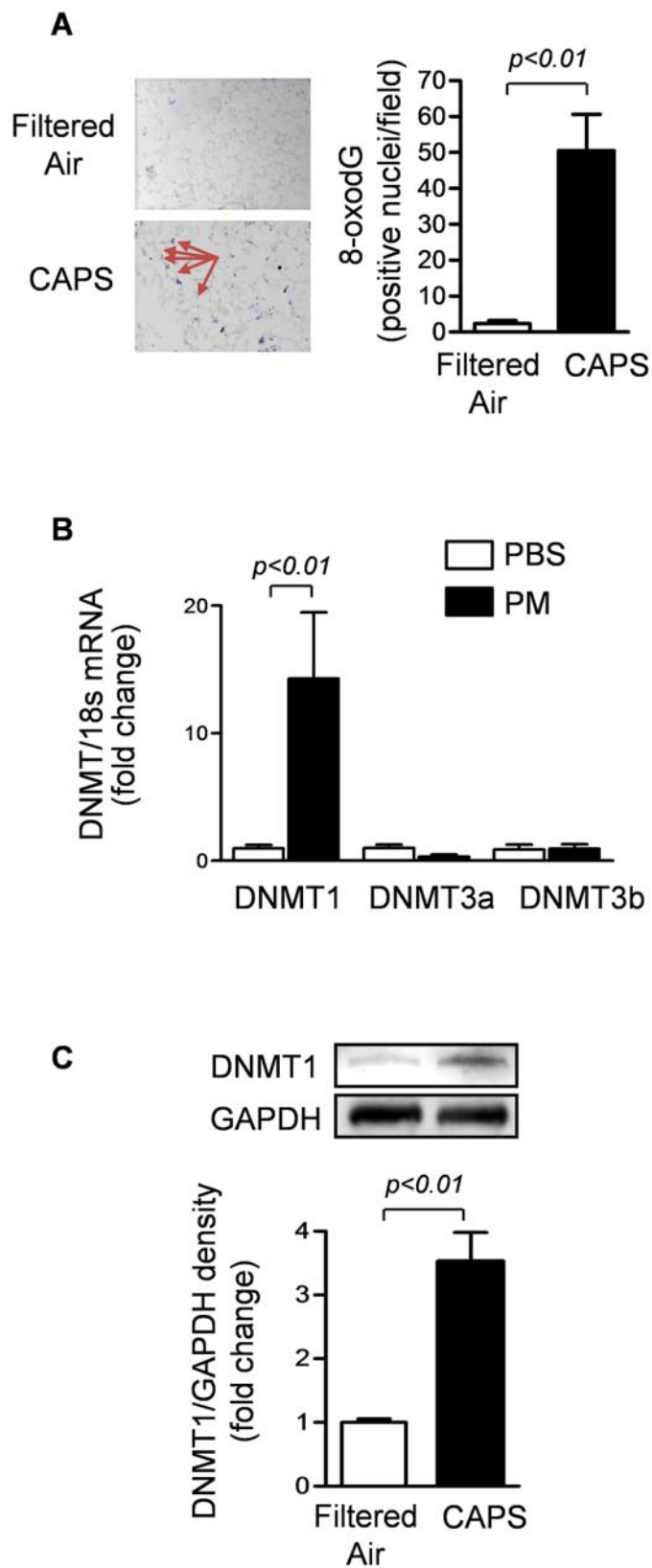


Figure 6 | Exposure to Concentrated Ambient $PM_{2.5}$ (CAPS) induces oxidant stress and increases DNMT1 mRNA and protein in the lungs of mice. Mice were exposed to concentrated ambient $PM_{2.5}$ ($PM_{2.5}$) or to filtered air for 3 days and (A) lung sections were stained for the presence of 8-oxodG (representative positive nuclei indicated by arrows, left panel), the bar graph represents the number of positive cells in 10 random fields from each section (200X) (B) In lung homogenates from mice exposed to concentrated ambient $PM_{2.5}$ ($PM_{2.5}$) or to filtered air for 9 weeks, mRNA

levels of DNMT1, DNMT3a or DNMT3b (RT-qPCR) and (C) DNMT1 protein (immunoblot) were measured. $N=4$ for all measures, P values (two tailed t-test) are indicated in italics above the bars.

Methods

Animals and alveolar type-2 cell isolation and cell culture. The protocol for the use of mice was approved by the Animal Care and Use Committee at Northwestern University. Six- to 8-wk-old (weighing 20–25 g)-male-C57BL/6 mice (wild-type mice) were purchased from Charles River Labs (Roanoke, Illinois). Type II alveolar epithelial cells (AECs) were isolated from pathogen-free male mice as previously described³⁸. Briefly, the lungs were perfused via the pulmonary artery, lavaged and digested with elastase ($3 U ml^{-1}$; Worthington Biochemical, Freehold, NJ). Alveolar type II cells were purified by differential adherence to immunoglobulin G and cell viability was assessed by trypan-blue exclusion ($>95\%$). Cells were resuspended in Dulbecco's modified Eagle's medium (Sigma-Aldrich Inc., St. Louis, MO) containing 10% fetal bovine serum (Hyclone, Logan, UT) with 2 mM glutamine, $100 U ml^{-1}$ penicillin, $0.25 \mu g ml^{-1}$ amphotericin B, and $100 \mu g ml^{-1}$ streptomycin. Cells were seeded in permeable transwell supports and maintained in air-liquid interface from the second day of culture until the end of the experiment while incubated in a humidified atmosphere of 5% CO_2 and 95% air at $37^\circ C$. The purity of the AECs was determined to be $90 \pm 5\%$ by immunostaining for surfactant protein C.

Reagents. Chemicals: The mitochondrially targeted anti-oxidant Mito-carboxy proxyl (Mito-CP) is a synthetic superoxide dismutase mimetic that accumulates in the mitochondria and has been previously described^{35,39}. SP600125 was purchased from Calbiochem (Darmstadt, Germany). 5-azacytidine (5-aza) was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies: Anti-phosphorylated and total JNK antibodies were purchased from Cell Signaling (Boston, MA, catalogue numbers 4668 and 9252, respectively) Anti-c-Jun was purchased from Sigma-Aldrich (catalogue number J4399). Anti 8-OxodG was purchased from Trevigen (Gaithersburg, MD, catalogue number 4354-MC-50). Anti-DNMT1 was purchased from ABCAM (Cambridge, MA, catalogue number AB11891).

Particulate Matter (PM). The PM for *in vitro* experiments was obtained from the National Institutes of Standards and Technology (Gaithersburg, MD, USA; NIST SRM 1649: Filter-Based Fine Particulate Material). The characteristics of the PM have been previously described⁴⁰. For *in vivo* experiments, concentrated ambient $PM_{2.5}$ (CAPS) was obtained using a Versatile Aerosol Concentration and Enrichment System (VACES) as previously described^{41–43}. The VACES concentrates $PM_{2.5}$ collected from the ambient air outside our laboratory, which is located in an urban environment with high levels of traffic. Wild-type mice were placed in the exposure chamber for 8 hours daily, five days per week for up to 9 weeks in the autumn of 2010. Control mice were identically treated except that a teflon filter was placed in the chamber inlet. The mean particle concentration in the chamber was monitored in real-time using a TSI 3775 particle counter.

Mitochondrial ROS production. We employed a mitochondrial matrix localized oxidant-sensitive ratiometric probe (mito-Ro-GFP) as previously described¹⁷. Primary murine alveolar epithelial cells were infected with 5 PFU/cell of an adenovirus containing the probe 48 h before exposure to PM. Oxidation of the mito-Ro-GFP probe was assessed by removing the cells from the plate using trypsin, and transferring equal aliquots of the resulting suspension to tubes containing medium alone or medium containing 1 mM dithiothreitol (DTT) or 1 mM hydrogen peroxide (H_2O_2). The ratio of fluorescence (emission of 535 nm) at excitations of 405 and 488 nm was measured by flow cytometry in 5,000 cells/condition using a DakoCytometry CyAn high speed multilaser droplet cell sorter. The oxidation state was calculated as the completely reduced ratio (dithiothreitol) less the untreated value divided by the difference in the ratio observed with 1 mM dithiothreitol and 1 mM H_2O_2 .

Apoptosis. Apoptosis was measured with a commercially available double sandwich colorimetric ELISA that detects nucleosomal fragmentation (Roche Applied Science, Cat # 11774425001) according to the manufacturer's instructions.

JNK activation and JNK Kinase assay. The phosphorylation of JNK was assessed by protein immunoblot as previously described¹⁷. Kinase assays to measure the activity of c-jun were performed using a commercially available assay (Cell Signaling; catalog number 9810)⁴⁴. Briefly, cell lysates were obtained and incubated with agarose beads linked to a c-jun fusion protein. The resulting c-jun/JNK complexes were precipitated by centrifugation and sequential washing of the beads. The beads were then incubated with 2 μg of myelin basic protein and 100 μM ATP for 60 min at $30^\circ C$ in 35 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM EGTA, 1 mM $CaCl_2$, and 10 mM β -glycerolphosphate. The reactions were stopped and c-jun was released from the beads by adding SDS sample buffer and boiling the samples. The proteins were separated by SDS-PAGE, and detected by immunoblot.

8-oxo-dG Immunostaining. After 9 weeks of exposure to concentrated ambient $PM_{2.5}$, isolated mouse lungs were inflated with 4% paraformaldehyde ($15 cm H_2O$) then fixed for 24 hours and paraffin embedded and cut in 5 μm sections. Tissues were deparaffinized and treated with Proteinase K for 20 minutes and the slides were placed in citrate buffer on a hot plate for 2–5 minutes for antigen retrieval. The slides



were then left to cool at room temperature, rinsed with deionized water and blocked for 1 hr with 1% goat serum and then incubated overnight with Anti-8-oxo-dG Monoclonal Antibody (1 : 100). The slides were then treated with goat anti-mouse IgG conjugated to biotin at 37°C for 60 minutes and then incubated with AP-conjugated streptavidin at 37°C in a light-tight box in a humid chamber for 60 minutes. Slides were incubated with 3,3' Diaminobenzidine (DAB) for 15 minutes, rinsed with TBS-T, and mounted. The tissues were imaged with a Nikon Eclipse T2000 microscope and the number of positive nuclei per field was counted with the ImageJ software (NIH).

Nucleic Acid Isolation and Methylation-Specific PCR. Lung DNA was isolated from paraffin embedded samples by hydrating a 20 μ m section with xylene followed by two passes of 100% ethanol, one of 90%, 80% and 70% ethanol followed by digestion with proteinase K in 1% SDS. AT2 cell DNA was directly extracted by affinity columns using the Nucleospin tissue kit, following the manufacturer's recommendations (Clontech, Mountain View, CA). The methylation state of the p16 gene was determined by methylation-specific PCR (MSP) as described elsewhere^{8,45}. Briefly, genomic DNA was modified by treatment with sodium bisulfite, which converts all unmethylated cytosines to uracil, then to thymidine during the subsequent PCR step. Before PCR, 200 ng of purified DNA was treated 15 minutes with 0.3 M NaOH, and treated with a solution containing 5 M sodium bisulfite, 2.5 M sodium metabisulfite and 100 mM hydroquinone for 2.5 hr at 50°C. The modified DNA was desalted, precipitated with ethanol, and finally resuspended in 20 μ l of Tris-EDTA buffer. Two sets of primers were used to amplify each region of interest: one pair recognizes a sequence in which CpG sites are unmethylated (bisulfite modified to UpG), and the other recognizes a sequence in which CpG sites are methylated (unmodified by bisulfate). Treated DNA (200 ng) was used as the template and DNA amplification was measured using SYBR Green qPCR by the delta-delta Ct ($\Delta\Delta$ Ct) method using the following primer sequences⁴⁵:

Unmethylated p16:

Forward: AAT TTG AGG AGA GTT ATT TG.

Reverse: AAA TCA AAA TAC AAC CAA AAA A.

p16:

Forward: AAT TCG AGG AGA GTT ATT TG.

Reverse: AAA TCG AAA TAC GAC CGA AA.

Unmethylated MMP2:

Forward: AGAGATTTTAGGGTGATATGTGG

Reverse: CACCAAAAACTTAATAATAACAAT

Methylated MMP2:

Forward: AGTTAGAGATTTTAGGGTGATACGC

Reverse: GCCGAAAACTTAATAATAACGAT

Real-time reverse transcriptase PCR measurement of RNA. Wild-type mice were exposed to concentrated PM_{2.5} or filtered air for 9 weeks and the lungs were harvested for isolation of total RNA using a commercially available system (TRIzol, Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Primary alveolar epithelial cells were exposed for 10 days to PBS or 5 μ g/cm² of PM_{2.5} and the resulting mRNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase and random decamers, using a commercially available system, according to the manufacturer's instructions (Ambion, Austin TX, USA). RT-qPCR reactions were performed using IQ SYBR Green-superscript (Bio-Rad Laboratories) with the primers listed below and analyzed on an IQ5 Real-Time PCR Detection System. Cycle threshold (C_t) values were normalized to C_t values for ribosomal protein 18 s, and data were analyzed using the $\Delta\Delta$ Ct method⁴⁶. The primer sequences used were previously described³² and are the following:

DNMT1:

Forward: CCA AGC TCC GGA CCC TGG ATG TGT;

Reverse: TGA CAC CAT CTG TTC TTT CAG CTT CAC.

DNMT3a:

Forward: CGA GGC CGG TAG TAG TCA CAG TAG;

Reverse: GCA CCT ATG GGC TGC TGC GAA GAC G.

DNMT3b:

Forward: CTG CCT CCA ATC ACC AGG TCG ATT G;

Reverse: CAA GGA GGG CGA CAA CCG TCC ATT.

18 s: forward:

TGG CTC ATT AAA TCA GTT ATG GT,

Reverse: GTC GGC ATG TAT TAG CTC TAG

Statistical Analysis. Data are presented as means \pm SEM. Student's t tests were performed to compare experimental data with appropriate controls (as indicated in each figure legend). For comparisons involving more than two groups, we performed an analysis of variance (ANOVA). When the ANOVA indicated a significant difference, individual differences between groups were explored with Dunnett or Bonferroni corrected t-tests. Statistical significance was determined at a value of $P \leq 0.05$.

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Author contributions

SS, AG, DU, KAR and SEC performed the experimental work. KMR provided the primary alveolar epithelial cells and ensured their quality. JJ and BK provided key reagents for the study (Mito-CP and TPP). AOV, NSC, assisted in study design and data analysis. SS, GM, AVR and GRSB designed the study and performed the analysis. SS and GRSB prepared the manuscript. All authors reviewed and commented on the paper.

Additional information

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