TITLE:

Paternal cigarette smoke alters DNA methylation in sperm and gene expression in offspring brain

SHORT TITLE:

Paternal smoking, sperm DNA methylation and impacts in offspring

AUTHORS:

†,*Patrick J Murphy[1,3], †Jingtao Guo[2,3], Timothy G Jenkins[2], Emma R James[2,6], John R Hoidal[4], Thomas Hueckstead[4], James M Hotaling[2], Douglas T Carrell[2,5,6], *Bradley R Cairns[3], *Kenneth I Aston[2]

† These authors contributed equally to this work.

* Corresponding authors: kiaston@utah.edu, patrick_murphy@urmc.rochester.edu, brad.cairns@hci.utah.edu

1 Departments of Biomedical Genetics, Wilmot Cancer Institute, University of Rochester Medical Center, Rochester NY, USA.

2 Andrology and IVF Laboratories, Department of Surgery, University of Utah School of Medicine, Salt Lake City, Utah, USA

3 Howard Hughes Medical Institute, Department of Oncological Sciences and Huntsman Cancer Institute, University of Utah School of Medicine, Salt Lake City, Utah, USA

4 Department of Internal Medicine, University of Utah School of Medicine and Salt Lake VA Medical Center, Salt Lake City, Utah, USA

5 Department of Obstetrics and Gynecology, University of Utah School of Medicine, Salt Lake City, Utah, USA

6 Department of Genetics, University of Utah School of Medicine, Salt Lake City, Utah, USA

ABSTRACT:

Background: There is growing evidence that paternal pre-conception cigarette smoke (CS) exposure is associated with increased risk of behavioral disorders and cancer in offspring. The aim of the current study was to evaluate the impact of paternal pre-conception CS exposure on sperm DNA methylation and offspring phenotype.

Methodology/Principal: To characterize the effects of CS exposure on the sperm epigenome and offspring neurodevelopment, we exposed male mice to CS and bred exposed and control males to unexposed females and subsequently evaluated sperm DNA methylation in sires and frontal cortex DNA methylation and gene expression in offspring. We further investigated the role of oxidative stress on sperm epigenetic changes using a mouse model (*Nrf2*$^{-/-}$) with impaired antioxidant capacity. Lastly, we evaluated the capacity for sperm DNA methylation to recover following removal of CS for 1-5 spermatogenic cycles (28-171 days).

Conclusions/Significance: Smoking significantly impacts sperm DNA methylation as well as DNA methylation and gene expression in offspring. These changes were largely recapitulated in *Nrf2*$^{-/-}$ mice independent of smoke exposure. Recovery experiments indicated that about half of differentially methylated regions returned to normal within 28 days of removal from smoke exposure, however additional recovery following longer periods was not observed. We present strong evidence that cigarette smoke exposure induces paternally mediated, heritable epigenetic changes. Parallel studies performed in *Nrf2*$^{-/-}$ mice provide evidence for oxidative stress as the predominant underlying mechanism for smoke-induced

epigenetic changes to sperm along with the associated effects in offspring. Lastly, recovery experiments indicate that while many epigenetic changes are corrected following removal from smoke exposure, aberrant methylation persists at a significant number of regions even after five spermatogenic cycles

KEYWORDS: sperm DNA methylation, epigenetics, gene expression, frontal cortex, smoking, heritable, cigarette**,** oxidative stress

INTRODUCTION:

Cigarette smoke (CS) exposure is a global epidemic with significant health consequences. In a recent study it was estimated that more than one third of the world's population is regularly exposed, directly or indirectly, to tobacco smoke (1). Further, it was estimated that in 2004 involuntary exposure to tobacco smoke caused 603,000 premature deaths and a loss of 10.9 million disability adjusted life years, and an estimated six million annual deaths are attributable to tobacco smoke exposure (1). Tobacco smoke contains more than 4000 chemicals including a myriad of known carcinogens. The health consequences of smoke exposure are significant and include numerous diseases and dysfunctions of the respiratory tract, increased risk of multiple types of cancer and increased incidence of cardiovascular disease (2, 3). Tobacco smoke exposure is a global problem, the implications of which are becoming increasingly apparent. However, little is known about the impact of paternal exposure to cigarette smoke (CS) on sperm and implications of pre-conception paternal CS on offspring health (4).

Male fertility rates have steadily declined in developed countries over the past half-century (5-7), and metabolic disorders have steadily increased over a similar period (8, 9). These trends are due to a variety of factors, but increased exposure to environmental toxins is likely a significant contributor. Tobacco smoke exposure, which negatively affects semen quality (10, 11), might contribute to these trends. Moreover, *in utero* exposure to maternal tobacco smoke increases the risk of obesity and hypertension in offspring (12, 13). A more complete understanding of the effects of preconception paternal tobacco smoke exposure on offspring is of critical relevance to public health.

Although understudied, there is evidence for paternally transmitted effects of CS exposure to offspring. The negative impacts of CS on semen parameters are well established. Smoking is associated with an accumulation of cadmium and lead in seminal plasma, reduced sperm count and motility, and increased morphological abnormalities in sperm (10, 11). In addition, reduced reproductive potential has been reported in tobacco smoke-exposed mice (14) and humans (15). Although the impact of tobacco smoke exposure on germ cell genetics and epigenetics has received surprisingly little attention, recent evidence demonstrates strong effects on the adult male germline. Adult male mice exposed to sidestream tobacco smoke display significant increases in sperm DNA mutations at expanded simple tandem repeats (ESTRs) (16), as well as more frequent aberrations in sperm chromatin structure and elevated sperm DNA damage (14). In contrast, CS-exposed male

106 mice exhibit no measurable increase in somatic cell chromosome damage,
107 indicating that germ cells may be more prone to environmentally-induced genetic
108 and/or epigenetic insults compared with somatic cells. Destabilization of ESTRs in
109 the adult male germline is well documented to be associated with
110 transgenerational effects in the mouse genome (17-21).
111
112 The International Association for Research on Cancer recently declared that
113 *paternal* smoking *prior* to pregnancy is associated with a significantly elevated risk
114 of leukemia in the offspring (22), suggesting CS-induced genetic or epigenetic
115 changes occur in sperm that are transmitted to offspring. We recently reported
116 altered sperm DNA methylation (DNAme) patterns in men who smoke (23). In
117 addition, smoking has been clearly shown to modify DNAme patterns and gene
118 expression in somatic tissues in individuals exposed to first- or second-hand
119 tobacco smoke (24-26) as well as in newborns of smoking mothers (27-29). A
120 growing body of evidence suggests that CS exposure could have negative health
121 consequences not only for the exposed fathers, but also for their offspring.
122
123 Results of these studies motivated us to investigate the impact of smoking on
124 mouse sperm DNAme as well as the potential transmission of those effects to
125 offspring. Additionally, we aimed to explore the dynamics of sperm epigenetic
126 changes after withdrawal from smoke, to determine whether DNAme changes
127 recover following removal of the insult for more than five complete cycles of
128 spermatogenesis (171 days; Figure 1).

Lastly, to investigate the mechanism underlying CS-induced alterations to the sperm epigenetic profile, we used a knockout mouse model to assess the role of antioxidant capacity on smoke-induced alterations to the methylome. The nuclear factor (erythroid-derived 2)-like 2 (NRF2) pathway is the primary cellular defense against the cytotoxic effects of oxidative stress. Thus, to investigate the mode by which CS induced epigenetic changes might occur, we utilized the *Nrf2*$^{-/-}$ mouse model, which has compromised antioxidant capacity.

RESULTS:

Experimental Design and phenotypic effects:

Male mice were assigned to CS- exposed or non-exposed groups (n = 10-12 per group). The CS animals were exposed to the body mass-adjusted equivalent of 10-20 cigarettes per day, 5 days per week over a period of 60 days. CS- exposed and control mice were bred to unexposed females, and offspring were analyzed for phenotypic and molecular measures.

In agreement with the literature, both WT and *Nrf2*$^{-/-}$ mice that were exposed to CS weighed significantly less than non-exposed control animals (TABLE 1). Sperm concentration and motility were not significantly impacted by CS exposure, sperm concentration was lower in *Nrf2*$^{-/-}$ than WT males, and conception was significantly delayed in CS-exposed *Nrf2*$^{-/-}$mice compared with unexposed *Nrf2*$^{-/-}$mice (TABLE 1). Neither growth trajectories nor sperm

parameters were different in F1 animals based on paternal CS exposure (TABLE 2).

Smoking-induced DNAme changes in F0 sperm were either maintained, recovered, or variable, depending on the locus.

Initially, we performed reduced representation bisulfite sequencing (RRBS) to explore the effects of smoking on F0 sperm DNAme patterns (sperm collected within 3 days of completing a 60-day smoking treatment), and we examined whether CS-induced methylation changes recover to baseline unexposed levels following removal of CS exposure (28, 103 and 171 days after smoking treatment; n = 10 per group).

At individual CpG sites, we found changes in DNA methylation at a large number of CpGs (Figure 2A & Figure S1A) with essentially equal representation of sites that lost methylation and gained methylation as a consequence of smoke exposure. In addition, we found that the number of differentially methylated CpGs declined only slightly after removal of CS exposure for 28-171 days (Figure 2A).

We partitioned differentially methylated CpGs into three groups: 1) shared, meaning differentially methylated CpGs that were maintained between the treatment group and the respective recovery group, 2) recovered, meaning CpGs that were differentially methylated after initial exposure but were no longer differentially methylated in the recovery group, and 3) new, meaning CpGs that

were not differentially methylated after initial exposure but emerged as newly differentially methylated in the recovery groups. These classes were assessed separately for CpGs that initially lost methylation verses those that initially gained methylation relative to the control. Interestingly, for CpGs that lost methylation, the three groups were relatively evenly represented across recovery times, with only a slight over-representation of shared CpGs. For CpGs that gained methylation, there was a slight bias toward the emergence of new differentially methylated CpGs across recovery groups (Figure 2B).

Given that DNAme status of CpG regions likely has a greater capacity to confer functional effects compared with individual sites, we subsequently binned individual CpG sites into regions based on their proximity (see methods). We then performed analyses similar to those performed for individual CpGs. Interestingly, changes in DNA methylation occurred at far fewer regions compared with individual CpGs (Figure S1B), and we found strong evidence for recovery of smoke-induced sperm DNAme changes following a recovery period. Strikingly, the majority of DNAme regions that recovered returned to control levels within 28 days of removal of smoke exposure, with no evidence for additional correction following longer recovery periods (Figure 2C). In contrast with the individual CpG data, we found that far fewer new differentially methylated regions (DMRs) emerged during the recovery period (Figure 2D).

Regions of high and low DNAme recovered, and changes were associated with Nrf2-mediated effects.

We then sought to understand epigenetic properties that might impact the ability of specific regions to recover following removal of the CS, including the initial methylation status. For this analysis, we classified all DMRs into six classes, based on their dynamics during recovery (shared, recovered or new) and their direction of change (increase or decrease; Figure 3A). We discovered that the initially hypomethylated DMRs in which methylation increased with smoke exposure displayed a higher likelihood of recovery, and initially hypermethylated DMRs that decreased in methylation were likewise more likely to recover (Figure 3A). Regions of intermediate DNAme were less likely to recover across all groups. In addition, we observed that recovered DMRs that initially decreased in methylation level show lower variation compared to all other groups (Figure S2A), thus suggesting that regions of extreme hyper- and hypo-DNAme were less likely to change after CS exposure, and when changes did occur, these regions were more likely to recover.

We hypothesized that CS- induced alterations to the methylome might depend on antioxidant capacity, and that reducing antioxidant capacity might mimic or enhance smoke-induced changes to sperm DNAme. This led us to examine the changes of sperm DNAme in *Nrf2*$^{-/-}$ mice (compared to WT mice), as well as the changes in sperm DNAme in CS-exposed compared with unexposed *Nrf2*$^{-/-}$ mice. We initially found that the degree of DNA methylation changes, and the number

of sites impacted, were similar in *Nrf2*$^{-/-}$ mice compared with WT (Figure S3A). We were surprised to find that the CS-induced DNAme changes in WT sperm were largely recapitulated in *Nrf2*$^{-/-}$ sperm irrespective of CS exposure (Figure 3B). Notably, the sperm DNAme changes observed in unexposed *Nrf2*$^{-/-}$ mice were similar to changes observed in *Nrf2*$^{-/-}$ mice that were exposed to CS, with no evidence for an enhanced effect attributable to CS. This correlation in the degree of DNAme change was consistent when assessed genome-wide, and we did not observe this correlation when comparing our data to other datasets evaluating the effect of other environmental factors (vinclozolin exposure and protein restricted diet) (30, 31) on sperm DNAme (Figure 3C).

Smoking-induced DNAme changes in F1 brains were regulated by oxidative stress

CS-induced epigenetic changes in sperm might correspond with an affect in offspring if they are maintained through fertilization, or if those DNAme changes are associated with an alternative, unidentified inheritance mechanism. Thus, we aimed to test whether paternal CS exposure could impact the next generation (F1). Given that brain and nervous systems were previously reported to be sensitive to preconception paternal exposures (32-35), we investigated DNAme by RRBS in the prefrontal cortex of F1 mice derived from CS-exposed WT and *Nrf2*$^{-/-}$ sires compared to the offspring of unexposed males (n = 8 per group). Similar to observations in F0 sperm, we found that paternal smoking altered DNAme patterns in offspring brains, and these DNAme changes were highly

243 similar to brain DNAme changes observed in offspring of both unexposed *Nrf2*$^{-/-}$
244 males (r=0.609) (Figure 4A) and CS-exposed *Nrf2*$^{-/-}$ males (r=0.477). In
245 agreement with F0 sperm DNAme data, exposure to CS appeared to have no
246 additional impact on brain DNAme beyond the *Nrf2*$^{-/-}$ effect (Figure 4A - right).
247 These observations suggest that CS-induced DNAme changes that occur in F1
248 brains are mediated by paternal oxidative stress, a well-established effect of
249 NRF2 depletion (36, 37).
250
251 To investigate whether F0 CS-induced effects can be passed to F2, we
252 compared the smoke-associated DNAme changes observed in F0 WT sperm
253 with that of F1 sperm and found no correlation (r=-0.001; Figure 4B). We also
254 observed that paternal CS-induced DMRs in F1 brains and those in smoke-
255 exposed F0 sperm had minimal overlap (hypergeometric p-value =1; Figure 4C),
256 indicating a lack of maintained DNAme changes from sperm through fertilization,
257 into brain development. Accordingly, regions where DNA methylation changes
258 occurred are not marked by chromatin features that are known to be maintained
259 in mature sperm (38)(FIGURE S4). Regardless of whether changes in DNAme
260 are a marker of epigenetic inheritance, or a driver of epigenetic inheritance, these
261 results suggest that the effects of CS exposure do not persist through several
262 generations.
263
264 **Smoking causes high gene expression variations in F1 brains**

To further investigate the potential for phenotypic effects in offspring associated with paternal CS exposure, we performed RNA-seq of the F1 frontal cortex in the same animals as those assessed for frontal cortex DNAme. Interestingly, paternal CS exposure caused a globally elevated variation in gene expression in the F1 brains, which was also observed in the *Nrf2*$^{-/-}$ offspring (Figure 5A). In agreement with the F1 brain DNAme data, we found that the paternal CS-induced gene expression changes in WT were highly similar to the Nrf2$^{-/-}$ offspring, and the effects were not further elevated in the offspring of smoked Nrf2$^{-/-}$ mice (Figure 5B & S4D-E). Notably, this increased variation limited our ability to identify reliably differentially expressed genes. Instead, we ranked genes by changes in gene expression and performed gene ontology (GO) analysis. We found that gene transcripts associated with classes including neuropeptide receptors and hormone activity were over-represented in the offspring of CS-exposed males (Figure 5C), while gene transcripts associated with gene classes including immune response and metabolism were under-represented (Figure 5D).

DISCUSSION:

Recent studies have demonstrated that paternal preconception exposures to a variety of pharmacologic agents and pollutants, including nicotine, THC, morphine, and benzo[a]pyrene effect offspring phenotype, and often confer neurobehavioral consequences (32-35, 39-42). In some cases, the impacts are inherited transgenerationally (40, 41). The direct assessment of

transgenerational inheritance through F2 was beyond the scope of the current study, however the absence of correlation between DMRs in F1 sperm and F2 sperm suggests that the affects we observed in F1 frontal cortex are not likely to be maintained in a second generation, although an alternate non-DNA-methylation associated mechanism for transgenerational inheritance mediated through noncoding RNAs or chromatin structure cannot be ruled out. Our data suggest that in our experimental model, oxidative stress is the major contributor to sperm DNA methylation changes and subsequent effects in the offspring. This does not exclude the possibility that other factors, such as low-level nicotine exposure might also contribute to DNAme effects (32, 33, 39, 42), but we suspect these additional factors are only minor contributors, based on the absence of transgenerational effects in our system.

CS exposure clearly impacts sperm DNAme patterns, however it is reassuring that many of the DNAme effects that occurred at high CpG dense regions were corrected within 28 days. Genomic regulatory regions (such as promoters and enhancers) tend to have high CpG density in the mouse. Thus, DNA methylation changes at these regions, which might otherwise impact gene regulation, are likely to be relatively short-lived. These data are in agreement with previous studies that found that CS exposure significantly impacts DNA methylation patterns in whole blood, and CS-associated methylation changes are largely corrected following smoking cessation in a time-dependent manner (43, 44).

We observed recovery after just twenty-eight days, which corresponds to just under the duration of a full spermatogenic cycle in mice of 30 days. In man, a spermatogenic cycle is 67 days, and additional research is required to characterize the similarities and differences in the dynamics of sperm DNAme alterations between mice and men. Additionally, it is not yet known whether the corrections observed following removal of CS exposure ameliorate the affects observed in offspring, or whether those effects are driven by the minority of regions that persist following CS removal. Addressing these unknowns will be of great interest for follow-up studies.

Interestingly, we found that regions of extreme hyper- and hypo-DNAme were less likely to be impacted by CS exposure, and when changes did occur, these regions were more likely to recover. This is consistent with a recent monozygotic twin study that investigated differences in blood DNAme in twins discordant for smoking (45). Additionally, our observation that individual CpGs did not recover after smoke exposure to near the same degree as more CpG dense regions suggests that CpG density might provide a degree of “buffering” against environmental insults. If genomic regulatory function were held by individual CpGs rather than by clusters of CpGs, minor variations or fluctuation in DNAme levels might have significant impacts on physiology. Instead, our data suggests that regions of higher CpG density, which are often found at gene regulator regions like promoters (46), vary less in DNAme level than individual CpGs, and therefore, these regions may be able to withstand a higher degree of

334 environmental insult without having lasting effects on physiology. This robustness
335 in function might explain in part why genomic regulatory regions have a high
336 degree of CpG density.
337
338 We investigated the impact of CS exposure in the oxidative stress-compromised
339 *Nrf2*$^{-/-}$ mouse strain with the expectation that we would observe more profound
340 effects of CS exposure on sperm epigenetic changes. Remarkably, while we did
341 not observe evidence for elevated susceptibility to CS-induced sperm DNAme
342 changes in *Nrf2*$^{-/-}$, we found the CS-effects observed in WT animals were
343 commensurately recapitulated in *Nrf2*$^{-/-}$ mice independent of CS exposure,
344 suggesting that elevated oxidative stress is the primary mechanism for CS-
345 mediated sperm epigenetic changes. These findings were remarkably consistent
346 with the observations of offspring frontal cortex DNAme and gene expression
347 changes as well. While CS-exposure may represent among the most extreme
348 examples of environmental insults that induce oxidative stress, the list of
349 environmentally relevant exposures that impact oxidative stress are innumerable,
350 and the results presented here indicate that all such exposures could potentially
351 impact the epigenetic status of the paternal germline and thus offspring
352 phenotype. In addition, our work provides strong evidence that *Nrf2*$^{-/-}$ mice can
353 serve as a good animal model to study CS-exposure induced effects. Further
354 studies may focus on investigating the molecular mechanisms underlying NRF-
355 mediated DNAme alterations.
356

Lastly, by comparing the DNAme and gene expression patterns in the frontal cortex between offspring of male mice exposed to CS with those not exposed, we found strong evidence for an impact of paternal smoking on offspring phenotype. In keeping with data obtained from *Nrf2*$^{-/-}$ mice, we suggest that oxidative stress agents might contribute to behavioral or developmental impacts in offspring. Notably however, our data showed little overlap of DNAme changes in the F1 frontal cortex with DNAme changes in the F0 sperm. While this was somewhat expected, as DNAme state undergoes dramatic reprogramming during early development and neuronal differentiation, it is important to highlight — direct mitotic inheritance of DNAme state is unlikely to mechanistically contribute to the oxidative stress effects we observed in F1 mice. Furthermore, we propose that DNAme may be more accurately described as a marker of epigenetic inheritance and not a mechanistic driver in transmitting environmental impacts between generations. Additional studies are necessary to confirm this and to distinguish other epigenetic features as markers or drivers, including chromatin status and small RNAs, which could play a significant role in inheritance. Further, the observation that CS-associated sperm DNAme changes were not identified in the sperm of the F1 generation offers reassuring evidence that the changes observed in F1 animals likely would not persist in the F2 generation. However, direct studies to evaluate the potential for transgenerational impacts of CS exposure are warranted. The implications of this for human spermatogenesis and transgenerational inheritance also warrant further investigation.

Here we present strong evidence for: 1) a significant impact of CS-exposure on the male germline, 2) relatively rapid partial correction of CS-induced sperm DNAme alterations following removal of CS exposure, 3) the implication of oxidative stress as the primary mechanism for CS-induced sperm DNAme changes and 4) an impact of paternal CS exposure on offspring phenotype based on frontal cortex DNAme and gene expression patterns. These data have important implications in characterizing the potential mechanisms that underlie the elevated risk observed for paternal CS exposure to offspring health. In addition, the findings reported here are likely more broadly applicable to understanding the risks of other environmental exposures that induce oxidative stress such as air pollution and some chemical exposures.

While additional studies are necessary to fully characterize the impacts of CS exposure on offspring phenotype, the mechanisms involved in heritability of pre-conception paternal exposures, and the phenotypic impacts of the observed epigenetic and gene expression changes in offspring, the current study significantly expands our understanding of the impacts of paternal CS exposure on offspring and the underlying mechanism of CS induced epigenetic changes to sperm.

MATERIALS AND METHODS:

Animals:

Animal care:

All animal experiments were performed under protocols that were approved by the University of Utah Institutional Animal Care and Use Committee (protocol # 14-11006). All animals were obtained from Jackson Laboratories (Bar Harbor, ME, USA)

Smoke exposure:

All CS-exposed and control mice were age matched and smoking was initiated between 6 and 7 weeks of age. Mice were exposed to CS using a Teague Model TE-10 (Teague Enterprises, Woodland, CA) smoking machine, which produces a combination of side-stream and mainstream CS. A pump on the machine "puffs" each 3R4F University of Kentucky research cigarette for 2 seconds for a total of 9 puffs before ejection. The 2.5 hour daily exposure occurred for 5 consecutive days per week over a period of 60 days. The smoking chamber atmosphere was periodically sampled to confirm total particulate matter concentrations of approximately 150 mg/m^3, the human equivalent of smoking approximately 10-20 cigarettes per day (47).

Smoking and recovery experiments:

To characterize the impact of smoke exposure on the sperm DNA methylome, and the capacity for smoke-induced sperm DNA methylation alterations to recover following removal of the insult, we exposed 40 C57BL/6J (Jackson Labs Stock # 000664) to cigarette smoke for comparison against 10 age-matched, non-smoked controls. Ten CS-exposed mice and the 10 non-exposed controls

were euthanized and tissues collected within three days of the CS exposure period. Subsequent "recovery" groups of 10 CS-exposed animals were euthanized 28, 103 and 171 days after the exposure period (corresponding to approximately 0.8, 3 and 5 spermatogenic cycles). In addition to experiments with wild-type animals, ten age-matched *Nrf2*$^{-/-}$ mice on a C57BL/6J genetic background (Jackson Labs Stock # 017009) were exposed to the same doses of CS for the same time period, and ten age-matched unexposed *Nrf2*$^{-/-}$ mice were utilized as controls.

Offspring transmission experiments:

Founder mice for heritability experiments included wild type C57BL/6J mice (Jackson Labs Stock # 000664) that were exposed and not exposed to CS (n = 10-12 per group). Approximately one week after the exposure period, exposed and control males were introduced to 6-week old CAST/EiJ female mice (Jackson Labs Stock # 000928), and pairs were kept together until F1 litters were born, or for 7 weeks without conceiving, whichever came first. The motivation for outcrossing males to CAST/EiJ females was to leverage polymorphic alleles to enable attributing reads to a specific parent, however due to the large average spacing of informative SNPs in the CAST strain and the short sequencing reads inherent in Illumina sequencing we were unable to classify the large majority of reads based on parent-of-origin. We therefore analyzed the data without regard to parent-of-origin. F1 litters were weaned at approximately 21 days of age, and pups were regularly weighed until they were euthanized. F1 animals were

448 euthanized at 14-17 weeks of age, and heart, lung, liver, kidney, brain, testis and
449 epididymal sperm were collected for molecular studies.
450
451 **Animal phenotyping:**
452 Following epididymal sperm extraction, sperm count and motility were assessed
453 in CS-exposed and control F0 animals as well as F1 offspring. In addition, time to
454 conception and litter size were compared between F0 groups. F1 offspring were
455 evaluated for growth trajectory. For statistical analysis of growth trajectories
456 between groups, animal weights were plotted against age for all pups within a
457 group (C57BL/6J or *Nrf2*$^{-/-}$). Models to fit the data were tested, and a logarithmic
458 model generally yielded the highest r^2. Theoretical weights were calculated for
459 each weight event based on the model generated, and differences between
460 theoretical and actual weight were calculated. A mean of average differences
461 within an individual across weight events was calculated for each animal, and
462 unpaired student's t-test was used to compare these differences between
463 smoked and non-smoked animals within each group. Differences in animal
464 weights and sperm parameters were evaluated using two-tailed Student's t-test,
465 and two-tailed Fisher's Exact tests were used to evaluate weekly differences in
466 conception between groups. $P < 0.05$ was considered significant.
467
468 **Molecular analyses:**
469 **Sperm collection and DNA extraction:**

Sperm was collected from the cauda epididymis and vas deferens immediately after euthanasia by scoring the tissue along the length of the tubules with a 28-G needle and gently pressing the tissue to expel the sperm mass. Tissues were then placed in a center-well dish in equilibrated Quinn's medium supplemented with FBS in a humidified CO_2 incubator for one hour. Following the swim out period, sperm concentration and motility were assessed on a Makler chamber and sperm were snap frozen in liquid nitrogen. Samples were subsequently thawed and subjected to a stringent somatic cell lysis protocol to ensure a pure population of sperm. Briefly, samples were passed through a 40 µM filter to remove cell and tissue clumps followed by two 14 ml washes with ddH_2O and incubation for at least 60 minutes in somatic cell lysis buffer (0.1% SDS, 0.5% Triton X in ddH_2O) at 4º C. Following somatic cell lysis and visual confirmation of the absence of contaminating cells, sperm DNA was extracted using the Qiagen AllPrep Universal kit. Samples in cell lysis buffer were passed through a 28-gauge syringe multiple times to disrupt sperm membranes and liberate nucleic acids prior to extraction.

Frontal cortex dissection and nucleic acid extraction:

Following euthanasia of F1 males (n = 8 per group), left brain hemispheres were dissected and placed in PaxGene tissue stabilizer and after 24 hours, fixed in PaxGene fixative and stored at -80º C. Samples were subsequently thawed and frontal cortex dissected under a stereo microscope according to the method described by Chiu et al. (48). Tissue was then disrupted using a microcentrifuge

pestle, and RNA and DNA were extracted using the Qiagen AllPrep Universal kit according to manufacturer's protocols.

RRBS library construction:

Following DNA extraction, Bioo Scientific NEXTflex Bisulfite Library Prep Kit for Illumina Sequencing was used for library preparation. To maximize coverage, we employed two separate restriction digests with MspI and TaqαI. Following digestion, products were pooled, and Klenow Fragment was utilized to create 3'A overhangs. DNA was subsequently purified with Zymo DNA Clean and Concentrate Columns followed by ligation of Methylated Illumina PE Adapters and Ampure purification with SPRI beads. Purified products were Sodium Bisulfite Converted using ZymoResearch EZ DNA Methylation Gold Kit, and libraries were amplified over 20 cycles using Platinum Taq DNA polymerase, followed by a final Ampure purification and confirmation of library size range on a 2% agarose gel. DNA was submitted to the Huntsman Cancer Institute High Throughput genomic core for sequencing on a Hi-Seq 2500 using 50 cycle-single read chemistry. Four to six samples were sequenced per lane for a minimum of 35-million reads per sample.

RNAseq library construction:

RNA extracted from F1 frontal cortices (n = 8 per group) was subjected to Illumina TruSeq Stranded RNA kit with Ribo-Zero Gold library preparation and subsequently sequenced on a Hi-Seq 2500 using 50 Cycle-Single Read

Sequencing v4. Eight samples were sequenced per lane for a minimum of 25-million reads per sample.

Bioinformatics analyses:

For genome wide DNA methylation analysis, sequence data from RRBS libraries was aligned to the mouse mm10 genome using the Bismark pipeline with special attention to RRBS specific issues, as noted in the Bismark User Guide and the Bismark RRBS Guide. Only CpGs where read coverage was greater than 8 for at least 4 biological replicates were considered “scoreable” for downstream analysis. Only CpGs with more than 5% change in methylation relative to control samples were classified as differentially methylated. When considering DMRs, only regions greater than 50 base-pairs in length with 3 or more scorable CpGs were analyzed. Then, one third of the CpGs within each analyzed region needed to be differentially methylated in order for a given region to be under consideration as a DMR. Finally, qualifying regions were classified as bonafide DMRs if there was more than 5% change in methylation relative to control samples. For genome wide gene expression analysis, sequencing data from RNASeq libraries was aligned using Novoalign. Aligned splice junction were converted to genomic coordinates and low quality and non-unique reads were further parsed using SamTranscriptomeParser (USeq; v8.8.8) under default settings. Stranded differential expression analysis was calculated with the USeq program DefinedRegionDifferentialSeq, which utilizes DESeq2 and the reference mm10. Normalized read count tables were then analyzed in R, along with all

539 DNA methylation data. Integration and parsing of bed files or tables was
540 performed in R. Generation of all figures and statistical analyses was
541 accomplished using standard methods in R, with the exception of aggregate
542 histone modification profiles, which were generated using Deeptools. Gene
543 ontology analysis was performed using DAVID Functional Annotation
544 Bioinformatics Resources.

545 **REFERENCES**

546 1. Oberg M, Jaakkola MS, Woodward A, Peruga A, Pruss-Ustun A. Worldwide
547 burden of disease from exposure to second-hand smoke: a retrospective analysis of
548 data from 192 countries. Lancet. 2011;377(9760):139-46.
549 2. DiFranza JR, Aligne CA, Weitzman M. Prenatal and postnatal environmental
550 tobacco smoke exposure and children's health. Pediatrics. 2004;113(4 Suppl):1007-
551 15.
552 3. Moritsugu KP. The 2006 Report of the Surgeon General: the health
553 consequences of involuntary exposure to tobacco smoke. Am J Prev Med.
554 2007;32(6):542-3.
555 4. Laubenthal J, Zlobinskaya O, Poterlowicz K, Baumgartner A, Gdula MR,
556 Fthenou E, et al. Cigarette smoke-induced transgenerational alterations in genome
557 stability in cord blood of human F1 offspring. FASEB journal : official publication of
558 the Federation of American Societies for Experimental Biology. 2012;26(10):3946-
559 56.
560 5. Rolland M, Le Moal J, Wagner V, Royere D, De Mouzon J. Decline in semen
561 concentration and morphology in a sample of 26 609 men close to general
562 population between 1989 and 2005 in France. Human reproduction. 2012.
563 6. Priskorn L, Holmboe SA, Jacobsen R, Jensen TK, Lassen TH, Skakkebaek NE.
564 Increasing trends in childlessness in recent birth cohorts - a registry-based study of
565 the total Danish male population born from 1945 to 1980. International journal of
566 andrology. 2012;35(3):449-55.
567 7. Swan SH, Elkin EP, Fenster L. Have sperm densities declined? A reanalysis of
568 global trend data. Environmental health perspectives. 1997;105(11):1228-32.
569 8. Ford ES, Giles WH, Mokdad AH. Increasing prevalence of the metabolic
570 syndrome among u.s. Adults. Diabetes Care. 2004;27(10):2444-9.
571 9. Ford ES, Giles WH, Dietz WH. Prevalence of the metabolic syndrome among
572 US adults: findings from the third National Health and Nutrition Examination
573 Survey. JAMA : the journal of the American Medical Association. 2002;287(3):356-9.
574 10. Kiziler AR, Aydemir B, Onaran I, Alici B, Ozkara H, Gulyasar T, et al. High
575 levels of cadmium and lead in seminal fluid and blood of smoking men are

576 associated with high oxidative stress and damage in infertile subjects. Biological
577 trace element research. 2007;120(1-3):82-91.
578 11. Kulikauskas V, Blaustein D, Ablin RJ. Cigarette smoking and its possible
579 effects on sperm. Fertility and sterility. 1985;44(4):526-8.
580 12. Cupul-Uicab LA, Skjaerven R, Haug K, Melve KK, Engel SM, Longnecker MP. In
581 utero exposure to maternal tobacco smoke and subsequent obesity, hypertension,
582 and gestational diabetes among women in the MoBa cohort. Environmental health
583 perspectives. 2012;120(3):355-60.
584 13. Behl M, Rao D, Aagaard K, Davidson TL, Levin ED, Slotkin TA, et al. Evaluation
585 of the Association between Maternal Smoking, Childhood Obesity, and Metabolic
586 Disorders: A National Toxicology Program Workshop Review. Environmental health
587 perspectives. 2012.
588 14. Polyzos A, Schmid TE, Pina-Guzman B, Quintanilla-Vega B, Marchetti F.
589 Differential sensitivity of male germ cells to mainstream and sidestream tobacco
590 smoke in the mouse. Toxicology and applied pharmacology. 2009;237(3):298-305.
591 15. Fuentes A, Munoz A, Barnhart K, Arguello B, Diaz M, Pommer R. Recent
592 cigarette smoking and assisted reproductive technologies outcome. Fertility and
593 sterility. 2010;93(1):89-95.
594 16. Marchetti F, Rowan-Carroll A, Williams A, Polyzos A, Berndt-Weis ML, Yauk
595 CL. Sidestream tobacco smoke is a male germ cell mutagen. Proceedings of the
596 National Academy of Sciences of the United States of America. 2011;108(31):12811-
597 4.
598 17. Glen CD, Dubrova YE. Exposure to anticancer drugs can result in
599 transgenerational genomic instability in mice. Proceedings of the National Academy
600 of Sciences of the United States of America. 2012;109(8):2984-8.
601 18. Dubrova YE, Hickenbotham P, Glen CD, Monger K, Wong HP, Barber RC.
602 Paternal exposure to ethylnitrosourea results in transgenerational genomic
603 instability in mice. Environmental and molecular mutagenesis. 2008;49(4):308-11.
604 19. Barber RC, Dubrova YE. The offspring of irradiated parents, are they stable?
605 Mutation research. 2006;598(1-2):50-60.
606 20. Dubrova YE. Radiation-induced transgenerational instability. Oncogene.
607 2003;22(45):7087-93.
608 21. Barber R, Plumb MA, Boulton E, Roux I, Dubrova YE. Elevated mutation rates
609 in the germ line of first- and second-generation offspring of irradiated male mice.
610 Proceedings of the National Academy of Sciences of the United States of America.
611 2002;99(10):6877-82.
612 22. Secretan B, Straif K, Baan R, Grosse Y, El Ghissassi F, Bouvard V, et al. A
613 review of human carcinogens--Part E: tobacco, areca nut, alcohol, coal smoke, and
614 salted fish. Lancet Oncol. 2009;10(11):1033-4.
615 23. Jenkins TG, James ER, Alonso DF, Hoidal JR, Murphy PJ, Hotaling JM, et al.
616 Cigarette smoking significantly alters sperm DNA methylation patterns. Andrology.
617 2017.
618 24. Kohli A, Garcia MA, Miller RL, Maher C, Humblet O, Hammond SK, et al.
619 Secondhand smoke in combination with ambient air pollution exposure is
620 associated with increasedx CpG methylation and decreased expression of IFN-

621 gamma in T effector cells and Foxp3 in T regulatory cells in children. Clin
622 Epigenetics. 2012;4(1):17.
623 25. Bosse Y, Postma DS, Sin DD, Lamontagne M, Couture C, Gaudreault N, et al.
624 Molecular signature of smoking in human lung tissues. Cancer research.
625 2012;72(15):3753-63.
626 26. Word B, Lyn-Cook LE, Jr., Mwamba B, Wang H, Lyn-Cook B, Hammons G.
627 Cigarette Smoke Condensate Induces Differential Expression and Promoter
628 Methylation Profiles of Critical Genes Involved in Lung Cancer in NL-20 Lung Cells In
629 Vitro: Short-Term and Chronic Exposure. International journal of toxicology. 2012.
630 27. Breton CV, Byun HM, Wenten M, Pan F, Yang A, Gilliland FD. Prenatal tobacco
631 smoke exposure affects global and gene-specific DNA methylation. Am J Respir Crit
632 Care Med. 2009;180(5):462-7.
633 28. Perera F, Herbstman J. Prenatal environmental exposures, epigenetics, and
634 disease. Reproductive toxicology. 2011;31(3):363-73.
635 29. Joubert BR, Haberg SE, Nilsen RM, Wang X, Vollset SE, Murphy SK, et al. 450K
636 epigenome-wide scan identifies differential DNA methylation in newborns related to
637 maternal smoking during pregnancy. Environmental health perspectives.
638 2012;120(10):1425-31.
639 30. Holland ML, Lowe R, Caton PW, Gemma C, Carbajosa G, Danson AF, et al.
640 Early-life nutrition modulates the epigenetic state of specific rDNA genetic variants
641 in mice. Science. 2016;353(6298):495-8.
642 31. Brieno-Enriquez MA, Garcia-Lopez J, Cardenas DB, Guibert S, Cleroux E, Ded
643 L, et al. Exposure to endocrine disruptor induces transgenerational epigenetic
644 deregulation of microRNAs in primordial germ cells. PLoS One.
645 2015;10(4):e0124296.
646 32. Dai J, Wang Z, Xu W, Zhang M, Zhu Z, Zhao X, et al. Paternal nicotine exposure
647 defines different behavior in subsequent generation via hyper-methylation of mmu-
648 miR-15b. Sci Rep. 2017;7(1):7286.
649 33. Hawkey AB, White H, Pippen E, Greengrove E, Rezvani AH, Murphy SK, et al.
650 Paternal nicotine exposure in rats produces long-lasting neurobehavioral effects in
651 the offspring. Neurotoxicol Teratol. 2019;74:106808.
652 34. Pachenari N, Azizi H, Semnaniann S. Adolescent Morphine Exposure in Male
653 Rats Alters the Electrophysiological Properties of Locus Coeruleus Neurons of the
654 Male Offspring. Neuroscience. 2019;410:108-17.
655 35. Levin ED, Hawkey AB, Hall BJ, Cauley M, Slade S, Yazdani E, et al. Paternal
656 THC exposure in rats causes long-lasting neurobehavioral effects in the offspring.
657 Neurotoxicol Teratol. 2019;74:106806.
658 36. Venugopal R, Jaiswal AK. Nrf2 and Nrf1 in association with Jun proteins
659 regulate antioxidant response element-mediated expression and coordinated
660 induction of genes encoding detoxifying enzymes. Oncogene. 1998;17(24):3145-56.
661 37. Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, et al. Transcription
662 factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in
663 macrophages. J Biol Chem. 2000;275(21):16023-9.
664 38. Jung YH, Sauria MEG, Lyu X, Cheema MS, Ausio J, Taylor J, et al. Chromatin
665 States in Mouse Sperm Correlate with Embryonic and Adult Regulatory Landscapes.
666 Cell Rep. 2017;18(6):1366-82.

667 39. Vallaster MP, Kukreja S, Bing XY, Ngolab J, Zhao-Shea R, Gardner PD, et al.
668 Paternal nicotine exposure alters hepatic xenobiotic metabolism in offspring. Elife.
669 2017;6.
670 40. Zhang W, Yang J, Lv Y, Li S, Qiang M. Paternal benzo[a]pyrene exposure alters
671 the sperm DNA methylation levels of imprinting genes in F0 generation mice and
672 their unexposed F1-2 male offspring. Chemosphere. 2019;228:586-94.
673 41. Viluksela M, Pohjanvirta R. Multigenerational and Transgenerational Effects
674 of Dioxins. Int J Mol Sci. 2019;20(12).
675 42. McCarthy DM, Morgan TJ, Jr., Lowe SE, Williamson MJ, Spencer TJ, Biederman
676 J, et al. Nicotine exposure of male mice produces behavioral impairment in multiple
677 generations of descendants. PLoS Biol. 2018;16(10):e2006497.
678 43. Tsaprouni LG, Yang TP, Bell J, Dick KJ, Kanoni S, Nisbet J, et al. Cigarette
679 smoking reduces DNA methylation levels at multiple genomic loci but the effect is
680 partially reversible upon cessation. Epigenetics. 2014;9(10):1382-96.
681 44. Zeilinger S, Kuhnel B, Klopp N, Baurecht H, Kleinschmidt A, Gieger C, et al.
682 Tobacco smoking leads to extensive genome-wide changes in DNA methylation.
683 PLoS One. 2013;8(5):e63812.
684 45. Busche S, Shao X, Caron M, Kwan T, Allum F, Cheung WA, et al. Population
685 whole-genome bisulfite sequencing across two tissues highlights the environment
686 as the principal source of human methylome variation. Genome Biol. 2015;16:290.
687 46. Portela A, Esteller M. Epigenetic modifications and human disease. Nat
688 Biotechnol. 2010;28(10):1057-68.
689 47. Barrett EG, Wilder JA, March TH, Espindola T, Bice DE. Cigarette smoke-
690 induced airway hyperresponsiveness is not dependent on elevated immunoglobulin
691 and eosinophilic inflammation in a mouse model of allergic airway disease. Am J
692 Respir Crit Care Med. 2002;165(10):1410-8.
693 48. Chiu K, Lau WM, Lau HT, So KF, Chang RC. Micro-dissection of rat brain for
694 RNA or protein extraction from specific brain region. J Vis Exp. 2007(7):269.
695

696

TABLE 1 - Sperm characteristics, growth rates and conception data for F0 smoke-exposed and control animals

	M/ml sperm	Total motility	Days to conception	# conceived w/in 1 week	# conceived w/in 2 weeks	# conceived w/in 3 weeks	# conceived w/in 4 weeks	# never conceived	Pre-smoke weight	Weight after 4 wks treatment	Weight after 8 wks treatment
CN	48.4	58.7	6.5	9.0	9.0	10.0	10.0	2.0	20.1	24.9	27.7
SN	40.2	56.5	11.8	3.0	3.0	4.0	5.0	6.0	21.4	21.1	22.7
CN vs SN p val	0.110	0.526	0.204	**0.039**	**0.039**	**0.036**	0.089	0.193	0.247	**0.001**	**<0.0001**
CWT	55.2	55.4	5.8	6.0	8.0	8.0	8.0	4.0	21.6	25.8	28.6
SWT	51.8	54.8	5.4	6.0	6.0	7.0	7.0	5.0	21.0	21.1	22.4
CWT vs SWT p val	0.618	0.868	0.908	1.000	0.608	1.000	1.000	1.000	**0.029**	**<0.0001**	**<0.0001**
NRF	44.5	57.6	8.3	12.0	12.0	14.0	15.0	8.0	20.9	23.0	25.2
WT	53.5	55.1	5.6	12.0	14.0	15.0	15.0	9.0	21.3	23.2	25.2
NRF vs WT p val	**0.039**	0.306	0.261	1.000	0.773	1.000	1.000	1.000	0.444	0.757	0.975
Smoke	46.2	55.6	8.1	9.0	9.0	11.0	12.0	11.0	21.0	21.1	22.5
No Smoke	51.8	57.0	6.2	15.0	17.0	18.0	18.0	6.0	21.4	25.6	28.4
Smoke vs No Smoke p val	0.207	0.556	0.432	0.148	**0.042**	0.075	0.135	0.135	0.221	**<0.0001**	**<0.0001**

Abbreviations: CN-control *Nrf*$^{-/-}$, SN-smoke-exposed *Nrf*$^{-/-}$, CWT-control wild type animals, SWT-smoke-exposed wild type animals.

705 **TABLE 2 –** Sperm characteristics, litter size and growth rates for F1 offspring of smoke-

706 exposed and control animals

	Litter size	M/ml sperm	Total sperm motility	Postnatal weight (g) week 4	Postnatal weight (g) week 6	Postnatal weight (g) week 10
CN	5.3	25.7	36.1	11.6	16.4	17.4
SN	6.0	26.5	33.0	12.3	16.6	18.7
CN vs SN p val	0.379	0.851	0.409	0.351	0.727	0.100
CWT	5.0	31.3	27.3	12.1	16.2	17.4
SWT	5.2	33.6	34.0	12.7	16.8	17.8
CWT vs SWT p val	0.723	0.708	0.207	0.254	0.260	0.354
NRF	5.5	26.0	34.0	11.8	16.4	17.6
WT	5.1	32.5	30.5	12.5	16.4	17.5
NRF vs WT p val	0.356	0.063	0.264	0.194	0.979	0.828
Smoke	5.6	29.8	32.0	12.5	16.7	18.0
No Smoke	5.2	27.3	33.2	11.7	16.3	17.4
Smoke vs No Smoke p val	0.424	0.469	0.700	0.104	0.288	0.105

707

708 Sperm characteristics, litter size and growth rates for F1 offspring of smoke-exposed and control

709 animals. Abbreviations: CN-control *Nrf*$^{-/-}$, SN-smoke-exposed *Nrf*$^{-/-}$, CWT-control wild type

710 animals, SWT-smoke-exposed wild type animals.

711

712

713 **Figure 1 Schematic of study design.** Six to seven-week-old WT and *Nrf2*$^{-/-}$
714 mice were assigned to one of two groups: CS-exposed and non-exposed
715 controls. Following 60 days of CS exposure, mice were bred to unexposed
716 CAST/EiJ female mice. Groups of animals were euthanized and tissues collected
717 3, 28, 103, and 171 days after removal from CS exposure. Sperm DNA
718 methylation analysis was performed by RRBS on F0 exposed and control
719 animals. Offspring derived from exposed and control C57BL/6J males were
720 euthanized at 14-17 weeks of age, and tissues were collected. DNA methylation
721 analysis was performed on sperm and frontal cortex to investigate the impact of
722 paternal smoking status on methylation patterns in offspring. In addition, RNAseq
723 was performed on frontal cortex tissue to investigate the association between
724 paternal smoking and neural gene expression.
725
726 **Figure 2 Summary of the recovery, persistence or emergence of**
727 **differentially methylated loci (A and B) and regions (C and D) following a**
728 **recovery period.** A) Quantitative data indicate that the number of differentially
729 methylated loci in CS-exposed mice compared with age matched controls does
730 not diminish following a recovery period of up to 171 days. B) When considering
731 only loci that lost methylation in the CS-exposed group, about one third of
732 differentially methylated loci persisted for the entire recovery period (white), one
733 third returned to baseline levels (blue) and one third emerged as differentially
734 methylated following a recovery period (pink). Contrastingly, for loci that gained
735 methylation as a result of CS exposure, a smaller fraction of differentially

methylated CpGs persisted of recovered while nearly half of differentially methylated loci emerged during the recovery period. C) Unlike the case of recovery at the CpG level, when differentially methylated regions were analyzed, a significant proportion (about half) of regions recovered within 28 days without CS exposure, with no evidence for additional recovery following longer periods of recovery. D) The majority of DMRs observed prior to recovery were either maintained across the recovery period or returned to baseline levels, with only a small fraction of DMRs emerging during the recovery period. Of note, regions that gained methylation in CS-exposed animals were less likely to recover and more likely to emerge as a new DMR during the recovery period compared with DMRs that lost methylation. Timepoint 1 = 28-day recovery group, timepoint 2 = 103-day recovery group, and timepoint 3 = 171-day recovery group. All comparisons were to the 3-day recovery group.

Figure 3 DMR recovery and comparison of DMRs in WT mice with CS-exposed and control *Nrf2*$^{-/-}$ mice as well as previously published sperm DMRs. A) Impact of the initial methylation status and direction of change on methylation recovery. The hypomethylated DMRs (<25% DNAme) in which methylation increased and the hypermethylated DMRs (> 75% DNAme) in which methylation decreased with smoke exposure were more likely to recover. Regions displaying methylation between 25% and 75% Regions of intermediate DNAme were less likely to recover across all groups. Timepoint 1 = 28-day recovery group, timepoint 2 = 103-day recovery group, and timepoint 3 = 171-day

759 recovery group. All comparisons were to the 3-day recovery group. B) Heatmap
760 illustrating the significant similarity between CS-associated DMRs identified in
761 WT mice and DMRs associated with the *Nrf2*$^{-/-}$ genotype, apparently independent
762 of CS-exposure status. C) A high correlation in DMRs was observed between
763 CS-exposed WT mice and *Nrf2*$^{-/-}$ whether or not they were exposed to CS. No
764 correlation was observed between DMRs identified in the current study
765 compared with previously published DMRs associated with vinclozolin exposure
766 (VD2) and protein restricted diet (PR).
767
768 **Figure 4 Impact of paternal CS-exposure and genotype on F1 brain and**
769 **sperm DNAme.** A) A highly significant correlation was observed in F1 brain
770 DNAme changes induced by CS exposure in WT sires (x-axis) compared with
771 DNAme changes associated *Nrf2* status irrespective of CS exposure (left 2
772 panels), however the correlation disappeared when evaluating differential
773 methylation in *Nrf2*$^{-/-}$ offspring based on CS exposure status compared with CS-
774 associated DNAme changes in offspring sired by WT mice. B) The DNAme
775 changes observed in F0 sperm were not observed in the sperm of F1 offspring,
776 suggesting the CS-associated effects likely do not confer risk transgenerationally.
777 C) Likewise, no significant overlap was observed in differentially methylated
778 regions in F0 sperm compared with differentially methylated regions in F1 frontal
779 cortex.
780

Figure 5 Frontal cortex gene expression variation and correlation between WT and *Nrf2*$^{-/-}$ offspring in relation to smoking status. A) Variation in F1 frontal cortex gene expression was significantly higher in WT CS-exposed animals compared with WT controls (left panel) suggesting stochastic dysregulation of gene expression in paternal CS-exposed offspring and offspring of mice with reduced antioxidant capacity. Likewise, variation was significantly higher in *Nrf2*$^{-/-}$ controls compared with WT controls (right panel). B) A significant correlation was observed between differentially expressed genes associated with *Nrf2*$^{-/-}$ genotype, with (left panel) or without CS exposure (center panel) and CS-exposure-induced differential gene expression. There is no association gene expression impacted by smoking status in *Nrf2*$^{-/-}$ offspring (right panel). C) Gene ontology analysis of significantly upregulated and D) downregulated genes associated with paternal CS exposure indicated overrepresentation of several gene families.

Figure S1 Scatter plots of CpG-level and regional sperm DNAme in CS-exposed vs control WT mice across the recovery timeline. A) DNAme at all CpGs across the genome B) Regional DNAme comparisons. Broad genome-wide changes in DNAme, either at individual CpGs of within genomic regions, are not observed. Further, the methylation at individual CpGs deviates much more broadly than regional DNAme, supporting the role of increased CpG density in buffering environmentally-induced DNAme. Smoked WT = samples collected 3

803 days after removal from CS, recovery 1 = 28-day recovery group, recovery 2 =
804 103-day recovery group, and recovery 3 = 171-day recovery group.
805
806 **Figure S2 Scatter plots of regional sperm DNAme for control and smoked**
807 **NRF versus control and smoked WT.** Like the distributions observed in figure
808 S1, these plots indicate that CS exposure and *Nrf*$^{-/-}$ do not induce broad genome-
809 wide changes in sperm DNA methylation.
810
811 **Figure S3 DNAme variation in shared, recovered and new DMRs as a**
812 **function of CpG density across recovery timepoints.** A) In every category of
813 DMR (shared, recovery or new) variation diminished as CpG density of a region
814 increased. The impact of CpG density on variation was particularly apparent for
815 regions in which DNAme decreased as a result of CS exposure and later
816 recovered to baseline. B) DMRs that displayed increased DNAme in CS-exposed
817 animals and later recovered were generally regions of lower CpG density, while
818 DMRs that gained methylation and subsequently recovered were generally at
819 regions of higher CpG density. Timepoint 1 = 28-day recovery group, timepoint 2
820 = 103-day recovery group, and timepoint 3 = 171-day recovery group. All
821 comparisons were to the 3-day recovery group.
822
823 **Figure S4 Chromatin properties at F0 sperm DMRs and variation in F1**
824 **frontal cortex gene expression.** A) Chromatin accessibility was not predictive
825 of the propensity of DMRs to recover or be maintained after removal of CS

826 exposure. B and C) Localization of H3K4me3 and H3K27me3 was likewise not
827 associated with DMR recovery or maintenance. D) Scatter plot of F1 frontal
828 cortex variation in gene expression in CS-exposed versus control *Nrf*$^{-/-}$ mice,
829 demonstrating that paternal CS exposure did not further elevate the increased
830 gene expression variation in *Nrf*$^{-/-}$ offspring beyond the effects of genotype alone
831 illustrated in Figure 5A. E) Scatter plot of F1 frontal cortex gene expression
832 variation in CS-exposed WT offspring versus control *Nrf*$^{-/-}$ demonstrating that
833 control *Nrf*$^{-/-}$ offspring exhibit a similar degree of variation as CS-exposed WT
834 animals.
835

LIST OF ABBREVIATIONS: DMR-differentially methylated regions; DNAme-DNA methylation; CS-cigarette smoke; CN-control *Nrf2*$^{-/-}$; SN-smoke-exposed *Nrf2*$^{-/-}$, CWT-control wild type animals; SWT-smoke-exposed wild type; VD2-vinclozolin exposure; PR-protein restricted diet; ESTR-expanded simple tandem repeat; NRF2- nuclear factor (erythroid-derived 2)-like 2; GO-gene ontology

Ethics approval and consent to participate: All animal experiments were performed under protocols that were approved by the University of Utah Institutional Animal Care and Use Committee (protocol # 14-11006).

Consent for publication: Not applicable

Availability of data and material: The datasets used and/or analyzed during the current study have been deposited in NCBI's Gene Expression Omnibus
are publicly available GEO Submission (GSE133742) [NCBI tracking system and are accessible through GEO Series accession number GSE133742 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133742).
Sperm DNA methylation datasets used for comparison to data generated in the current study are likewise available through GEO: GSE7261 and GSE65784.

Competing interests: The authors declare that they have no competing interests.

Funding: This work was supported by a grant from the Eunice Kennedy Shriver National Institute of Child Health and Human Development, USA (R01HD082062).

Authors' contributions: PJM directed RRBS experiments, analyzed RRBS and RNAseq datasets and assisted in manuscript preparation. JG analyzed RRBS and RNAseq datasets, prepared figures and assisted in manuscript preparation. TGJ assisted in study design and animal experiments as well as data analysis and interpretation. JRH assisted with initial study design, and data interpretation, TH assisted with animal experiments including CS treatments and animal phenotyping. JMH assisted with study design and manuscript preparation. DTC assisted with initial study design, data interpretation and manuscript preparation. BRC assisted with study design, data analysis and interpretation and manuscript preparation. KIA assisted in study design, animal experiments, molecular experiments and data analysis and took the primary role in manuscript preparation. All authors read and approved the final manuscript.

Acknowledgements: Not applicable

Figure 1

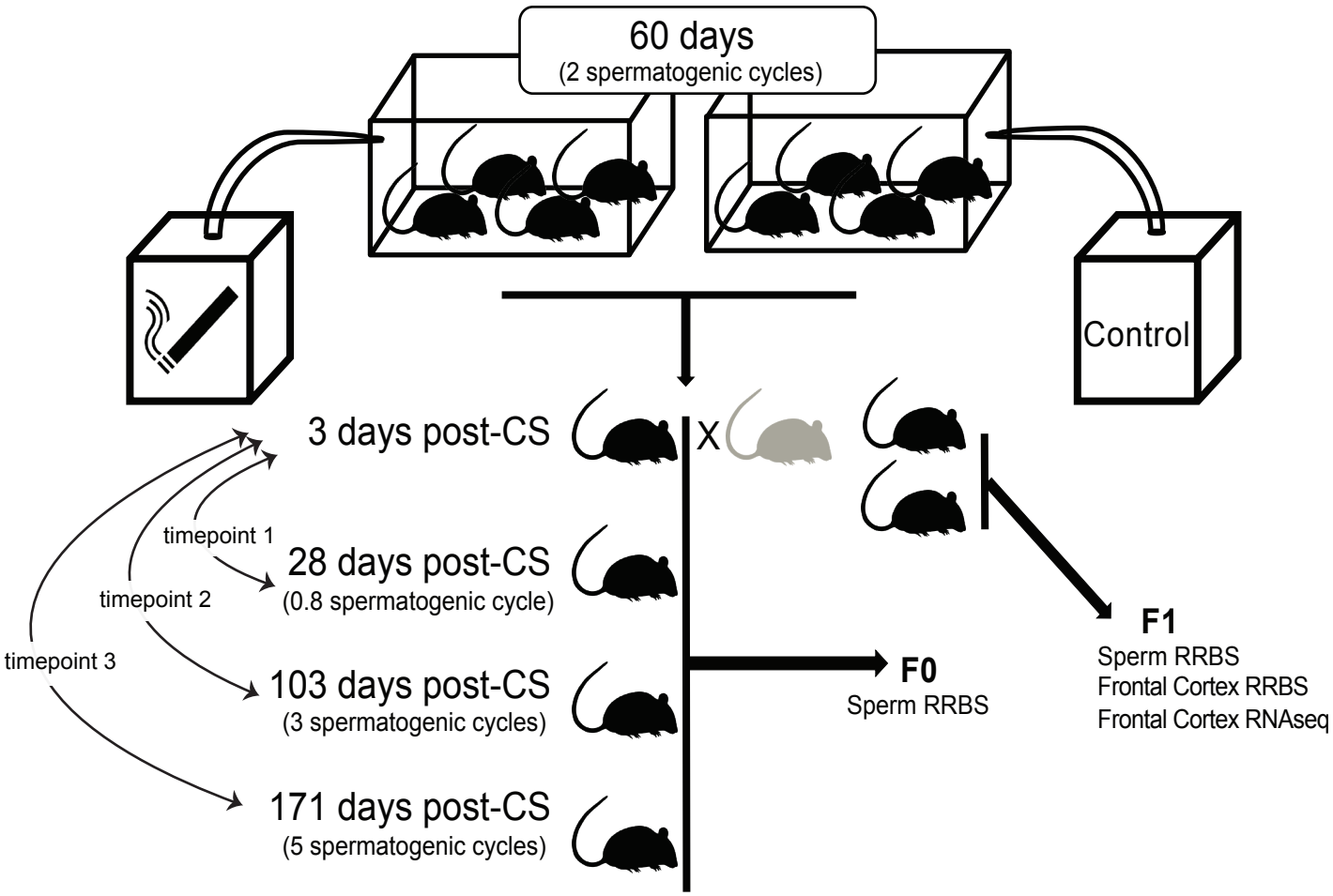


Figure 2

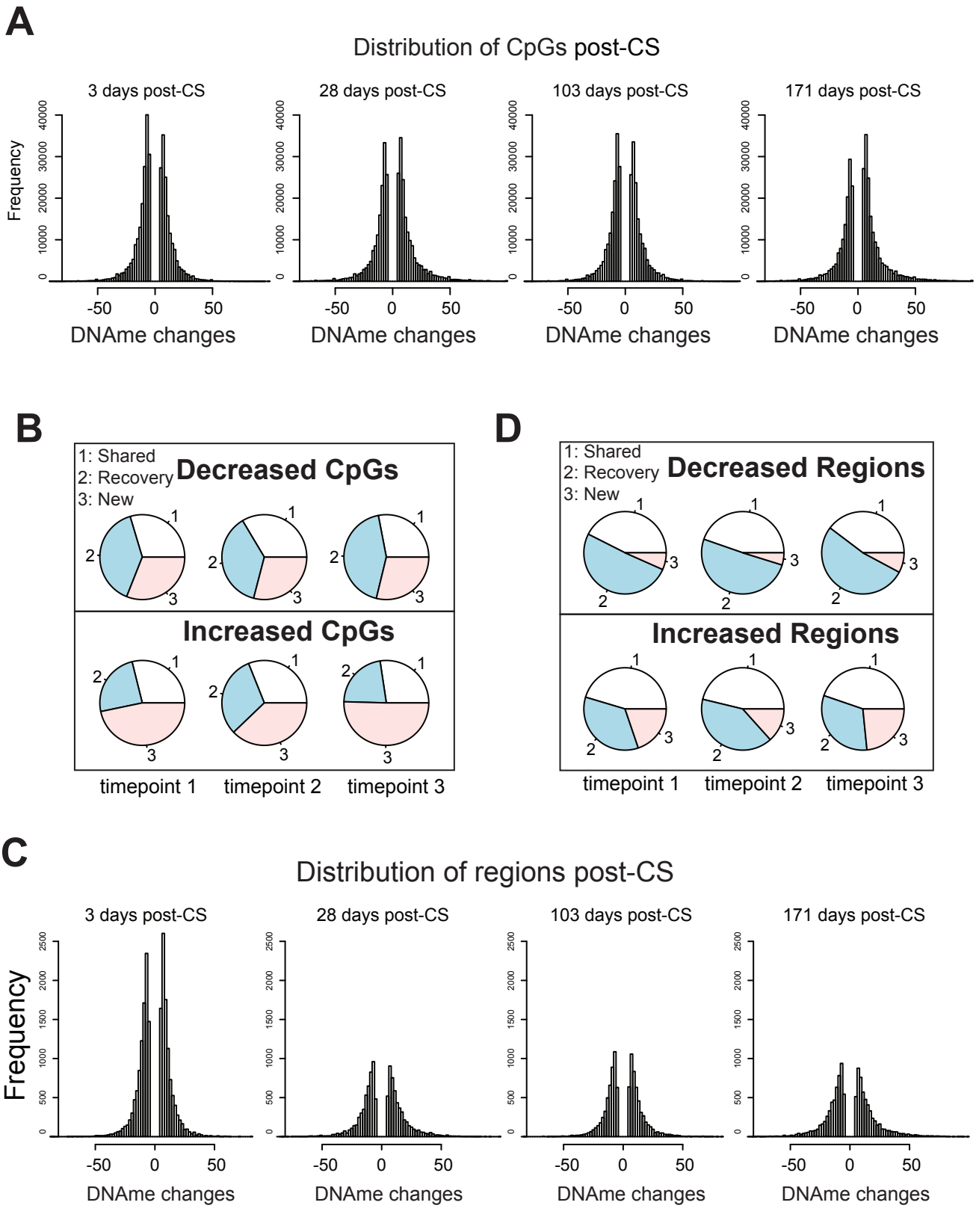


Figure 3

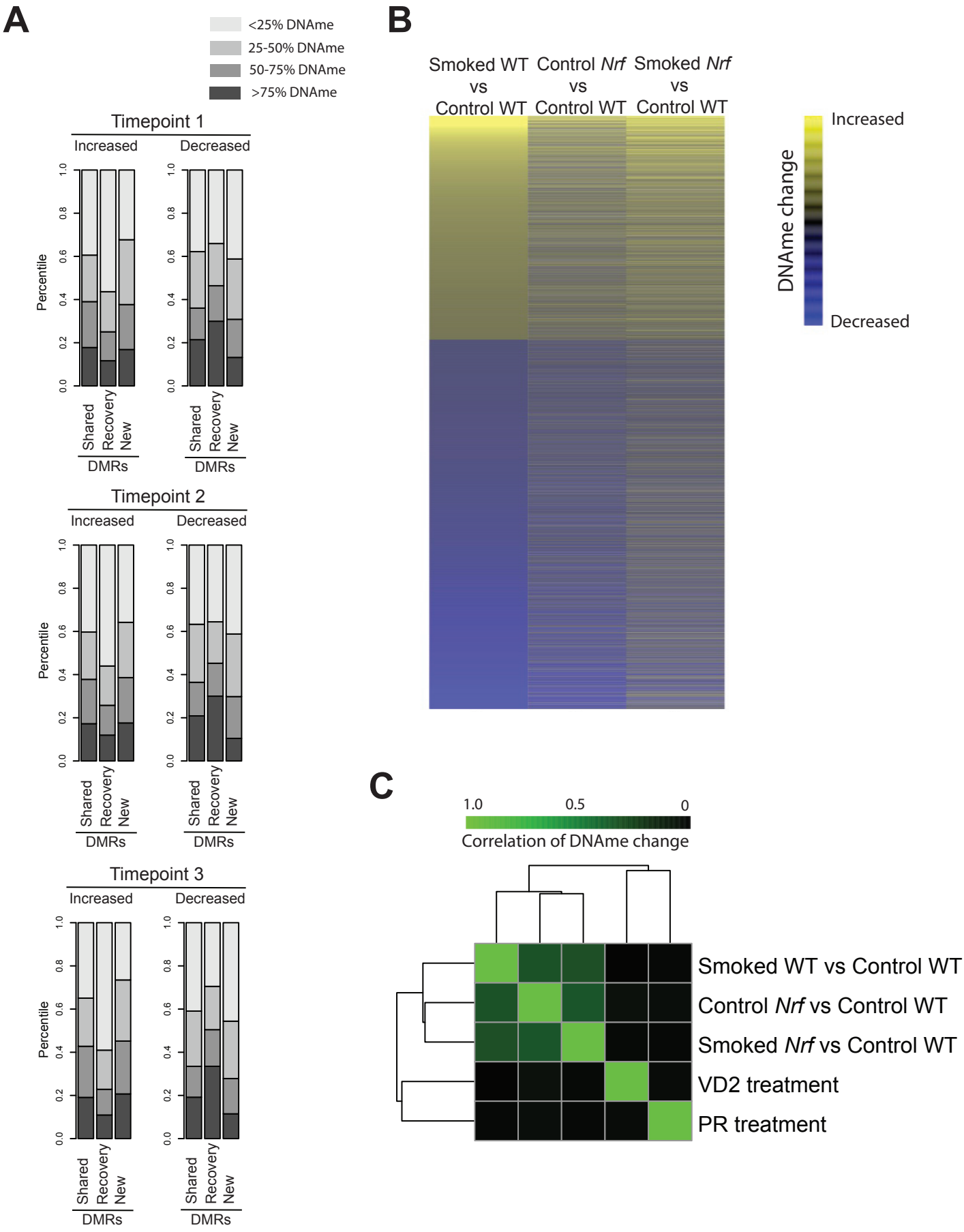


Figure 4

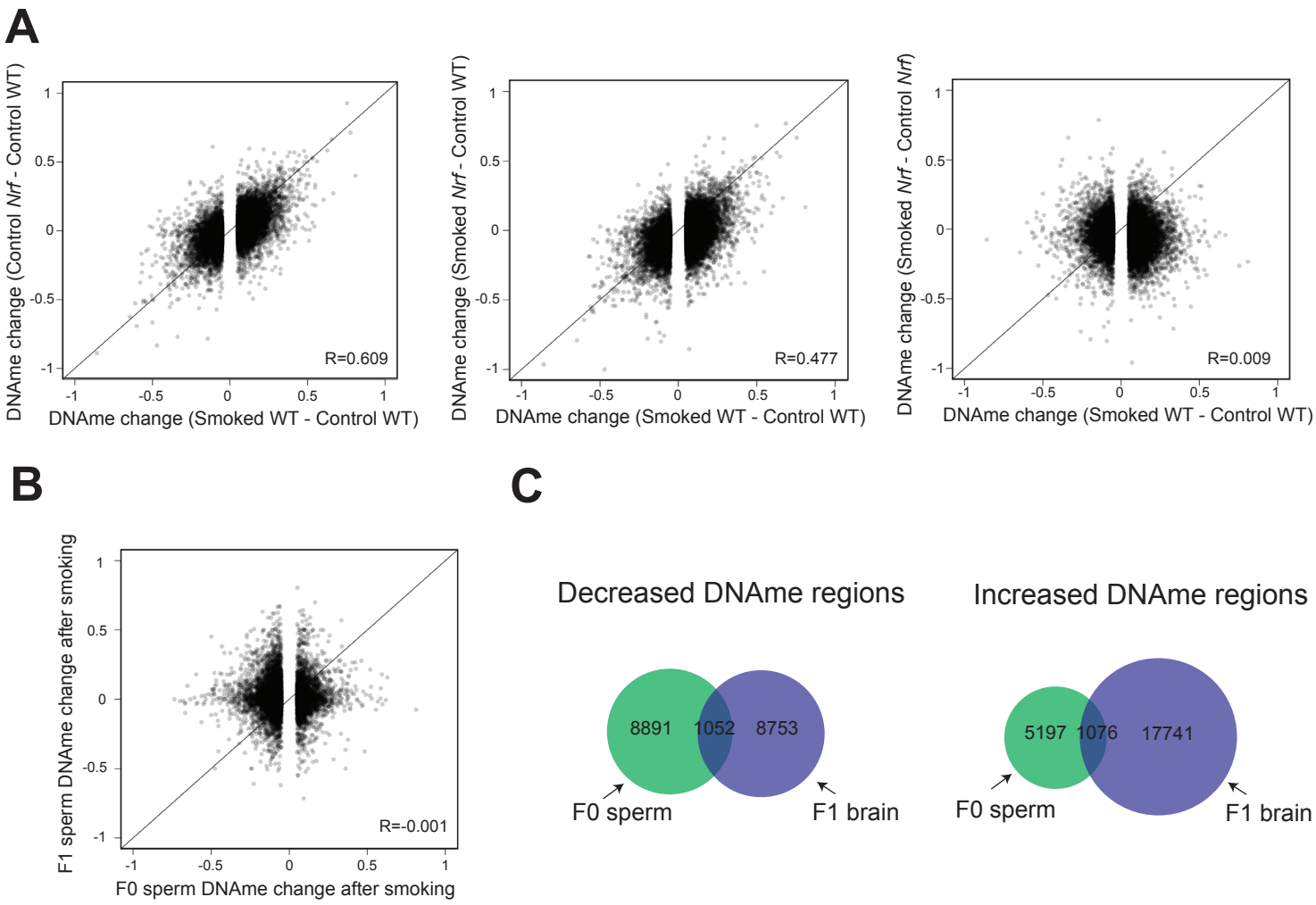


Figure 5

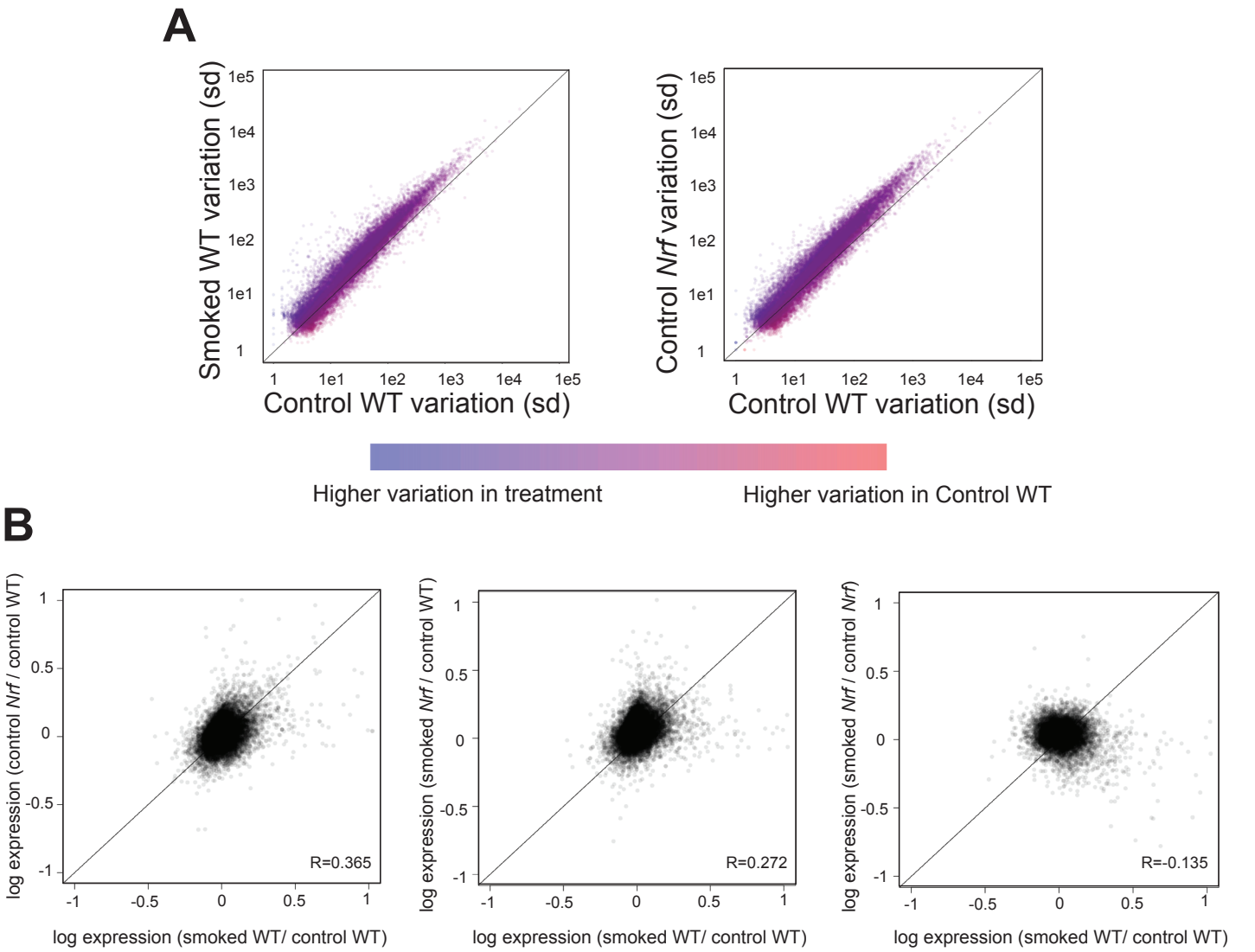


Figure S1

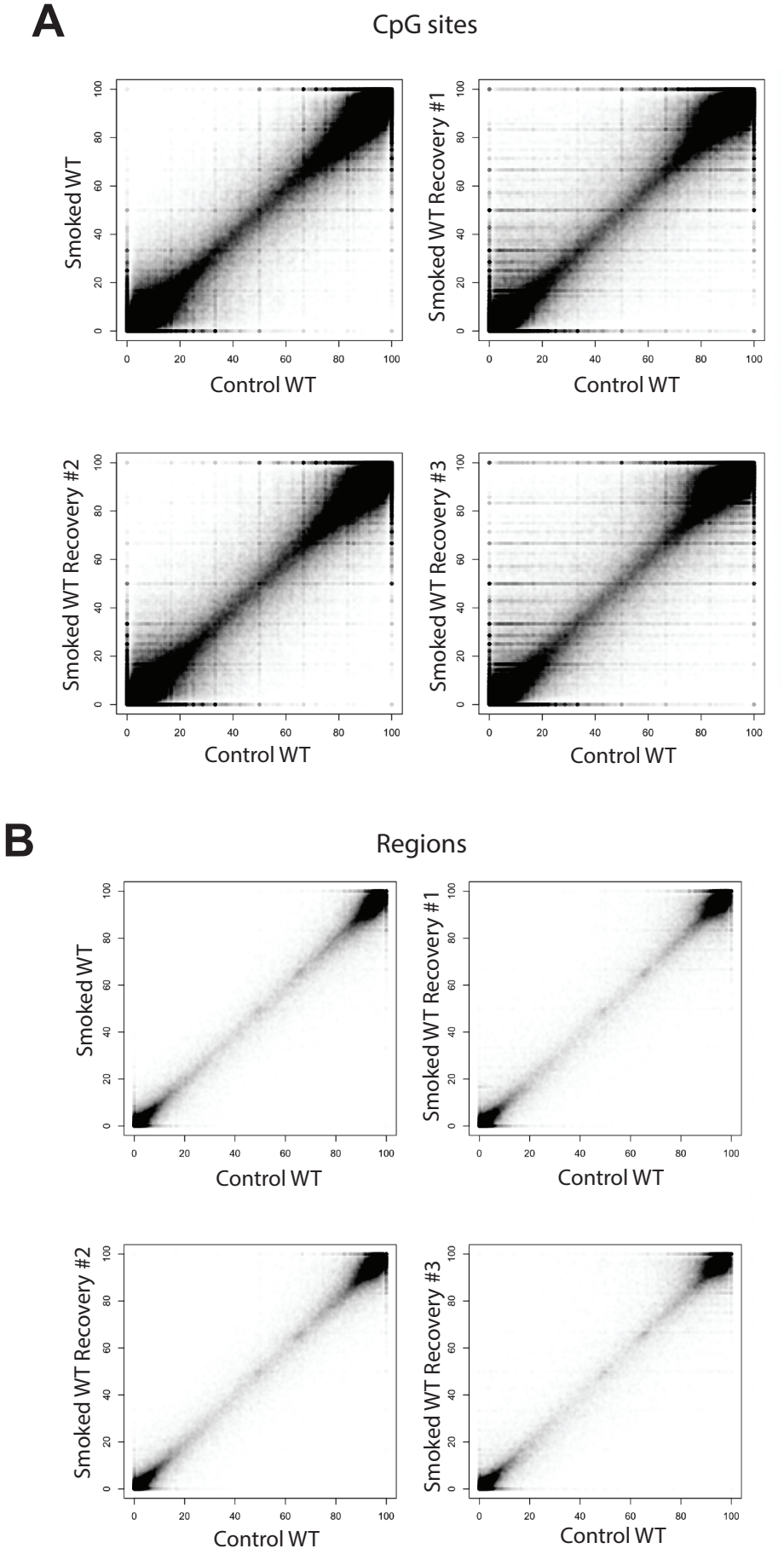
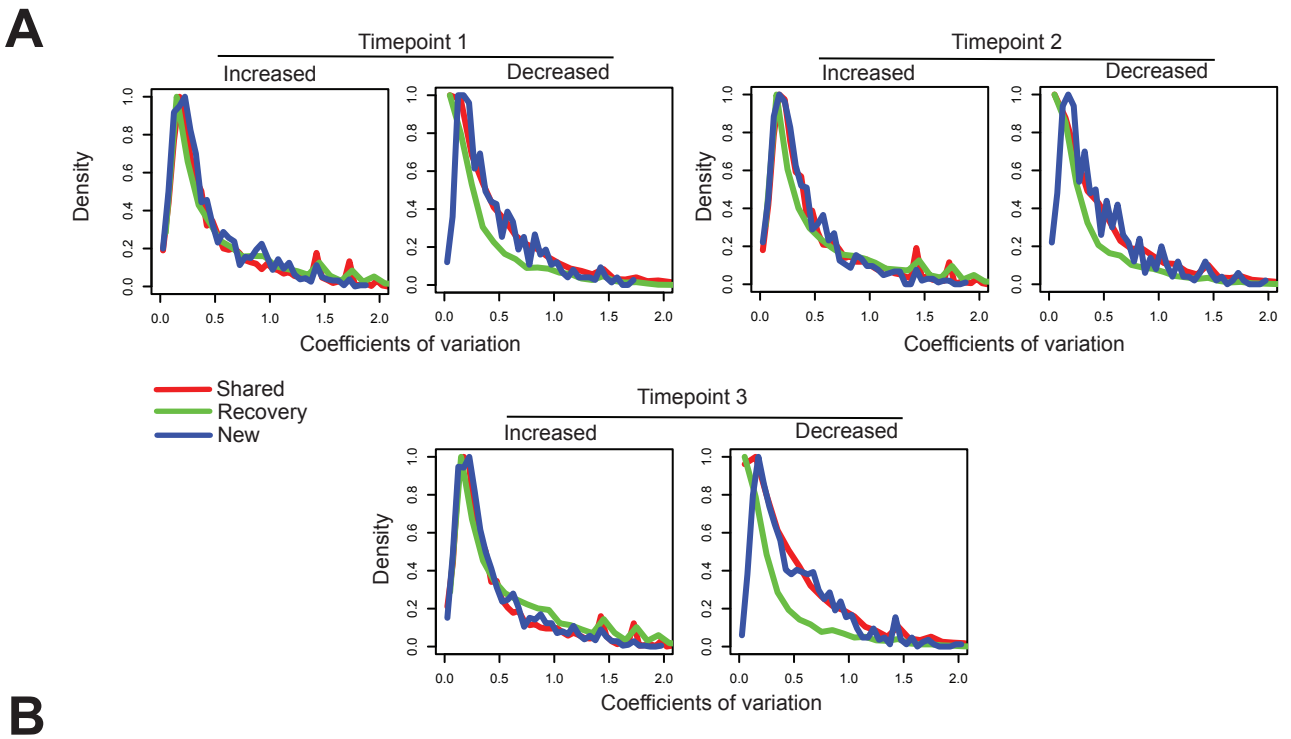


Figure S2



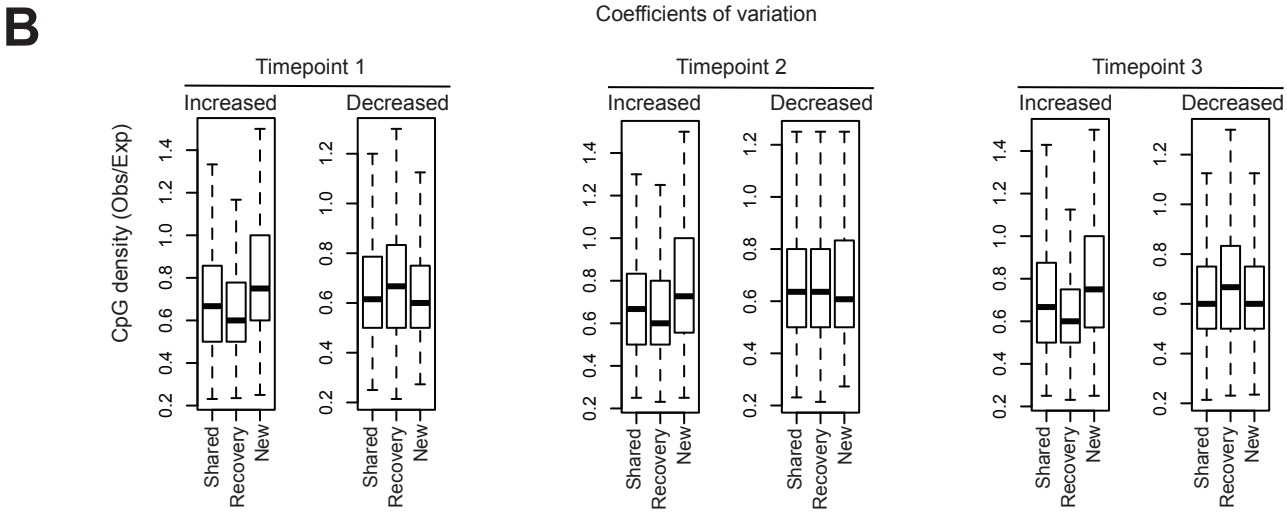


Figure S3

A

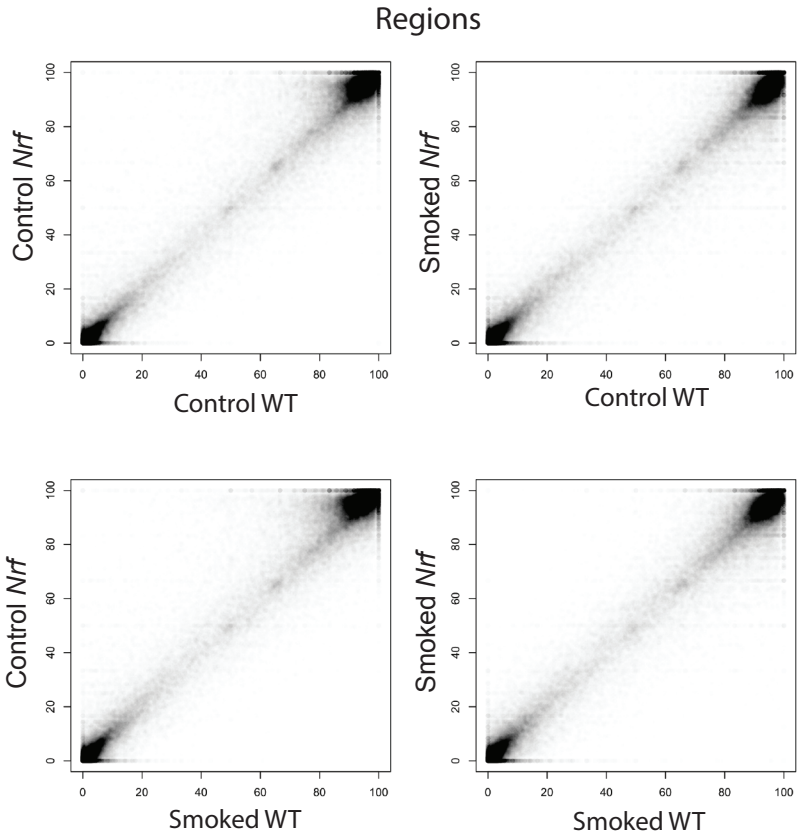


Figure S4