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Adolescent chronic intermittent toluene inhalation dynamically regulates the transcriptome and neuronal methylome within the rat medial prefrontal cortex

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Abstract

Inhalants containing the volatile solvent toluene are misused to induce euphoria or intoxication. Inhalant abuse is most common during adolescence and can result in cognitive impairments during an important maturational period. Despite evidence suggesting that epigenetic modifications may underpin the cognitive effects of inhalants, no studies to date have thoroughly investigated toluene-induced regulation of the transcriptome or discrete epigenetic modifications within the brain. To address this, we investigated effects of adolescent chronic intermittent toluene (CIT) inhalation on gene expression and DNA methylation profiles within the rat medial prefrontal cortex (mPFC), which undergoes maturation throughout adolescence and has been implicated in toluene-induced cognitive deficits. Employing both RNA-seq and genome-wide Methyl CpG Binding Domain (MBD) Ultra-seq analysis, we demonstrate that adolescent CIT inhalation (10 000 ppm for 1 h/day, 3 days/week for 4 weeks) induces both transient and persistent changes to the transcriptome and DNA methylome within the rat mPFC for at least 2 weeks following toluene exposure. We demonstrate for the first time that adolescent CIT exposure results in dynamic regulation of the mPFC transcriptome likely relating to acute inflammatory responses and persistent deficits in synaptic plasticity. These adaptations may contribute to the cognitive deficits associated with chronic toluene exposure and provide novel molecular targets for preventing long-term neurophysiological abnormalities following chronic toluene inhalation.

KEYWORDS

adolescence, DNA methylation, epigenetics, inhalant misuse, toluene, volatile solvent abuse

1 | INTRODUCTION

Inhalation of volatile organic solvents, such as toluene, is a significant public health concern especially within adolescent populations, with an estimated lifetime prevalence of use up to 20%.[1] This is driven, in part, by the fact that products containing toluene (i.e., paints, glues and aerosols) are cheap, legal and readily accessible and provide a rapid yet transient feeling of euphoria.[2] Although discrete incidents of inhalant abuse generally occur for a short duration, ranging from minutes to 1 h, this behaviour is often repeated over time and extends for periods greater than 1 year, such that exposure becomes chronic but intermittent.[2] Adolescent inhalant abuse is a predictor of substance use disorders later in life and has a significant comorbidity with, and is predictive of, neuropsychiatric disorders including depression and anxiety.[3] Additionally, adolescent inhalant abuse is associated with neurocognitive impairments,[4] highlighting the effect of this behaviour on the brain. The harms associated with inhalant abuse are of particular concern given its association with adolescence, a critical period of brain maturation. In adolescence, dynamic reorganisation of white and grey matter structures occurs, most notably in regions associated with cognitive and executive functions, such as the prefrontal cortex (PFC).[5] Consequently, the negative implications of adolescent inhalant abuse likely relate to the vulnerability of the maturing brain.[6,7]

In rodents and non-human primates, toluene in isolation is commonly used as a preclinical model of inhalant abuse with evidence of conditioned place preference (CPP), self-administration and increased firing of dopaminergic neurons within the ventral tegmental area (VTA).[8,9] This leads to increased dopamine levels within the PFC and striatum and induces adaptations within mesocorticolimbic circuitry following acute and chronic inhalation. Although these mechanisms are unknown, studies using other drugs of abuse have established that such neuroadaptations are the result of long-term modifications of synaptic efficacy and neuronal circuitry function requiring dynamic and long-lasting changes in gene expression.[10] Such changes involve epigenetic modifications, which dynamically regulate gene expression within the brain in response to various environmental stimuli, including drugs of abuse.[11] In particular, DNA methylation serves a prominent role in neuronal function throughout life.[12] Experience-dependent regulation of 5-methylcytosine (5mC) and hydroxymethylcytosine (5hmC) is required, not only for synaptic plasticity[13] but also the formation, maintenance, reconsolidation and extinction of fear memories,[14,15] associative reward learning,[16] and the rewarding properties of drugs of abuse.[17,18]

Toluene inhalation in rats dynamically alters messenger RNA (mRNA) expression within the striatum[19] and global H3 and H4 acetylation within the VTA, nucleus accumbens (NAc) and dorsal dentate gyrus.[20,21] Aberrant DNA methylation is also observed in blood samples from solvent-exposed humans.[22] Such changes may subsequently mediate persistent toluene-induced neuroadaptations previously observed in preclinical models of inhalant abuse.[23–25] Despite this, no studies have thoroughly investigated toluene-induced regulation of the transcriptome or discrete epigenetic modifications within the brain.

To address this knowledge gap, the aim of the present study was to comprehensively analyse molecular adaptations within the brain using a preclinical model of chronic adolescent inhalant abuse. We focused our analysis on the medial PFC (mPFC) due to previous reports of PFC-dependent cognitive deficits and neuroadaptations in this model,[23,26] and others,[27] as well as the vulnerability of the mPFC to toluene-induced adaptions during adolescence.[6,24] We hypothesised that exposure to chronic intermittent toluene (CIT) during adolescence would result in dynamic regulation of the mPFC transcriptome, in part via epigenetic mechanisms, specifically DNA methylation. Moreover, to investigate the reversibility and/or persistence of such changes, analysis was conducted both 3 and 14 days following exposure enabling the identification of transient, persistent and/or emergent patterns of CIT-induced molecular adaptations. To our knowledge, this is the first study to comprehensively analyse toluene-induced regulation of the transcriptome and assess DNA methylation profiles within the adolescent brain following CIT exposure in rats.

2 | MATERIALS AND METHODS

2.1 | Animals

Adolescent male Wistar rats [n = 50, postnatal day (PND) 24—where adolescence ranges from PND 21 to 60; see Duncan et al[28]] were obtained from the Australian Resources Centre (Perth, Australia). Rats were pair-housed, maintained on a 12-h light/dark cycle (light phase 7:00–19:00) with food and water ad libitum and acclimatised for 3 days prior to experimentation. All experiments were performed in accordance with the *Prevention of Cruelty to Animals Act 1986* under the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

2.2 | Toluene inhalation

Exposure to 10 000 parts per million (ppm) vaporised liquid toluene (1.08389, purity 99.8%, Merck, Bayswater, Vic., Australia) was conducted as previously described[26]; this model is well-validated with consistent behavioural effects.[25,26,29] Briefly, exposure was conducted in chambers constructed using toluene-resistant materials and the concentration (10 000 ± 100 ppm) of toluene verified using a calibrated inline gas chromatography system (Shimadzu Corporation, Kyoto, Japan). Chambers of similar design but exposed to room air were utilized for control animals (0 ppm exposure). Rats were acclimatized to the laboratory for 1 h before exposure to toluene or air during which time their body weights were recorded. Rats were randomly assigned to inhale either room air (n = 24) or toluene (10 000 ppm, n = 26) for 1 h/day, three alternating days/week, for 4 weeks (PNDs 27–52). This exposure paradigm was employed to reflect the human

pattern of toluene abuse at a relevant concentration[2] where the sensitivity to toluene is approximately equivalent in both species.[30] After 1 h, rats were placed back into their home cages and isolated from other rats for 1 h to avoid olfactory stimulation by toluene scent on the fur before being returned to their holding rooms. Exposures were conducted at room temperature (~21°C) under normal lighting, and each rat was exposed at the same time each day (2–4 h into the light cycle).

2.3 | Tissue collection and mPFC isolation

To assess the acute, persistent and emergent effects of adolescent CIT exposure, tissue was collected 3 or 14 days following the final toluene exposure (Figure 1A). Rats were euthanised (Lethabarb, 1 ml kg^{-1} ip), decapitated and the brains rapidly dissected, weighed, snap frozen on liquid nitrogen and stored at −80°C. Tissue was collected between 9:00 and 14:00 (light phase) and counter-balanced for time of day between treatment groups. The mPFC was micro-dissected using tissue punches from serial 350-µm cryosections and tissues homogenised in nuclear extraction buffer (0.32-M sucrose; 5-mM $CaCl_2$; 3-mM Mg $(Ac)_2$; 0.1-mM EDTA; 10-mM Tris–HCl pH 8; Protease inhibitor cocktail (Roche); 0.3% Triton-X-100), a 100-µl aliquot taken for total RNA extraction and remaining mPFC homogenate was employed to isolate neuronal (NeuN+) nuclei via fluorescence-activated cell sorting (FACS).

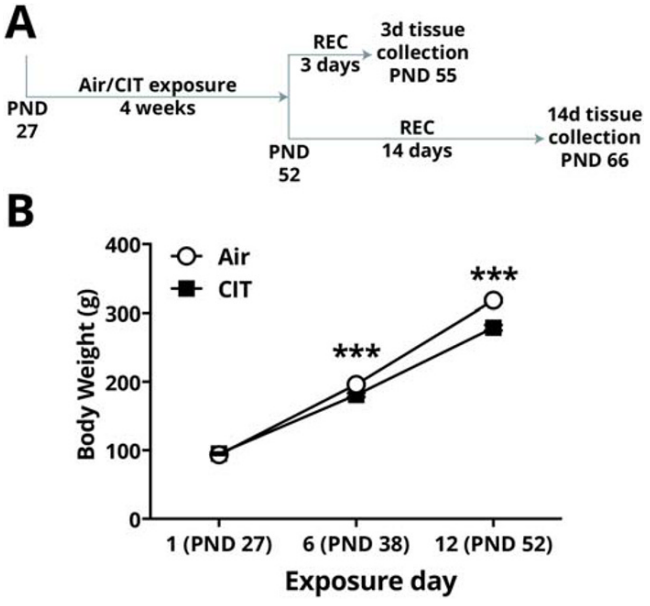

FIGURE 1 Adolescent CIT exposure attenuates body weights throughout the exposure period. A, Experimental timeline. B, Grouped body weights of rats exposed to adolescent CIT (n = 26) were significantly reduced compared with air-exposed rats (n = 24) by the sixth exposure day [postnatal day (PN) 38], with significantly decreased weights compared with rats exposed to air at the cessation of the exposure period (PN 52) ($^{***}p < 0.001$, air vs. CIT; two-way repeated measures (RM) analysis of variance (ANOVA) with Holm–Sidak post hoc analysis). Data are mean ± SEM. 3d, 3 days; 14d, 14 days; CIT, chronic intermittent toluene; REC, recovery

2.4 | Total RNA extraction and RNA-seq library preparation

Total RNA was extracted from 100 µl of mPFC homogenate via Trizol LS as per manufacturer's instructions and stored at −80°C (Thermo Fischer). All RNA samples were quantified via Qubit HS RNA assays (Thermo Fischer) and had RNA integrity number (RIN) > 8 as assessed with Agilent 2100 Bioanalyzer total RNA assay (Agilent Technologies). Total RNA (400 ng) derived from mPFC homogenate of a subset of animals (n = 10/group) was employed to construct paired-end (PE) RNA-seq libraries with the Illumina Truseq™ RNA V2 LT sample prep kit (Illumina, USA) as per manufacturer's instructions. The size and concentration of the PE RNA-seq libraries were verified on an Agilent 2100 Bioanalyzer and sequenced on an Illumina Hiseq2000.

2.5 | Validation of RNA-seq

Quantitative polymerase chain reaction (qPCR) was employed to validate RNA-seq data. Total RNA (150 ng) from the mPFC was employed for complementary DNA (cDNA) synthesis using the Superscript VILO cDNA synthesis kit (Thermo Fischer) as per manufacturer's instructions. Determination of relative gene expression was conducted using the $2^{-\Delta\Delta CT}$ method employing exon spanning primers and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as an endogenous control, which was chosen based on its stability and lack of significant differences in expression between groups in RNA-seq data. Primer efficiencies were confirmed to be 90%–110% and are listed in the supporting information. Subsequent qPCR was conducted on a ViiA™ 7 Real-Time PCR system (Applied Biosystems, USA) using SYBR® Green (Applied Biosystems, USA). All data are expressed as a fold change relative to air-exposed controls within each group. For a given gene, mean C_T values that were two standard deviations from the mean were deemed statistical outliers and excluded from analyses resulting in n = 8–10 per group.

2.6 | MBD-ultra seq library preparation

Neuronal nuclei (NeuN+) were isolated via FACS from mPFC homogenate (n = 5–7 group) employing the Methyl CpG Binding Domain (MBD) Ultra-seq method exactly as previously described.[17,31] Following FACS isolation of NeuN+, neuronal gDNA was extracted via phenol chloroform extraction, quantified with Qubit HS double stranded DNA (dsDNA) assays (Thermo Fischer) and stored at −20°C, 100 ng of purified neuronal gDNA was then employed to generate MBD Ultra-Seq libraries as previously described.[17,31] PE Libraries were sequenced on an Illumina HiSeq2000.

2.7 | Bioinformatic analysis

For more details, see supporting information.

2.8 | Statistical analysis

Body weights throughout exposure were analysed using two-way repeated measures (RM) analysis of variance (ANOVA) with treatment and time as factors. Holm–Sidak post hoc comparisons were employed where main effects were observed. Body and brain weights 3 and 14 days following the exposure period and relative qPCR data were analysed with unpaired *t* tests. All data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Significance was accepted at $p < 0.05$.

3 | RESULTS

3.1 | Adolescent CIT exposure retards body weight gain throughout the exposure period

Analysis of grouped body weights throughout the exposure period revealed a main effect of treatment ($F_{(1,48)}=22.85$, $p < 0.001$) and time ($F_{(2,110)} = 9{,}027.81$, $p < 0.001$), as well as a significant treatment × time interaction ($F_{(2,96)} = 92.31$, $p < 0.001$; Figure 1B). Post hoc analysis revealed that body weights were reduced in CIT-exposed rats from the sixth exposure day ($p < 0.001$), confirming results from previous studies employing this model[26,29] and consistent with meta-analysis.[32] Body weights remained significantly reduced in CIT-exposed rats at 3 days (Air 343.1 ± 4.7; CIT 302.2 ± 5.9 g, $p < 0.001$; PND 55) and 14 days (Air 405.3 ± 8.4; CIT, 369.4 ± 7.8 g, $p < 0.01$; PND 66) following the exposure period. No significant differences in brain weight were observed at either time point (3 days, Air 1.86 ± 0.02 g, CIT 1.84 ± 0.02 g; 14 days, Air 1.95 ± 0.02 g; CIT 1.93 ± 0.02 g).

3.2 | Adolescent CIT exposure induces dynamic regulation of the mPFC transcriptome

To investigate CIT-induced changes of the mPFC transcriptome, we conducted RNA-seq at both 3 and 14 days following the final toluene exposure. Both time points were analysed independently employing the between-subjects factor of treatment (air or CIT). Analysis of the mPFC transcriptome 3 days following adolescent CIT exposure revealed 473 differentially expressed genes (DEGs) between CIT and air-exposed rats with a predominance of downregulated (381), compared

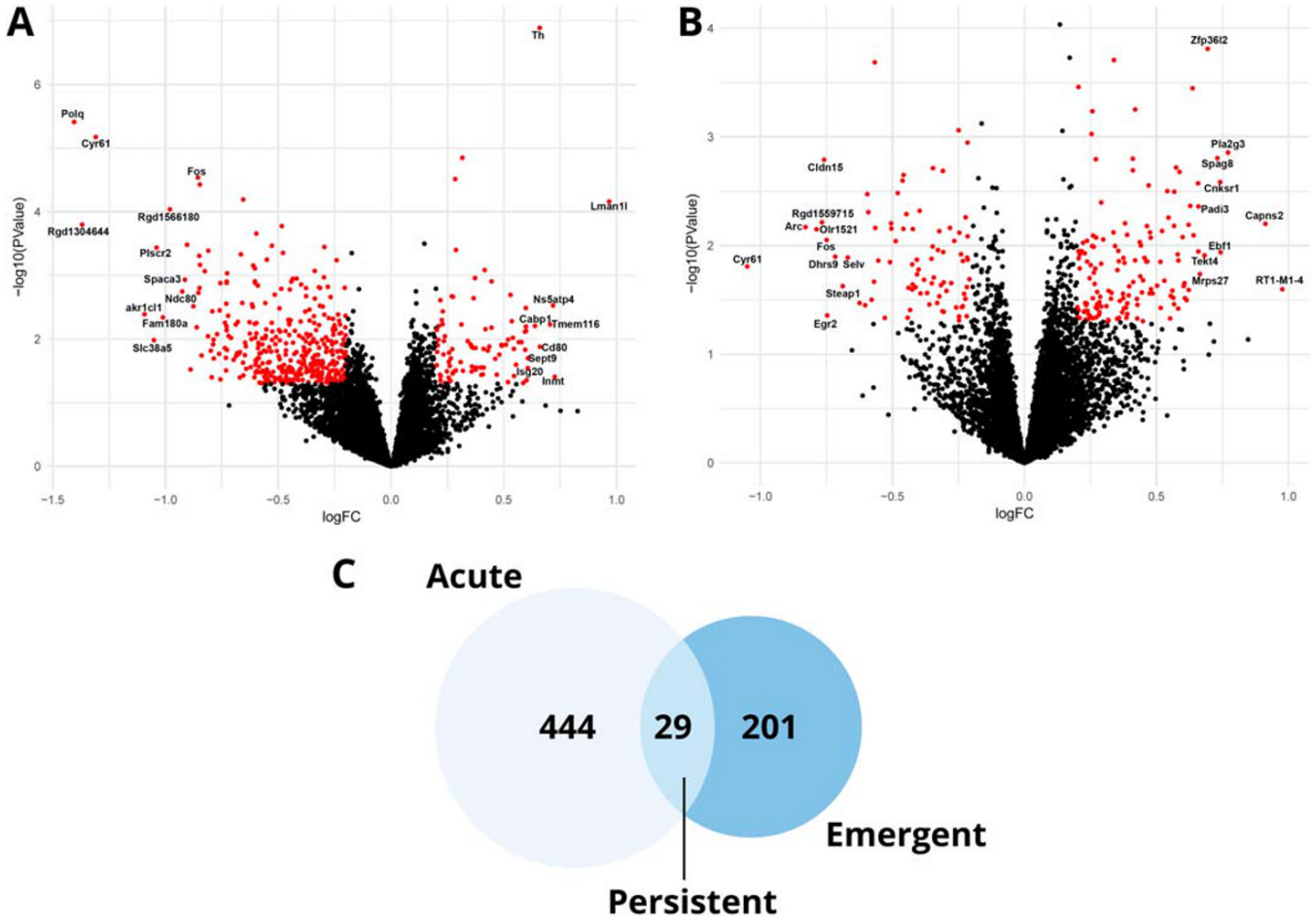

FIGURE 2 Differentially expressed genes (DEGs) within the medial prefrontal cortex (mPFC) following adolescent CIT exposure. DEGs identified after 3 days (A) and 14 days (B) of CIT ($n = 10$) compared with air-exposed rats ($n = 10$). (C) Venn diagram depicting acute (3 days only), persistent (3 and 14 days) or emergent (14 days only) DEGs within the mPFC. Red data points indicate DEGs (p value < 0.05 and logFC ± 0.2). CIT, chronic intermittent toluene

with upregulated (92), transcripts (Figure 2A, Table S1). In contrast, ongoing abstinence from CIT exposure for 14 days resulted in fewer DEGs (230) within the mPFC and a predominance of upregulated (147), opposed to downregulated (83), transcripts between CIT and air-exposed rats (Figure 2B, Table S2). To assess the dynamic nature of CIT-induced regulation of the mPFC transcriptome, we grouped DEGs into acute (3 days only), persistent (both 3 and 14 days) or emergent categories (14 days only). Adolescent CIT exposure resulted in 444 acute DEGs (361 downregulated and 83 upregulated), with a small subset of persistent DEGs observed (17 downregulated, 6 upregulated and 4 dynamically regulated); 201 emergent DEGs were also identified (138 upregulated and 63 downregulated; Figure 2C). Several acute, persistent and emergent DEGs were validated via qPCR, which represented genes of interest (e.g., *Th* and *Dnmt3b*), immediate early genes (IEGs) and synaptic plasticity-associated genes (e.g., *Igf2*, *Igfbp2*, *Fos* and *FosB*), as well as extracellular matrix (ECM) receptor interactors (*Fmod* and *Mmp9)* and blood–brain barrier (BBB) components (e.g., *Aqp9*) relevant to the functional annotation analysis below (Figure 3).

To elucidate the functional significance of CIT-induced transcript expression, we conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and gene ontology analysis of DEGs via Generally Applicable Gene Set Enrichment (GAGE) analysis, which considers all genes expressed in a given RNA-seq data set for a more robust analysis. Acutely, DEGs were enriched for KEGG pathways relating to extracellular signalling (Table 1) such as cell adhesion molecules (CAMs; *Cldn5*, *Cldn3*, *Cldn15*, *Esam* and *Icam2*); neuroactive ligand-receptor interaction including many neuromodulatory receptors such as downregulation of genes encoding the thyrotropin releasing hormone receptor (*Trhr*), Hypocretin (Orexin) Receptor 2 (*Hcrtr2*) and leptin receptor (*Lepr*) and upregulation of the adrenoreceptor genes *Adra2b* and A*dra2c*, as well as neuropeptide Y receptor 2 gene *Npy2r*. The *Nr3c1* gene encoding the glucocorticoid receptor (GR) was downregulated, although with a modest $\log_2$ FC of −0.15. Moreover, ECM-receptor interacting gene networks were modulated, including upregulation of metalloproteinase 9 (*Mmp9*) mRNA, and downregulation of TIMP metallopeptidase inhibitor 1 (*Timp1*) and fibromodulin (*Fmod*) mRNA.

Downregulated DEGs at 3 days were also enriched for molecular functions relating to RNA polymerase II transcription factor activity including numerous transcription factors critical for neuronal plasticity subserving learning and memory processes. This network included genes encoding the calmodulin-binding transcription activator 1 (*Camta1*), and IEGs including neuronal PAS domain protein 4 (*Npas4*), early growth response 1 and 2 (*Egr1*, *Egr2*), activity regulated cytoskeleton associated protein (*Arc*), nuclear receptor family members *Nr4a1* and *Nr4a2*, Sp1 transcription factor (*Sp1*) and components of the AP-1 complex (i.e, *Jun*, *Fos* and *Fosb*) (Table 1).[33] Enrichment of downregulated DEGs at 3 days was also prominent among Gene Ontology (GO) terms relating to the regulation of vasculature development and angiogenesis including nitric oxide synthase 3 (*Nos3*), Elastin (*Eln*) and Collagen, type III, alpha-1 (*Col3a1*).

No significant enrichment for functional categories was observed for upregulated DEGs at 3 days or any DEGs at 14 days; however, several IEGs were persistently downregulated at 14 days (e.g., *Stat4*, *Fos*, *FosB*, *Arc*, *JunB*, *Egr2* and *Dusp1*).

Adolescent CIT exposure reduced the expression of genes encoding DNA methylation machinery with acute downregulation of

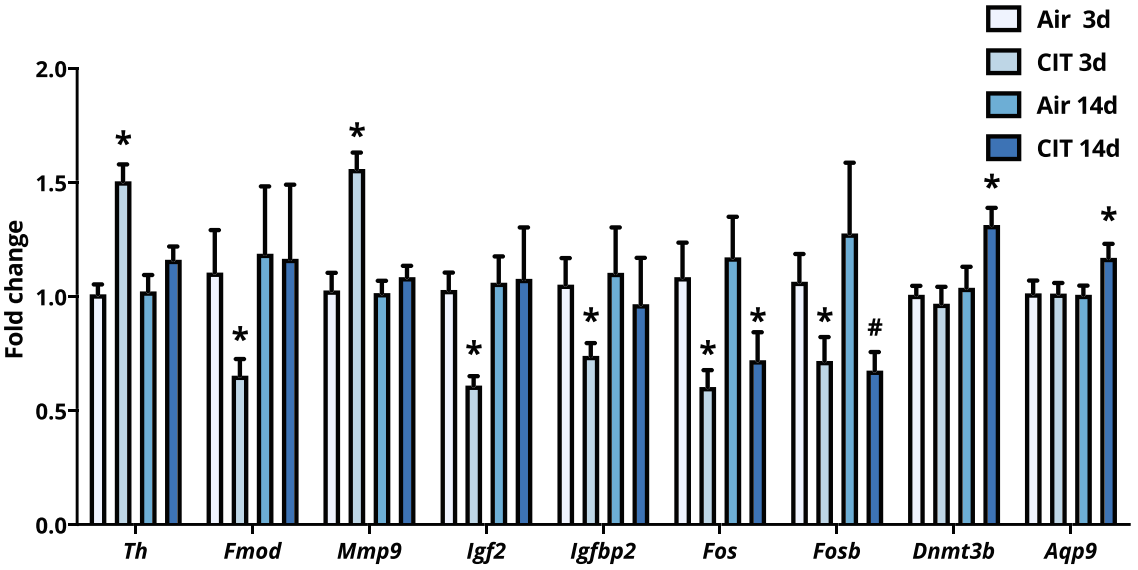

FIGURE 3 Quantitative polymerase chain reaction (qPCR) validation of RNA-seq data qPCR validation of differentially expressed genes (DEGs) identified with RNA-seq between air and chronic intermittent toluene (CIT)-exposed rats within the medial prefrontal cortex following adolescent CIT exposure. Data are mean ± SEM normalised to air control group at each time point. N = 8–10 per group; $^{*}p < 0.05$; $^{\#}p < 0.10$ unpaired t test. *Aqp9*, Aquaporin 9; *Cyr61*, Cysteine-rich angiogenic inducer 61; *Dnmt3b*, DNA methyltransferase 3 beta; *Fmod*, Fibromodulin; *Fos*, FBJ murine osteosarcoma viral oncogene homolog; *FosB*, FBJ murine osteosarcoma viral oncogene homolog B; *Igf2*, Insulin-like growth factor 2; *Igfbp2*, Insulin-like growth factor binding protein 2; *Mmp9*, Matrix metallopeptidase 9; *Th*, Tyrosine hydroxylase

TABLE 1 GAGE pathway and functional enrichment analysis of differentially expressed genes (DEGs) within the medial prefrontal cortex 3 days following adolescent chronic intermittent toluene exposure

GAGE pathway analysis for 3-day DEGs	
KEGG signalling pathways	**Adjusted *p* value**
Cell adhesion molecules	0.037
Neuroactive ligand-receptor interaction	0.037
Complement and coagulation cascades	0.037
ECM-receptor interaction	0.046
GAGE functional enrichment analysis for 3-day downregulated DEGs	
GO: Biological processes	
RNA polymerase II transcription factor activity, sequence-specific DNA binding	0.007
Transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding	0.024
GO: Molecular function	
Vasculature development	5.64E-06
Blood vessel development	5.64E-06
Cardiovascular system development	5.65E-06
Blood vessel morphogenesis	3.84E-05
Angiogenesis	>0.001
Regulation of vasculature development	0.005
Regulation of angiogenesis	0.017
Regulation of cell migration	0.062
Response to mechanical stimulus	0.062
Tissue morphogenesis	0.069

Note. KEGG pathway and GO terms represent nonoverlapping terms with false detection rate corrected *p* values < 0.1.
Abbreviations: ECM, extracellular matrix; DEGs, differentially expressed genes; GAGE, Generally Applicable Gene Set Enrichment; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Gadd45g encoding growth arrest and DNA-damage-inducible 45, γ (GADD45γ), persistent downregulation of *Gadd45b* encoding GADD45β and emergent upregulation of *Dnmt3b* encoding the *de novo* DNA methyltransferase DNMT3B. Notably, the novel IEG, *Cyr61*, was recently shown to be regulated upon learning in the mouse PFC in part via GADD45γ[34] and was one of the most prominently downregulated genes 3 days ($\log_2$ FC = −1.3) following adolescent CIT exposure and remained decreased 14 days ($\log_2$ FC = −1.1) following CIT exposure.

3.3 | Adolescent CIT exposure dynamically regulates neuronal DNA methylation within the mPFC

Considering the regulation GADD45 family members and the emergent upregulation of *Dnmt3b* following adolescent CIT exposure, we hypothesised that CIT-induced alterations in DNA methylation would be present within the mPFC, which may, in part, contribute to CIT-induced regulation of the mPFC transcriptome. Therefore, we employed MBD Ultra-Seq[17,31] to analyse genome-wide DNA methylation profiles within neurons of the mPFC following adolescent CIT exposure. MBD Ultra-seq resulted in an average of ∼46 million reads with high mapping quality (>Q20) per biological replicate. We identified a total of 18 683 high confidence 5mC peaks in the MBD Ultra-seq data from which statistical comparisons were undertaken to identify differentially methylated regions (DMRs) following adolescent CIT exposure. Employing high stringency criteria, we identified 5130 DMRs at 3 days and 8906 DMRs at 14 days following adolescent CIT exposure (Figure 4A,B). At both time points, adolescent CIT exposure resulted in a predominance of hypermethylation with only 47 (3 days) and 109 (14 days) hypomethylated DMRs identified (Figure 4A,B). Indeed, further categorisation of DMRs (see supporting information) identified 1570 acute DMRs (1224 hypermethylated and 39 hypomethylated loci), 3560 persistent DMRs (1552 hypermethylated and 8 hypomethylated loci) and 4742 emergent DMRs (4643 hypermethylated and 99 hypomethylated; note increased number of DMRs at 14 days compared with the total of persistent and emergent DMRs is due to the ≥5 read cut-off at both time points for persistent DMRs; Figure 4C).

Database for Annotation, Visualization and Integrated Discovery (DAVID) pathway and gene ontology analysis revealed minimal overlap of DEG-associated gene networks with genes overlapping or proximal (±10 kb) to CIT-induced DMRs [acute (557 genes), persistent (734 genes) and emergent (1,696 genes), Table 2]. Enrichment of DMRs was observed within gene networks related to neuronal function including acute DMRs proximal to structural molecular activity networks (e.g., *Cldn6*, *Cldn9*, *Cav1* and *Krt4*), persistent DMRs to calcium ion binding gene networks (e.g., *Kcnip1* and *Hpcal1*) and emergent DMRs enriched for calcium signalling (e.g., *Hpcal1* and *Cacna1a*), and neuroactive ligand-receptor interaction (e.g., *Nr3c1*, *Gabrr1*, *Gabrb1*, *Grm2*, *Grm4*, *Chrna1* and *Chrna2*).

To assess whether CIT-induced changes in neuronal 5mC may correlate with the observed changes in the mPFC transcriptome, we conducted hypergeometric distribution analysis for DEGs with overlapping DMRs within intragenic, promoter (2 kb upstream of TSS) or proximal intergenic regions (2–10 kb upstream of TSS) at both 3- and 14-day time points. In agreement with the accumulation of DMRs at 14 days, we identified a modest but significant overlap of DEGs (11 downregulated and 19 upregulated, Table S3) with proximal DMRs 10 kb upstream of the transcription start site (TSS; p = 0.042) at this time point with no other significant effects observed. This overlap accounted for 13% of the DEGs identified at 14 days and includes IEGs such as *Egr2* (hypermethylated DMR ∼ 6 kb upstream of TSS) and the DNA demethylation factor *Gadd45b* (hypermethylated DMR ∼ 7 kb upstream of TSS). Moreover, the same DMR identified upstream of *Gadd45b* was also hypermethylated at the 3-day time point albeit at nominal (trend) significance [false discovery rate (FDR) = 0.057].

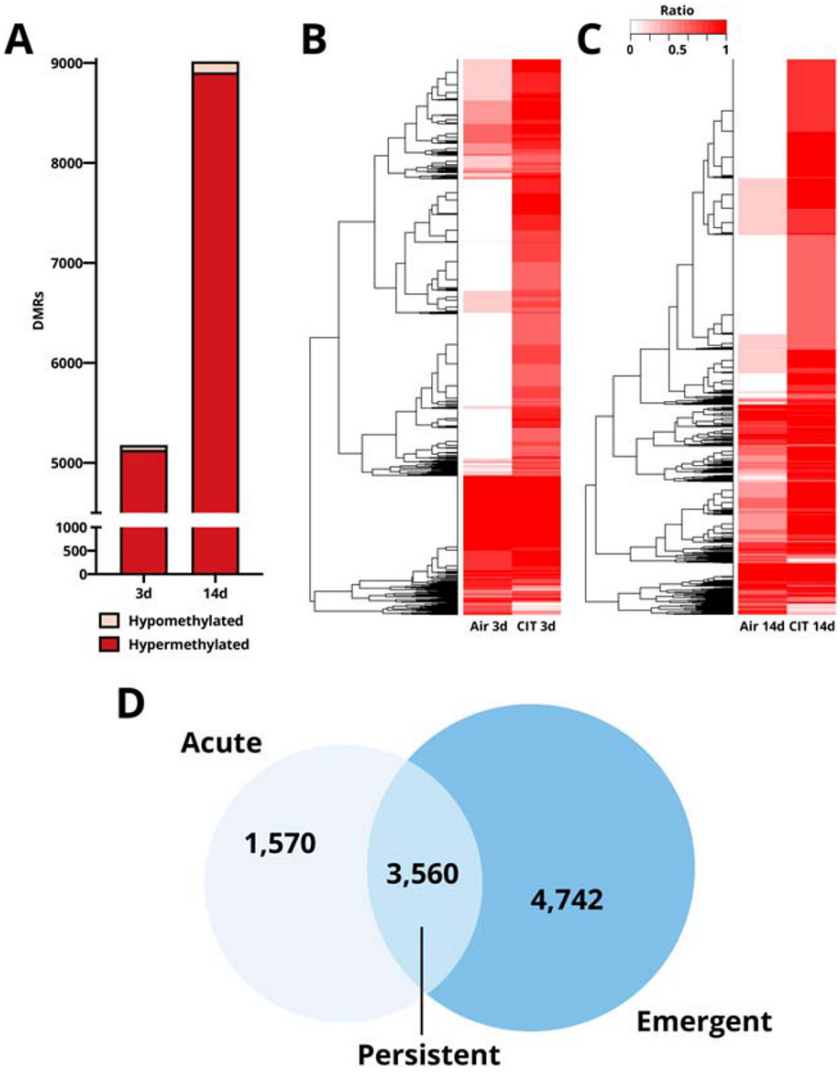

FIGURE 4 Differentially methylated regions (DMRs) within neurons of the medial prefrontal cortex (mPFC) following adolescent chronic intermittent toluene (CIT) exposure. (A) Number of DMRs identified at the 3- and 14-day time points depicting a predominance of hypermethylated loci following adolescent CIT exposure. (B, C) Representative heat map depictions of 5-methylcytosine (5mC) enrichment at differentially methylated regions (FDR < 0.1) between air and CIT-exposed rats identified at 3- (B) and 14-day (C) time points. The ratio represents the number of animals within a group with an enrichment of 5mC at a given DMR where white indicates no biological replicates and red indicates all biological replicates displayed an enrichment of 5mC. (D) Venn diagram depicting total number of acute (3 days only), persistent (3 and 14 days) or emergent (14 days only) DMRs within neurons of the mPFC

4 | DISCUSSION

Here, we demonstrate for the first time that adolescent CIT exposure induces dynamic regulation of the mPFC transcriptome and neuronal DNA methylation landscape in the rodent brain, which persist into early adulthood. Acutely following adolescent CIT exposure, prominent downregulation of IEGs important for synaptic plasticity, as well as genes associated with CAMs and vascular organisation was observed. Following longer term abstinence from toluene exposure, hundreds of emergent DEGs were observed within the mPFC, with persistent downregulation of IEGs critical for neuronal function and dysregulation of genes encoding DNA methylation machinery. Interestingly, we identified predominant hypermethylation within neurons of the mPFC following adolescent CIT exposure, with ongoing accumulation of hypermethylated DMRs throughout abstinence, in line with the emergent upregulation of Dnmt3b mRNA encoding the de novo DNA methyltransferase DNMT3B. Thus, adolescent CIT exposure appears to induce persistent aberrant de novo methylation within neurons of the mPFC. Despite the modest overlap of DMRs and DEGs observed, DEGs such as *Egr2*, *Cyr61*, *Gadd45b* and *Camta1* were associated with CIT-induced DMRs implying toluene-induced epigenetic modulation of a subset of genes integral for neuronal function.

Acutely following adolescent CIT exposure, we identified an enrichment of altered gene networks mediating transcriptional regulation within the mPFC. This included several IEGs with critical roles in homeostatic and stimulus-induced synaptic plasticity[33] that are of particular interest considering the PFC-dependent cognitive deficits previously observed in our model[23,26] and others following adolescent toluene exposure.[27] For example, the neuronal specific IEG, *Npas4*, mediates aspects of homeostatic plasticity and excitation/inhibition balance within the cortex and hippocampus and is critical for learning-induced plasticity[35] via stimulus-induced induction of downstream gene networks.[36] Genetic or viral-mediated reductions in *Npas4* in the rodent brain also result in significant cognitive deficits and a loss of stimulus induced transcription,[37] and adolescent deficiency of *Npas4* is associated with impairments in object recognition.[38] Thus, persistent downregulation of *Npas4* within the PFC following adolescent CIT exposure may impair homeostatic and learning-induced plasticity within the PFC via the reduction of activity-induced transcriptional

TABLE 2 DAVID pathway and functional enrichment analysis of hypermethylated differentially methylated regions (DMRs) within the medial prefrontal cortex

DAVID pathway analysis for DMRs	
KEGG pathways	**Adjusted *p* value**
Persistent DMRs	
Osteoclast differentiation	0.092
Emergent DMRs	
Complement and coagulation cascades	0.051
Calcium signalling pathway	0.106*
Gastric acid secretion	0.132*
ABC transporters	0.140*
Dorso-ventral axis formation	0.155*
Neuroactive ligand-receptor interaction	0.187*
N-glycan biosynthesis	0.190*
DAVID functional enrichment analysis for DMRs	
GO: Biological processes	
Acute DMRs	
Structural molecule activity	0.126*
Emergent DMRs	
Positive regulation of cell migration	0.005
Sodium ion transport	0.131*
Blood coagulation	0.018
GO: Molecular function	
Persistent DMRs	
Calcium ion binding	0.176*

Note. KEGG pathway and GO terms represent nonoverlapping terms with Benjamini–Hochberg corrected *p* values < 0.2.
Abbreviations: DAVID, Database for Annotation, Visualization and Integrated; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.
*Denotes nominally significant enrichment.

networks, which may, in part, mediate the deficits in reward processing and cognitive flexibility previously observed in our model.[23,26]

Downregulation of IEGs encoding AP-1 components, *Fos*, *JunB* and *FosB*, following adolescent CIT exposure further support this notion. The role of AP-1 components in learning and drug-induced transcriptional activation and subsequent plasticity is well established.[39] AP-1 components, specifically cFos, induce rapid chromatin state changes within regulatory elements of target gene networks to drive transcription in response to neuronal activity.[40] Thus, decreased levels of AP-1 components following adolescent CIT exposure may result in impaired activity-induced transcription within the mPFC. Moreover, we observed persistent downregulation of other IEGs such as *Egr1*, *Egr2* and Nr4a family members. Similar long-term downregulation of these IEGs is also observed within the rat mPFC up to 3 weeks following exposure to chronic intermittent ethanol vapour inhalation,[41] associated with increased global 5mC and escalation of ethanol seeking.[42]

Additional gene networks associated with activity-induced regulation and cognition were found to be dysregulated following adolescent CIT exposure. For example, Gadd45g, a GADD45 family demethylase, was recently demonstrated to be induced upon fear learning in the mouse mPFC and was involved in the delayed or second wave (i.e., ~5 h post stimulus) of IEG activation, including *Npas4*, *Fos*, *Arc* and *Cyr61* in this study.[34] *Cyr61* was further shown to be a novel IEG associated with the consolidation of cued-fear memory in this context.[34] Considering the persistent downregulation of *Gadd45g* and *Cyr61* mRNA within the mPFC following adolescent CIT exposure in the present study, as well as downregulation of an extended network of plasticity-associated IEGs, adolescent CIT exposure may impair the activity-dependent transcriptional efficacy of mPFC neurons, likely affecting stimulus-induced plasticity and thus learning and memory processes, consistent with previously observations in the CIT model.[23,26] Recent evidence further supports this notion as adolescent CIT exposure in rats induced deficits in behavioural flexibility in PFC-dependent risk/reward-based decision-making tasks, which was associated with altered activity of mPFC neurons.[27] Moreover, CIT-induced downregulation of critical plasticity genes may also reflect altered mPFC physiology as two acute exposures to toluene (10 500 ppm) in adolescent but not adult rats alter the intrinsic excitability of mPFC neurons projecting to the NAc.[6] Thus, future studies that focus on the consequences of adolescent CIT exposure upon mPFC neurophysiology and the responsivity of the mPFC transcriptome to subsequent stimuli (e.g., additional toluene exposure or learning) are warranted, particularly in light of recent findings that *N*-methyl-D-aspartate (NMDA)-mediated plasticity within the mPFC may underlie the cognitive deficits associated with CIT.[43] Moreover, future studies should functionally validate the role of identified candidate genes in CIT-induced cognitive deficits employing viral-mediated gene knock-down or overexpression within the mPFC, although this is beyond the scope of the present study.

Adolescent CIT exposure RNA-seq identified upregulation of mRNA encoding the de novo DNA methyltransferase DNMT3B and reduced Gadd45b, and Gadd45g mRNA encoding the DNA demethylation family members GADD45β, and GADD45γ, following adolescent CIT exposure. Stimulus-induced regulation of DNA methylation machinery, such as following learning or drug exposure, is mediated by neuronal activity.[13] Thus, because toluene activates neurons within the mPFC,[44] it is likely that regulation of DNA modifying enzymes, and thus 5mC, occurs repeatedly throughout the exposure period in our model. As such, adolescent CIT exposure may predominantly stimulate de novo methylation, in line with the predominance of hypermethylated DMRs. The continued accumulation of hypermethylated DMRs agrees with upregulated Dnmt3b mRNA and downregulation of several Gadd45 family demethylases. As is observed for other drugs of abuse, adolescent CIT exposure appears to induce hypermethylation within the mPFC, an effect which persists into adulthood.[42,45]

Widespread hypermethylation following adolescent CIT exposure does not, however, readily explain many of the gene expression changes observed in this study. One caveat that may underlie this

disparity is that although mPFC RNA and neuronal gDNA were derived from a subset of the same animals, due to technical limitations, RNA-seq libraries were prepared from heterogeneous mPFC lysate rather than from isolated neuronal cells. Thus, the modest association of DEGs and DMRs may be explained from the heterogeneous source of the RNA-seq data compared with the neuronal source of the MBD Ultra-seq data set. Another explanation is that the majority of CIT-induced DMRs are observed within intergenic regions, and association with distal regulatory elements of DEGs, such as enhancers, may have been overlooked in the present study. Further investigation into the chromatin state at CIT-induced DMRs would be informative, although limited by the availability of ChIP-seq data and resources for the rat genome. MBD Ultra-seq is also limited to the analysis of 5mC within the CpG context such that potential regulation of abundant 5mCH (H = A, C, or T) or even 5hmC profiles following CIT exposure were missed in this study. Furthermore, CIT-induced methylation changes may mediate long-term metaplastic changes to transcriptional regulation with only minor modulation of basal mRNA levels. Indeed, cocaine self-administration induces DNA methylation changes within the rodent mPFC that alters subsequent cocaine-induced transcription.[17] Thus, future experiments analysing toluene-induced transcriptional activation following adolescent CIT exposure would be of interest to ascertain whether CIT-induced methylation does indeed alter the inducibility of the mPFC transcriptome.

The transcriptome and DNA methylation changes observed in this study may in part be mediated via downstream consequences of adolescent CIT exposure rather than CIT exposure itself. For example, persistent and emergent gene expression changes and ongoing accumulation of 5mC may represent homeostatic compensation within the mPFC following adolescent CIT exposure or reflect the effects of protracted 'withdrawal' from CIT exposure. Withdrawal has been reported in human toluene abusers,[46] as well as following continuous solvent inhalation in mice.[47] Although these factors are likely to interact, discerning the effects of the potential molecular adaptations resulting from CIT-exposure itself compared with potential withdrawal and/or homeostatic compensation will be of interest in future studies and may further elucidate efficacious therapeutic targets to facilitate the resolution of the cognitive/behavioural impairments following chronic inhalant abuse. It is also possible that the significant body weight impairments seen following CIT exposure, which are consistent with prior observations in this model,[29] may influence gene expression changes. Although this question cannot be answered in this study, and is a limitation on the interpretation of the findings, future studies that control for the effects of body weight via strategies such as pair-feeding may elucidate any interactions.

Toluene exposure is known to interact with the stress response system via modulation of the hypothalamic–pituitary–adrenal (HPA) axis.[29,48] We have also previously observed increased *Crh* mRNA expression (encoding corticotropin-releasing hormone) within the hypothalamus, increased circulating adrenocorticotropic hormone without altered corticosterone levels, and adrenal hypertrophy following adolescent CIT exposure in rats.[29,49] In the present study, we identified a modest decrease in Nr3c1 mRNA expression (encoding GR) within the mPFC acutely following CIT exposure associated with a persistent intronic DMR, although mRNA levels had normalised by 14d, consistent with chronic inhalation of ethanol vapour.[50] Thus, although we cannot rule out the contribution of the stress-induced changes in the mPFC transcriptome, our findings are in line with ethanol vapour-induced changes in GR expression such that adolescent CIT exposure only acutely modulates GR expression (and likely GR signalling) as a consequence of the exposure itself, which normalises upon abstinence.

Similarly, we observed modulation of gene networks related to ECM components, blood vessel development and angiogenesis acutely following CIT exposure, which normalised by 14 days. As toluene is a known neurotoxin affecting both white and grey matter structures, such changes may indicate CIT-induced effects upon neuroinflammatory processes and BBB integrity. For example, CIT exposure acutely increased Mmp9 mRNA, which is a known inflammatory-mediator affecting BBB integrity via cleavage of ECM components, such as claudin family members that were also acutely downregulated following adolescent CIT exposure (e.g., *Claudin-5*).[51] Additionally, decreased Timp1 mRNA, which encodes TIMP1, one of the major MMP9 inhibitors within the brain[52] implies increased pro-inflammatory MMP9 signalling acutely following adolescent CIT exposure. However, although adolescent CIT exposure may acutely increase neuroinflammatory processes, these processes appear to normalise by 14 days abstinence. Ultimately, the long-term behavioural and cognitive deficits previously observed in this model[23,26] are more likely the consequence of persistent and emergent adaptions with the mPFC, as seen in the current study.

5 | CONCLUSIONS

This study provides novel insights into the molecular adaptions induced by chronic adolescent toluene inhalation within the rat mPFC. We demonstrate for the first time that adolescent CIT exposure results in dynamic regulation of the mPFC transcriptome likely relating to acute inflammatory responses and persistent deficits in synaptic plasticity. Ongoing hypermethylation of the neuronal genome further emphasises the persistent nature of CIT-induced adaptions within the mPFC, which may perpetuate aberrant transcriptional states into adulthood. Ultimately, such adaptations may contribute to the cognitive deficits previously observed following chronic toluene exposure and provide novel molecular targets for preventing long-term neurophysiological abnormalities following chronic toluene inhalation.

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AUTHORS CONTRIBUTION

JRD, AD, TB and AJL conceptualised and designed the experiments. AD, RC, JRD and AJL wrote the manuscript. AD and JRD conducted all animal experiments analysed by AD. AD processed all tissue, and AD and JE conducted RNA-seq experiments. AD, DBA and XL conducted MBD Ultra-seq experiments. JE and VM sequenced all libraries. QZ analysed all RNA-seq and MBD Ultra-seq data. SR conducted GAGE enrichment analysis. AD conducted qPCR experiments analysed by AD.

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