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ORIGINAL ARTICLE

Elevated 5-hydroxymethylcytosine in the Engrailed-2 (*EN-2*) promoter is associated with increased gene expression and decreased MeCP2 binding in autism cerebellum

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Epigenetic mechanisms regulate programmed gene expression during prenatal neurogenesis and serve as a mediator between genetics and environment in postnatal life. The recent discovery of 5-hydroxymethylcytosine (5-hmC), with highest concentration in the brain, has added a new dimension to epigenetic regulation of neurogenesis and the development of complex behavior disorders. Here, we take a candidate gene approach to define the role 5-hmC in Engrailed-2 (*EN-2*) gene expression in the autism cerebellum. The EN-2 homeobox transcription factor, previously implicated in autism, is essential for normal cerebellar patterning and development. We previously reported *EN-2* overexpression associated with promoter DNA hypermethylation in the autism cerebellum but because traditional DNA methylation methodology cannot distinguish 5-methylcytosine (5-mC) from 5-hmC, we now extend our investigation by quantifying global and gene-specific 5-mC and 5-hmC. Globally, 5-hmC was significantly increased in the autism cerebellum and accompanied by increases in the expression of *de novo* methyltransferases *DNMT3A and DNMT3B*, ten-eleven translocase genes *TET1 and TET3*, and in 8-oxo-deoxyguanosine (8-oxo-dG) content, a marker of oxidative DNA damage. Within the *EN-2* promoter, there was a significant positive correlation between 5-hmC content and *EN-2* gene expression. Based on reports of reduced MeCP2 affinity for 5-hmC, MeCP2 binding studies in the *EN-2* promoter revealed a significant decrease in repressive MeCP2 binding that may contribute to the aberrant overexpression of *EN-2*. Because normal cerebellar development depends on perinatal *EN-2* downregulation, the sustained postnatal overexpression suggests that a critical window of cerebellar development may have been missed in some individuals with autism with downstream developmental consequences. Epigenetic regulation of the programmed on-off switches in gene expression that occur at birth and during early brain development warrants further investigation

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INTRODUCTION

Engrailed-2 (*EN-2*) is a developmentally regulated homeobox gene that is essential for Purkinje cell maturation and normal cerebellar development and patterning.[1] We recently reported for the first time that alterations in DNA and histone lysine methylation within the *EN-2* promoter region were associated with *EN-2* overexpression in the post-mortem autism cerebellum.[2] These findings were unusual in that an increase in *EN-2* gene expression and protein level was associated with promoter DNA *hyper*methylation that could be partially explained by a significant decrease in repressive histone H3 lysine 27 trimethylation (H3K27) in the same region. Previous studies have considered *EN-2* to be an autism-susceptibility gene based on family linkage studies indicating an increased transmission of *EN-2* polymorphic variants from parents to affected children[3] and additionally on similarities between autism cerebellar abnormalities and cerebellar abnormalities in EN-2 transgenic rodent models.[4,5] In mice, *En-2* is highly expressed in Purkinje cells during fetal and early postnatal development acting primarily as a transcriptional repressor until it is down-regulated during the perinatal period.[6] *EN-2* is also expressed in hindbrain nuclei involved in the development of serotonin (raphe nucleus) and norepinephrine (locus coeruleus) neurotransmitter systems that have been implicated in autism.[7,8] It is important to note that the programed timing of Purkinje cell maturation and cerebellar patterning is critically dependent on perinatal *EN-2* downregulation,[6,9] which is obviated by sustained *EN-2* over-expression.[9,10] Our finding of sustained *EN-2* gene overexpression in the postnatal autism cerebellum suggests that a critical developmental window for normal downregulation may have been missed in some individuals with autism.

DNA methylation as 5-methylcytosine (5-mC) has long been considered a highly stable epigenetic mark that is established during embryogenesis and early fetal development and serves to define tissue-specific cell identity by dense DNA methylation and silencing of functionally irrelevant genes. This initial notion has been modified by reports of dynamic gene-specific and genome-wide loss of 5-mC (DNA hypomethylation) and altered gene expression in response to endogenous or exogenous environmental signals during postnatal development, aging and carcinogenesis.[11–13] However, the elucidation of the molecular mechanisms underlying DNA demethylation has proved to be a challenge. The recent discovery that 5-hydroxymethylcytosine (5-hmC) is an intermediate in the oxidative demethylation of 5-mC via ten-eleven-translocase (TET) family of dioxygenases has added

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a new dimension to the epigenetic repertoire and cell-specific regulation of gene expression.[14,15]

Whereas 5-hmC is present in genomic DNA of all cells, it is most highly concentrated in mammalian neurons relative to other cell types with one of the highest levels in cerebellar Purkinje cells where 5-hmC was first isolated.[16] Genome-wide 5-hmC profiling has revealed that 5-hmC distribution is highly variable in the brain and can be concentrated in 5′ enhancer regions, gene bodies or CpG island shores depending on cell type, gene activation and/or differentiation stage.[17–19] For example, 5-hmC content is 0.6% in Purkinje cells but only 0.2% in granule cells underscoring the cell type specificity of 5-hmC distribution.[16] Genomic mapping of 5-hmC during mouse neurodevelopment found that it is enriched in the body of genes with the high CpG density, whereas genes with moderate or low CpG density accumulate 5-hmC in distal and proximal promoter regions.[18] Nonetheless, reports of the stable presence of 5-hmC in the postnatal cerebellum and hippocampus[17,20] suggest that this modified base may have additional roles beyond that of a transient intermediate during TET-mediated 5-mC demethylation. Thus, it is highly likely that 5-hmC, as the sixth base in DNA, functions as an independent epigenetic mechanism in chromatin remodeling and gene expression regulation, although much more research will be required before definitive conclusions are possible.

The sequence-specific accumulation of 5-hmC has been associated with gene activation during neuronal neurogenesis, differentiation and aging.[17,19,20] Interestingly, several reports have shown that a stable increase in 5-hmC can occur without substantial reduction of 5-mC content (demethylation).[21–23] The possibility of 5 mC to 5-hmC conversion under conditions of oxidative stress has received some theoretical support.[24–26] For example, hydroxyl radical generation with Fenton reaction chemistry resulted in the conversion of 5-mC to 5-hmC in liver microsomes;[24] however, similar demonstration in brain DNA has not been reported. Further, 5-mC and other oxidatively modified DNA bases have been shown to decrease MeCP2-binding affinity. An *in vitro* study using the electrophoretic mobility assay with an oligonucleotide duplex containing an MeCP2 recognition sequence demonstrated that the replacement of 5-mC with 5-hmC or 8-oxo-dG on one or both strands of the duplex significantly reduced MeCP2 binding, suggesting that oxidatively damaged bases could alter the epigenomic landscape and gene expression.[25] However, conflicting evidence was recently reported using a similar *in vitro* oligonucleotide electrophoretic mobility assay assay and a synthetic N-terminal fragment of human MeCP2 and found that MeCP2 binds with equal affinity to both 5 mC and 5 hmC.[27] It is likely that the *in vivo* binding affinity of MeCP2 to 5-hmC may vary depending on its interaction with the chromatin environment and/or the specific region and DNA sequence; however, this possibility will require much more research using neuronal cell populations rather than *in vitro* oligonucleotide binding.

It is important to note that previous methods for quantifying CpG methylation in DNA, including bisulfite sequencing and methyl-sensitive restriction methodologies, are not capable of distinguishing 5-hmC from 5-mC.[28] Therefore, published studies of DNA methylation density may need to be repeated with the newer methodology capable of independently measuring 5-hmC and 5-mC content in DNA. In the present report, we refine and extend our previous investigation of *EN-2* gene overexpression in the autism cerebellum by quantifying both 5-mC and 5-hmC in order to better define the contribution of 5-hmC to the global and *EN-2* gene-specific DNA *hyper*methylation we previously reported.[2] In addition, we examine the relationship between global 5-hmC content and 8-oxo-dG as an indication of cerebellar oxidative stress as well as the expression of *TET1-3* and DNA methyltransferase genes as potential contributors to altered 5-hmC and 5-mC content. Finally, in an attempt to better explain potential mechanisms underlying *EN-2* overexpression, we quantify MeCP2 binding and its correlation with *EN-2* expression.

MATERIALS AND METHODS

Post-mortem cerebellum

Nuclei and DNA were isolated from frozen 1 square centimeter blocks of cerebellar cortex from 13 autism and 13 unaffected control individuals obtained from the National Institute for Child Health and Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD, USA) and from the Autism Tissue Program at the Harvard Brain Tissue Resource Center (Belmont, MA, USA). All case donors had a confirmed diagnosis of autism (not PDD-NOS, Asperger's, Rett or Fragile X) based on Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) and Autistic Diagnostic Interview Revised. Integrity of RNA and DNA was confirmed for all samples. Control donors had no previous medical history of neurologic disorders, seizures or mental retardation. Autism and control groups were matched as closely as possible for post-mortem interval, age, gender, race and cause of death (details in Results section). Matched pairs and ID numbers have been previously published in our previous publication.[2]

Determination of global 5-mC, 5-hmC and 8-oxo-dG content

DNA was extracted from frozen cerebellum blocks using the Puregene DNA Purification kit (Qiagen, Valencia, CA, USA). The levels of 8-oxodG, 5 mC and 5 hmC in cerebellar DNA were measured using liquid chromatography combined with electrospray tandem mass spectrometry (LC/MS/MS) as previously detailed.[2]

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from brain tissues using TRI Reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The levels of *EN-2, TET1, TET2, TET3, DNMT1 and DNMT3A and 3B* gene transcripts were determined with qRT-PCR using TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's protocol. Relative quantification of gene expression was performed by using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control. The $2^{-\Delta Ct}$ method was used for calculating the relative amount of the target RNA.

Hydroxymethylated DNA immunoprecipitation (hMeDIP) analysis of 5-hmC within the EN-2 promoter and gene body

There are two CpG islands within the *EN-2* gene promoter. One includes the TSS and the other is upstream from the transcription start site (TSS). Location and primers for both CpG islands and the gene body are provided in Supplementary Table 1. The hMeDIP analysis of 5-hmC was carried out within both CpG islands and in the gene body as described in the protocol for the Novel Diagenode hMeDIP kit (Diagenode, NJ, USA). Briefly, 1 μg of genomic DNA fragments were immunoprecipitated with 2.5 μg of mouse monoclonal 5-hmC antibody, or with 2.5 μg of mouse IgG per tube on the magnetic beads. DNA from the antibody-bound fractions was purified with Proteinase K in DNA isolation buffer and stored at − 20 °C. DNA was subjected to qPCR analysis. Percent enrichment was calculated by $100x\ 2^{(\Delta Ct\ adjusted\ input\ -\Delta Ct\ enriched)}$. Input DNA ΔCt value was adjusted from 10 to 100% equivalent to subtracting 3.32 ΔCts or $\log_2 10$.

Analysis of 5-mC and 5-hmC within EN-2 gene promoter

The levels of 5-mC and 5-hmC within *EN-2* promoter and body were analyzed exactly as described in the EpiMark 5-hmC and 5-mC Analysis Kit (New England BioLabs, Ipwich, MA, USA). Briefly, immunoprecipitated DNA (50 ng) was analyzed using qPCR with forward primer 5′-AACG GGGTTCCCGGGTCAGT-3′ and reverse primer 5′-GAACGACCGCCGCCCTC AAG-3′ and spanning − 150 to − 41 relative to the TSS. To determine the methylation status of inner C in CCGG sites, a calculation was carried out using the formula described in the Epimark analysis kit. The extent of *EN-2* promoter 5-mC methylation was also measured by the McrBC assay as described previously.[2,29]

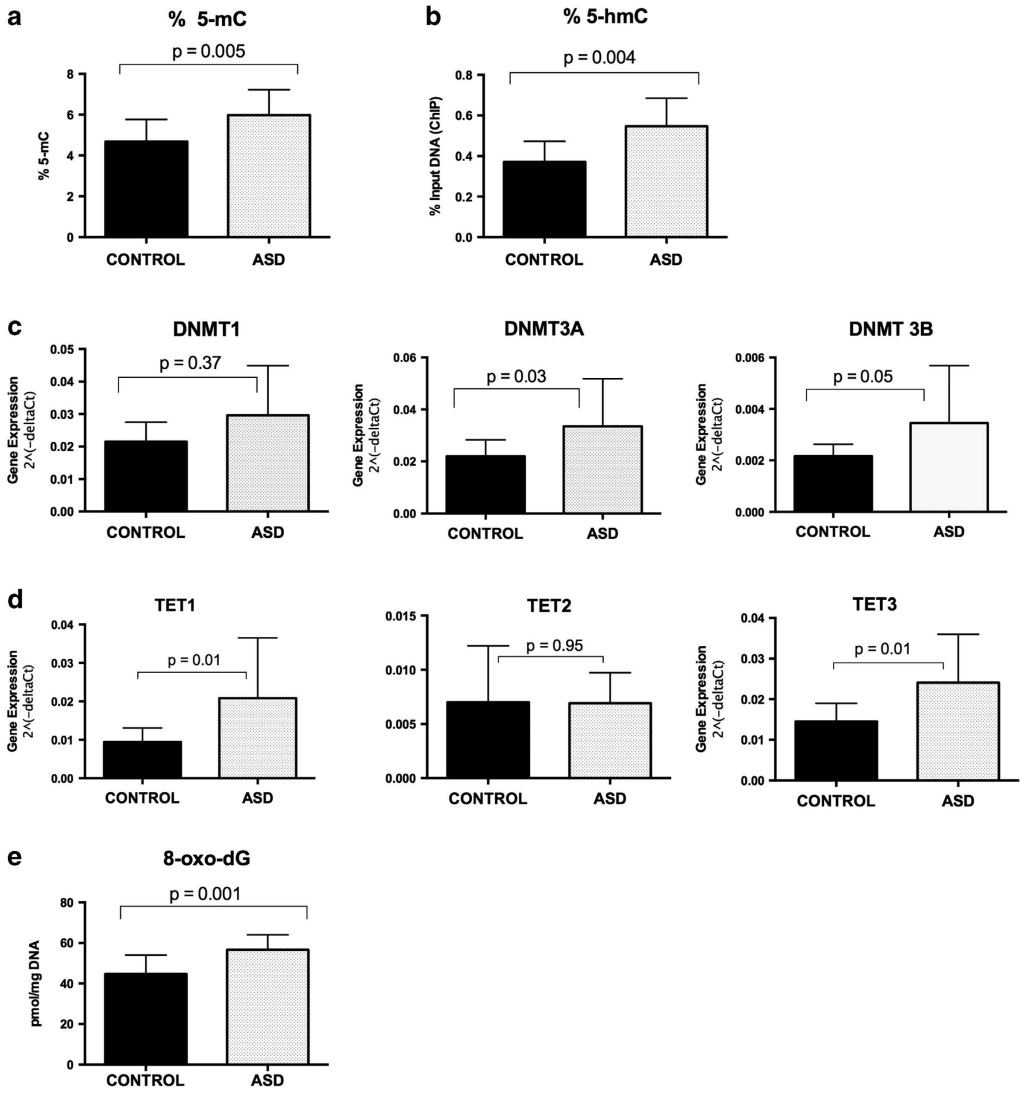

Figure 1. Global alterations in cerebellar levels of 5-mC (**a**), 5-hmC (**b**), DNA methyltransferases DNMT1, DNMT3A and DNMT3B (**c**), TET1, TET2, TET3 (**d**) and 8-oxo-dG (**e**) in 13 autism (ASD) and 13 matched control cerebellar samples. Results are expressed as the mean and s.d.'s with associated *P*-values.

Chromatin immunoprecipitation assay

The level of MeCP2 at the *EN-2* promoter region was determined by chromatin immunoprecipitation assay using primary anti-MeCP2 antibody (Active Motif, Carlsbad, CA, USA). Purified immunoprecipitated DNA and input DNA were subsequently co-amplified with qPCR using the same primers utilized for hMeDIP assay described above. All assays were run in triplicate and data expressed as the mean (± s.e.) percent input DNA after adjusting for total input DNA: $100x2^{(\text{adjusted input } Ct - IP\ Ct)}$.

Statistical analyses

All statistical analyses were performed using Graphpad Prism software (La Jolla, CA, USA). Normal distribution of the data was determined using the Kolmogorov–Smirnov test. Results are expressed as means ± s.d. with statistical significance set at 0.05. The mean differences between case and control groups were evaluated using the Student's *t*-test for data that were normally distributed and the nonparametric Mann–Whitney *U*-test was used for data that were not normally distributed. Relationships between variables were determined using linear regression analysis within the Graphpad Prism software.

RESULTS

Cerebellar tissue sample matching and selection

Careful case–control matching resulted in no mean differences in age, gender, race or post-mortem interval between the autism and control groups ($P > 0.05$). The mean age was 15.5 ±9.5 years in the autism group (eight children under 18 years and five adults) and 15.8 ± 8.6 years (nine children under 18 years and four adults) in the control group. In both groups, 69% (9/13) of individuals were male. The mean post-mortem interval was 18.4 ± 5.7 h for the autism samples and 15.7 ± 6.2 h in the control samples. Age, gender, race and post-mortem interval were the primary variables matched between groups. An attempt was made to match the cause of death as closely as possible but, given limited sample availability, we made the best matches possible. For example, asphyxia, smoke inhalation or aspiration in cases were matched to asthma and drowning in controls. Head trauma was matched with brain bruising and gastrointestinal (GI) bleeding matched with multiple injuries.

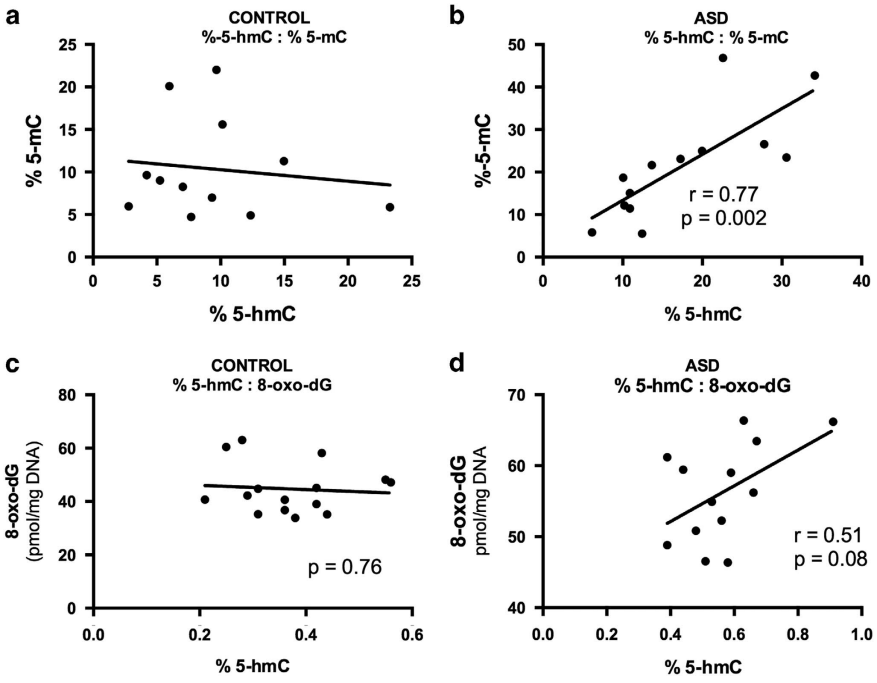

Figure 2. Panels **a**, **b** depict the correlations between 5-mC and 5-hmC in matched control and autism cerebellar samples, respectively, with associated correlation coefficients and *P*-values. Panels **c**, **d** show the correlation between 5-hmC and 8-oxo-dG in control and autism samples, respectively, with no correlation within the control samples and marginally significant positive association ($P = 0.08$) in the autism samples.

Global 5-hmC, 5-mC, expression of DNA methyltransferases, TET dioxygenases and 8-oxo-dG in autism and the control cerebellum

It is well accepted that standard bisulfite and enzymatic assays previously used to quantify levels of 5-mC in DNA cannot distinguish 5-mC from 5-hmC.[28] As 5-hmC is now emerging as a significant and independent epigenetic mark, it becomes imperative to redefine previous reports of global and gene-specific alterations in DNA methylation and associated alterations in gene expression in terms of the independent contributions of both 5-mC and 5-hmC. We previously reported global and *EN-2* gene-specific elevation in 5-mC that was associated with increased gene expression using standard methodology that does not distinguish the contribution of 5-hmC.[2] To refine our determination of global DNA cytosine methylation, we separated and quantified global 5-mC and 5-hmC bases in the same cerebellar DNA using LC/MS/MS and expressed the results as percent 5-mC and 5 hmC/total cytosine content. As shown in Figure 1a and b, we observed a significant increase in *both* 5-mC and 5-hmC in the autism cerebellum relative to the control samples. The level of 5-mC was $5.9 \pm 1.2\%$ in 13 autism cerebellum compared with $4.7 \pm 1.1\%$ in 13 control samples ($P = 0.005$) and the level of 5-hmC was $0.57 \pm 0.14\%$ in the autism samples and $0.37 \pm 0.10\%$ in control samples ($P = 0.004$). We then measured the expression of *DNMT1, DNMT3A and DNMT3B* as possible reasons for unexpected parallel increase in both 5-mC and 5-hmC. As shown in Figure 1c, *DNMT3A and DNMT3B* expression levels were significantly increased in the autism cerebellum relative to control samples, suggesting a compensatory increase in *de novo* DNA methylation in the autism cerebellum.[30] In contrast, there was no significant difference in DNMT1 expression. We next evaluated the expression of the *TET* family of dioxygenases (*TET1-3*) to determine whether the elevation in 5-hmC content could be related to an increase in TET-mediated conversion of 5-mC to 5-hmC. As shown in Figure 1d, the results indicated a significant increase in both TET1 and TET3 expression with no significant difference in TET2 in case compared with control cerebellum. Thus, it is possible that a sustained increase in TET1 and TET3 expression contributed to the increase in 5-hmC in the EN-2 promoter. However, if this were the case, one might expect a concomitant decrease in 5-mC via the TET-mediated 5-mC demethylation pathway instead of the increase in 5-mC observed. Finally, given previous reports of possible conversion of 5 mC to 5-hmC under conditions of oxidative stress,[24,26] we assessed the relative pro-oxidant environment in autism and control cerebellum by quantifying 8-oxo-dG in DNA, a well-established marker of oxidative DNA damage and oxidative stress.[31] Figure 1e confirms a significant increase in 8-oxo-dG content in the autism cerebellum relative to control ($P = 0.001$). Figure 2 shows a strong positive correlation ($P = 0.002$) between 5-mC and 5-hmC in the autism cerebellum (Figure 2b) that was not significant in control samples (Figure 2a). A marginally significant correlation ($P = 0.08$) was observed between 5-hmC and 8-oxo-dG in autism (Figure 2d) but not control cerebellar samples (Figure 2c).

5-hmC level within the upstream EN-2 CpG island promoter and gene body

In our initial epigenetic evaluation of the developmentally regulated *EN-2* gene, we reported promoter *hyper*methylation that was unexpectedly associated with an increase in gene expression and level of EN-2 protein.[2] Genomic mapping reports of 5-hmC distribution during rodent and human neurodevelopment have linked elevated 5-hmC content in the promoter and gene body with gene activation and overexpression.[18,27,32] Therefore, it was of considerable interest to quantify 5-hmC content and determine its association with 5-mC content within the overexpressed *EN-2* gene. Figures 3a and b show the level of 5-hmC in the upstream *EN-2* promoter region using two independent assays (Epimark and 5-hMeDIP, respectively). Both assays confirmed a significant increase in 5-hmC content within the upstream *EN-2* promoter region. In contrast, both assays found no significant difference in 5-hmC content within the proximal promoter in control samples (data not shown). Within the gene body (+1861 to +1996), the level of 5-hmC was significantly elevated (0.55 ± 0.26 in autism and 0.31 ± 0.33 in control samples

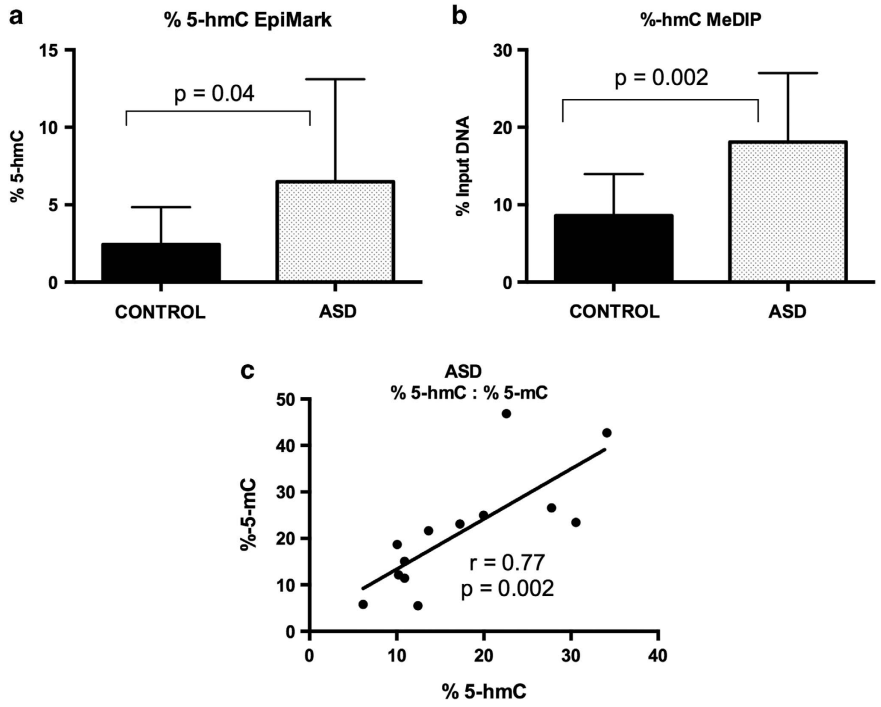

Figure 3. The mean increase in 5-hmC levels in the *EN-2* promoter in autism compared control cerebellar samples using two independent assays: **a** shows the relative increase using the Epimark assay and **b** shows similar increase using the hMeDIP assay (means ± s.d.). Panel **c** shows the highly significant positive association between 5-hmC and 5-mC ($P = 0.002$).

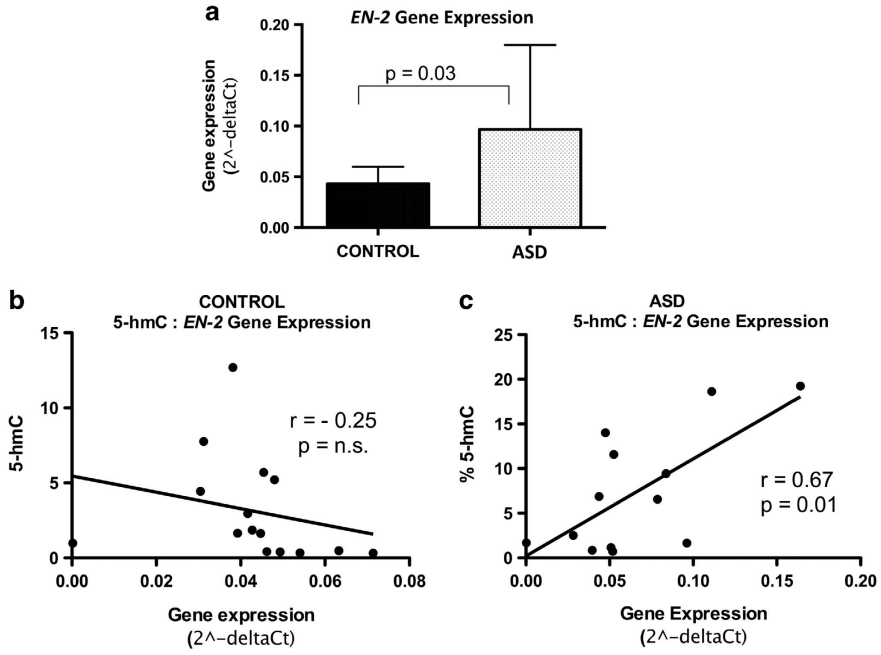

Figure 4. Panel **a** confirms the significant increase in *EN-2* gene expression in autism compared with control cerebellar samples. The positive association between 5-hmC and gene expression in the autism samples is shown in **c** ($P = 0.01$) that was not present in control samples (**b**).

($P = 0.03$), albeit at a fraction (3%) of 5-hmC content in the 5′ distal promoter CpG island. Within the 5′ promoter CpG island, there was a highly significant positive correlation between 5-mC and 5-hmC as shown in Figures 3c ($r = 0.77$, $P = 0.002$) similar to the positive relationship observed globally in the cerebellum (Figure 2b). The 5-hmC/5-mC ratio was 0.87 in autism and 0.42 in control samples ($P = 0.03$).

Association between 5-hmC content and EN-2 gene expression

On the basis of recent reports of a positive association between actively transcribed neurodevelopmental genes and the abundance of 5-hmC in defined 5′ promoter regions and in gene bodies,[17,19,32] we interrogated the relationship between the level of 5-hmC in the *EN-2* upstream CpG island promoter region and in the gene body with *EN-2* gene expression. As shown in Figure 4a,

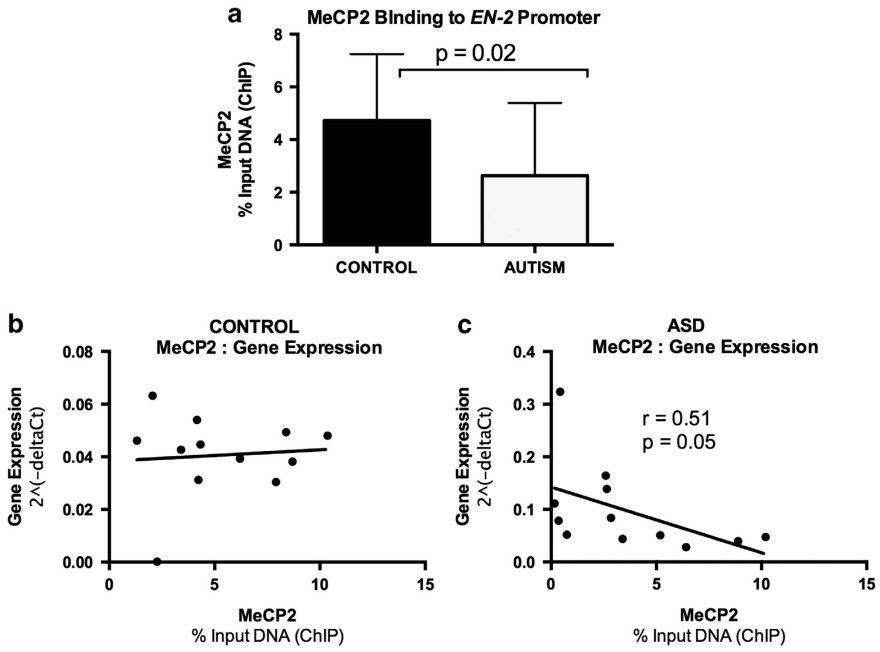

Figure 5. Panel **a** confirms the significant elevation in MeCP2 within the *EN-2* promoter and **c** shows the negative correlation between MeCP2 and *EN-2* gene expression ($P = 0.05$) that was not present in control samples (**b**).

EN-2 gene expression was significantly increased in the autism cerebellum compared with control as previously reported.[2] Further, a highly significant positive correlation ($r = 0.67$, $P = 0.01$) was found between 5-hmC content and *EN-2* gene expression in the 5′ promoter CpG island in autism but not in control samples (Figures 4c and b, respectively). In contrast to previous reports, the level of 5-hmC in the *EN-2* gene body was not correlated with EN-2 gene expression (data not shown).

MeCP2 binding to the EN-2 promoter and correlation between EN-2 gene expression and MeCP2 binding

When MeCP2 binding is reduced, the recruitment of chromatin-modifying enzymes that promote chromatin condensation and gene repression are concomitantly reduced. A reduction in MeCP2 binding, whether because of MeCP2 mutation as in Rett syndrome or reduced 5-hmC affinity, has been associated with aberrant increase in gene expression[33,34] and could offer a partial explanation for the unexpected increase in *EN-2* expression we previously observed despite the increase in 5-mC content (DNA hypermethylation). To address this possibility, we quantified the extent of MeCP2 binding to the *EN-2* promoter region using a chromatin immunoprecipitation-based approach with the same primer sets employed for hMeDIP. Figure 5a reveals a highly significant ($P = 0.02$) decrease in MeCP2 binding to the same 5′ promoter region that contained elevated levels of 5-hmC (Figure 3b) in autism relative to control samples. Given the significant decrease in repressive MeCP2 binding in the *EN-2* promoter, it was important to determine whether reduced binding would be correlated with increased *EN-2* gene expression. Figure 5b shows the correlation between MeCP2 binding and *EN-2* expression in the *EN-2* promoter region in the autism and control cerebellum. The significant inverse relationship ($P = 0.05$) suggests that reduced MeCP2-mediated gene repression may have contributed to persistent *EN-2* gene overexpression in the autism samples. Recently, a novel association between MeCP2 binding and histone H3K27 trimethylation was reported, suggesting that histone marks may influence nucleosomal MeCP2 binding and that the two chromatin-modulating proteins may work in concert with DNA methylation to repress gene expression by maintaining chromatin in a closed inaccessible conformation.[35–37] In our previous report, we showed that histone H3K27me3 binding was significantly reduced in the *EN-2* promoter in autism but not control samples.[2] Thus, an interactive decrease in both repressive marks may contribute to aberrant overexpression of *EN-2* in the autism cerebellum.

DISCUSSION

Using an autism candidate gene approach, we define for the first time global and gene-specific 5-hmC content in the *EN-2* gene and explore the relationship between the 5-hmC level and gene expression in a chromatin context and the potential role of oxidative stress in the generation of 5-hmC. Our results confirm an epigenetic role for 5-hmC that is distinct from 5-mC and suggest that the increase in 5-hmC content and decrease in both MeCP2 and histone H3K27me3 binding may alter local chromatin conformation to facilitate enhancer binding and the persistent upregulation of *EN-2* gene expression in the postnatal autism cerebellum.

The significant positive correlation between 5-hmC and 5-mC both globally and within the *EN-2* promoter was unexpected. If the increase in 5-hmC derived solely from TET enzyme-mediated oxidative demethylation of 5-mC, one might expect an inverse correlation between these two modified cytosines as previously observed.[17,27,38] However, an increase in both epigenetic modifications simultaneously suggests that different cytosines were targeted by TET1/3 and DNMT3A/B. The increase, rather than decrease, in 5-mC plus the stable accumulation 5-hmC strongly suggest an independent epigenetic role for 5-hmC beyond 5-mC demethylation in the postnatal autism cerebellum. Indeed, the expression of *EN-2* was highly correlated with 5-hmC levels in the upstream promoter CpG island (Figure 3c) suggesting that 5-hmC accumulation is mechanistically related to gene upregulation. A similar positive association between global levels of 5-mC and 5-hmC has been recently reported in the temporal cortex of Alzheimer's patients.[21] Other studies using genome-wide mapping of 5-hmC distribution have also reported an increase in 5-hmC density associated with increased gene expression but, in contrast, find a stronger association with 5-hmC in gene bodies

than in promoters,[17,27] whereas others have reported 5-hmC accumulation in promoters and upstream enhancer regions.[39,40] These divergent results suggest that 5-hmC density and function appears to vary with cell type, differentiation stage and gene-specific function.

The significant increase in *DNMT3A* and *DNMT3B* expression together with an increase in *TET1* and *TET3* expression may offer clues to the unexpected positive relationship between 5-mC and 5-hmC. Although the increase in *TET1/3* expression may contribute to the increase in 5-hmC in the EN-2 promoter, it was not associated with a decrease in total 5 mC content. We hypothesize that the upregulation of *de novo* methyltransferases DNMT3A and DNMT3B could be an abortive attempt to downregulate the persistent elevation in *EN-2* expression during early cerebellar development. Although *de novo* DNMT3A normally functions to establish and maintain DNA methylation patterns, Hashimoto *et al*.[41] have shown that DNMT3A but not DNMT1 can methylate hemihydroxylated 5-hmC and may provide a mechanism for the maintenance of 5-hmC levels during replication and DNA repair. Thus, the increase in *de novo* methyltransferases in the autism cerebellum may have a dual role in maintaining both 5-hmC and 5-mC methylation within the *EN-2* gene. Clearly, the simultaneous increase in both 5-mC and 5-hmC within the same *EN-2* upstream promoter sequence indicates that different CpG sites were targeted for 5-mC and 5-hmC by DNMT3A/B and TET1/3, respectively, and may explain the parallel increase in both cytosine modifications.

During prenatal cerebellar development, *EN-2* homeobox transcription factor is normally upregulated acting primarily as a repressor of gene expression and is then downregulated in the late prenatal and early postnatal period.[6,10] The programed upregulation and downregulation of *EN-2* expression during cerebellar development is reminiscent of other neurodevelopmental windows that open and close in a precisely programed temporal sequence that is essential for normal brain development.[42,43] Indeed, autism has been proposed to be a 'critical period' disorder.[44] Early life is thought to be a period with increased sensitivity to epigenetic regulation of the dynamic alterations in gene expression during brain development when dysregulation can contribute to abnormal behavior phenotypes.[43] Inappropriate delay or failure to close a critical window can disrupt the hierarchal progression of downstream critical periods and negatively affect structural organization and neuronal synchronization between regions.[44] The persistent postnatal elevation in *EN-2* expression suggests that this developmental window failed to close appropriately during early cerebellar development and in this way could contribute to the several structural and neuronal abnormalities reported in the autism cerebellum.[45,46]

The non-enzymatic oxidation of 5-mC to 5-hmC cannot be ruled out as a mechanism for 5-hmC accumulation in the autism cerebellum. In our previous publication, we reported a significant decrease in glutathione antioxidant/detoxification capacity (GSH/GSSG) and an increase in DNA and protein oxidative damage (8-oxo-dG and nitrotyrosine, respectively) using the same cerebellar samples analyzed in the present study. Other investigators have reported similar markers of oxidative stress in the autism cerebellum.[47,48] Whereas precedent exists for a non-enzymatic oxidative conversion of 5-mC to 5-hmC in the production of 5-hmC in liver microsomes with exposure to hydroxyl radicals,[24] this intriguing observation requires confirmation in cellular DNA. The DNA adduct 8-oxo-dG is an established marker of oxidative DNA damage generated by excessive hydroxyl ion exposure that exceeds repair capacity and confirms a chronic pro-oxidant microenvironment in the autism cerebellum.[49] The positive correlation between 8-oxo-dG and 5-hmC provides indirect support for possible free radical oxidation of 5-mC to 5-hmC; however, this provocative alternative mechanism for 5-hmC generation will require further focused investigation. Finally, it should be noted that reduced binding of MeCP2 to the oxidized bases 5-hmC and 8-oxo-dG *in vitro* has been reported.[25]

A decrease in MeCP2-binding affinity to 5-hmC compared with 5-mC has been reported by several investigators using genome-wide profiling or *in vitro* binding affinity assays.[17,25] Our *in vivo* finding of a significant decrease in MeCP2 binding to the same promoter sequence that contained increased 5-hmC content in the autism cerebellum is consistent with these reports but inconsistent with a recent *in vitro* finding that MeCP2 has equal binding affinity for both 5-hmC and 5-mC.[27] Using a similar candidate gene approach to explore MeCP2 binding and 5-hmC content in the autism cerebellum, Zhubi *et al*.[38] examined *GAD1* and *RELN* genes previously shown to be downregulated in this region. These investigators found an increase in 5-hmC and *TET1* expression associated with a decrease in 5-mC and an unexpected increase in MeCP2 binding in the promoters of these down-regulated genes. Although an increase in MeCP2 binding is consistent with repression of gene expression, it is not consistent with reports of gene activation and increased gene expression with elevated 5-hmC. The positive association between 5-hmC and gene expression in the present study are more consistent with gene upregulation as observed with the MeCP2 mutation in Rett syndrome.[34,50] These contrasting results in the autism cerebellum emphasize the plurality of gene expression alterations associated with MeCP2 binding that appears to vary with developmental context, local microenvironment and gene activation or inactivation status *in vivo*. Further, the results underscore the importance of re-examining previous reports of DNA hypermethylation associated with altered gene expression in the autism brain that use methodology that is incapable of distinguishing 5-hmC from 5-mC.

Highly relevant to the observed positive association between 5-hmC level and *EN-2* expression shown in Figure 4b, Szulwach *et al*.[17] demonstrated the presence of a developmentally regulated 5-hmC-enriched area (differential 5-hmC region, DHMR) in the mouse cerebellum directly upstream of the *En-2* gene that overlapped with a Pax 2/5/8-regulated enhancer element. Supporting this observation, Song *et al*.[51] confirmed the presence of several PAX-binding sites in the mouse upstream *En-2* enhancer region that were essential for *En-2* activation and, in addition, demonstrated strong evolutionary conservation in this region. Enhancers are transcriptional regulatory regions that have been shown to be modulated by epigenetic modifications during development.[52] Recently, whole-genome analysis with base resolution of 5-hmC and 5-mC in the human brain reported increased 5-hmC at poised enhancer regions.[40] Further, 5-hmC accumulation has been shown to be an early event in enhancer activation during neuronal differentiation in mouse P19 cells.[53] On the basis of these reports, we searched the *EN-2* upstream CpG island sequence for PAX5-binding sites using the JASPAR transcription factor database (jaspar.genereg.net/cgi-bin/jaspar_db.pl?rm=browse&db=core&tax_group=vertebrates). Our search yielded six PAX5-binding sites within the *EN-2* upstream CpG island in the human cerebellum. In addition, we found a CCAAT enhancer-binding protein beta-binding site within our primer sequence.[54] The presence of several enhancer-binding sites together with the significant increase in 5-hmC in this region suggests that the positive association of 5-hmC with gene expression may be because of epigenetic facilitation of enhancer activation. Taken together, our data also support the possibility that the increase in 5-hmC accompanied by reduced MeCP2 and histone H3K27me3 binding collaborate to promote a more open chromatin configuration that facilitates enhancer protein binding and increased *EN-2* expression.

Our study was limited by methodology that does not provide base resolution, which would identify the exact location of the modified cytosines within the promoter and gene body regions. The Epimark assay targets cytosines within the CCGG sequences

only, whereas a monoclonal antibody to 5-hmC with hMeDIP quantifies the total 5-hmC content in defined regions. Nonetheless, both assays independently confirmed the increase in 5-hmC content and reduced 5-hm5-mC ratio in the *EN-2* promoter. Another limitation was the report of *TET* gene expression rather than TET activity. An increase in 5-hmC could reflect and increase in TET2 activity independent of gene expression.

In summary, our results indicate that elevated 5-hmC in the *EN-2* promoter is associated with a significant decrease in repressive MeCP2 and histone H3K27me3 that appear to override 5-mC hypermethylation. These epigenetic alterations would be expected to relax enhancer region chromatin and facilitate enhancer binding and promote sustained upregulation of *EN-2* expression. As perinatal *EN-2* downregulation is essential for normal Purkinje cell differentiation and cerebellar patterning, the persistent postnatal overexpression of *EN-2* suggests that the closing of this programed developmental window may have been missed in some individuals with autism because of epigenetic abnormalities. The potential role of epigenetic switches in the opening and closing of critical windows during fetal and postnatal development warrants further gene-specific investigation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DISCLAIMER

The views expressed in this paper do not necessarily represent those of the US Food and Drug Administration.

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