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## Cytosine modifications in neurodevelopment and diseases

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### Abstract

DNA methylation has been studied comprehensively and linked to both normal neurodevelopment and neurological diseases. The recent identification of several new DNA modifications, including 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), has given us a new perspective on the previously observed plasticity in 5mC-dependent regulatory processes. Here we review the latest research into these cytosine modifications, focusing mainly on their roles in neurodevelopment and diseases.

### Keywords

DNA methylation (5mC); 5-hydroxymethylcytosine (5hmC); 5-formylcytosine (5fC); 5-carboxylcytosine (5caC); DNA methyltransferases (DNMTs); ten-eleven translocation (TET); neurodevelopment; neurological; neurodegenerative diseases

### Introduction

In the early 1900s, genetics and developmental biology were considered to be two separate entities [1]. Conrad Waddington first coined the term “epigenetics” in the middle of the twentieth century, derived from the Greek words for “over” or “above” genetics, to describe the molecular events involved in early undifferentiated embryonic development, linking the two important fields together [2]. The current definition of epigenetics is the study of heritable changes in gene expression and function that do not alter DNA sequence [3,4]. There are currently three well-characterized epigenetic mechanisms, cytosine modifications, histone modifications, and ATP-dependent chromatin remodeling, of which modification of cytosines is the only mechanism that directly imposes on DNA [5–8]. DNA methylation was first proposed to play an important role in long-term memory formation [9] and remained the major DNA covalent modification to influence transcriptional states, and ultimately cellular identity. Methylation on the fifth position of cytosine (5mC) typically occurs in the context of regions that contain a high frequency of CG dinucleotides in the mammalian genome and plays pivotal roles in the regulation of gene expression, chromatin structure, gene imprinting, X-chromosome inactivation, and genomic stability [10–12]. The exceptions are CpG islands, which are frequently located alongside gene promoters and usually remain unmethylated. DNA methylation is often associated with a gene repressive environment, and maintaining proper DNA methylation status is essential for normal development, with aberrant DNA methylation patterns frequently being linked to the pathogenesis of numerous diseases, including neurological disease and cancer [6,13–15]. Three well-defined DNA methyltransferases (DNMTs) are responsible for preserving or generating this marker. DNMT1 maintains DNA methylation during the cell cycle by copying the existing pattern of hemi-methylated DNA to their daughter strands during DNA replication. DNMT3A and 3B,

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in contrast, create new methylation loci by coordinating with different interacting partners, including histone modifiers or transcription repressors, to achieve their specificity, thereby acting as *de novo* methyltransferases [16,17]. Aside from the known DNMT characters, DNA methylation can also be influenced by non-canonical DNMT functions or their co-factors [18,19]. DNA methylation can be recognized by a spectrum of protein “readers,” such as methyl-CpG binding protein 2 (MeCP2) and methyl-CpG-binding domain proteins 1-4 (MBD1-4) [20,21]. The aberrant expression of these proteins often has severe consequences, such as neurological disorders and cancer, emphasizing the importance of the correct interpretation of DNA methylation markers [22–24] (Fig 1). The respective roles of DNMTs and MBDs in neurodevelopment and disease have been extensively characterized and will be discussed in the following sections.

Another DNA modification, 5-hydroxymethylcytosine (5hmC), was initially identified in bacteriophage in 1953, the same year Watson and Crick proposed the DNA structure [25]. 5hmC was found in mammalian genomes in 1972 [26]; however, the mechanisms and proteins responsible for generating this marker remained unknown. In 2009, Rao and colleagues demonstrated that ten-eleven translocation 1 (TET1), a 2-oxoglutarate (2OG)- and Fe (II)-dependent enzyme, catalyzes conversion of 5mC to 5hmC [27]. Subsequent studies revealed that TET1 could further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), giving us a completely new perspective on the plasticity of 5mC-dependent processes [28–30]. It is of particular interest that the overall 5hmC level varies between tissues, with approximately ten times more in brain tissues like Purkinje neurons [31–35], as well as embryonic stem cells (ESCs), compared to other tissues [28,36–38]. This differential distribution points to the possible functional importance of this DNA modification in development and neuronal activity.

In this review, we summarize the current knowledge of and advances in the molecular mechanisms of cytosine modifications, with a particular focus on the impact they have on neurodevelopment and human diseases.

## DNA methylation in neurodevelopment and neurological disorders

### Roles of DNA methyltransferases: the writers

The adult mammalian central nervous system (CNS) was once believed to never generate new neurons, but recent research has proved that thousands of new neurons are actually generated every day, primarily derived from the adult neural stem cells (NSCs) located in the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the subventricular zone (SVZ) of the lateral ventricle [14]. In this case, adult neurodevelopment can be viewed as classic stem cell differentiation, and thus involves precise epigenetic control. On the other hand, DNA methylation is known to be critical in synaptic plasticity related to long-term learning and memory in mature neurons, likely owing to regulation of specific gene expression [39,40]. It is intriguing that the expressions of three DNMTs show differential patterns in various brain tissues and in the developmental stage, pointing to their distinctive roles in neuronal development and function [41,42]. For example, *Dnmt1* mRNA is ubiquitously expressed in both dividing neural precursor cells and postmitotic neurons in mouse brain, consistent with their important role in maintaining DNA methylation patterns throughout cell replication [42]. In contrast, *Dnmt3a* and *3b* show temporally and spatially different expression during neurodevelopment. *Dnmt3b* is robustly expressed in SVZ between embryonic days (E) 10.5 and 13.5, but becomes virtually undetectable in the CNS after E15.5, whereas *Dnmt3a* starts to be expressed in SVZ neural stem cells from E10.5 to E17.5 and can be detected predominantly in postnatal neurons from almost all brain regions [41]. These interesting observations suggest distinct, non-overlapping roles for the different

Dnmts in brain function and highlight the importance of DNA methylation in prenatal and postnatal neurodevelopment.

Mutations in any of the three major Dnmts in mice lead to embryonic lethality [16,43], thus conditional knockout of Dnmts in mice has been applied to study their role in the CNS [44–46]. For example, conditional ablation of Dnmt1 in the dorsal forebrain results in the failure to develop somatosensory barrel cortex, and thalamocortical long-term potentiation is also impaired [44]. In a separate report, Dnmt1 deletion in neural progenitor cells induced derepression of astroglial marker genes, as well as genes involved in JAK-STAT signaling, indicating the importance of DNA methylation in controlling astroglial differentiation [47]. Unlike the deletion of Dnmt1 and Dnmt3b, mice that lack functional Dnmt3a in the CNS appear to be grossly normal at birth, but die prematurely with the acquisition of developmental mental defects [45]. In a separate study, Wu et al demonstrated that Dnmt3a deletion leads to impaired postnatal neurogenesis at both neurogenic zones. This is confirmed by 10-fold fewer neurons being differentiated *in vitro* from Dnmt3a-null neural stem cells compared to wild-type. Genome-wide Dnmt1-binding and DNA methylation analysis using WT or Dnmt3a-null postnatal neural stem cells revealed that Dnmt3a methylates intergenic regions and gene bodies flanking proximal promoters. Surprisingly, Dnmt3a knockout induces the silencing of genes related to neurogenic activity, and Dnmt3a occupancy prevents Polycomb repressive complex recruitment [46]. However, the possibility remains that the Dnmt3a-dependent nonproximal promoter methylation can functionally antagonize Polycomb deposition of trimethylation of histone H3 lysine 27 (H3K27me3). Nonetheless, this interesting observation implies that Dnmts might have dual functions in neurodevelopment. With regard to long-term plasticity maintenance, neither Dnmt1 nor Dnmt3a knockout alone, but their double knockout (DKO) in mouse forebrain leads to profound deficits in learning and memory [39]. All this evidence points to a mechanism whereby individual Dnmts play distinctive roles in early neurodevelopment, and they orchestrate together to maintain long-term proper neuronal functions.

Both genetic and epigenetic alterations have been studied extensively in cancer and are found to be critical in carcinogenesis and tumor progression [48,49]. This powerful evidence serves as an excellent reference for dissecting epigenetic mechanisms in neuronal disease. In normal cells, DNA methylation occurs largely on repetitive sequences to maintain genomic stability, and promoter CpG islands are usually unmethylated. These features are often reversed in cancer cells, with promoter CpG islands becoming hypermethylated, thereby repressing a large set of tumor-suppressing genes, and repetitive sequences showing hypomethylation, resulting in chromosome instability and activation of transposable elements [50–52]. Recent genome-wide DNA methylome analysis in cancer cells indicates that most affected CpG island genes are already silenced prior to aberrant DNA hypermethylation, which is reminiscent of normal development, where gene silencing precedes promoter CpG methylation [53]. Mechanisms other than DNA methylation appear to be involved in transcription control on these genes. In fact, DNA methylation orchestrates with other epigenetic mechanisms, such as histone modifications, to influence cellular activity and disease onset [13,51,52].

Given the critical functions of DNMTs mentioned in the previous section, it is not surprising that several neurological disorders are caused by mutations within DNMTs. For example, hereditary sensory and autonomic neuropathy type 1 (HSAN1) is an autosomal dominant neurodegenerative disorder involving loss of sensation and various neuropathies in the third or fourth decade of life [13]. A recent study identified mutations on the DNMT1 sequence, and these mutations lead to premature degradation of mutant proteins and impair DNMT1 cellular activity. Since DNMT1 primarily maintains methylation patterns during the cell cycle, these mutations likely affect gene transcription due to the loss of specific promoter

methyations through cell replication and may progressively impair neuronal differentiation, migration, and central neural connections [54]. Notably, the fact that DNMT1 mutation also affects postmitotic neurons in HSAN1 patients led to the interesting notion that DNMT1 might also be involved in modulating DNA methylation in a cell cycle-independent manner [55]. Mutations in DNMT1 are also reported in autosomal dominant cerebellar ataxia, deafness, and narcolepsy (ADCA-DN), with their locations in close proximity to the mutations in HSAN1 [56]. These findings highlight the functional importance of DNMT1 in the maintenance of normal neuronal activity.

The immunodeficiency, centromere instability, facial anomalies (ICF) syndrome is characterized mainly by polymorphic mutations on the *de novo* methyltransferase, DNMT3B [57,58]. These patients suffer from impaired cellular immunity and unusual facial features [51]. DNMT3B-mutated cells show hypomethylation and imbalances in histone markers on pericentromeric repeats [16]. Subsequent global gene profiling revealed that a large set of genes related to immune function, development, and neurogenesis are de-repressed. These genes are associated with loss of DNA methylation, repressive H3K27me3 marker, and Polycomb repressive complex [59]. Another syndrome closely related to ICF, ICF2, was recently linked to mutations of the zinc-finger- and BTB (bric-a-brac, tramtrack, broad complex)-domain-containing 24 (*ZBTB24*) gene [60,61]. Mechanistically, ZBTB24 is a putative DNA-binding protein and may be involved in juxtacentromeric DNA methylation [60]. DNMT3A has not yet been linked to neuronal diseases, although its deletion does impair postnatal neurogenesis [46], but it is strongly associated with acute myeloid leukemia [62,63].

### Roles of methyl-CpG binding proteins (MBPs): the readers

Since DNA methylation plays a critical role in neurodevelopment, it is feasible that the methylation “reader,” methyl-binding proteins, may also be indispensable to correctly interpreting existing modifications [21]. Indeed, methyl-CpG binding protein 1 (MBD1) appears to be an important regulator of neural stem cells. MBD1-null mice show reduced neuronal differentiation and increased genomic instability. In addition, MBD1 knockout also affects postnatal neurogenesis in the SGZ, indicating its dual roles in both neurogenesis and the maintenance of long-term brain function [64]. Nevertheless, in this particular study, the adult NSCs from wild-type mice already exhibited 21.3% aneuploidy, and NSCs from MBD1 null mice enhanced this trend by an additional 20%. Hence, further experiments can be performed in the future to rule out indirect effects caused by the specific cell type used here. One proposed mechanism for MBD1 is that it directly binds to the hypermethylated promoter of basic fibroblast growth factor 2 (*Fgf2*), an essential growth factor for neural development. MBD1 deletion results in *Fgf2* promoter hypomethylation and differentiation arrest [65]. Since this study was performed in cultured adult NSCs, further studies should be conducted to confirm this observation *in vivo*.

Methyl-CpG binding protein 2 (MeCP2) was first identified about two decades ago for its ability to recognize and bind to methylated DNA [66]. Quantitative analysis of MeCP2 revealed its broad expression in various tissues, with the highest levels in brain, lung, and spleen. Expression of MeCP2 in brain shows temporal and spatial order and is correlated with the maturation and function of the central nervous system [67]. MeCP2 is one of the best-characterized MBPs [24,68] and has been implicated in the regulation of global histone modification [69], target gene repression [70,71] or activation [23,72], long-range interaction between distant regions of the genome [73], and the regulation of alternative splicing [74]. The MeCP2 gene is X-linked, with one copy inactivated during dosage compensation [75]. Therefore, mutations induce the premature death of males in their first two years of life [76]. Females with the same mutations survive longer, but develop a severe

progressive neurodevelopmental disorder called Rett syndrome (RTT) [77]. It appears that precise control of the MeCP2 level is important, as increasing copy numbers of MeCP2 are found to cause mental retardation, autism, and psychiatric symptoms [78]. A number of mouse models with MeCP2 mutations or conditional knockout have been created [24,68]. These mice recapitulate the RTT phenotypes, confirming the key role of MeCP2 in RTT. Post-translational modifications are suspected of playing a key part in the regulation of MeCP2 behavior. For instance, brain-specific MeCP2 phosphorylation has been linked to activity-dependent brain-derived neurotrophic factor (Bdnf) transcription, a growth factor that supports neuron growth and differentiation [79]. Recent genome-wide profiling of MeCP2 revealed that MeCP2 S421 phosphorylation occurs globally in response to neuronal stimulation and participates in dendritic development and key neurological responses [80].

There have also been efforts to rescue the RTT phenotypes in a mouse model by re-expressing functional MeCP2 proteins. Indeed, tamoxifen-induced MeCP2 expression in MeCP2 null mice could reverse neurological phenotypes, although some mice experienced rapid death, possibly due to overinduction of MeCP2 [81]. This observation suggests that the neuronal damage of RTT is reversible. The authors of this study proposed a model wherein DNA methylation patterns are preserved, even in the absence of MeCP2, and replenishment of MeCP2 can thus restore neuronal activity. These observations, along with others [82], shed new light on potential therapeutic treatments for RTT patients.

Numerous efforts have also been put into identifying the 5hmC or even 5fC and 5caC readers. It turns out that many MBPs also possess the capacity to recognize 5hmC. For example, a recent study purified distinct types of neuronal cells and found that both 5mC and 5hmC show a strong cell type-related bias. These investigators also identified MeCP2 as a 5hmC-binding protein, establishing a dual role for MeCP2 in the orchestration of neuronal plasticity by coordinating different cytosine modifications [31]. In addition, Yildirim et al demonstrated the Methyl-CpG binding protein 3 (MBD3) co-localizes with Tet1 and 5hmC in ESCs, and MBD3 showed a preference to bind to 5hmC over 5mC [83]. Furthermore, Vermeulen and colleagues systematically characterized the protein readers to a variety of different cytosine modifications in ESCs, neuronal progenitor cells (NPCs), and adult mouse brain tissue. Their study revealed a protein list that overlaps between various modifications, including MeCP2 and some methyl-CpG binding proteins. Remarkably, some readers displayed strong tissue or modification specificity, suggesting that correct and specific interpretation of the cytosine modifications is critical for normal development and homeostasis [84].

### DNA methylation in repetitive elements

As discussed above, the deletion or mutation of either DNMTs or MBPs leads to aberrant DNA methylation patterns in brain, and eventually neurodevelopmental deficiency. Among many mechanisms proposed, the activation of retrotransposons is one attractive model [85]. An engineered human long interspersed element 1 (LINE-1 or L1) has been found to retrotranspose in neuronal precursors derived from rat hippocampus neural stem cells [86]. Subsequent research confirmed this by detecting an increased copy number of endogenous L1 in several regions of human brain. Bisulfite sequencing revealed 5'UTR hypomethylation in human brain tissues compared to matched skin samples, highlighting the importance of DNA methylation in this process, which is consistent with the notion that DNA hypermethylation occurs in the repetitive elements to maintain genomic stability [87]. The transposable elements may help fine-tune gene expression in the brain in a single cell-based manner to achieve specific and complicated neuronal responses [14].

## Other DNA methylation-related neurodevelopmental and neurodegenerative diseases

A common feature of repeat expansion diseases, including many common neurological disorders like fragile X mental retardation syndrome (FXS) and Huntington's disease (HD), is trinucleotide repeats on the coding region, untranslated region, or even introns of certain genes [88,89]. Among different mechanisms of disease formation, RNA gain-of-function toxicity has been reported extensively from the repeat elements either interfering with alternative splicing (e.g., in myotonic dystrophy, DM) or disrupting the balance and availability of RNA-binding proteins (e.g., fragile X tremor ataxia syndrome, FXTAS) [88–91]. Nonetheless, a recent study suggests that polyglutamine-expanded HTT can alter DNA methylation at both promoter-proximal regions bearing low CpG content, as well as distal regulatory regions [92]. Notably, trinucleotide repeat instability on the genome has also been proposed [93]. Since DNA methylation occurs primarily in CG dinucleotides, some of the diseases containing non-methylatable repeats, such as CAG in HD, spinal and bulbar muscular atrophy (SBMA), several forms of spinocerebellar ataxia (SCA1, 2, 3, 6, 7, 8, 12 and 17), and CTG in myotonic dystrophy type 1 (DM1), may not be affected [51,89]. However, methylatable CGG repeats do occur in several diseases, including FXS and FXTAS, and may contribute to the onset and progression of those diseases. The molecular basis of FXS has been attributed to the CGG repeats within the 5' untranslated region of the *FMRI* gene [94], with extensive repeats causing silencing of the transcripts [95]. DNA methylation has been clearly taken into account for the mechanism of *FMRI* gene silencing [96]. There are also other repeat-related diseases, such as syndromic/non-syndromic X-linked mental retardation, oculopharyngeal muscular dystrophy with GCG repeats, and myoclonic epilepsy of Unverricht and Lundborg with CCCC GCCCGCG repeats, which could also be subject to DNA methylation, although whether DNA methylation plays a part in those diseases remains to be determined [89].

In recent decades, DNA methylation has emerged unequivocally as a key player in normal neurodevelopment and numerous neurological disorders. DNA methylation inhibitors, such as 5-aza-2'-deoxycytidine, are already undergoing clinical trials for some of these diseases, which could give us insight into reversing disease phenotypes [52]. Taken together, there is mounting and compelling evidence that the DNA methylation machinery, including DNA methyltransferases and MBPs, is critical in prenatal and postnatal neurodevelopment. Deletion or mutation of these players can cause skewed neuronal activity and lead to neurological phenotypes.

## DNA 5-hydroxymethylation in neurodevelopment and disease: a new perspective

### Is 5-hydroxymethylcytosine an intermediate or stable cytosine modification?

DNA methylation has been studied exhaustively for decades, with relatively well-established protein players to generate or interpret this marker. In contrast, how DNA methylation is dynamically regulated, especially the machinery of passive or active DNA demethylation and the identity of demethylases, had not been described until very recently [33,97–100]. The discovery that the DNA methylation eraser ten-eleven translocation 1 (TET1, one of the three TET proteins in mammals) can oxidize 5mC to 5-hydroxymethylcytosine (5hmC) [27] has attracted broad attention and led to a flurry of studies within the last several years. The development of next-generation sequencing allows scientists to characterize this new cytosine modification genome-wide and postulate its function in the transcription state by analyzing its genomic distribution and associated protein factors. In the meantime, two further 5hmC oxidation products by TET proteins, 5-formylcytosine and 5-carboxylcytosine, were also found to exist in mouse embryonic stem cells (mESC), mouse tissue, and human cells shortly after 5hmC was shown to be converted

from 5mC [29,30,101]. The establishment of various cytosine modifications raises an intriguing question: are either of the oxidized 5mC derivatives an intermediate in the active DNA demethylation process, or are they actually novel stable cytosine modifications that participate in epigenetic regulation?

Several lines of evidence indicate that different cytosine modifications are actually unified in a cycle to ensure their dynamic regulation in response to developmental cues. By investigating DNA demethylation in the dentate gyrus of adult mouse brain, Guo et al demonstrated that 5hmC converted by TET1 from 5mC is more prone to undergo deamination than 5mC by the AID (activation-induced deaminase)/APOBEC (apolipoprotein B mRNA-editing enzyme complex) family of cytidine deaminases. The deamination product, 5-hydroxymethyluracil (5hmU), triggers the base-excision repair (BER) pathway to be turned back to 5mC to complete the demethylation cycle. This relatively short pathway, which is not involved in 5fC and 5caC formation, is important for neuronal activity-induced, region-specific, active DNA demethylation [102]. An alternative pathway has also been proposed recently, based on the fact that TET proteins can further oxidize 5hmC to 5fC and 5caC. 5caC can be successively excised by thymine DNA glycosylase (TDG) to generate an abasic site, which can then be repaired to a cytosine by the BER pathway [29,30,101,103]. These findings elucidate plausible models for active DNA demethylation, although their precise dynamics, cellular specificity, and associated functions remain to be determined. Furthermore, simultaneous passive DNA demethylation may also be important in basic biological activities like development. It has been suggested that DNMT1, the only DNA methyltransferase that copies the existing DNA methylation to the daughter strand during DNA replication, has dramatically more affinity for hemi-methylated DNA than hemi-hydroxymethylated DNA [104]. This could easily result in the loss of inheritable DNA methylation if corresponding sites have been converted to 5hmC/5fC/5caC before cell replication [105]. It has long been known that the paternal but not the maternal genome in a zygote endures global DNA demethylation before the first mitosis, but the mechanisms behind this have remained elusive [106,107]. A recent report revealed the 5mC of the paternal genome can be converted to 5hmC by TET3, the only one of the TET proteins expressed in zygote. Tet3-deficient zygotes fail to convert 5mC to 5hmC, impairing key epigenetic reprogramming genes, such as Oct4 or Nanog expression, thereby affecting normal embryonic development [108]. These findings taken together argue that 5hmC and its subsequent oxidation products may serve as an intermediate for the DNA demethylation process.

5hmC, on the other hand, is also implicated as a stable cytosine modification that has a long half-life in the mammalian genome, especially in neuronal cells, as demonstrated by us and others [31–33]. For example, Song et al used selective chemical labeling combined with high-throughput sequencing to map 5hmC genome-wide in several cell lines and mouse brain and showed that intragenic 5hmC enrichment correlated with the expression of genes linked to age-related neurodegenerative diseases. In addition, the age-dependent acquisition of 5hmC is also seen [33]. Szulwach et al have specifically mapped the genome-wide distribution of 5hmC in mouse hippocampus and cerebellum at three different ages, allowing accurate comparison of 5hmC levels and distribution at different stages of postnatal neurodevelopment [32]. 5hmC levels were found to be significantly higher in both the cerebellum and hippocampus of adult mice at 6 weeks and 1 year of age than postnatal day 7 mice. Two findings are important to note here. First, the gain of 5hmC level in a number of neurodevelopmentally activated genes did not result in a concomitant loss of 5mC, arguing that 5hmC is not solely the oxidation product of 5mC, and newly formed 5hmC can also occur on non-CpG cytosines. Second, tissue-specific differentially hydroxymethylated regions (DhMRs), for instance, cerebellum- but not hippocampus-specific 5hmC territories associated with different ages, were identified. Interestingly, more than 6000 DhMRs found

in 6-week-old but not P7 mouse cerebellum persists until 1 year of age, arguing that 5hmC is a stable cytosine modification in brain tissues. Moreover, a RTT mouse model with MeCP2 knockout displayed increased 5hmC levels. This is consistent with the notion that MeCP2 serves as a protective mechanism by binding to 5mC, and its depletion leads to the conversion of 5mC to 5hmC [32]. A recent study further purified distinct types of neuronal cells and found that both 5mC and 5hmC show a strong cell type-related bias. These investigators also identified MeCP2 as a 5hmC-binding protein, establishing a dual role for MeCP2 in the orchestration of neuronal plasticity by coordinating different cytosine modifications [31].

Based on the evidence presented here, one feasible proposal is that 5hmC has dual roles, being either a DNA demethylation intermediate or a stable epigenetic marker. In some developmental stages that require rapid DNA demethylation to reset the epigenome, such as in zygote or primordial germ cells (PGCs), the available TET proteins (TET3 in zygote and TET1 or TET2 in PGCs) rapidly convert 5mC to 5hmC and all the way back to unmethylated cytosine, possibly coordinating with a number of their cofactors to ensure proper gene expression and facilitate normal development [108–110]. The rationale behind this epigenetic reshuffling during embryonic development likely accounts for the re-establishment of a proper epigenetic state adapted from paternal and maternal genome to ensure normal development, and the expression of Tet proteins appears to be the decisive factor for 5hmC abundance and distribution. In postnatal development, however, some 5hmC derived from 5mC can be quickly turned back into unmethylated cytosine, whereas a significant and increasing portion of 5hmC during aging persists for a long time, even throughout the entire life span, to control long-term gene expression. Thus, the rationale for stable 5hmC is possibly related to transcriptional control of specific genes that are important for tissue-specific events. This model is particularly apt for neuronal cells, given the extraordinarily high level of 5hmC in those cells, likely reflecting its unique function in neuronal activity. However, there were no expression differences in Tet proteins seen during postnatal neurodevelopment, suggesting additional co-factors or mechanisms may be involved in regulating 5hmC level [32].

### **Requirements of 5hmC and the TET proteins in neurodevelopment and implications in neurological diseases**

Our understanding of the correlation between TET proteins, 5hmC, and neurodevelopment is still in its infancy. Although the mechanisms of active DNA demethylation have been speculated about for years, there are many factors involved in this process [111]. One of these is growth arrest and DNA damage-inducible protein 45 (Gadd45) [112,113]. Knockdown of Gadd45a promotes global DNA demethylation, with a requirement for the DNA repair endonuclease XPG [112]. Gadd45b was found in mouse hippocampus as an early responder to various stimuli that promotes adult neurogenesis. Gadd45b induces promoter DNA demethylation of several genes involved in neurogenesis, including brain-derived neurotrophic factor (Bdnf) and fibroblast growth factor (Fgf) [113]. Although a direct interaction between Gadd45 and TET protein has not been reported, it is possible that TET proteins could be involved in Gadd45-mediated DNA demethylation, thereby affecting neuronal development. Another enzyme family, isocitrate dehydrogenases (IDHs), catalyzes oxidative decarboxylation of isocitrate to produce  $\alpha$ -KG, which is required for TET oxygenase activity [114]. IDH deletions or mutations cause global 5hmC loss and are associated with numerous cancer types, including lower-grade diffuse astrocytic glioma [115–118]. Three recent reports simultaneously demonstrated the direct interaction between TET proteins and O-linked N-acetylglucosamine (O-GlcNAc) transferase (Ogt). TET proteins are required for the recruitment of Ogt to chromatin, and Ogt can then GlcNAcylate host cell factor 1 (HCF1), a component of the H3K4 methyltransferase SET1/COMPASS



complex, as well as trigger histone2B Serine 112 GlcNAcylation [119–121]. Interestingly, a previous study showed overexpression of Ogt increases the percentage of neurons exhibiting axon branching and the numbers of axonal filopodia [122]. In brain, Ogt is seen mostly in neuronal cell bodies and processes, arguing for its functional importance in neurodevelopment and neuronal activity [123]. The detailed molecular mechanisms of Ogt-TET proteins, as well as 5hmC, in neurodevelopment and possibly neuronal diseases remain to be determined.

One report looked at the dynamic change in TET proteins and 5hmC in neurogenesis and found an increased 5hmC level during neuronal differentiation from neural stem cells [124]. 5hmC is enriched in the gene bodies of activated neuronal function-related genes, and no profound cytosine demethylation was seen, confirming 5hmC is stable in neuronal cells. These investigators also show a negative correlation of 5hmC with H3K27me3 and its effector Polycomb protein complex [124]. These data confirmed the critical role of 5hmC and TET proteins in neurodevelopment, as also shown in *Xenopus* [125].

### Conclusions and perspectives

The field of cytosine modifications is progressing rapidly, thanks to the development of high-throughput sequencing methods that allow scientists to precisely investigate their detailed distribution genome-wide, and the build-up of cytosine modification variants in recent years has attracted vast attention. One of the most important future tasks certainly is to understand the dynamic regulation, distribution, and conversion of cytosine modifications. The evidence described in this review offers strong support for the idea that cytosine modifications are one of the most decisive epigenetic mechanisms influencing normal neurodevelopment, and its aberrant regulation can lead to a variety of neurological disorders. Often, multiple epigenetic mechanisms, such as cytosine modification, histone modification, or chromatin remodeling, cooperate and orchestrate the transcription state and ultimately govern cell identity, tissue development, and its normal function [36,37]. Given the high enrichment of 5hmC in the CNS, it will be interesting to explore the crosstalk among various epigenetic mechanisms, especially the newly defined 5hmC, in neuronal activity, and ultimately to develop novel therapeutic approaches for neurological disorders. Therefore, we have our eye focused on 5hmC for the future.

Although there has been significant progress on 5hmC and TET proteins just since 2009, in comparison with the relatively more well-developed 5mC field, several important questions remain unanswered. For example, the exact correlation of TET proteins and 5hmC with gene expression remains elusive and controversial. Proteomic analysis has revealed several TET-binding proteins. Among these, Ogt [119–121], Nanog [126], and PARP1 [127] may be involved in TET-mediated gene activation. In comparison, SIN3A could be serving as a co-repressor for TET-mediated gene silencing [128] (Fig. 2). The dual roles of TET proteins in transcription require further investigation. Tet1 or Tet2 knockdown in mESCs does not appear to affect their self-renewal, although the global 5hmC level is reduced, and the differentiation ability of the cells is skewed [126,128–130]. In addition, TET1 knockout mice appear grossly normal, but with skewed differentiation toward trophoblast *in vitro* [131]. Recently, efforts have also been made to generate Tet1 and Tet2 double knockouts (DKO). Surprisingly, viable and overtly normal DKO mice were obtained, although a fraction of DKO embryos displayed midgestation abnormalities [132]. These observations point to the possibility of a partially redundant role of different TET proteins. Future studies are needed to uncover the precise roles of TET proteins and 5hmC in stem cell pluripotency and differentiation.

Given the unusually high 5hmC level in brain tissues, it could be critical in maintaining normal neuronal homeostasis and functions. Therefore, it is reasonable to speculate that

there are aberrant 5hmC levels and distributions in neurological and neurodegenerative disorders. In fact, a recent publication demonstrated genome-wide loss of 5hmC in HD, and their DhMR-annotated genes are associated with canonical pathways related to neuronal development and differentiation [133]. It will be very interesting to investigate the roles and regulations of 5hmC, its writers, readers, and erasers in other neuronal disorders, as well as normal neuronal activities.

The roles of 5fC and 5caC, including whether they are also stable in the genome, have gone uninvestigated because they are not at all abundant in the genome, and we lack a specific method to enrich and map them. We and others recently solved this issue by using either a chemical labeling or antibody enrichment method to globally map these two cytosine modifications. 5fC has been found at poised enhancers among other gene regulatory elements [134], as well as in repetitive elements [135]. Future work will be needed to address their precise role in gene expression.

Evidence accumulated in the past decade has revealed the critical role of DNA methylation in neurological diseases; however, a direct correlation between TET proteins, 5hmC, and neurological diseases has not been reported, although we now know they clearly play a role in leukemia [114,136–138] and melanoma [117]. In addition, unlike DNA methylation and many histone modifications, chemical compounds that can manipulate the 5hmC level *in vivo* have not been systematically explored, despite isolated reports that ascorbate (vitamin C) may be one of them [139]. Establishing such a compound library would be a major benefit for future clinical trials on 5hmC-related neurological diseases.

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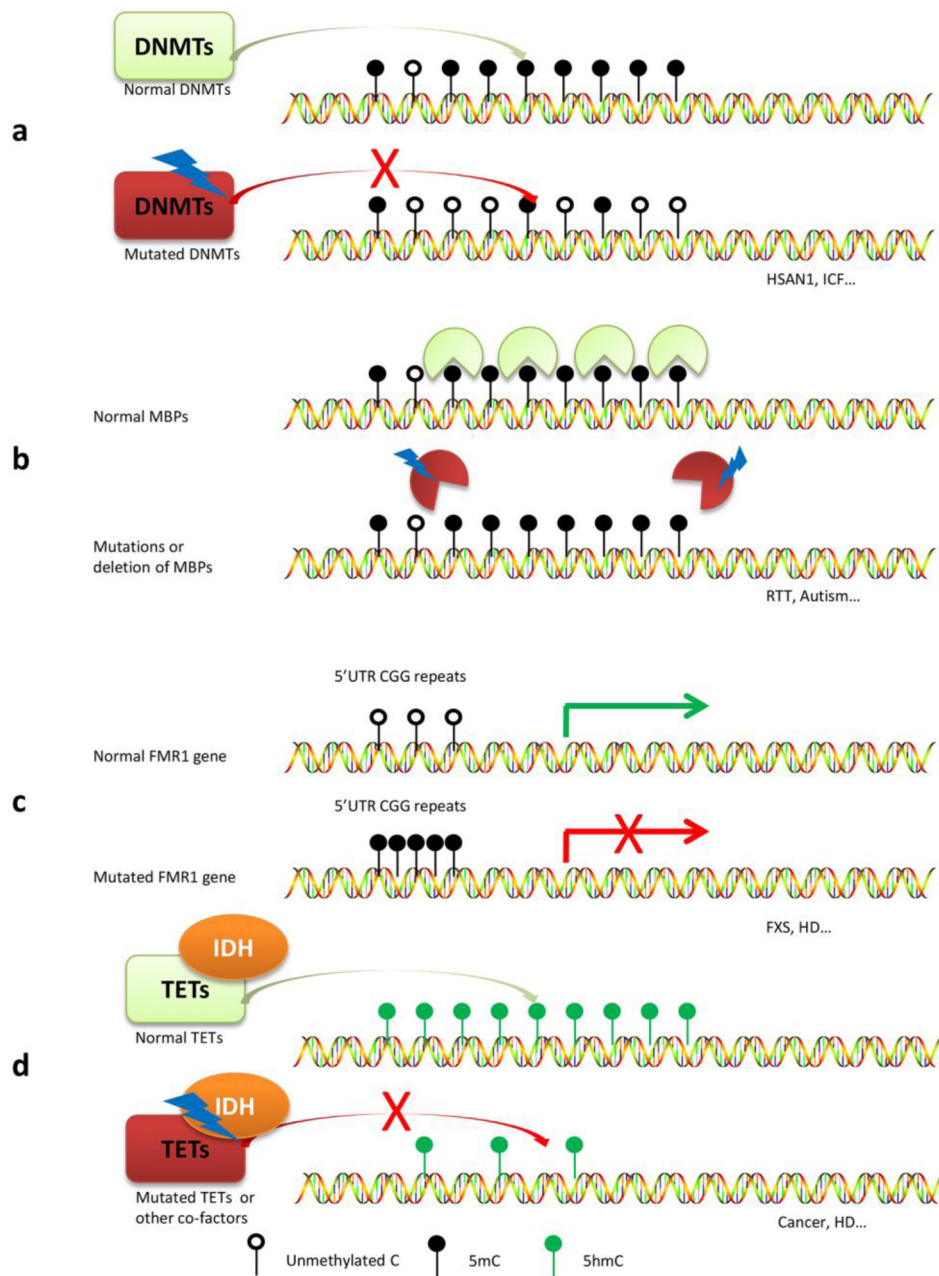


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**Figure 1. Molecular mechanisms for cytosine modification-related pathogenesis**

**a.** Mutations of DNMT1 or DNMT3 reduce 5mC levels and influence gene transcription or genomic stability. **b.** Mutations of MBPs reduce their 5mC binding affinity and trigger severe neurological diseases. **c.** The expanded CGG repeats on mutated FMR1 genes could possibly be methylated and inhibit FMR1 transcription. **d.** Mutations of TET proteins or their co-factors, such as IDH, could reduce the global 5hmC level, as seen in cancer or HD.

**Table 1**

Summary of key proteins discussed in this review and their related diseases or neurological phenotypes.

Categories	Molecular mechanisms	Related diseases or phenotypes	References
DNMTs	<i>DNMT1</i> mutations	Hereditary sensory and autonomic neuropathy type 1 (HSAN1); autosomal dominant cerebellar ataxia, deafness, and narcolepsy (ADCA-DN).	[54–56]
DNMTs	<i>DNMT3a</i> functional mutant or deletion	Acquisition of developmental mental defects; impaired postnatal neurogenesis.	[46]
DNMTs	<i>DNMT3b</i> mutations	The immunodeficiency, centromere instability, facial anomalies (ICF) syndrome.	[51],[57–58]
MBPs	<i>MBD1</i> deletion	Reduced neuronal differentiation and increased genomic instability.	[64–65]
MBPs	<i>MeCP2</i> mutations or overexpression	Rett syndrome (RTT); mental retardation, autism, and psychiatric symptoms.	[66–78]
Methylatable CGG repeats	Potential possibility of CGG methylation on <i>FMR1</i> genes	Fragile X mental retardation syndrome (FXS); fragile X tremor ataxia syndrome (FXTAS)	[51], [88–91],
TETs	<i>TET2</i> mutations	Leukemia; loss of 5hmC	[114], [135–137]
5mC excision	<i>Gadd45b</i> deletion	Induced proliferation of NPCs and dendritic growth of newborn neurons; loss of 5hmC	[113]
TET co-factors	<i>IDH</i> deletion or mutation	Various cancer types; loss of 5hmC	[115–118]
TET co-factors	<i>Ogt</i> overexpression	Elevation of neurons exhibiting axon branching and the numbers of axonal filopodia	[122]