

# Hydroxylation of 5-Methylcytosine by TET1 Promotes Active DNA Demethylation in the Adult Brain

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## SUMMARY

Cytosine methylation is the major covalent modification of mammalian genomic DNA and plays important roles in transcriptional regulation. The molecular mechanism underlying the enzymatic removal of this epigenetic mark, however, remains elusive. Here, we show that 5-methylcytosine (5mC) hydroxylase TET1, by converting 5mCs to 5-hydroxymethylcytosines (5hmCs), promotes DNA demethylation in mammalian cells through a process that requires the base excision repair pathway. Though expression of the 12 known human DNA glycosylases individually did not enhance removal of 5hmCs in mammalian cells, demethylation of both exogenously introduced and endogenous 5hmCs is promoted by the AID (activation-induced deaminase)/APOBEC (apolipoprotein B mRNA-editing enzyme complex) family of cytidine deaminases. Furthermore, Tet1 and Apobec1 are involved in neuronal activity-induced, region-specific, active DNA demethylation and subsequent gene expression in the dentate gyrus of the adult mouse brain *in vivo*. Our study suggests a TET1-induced oxidation-deamination mechanism for active DNA demethylation in mammals.

## INTRODUCTION

The mammalian genome is methylated predominantly at the C5 position of cytosine bases within CpG dinucleotides (Suzuki and Bird, 2008; Zhu, 2009), whereas non-CpG methylation has been observed at relatively low levels in pluripotent stem cells (Lister et al., 2009; Ramsahoye et al., 2000). 5mCs, especially when clustered, are important transcriptional silencers at gene promoters and endogenous retrotransposons in the genome (Jaenisch and Bird, 2003). DNA methylation also plays critical roles in tissue-specific gene expression, X chromosome inactivation, gene imprinting, and nuclear reprogramming (Bonasio

et al., 2010; Feng et al., 2010b). Cytosine methylation is catalyzed by a family of DNA methyltransferases (DNMTs), and deficiencies in these enzymes result in profound developmental defects (Goll and Bestor, 2005; Reik, 2007).

During early development, the mammalian DNA methylome is dramatically reprogrammed at two stages (Feng et al., 2010b; Gehring et al., 2009). In the zygote, DNA methylation of the paternal, but not maternal, pronucleus is rapidly lost at the genome-wide scale (Mayer et al., 2000; Oswald et al., 2000; Surani et al., 2007). In primordial germ cells, DNA methylomes undergo a second wave of reprogramming, resulting in a large decrease in the global 5mC level (Hajkova et al., 2010; Surani et al., 2007). In contrast, postdevelopmental DNA demethylation occurs in a highly locus-specific fashion and has been shown to regulate gene expression in various tissues (Ma et al., 2009a; Wu and Zhang, 2010; Zhu, 2009). In addition, DNA demethylation is required for epigenetic resetting during somatic reprogramming by nuclear transfer (Simonsson and Gurdon, 2004), cell fusion (Bhutani et al., 2010), or transcription factor-based derivation of induced pluripotent stem cells (Mikkelsen et al., 2008). In the adult mammalian brain, whereas the DNA epigenome is stable at the genome-wide level (Ma et al., 2009b), emerging evidence suggests the presence of active DNA modifications at specific genomic loci, and these modifications are critical for certain types of brain plasticity (Day and Sweatt, 2010; Ma et al., 2010). For example, deletion of both *Dnmt1* and *Dnmt3a* leads to decreased CpG methylation at several genomic loci and impacts synaptic functions of adult forebrain neurons (Feng et al., 2010a). Deletion of *Gadd45b* abolishes neuronal activity-induced DNA demethylation in the adult mouse dentate gyrus at specific genomic loci, including brain-derived neurotrophic factor (*Bdnf*) and fibroblast growth factor 1 (*Fgf1*) promoters, and attenuates activity-induced adult hippocampal neurogenesis (Ma et al., 2009b). Pharmacological inhibition of DNA methylation changes also affects synaptic plasticity, learning, and memory (Day and Sweatt, 2010), further suggesting the importance of epigenetic DNA modification mechanisms in the adult nervous system.

Although DNA demethylation in mammals has been observed both at the genome-wide and locus-specific levels, mechanisms underlying the removal of methyl groups from the genomic DNA have been under intensive debate (Ooi and Bestor, 2008). Several

mammalian proteins have been reported to exhibit DNA demethylase activity; however, such activities remain controversial (Wu and Zhang, 2010). Emerging evidence has implicated DNA repair as a potential mechanism for active DNA demethylation (Gehring et al., 2009; Ma et al., 2009a; Zhu, 2009). In plants, a group of 5mC-specific DNA glycosylases, including ROS1, DME, and DML2/3, can specifically excise 5mC to initiate the base excision repair (BER) to achieve active DNA demethylation (Zhu, 2009). However, no ortholog of these 5mC-specific DNA glycosylases has been identified in mammalian genomes. Alternatively, it has been proposed that glycosylation of 5mC may be preceded by deamination of 5mC and generation of a T:G mismatch, which can be efficiently repaired by mammalian glycosylases TDG and MBD4 (Gehring et al., 2009; Morgan et al., 2004; Rai et al., 2008). One cytidine deaminase, activation-induced deaminase (*AID*), is required for *OCT4* and *NANOG* promoter demethylation during in vitro nuclear reprogramming (Bhutani et al., 2010). *Aid* null mice also exhibit significant defect in genome-wide DNA demethylation in primordial germ cells (Popp et al., 2010). However, *AID* is unable to act on double-stranded DNA (dsDNA), and the 5mC reactivity for *AID* deamination in vitro is much lower than unmethylated cytosines (Bransteitter et al., 2003; Conticello et al., 2007; Di Noia and Neuberger, 2007; Larjani et al., 2005), raising the question of whether *AID*, if it does initiate demethylation, directly deaminates 5mCs in vivo.

TET proteins, a group of Fe(II)/2-oxoglutarate-dependent dioxygenases, have recently been identified as 5mC hydroxylases that oxidize 5mCs to produce 5hmCs (Ito et al., 2010; Tahiliani et al., 2009). 5hmC may achieve passive DNA demethylation by excluding DNMT1, which maintains symmetric CpG methylation during DNA replication (Valinluck and Sowers, 2007). Interestingly, Tet1 knockdown in mouse embryonic stem cells (ESCs) results in *Nanog* promoter hypermethylation and defects in ESC self-renewal (Ito et al., 2010). 5hmC has also been hypothesized as a potential intermediate for active DNA demethylation (Bonasio et al., 2010; Feng et al., 2010b; Hajkova et al., 2010; Ito et al., 2010; Wu and Zhang, 2010; Xu et al., 2011). However, direct evidence for active demethylation of 5hmC-containing DNA is lacking. In two recent studies, loss-of-function mutations of TET2 appeared to display opposite effects on DNA methylation status (Figueroa et al., 2010; Ko et al., 2010), further raising the question of a general role of TET proteins and 5hmC in DNA demethylation. Notably, 5hmCs exist in relative abundance in various brain regions (Kriaucionis and Heintz, 2009). The physiological function of 5hmC and TET proteins in the brain remains to be determined.

Here, we show that overexpression of TET1 in human cells reactivates a methylation-silenced plasmid reporter and promotes DNA demethylation of both exogenous nonreplicable methylated reporter plasmids and multiple endogenous genomic loci. We provide direct evidence that human cells possess a robust demethylating activity toward 5hmC-containing DNA, which is DNA replication independent and requires an intact BER pathway. Furthermore, *AID/APOBEC* cytidine deaminases promote 5hmC demethylation both in cultured human cells and in the adult mouse brain. Similar to deamination, 5hmC demethylation is processive, transcription dependent, and strand biased. Finally, Tet1 is both sufficient and required for neuronal activity-

induced, region-specific, and active DNA demethylation in the adult mouse brain in vivo. Our study identified TET1 as a critical factor to initiate an oxidation-deamination mechanism underlying active DNA demethylation in mammals.

## RESULTS

### TET1 Promotes DNA Demethylation in Human Cells

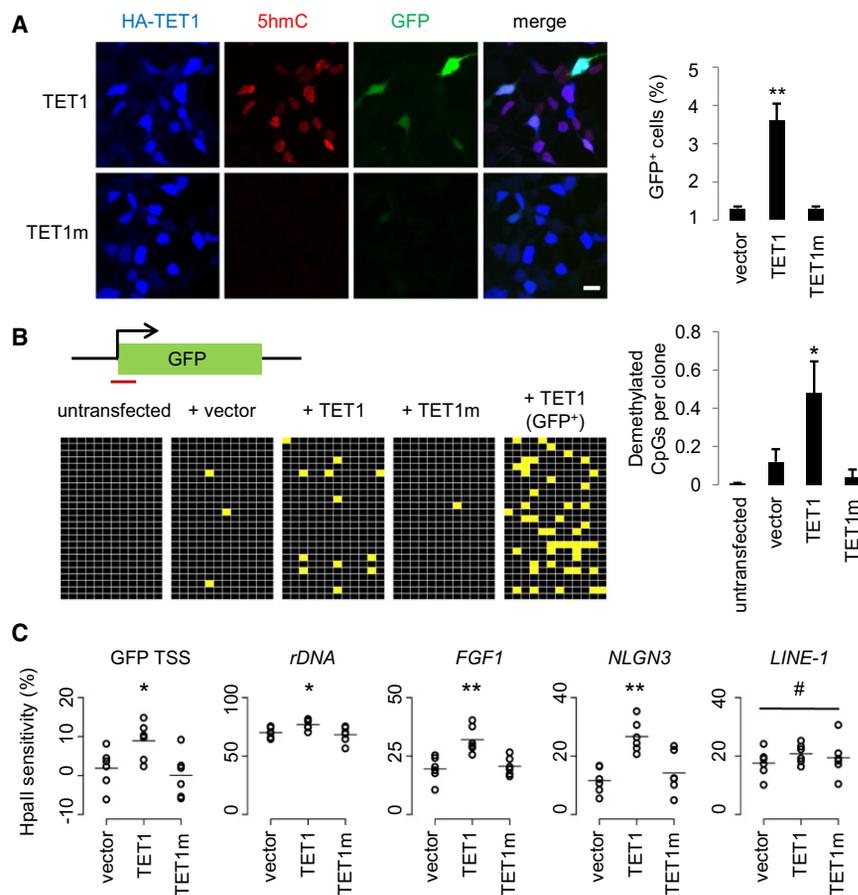
We first confirmed the 5mC hydroxylase activity of human TET1 by analysis of purified genomic DNA from HEK293 cells overexpressing the HA-tagged human TET1 catalytic domain (aa1418–2136, referred to as TET1) using immunoblotting and immunocytochemistry (Figures 1A and Figure S1A available online). The specificity of 5hmC antibodies was confirmed by immunoblotting of linear DNA that contained almost exclusively unmethylated cytosines, 5mCs, or 5hmCs (Figure S1B). To examine whether hydroxylation of 5mC by TET1 promotes active DNA demethylation, we utilized a reporter assay using an in vitro methylated GFP-expressing plasmid (Figure S1C) (Ma et al., 2009b). Complete methylation of all CpGs on the plasmid by the bacterial methyltransferase *SssI* resulted in strong silencing of GFP expression after transfection into HEK293 cells (Figure 1A). Overexpression of TET1, but not a catalytically inactive TET1 mutant (H1671Y/D1673A; TET1m), promoted reactivation of GFP expression, resulting in a significant increase in the number of GFP<sup>+</sup> cells as quantified by FACS (Figure 1A). Thus, TET1 hydroxylase activity promotes the reversal of the silencing effect of cytosine methylation.

Cytosine methylation near transcription start sites (TSSs) of genes is known to be critical for transcriptional silencing (Suzuki and Bird, 2008). Bisulfite sequencing around the GFP TSS showed very sparse CpG demethylation 48 hr after transfection of *SssI*-methylated plasmids into HEK293 cells (Figure 1B). Expression of TET1, but not TET1m, significantly enhanced the demethylation of these CpGs (Figure 1B). As expected, sorted GFP<sup>+</sup> cells with TET1 expression displayed much increased CpG demethylation (Figure 1B). We also used an independent approach to measure CpG methylation levels based on quantitative PCR (qPCR) after *HpaII* digestion, which is blocked by both CpG methylation and hydroxymethylation (Jin et al., 2010). Quantitative analysis showed a significant increase of *HpaII* sensitivity around GFP TSS in the presence of TET1, but not TET1m (Figure 1C and Table S1). Because the reporter plasmid is incapable of replication in HEK293 cells, the DNA demethylation observed around the GFP TSS is active in nature.

We next examined whether 5mC hydroxylation also promotes demethylation of the endogenous genomic DNA. Indeed, overexpression of TET1, but not TET1m, significantly increased *HpaII* sensitivity at multiple loci from distinct genomic subregions (Figure 1C), including promoters of ribosomal DNA (*rDNA*), *FGF1*, and neuroligin-3 (*NLGN3*), but not at long interspersed nonrepetitive elements (*LINE-1s*). Thus, TET1 overexpression also promotes region-specific DNA demethylation at endogenous genomic loci in human cells.

### 5hmC in Both CpG and CpH Contexts Can Be Demethylated in Human Cells

To ascertain whether TET1-induced DNA demethylation is mediated by 5hmC generation, we examined whether premodified



### Figure 1. TET1 Catalyzes 5mC Hydroxylation and Promotes Demethylation of Exogenous Sssl-Methylated Reporter Plasmids and Endogenous Genomic Loci

(A) Reactivation of methylation-silenced reporter plasmids by TET1. Shown on the left are sample images of immunostaining of HEK293 cells cotransfected with Sssl-methylated GFP expression plasmids and expression constructs for TET1 or TET1m. Scale bar, 10  $\mu$ m. Shown on the right is a summary of quantification of GFP<sup>+</sup> cells as measured by FACS. Values represent mean  $\pm$  SEM. n = 3; \*\*p < 0.01; Student's t test.

(B) Bisulfite sequencing analysis of reporter plasmids. Shown on the top is a schematic diagram of the region around GFP transcription start site (TSS) for bisulfite sequencing (indicated by the red line). Shown on the left are illustrations of methylation status of CpGs within the sequenced region. Black, methylated; yellow, unmethylated. Note the much higher level of demethylation in GFP<sup>+</sup>-sorted cells. Shown on the right is a summary of mean numbers of CpGs that are demethylated within each clone. Values represent mean  $\pm$  SEM. n = 3; \*p < 0.05; Student's t test.

(C) Effects of TET1 and TET1m overexpression on the HpaII sensitivity of the GFP TSS region and several endogenous genomic loci in HEK293 cells. Open circles represent data from individual experiments, and lines represent mean values. \*\*p < 0.01, \*p < 0.05, and #p > 0.1; Student's t test. See also Figure S1 and Table S1.

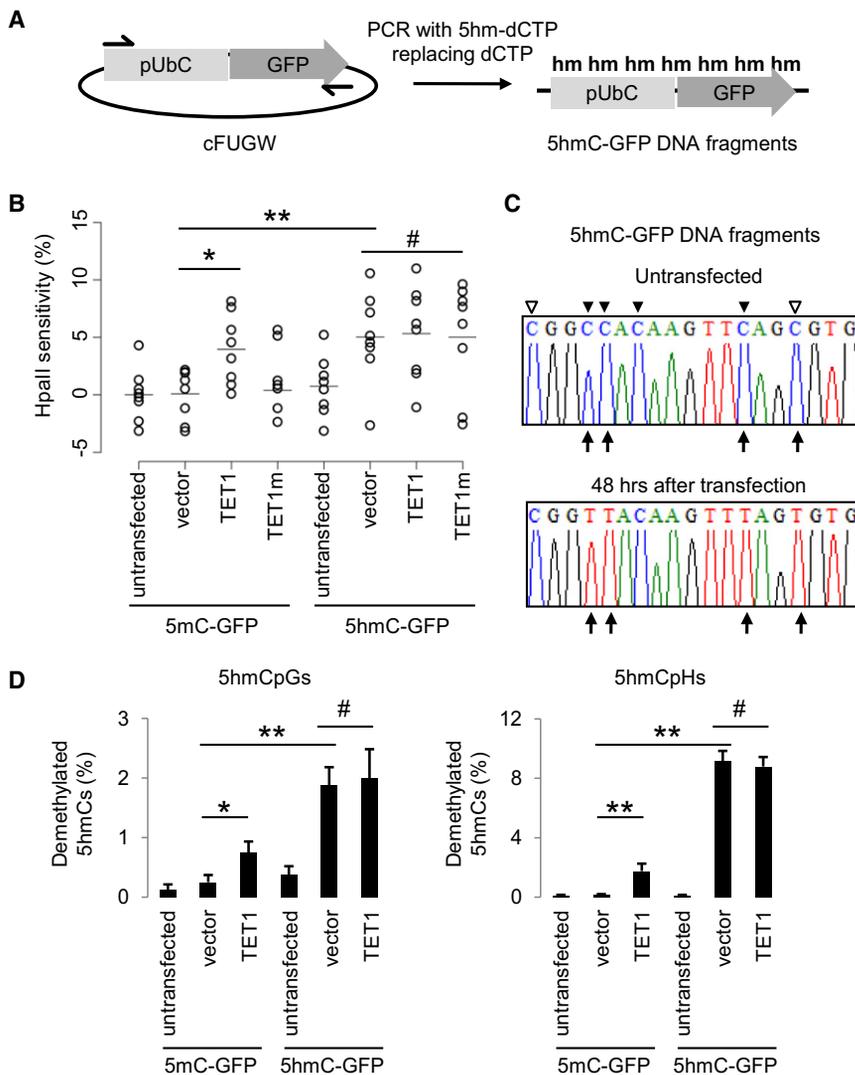
5hmC-containing DNA is demethylated in human cells. By replacing deoxycytidine triphosphates (dCTPs) with either 5-methyl-deoxycytidine triphosphates (5mdCTPs) or 5-hydroxymethyl-deoxycytidine triphosphates (5hmdCTPs) in PCR reactions, we generated linear GFP expression cassettes in which almost all cytosines, except for primer regions, are either 5mCs or 5hmCs ( $\sim$ 99.3% of all cytosines; Figure 2A, Figure S2A, and Table S1). As expected, HpaII can digest neither fully methylated nor fully hydroxymethylated DNA fragments (Figure S2B). Overexpression of TET1, but not TET1m, led to significant HpaII sensitivity near GFP TSS in 5mC-GFP DNA after transfection into HEK293 cells (Figure 2B), which required demethylation of all four cytosines in the CCGG motif. Importantly, HpaII sensitivity at the same position arose on 5hmC-GFP DNA after transfection and was not further increased by TET1 overexpression (Figure 2B). These results suggest that TET1-induced DNA demethylation is a direct result of 5hmC generation in human cells.

To directly quantify cytosine demethylation, we carried out bisulfite sequencing analysis of 5mC-GFP and 5hmC-GFP DNA using a modified protocol (Table S1). Consistent with HpaII digestion results, bisulfite sequencing of 5mC-GFP DNA showed negligible demethylation after transfection into HEK293 cells (Figures 2C and 2D). Expression of TET1 led to  $\sim$ 0.8% of 5mCpGs demethylated in each 500 bp clone (Figure 2D). Surprisingly, 5mCs in non-CpG contexts (5mCpH, H = A/C/T) showed an

even higher frequency of demethylation in the presence of TET1 (Figure 2D). Importantly, robust demethylation occurred on transfected 5hmC-GFP DNA, with  $\sim$ 2% of 5hmCpGs and  $\sim$ 9% of 5hmCpHs demethylated in each clone, which accounts for  $\sim$ 8.5% of all modified cytosines in this region (Figure 2D and Figure S2C). Collectively, these results demonstrate the presence of a DNA replication-independent 5hmC-demethylating activity and an unexpected capacity for active demethylation of 5hmCs in non-CpG contexts in mammalian cells.

### 5hmC Demethylation Requires the BER Pathway

To characterize the mechanism underlying 5hmC demethylation, we used pharmacological inhibitors to examine the potential involvement of the BER pathway, which has been implicated for DNA demethylation in vertebrates (Gehring et al., 2009; Ma et al., 2009a). ABT-888 (ABT) inhibits poly(ADP-ribose) polymerases (PARPs), which signals single-strand breaks (Donawho et al., 2007; Purnell and Whish, 1980), whereas CRT0044876 (CRT) inhibits apurinic/aprimidinic endonuclease APE1 (Madhusudan et al., 2005). Both enzymes are critical components in the BER pathway, and these two inhibitors block DNA demethylation in mouse zygotes (Hajkova et al., 2010). Interestingly, HpaII sensitivity around GFP TSS on 5hmC-GFP DNA was abolished in the presence of ABT and CRT (Figure 3A). Bisulfite sequencing analysis further confirmed the reduced DNA demethylation of



**Figure 2. 5hmC-Containing DNA Is Demethylated in HEK293 Cells**

(A) A schematic diagram of a PCR-based approach to generate 5mC- or 5hmC-containing DNA fragments that contain an ubiquitin promoter (pUbC) followed by the GFP open reading frame. (B) Summary of CpG methylation levels of GFP TSS regions under different conditions as quantified by HpaII sensitivity. Open circles represent data from individual experiments, and lines represent mean values. \*\* $p < 0.01$ , \* $p < 0.05$ , and # $p > 0.1$ ; Student's *t* test.

(C and D) Bisulfite sequencing analysis of 5mC-/5hmC-GFP DNA fragments under different conditions. Shown in (C) are sample traces from bisulfite sequencing of 5hmC-GFP DNA before or after transfection. Open and filled triangles indicate cytosines in CpG and CpH contexts, respectively. Arrows indicate demethylated 5hmCs. Shown in (D) are quantifications of demethylated CpGs (left) and CpHs (right) on 5mC-/5hmC-GFP DNA. Values represent mean  $\pm$  SEM.  $n = 3$ ; \*\* $p < 0.01$ , \* $p < 0.05$ , and # $p > 0.1$ ; Student's *t* test. See also Figure S2.

glycosylases (Figure 3D). Thus, 5hmC does not appear to be directly recognized and removed by any of these glycosylases under our experimental conditions.

#### AID/APOBEC Deaminases Facilitate 5hmC Demethylation

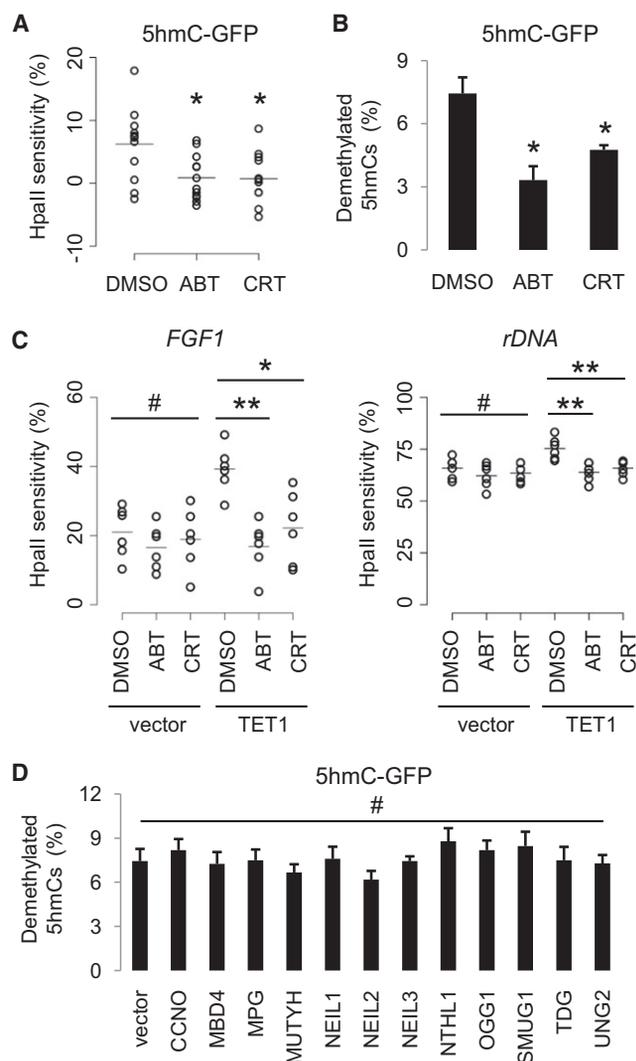
Which pathway could process 5hmC in mammalian cells? Previous studies have suggested that coupling between the AID/APOBEC family of cytidine deaminases and T:G mismatch glycosylases may mediate DNA demethylation in vertebrate cells (Morgan et al., 2004; Rai et al., 2008). However, 5mCs appear to be less favorable substrates than unmethylated

cytosines for AID in vitro (Bransteitter et al., 2003; Larjani et al., 2005), suggesting that AID might not directly deaminate 5mCs during the process of active DNA demethylation. Therefore, we examined whether the AID/APOBEC family of cytidine deaminases might play a role in 5hmC demethylation. Interestingly, AID overexpression led to a significant decrease of 5hmC levels induced by TET1 in HEK293 cells (Figures 4A and 4B and Figure S4A). Bisulfite sequencing analysis of transfected 5hmC-GFP DNA showed that AID significantly increased 5hmC demethylation (Figure 4C). Importantly, these demethylated cytosines were not unrepaired deamination products because sequences of nonbisulfite-converted 5hmC-GFP DNA cotransfected with AID showed no significant level of C-to-T transitions (Figure S4C).

Does the demethylation-promoting effect of AID specifically target 5hmCs or both 5hmCs and 5mCs? Overexpression of AID showed no significant effect on the demethylation of 5mC-GFP DNA (Figure 4C). Immunostaining also did not show any

5hmC-GFP DNA after treatment of these two inhibitors (Figure 3B). In addition, TET1-induced demethylation of endogenous genomic loci, including *FGF1* and *rDNA* promoters, was abolished by ABT and CRT (Figure 3C). Neither of the inhibitors interfered with the TET1 hydroxylase activity, as indicated by largely unchanged overall 5hmC levels in HEK293 cells with TET1 overexpression (Figure S3A). Taken together, these results suggest that the BER pathway is required for demethylation of both exogenous 5hmC and endogenous loci after TET1 overexpression.

The central components of the BER pathway include DNA glycosylases that recognize and remove specific types of damaged or mismatched DNA bases (Wood et al., 2005). Overexpression of each of the 12 known human DNA glycosylases in HEK293 cells (Figure S3B) did not lead to any noticeable decrease of 5hmC levels in the genomic DNA, as measured by immunoblotting and immunocytochemistry (Figures S3C and S3D). Similarly, no significant enhancement of demethylation of transfected 5hmC-GFP DNA was detected after overexpression of these



**Figure 3. BER Enzyme Activities Are Required for 5hmC Demethylation**

(A and B) Effects of PARP1 inhibitor ABT-888 (50  $\mu$ M) and APE inhibitor CRT0044876 (50  $\mu$ M) on demethylation of transfected 5hmC-GFP DNA fragments. Shown in (A) is a summary of HpaII sensitivity assay. Open circles represent data from individual experiments, and lines represent mean values. \* $p < 0.05$ ; Student's *t* test. Shown in (B) is a summary of bisulfite sequencing analysis. Values represent mean  $\pm$  SEM.  $n = 3$ ; \* $p < 0.05$ ; Student's *t* test.

(C) Effects of ABT and CRT on TET1-induced increases of HpaII sensitivity of *FGF1* (left) and *rDNA* (right) promoters. Open circles represent data from individual experiments, and lines represent mean values. \*\* $p < 0.01$ , \* $p < 0.05$ , and # $p > 0.1$ ; Student's *t* test.

(D) Effects of overexpression of human DNA glycosylases on demethylation of 5hmC-GFP DNA fragments. Shown is a summary of bisulfite sequencing analysis of 5hmC-GFP DNA after cotransfection with one of the 12 human DNA glycosylase cDNAs. Values represent mean  $\pm$  SEM.  $n = 3$ ; # $p > 0.1$ ; one-way ANOVA.

See also Figure S3.

noticeable change in genomic 5mC levels in AID-overexpressing cells (Figure S4B). As expected, coexpression of AID and TET1 led to a significant increase in demethylation of 5mC-GFP DNA (Figure 4C). Thus, AID appears to specifically promote 5hmC

demethylation but does not directly deaminate 5mCs to induce demethylation.

We next examined whether other cytidine deaminases of the AID/APOBEC family also promote demethylation of 5hmCs. Overexpression of mouse *Apobec1* (*mApobec1*), human *APOBEC2*, *APOBEC3A*, *3C*, and *3E*, but not *APOBEC3B* or *3G*, significantly increased demethylation of 5hmC-GFP DNA after transfection into HEK293 cells (Figure 4D and Figure S4D), suggesting a general property of the majority of AID/APOBEC deaminases in promoting 5hmC demethylation. Notably, *APOBEC1* and *APOBEC2* have both been implicated in active DNA demethylation in vertebrates (Morgan et al., 2004; Rai et al., 2008).

Deamination of 5hmC would produce 5-hydroxyluracil (5hmU). Though there is no detectable endogenous 5hmU in HEK293 cells, expression of TET1 resulted in a significant amount of 5hmU (Figure 4E and Figure S4E). Direct transfection of 5hmC-GFP DNA into HEK293 cells also led to a significant amount of 5hmU, which was decreased by coexpression of 5hmU glycosylase SMUG1 (Boorstein et al., 2001)(Figure 4E). In addition, overexpression of strong enhancers of 5hmC demethylation, including *mApobec1*, *APOBEC3A*, and *3C*, appeared to produce higher levels of 5hmU (Figure S4F). Expression analysis of HEK293 cells revealed the presence of many AID/APOBEC deaminases and DNA glycosylases (Figure S4G and Table S1), including known 5hmU glycosylases SMUG1, TDG (Baker et al., 2002), NEIL1, and NTHL1 (Zhang et al., 2005).

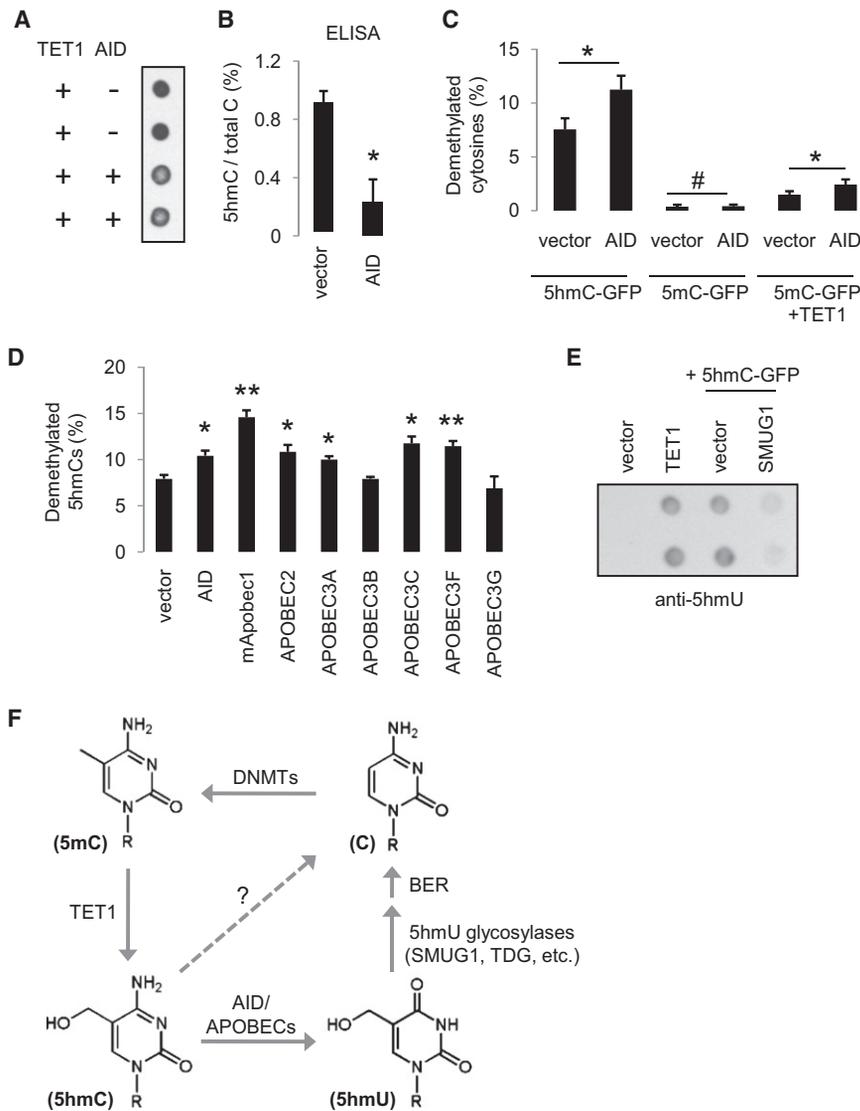
Collectively, these results suggest the following model for active DNA demethylation in mammalian cells (Figure 4F): 5mCs are first oxidized to 5hmCs by TET proteins. 5hmCs are then deaminated by AID/APOBEC deaminases into 5hmU. Finally, 5hmU can be excised by 5hmU glycosylases and repaired by the BER pathway with unmethylated cytosines.

### 5hmC Demethylation Recapitulates Properties of AID-Mediated Deamination

Much effort has been made to characterize catalytic properties of AID, with a focus on the involvement of AID-mediated deamination in somatic hypermutation in B cells (Di Noia and Neuberger, 2007). If active 5hmC demethylation entails deamination, does it exhibit known catalytic features of AID-mediated deamination?

First, AID-mediated deamination is a processive reaction (Pham et al., 2003). Extensive bisulfite sequencing of 174 clones of 5hmC-GFP DNA after transfection showed a broad distribution of numbers of demethylated 5hmCs in individual clones, which significantly deviated from a distributive Poisson model ( $p < 2 \times 10^{-26}$ ; Figure 5A). In three clones, 67%, 73%, and 86% of all 5hmCs on each clone were demethylated, indicating high processivity of 5hmC demethylation.

Second, AID exhibits DNA sequence selectivity for deamination with hot spots (WRC motifs; W = A/T; R = A/G) and cold spots (SYC motifs; S = C/G; Y = C/T) (Pham et al., 2003). To minimize the nonspecific targeting effect of the processive activity, only single demethylated 5hmCs at least 5 nucleotides away from any other demethylated 5hmCs were analyzed. The observed frequency of 5hmC demethylation at WRC motifs was 2.4-fold higher than that at SYC motifs (Figure 5B), indicating a similar sequence preference between 5hmC demethylation and AID-mediated deamination. Notably, the observed sequence preference of



**Figure 4. AID/APOBEC Deaminases Promote 5hmC Demethylation**

(A and B) Overexpression of AID results in a decrease in the abundance of 5hmCs in genomic DNA samples from HEK293 cells as monitored by immunoblotting (A) and ELISA (B) using anti-5hmC antibodies.

(C) Effects of AID on demethylation of 5hmC-GFP and 5mC-GFP DNA. Values represent mean  $\pm$  SEM.  $n = 3$ ;  $*p < 0.05$  and  $^{\#}p > 0.1$ ; Student's *t* test.

(D) Effects of overexpression of AID/APOBEC deaminases on 5hmC demethylation as measured by bisulfite sequencing. Values represent mean  $\pm$  SEM.  $n = 3$ ;  $**p < 0.01$  and  $^{\#}p < 0.05$ ; Student's *t* test.

(E) Immunoblotting analysis of 5hmU levels in HEK293 cells after TET1 expression or transfection of 5hmC-GFP DNA, with or without the expression of 5hmU glycosylase SMUG1.

(F) An oxidation-deamination-BER model of TET1-induced active DNA demethylation in mammalian cells.

See also Figure S4.

strand (Chaudhuri et al., 2003). This prompted us to discriminate demethylation of 5hmC-GFP DNA on two opposite strands of DNA with bisulfite sequencing analysis (Figure S5C). Indeed, the average frequency of demethylation on the untranscribed strand of 5hmC-GFP DNA is 2.6-fold higher than that on the transcribed strand at 48 hr after transfection (Figure 5D). This strand preference was not due to an asymmetric distribution of cytosines because it was observed in both CpGs and symmetrically distributed CpGs (Figure 5D).

Taken together, these results reveal an unexpected similarity between two seemingly unrelated processes—5hmC demethylation and AID-mediated somatic

5hmC demethylation is less robust than that of AID deamination of unmethylated cytosines *in vitro* or in somatic hypermutation, consistent with the notion that other AID/APOBEC deaminases with different sequence selectivity may also contribute to 5hmC demethylation.

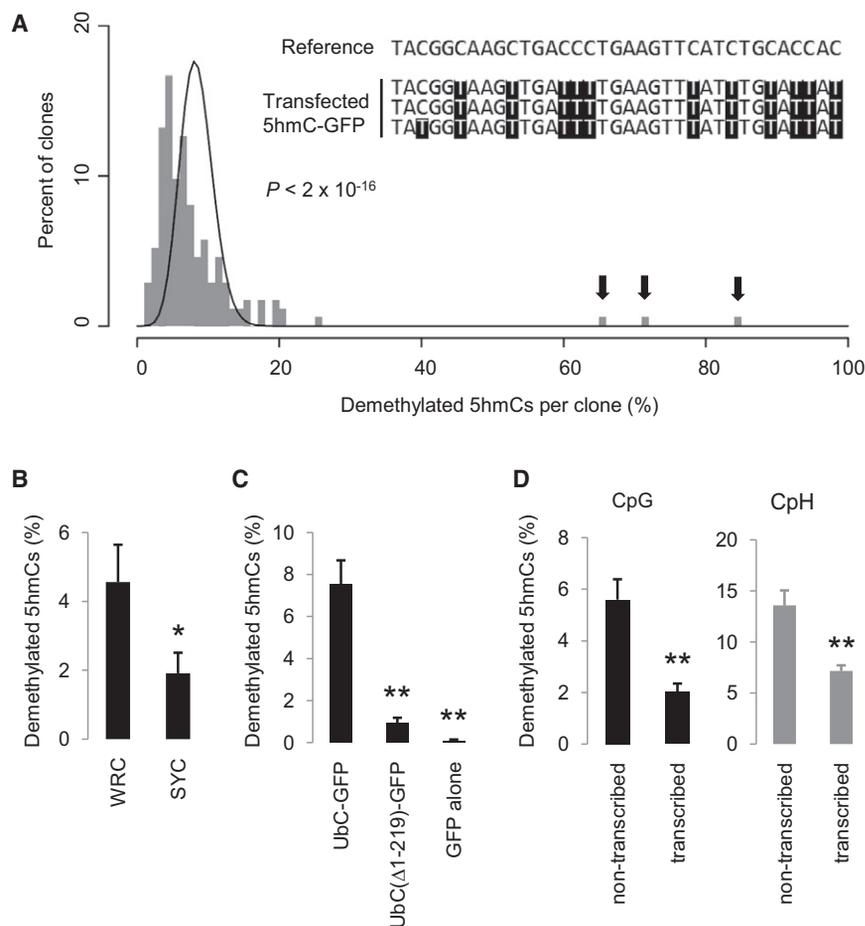
Third, transcription has been shown to target AID to specific genomic loci for deamination (Chaudhuri et al., 2003). We generated two variants of 5hmC-GFP DNA fragments, one with a truncated UbC-promoter and one with no promoter (Figure S5A), which exhibited either much reduced or no detectable GFP expression after transfection (Figure S5B). Concomitantly, demethylation of these truncated 5hmC-GFP DNA fragments was almost completely abolished (Figure 5C), suggesting that sufficient transcriptional activity is a prerequisite for 5hmC demethylation.

Finally, it has been shown that, on a transcribed dsDNA substrate, AID preferentially deaminates the untranscribed

hypermutation—and further support the role of deamination in active 5hmC demethylation.

**TET1 and AID/APOBEC Deaminases Promote Region-Specific DNA Demethylation in the Adult Mouse Brain**

We next examined whether the TET1-induced oxidation-deamination mechanism regulates active DNA demethylation in mature neurons of the adult mouse brain *in vivo*. Notably, 5hmC has been shown to be abundant in various adult brain regions, including the hippocampal dentate gyrus (Münzel et al., 2010). We stereotaxically injected adeno-associated viruses (AAVs) overexpressing TET1, or TET1m, into the adult mouse dentate gyrus and measured 5hmC levels in microdissected dentate gyrus tissue 1 week after viral injection (Figure S6A). Despite the presence of a high basal level of 5hmCs (1.35% of all cytosines), overexpression of TET1, but not TET1m, further increased the 5hmC level by 43% (Figure 6A). On the other hand, AAV-mediated



### Figure 5. 5hmC Demethylation Recapitulates Known Properties of AID-Catalyzed Deamination

(A) A histogram of clone distribution of the percentage of demethylated 5hmCs. A Poisson distribution with  $\lambda = 8.4\%$  is shown as a black line. Arrows indicate three highly processively demethylated 5hmC-GFP clones. A small portion of the reference sequence and the bisulfite sequencing results for these three clones are shown in the inset.

(B) Occurrence of single demethylated 5hmCs at WRC and SYC motifs. WRC motif: W = A/T; R = A/G. SYC motif: S = C/G; Y = C/T. Only demethylated 5hmCs that are at least 5 nucleotides away from any other demethylated 5hmCs are analyzed. Values represent mean  $\pm$  SEM.  $n = 3$ ; \* $p < 0.05$ ; Student t test.

(C) Demethylation of promoter-truncated 5hmC-GFP variants. The same region in the GFP open reading frame is bisulfite sequenced for all variants. Values represent mean  $\pm$  SEM.  $n = 3$ ; \*\* $p < 0.01$ ; Student t test.

(D) Demethylation of 5hmCs in the CpG (left) and CpH (right) contexts on two opposite strands. Demethylated 5hmCs of the forward (untranscribed) strand and the reverse (transcribed) strand are represented by C-to-T and G-to-A transitions in bisulfite sequencing (Figure S5C), respectively. Values represent mean  $\pm$  SEM.  $n = 3$ ; \*\* $p < 0.01$ ; Student's t test.

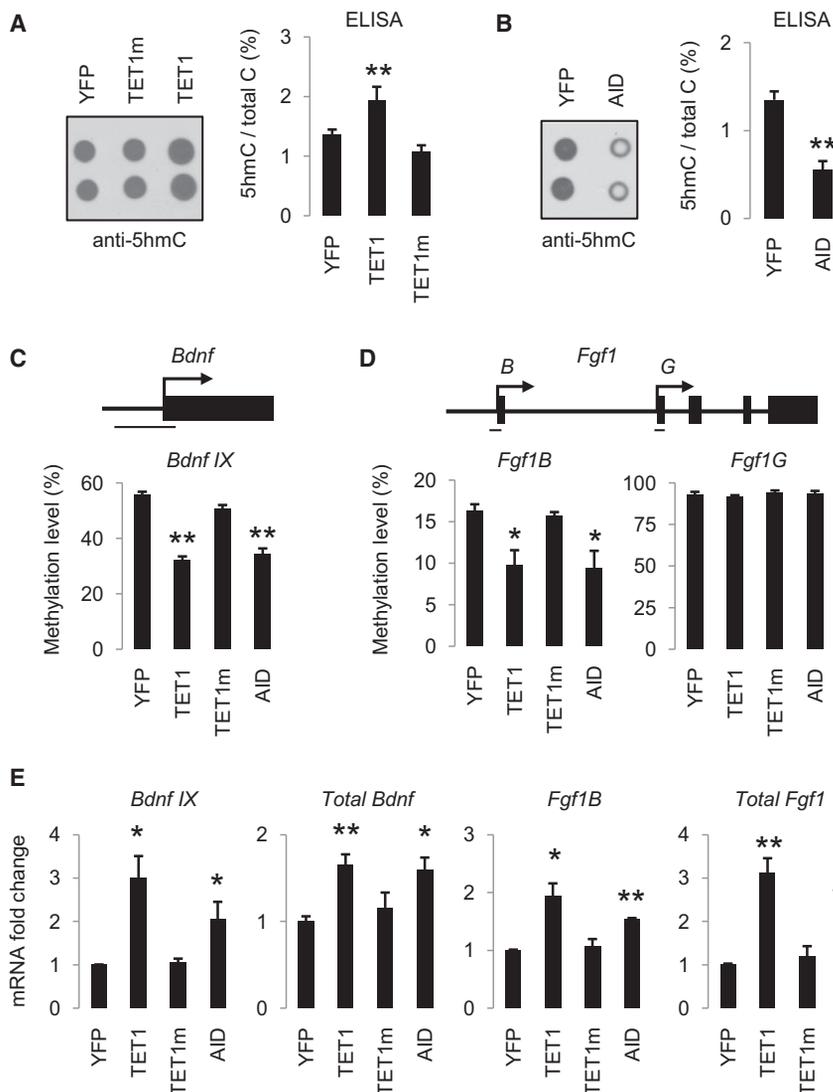
See also Figure S5.

overexpression of AID significantly decreased the endogenous level of 5hmCs by 59% in the adult dentate gyrus (Figure 6B), supporting its role in 5hmC removal in vivo.

To determine whether TET1 and AID promote DNA demethylation in the adult brain in vivo, we examined the CpG methylation status of promoter IX of *Bdnf* (*Bdnf IX*) and a brain-specific promoter of *Fgf1* (*Fgf1B*), two endogenous loci that have been shown to exhibit neuronal activity-induced active DNA demethylation in the adult dentate granule cells (Ma et al., 2009b). AAV-mediated overexpression of TET1 or AID, but not TET1m, led to significant decreases in the CpG methylation levels at these two genomic loci in dentate neurons from multiple animals (Figures 6C and 6D and Figures S6B and S6C). In contrast, the CpG methylation status of a nonneuronal promoter of *Fgf1* (*Fgf1G*) was not altered by these manipulations (Figure 6D and Figure S6D). Consistent with our previous finding that the methylation status of *Bdnf IX* and *Fgf1B* promoters regulates their gene expression (Ma et al., 2009b), both TET1- and AID-induced demethylation was accompanied by a significant upregulation in transcript levels of these two gene isoforms in vivo (Figure 6E). Taken together, these results suggest that TET1 and AID are sufficient to promote CpG demethylation of specific endogenous genomic loci and to increase expression of associated genes in the adult mouse brain.

### Tet1 and Apobec1 Are Involved in Neuronal Activity-Induced DNA Demethylation in the Adult Mouse Brain

Our previous studies have shown that synchronous activation of adult dentate granule neurons in vivo by electroconvulsive stimulation (ECS) leads to CpG demethylation of *Bdnf IX* and *Fgf1B* promoters in these neurons within 4 hr but has no effect on the *Fgf1G* promoter (Ma et al., 2009b). To assess whether Tet1 is required for neuronal activity-induced DNA demethylation in vivo, we injected engineered AAVs that express short-hairpin RNAs (shRNAs) to knock down the endogenous *Tet1* expression in the adult dentate gyrus (Figure S7A). The two shRNAs against mouse *Tet1* have been previously characterized (Table S1) (Ito et al., 2010). We further confirmed the effectiveness of these two shRNAs in knocking down endogenous mouse *Tet1* both in vitro and in vivo (Figure S7B). Interestingly, expression of either shRNA against mouse *Tet1*, but not the control shRNA, completely abolished ECS-induced demethylation of both *Bdnf IX* and *Fgf1B*, but not *Fgf1G*, promoters in dentate neurons from multiple animals (Figures 7A–7C and Figure S7E). ECS-induced expression of these two gene isoforms was also abolished (Figures 7D and 7E). Thus, endogenous Tet1 is required for neuronal activity-induced, region-specific, active DNA demethylation and gene expression in the adult brain.



**Figure 6. TET1 and AID Regulate Endogenous 5hmC Levels and Promote Region-Specific DNA Demethylation in the Adult Mouse Brain**

(A and B) Effects of AAV-mediated overexpression of control YFP, TET1, TET1m (A), and AID (B) on the endogenous 5hmC levels as measured by immunoblotting (left) and ELISA (right). Engineered AAV viruses were stereotactically injected into the dentate gyrus of adult mice, and dentate gyrus tissue was microdissected 1 week later for analysis. Values represent mean  $\pm$  SEM.  $n = 3$  animals for each condition; \*\* $p < 0.01$ ; Student's  $t$  test.

(C and D) Effects of YFP, TET1, TET1m, and AID overexpression on methylation levels of *Bdnf* IX (C), *Fgf1B*, and *Fgf1G* (D) alternative promoters in the adult dentate gyrus. Shown on top are diagrams of promoters, and bars indicate regions for bisulfite sequencing analysis. Values represent mean  $\pm$  SEM.  $n = 3$  animals for each condition; \*\* $p < 0.01$  and \* $p < 0.05$ ; Student's  $t$  test.

(E) Effects of YFP, TET1, TET1m, and AID overexpression on specific isoform and total expression levels of *Fgf1* and *Bdnf* in the adult dentate gyrus. Same groups of animals as in (C) and (D) were examined. Values represent mean  $\pm$  SEM. \*\* $p < 0.01$  and \* $p < 0.05$ ; Student's  $t$  test. See also Figure S6.

To directly examine whether 5hmCs can be demethylated in neurons, we transfected 5hmC-GFP DNA fragments into primary neurons in culture. Bisulfite sequencing analysis showed significant demethylation of 5hmC DNA in both CpG ( $\sim 5\%$ ) and CpH ( $\sim 6\%$ ) contexts. Interestingly, the numbers of demethylated 5hmCs in individual clones also exhibited a broad distribution, suggesting high processivity (Figure 7F).

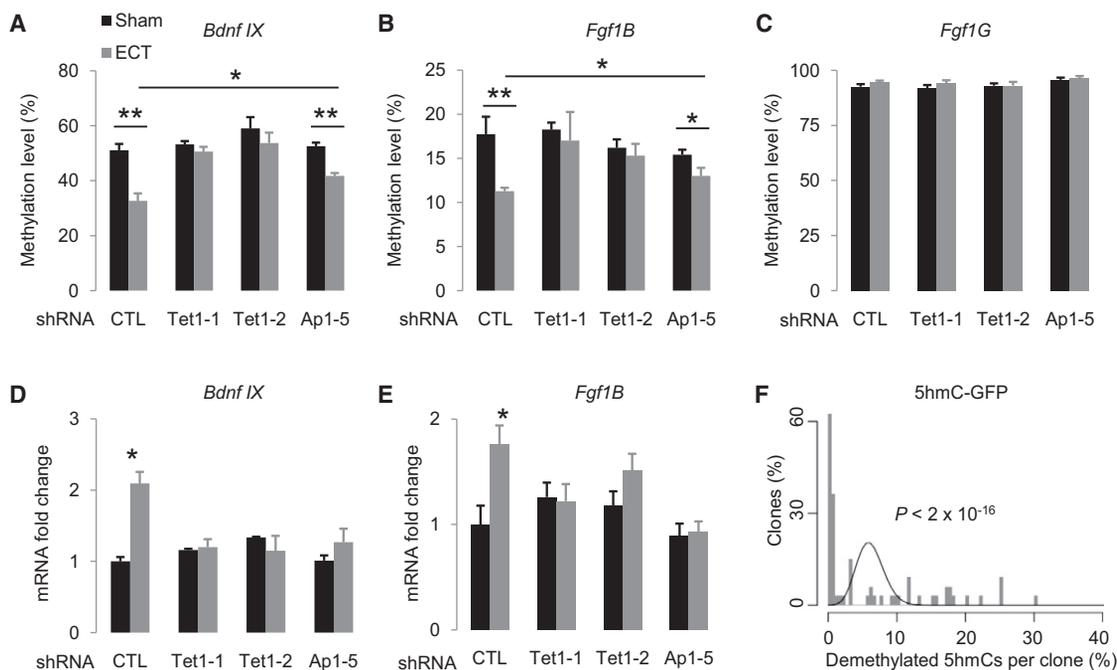
To identify the endogenous deaminase that may contribute to neuronal activity-induced DNA demethylation in vivo, we examined the expression of *Aid*/*Apobec* genes under normal conditions and 4 hr after ECS in the adult dentate gyrus. Whereas *Aid*, *Apobec2*, and *Apobec4* appeared to be absent, *Apobec1* and *Apobec3* were expressed in the adult dentate gyrus at similar levels with or without ECS (Figure S7C). We obtained one shRNA (Ap1-5) that achieved effective knockdown of endogenous *Apobec1* expression in culture and in the adult dentate gyrus after AAV-mediated expression (Figure S7D and Table S1). Interestingly, knockdown of *Apobec1* significantly reduced ECS-

induced demethylation of both *Bdnf* IX and *Fgf1B* promoters in dentate neurons from multiple animals in comparison to those expressing the control shRNA (Figures 7A–7C and Figure S7E). Furthermore, ECS-induced expression of these two gene isoforms was completely abolished (Figures 7D and 7E). It remains to be determined whether *Apobec3* also plays a role in neuronal activity-induced DNA demethylation and expression of associated genes in the adult brain.

Taken together, these results demonstrated that neurons possess a significant capacity to demethylate 5hmCs and suggest that Tet1/*Apobec1*-mediated oxidation-deamination mechanism plays a critical role in neuronal activity-induced, region-specific, active DNA demethylation in the adult mouse brain.

## DISCUSSION

Accumulating evidence supports the existence of active DNA demethylation in vertebrate cells, yet the underlying molecular mechanisms remain unclear (Wu and Zhang, 2010). The discovery of the TET family of 5mC hydroxylases has raised the question of its potential role in active DNA demethylation. Here, we identify TET1-catalyzed 5mC hydroxylation as a key initiating step in DNA excision repair-based active DNA demethylation in mammalian cells both in vitro and in vivo. By introducing fully modified linear dsDNA substrates with 5hmCs into HEK293 cells



**Figure 7. Tet1 and Apobec1 Are Involved in Neuronal Activity-Induced Region-Specific Demethylation in the Adult Mouse Brain**

(A–C) Effects of AAV-mediated shRNA knockdown of endogenous Tet1 and Apobec1 on ECS-induced CpG demethylation of *Bdnf IX* (A), *Fgf1B* (B), and *Fgf1G* (C) promoters. Engineered AAV viruses expressing control shRNA (CTL), two shRNAs against mouse *Tet1* (Tet1-1 and Tet1-2), and one shRNA against mouse *Apobec1* (Ap1-5) were stereotactically injected into the dentate gyrus of adult mice. One week after viral injection, animals were subjected to ECS or sham treatment. Dentate gyrus tissue was microdissected 4 hr later for analysis. Values represent mean  $\pm$  SEM.  $n = 3$ –6 animals for each condition; \*\* $p < 0.01$  and \* $p < 0.05$ ; Student's *t* test.

(D and E) Effects of Tet1 and Apobec1 knockdown on ECS-induced isoform-specific expression of *Bdnf* and *Fgf1* in the adult dentate gyrus. Same groups of animals as in (A–C) were examined. Values represent mean  $\pm$  SEM. \* $p < 0.05$ ; Student's *t* test.

(F) Demethylation of 5hmC-GFP DNA fragments after transfection into primary hippocampal neurons in culture. Shown is a histogram of clone distribution of the percentage of demethylated 5hmCs in 5hmC-GFP DNA fragments retrieved 7 days after transfection. A distributive Poisson model ( $\lambda = 6.2\%$ ) is shown by a black curve.

See also Figure S7.

and primary mouse neurons, we demonstrate that mammalian cells possess a robust, active demethylating machinery targeting 5hmC-containing DNA in both CpG and non-CpG contexts. We further provide mechanistic insights into this process by pharmacological inhibition of key DNA repair enzymes and genetic manipulations of core enzymes in the BER pathway, including DNA glycosylases and AID/APOBEC deaminases. In cultured human cells, AID/APOBEC deaminases specifically promote 5hmC demethylation but have no apparent effect on 5mCs. In the adult mouse brain, AID facilitates the removal of endogenous 5hmCs, and furthermore, both TET1 and AID promote region-specific DNA demethylation in dentate neurons. Finally, loss-of-function experiments establish an important role of endogenous *Tet1* and *Apobec1* in neuronal activity-induced DNA demethylation and expression of associated genes in dentate neurons in the adult mouse brain. Together, our results delineate a TET1/APOBEC-mediated oxidation-deamination mechanism underlying active, region-specific DNA demethylation in mammals. Previous studies have implicated either 5hmC or deaminated 5mC as the potential key intermediate for DNA demethylation. Our work thus reconciles those previous studies and suggests a convergence of two seemingly separate mechanisms into one single

cooperative pathway for BER-mediated completion of active DNA demethylation.

Oxidation of 5mCs had been previously proposed to cause passive DNA demethylation by preventing DNMT1 recognition in vitro, which recognizes hemi-methylated CpGs after DNA replication to maintain a symmetric methylation pattern (Valinluck and Sowers, 2007). We present evidence for an unequivocal role of 5mC hydroxylation in promoting DNA demethylation in a DNA replication-independent manner by using nonreplicating SssI-methylated plasmids and linear premodified dsDNA substrates. Consistent with a role in active DNA demethylation, TET proteins are expressed in mouse primordial germ cells (Hajkova et al., 2010) and in the dentate gyrus of the adult mouse brain (Szwagierczak et al., 2010), where genome-wide and locus-specific DNA demethylation occur (Hajkova et al., 2010; Ma et al., 2009b), respectively. The adult dentate gyrus provides a relative homogenous population of postmitotic neurons in large numbers (Ma et al., 2009b), which is particularly important for epigenetic analysis because each diploid cell displays only two locus-specific binary modifications. Although mRNAs of all three *Tet* genes are detected in the mouse hippocampus (Szwagierczak et al., 2010), specific knockdown of *Tet1* abolished neuronal activity-induced

promoter demethylation of *Bdnf IX* and *Fgf1B*, suggesting nonredundant roles of different TET proteins in DNA demethylation. All three TET proteins exhibit 5mC hydroxylase activity (Ito et al., 2010), and it will be interesting to explore their roles in genome-wide and region-specific DNA demethylation in different cell types.

Previous studies (Hajkova et al., 2010; Rai et al., 2008) and our current result support a role of BER in active DNA demethylation, yet none of the known mammalian DNA glycosylases has been shown to possess 5hmC glycosylase activity. Instead, our result suggests that 5mCs, once converted to 5hmCs by TET proteins, are processed by AID/APOBEC deaminases, a family of Zn<sup>2+</sup>-dependent cytidine deaminases that have been implicated in active DNA demethylation in vertebrates (Bhutani et al., 2010; Conticello et al., 2007). AID appears to specifically promote demethylation of 5hmCs, but not 5mCs in mammalian cells. In a recent study of reprogramming-associated demethylation of *OCT4* promoter, AID was shown to occupy its target loci even before demethylation was initiated (Bhutani et al., 2010), suggesting a preceding event to AID catalysis. In support of a role of AID/APOBEC deaminases in TET1-induced DNA demethylation, 5hmC demethylation shares many known characteristics of AID-induced deamination, including high processivity, sequence selectivity, transcription dependency, and DNA strand preference. Interestingly, the 5hmC demethylation-enhancing ability is not limited to AID but is also present in multiple APOBEC deaminases. Our results do not rule out the possibility that AID/APOBEC deaminases may act on tertiary intermediates rather than on 5hmCs directly, nor can we eliminate the possibility of other pathways that process 5hmCs independently of AID/APOBEC deaminases in mammalian cells (Figure 4F). AID/APOBEC deaminases are usually expressed in a highly restricted and tissue-specific manner and exhibit distinct sequence selectivity for deamination (Conticello et al., 2007); therefore, different deaminases may mediate 5hmC demethylation cooperatively with their individual sequence preferences. Previous in vitro analysis has revealed different capacities of AID/APOBEC family members in deaminating unmethylated cytosines and 5mCs (Conticello et al., 2007). Future studies of in vitro 5hmC deamination will provide critical insight into the role of AID/APOBEC family members in DNA demethylation.

In summary, our results uncover a role for TET1, through conversion of 5mC into a critical intermediate product 5hmC, in promoting active DNA demethylation in mammalian cells both in vitro and in vivo. In addition to a well-established association with cancer (Jones and Baylin, 2002), accumulating evidence implicates epigenetic aberrations in DNA methylation in neurodegenerative diseases (Urduinguio et al., 2009) and psychiatric disorders (Feng and Fan, 2009). Identification of a role for TET1 and 5hmC in neuronal activity-induced DNA demethylation will enhance our understanding of molecular mechanisms underlying dynamic changes of DNA methylation in the adult nervous system and will provide therapeutic targets for novel treatments.

## EXPERIMENTAL PROCEDURES

### DNA Constructs

Human open reading frame entry clones were either obtained from Johns Hopkins HIT Center or cloned from a human cDNA library. Fully modified

linear UbC-GFP fragments and promoter-truncated variants were generated using dCTP, 5mdCTP (Amersham), or 5hmCCTP (Biolone) in PCR amplifications (Table S1C). AAV gene delivery vectors were constructed by cloning the EF1a-Gene-WPRE and U6-shRNA-EF1a-EYFP-WPRE cassette (Ge et al., 2006) into an AAV backbone. The efficacy of shRNAs against mouse *Tet1* and *Apoec1* (Table S1E) were tested in cell culture after electroporation and confirmed in adult mouse dentate gyri after AAV-mediated expression.

### Methylated Reporter Assay and Quantitative Analysis of DNA Methylation

NIT-GFP plasmid was used for methylation reporter assay as previously described (Ma et al., 2009b). The methylation-sensitive restriction assay was carried out using a StepOnePlus Real-Time PCR system (Applied Biosystems; Table S1A). HpaII sensitivity of a CCGG site was calculated by  $[1 - 2^{Ct(\text{mock}) - Ct(\text{HpaII})}] \times 100\%$ . Bisulfite (Zymo)-treated DNA was used as a template for PCR amplification of the region of interest as previously described (Table S1B) (Ma et al., 2008). Primers targeting nonbisulfite converted sequences were used in bisulfite sequencing analysis of 5hmC-GFP DNA fragments. Although these primers do not selectively amplify either strand, demethylation of the forward (nontranscribed) strand would appear as C-to-T transitions in Sanger sequencing results, whereas G-to-A transitions represent demethylation of the reverse (transcribed) strand (Figure S5C).

### Detection and Quantification of 5hmC and 5hmU by Immunoblotting, ELISA, and Immunocytochemistry

DNA samples were applied onto Hybond-N<sup>+</sup> membrane (Amersham), cross-linked by a UV stratalinker 1800 (Stratagene), and subjected to immunoblotting using antibodies against 5hmC (Active motif; 1:10,000) or against 5hmU (Abcam; 1:2000). For ELISA quantification of 5hmC, DNA standards and samples were immobilized on a 96-well plate using Reacti-Bind DNA coating solution (Thermo Scientific). Chemiluminescent signals were developed using a TMB substrate (Thermo Scientific) and monitored by SpectraMax Plus 384 plate reader (Molecular Devices). Immunocytochemistry used the following primary antibodies: anti-HA (rat; 1:500; Roche); anti-5hmC (rabbit; 1:500; ActiveMotif); anti-5mC (mouse; 1:250; Eurogentec); and anti-V5 (goat; 1:500; Abcam).

### Methylation and Gene Expression Analysis of the Adult Mouse Dentate Gyrus

Adult mice (8- to 10-weeks-old, male, C57BL/6 background) were used for analysis in accordance with protocols approved by the Institutional Animal Care and Use Committee. High titers of engineered AAV2/9 were stereotaxically injected into the dentate gyrus of adult mice as previously described (Ge et al., 2006). Mice were used 1 week after viral injection for analysis. ECS was administered as previously described (Ma et al., 2009b). Sham animals were similarly handled in parallel without the current delivery. Animals were analyzed at 4 hr after ECS. DNA methylation and gene expression were carried out as previously described (Tables S1B and S1D) (Ma et al., 2009b).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at doi:10.1016/j.cell.2011.03.022.

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