that is enriched within active genes as well as accessible chromatin in the nervous system (Mellén et al., 2012). More recently, it has been shown that 5mC, 5hmC, 5fC, and 5caC recruit distinct sets of proteins in a dynamic and cell-type-dependent manner (Spruijt et al., 2013). This result suggests that 5hmC, 5fC, and 5caC may recruit transcription regulators in certain cell types, as well as DNA repair proteins that may also be involved in DNA demethylation.

5hmC and the TET family of enzymes function in a diverse set of biological processes, including embryogenesis and differentiation (Cimmino et al., 2011; Dawlaty et al., 2013; Guo et al., 2011; Hahn et al., 2013; Koh et al., 2011; Wu and Zhang, 2014). 5hmC and TET family enzymes also play critical roles in the development of cancer (Cimmino et al., 2011). TET1 was first identified as part of a fusion gene with mixed lineage leukemia in patients with acute myeloid leukemia (AML) (Ono et al., 2002). Furthermore, it has been reported that TET2 is frequently inactivated by mutations or deletions in various myeloid leukemias, resulting in the downregulation of 5hmC levels (Delhommeau et al., 2009). It is also known that isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) are mutated in AML, glioma, sarcoma, and melanoma (Dang et al., 2009; Parsons et al., 2008; Ward et al., 2010). Whereas wild-type IDHs generate α-ketoglutarate, mutated IDHs produce an oncometabolite, 2-hydroxyglutarate, which inhibits the TET family of enzymes and thereby interferes with the conversion of 5mC to 5hmC (Figueroa et al., 2010; Xu et al., 2011). More recently, it has been reported that transcriptional downregulation rather than mutation of TET family enzymes or IDH can result in the depletion of 5hmC in many cancers, including melanoma, breast, lung, and prostate cancers (Hsu et al., 2012; Lian et al., 2012).

In the present study, we examined the levels of 5hmC and TET1 in glioblastoma, the most malignant type of brain tumor, which has a median survival of approximately 1 year (Furnari et al., 2007; Stupp et al., 2005). We found that, in contrast to previous studies of other tumor types (Cimmino et al., 2011), proneural glioblastoma contain high levels of 5hmC and TET1 and that TET1-mediated production of 5hmC is required for glioblastomagenesis. Furthermore, we found that 5hmC recruits chromatin target of PRMT1 (CHTOP) associated with the methylosome (Friesen et al., 2001), which methylates arginine 3 on histone H4 (H4R3) and activates the transcription of cancer-related genes.

RESULTS

Glioblastoma Cells Contain Elevated Levels of 5hmC and TET1

Glioblastoma cells cultured in serum-free conditions form spheres and retain stem-cell-like properties and tumorigenicity (Lee et al., 2006). We have previously described the isolation and culture of glioblastoma cells from seven patients, designated GB1~GB5, GB11, and GB13 (Koyama-Nasu et al., 2013). DNA array analysis revealed that GB2~GB5, GB11, and GB13 resembled proneural glioblastoma cells, whereas GB1 resembled the mesenchymal type (Lottaz et al., 2010; Figure S1A). Furthermore, we performed quantitative RT-PCR (qRT-PCR) analyses to examine the expression levels of marker

genes indicating subtypes of glioblastoma in patient samples (GBM3–GBM7) as well as in glioblastoma cell lines (GB1–GB5, GB8, and GB21). We found that glioblastoma patient samples and cell lines were classified into four subtypes: proneural, neural, classical, and mesenchymal (Figure S1B). In addition, we found that the glioblastoma cell lines and patient samples used in this study do not have mutations in either IDH1 (R132) or IDH2 (R172; Figure S1C).

Previous studies have reported that 5hmC was strongly depleted in tissue stem cell compartments and human cancers (Haffner et al., 2011; Hsu et al., 2012; Kraus et al., 2012; Lian et al., 2012; Müller et al., 2012; Orr et al., 2012). However, our dot blot analysis showed that GB2~GB5, GB11, and GB13 cells, but not GB1 cells, contained elevated levels of 5hmC compared to HeLa, U87MG, and human neural progenitor cell lines (hNPCs) (Figure 1A). Furthermore, absolute mRNA quantification using qRT-PCR analyses revealed that TET1 expression levels were significantly higher than those of the other TET family members (TET2 and TET3) in GB2~GB5, GB11, and GB13 cells and in hNPCs, but not in GB1 cells (Figure 1B). Moreover, using a stable isotope-labeled 5hmC as an internal standard, we quantified the genomic content of 5hmC in glioblastoma cells by liquid chromatography-mass spectrometry (LC-MS). We found that 5hmC accounts for about one percent of all genomic cytosines in glioblastoma cells (Figure 1C). We also performed dot blot analysis of glioblastoma patient samples and found that the levels of 5hmC and TET1 expression were upregulated in proneural glioblastoma, but not in other subtypes (Figures 1D, 1E, and S1D–S1F). Thus, upregulation of TET1 may be responsible for the elevated levels of 5hmC in the proneural subtypes.

TET1-Mediated Production of 5hmC Is Required for Glioblastomagenesis

We next examined whether the TET family of genes is involved in the proliferation and tumorigenicity of glioblastoma cells. Infection of GB2 cells with a lentivirus expressing a small hairpin RNA (shRNA) or small interfering RNA (siRNA) targeting TET1 resulted in a significant decrease in their growth (Figures 2A and S2A). Knockdown of TET1 also suppressed sphere formation of GB3~GB5 cells (Figure 2B). Furthermore, we found that overexpression of wild-type, but not of catalytic mutant TET1, restored the growth and sphere formation of GB2 cells in which TET1 had been knocked down (Figures 2A, 2C, and S2B). Consistent with these results, overexpression of wild-type TET1, but not of mutant TET1, restored the decreased levels of 5hmC caused by knockdown of TET1 (Figure S2C). These results suggest that TET1 is important for the proliferation of glioblastoma cells. We next infected GB2 cells with lentivirus-expressing shRNAs directed against TET1 and intracranially transplanted these into immunodeficient mice. Mice receiving the shRNA-expressing GB2 cells survived significantly longer than those receiving GB2 cells infected with a control lentivirus (Figure 2D). Histopathological analysis of tumor xenografts demonstrated that knockdown of TET1 inhibited glioblastoma progression, whereas control GB2 cells formed invasive glioblastoma (Figure 2E). Moreover, we found that overexpression of TET1 restored the tumorigenicity of GB2 cells in which TET1 had been knocked down (Figure 2D). These results suggest that
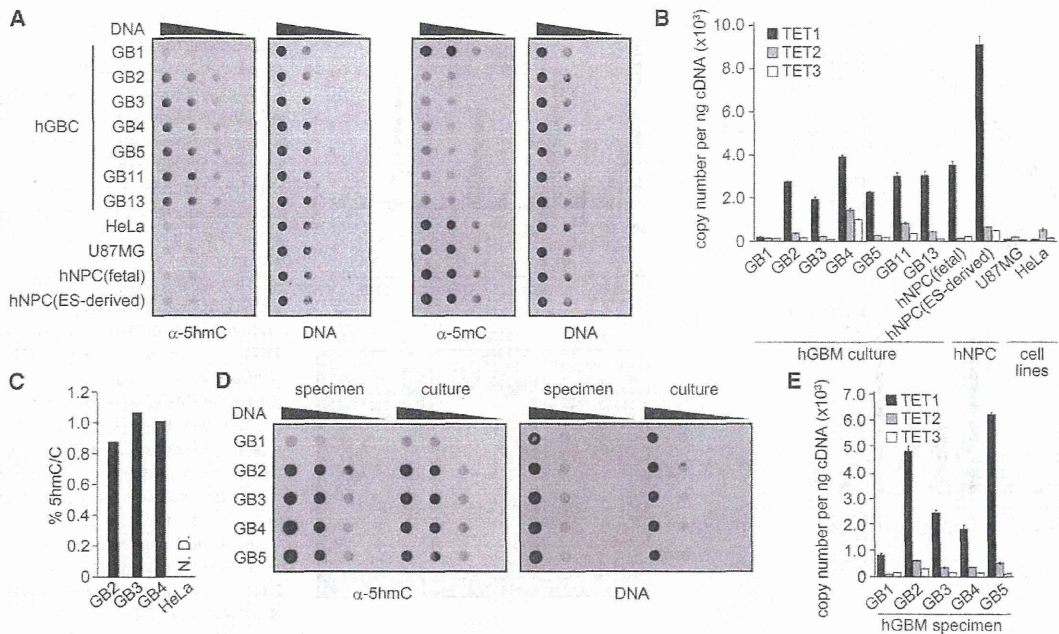
Figure 1. 5hmC and TET1 Levels in Human Glioblastoma Cells
(A) Dot blot analysis of genomic 5hmC and 5mC in human glioblastoma cells and NPCs.
(B) Absolute expression levels of the TET family of genes were determined by qRT-PCR. Copy numbers were calculated according to the standard curves generated by known quantities of plasmids containing the TET family of genes. Data show the means ± SD of three independent experiments.
(C) LC-MS quantification of 5hmC in genomic DNA isolated from glioblastoma cells and HeLa cells. N.D., not detected.
(D) Dot blot analysis of genomic 5hmC in primary glioblastoma samples and cultured cells.
(E) Absolute expression levels of the TET family of genes in primary glioblastoma samples as determined by qRT-PCR. Data show the means ± SD of three independent experiments.
See also Figure S1.

TET1 plays a critical role in the tumorigenicity of glioblastoma cells.

TET1-Catalyzed Enrichment of 5hmC Is Critical for the Expression of Cancer-Related Genes

To identify hydroxymethylated loci on a genomic scale, we immunoprecipitated 5-hydroxymethylated DNA using a 5hmC-specific antibody and analyzed the DNA by high-throughput sequencing (hMeDIP-seq). We found that the number of 5hmC peaks was greater in the genome of GB2 cells compared to the hNPC genome (Figure 3A). 5hmC was relatively enriched within the intragenic regions and promoters, as previously observed in mouse embryonic stem cells (Figures 3B and 3C). Gene ontology analysis revealed that genes encoding components of cancer-related signaling pathways and neuronal functions are overrepresented among the hydroxymethylated genes (Table S1). Furthermore, we identified intragenic 5hmC peaks in the *EGFR*, *AKT3*, *CDK6*, *CCND2*, and *BRAF* genes (Figure 3D). hMeDIP-qPCR assays to detect 5hmC in these five genes confirmed the results of hMeDIP-seq analyses (Figure 3E). By contrast, we could not detect 5hmC enrichment in these genes in hNPCs. To elucidate the significance of elevated 5hmC content in these genes in glioblastoma cells, we examined the effect of TET1 knockdown on their expression. We found that knock-

down of TET1 resulted in decreased expression of these five genes (Figure 3F). Furthermore, we found that overexpression of wild-type, but not of mutated, TET1 restored the expression of these genes in GB2 cells in which TET1 had been knocked down. Thus, TET1-mediated enrichment of 5hmC may be critical for their expression. EGFR and AKT3 are core components of the RTK/RAS/PI(3)K-signaling pathway, whereas CDK6 and CCND2 are key regulators of the RB-signaling pathway. These two signaling pathways, together with the p53-signaling pathway, are known to be frequently altered in glioblastoma. Our results therefore suggest that the TET1-catalyzed enrichment of 5hmC in these genes may drive the tumorigenicity of glioblastomas.

5hmC Interacts with CHTOP in Glioblastoma Cells

Based on the above findings, we hypothesize that 5hmC is not just an intermediate in DNA demethylation but functions as a *cis*-acting modulator of gene activity. Consistent with this notion, it has recently been reported that enrichment of 5hmC is not associated with DNA demethylation during neurogenesis (Hahn et al., 2013). To test our hypothesis, we attempted to identify proteins that interact with 5hmC. We prepared synthetic double-stranded DNA oligonucleotides that contain three modified CpG residues (Figure S3A). We incubated
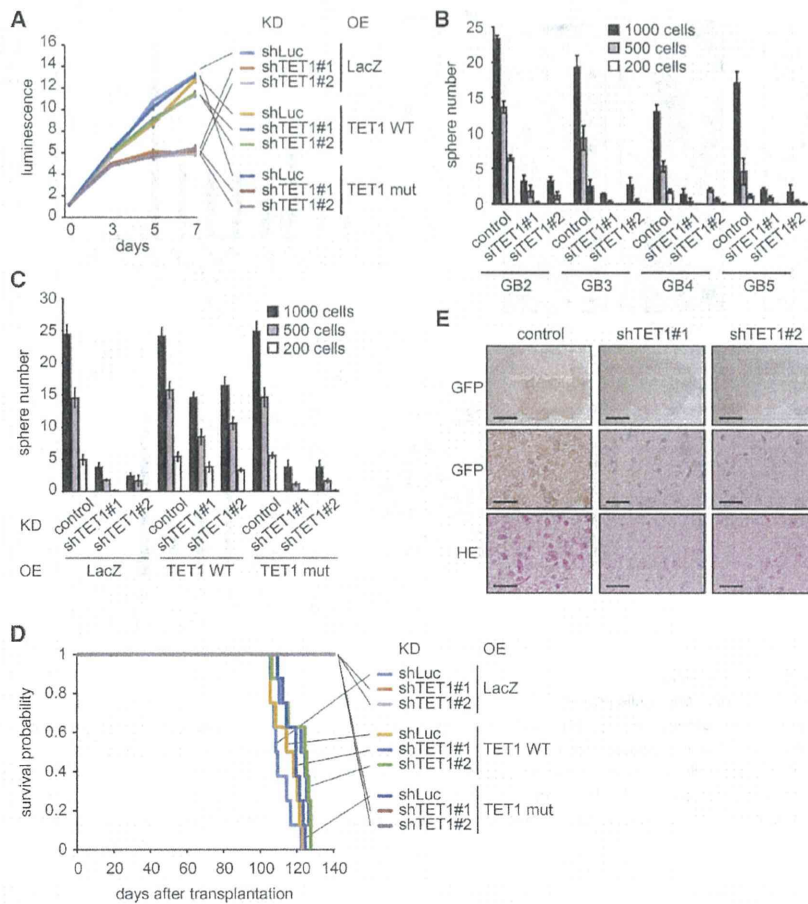
Figure 2. TET1 Is Required for the Tumorigenicity of Glioblastoma Cells

(A) Growth curves of GB2 cells infected with a lentivirus expressing shRNA targeting TET1 and/or a lentivirus expressing wild-type or mutant TET1. Data show the means ± SD of three independent experiments. KD, knockdown. OE, overexpression.

(B) The numbers of spheres of glioblastoma cells transfected with siRNA targeting TET1. Data show the means ± SD of three independent experiments.
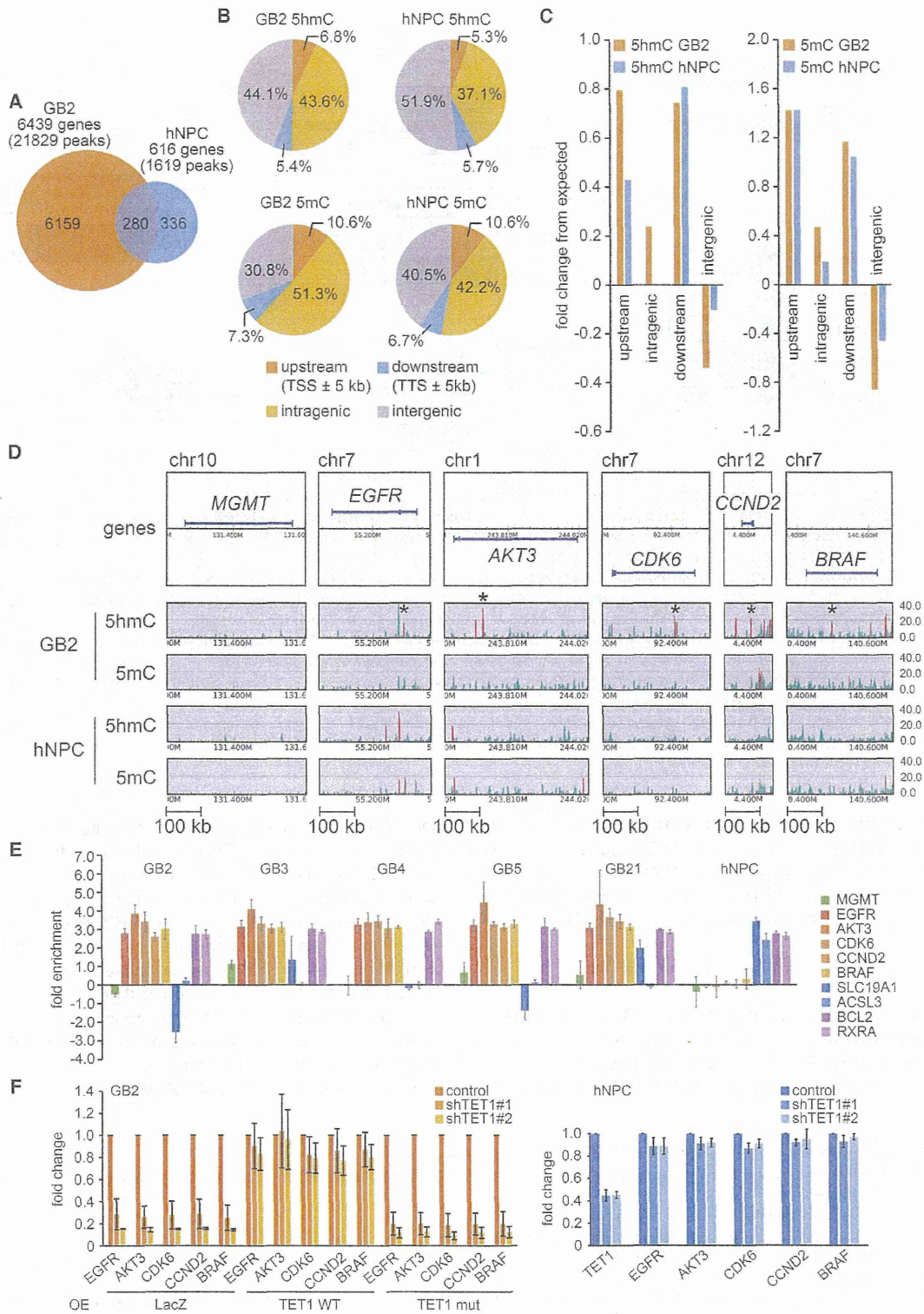
(C) The number of spheres of GB2 cells infected with a lentivirus expressing shRNA targeting TET1 and/or a lentivirus expressing wild-type or mutant TET1. Data show the means ± SD of three independent experiments.

(D) Kaplan-Meier survival curves of mice transplanted with 1.0×10^4 GB2 cells infected with a lentivirus expressing shRNA targeting TET1 or a lentivirus expressing wild-type or mutant TET1.

(E) Histological examination of tumors developed in the mice in (D). At day 100, tissue sections were stained with hematoxylin and eosin (HE) or anti-GFP antibody. The scale bars represent 2 mm (upper panels) and 50 μm (lower panels). See also Figure S2.

3′-biotin-tagged oligonucleotides with nuclear extracts from GB2 cells. The bound proteins were precipitated with streptavidin-Dynabeads and subjected to LC-MS (Figure S3B). Among the coprecipitated proteins identified (Table S2), we focused our attention on CHTOP, because it preferentially bound to the 5hmC-containing oligonucleotide versus the unmodified or 5mC-containing oligonucleotide (Figure 4A, upper panel). CHTOP is a chromatin-associated protein that is involved in transcriptional regulation. It contains a glycine- and arginine-rich (GAR) region, which interacts with RNA or DNA directly or in combination with other nucleotide-binding proteins (Rajyaguru and Parker, 2012). We generated recombinant CHTOP using a baculovirus system (Figure S3C) and confirmed that CHTOP preferentially bound to 5hmC in vitro (Figure 4A, lower panel). Moreover, we performed electrophoretic mobility shift assays and found that CHTOP preferentially binds to the 5hmC-containing oligonucleotide (Figure S3D, left panel). Addition of an anti-CHTOP antibody induced a supershift of the band (Figure S3D, middle panel), suggesting that the band detected may indeed represent a complex consisting of CHTOP and the 5hmC-containing oligonucleotide. By contrast, MBD1 specifically interacted with the 5mC-containing oligonucleotide (Figure S3D, right panel).

CHTOP was first identified as a target of protein arginine methyltransferases, especially PRMT1 and PRMT5 (van Dijk et al., 2010; Zullo et al., 2009). PRMT1 catalyzes the generation of both monomethylarginine and asymmetrical dimethylarginine residues, whereas PRMT5 generates monomethylarginine and symmetrical dimethylarginine residues (Bedford and Clarke, 2009; Martin and Zhang, 2005). Immunoblotting analysis of CHTOP with antibody against asymmetrical or symmetrical dimethylarginine revealed that CHTOP is asymmetrically, but not symmetrically, dimethylated at arginine residues in human embryonic kidney 293FT (HEK293FT) cells (Figure 4B). Knockdown of PRMT1 using siRNA resulted in a reduction in the asymmetrical methylation of arginine residues in CHTOP. PRMT1 knockdown also led to a reduction in CHTOP, especially the upper band of the doublet. In addition, as reported previously (van Dijk et al., 2010), incubation of purified glutathione S-transferase (GST)-PRMT1 and GST-CHTOP in vitro resulted in the asymmetrical methylation of CHTOP (Figure 4C). The asymmetrical methylation of CHTOP retarded its migration, suggesting that the upper band of CHTOP detected in vivo may represent the asymmetrically dimethylated form. We speculate that arginine methylation of CHTOP may increase its molecular weight and reduce its positive charge, causing its retarded migration in SDS-PAGE.

We next examined the effect of arginine methylation on the 5hmC-binding activity of CHTOP. We found that knockdown of PRMT1 suppressed the 5hmC-binding activity of CHTOP (Figure 4D). By contrast, knockdown of PRMT5 barely affected the arginine methylation or 5hmC-binding ability of

A

GB2
6439 genes
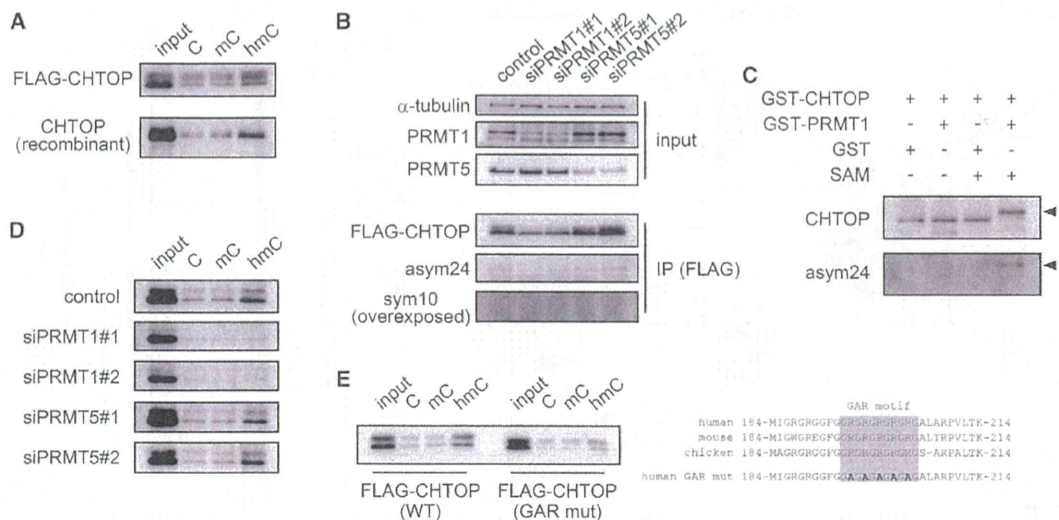(21829 peaks)

hNPC
616 genes
(1619 peaks)

6159   280   336

B

GB2 5hmC
—6.8%
44.1%   43.6%
5.4%

hNPC 5hmC
—5.3%
51.9%   37.1%
5.7%

GB2 5mC
—10.6%
30.8%   51.3%
7.3%

hNPC 5mC
—10.6%
40.5%   42.2%
6.7%

■ upstream (TSS ± 5 kb)   ■ downstream (TTS ± 5kb)
■ intragenic   ■ intergenic

C

■ 5hmC GB2   ■ 5hmC hNPC
■ 5mC GB2   ■ 5mC hNPC

D

genes: chr10 *MGMT* | chr7 *EGFR* | chr1 *AKT3* | chr7 *CDK6* | chr12 *CCND2* / *CDK6* | chr7 *BRAF*

GB2 5hmC / 5mC; hNPC 5hmC / 5mC

100 kb (×6)

E

GB2   GB3   GB4   GB5   GB21   hNPC

■ MGMT ■ EGFR ■ AKT3 ■ CDK6 ■ CCND2 ■ BRAF ■ SLC19A1 ■ ACSL3 ■ BCL2 ■ RXRA

F

GB2   ■ control ■ shTET1#1 ■ shTET1#2

EGFR AKT3 CDK6 CCND2 BRAF | EGFR AKT3 CDK6 CCND2 BRAF | EGFR AKT3 CDK6 CCND2 BRAF
OE   LacZ   TET1 WT   TET1 mut

hNPC   ■ control ■ shTET1#1 ■ shTET1#2
TET1 EGFR AKT3 CDK6 CCND2 BRAF

(legend on next page)

Figure 4. 5hmC Is Associated with CHTOP in Glioblastoma Cells

(A) CHTOP preferentially binds to 5hmC. Biotin-tagged oligonucleotides containing the indicated nucleotide modifications were incubated with lysates from HEK293FT cells transfected with FLAG-CHTOP (upper panel) or recombinant CHTOP generated using the baculovirus system (lower panel). C, unmodified cytosine; mC, 5-methylcytosine; hmC, 5-hydroxymethylcytosine.

(B) CHTOP is asymmetrically dimethylated by PRMT1, but not symmetrically by PRMT5. Lysates from HEK293FT cells transfected with FLAG-CHTOP and siRNA targeting PRMT1 or PRMT5 were subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblotting with the indicated antibodies: asym24, antibody against asymmetrically dimethylated arginine; sym10, antibody against symmetrically dimethylated arginine.

(C) PRMT1 asymmetrically dimethylates CHTOP in vitro. GST-CHTOP was incubated with GST-PRMT1 in vitro and subjected to immunoblotting analysis with the indicated antibodies. The upper band indicated by the arrowhead represents asymmetrically dimethylated CHTOP.

(D) PRMT1-mediated methylation of CHTOP is required for its binding to 5hmC. Lysates from HEK293FT cells transfected with FLAG-CHTOP and siRNA against PRMT1 or PRMT5 were subjected to pull-down assays as in (A).

(E) CHTOP binds to 5hmC via its GAR motif. Lysates from HEK293FT cells transfected with FLAG-tagged wild-type or GAR mutant CHTOP were subjected to pull-down assays as in (A). Sequences of the GAR motif of CHTOP are shown in the right panel. WT, wild-type.

See also Figure S3 and Table S2.

CHTOP. Consistent with these results, a mutant CHTOP, in which five arginine residues in the GAR region were replaced with alanine, did not interact with 5hmC (Figure 4E). These results suggest that PRMT1-mediated arginine methylation is critical for the 5hmC-binding activity of CHTOP.

CHTOP Associated with 5hmC Recruits the Methylosome

To further elucidate the function of CHTOP, we analyzed its associated proteins in cell extracts. FLAG-CHTOP was overexpressed in HEK293FT cells and immunoprecipitated with anti-FLAG antibody followed by SDS-PAGE and silver stain- ing. When coimmunoprecipitated proteins were analyzed by LC-MS, we found that PRMT1, PRMT5, methylosome protein 50 (MEP50), and enhancer of rudimentary homolog (ERH) were associated with CHTOP (Figure 5A; Table S3). Pull-down assays using lysates from GB2 cells infected with a lentivirus expressing wild-type or GAR mutant CHTOP confirmed that CHTOP was associated with these proteins (Figure 5B). Furthermore, we found that these proteins preferentially bind to 5hmC-containing oligonucleotides (Figure 5C). These proteins are components of an arginine methyltransferase complex, termed the methylosome (Friesen et al., 2001), which is involved in several cellular processes, including the

Figure 3. 5hmC Is Enriched in Genes Involved in Glioblastomagenesis

(A) Venn diagram showing the number of 5-hydroxymethylated genes in GB2 cells and NPCs. (h)MeDIP-seq was performed with cell extracts using antibodies specific for 5mC or 5hmC.

(B) Association of all peaks identified with various genomic features.

(C) 5hmC and 5mC are enriched in intragenic and upstream regions in GB2 cells.

(D) 5mC and 5hmC profiles of the genes involved in glioblastomagenesis. Regions in which signals were significantly enriched are marked in red. Asterisks indicate the peaks confirmed by hMeDIP-qPCR in (E).

(E) hMeDIP-qPCR assays were performed with glioblastoma cells and NPCs using anti-5hmC antibody. SLC19A1 and ACSL3 are positive controls for NPCs. BCL2 and RXRA are positive controls for both glioblastoma cells and NPCs. Data show the means ± SD of three independent experiments.

(F) qRT-PCR analysis of the indicated genes in GB2 cells and NPCs infected with a lentivirus expressing shRNA targeting TET1 and/or a lentivirus expressing wild-type or mutant TET1. Data are the mean ± SD of three independent experiments. OE, overexpression.
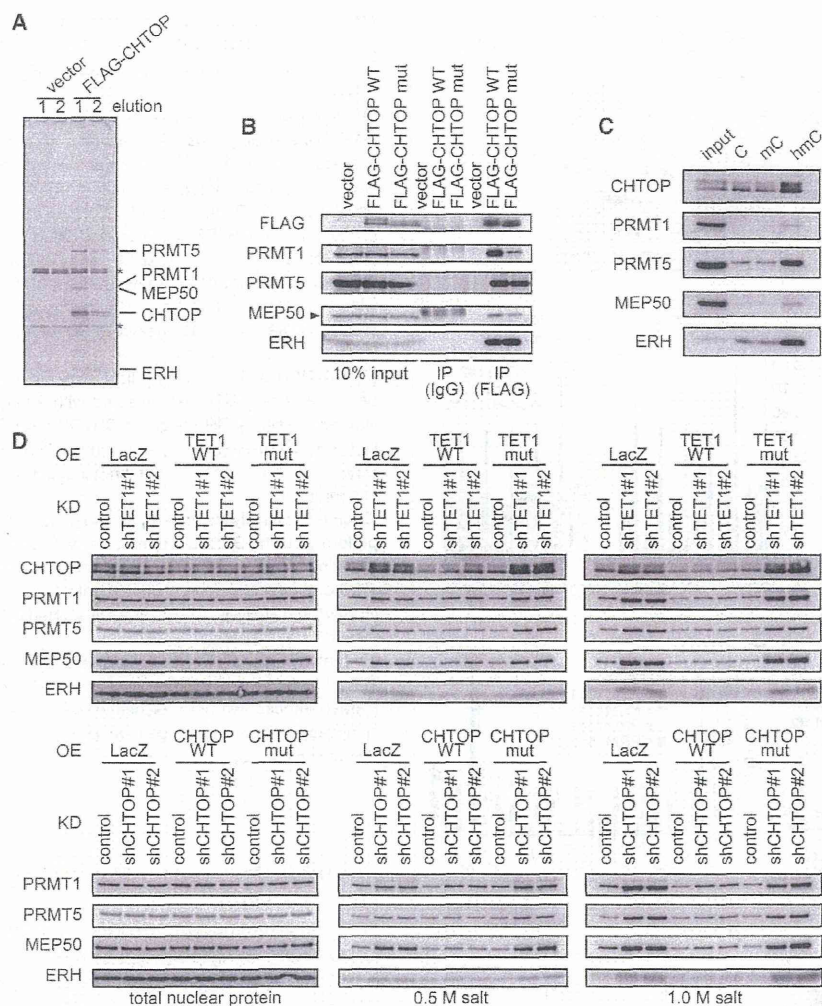
See also Table S1.

Figure 5. CHTOP Associated with the Methylosome Binds to 5hmC

(A) CHTOP is associated with the methylosome. Lysates from HEK293FT cells transfected with FLAG-CHTOP were subjected to immunoprecipitation with anti-FLAG antibody followed by SDS-PAGE and silver staining, and coprecipitated proteins were identified by mass spectrometry. Asterisks indicate immunoglobulin chains.

(B) CHTOP is associated with the methylosome in GB2 cells. The immunoprecipitates prepared as in (A) were subjected to immunoblotting analysis with the indicated antibodies.

(C) CHTOP and the methylosome preferentially bind to 5hmC in GB2 cells.

(D) Immunoblotting analysis of methylosome components extracted with salt buffer from the chromatin fractions prepared from GB2 cells infected with a lentivirus expressing the indicated shRNAs and/or a lentivirus expressing wild-type or mutant TET1 (upper panels) or CHTOP (lower panels). KD, knockdown.

See also Figure S4 and Table S3.

suggest that CHTOP associated with 5hmC functions as a recruiter of the methylosome.

H4R3 Is Methylated by PRMTs in a CHTOP- and/or TET1-Dependent Manner

We next investigated whether H4R3 is methylated by PRMTs in a CHTOP- and/ or TET1-dependent manner. Immunoblotting analysis revealed that knockdown of either TET1 or CHTOP resulted in a reduction in the amounts of both H4R3me2a and H4R3me2s (Figure 6A). We also performed chromatin immunoprecipitation (ChIP) assays with antibodies against PRMT1, PRMT5, H4R3me2a, and H4R3me2s. We found that these are associated with the *EGFR*, *AKT3*, *CDK6*, *CCND2*, and *BRAF* genes, but not the *HPRT1* gene (Figure 6B). Knockdown of either CHTOP or TET1 reduced the association of PRMT1 and PRMT5 with these genes (Figure 6B). Furthermore, we found that knockdown of either CHTOP or TET1 resulted in decreases in both the asymmetrical and symmetrical dimethylation of H4R3 (Figure 6B), whereas knockdown of PRMT1 or PRMT5 led to a decrease in the asymmetrical or symmetrical dimethylation of H4R3, respectively (Figure S5A). We also found that knockdown of either TET1 or CHTOP led to an increase in the trimethylation of H3K27, whereas the trimethylation of H3K36 and the acetylation of H4 were not affected (Figure S5B). Consistent with these results, knockdown of either CHTOP or PRMT1 suppressed the expression of these genes (Figures 6C and 6D), whereas knockdown of PRMT5 barely changed their expression, except for *CCND2*.

Consistent with the above findings, RNA sequencing (RNA-seq) analyses revealed that knockdown of TET1,
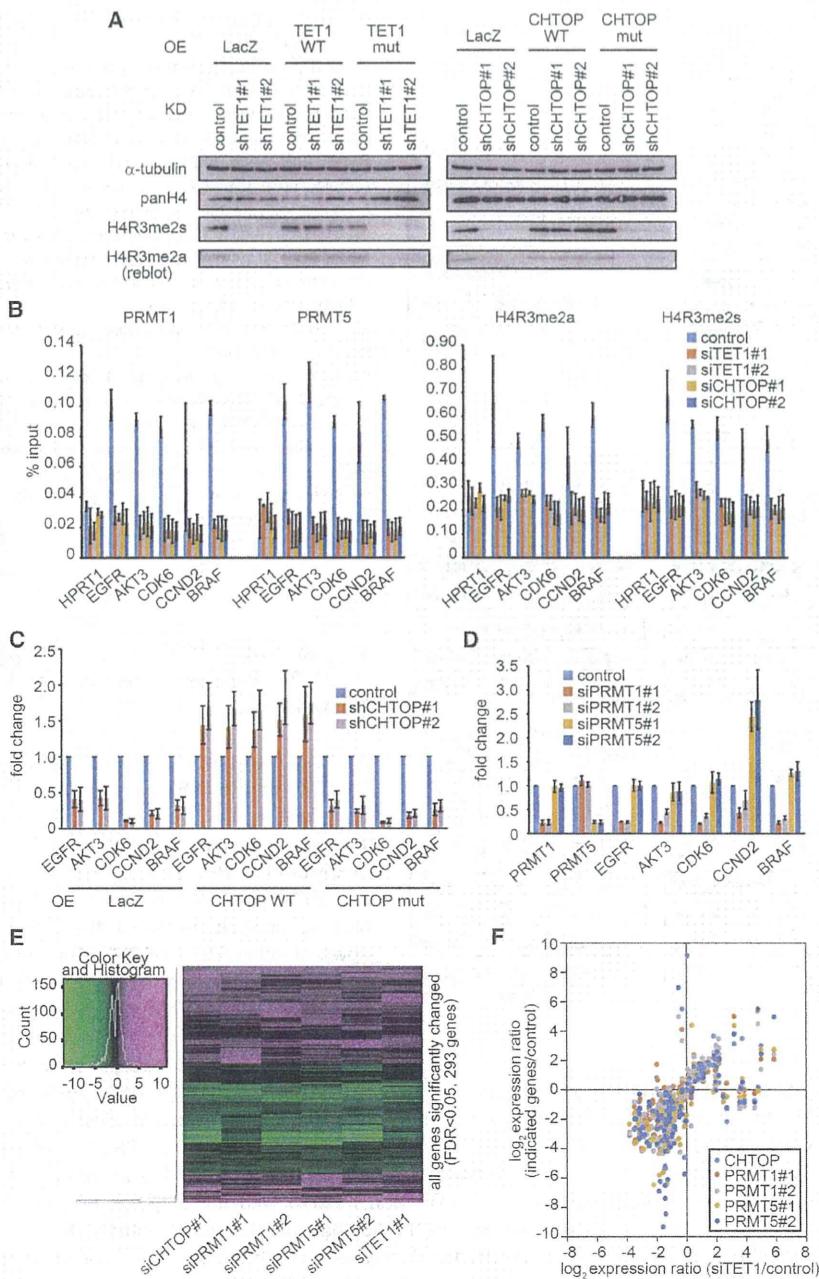
regulation of gene expression and RNA processing. Of particular note, it has been reported that PRMT1-mediated dimethylation of H4R3 is important for subsequent histone modifications such as acetylation and thereby is involved in modulating transcriptional activation (Huang et al., 2005; Wang et al., 2001).

To examine whether CHTOP is required for the association of the methylosome with chromatin, chromatin samples prepared from GB2 cells were incubated with NaCl and extracted proteins were analyzed by immunoblotting analysis (Meshorer et al., 2006). We found that knockdown of CHTOP resulted in increased amounts of PRMT1, PRMT5, MEP50, and ERH extracted from the chromatin fraction (Figures 5D, S4A, and S4B). We also found that knockdown of TET1 led to the increased extraction of these proteins, as well as increased extraction of CHTOP. In addition, the increased extraction of these proteins caused by knockdown of TET1 or CHTOP could be suppressed by overexpression of wild-type TET1 or CHTOP, but not of mutant TET1 or CHTOP. These results

Figure 6. The CHTOP-Methylosome Complex Associated with 5hmC Methylates H4R3 and Transactivates Cancer-Related Genes

(A) Immunoblotting analysis of H4R3me2a and H4R3me2s in GB2 cells infected with a lentivirus expressing the indicated shRNAs and/or a lentivirus expressing wild-type or mutant TET1 (left panels) or CHTOP (right panels).

(B) PRMTs and H4R3me2 are associated with 5hmC-enriched loci in a TET1- and CHTOP-dependent manner. ChIP assays were performed with GB2 cells transfected with the indicated siRNAs. Data show the means ± SD of three independent experiments.

(C) qRT-PCR analyses of the indicated genes were performed using GB2 cells infected with a lentivirus expressing shRNA targeting CHTOP and/or a lentivirus expressing wild-type or mutant CHTOP. Data show the means ± SD of three independent experiments.

(D) qRT-PCR analyses of the indicated genes were performed using GB2 cells transfected with siRNA targeting PRMT1 or PRMT5. Data show the means ± SD of three independent experiments.

(E) RNA-seq analysis of GB2 cells transfected with the indicated siRNAs.

(F) Scatterplot comparing transcriptome between TET1 knockdown cells and CHTOP, PRMT1, or PRMT5 knockdown cells. Genes whose expression was significantly changed are shown.

See also Figure S5.

CHTOP Is Required for Maintaining 5hmC Levels

We next examined the expression levels of CHTOP in glioblastoma cells and hNPCs. We found that GB2~GB5, GB11, and GB13 cells, but not GB1 cells, express somewhat higher levels of CHTOP than hNPCs (Figure S6A). Furthermore, we found that knockdown of CHTOP results in a reduction in global 5hmC levels in GB2 cells (Figure S6B). We have also found that overexpression of wild-type, but not of GAR mutant, CHTOP restores 5hmC levels. These results suggest that CHTOP is required for maintaining 5hmC levels and that upregulation of CHTOP as well as TET1 is responsible for the elevated levels of 5hmC in glioblastoma cells. This may be one of the reasons why 5hmC levels are not elevated in hNPCs, in which only TET1, but not CHTOP, are upregulated.

CHTOP Is Required for the Tumorigenicity of Glioblastoma Cells

Finally, we investigated the importance of CHTOP in the proliferation and tumorigenicity of glioblastoma cells. We found that knockdown of CHTOP led to decreases in sphere formation of
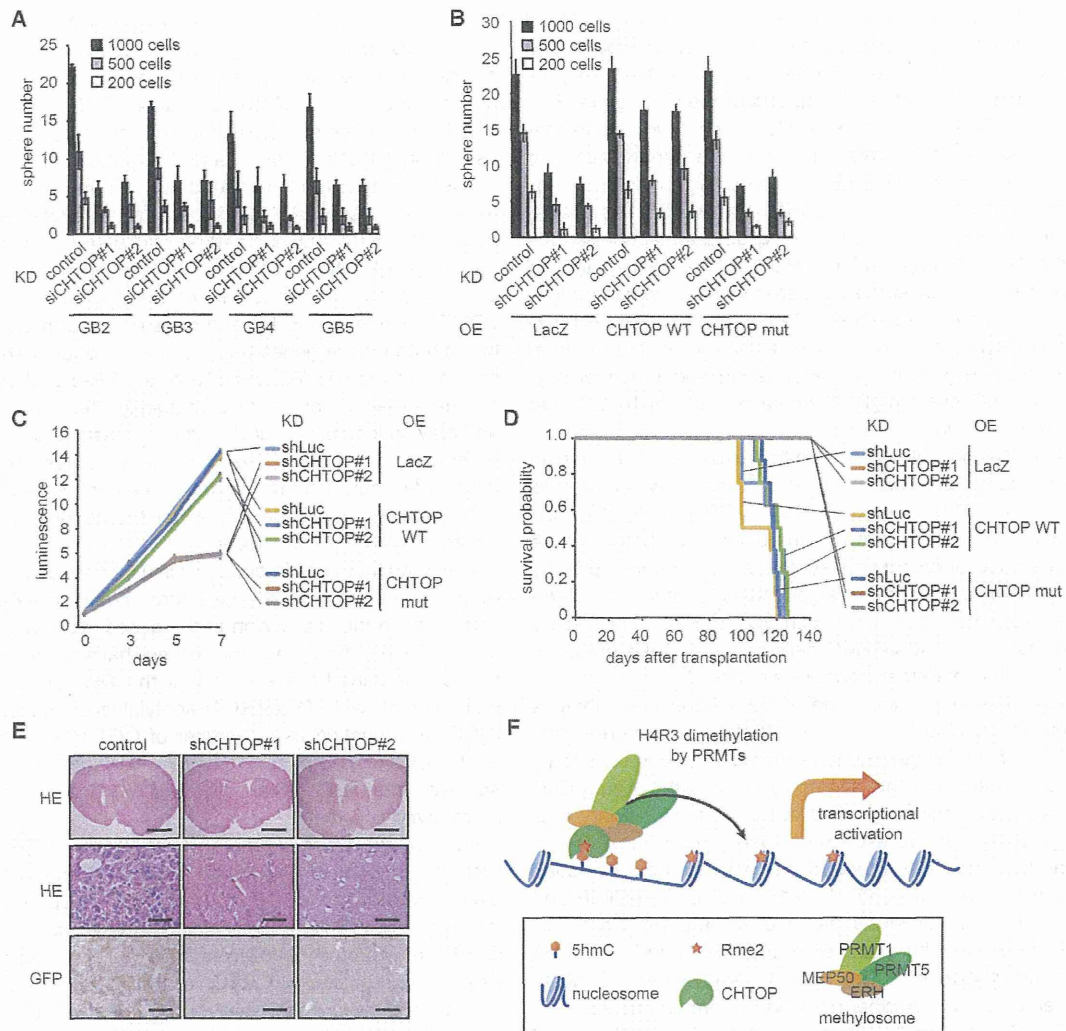
CHTOP, PRMT1, or PRMT5 resulted in similar changes in gene-expression patterns in GB2 cells (Figures 6E and S5C) and that the gene-expression profile of TET1 knockdown cells strongly correlated with the profiles of cells in which the other genes were knocked down (Figure 6F). In addition, we found that 5hmC was overrepresented in the promoters and intragenic regions of approximately 30% of genes whose expression was significantly changed (false discovery rate < 0.05).

Figure 7. CHTOP Is Required for the Tumorigenicity of Glioblastoma Cells

(A) The number of spheres of glioblastoma cells transfected with siRNA targeting CHTOP. Data show the means ± SD of three independent experiments.

(B) The number of spheres of GB2 cells infected with a lentivirus expressing shRNA targeting CHTOP and/or a lentivirus expressing wild-type or mutant CHTOP. Data show the means ± SD of three independent experiments.

(C) Growth curves of GB2 cells infected with a lentivirus expressing shRNA targeting CHTOP and/or a lentivirus expressing wild-type or mutant CHTOP. Data show the means ± SD of three independent experiments.

(D) Kaplan-Meier survival curves of mice transplanted with 1.0×10^4 GB2 cells infected with a lentivirus expressing shRNA targeting CHTOP and/or a lentivirus expressing wild-type or mutant CHTOP.

(E) Histological examination of tumors developed in the mice in (D). At day 100, tissue sections were stained with HE or anti-GFP antibody. The scale bars represent 2 mm (upper panels) and 50 μm (lower panels).

(F) Schematic representation of 5hmC-mediated transcriptional activation. For details, see text.

See also Figure S6.

GB2~GB5 cells (Figures 7A and 7B) and GB2 cell proliferation (Figure 7C). These phenotypes were rescued by overexpression of wild-type CHTOP, but not by GAR mutant CHTOP. Mice receiving shCHTOP-expressing GB2 cells survived significantly longer than control mice (Figures 7D and 7E). Furthermore, we found that overexpression of CHTOP restored the tumorigenicity of shCHTOP-expressing GB2 cells (Figure 7D). These results suggest that 5hmC serves as a recruitment signal for the

CHTOP-methylosome complex, which in turn methylates H4R3 and thereby activates the transcription of genes required for glioblastomagenesis (Figure 7F).

DISCUSSION

It is well known that glioblastomas contain genetic alterations in the p53, RB, and RTK pathways (Chen et al., 2012; Parsons

et al., 2008; Cancer Genome Atlas Research Network, 2008), as well as other genes, including the neurofibromatosis type 1 gene (Cancer Genome Atlas Research Network, 2008), the NF-κB inhibitor α gene (Bredel et al., 2011), and/or the IDH1 gene (Parsons et al., 2008). In the present study, we have shown that epigenetic alterations are also critical for the tumorigenicity of glioblastoma cells. We showed that proneural glioblastoma contains elevated levels of 5hmC and TET1 and that TET1-mediated production of 5hmC is required for glioblastomagenesis. We found that knockdown of TET1 in glioblastoma cells resulted in decreases in their proliferation, sphere formation, and tumorigenicity. Furthermore, we demonstrated that ectopic overexpression of wild-type, but not of a catalytically inactive mutant, TET1 restored the growth, sphere formation, and tumorigenicity, as well as 5hmC levels in glioblastoma cells in which TET1 had been knocked down.

Our results are in striking contrast to previous studies showing that 5hmC levels are markedly reduced in many tumor cells (Haffner et al., 2011; Kraus et al., 2012; Müller et al., 2012; Orr et al., 2012). This may be due to the differences in the culture conditions used and/or tumor subtypes. We cultured primary glioblastoma cells in serum-free conditions to retain their stem-cell-like properties and tumorigenicity. Indeed, our results showed that HeLa and U87MG cells cultured in the presence of serum contain extremely low levels of 5hmC.

Although most of previous studies did not consider subtypes of glioblastoma, Noushmehr et al. (2010) have reported that the IDH1 mutation is highly enriched in recurrent and secondary proneural glioblastoma and is strongly linked to the glioma-CpG island methylator phenotype (G-CIMP). Thus, secondary proneural glioblastomas are expected to have lower 5hmC levels. However, they have also reported that only a minor population of primary glioblastoma patients (<10%) display G-CIMP and IDH1 mutations. In this study, we focused on primary glioblastomas that can be cultured in serum-free media and found that most of the glioblastoma cell lines we established belong to the proneural subtype. Furthermore, we performed dot blot analysis of 5hmC using glioblastoma specimens of all subtypes and found that primary proneural glioblastomas that do not have an IDH1 mutation have markedly higher levels of 5hmC compared to other subtypes. We speculate that 5hmC may function differently at the molecular level in primary proneural glioblastoma compared to other subtypes of glioblastoma and other tumor cell types, and accordingly, 5hmC may recruit different molecules and elicit different downstream signals in proneural glioblastoma cells than it does in other tumor cells.

We have shown that 5hmC is not simply a demethylation intermediate but rather functions itself as an epigenetic mark modulating gene expression. Our results are consistent with previous findings showing that 5hmC is stably present in the genomes of ESCs and neuronal cells (Ficz et al., 2011; Guo et al., 2011; Hahn et al., 2013; Wu et al., 2011) and that enrichment of 5hmC is not necessarily associated with DNA demethylation (Hahn et al., 2013). Furthermore, Mbd3/NURD and MeCP2 have been reported to bind to both 5hmC and 5mC (Mellén et al., 2012; Yildirim et al., 2011). A systematic analysis of 5hmC-binding proteins using quantitative mass-spectrometry-based proteomics has identified a number of specific 5hmC-binding proteins

(Spruijt et al., 2013). These reported results suggested that 5hmC, 5fC, and 5caC may recruit transcription regulators in certain cell types, as well as DNA repair proteins, which may also be involved in DNA demethylation. CHTOP was not identified in this latter study, presumably because this laboratory used mouse ESCs, mNPCs, and adult mouse brain tissue.

We found that TET1-mediated enrichment of 5hmC is critical for the expression of a number of cancer-related genes such as *EGFR*, *AKT3*, *CDK6*, *CCND2*, and *BRAF*. Furthermore, we found that CHTOP associated with 5hmC recruits the methylosome and that a component of the methylosome complex, PRMT1, methylates H4R3 and transactivates these genes. We investigated these genes because their products are key components of the RTK/RAS/PI(3)K- or the RB-signaling pathways and have been reported to be frequently altered in glioblastoma and play critical roles in glioblastomagenesis (Chen et al., 2012; Parsons et al., 2008; Cancer Genome Atlas Research Network, 2008). We would like to investigate whether hydroxymethylation of these five genes is necessary for the tumorigenicity of glioblastoma cells in future studies.

We found that TET1 knockdown in GB2 cells alters expression of many genes that do not have 5hmC peaks. It is possible that alterations in the expression of the genes that have 5hmC may affect that of other genes that do not have 5hmC. In addition, previous studies have shown that the TET family of proteins can interact with O-linked N-acetylglucosamine transferase (OGT) and function as a recruiter of OGT to chromatin (Chen et al., 2013; Deplus et al., 2013; Vella et al., 2013). We therefore speculate that TET1 has an important role that is independent of its catalytic activity.

Interestingly, we found that knockdown of CHTOP results in a reduction in global 5hmC levels in glioblastoma cells, whereas overexpression of CHTOP restores 5hmC levels. Thus, CHTOP may be required for maintaining 5hmC levels. For example, CHTOP may protect 5hmC from demethylation. Our results suggest that upregulation of CHTOP as well as TET1 may be responsible for the elevated levels of 5hmC observed in glioblastoma cells. We would like to investigate this issue in more detail in future studies. In addition, we found that hNPCs express somewhat lower levels of CHTOP than glioblastoma cells. It is therefore possible that reduced expression of CHTOP in hNPCs contributes, at least in part, to the poor expression of 5hmC, despite high TET1 expression in these cells.

Glioblastoma stem cells are subsets of glioblastoma cells that possess the capability of self-renewal and exhibit extensive tumorigenicity (Gilbertson and Rich, 2007; Lathia et al., 2011; Singh et al., 2004). Glioblastoma stem cells have been reported to be resistant to both chemotherapy and radiotherapy and thus are responsible for the poor prognosis of glioblastoma (Bao et al., 2006; Chen et al., 2012). In this study, we utilized glioblastoma cells cultured in serum-free medium, which enriches for glioblastoma stem cells (Lee et al., 2006). Thus, it is possible that 5hmC is critical for the tumorigenicity of glioblastoma stem cells. To test this possibility, we need to analyze the CD15- and/or CD133-positive stem cell population (Singh et al., 2004; Son et al., 2009). It also remains to be examined whether 5hmC is required specifically for glioblastoma stem cells or required for both glioblastoma stem and nonstem cells.

In conclusion, we found that 5hmC recruits the CHTOP-methylosome complex, which methylates H4R3 and transactivates cancer-related genes. Of particular interest is the fact that knockdown of TET1 as well as CHTOP results in the strong suppression of glioblastoma cell tumorigenicity. We therefore speculate that TET1 could be a promising molecular target for glioblastoma therapy. Because TET1-deficient mice are viable and fertile (Dawlaty et al., 2011, 2013), compounds targeting TET1 would be expected to have few serious side effects.

EXPERIMENTAL PROCEDURES

Antibodies

Antibodies used in immunoblot, ChIP, and (h)MeDIP assays are listed in Table S4.

Cell Culture

Following informed consent, tumor samples classified as primary glioblastoma were obtained from patients undergoing surgical treatment at the University of Tokyo Hospital, as approved by the Institutional Review Board. Mouse experiments were also approved by the Institutional Review Board. Tumors were washed and mechanically and enzymatically dissociated into single cells. Tumor cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Life Technologies) containing B27 supplement minus vitamin A (Life Technologies), epidermal growth factor, and fibroblast growth factor 2 (20 ng/ml each; Wako Pure Chemicals Industries). Fetal and ESC-derived hNPCs were purchased from Lonza and Millipore, respectively, and cultured under the same conditions. HEK293FT, HeLa, and human glioblastoma U87MG cells were cultured in DMEM (Nissui) containing 10% fetal bovine serum. GB2 cells transfected with siRNA targeting TET1 or control were seeded into a 96-well plate at the indicated cell number. The number of spheres was counted after 7 days. Cell viability was measured by CellTiter-Glo (Promega) according to the manufacturer's instructions.

RNAi

For lentivirus production, the lentiviral vector CS-Rfa-CG harboring an shRNA driven by the H1 promoter was transfected with the packaging vectors pCAG-HIV-gp and pCMV-VSV-G-RSV-Rev into HEK293FT cells using Lipofectamine 2000 Transfection Reagent (Life Technologies). All plasmids were kindly provided by H. Miyoshi (RIKEN BioResource Center). Virus supernatants were purified by ultracentrifugation at 25,000 rpm at 4°C for 90 min (SW28 rotor; Beckman Coulter Genomics). The target sequences for shRNAs are as follows: shTET1 no.1: 5′-GCATATTCCTTTGAAATAA-3′; shTET1 no. 2: 5′-GAACTAAACAAGATTAAGT-3′; shCHTOP no. 1: 5′-CTAAATGAGCGCTTTA CTA-3′; shCHTOP no. 2: 5′-CCAAGATGTCTCTAAATGA-3′. The infection efficiency of the lentiviruses was more than 95%, as judged by GFP or Venus fluorescence. Transfection of Stealth siRNA duplexes targeting human TET1 (Life Technologies; catalog no. 10620318 and 10620319) or Silencer Select Pre-Designed siRNA targeting human CHTOP (Life Technologies; catalog no. s25092 and s25093) were performed using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions.

RNA Isolation and qRT-PCR

Total RNA was prepared by the NucleoSpin RNA Clean-up kit (Macherey-Nagel) and reverse transcribed with PrimeScript Reverse Transcriptase (TaKaRa). qPCR reactions were performed with Sybr Green I using a LightCycler480 (Roche Applied Science). The results were normalized with the detected values for TATA box-binding protein mRNA. Absolute mRNA levels of the TET family of genes were determined according to the standard curves generated by serial dilutions of plasmids containing TET1~TET3. Primers used in qRT-PCR are shown in Table S5.

(h)MeDIP

Cells were digested with proteinase K and RNase A, and genomic DNA was purified by phenol/chloroform extraction. Purified genomic DNA was sonicated to 200–500 bp with a Handy Sonic (TOMY). (h)MeDIP was performed as described previously (Weber et al., 2005) with minor modifications. Briefly, 4 μg of fragmented genomic DNA was immunoprecipitated with 4 μl of polyclonal antibody against 5hmC (Active Motif) or 4 μg of monoclonal antibody against 5mC (Eurogentec) at 4°C overnight in a 500 μl of IP buffer (10 mM sodium phosphate [pH 7.0], 140 mM NaCl, and 0.05% Triton X-100). The mixture was incubated with 30 μl of Dynabeads Protein G at 4°C for 2 hr and washed three times with 1 ml of IP buffer. The beads were suspended in 20 μg of proteinase K and incubated at 55°C for at least 3 hr. Immunoprecipitated DNA was purified by phenol/chloroform extraction followed by isopropanol precipitation.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.071.

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