

their proliferation (Fig. 3A). By contrast, overexpression of PCDH10 did not affect proliferation of T98G or U251 cells, which do not express detectable levels of PCDH10 protein (Supplementary Fig. 2). We also performed AnnexinV assays and found that knockdown of PCDH10 induced apoptosis of GB2 cells (Fig. 3B and C). We next performed sphere formation assays to clarify the role of PCDH10 in the self-renewal capacity of glioblastoma cells. We found that knockdown of PCDH10 by shRNA resulted in significant inhibition of sphere formation (Fig. 3D and E). Thus, PCDH10 may be critical for the proliferation, survival and self-renewal of glioblastoma cells.

3.4. Knockdown of PCDH10 suppresses the tumorigenicity of glioblastoma cells

To clarify the role of PCDH10 in the tumorigenicity of glioblastoma cells, we orthotopically transplanted GB16 cells that had been infected with a lentivirus expressing GFP and an shRNA targeting PCDH10 into the frontal lobe of nude mice. We found that the mice transplanted with PCDH10-knockdown GB16 cells survived longer than control mice (sh PCDH10#2, log-rank test $p = 0.042$; sh PCDH10#4, log-rank test $p = 0.00031$) (Fig. 4A). Histological studies revealed that all mice transplanted with

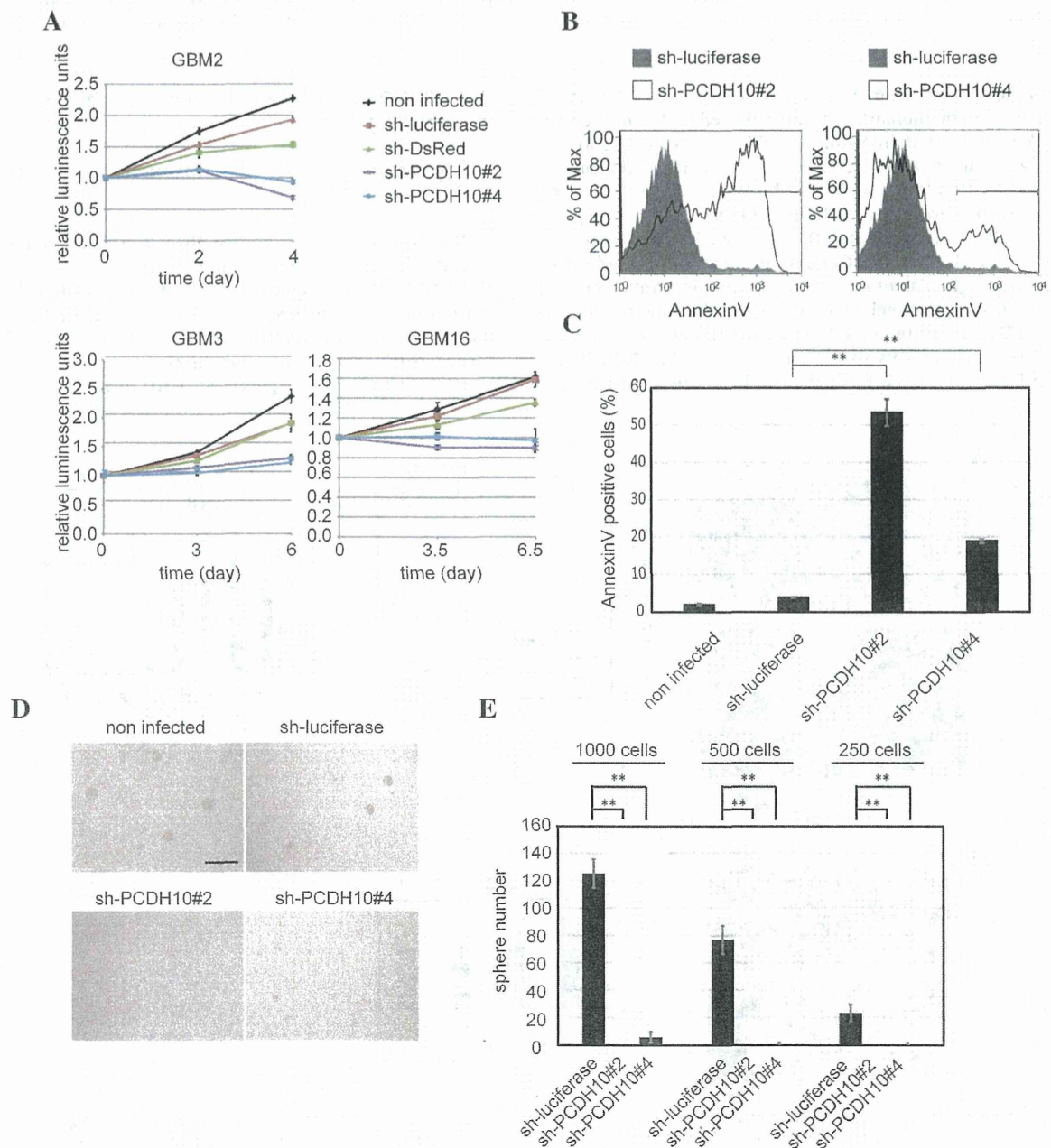


Fig. 3. PCDH10 is required for the proliferation, survival and self-renewal of glioblastoma cells. (A) Cell Titer-Glo assays were performed with GB2, GB3 or GB16 cells infected with a lentivirus expressing an shRNA targeting PCDH10 (MOI = 3). Results are shown as average of 5 wells \pm SD. (B) AnnexinV assays were performed with GB2 cells that had been infected with a lentivirus expressing an shRNA targeting PCDH10 for 8 days. (C) AnnexinV-positive populations in (B) are shown. Results are shown as the mean \pm SD ($n = 3$). At least 5000 cells were analyzed per sample. $**p < 0.01$. (D and E) Sphere formation assays were performed with GB2 cells that had been infected with a lentivirus expressing an shRNA targeting PCDH10 for 17 days. (D) Bright field images of spheres. (E) Results are shown as average of 6 wells \pm SD. $**p < 0.01$.

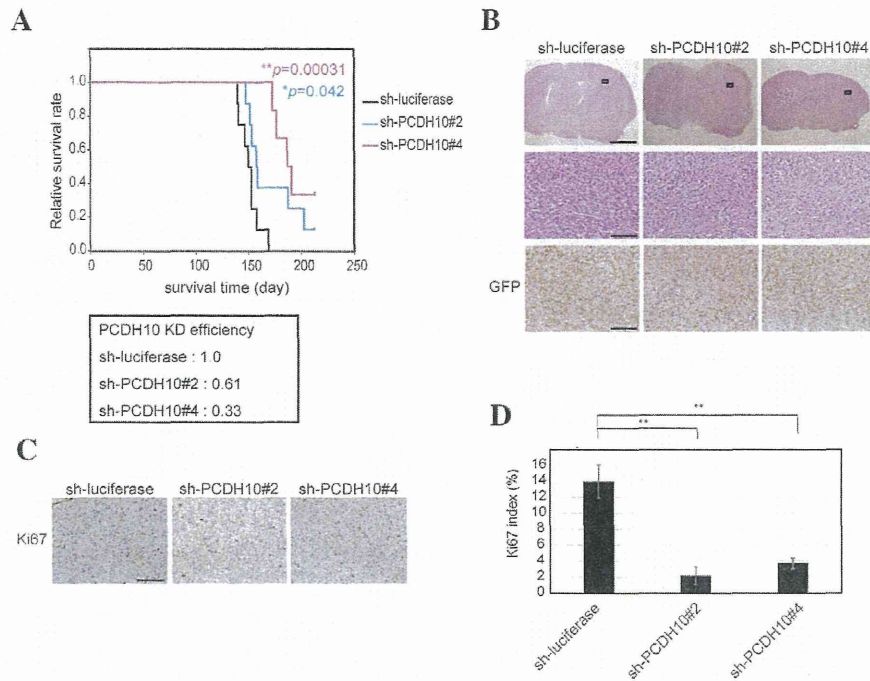


Fig. 4. PCDH10 is required for the tumorigenicity of glioblastoma cells. (A) Kaplan–Meier survival curves of mice transplanted with 1.0×10^4 GB16 cells infected with a lentivirus expressing GFP and shRNA targeting PCDH10 or luciferase (control) (MOI = 1; $n = 8$ for shPCDH10#2 and luciferase; $n = 6$ for shPCDH10#4). (B) Histological analysis of tumors harvested from the mice in (A). Tissue sections were stained with HE or anti-GFP antibody. General views (Upper Panel; Scale bar, 2.0 mm) and magnified views (Middle and Lower panels; Scale bars, 100 μm) of the region around the corpus callosum in the right hemisphere in the right hemisphere (near the injection point, black squares in the upper panel). (C and D) Immunohistochemical analysis of tumors harvested from the mice in (A). Tissue sections were stained with anti-GFP and anti-Ki-67 antibodies. Scale bars, 100 μm . (D) Ki67 indices of the brain sections. 1.0×10^3 cells were counted in each field. Results are shown as average of 6 fields \pm SD. ** $p < 0.01$.

GB16 cells had developed tumors with diffuse infiltration into surrounding brain tissues, one of the hallmark features of glioblastoma (Fig. 4B). The density of GFP-expressing cells (injected tumor cells) was slightly lower in the brains of the mice transplanted with PCDH10-knockdown GB16 cells compared to those of control mice (Fig. 4B). Furthermore, we observed that the number of Ki67-positive cells was significantly reduced in the brains of the mice transplanted with PCDH10-knockdown GB16 cells compared to those of control mice (sh-luciferase, $14.0 \pm 2.0\%$; sh-PCDH10#2, $2.2 \pm 1.1\%$; sh-PCDH10#4, $3.7 \pm 0.64\%$) (Fig. 4C and D). These results suggest that knockdown of PCDH10 suppresses the proliferation of glioblastoma cells and extends the survival of tumor-bearing mice.

These results show that PCDH10 is required for the proliferation and tumorigenicity of glioblastoma cells. This suggests that PCDH10 may function differently at the molecular level in glioblastoma cells compared to other tumor cell types. This may mean that PCDH10 associates with different molecules and elicits different downstream signals in glioblastoma cells than it does in other tumor cells. The molecular mechanisms underlying this functional difference remain to be elucidated. It also remains to be investigated whether PCDH10 expression levels are associated with the classification and prognosis of glioblastoma patients. Finally, we speculate that PCDH10 may be a potential therapeutic target for glioblastoma. In particular, monoclonal antibodies that target the extracellular domain of PCDH10 could hold promise as novel anti-tumor reagents.

Acknowledgments

This work was supported by Research Program of Innovative Cell Biology by Innovative Technology (Integrated Systems Analysis of Cellular Oncogenic Signaling Networks), Grants-in-Aid for Scientific Research on Innovative Areas (Integrative Research on

Cancer Microenvironment Network), Project for Development of Innovative Research on Cancer Therapeutics, Grant-in-Aid for Scientific Research (C-23592117) from the Japan Society for the Promotion of Science (M. Nakada) and Foundation for Promotion of Cancer Research (M. Nakada), Takeda Science Foundation, Kato Memorial Bioscience Foundation and in part by Global COE Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms), MEXT, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.12.138>.

References

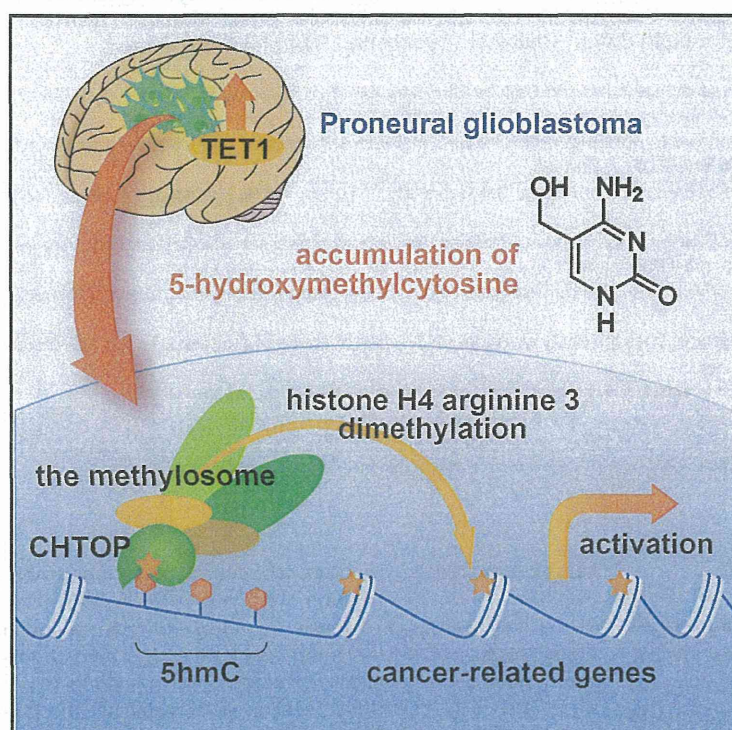
- [1] Q. Wu, T. Maniatis, A striking organization of a large family of human neural cadherin-like cell adhesion genes, *Cell* 97 (1999) 779–790.
- [2] S. Hirano, M. Takeichi, Cadherins in brain morphogenesis and wiring, *Physiol. Rev.* 92 (2011) 597–634.
- [3] W.V. Chen, T. Maniatis, Clustered protocadherins, *Development* 140 (2013) 3297–3302.
- [4] S. Hirano, Q. Yan, S.T. Suzuki, Expression of a novel protocadherin, OL-protocadherin, in a subset of functional systems of the developing mouse brain, *J. Neurosci.* 19 (1999) 995–1005.
- [5] S. Nakao, A. Platek, S. Hirano, M. Takeichi, Contact-dependent promotion of cell migration by the OL-protocadherin–Nap1 interaction, *J. Cell Biol.* 182 (2008) 395–410.
- [6] M. Uemura, S. Nakao, S.T. Suzuki, M. Takeichi, S. Hirano, OL-protocadherin is essential for growth of striatal axons and thalamocortical projections, *Nat. Neurosci.* 10 (2007) 1151–1159.
- [7] K. Miyamoto, T. Fukutomi, S. Akashi-Tanaka, T. Hasegawa, T. Asahara, T. Sugimura, T. Ushijima, Identification of 20 genes aberrantly methylated in human breast cancers, *Int. J. Cancer* 116 (2005) 407–414.
- [8] J. Ying, H. Li, T.J. Seng, C. Langford, G. Srivastava, S.W. Tsao, T. Putti, P. Murray, A.T. Chan, Q. Tao, Functional epigenetics identifies a protocadherin PCDH10 as a candidate tumor suppressor for nasopharyngeal, esophageal and multiple other carcinomas with frequent methylation, *Oncogene* 25 (2006) 1070–1080.

- [9] X. Zhong, Y. Zhu, J. Mao, J. Zhang, S. Zheng, Frequent epigenetic silencing of PCDH10 by methylation in human colorectal cancer, *J. Cancer Res. Clin. Oncol.* 139 (2013) 485–490.
- [10] J. Ying, Z. Gao, H. Li, G. Srivastava, P.G. Murray, H.K. Goh, C.Y. Lim, Y. Wang, T. Marafioti, D.Y. Mason, R.F. Ambinder, A.T. Chan, Q. Tao, Frequent epigenetic silencing of protocadherin 10 by methylation in multiple haematologic malignancies, *Br. J. Haematol.* 136 (2007) 829–832.
- [11] J. Yu, Y.Y. Cheng, Q. Tao, K.F. Cheung, C.N. Lam, H. Geng, L.W. Tian, Y.P. Wong, J.H. Tong, J.M. Ying, H. Jin, K.F. To, F.K. Chan, J.J. Sung, Methylation of protocadherin 10, a novel tumor suppressor, is associated with poor prognosis in patients with gastric cancer, *Gastroenterology* 136 (2009), 640–651 e641.
- [12] J.G. Ma, Z.K. He, J.H. Ma, W.P. Li, G. Sun, Downregulation of protocadherin-10 expression correlates with malignant behaviour and poor prognosis in human bladder cancer, *J. Int. Med. Res.* 41 (2013) 38–47.
- [13] J. Chen, R.M. McKay, L.F. Parada, Malignant glioma: lessons from genomics, mouse models, and stem cells, *Cell* 149 (2012) 36–47.
- [14] R. Koyama-Nasu, Y. Nasu-Nishimura, T. Todo, Y. Ino, N. Saito, H. Aburatani, K. Funato, K. Echizen, H. Sugano, R. Haruta, M. Matsui, R. Takahashi, E. Manabe, T. Oda, T. Akiyama, The critical role of cyclin D2 in cell cycle progression and tumorigenicity of glioblastoma stem cells, *Oncogene* 32 (2013) 3840–3845.
- [15] J. Lee, S. Kotliarova, Y. Kotliarov, A. Li, Q. Su, N.M. Donin, S. Pastorino, B.W. Purow, N. Christopher, W. Zhang, J.K. Park, H.A. Fine, Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines, *Cancer Cell* 9 (2006) 391–403.
- [16] S.M. Pollard, K. Yoshikawa, I.D. Clarke, D. Danovi, S. Stricker, R. Russell, J. Bayani, R. Head, M. Lee, M. Bernstein, J.A. Squire, A. Smith, P. Dirks, Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens, *Cell Stem Cell* 4 (2009) 568–580.
- [17] M. Nakada, S. Nakada, T. Demuth, N.L. Tran, D.B. Hoelzinger, M.E. Berens, Molecular targets of glioma invasion, *Cell. Mol. Life Sci.* 64 (2007) 458–478.

Cell Reports

5-Hydroxymethylcytosine Plays a Critical Role in Glioblastomagenesis by Recruiting the CHTOP-Methylosome Complex

Graphical Abstract



Authors

Hiroki Takai, Koji Masuda, ..., Katsuhiko Shirahige, Tetsu Akiyama

Correspondence

akiyama@iam.u-tokyo.ac.jp

In Brief

The development of cancer is driven not only by genetic mutations but also by chromatin and DNA modification changes. Takai et al. now show that proneural glioblastomas contain high levels of 5hmC and TET1. Production of 5hmC is required for the tumorigenicity of glioblastoma cells. Furthermore, 5hmC recruits the CHTOP-methylosome complex to selective sites on the chromosome, where it methylates H4R3 and activates the transcription of cancer-related genes.

Highlights

Glioblastoma cells contain elevated levels of 5hmC and TET1

TET1-mediated production of 5hmC is required for glioblastomagenesis

5hmC recruits the CHTOP-methylosome complex

The CHTOP-methylosome complex methylates H4R3 and trans-activates cancer-related genes



Takai et al., 2014, Cell Reports 9, 48–60
 October 9, 2014 ©2014 The Authors
<http://dx.doi.org/10.1016/j.celrep.2014.08.071>

CellPress

5-Hydroxymethylcytosine Plays a Critical Role in Glioblastomagenesis by Recruiting the CHTOP-Methylosome Complex

Hiroki Takai,¹ Koji Masuda,² Tomohiro Sato,¹ Yuriko Sakaguchi,³ Takeo Suzuki,³ Tsutomu Suzuki,³ Ryo Koyama-Nasu,¹ Yukiko Nasu-Nishimura,¹ Yuki Katou,² Haruo Ogawa,⁴ Yasuyuki Morishita,⁵ Hiroko Kozuka-Hata,⁶ Masaaki Oyama,⁶ Tomoki Todo,⁷ Yasushi Ino,⁷ Akitake Mukasa,⁷ Nobuhito Saito,⁷ Chikashi Toyoshima,⁴ Katsuhiko Shirahige,² and Tetsu Akiyama^{1,*}

¹Laboratory of Molecular and Genetic Information, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

²Laboratory of Genome Structure and Function, Research Center for Epigenetic Disease, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

³Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

⁴Laboratory of Membrane Proteins, Center for Structural Biology of Challenging Proteins, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

⁵Department of Molecular Pathology, Graduate School of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

⁶Medical Proteomics Laboratory, Institute of Medical Science, The University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

⁷Department of Neurosurgery, The University of Tokyo Hospital, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

*Correspondence: akiyama@iam.u-tokyo.ac.jp

<http://dx.doi.org/10.1016/j.celrep.2014.08.071>

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

SUMMARY

The development of cancer is driven not only by genetic mutations but also by epigenetic alterations. Here, we show that TET1-mediated production of 5-hydroxymethylcytosine (5hmC) is required for the tumorigenicity of glioblastoma cells. Furthermore, we demonstrate that chromatin target of PRMT1 (CHTOP) binds to 5hmC. We found that CHTOP is associated with an arginine methyltransferase complex, termed the methylosome, and that this promotes the PRMT1-mediated methylation of arginine 3 of histone H4 (H4R3) in genes involved in glioblastomagenesis, including *EGFR*, *AKT3*, *CDK6*, *CCND2*, and *BRAF*. Moreover, we found that CHTOP and PRMT1 are essential for the expression of these genes and that CHTOP is required for the tumorigenicity of glioblastoma cells. These results suggest that 5hmC plays a critical role in glioblastomagenesis by recruiting the CHTOP-methylosome complex to selective sites on the chromosome, where it methylates H4R3 and activates the transcription of cancer-related genes.

INTRODUCTION

Covalent modifications of DNA and histones influence transcriptional activity and the timing of DNA replication, thereby regu-

lating cell proliferation, survival, self-renewal, and tumorigenesis (Goldberg et al., 2007; Sasaki and Matsui, 2008). Methylation at the five position of cytosine is one of the most abundant modifications of DNA and is required for the regulation of gene expression, genome stability, and genomic imprinting (Baylin and Jones, 2011). This modification is mediated by the DNA methyltransferase family of proteins and occurs predominantly in CpG dinucleotides (Bird, 2001; Jones, 2012). It has recently been shown that 5mC is oxidized to 5-hydroxymethylcytosine (5hmC) by the ten-eleven translocation (TET) family of Fe(II) and 2-oxoglutarate-dependent DNA dioxygenases, TET1~TET3 (He et al., 2011; Ito et al., 2011; Tahiliani et al., 2009). 5hmC is found in diverse cell types and developmental stages, including embryonic stem cells and Purkinje cells (Cimmino et al., 2011; Dawlaty et al., 2013; Guo et al., 2011; Hahn et al., 2013; Koh et al., 2011; Wu and Zhang, 2014). More recently, 5hmC has been shown to be successively oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by the TET family of enzymes (He et al., 2011; Ito et al., 2011; Shen et al., 2014; Wu and Zhang, 2014). 5hmC, 5fC, and 5caC are assumed to be intermediates in DNA demethylation, and 5fC and 5caC can be converted to unmodified cytosine by thymine-DNA glycosylase (TDG) and by the base excision repair pathway (Guo et al., 2011; He et al., 2011).

It has recently been shown that 5hmC acts not only as an intermediate of DNA demethylation but also as an epigenetic mark that recruits DNA-binding proteins. For example, it has been shown that the Mbd3/NURD complex regulates expression of 5hmC-marked genes in embryonic stem cells (ESCs) (Yildirim et al., 2011). It has also been reported that MeCP2 binds to 5hmC

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

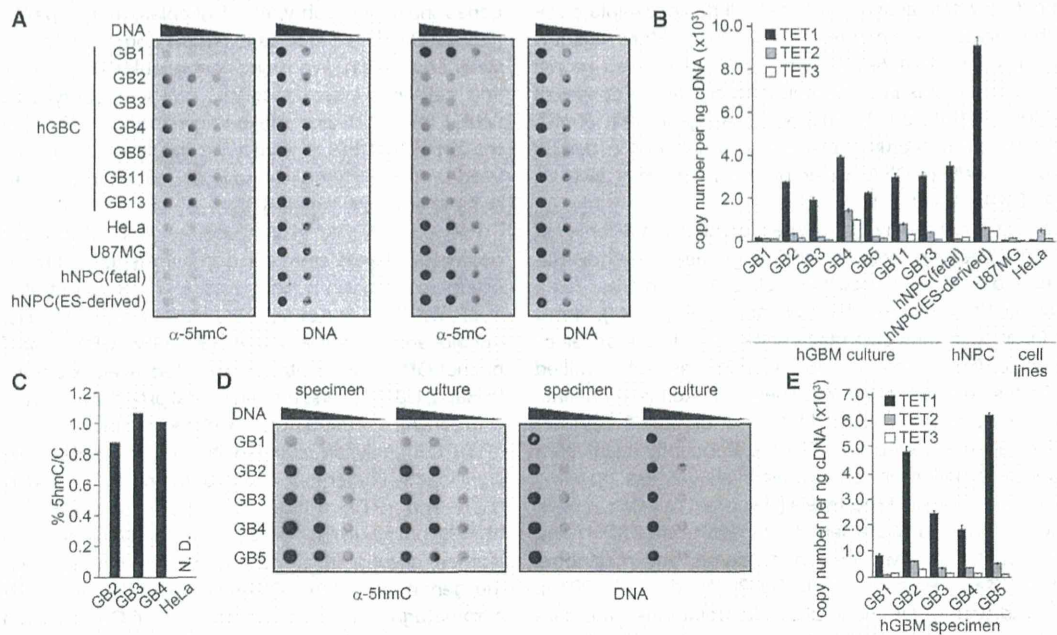


Figure 1. 5hmC and TET1 Levels in Human Glioblastoma Cells

(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 \pm 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 \pm 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

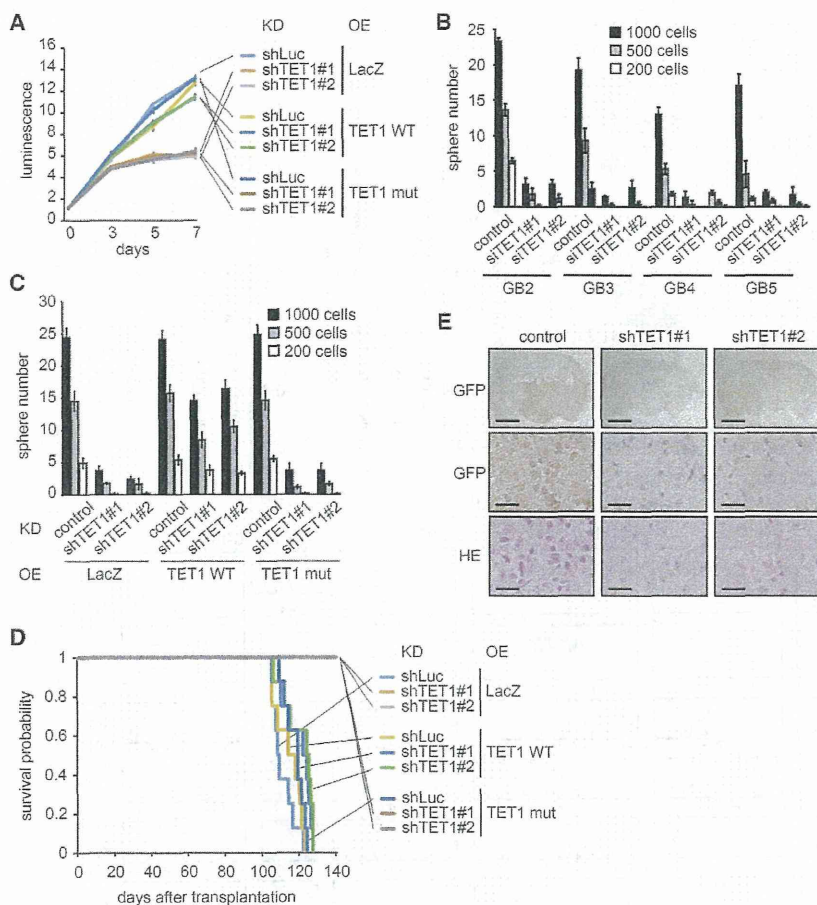


Figure 2. TET1 Is Required for the Tumorigenicity of Glioblastoma Cells

(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 \pm 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 \pm SD of three independent experiments.

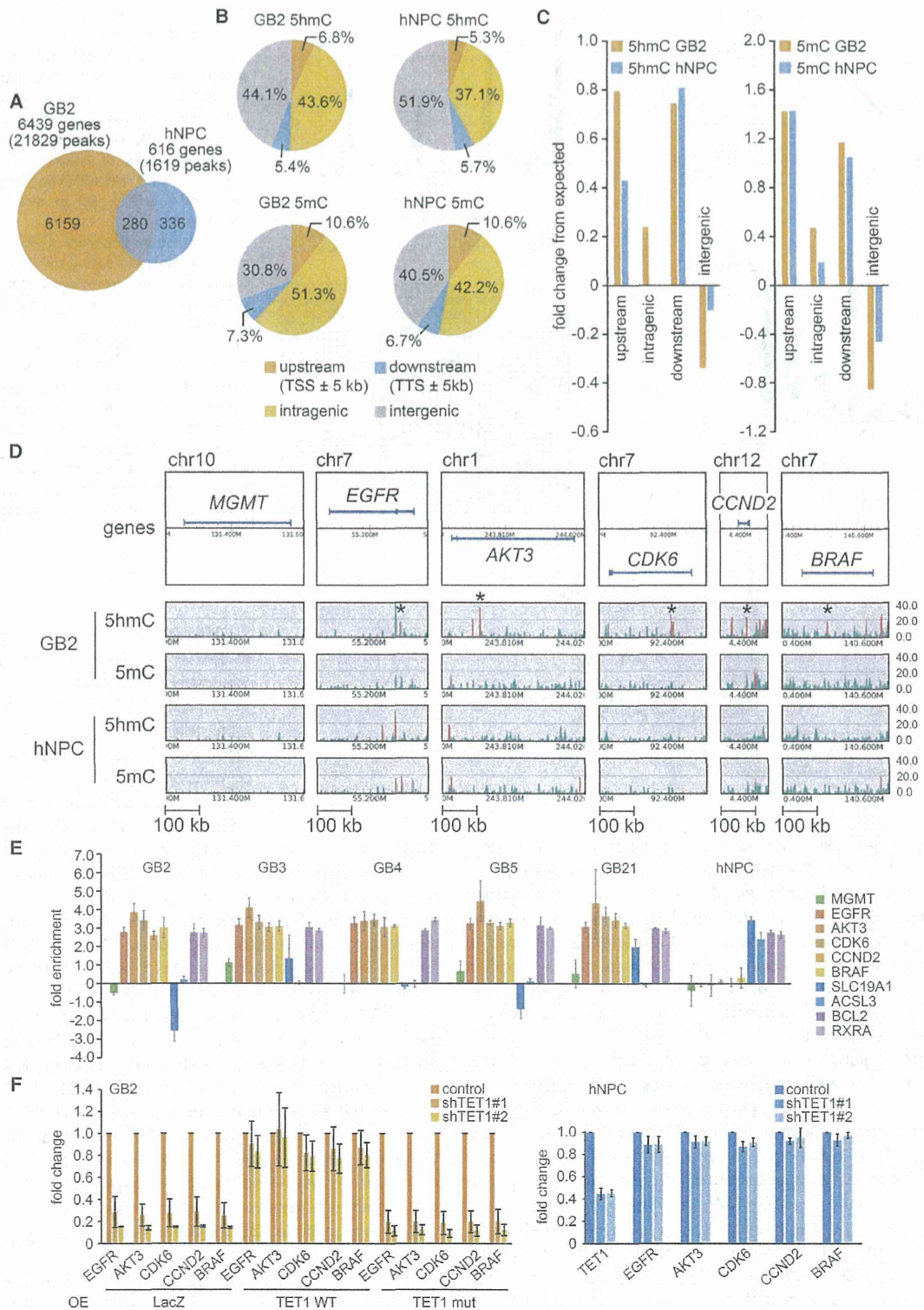
(C) The number of spheres of GB2 cells infected with a lentivirus expressing shRNA targeting TET1 and/or a lentivirus expressing wild-type or mutant TET1. Data show the means \pm SD of three independent experiments.

(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 μ m (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 asymmetrical, 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 asymmetrical 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



(legend on next page)

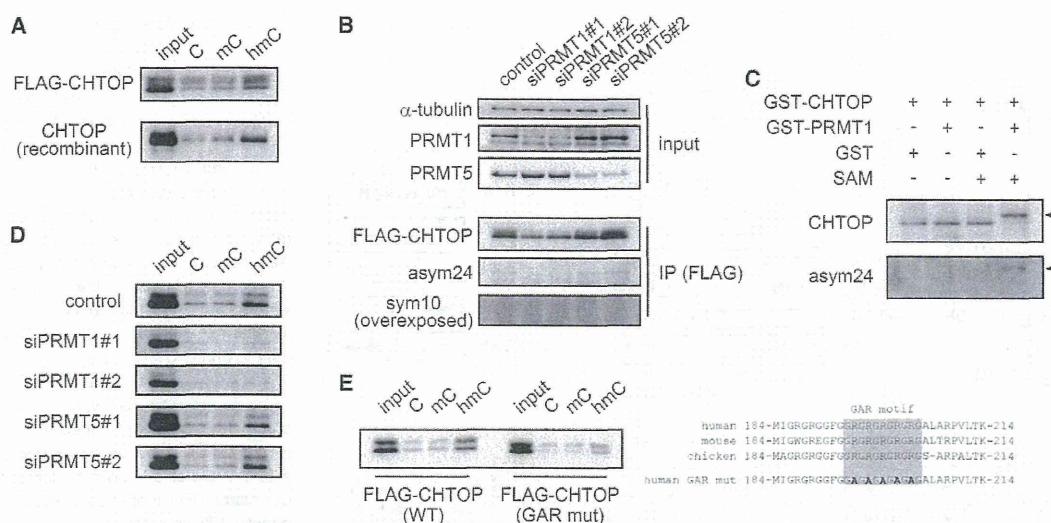


Figure 4. 5hmC Is Associated with CHTOP in Glioblastoma Cells

(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, methyltransferase 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 \pm 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 \pm SD of three independent experiments. OE, overexpression.

See also Table S1.