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LABORATORY INVESTIGATION

SPINT2 is hypermethylated in both *IDH1* mutated and wild-type glioblastomas, and exerts tumor suppression via reduction of c-Met activation

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Abstract

Purpose Both *IDH1-mutated* and *wild-type* gliomas abundantly display aberrant CpG island hypermethylation. However, the potential role of hypermethylation in promoting gliomas, especially the most aggressive form, glioblastoma (GBM), remains poorly understood.

Methods We analyzed RRBS-generated methylation profiles for 11 $IDH1^{WT}$ gliomas (including 7 GBMs), 24 $IDH1^{MUT}$ gliomas (including 6 GBMs), and 5 normal brain samples and employed TCGA GBM methylation profiles as a validation set. Upon classification of differentially methylated CpG islands by *IDH1* status, we used integrated analysis of methylation and gene expression to identify *SPINT2* as a top cancer related gene. To explore functional consequences of *SPINT2* methylation in GBM, we validated *SPINT2* methylation status using targeted bisulfite sequencing in a large cohort of GBM samples. We assessed DNA methylation-mediated *SPINT2* gene regulation using 5-aza-2′-deoxycytidine treatment, *DNMT1* knockdown and luciferase reporter assays. We conducted functional analyses of *SPINT2* in GBM cell lines in vitro and in vivo.

Results We identified *SPINT2* as a candidate tumor-suppressor gene within a group of CpG islands (designated G_T-CMG) that are hypermethylated in both $IDH1^{MUT}$ and $IDH1^{WT}$ gliomas but not in normal brain. We established that *SPINT2* down-regulation results from promoter hypermethylation, and that restoration of *SPINT2* expression reduces c-Met activation and tumorigenic properties of GBM cells.

Conclusions We defined a previously under-recognized group of coordinately methylated CpG islands common to both $IDH1^{WT}$ and $IDH1^{MUT}$ gliomas (G_T-CMG). Within G_T-CMG, we identified *SPINT2* as a top cancer-related candidate and demonstrated that *SPINT2* suppressed GBM via down-regulation of c-Met activation.

Keywords Glioblastoma · CpG island methylation · *SPINT2* · Tumor suppressor · HGF/c-Met

Introduction

Glioblastoma multiforme (GBM) is the most prevalent and most lethal form of brain cancer [1, 2], affecting 15,000 new patients yearly in the United States. Median survival

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with this type of cancer is 14.6 months, with only 3–5% of patients surviving over 5 years post-diagnosis [3]. This poor prognosis likely results from genetic and epigenetic influences, which vary between discrete subsets of patients [4, 5].

The silencing of endogenous tumor-suppressor genes by methylation of discrete CpG islands located within their promoter regions has been identified as a key process in gliomagenesis [1, 6, 7]. Following the recent discovery that somatic mutations to the isocitrate dehydrogenase 1 and 2 (*IDH1/2*) genes are present in a number of human cancers [8–14] and a majority of secondary glioblastomas [1, 15], genome-wide DNA methylation profiling of GBM and lower grade gliomas has identified several distinct methylation patient clusters, most notably the IDH^{MUT}-associated glioma CpG island methylator phenotype (G-CIMP) [5, 6, 16–18].

Enhanced c-Met activation via HGF has been reported to promote growth, angiogenesis, invasion, and stem cell survival in GBM [19–24]. Serine Protease Inhibitor, Kunitz Type 2 (SPINT2) is a major inhibitor of hepatocyte growth factor activator (HGFA). HGFA is the primary enzyme catalyzing the conversion of pro-HGF to the active c-Met ligand HGF [25, 26]. While *SPINT2* hypermethylation has been previously reported in several cancers [27–30], reports of *SPINT2* hypermethylation in GBM have been limited [30, 31].

By performing methylation profiling of patient glioma samples, we confirmed a large set of CpG islands coordinately methylated in both $IDH1^{WT}$ and $IDH1^{MUT}$ gliomas (abbreviated as G_T-CMG), which was potentially recognizable in other published methylomic datasets [5, 16, 17] but had yet to be clearly delineated. By applying unbiased bioinformatic criteria to G_T-CMG, we identified *SPINT2* as one of the top candidate tumor-suppressor genes that was hypermethylated and downregulated in $IDH1^{WT}$ GBMs. Furthermore, we confirmed that CpG island promoter methylation silenced *SPINT2*, and restoration of *SPINT2* suppressed growth and migration of GBM cells by downregulating c-Met activation. Thus, our data supports a clinically relevant model for c-Met activation in GBM, in which *SPINT2* methylation/downregulation releases the suppression of serine proteases such as HGFA on pro-HGF conversion and enables overactive c-Met activation.

Materials and methods

Details regarding cell cultures and pharmacological treatments, patient glioma specimens, methylation and expression data, in vitro and in vivo protocols and all data analyses are detailed in Online Resource 1_Supplemental Materials and Methods.

Results

G_T-CMG: a group of CpG islands coordinately methylated in both $IDH1^{WT}$ and $IDH1^{MUT}$ gliomas

In order to classify groups of hypermethylated islands in terms of *IDH1* genotype, we used our reduced representation bisulfite sequencing (RRBS) data to identify differentially methylated CpG islands depicted in a heatmap (Online Resource 2_Suppl. Figure 1a). First, as expected, gliomas demonstrated abundant hypermethylation as compared to normal brain. Instead of looking for methylation patient clusters (or CIMPs), we observed three sets of differentially methylated CpG islands based on whether they were methylated in $IDH1^{MUT}$, $IDH1^{WT}$, or both (Online Resource 2_Suppl. Figure 1a). We defined these groups as a Coordinately Methylated Group (CMG) of CpG islands in order to distinguish them from a CIMP. Thus, we designated: (1) Glioma-tumor-CMG (G_T-CMG) as the set containing CpG islands methylated in both $IDH1^{WT}$ and $IDH1^{MUT}$ gliomas; (2) Glioma-$IDH1^{MUT}$-CMG (G_M-CMG) as the set containing CpG islands methylated in $IDH1^{MUT}$ gliomas only; and (3) Glioma-$IDH1^{WT}$-CMG (G_W-CMG) as the set containing CpG islands methylated in $IDH1^{WT}$ gliomas only (Online Resource 3_Suppl. Table 1). G_T-CMG consisted of 1743 CpG islands exhibiting hypermethylation across both $IDH1^{WT}$ and $IDH1^{MUT}$ gliomas. G_M-CMG exhibited hypermethylation in only $IDH1^{MUT}$ samples and consisted of 1421 CpG islands, which as expected exhibited high overlap with G-CIMP in $IDH1^{MUT}$ versus $IDH1^{WT}$ GBMs, with 84.4% overlap (Online Resource 3_Suppl. Tables 2, 3). Representing a much smaller group, G_W-CMG consisted of 137 CpG islands hypermethylated in only $IDH1^{WT}$ samples (Online Resource 3_Suppl. Table 1).

In order to validate the CMG modules observed in our RRBS data in an independent dataset, methylation array data for 422 GBM samples (282 $IDH1^{WT}$, 27 $IDH1^{MUT}$, 113 unknown *IDH1*) obtained directly from TCGA (https://portal.gdc.cancer.gov/projects/TCGA-GBM) were similarly analyzed. This included 282 and 140 samples, generated via the Illumina Infinium Human Methylation 27 and 450 arrays, respectively. Because the TCGA GBM methylation data lacked normal brain samples, Illumina Infinium Human Methylation 450 platform data for 12 normal tissue samples were obtained from previously published work by Nardone et al. [32]. Similar to our RRBS results, comparisons of $IDH1^{WT}$ GBM, $IDH1^{MUT}$ GBM, and normal samples resulted in 3 distinct groups of CpG islands: G_T-CMG, with 3115 CpG islands; G_M-CMG, with 293 CpG islands; and G_W-CMG, with 210 CpG islands (Online Resource 2_Suppl. Figure 1b; Online Resource 3_Suppl.

Table 4). In addition to validating the presence of the three groups observed in the RRBS data, we also observed a small group of CpG islands that were hypomethylated in tumors versus normal.

We further validated our CMG classification by selecting 9 G_T-CMG and 2 G_W-CMG genes/CpG islands and performed targeted bisulfite sequencing (BiSeq) on patient GBM samples (Online Resource 4_Suppl. Table 5).

Identification of candidate tumor-suppressor genes within G_T-CMG by integrated analysis of expression and methylation

In order to identify candidate tumor suppressors within G_T-CMG, we applied bioinformatic filtering based on CpG island position within the gene and gene expression. Using genome annotation data downloaded directly from the UCSC genome browser (https://genome.ucsc.edu), we found 496 of 1743 G_T-CMG CpG islands overlapping with promoter regions of known RefSeq genes. Differential gene expression between 573 GBM samples and 10 normal brain samples was determined using TCGA GBM gene expression array data (Online Resource 2_Suppl. Figure 1b; Online Resource 4_Suppl. Table 6). Of the 496 promoter-associated CpG islands, this filter yielded 58 corresponding genes exhibiting down-regulation in a tumor versus normal comparison, with a minimum threefold change and $p < 1.0 \times 10^{-5}$. The top 10 genes, ranked by the significance of differential methylation, are shown in (Online Resource 4_Suppl. Table 7).

SPINT2 is one of the top cancer-related G_T-CMG candidates with down-regulated gene expression in GBM patient tumors and cell lines

Identified within the top 10 G_T-CMG genes (Online Resource 4_Suppl. Table 7), *SPINT2* is an upstream regulator of the HGF/c-Met pathway [33] whose silencing may result in overactive c-Met activation by HGF. The *SPINT2*-associated CpG island revealed differential methylation of 0.38 ($p = 8.3 \times 10^{-14}$) in gliomas as compared to normal samples in our RRBS screening (Online Resource 4_Suppl. Table 7). This is confirmed by inspection of RRBS data for *SPINT2* CpG islands for gliomas (Online Resource 2_Suppl. Figure 2) including GBMs (Fig. 1a). We further validated tumor hypermethylation of the *SPINT2* promoter associated CpG island using BiSeq, where we found hypermethylation in 47/74 $IDH1^{WT}$ GBM, 8/8 $IDH1^{MUT}$ GBM and 0/12 normal samples (Online Resource 4_Suppl. Table 5). As found from our filter, TCGA array data for 145 GBM (115 $IDH1^{WT}$, 30 $IDH1^{MUT}$) and 10 normal samples revealed 5.03-fold downregulation ($p = 6.5 \times 10^{-10}$) of *SPINT2* gene expression in GBMs (Online Resource 4_Suppl. Table 7), and the downregulation was associated with DNA hypermethylation (Fig. 1b). To confirm *SPINT2* down-regulation in GBMs, we measured *SPINT2* gene expression by qPCR in 10 normal brain tissues and 22 $IDH1^{WT}$ GBM samples and found *SPINT2* gene expression was significantly lower in the hypermethylated GBM samples, compared to unmethylated GBM samples (Fig. 1c). We did not test $IDH1^{MUT}$ samples since they all appear to be methylated. Interestingly, even unmethylated GBMs had lower expression of *SPINT2* compared to normal brain (Fig. 1b, c).

Silenced *SPINT2* can be re-expressed by pharmacological and genetic disruption of DNA methyltransferases

We determined methylation status and gene expression of *SPINT2* by BiSeq and qRT-PCR, respectively, in 6 GBM and 2 non-neoplastic cell lines (hTERT-immortalized astrocytes and HEK-293T cells). We found that *SPINT2* was hypermethylated in all tested GBM cell lines and unmethylated in the 2 non-malignant cell lines. As expected, *SPINT2* was highly expressed in the 2 non-malignant cell lines but significantly downregulated in GBM cell lines. In addition, *SPINT2* was hypermethylated in 7 patient-derived GBM neurosphere lines and all 7 lines demonstrated silenced *SPINT2* gene expression (Fig. 2a).

To investigate whether methylation was responsible for silenced *SPINT2* expression, we treated GBM cell lines (U251, T98G, LN18) harboring *SPINT2* hypermethylation with 5-aza-2′-deoxycytidine (5-aza-CdR), a pharmacological demethylating agent. The treatment resulted in a substantial increase (> 100-fold change) in *SPINT2* mRNA expression as compared to non-treated cells (Fig. 2b). Interestingly, NHA cells also showed a modest increase in expression. Moreover, we found that increased *SPINT2* expression was dose-dependent (Online Resource 2_Suppl. Figure 3a). As expected, SPINT2 protein levels were also upregulated in a dose-dependent manner after 3 days of treatment with 5-aza-CdR (Fig. 2c). To provide further evidence, we treated U251 cells with a low dose of 5-aza-CdR for 9 days, and found that *SPINT2* expression was greatly increased (Online Resource 2_Suppl. Figure 3b), and that this increase was associated with demethylation of the *SPINT2* promoter (Online Resource 2_Suppl. Figure 3c). In addition to pharmacological treatment, we conducted siRNA-based knockdown of *DNA (Cytosine-5)-Methyltransferase 1* (*DNMT1*) in LN18 cells and observed a threefold increase in the expression of *SPINT2* as compared to nonspecific siRNA-treated cells (Fig. 2d). Taken together, our in vitro data combined with clinical sample data strongly suggests that *SPINT2* gene expression is regulated by promoter CpG island methylation.

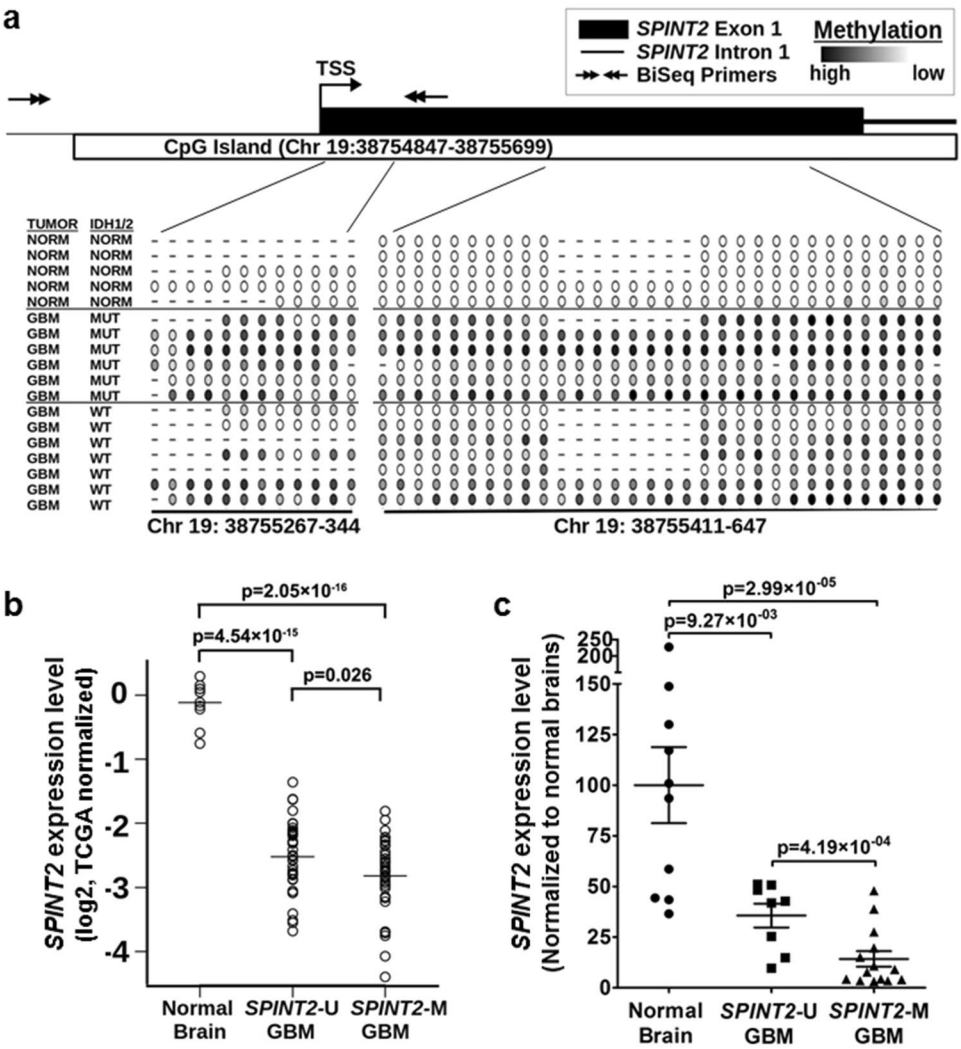

Fig. 1 *SPINT2* is hypermethylated and downregulated in GBMs compared to normal brain tissues. **a** Methylation profile of the *SPINT2* associated CpG island via RRBS. Upper: map of the *SPINT2* promoter region, showing position of CpG island, transcription start site (arrow), exon 1 (shaded box) and BiSeq primers (double headed arrows, Chr 19: 38,754,739–38,755,328); Lower: representative CpG site methylation pattern of $IDH1^{WT}$ gliomas or $IDH1^{MUT}$ GBMs and normal brain determined by RRBS. **b** Analysis of TCGA data demonstrates that *SPINT2* expression was down-regulated in *SPINT2* hypermethylated versus unmethylated GBMs, $p = 0.026$. **c** In our set of GBM samples, *SPINT2* expression measured by qPCR was also down-regulated in *SPINT2* hypermethylated versus unmethylated GBMs as determined by targeted BiSeq. $p = 4.19 \times 10^{-4}$

SPINT2 promoter activity is regulated by DNA methylation

To directly demonstrate regulation of *SPINT2* promoter activity by methylation, we generated 4 reporter constructs by inserting the *SPINT2* promoter and 3 control promoters (*CMV, SV40, HSV TK*) into the promoter free pGL4.17 reporter plasmid. The luciferase assay showed that *SPINT2* promoter activity was comparable to the control promoters, indicating that the *SPINT2* promoter was highly active in driving transcription (Online Resource 2_Suppl. Figure 4a). Transfection of NHA cells also demonstrated that the *SPINT2* promoter was highly active (Online Resource 2_Suppl. Figure 4b).

To determine the role of methylation in regulation of *SPINT2* promoter activity, we treated the three plasmid constructs in vitro with *HhaI* methyltransferase, which specifically methylates the first cytosine of GCGC DNA sites. We used the *SV40* promoter construct, lacking GCGC sites, as a negative control for methylation-dependent expression regulation; we used the *TK* promoter construct, containing multiple GCGC sites, as a positive control (Online Resource 2_Suppl. Figure 5a). Treated constructs were then transfected into 293T and NHA cells. As expected, *HhaI* methylation modification did not alter *SV40* promoter activity. In contrast, *HhaI* methylation dramatically reduced *SPINT2* and *TK* promoter activities in NHA and 293T cells (Fig. 2e and Online Resource 2_Suppl. Figure 5b). These results demonstrated direct regulation of *SPINT2* transcription via promoter methylation.

SPINT2 exerts tumor-suppressive properties in GBM cell lines

To determine whether *SPINT2* could exert tumor suppression, we achieved stable *SPINT2* overexpression by retroviral infection (pLPCX, Clontech) in LN18 and U87 cells. These cell lines were selected because both demonstrated

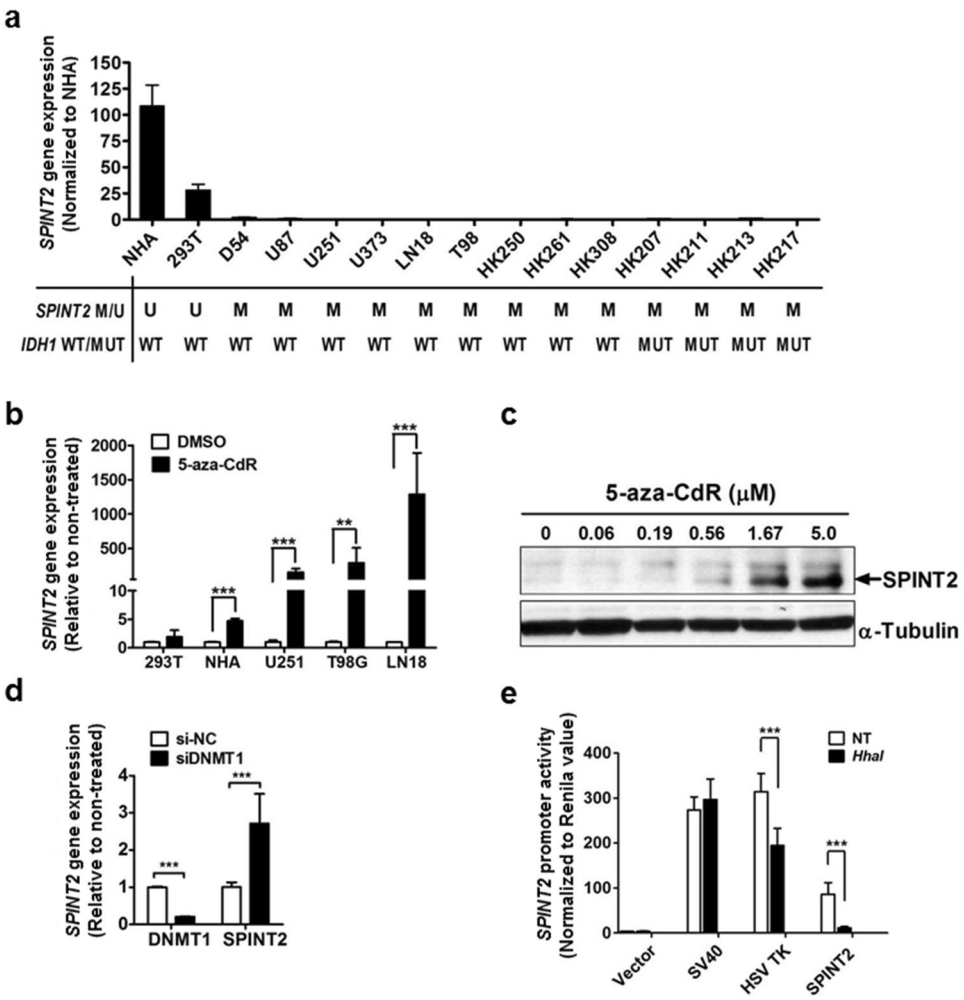

Fig. 2 *SPINT2* expression is regulated by promoter methylation. **a** *SPINT2* was downregulated in promoter-methylated GBM cell lines and GBM neurosphere lines measured by qPCR. NHA and 293T are non-malignant cell lines; D54, U87, U251, U373, LN18 and T98 are GBM cell lines; HK207, HK250, HK261, HK308, HK211, HK213 and HK217 are GBM patient-derived neurospheres. **b** *SPINT2* expression was upregulated in hypermethylated cell lines by treatment with demethylating agent 5-aza-CdR. *SPINT2* expression was measured by qPCR in 293T, NHA, U251, T98G and LN18 cells treated with DMSO or 5-aza-CdR for 72 h (n = 3, mean ± SEM). **c** SPINT2 protein was upregulated in LN18 cells treated with 5-aza-CdR for 72 h (n = 3, representative figure shown). **d** *DNMT1*-siRNA transfection, three times every 2 days, achieved 80% downregulation of *DNMT1*, while *SPINT2* gene expression was upregulated around threefold in LN18 cells (n = 2, mean ± SEM). **e** *SPINT2* promoter activity was decreased by DNA methylation modification by *HhaI* in NHA cells. *SV40* promoter, lacking "GCGC" sites was used as a negative control. *HSV TK* promoter, containing multiple "GCGC" sites, was used as a positive control. The *Renilla* luciferase vector was used as an internal control. *SPINT2* promoter activity was measured as the mean value of Firefly/Renilla luciferase activity (n = 3, mean ± SEM). *p < 0.05; **p < 0.01; ***p < 0.001

simultaneous expression of *HGF* and *c-Met* (Online Resource 2_Suppl. Figure 6). We confirmed *SPINT2* overexpression by Western blot (Fig. 3a). As compared to pLPCX-control cells, Transwell invasion assays (Fig. 3b) showed that *SPINT2* overexpression strongly arrested cell invasion of LN18 cells. In addition, *SPINT2* overexpression clearly inhibited cell migration as measured by the wound healing assay in LN18 cells (Fig. 3c). *SPINT2* overexpression significantly reduced cell proliferation as measured by the MTT assay both in LN18 (Fig. 3d) and U87 cells (Fig. 3e). *SPINT2* overexpression also significantly reduced cell colony formation as measured by the colony growth assay in LN18 (Fig. 3f) and U87 cells (Fig. 3g), and anchorage-independent growth as demonstrated by soft agar growth assays in LN18 (Fig. 3h) and U87 cells (Fig. 3i). By showing reduced cell invasion, migration, and proliferation, these assays demonstrate the tumor-suppressive properties of *SPINT2* overexpression in vitro.

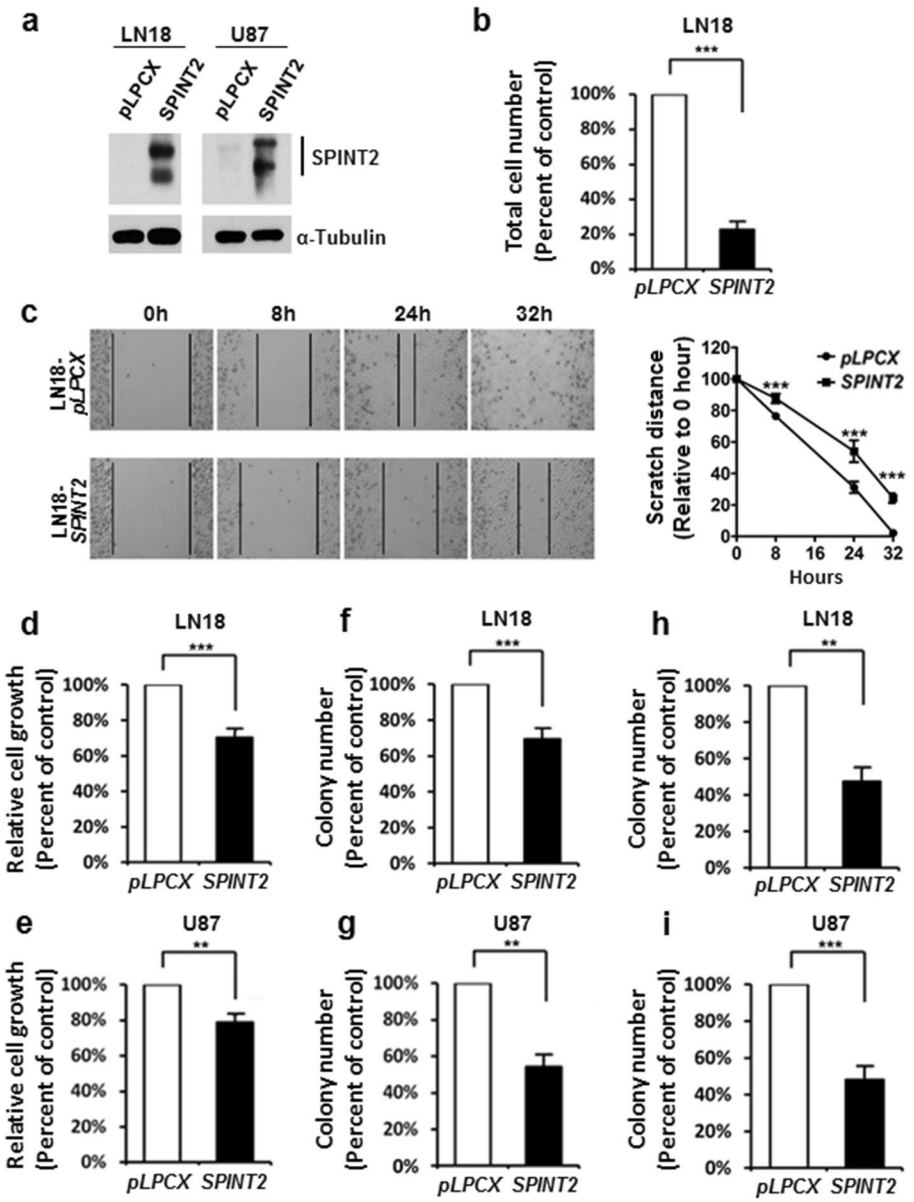

Fig. 3 *SPINT2* overexpression blocks glioma cell invasion, migration and proliferation. **a** Stable overexpression of *SPINT2* in LN18 and U87 cell lines was achieved by retroviral infection and puromycin selection. **b** *SPINT2* overexpression reduced LN18 cell invasion in the Transwell migration assay (n = 6, mean ± SEM). **c** *SPINT2* overexpression inhibited the migration of LN18 cells in the wound healing assay (n = 6, mean ± SEM). **d, e** *SPINT2* overexpression reduced cell proliferation (MTT assay) in LN18 (n = 8) and U87 (n = 3) cells (mean ± SEM). **f, g** *SPINT2* overexpression reduced colony formation in LN18 (n = 7) and U87 (n = 3) cells (mean ± SEM). **h, i** *SPINT2* overexpression reduced anchorage-independent growth in LN18 (n = 3) and U87 (n = 4) cells (mean ± SEM). *p < 0.05; **p < 0.01; ***p < 0.001

Conditioned medium from *SPINT2*-overexpressing cells (SPINT2-CM) and recombinant SPINT2 (rSPINT2) application reduce c-Met phosphorylation and growth in GBM cell lines

Based on previous reports that secreted SPINT2 can inhibit HGFA-mediated conversion of pro-HGF to active HGF [33, 34], we tested whether GBM cells shed SPINT2 proteins extracellularly by harvesting starvation-derived conditioned medium (CM) from LN18 cells (FBS-free, 24 h starvation). Western blot analysis of concentrated proteins (molecular weight ≥ 10 kD) demonstrated the presence of SPINT2 protein (~ 30 kD) in the conditioned medium (Fig. 4a). In comparison to protein extracts of LN18 cells, concentrated CM proteins, as expected, did not contain α-tubulin protein, excluding the possibility that conditioned medium was contaminated with cell-derived SPINT2 (Fig. 4a). By applying starvation-derived CM from LN18-*SPINT2* cells (SPINT2-CM) to LN18-*pLPCX* cells, we found that SPINT2-CM inhibited c-Met phosphorylation in LN18-*pLPCX* cells in the presence of FBS/HGF (Fig. 4b) (note that serum provides bovine HGFA [26, 28]). SPINT2-CM treated LN18-*pLPCX* cells (Fig. 4c) and U87-*pLPCX* cells (Fig. 4d) showed reduced growth compared with pLPCX-CM treated cells. SPINT2-CM treated LN18-*pLPCX* cells also showed reduced cell invasion in the Transwell migration assay (Fig. 4e). Similarly, recombinant SPINT2 (rSPINT2) was found to reduce c-Met phosphoryation compared with vehicle to a level approaching that of the c-Met receptor inhibitor, iMet (Fig. 4f, upper panel). As expected, treatment

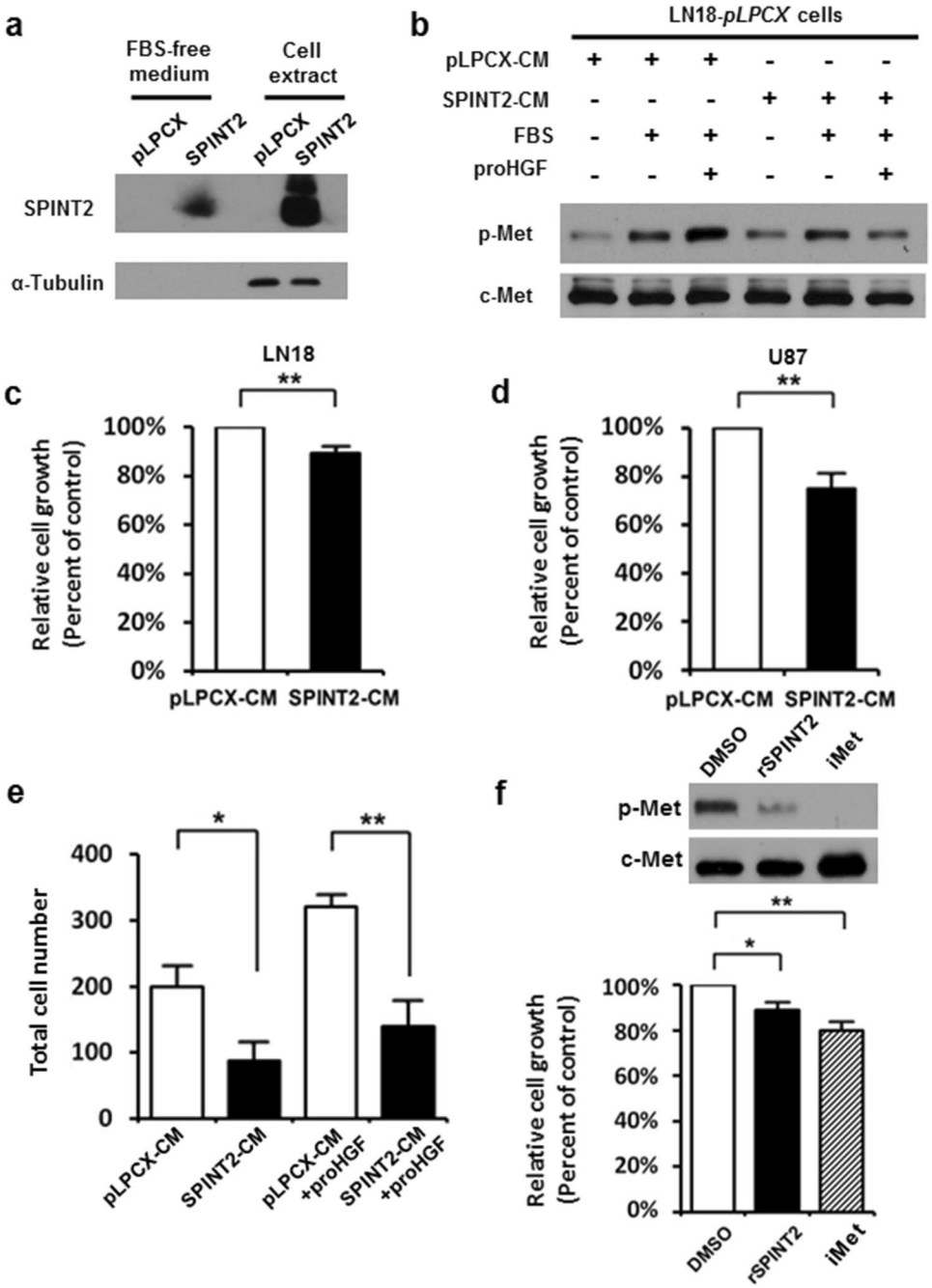

Fig. 4 SPINT2 protein diminishes c-Met activation, cell proliferation and invasion in LN18 cells. **a** Starvation-derived conditioned medium of LN18-*SPINT2* cells contained SPINT2 protein indicating shedding of soluble SPINT2 into medium by glioma cells (n = 4, representative figure shown). **b** Conditioned medium from LN18-*SPINT2* cells suppressed c-Met activation in LN18-*pLPCX* cells. pLPCX-CM, serum-starvation-conditioned medium from LN18-*pLPCX* cells; SPINT2-CM, serum-starvation-conditioned medium from LN18-*SPINT2* cells (n = 3, representative figure shown). **c** Conditioned medium from LN18-*SPINT2* cells suppressed cell proliferation of LN18-*pLPCX* cells (n = 3, mean ± SEM). **d** Conditioned medium from LN18-*SPINT2* cells suppressed cell proliferation of U87-*pLPCX* cells (n = 4, mean ± SEM). **e** Conditioned medium from LN18-*SPINT2* cells suppressed cell invasion of LN18-*pLPCX* cells in the Transwell migration assay (n = 3, mean ± SEM). **f** Treatment with rSPINT2 suppressed c-Met activation in LN18 cells (upper panel; n = 3, representative figure shown). Treatment with rSPINT2 suppressed cell proliferation in LN18 cells measured by MTT assay (lower panel; n = 3, mean ± SEM). *p < 0.05; **p < 0.01; ***p < 0.001

with rSPINT2 also significantly reduced LN18 cell growth compared with vehicle-treated cells, and comparably with iMet (Fig. 4f, lower panel). A reduction in c-Met activation was also noted in SPINT2-CM treated *SPINT2*-overexpressing LN18 cells (Online Resource 2_Suppl. Figure 7).

SPINT2 overexpression inhibits tumor growth in vivo

To determine whether *SPINT2* overexpression affects GBM growth and influences survival in vivo, we xenografted U87 cells intracranially into 12 NSG mice (6 U87-*SPINT2*, 6 U87-*pLPCX*) and assessed tumor burden using overall survival (OS) as the primary endpoint. Using Kaplan–Meier analysis, we found that the median OS of mice injected with U87-*SPINT2* cells (20 days) exceeded those of mice injected with U87-*pLPCX* cells (15.5 days; log-rank, p = 0.0016; Fig. 5a). Intracranially xenografted LN-18 cells failed to generate tumors (data not shown). We then xenografted 10^7 LN18 cells into the flank of 10 NSG mice (5 LN18-*SPINT2*, 5 LN18-*pLPCX*), and found that 2 out of 5 mice from the LN18-*SPINT2* group grew tumors as compared to

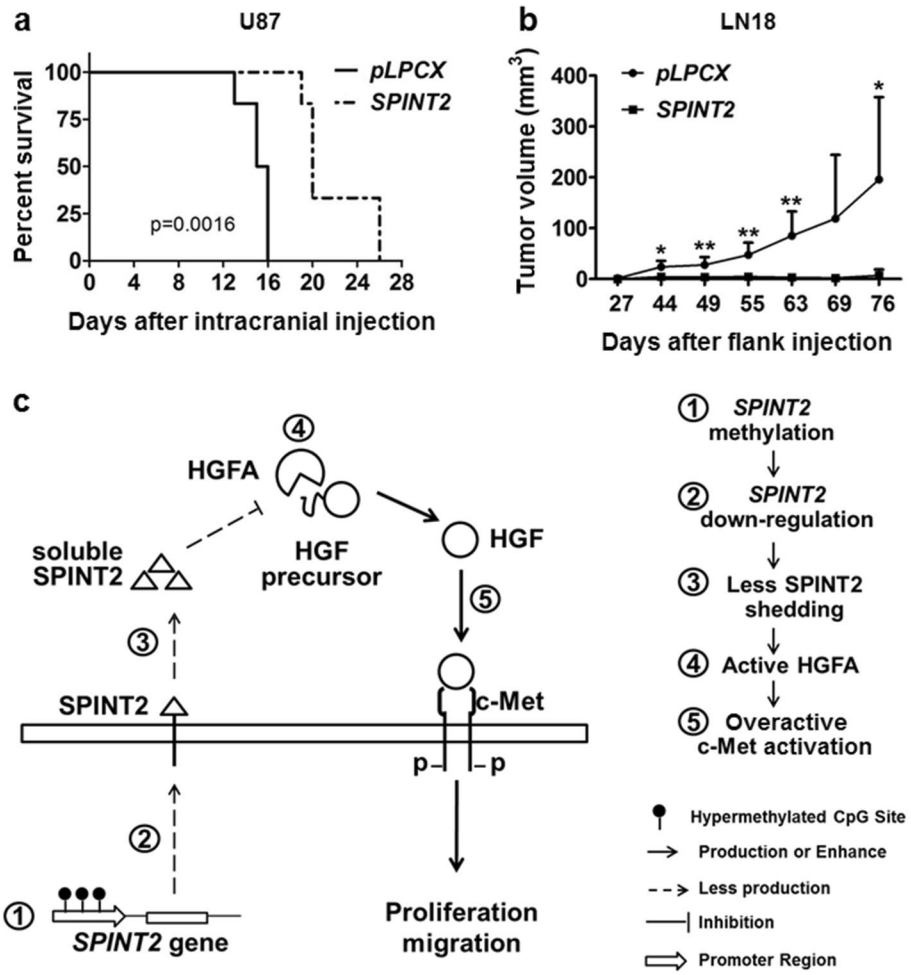

Fig. 5 *SPINT2* overexpression diminishes tumor growth in vivo and prolonged survival of mice with intracranial transplantation. **a** *SPINT2* overexpression prolonged the survival of NSG mice. U87 cells were intracranially transplanted into NSG mice. Tumor burden was assessed by overall survival (OS) analysis (Kaplan–Meier analysis). The median OS of mice injected with U87-*pLPCX* control cells was 15.5 days, the median OS of mice injected with U87-*SPINT2* cells was 20 days, p = 0.0016 (log-rank; n = 6). **b** *SPINT2* overexpression inhibited tumor formation and growth in mice transplanted subcutaneously with LN18 cells. Tumor size was measured with a caliper (n = 5). **c** Schematic model summarizing the impact of *SPINT2* methylation on the regulation of c-Met in GBM. *SPINT2* promoter methylation downregulates *SPINT2* expression. As less SPINT2 is available to inhibit HGFA, HGFA maximally converts pro-HGF into HGF which then activates c-Met in GBM cells. *p < 0.05; **p < 0.01; ***p < 0.001

the 5 out of 5 mice from control LN18-*pLPCX* group (data not shown). LN18-*SPINT2* cells formed smaller tumors (n = 5) as compared to the control mice as assessed over 76 days post-transplantation (Fig. 5b). The two LN18-*SPINT2* tumors also displayed a reduced rate of growth (data not shown).

Conclusions

In this study, we defined and confirmed a group of coordinately methylated CpG islands (G_T-CMG) common to both *IDH1*^{WT} and *IDH1*^{MUT} gliomas including GBMs. Within G_T-CMG, we identified *SPINT2* as a top cancer-related candidate and demonstrated that *SPINT2* exerts tumor-suppressive properties in GBM via inhibition of c-Met activation. The value of defining G_T-CMG is to acknowledge a group of genes that has been overlooked in the G-CIMP paradigm. Recognition of G_T-CMG contributes an orthogonal view of the CpG island methylation landscape of gliomas [5, 16–18, 35], ultimately to enable a better understanding of the generation of aberrant methylation and its subsequent contribution to glioma formation and progression. For example, it has recently been noted that a subset of *IDH1*^{MUT} tumors more closely resemble the methylation profile of *IDH1*^{WT} at recurrence [36]. The existence of G_T-CMG strongly suggests that aberrant hypermethylation in gliomas can occur via multiple mechanisms, possibly separated into *IDH1*^{MUT}-dependent and -independent mechanisms. Comparison of methylation at specific CpG sites for G_T-CMG CpG islands in the context of *IDH1*^{WT} and *IDH1*^{MUT} gliomas may provide important insight into these mechanisms.

Through multiple lines of evidence, we have demonstrated that *SPINT2* gene expression is suppressed by methylation of its associated CpG island. Moreover, through a series of in vitro and in vivo experiments, we show that overexpression of *SPINT2* exerts tumor suppression in GBM cell lines. These results corroborate similar findings previously reported in GBM cells [30, 31]. In our study, we demonstrated that cell surface or soluble *SPINT2* suppressed the proliferation and invasion of tumor cells via downregulation of c-Met activation.

SPINT2 has been found to be hypermethylated in patient tumors from several other cancer types [27, 29, 37].

Evidence for *SPINT2* hypermethylation is more limited in the context of GBM patient tumors [30, 31]. Kongkham and colleagues [37] identified *SPINT2* hypermethylation and associated reductions in *SPINT2* mRNA expression in medulloblastoma cell lines, and demonstrated reductions in cell growth and motility following forced *SPINT2* re-expression in these cell lines. They also demonstrated increased overall survival in *SPINT2*-overexpressing murine medulloblastoma xenografts. Lee and colleagues [31] examined *SPINT2* promoter hypermethylation in GBMs and GSC lines and observed associated reductions in *SPINT2* mRNA expression in GSC lines; they similarly demonstrated reduced cell growth and migration following forced *SPINT2* expression in U87 cells. A study published as this manuscript was being prepared also identified hypermethylation of the *SPINT2* gene in human glioma-derived cell lines and high-grade glioma tissue samples [38].

The overall clinical significance of our findings is in the apparent intersection with the HGF/c-Met pathway. In GBM, although c-*Met* mutations only occur in 1.6% of GBM patients [5], *HGF* and c-*Met* are frequently overexpressed, and one-third of patients simultaneously overexpress both *HGF* and c-*Met*. This provides further evidence corroborating other recent work suggesting that HGF/c-Met signaling is important in GBM [16, 17, 20, 39–41]. The downstream effectors of the c-Met pathway have been well-characterized in prior studies [42–45]. In preclinical models, inhibition of c-Met or downregulation of HGF and c-Met can suppress GBM growth [22, 46], and targeted c-Met therapies have been tested in three phase II clinical trials for adult GBM [47, 48]. However, none of these trials has demonstrated clear clinical benefit, possibly due to the inability to predict those patients most likely to benefit from c-Met targeted therapy. In this regard, our data suggest that *SPINT2* methylation may be associated with c-Met activation and serve as a biomarker indicative of c-Met activation in GBM.

In conclusion, this study defines a set of hypermethylated genes, G_T-CMG, that contains a potential therapeutic target, *SPINT2*. Based on our data, we formulate a model in which *SPINT2* downregulation may promote GBM progression via unregulated c-Met activation by HGF (Fig. 5c). This model suggests that targeting c-Met activation via blocking HGFA-mediated activation of HGF is potentially an effective strategy to treat GBM with hypermethylated *SPINT2*.

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Compliance with ethical standards

Conflict of interest Author Byram Ozer has received an honorarium for a one-time consultation with Neurocore, 2015. Author Timothy Cloughesy reports personal fees from Pfizer, Tocagen, Roche, Novocure, Nektar, VBL, ABBVIE, Upshire Smith, Notable Labs, Oxigene, NewGen, Agios, Cortice, MedQia, PRoNai, Wellcome Trust, Merck, Insys, Human Longevity, Sunovion, Boston Biomedical, Alexion, Novogen, VBI, BMS, GW Pharma, Bioclinica, Amgen outside the submitted work. Dr. Cloughesy is a board member of the Global Coalition for Adaptive Research 501c3 and the PI for GBM Agile. Dr. Cloughesy has stock options for Notable Labs. Author Albert Lai has received Honoraria from Merck, Genentech, Abbvie, and Optune.

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