SETDB1 Promotes the Growth of Glioma via CSF-1-dependent Macrophage Recruitment by Activating the AKT/mTOR Signaling Pathway

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Research

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Abstract

**Background:** Glioma is a common disease of the central nervous system (CNS), with high morbidity and mortality. Among the infiltrates in the tumor microenvironment, tumor-associated macrophages (TAMs) are abundant and they are significant factors in glioma progression. However, the exact details of disease progression have yet to be determined.

**Methods:** The clinical relevance of SETDB1 was analyzed by immunohistochemistry, real-time PCR and Western blotting and of glioma cancer tissues. Tumor cell proliferation, migration and invasion were investigated by MTS assay, colony formation assay, xenograft, wound healing and Transwell assay. The relationship between SETDB1 and CSF-1, as well as TAMS was examined by Western blotting, real-time PCR and syngeneic mouse model.

**Results:** This work shows the presence and upregulation of SETDB1 in glioma and its relationship with disease prognosis. Gain and loss of function approaches showed the inhibition of apoptosis and the promotion of growth, migration and invasion of the disease with SETDB1 overexpression and converse effects with SETDB1 silencing *in vitro*. Mechanistically, SETDB1 promotes CSF-1 expression by activating the AKT/mTOR signaling pathway. This leads to macrophage recruitment in the tumor, leading to tumor growth.

**Conclusion:** This study clarifies the modulation of tumor functions by SETDB1 and hence presents a future avenue for treating glioma.

Introduction

Glioma is a tumor type that derived from glial cells, with a high incidence, high recurrence rate, and poor prognosis[1]. Previous research has demonstrated that gliomas account for 47.1% of primary malignant brain and other central nervous system tumors, of which glioblastoma is the main type of gliomas, accounting for about 56.1% of cases[2, 3]. The treatment process for glioma includes surgery followed by radiotherapy, with or without temozolomide (TMZ) chemotherapy[4, 5].

Tumor progression and metastasis involve the several stromal cells that are not malignant, such as tumor-associated macrophages (TAMs) of the hematopoietic lineage, which are abundant migratory cells[6, 7]. The density of these cells has been shown to be related to the prognosis of several types of cancer, such as glioma[8, 9]. The heterogeneous nature of these cells and their ability to show different responses to cues from the environment is indicative of their roles in cancer progression[10, 11]. This microenvironment is modulated by the chemokine profile at the tumor site, as this influences macrophage differentiation to hence affect the advancement of glioma[12, 13]. Among these chemokines, colony stimulating factor 1 (CSF-1) is a ubiquitously produced factor seen in many tumors (including glioma) that is vital for metastasis[14]. This factor causes the recruitment of TAMs and other cell subsets to influence the processes of inflammation, angiogenesis, proliferation and evasion of the immune
The use of anti-CSF-1 antibodies has been shown to decrease the *in vivo* tumor burden by 96%, according to preclinical cancer models[17].

SETDB1 (SET domain bifurcated 1) is encoded by the approximately 38.6 kb long *SETDB1* gene located on human chromosome 1q21.3[18]. This protein is a member of the methyltransferase family of SET-domains (Su(var)3-9, E(z), Trithorax) that function by silencing genes or inhibiting transcription via H3K9 trimethylation[19]. SETDB1 is linked to embryonic development and is also a candidate for early Huntington disease therapy[20]. Recently, research pointed out that SETDB1 is expressed at abnormal and high levels in melanoma, ovarian cancer, lung cancer, and breast cancer[20-24]. The involvement and function of SETDB1 in glioma have yet to be well studied, which calls for studies in this direction.

In the current study, we identified that SETDB1 was markedly upregulated in glioma and displayed a significant association with the clinicopathological characteristics and survival of glioma patients. Overexpressing SETDB1 boosted the transcription of CSF-1 by activating the AKT/mTOR signaling pathway. Furthermore, SETDB1 induced CSF-1 expression in glioma cells leading to the recruitment of TAMs and subsequent tumor growth. These finding indicate the role of SETDB1 in both oncogenesis and TAM recruitment in glioma pathogenesis.

**Material And Methods**

**Cell lines and culture**

American Type Culture Collection (ATCC, Manassas, VA, USA) was the source of glioma cell lines: U87, U251, H4, A172, U118, LN229, SHG-44 and GL261. Cell culture was performed in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (HyClone, Thermo, USA) at 37°C and 5% CO₂. NHAs (Normal human astrocytes) were procured from Lonza (Switzerland) and cultured as per supplied instructions.

**Human glioma samples**

Glioma tissues (n = 40) and neighboring healthy tissues (n = 40) were surgically excised at The People’s Hospital of China Medical University and the First Hospital of China Medical University. Table 1 displays the clinicopathological features of the patients. Regular follow-up was performed for patients along with informed consent. Other data included relapse-free survival and overall survival (OS). This work received approval from the ethics committee of The People’s Hospital of China Medical University and the First Hospital of China Medical University.

**Immunohistochemistry (IHC)**

In accordance with previous works[25, 26], staining of tissues was performed with a SETDB1 antibody (Sigma-Aldrich, USA). The scoring of cells was performed in accordance with the following guide: 0 (no positive staining); 1 (1 to 25% positive cells); 2 (26 to 50% positive cells); 3 (51 to 75% positive cells); and
4 (> 75% positive cells). The scoring for staining intensity was performed as follows: 0, negative; 1, weak; 2, moderate; and 3, high. The formula for SETDB1-positive cells was as follows: staining intensity score × percentage. This score involved both the nucleus and cytoplasm and was analyzed by two pathologists.

**Cell migration and invasion assay**

The migration assay used Falcon cell culture inserts from BD (Franklin, USA). The invasion assay utilized a BioCoat™ Matrigel™ Invasion Chamber (BD) in accordance with the instructions of the manufacturer. A phase contrast microscope was utilized to count cells; the average of 5 various fields/well was considered.

**MTS analysis**

A 96-well plate seeded with cells at 5000 cells/well. AMultiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific) was employed to measure the absorbance at 570 nm for three independent assays.

**RNA extraction and real-time PCR**

Real-time PCR was performed as previously described[27, 28]. Briefly, TRizol (Invitrogen, USA) was utilized for the extraction of total RNA. cDNA was synthesized with thePrimeScript™ RT reagent kit (Takara, Dalian) in accordance with the instructions of the manufacturer. SYBR Premix ExTaq II (Takara, Dalian) was utilized for real-time PCR of this cDNA on an ABI PRISM 7300 (Applied Biosystems, USA) to analyze the chemokines of the immune system. GAPDH was used to normalize mRNA levels. The primers used are listed in Table 2.

**Western blotting**

Western blotting was performed as previously described[29, 30]. Briefly, cell lysis was performed with the RIPA buffer protein extraction reagent (Pierce, Rockford, IL, USA) containing a protease inhibitor cocktail (Roche, USA). The proteins were resolved by 10% SDS-PAGE followed by transfer to polyvinylidene fluoride (PVDF) membranes (Sigma-Aldrich). Next, the membranes were blocked using 5% bovine serum albumin (BSA) and incubated with primary antibodies at 4°C overnight. Appropriate secondary antibodies were later added and then visualized by using an ECL chemiluminescence kit. The primary antibodies used are listed as follows: SETDB1 (HPA018142, Sigma-Aldrich), cleaved caspase 3 (9661, Cell signaling technology), cleaved caspase 8 (9748, Cell signaling technology), slug (9585, Cell signaling technology), vimentin (5741, Cell signaling technology), E-cadherin (14472, Cell signaling technology), mTOR (2983, Cell signaling technology), p-mTOR (5536, Cell signaling technology), AKT (4685, Cell signaling technology), p-AKT (4060, Cell signaling technology), CSF-1 (3155, Cell signaling technology), β-actin (3700, Cell signaling technology).

**Cell isolation and differentiation**
The preparation of human monocytes from buffy coats of healthy volunteers was performed using Ficoll-Hypaque (Pharmacia Corporation) for 50 min at 400 g. Twenty-four-well plates were seeded with $2 \times 10^6$ cells/mL in RPMI 1640 medium containing 10% heat inactivated human AB serum, 50 U of penicillin/mL, 50 U of streptomycin/mL, 2 mM L-glutamine, and 100 ng/mL human M-CSF (which allows differentiation into macrophages). Warm medium was used to gently wash away non-adherent cells 6 days post-culture. CD14$^+$ macrophages were found to account for greater than 95% of the adherent cells. The activation of these monocytes to macrophages in vitro involved the treatment of $2 \times 10^6$ cells/L with 25 $\mu$g/mL lipopolysaccharide (LPS, Sigma-Aldrich) to produce M1-polarized macrophages and 45 ng/mL recombinant human interleukin-4 (IL-4; R&D) to produce M2-polarized macrophages. Flow cytometry was employed to detect the formation of macrophages. For the following in vitro assays, cells were cultured for 24 hours with RPMI media minus supplements and meticulously washed with PBS prior to the experiments.

**Clone formation assay**

Six-well plates were used to plate single-cell suspensions at a density of 1,000 cells per plate. Every 3 days, the culture medium was replaced. After allowing 2 weeks for clone formation, fixation and staining of the clones was performed using 0.1% crystal violet/40% methanol. Microscopy was performed to count colonies with >50 cells/colony.

**Animal experiments**

The syngeneic glioma mouse model was generated in accordance with previous reports[31, 32]. Briefly, 2% isoflurane in O$_2$ was used to sedate C57BL/6J mice (4-6 weeks old). the addition of $5 \times 10^5$ GL261-EV and GL261-SETDB1 cells in 100 $\mu$L of PBS was performed followed by subcutaneous injection in the flanks of C57BL/6J mice. The mice were sacrificed after 6 weeks and tumor weights were assessed.

For the subcutaneous model, the addition of $5 \times 10^5$ U251-EV, U251-SETDB1, U87-shCon or sh SETDB1 cells in 100 $\mu$L of PBS was performed followed by subcutaneous injection in the flanks of nude mice. The mice were sacrificed 15 days and tumor weights were assessed. The mice were kept at the Mouse Experimentation Core premises of the China Medical University.

**Statistical analysis**

The mean ± standard deviation (SD) was used to represent the data of triplicate assays. Student's t-test was applied to assess significant differences between groups. Repeated measures analysis of variance was performed to assess variations between tumor parameters (growth rate and cell growth) of the animals.

**Results**

**SETDB1 is frequently overexpressed and predicts poor glioma prognosis**
To investigate the role of SETDB1 in glioma cells, the level of SETDB1 was assessed in glioma patients. IHC and real-time PCR results demonstrated that SETDB1 was evidently increased in tumor samples compared with surrounding healthy tissues (Figure 1A and 1B). Next, real-time PCR was employed to assess the expression of SETDB1 mRNA in cell lines. Higher levels of SETDB1 were detected in the glioma lines in comparison with NHAs (Figure 1C). In addition, Western blotting showed that SETDB1 levels were higher in glioma cells than in NHAs cells (Figure 1D).

This was followed by studying the effect of this SETDB1 overexpression in the clinic. Increased mRNA expression of SETDB1 was evidently linked to shorter relapse-free glioma survival, as shown by Kaplan-Meier curves (Figure 1E). Our findings indicate that age and sex had no association with SETDB1 expression and TNM stage were found to be linked with the protein expression (Table 1). SETDB1 was also found to serve as an independent factor for prognosis and shorter relapse-free survival, as shown by the multivariate Cox regression analysis (Table 3). Overall, high expression of SETDB1 was found to serve as a prognostic factor for glioma.

**SETDB1 boosts the growth of glioma and inhibits apoptosis**

The examination of the biological functions of SETDB1 involved overexpressing the protein using cDNA in or silencing it using SETDB1-specific small hairpin RNAs (shSETDB1) using lentiviral vectors in U87 and U251 cells. Lentiviruses with empty vector (EV) or negative control shRNA (shCon) served as controls in the transduction assays. The following changes in expression were assessed using real-time PCR and Western blotting (Figure 2A, 2B, S1A and S1B). Increased SETDB1 expression caused an evident increase in viability and clone formation in the cell lines studied compared with those in the controls (Figure 2C and 2D). SETDB1 silencing caused the opposite results: decreased cell division and clone formation (Figure S1C-S1F). Next, the effect of this ectopic expression on apoptosis was studied; increased expression of SETDB1 noticeably reduced the levels of the active (cleaved) forms of caspase-8 and caspase-3 and hence decreased apoptosis in the cell lines mentioned previously in the presence or absence of TRAIL (Figure 2E). The opposite results were seen when silencing SETDB1, as indicated by increased sensitivity to TRAIL treatment and increased apoptosis and expression of its markers in U251 cells (Figure S1G).

Further investigation involved the subcutaneous injection of U251-EV and U251-SETDB1 into nude mice. This ectopic SETDB1 expression caused a conspicuous growth increase in tumors in the animals (Figure 2F). SETDB1 overexpression was confirmed by Western blotting (Figure 2G). Ki-67 assays showed that U251-SETDB1 xenografts displayed increased numbers of proliferating cells (Figure 2H). However, knockdown of SETDB1 caused an evident decrease in xenograft size (Figure S1H and S1I). Markedly decreased cell proliferation was observed in U87-shSETDB1 xenografts according to Ki-67 staining results (Figure S1J). Overall, SETDB1 is vital in the oncogenesis and advancement of glioma.

**SETDB1 increases migration and invasion in glioma cells**

Assays to assess the abilities of cells to migrate and invade were performed to examine the metastasis potential in vitro. The ability of cells to migrate and invade was augmented in the presence of ectopic
SETDB1 expression in the U87 and U251 cell lines compared with the control (Figure 3A and 3C). Upregulation of Slug and Vimentin (mesenchymal markers) and downregulation of E-cadherin (an epithelial marker) were shown by Western blotting (Figure 3E). The opposite results were observed for the same cell lines that were subjected to silencing of SETDB1, which decreased the ability of the cells to invade (Figure 3B and 3D), decreased Slug and Vimentin and increased E-cadherin (Figure 3F). The above data suggest regulation of glioma metastasis by SETDB1.

**Increased SETDB1 promotes CSF-1 secretion from glioma**

The levels of cytokines and chemokines CSF-1, TGF-β, IL-4, IL-13, and VEGF involved in the infiltration of TAMs were assessed through real-time PCR and enzyme-linked immunosorbent assay (ELISA) for SETDB1 in glioma lines. U87 and U251 glioma cells displayed elevated profiles of CSF-1 mRNA and proteins in the presence of SETDB1 overexpression (Figure 4A, 4B and 4E), while knockdown caused the opposite effects (Figure 4C, 4D and 4F). As expected, CSF-1 expression significantly correlated with CD163 and SETDB1 expression in glioma samples (Figure 4G and 4H). These observations are indicative of higher CSF-1 secretion due to elevated levels of SETDB1, which is in turn connected to increased TAMs in glioma.

**SETDB1 promoted the secretion of CSF-1 by activating the AKT/mTOR signaling pathway**

Next, the potential involvement of the AKT/mTOR signaling pathway in the CSF-1 secretion caused by SETDB1 was assessed. Increased AKT and mTOR phosphorylation was shown by Western blotting when SETDB1 was overexpressed in glioma lines, and this effect was reversed by administration of MK-2206 (Figure 5A and 5B). Similarly, knockdown of AKT expression using siRNA attenuated the increase of CSF-1 due to SETDB1 overexpression (Figure 5C and 5D). To decipher the SETDB1/AKT/mTOR/CSF-1 axis in vivo, we performed Western blotting analysis on the tumor tissue from the xenograft model. We found that SETDB1 knockdown significantly reduced AKT activation and CSF-1 protein expression in the xenograft model, while SETDB1 overexpression remarkably promoted AKT activation and CSF-1 protein levels in xenograft tumor tissues (Figure 5E and 5F). Overall, these observations suggest the involvement of AKT/mTOR signaling in the increase in SETDB1-mediated CSF-1 synthesis. Hence, this axis can serve as a prognostic marker in patients.

**SETDB1 promotes recruitment and polarization of macrophages via CSF-1**

The involvement of this SETDB1-CSF-1 axis in macrophage infiltration was assessed by an *in vitro* migration assay using conditioned media (CM) from glioma cells overexpressing SETDB1. Compared with control CM, CM from these cells was found to clearly augment the recruitment of macrophages (Figure 6A). Further, this recruitment was evidently suppressed when a CSF-1 antagonist was administered to macrophages. Overall, these results suggest that macrophage recruitment is mediated by SETDB1 via CSF-1 induction.
Next, the effect of SETDB1 on macrophage polarization via CSF-1 was assessed. The CM from gliomacell lines overexpressing SETDB1 caused elevated expression of CD126 (a TAM marker) mRNA compared to that in cells cultured with control CM, while there was no change in the expression of CD68 (a macrophage marker) (Figure 6B). Flow cytometry showed a similar protein profile (Figure 6C). The cytokine mRNAs and proteins that are representative of TAMs, IL-10, CCL17, and CCL22, were noticeably higher in TAMs incubated with CM from gliomacell lines with high SETDB1 expression than in TAMs incubated with CM from control cells (Figure 6D and 6E). Overall, these data suggest a role for CSF-1 and SETDB1 in promoting macrophage entry and differentiation.

**SETDB1 promotes tumor growth in a macrophage-dependent manner**

To demonstrate whether the oncogenic roles of SETDB1 in glioma are macrophage-dependent, we investigated the susceptibility of macrophages to liposomal clodronate treatment. For this, we overexpressed SETDB1 in the mouse glioma cell line GL261, which showed faint SETDB1 protein expression (Figure 7A). Next, we performed asyngeneic mouse model study with injection of SETDB1-overexpressing and control cell lines into the C57BL/6J mice. Prior to injection, liposomes containing clodronate or PBS were administered for two weeks and continued for three additional weeks after tumor implantation (Figure 7B). The former caused a decrease in F4/80+ macrophages in the glioma tumors of the animals (Figure 7C and 7D). In addition, we noticed more macrophage infiltration in the SETDB1-overexpressing group than in the control group (Figure 7E). The increased tumor burden induced by SETDB1 overexpression was largely attenuated in mice that received clodronate liposomes (Figure 7F), suggesting that the tumor-promoting effects of SETDB1 in glioma are, at least in part, mediated by macrophage infiltration of the tumor microenvironment.

**Discussion**

The progression of glioma involves the role of interconnected glioma cells and TAMs in the tumor microenvironment[33]. This increase in infiltrates is connected to the poor prognosis of glioma[5]. The function of this system is yet to be characterized; thus, further studies are warranted to identify such patterns to rapidly unearth potential molecules that may serve as therapeutic tools[9]. Herein, for the first time, we reveal that SETDB1 is involved in the modulation of the tumor microenvironment and, hence, glioma progression. SETDB1 was found to promote CSF-1 expression by activating the AKT/mTOR pathway. Moreover, SETDB1-induced CSF-1 modulated the tumor microenvironment by recruiting TAMs to glioma tissues, leading to tumor growth. These findings reveal opportunities for research on the role of SETDB1 in disease progression.

SETDB1 functions as a histone methyltransferase to cause histone H3K9 trimethylation, which is involved in the formation of heterochromatin[34]. These H3K9 and H3K27 sites are connected to transcriptional regulation and epigenetics[35]. This presents an opportunity to target epigenetic modifiers such as SETDB1 to treat malignancies. Research has identified the overexpression of SETDB1 in many
malignancies, such as glioma, melanoma, prostate cancer, glioma, and breast cancer (BRC), which was linked to cancer cell division as well as metastasis[19, 22, 34]. However, a complete picture is lacking in this area of cancer studies.

The association of TAMs with cancer cells in the tumor microenvironment has been the focus of studies[36, 37]. These cells have been shown to synthesize several factors that modulate cancer cell division and angiogenesis according to recent studies[38]. Particularly, the presence of symbiosis between macrophages and tumor cells has been shown by experiments where coculture of these cells caused the degradation of collagen[39]. The results from this work highlight several novel features of the mechanisms underlying glioma.

Such features of TAMs have facilitated research on targeting these cells to address disease. CSF-1 and its receptor, colony-stimulating factor 1 receptor (CSF-1R), is a aspect that has been in the spotlight and is seeing developments in clinical research[40]. One feature that is encouraging here is identification of safe applications of immunotherapeutic or standard treatment tools[41-43]. Such promising activity has been demonstrated in autocrine CSF-1-based benign diffuse-type tenosynovial giant cell tumors[44, 45]. In the case of malignant disorders, reports from clinical perspectives have yet to be explored.

Continuous research facilitates the understanding that distinct macrophage features, such as functions and phenotypes, are a reflection of various signals (for differentiation, polarization, survival or recruitment) in a tissue-specific environment[46]. The implication here is that targeting TAMs for therapy would show variation according to the organ in which the cells are present[47, 48]. This work showed that increased CSF-1 was caused by SETDB1. The former molecule is synthesized by tumor cells and is involved in tumor progression and TAM infiltration; the origin of CSF-1 can be traced back to circulating monocytes in the blood vessels of the tumor. This provides the possibility that CSF-1 and its sustained production could serve as a target for efficient disease treatment. This work showed that the increase in CSF-1 was mediated by SETDB1 via AKT/mTOR activation. Hence, this work proposes a molecular mechanism for CSF-1 overexpression in glioma, opening up the possibility for this molecule or its receptor to be a target in patients with SETDB1-overexpressing glioma.

**Conclusion**

This work shows the overexpression of SETDB1 and its relevance in glioma via activating the synthesis of CSF-1 and modulating the tumor microenvironment. The study of the precise function of this SETDB1/CSF-1/TAM axis in glioma could support research on identifying appropriate therapies to target glioma.

**List Of Abbreviations**

ATCC: American Type Culture Collection; BSA: bovine serum albumin; CM: conditioned media; CNS: central nervous system; CSF-1: colony stimulating factor 1; CSF-1R: colony-stimulating factor 1 receptor;
DMEM: Dulbecco's modified Eagle's medium; ELISA: enzyme-linked immunosorbent assay; EV: empty vector; FBS: fetal bovine serum; IHC: Immunohistochemistry; NHAs: Normal human astrocytes; OS: overall survival; PVDF: polyvinylidene fluoride; SD: standard deviation; SETDB1: SET domain bifurcated 1; TAMs: tumor-associated macrophages; TMZ: temozolomide.

Declarations

Acknowledgements

None

Authors’ contributions

SH, WZ, TG, JZ and FL were involved in the experimental design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. SH, WZ and FL were involved in the experimental design and acquisition of xenografts. SH and FL were involved in the study conception and design, analysis and interpretation of data, revision of the manuscript, and study supervision.

Funding

None

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

This study was approved by the Ethical Committee of The People's Hospital of China Medical University (The People's Hospital of Liaoning Province).

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Competing interests

The authors declare that they have no conflicts of interest.

References


29.


Tables
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Table 3. Cox regression analysis for relapse-free survival in glioma patients

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Figures

A

B

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D

E

Figure 1
SETDB1 was upregulated in glioma tissues and corelative with poor tumor progression. (A) Representative IHC staining of SETDB1 protein expression in glioma tumor tissues (T) and adjacent normal tissue (N) of three patients. Scale bar: 50 µm. (B) mRNA level of SETDB1 was analyzed by real-time PCR in glioma tumor tissues (T) and adjacent normal tissue (N) of patients. (C) Western blotting of SETDB1 expression in NHAs and indicated glioma cells. (D) mRNA level of SETDB1 was analyzed by real-time PCR in NHAs and indicated glioma cells. (E) Kaplan-Meier survival analysis indicated that glioma patients with high expression of SETDB1 had worse relapse-free survival.

Figure 2

SETDB1 promotes cell growth, and apoptosis in vitro and in vivo. (A) mRNA level of SETDB1 in indicated cells transfected with SETDB1 plasmid or control vector. (B) Western blotting of SETDB1 in indicated cells transfected with SETDB1 plasmid or control vector. (C) MTS of indicated cells transfected with SETDB1 plasmid or control vector. (D) Colony formation of indicated cells transfected with SETDB1 plasmid or control vector. (E) Western blotting of indicated proteins in U251 cells transfected with SETDB1 plasmid or control vector treated with 50 ng/mL TRAIL for 24 hours. (F) Ectopic expression of SETDB1 accelerated growth of U251 xenografts in nude mice (n=6) as compared to controls. (G) Western blotting of ectopic expression of SETDB1 in tumors from U251-SETDB1 groups. (H) Representative images of Ki67-positive cells in vector and SETDB1 transfected tumors. Scale bar: 50 µm. Results were expressed as means ± SD of 3 independent experiments. **, P<0.01.
Figure 3

SETDB1 promotes invasion, migration and EMT of glioma cells. (A) Representative images of matrigel invasion assay revealed that ectopic expression of SETDB1 promoted glioma cells invasion. (B) Representative images of matrigel invasion assay revealed that downregulation of SETDB1 suppressed glioma cells invasion. (C) Representative images of wound-healing assay indicated that ectopic expression of SETDB1 promoted cell migration in glioma cells. (D) Representative images of
wound-healing assay indicated that downregulation of SETDB1 suppressed cell migration in gliomacells.

(E) Western blotting of mesenchymal markers (Slug and Vimentin) and epithelial markers (E-cadherin) in cells transfected with SETDB1 or control plasmid. (F) Western blotting of mesenchymal markers (Slug and Vimentin) and epithelial markers (E-cadherin) in cells transfected with stable shRNA against SETDB1. Results were expressed as means ± SD of 3 independent experiments. **, P<0.01.
SETDB1 regulates CSF-1 level in glioma cells. (A) Real-time PCR for mRNA level of genes coding for tumor-associated macrophage (TAMs) recruitment associated cytokines in U87 cells with SETDB1 overexpression. (B) Real-time PCR for mRNA level of genes coding for tumor-associated macrophage (TAMs) recruitment associated cytokines in U251 cells with SETDB1 overexpression. (C) Real-time PCR for mRNA level of genes coding for tumor-associated macrophage (TAMs) recruitment associated cytokines in U87 cells with SETDB1 knockdown. (D) Real-time PCR for mRNA level of genes coding for tumor-associated macrophage (TAMs) recruitment associated cytokines in U251 cells with SETDB1 knockdown. (E) Enzyme-linked immunosorbent assay (ELISA) analysis of CSF-1 concentration in the supernatants of cultured glioma cells with SETDB1 overexpression. (F) Enzyme-linked immunosorbent assay (ELISA) analysis of CSF-1 concentration in the supernatants of cultured glioma cells with SETDB1 knockdown. (G) TCGA database indicated the correlation of CD163 and CSF-1 in glioma tissues. (H) TCGA database indicated the correlation of SETDB1 and CSF-1 in glioma tissues. Results were expressed as means ± SD of 3 independent experiments. **, P<0.01.
Figure 5

PI3K/AKT signaling pathway mediated SETDB1-induced CSF-1 induction. (A) Western blotting of indicated proteins in U87 cells transfected with SETDB1 overexpression with or without MK-2206 pretreatment. (B) Western blotting of indicated proteins in U251 cells transfected with SETDB1 overexpression with or without MK-2206 pretreatment. (C) Western blotting of indicated proteins in U87 cells transfected with SETDB1 overexpression with or without siRNA against AKT. (D) Western blotting of
indicated proteins in U251 cells transfected with SETDB1 overexpression with or without siRNA against AKT. (E) Western blotting of indicated proteins in control and SETDB1 overexpression xenograft tumors. (F) Western blotting of indicated proteins in control and SETDB1 knockdown xenograft tumors.

Figure 6

SETDB1 promotes macrophage recruitment and polarization. (A) Transwell migration assay of macrophage by CM from glioma cells. (B) Real-time PCR for the expression levels of CD68 and CD163 in macrophages treated with CM from glioma cells as indicated. (C) Flow cytometry analysis for the expression levels of CD163 in macrophages treated with CM from glioma cells as indicated. (D) Real-time PCR for the mRNA expression of tumor-associated macrophage (TAM) characteristic cytokines in THP-1 macrophages treated with CM from glioma cells as indicated. (E) Enzyme-linked immunosorbent assay (ELISA) for the secretion of tumor-associated macrophage (TAM) characteristic cytokines in THP-1 macrophages treated with CM from glioma cells as indicated. Results were expressed as means ± SD of 3 independent experiments. **, P<0.01.
Figure 7

SETDB1 Promotes tumor growth in a macrophage-dependent manner. (A) Western blotting of SETDB1 overexpression efficiency in GL261 cells. (B) Treatment scheme depicting intraperitoneal application of clodronate liposomes or phosphate-buffered saline (PBS) liposomes in C57BL/6J mice for 4 weeks. GL261 cells were administered at the end of week 2 and animals were sacrificed 2 weeks later. (C) Representative immunohistochemistry of F4/80+ cells in sections from tumors obtained from C57BL/6J mice treated with clodronate liposomes or PBS liposomes. Scale bar: 50 µm. (D) Tumor volume of vector and SETDB1 overexpression tumors from clodronate-liposome or PBS-liposome-treated mice (n=6 per group). (E) Tumor weight of vector and SETDB1 overexpression tumors from clodronate-liposome or PBS-liposome-treated mice (n=6 per group).

Supplementary Files

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- Supplementaldata.docx