Histone acetyltransferase KAT6A upregulates PI3K/AKT signaling through TRIM24 binding

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Conflict of interest

All authors declare no conflict of interest.

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Abstract

Lysine acetyltransferase KAT6A is a chromatin regulator that contributes to histone modification and cancer, but the basis of its actions are not well understood. Here we identify a KAT6A signaling pathway that facilitates glioblastoma (GBM) where it is upregulated. KAT6A expression was associated with GBM patient survival. KAT6A silencing suppressed cell proliferation, cell migration, colony formation and tumor development in an orthotopic mouse xenograft model system. Mechanistic investigations demonstrated that KAT6A acetylates lysine 23 of histone H3 (H3K23), which recruits the nuclear receptor binding protein TRIM24 to activate PIK3CA transcription, thereby enhancing PI3K/AKT signaling and tumorigenesis. Overexpressing activated AKT or PIK3CA rescued the growth inhibition due to KAT6A silencing. Conversely, the pan-PI3K inhibitor LY294002 abrogated the growth-promoting effect of KAT6A. Overexpression of KAT6A or TRIM24, but not KAT6A acetyltransferase activity-deficient mutants or TRIM24 mutants lacking H3K23ac binding sites promoted PIK3CA expression, AKT phosphorylation and cell proliferation. Taken together, our results define an essential role of KAT6A in glioma formation, rationalizing its candidacy as a therapeutic target for GBM treatment.
Introduction

Glioblastoma (GBM) is the most common primary malignant cancer of the central nervous system with a grim median survival of less than 1.5 years upon diagnosis (1). Recent global genomic and transcriptome analyses reveal that the epigenetic landscape is deregulated in cancer, including glioma, partially due to aberrant histone modifications and mutations (2-4). However, the mechanisms by which histone modifiers regulate gliomagenesis are still being elucidated.

Lysine acetyltransferase 6A (KAT6A, also known as MYST3 and MOZ) is a MYST-family histone acetyltransferase, which controls fundamental cellular processes, including gene transcription (5), cellular senescence (6), cardiac septum development (7), memory T cell diversity (8), and maintenance of normal hematopoietic stem cells (9). Dysregulation of the KAT6A histone acetyltransferase activity or aberrant expression of KAT6A has been associated with oncogenesis in leukemia (10-13) and breast cancer (14). KAT6A's oncogenic activity was first identified as a recurrent fusion partner of the CREB binding protein (CBP) as a consequence of gene translocation, t(8;16)(p11;p13) in the FAB M4/M5 subtype of acute myeloid leukemia (AML) (13). Then, the KAT6A-TIF2 fusion was identified as a consequence of another translocation, inv(8)(p11;p13) in AML. This AML fusion event requires the KAT6A nucleosome binding motif and TIF2-mediated recruitment of CBP (10). KAT6A is composed of tandem PHD fingers, a MYST domain, an acidic region, and a
Ser/Met (SM)-rich domain (15). KAT6A binds and acetylates histones via its MYST domain (15). In addition, KAT6A and the related factor Lysine acetyltransferase 6B (KAT6B, also known as MORF) form a tetrameric complex with ING5 (Inhibitor of growth 5), EAF6 (Esa1-associated factor 6 ortholog), and the bromodomain-PHD finger protein BRPF1 (15). Increasing evidence suggests that KAT6A is implicated in regulating tumor progression (16), but mechanisms that facilitate its oncogenic activity independent of gene fusion remain elusive.

In this study, we firstly examined KAT6A expression in glioma cells and clinical GBM specimens. We then assessed the role of KAT6A in cell proliferation, cell migration, and tumor growth in gliomas using cell culture and orthotopic xenograft models. Finally, we determined the mechanisms by which KAT6A regulates glioma tumorigenesis.
Materials and Methods

Cell lines

LN428, LN340, U87, LN229, D54, T98G, U251 and LN444 GBM cells were from ATCC (Manassas, VA, USA), and were cultured in 10% FBS/DMEM. All cell lines in this study were authenticated using STR DNA fingerprinting, most recently in March 2017 by Shanghai Biowing Applied Biotechnology Co., Ltd (Shanghai, China), and mycoplasma infection was detected using LookOut Mycoplasma PCR Detection kit (Sigma-Aldrich). Only lower-passage cell lines were used for the study.

Plasmids. Flag-TRIM24 was a gift from Michelle Barton (Addgene plasmid # 28138) (17), pCIG-PIK3CA was a gift from Joseph Gleeson (Addgene plasmid # 73056) (18), and pLNCX-Myr-AKT was a gift from Joan Brugge (Addgene plasmid # 17245) (19). Wild-type KAT6A cDNA was amplified from U87 cells, and then inserted into a pcDNA3 vector (Invitrogen). KAT6A<sup>G657E</sup>, KAT6A<sup>C543G/G657E</sup> and TRIM24<sup>F979A/N980A</sup> point mutations were generated using a site-directed mutagenesis kit (Invitrogen) following the manufacturer’s protocol. KAT6A shRNAs were purchased from Genechem, Inc (Shanghai, China).

Immunoprecipitation (IP) and Western blotting (WB) assays. Cells were lysed in an IP buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM NaF, 1% Triton X-100 and protease inhibitor cocktail) at 4°C for 30 min. The lysates were
centrifuged, and then protein concentrations were determined. Equal amounts of cell lysates were immunoprecipitated with specific antibodies and protein G-agarose beads (Invitrogen). Standard WB was done with antibody against β-actin (I-19), Histone H3 (FL-136) (Santa Cruz Biotechnology); Flag (Sigma-Aldrich); acetyl-Histone H3 (Lys23) (#07-355, Millipore-Upstate); TRIM24 (#14208-1-AP, Proteintech Group); KAT6A (H00007994-M07, Abnova); AKT (#4060S), phospho-AKT (#9272S), acetyl-Histone H3 (Lys14)(D4B9), and acetyl-Histone H3 (Lys9)(C5B11) (Cell Signaling Technology); BRPF1 (PA5-27783), KAT6B (PA5-37101), and PIK3CA (4F3) (ThermoFisher Scientific); KAT5/Tip60 (C-7, sc-166323, Santa Cruz Biotechnology).

**Cell proliferation and migration assays.** Cell proliferation analysis was performed using a WST-1 assay kit (Roche) and cell migration analysis was performed using a Boyden chamber as previously described (20). Briefly, cells were seeded in medium, split, and detected with a WST-1 assay kit. For cell migration analysis, cells were serum-starved for 24 h, washed with PBS and resuspended in DMEM plus 0.1% FBS. Then, cells were placed into the top compartment of a Boyden chamber and the bottom chamber was added with 10% FBS/DMEM. After 16-18 h, the membrane was fixed, stained and analyzed.

**Colony formation assay.** Soft agar colony formation assays were performed as we previously described (21). Briefly, 10,000 cells were seeded in a 0.4% Noble Agar top layer with a bottom layer of 0.8% Noble Agar in each of the triplicate wells of a 12-well plate. Cell
culture media was changed every 3 days thereafter. Colonies were scored after 2-3 weeks and data were analyzed.

**shRNA knockdown and transfection.** Lentiviruses were produced by co-transfecting various cDNA and packaging plasmids into HEK293T cells using Lipofectamine 2000 reagent according to manufacturer’s instruction (#52758, Invitrogen). Forty-eight hours after transfection, the viral-containing supernatants were filtered and added into the culture media supplemented with 8 μg/ml polybrene. Transduced human GBM cells were harvested and expression of exogenous proteins was validated by WB.

**RNA isolation and qRT-PCR.** Total RNA was isolated from cells with Trizol Plus RNA Purification Kit (Thermo Fisher Scientific) for reverse transcription with Reverse Transcription Kit (Takara) according to the manufacturer’s instructions. qRT-PCR was performed with the Power SYBR Green Master Mix (Life Technologies) on the Applied Biosystems StepOne Plus Real-Time Thermal Cycling Block. Results were analyzed using the $2^{-({\Delta\Delta Ct})}$ method. Primers are listed in Table S1.

**ChIP-qPCR.** ChIP was performed using the Chromatin Immunoprecipitation Kit (Millipore-Upstate) according to the manufacturer’s instructions. Immunoprecipitated DNA was purified after phenol extraction and was used for qRT-PCR. Primers are listed in Table S1.
Tumorigenesis studies. All animal experiments were performed in accordance to a protocol approved by Shanghai Jiao Tong University Institutional Animal Care and Use Committee (IACUC). Athymic (Ncr nu/nu) female mice at an age of 6-8 weeks (SLAC, Shanghai, China) were randomly divided into 5 per group. 5 x 10^5 U87 GBM cells were stereotactically implanted into the brains to generate orthotopic xenografts. Mice were euthanized when neuropathological symptoms developed. Tumor volumes were measured and estimated as \((W^2 \times L) / 2, W < L\) (22).

Immunohistochemical (IHC) staining of human glioma specimens. All the work related to Human tissues were performed under the Institutional Review Board approved protocols approved at Shanghai Jiao Tong University, according to the Declaration of Helsinki, and the investigators obtained informed written consent from the subjects (wherever necessary). Human tissues including 13 WHO Grade II, 22 WHO Grade III, and 111 primary human GBM specimens were collected from Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China. The tissue sections from paraffin-embedded de-identified human glioma specimens were stained with antibodies against KAT6A (1:50). IHC staining was scored as 0-3 according to the percentage of positive cells, as previously described (23) Two separate individuals who were blinded to the slides examined and scored each sample.
**Statistics.** GraphPad Prism version 5.0 for Windows (GraphPad Software Inc., San Diego, CA, USA) was used to perform one-way analysis of variance (ANOVA) with Newman-Keuls post-hoc test or an unpaired two-tailed Student's *t*-test. Kaplan-Meier survival analysis was carried out using log-rank tests. A Spearman’s rank correlation analysis was used to investigate the correlation of gene expression levels in human clinical GBM specimens. A *P*-values of less than 0.05 was considered significant.
Results

**KAT6A expression is prognostic for clinical GBM**

To identify if KAT6A is important for glioma progression, we first assessed expression of KAT6A in clinical specimens of patients. We downloaded two GBM microarray datasets, GDS1962 (24) and GSE7696 (25), and examined KAT6A mRNA expression in clinical GBM samples and normal brain tissue. As shown in Fig. 1A and 1B, KAT6A was expressed at higher levels in GBM compared with normal brain tissue.

Then, we performed IHC staining assays in the total 146 clinical brain tumors, including 13 WHO grade II, 22 WHO grade III and 111 GBM. As shown in Fig. 1C, KAT6A staining was negative or weak in WHO grade II tumors. KAT6A was expressed in the majority of high grade gliomas (WHO grade III and GBM tumors), and was particularly high in GBM (Fig. 1C and 1D), suggesting that the levels of KAT6A expression are correlated with glioma grade.

Last, we examined the relationship of KAT6A expression and GBM patient survival by Kaplan–Meier survival analysis. As shown in Fig. 1E, Kaplan–Meier survival analysis revealed a statistically significant worse prognosis for GBM patients with high KAT6A protein levels compared with those with low. The median patient survival times of these patients were 9.97 and 13.53 months, respectively ($P < 0.05$). Taken together, these observations strongly indicate that upregulation of KAT6A was closely associated with
progression and poor prognosis in GBM patients.

**Knockdown of KAT6A inhibits glioma cell proliferation, migration, colony formation, and tumor growth**

To demonstrate the role of KAT6A in glioma tumorigenesis, we detected the levels of KAT6A protein and mRNA expression in LN428, LN444, LN340, T98G, LN229, U87, and D54 GBM cells. As shown in Fig. 2A and Fig. S1A, KAT6A was ubiquitously expressed in all GBM cells, and expressed relatively higher in LN229 and U87 GBM cells compared with other cells. Then, we used lentivirus-mediated short hairpin RNAs (shRNAs) of KAT6A or a control shRNA to deplete KAT6A in U87 and LN229 GBM cells (Fig. 2B and Fig. S1B). As shown in Fig. 2C to 2G, knockdown of endogenous KAT6A significantly inhibited cell proliferation, cell migration and colony formation in soft agar in both GBM cells.

To further determine whether KAT6A is critical for glioma tumorigenesis, we employed an orthotopic GBM model as we previously described (26). U87 GBM cells transduced with shKAT6A-1, shKAT6A-2 or shControl were separately implanted intracranially to generate orthotopic xenografts in immunocompromised mice. Then, the effects of KAT6A depletion on glioma tumorigenesis were assessed. Compared with the control xenograft models, knockdown of KAT6A significantly inhibited glioma tumor growth (Fig. 2H and 2I). These data support that KAT6A is critical for cell proliferation, cell migration, and tumor growth in gliomas.
**KAT6A regulates AKT activity**

Since AKT activation is important for histone acetyltransferase function (27-30) and glioma cell proliferation (26), we determined the effect of KAT6A knockdown on phosphorylation of AKT (p-AKT) in U87 and LN229 GBM cells. As shown in Fig. 3A, knockdown of KAT6A markedly decreased AKT phosphorylation (Fig. 3A), suggesting that KAT6A regulates Akt activity in gliomas.

To support that KAT6A mediates AKT activity, we overexpressed a constitutively activated (CA) AKT (Myr-AKT) mutant in LN229 GBM cell with KAT6A shRNAs or a control shRNA. As shown in Fig. 3B to 3D, compared with the control, overexpression of the CA AKT mutant in LN229/shC cells promotes AKT phosphorylation (Fig. 3B), cell proliferation (Fig. 3C) and colony formation (Fig. 3D). Moreover, overexpression of the CA AKT mutant in LN229/shKAT6A cells markedly restored AKT phosphorylation (Fig. 3B), cell proliferation (Fig. 3C) and colony formation in soft agar (Fig. 3D) inhibited by KAT6A knockdown. In order to rule out non-specific changes or off-target effects that may account for our results, we employed direct genetic manipulations using CRISPR technology to directly validate the functions of KAT6A. We constructed KAT6A-knockout U87 and LN229 GBM cells using two different sgRNAs, and found that KAT6A knockout markedly impaired AKT phosphorylation in both GBM cells compared with the controls (Fig. 3E). Overexpression of the CA AKT mutant also restored KAT6A knockout-inhibited AKT phosphorylation (Fig. 3F), cell proliferation (Fig. 3G), and colony formation in soft agar (Fig. 3H) in LN229 cells. This data
demonstrates that KAT6A regulates AKT activity to promote glioma cell proliferation.

**KAT6A upregulates PIK3CA expression in gliomas to activate PI3K/AKT signaling**

As PI3K signaling pathway has a critical role in driving tumor initiation and progression and AKT is the dominant effector of PI3K signaling (31), we assessed the effects of KAT6A knockdown on expression of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) in U87 and LN229 GBM cells. As shown in Fig. 4A and 4B, KAT6A knockdown markedly inhibited expression levels of PIK3CA protein and mRNA in both GBM cells. We also detected mRNA expression levels of KAT6A homologous KAT6B and KAT5 in U87 and LN229 GBM cells, and found that their expression are relatively lower than that of KAT6A in U87 and LN229 GBM cells (Fig. S2A). Knockdown of KAT6B or KAT5 did not impair PI3KCA protein (Fig. S2B and S2D) and mRNA (Fig. S2C and S2E) expression but increased AKT phosphorylation (Fig. S2B and S2D). Moreover, ChIP-qPCR assays using antibodies against KAT6A or its partner BRPF1 (Fig. 4C and 4D) demonstrated that KAT6A and BRPF1 bind to the PIK3CA promoter. These data suggest that KAT6A mediates PIK3CA expression to regulate glioma cell proliferation.

To validate that KAT6A regulates PIK3CA expression in glioma cells, we overexpressed PIK3CA in LN229 GBM cells with KAT6A shRNAs or a control shRNA. As shown in Fig. 4E to 4G, compared with the control, overexpression of PIK3CA in LN229/shC cells promotes AKT phosphorylation (Fig. 4E), cell proliferation (Fig. 4F) and colony formation (Fig. 4G).
Furthermore, PIK3CA overexpression in LN229/shKAT6A cells markedly restored AKT phosphorylation (Fig. 4E), cell proliferation (Fig. 4F) and colony formation in soft agar (Fig. 4G) inhibited by KAT6A knockdown. We also downloaded expression data of PIK3CA and KAT6A in 547 GBM samples from The Cancer Genome Atlas (TCGA), and found that PIK3CA expression is correlated with KAT6A expression (Fig. 4H). These data suggest that KAT6A upregulates PIK3CA expression to activate PI3K/AKT signaling pathway, resulting in enhancing glioma tumorigenesis.

**KAT6A acetyltransferase activity is required for activating PI3K/AKT signaling**

To determine potential mechanisms by which KAT6A regulates PI3K/AKT signaling, we detected histone H3 acetylation using WB assays. H3K9, H3K14, and H3K23 were reported as targets of KAT6A/KAT6B (16). As shown in Fig. 5A, compared with the control, histone H3, knockdown of KAT6A decreased the acetylation levels of H3K23, H3K9 and H3K14. Compared with H3K9ac and H3K14ac, the acetylation levels of H3K23 were particularly suppressed by KAT6A knockdown in LN229 and U87 GBM cells.

Since the functions of H3K23 acetylation (H3K23ac) mediated by KAT6A in solid tumors is not well established, we focused to determine H3K23ac roles in KAT6A-regulated glioma cell proliferation. To examine whether KAT6A acetyltransferase activity is required for activating PI3K/AKT signaling, we generated two KAT6A acetyltransferase activity deficient mutants, KAT6A^{G657E} and KAT6A^{C543G/G657E} (10, 14). As shown in Fig. 5B to 5D,
overexpression of KAT6A wild type (WT) in LN229 GBM cells promoted PIK3CA expression, H3K23 acetylation, Akt phosphorylation, cell proliferation, and colony formation compared with the control, empty vector (EV). However, overexpression of KAT6A\(^{G657E}\) or KAT6A\(^{C543G/G657E}\) mutant had no effects on PIK3CA expression, H3K23 acetylation, AKT phosphorylation, cell proliferation, and colony formation (Fig. 5B to 5D). This data demonstrates that KAT6A acetyltransferase activity is important for activating PI3K/AKT signaling.

Then, we investigated whether treatment with PI3K inhibitor LY294002 suppresses KAT6A-promoted cell proliferation. As shown in Fig. 5E, LY294002 significantly inhibited AKT phosphorylation in LN229 GBM cells transfected KAT6A wild type or G657E mutation compared with the EV controls, whereas expression levels of PIK3CA and H3K23ac were unaffected by LY294002 treatment. Importantly, compared with the EV control, LY294002 significantly inhibited KAT6A WT, but not KAT6A-G657E mutation, overexpression-promoted AKT phosphorylation in LN229 GBM cells (Fig. 5E). We further examined the impact of LY294002 on in vitro KAT6A-promoted cell proliferation and colony formation, and found that LY294002 markedly suppressed glioma cell proliferation and colony formation of KAT6A WT-overexpressing, but not KAT6A-G657E mutation-overexpressing, GBM cells compared with the EV control (Fig. 5F and 5G). This data further supports that KAT6A acetyltransferase activity is required for the PI3K/AKT signaling pathway activation.
KAT6A promotes H3K23ac association with TRIM24 to mediate PI3K/AKT signaling

Tripartite motif-containing 24 (TRIM24, also known as TIF 1α) is a member of the transcription intermediary factor family (32), and was reported to bind with H3K23ac to regulate the development of breast cancer (33) and prostate cancer (34). Moreover, TRIM24 as a transcriptional activator upregulates PIK3CA mRNA expression and activates PI3K/AKT signaling pathway in gliomas (35). We hypothesized that KAT6A promotes H3K23 acetylation, and then enhances the association of TRIM24 and H3K23ac. Consequently, TRIM24 functions as a transcriptional activator to upregulate PIK3CA expression, leading to activation of PI3K/AKT signaling, thereby enhancing glioma tumorigenesis. To test this hypothesis, we first performed IP analysis in U87 and LN229 GBM cells with a KAT6A shRNA or a control shRNA. As shown in Fig. 6A, TRIM24 interacted with H3K23ac in both GBM cells. However, knockdown of KAT6A markedly attenuated the association of TRIM24 and H3K23ac.

Next, we performed ChIP-qPCR assays on PIK3CA using antibodies directed against TRIM24 and H3K23ac to determine whether TRIM24 and H3K23ac bind to the PIK3CA promoter in LN229 GBM cells with, or without a KAT6A shRNA. As shown in Fig. 6B, both TRIM24 and H3K23ac could bind to the PIK3CA promoter at -548~-251 locus as previously reported (35). Compared with the control (shC), KAT6A knockdown significantly inhibited the binding of TRIM24 and H3K23ac with the PIK3CA promoter. This data shows that
KAT6A regulates PIK3CA expression through the TRIM24/H3K23ac complex.

To further support that TRIM24 is important for KAT6A-regulated PI3K/AKT signaling, we overexpressed Flag-tagged TRIM24 WT or F979A/N980A mutant (F979A/N980A), which inhibits H3K23ac association with TRIM24 (33), or an EV in LN229 GBM cells with a KAT6A shRNA or a control shRNA. As shown in Fig. 6C, compared with the EV control, overexpression of Flag-TRIM24 WT promoted PIK3CA expression and Akt phosphorylation but not H3K23 acetylation in LN229/shC cells, whereas overexpression of Flag-TRIM24 WT did not affect PIK3CA expression, AKT phosphorylation, and H3K23 acetylation in LN229/shKAT6A cells (Fig. 6C). Overexpression of Flag-TRIM24-F979A/N980A mutant did not impair PIK3CA expression, AKT phosphorylation, and H3K23 acetylation in both LN229/shC and LN229/shKAT6A cells compared with the EV controls, respectively (Fig. 6C). Moreover, ChIP-qPCR analyses on PIK3CA showed that overexpression of Flag-TRIM24 WT promoted the binding of TRIM24 and H3K23ac with PIK3CA promoter in LN229/shC cells but not in LN229/shKAT6A cells (Fig. 6D), whereas overexpression of Flag-TRIM24-F979A/N980A mutant had no effects on the binding of TRIM24 and H3K23ac with PIK3CA promoter (Fig. 6D). Consistently, overexpression of Flag-TRIM24 WT promotes cell proliferation and colony formation in LN229/shC cells but not in LN229/shKAT6A cells (Fig. 6E and 6F), while overexpression of Flag-TRIM24-F979A/N980A mutant did not alter these growth characteristics (Fig. 6E and 6F). Taken together, our data demonstrate that KAT6A promotes glioma cell proliferation.
and tumor growth through H3K23ac/TRIM24-mediated PI3K/AKT signaling pathway.
Discussion

In this study, we described a novel mechanism by which KAT6A-upregulated PI3K/AKT signaling through TRIM24 binding is critical for cell proliferation and tumor growth in gliomas (Fig. 7). KAT6A promotes H3K23 acetylation and association with TRIM24, leading to increased PIK3CA expression and PI3K/Akt signaling activation, resulting in enhanced glioma tumorigenesis.

Our data demonstrate that KAT6A functions as an oncogene in gliomas. The mammalian KAT6A was firstly identified in AML patients (13). Previous work identified KAT6A interactions with ING5, EAF6, and BRPF1, -2, and -3 (36, 37) to form a macromolecular complex to regulate normal hematopoietic stem cell maintenance (9) and AML development (36). Recent KAT6A was revealed to be amplified and overexpressed in breast cancers, and depletion of KAT6A markedly inhibited cell proliferation and tumor growth in breast cancer through regulating estrogen receptor α expression (ERα) (14). Here, we show that KAT6A promotes glioma tumorigenesis. Expression of KAT6A was upregulated in GBM samples. Moreover, evaluation of GBM patient samples revealed a negative correlation between KAT6A expression and survival in glioma patients. Knockdown of KAT6A by shRNAs inhibited glioma cell proliferation, cell migration in vitro and glioma tumorigenicity. These results show that KAT6A is critical for glioma tumorigenesis.
Our results also suggest that KAT6A mediates gliomagenesis through PI3K/AKT signaling pathway. Although KAT6A has long been linked to the regulation of cell proliferation in normal and cancer cells (14, 38), the mechanisms by which KAT6A regulates cell proliferation in solid tumors are still unclear. Inactivating Sas3 (KAT6A homologous in yeast) acetyltransferase activity resulted in the accumulation of cells at the G2/M phase (38). Enok (KAT6A homologous in *Drosophila*) promotes the G1/S transition by interacting with the Elg1 proliferating cell nuclear antigen (PCNA)-unloader complex (39). Our data show that knockdown of *KAT6A* inhibited glioma cell proliferation and tumor growth through PI3K/AKT signaling. Overexpression of PIK3CA or CA AKT restored *KAT6A* knockdown-inhibited cell proliferation. KAT6A and its partner BRPF1 bind with PIK3CA and up-regulate *PIK3CA* transcription in glioma cells. In contrast to a previous report in prostate cancer (28), we observed that knockdown of *KAT6B* or *KAT5* inhibited AKT phosphorylation but did not affect PIK3CA expression in glioma cells, suggesting that KAT6B and KAT5 may regulate PI3K/AKT signaling through different pathways in gliomas. Taken together, our results demonstrate that histone acetyltransferases regulate cell proliferation through PI3K/AKT signaling pathway.

Our results further suggest that KAT6A upregulates PI3K/AKT signaling pathway through H3K23ac-binding TRIM24 transcriptional activation. KAT6A can acetylate nonhistone and histone substrates in mammals, and functions as a positive or negative regulator (16).
KAT6A interacts with transcriptional factors, such as p53 and RUNX1, to activate gene transcriptions by acetylating histones near target gene promoters (15, 40). KAT6A can interact with and promote p53 acetylation, which enhances the p53 activity to drive p21 expression and subsequent senescence (41, 42). KAT6A also has been shown to regulate the expression of ER$\alpha$ by directly binding to its promoter via H3K9 acetylation in breast cancer cells (14). On the other hand, KAT6A is required for the expression of several repressors of the INK4A-ARF pathway by maintaining the H3K9ac levels but not the H3K14ac or H3K23ac levels at those gene loci (6). Here, we found that KAT6A significantly promotes H3K23 acetylation in glioma cells compared with H3K9 and H3K14 acetylation. KAT6A-induced H3K23 acetylation enhances the association of TRIM24 with chromatin. Consistent with the previous report on TRIM24 signaling (35), our results reveal that overexpression of KAT6A or TRIM24 upregulates PIK3CA transcription in glioma cells to activate PI3K/AKT signaling pathway, whereas overexpression of KAT6A acetyltransferase activity deficient mutants or TRIM24 binding mutant with H3K23ac does not. Our data support that KAT6A-regulated PI3K/AKT signaling pathway depends on its acetyltransferase activity and H3K23/TRIM24 complex.

In summary, our findings identify KAT6A as a potential target for treatment of highly malignant gliomas. Our data reveal a mechanism by which KAT6A promotes glioma proliferation through H3K23ac/TRIM24-PI3K/AKT pathway. The newly elucidated roles of KAT6A in glioma proliferation also contribute to better understanding the regulation of
KAT6A functions in human diseases.
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Figure Legends

**Figure 1 KAT6A expression is correlated with glioma progression.** A and B, Expression levels of *KAT6A* mRNA are significantly higher in GBM samples compared with normal brain tissues. Expression data of *KAT6A* mRNA were downloaded from the GDS1962 dataset (24) and the GSE7696 dataset (25) and analyzed. C, IHC staining of KAT6A in clinical glioma specimens. Scale bars: 50 μm. Arrows, positive staining. Data are representative from two independent experiments with similar results. D, Quantitative analysis of KAT6A protein expression in C. E, Kaplan-Meier analysis of patients with high KAT6A protein-expressing GBM versus low KAT6A protein-expressing GBM. Statistical analysis was performed by log-rank test in a GraphPad Prism version 5.0 for Windows. Median survival (in months) for low KAT6A (13.53) and high KAT6A (9.97) protein expression. Black bars, censored data. Error bars ± SD. *, *P*<0.05. ***, *P*<0.05. Data are representative from two independent experiments with similar results.

**Figure 2 Knockdown of KAT6A inhibits glioma cell proliferation, migration, colony formation in soft agar, and tumor growth.** A, Western blotting (WB) analysis of KAT6A protein expression in GBM cells. β-actin was used as a control. B, WB analysis of KAT6A knockdown using two different shRNAs (shKAT6A-1 and shKAT6A-2) or a control shRNA (shC). C, Effects of KAT6A knockdown on GBM cell proliferation. D, Representative cell migration images using a Boyden chamber assay of U87 and LN229 GBM cells with shC,
shKAT6A-1 or shKAT6A-2. Scale bars: 400 μm. E, Quantification of GBM cell migration in D. F, Representative soft agar colony formation images. Scale bars: 300 μm. G, Quantification of soft agar colonies in F. H, Representative hematoxylin and eosin (H&E) staining images of mouse brain sections with U87/shC, U87/shKAT6A-1 or U87/shKAT6A-2 tumors. Brains were harvested at 6-7 weeks after transplantation. Scale bars: 1 mm. Data were from two independent experiments with 5 mice per group with similar results. I, Quantification of tumor size in H. Data are representative from two independent experiments with similar results. Error bars ± SD. *, P < 0.05. **, P < 0.01.

**Figure 3** KAT6A depletion inhibits AKT activation. A, WB analysis of effects of KAT6A knockdown on AKT activation. B, Effects of overexpression of a constitutively activated (CA) Akt (Myr-AKT) mutant on KAT6A knockdown-inhibited Akt activation. C and D, Effects of overexpression of Myr-AKT on KAT6A knockdown-inhibited glioma cell proliferation (C) and soft agar colony formation (D). (E) WB analysis of effects of KAT6A knockout using two different sgRNAs on Akt phosphorylation. EV, empty vector. (F) Effects of overexpression of a CA AKT (Myr-AKT) mutant on KAT6A knockout-inhibited Akt activation. (G and H) Effects of overexpression of Myr-AKT on KAT6A depletion-inhibited glioma cell proliferation (G) and soft agar colony formation (H). Error bars ± SD. *, P < 0.05. Data are representative from two independent experiments with similar results.

**Figure 4** KAT6A upregulates PIK3CA expression in glioma cells to activate PI3K/AKT
signal. A, Effects of KAT6A knockdown on PIK3CA protein expression in U87 and LN229 GBM cells. B, qRT-PCR analysis of effects of KAT6A knockdown on PIK3CA mRNA expression. C and D, ChIP-qPCR assays of the binding of KAT6A (C) and BRPF1 (D) with the PIK3CA promoter. After ChIP using antibodies against KAT6A, BRPF1, or control IgG, qPCR assays were performed using primers corresponding to five different loci of the PIK3CA promoter. E-G. Overexpression of PIK3CA rescues KAT6A knockdown-inhibited AKT activation (E), glioma cell proliferation (F), and soft agar colony formation (G). H, Correlation of expression between PIK3CA and KAT6A in 547 GBM samples from The Cancer Genome Atlas (TCGA) dataset. Data are representative from two independent experiments with similar results. Error bars ± SD. *, P < 0.05. **, P < 0.01.

Figure 5 KAT6A acetyltransferase activity is required for activating PI3K/AKT signaling. A, WB analyses of effects of KAT6A knockdown on the acetylation of H3K23, H3K9, H3K14 and KAT6A expression. Histone H3 and β-actin were used as controls. B, Overexpression of KAT6A wild type but not acetyltransferase activity deficient mutant, G657E or C543G/G657E, restored KAT6A knockdown-inhibited activation of PI3K/AKT signaling pathway. C and D, Effects of overexpression of KAT6A wild type, acetyltransferase activity deficient mutant, G657E or C543G/G657E, on cell proliferation (C) and colony formation in soft agar in LN229 GBM cells (D). E-G, Inhibition of PI3K suppressed KAT6A-stimulated AKT phosphorylation (E), cell proliferation (F), and colony formation (G). Data are representative from two independent experiments with similar
results. Error bars ± SD. *, P < 0.05.

Figure 6 KAT6A promotes H3K23ac association with TRIM24 to mediate PI3K/AKT signaling. A, Immunoprecipitation (IP) and WB analyses of effects of KAT6A knockdown on H3K23ac association with TRIM24. B, ChIP-qPCR assays of the binding of TRIM24 and H3K23ac with the PIK3CA promoter. C, Overexpression of Flag-TRIM24 wild type but not the binding mutant of TRIM24 with H3K23ac, F979A/N980A, promoted PIK3CA expression and AKT activation. D, Effects of overexpression of Flag-TRIM24 wild type or F979A/N980A mutant on the binding of TRIM24 and H3K23ac with PIK3CA promoter. E and F, Effects of overexpression of Flag-TRIM24 wild type or F979A/N980A mutant on cell proliferation (E) and colony formation in soft agar in LN229 GBM cells (F). Data are representative from two independent experiments with similar results. Error bars ± SD. *, P < 0.05.

Figure 7 A working model for KAT6A-regulated glioma tumorigenesis. KAT6A/MOZ interacts with ING5, EAF6 and BRPF1, and highly promotes H3K23 acetylation, and recruits TRIM24 binding with PIK3CA promoter to upregulate its transcription, resulting in PI3K/AKT signaling pathway activation and glioma tumorigenesis.
Figure 1

(A) Relative KAT6A mRNA expression in normal brain (n=23) and GBM (n=81).

(B) Relative KAT6A mRNA expression in normal brain (n=4) and GBM (n=80). 

(C) Immunohistochemical staining for KAT6A in Grade II, Grade III, and GBM.

(D) Relative KAT6A protein expression in Grade II (n=13), Grade III (n=22), and GBM (n=111).

(E) Percent survival for high (n=82) and low (n=29) KAT6A expression, with p < 0.05.
Figure 2

A

B

C

D

E

F

G

H

I

Figure 2

A

B

C

D

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H

I
Figure 3

A

B

C

D

E

F

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H
Figure 5

A

B

C

D

E

F

G

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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Figure 6

A. Western blot analysis showing expression levels of H3K23ac, TRIM24, and KAT6A in U87 and LN229 cells treated with shC, shKAT6A, or shC/KAT6A complexes. 

B. ChIP-qPCR results for TRIM24 and H3K23ac promoter regions in U87 and LN229 cells treated with shC or shKAT6A. 

C. Western blot analysis for Flag-TRIM24, PIK3CA, p-Akt, Akt, H3K23ac, and b-actin in LN229 cells treated with WT or F979A/N980A F979A/N980A complexes.

D. ChIP-qPCR results for PIK3CA promoter region in LN229 cells treated with shKAT6A or with shKAT6A plus WT or F979A/N980A complexes.

E. Relative cell proliferation assay showing the effect of shKAT6A or shKAT6A plus WT or F979A/N980A complexes on LN229 cell proliferation.

F. Relative soft agar colony assay showing the effect of shKAT6A or shKAT6A plus WT or F979A/N980A complexes on colony formation in LN229 cells.
Figure 7

ING5, EAF6, BRPF1, KAT6A/ MOZ

Histone H3

TRIM24 → PIK3CA

K29ac, K23ac, K14ac, K9ac

PI3K/Akt

Glioma tumorigenesis
Histone acetyltransferase KAT6A upregulates PI3K/Akt signaling through TRIM24 binding

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