Nuclear GSK3β promotes tumorigenesis by phosphorylating KDM1A and inducing its deubiquitylation by USP22

Aidong Zhou¹,⁷, Kangyu Lin¹,⁷, Sicong Zhang¹,², Yaohui Chen¹, Nu Zhang³, Jianfei Xue¹, Zhongyong Wang¹, Kenneth D. Aldape⁴,⁸, Keping Xie²,⁵, James R. Woodgett⁶ and Suyun Huang¹,²,⁹

Emerging evidence has shown that GSK3β plays oncogenic roles in multiple tumour types; however, the underlying mechanisms remain largely unknown. Here, we show that nuclear GSK3β is responsible for the accumulation of the histone demethylase KDM1A and critically regulates histone H3K4 methylation during tumorigenesis. GSK3β phosphorylates KDM1A Ser683 upon priming phosphorylation of KDM1A Ser687 by CK1α. Phosphorylation of KDM1A induces its binding with and deubiquitylation by USP22, leading to KDM1A stabilization. GSK3β- and USP22-dependent KDM1A stabilization is required for the demethylation of histone H3K4, thereby repressing BMP2, CDKN1A and GATA6 transcription, which results in cancer stem cell self-renewal and glioblastoma tumorigenesis. In human glioblastoma specimens, KDM1A levels are correlated with nuclear GSK3β and USP22 levels. Furthermore, a GSK3 inhibitor, tideglusib, sensitizes tumour xenografts to chemotherapy in mice via KDM1A downregulation and improves survival. Our findings demonstrate that nuclear GSK3β- and USP22-mediated KDM1A stabilization is essential for glioblastoma tumorigenesis.

Histone methylation plays an important role in the dynamic transitions of chromatin structure and critically regulates gene transcription, which contribute to tumour progression. It has been shown that GSK3 is involved in the regulation of histone methylation including H3K4 methylation in the promoter regions of multiple genes. However, the molecular mechanisms for GSK3 in mediating alterations of histone methylation remain to be defined.

Lysine-specific histone demethylase 1A (KDM1A, also known as LSD1) is the first identified lysine-specific histone demethylase. As a key component of various transcriptional co-repressor complexes, KDM1A selectively removes the methyl from H3K4me1/2 and mediates gene repression. During cell cycle progression, KDM1A is hyperphosphorylated in tumour cells by an unknown mechanism. Moreover, like GSK3β, the function of KDM1A shows tumour type specificity. In breast carcinomas, KDM1A is downregulated and introduction of KDM1A inhibits cancer metastatic potential in vivo. In contrast, KDM1A was elevated in a variety of cancers, including glioblastoma, and plays important roles in tumour progression.

Glycogen synthase kinase 3 (GSK3) exists as two isoforms (α and β) that play central roles in many cellular and physiological processes. In mice, knockout of Gsk3β leads to death before or at birth, and embryonic fibroblasts derived from Gsk3β-null animals are sensitized to apoptosis. In malignancies, GSK3β functions are tumour type specific. GSK3β was recognized as a tumour suppressor by inactivating growth-promoting pathways such as those mediated by β-catenin and c-Myc proteins. However, growing evidence indicates that GSK3β has tumour-promoting roles in diverse cancers, such as bladder cancer, osteosarcoma, leukaemia and glioblastoma. Moreover, a high nuclear GSK3β level has been associated with high-grade tumours and poor prognosis, and growth factors induce GSK3β nuclear translocation. However, the roles and underlying mechanisms for nuclear GSK3β in tumorigenesis remain largely unknown.
Figure 1 GSK3β stabilizes KDM1A by decreasing its ubiquitylation. (a) Immunoblotting analysis of nuclear GSK3β and KDM1A in glioma stem cells (GSCs) (GSC11, GSC20 and GSC23), glioblastoma cell lines (LN229, HF U-251 and U87), and non-GSC glioma cell lines (H5683 and SW1783). (b) Immunoblotting analysis of KDM1A in Gsk3β−/− or Gsk3β+/− MEFs. KDM1A levels were quantified by scanning densitometry and the results are expressed as fold change relative to Gsk3β−/− MEFs. (c) 293T cells were transfected with the indicated plasmids and KDM1A levels were assessed by immunoblotting. KDM1A fold changes were determined as in (b). (d) GSC11 cells were transfected with Gsk3β siRNAs and then treated with or without 20 μM MG132 for 6 h. Cell lysates were analysed by immunoblotting. (e) Gsk3β−/− or Gsk3β+/− MEFs were treated with 50 μg/ml cycloheximide (CHX) for the indicated times and cell lysates were examined by immunoblotting (left panel). KDM1A band intensity was quantified and the results are expressed as KDM1A levels relative to untreated cells (mean ± s.d., n=3 independent experiments, paired Student’s t-test, right panel). *P<0.01. (f) GSC11 cells were transfected with control or Gsk3β siRNA and then treated with CHX as indicated. Western blot band intensity of KDM1A was quantified (mean ± s.d., n=3 independent experiments, student’s t-test) (h). (g) Frozen tissue sections from human glioblastomas (n=15) were immunofluorescence double-stained with anti-KDM1A and anti-GSK3β antibodies. Representative images of two tumours are shown (g). Insets: high-magnification images. Scale bars, 50 μm. In five randomly selected microscope fields of each tumour, the percentages of nuclear GSK3β/KDM1A double-stained cells and nuclear GSK3β-negative but KDM1A-positive cells were analysed and compared (mean ± s.e.m., n=15 glioblastomas, unpaired Student’s t-test) (h). (i) 293T cells were transfected with the indicated plasmids and then treated with MG132. Cell lysates were immunoprecipitated by a HA-tag antibody and then analysed by immunoblotting. (j) GSC11 cells were transfected with HA-Ubi plus control or Gsk3β siRNA and then treated with MG132. Cell lysates were immunoprecipitated using an anti-KDM1A antibody and then subjected to immunoblotting. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
Until now, the mechanisms that lead to KDM1A dysregulation in these tumours remain unclear.

In this report, we have found that high-level nuclear GSK3β is responsible for the accumulation of KDM1A by inducing its binding with and deubiquitylation by USP22. We have demonstrated that the GSK3β-USP22–KDM1A axis is critical for gliomagenesis and targeting this axis is a potential therapeutic strategy against glioblastoma.

RESULTS

GSK3β stabilizes KDM1A protein by decreasing its ubiquitylation

We first assessed the relationship between GSK3β and KDM1A expression (Supplementary Fig. 1a) and found that KDM1A expression was sharply decreased after Gsk3β knockout, but rescued by reconstituted expression of GSK3β (Fig. 1a and Supplementary Fig. 1a). Likewise, KDM1A levels were elevated by wild-type GSK3β (GSK3β-WT) or constitutively active GSK3β (GSK3β-CA), but not by kinase-defective GSK3β (GSK3β-KD) (Fig. 1c). In contrast, KDM1A levels in Gsk3β short interfering RNA (siRNA)-transfected GSC11 cells were lower than those in control cells (Supplementary Fig. 1b). Knockdown of GSK3α, the other GSK3 isoform, also decreased KDM1A levels but to a lesser degree (Supplementary Fig. 1c), suggesting partial redundancy with GSK3β playing the major role. However, we found no difference in KDM1A messenger RNA levels between Gsk3β+/+ and Gsk3β−/− MEFs (Supplementary Fig. 1d). Moreover, there is no difference in KDM1A levels between β-catenin knockout and β-catenin wild-type MEFs (Supplementary Fig. 1e), indicating that GSK3β regulates KDM1A in a manner that is independent of Wnt/β-catenin signalling.

KDM1A has been reported to be subject to proteasome degradation14, and we confirmed this result (Supplementary Fig. 1f). In GSC11, the proteasome inhibitor MG132 reversed the inhibiting effect of GSK3β knockdown on KDM1A (Fig. 1d). Furthermore, KDM1A stability was decreased in Gsk3β−/− MEFs compared with that in Gsk3β+/+ MEFs (Fig. 1e). Likewise, knockdown of GSK3β in GSC11 cells promoted KDM1A degradation (Fig. 1f). These results were confirmed in 293T cells transfected with control vector or GSK3β-CA (Supplementary Fig. 1g). We next performed immunofluorescence-based co-expression assays in glioblastoma specimens. The results showed that the percentages of nuclear GSK3β/KDM1A double-stained cells are significantly higher than that of nuclear GSK3β-negative but KDM1A-positive cells (Fig. 1g,h, P < 0.001), suggesting that KDM1A co-expresses with nuclear GSK3β in tumour cells.

We assessed the effect of GSK3β on the ubiquitylation of KDM1A. Compared with control vector, transfection of GSK3β-WT and GSK3β-CA greatly inhibited KDM1A ubiquitylation in the presence of MG132, whereas GSK3β-KD had almost no effect (Fig. 1i). Moreover, knockdown of GSK3β in GSC11 cells increased KDM1A ubiquitylation (Fig. 1j). Together, these results support the notion that GSK3β stabilizes KDM1A by decreasing its ubiquitylation.

GSK3β phosphorylates KDM1A Ser683 after priming phosphorylation by casein kinase 1α

The amino acid sequence of KDM1A contains several potential GSK3β phosphorylation sites harbouring a canonical -S/T×××S/T- motif (Fig. 2a and Supplementary Fig. 2a). Treatment with lithium chloride (a GSK3 inhibitor) decreased the level of phospho-serine/threonine on KDM1A in GSC11 cells (Fig. 2b). Moreover, GSK3β is localized in both the cytoplasm and nucleus and co-localized with KDM1A in the nuclei in glioblastoma cells and GSCs (Supplementary Fig. 2b). Furthermore, nuclear GSK3β and KDM1A bound to each other in GSC11 cells (Fig. 2c), and the amine oxidase-like (AOL) domain (residues 522–852) of KDM1A is required for the binding of KDM1A with GSK3β (Fig. 2d).

To identify the target sites of GSK3β, we mutated serine/threonine to alanine at Ser31, Thr389, Thr542, Thr607, Ser683 or Ser781 of KDM1A. Mutation of Ser683 to alanine (S683A) increased ubiquitylation and degradation of KDM1A (Supplementary Fig. 2d). In contrast, mutation of Ser683 to aspartic acid (S687D), which mimics Ser683 phosphorylation, stabilized KDM1A (Supplementary Fig. 2e). These results, which are in line with our finding that GSK3β binds to residues 522–852 (Fig. 2d), indicate that Ser683 may be phosphorylated by GSK3β and is required for the regulation of KDM1A ubiquitylation.

Substrate phosphorylation by GSK3β usually requires a prior phosphorylation by a priming kinase to form the -S/T×××pS/T- motif. Amino acid sequence analysis predicts with high probability that Ser687 is phosphorylated by casein kinase 1 (CK1) (Fig. 2a). Moreover, treatment of GSC11 and HF U-251 cells with D4476, a CK1 inhibitor25, potentiated lithium chloride’s repression on KDM1A expression (Supplementary Fig. 2e). Using tagged proteins ectopically expressed in 293T cells or endogenous proteins from GSC11 cells, we found that KDM1A interacts with CK1α, but not with CK1δ or CK1ε (Fig. 2c and Supplementary Fig. 2f,g). Furthermore, KDM1A levels are decreased only by knockdown of CK1α, but not other CK1 isoforms (Supplementary Fig. 2h).

We next performed in vitro kinase assays and found that GSK3β phosphorylated wild-type KDM1A in the presence of CK1α, but not in its absence (Fig. 2e). However, we did not detect phosphorylation using the S683A/S687A mutant (Fig. 2e). Moreover, using a specific antibody against phospho-KDM1A-Ser683 (pKDM1A-Ser683), we detected KDM1A phosphorylation only in 293T cells transfected with wild-type KDM1A, but not KDM1A S683A (Fig. 2f, lane 2 versus lane 4). In addition, the pKDM1A-Ser683 level was upregulated by GSK3β-CA in the cells transfected with wild-type KDM1A, but not KDM1A S683A (Fig. 2f). Furthermore, endogenous pKDM1A-Ser683 was detected in GSC11 and HF U-251 cells, whereas inhibition of GSK3β or CK1 decreased endogenous pKDM1A-Ser683 levels (Fig. 2g). These results consistently indicated that priming phosphorylation by CK1α at Ser687 facilitates GSK3β phosphorylation of KDM1A Ser683, which then represses KDM1A ubiquitylation.
GSK3β-dependent phosphorylation of KDM1A promotes the deubiquitylation and stabilization of KDM1A by ubiquitin-specific protease 22

As Ser683 phosphorylation decreased KDM1A ubiquitylation, we hypothesized that GSK3β functions in cooperation with an unknown deubiquitylase (DUB) to deubiquitylate KDM1A. We therefore screened a panel of DUBs in which the complementary DNA plasmids of 23 DUBs were transfected into 293T cells, and found that USP15, USP21, USP22 and USP28 upregulated KDM1A levels (Supplementary Fig. 3a). However, of those four DUBs, only USP22 substantially decreased KDM1A ubiquitylation (Supplementary Fig. 3b–d). This result was further confirmed under denaturing conditions (Fig. 3a). Moreover, using an in vitro deubiquitylation assay, we found that KDM1A ubiquitylation was decreased by incubating with recombinant USP22, suggesting that USP22 deubiquitylates KDM1A directly (Fig. 3b). Furthermore, knockdown of USP22 in GSC11 cells increased KDM1A ubiquitylation (Fig. 3c).

We next assessed the effect of USP22 on KDM1A stability. In GSC11 cells, USP22 knockdown decreased KDM1A protein level (Fig. 3d), but not KDM1A mRNA (Supplementary Fig. 3e).

**Figure 2** GSK3β phosphorylates KDM1A Ser683 after the phosphorylation priming by CK1α. (a) Amino acid sequence conservation in different species of the motif in KDM1A targeted by GSK3β. (b) GSC11 cells were treated with 20 mM sodium chloride or lithium chloride for 6 h in the presence of MG132. Cell lysates were immunoprecipitated using an anti-KDM1A antibody and then subjected to immunoblotting analysis using an antibody against pKDM1A-Ser683. Normal rabbit immunoglobulin G (IgG) was used as the isotype control. (c) Reciprocal interaction of KDM1A with GSK3β and CK1α. GSC11 cell nuclear extracts were immunoprecipitated using antibodies against KDM1A, GSK3β and CK1α, and then subjected to immunoblotting analysis. Inputs correspond to 5% nuclear extracts used for immunoblotting. The domains in KDM1A are indicated (SWIRM, amine-oxidase-like (AOL) and Tower). (e) In vitro kinase assays were performed by incubating purified active GSK3β and/or CK1α with recombinant wild-type KDM1A or KDM1A-S683A in the presence of [γ-32P]ATP. The resultant products were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. (f) Flag–KDM1A-WT or Flag–KDM1A-S683A was co-transfected with or without GSK3β into 293T cells, and then treated with MG132 for 6 h. Cell lysates were immunoprecipitated using an anti-Flag antibody and then analysed by immunoblotting using a specific antibody against pKDM1A-Ser683. (g) GSC11 or HF U-251 cells were treated with lithium chloride or D4476 for 6 h in the presence of MG132, and the cell lysates were analysed by immunoblotting using an antibody against pKDM1A-Ser683. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
**Figure 3** USP22 is a deubiquitylase of KDM1A. (a) 293T cells were transfected with the indicated plasmids and then treated with MG132 for 6 h. Cell lysates were immunoprecipitated using an anti-Flag antibody and then washed by ubiquitylation wash buffer containing 1 M urea. The resulting immunoprecipitates were analysed by immunoblotting. (b) HF U-251 cells were transfected with Flag–KDM1A and HA–Ubi and then treated with MG132 for 6 h. Cell lysates were immunoprecipitated using an anti-Flag antibody. The purified KDM1A–Ub
\(^n\) was incubated with 10 ng or 100 ng recombinant GST–USP22 proteins in a deubiquitylation buffer, and the resulting reactions were subjected to immunoblotting analysis. (c) GSC11 cells were transfected with HA–Ubi plus control siRNA or USP22 siRNA. Cell lysates were immunoprecipitated using an anti-KDM1A antibody and then analysed by immunoblotting. (d) GSC11 cells were transfected with two independent siRNAs targeting USP22, and levels of USP22 and KDM1A were detected by immunoblotting. (e) GSC11 cells were transfected with control siRNA or USP22 siRNA and then treated with CHX for the indicated times. Cell lysates were analysed by immunoblotting (left panel). Band intensities of KDM1A were quantified and the results are expressed as KDM1A levels relative to untreated cells (mean ± s.d., \(n = 3\) independent experiments, paired Student’s \(t\)-test, right panel). \(^*\)\(P < 0.01\). (f) Immunoblotting analysis of the nuclear levels of GSK3β, USP22 and KDM1A in eleven cell lines. Quantification of western blot bands of each protein from three independent experiments is shown in Supplementary Fig. 3h. (g,h) Frozen tissue sections from human glioblastomas \((n = 15)\) were stained as in Fig. 1 g,h with anti-KDM1A and anti-USP22 antibodies. Representative images of two tumours are shown (g). Scale bars, 50 \(\mu\)m. In five randomly selected microscope fields of each tumour, the percentages of nuclear USP22/KDM1A double-stained cells and nuclear USP22-negative but KDM1A-positive cells were analysed and compared (mean ± s.e.m., \(n = 15\) glioblastomas, unpaired Student’s \(t\)-test) (h). Unprocessed original scans of blots are shown in Supplementary Fig. 8.
Downregulation of KDM1A protein by USP22 knockdown was due to the decreased KDM1A stability (Fig. 3e). In contrast, USP22 overexpression in 293T cells increased KDM1A stability (Supplementary Fig. 3f). Moreover, in a panel of cell lines, the levels of nuclear USP22 and KDM1A were directly correlated (Fig. 3f and Supplementary Fig. 3g). The levels of nuclear GSK3β were also directly correlated in most of the cell lines (Fig. 3f and Supplementary Fig. 3g). In glioblastoma specimens, the percentages of nuclear USP22 and KDM1A were directly correlated (Fig. 3f and Supplementary Fig. 3g). Moreoever, in a panel of cell lines, the levels of nuclear GSK3β were also directly correlated in most of the cell lines (Fig. 3f and Supplementary Fig. 3g). Furthermore, in GSCs, KDM1A and USP22 were co-localized in nuclei (Supplementary Fig. 3h). In glioblastoma specimens, the percentages of nuclear USP22/KDM1A double-stained cells were significantly higher than that of nuclear USP22-negative but KDM1A-positive cells (Fig. 3g,h, P < 0.001), suggesting that nuclear KDM1A co-expresses with USP22 in tumour cells. These results indicate that USP22 deubiquitylates and stabilizes KDM1A.

We sought to determine whether the deubiquitylation of KDM1A by USP22 depends on the phosphorylation of KDM1A by GSK3β. Using a series of KDM1A deletion mutants, we found that the A0L domain of KDM1A was required for the binding of KDM1A with USP22 (Fig. 4a). Coincidently, the A0L domain of KDM1A was required for the binding of KDM1A with GSK3β (Fig. 4d), suggesting a possible link between KDM1A phosphorylation and deubiquitylation. Moreover, GSK3β knockdown in GSC11 cells attenuated the interaction between endogenous USP22 and KDM1A (Fig. 4b), which was confirmed in 293T cells by transfection of GSK3β-KD as compared with GSK3β-CA (Supplementary Fig. 4). In addition, deubiquitylation of KDM1A by USP22 was attenuated after GSK3β knockdown (Fig. 4c). Furthermore, KDM1A S683A significantly decreased the interaction between USP22 and KDM1A in...
Figure 5 GSK3β and USP22 repress KDM1A target genes and are required for the maintenance of GSCs. (a) GSC11 cells were transfected with control shRNA, KDM1A shRNA, Gsk3β shRNA or USP22 shRNA and ChIP assays were performed using an anti-H3K4me2/3 antibody. The immunoprecipitated DNA was analysed by real-time PCR using specific primers in the promoter of BMP2, CDK1A or GATA6. ACTB promoter was used as a negative control. Values are the percentage to input (mean ± s.e.m., n = 3 independent experiments, two-tailed Student’s t-test; *P < 0.01. (b) BMP2, CDK1A and GATA6 mRNA levels were analysed by real-time PCR in GSC11 and GSC20 cells stably expressing the indicated shRNAs. Values were normalized to that in control cells (mean ± s.e.m., n = 3 independent experiments, two-tailed Student’s t-test). ACTB was used as a negative control and GAPDH was used as an internal control; *P < 0.01. (c) GSC11 cells stably expressing the indicated shRNAs were analysed by immunoblotting using BMP2, CDK1A and GATA6 antibodies. (d) Limiting dilution assays by plating decreasing numbers of GSC11 cells showed the frequencies of neurosphere formation. The frequencies of neurosphere formation were calculated as 1/x, where x is the average cell number. The significance of the difference between the indicated groups was determined by χ² test (n = 3 independent experiments). Control shRNA versus KDM1A shRNA, P = 2.39 × 10⁻¹³; KDM1A shRNA versus KDM1A shRNA + shR, P = 5.36 × 10⁻¹³; control shRNA versus USP22 shRNA, P = 5.55 × 10⁻¹¹; USP22 shRNA versus USP22 shRNA + KDM1A, P = 1.85 × 10⁻³. (e) GSC11 cells expressing KDM1A shRNA were reconstituted by the expression of shRNA-resistant KDM1A (KDM1A-shR). (f) GSC11 cells expressing USP22 shRNA were reconstituted by the expression of wild-type KDM1A. (g) GSC11 cells expressing Gsk3β shRNA were reconstituted by the expression of KDM1A-S683D or KDM1A-S683A. Cell extracts in e–g were assessed by immunoblotting. (h) Limiting dilution assay analysis of GSC11 cells showed the frequencies of neurosphere formation. The significance of the difference between the indicated groups was determined by χ² test (n = 3 independent experiments). Control shRNA versus Gsk3β shRNA, P = 8.9 × 10⁻¹¹; Gsk3β shRNA versus Gsk3β shRNA + S683D, P = 6.54 × 10⁻³; Gsk3β shRNA versus Gsk3β shRNA + S683A, P = 0.215. (i) Representative photographs of primary or secondary neurosphere formation of GSC11 cells expressing control shRNA, Gsk3β shRNA, Gsk3β shRNA + KDM1A-S683D, or Gsk3β shRNA + KDM1A-S683A. Scale bars, 500 μm. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
Figure 6 The GSK3β-USP22–KDM1A axis is required for gliomagenesis of GSCs and associated with the grade of glioma malignance in human glioma specimens. (a) GSC11 cells (5 × 10^5 cells per mouse) stably expressing control shRNA, KDM1A shRNA, KDM1A shRNA + KDM1A-shR, USP22 shRNA, or USP22 shRNA + KDM1A-WT were intracranially injected into nude mice. (b) GSC11 cells expressing control shRNA, Gsk3β shRNA, Gsk3β shRNA + KDM1A-S683D, or Gsk3β shRNA + KDM1A-S683A were intracranially injected into nude mice. In a and b, six weeks after injection, the mice were humanely killed, and tumour growth was assessed. The haematoxylin and eosin (H&E)-stained sections show representative tumour xenografts. Tumour volumes were calculated (mean ± s.d., n = 5 mice for each group, one-way ANOVA test). Data show two independent experiments (Exp. 1 and Exp. 2). (c,d) Immunohistochemistry assays using anti-GSK3β, anti-KDM1A and anti-USP22 antibodies were performed on 95 human glioblastoma specimens. Representative images of three tumours are shown. Insets in c: high-magnification images corresponding to the areas marked by yellow dotted lines. Scale bars, 100 μm. Staining of nuclear GSK3β, KDM1A or USP22 was scored on a scale of 1–12. The correlation of nuclear GSK3β, KDM1A and USP22 was statistically significant among different specimens (n = 95 glioblastomas, top panel, r = 0.6048, P < 0.001; bottom panel, r = 0.6784, P < 0.001; Pearson correlation test). Note that the scores of some samples overlap (d). (e) Immunohistochemistry analysis of the expression of GSK3β, anti-KDM1A and anti-USP22 in human glioma specimens. Human grade II astrocytoma specimens (n = 50) were immunohistochemically stained with anti-KDM1A, USP22 or GSK3β antibody, and expression scores of KDM1A, USP22 or nuclear GSK3β were compared with those in 95 glioblastoma (GBM) specimens as in (d) (n = 50 astrocytomas and n = 95 glioblastomas, unpaired Student’s t-test). Source data for a,b can be found in Supplementary Table 3.
the presence of GSK3β-CA (Fig. 4d). Accordingly, the mutation also attenuated the deubiquitylation of KDM1A by USP22 in the presence of GSK3β-CA (Fig. 4e). These results demonstrate that GSK3β-dependent phosphorylation of KDM1A is critical for the binding of KDM1A with and deubiquitylation by USP22.

GSK3β and USP22 regulate KDM1A target gene expression and maintain the stemness of GSCs through KDM1A

KDM1A has been reported to regulate a series of genes associated with cell proliferation, stem cell pluripotency and differentiation, including BMP2, CDKN1A and GATA6. In GSCs, knockdown of KDM1A, GSK3β and USP22 decreased the binding of KDM1A to the promoter of those target genes (Supplementary Fig. 5a). Moreover, KDM1A knockdown increased H3K4me2 as well as the permissive H3K4me3 levels in the promoters of BMP2, CDKN1A and GATA6 (Fig. 5a), and this led to the increased levels of their mRNAs and proteins (Fig. 5b,c). Knockdown of GSK3β or USP22 also led to the accumulation of both H3K4me2 and H3K4me3 levels in BMP2, CDKN1A and GATA6 promoters (Fig. 5a), and decreased their mRNA and protein levels (Fig. 5b,c), suggesting that GSK3β and USP22 epigenetically repress the expression of those genes via KDM1A.

We further investigated the roles of KDM1A in regulating GSC stemness. Knockdown of KDM1A in GSCs decreased the frequency of neurosphere formation as determined by limiting dilution assays (Fig. 5d) and Supplementary Fig. 5b), and decreased the sphere formation efficiency as determined by primary and secondary neurosphere assays (Supplementary Fig. 5c,j). Moreover, following KDM1A knockdown, the stem cell markers Nestin, Oct4 and CD133 were decreased, whereas the differentiation marker Tuj1 was increased (Fig. 5e and Supplementary Fig. 5f). Likewise, knockdown of USP22 inhibited GSC stemness (Fig. 5d,f and Supplementary Fig. 5g), and exogenous KDM1A rescued the effect of USP22 depletion on GSC stemness (Fig. 5d,f and Supplementary Fig. 5g). These results suggest that USP22 promotes the stemness of GSCs through KDM1A.

In GSC11 cells, knockdown of GSK3β inhibited the expression of the stem cell markers but increased the expression of the differentiation marker (Fig. 5g and Supplementary Fig. 5h), and substantially decreased the frequency and efficiency of sphere formation (Fig. 5i and Supplementary Fig. 5i). We next overexpressed KDM1A-S683D or -S683A in GSK3β-depleted cells. Although KDM1A mRNA levels were similar in KDM1A-S683D- and KDM1A-S683A-transfected cells (Supplementary Fig. 5i), only KDM1A-S683D overexpression greatly increased the KDM1A protein in GSK3β-depleted cells (Fig. 5g). Moreover, the inhibitory effect of GSK3β depletion on GSC stemness was rescued by KDM1A-S683D, but not -S683A (Fig. 5g-i and Supplementary Fig. 5j). These results suggest that the GSK3β-dependent phosphorylation of KDM1A is important for maintaining the stemness of GSCs.

GSK3β and USP22 promote the tumorigenicity of GSCs through KDM1A

Using a mouse model, we investigated whether GSK3β and USP22 regulate the tumorigenicity of GSCs through KDM1A. All mice intracranially injected with control GSC11 cells developed tumours with characteristic glioblastoma features (Fig. 6a). In contrast, depletion of KDM1A in GSC11 cells abrogated brain tumour formation, which was rescued by shRNA-resistant KDM1A (KDM1A-shR; Fig. 6a). Likewise, depletion of USP22 in GSC11 cells abrogated brain tumour formation (Fig. 6a), but the inhibitory effects of USP22 depletion was rescued by KDM1A expression (Fig. 6a).

We next determined the role of GSK3β-dependent phosphorylation of KDM1A at Ser683 in promoting tumorigenesis. GSK3β depletion in GSC11 cells almost abrogated brain tumour formation (Fig. 6b). Moreover, the inhibitory effect of GSK3β depletion on tumorigenicity was rescued by KDM1A-S683D, but not -S683A (Fig. 6b). Immunohistochemical analysis showed that KDM1A expression in GSK3β-depleted tumour was recovered by KDM1A-S683D, but not by -S683A (Supplementary Fig. 6). These results suggest that GSK3β-dependent phosphorylation of KDM1A is necessary for promoting the tumorigenicity of GSCs.

KDM1A expression is correlated with nuclear GSK3β and USP22 expression, and nuclear GSK3β/USP22/KDM1A expression is associated with grade of glioma

To determine the potential clinical relevance of our findings, we assessed the expression of GSK3β, USP22 and KDM1A proteins in serial sections of 95 human glioblastoma (WHO grade IV) specimens. Expression levels of KDM1A were significantly correlated with those of nuclear GSK3β and USP22 (Fig. 6c,d: upper panel, r = 0.6048, P < 0.0001; lower panel, r = 0.6784, P < 0.0001). We next examined whether the levels of nuclear GSK3β, USP22 and KDM1A correlated with the grade of glioma malignance using the same 95 glioblastomas specimens with 50 low-grade astrocytoma (WHO grade II) specimens. The levels of nuclear GSK3β, USP22 and KDM1A were significantly lower in the 50 astrocytomas than in the 95 glioblastomas (Fig. 6e). These results strongly suggest that dysregulated KDM1A expression in glioblastoma is driven by high-level nuclear GSK3β and USP22.

The GSK3 inhibitor tideglusib attenuates the tumour-initiating ability of GSCs and sensitizes GSC-derived glioblastoma xenografts to TMZ in mice

We next assessed the effect of GSK3β inhibition on GSCs. Tideglusib is an irreversible and non-adenosine triphosphate (ATP)-competitive GSK3 inhibitor. Tideglusib treatment of GSC11 cells decreased the level of phosphorylated tau, a well-established GSK3β substrate (Fig. 7a). Moreover, tideglusib treatment repressed KDM1A (Fig. 7a), which confirmed our finding that GSK3β stabilizes KDM1A. Furthermore, because it decreased CD133, Nestin and Oct4 expression but increased Tuj1 expression, tideglusib treatment led to the loss of GSC features (Fig. 7b) and Supplementary Fig. 7b). Tideglusib also attenuated GSC neurosphere formation (Fig. 7c). Importantly, tideglusib was not toxic to normal human astrocytes (Supplementary Fig. 7c).

In glioblastoma, GSCs are believed to be the main cause of temozolomide (TMZ) resistance. Therefore, we sought to determine whether tideglusib inhibits GSC self-renewal to sensitize GSCs to TMZ. Tideglusib significantly repressed GSC viability and sensitized the cells to TMZ treatment in vitro (Fig. 7c,e). Given the good...
Figure 7 The GSK3 inhibitor tideglusib attenuates GSC self-renewal and enhances the effect of TMZ on GSC proliferation. (a) GSC11 cells were treated with the indicated concentrations of tideglusib for 6 h (left panel) or with tideglusib at a final concentration of 5 μM for the indicated times (right panel). Cell lysates were analysed by immunoblotting using the indicated antibodies. Data were quantified and the results are expressed as fold change relative to control cells. (b) GSC11 cells were treated with 2.5 μM or 5 μM tideglusib for 36 h, and cell lysates were assessed by immunoblotting. (c) Extreme limiting dilution assay by plating decreasing numbers of GSC11 cells showed the frequencies of neurosphere formation in GSC11 cells treated with 2.5 μM or 5 μM tideglusib. Dimethyl sulfoxide (DMSO) vehicle was used as a control. The significance of the difference between the indicated groups was determined by χ² test (n = 3 independent experiments). (d) Primary neurosphere formation was assessed in GSC11 cells treated with 2.5 μM or 5 μM tideglusib for 10 d. Representative images are shown (upper panel). Scale bars, 500 μm. Neurosphere formation efficiency (spheres/cells plated) was quantified (lower panel, mean ± s.e.m., n = 3 independent experiments, paired Student’s t-test). *P < 0.05, **P < 0.001. (e) GSC11 (top) and GSC20 (bottom) cells were treated with different concentration of tideglusib for 7 d. IC₅₀ values for each cell line were calculated using the GraphPad Prism 6 software (mean ± s.e.m., n = 3 independent experiments). *P < 0.05, **P < 0.001. (f) GSC11 and GSC20 cells were treated with DMSO, temozolomide (TMZ; 100 μM), tideglusib (2.5 μM), or TMZ plus tideglusib for the indicated times, and cell proliferation was assessed by XTT assays (mean ± s.e.m., n = 3 independent experiments, paired Student’s t-test). P values were analysed by comparing TMZ or tideglusib alone versus the combination of the two and shown. Unprocessed original scans of blots are shown in Supplementary Fig. 8.

Next, we assessed the effects of tideglusib alone, TMZ alone, and tideglusib plus TMZ on the survival of GSC11- or GSC20-glioblastoma-bearing mice. TMZ alone had a modest effect on survival (Fig. 8c), and tideglusib alone had a better effect on survival than TMZ alone (Fig. 8c). However, TMZ plus tideglusib significantly improved the survival of the GSCs-glioblastoma-bearing mice as compared with TMZ or tideglusib alone (Fig. 8c). Certainly, TMZ combined with tideglusib significantly extended the overall survival of GSC11-glioblastoma-bearing mice as compared with the no-treatment control (median survival duration of 83 d versus 49 d).
Figure 8 Tideglusib attenuates the tumour-initiating ability of GSCs and sensitizes GSC-derived xenografts to TMZ. (a) GSC11 or GSC20 cells (5 × 10⁵ cells per mouse) were intracranially injected into nude mice. One day after cell injection, mice were intraperitoneally injected with TMZ (20 mg kg⁻¹ d⁻¹) or tideglusib (25 mg kg⁻¹ d⁻¹) every other day for 30 d. For combinatorial treatment, mice received injections of TMZ or tideglusib on alternating days for 30 d. Mice were humanely killed 6 weeks after the injection of GSC11 cells or 8 weeks after the injection of GSC20 cells. H&E-stained sections show representative tumour xenografts. Scale bar, 50 μm. Tumour volumes were calculated (mean ± s.d., n=8 mice for each group, one-way ANOVA test). (b) Consecutive sections from tumour xenografts derived from GSC11 cells were immunostained using the indicated antibodies. Representative microphotographs of immunostaining for each group are shown. Insets: high-magnification images corresponding to the areas marked by yellow dotted lines. Scale bars in a, b, 50 μm. (c) GSC11 (left) or GSC20 (right) cells (5 × 10⁵ cells per mouse) were intracranially injected into nude mice. Mice were treated as in a. The survival of mice was evaluated (n=8 mice for each group, Kaplan–Meier model with two-sided log-rank test). P values were analysed by comparing TMZ or tideglusib alone versus the combination of the two. (d) Illustration of the GSKβ–USP22–KDM1A axis in the regulation of tumorigenesis. Nuclear GSKβ phosphorylates KDM1A after the priming phosphorylation by CK1α. KDM1A phosphorylation enhances its binding and deubiquitylation by USP22, leading to KDM1A stabilization. GSK3β-dependent stabilization of KDM1A promotes the demethylation of histone H3K4 and represses the transcription of BMP2, CDKN1A and GATA6, which in turn promotes glioblastoma tumorigenesis.
as well as survival of GSC20-glioblastoma-bearing mice (89 d versus 58 d) (Fig. 8c). Together, these results indicate that tideglusib sensitizes GSCs-derived xenografts to TMZ and support clinical application of this combinatorial targeted therapy for treatment of glioblastoma.

**DISCUSSION**

In this study, we have identified a critical role for GSK3β in KDM1A accumulation via phosphorylating KDM1A and thus enhancing the binding of KDM1As with and deubiquitylation by USP22, which leads to GSC self-renewal and glioblastoma tumorigenesis (Fig. 8d). Our mechanistic and clinical findings establish that the GSK3β-USP22-KDM1A axis is critical for glioblastoma tumorigenesis and targeting this axis is a potential therapeutic strategy against glioblastoma.

The function of GSK3β in malignancy is affected by the context with substrate as well as tumour type. GSK3β phosphorylates a series of substrates, including β-catenin and Myc, which leads to their degradation and/or inactivation, thus inhibiting signals that would otherwise promote cell proliferation and self-renewal. On the other hand, GSK3β also promotes cell survival by activating multiple pro-proliferative pathways or factors, including mammalian target of rapamycin (mTOR), nuclear factor kB (NF-kB), STAT3 and C/EBPβ. Interestingly, GSK3 inhibition leads to NF-kB target gene repression partially by affecting histone methylation of the target promoters. Our present study has strongly suggested that the GSK3β-USP22-KDM1A axis may underlie this action. Moreover, GSK3β-mediated phosphorylation of substrates also stabilizes target proteins by an unknown mechanism. Present study identified that KDM1A phosphorylation by GSK3β promotes the binding of KDM1A with and then deubiquitylation by USP22, which provides a direct link between substrate phosphorylation and deubiquitylation.

As a reverse procedure of ubiquitylation, protein deubiquitylation is a highly controlled process. Recent studies reported that phosphorylation of the deubiquitylase OTULIN negatively regulates its interaction with HOIP and thus promotes HOIP ubiquitylation. In the current study, we found that substrate phosphorylation affects its recognition by a DUB. Specifically, KDM1A phosphorylation by GSK3β increases the association of KDM1A with and then deubiquitylation by USP22. In contrast, USP28, a previously identified DUB for KDM1A in breast cancer cells, did not substantially affect KDM1A ubiquitylation in our system. Furthermore, previous studies show that USP22 is involved in the regulation of cancer stem cell and required for tumour progression in some cancers. So far, only a few substrates of USP22 have been identified, and little is known about the functions of USP22 in GSCs and glioma tumorigenesis. Our present study demonstrates that USP22 is highly expressed in glioblastoma, and USP22 knockdown suppresses the stemness and tumour-initiating ability of GSCs.

Tideglusib is a selective non-ATP-competitive GSK3 inhibitor with good blood–brain barrier penetration and is now in a phase II trial for the treatment of Alzheimer’s disease. Our study demonstrates that tideglusib has potent therapeutic efficacy against glioblastoma in a preclinical model. Specifically, tideglusib inhibits GSC self-renewal and sensitizes glioblastoma to TMZ in vivo but has little effect on the proliferation of normal astrocytes. The tumour specificity of tideglusib may be due to the elevated nuclear GSK3β levels in glioblastoma cells, which leads to upregulation of KDM1A target tumour suppressors.

Of these targets, BMP2 sensitizes glioblastoma stem-like cells to TMZ by inhibiting hypoxia-inducible factor 1α stability, and CDKN1A is a differentiation marker and inhibits the tumorigenicity of glioblastoma cells. CDKN1A is an inhibitor of glioma stem cell proliferation, and is involved in determining the fate of glioblastoma cells after TMZ treatment.

In conclusion, we show that GSK3β phosphorylates and stabilizes KDM1A by promoting recruitment of USP22, leading to repression of KDM1A target genes. Moreover, disruption of the GSK3β–USP22–KDM1A axis by tideglusib represses glioma tumorigenesis, sensitizes glioblastoma xenografts to TMZ treatment, and improves mouse survival, suggesting a potential therapeutic strategy against glioblastoma.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

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**AUTHOR CONTRIBUTIONS**

S.H. and A.Z. conceived the project and designed the study; A.Z. and K.L. performed most of the experiments under the supervision of S.H.; S.Z. and Y.C. assisted in some experiments; J.X. and Z.W. assisted in the mouse experiments; N.Z., K.D.A., K.K. and J.R.W. provided reagents and conceptual advice; S.H. and A.Z. wrote the manuscript. All authors discussed the results and commented on the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Regents and antibodies. Antibodies against human KDM1A (ab17721 and ab90966), CK16 (ab48031), GATA6 (ab26200) and USP22 (ab4812) were from Abcam. Antibodies against GST (sc-138), Tuj1(sc-58888), CK16 (sc-6477), CK1e (sc-6471), BMP2 (sc-6895), CDKN1A (sc-397) and Tubulin (sc-5206) were from Santa Cruz Biotechnology. Antibodies against human GSK3β (9315), GSK3α (9338), CD133 (3663), phospho-GSK3β-S9 (9336), Oct4 (2750), H3Kme2ac (9725 and 9751) and Myc-tag (2276) were from Cell Signaling Technology. Antibodies against phospho-serine/threonine (612548) and Nestin (611658) were from BD Transduction Laboratories. Antibodies against haemagglutinin (HA)-Tag and Flag-Tag were from Sigma. Hoechst 33342, Alexa Fluor 488 goat anti-rabbit antibody, and anti-mouse secondary antibodies were from Jackson ImmunoResearch and antibodies.

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shRNA and siRNA. Lentiviral shRNA plasmids were from Sigma. The shRNAs against human KDM1A (NM_015031) correspond to the coding sequences (CDSs) from 1311 to 1332 (KDM1A shRNA1, TRCN0000046071) and from 1975 to 1995 (KDM1A shRNA2, TRCN0000064072) relative to the first nucleotide of the start codon. The shRNAs against human USP22 (NM_015276) correspond to the CDSs from 545 to 565 (USP22 shRNA1, TRCN0000296868) and from 1366 to 1385 (USP22 shRNA2, TRCN0000296867). The shRNAs against human GSK3β (NM_002093) correspond to the CDSs from 1838 to 1848 (GSK3β shRNA1, TRCN0000399999) and from 1058 to 1078 (GSK3β shRNA2, TRCN0000395846). The siRNAs against USP22 and GSK3β have the same target sequences, respectively. All siRNAs were synthesized by Sigma. Supplementary Table 2 contains detailed information about the siRNA sequences.

Cell culture, transfection and treatment. NHAs-E6/E7 (normal human astrocytes immortalized with human telomerase reverse transcriptase and infected with retrovirus E6/E7), MEFS, and 293T, 293FT, LN229, HF U-251, SH-SY5Y, SW1738 and U87 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (HyClone). GSC11, GSC20, GSC23 and GSC262 human immortalized with human telomerase reverse transcriptase and infected with retrovirus were maintained in 1311 medium supplemented with 10% FBS. All cell lines were authenticated by short tandem repeat profiling and were routinely tested for misidentified cell lines that is maintained by ICLAC and NCBI Biosample. Cell lines were provided by Y. Shi (Harvard University, USA).

Plasmid constructs and mutagenesis. USP2, USP4, USP5, USP8, USP13, USP14, USP16, USP18, USP25, USP26, USP29, USP30, USP39, USP46 and USP48 expression plasmids were provided by J. Yang (Texas Children’s Cancer Center, USA). Flag-HA–USP1, Flag-HA–USP2, Flag-HA–USP12 and Flag-HA were provided by H.-K. Lin (MD Anderson Cancer Center, USA). Myc-USP11, Flag-USP12 and Flag-USP28 were from Addgene. Myc-GSK3β-WT, Myc-GSK3β-CA and Myc-GSK3β-KD were provided by M.-C. Hung (MD Anderson Cancer Center, USA). Flag–KDM1A was provided by Y. Shi (Harvard University, USA). USP22 cDNA from Flag–HA–USP22 plasmid was further cloned into pcDNA3-H vector and pGEX–4T1 vector.

Deletion mutants of KDM1A were constructed into 3× Flag vector. Site-directed mutagenesis (in KDM1A V10A) was introduced using the QuikChange site-directed mutagenesis kit (Agilent Technologies). GST-tagged KDM1A–shRNA plasmids were from AbbVie. E2A–KDM1A–shRNA plasmids were obtained with cloning the splicable vector into a pLVX–IRE–Hyg vector. The cDNAs of three ClKO isomers were amplified and cloned into pcDNA3–HA vector. All plasmids were confirmed by DNA sequencing. Supplementary Table 2 contains detailed information about the sequence of the used primers.

Generation of lentiviral stable cell lines. To generate lentiviral stable cells expressing shRNAs, we transfected 293FT cells in 60-mm dishes with pLKO.1–shRNA plasmids and the ViraPower Lentiviral Packaging Mix (Life Technologies). Twenty-four hours after transfection, the supernatant was collected, filtered, and used to infect GSCs cultured in 6-well plates. Thirty-six hours after infection, cells were selected with puromycin (10 μg ml⁻¹) for 1 week. For the generation of stable cells expressing KDM1A–shRNA, KDM1A–S683D or –S683A, GSCs were transduced with lentivirus expressing KDM1A–shRNA, KDM1A–S683D or –S683A–CDNA and then selected by hygromycin (200 μg ml⁻¹) for 1 week.

Extreme limiting dilution assay and neurosphere formation. In vitro extreme limiting dilution assay was performed as described previously². Briefly, GSCs expressing different constructs were dissociated to single cells and then plated in 96-well plates at a cell number of 1, 5, 10 or 25 cells per well. Wells with no neurosphere were counted for each group after 10 d. Extreme limiting dilution assays were analysed using software available at http://bioinfo.wehi.edu.au/software/elds²⁵. Primary and secondary neurosphere assays were performed as we described previously². Briefly, dissociated single cells were plated at a density of 1 cell μl⁻¹, and the spheres that formed after 10 d were counted. For the secondary neurosphere assay, established tumourspheres were dissociated into single cells and plated at a clonal density of 1 cell μl⁻¹, and the spheres that formed after 10 d were counted.

Nuclear protein extraction, immunoprecipitation and immunoblotting. Nuclear proteins were extracted using the CellLytic NuCLEAR Extraction Kit (Sigma) according to the manufacturer’s instructions. The extraction of total proteins was performed using immunoprecipitation lysis buffer (25 mM Tris·HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate (SDS), protease and phosphatase inhibitors). Immunoprecipitation and immunoblotting with corresponding antibodies was performed as described previously²⁶. All experiments were independently repeated three times.

GST-tagged protein purification. GST–KDM1A–WT, GST–KDM1A–S683A/S687A and GST–USP22 in the bacterial expression plasmid pGEX–4T-1 were expressed in Escherichia coli BL21 under 0.5 mM isopropyl β-D-thiogalactopyranoside at 16 °C. Cells were resuspended in BugBuster Protein Extraction Reagent (Novagen). The proteins were purified using glutathione beads (Sigma) according to the manufacturer’s protocol.

In vitro kinase assay. The purified GST–KDM1A–WT or GST–KDM1A–S683A/S687A fusion protein as a substrate was incubated with 10 ng of purified GSK3β and CK1α (SignalChem) in kinase assay buffer containing 2 μCi [γ-³²P]ATP per reaction. The kinase reaction was performed at 30 °C for 30 min, and the reaction was terminated by adding SDS sample buffer. The resultant product was then subjected to SDS–polyacrylamide gel electrophoresis and autoradiography.

In vivo and in vitro detection of KDM1A ubiquitlation. Cells were co-transfected with the indicated plasmids for 48 h and then treated with the proteasome inhibitor MG132 (20 μM) for 6 h. Cells were lysed using RIPA lysis buffer (50 mM Tris-base pH 6.8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 10 mM NaF, 10 mM diethiothreitol (DTT), 0.2 mM NaVO₃, 1% cocktail protease inhibitors, 1 mM phenylmethylsulfonyl fluoride (PMSF)). Cell lysates were immunoprecipitated using the indicated antibodies and washed three times with RIPA buffer. To exclude nonspecific ubiquitin-modified species from the KDM1A complex, we washed the immunoprecipitates three times using a ubiquitination wash buffer (50 mM Tris-base pH 6.8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1 M urea, 1 mM N-ethylmaleimide (NEM), and protease inhibitors).²⁶

In vitro deubiquitylation of KDM1A by USP22 was performed as described previously³. HF U-251 cells were transfected with Flag–KDM1A and HA–UbI expression vectors. After treatment with MG132 for 6 h, KDM1A protein containing ubiquitylated KDM1A was purified from the cell lysates using Flag–beads and washed extensively using Flag lysis buffer (50 mM Tris·HCl pH 7.8, 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% sarkosyl, 1 mM DTT, and protease inhibitors) and the remaining ubiquitin was removed using the wash cocktail. The proteins were then eluted with Flag–peptides (Sigma) in BC100 buffer (25 mM Tris·HCl pH 7.8, 100 mM NaCl). The recombinant GST–tagged USP22 protein was expressed in BL21 cells and purified using GST beads. For the deubiquitylation assay reaction in vitro, the ubiquitylated KDM1A protein was incubated with different amount of recombinant USP22 protein (10 ng or 100 ng) in a deubiquitylation buffer (50 mM Tris·HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol) for 2 h at 37 °C. The result reactions were subjected to immunoblotting analysis.

In vitro cell viability assay. NHAs, GSC11 cells, or GSC20 cells in 96-well plates were treated with TMZ (100 μM), tigedulib (2.5 or 5 μM), or both tigedulib and TMZ
for the indicated numbers of days. Cell viability was analysed using an XTT assay kit (Sigma) according to the manufacturer’s instructions.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChIP) was performed as described previously\(^2\). Briefly, 2 × 10^6 cells were used for each reaction. The resulting precipitated DNA samples were used for quantitative polymerase chain reaction (PCR) analysis.

**Quantitative PCR.** Total RNA was extracted using Trizol reagent (Invitrogen) and reverse-transcribed using the iScript Reverse Transcription Supermix (Bio-Rad) according to the manufacturer’s instructions. The reverse-transcribed cDNA products were used for quantitative PCR analysis using SYBR Select Master Mix (Life Technologies). All quantitative PCR results are mean ± s.e.m. from three independent experiments. Supplementary Table 2 contains detailed information about the sequence of the used primers.

**Immunohistochemical and immunofluorescence analysis.** Anonymous archived human glioma specimens (90 grade II astrocytomas and 95 glioblastomas) were obtained from The University of Texas MD Anderson Cancer Center under a protocol approved by the Institutional Review Board. All tissue samples were collected in compliance with informed consent policy. For antibody staining, tissue slides were deparaffinized, rehydrated through an alcohol series, and then stained with primary antibodies against human KDM1A (ab17721, Abcam), USP22 (ab4812, Abcam), phospho-GSK3β (9336) and GSK3β (9315, Cell Signalling Technology) using standard procedures\(^2\). To quantify GSK3β, KDM1A and USP22 expression, we measured the immunostaining scores of GSK3β, KDM1A or USP22 in human glioblastoma tissues as described previously\(^2\). Briefly, staining of nuclear GSK3β, KDM1A or USP22 was scored according to the percentage of cells with positive nuclear staining and to the staining intensity. We assigned the percentage score as follows: 0 if no cell had nuclear staining, 1 if 0–25% of cells had nuclear staining, 2 if 25–50%, 3 if 50–75%, 4 if more than 75% of cells had nuclear staining. We scored the staining intensity as 0 for negative, 1 for weak, 2 for moderate, and 3 for strong. The total score was obtained by multiplying the percentage score by the intensity score. Two individuals who were both blinded to the slides examined and scored each sample. The final score was the mean value of the two scores provided by the individuals.

For immunochemistry analysis of tissue sections, 15 frozen glioblastoma sections were fixed with paraformaldehyde and incubated with antibodies against KDM1A (ab90966, Abcam) and GSK3β (9315, Cell Signalling Technology) or KDM1A and USP22 (ab4812, Abcam) at 4 °C overnight, followed by staining with Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse IgG for 1 h at room temperature. Coverslips were mounted on slides using mounting medium with DAPI (H-1200, Vector Laboratories). Immunofluorescence images were acquired using a deconvolutional microscope (Zeiss). The percentages of nuclear GSK3β/KDM1A double-stained cells and nuclear GSK3β-negative but KDM1A-positive cells were analysed and compared in 5 random selected microscope fields of 15 specimens (75 microscope fields in total).

For immunofluorescence analysis of cultured cells, GSCs were grown on chamber slides precoated with poly(L-lysine). Cells were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.1% Triton X-100 for 5 min, and blocked with 1% BSA in PBS. Immunostaining was performed using the primary antibodies indicated in the related figures, and the above secondary antibodies.

**Intracranial tumour-cell injection.** All animal experiments were approved by MD Anderson’s Institutional Animal Care and Use Committee. The sample sizes of the animals were justified by statistical considerations and statistical power analyses. The animals were randomized to different experimental groups. The investigators were blinded to allocation during experiments and outcome assessment. GSC11 or GSC20 cells (5 × 10^6 per mouse) were injected intracranially into 6- to 8-week-old male nude (nu/nu) mice (5 mice for each group) as described previously\(^2\). At the end of the experiments, the mice were humanely killed, and each mouse’s brain was harvested, fixed in 4% formaldehyde, and embedded in paraffin. Tumour formation was determined by histologic analysis of tissue sections stained with haematoxylin and eosin. Tumour volumes were calculated using the formula V = (a/b) × a^2 × b, where a and b are the tumour’s short axis and long axis, respectively.

For in vivo therapeutic experiments, 1 d after intracranial implantation of tumour cells into nude mice (8 mice for each group), tideglusib (25 mg kg\(^{-1}\) d\(^{-1}\)) or TMZ (20 mg kg\(^{-1}\) d\(^{-1}\)) in a vehicle of dimethyl sulfoxide/polyethylene glycol 300 (Sigma, 1:4 ratio) was injected intraperitoneally every other day for 30 d. For combinatorial treatment, mice received injections of TMZ or tideglusib on alternating days for 30 d. The vehicle alone was used for the negative control group. In a set experiment to analyse mouse survival, animals were humanely killed when they were moribund; the remaining animals were humanely killed 90 d after tumour-cell injection.

**Statistics and reproducibility.** We assessed differences in the human glioblastoma data using the Pearson correlation test, in the in vitro data using the two-tailed Student’s t-test, and in the in vivo data using one-way analysis of variance. Survival analysis was conducted using the Kaplan–Meier model with a two-sided log-rank test. The results for statistical significance tests are included in the legend of each figure. The results in Figs 1e,f, 3e, 5a,b,d,h and 7cf, and Supplementary Figs 2d, 3e,g were independently reproduced three times with similar results. The in vivo mouse studies in Fig. 6a,b contain five mice for each group, and were independently performed two times with similar results. The in vivo mouse studies in Fig. 7d,f contain eight mice for each group. The results in Supplementary Figs 1d and 7c were independently performed two times. All other representative images have been independently reproduced three times with similar results. A P value of less than 0.05 was considered statistically significant.

**Data availability.** The source data for Fig. 6a,b and Supplementary Figs 1d and 7c have been provided as Statistics Source Data (Supplementary Table 3). All data that support the conclusions are available from the authors on request, and/or available in the manuscript itself.

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Supplementary Figure 1 GSK3β stabilizes KDM1A by inhibiting the proteasome degradation pathway. (a) KDM1A protein levels were detected in GSK3β knockout (GSK3β+/−) mouse embryonic fibroblasts (MEFs), GSK3β knockout (GSK3β−/−) MEFs, and GSK3β−/− MEFs with reconstituted expression of GSK3β. (b) Effect of GSK3β knockdown on KDM1A expression. GSC11 cells were transfected with GSK3β siRNAs, and KDM1A expression was detected by immunoblotting. (c) Effect of GSK3α knockdown on KDM1A expression. KDM1A protein levels were detected in GSC11 cells after transfection of GSK3α siRNAs. (d) KDM1A mRNA levels were detected in GSK3β+/− and GSK3β−/− MEFs. GAPDH was used as an internal control. (e) KDM1A protein levels were detected in β-catenin+/+ and β-catenin−/− MEFs. (f) KDM1A is regulated by the ubiquitin-proteasome pathway. HS683 cells were treated with cycloheximide (CHX) with or without the proteasome inhibitor MG132 for the indicated times and KDM1A levels were detected by immunoblotting. (g) 293T cells were transfected with Flag-KDM1A and the constitutively active form of GSK3β (GSK3β-CA) or the control vector, and then treated with CHX as indicated. Expression of KDM1A was detected by immunoblotting. Fold change of western blot bands was determined as described in Fig. 1b and shown. Source data of Supplementary Fig. 1d can be found in Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 8.
Supplementary Figure 2 GSK3β phosphorylates KDM1A serine 683 after priming phosphorylation by CKIα. (a) The amino acid sequence of KDM1A shows the sites potentially targeted by GSK3β. (b) Immunofluorescence assays were performed in HFU-251 MG and GSC11 cells using antibodies against KDM1A and GSK3β. Scale bar, 50 μM. (c) Effects of mutations of various Ser/Thr residues on the ubiquitination of KDM1A. 293T cells were transfected with HA-Ubi, Myc-GSK3β, and wild-type Flag-KDM1A or various mutants with serine/threonine-to-alanine mutations. Cells were treated with MG132 for 4 h, harvested, and then lysed for immunoprecipitation (IP) analysis. (d) 293T cells were transfected with wild-type Flag-KDM1A (WT) or mutant with serine 683-to-alanine (S683A) or aspartic acid (S683D) mutation. Cells were treated with CHX for the indicated times. Band intensity of KDM1A from western blots was quantified as described in Fig. 1b (mean ± s.d., n=3 independent experiments, two-tailed Student’s t-test). *P<0.01. (e) GSC11 or HFU-251 MG cells were treated with the CKI inhibitor D4476 or D4476 plus LiCl for 6 h. Cell lysates were subjected to immunoblotting analysis. Fold change of western blot bands was determined as described in Fig. 1b and shown. (f) Reciprocal interaction between Flag-KDM1A and HA-CKIα. 293T cells were transfected with Flag-KDM1A and HA-CKIα, HA-CKIβ, or HA-CKIε plasmids. Cell lysates were immunoprecipitated with the HA-tag (left panel) or Flag-tag antibody (right panel) and then analyzed by immunoblotting using the indicated primary antibodies. Blots were incubated with a HRP-conjugated secondary antibody (light chain specific). (g) Reciprocal interaction of endogenous KDM1A with CKIα. GSC11 cell lysates were immunoprecipitated using antibodies against CKIα, CKIδ, and CKIε (left panel), or KDM1A (right panel). The immunoprecipitates were subjected to immunoblotting analysis using the indicated primary antibodies and a HRP-conjugated secondary antibody (light chain specific). Inputs correspond to 2% total cell lysates. (h) HF-U251 MG cells were transfected two independent siRNAs against CKIα, CKIβ, or CKIε, and then the expression of KDM1A was detected by western blotting. Uncropped images of blots are shown in Supplementary Fig. 8.
Supplementary Figure 3 USP22 deubiquitinates and stabilizes KDM1A. (a) Effect of different deubiquitinating enzymes (DUBs) on the expression of KDM1A. 293T cells were transfected with different DUBs and then lysed for immunoblotting to detect KDM1A expression. (b) Effect of various DUBs on the ubiquitination of KDM1A. 293T cells were transfected with Flag-KDM1A, Myc-Ubi, and USP15, USP21, USP22, or USP28 and then treated with MG132 for 6 h before harvest. Cell lysates were immunoprecipitated using an anti-KDM1A antibody and then subjected to immunoblotting analysis using the indicated antibodies. (c,d) 293T cells were transfected with Flag-KDM1A, Myc-Ubi, and HA-USP22 or Flag-USP28. Cell lysates were immunoprecipitated using antibodies against Myc-tag (c) or KDM1A (d). (e) Knockdown of USP22 does not affect KDM1A mRNA level. GSC11 cell were transfected with control siRNA or USP22 siRNA and KDM1A mRNA were detected by real-time PCR. Values were normalized to that in control (mean ± s.e.m., n=3 independent experiments, two-tailed Student’s t-test). GAPDH mRNA was used as an internal control. P>0.05. (f) 293T cells were transfected with HA-USP22 or Flag-USP28 plasmid and then treated with 50 μg/mL CHX for the indicated times. Cell lysates were subjected to immunoblotting analysis as indicated. (g) The expression levels of nuclear KDM1A, USP22 and GSK3β in Fig. 3f were determined by quantification of the intensity of western blot bands, using the Lamin B for normalization and the results are expressed as level relative to NHA-E6/E7 cells. Then the correlation of nuclear KDM1A/USP22 and KDM1A/GSK3β levels in different cell lines was analyzed (mean ± s.e.m., n=3 independent experiments, Pearson correlation test). (h) Immunofluorescence assay was used to analyze the co-localization of KDM1A and USP22 in GSC11 cells. Scale bar, 20μm. Uncropped images of blots are shown in Supplementary Fig. 8.
Supplementary Figure 4 GSK3β promotes the binding of KDM1A with USP22. 293T cells were transfected with Flag-KDM1A, HA-USP22, and GSK3β-CA or GSK3β-KD and then treated with MG132 for 6 h before harvest. Cell lysates were immunoprecipitated with antibodies against (a) HA or (b) KDM1A and then analyzed by immunoblotting using the indicated antibodies. Uncropped images of blots are shown in Supplementary Fig. 8.
Supplementary Figure 5 GSK3β and USP22 promote stem cell self-renewal through KDM1A. (a) ChIP assays were performed in GSC11 cells transfected with control shRNA, shRNA targeting KDM1A, GSK3β or USP22 using an antibody against KDM1A and primers in the promoter regions of BMP2, CDKNA1, and GATA6. ACTB promoter was used as a negative control. Values are the percentage to input (mean ± s.e.m., n=3 independent experiments, two-tailed Student’s t-test). *P<0.01. (b) KDM1A expression was detected by immunoblotting in GSC11 cells stably expressing two individual shRNAs targeting KDM1A. (c) Primary (1st) and secondary (2nd) neurosphere formation were assessed in GSC11 and GSC20 cells using two different KDM1A shRNA. Scale bar, 500 μm. (d) Effects of KDM1A knockdown on the expression of stem cell self-renewal and differentiation markers. GSC11 and GSC20 cells expressing KDM1A shRNAs were analyzed by immunoblotting using the indicated antibodies. (e) Immunofluorescence assays were performed to detect the expression of self-renewal and differentiation markers in GSC11 cells expressing KDM1A shRNAs. Scale bar, 100 μm. (f) GSC11 cells overexpressing KDM1A shRNAs were reconstituted by the expression of shRNA-resistant KDM1A (ShR), and neurosphere formation was assessed. Scale bar, 500 μm. (g) USP22 knockdown inhibits neurosphere formation through KDM1A. GSC11 cells overexpressing USP22 shRNAs were reconstituted by the expression of wild-type KDM1A, and neurosphere formation was assessed. Scale bar, 500 μm. (h) GSC11 cells stably expressing two different shRNAs targeting GSK3β were analyzed by immunoblotting. (i) mRNA levels of KDM1A in GSC11 cells expressing sh-control, sh-GSK3β, sh-GSK3β+KDM1A S683D, and sh-GSK3β+KDM1A S683A were analyzed by real-time PCR (mean ± s.e.m., n=3 independent experiments, two-tailed Student’s t-test). (j) The secondary neurosphere formation efficiency (spheres/cells plated) of GSC11 cells stably expressing the indicated shRNAs or proteins were calculated (mean ± s.e.m., n=3 independent experiments, two-tailed Student’s t-test). *P<0.05. Uncropped images of blots are shown in Supplementary Fig. 8.
Supplementary Figure 6 Immunohistochemical analysis of GSK3β and KDM1A in mouse brain tumors derived from GSC11 cells expressing Sh-Control, Sh-GSK3β, Sh-GSK3β+KDM1A S683D, or Sh-GSK3β+KDM1A S683A. Insets: high magnification images corresponding to the areas marked by yellow dot lines. Scale bar for H&E staining, 50 μm; Scale bar for IHC, 25 μm.
Supplementary Figure 7 Tideglusib inhibits GSC self-renewal and tumor formation. (a) Effect of tideglusib on Tau phosphorylation. GSC11 cells were treated with the indicated concentrations of tideglusib for 6 h (left panel) or with tideglusib at a final concentration of 2.5 µM for the indicated times (right panel) in the presence of MG132. Cell lysates were analyzed by western blotting using antibodies against Tau or phospho-Tau (Ser396). Fold change of western blot bands was determined as described in Fig. 1b and shown. (b) Effect of tideglusib on the expression of stem cell self-renewal and differentiation markers. GSC11 and GSC20 cells were treated with 5 µM tideglusib for 48 h, and Oct4, CD133, and Tuj-1 expression was analyzed by immunofluorescence. Scale bar, 50 µm. (c) NHAs were treated with 5 µM tideglusib for the indicated times, and cell viability was analyzed using XTT assays. (d) Schedule for animal treatment. For treatment with TMZ (20 mg/kg/d) or tideglusib (25 mg/kg/d) alone, mice were intraperitoneally injected every other day. For combinatorial treatment, mice received injections of TMZ or tideglusib on alternating days for 30 d. Source data of Supplementary Fig. 7c can be found in Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 8.
Supplementary Figure 8 Uncropped images of blots. Uncropped images of scanned western blots shown in Figures and Supplementary Figures are provided.
Supplementary Figure 8 continued
Supplementary Figure 8 continued
Supplementary Figure 8 continued
Supplementary Figure 8 continued
Supplementary Table Legends

- **Supplementary Table 1** Detail information about the antibodies used in the study.
- **Supplementary Table 2** Detail information about the sequence of siRNAs and primers used in the study.
- **Supplementary Table 3** Statistics source data of Fig.6a,b, Supplementary Fig.1d, and Supplementary Fig.7c.