GINS1 promotes the proliferation and migration of glioma cells through USP15-mediated deubiquitination of TOP2A

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Highlights
GINS1 expression was upregulated and associated with poor clinical outcome in glioma
GINS1 aggravated glioma malignant phenotypes in vitro and in vivo
GINS1 physically interacts with TOP2A
GINS1 regulates glioma progression through USP15-mediated deubiquitination of TOP2A

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GINS1 promotes the proliferation and migration of glioma cells through USP15-mediated deubiquitination of TOP2A

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SUMMARY

GINS1 is a GINS complex subunit that functions along with the MCM2-7 complex and Cdc45 in eukaryotic DNA replication. Despite the significance of the GINS complex in the switch between quiescence and proliferation of glioma cells inside and outside the perinecrotic niche, the biological functions and the underlying mechanism of GINS1 remain unclear. Unlike in normal cells and tissues, GINS1 expression level was significantly upregulated in glioma cells and tissues. High expression of GINS1 predicted an advanced clinical grade and a poor survival. Functional assays revealed that GINS1 aggravated glioma malignant phenotypes in vitro and in vivo. Mechanistically, this study identified that GINS1 physically interacts with TOP2A. GINS1 promotes glioma cell proliferation and migration through USP15-mediated deubiquitination of TOP2A protein. Our results delineate the clinical significance of GINS1 in glioma and the regulatory mechanisms involved in glioma cell proliferation and migration. This work provides potential therapeutic targets for glioma treatment.

INTRODUCTION

Glioma is the most common malignancy in the CNS, accounting for approximately 30% and 80% of all major nervous tumor cases and brain tumor cases, respectively (Siegel et al., 2021; Miller et al., 2021). Glioma is a lethal and aggressive cancer with high mortality and morbidity. According to the World Health Organization (WHO), gliomas are divided into four grades: pilocytic astrocytoma (WHO grade I), diffuse astrocytoma (WHO grade II), anaplastic astrocytoma (WHO grade III), and glioblastoma multiform (WHO grade IV) (Kris-tensen et al., 2019; Louis et al., 2016). Despite improvements and advances made in recent years in glioma treatment options, including surgical resection, radiation therapy, and chemotherapy, the prognosis of patients with glioma is still dismal (Silantyev et al., 2019). It has been estimated that the 5-year survival rate of patients with glioma is extremely low, which is even less than 10% for those with advanced WHO grades (Wen et al., 2020). Therefore, it is essential to uncover the detailed molecular mechanisms in glioma tumorigenesis and metastasis.

GINS family has four members, GINS5, GINS1, GINS2, and GINS3 (Go, Ichi, Nii, and San in Japanese for five, one, two, and three, respectively, in English). GINS1, also known as Partner of SLD Five 1 (PSF1), forms a heterotetramer complex with SLDS, PSF2, and PSF3. Through recruiting Cdc45 and DNA polymerase, this complex is essential for the initiation and elongation of DNA replication (Chang et al., 2007; Gambus et al., 2006; Kanemaki et al., 2003). GINS1 was initially found to be predominantly expressed in highly proliferating cells instead of mature cells. Later on, it was found that GINS was involved in the tumorigenesis of various cancers, including breast cancer, colorectal cancer, and hepatocellular carcinoma (Toda et al., 2020; Bu et al., 2020; Li et al., 2021). Although growing evidence has shown that the GINS complex serves as a switch between quiescence and proliferation of glioma cells inside and outside the perinecrotic niche (Kimura et al., 2019), the specific function of GINS1 and the underlying mechanism in glioma remain largely unknown.

Topoisomerases play a vital role in DNA world, being essentially involved in administering DNA topology (Wang, 2002). Topoisomerases show elevated levels of expression in tumor cells. There are total of six topoisomerases in humans, which include two enzymes in each subfamily: IA (TOP3A and TOP3B), IB (TOP1 and mitochondrial TOP1), and IIA (TOP2A and TOP2B) (Cuya et al., 2017; Hou et al., 2020). Topoisomerase
II alpha (TOP2A) is an enzyme involved in DNA maintenance and repair as well as cell proliferation (Albandine et al., 2009). By creating a DNA-linked protein gate through which another intact DNA duplex passes, TOP2A mainly supports DNA decoling, chromosome segregation during cell cycle anaphase, and DNA replication that are necessary processes for cell proliferation (Schindbeck et al., 2010), so TOP2A is also a cell proliferation marker in normal and neoplastic tissues (Shvero et al., 2008). TOP2A plays a critical role in glioma cell proliferation and migration (Song et al., 2018; Deguchi et al., 2017). In malignant cells, TOP2A protein overexpression might reflect the proliferative advantage of these cells and qualitative alterations caused by malignant transformation and dedifferentiation (Nakopoulou et al., 2001). The immunohistochemical (IHC) method for in situ determination of TOP2A has been extensively validated to reflect the exact enzyme activity closely in formalin-fixed paraffin-embedded human tissues, leading to the prognostic and predictive importance of this test in other neoplasms (Shamaa et al., 2008). Posttranslational modifications of TOP2A, including ubiquitylation, SUMOylation, and phosphorylation, have been implicated in the regulation of TOP2A localization, turnover, and activities (Ntiss, 2009; Chen et al., 2013; Vos et al., 2011). TOP2A ubiquitination by BMI1/RING1A or APC/C-Cdh1 promotes its degradation, while its ubiquitination by BRCA1 stimulates its decatenation activity and its proteasomal degradation (Alchanati et al., 2009; Egueren et al., 2014; Lou et al., 2005; Shinagawa et al., 2008). The reversal posttranslational modification of ubiquitylation, deubiquitination, is also important in the regulation of TOP2A function (Chen et al., 2015). Despite the importance of ubiquitylation on TOP2A function and its steady-state levels, the mechanisms that mediate its deubiquitination in glioma cell proliferation and migration have not yet been identified.

Ubiquitination is a posttranslational protein modification process that attaches single or multiple ubiquitin molecules to a target protein and affects its stability and function. Ubiquitin conjugation is mediated by the sequential action of three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s) (Hershko and Ciechanover, 1998). Lysine (K) 48-linked polyubiquitin chains are the best-studied ubiquitin chains that target proteins for proteasomal degradation, whereas some other ubiquitin chains, such as the K63-linked polyubiquitin chains, can mediate nondegradative functions (Chen and Sun, 2009). The specificity of protein ubiquitination is regulated by E3 ubiquitin ligases, which exist in large numbers in mammalian cells and mediate substrate recognitions (Li et al., 2008). Ubiquitination is a reversible reaction since the ubiquitin chains can be cleaved by a large family of ubiquitin-specific proteases, termed deubiquitinases (Reyes-Turcu et al., 2009; Zou et al., 2014). Deubiquitinases can have specificity for both protein substrates and ubiquitin chains, thereby bringing in a different level of diversity in the regulation of ubiquitination. Deregulation of the deubiquitination process is frequently associated with tumorigenesis (Sun et al., 2020; Mevissen and Komander, 2017). Among deubiquitinases, ubiquitin-specific protease 15 (USP15) is a member of the largest subfamily of cysteine protease deubiquitinases (Mevissen and Komander, 2017). USP15 was previously reported to regulate TGF-β signaling and cancer cell survival. USP15 is aberrantly regulated or mutated in many human cancers, including glioma (Vos et al., 2009; Inui et al., 2011; Eichhorn et al., 2012). However, the detailed function of USP15, particularly in the regulation of glioma cell proliferation and migration, has remained unclear.

In the current study, we revealed that GINS1 was significantly upregulated in glioma cells and tissues. High expression of GINS1 predicted an advanced clinical grade and a poor survival. GINS1 exacerbated glioma malignant phenotypes in vitro and in vivo. Mechanistically, this study identified that GINS1 physically interacts with TOP2A and that GINS1 promotes glioma cell proliferation and migration through USP15-mediated deubiquitination of the TOP2A protein. Thus, our results, for the first time, identified a GINS1-USP15-TOP2A axis that regulates glioma cell proliferation and migration and will provide a potential therapeutic strategy for glioma.

RESULTS
GINS1 expression was significantly upregulated in glioma and associated with poor clinical glioma outcomes

Microarray expression profiling was performed to identify differentially expressed genes (DEGs) in 5 pairs of human glioma tissue specimens and corresponding adjacent nontumor tissue specimens. Several notably upregulated DEGs and downregulated DEGs existed between human glioma tissue specimens and corresponding adjacent nontumor tissue specimens (Figures 1A–1C). Eight candidate genes (SGK223, BORCS8, GINS1, UBDT2, THOC3, RFC4, HOXD4, and ANXA2P2) were further identified based on the Cancer Genome Atlas (TCGA) database and gene chip. High-content screening (HCS) analysis was introduced to verify these eight candidate genes in U251 cells. Among them, only GINS1 silencing outstandingly inhibited glioma cell proliferation (Figures 1D–1F), suggesting that GINS1 might be involved in glioma progression.
To explore GINS1 expression in glioma, we first used TCGA database to show that GINS1 expression was increased in glioma (n = 156), compared with other normal brain tissues (n = 5) (Figure 2A). We then compared GINS1 expression level between four human glioma cell lines and one normal human glial cell line, HEB by RT-PCR and Western blot analyses. We found that GINS1 mRNA and protein levels were upregulated in four human glioma cell lines, T98G, U251, U373, and A172, compared with HEB (Figures 2B and 2C). Next, we sought to examine the expression level of GINS1 first in four pairs and then in 80 pairs of our human glioma tissue and adjacent noncancerous tissues that we collected. GINS1 expression levels detected by Western blotting was increased more significantly in four human glioma tissue samples, compared with levels in four adjacent noncancerous tissues (Figure 2D). Furthermore, RT-PCR and Western blot analyses were utilized to detect GINS1 expression in 80 paired glioma and adjacent noncancerous tissues. The expression patterns of GINS1 between these 80 paired samples were found to be similar to that of four paired samples. The expression level of GINS1 is positively correlated with the tumor clinical stage (Figures 2E and 2F). In order to examine the spatial expression level of GINS1, we next used tissue microarrays and IHC to analyze the protein level of GINS1 between glioma tissues and normal brain samples. Similar to the mRNA results, the IHC staining intensity of GINS1 protein was positively correlated with the tumor clinical stage (Figure 2G). GINS1 protein expression in glioma tissues was also higher than that in matched adjacent noncancerous tissues (Figure 2H). Kaplan–Meier survival analysis from TCGA and CGGA databases showed that patients with glioma with high GINS1 expression had poorer overall survival (OS), progression-free survival (PFS), and disease-free survival rates (DFS) than patients with glioma with low GINS1 expression (Figures 2I–2K and S1A–S1C). Similar results were obtained in the specimens of the validation cohort of patients with glioma (Figures S1D and S1E). The clinicopathological analysis indicated that age and tumor stage were also associated with the GINS1 variable (chi-square test, p = 0.001). In contrast, cell differentiation grade of glioma and gender exhibited no significant correlation with GINS1 expression level (Table 1). These data indicated that GINS1 was upregulated in glioma and correlated with the clinicopathological features and poor prognosis of patients with glioma.
GINS1 silencing suppresses glioma cell proliferation, migration, and invasion, but promotes glioma cell apoptosis in vitro and in vivo

Two lentiviruses expressing shRNAs were constructed to silence GINS1 expression in U251 and T98G cells to investigate the functional role of GINS1 in glioma cells (Figures 3A–3D). The MTT assay demonstrated that GINS1 silencing markedly inhibited cell proliferation (Figures 3E, 3F, S2A, and S2B). Colony formation assays indicated that silencing GINS1 expression resulted in much smaller and fewer colonies (Figures 3G and 3H). In addition, Transwell assay and wound healing assay showed that GINS1 silencing dramatically decreased glioma cell migration and invasion, respectively (Figures 3I and 3J). Given that matrix metalloproteinase 9 (MMP-9) protein and EMT-related proteins participated in tumor cell proliferation, migration,
and invasion, the protein levels of MMP9, N-cadherin, Vimentin, and E-cadherin in GINS1-silenced U251 and T98G cells were measured. GINS1 silencing decreased the expression levels of N-cadherin, Vimentin, and MMP9 but enhanced the E-cadherin expression level in U251 and T98G cells (Figures 3K and 3L). These observations demonstrate that GINS1 silencing suppresses the proliferation, migration, and invasion of glioma cells. Then, the impact of GINS1 on cell apoptosis was assessed through flow cytometry. After GINS1 silencing, the percentage of apoptotic cells was significantly increased (Figures 3M and 3N). Additionally, GINS1 silencing decreased the expression levels of the antiapoptotic proteins Bcl-2 and Bcl-xl but enhanced the proapoptotic protein BAX in U251 and T98G cells (Figures 3O and 3P). Furthermore, we found that GINS1 silencing has no influence on the proliferation, migration, invasion, and apoptosis of the normal glial cell HEB (Figures S3A–S3I). The xenograft tumor model was generated to determine whether GINS1 contributes to tumor progression in vivo. GINS1 shRNA (shGINS1#1)-transfected tumor cells and control shRNA (shCtrl)-transfected cells were established and then injected subcutaneously into the right dorsal flanks of the nude mice. After 5 weeks of injection, the tumor volume and tumor weight of the shGINS1#1 group were significantly reduced compared to those of the shCtrl group (Figures 4A–4C). We found that downregulated Ki67 expression levels were detected in the GINS1-silenced group by immunohistochemistry (Figure 4D). These findings indicated that GINS1 silencing suppressed glioma cell proliferation, migration, and invasion, but promoted glioma cell apoptosis in vitro and in vivo.

GINS1 overexpression promotes the proliferation, migration, and invasion of glioma cells, but suppresses apoptosis of glioma cells in vitro and in vivo

To further explore the biological role of overexpressed GINS1 in glioma, the glioma cell line A172 with GINS1 overexpression was established (Figures 4E and 4F). MTT assay and colony formation assay showed that GINS1 overexpression significantly promoted glioma cell proliferation (Figures 4G, 4H, and S2C), while Transwell assay and wound healing assay proved that GINS1 overexpression dramatically increased the invading and migrating abilities of glioma cells, respectively (Figures 4I and 4J). Next, the effects of GINS1 overexpression on cell apoptosis were further investigated. The percentage of apoptotic cells significantly decreased in GINS1-overexpressing A172 cells (Figure 4K). Figures 4L and 4M show that GINS1-overexpressing (GINS1 cDNA-transfected) A172 cells displayed higher MMP9, N-cadherin, Vimentin, Bcl-2, and Bcl-xl expression levels and lower levels of E-cadherin and BAX than control plasmid (pcDNA3.1)-transfected A172 cells (Figures 4L and 4M). Glioma cells with an endogenous or overexpressed GINS1 expression level were subcutaneously injected into the left flank of nude mice under the same conditions used for a xenograft tumor model of GINS1-silenced glioma cells to investigate the biological role of overexpressed GINS1 in glioma in vivo. The tumors formed by GINS1-overexpressing A172 cells had larger tumor volumes and heavier tumor weights than those induced by control plasmid (pcDNA3.1)-transfected A172 cells (Figures 4N–4P). In addition, the Ki67 expression level was upregulated in the tumors formed by GINS1-overexpressing glioma cells compared with those induced by control plasmid (pcDNA3.1)-transfected A172 cells (Figure 4Q). These above results revealed that overexpressed GINS1

Table 1. Correlation between GINS1 expression and clinicopathological characteristics of the patients with glioma

| GINS1 expression | Cases | High (%) | Low (%) | P     |
|------------------|-------|----------|---------|-------|
| Age (years)      |       |          |         |       |
| ≤ 50             | 121   | 90 (74.38) | 31 (25.62) | 0.001 |
| > 50             | 52    | 44 (84.62) | 8 (15.38) |       |
| Gender           |       |          |         | 0.199 |
| Male             | 109   | 84 (77.06) | 25 (22.94) |       |
| Female           | 64    | 33 (51.56) | 31 (48.44) |       |
| Differentiation  |       |          |         |       |
| Well-moderate    | 81    | 55 (67.90) | 26 (32.1) | 0.211 |
| Poor             | 92    | 62 (67.39) | 30 (32.61) |       |
| Tumor stages     |       |          |         | 0.001 |
| I + II           | 102   | 7 (6.73) | 97 (93.27) |       |
| III + IV         | 71    | 72 (96.00) | 3 (4.00) |       |
significantly promoted the proliferation, migration, and invasion of glioma cells, but suppressed apoptosis of glioma cells in vitro and in vivo.

**GINS1 physically interacts with TOP2A, while GINS1 induces glioma cell proliferation and migration in a TOP2A-dependent manner**

We have proven that GINS1 silencing can suppress glioma cell proliferation and migration abilities. However, the specific regulatory mechanism signifying these effects is still unclear. Therefore, we performed affinity purification, silver staining, and LC-MS/MS assays to identify GINS1-associated proteins. The results revealed that TOP2A was a potential GINS1-interacting protein (Figure 5A) and verified by immunoprecipitation (Figure 5B), co-immunoprecipitation analysis (Figure 5C), and GST pull-down experiments of immunoprecipitated samples (Figure 5D). Detailed results from the mass spectrometric analysis are provided in Table S4. These experiments suggested that GINS1 physically interacts with TOP2A. TOP2A is an enzyme that controls the DNA topology and plays a critical role in glioma cell proliferation and migration (Albadine et al., 2009; Song et al., 2018; Deguchi et al., 2017). Thus, we examined whether GINS1 exerts oncogenic effects on glioma cell proliferation and
Migration in a TOP2A-dependent manner. To explore the role of GINS1 in regulating TOP2A expression, we examined TOP2A protein levels in cells by varying GINS1 expression levels. GINS1 depletion significantly decreased TOP2A protein level in U251 cells, whereas GINS1 overexpression considerably elevated TOP2A protein levels in A172 cells (Figure 5E). Notably, no statistically significant changes in TOP2A mRNA levels were observed between these groups (Figure 5F), which suggests that GINS1 modulates TOP2A expression posttranscriptionally. Similarly, exogenous GINS1 expression in HEK293T cells dramatically elevated...
exogenous TOP2A protein levels in a dose-dependent manner (Figure 5G). In addition, GINS1 expression levels were positively correlated with TOP2A protein levels in four glioma clinical specimens and glioma cells of five cell lines (Figures 5H and 5I). Then, we speculated that GINS1 might regulate the degradation pathway of the TOP2A protein. The autophagolysosome and ubiquitin–proteasome pathways are the main protein degradation pathways, so glioma cells were treated with ubiquitin–proteasome and autophagolysosome pathway inhibitors MG132 and chloroquine diphosphate (CQ), respectively. The results showed that MG132 could, to a certain extent, reverse GINS1 regulation of TOP2A protein (Figure 5J). The TOP2A abundance in glioma cells treated with cycloheximide (CHX), a de novo protein synthesis inhibitor, was detected to examine whether GINS1 regulates TOP2A protein stability. GINS1 overexpression in A172 cells significantly increased the
stability of TOP2A (Figure 5K). Accordingly, the silencing of GINS1 in U251 cells greatly destabilized the TOP2A protein (Figure 5L). The role of GINS1 in TOP2A ubiquitination was further identified. In the presence or absence of MG132, enforced GINS1 expression inhibited TOP2A ubiquitination, whereas GINS1 silencing promoted TOP2A ubiquitination (Figures 5M and 5N). The above data all showed that GINS1 stabilized the TOP2A protein by inhibiting its ubiquitination.

To further explore whether GINS1 induces the proliferation and invasion of glioma cells in a TOP2A-dependent manner, either a specific TOP2A plasmid or shRNA was utilized to conduct studies on U251 and A172 cells. The effects of TOP2A shRNA and plasmid on proliferation and migration were also examined. TOP2A shRNA impaired cell proliferation in GINS1-overexpressing A172 cells. In contrast, the TOP2A plasmid rescued the diminished cell proliferation in GINS1-silenced U251 cells (Figures 5O and 5P). A Transwell assay was then conducted to explore the effect of TOP2A plasmid or shRNA on glioma cell migration. The results showed that TOP2A shRNA attenuated cell migration in GINS1-overexpressing A172 cells, whereas TOP2A plasmid recovered the diminished cell migration in GINS1-silenced U251 cells.
Furthermore, we used Western blotting to analyze the protein levels of MMP9, N-cadherin, Vimentin, and E-cadherin in GINS1-silenced U251 and GINS1-overexpressing A172 cells. Figure 5S shows that TOP2A shRNA impaired the protein levels of MMP9, N-cadherin, and Vimentin, but recovered E-cadherin expression in GINS1-overexpressing A172 cells, while TOP2A plasmid restored the protein levels of MMP9, N-cadherin, and Vimentin, but attenuated E-cadherin expression in GINS1-silenced U251 cells (Figure 5S). These observations demonstrate that GINS1 induced the proliferation and migration of glioma cells in a TOP2A-dependent manner.

GINS1 promotes glioma cell proliferation and migration by USP15-mediated deubiquitination of TOP2A

Posttranslational modifications, including deubiquitination, are important regulators of TOP2A function (Chen et al., 2015). GINS1 mainly serves as a relevant factor of DNA replication and is unlikely to act...
directly as a deubiquitinase to mediate TOP2A ubiquitination. Therefore, it was speculated that some deubiquitinases might be responsible for the TOP2A ubiquitination induced by GINS1. mRNA microarray analysis was conducted using total RNA isolated from U251 shCtrl- and U251 shGINS1-transfected cells (n = 3), and USP15 was screened as the top of the differentially expressed deubiquitinase genes (Figure 6A). Then, the influence of USP15 on TOP2A ubiquitination was identified. The results revealed that USP15 significantly diminished TOP2A ubiquitination, suggesting that USP15 might be a potential TOP2A deubiquitinase (Figure 6B). Simultaneously, USP15 protein expression was actively correlated with GINS1 protein levels in glioma cell lines and tissues (Figures 6C and 6D). Moreover, USP15 knockdown enhanced the TOP2A inhibition induced by GINS1 silencing and USP15 overexpression promoted the upregulation of TOP2A expression induced by GINS1 overexpression (Figures 6E and 6F). Next, the effect of USP15 on TOP2A protein expression was examined. As shown in Figure 6G, TOP2A depletion in U251 cells significantly reduced USP15 protein expression. In contrast, enhanced TOP2A expression in A172 cells dramatically upregulated USP15 protein levels (Figure 6G). Furthermore, the impact of USP15 on TOP2A ubiquitination was assessed. USP15 overexpression significantly promoted the endogenous ubiquitination of TOP2A in A172 cells (Figure 6G). We further investigated the interaction between USP15 and TOP2A. Reciprocal co-immunoprecipitation analysis demonstrated that endogenous USP15 and TOP2A bound to each other in A172 cells (Figure 6H). We also assessed the impact of USP15 on TOP2A ubiquitination. Overexpression of USP15 markedly reduced K48-linked ubiquitination, but not K63-linked ubiquitination (Figures 6K and 6L). Therefore, USP15 interacts with TOP2A and prevents its K48-linked ubiquitination. The above findings showed that TOP2A regulation by GINS1 was dependent on USP15-mediated deubiquitination in glioma cells. In addition, USP15 overexpression reversed the inhibitory effects of GINS1 silencing on proliferation and migration ability of in U251 cells, whereas USP15 knockdown impaired the stimulatory effects of GINS1 overexpression on proliferation and migration ability of A172 cells (Figures 6M–6P). Consistently, USP15 shRNA impaired the protein levels of MMP9, N-cadherin, and Vimentin, but recovered E-cadherin expression in GINS1-overexpressing A172 cells, while the USP15 plasmid restored the protein levels of MMP9, N-cadherin, and Vimentin, but attenuated E-cadherin expression in GINS1-silenced U251 cells (Figure 6Q). Overall, these results revealed that GINS1 promotes glioma cell proliferation and migration by USP15-mediated deubiquitination of TOP2A.

Figure 8. Schematic diagram of the molecular mechanism by which GINS1 promotes the proliferation and migration of glioma cells

Schematic diagram of the molecular mechanism by which GINS1 promotes the proliferation and migration of glioma cells. GINS1 physically interacts with TOP2A, and TOP2A physically interacts with USP15. GINS1 promotes glioma cell proliferation and migration through USP15-mediated ubiquitination of the TOP2A protein.
Both TOP2A and USP15 are upregulated in glioma and correlated with poor prognosis

The above data revealed that TOP2A and USP15 led to the oncogenicity of GINS1. Thus, the expression and correlation of TOP2A and USP15 in clinical glioma specimens were examined. The expression levels of TOP2A and USP15 were markedly higher in glioma tissues than in the corresponding adjacent normal brain tissues. They showed a close correlation, as determined by IHC staining, Western blotting, and RT-PCR analysis (Figures 7A–7D). The GEPIA database also indicated that the expression levels of TOP2A and USP15 were higher in glioma tissues than in normal brain tissues (Figures S4A and S4B). Furthermore, the TCGA and CGGA survival data from Kaplan–Meier Plots revealed that patients with glioma with high TOP2A or USP15 expression had poorer overall survival (OS) rate, progression-free survival (PFS) rate, and disease-free survival (DFS) rate than patients with glioma with low TOP2A or USP15 expression (Figures 7E–7J and S4C–S4H). These observations suggested that higher TOP2A and USP15 expression reflected a poor glioma prognosis. In conclusion, TOP2A and USP15 were upregulated in glioma, and a good correlation predicted poor prognosis, reinforcing our proposal that GINS1 promotes glioma cell proliferation and migration by USP15-mediated TOP2A deubiquitination.

DISCUSSION

GINS1 mainly acts as a DNA replication-relevant factor that has been implicated in the progression of multiple types of tumors. This study explored whether GINS1 was upregulated in glioma cells and specimens. High expression of GINS1 predicted an advanced clinical grade and poor survival probability. Subsequent loss-of-function and gain-of-function experiments in vitro and in vivo showed that GINS1 significantly promoted cancer cell proliferation and migration, indicating the carcinogenic role of GINS1 in regulating glioma progression. Consistent with these results, recent studies have revealed that GINS1 serves as an oncogene to induce breast cancer, colorectal cancer, and hepatocellular carcinoma (Toda et al., 2020; Bu et al., 2020; Li et al., 2021). Notably, the underlying mechanism by which GINS1 functions as a glioma proliferation and migration promoter is to physically interact with TOP2A, while USP15 directly deubiquitinates TOP2A.

TOP2A is an enzyme that controls and alters DNA topologic states. TOP2A plays a crucial role in proliferation, metastasis, tumor recurrence, and metabolism and it is strictly controlled at the transcriptional, translational, and posttranslational levels (Albadine et al., 2009; Chen et al., 2013). In response to cellular stress, TOP2A protein levels change rapidly, primarily dependent on protein stability regulation (Shvero et al., 2008). Enhanced TOP2A expression was found in several cancers, including glioma (Albadine et al., 2009; Schindlbeck et al., 2010; Shvero et al., 2008; Song et al., 2018; Deguchi et al., 2017). In our study, we found that TOP2A mRNA and protein levels were upregulated in glioma tissues compared to those in adjacent normal brain tissues, that higher TOP2A expression reflected a poor glioma prognosis and that TOP2A overexpression promoted cell proliferation and migration. Importantly, the key findings of this study showed that GINS1 physically interacts with TOP2A, while GINS1 induces glioma cell proliferation and migration in a TOP2A-dependent manner. GINS1 overexpression significantly increased TOP2A protein levels to promote glioma cell proliferation and migration. Although GINS1 did not function as a deubiquitinase to impede TOP2A ubiquitination, we identified USP15 as the deubiquitinase that interacted with and directly deubiquitinates TOP2A.

USP15 belongs to a subfamily of deubiquitinases and is a widely expressed deubiquitinase that has been implicated in diverse cellular processes in cancer (Reyes-Turcu et al., 2009). USP15 has been shown to deubiquitinate receptor-activated SMADs and TRIM25 and antagonize Parkin-mediated mitochondrial ubiquitination, and USP15 regulates the TGF-β pathway and is a key factor in glioblastoma pathogenesis (Eichhorn et al., 2012; Pauli et al., 2014; Inui et al., 2011). Ubiquitination is an important cellular mechanism for targeted degradation of proteins, which is instrumental for various cellular processes, including but not limited to, transcription, cell proliferation progression, programmed cell death, and antigen presentation (Sun et al., 2020). Here, we identified USP15 as a deubiquitinase that interacted with and deubiquitinated TOP2A directly. Surprisingly, prior studies suggest that USP15 regulates TOP2A to maintain genome integrity, which is in agreement with our results (Fielding et al., 2018). Our data also revealed that USP15 mRNA and protein levels were upregulated in glioma tissues compared to those in adjacent normal brain tissues, that higher USP15 expression reflected a poor glioma prognosis and that USP15 overexpression promoted cell proliferation and migration. Moreover, USP15 knockdown reversed the TOP2A inhibition induced by GINS1 silencing and the upregulation of TOP2A expression induced by GINS1 overexpression. USP15 overexpression significantly reduced the endogenous ubiquitination of TOP2A, and silencing endogenous
USP15 promoted TOP2A ubiquitination. Importantly, similar to other deubiquitinases (Zhong et al., 2013), we found that USP15 deubiquitinates K48-linked TOP2A ubiquitination. In addition, USP15 overexpression reversed the inhibited proliferation and migration ability of glioma cells induced by GINS1 silencing in U251 cells, whereas USP15 knockdown impaired the elevated proliferation and migration ability of glioma cells induced by GINS1 overexpression in A72 cells. These results collectively suggest that GINS1 promotes glioma cell proliferation and migration by USP15-mediated TOP2A deubiquitination.

In summary, our integrated analyses have identified for the first time that a GINS1-USP15-TOP2A axis represents a critical mechanism in the proliferation and migration of glioma cells (Figure 8). Hence, blocking this axis may be a potent therapeutic strategy against glioma.

Limitations of the study
Although some significant discoveries were presented in this study, there are several limitations that must be mentioned. For example, we utilized subcutaneous xenograft tumor model, but not an intracranial orthotopic xenograft tumor model, to show the carcinogenic role of GINS1 in regulating glioma progression. However, no intracranial tumor xenograft model has been developed to assess the downregulation and overexpression of GINS1 in orthotopic tumorigenic properties. This limitation requires further investigation. Despite these limitations, we revealed that GINS1 promotes the proliferation and migration of glioma cells by USP15-mediated deubiquitination of TOP2A.

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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104952.

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We declare that they have no competing interests.

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**STAR METHODS**

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| **Antibodies**       |        |            |
| Antibodies           |        |            |
| Anti-GINS1/PSF1      | Abcam  | Cat#ab183524; RRID:AB_2922402 |
| Anti-TOP2A          | Abcam  | Cat#ab12318; RRID:AB_299006    |
| Anti-USP15           | Abcam  | Cat#ab71713; RRID: AB_2304348  |
| Anti-USP15           | Abcam  | Cat#ab97533; RRID: AB_10678830  |
| Anti-PRPF8           | Abcam  | Cat#ab24550; RRID: AB_448137    |
| Anti-VCP            | Abcam  | Cat#ab109240; RRID:AB_10862588  |
| Anti-EBP1           | Abcam  | Cat#ab180602; RRID:AB_2922403  |
| Anti-TOP2A          | Cell Signaling Technology | Cat#12286; RRID: AB_2797871 |
| Anti-MMP9           | Cell Signaling Technology | Cat#13667; RRID: AB_2798289 |
| Anti-E-cadherin     | Cell Signaling Technology | Cat#14472; RRID: AB_2728770 |
| Anti-N-cadherin     | Cell Signaling Technology | Cat#13116; RRID: AB_2687616 |
| Anti-Vimentin       | Cell Signaling Technology | Cat#5741; RRID: AB_10695459 |
| Anti-Bcl-2          | Cell Signaling Technology | Cat#15071; RRID: AB_2744528 |
| Anti-Bcl-xl         | Cell Signaling Technology | Cat#2764; RRID: AB_2228008 |
| Anti-BAX           | Cell Signaling Technology | Cat#5023; RRID: AB_10557411 |
| Anti-Myc-Tag        | Cell Signaling Technology | Cat#2276; RRID: AB_331783 |
| Anti-Flag          | Cell Signaling Technology | Cat#14793; RRID: AB_2572291 |
| Anti-Ubiquitin      | Cell Signaling Technology | Cat#3936; RRID: AB_331292 |
| Anti-NPM           | Cell Signaling Technology | Cat#3542; RRID: AB_2155178 |
| Anti-Ki67          | Cell Signaling Technology | Cat#9449; RRID: AB_2797703 |
| Anti-GINS1         | Invitrogen | Cat#PAS-50621; RRID:AB_2636074 |
| Anti-β-Actin       | Sigma-Aldrich | Cat#A1978; RRID: AB_476692 |
| Anti-rabbit IgG, HRP-linked Antibody | Cell Signaling Technology | Cat#7074; RRID: AB_2099233 |
| Anti-mouse IgG, HRP-linked Antibody | Cell Signaling Technology | Cat#7076; RRID: AB_330924 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| DMEM                | Invitrogen | Cat#11965092 |
| FBS                 | Gibco    | Cat#12664025C |
| PBS                 | Gibco    | Cat#14190144 |
| 0.25%Trypsin-EDTA  | Gibco    | Cat#25200056 |
| Cycloheximide       | Selleck Chemicals | Cat#57418 |
| MG132               | Selleck Chemicals | Cat#S2619 |
| chloroquine diphosphate | Selleck Chemicals | Cat# S4157 |
| GINS1 knockdown     | GeneChem | N/A |
| TOP2A knockdown     | GeneChem | N/A |
| USP15 knockdown     | GeneChem | N/A |
| GINS1 plasmid       | GeneChem | N/A |
| TOP2A plasmid       | GeneChem | N/A |
| USP15 plasmid       | GeneChem | N/A |
| Trizol              | Invitrogen | Cat#15596018 |
| RIPA lysis buffer   | Beyotime | Cat#P0013C |
| SDS–PAGE            | Beyotime | Cat#P0014A |
| 0.22-μm nitrocellulose membrane | Pall | Cat#S80209 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Non-fat milk        | Beyotime | Cat#P0216-300g |
| TBS                 | Beyotime | Cat#P0228 |
| Tween 20            | Beyotime | Cat#ST825-100mL |
| Chemiluminescent ECL Plus reagents | Millipore | Cat#WBKLS0500 |
| crystal violet      | Beyotime | Cat#C0121-500mL |

**Critical commercial assays**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| SYBR Green PCR kit  | ThermoFisher | Cat#4309155 |
| ImmunoPure Metal enhanced DAB substrate kit | ThermoFisher | Cat#34065 |
| The Pierce™ Classic Magnetic IP/Co-IP Kit | ThermoFisher | Cat#88804 |
| MTT assay kit       | Beyotime | Cat#C0009S |
| BCA assay kit       | Beyotime | Cat#P0012 |

**Experimental models: Cell lines**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HEB cell line       | ATCC   | N/A        |
| T98G cell line      | ATCC   | N/A        |
| U251 cell line      | ATCC   | N/A        |
| U373 cell line      | ATCC   | N/A        |
| A172 cell line      | ATCC   | N/A        |

**Experimental models: Organisms/strains**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| BALB/c nude mice    | SIMM,CAS | N/A        |

**Oligonucleotides**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| GINS1 primers: Sangon Biotech | N/A |
| R: 5'-AAAGTCAAGTGGACGAAAG-3' |
| F: 5'-CATTGGCAAGACGCATAC-3' |
| TOP2A primers: Sangon Biotech | N/A |
| R: 5'-GCCGGCCCTCTTATCTTCTCC-3' |
| F: 5'-CACCTAAGTGCACAGTTCC-3' |
| USP15 primers: Sangon Biotech | N/A |
| R: 5'-GCTCTTCTTTCTTTCCTC-3' |
| F: 5'-TGCCCTACTTCCAACCTTCTC-3' |
| GAPDH primers: Sangon Biotech | N/A |
| R: 5'-GCTTCTTCTTTCTTCTC-3' |
| F: 5'-CCAGGTGGTCTCCCTCTGGA-3' |
| R: 5'-GCTGAGCCAAATCGTTGT-3' |

**Software and algorithms**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| ImageJ              | National Institutes of Health | https://imagej.nih.gov/ij/ |
| SPSS 19.0           | SPSS | http://www.spss.com.cn |
| Graphpad prime 8.0  | Graphpad | https://www.graphpad.com/ |
| Adobe Illustrator CS6 | Adobe | https://www.adobe.com/products/illustrator/ |

**Other**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| CGGA - the Chinese Glioma Genome Atlas | Beijing Tiantan Hospital, Capital Medical University | http://www.cgga.org.cn/index.jsp |
| TCGA - the Cancer Genome Atlas | HOME for Researchers | https://www.aclbi.com/static/index.html#tcga |

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RESOURCES AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kun Lv (lvkun315@126.com).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This paper analyzes existing, publicly available data. The accession number and link are listed in the key resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human tissue samples
173 pairs of adjacent nontumor tissues and glioma samples were collected from the Department of Neurosurgery of the First Affiliated Hospital, Wannan Medical College (Wuhu, Anhui, P. R. China) from February 2008 to October 2011. According to the WHO guidelines, two pathologists evaluated all specimens. All tissues obtained at the surgery were directly preserved in liquid nitrogen. Patients received no systemic treatment before the surgery. Patient consent was obtained. All surveys and experiments were approved by the Ethic Committee for Clinical Research of the First Affiliated Hospital of Wannan Medical College. The clinical information of the samples is summarized in Table S5.

Cell culture and reagents
Human normal glial cell and glioma cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cell lines were characterized by DNA fingerprinting, cell vitality detection, isozyme detection, and mycoplasma detection. The last cell characterization was performed in December 2020. The five cell lines were cultured in high-glucose Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Bethesda, MD, USA) and pen/strep antibiotics. All cells were incubated in 5% CO2 at 37°C. Cycloheximide, proteasome inhibitor MG132, and chloroquine diphosphate were purchased from Selleck Chemicals (Houston, TX, USA).

Xenografted tumor model
The xenografted tumor model experimental protocol was approved by the Wannan Medical College Animal Experimental Ethics Committee. Five-week-old BALB/c nude mice (male/female ratio 1:1) were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Science and maintained under specific pathogen-free conditions. For the tumorigenesis assay, 6 × 10⁶ cells (U251-shGINS1 cells, U251-shCtrl cells, A172-GINS1 cDNA cells and A172-pcDNA3.1 cells) in 200 µL of PBS were implanted subcutaneously into the right dorsal flank of the mice (4 mice for each group). All mice were sacrificed 4 weeks after inoculation. Tumor volume was calculated with the following formula: Volume = 0.5 × length × width².

METHOD DETAILS

Cell transfection
The lentivirus constructs of GINS1 knockdown, TOP2A knockdown, USP15 knockdown, GINS1 plasmid, TOP2A plasmid and USP15 plasmid were obtained from GeneChem (Shanghai, China). Glioma cell lines
were plated in 6-well microplates at 50% confluence and infected with lentivirus-mediated knockdown shRNA or scrambled control shRNA in U251 and T98G cells, respectively. For plasmid transfection, A172 cells were seeded in 6-well microplates and transfected with control vector plasmid or GINS1 cDNA, TOP2A cDNA, or USP15 cDNA when the cell density reached 70–80% confluence. The above cell transfection protocol was based on the manufacturer’s instructions. Stable transduction pools were generated by puromycin selection for two weeks. The cells were cultured after transfection and harvested for the following experiments.

**Quantitative real-time polymerase chain reaction (qRT–PCR)**

With TRIzol reagent, total RNA was extracted from cells or tissues. According to the manufacturer’s instructions, the qRT–PCR assay was performed using the SYBR Green PCR kit. Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method. Each sample was repeated in triplicate and analyzed using relative quantification method.

**Western blotting assay and immunoprecipitation (IP) analysis**

The proteins from cells or tissues, the protein concentration, and the 10% SDS–PAGE isolated protein were extracted with RIPA lysis buffer, measured with a BCA assay kit, and transferred to a 0.22-μm nitrocellulose membrane. The membranes were blocked at room temperature with 5% non-fat milk for 2 h. Then, each membrane was incubated with one of the indicated specific primary antibodies. The membranes were washed with 0.1% TBST three times for 5 min and then incubated with anti-mouse or anti-rabbit IgG HRP-linked secondary antibody for 2 h. Each was washed with 0.1% TBST three times for 5 min. Chemiluminescent ECL Plus reagents were added to visualize the reaction products. The membranes, the band intensity, and the protein levels were scanned with Tanon 5200, measured by densitometry using Quantity One Software, and normalized to that of β-actin. All experiments were repeated independently thrice, and representative results are shown.

**Immunohistochemical (IHC) staining**

Paraffin-embedded mouse tumor tissues was cut into 5-μm-thick section for the IHC analysis of GINS1, TOP2A and USP15 protein. After baking for 2 h at 60°C, the tissues were immersed in xylene for deparaffinization and rehydrated with a series of ethanol at decreased concentrations. Then, the tissue sections were incubated with the following one of primary antibodies for 12 h at 4°C. The tissue sections were washed with PBS and incubated with biotinylated secondary antibody for 2 h at room temperature the next day. Finally, tissue sections were treated with an ImmunoPure Metal enhanced DAB substrate kit in accordance with the manufacturers’ instructions.

**Cell proliferation assay**

Cell proliferation assays were performed with MTT assays according to the manufacturer’s instructions. In brief, 2×10^3 glioma cells from each group were collected and inoculated into 96-well microplates. Then, the cells were incubated with 50 μL MTT solution for 3 h. At 490 nM, the absorbance value of each well was measured by a microplate reader. Six replicates were set up in each group, and then the mean value was taken.

**Colony formation assay**

Glioma cells were plated in 6-well microplates and cultured in the appropriate culture medium for 2 weeks. The culture medium was changed every 3 days. Cell colonies were stained with 1% crystal violet and then counted.

**Cell migration and invasion assays**

Transwell assays and wound healing assays were used to measure the migration and invasion of glioma cells. For transwell assays, glioma cells in each group were inoculated into the upper chamber of the Transwell chamber (BD Biosciences, USA) at the density of 4×10^4 cells/well. Besides, 500 μL culture medium containing 10% fetal bovine serum was added to the 24-well culture plate of the lower chamber. After 24 h of routine culture, the chamber was removed, and cells from the upper layer of microporous membranes were wiped with cotton swabs. After that, cells were immobilized in 4% paraformaldehyde solution for 10 min at room temperature and stained with 0.5% crystal violet solution (Sigma-Aldrich; Merck KGaA) for 15 min. In the last step, 5 visual fields were randomly selected and observed under an optical microscope (Nikon,
Japan) to count the number of cells invading the sub-layer of the microporous membranes of the chamber.

For wound healing assays, when the transfected glioma cells were maintained in a 6-well plate, achieving 90-95% confluence, scratches were generated using the micropipette tips. The wound state was observed at 0 and 72 h after scratching with an X71 inverted microscope (Olympus, Tokyo, Japan) (Liu et al., 2021; Yang et al., 2021).

**Co-immunoprecipitation and mass spectrometry**

The Pierce Classic Magnetic IP/Co-IP Kit was used to examine proteins bound to GINS1. Cells (1 × 10⁷) were washed in PBS twice, and then 500 μL IP lysis buffer was added to lyse cells. One-tenth of the supernatant was saved as the input. To the remaining supernatant, 5 μg FLAG antibody-magnetic bead complex was added, and then samples were incubated at 4°C overnight. Magnetic beads were washed five times with buffer containing 10 μg/mL RNAase A before being eluted with 100 μL elution buffer. Protein samples (20 μL) were boiled in LDS buffer for Western blotting, and 80 μL protein samples were analyzed by label-free LC/MS.

**Glutathione S-transferase pull-down experiments**

Glutathione S-transferase fusion constructs were expressed in *Escherichia coli* BL21 cells, and crude bacterial lysates were prepared by sonication in cold PBS in the presence of a protease inhibitor mixture. *In vitro* transcription and translation experiments were performed with rabbit reticulocyte lysate. In GST pull-down assays, ~10 μg of the appropriate GST fusion protein was mixed with 5–8 μL of the *in vitro* transcribed/translated products and incubated in binding buffer. The binding reaction was then added to 30 μL of glutathione Sepharose beads and mixed at 4°C for 2 h. The beads were washed five times with binding buffer, resuspended in 30 μL of 2× SDS–PAGE loading buffer, and resolved on 12% gels. Protein bands were detected with specific antibodies by Western blotting.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All data from three independent experiments are expressed as the mean ± SD, and statistical analyses were performed using SPSS 19.0 software. Student’s t test was used to examine the differences between the two groups, and ANOVA was used to investigate the differences in multiple groups. The chi-square test was applied to examine the relationship between GINS1 levels and clinicopathological characteristics. Overall survival, progression-free survival and disease-free survival rates were analyzed using the Kaplan–Meier plot and tested via the log rank test. Statistics were regarded as significant when *, p < 0.05.