Oncogenic Activity of Glucocorticoid Receptor β Is Controlled by Ubiquitination-Dependent Interaction with USP49 in Glioblastoma Cells

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ABSTRACT

Previous studies have demonstrated that glucocorticoid receptor β (GRβ) functions as an oncoprotein, regulating the malignant phenotypes and stem-like cell maintaining in human glioblastoma (GBM). Of the glucocorticoid receptor (GR) isoforms, GRβ and GRα are highly homologous, though the mechanism underlying the distinct functions of these two isoforms in GBM has not been clarified. Here by establishing a carboxyl-terminal (COOH-terminal) deletion mutant, we determined that GRβ can be ubiquitinated. We also found that its COOH terminal is essential for this ubiquitination. The mutation of a lysine to arginine at residue 733 (K733R) blocked the ubiquitination of GRβ, indicating that K733 is a key site for ubiquitination. Using K733R to establish nonubiquitinated GRβ, we demonstrated that ubiquitination not only regulates the stability and nuclear translocation of GRβ, but is also a vital mechanism for its oncogenic functions *in vitro* and

in vivo. Protein interaction assay further indicated that ubiquitin-specific protease 49 (USP49) is a GRβ-binding protein and the interaction depends on GRβ ubiquitination. USP49 knockdown resulted in a decrease of cell proliferation, invasion, and an increase of cell apoptosis. More importantly, USP49 knockdown increased ubiquitination and amplified the oncogenic effects of GRβ, confirming the decisive role of ubiquitination on GRβ carcinogenicity. Taken together, these findings established that ubiquitination is a vial process for GRβ the execution of oncogenic functions in GBM and that the K733 site is crucial for ubiquitination of GRβ.

Implications: This work is the first identify of the activation GRβ by a single lysine point-mediated ubiquitination and proteasome degradation, which determines its oncogenic functions in GBM.

Introduction

Glioblastoma (GBM) is a malignant primary brain tumor with the third highest mortality rate among all human tumors. The mechanisms underlying the proliferation, invasion, and recurrence of GBM are largely unknown, limiting progress in the area of GBM therapeutics (1–4). Therefore, it is of great importance to investigate key molecules, signal pathways, intervention targets, and interactions involving the regulation of the malignant biological process of GBM. The glucocorticoid receptor (GR), a member of nuclear hormone receptor superfamily of transcription factors, regulates multiple physiologic functions, such as inflammatory responses (5), cellular proliferation (6), and differentiation in target tissues (7). Through alternative splicing of exons, the human GR gene generates two major isoforms, GRα and GRβ (8, 9). These two isoforms mediate almost

entirely different sets of actions (10). In GBM, we have previously demonstrated that GRα is a nontumor phenotypic regulatory protein, while GRβ is an onco-protein which regulates the pathogenesis and biological characteristics of GBM cells and other stem-like cells (11, 12). Unlike GRα which depend on ligand-binding for activation, GRβ do not require ligand-binding and are instead preferentially expressed in the nucleus of GBMs. GRα and GRβ are identical from the carboxyl-terminus (COOH-terminus) through the 728th residue, with GRα having an additional 50 amino acids and GRβ having an additional 15 amino acids (13). The difference in cellular distribution and function between the two isoforms may be due to the difference in the ligand-binding domain (LBD), though the precise mechanisms underlying the regulation of GRβ functions remain to be clarified.

Posttranslational modifications (PTM) play a critical role in signal transduction, protein stability and, protein–protein recognition and interaction (14–16). At present, most studies on GR PTMs focus on GRα. It is known that GRα can be modified by several PTMs at distinct sites, including phosphorylation, acetylation, ubiquitination, and SUMOylation (17–19). However, there is lacking evidence regarding the PTMs of GRβ and the impact of PTM on GRβ functions. Among the PTMs, acetylation, ubiquitination, and SUMOylation have been known to occur on lysine (K) residues. Through online prediction tools and the establishment of COOH-terminus mutants, we reveal that GRβ is modified by ubiquitination, and that this PTM is mainly dependent on the lysine residues at position 733. Using a K733R mutant to establish nonubiquitinated GRβ, we demonstrate that ubiquitination determines the stability and nuclear localization of GRβ as well as its onco-functions. Furthermore, we reported that ubiquitin-specific protease 49 (USP49) binds ubiquitinated GRβ and regulates GRβ ubiquitination and degradation. Finally, we demonstrate that increased ubiquitination induced by USP49 knockdown amplifies the oncogenic effects of GRβ.

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Materials and Methods

Cell culture and reagents

The human GBM cell lines U87 (RRID:CVCL_0022), U118 (RRID:CVCL_0633) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Science (Shanghai, China) and cultured in DMEM (HyClone) supplemented with 10% FBS (Biological Industries). Cell lines were authenticated by short tandem repeat (STR) analysis and tested for mycoplasma contamination.

Vector construction and transduction

For GRβ full-length, deletion, and point mutant expression constructs (GR727, GRβ K733R), cDNA was amplified by PCR from the plasmid hGRβ-pOTB7 (YRBIO) and verified by DNA sequencing as previously reported (11). The fragments were cloned into lentiviral vector GV218 established by Genechem fused with GFP. For GRα expression construct, cDNA was amplified from hGRα-pOTB7 (YRBIO), verified by DNA sequencing, and cloned into lentiviral vector GV147 established by Genechem fused with red fluorescent protein (RFP). Full-length cDNA-encoding human USP49 was cloned into lentiviral vector GL102 established by OBIO Technology with Flag-tagged. GRβ, GR727, GRβ K733R stable expressing cells were derived from U87 cells infected with the indicated lentiviral constructs and enriched by puromycin selection. The purity of stable cell-line expression indicated lentiviral constructs were confirmed by flow cytometry (Becton Dickinson) and reached over 90%. For intracranial xenograft assay, the indicated U87 stable cells were infected with lentivirus carrying the firefly luciferase gene (H7656; OBIO Technology) and the expression efficiency was confirmed by luciferase assay. The siRNAs were used as follows: GRβ siRNA 5′-GGCUUUU-CAUUAAAUGGGAtt -3′, GRβ scrambled siRNA: 5′-UUAAA-GUGCGAUCUGUUAGtt-3′; USP49 siRNA: 5′-GCCGUAAU-CAUCGAGAGAAGtt-3′, USP49 scrambled siRNA: 5′-GAGAGGU-CAUCAGAGACUACtt-3′. The siRNAs were synthesized by Gene Pharma, and the scramble siRNAs served as negative control (NC). For siRNA transfections, cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX transfection reagent (Invitrogen). Cells were collected for the following assays after 48 hours of transfection.

Western blot analysis

For Western blot analysis, cells were harvested and lysed in RIPA lysis buffer (Cell Signal Technology). To investigate subcellular distribution of proteins, nuclear and cytoplasmic fractions were enriched using Nuclear and Cytoplasmic Protein Extraction Kit (CoWin Bioscience). Western blot assays were followed according to an established protocol (20). Antibody used included mouse anti-GFP (Abmart), rabbit anti-acetyl Lysine (Abcam), rabbit anti-SUMO1 (Abcam), rabbit anti-Lamin B1 (Abcam), mouse anti-Ub (Santa Cruz), rabbit anti-USP49 (NOVUS), mouse anti-Flag (Proteintech), mouse anti-GAPDH (Thermo Fisher Scientific), mouse anti–β-tubulin (Thermo Fisher Scientific), rabbit anti-GRα (Abcam), rabbit anti-GRβ (Abcam). For protein stability detections, cells were treated with proteasome inhibitor MG132 (20 μmol/L, Sigma-Aldrich) for 6 hours or protein synthesis inhibitor Cycloheximide (CHX, 100 μg/mL, Sigma-Aldrich) for indicate time before harvest.

Immunoprecipitation

Immunoprecipitation experiment was performed to analyze the protein interactions or indicated PTMs as previously described (20). In brief, cell lysates were prepared in RIPA buffer and precipitated using anti–DDDDK-tag mAb-Magnetic Beads (MBL) or anti-GFP mAb-Magnetic Beads (MBL). Precipitated products were analyzed by Western blot using indicated antibodies. For the ubiquitination assay, cells were treated with MG132 (20 μmol/L) for 6 hours before collecting. The targeting protein ubiquitination was detected by mouse anti-Ub antibody (Santa Cruz Biotechnology).

qRT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen). First-strand cDNA was generated using HiFiScript cDNA synthesis Kit (CoWin Bioscience) according to the manufacture's protocol. qPCR analyses were conducted to quantitate mRNA relative expression using Real SYBR Mixture (CoWin Bioscience), with *GAPDH* as an internal control. The primers used were as follows. *USP49*: 5′-GGAGAATC-TACGCTTGTGACCAG-3′ (forward), 5′- CGGAGAACCTGAGG-TAGTCTGT-3′ (reverse); *GRβ*: 5′-TGTGTTTTGCTCCTGATCT-GA-3′ (forward), 5′-TGAGATGTGCTTTCTGGTTTT-3′ (reverse); *GRα*: 5′-TGTGTTTTGCTCCTGATCTGA-3′ (forward), 5′-CAGC-TAACATCTCGGGGAAT-3′ (reverse); *GAPDH*: 5′-TGTTGCCAT-CAATGACCCCTT-3′ (forward), 5′-CTCCACGACGTACTCAGCG-3′ (reverse).

Cell growth assay and colony formation assay

For cell growth assays, a total of 1,000 cells were seeded into 96-well plates, and monitored by Cell Counting Kit-8 (Bimake,) according to the manufacture's protocol at indicated time points. For colony formation assays, 1,000 cells were seeded into 6-well plates and maintained in a complete medium for 14 days. The colonies were fixed with 4% paraformaldehyde (PFA; Sangon) and stained with 0.1% crystal violet (Sangon), the number of colonies was counted using an inverted microscope.

Three-dimensional cell invasion assay

Three-dimensional (3-D) cell culture chip (AIM Biotech) were used in this study, and the assay proposed according to the manufacturer's instructions. In brief, type I rat tail collagen (Corning) at a concentration of 2.5 mg/mL following the addition of 10 × PBS (Gibco) with phenol red (Sigma-Aldrich) with pH adjusted using NaOH (Sigma-Aldrich). The collagen solution was filled into the middle gel channel and incubate the chip for half an hour in 37°C to allow polymerization of collagen. Then cells (2.0×10^6/mL) with serum-free DMEM medium were added into the left medium channel, while DMEM containing 20% FBS was added into the right medium channel to create a serum gradient across the collagen gel which triggers cell invasion through collagen gel. The chips were cultured and 20% FBS in the right medium channel was refreshed every 24 hours to maintain the serum gradient. After 3 days of culture, cells were fixed with 4% PFA and stained with Hoechst 33342 (Invitrogen). The staining was visualized using Axio Vert.A1 microscope (ZEISS), the number of cells invaded into the gel channel were calculated and statistics with Image J software (RRID:SCR_003070 NIH, Bethesda, Maryland).

Apoptosis experiments

Cell apoptosis was assayed using a commercial Annexin V-Alexa Fluor 647/PI apoptosis detection Kit (Fcmacs Biotech). Briefly, cells were incubated in complete culture medium for 12 hours, and then treated with Etoposide (30 μmol/L, Selleck Chemicals Houston) or Doxorubicin (Dox, 2 μmol/L, Selleck Chemicals) for another 24 hours. Then, cells were harvested and stained according to the instructions. The percentages of Annexin V and/or propidium iodide (PI)–positive cells were determined by flow cytometry (Becton Dickinson). Annexin

V single positive and Annexin V/PI double positive cells were counted as apoptotic cells.

LC/MS-MS assay

U87 cells stable expressing GRβ-GFP, K733R-GFP, and their GFP control were collected and lysed in RIPA buffer and precipitated by anti-GFP mAb-Magnetic Beads (MBL) overnight at 4°C. Precipitated products were washed and separated by SDS-PAGE gel, the integrated electrophoretic bands were cut off and subjected to in-gel digestion with trypsin (Gibco). The peptides were filtered through C18 to remove excess salt, and solubilized with a Loading Buffer. The solubilized peptides were analyzed and identified by LC-MS/MS on a mass spectrometer, and the results were compared by numerous protein databases.

Tumor xenografts in nude mice

Four-week-old male BALB/c nude mice (RRID:MGI:3806503) were purchased from the Changzhou Cavens Laboratory Animal Co. Ltd., and maintained under specific pathogen-free conditions at Wuxi People's Hospital. For intracranial xenograft assay, cells stably expressing the firefly luciferase gene and indicated GRβ construct were transplanted into the brain (5×10^5 cells in 5 μL DMEM per mouse). After 30 days of transplantation, tumor growth was monitored via bioluminescent imaging using IVIS Spectrum system and quantified by Living Image Software, as described previously (20). For subcutaneous xenograft assay, 5×10^6 cells in 100 μL DMEM were injected subcutaneously into the left flanks of mice. After 28 days of transplantation, tumor growth was recorded every 2 days with a caliper and tumor volume was calculated as $a \times b^2 \times 0.5$ (a, longest diameter; b, shortest diameter). After 40 days of transplantation, mice were euthanized and tumors were collected, weighed, and analyzed. In order to detect the proliferation of tumors *in vivo*, mice were intraperitoneally injected with 0.4 mL EdU (10 mmol/L in PBS) 12 hours before sacrifice. After sacrifice, the mouse brains or subcutaneous tumor tissues were collected and embedded in optimal cutting temperature compound and subjected to EdU labeling. All animal care and handling procedures were performed in accordance with the NIH's Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Review Board of Nanjing Medical University.

EdU labeling

EdU labeling assay was performed to examine the DNA replication as described previously (20). Briefly, the tumor xenografts were collected, fixed with 4% PFA, and sectioned. The staining procedure was performed according to the manufacturer's instructions for the Alexa Fluor 594 Click-iT EdU Imaging Kit (Thermo Fisher Scientific). The coverslips or sections were washed, mounted, and examined using lazer scanning confocal microscope (Leica Microsystems GmbH). Positive cells were quantified ($n = 10$) randomly selected fields per mouse.

Statistical analyses

All experiments were independently repeated at least three times. Data are expressed as mean ±SD. The difference between 2 independent samples or multiple groups were determined by Student t test or one-way ANOVA followed by a Student–Newman–Keuls multiple comparison test (SNK test) respectively, with statistical significance at $P < 0.05$. SPSS 20.0 package (RRID:SCR_002865, IBM) and GraphPad Prism 6.0 software (RRID:SCR_002798, GraphPad Software) were used to carry out all statistical analyses and data graphing respectively.

Results

K733 determined the ubiquitin of GRβ

Lysine (K) is known to undergo a variety of PTMs, such as acetylation, SUMOylation, and ubiquitination. GPS-SUMO (http://sumosp.biocuckoo.org/online.php), an online PTMs tool, was applied to predict and compare the potential SUMOylation sites between GRα and GRβ. As shown in **Fig. 1A**, GRα and GRβ share three predicted lysine sites for SUMOylation in the common amino acid sequence (amino acid residues 1–727). The prediction tools additionally indicated that GRβ has an independent candidate site at K733. To explore the PTMs possibly occurring at K733, we constructed GFP-tagged lentivirus constructs, including a GRβ, GR727 mutant lacking a COOH-terminus from the amino acid of 728, as well as a K733R mutant containing a site mutation that converts the 733 lysine to arginine (R; **Fig. 1B**). Using these constructs, we examined whether GRβ is SUMOylated via an immunoprecipitation (IP) experiment with a GFP antibody in U87 stable expressing cells. However, no detectable SUMOylation was found in GRβ, nor in GR727 or K733R constructs (**Fig. 1C**). Subsequently, we examined acetylation and ubiquitin of GRβ in U87 cells expressing ectopic GRβ, GR727, or K733R. As shown in **Fig. 1D**, no significant acetylation was detected in GRβ-, GR727-, or K733R-expressing cells. In contrast, we detected a ubiquitination modification of GRβ, a modification reduced by the removal of the COOH-terminal region or by mutation of K733, indicating a vital site in regulating the ubiquitination of GRβ (**Fig. 1E**). These data suggest that K733R may be a useful tool for studying the ubiquitination of GRβ.

Ubiquitination regulates GRβ stability and cellular localization

It's well-known that ubiquitination can regulate the stability, localization, and activity of target proteins. To determine whether ubiquitination affects the stability of GRβ, MG132 was added in ectopic GRβ-expressing U87 cells for 6 hours to inhibit the proteasome-dependent degradation pathway. As shown in **Fig. 2A**, MG132 treatment resulted in an increase of GRβ-GFP expression without affecting GFP expression in U87 cells with ectopic GFP. Protein half-life analysis via CHX to inhibit protein synthesis indicated that K733R extended the half-life of GRβ, confirming ubiquitination regulates GRβ stability (**Fig. 2B**). Since GRβ has been shown to be a nuclear functional protein (11, 12), whether ubiquitination affects the nuclear localization of GRβ was subsequently determined (**Fig. 2C**). Calculating the relative ratio of nuclear GRβ to cytoplasm GRβ, we observed that GRβ is preferentially expressed in the nucleus, while GR727 and K733R demonstrated opposite nuclear cytoplasmic distribution. Collectively, these data uncovered that ubiquitination plays important roles in regulating GRβ stability and cellular distribution.

Ubiquitination determines onco-functions of GRβ in GBM

Given the evidence in our published studies that GRβ functions as a tumor promoter in GBM (11, 12), we confirmed the actions of GRβ in U87 and U118 cells using the siRNA specifically targeting GRβ (12). It indicated that GRβ knockdown (KD) resulted in a significant decrease of cell proliferation and invasion, and a promotion of doxorubicin (Dox)–induced apoptosis (Supplementary Fig. S1). Next, to explore whether ubiquitination affects biological function of GRβ in GBM, a K733R mutant was used as a tool for study of deubiquitination of GRβ. U87 and U118 cells expressing ectopic GRβ-GFP, K733R-GFP, and GFP were established for the following functional studies. A cell growth assay showed that ectopic GRβ expression endowed U87 and
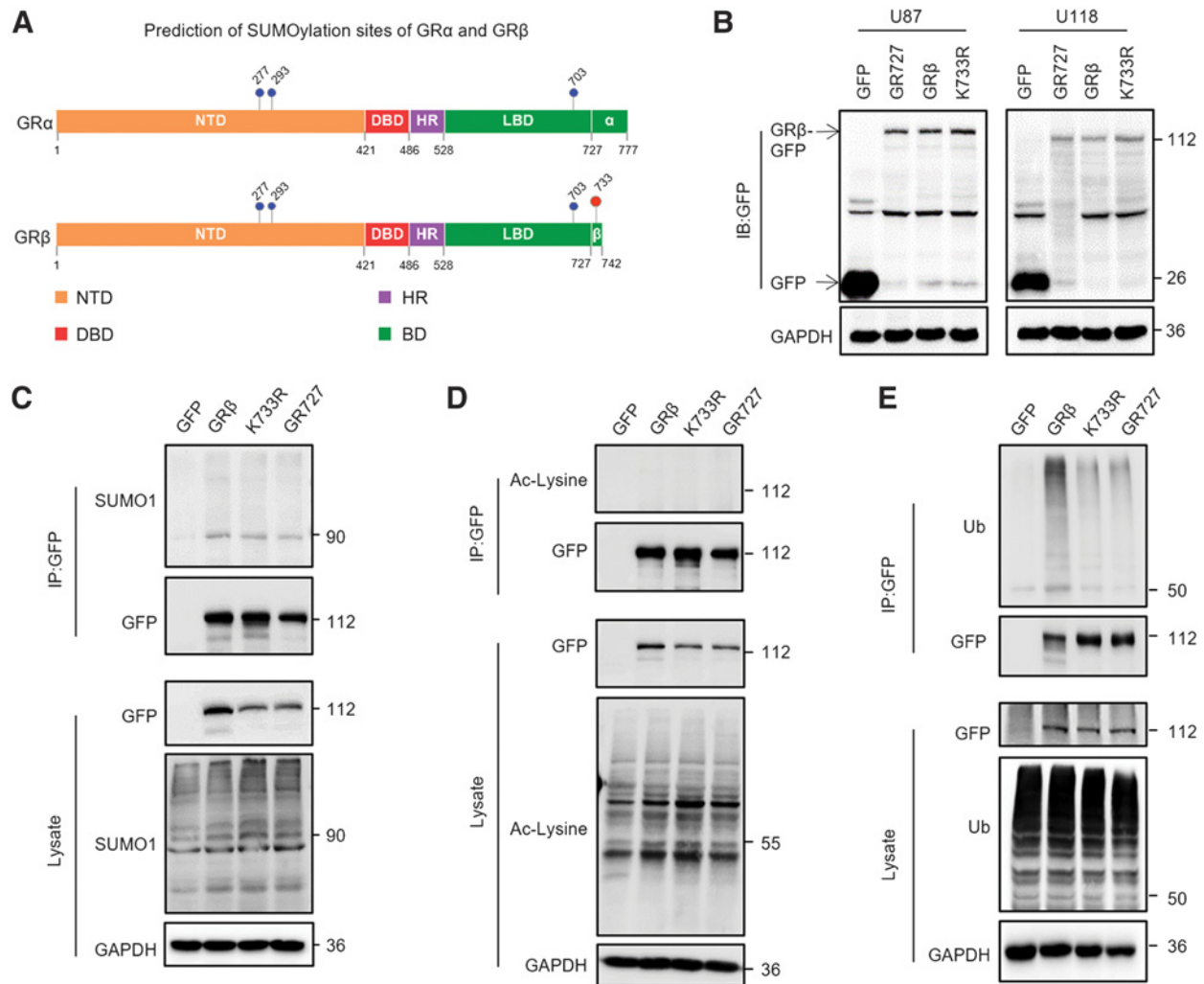
Figure 1.
K733 is a decisive site for GRβ ubiquitination. **A,** Diagram showing the prediction of SUMOylation sites of GRα and GRβ. HR, hinge region; NTD, N-terminal transactivation domain. **B,** Western blot illustrating the ectopic expression of indicated constructs in U87 and U118 cells mediated by lentivirus with GFP antibody. IB, immunoblot. **C,** SUMOylation analysis of GRβ and indicated mutants. Proteins derived from U87 cells expressing indicated constructs were immunoprecipitated by GFP antibody followed by immunoblots using SUMO1 antibody. IP, immunoprecipitation. **D,** Acetylation analysis of GRβ and its indicated mutants. Acetylated-Lysine (Ac-Lysine) antibody was used to detect the acetylation of GRβ. **E,** Ubiquitination analysis of GRβ and its indicated mutants. U87 cells expressing indicated constructs were incubated with MG132 (20 μmol/L) for 6 hours before harvest. Ubiquitin (Ub) antibody was used to detect the ubiquitination of GRβ.

U118 cells with stronger amplification ability, while K733R mutation reversed the cell-growth promotion of GRβ (**Fig. 3A**). Colony formation assay further confirmed this finding (**Fig. 3B**). To investigate whether K733R affects GBM cell invasion, a 3D-invasion chip was applied (**Fig. 3C**). In this assay, serum gradient across the collagen gel induced cell invasion through collagen gel, and the invasion can be quantified by the number and distance of invading cells through collagen gel. This assay closely mimics *in vivo* conditions where interactions between cells and extracellular matrixes are accounted for. As shown in **Fig. 3C**, ectopic GRβ enhanced cell invasion both in cell number and migrating distance while, K733R-expressing mutants lost the invasion enhancements of GRβ. Since U87 and U118 cells, as well as cells expressing ectopic GRβ or K733R, did not undergo obvious apoptosis under normal culture conditions (Supplementary Fig. S2A), Etoposide and Dox, two widely used alkylating agents were

applied to induce cell apoptosis. GRβ overexpression showed a protective effect against Etoposide or Dox-induced apoptosis while K733R demonstrated an inverse effect (**Fig. 3D**; Supplementary Fig. S2B). To further verify the *in vitro* findings, an intracranial xenograft tumor growth assay was performed in nude mice (**Fig. 3E**). It indicated that ectopic GRβ significantly increased tumor growth and resulted in more EdU-labeled cells in tumor tissues. Although tumors derived from K733R-expressing cells were bigger than those derived from Mock expressing cells, the tumorigenesis effect of K733R was markedly impaired as compared with GRβ (**Fig. 3E–G**). Moreover, subcutaneous xenograft tumor growth assay verified the results of intracranial xenograft assay (Supplementary Fig. S3). Collectively, these data suggest that K733-mediated ubiquitination is a vital biological process for GRβ to execute onco-functions in GBM.
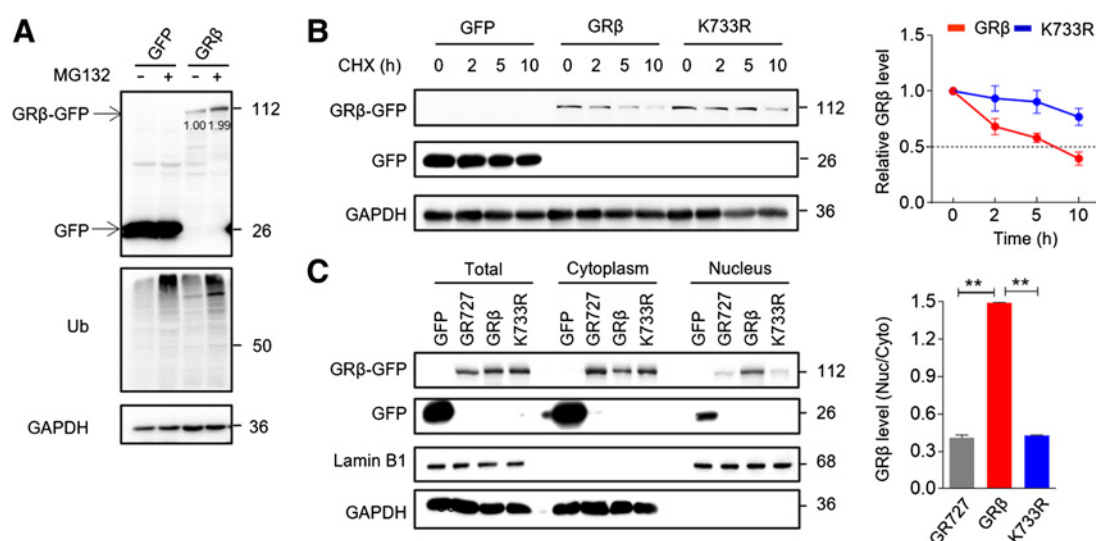
Figure 2.
Ubiquitination affects GRβ stability and nuclear localization. **A,** Western blot analysis of GRβ expression in U87 cells with ectopic GFP or GRβ-GFP expression. Cells were treated with MG132 (20 μmol/L) for 6 hours before harvest. Ubiquitin (Ub) and GAPDH served as loading controls. The quantification of GRβ expression was listed below. **B,** Western blot analysis of GRβ expression in U87 cells with ectopic GFP or GRβ-GFP expression. Cells were treated with CHX (100 μg/mL) for indicated hours before harvest (left). The alteration of relative GRβ protein level was shown in the right (mean ±SD, $n = 3$). h, hours. **C,** Western blot analysis of cellular GRβ expression in U87 cells with ectopic expression of indicated constructs (left). GAPDH and Lamin B1 served as cytoplasmic and nuclear marker, respectively. Nuc, nuclear; Cyto, cytoplasmic. The nuclear GRβ level relative to cytoplasmic GRβ were shown in the right ($n = 3$, mean ±SD, one-way ANOVA with SNK test. **, $P < 0.01$).

USP49 binds ubiquitinated GRβ and decreases its ubiquitination

To identify the potential target proteins regulating the ubiquitination of GRβ, an IP experiment was followed with a LC/MS-MS analysis. Briefly, GRβ or K733R mutants and their binding proteins were enriched by GFP-conjugated magnetic beads (**Fig. 4A**). The LC/MS-MS analysis uncovered that GRβ binds many proteins in GBM cells. Among the top six binding proteins, GRβ binds GR itself and HSP90, a known chaperone of GR (**Fig. 4B**). This indicated satisfactory specificity of the IP for GRβ enrichment. Among the top six GRβ-binding proteins, USP49 was inhibited from K733R binding proteins. Screening of additional GRβ-binding proteins not enriched in K733R-expressing mutants, USP49 ranked the largest reduction in binding concentration (**Fig. 4C**). Through IP analysis, we further confirmed the interaction between USP49 and GRβ, particularly that this interaction was blocked by the mutation of K733R (**Fig. 4D**). Furthermore, it confirmed that USP49 binds specifically to GRβ but not GRα (**Fig. 4E**), suggesting that the binding of USP49 depends specifically on the COOH-terminal ubiquitination state of the GRβ. USP49 is one of ubiquitin-specific peptidases (USP) which oppose the action of ubiquitinating enzymes (21). Subsequently, we examined whether USP49 could modulates the ubiquitination level of GRβ in GBM cells directly. As shown in **Fig. 4F**, the ubiquitination of GRβ was increased upon USP49 knockdown, which correspondingly induced a decrease of GRβ in total lysate (left panel). Accordingly, USP49 overexpression exerted an opposite effect on GRβ (right panel). Since ubiquitination plays important roles in regulating GRβ cellular distribution, we determined whether USP49 could affect cytoplasmic/nuclear distribution of GRβ. As shown in **Fig. 4G**, USP49 KD resulted in an increase of GRβ in nucleus and a decrease in cytoplasm (left panel). Similarly, the opposite result was observed in cell-containing ectopic USP49 (right panel). Meanwhile, the cellular distribution of endogenous GRβ was also modulated by USP49 (Supplementary Fig. S4). Collectively,

these observations demonstrated that USP49 is a USP targeting ubiquitinated GRβ.

USP49 contributes oncogenic functions of GRβ in GBM

Next, we investigated the significance of USP49 mediated deubiquitination for the functional execution of GRβ in GBM. As shown in Supplementary Fig. S5A and **Fig. 5A**, USP49 KD with specific siRNA resulted in a significant growth inhibition of U87 cells. Ectopic GRβ promoted cell growth and rescued the growth inhibition induced by USP49 KD. While not expected, there was no comparable difference in cell number between cells expressing ectopic GRβ with or without USP49 knockdown. The growth promotion of GRβ induced by GRβ overexpression was enhanced in cells with USP49 knockdown, suggesting USP49 knockdown reinforces the growth-promoting effect of GRβ (**Fig. 5B**). A 3D invasion assay showed that USP49 KD led to a significant inhibition of cell invasion. Similarly, GRβ overexpression reversed the inhibition of USP49 knockdown (**Fig. 5C** and **D**). To explore whether USP49 affects cell survival in response to chemotherapy, U87 cells were incubated with Etoposide or Dox for 24 hours, followed by apoptotic assay using flow cytometry (**Fig. 5E**; Supplementary Fig. S5B). This demonstrated that USP49 knockdown significantly increased cell apoptosis, while GRβ introduction overcame this effect. The protection of GRβ against cell apoptosis was further demonstrated in cells undergoing USP49 knockdown (**Fig. 5F**; Supplementary Fig. S5C). Taken together, these results suggest that USP49 acts as an oncogene in GBM and its deubiquitination activity impedes GRβ from producing excessive cancer-promoting effects.

Discussion

GRβ shares N-terminal and DNA-binding domains (DBD) with GRα but possesses a unique LBD. The unique COOH-terminus of the LBD endows GRβ with functions and cellular localization that
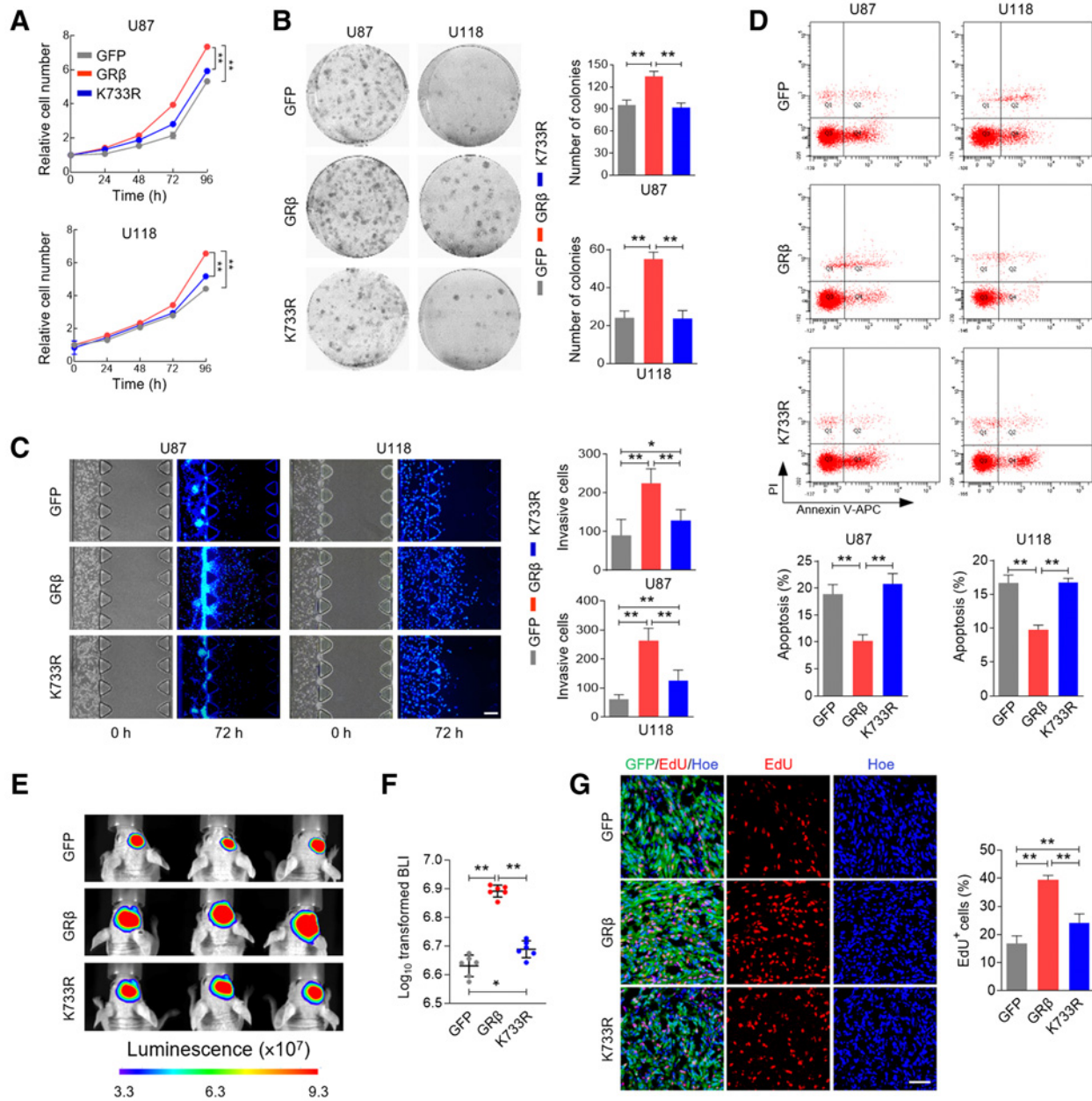
Figure 3.
Ubiquitination is essential for onco-functions of GRβ in GBM. **A,** Cell growth assay of U87 cells expressing indicated ectopic constructs ($n = 6$, mean ±SD, one-way ANOVA with SNK test, **$P < 0.01$). **B,** Colony formation analysis of U87 cells expressing indicated ectopic constructs ($n = 3$, mean ±SD, one-way ANOVA with SNK test; **, $P < 0.01$). **C,** 3D invasion assay of U87 cells expressing indicated ectopic constructs, Scale bar: 100 μm ($n = 9$, mean ±SD, one-way ANOVA with SNK test; *, $P < 0.05$, **, $P < 0.01$). **D,** Cell apoptosis assay of indicated cells by flow cytometry. Cells were treated with Etoposide (30 μmol/L) for 24 hours and apoptotic cells were measured by Annexin V/PI staining followed with flow cytometry ($n = 3$, mean ±SD, one-way ANOVA with SNK test; **, $P < 0.01$). APC, allophycocyanin. **E,** Bioluminescence images (BLI) of the intracranial xenografts derived from the implantation of indicated U87 cells after 30 days. **F,** Quantification of tumor volume ($n = 6$, mean ±SD, one-way ANOVA with SNK test; *, $P < 0.05$; **, $P < 0.01$). **G,** Representative EdU labeling images and quantification of EdU+ cells in xenografts, Scale bar: 50 μm ($n = 6$, mean ±SD, one-way ANOVA with SNK test, **, $P < 0.01$).

distinguish it from GRα (22), particularly the regulation of tumorigenesis and progression of GBM (11, 12). A novel finding of the present study was that a ubiquitin modification at the COOH-terminus was responsible for the unique functions of GRβ in GBM. This ubiquitination affected GRβ stability and nuclear localization, factors contributing critically to its role in cancer-promotion effects.

GR can be modified by a variety of PTMs including phosphorylation, acetylation, ubiquitylation, and SUMOylation, all of which affect functions and phenotypes of GR in distinct tumor cells (23, 24). To date, most efforts have been focused on GRα modifications and the implications of GRβ PTMs have not been reported. Through proteomic modification using GRβ deletion or point mutants, the present
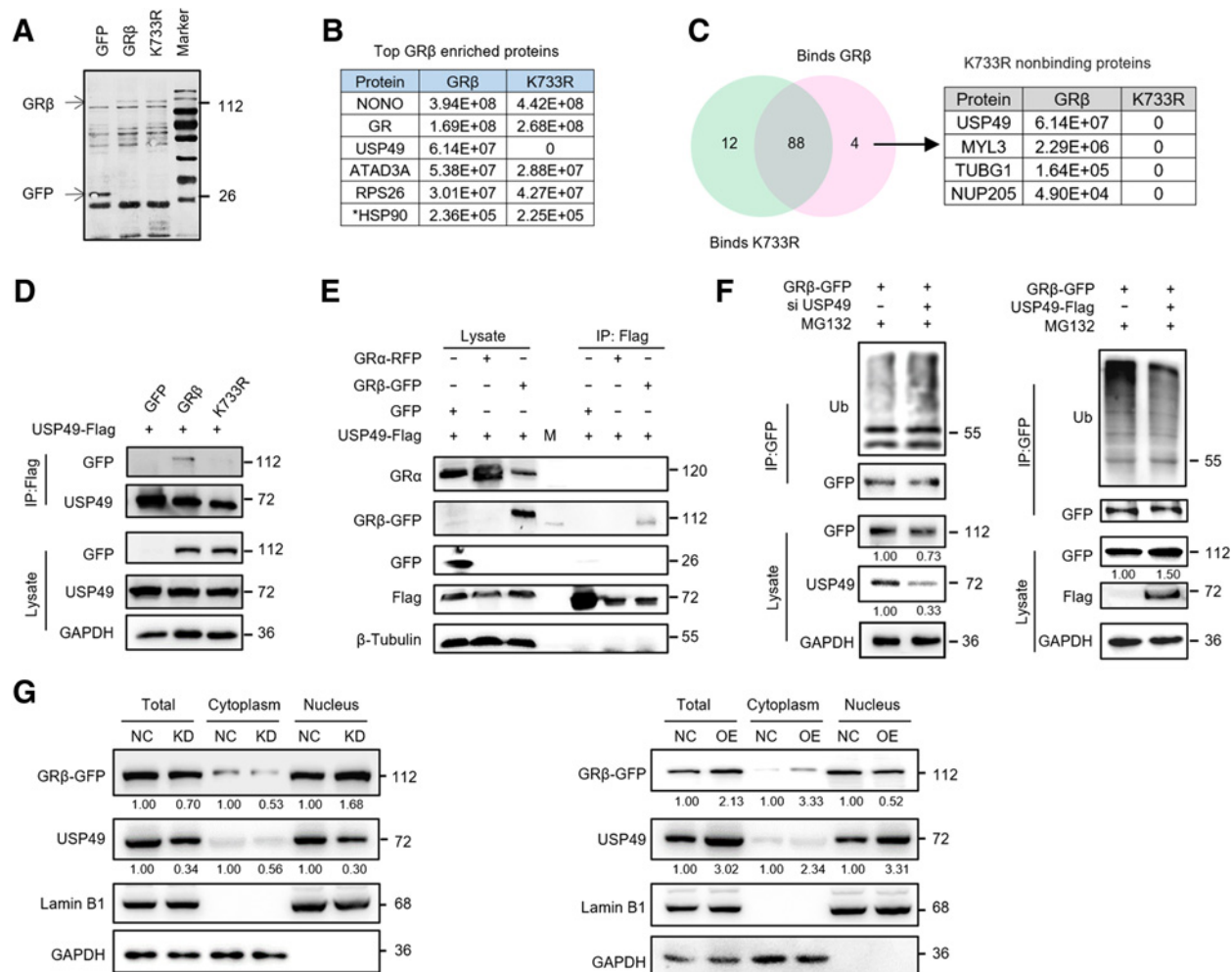
Figure 4.
USP49 binds GRβ and influences its ubiquitination level. **A,** Proteins binding to GRβ, K733R, and GFP were enriched by GFP antibody-labeled precipitation. **B,** LC/MS-MS identified the top six proteins binding to GRβ. **C,** Screening for proteins that bind GRβ but do not bind K733R. **D,** IP analysis of the interaction between GRβ and USP49 in indicated U87 cells with ectopic USP49-Flag. **E,** IP analysis of the interaction between USP49 and GRβ or GRα in indicated U87 cells with ectopic USP49-Flag. M represents protein ladder. **F,** Analyzing the ubiquitination and expression of GRβ using IP and Western blot with the indicated antibodies. GRβ stably expressing U87 cells were transfected with USP49 siRNA for 48 hours (left) or USP49-Flag vector (right panel) before harvest. The quantification of GRβ and USP49 expression were listed below. **G,** Western blot analysis of cellular GRβ expression in GRβ stably U87 cells with USP49 knockdown (left) or ectopic USP49-Flag (right). The quantification of GRβ and USP49 expression were listed below. GAPDH and Lamin B1 served as cytoplasmic and nuclear marker, respectively. NC, negative control; OE, overexpression; KD, knockdown.

study reports a novel finding that ubiquitination of GRβ in GBM cells is determined by a single amino acid residue at K733. Moreover, using the K733R mutation as a nonubiquitinated version of GRβ, we determined that ubiquitination is a vital PTM for GRβ functioning in GBM. Ubiquitination regulates multiple biological processes by modifying target protein stability, subcellular localization, and trafficking (25, 26). The current observations indicated that ubiquitination simultaneously induces proteasomal degradation and nuclear translocation of GRβ. It is generally believed that ubiquitination-mediated protein degradation leads to the functional loss or inactivation target proteins; one typical example is p53 (27). The effects of ubiquitination on GRβ in GBM seem to be a contradictory process. The proteasome degradative pathway plays important roles in regulating steroid receptor action and exerting distinct effects on individual receptor types (28). GRα undergoes proteasome-dependent degradation and turnover

upon ligand-binding as a vital process for its cellular translocation, and following transactivation and transrepression (17, 19, 29, 30). Unique from other steroid receptors whose transcriptional activity are reduced upon ubiquitination processes, the transcriptional activity of GRα is enhanced upon entering the ubiquitin-proteasome degradation pathway (31). The functional mechanisms recruited by ubiquitination in GRα can be extrapolated to predict the effects of ubiquitination on GRβ activity, though it remains unclear how GRβ can be spontaneously activated and undergo ubiquitination in a ligand-free state. Ultimately, we propose that the dynamic between ubiquitination and deubiquitination is a crucial mechanism to maintain the functions of GRβ in GBM.

USP49 is a USP participating in various biological or pathologic processes (32, 33). In human pancreatic cancer, USP49 acts as a tumor suppressor and increases the chemotherapy response in a FKBP51-

Figure 5.
USP49 knockdown enhances onco-functions of GRβ. **A,** Cell growth assay of indicated stable U87 cells with or without USP49 KD. Cells were transfected with USP49 siRNA for 48 hours and cell growth was assayed by CCK-8 ($n = 6$, mean ±SD, one-way ANOVA with SNK test; **, $P < 0.01$). ns, not significant. **B,** Comparing the fold change of cell growth between the indicated groups ($n = 6$, mean ±SD, Student t test; **, $P < 0.01$). **C,** 3D invasive assay of indicated stable U87 cells with or without USP49 knockdown, Scale bar: 100 μm ($n = 6$, mean ±SD, one-way ANOVA with SNK test; *, $P < 0.05$; **, $P < 0.01$). **D,** Comparing the fold change of invasion between indicated groups ($n = 6$, mean ±SD, Student t test; **, $P < 0.01$). **E,** Apoptosis assay of indicated stable U87 cells with or without USP49 knockdown. Cells were treated with Etoposide (30 μmol/L) for 24 hours and apoptotic cells were measured by Annexin V/PI staining followed with flow cytometry analysis ($n = 3$, mean ±SD, one-way ANOVA with SNK test; **, $P < 0.01$). **F,** Comparing the fold change of apoptosis between the indicated groups ($n = 3$, mean ±SD, Student t test; *, $P < 0.05$).
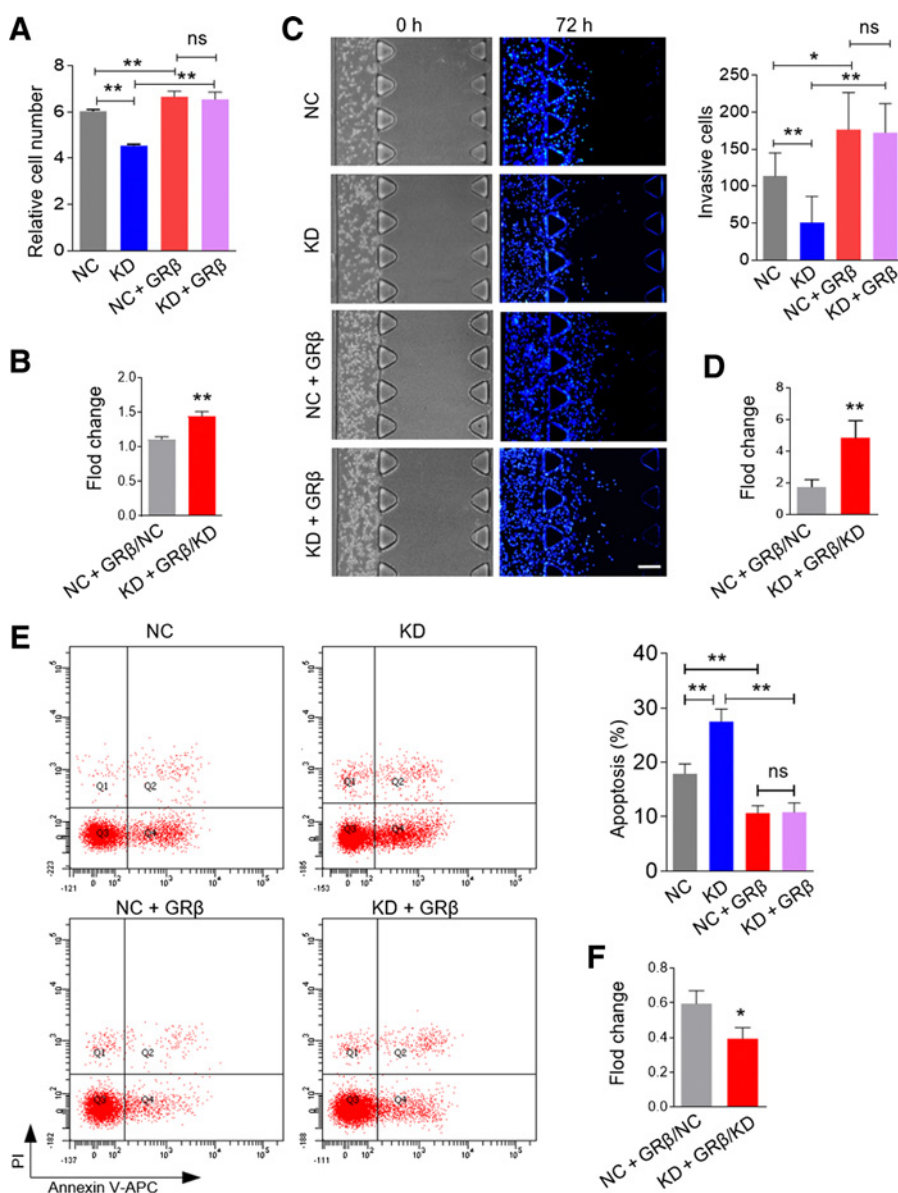
AKT–dependent manner (34). By stabilizing PTEN, USP49 inhibits non–small cell lung cancer growth (35). Moreover, it was reported that USP49 acts as a tumor suppressor by forming a positive feedback loop with p53 (36). The present study showed that USP49 promotes cell proliferation, invasion, and antiapoptosis, suggesting a role as a tumor promoter in GBM. The opposite roles of USP49 in various cancers is thought to be driven by the typical tumor microenvironment or upstream signaling pathways.

Although the current study observed that USP49 is a key enzyme that regulates GRβ deubiquitination, the mechanisms regulating GRβ ubiquitination remain unclear. Ubiquitination is involved in protein–protein interaction, protein trafficking, and protein biological processes. Ubiquitination is mediated by the sequential action of three enzymes: E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase; ref. 37). The E3 ligases are thought to determine the substrate specificity of the ubiquitination reactions. Identification of E3 ubiquitin ligases is extensively investigated (38).

Several E3 proteins which potentially bind and associate with GRβ for ubiquitination have been identified in the present study through IP–mass spectrometry (MS) analysis. Although K733 is a modification that determines the ubiquitination of GRβ, there is no evidence that it is a decisive site that affects the binding of E3 ligase to GRβ, therefore, making it difficult to draw candidate E3 ligase from the comparative analysis of GRβ- and K733R-binding proteins. Despite that, the important findings of this study not only clarify ubiquitination mechanisms occurring in GRβ at the K733 site, but also demonstrate the essential role of ubiquitination in the cancer promotion of GRβ in GBM.

In conclusion, this study discloses a novel finding that GRβ has a unique ubiquitination site at K733, a site which modulates the important roles of GRβ in GBM. In addition, it provides a tool for future functional and mechanistic studies of GRβ via the K733R mutant. Overall, this study provides new insights into the activity mechanisms of GRβ in GBM.

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References

1. Jacob F, Salinas RD, Zhang DY, Nguyen PTT, Schnoll JG, Wong SZH, et al. A patient-derived glioblastoma organoid model and biobank recapitulates inter- and intra-tumoral heterogeneity. Cell 2020;180:188–204.
2. Qazi MA, Vora P, Venugopal C, Sidhu SS, Moffat J, Swanton C, et al. Intra-tumoral heterogeneity: pathways to treatment resistance and relapse in human glioblastoma. Ann Oncol 2017;28:1448–56.
3. Erasimus H, Gobin M, Niclou S, Van Dyck E. DNA repair mechanisms and their clinical impact in glioblastoma. Mutat Res Rev Mutat Res 2016;769:19–35.
4. Yan C, Kong X, Gong S, Liu F, Zhao Y. Recent advances of the regulation roles of MicroRNA in glioblastoma. Int J Clin Oncol 2020;25:1215–22.
5. Liu N, Liu JT, Ji YY, Lu PP. Rosiglitazone regulates c-reactive protein-induced inflammatory responses via glucocorticoid receptor-mediated inhibition of p38 mitogen-activated protein kinase-toll-like receptor 4 signal pathway in vascular smooth muscle cells. J Cardiovasc Pharmacol 2011;57:348–56.
6. Cook PW, Swanson KT, Edwards CP, Firestone GL. Glucocorticoid receptor-dependent inhibition of cellular proliferation in dexamethasone-resistant and hypersensitive rat hepatoma cell variants. Mol Cell Biol 1988;8:1449–59.
7. Liu K, Zhang X, Wei W, Liu X, Tian Y, Han H, et al. Myostatin/SMAD4 signaling-mediated regulation of miR-124–3p represses glucocorticoid receptor expression and inhibits adipocyte differentiation. Am J Physiol Endocrinol Metab 2019;316:E635–45.
8. Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, et al. Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 1985;318:635–41.
9. Ramos-Ramirez P, Tliba O. Glucocorticoid receptor beta (GRbeta): beyond its dominant-negative function. Int J Mol Sci 2021;22:3649.
10. Oakley RH, Sar M, Cidlowski JA. The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. J Biol Chem 1996;271:9550–9.
11. Yin Y, Zhang X, Li Z, Deng L, Jiao G, Zhang B, et al. Glucocorticoid receptor beta regulates injury-mediated astrocyte activation and contributes to glioma pathogenesis via modulation of beta-catenin/TCF transcriptional activity. Neurobiol Dis 2013;59:165–76.
12. Wang Q, Lu PH, Shi ZF, Xu YJ, Xiang J, Wang YX, et al. Glucocorticoid receptor beta acts as a co-activator of T-cell factor 4 and enhances glioma cell proliferation. Mol Neurobiol 2015;52:1106–18.
13. Encio IJ, Detera-Wadleigh SD. The genomic structure of the human glucocorticoid receptor. J Biol Chem 1991;266:7182–8.
14. Scott A, Song J, Ewing R, Wang Z. Regulation of protein stability of DNA methyltransferase 1 by post-translational modifications. Acta Biochim Biophys Sin 2014;46:199–203.
15. Warden SM, Richardson C, O'Donnell J Jr, Stapleton D, Kemp BE, Witters LA. Post-translational modifications of the beta-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization. Biochem J 2001;354:275–83.
16. Rahimi N, Costello CE. Emerging roles of post-translational modifications in signal transduction and angiogenesis. Proteomics 2015;15:300–9.
17. Malyukova A, Brown S, Papa R, O'Brien R, Giles J, Trahair TN, et al. FBXW7 regulates glucocorticoid response in T-cell acute lymphoblastic leukaemia by targeting the glucocorticoid receptor for degradation. Leukemia 2013;27:1053–62.
18. Hua G, Ganti KP, Chambon P. Glucocorticoid-induced tethered transrepression requires SUMOylation of GR and formation of a SUMO-SMRT/NCoR1-HDAC3 repressing complex. Proc Natl Acad Sci U S A 2016;113:E635–43.
19. Wilkinson L, Verhoog N, Louw A. Novel role for receptor dimerization in post-translational processing and turnover of the GRalpha. Sci Rep 2018;8:14266.
20. Jiao J, Zhang R, Li Z, Yin Y, Fang X, Ding X, et al. Nuclear Smad6 promotes gliomagenesis by negatively regulating PIAS3-mediated STAT3 inhibition. Nat Commun 2018;9:2504.
21. Huang OW, Cochran AG. Regulation of deubiquitinase proteolytic activity. Curr Opin Struct Biol 2013;23:806–11.
22. Kino T, Manoli I, Kelkar S, Wang Y, Su YA, Chrousos GP. Glucocorticoid receptor (GR) beta has intrinsic, GRalpha-independent transcriptional activity. Biochem Biophys Res Commun 2009;381:671–5.
23. Duma D, Cidlowski JA. Generating diversity in glucocorticoid receptor signaling: mechanisms, receptor isoforms, and post-translational modifications. Horm Mol Biol Clin Investig 2010;3:319–28.
24. Wang Z, Garabedian MJ. Modulation of glucocorticoid receptor transcriptional activation, phosphorylation, and growth inhibition by p27Kip1. J Biol Chem 2003;278:50897–901.
25. Swatek KN, Komander D. Ubiquitin modifications. Cell Res 2016;26:399–422.
26. Mussell A, Shen H, Chen Y, Mastri M, Eng KH, Bshara W, et al. USP1 regulates TAZ protein stability through ubiquitin modifications in breast cancer. Cancers 2020;12:3090.
27. Hock AK, Vousden KH. The role of ubiquitin modification in the regulation of p53. Biochim Biophys Acta 2014;1843:137–49.
28. Rodriguez-Gonzalez A, Cyrus K, Salcius M, Kim K, Crews CM, Deshaies RJ, et al. Targeting steroid hormone receptors for ubiquitination and degradation in breast and prostate cancer. Oncogene 2008;27:7201–11.
29. Oakley RH, Cidlowski JA. Cellular processing of the glucocorticoid receptor gene and protein: new mechanisms for generating tissue-specific actions of glucocorticoids. J Biol Chem 2011;286:3177–84.
30. Wallace AD, Cao Y, Chandramouleeswaran S, Cidlowski JA. Lysine 419 targets human glucocorticoid receptor for proteasomal degradation. Steroids 2010;75:1016–23.
31. Wang X, DeFranco DB. Alternative effects of the ubiquitin-proteasome pathway on glucocorticoid receptor down-regulation and transactivation are mediated by CHIP, an E3 ligase. Mol Endocrinol 2005;19:1474–82.
32. Zhang Z, Jones A, Joo HY, Zhou D, Cao Y, Chen S, et al. USP49 deubiquitinates histone H2B and regulates cotranscriptional pre-mRNA splicing. Genes Dev 2013;27:1581–95.
33. Ye L, Zhang Q, Liuyu T, Xu Z, Zhang MX, Luo MH, et al. USP49 negatively regulates cellular antiviral responses via deconjugating K63-linked ubiquitination of MITA. PLoS Pathog 2019;15:e1007680.
34. Luo K, Li Y, Yin Y, Li L, Wu C, Chen Y, et al. USP49 negatively regulates tumorigenesis and chemoresistance through FKBP51-AKT signaling. EMBO J 2017;36:1434–46.

35. Shen WM, Yin JN, Xu RJ, Xu DF, Zheng SY. Ubiquitin specific peptidase 49 inhibits non-small cell lung cancer cell growth by suppressing PI3K/AKT signaling. Kaohsiung J Med Sci 2019;35:401–7.

36. Tu R, Kang W, Yang X, Zhang Q, Xie X, Liu W, et al. USP49 participates in the DNA damage response by forming a positive feedback loop with p53. Cell Death Dis 2018;9:553.

37. Friend SF, Deason-Towne F, Peterson LK, Berger AJ, Dragone LL. Regulation of T cell receptor complex-mediated signaling by ubiquitin and ubiquitin-like modifications. Am J Clin Exp Immunol 2014;3:107–23.

38. Kanack A, Vittal V, Haver H, Keppel T, Gundry RL, Klevit RE, et al. UbcH5 interacts with substrates to participate in lysine selection with the E3 ubiquitin ligase CHIP. Biochemistry 2020;59:2078–88.