Nuclear GSK3β induces DNA double-strand break repair by phosphorylating 53BP1 in glioblastoma

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Abstract. Glioblastoma is the most malignant and lethal subtype brain tumors with high risk of recurrence and therapeutic resistance. Emerging evidence has indicated that glycogen synthesis kinase 3 (GSK3)β plays oncogenic roles in multiple tumor types; however, the underlying mechanisms remain largely unknown. It has also been demonstrated that p53 binding protein 1 (53BP1) plays a central role in DNA double-strand break (DSB) repair. This study aimed to reveal the significance of GSK3β translocation from the cytoplasm to the nucleus, and to determine whether GSK3β induces DNA DSB repair in the nuclei of glioblastoma cells via phospho-53BP1. By performing *in vitro* experiments, we found that GSK3β translocated from the cytoplasm to the nucleus, and it then bound to 53BP1 following exposure to IR (IR). In addition, 53BP1-mediated DNA DSB repair was observed to be abrogated by the inhibition of GSK3β. Further experiments on the phosphorylation site of 53BP1 by GSK3β revealed that the S/T-Q motif may play a critical role. Importantly, our *in vivo* and *in vitro* data clearly indicated that GSK3β induced the phosphorylation of 53BP1 at the Ser166 site. Moreover, brain tumor xenograft models revealed that following exposure to IR plus SB216763, a specific GSK3β inhibitor, tumor growth was markedly inhibited and the survival of mice markedly increased. Based on these results, we concluded that the phosphorylation of 53BP1 by GSK3β was indispensable for DNA DSB repair. Our study also suggested that the inhibition of GSK3β by SB216763 significantly inhibited the proliferation and induced the apoptosis of glioblastoma cells. Taken together, our data indicate that GSK3β, a key phosphorylation protein for 53BP1, may be a potential target for enhancing the sensitivity of glioblastoma cells to radiation.

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Introduction

Glioblastoma multiforme (GBM), a neoplasm of the central nervous system (CNS), is a highly aggressive and the most malignant subtype of glioma (1). Attention was drawn to GBM due to its poor 2-year survival rate and high mortality rate. Although radiotherapy following surgical resection of the tumor remains the most effective treatment for GBM, this is still palliative as the majority of tumors recur (2). Various factors, including vasculogenesis, contribute to the recurrence (3-5). Among these, the main explanation for recurrence is the activation of DNA double strand break (DSB) repair following ionizing radiation (IR) (6). Evidence has accumulated over the past decade indicating that p53 binding protein 1 (53BP1) plays a central role in response to DNA damage recognition and repair (7,8). Moreover, the robust silencing of the 53BP1 gene sensitizes glioma cells to IR both *in vitro* and *in vivo*, suggesting the critical role of 53BP1 in radiotherap (9). Thus, molecules targeting the regulation of 53BP1 phosphorylation and activation may provide a therapeutic strategy with which to combat radioresistance. Studies on the structure of 53BP1 have revealed 3 highly conserved domains of 53BP1,

namely the BRCA1 carboxy-terminal (BRCT) repeats, the tandem tudor domain and 28 amino-terminal Ser/Thr-Gln (S/T-Q) sites. Upon IR, the S/T-Q site of 53BP1 is partially phosphorylated by the ataxia-telangiectasia mutated (ATM) kinase (7,10). Therefore, the question of whether there are other factors regulating the phosphorylation and activation of 53BP1 remains unresolved.

There is emerging evidence to suggest that glycogen synthase kinase 3β (GSK3β), a multifunctional serine/threonine kinase, is a key regulator of numerous signaling pathways, such as cell cycle control, proliferation, differentiation and apoptosis (11,12). Increasing knowledge has revealed the intriguing regulatory role of GSK3β in a diverse array of prevalent diseases, such as hypertrophy, diabetes and Alzheimer's disease (13,14). Previous studies on tumors have suggested that GSK3β is one of the driving forces behind tumorigenesis (15,16) and it plays critical role in multiple tumor types (17-19). Described as a tumor-promoting factor, GSK3β is interconnected with several critical pathways associated with tumors, such as the Wnt/β-catenin (19) and PI3K/PTEN pathways (20). In glioblastoma, studies have indicated the oncogenic role of GSK3β (21,22). More importantly, nuclear GSK3β is of particular interest as it regulates a number of transcription factors and is deemed to be a hallmark of high-grade tumors (23,24).

In this study, our findings demonstrated that IR induced the nuclear translocation of GSK3β, which was responsible for the activation of 53BP1, a key DNA damage response protein. Furthermore, a high level of nuclear GSK3β sensitized glioblastoma cells to IR via the phosphorylation of 53BP1 at serine 166. *In vivo* experiments revealed that the inhibition of GSK3β following IR may lead to a decreased tumor volume and an increased survival rate. Therefore, SB216763, a GSK3β specific inhibitor, may overcome radioresistance and may provide a therapeutic strategy for malignant brain tumors.

Materials and methods

Reagents, kits and antibodies. The GSK3 specific inhibitor, SB216763 (Cat. no. S3443), 3-(4,5-dimetrylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Cat. no. M5655), dimethyl sulfoxide (DMSO, Cat. no. D2650), paraformaldehyde (PFA, Cat. no. 16005), DAPI (Cat. no. D9542), protein A sepharose (Cat. no. P3391), HEPES (Cat. no. H3375), Triton X-100 (Cat. no. H9284), 2 mmol/l sodium orthovanadate (Na3VO4; Cat. no. S6508), sodium fluoride (Cat. no. S7920), 1 mmol/l edetic acid (Cat. no. E9884), PMSF (Cat. no. 78830), aprotinin (Cat. no. A11530), leupeptin (Cat. no. L2884) and rabbit polyclonal anti-Flag antibody (Cat. no. F7425) were purchased from Sigma-Aldrich (St. Louis, MO, USA). KU-55933 (Cat. no. S1092) was purchased from Selleck Chemicals (Houston, TX, USA). Rabbit polyclonal Mre11 antibody (Cat. no. 4895) was purchased from Cell Signaling Technology (Danvers, MA, USA). [γ-P32]ATP was purchased from Perkin-Elmer (Foster City, CA, USA). The nuclear and cytosolic protein extraction kit (Cat. no. AKR-171) was purchased from Cell Biolabs (San Diego, CA, USA). Mouse monoclonal GSK3β antibody (Cat. no. ab93926), rabbit polyclonal 53BP1 antibody (Cat. no. ab36823), rabbit polyclonal γ-H2AX (phosphor-S139) antibody (Cat. no. ab11174) and rabbit polyclonal GST antibody (Cat. no. ab19256) were purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal Lamin A antibody (Cat. no. sc-7292), mouse monoclonal β-actin antibody (Cat. no. sc-47778) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse monoclonal NBS1 antibody was purchased from Novus Biologicals (Littleton, CO, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG polyclonal antibody (Cat. no. 115-035-003), HRP-conjugated goat anti-rabbit polyclonal IgG (Cat. no. 111-035-003), FITC-conjugated goat anti-mouse IgG polyclonal antibody (Cat. no. 115-005-003) and FITC-conjugated goat anti-rabbit IgG polyclonal antibody (Cat. no. 111-095-003) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Cell culture, cell survival and DNA damage assay. The human glioblastoma cell line, U87, was obtained from the American Type Culture Collection (Cat. no. HTB-14; ATCC, Manassas, VA, USA). Short tandem repeat (STR) DNA profiling of the U87 cells was performed using the Cell ID System (Cat. no. G9500; Promega, Madison, WI, USA), indicating that the U87 cells used in this study were from ATCC. Although the U87 cell line from ATCC was not the original cell line from the University of Uppsala (25), the cells are still widely used in the study of glioblastoma (26,27). Previous studies have demonstrated that GSK3 is expressed in primary GBM (28) and U87 (ATCC) cells (24,29). Furthermore, *in vivo* U87 (ATCC) xenograft glioblastoma mouse model analyses on GSK3 have revealed that the combination of GSK3 inhibitor with carboplatin, a DNA damage-inducing reagent, results in a significantly reduced tumor volume (22). Moreover, U87 cells can be engineered with various expression vectors. Thus, we decided to use the U87 cells from ATCC in this study in both *in vitro* and *in vivo* experiments. The cells were cultured in DMEM containing 10% FBS, 100 μg/ml streptomycin (Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin (Life Technologies) and 0.03% L-glutamine (Sigma-Aldrich).

The U87 cells at logarithmic growth phase were seeded in a 96-well plate and incubated at 37˚C for 24 h, and GSK3 inhibitor 20 μM SB216763 and control solvent were added followed by incubation at 37˚C for a further 24 h. Subsequently, 0.05 mg (10 μl of 5 mg/ml) MTT was added to each well and incubated at 37˚C for 4 h, and the medium was then removed followed by shaking thoroughly for 1 h. Finally, termination buffer was added to each well. The absorbance at 570 nm was measured using a spectrophotometer (Model 3550 Microplate Reader, Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was determined as follows: Cell viability (%) = [OD 570 nm (drug)/OD 570 nm (control)] x100%.

8-Hydroxy-2'-deoxyguanosine (8-OHdG) is a modified nucleotide base and a by-product of DNA damage that is excreted upon DNA repair. By measuring the levels of 8-OHdG, DNA damage percentages were determined using a commercial ELISA kit (Cat. no. ADI-EKS-350; Enzo Life Sciences, Ann Arbor, MI, USA).

Immunofluorescence staining. The U87 cells were seeded into 6-well culture plate at a density of $4x10^5$ cells/well and cultured for 24 h. For the 53BP1 knockdown group, cells were transfected with 5 pM ON-TARGET plus Human

53BP1 siRNA pool (Cat. no. L-003548-00-0005, Dharmacon, Lafayette, CO, USA) by DharmaFect (Cat. no. T-2001-01, Dharmacon) for 48 h. The cells were fixed with 4% PFA for 30 min at room temperature, and then permeabilized by 1% Triton-X 100. After blocking with 5% BSA for 1 h at room temperature, the cells with incubated with primary antibody (mouse monoclonal GSK3 antibody, rabbit polyclonal γ-H2AX (phospho-S139) antibody and mouse monoclonal NBS1 antibody) overnight at 4˚C. The cells were then incubated with corresponding secondary antibody (FITC-conjugated goat anti-mouse IgG polyclonal antibody and FITC-conjugated goat anti-rabbit IgG polyclonal antibody) for 2 h at room temperature. After staining with DAPI, the cells were washed and analyzed immediately under a fluorescence microscope (Olympus, Tokyo, Japan). All experiments were carried out in triplicate. Experiments were repeated 3 times, and data are representative of replicate experiments.

Immunoprecipitation. The U87 cells were treated with 20 μM SB216763 for 24 h. For the GSK3β knockdown group, cells were transfected with 5 pM ON-TARGET plus Human GSK3β siRNA pool (Cat. no. LU-003010-00-0005, Dharmacon, Lafayette, CO, USA) by DharmaFect (Dharmacon) for 48 h. For the knockdown control group, cells were transfected with 5 pM scramble siRNA (Cat. no. D-001810-10-05, Dharmacon) by DharmaFect (Dharmacon) for 48 h, and the cells were then harvested. The cell pellets were resuspended with lysis buffer and lysed at 4˚C for 15 min. The lysis buffer consists of 50 mmol/l HEPES (pH 7.4), 1% Triton X-100, 2 mmol/l Na_3VO_4, 100 mmol/l sodium fluoride, 1 mmol/l edetic acid, 1 mmol/l PMSF, 10 mg/l aprotinin and 10 mg/l leupeptin. Following centrifugation at 12,000 x g for 15 min, the protein content of the supernatant was determined by Bradford protein assay (Cat. no. P0006; Beyotime, Suzhou, China). In addition, protein A Sepharose beads were incubated with GSK3β antibody or 53BP1 antibody at overnight 4˚C. The the cell supernatant was then incubated with 30 mg protein A Sepharose beads at overnight 4˚C, and performed western blot analysis was performed as described below.

Western blot analysis. The U87 cells were treated with 20 μM SB216763 for 24 h. For the GSK3β knockdown group, cells were transfected with 5 pM ON-TARGET plus human GSK3β siRNA pool (Cat. no. LU-003010-00-0005, Dharmacon) by DharmaFect (Dharmacon) for 48 h. For the knockdown control group, cells were transfected with 5pM scramble siRNA (Cat. no. D-001810-10-05, Dharmacon) by DharmaFect (Dharmacon) for 48 h, and the cells were then collected and the cell pellets were resuspended. Nuclear and cytoplasmic proteins were extracted using the nuclear and cytosolic protein extraction kit (Cat. no. AKR-171; Cell Biolabs) according to the manufacturer's instructions. The protein content was determined by Bradford protein assay. Equal amounts of the total protein were separated by 4-12% NuPAGE Bis-Tris gels (Cat. no. NP0327BOX, Life Technologies) and transferred onto PVDF membranes (Cat. no. ISEQ00010, Millipore, Bedford, MA, USA), and the membranes were soaked in 5% BSA (Cat. no. V900933, Sigma-Aldrich). The proteins were then detected using primary (mouse monoclonal GSK3β antibody, rabbit polyclonal 53BP1 antibody, mouse monoclonal Lamin A antibody, rabbit polyclonal anti-Flag antibody and rabbit polyclonal GST antibody) and secondary antibodies (HRP-conjugated goat anti-mouse IgG polyclonal antibody and HRP-conjugated goat anti-rabbit polyclonal IgG), and then visualized with ECL (Cat. no. 345818, Millipore). The experiments were repeated 3 times, and data are representative of replicate experiments.

Plasmids. The cDNA encoding full-length 53BP1 was obtained from the U87 cells by reverse transcription-polymerase chain reaction using a Reverse Transcript System kit (Cat. no. A3500, Promega), and then inserted into the pBluescript bacterial expression vector, which was purchased from Addgene (Cambridge, MA, USA). pGST-53BP1-N1, pGST-53BP1-N2, pGST-53BP1-C1 and pGST-53BP1-BRCT (Fig. 4D) were respectively constructed by ligating a NdeI-EcoRI fragment of full-length 53BP1 to pBluescript. The GST-fused 53BP1 constructs were overexpressed in *E. coli* BL21 (DE3) cells (Cat. no. CB101, Tiangen Biotech, Beijing, China) by induction with 0.4 mM IPTG, and then purified with glutathione Sepharose beads (GE Healthcare, Piscataway, NJ, USA). Point mutations for the replacement of Ser (S) with Ala (A) were performed by using a Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and all of the mutations were confirmed by DNA sequencing analysis. Flag tagged 53BP1 was constructed in pcDNA3, and Flag tagged GSK3 was constructed in pBABE-puro.

In vivo phosphorylation assay. For the analysis of 53BP1 labeling with [γ-^{32}P]ATP, the U87 cells were washed twice with phosphate-free Dulbecco's modified Eagle's medium and subsequently incubated with 15 mCi [γ-^{32}P]ATP for 3 h.

For irradiation of the cells, the cells were irradiated with a calibrated Shepherd and Associates Mark I self-shielded ^{137}Cs y irradiator (JL Shepherd and Associates Inc., San Fernando, CA, USA), at a dose rate of 1.84 Gy/min. As a control, the mock irradiation (0 Gy) was performed by placing the plates containing cells in the irradiator for the designated time period without turning on the machine. Following stimulation of the cells with 5 Gy IR, we immunoprecipitated GSK3β or 53BP1 as described previously. For treatment with λ-PPase (Cat. no. B0753S, New England Biolabs, Beverly, MA, USA), the cells were washed in ice-cold PBS and harvested in λ-PPase buffer supplemented with protease inhibitors. The cells were then sonicated on ice (Sonicator 4000, Misonix, Farmingdale, NY, USA), and treated with 800 units λ-PPase for 30 min at 30˚C. The proteins were separated by 4-12% NuPAGE Bis-Tris gels and scanned on a Typhoon phosphorimager (Typhoon 9200 instrument, GE Healthcare Sciences).

In vitro phosphorylation assay. The Flag-tagged GSK3 vector (Camod Biological, Beijing, China) was transfected into 293T cells (ATCC, Manassas, VA, USA) using Lipofectamine 2000 and Opti-MEM (both from Life Technologies). At 48 h after transfection, the cells were lysed by sonication in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM β-glycerophosphate, 150 mM NaCl, 10% glycerol, 1% Tween-20, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2 mg/ml aprotinin, 2 mg/ml leupeptin and 1 mM DTT. Following centrifugation at 10,000 x g for 10 min, Flag-tagged proteins were immunoprecipitated

with anti-Flag M2 antibodies (Sigma-Aldrich) and protein A Sepharose. Immunoprecipitated Flag tagged GSK3β was bound to protein A Sepharose and 1 μg of GST-53BP1 fragment substrates described above in 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 25 mM ATP, and 15 mCi [γ-^{32}P] ATP for 30 min at 30˚C. The phosphorylated 53BP1 proteins were separated by 4-12% NuPAGE Bis-Tris gels and scanned on a Typhoon phosphorimager (Typhoon 9200 instrument, GE Healthcare Sciences).

Tumor xenograft model. To establish tumor xenografts, $5x10^6$ U87 cells were injected into BALB/c nude male mice (5-6 weeks old, 16-18 g body weight, purchased from the Affiliated Laboratory Animal Center of Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital, Chengdu, China). Following implantation, the tumors were allowed to grow to a size of 100-550 mm^3. No mouse bearing multiple tumors was found in our study. The mice are randomly divided into 4 groups (n=80 in total and n=20 per group) as follows: i) the control group, in which mice with U87-derived tumors were left untreated; ii) the IR group, in which mice received IR 25 Gy following tumor formation by U87 cells; iii) the SB216763 group, in which mice were intraperitoneally administered 5 mg/kg SB216763 following tumor formation by U87 cells; iv) the SB216763 + IR group, in which mice intraperitoneally were administered 5 mg/kg SB216763 and subjected to IR 25 Gy following tumor formation by U87 cells. Following treatment, mouse body weight was measured every 2 days. Subsequently, 10 mice in each group were sacrificed, and 10 mice in each group were used for survival analysis. The subcutaneous tumors were removed from the sacrificed mice, weighed and fixed for immunofluorescence staining described above. In brief, tumors were fixed in 10% formalin (Cat. no. HT-5011, Sigma-Aldrich) for 24 h at room temperature, dehydrated, embedded in paraffin (Cat. no. A601888, Sangon Biological, Shanghai, China) and sectioned (5 μm). To detect the apoptosis, transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using Roche fluorescence Dead End kit (Cat. no. 11684795910, Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. The cells were analyzed and images were captured using a fluorescence microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan). In addition, the volume of the tumors was determined in 3 dimensions with venire calipers according to the following formula: Tvol = length x width x depth x 0.5. Animal handling was in accordance with the Ethics Committee of Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital, and all animals were kept in a 12 h light/dark cycle with free access to water and food (26˚C and 40-60% humidity), which is in accordance with IVC requirements at the Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital.

Statistical analysis of the data. All data are expressed as means ± SEM from at least 3 independent experiments. Statistical significance between multiple groups was determined by ANOVA with a Bonferroni post-hoc test, and between 2 groups using a Student's t-test. Survival analyses were carried out using Kaplan-Meier curves. The experiments were repeated 3 times, and data are representative of replicate experiments. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Nuclear translocation of GSK3β and its binding with 53BP1. Based on the previous data indicating that GSK3β mRNA and protein levels are increased in glioblastoma (28), we hypothesized that the inhibition of GSK3β may enhance the sensitivity of U87 cells to IR. As predicted, the proliferation rates of the U87 cells treated with 20 μM SB216763, a specific GSK3β inhibitor, were significantly decreased (Fig. 1A), indicating that the inhibition of GSK3β may be a potential radiosensitive therapeutic method in the treatment of GBM after IR. Accumulating evidence indicates that GSK3β is predominantly a cytosolic protein in glioblastoma cells and that nuclear GSK3β promotes tumorigenesis in glioblastoma stem cells (30). Therefore, in this study, to ascertain the distribution of GSK3β in glioblastoma cells, we performed western blot analysis using the U87 cells. As shown in Fig. 1B, 5 of the cells exposed to Gy IR had a higher expression level of GSK3β in the nucleus compared with that of the control cells. Furthermore, the results were validated by immunofluorescence staining of the glioblastoma cells upon exposure to IR; GSK3β was found present in the nucleus following the exposure of the cells to 5 Gy IR (Fig. 1C). Taken together, our findings indicated that IR was able to promote the translocation of GSK3β from the cytosol to the nucleus in U87 cells.

As it has been reported that nuclear GSK3β promotes tumorigenesis in glioma stem cells and regulates several transcription factors, we hypothesized that it may bind with nuclear protein 53BP1, a key regulator in DSB repair, to initiate recurrence. To elucidate the mechanisms responsible for GSK3β-mediated DNA DSB repair, a series of immunoprecipitation experiments were performed. As shown in Fig. 2A, 1 h following exposure to 5 Gy IR, the nuclear shift of GSK3β contributed to the binding with 53BP1, and ultimately led to DNA DSB repair, which was chemically abrogated by SB216763, a specific GSK3 inhibitor. As shown in Fig. 2B, the binding of GSK3β with 53BP1 was also abolished by the silencing of GSK3β by siRNA, indicating that following exposure to 5 Gy IR, GSK3β translocates to the nucleus, binds to 53BP1 and further initiates DNA DSB repair.

Inhibition of GSK3β abolishes 53BP1-mediated DSB repair. As discussed above, as SB216763, a specific GSK3β inhibitor, evidently decreased the proliferation rates of the U87 cells, and the translocation of GSK3β into the nucleus was followed by binding to 53BP1 and subsequently DSB repair, we evaluated whether the inhibition of GSK3β would abolish 53BP1-mediated DSB repair. As shown in Fig. 3A, accompanied by the inhibitory effects of SB216763 and GSK3β siRNA on GSK3β, DSB repair key factors, including γ-H2AX, Mre11 and NBS1 were dispersed in the nucleus, indicating that the inhibition of GSK3β did not induce the formation of the MRN complex, which is indispensable for DSB repair (31). The results were further confirmed though the assessment of DNA damage percentages and proliferation rates. DNA damage percentages were significantly increased (Fig. 3B), while the proliferation rates were markedly decreased (Fig. 3C)

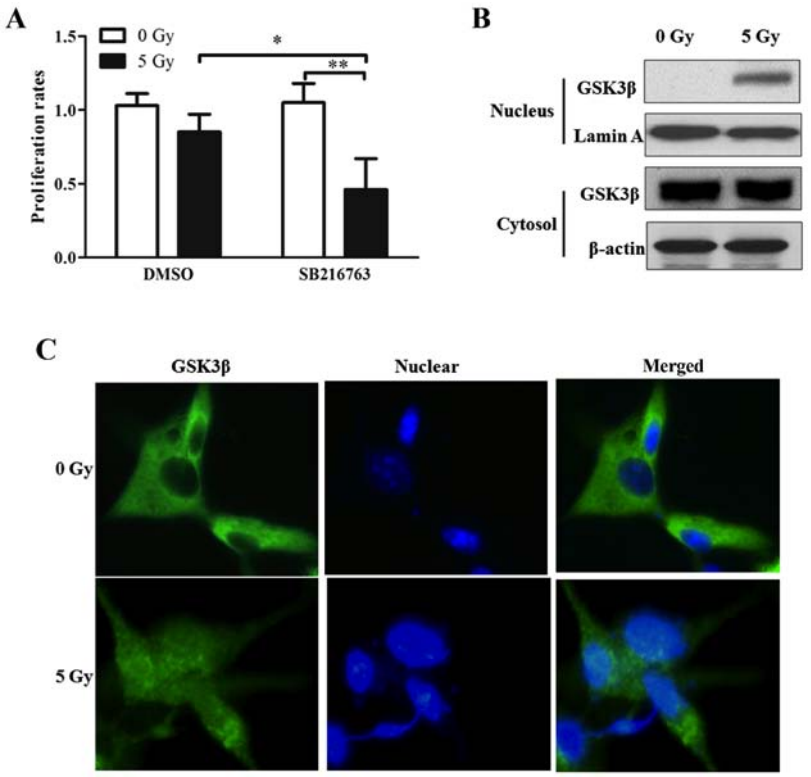

Figure 1. GSK3β translocates into nucleus following ionizing radiation (IR). (A) Proliferation rates of U87 cells treated with SB216763 (GSK3β inhibitor); *P<0.05 vs. the control. Data are the means ± SEM from 3 independent experiments. (B) Expression level of GSK3β in the nucleus and cytosol following treatment with IR. (C) Immunofluorescence staining images of GBM cells with or without treatment with IR.

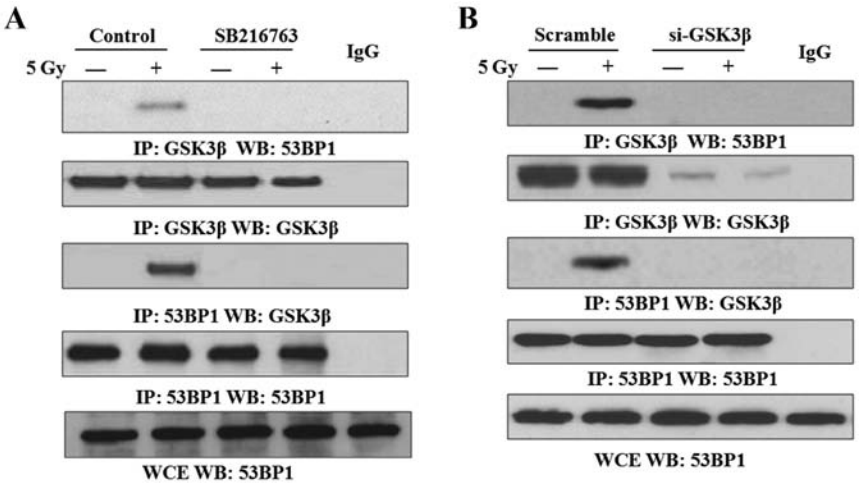

Figure 2. GSK3β binds with 53BP1 upon exposure to ionizing radiation (IR). Co-immunoprecipitation of GSK3β with 53BP1 was performed by protein A/G beads incubated with anti-GSK3β antibody, and western blot analysis was then performed to detect the immunoprecipitated 53BP1 with anti-53BP1 antibody. With the addition of GSK3β specific inhibitor (A) SB216763 and (B) siRNA, the binding of 53BP1 with GSK3β was abolished. Co-immunoprecipitation of 53BP1 with GSK3β was also performed and the GSK3β protein levels were determined by western blot analysis. WCE, whole cell extract.

following treatment of SB21676 or transfection with GSK3β siRNA, suggesting that 53BP1-mediated DSB repair may be abrogated by the inhibition of GSK3β.

GSK3β induced the phosphorylation of 53BP1. It has been previously demonstrated that GSK3β may co-localize with 53BP1, which contains several potential GSK3β phosphorylation sites involving a canonical S/T-Q motif (7), suggesting the phosphorylation and activation of 53BP1 by GSK3β. In this study, to address the interactive mechanisms between GSK3β and 53BP1, we observed whether 53BP1 could be phosphorylated endogenously by GSK3β *in vivo*. As demonstrated in Fig. 4A, 53BP1 was phosphorylated by GSK3β following exposure to 5 Gy IR, which was be abrogated by treatment with SB216763. λ-PPase, a phosphotransferase inhibitor, also abolished the GSK3β-induced phosphorylation of 53BP1. The results were further substantiated *in vivo* phosphorylation by the silencing of GSK3β by GSK3β siRNA and phosphotransferase

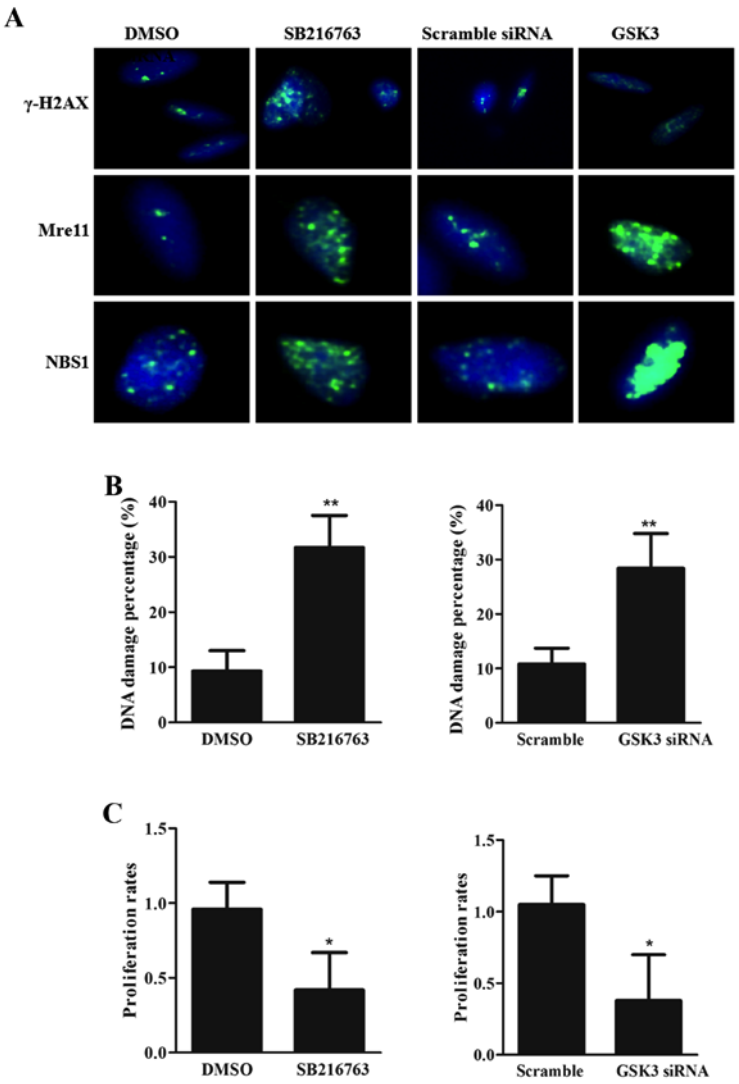

Figure 3. Inhibition of GSK3 abolishes 53BP1-mediated DNA repair. (A) Immunofluorescence staining images of γ-H2AX, Mre11 and NBS1 in the nucleus of cells treated with control solvent (DMSO), SB216763 (GSK3β inhibitor), scramble siRNA and GSK3β siRNA. (B) DNA damage percentage of U87 cells treated with SB216763 (left panel) and GSK3β siRNA (right panel); $^{**}P<0.01$ vs. control. (C) Proliferation rates of U87 cells treated with SB216763 (left panel) and GSK3β siRNA (right panel); $^{*}P<0.05$ vs. control group, as shown by the Student's t-test. Data are the means ± SEM from 3 independent experiments.

restraint by λ-PPase (Fig. 4B), and *in vitro* via the inhibition of GSK3β by SB216763 (Fig. 4C). To characterize the phosphorylation site of 53BP1 *in vitro*, we generated NH2-terminal (N1, N2, N3 and N4) and COOH-terminal (C1 and BRCT) 53BP1 proteins fused with GST (Fig. 4D). The phosphorylation blot was detected when the NH2-terminal 1 (N1) of 53BP1 was used as a substrate. Therefore, it was obvious that the phosphorylation site of 53BP1 is at the N1 fragment via phosphorylation *in vivo* (Fig. 4E), which was in accordance with phosphorylation *in vitro* (Fig. 4F). In summary, our *in vivo* and *in vitro* phosphorylation data clearly indicated that GSK3β induced the phosphorylation of 53BP1 at the N1 site.

As demonstrated above, GSK3β bound to 53BP1 following its translocation into the nucleus, and GSK3β is critical to 53BP1-mediated DSB repair. Although we found that the phosphorylation domain of 53BP1 may reside at the NH2-terminal, we wished to determine which amino acid was phosphorylated by GSK3β. It has been demonstrated that Ser25, Thr302, Ser831, Ser166, Ser176/Ser178 and Ser452 may be potential phosphorylation sites of 53BP1 upon IR treatment (32). In this study (Fig. 4E and F), we demonstrated that the phosphorylation site should be from 1 to 301 amino acids, and Ser25 was a typical ATM phosphorylation site (33); thus, we hypothesized that the phosphorylation sites of 53BP1 may be Ser166 or Ser176/Ser178. Thereby, we mutated Ser166 and Ser176, which are canonical phosphorylation sites, and observed which mutation of 53BP1 may abolish its binding with GSK3β. As shown in Fig. 5A, GSK3β bound to 53BP1 in the nucleus without the inhibition of SB216763, and this effect was abrogated by the mutation of 53BP1 at S166A. But for S176A 53BP1, in the absence of GSK3β inhibitor, S176A 53BP1 could still bind with GSK3β, indicating that Ser176 was not the phosphorylation site of 53BP1 by GSK3β. To address the effects of the S166A mutation, we observed whether the S166A mutation could negatively regulate the phosphorylation of 53BP1. The U87 cells were pre-treated with 10 μM KU-55933 for 48 h, and then co-treated with with 20 μM SB216763 for a further 24 h piror to exposure to IR. We found that the phosphorylation of 53BP1 was abrogated due to the S166A mutation. And for S176A 53BP1, it could still be phosphorylated, revealing that Ser176 was not the

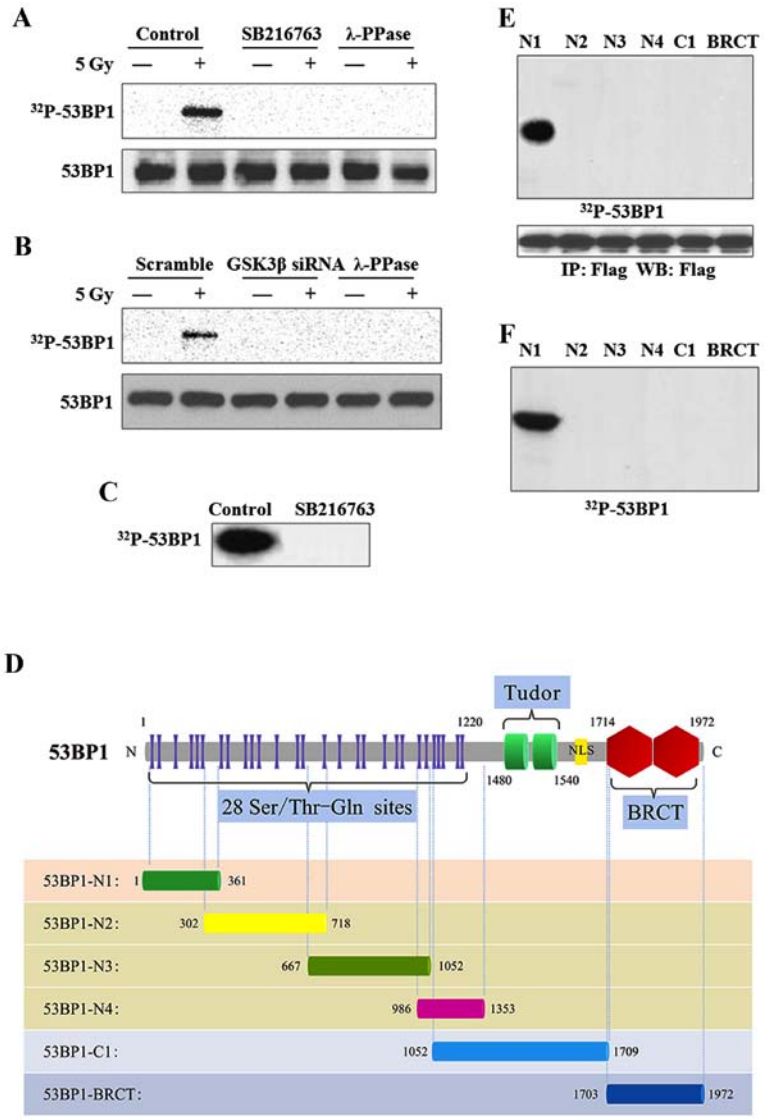

Figure 4. 53BP1 is phosphorylated by GSK3β at the N1 fragment following exposure to IR. (A) *In vivo* phosphorylation analysis of 53BP1 in the presence of the ATM inhibitor, KU-55933. The phosphorylation of 53BP1 was abrogated by GSK3β inhibitor and abolished by λ-PPase. (B) *In vivo* phosphorylation assay of 53BP1 with scramble siRNA or with GSK3β siRNA. (C) *In vitro* phosphorylation assay of 53BP1 by GSK3β. (D) The schematic map of 6 fragments of 53BP1, N1, N2, N3, N4, C1 and BRCT, which were cloned on an expression vector. (E) Endogenous 53BP1 was knocked down by siRNA and the cells were transfected with vectors expressing fragments of 53BP1 as a substrate. *In vivo* phosphorylation assay was performed to assess the phosphorylation site of 53BP1. (F) GST fused 53BP1 fragments were purified and *in vitro* phosphorylation was performed.

phosphorylation site of 53BP1 by GSK3β (Fig. 5B). In addition, we mixed GST immobilized mutational 53BP1 proteins with purified Flag tagged GSK3β in the presence or absence of IR, and found that GSK3β could not phosphorylate 53BP1 due to the S166A mutation (Fig. 5C). Taken together, the data above explicitly confirmed that the mutation at S166A abrogated the phosphorylation of 53BP1 by GSK3β, and consequently abolished the binding of these two factors.

Mutation at S166A abolishes 53BP1-mediated DSB repair. As mentioned above, 53BP1, primarily exists in the nucleus in GBM cells, and is essential for DSB repair. Therefore, we hypothesized that the mutation of 53BP1 may negatively affect 53BP1-mediated DSB repair. As predicted, DSB repair factors, including γ-H2AX, Mre11 and NBS1 were dispersed in the nucleus via the mutation at S166A and the silencing of 53BP1 (Fig. 6A), which suggested the failure of the MRN complex formation, resulting in the restraint of 53BP1-mediated DSB repair. The results were further ascertained through statistical analysis, as shown in Fig. 6B and C. Notably, the cell proliferation rates were decreased, whereas the DNA damage percentages were increased due to the mutation at S166A and the silencing of 53BP1, indicating that the mutation at S166A abolished 53BP1-mediated DSB repair.

Inhibition of GSK3β increases the radiosensitivity of brain tumor xenografts. To examine the effects of GSK3β inhibition on the radiosensitivity of brain tumor xenografts, we examined the growth rates of these tumors with or without SB216763 treatment. As shown in Fig. 7A and B, the calculation of the tumor volume and weight revealed that treatment with SB216763 plus IR markedly inhibited tumor growth compared to treatment with IR treatment alone, SB216763 treatment alone and treatment with normal saline (control);

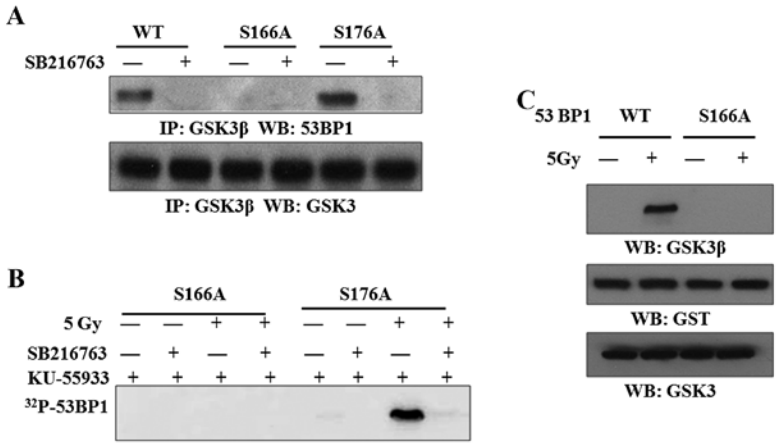

Figure 5. Mutation at S166A abrogates the phosphorylation of 53BP1 by GSK3β. (A) *In vivo* co-immunoprecipitation of GSK3β and mutational 53BP1 was performed with the addition of SB216763 or control solvent. Western blot analysis was performed by anti-53BP1 antibody. (B) The ATM kinase inhibitor, KU-55933, was added to the cell medium, and *in vivo* phosphorylation assay of mutational 53BP1 with or without SB216763 was performed. (C) Immobilized GST-53BP1 proteins were mixed with purified Flag-GSK3 proteins in the presence or absence of IR.

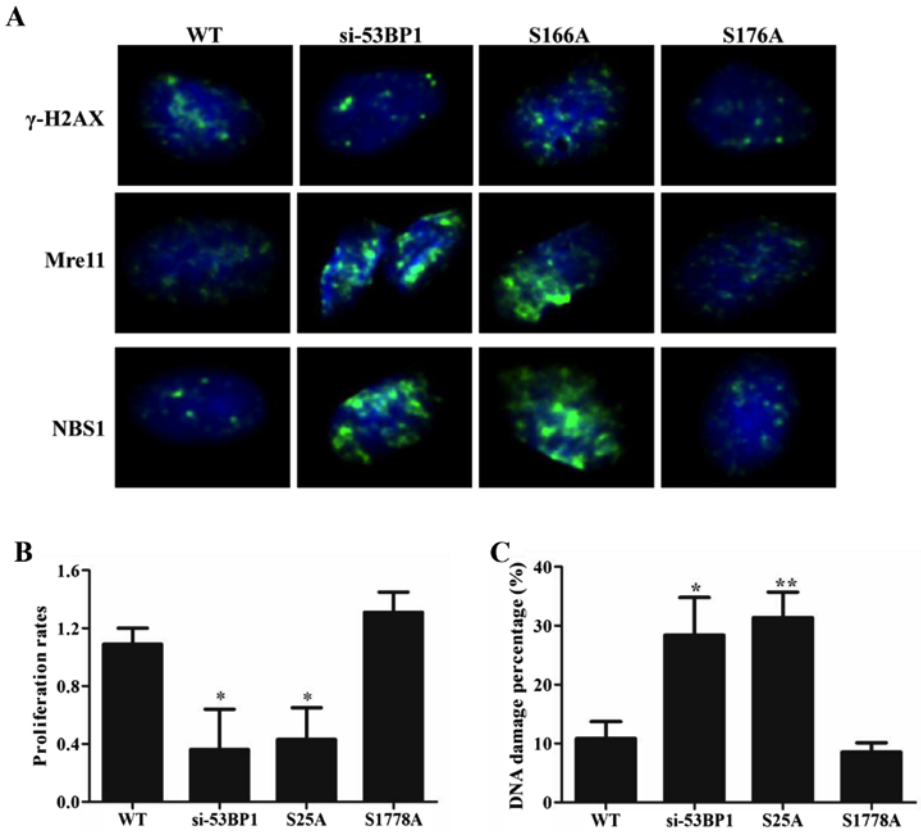

Figure 6. Mutation at S166A abolishes 53BP1-mediated DSB repair. (A) Immunofluorescence staining images of γ-H2AX, Mre11 and NBS1 in the nucleus of cells treated with wild-type 53BP1, si-53BP1, S166A and S176A mutation of 53BP1. (B) Proliferation rates and (C) DNA damage percentages of U87 cells were assessed. *P<0.05 and **P<0.01 vs. the wild-type 53BP1 group, as shown by one-way ANOVA with with Bonferroni post-hoc test. Data are the means ± SEM from 3 independent experiments.

this was further confirmed by the decreased tumor size of the tumors obtained from the mice treated with SB216763 plus IR (Fig. 7D). Furthermore, judged by the body weights and survival curve, treatment with SB216763 plus IR led to a marked recovery in body weight (Fig. 7E) and prolonged the lifespan of mice with brain tumor xenografts compared with those of the control group (Fig. 7C). In addition, as shown in Fig. 7F, a greater number of apoptotic cells was observed following treatment with SB216763 plus IR, as shown by TUNEL labeling. Taken together, based on the qualitative and quantitative analysis above, the inhibition of GSK3β via SB216763 plus IR treatment enhyanced the radiosensitivity of brain tumor xenografts.

Molecular signaling pathway for GSK3β- and 53BP1-mediated DSB repair. The mutual effects between

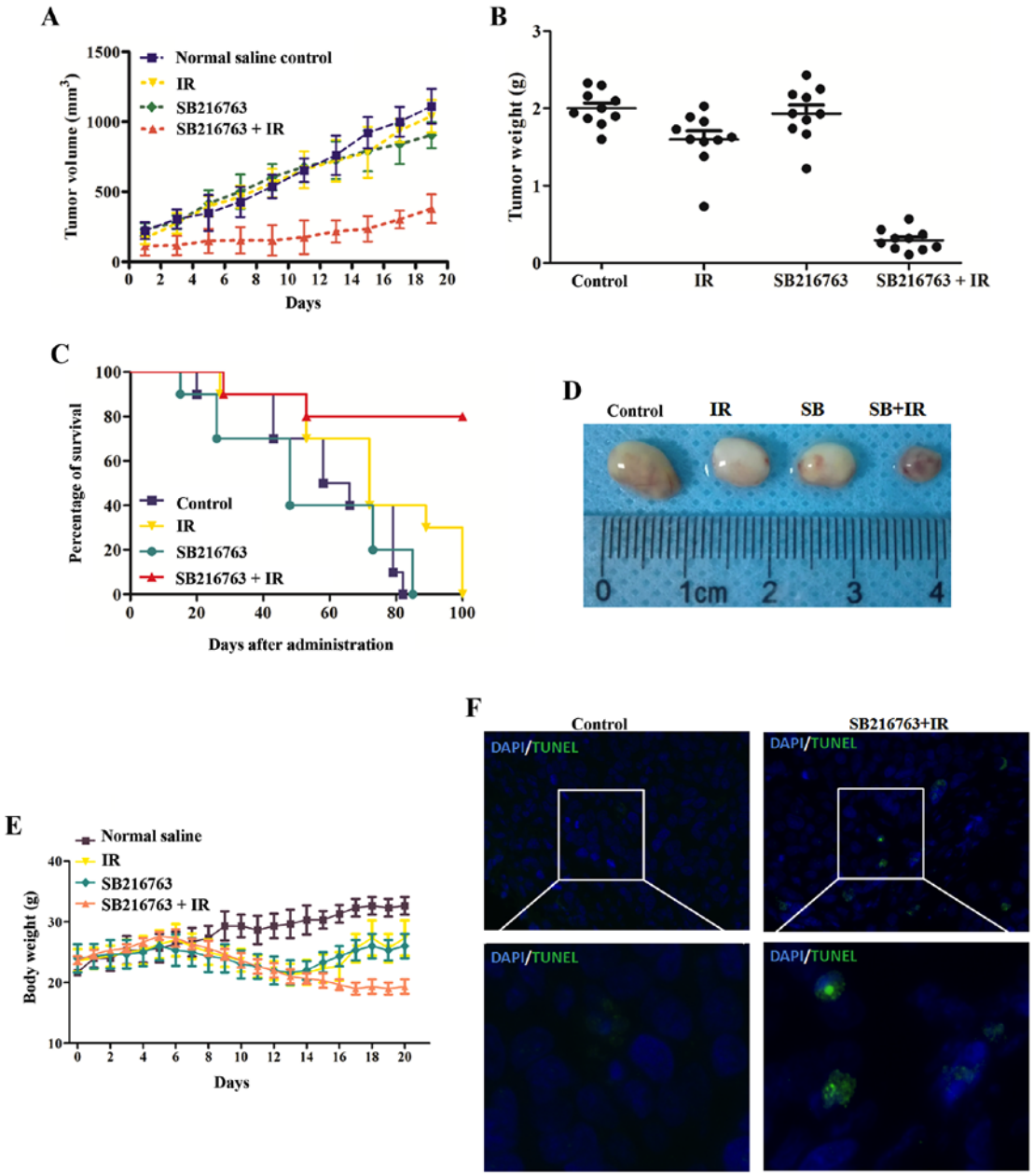

Figure 7. Inhibition of GSK3 enhances the radiosensitivity of brain tumor xenografts. (A) Tumor volume were determined in the control group, ionizing radiation (IR) group, SB216763 group and SB21676 plus IR group (n=10, P<0.05 by ANOVA with Bonferroni post-hoc test). (B) Tumor weights of each group (n=10, P<0.05 by ANOVA wtih Bonferroni post-hoc test). (C) Survival curve of each group (n=10, P<0.05 by Kaplan-Meier). (D) Representative image of xenograft brain tumors. (E) Body weights of each group (n=10, P<0.05 by ANOVA wtih Bonferroni post-hoc test). (F) Immunofluorescence staining images of TUNEL assay of apoptosis with DNA double-strand breaks in cells of the control group or the SB216763 plus IR group. Data are means ± SEM from 3 independent experiments.

GSK3β and 53BP1 on DSB repair are shown in Fig. 8. On the basis of IR treatment, GSK3β, highly expressed in GBM cells, translocated from the cytoplasm to the nucleus. Co-localized with 53BP1, predominantly existing in the nucleus, GSK3β bound with it by inducing the phosphorylation of 53BP1. As a result, 53BP1-mediated DSB repair was initiated via the stimulation of the MRN complex formation, resulting in the radioresistance of GBM cells. On the other hand, SB216763, a potent and specific GSK3β inhibitor, chemically abrogated the translocation of GSK3β and ultimately led to the apoptosis of GBM cells. In a word, GSK3β and 53BP1 played a central role in DSB repair and the radiosensitivity of GBM cells.

Discussion

It has been widely acknowledged that with a poor 2-year survival rate, GBM accounts for 80% of all high-grade primary CNS cancers and is the leading cause of cancer-related mortality (1,2). The standard therapy for GBM consists of surgical resection followed by radiotherapy and chemotherapy (2). Although for patients with malignant glioma, chemotherapy has been given as an adjunct to radiotherapy or before radiotherapy, most of these combined treatments have limited success, and the recurrence of malignant glioma is still presumably high. Therefore, it is well established that

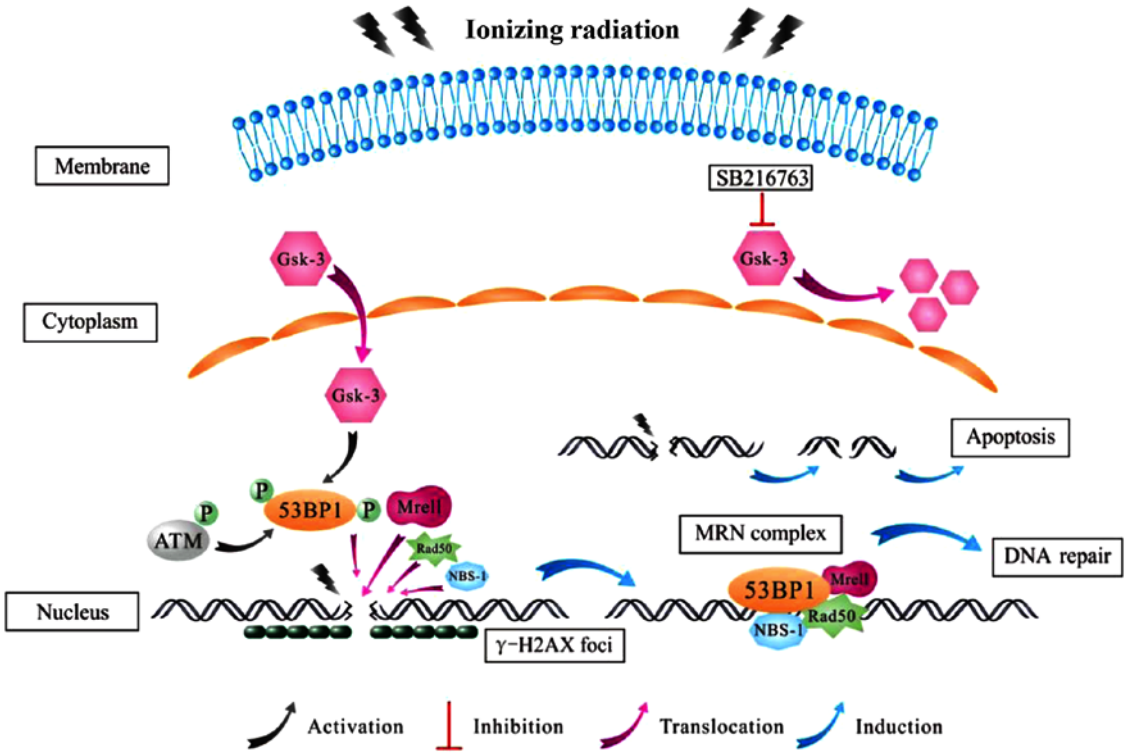

Figure 8. The diagram illustrates the regulation of 53BP1-mediated DSB repair via GSK3β. On the basis of exposure to ionizing radiation (IR), GSK3β, highly expressed in GBM cells, translocates from the cytoplasm to the nucleus. Co-localized with 53BP1, predominantly existing in the nucleus, GSK3β binds with it by inducing the phosphorylation of 53BP1. As a result, 53BP1-mediated DSB repair is initiated via the stimulation of the MRN complex formation, resulting in the radioresistance of GBM cells. On the other hand, SB216763, a potent and specific GSK3β inhibitor, chemically abrogates the translocation of GSK3β and ultimately leads to the apoptosis of GBM cells.

the development of effective therapeutic agents to improve the radiosensitivity of glioblastoma is of utmost importance. In the present study, we observed the nuclear translocation of GSK3β following IR, and the binding of GSK3β in the nucleus with 53BP1, a key DSB repair protein. Based on these observations, we hypothesized that the IR-induced GSK3β nuclear translocation may activate 53BP1 via phosphorylation at the S/T-Q motif. By cloning different fragments of 53BP1, we found that the phosphorylation site of 53BP1 resides at the N-terminal. The site mutation of Serine to Alanine of S/T-Q motif abrogated the GSK3β induced phosphorylation of 53BP1, which ultimately led to decreased DSB repair. Furthermore, data collected from tumor growth and survival curve analyses using animal models indicated that SB216763, a GSK3β specific inhibitor, enhanced the sensitivity of tumor xenografts to IR and may thus be a potential therapeutic drug for the treatment of GBM.

As a multifunctional serine-threonine (S/T) protein kinase found in all eukaryotes, GSK3 has a broad regulatory function, including metabolism, cell fate specification, cell division and cell death (34). There are two closely related isoforms, GSK3α and GSK3β, which are encoded by different genes and share nearly identical sequences in their kinase domains. GSK3β interconnects in multiple pathways, such as Wnt, Notch and G-protein coupled receptor (GPCR) signaling (35,36). The aberrant activation of the Wnt and Notch pathways, which is under strict spatial and temporal regulation, has been reported to be tightly associated with tumorigenesis (23). Therefore, by providing cancer cells with a higher self-renewal capacity, GSK3β play a fundamental and critical role in tumorigenesis on a subset of cancers. It has been shown that the downregulation of GSK3β induces the apoptosis of glioma cells (28) and nuclear GSK3β maintains stem cell-like characteristics of glioblastoma by phosphorylating KDM1A (24). However, the question of whether GSK3β is involved in DSB repair following IR remains unanswered. Thus, in this study, we performed cell proliferation assay, and found that GSK3β inhibitor possessed anti-proliferative capabilities following IR, suggesting that GSK3β may be directly or indirectly involved in the DSB repair signaling pathway. As GSK3β is predominantly a cytosolic protein, we proposed that, to mediate DSB repair, GSK3β should be shifted to the nucleus. Therefore, we focused our study on the cellular distribution of GSK3β following IR. Besides, a previous study on glioblastoma stem cells revealed the critical role of nuclear GSK3β in tumorigenesis (28). Therefore, judged from both the cell immunofluorescence staining images and the western blot analysis data of total nuclear protein, IR induced the translocation of GSK3β from the cytoplasm to the nucleus. Moreover, to elucidate the mechanisms responsible for GSK3β-mediated radioresistance, we performed co-immunoprecipitation assay and found the binding of 53BP1 with GSK3β, indicating that GSK3β may co-localize with 53BP1. We further hypothesized that the radioresistance of glioblastoma cells was mediated by the activation of 53BP1, which was phosphorylated by GSK3β.

53BP1 is an evolutionarily conserved DNA damage checkpoint protein, and is an important regulator of genome stability

that protects cells against DSBs (37). 53BP1 null mice are viable, but are highly tumor-prone, and are profoundly hypersensitive to IR, probably due to a defect in non-homologous end-joining (NHEJ) (38). Therefore, to study the mechanisms of radioresistance of glioblastoma, it is important to study 53BP1 as it may play a central role in the DSB repair after IR. It has been demonstrated that as a critical transducer of the DNA damage checkpoint, phosphorylated 53BP1 is re-localized to the nuclear foci upon ionizing irradiation (39). Accumulating evidence indicates that ATM-dependent phosphorylation is a prerequisite for the activation of 53BP1 at sites of DNA breaks and for G2/M arrest following exposure to irradiation (40). To note, a study on the phosphorylation sites of 53BP1 by ATM revealed that the only known *in vivo* 53BP1 phosphorylation site was Ser25 (41). Another study on the IR induced phosphorylation of 53BP1 suggested that Thr302, Ser831, Ser166, Ser176/Ser178 and Ser452 may be potential phosphorylation sites as well (32). Moreover, repair factors facilitating DNA repair, such as the Mre11-Rad50-Nbs1 (MRN) complex and phosphorylated γ-H2AX, are co-localized with 53BP1 (42). This localization is responsible for the recruit of ATM to the DNA damage sites (43). Therefore, whether the binding of GSK3β with 53BP1 causes the phosphorylation, activation and re-localization of 53BP1 remains to be determined. In this study, our *in vivo* phosphorylation experiments revealed that, in the presence of the ATM inhibitor KU-55933, 53BP1 could still be phosphorylated, further validating our hypothesis that there are additional redundant phosphorylation sites at the N terminus of 53BP1. Therefore, in the present study, we observed that the inhibition of GSK3β and ATM abrogated the phosphorylation of 53BP1, suggesting that GSK3β was one of the regulators for 53BP1.

For 53BP1, the NH2-terminus contains no functionally defined protein region, but has several Ser-Gln or Thr-Gln (S/T-Q) motifs, which are potential binding and phosphorylation sites for ATM or other kinase. As previously described, GSK3β is a multifunctional Serine-Threonine (S/T) protein kinase, which means that the NH2-terminus of 53BP1 may be the potential target sites for GSK3β. In fact, in this study, by cloning the NH2-terminus and COOH-terminus fragments of 53BP1 into an expression vector, we clearly demonstrated that the NH2-terminus of 53BP1 becomes hyperphoshorylated in a GSK3β-dependent manner in response to IR. Of note, there are approximately 60 amino acids overlapped in both the N1 and N2 fragments, and we did not observe any phosphorylation of the N2 fragments. Therefore, the phosphorylation sites of 53BP1 may be the N1 fragments from amino acid 1 to 301. The study by Jowsey *et al* revealed that Thr302, Ser831, Ser166, Ser176/Ser178 and Ser452 are potential phosphorylation sites of 53BP1 (32); thus, we hypothesized that GSK3β may phosphorylate 53BP1 at Ser166 or Ser176/Ser178. To find the phosphorylation sites of 53BP1 by GSK3β, we generated several mutated 53BP1, and found out that when Serine was mutated to Alanine at 166 site, the phosphorylation of 53BP1 was abrogated. The analysis of the DNA repair percentage and cell proliferation rates suggested that the phosphorylation of S166 was sufficient for mediating DNA DSB repair. Alternatively, the mutation of Ser166 to Ala166 markedly inhibited cell proliferation, indicating that 53BP1 phosphorylation at S166 was indispensible for DSB repair and cell survival.

To determine whether GSK3 inhibitor could sensitize tumors to IR, mice bearing established U87 tumors (100 mm^3) received either IR or SB216763 treatment prior to IR. On the basis of caliper measurements, although complete tumor regression did not occur in all mice, the mice treated with SB216763 prior to IR exhibited a marked decrease in both tumor volume and tumor weight. Besides, these mice also exhibited a markedly longer survival, indicating that specific GSK3β inhibitor enhanced the sensitivity of glioblastoma cells to IR. Furthermore, immunofluorescence staining images of the DSB breaks by TUNEL staining, which also represents apoptotic cells, clearly indicated that the inhibition of GSK3β inhibited DSB repair following IR. Therefore, based on the oncogenic roles of GSK3β in multiple tumor types, the inhibition of GSK3 may only inhibit tumor progression, but may also sensitize tumor xenografts to radiotherapy in mice and may improve survival.

In conclusion, based on our present findings, it may be possible to reverse the radioresistance of glioma cells with a specific inhibitor of GSK3β. Therefore, GSK3β inhibitor may be used as a novel a new drug in the treatment of human gliomas. We anticipate that with the gradual clarification of the molecular mechanisms of radioresistance in glioblastoma, our findings may pave the way in the near future for the development of a novel therapeutic drug by targeting tumor recurrence following radiation and enhancing the sensitivity of tumors to radiotherapy.

Acknowledgements

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Competing interests

The authors declare that they have no competing interests.

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