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SGEF is Regulated via TWEAK/Fn14/NF-κB Signaling and Promotes Survival by Modulation of the DNA Repair Response to Temozolomide

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Abstract

Glioblastoma (GB) is the highest grade and most common form of primary adult brain tumors. Despite surgical removal followed by concomitant radiation and chemotherapy with the alkylating agent temozolomide (TMZ), GB tumors develop treatment resistance and ultimately recur. Impaired response to treatment occurs rapidly, conferring a median survival of just fifteen months. Thus, it is necessary to identify the genetic and signaling mechanisms that promote tumor resistance in order to develop targeted therapies to combat this refractory disease. Previous observations indicated that SGEF (ARHGEF26), a RhoG specific guanine nucleotide exchange factor (GEF), is overexpressed in GB tumors and plays a role in promoting TWEAK-Fn14 mediated glioma invasion. Here, further investigation revealed an important role for SGEF in glioma cell survival. SGEF expression is up-regulated by TWEAK-Fn14 signaling via NF-κB activity while shRNA-mediated reduction of SGEF expression sensitizes glioma cells to TMZ-induced apoptosis and suppresses colony formation following TMZ treatment. Nuclear SGEF is activated following TMZ exposure and complexes with the DNA damage repair (DDR) protein BRCA1. Moreover, BRCA1 phosphorylation in response to TMZ treatment is hindered by SGEF knockdown. The role of SGEF in promoting chemotherapeutic resistance highlights a heretofore unappreciated driver, and suggests its candidacy for development of novel targeted therapeutics for TMZ refractory, invasive GB cells.

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Implication—SGEF, as a dual process modulator of cell survival and invasion, represents a novel target for treatment refractory glioblastoma.

Keywords

glioblastoma; survival; resistance; DNA damage; TMZ; RhoGTPase; GEFs; Fn14; SGEF; NF-κB; BRCA1

Introduction

Glioblastoma (GB) is the most common form of primary adult brain tumors characterized by a poorly delineated tumor mass resulting from highly invasive cells. The problem of resistance to the standard anti-proliferative treatment of concomitant radiotherapy with chemotherapy using the alkylating agent temozolomide (TMZ) is common, and actively invading cells survive the current therapeutic regimens. Glioma cells with the increased capacity for migration have a decreased expression of pro-apoptotic genes and are less sensitive to cytotoxic therapy-induced apoptosis (1–4); the knockdown of several pro-invasive gene candidates in GB decreases glioma cell migration rate and sensitizes the cells to cytotoxic therapy and importantly, therapy directed at mediators of invasion has been shown to increase chemotherapeutic sensitivity (5, 6).

An increased capacity for cell survival results from the multi-faceted regulation of pathways involved in promoting cell growth, replication and spread, and preventing apoptosis in response to cytotoxic insult (7). Treatment strategies of tumor irradiation and temozolomide administration in glioblastoma lead to the formation of DNA double strand breaks (DSBs), either directly, or via mismatch repair conversion of O(6)-methylguanine adducts into DSBs, respectively (8). DSBs are primarily repaired through two mechanisms, homologous recombination (HR) and non-homologous end-joining (NHEJ). HR repair makes use of a non-damaged homologous DNA template, and thus is characterized as an error free mechanism, while NHEJ has no homologous strand for template use resulting in sequence errors near the break point (9).

DNA repair is initiated via sensing of DSBs by three kinases: ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), and Chk2. Subsequently, the early phosphorylation of histone H2A.X (γH2A.X) by ATM occurs at damaged DNA foci and leads to the phosphorylation of mediator of DNA damage checkpoint protein 1 (MDC1), with subsequent chromatin remodeling and recruitment of DNA repair proteins (10). BRCA1 is one such key mediator of HR and NHEJ repair; after exposure to DNA damaging agents BRCA1 is rapidly phosphorylated by ATM, ATR, and Chk2, and relocated to sites of replication forks with γH2A.X foci, where it recruits further proteins including BRCA2 and Rad51 to mediate strand exchange toward DNA repair and cell survival (9).

One key driver in GB that has been characterized to promote both cell invasion and cell survival is the transmembrane receptor fibroblast growth factor inducible-14 (Fn14). Fn14 is a member of the tumor necrosis factor receptor superfamily with one known ligand, the tumor necrosis factor-like weak inducer of apoptosis (TWEAK). Signaling through Fn14 by its cytokine ligand TWEAK activates Rac1, Akt, and NF-κB-pathways, and has been shown

to promote increased cell invasion and resistance to cytotoxic therapy-induced apoptosis (3, 4, 11).

Here we show that the src-homology 3 domain containing GEF (SGEF) promotes cell survival in response to TMZ treatment. In GB tumors, SGEF has been shown to be significantly overexpressed, to be correlated with poor patient outcome, and to promote glioma cell migration (12). We report that SGEF expression is increased in a subset of TMZ-resistant derived primary GB xenografts and is up-regulated by TWEAK-Fn14 signaling via NF-κB activity. Moreover, levels of SGEF and Fn14 mRNA are positively correlated in GB tumor specimens. Depletion of SGEF impairs colony formation following TMZ treatment and increases cell susceptibility to TMZ-induced apoptosis. TMZ treatment of glioma cells both leads to increased nuclear activation of SGEF and the SGEF dependent phosphorylation of the DNA damage repair protein BRCA1. SGEF and BRCA1 are found in a complex upon TMZ treatment. SGEF may thus be an important mediator of pro-survival signaling in response to TMZ-induced DNA damage.

Materials and Methods

Cell culture conditions

Human astrocytoma cell lines U87, U118, and T98G (American Type Culture Collection), as well as primary glioblastoma xenograft cells (GBM & GBM TMZ-R lines) were maintained in DMEM (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA) at 37°C with 5% CO_2. For all assays with TWEAK treatment, cells were cultured in reduced serum (0.5% fetal bovine serum) for 16 h before stimulation with recombinant TWEAK at 100 ng/mL in DMEM + 0.1% bovine serum albumin for the indicated times.

Antibodies, plasmids, reagents, and Western blot analysis

A polyclonal SGEF antibody was purchased from Sigma (St. Louis, MO). A monoclonal tubulin antibody was purchased from Millipore (Billerica, MA). A polyclonal antibody for phospho-BRCA1 (Ser1524), and monoclonal antibodies for BRCA1, cleaved PARP, phospho-Histone H2A.X (Ser139), Histone H2A.X, Rabbit IgG (isotype control), Histone H3, and NF-κB p65 were purchased from Cell Signaling Technologies (Beverly, MA). Lipofectamine RNAiMax was purchased from Invitrogen. Human recombinant TWEAK was purchased from PeproTech (Rock Hill, NJ). Human placental laminin and temozolomide were obtained from Sigma. In certain experiments glioma cells were transiently transfected with either IκBα wild type (IκBαWT) or IκBαS32/36A mutant (IκBαM) super-repressor expressing plasmids (Addgene, Cambridge, MA) using the Effectene transfection protocol (Qiagen, Valencia, CA) for 24 h prior to culture in reduced serum medium (0.5% FBS DMEM) for 16 h with subsequent addition of TWEAK for 4 h. Plasmids: pGEX4T-1-RhoG(15A) was obtained from Dr. Keith Burridge (U. North Carolina-Chapel Hill).

For immunoblotting, cells were lysed in 2x SDS sample buffer (0.25 M Tris-HCl, pH 6.8, 10% SDS, 25% glycerol) containing 10 μg/mL aprotinin, 10 μg/mL leupeptin, 20 mM NaF,

2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using the BCA assay (Pierce) with bovine serum albumin as a standard. Thirty micrograms of total protein were loaded per lane and separated by SDS PAGE. After 4°C transfer, the nitrocellulose (Invitrogen) was blocked with either 5% nonfat milk or 5% BSA in Tris-buffered saline, pH 8.0, containing 0.1% Tween 20 (TBST) prior to addition of primary antibodies and followed with peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG. Protein was detected using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific) with a UVP BioSpectrum 500 Imaging System (Upland, CA). Densitometry was calculated via Image J software.

RNA isolation and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was isolated as previously described (1). cDNA was synthesized from 500 ng of total RNA in a 20 μL reaction volume using the SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen) for 50 minutes at 50°C, followed by 85°C for 5 minutes. qPCR analysis of SGEF (sense: 5'-TGC TGA AAG GAC AAG GAA CA-3'; anti-sense: 5'-GTA GTT TTG ATA CAG GAC AGC ATT-3') and histone H3.3 (sense: 5'- CCA CTG AAC TTC TGA TTC GC-3'; anti-sense: 5'-GCG TGC TAG CTG GAT GTC TT-3') mRNA levels was conducted using SYBR green (Roche) fluorescence for detection of amplification after each cycle with LightCycler analysis software and quantified as previously described (3).

Biotinylated electrophoretic mobility shift assay

T98G glioma cells were plated at a density of 3×10^6 in 100 mm^2 tissue culture dishes in normal growth medium. After 12 h, cells were cultured under reduced serum (0.5% FBS) for an additional 16 h before TWEAK (100 ng/mL) addition for 2 h. Isolation of cell nuclear protein was carried out using the NE-PER kit (Pierce) according to the protocol of the manufacturer. Protein-DNA complexes were detected using biotin end-labeled double-stranded DNA 23-mer probes containing the NF-κB binding sites within the SGEF promoter (NF-κB-SGEF wt target sequence: 5'- GTC TAG GAG GCA AAT CCC AGA AA −3'; NF-κB-SGEF mt target sequence: 5'- GTC TAG GAG CCA GAT CGC AGA AA −3'). The binding reactions were done using the LightShift kit (Pierce) according to the protocol of the manufacturer. Where indicated, 200-fold molar excess of unlabeled NF-κB-SGEF wt oligonucleotides or anti-p65 antibody was included. The reaction products were resolved by gel electrophoresis and detected by chemiluminescence according to the protocol of the manufacturer (Pierce).

Lentiviral production

Lentiviral vectors containing shRNA targeting SGEF (shSGEF-12 & shSGEF-13) or control empty vector (control) were obtained from Open Biosystems (Fisher Scientific, Pittsburgh, PA) and packaged for lentiviral production as previously described (12).

Clonogenic and apoptosis studies

Observations of colony forming capacity following cytotoxic insult were performed as described (13). Briefly, T98G, U87 and U118 cells stably expressing either control or

shRNA targeting SGEF were treated with TMZ (500 μM). In certain experiments cells were additionally transfected with siRNA targeting control luciferase or BRCA1 for 72 h prior to the addition of TMZ. Cells were trypsinized 24 h post-TMZ treatment and plated in triplicate in 6-well cell culture dishes at 250 cells per well. Colonies were allowed to grow until controls reached a 50 cell density (approximately 6–7 days) before being fixed briefly in a 10% (v/v) methanol 10% (v/v) glacial acetic acid solution, stained with a 0.5% (w/v) crystal violet solution and washed with de-ionized water. Apparent colonies were recorded, and surviving fractions were determined relative to the non-treated control for each cell line.

For apoptotic studies, T98G and U87 control or shSGEF cells were treated with TMZ (500μM) for 48 h and whole cell lysates were analyzed for cleaved PARP and caspase 3 by western blot. For chromatin condensation studies, apoptotic cells were evaluated by nuclear morphology of DAPI-stained cells as described previously (14). Briefly, glioma cells were plated onto 10-well slides pre-coated with 10ug/mL laminin. After 24 h cells were treated with TMZ (500μM) for an additional 48 h. Cells were fixed with 4% paraformaldehyde, stained with ProLong Gold Antifade Reagent with DAPI (Molecular Probes) and evaluated by nuclear morphology. Cells with condensed, fragmented chromatin were manually scored as apoptotic cells. At least three fields were evaluated per well, and data reported as apoptotic cells/total cells × 100.

Nucleotide-free GEF pulldowns

RhoG activity was measured as previously described using a GST-ELMO-NT fusion protein (15). Affinity pulldowns of active SGEF bound to RhoG were performed using a nucleotide free RhoG mutant (G15A) expressed and purified as described (16). Recombinant RhoG G15A-GST protein and GST-ELMO-NT were produced in Escherichia coli (BL21) cells. Cells were lysed in B-PER lysis buffer (Pierce) containing protease inhibitors, and purified with glutathione sepharose beads (GE Healthcare).

Isolation of cell nuclear protein was performed according to Guilluy et al (17). Briefly, 10^7 U87 cells were grown in 10 cm dishes before treatment with TMZ (500μM) for the indicated times. Cells were washed in ice-cold PBS containing protease inhibitors and lysed in a 1mL hypotonic solution (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT (freshly added before use)). Lysates were homogenized and centrifuged at ~300g for 5 minutes at 4 °C. Pellets were washed twice in 1.5 mL of a 30% (w/v) iodixanol solution and centrifuged at 10,000g at 4 °C. Supernatants were discarded and the pellets were resuspended in 300mL Rho GEF buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1% (v/v) Triton X-100, 1 mM DTT with protease inhibitors). Suspensions were sonicated briefly, centrifuged at 14,000g for 5 minutes at 4 °C, and the remaining nuclear fraction supernatants were quantified for protein concentration via BCA assay. Subsequently, equal amounts of total GST fusion protein were incubated with fresh nuclear protein lysate (1 mg) for one hour, and precipitated lysates were resuspended in 2X SDS buffer containing protease inhibitors and resolved with SDS-PAGE.

Subsequently, equal amounts of total GST fusion protein were then incubated with nuclear protein lysate (1 mg), and precipitated lysates were resolved with SDS-PAGE.

Immunoprecipitation

U87 cells were treated with 500 μM TMZ for the indicated times prior to lysis on ice in a buffer containing 10 mM Tris-HCl (pH 7.4), 0.5% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 2 mM sodium orthovanadate, 20 mM sodium fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Isolation of cell nuclear protein was carried out using the NE-PER kit (Pierce) according to the protocol of the manufacturer. Equivalent amounts of protein were pre-cleared and immunoprecipitated from the nuclear lysates using either SGEF or BRCA1 antibodies as indicated, or a control isotype-matched antibody, and then washed with lysis buffer followed by TX-100 buffer [10 mm HEPES (pH 7.4), 150 mm NaCl, 2 mm EDTA, 2 mm EGTA, 20 mm sodium fluoride, and 0.5% Triton X-100]. Samples were then re-suspended in 1x LDS sample buffer containing DTT and boiled, separated by SDS-PAGE, transferred to nitrocellulose for 1 h at 4 °C, and then proteins were detected using SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific).

Small-interfering RNA transfection

Small interfering RNA (siRNA) oligonucleotides specific for GL2 luciferase were described previously (18). BRCA1-specific siRNA target sequences are as follows: BRCA1-1 (5′-ACC ATA CAG CTT CAT AAA TAA-3’) and BRCA1–2 (5′- AAC CTA TCG GAA GAA GGC AAG-3’). Transient transfection of siRNA was performed using Lipofectamine RNAiMax. Cells were plated at 70% confluence in DMEM + 10% FBS without antibiotics and were transfected within 8 h of plating. The siRNA and Lipofectamine were diluted separately in Opti-MEM (Thermo Fisher Scientific). After 5 min, the mixtures were combined and incubated for 20 min at room temperature to enable complex formation. siRNA oligonucleotides were transfected at 50nM, and no cell toxicity was observed. Maximum inhibition of protein levels was achieved 48 to 72 h post-transfection.

Proliferation studies

Cell viability was determined via Alamar Blue. Briefly, glioma cells were plated in 96-well plates in quadruplicate for use as a standard curve of known cell counts, or plated in replicates of 8 at 3,000 cells per well to monitor proliferation over 72 h. After cell attachment of the standard curve, or 24 h post-attachment of experimental wells, cells were treated with 10% Alamar Blue (Trek Diagnostic Systems) for 5 h at 37°C. The absorbance was read at 560 nm and 595 nm and the cell viability was expressed as number of cells per well calculated relative to the standard curve for each line. Cell viability was repeatedly assessed daily over 72 h.

Immunofluorescence

Glioma cells were plated onto 10-well glass slides, pre-coated with 10 μg/mL laminin. Twenty-four hours later, cells were treated with 500 μM TMZ for 48 h and fixed in 4% formaldehyde/PBS, permeabilized with 0.1% Triton X-100 dissolved in PBS, and incubated with antibodies to BRCA1 or phospho-H2A.X (Ser139). Slides were mounted with ProLong Gold Antifade Reagent with DAPI (Molecular Probes). Images were collected using a Zeiss

LSM 510 microscope equipped with a 63x objective, ZEN 2009 image analysis software, and Adobe Photoshop CS3.

Gene expression analysis of SGEF and Fn14 correlation

Expression data generated using the Affymetrix U133 Plus 2.0 Array for 82 GB samples was downloaded from the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) for correlation of ARHGEF26 (SGEF) and TNFRSF12A (FN14) (19). The expression level of ARHGEF26 (SGEF) was calculated as the median of the three relative expression intensity values for the three probe sets annotated for ARHGEF26 (SGEF). There was a high correlation of all three ARHGEF26 (SGEF) probes sets to each other (Pearson correlation of .90 to .97). The relative expression intensity of the probe set 218368_s_at was used for TNFRSF12A (FN14) as it is the only probe set annotated for TNFRSF12A (FN14). The Pearson Product Moment Correlation was calculated using the R software code as supplied by (20).

Flow Cytometry

Kinetics of Histone H2A.X phosphorylation upon TMZ treatment in GB cells was quantified using flow cytometer analysis. P-H2A.X staining was performed as previously described (21) with minor modifications. Briefly, T98G NS Ctrl and T98G SGEF-12 cells were treated with 500 μM TMZ for 30 min, 2 hrs, and 8 hrs. Subsequently, cells were trypsinized and fixed in 70% ethanol overnight at 4°C. After washing with BSA-T-PBS (1% BSA/0.2% Triton X-100 in PBS), cells were incubated with phospho-H2AX antibody for 1 h at RT. After washing with BSA-T-PBS, cells were incubated with the Alexa Fluor ® 547-conjugated secondary antibody and thereafter with propidium iodide (PI). After staining with PI, the stained cells (gated on the basis of forward and side scatter profiles) were analyzed on a Caliber (Becton-Dickinson, San Jose, CA, USA) and data were processed using the FlowJo program. Rabbit IgG was used as isotype control to normalize for any non-specific signal.

Statistical analysis

Statistical analyses were done using the two-sample *t* test. $P < 0.05$ was considered significant.

Results

TWEAK-Fn14 signaling induces SGEF mRNA and protein expression via NF-κB

We previously reported that Fn14 signaling directs both pro-invasive and pro-survival responses in GB tumors via Rac1 and NF-κB, respectively (3, 4, 12). We also described a role for the novel GEF, SGEF, in the promotion of Fn14-directed increased cell motility whereby Fn14 signaling enacted SGEF-required downstream RhoG and subsequently Rac1 activation (12). Of note, an analysis of 82 primary GB tumor specimens in the publicly available REMBRANDT dataset revealed a positive association between Fn14 and SGEF expression across the tissues ($p < 0.001$) (Figure 1A). We have previously shown that, similar to Fn14, SGEF expression was inversely correlated to patient survival among primary GB tumors and that SGEF protein expression is highly increased in GB clinical

specimens (12). Thus, we sought to determine whether SGEF played an additional role in pro-survival signaling within GB cells. Given that there is a positive correlation between SGEF and Fn14 expression, we first analyzed whether Fn14 signaling played a role in the regulation of SGEF expression. SGEF expression is detected in T98G, A172 and U87 glioma cell lines, and minimally detected in U118 cells (Figure 1B). Stimulation of glioma cells with the TWEAK ligand resulted in increased SGEF mRNA and protein levels with increased levels apparent within two hours of treatment, indicating that SGEF expression is inducible following TWEAK-Fn14 interaction. (Figure 1C & D).

Since NF-κB is an important promoter of cell survival in GB tumors (3, 4, 22), and Fn14 pro-survival signaling is dependent upon NF-κB up-regulation of pro-survival gene transcripts (3), we next assessed whether the regulation of SGEF expression by TWEAK-Fn14 signaling required NF-κB. We analyzed the promoter sequence of SGEF and identified the presence of an NF-κB p65 consensus binding site at −2260 to −2238 base pairs upstream of the transcriptional start site including the 5' UTR. Using an electrophoretic mobility shift assay with wild-type and mutant NF-κB p65 consensus sequence oligonucleotides from the SGEF promoter region, we assessed whether p65 NF-κB binds to the SGEF promoter following treatment with TWEAK. Electrophoretic mobility of SGEF wild-type but not mutant sequences shifted consequent to nuclear lysate binding; the addition of an anti-p65 antibody confirmed the role of p65 in the shift (Figure 2A). To further determine whether TWEAK-Fn14 driven increase in SGEF expression is dependent upon NF-κB, we either transiently transfected T98G glioma cells with plasmids expressing either control vector or IκBαM, an upstream super-repressor of NF-κB, or pharmacologically inhibited NF-κB activation via the cell permeable peptide inhibitor SN50 or control SN50M, and analyzed SGEF mRNA or protein levels following treatment with TWEAK. NF-κB inhibition either by IκBαM (Figure 2B & C) or SN50 (Figure 2D & E) resulted in diminished SGEF mRNA and protein expression, respectively, indicating that NF-κB is required for TWEAK-Fn14 induction of SGEF.

Depletion of SGEF impairs colony formation following TMZ treatment and sensitizes cells to TMZ-mediated apoptosis

Since SGEF expression was up-regulated by TWEAK-Fn14 in an NF-kB dependent fashion, we sought to assess whether SGEF was important in pro-survival signaling in GB. To determine the importance of SGEF protein in response to TMZ, we utilized stable SGEF-depleted glioma cell lines, established via lentiviral-mediated transduction of either control (Ctrl) or shRNA targeting SGEF (SGEF-12 & SGEF-13) expressing vectors in T98G (Figure 3A), U87 and U118 (previously published, (12)) glioma cells. Stable depletion of SGEF in T98G, U87 or U118 glioma cells did not alter proliferation or cell cycle (Supplementary Figure 1). However, in glioma cells with stable depletion of SGEF, treatment with TMZ for forty-eight hours followed by assessment for cellular apoptosis revealed that TMZ-treated SGEF-depleted glioma cells showed increased chromatin condensation (Figure 3B) as well as elevated cleaved PARP and cleaved caspase 3 by immunoblot analysis (Figure 3C) in comparison to control TMZ treated cells. Validation of TMZ induced PARP cleavage in SGEF depleted glioma cells was performed using a specific antibody to the "cleaved" form of PARP (Supplementary Figure 2A). Therefore, knockdown

of SGEF protein increases TMZ-induced cytotoxicity. To further characterize the susceptibility to TMZ of glioma cells with stable SGEF knockdown, we treated T98G, U87 and U118 glioma cells for twenty-four hours with TMZ and measured colony growth formation. Cells depleted of SGEF by shRNA SGEF12 displayed significantly impaired colony formation after TMZ treatment as compared to control TMZ treated cells (Figure 3D). The depletion of SGEF in un-treated glioma cells did not alter the number of colony formation as compared to control TMZ treated cells (Figure 3D). Similar results were obtained for glioma cells depleted of SGEF expression by a second independent shRNA SGEF13 (Supplementary Figure 2B). Together, these data indicate that SGEF protein function is important in the recovery response following TMZ treatment.

TMZ treatment induces nuclear SGEF activity and SGEF dependent BRCA1 activity, and promotes SGEF in complex with BRCA1

TMZ treatment is known to result in the formation of double strand DNA breaks (DSB) (8). The phosphorylation of histone (γH2A.X) is one of the earliest responses to DSB. γH2A.X is involved in the recruitment of and localization of DNA repair proteins and thus this phosphorylation is indicative of DNA damage DSB foci (23). SGEF contains two nuclear localization sequences (Figure 4A) (24) and has previously been shown to be capable of nuclear localization, although the role of nuclear SGEF has not been described (25). We analyzed glioma cells over twenty-four hours of treatment with TMZ and assessed levels of H2A.X phosphorylation in control or SGEF depleted lines (Figure 4B). In both control and SGEF knockdown cells, increased phosphorylation of H2A.X is detected upon TMZ treatment within 8 and 24 h, indicating that SGEF does not play a role in preventing the formation of γH2A.X foci. However, SGEF knockdown cells do not show loss of γH2A.X foci beyond 24 h, as measured by immunofluorescent staining of γH2A.X, indicating altered kinetics of DNA damage response subsequent to TMZ-induced DSB (Supplementary Figure 3).

We next examined whether SGEF plays a role in the coordinated response to DNA damage. We first assessed whether the activity of SGEF is altered following TMZ treatment. U87 glioma cells treated for twenty-four hours with TMZ were fractionated for nuclear lysates, in which SGEF activity was determined using RhoG G15A nucleotide free mutant constructs (16). Treatment with TMZ resulted in increased SGEF activity in the nucleus (Figure 4C), further supporting a role for SGEF in the response to TMZ treatment. Use of functional site prediction analysis suggests that SGEF contains two phosphopeptide domain motifs at amino acids 493–497 (ASKKF) and 741–745 (ASHLF) (Figure 4A), which can directly interact with the BRCT (carboxy-terminal) domain of BRCA1. BRCT domains are present in several DNA damage response proteins (26), and the phosphorylation of BRCA1 occurs in response to DNA damaging agents (9). We therefore sought to determine if SGEF is important in BRCA1 activation following TMZ treatment. Glioma cells were treated for twenty-four hours with TMZ and the phosphorylation of BRCA1 was assessed between control and SGEF depleted lines (Figure 4B). The depletion of SGEF prevented TMZ-induced BRCA1 phosphorylation. We then assessed whether treatment with TMZ induces complex formation between SGEF and BRCA1. U87 glioma cells treated for twenty-four hours with TMZ were analyzed via immunoprecipitation of BRCA1 and immunoblot

analysis of SGEF. Minimal SGEF and BRCA1 co-immunoprecipitated from nuclear lysates of untreated cells, however TMZ treatment induced complex formation between SGEF and BRCA1 within thirty minutes, and maximal interaction was observed at eight hours (Figure 4D), along with increased binding to phosphorylated H2A.X. Thus SGEF may function in part to promote the BRCA1 response to DNA damage.

Depletion of BRCA1 impairs colony formation following TMZ treatment, which is not enhanced by concomitant SGEF depletion

Depletion of SGEF impairs cell survival following TMZ treatment (Figure 3D). To assess whether SGEF may promote a divergent response to TMZ treatment in addition to the promotion of a BRCA1-mediated response, we depleted BRCA1 via transient siRNA transfection (Figure 5A) in control glioma cells or glioma cells stably depleted of SGEF and treated the cells with TMZ. Depletion of BRCA1 impaired survival to TMZ treatment similar to that observed after SGEF depletion alone. The combination of BRCA1 depletion in SGEF depleted cells did not result in any further significant impairment of cell survival following TMZ treatment (Figure 5B). Given that there is no additive or synergistic effect of the dual knockdown of SGEF and BRCA1, the data support that SGEF works along the same pathway in concert with BRCA1 in the DNA damage repair response.

Discussion

In this study, we report a relationship between SGEF and both therapeutic resistance and cell survival. SGEF protein expression is induced in glioma cells by TWEAK-Fn14 signaling and dependent upon NF-κB. NF-κB signaling has been well characterized to promote both GB cell invasion and survival (3, 4, 22, 27). Moreover, elevated or constitutive NF-κB activity has been demonstrated in gliomas and correlates with increasing tumor grade (27, 28). NF-κB signaling has specifically been shown to protect cells against the standard of care treatments in GB. The inhibition of IκBα phosphorylation prevents NF-κB activity and sensitizes glioma cells to radiation treatment (29) and NF-κB is an important player in promoting resistance to O6-alkylation (30), a TMZ induced DNA damage modification.

TWEAK-Fn14 signaling is one notable pathway in glioma that utilizes Rac1 dependent NF-κB activation to promote cell invasion and cytotoxic therapy resistance with enhanced cell survival (3, 4, 31). To date, SGEF has been largely associated with a role in promoting cell motility; SGEF has been shown to promote the invasive capacity of HPV transformed tumor cells and actin cytoskeleton remodeling after salmonella infection (25, 32). In GB tumors, in addition to a role in the promotion of cell invasion, we have previously reported that SGEF is significantly overexpressed and is correlated with poor patient outcome (12). Here, we report the role of SGEF in promoting cell survival. We show that in glioblastoma, TWEAK binding to Fn14 fosters occupancy of the SGEF promoter by NF-κB , and that TWEAK-Fn14 up-regulation of SGEF mRNA and protein expression is dependent upon NF-κB function. The link between Fn14 and SGEF in GB is further supported on the basis of mRNA expression analysis indicating a strong positive correlation in expression of the two genes among a panel of primary tumor specimens in the publicly available REMBRANDT dataset of 82 GB tumors (Figure 1A). Of note, this correlation was not significant when

brain tumors of all grades were considered (data not shown) but was highly statistically significant within GB tumors alone, indicating the relationship between SGEF and Fn14 may be specific to malignant progression.

Our data suggest an important role for SGEF in the response of glioma cells to TMZ treatment. We showed that the shRNA-mediated depletion of SGEF does not affect cell proliferation or cell cycle (Supplementary Figure 1), but does impair the ability of glioma cells to form colonies following TMZ treatment and leads to glioma cell sensitization to TMZ-induced cell death via apoptosis. SGEF is known to contain two nuclear localization sequences (24), and has been reported to localize in the nucleus of cells (25). Here we show that TMZ treatment of glioma cells induces nuclear activity of SGEF in a time-dependent fashion. Moreover, SGEF is known to facilitate guanine nucleotide exchange for the GTPase RhoG (33) and RhoG has been shown to contain a nuclear localization sequence (34), the significance of which remains unknown to date. Our data indicate that RhoG becomes active in the nucleus, similar to SGEF, in response to TMZ treatment of glioma cells (Supplementary Figure 4). Therefore, nuclear RhoG may play a role in the SGEF pro-survival response to TMZ treatment.

Sequence analysis of SGEF revealed two BRCT binding domains with the potential for binding BRCA1. Here we demonstrate that SGEF is found in complex with BRCA1 following TMZ treatment, and that phosphorylation of BRCA1 is dependent upon SGEF. Thus, the decreased capacity of TMZ-induced BRCA1 phosphorylation in SGEF depleted glioma cells may help explain the observed significantly impaired capacity of glioma cells to recover colony formation and the increased apoptosis in TMZ-treated SGEF-depleted glioma cells. While our data showed that SGEF knockdown does not prevent γH2A.X foci formation as marked by H2A.X phosphorylation following TMZ addition within 24 h, longer assessment of the kinetics of DSB repair mechanism by immunofluorescent staining of γH2A.X showed defect in DSB repair in SGEF knockout cells beyond 36 to 72 h (Supplementary Figure 3). Interestingly, our data indicate that SGEF is required for BRCA1 recruitment to H2A.X foci following TMZ treatment (Supplementary Figure 5). The determination of the specific functional site responsible for SGEF interaction with BRCA1, along with the mechanism of SGEF-BRCA1 mediated glioma cell survival will be the focus of future studies. We thus propose a scheme of TWEAK-Fn14 inducible SGEF mRNA and protein expression dependent upon NF-κB nuclear translocation and activity, whereby increased SGEF levels promote a pro-survival phenotype in the face of TMZ treatment by promotion of BRCA1-γH2A.X DNA damage response activity (Figure 6).

BRCA1 has been shown to transiently interact at sites of damage or stalled replication forks with the role of homologous recombination. BRCA1 also functions in NHEJ, and S- and G2-M-phase checkpoints, however some reports suggest that BRCA1 preferentially promotes the error free HR pathway for DNA repair over NHEJ to preserve chromosome stability (9). Cancers known to have a deficiency of the HR repair proteins BRCA1 and BRCA2 display particular sensitivity to poly(ADP)-ribose polymerase (PARP) inhibition, a protein whose activity normally facilitates single strand damage repair. When unrepaired, these single strands are converted to double strand breaks during replication, which are then unable to be corrected due to a non-functioning HR system (35). Interestingly in gliomas,

the inhibition of HR via siRNA-mediated depletion of Rad51 or BRCA2 greatly sensitized glioma cells to TMZ, the effect of which was enhanced by concurrent PARP inhibition (36). These studies further support the notion that targeting the modulation of BRCA1 activity as regulated by SGEF expression may enhance cell killing in GB tumors, and future studies will address the potential for synergistic lethality in targeting this axis in combination with other inhibitors of DNA repair.

Tumor modulation of DNA repair pathways has been described as one main avenue of glioblastoma resistance to TMZ. Gene expression associated with promoter hypomethylation of the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) allows for notable inherent tumor resistance in glioblastomas. However epigenetic silencing of MGMT by promoter hypermethylation has been shown to occur in 30–60% of glioblastoma tumors, and it has been suggested that MGMT-deficient GB cells may be particularly susceptible to targeting HR in combination with additional DNA repair proteins including PARP inhibition (35). In addition, acquired TMZ resistance has been demonstrated by silencing of DNA mismatch repair genes through treatment-induced mutations which then allows the tumor cell to escape a futile repair process otherwise leading to cell death, while the epigenetic silencing of DNA base excision repair genes may predict TMZ sensitivity (35). Given the role of DNA repair pathways in the tumor cell response to TMZ treatment, we further characterized SGEF expression in the setting of TMZ chemoresistance using a panel of primary GB xenografts treated *in vivo* with TMZ to derive matched parental and TMZ-resistant (TMZ-R) pairs (37). Interestingly, protein expression of SGEF was found to be higher in the resistant lines versus the parental line in a subset (8/17) of samples (Supplementary Figure 6), suggesting in some GB tumors increased SGEF expression may result following exposure to TMZ. It is unknown whether the high expression of SGEF in these primary tumors directly confers resistance to TMZ, however the study of these lines is currently under investigation. Moreover, it is unknown why SGEF protein expression is elevated in only a subset of the TMZ-R GB xenografts. It is possible that genetic heterogeneity of the patient-derived parental and TMZ-resistant lines may play a role in perceived SGEF expression due to sampling bias, and this concern will also be addressed in future studies.

Interestingly, radial migration analysis of GBM14 and GBM14 TMZ-R primary xenografts revealed an elevated rate of cell migration in the GBM14 TMZ-R cells (data not shown). SGEF promotes cell migration and invasion in glioblastoma via activation of the Rho GTPase RhoG with subsequent RhoG-dependent activation of Rac1 and the formation of lamellipodia (12). Thus, NF-κB mediated increased SGEF expression may be one mechanism that facilitates the increased cell motility of TMZ-R glioma cells. Indeed, increased invasive capacity has been previously reported in glioma as a response to cytotoxic therapy. For example, it has been shown that radiation of glioblastoma leads to the enhanced cell invasive potential via activation of the Rho-PI-3K signaling pathway (38). In addition, the pro-invasive integrins, αvβ3 and αvβ5, have been demonstrated to mediate a pro-survival response in glioma to radiation through integrin-linked kinase and the RhoB GTPase (39), thus implying overlapping roles for mediators of cell motility with the promotion of cell survival. Of note, there have also been multiple reports of increased cell

invasiveness resulting from treatment with chemotherapeutic agents among several tumor types (40–42). Thus, the roles of invasion and survival are interconnected in the promotion of disease progression, and there is mounting evidence for overlap between these two processes (43). SGEF therefore presents a novel hub in the interrelated axes of tumor cell invasion and survival.

Despite advances in medical technology and treatment, GB prognosis has remained largely unchanged over the last several decades (44, 45). The ability of glioma cells to survive undeterred from current treatment strategies implies that new therapeutic avenues are necessary for treatment of this disease. There is accumulating evidence that combinatorial therapy that includes use of treatment modalities designed to hamper the DNA repair mechanisms of the cell may provide a significant added survival benefit to patients over the standard of care alone or when used in combination with inhibitors of other GB deregulated pathways (46–49). Moreover therapy aimed at mediators of invasion can also lead to increased chemotherapeutic sensitivity (5). Thus, pathways deregulated in GB that promote both TMZ resistance and cell motility represent novel therapeutic targets in future drug design. Our data support a role for SGEF in both the promotion of cell invasion and cell survival signaling within GB tumors and provide a rationale for targeting this signaling axis. Interestingly, there has been a recent report of the RhoJ GTPase in promoting melanoma chemoresistance by suppressing DNA damage sensing pathways including the uncoupling of ATR from its downstream effectors with resulting decreased DNA damage-induced apoptosis (50). Thus the role of GEFs and GTPases in chemoresistance via modulation of DNA repair mechanisms is an emerging field in which we have validated a role for SGEF in GB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

GB	glioblastoma
GEF	guanine nucleotide exchange factor
TMZ	temozolomide

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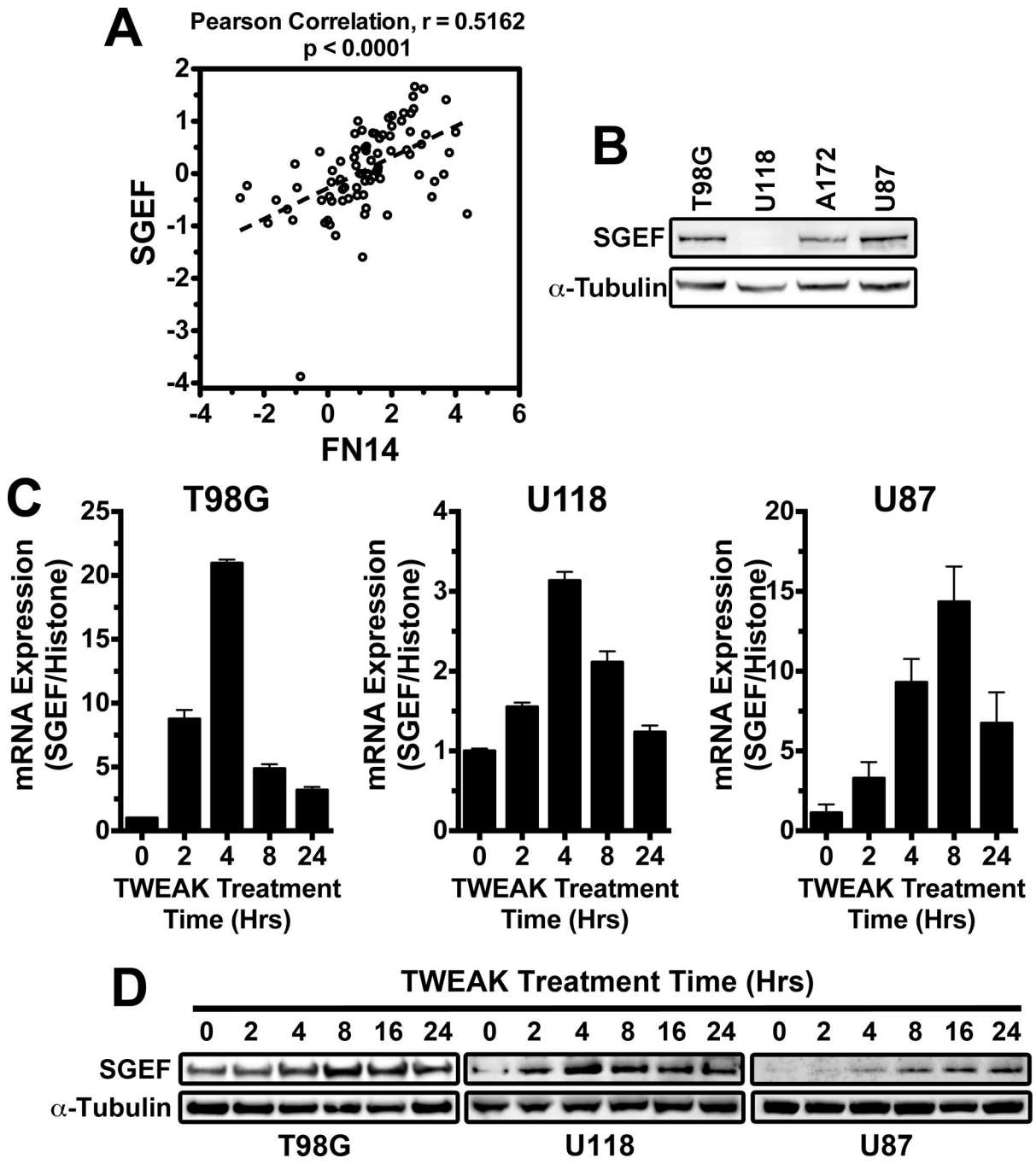

Figure 1. SGEF mRNA and protein expression is inducible via TWEAK cytokine stimulation

(A) SGEF and Fn14 mRNA expression from the publicly available REMBRANDT dataset of 82 GB tumors was accessed and assessed using the Pearson product moment correlation statistic ($p < 0.001$). (B) SGEF protein expression was assessed in serum-deprived glioma cell lines. (C & D) T98G, U118, and U87 glioma cells were cultured in reduced serum (0.5% FBS DMEM) for 16 hours prior to stimulation with TWEAK (100ng/mL) for the indicated times. SGEF mRNA (C) and protein (D) expression were analyzed via qPCR with

fold change relative to histone and via western blotting with the indicated antibodies, respectively. Data represent an average and SD of 3 replicates. (* $p < 0.01$).

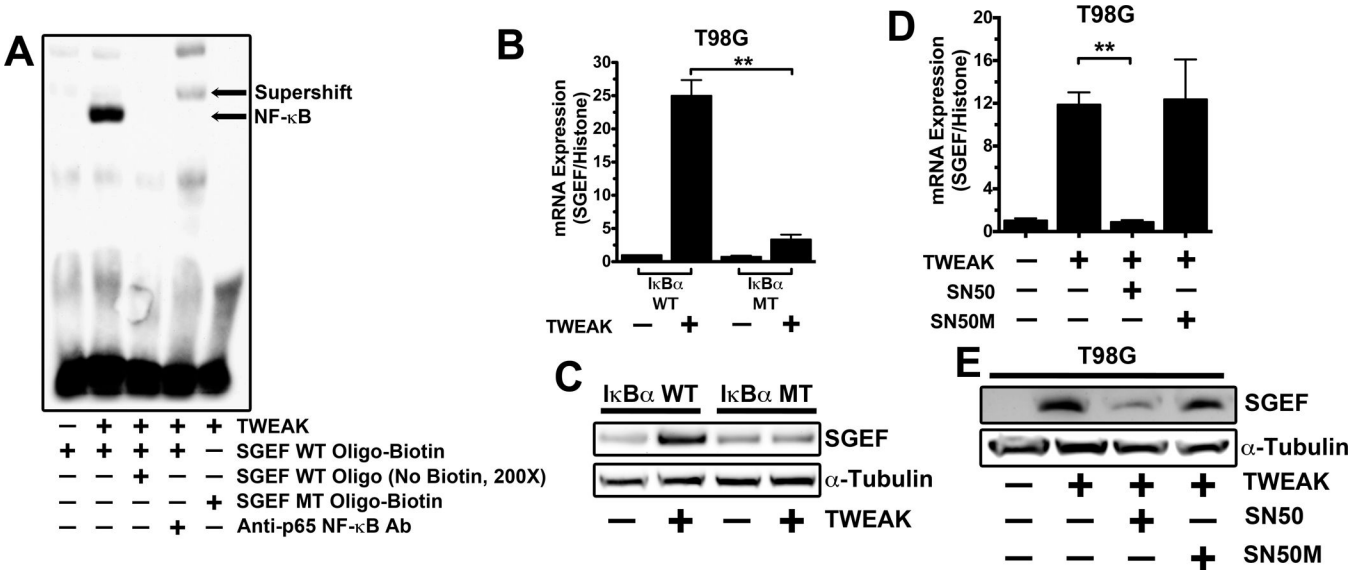

Figure 2. NF-κB binds to the SGEF promoter region upon TWEAK stimulation and TWEAK-Fn14 induction of SGEF expression is dependent upon NF-κB activity

(A) T98G cells were treated with TWEAK (100ng/mL). Nuclear proteins were isolated 2 hours post-treatment and incubated with biotin end-labeled, wild-type SGEF (SGEF wt) oligonucleotides containing the NF-κB consensus binding region. Proteins were also incubated with biotin end-labeled SGEF oligonucleotides containing mutated NF-κB consensus binding region (SGEF mt). In certain experiments, either 200-fold molar excess of unlabeled NF-κB-SGEF wt oligonucleotides or anti-p65 antibody was incubated with the nuclear lysates from TWEAK-treated cells. (B&C) T98G glioma cells were transiently transfected with either IκBα WT or IκBα MT super-repressor expressing plasmids for 24 hours, followed by serum starvation for an additional 16 hours (0.5% FBS DMEM), with subsequent TWEAK treatment in certain cases for 4 hours. (B) Total RNA was isolated and SGEF mRNA expression was analyzed via qPCR with fold change relative to histone H3.3. Data represent an average and SD of 3 replicates. (** $p < 0.01$). Protein Lysates were resolved via SDS-PAGE and immunoblot for SGEF protein expression (C). (D) T98G glioma cells were cultured for 16 hours in reduced serum medium (0.5% FBS DMEM) followed by pre-treatment with SN50 NF-κB inhibitor or control non-inhibiting SN50M as indicated for 1 hour with the subsequent addition of TWEAK to all dishes for 4 hours. Data represent an average and SD of 3 replicates. (** $p < 0.01$). Protein lysates were resolved via SDS-PAGE and probed with the indicated antibodies (E).

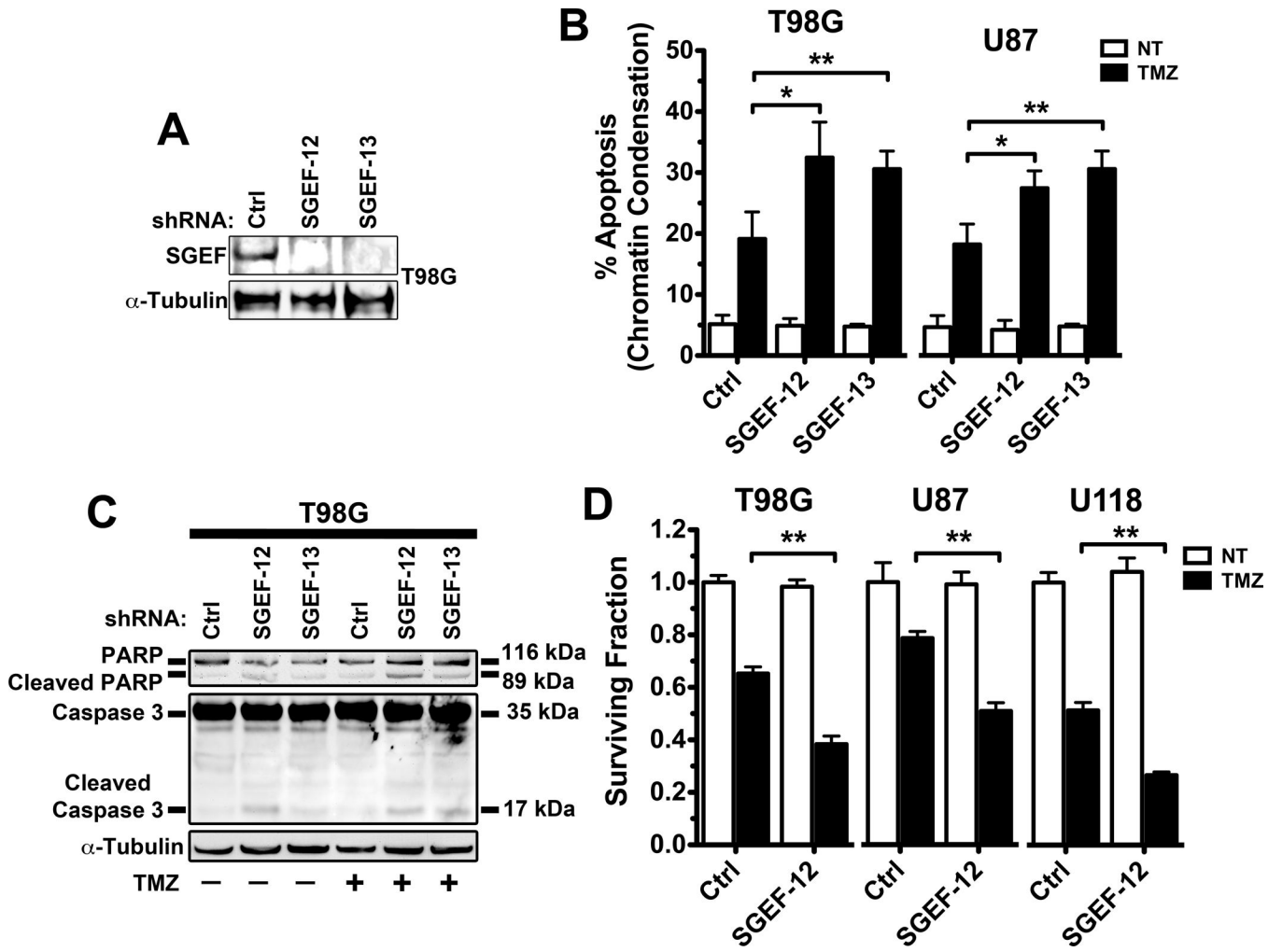

Figure 3. Depletion of SGEF impairs colony formation following TMZ treatment and sensitizes glioma cells to TMZ-induced apoptosis

(A) Glioma cells were stably transduced with lentiviruses expressing either control vector (Ctrl) or shRNA targeting SGEF (SGEF-12 & SGEF-13). (B & C) T98G and U87 glioma control cells (Ctrl) or shSGEF cells (SGEF-12 & SGEF-13) were treated for 48 hours with either control DMSO or TMZ (500μM), and were either (B) plated onto 10-well slides pre-coated with 10ug/mL laminin, stained for DAPI and counted for percent chromatin condensation, or (C) protein lysates were collected for immunoblotting with PARP and Caspase 3. (D) T98G, U87 and U118 control cells (Ctrl) or shSGEF cells were treated with either control DMSO or TMZ (500μM) for 48 hours followed by plating at a density of 250 cells in triplicate for clonogenic studies. Observable colonies were recorded approximately one week following plating.

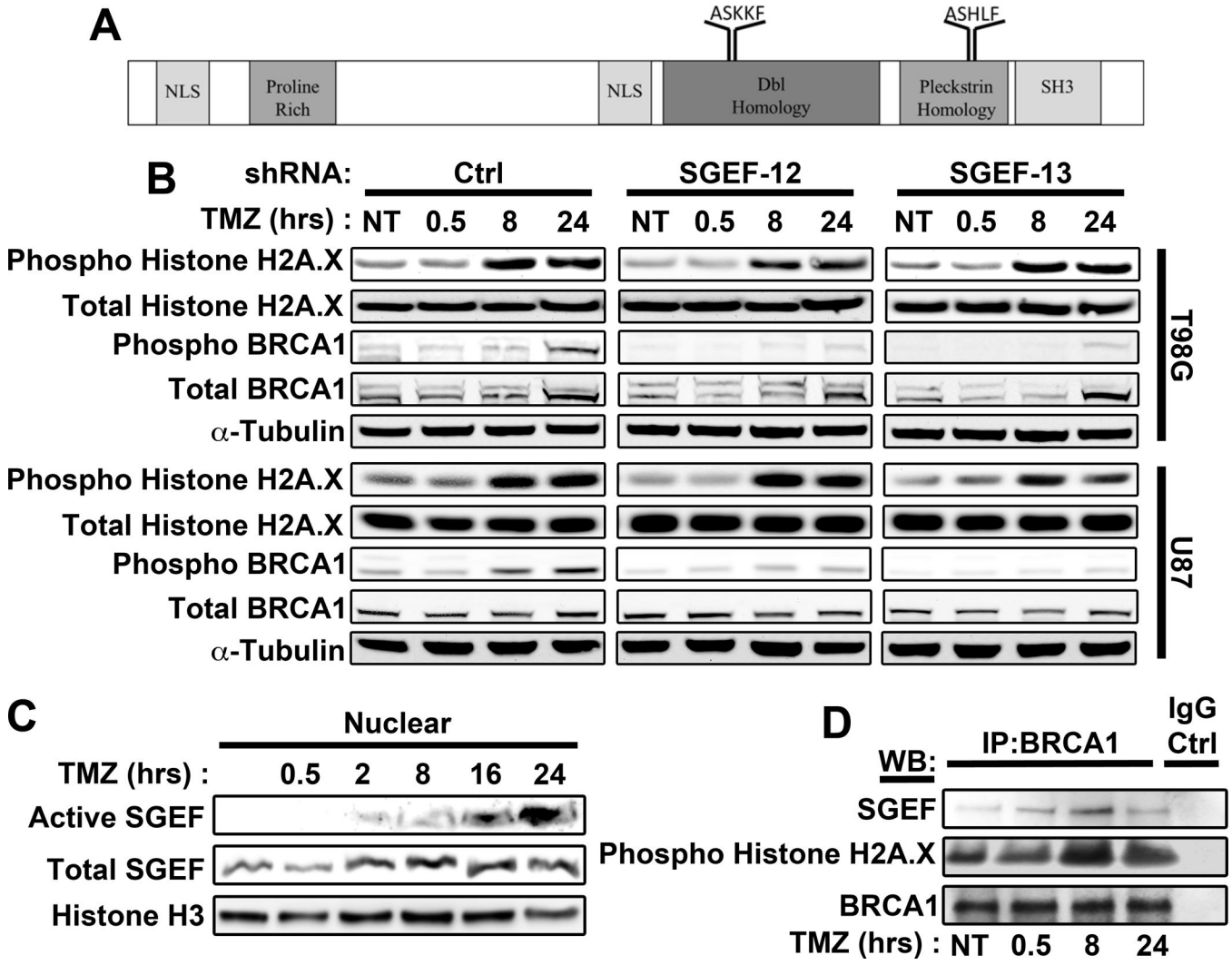

Figure 4. TMZ induces nuclear SGEF activity and SGEF dependent BRCA1 activity, and promotes SGEF in complex with BRCA1

(A) A diagrammatic representation of the SGEF polypeptide sequence, containing two BRCT domains (ASKKF and ASHLF) at aa 493–497 and aa 741–745. (B) U87 and T98G control cells (Ctrl) or shSGEF cells (SGEF-12 & SGEF-13) were treated with TMZ (500μM) for the indicated times, and protein lysates were analyzed by immunoblotting with phospho- and total-Histone H2A.X and phospho- and total-BRCA1, as well as tubulin antibodies. (C) U87 glioma cells were treated with TMZ (500μM) for the indicated times followed by isolation of nuclear proteins. SGEF activation in control and treated lysates was assessed using RhoG G15A-GST constructs with immunoblotting for antibodies as indicated. (D) Immunoblot analysis of SGEF and phospho-Histone H2A.X in nuclear lysates, immunoprecipitated with BRCA1-specific antibody, from U87 cells treated with TMZ for the indicated times.

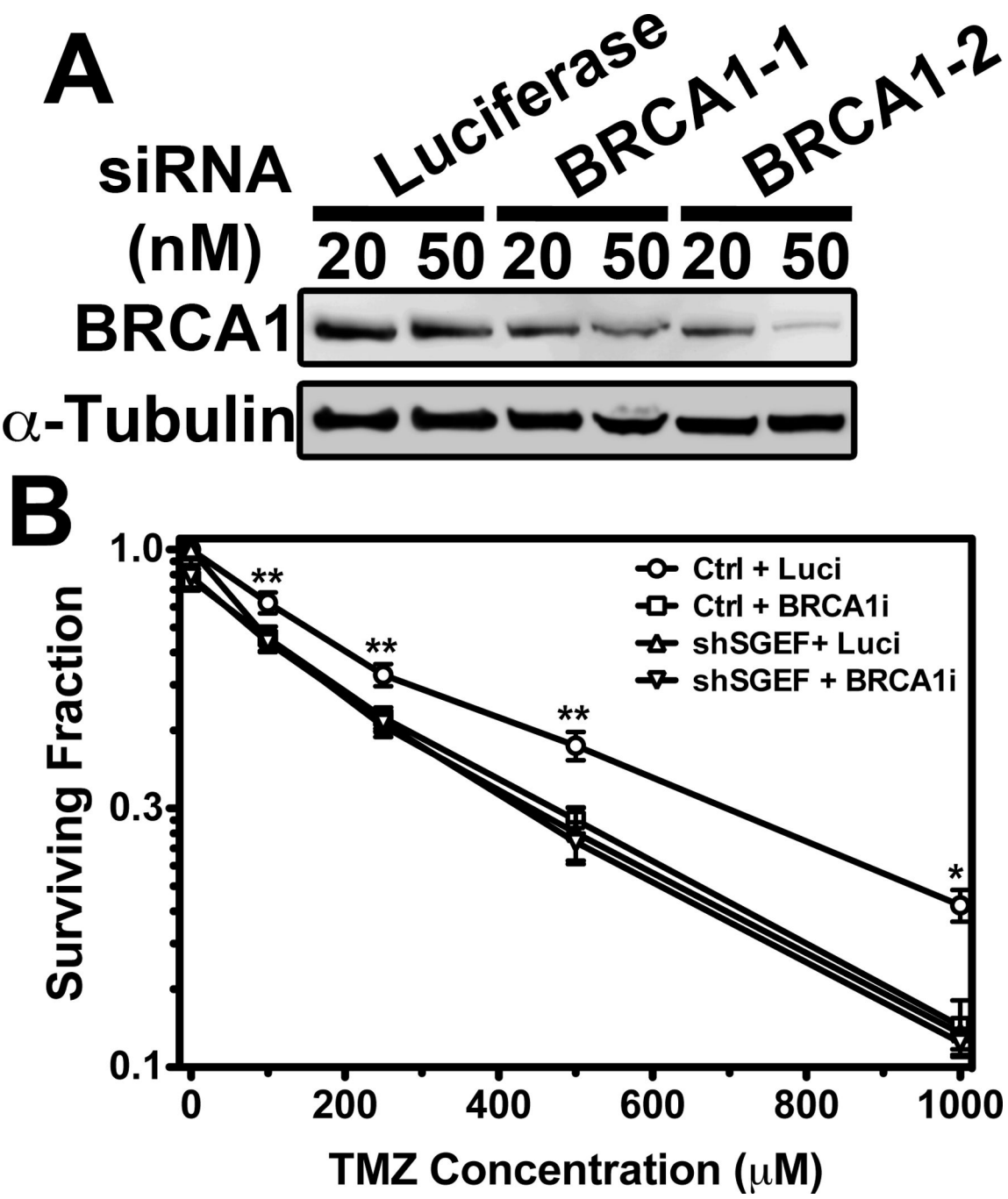

Figure 5. Depletion of BRCA1 impairs colony formation following TMZ treatment and is not enhanced by concurrent depletion of SGEF

(A) U87 cells were transfected with siRNA targeting either firefly luciferase (Luci) as a control or BRCA1 (BRCA1-1 & BRCA1-2) at 20 nM or 50nM concentrations. Protein knockdown was analyzed via immunoblot analysis for BRCA1. (B) Colony formation capacity of parental U87 cells and U87 shSGEF cells was evaluated after transfection with siRNA for either luciferase or BRCA1 (BRCA1-2) at 50nM and treatment at varying concentrations with TMZ. Cells were plated at a density of 250 cells in triplicate for clonogenic studies. Observable colonies were recorded approximately one week following

plating. Data reported as the surviving fraction represent an average and SD of 3 replicates. (** $p < 0.01$).

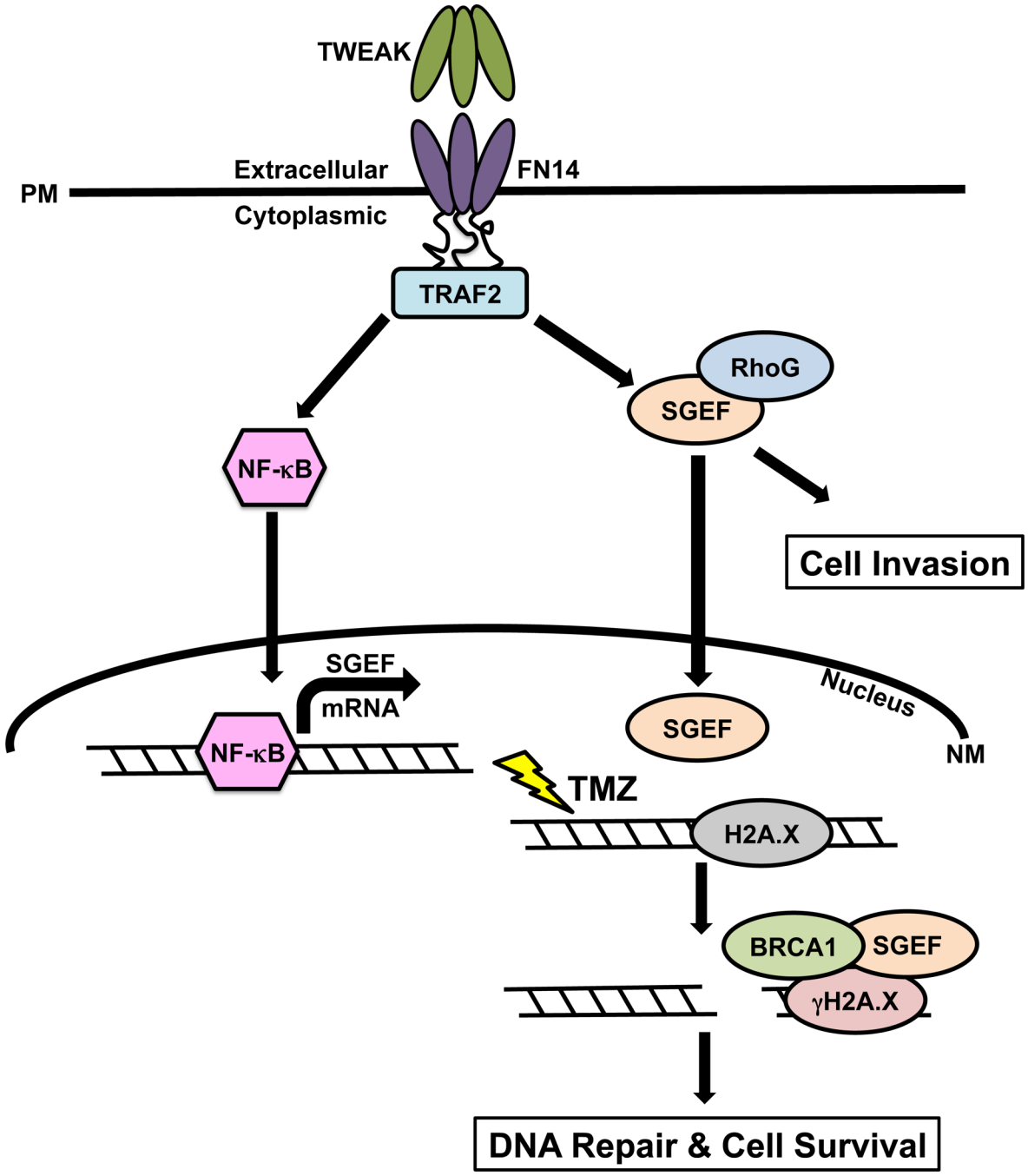

Figure 6. Schematic model of Fn14-NF- κB-dependent up-regulation of SGEF expression and SGEF pro-survival response to TMZ treatment

TWEAK interaction with the Fn14 receptor leads to the NF-κB mediated up-regulation of SGEF mRNA and protein expression. SGEF protein promotes glioma cell survival and is associated with TMZ resistance and DNA repair pathway activation via complex with BRCA1.