Published in final edited form as:

Glia. 2014 October ; 62(10): 1687–1698. doi:10.1002/glia.22708.

Molecular Targeting of TRF2 Suppresses the Growth and Tumorigenesis of Glioblastoma Stem Cells

Yun Bai^{a,b}, Justin D. Lathia^c, Peisu Zhang^b, William Flavahan^c, Jeremy N. Rich^d, and Mark P. Mattson^{b,*}

^aDepartments of Cell Biology, School of Basic Medical Sciences, Peking University Health Science Center, 38 XueYuan Road, Beijing, 100191, China

^bLaboratory of Neurosciences, National Institute on Aging Intramural Research Program, Baltimore, MD, USA

^cDepartment of Cellular and Molecular Medicine, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, USA

^dDepartment of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, USA

Abstract

Glioblastoma is the most prevalent primary brain tumor and is essentially universally fatal within two years of diagnosis. Glioblastomas contain cellular hierarchies with self-renewing glioblastoma stem cells (GSCs) that are often resistant to chemotherapy and radiation therapy. GSCs express high amounts of repressor element 1 silencing transcription factor (REST), which may contribute to their resistance to standard therapies. Telomere repeat-binding factor 2 (TRF2) stabilizes telomeres and REST to maintain self-renewal of neural stem cells and tumor cells. Here we show viral vector-mediated delivery of shRNAs targeting TRF2 mRNA depletes TRF2 and REST from GSCs isolated from patient specimens. As a result, GSC proliferation is reduced and the level of proteins normally expressed by postmitotic neurons (L1CAM and β 3-tubulin) is increased, suggesting that loss of TRF2 engages a cell differentiation program in the GSCs. Depletion of TRF2 also sensitizes GSCs to temozolomide, a DNA-alkylating agent currently used to treat glioblastoma. Targeting TRF2 significantly increased the survival of mice bearing GSC xenografts. These findings reveal a role for TRF2 in the maintenance of REST-associated proliferation and chemotherapy resistance of GSCs, suggesting that TRF2 is a potential therapeutic target for glioblastoma.

Introduction

Glioblastoma is the most devastating brain tumor with nearly all patients succumbing within 2 years of diagnosis. Tumor resection, chemotherapy and radiation treatments extend

*Corresponding author: Mark P. Mattson, Ph.D., Laboratory of Neurosciences, National Institute on Aging Intramural Research Program, 251 Bayview Blvd, Baltimore, Maryland 21224, USA. Telephone: 410 558-8463; Fax: 410 558-8465; mark.mattson@nih.gov.

Conflict of interest statement. The authors declare that they have no conflicts of interest to disclose.

survival minimally because of rapid recurrence of aggressive tumors (Preusser et al., 2011). Recent findings suggest a major role for so-called glioblastoma stem cells (GSC), a subpopulation of treatment-resistant cells, in tumor recurrence and invasiveness. Presumptive GSC isolated from patient tumors based upon their expression of CD133 exhibit resistance to chemotherapy and radiation and form aggressive tumors when grafted into the brains of nude mice (Singh et al., 2003; Bao et al., 2006; Wakimoto et al., 2009; Tamura et al., 2010). The molecular features of GSC are similar to those of neural progenitor cells (NPC), suggesting the possibility that they might arise from neural progenitor cells (Lottaz et al., 2010; Yan et al., 2011).

As with many other types of aggressive tumor cells, GSC often have mutations in proteins such as p53 and PTEN that normally trigger apoptosis (Hermisson et al., 2006; Zheng et al., 2008; Dasari et al., 2010; Sato et al., 2011), and they also have elevated levels of proteins that promote cell survival and proliferation including Bcl-2 (Ray and Banik, 2012) and Notch (Wang et al., 2010; Gursel et al., 2012; Harr et al., 2012). In addition, repressor element 1 silencing transcription factor (REST) is expressed in unusually high amounts in GSC, but its roles in their self-renewal and resistance to chemotherapy and radiation are unknown (Conti et al., 2012; Kamal et al., 2012). REST was initially discovered in neural progenitor cells of the developing nervous system wherein it represses the expression of numerous neuron-specific genes, thereby maintaining the progenitor cells in a self-renewing state (Chong et al., 1995; Ballas et al., 2005; Otto et al., 2007). REST is rapidly down-regulated in neural progenitors in response to differentiation signals resulting in the de-repression of neuronal genes and morphological and functional differentiation of neurons (Ballas et al., 2005). However, whether REST is a crucial factor for maintaining cancer stem cell self-renewal is not known, and there is even evidence that a reduction of REST levels is associated with growth of at least some types of non-neural cancers (Coulson, 2005) [23]. Consistent with complex roles for REST in cell immortality and differentiation are data showing that REST interacts with different sets of target genes in embryonic stem cells, neural progenitor cells and mature neurons (Sun et al., 2005)[24].

Telomere repeat-binding factor 2 (TRF2) is a critical component of the shelterin protein complex that protects and stabilizes telomeres (de Lange, 2005). TRF2 removal in proliferating human and mouse cells rapidly triggers a telomeric DNA damage response and cell-cycle arrest to promote either senescence or apoptosis, depending on the cell type and its physiological state (Karlseder et al., 1999). Data suggest that maintenance of telomeres by TRF2 contributes to the multidrug resistance of gastric carcinoma cell lines (Ning et al., 2006), although whether this is true in GSCs remains to be determined. GSCs can be classified as either telomerase-positive or telomerase-negative, with those lacking telomerase being capable of an alternative mechanism of telomere lengthening (Silvestre et al., 2011). However, TRF2 is expressed in all GSC regardless of their telomere status, suggesting the possibility of a telomere-independent role for TRF2 in glioblastoma pathogenesis.

In addition to its function in stabilizing telomeres, it was recently reported that TRF2 binds to REST, an interaction that may be critical for the maintenance of REST at RE-1 binding sites and suppression of differentiation or senescence of neural stem cells and tumor cells (Zhang et al., 2008). Indeed, experimental depletion of TRF2 or inhibition of its binding to

REST in neural tumor cells results in ubiquitin-proteasomal degradation of REST and expression of REST target genes (e.g., β 3-tubulin, BDNF and L1CAM) cessation of cell division and acquisition of phenotypic properties of differentiated neurons. Thus, in addition to protecting telomeres, TRF2 possesses a novel role in stabilization of REST that is required for controlling neural tumor and stem cell fate. The potential involvement of TRF2 in maintenance of GSC, and/or their resistance to chemotherapy is as yet unexplored. In the present study we employed lentiviral vectors and RNA interference technology to determine the impact of targeted depletion of TRF2 on the proliferation and tumor-generating activity of human GSCs, and their vulnerability to a chemotherapeutic agent.

Materials and Methods

Isolation of Glioblastoma Stem Cells

All tumor specimens were derived from primary glioblastoma surgical biopsy specimens obtained from patients undergoing resection for newly diagnosed or recurrent GBM in accordance with protocols approved by the Duke University Medical Center or Cleveland Clinic Foundation Institutional Review Boards. Written consent to utilize excess tissue for research was obtained from each patient, and de-identified tissues were used for all studies. Tumors were dissociated to single cells by a papain dissociation kit (Worthington Biochemical) as per manufacturer's protocol and cultured in vitro prior to use using previously reported culturing methods (Lee et al., 2006a; Li et al., 2009). The GBM cells used in the present study were sorted for CD133 status and the CD133+ population (GSCs) was used for experiments. Cells under passage 10 were used for all experiments.

Lentiviral shRNA construct production

All lentiviral constructs were generated as described previously (Li et al., 2009, Wang et al., 2008); the maps and shRNA sequences are shown in Supplemental Figure 1. Using Lipofectamine 2000 (Invitrogen), 293FT cells were co-transfected with packaging vectors psPAX2 and pCI-VSVG (Addgene) and lentiviral vectors directing expression of shRNAs specific to TRF2 (TRCN 0000004812 [**sh1**] and TRCN 0000004813 [**sh2**]) or a non-targeting control shRNA (**NT**) (Open Biosystems) to produce virus. The culture medium was changed 18 hours after transfection, and two days later viral supernatants were collected, filtered and concentrated by ultracentrifugation at 100,000 g for 3 hours.

Cell cycle analysis

Following TRF2 knockdown, 500,000 cells from each treatment group were filtered through a 30 μ m filter before fixation in 3 ml of 70% Ethanol at 4°C overnight. After fixation, the cells were pelleted and resuspended in 0.5ml of PBS. In order to remove any traces of free RNA that could bind with propidium iodide and interfere with data interpretation, each sample was incubated with 5 μ l of RNase A (10 μ g/ml) for 30 minutes at 37°C. The RNase was then inactivated by incubation for 5 minutes at 4°C. Following addition of 10 μ l of propidium iodide (PI), cells were analyzed by flow cytometry using a FACScan for DNA content.

Cell apoptosis analysis

Apoptotic cells were detected using the Apoptosis Detection Kit (Oncogene, San Diego, CA, USA) containing FITC labeled Annexin V (AV), according to the manufacturer's instruction. In brief, cells were rinsed with ice-cold PBS and then resuspended in 200 μ l of binding buffer. Ten microliters of AV-FITC stock solution was added to cell suspensions and incubated for 30 min at 4°C. The cells were then further incubated with 5 μ l PI and were immediately analyzed on a FACStar Plus flow cytometer (Becton–Dickinson, Mountain View, CA, USA). Approximately 10,000–20,000 cells in the tested sample were counted by the cytometer.

Growth curve and tumorsphere formation assays

GSC were infected with NT, sh1 or sh2 lentivirus and 3 days later were selected by incubation with 1 μ g/ml puromycin for 3 days (total of 6 days post infection). GSC were plated in 96-well plates in triplicate (5000 cells/well). Total viable cell numbers were then measured for 5 consecutive days using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega). For tumorsphere formation assays, 500 GSC were seeded per well in 96-well tissue culture plates. Eight wells were plated for each treatment condition. Analyses were performed at 7 days in culture; images of tumorspheres in each well were acquired and were then analyzed using Adobe Photoshop or NIH Image J software to quantify both the number of tumorspheres and the diameter of individual tumorspheres. All data presented are the results of three or four separate experiments.

Cell Proliferation Assays

Following TRF2 knockdown, BrdU was added to the cultures at a final concentration of 10 μ M, and 2 hours later the GSCs were fixed in 4% paraformaldehyde in PBS, processed for immunocytochemistry, and counterstained with PI to label the nucleus of all cells. Images were acquired in five randomly chosen microscope fields using a 25 $\times$ objective and dual channels for BrdU immunostaining (488 nm excitation and 510 nm emission) and PI (543 nm excitation and 590 nm emission). The BrdU⁺ and PI⁺ cells were counted simultaneously in each image, and the BrdU-labeling index was calculated as the number of BrdU⁺ cells divided by the total number of the cells (PI⁺). A minimum of three cultures for each condition were used in each experiment.

Immunoblotting

Cultured cells were solubilized in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis sample buffer, and the protein concentration in each sample was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Proteins (30 μ g of protein per lane) were then resolved in a 7.5%–10% SDS polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked with 4% nonfat milk in TBST (Tris-HCl based buffer with 0.2% Tween 20, pH 7.5) and then incubated in the presence of primary antibody overnight at 4 °C. Cells were then incubated for 1 hour in the presence of a 1:5,000 dilution of secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratory, West Grove, PA). Reaction product was visualized using an enhanced chemiluminescence

Western blot detection kit (Amersham Bioscience, Piscataway, NJ). The primary antibodies used were: a mouse monoclonal antibody against TRF2 (Imgenex); a goat polyclonal antibody against REST (P18, Santa Cruz); and antibodies against Olig2 (Millipore), p53 (Cell Signaling), β 3-tubulin (Sigma), L1CAM and actin (Sigma).

Drug cytotoxicity assay

These experiments were performed using methods described previously (Liu et al., 2006). The number of viable cells following drug treatment was assessed using a Cell Counting Kit-8 (CCK-8) following the manufacturer's protocol (Dojindo, Kumamoto, Japan). Briefly, 1×10^4 GSCs/well were plated in 96-well plates in 10% FBS/DMEM/F-12 culture medium and allowed to attach overnight. Cells were then exposed to the chemotherapeutic agent temozolomide (Sigma; temozolomide was prepared as a 100 mM stock solution in dimethylsulfoxide) at concentrations ranging from 0.5 to 2.0 mM for 48 h. After treatment, 10 μ l of CCK-8 solution was added to each well. After incubation at 37°C for 2 h in a humidified CO₂ incubator, absorbance at 540 nm was monitored using a SpectraMax Plus 384 microplate reader (Molecular Devices). Determinations were made in 8 wells for each experimental condition, and experiments were repeated at least twice.

Intracranial xenograft tumor formation assay

GSCs were infected with control (NT), sh1 or sh2 lentiviruses, were selected for puromycin resistance, and 5000 GSC were implanted into brains of athymic BALB/c nude mice by stereotaxic injection into the cerebral cortex. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of National Institutes of Health, and were approved by the Lab Animal Care Committee of Peking University. Mice were maintained until they developed severe neurologic signs and were unable to care for themselves, at which time they were euthanized. Mice were perfused transcardially with saline and 4% paraformaldehyde, and after post-fixing overnight the brains were embedded in paraffin. Brain sections (5 μ m thickness) were prepared, deparaffinized and then stained with hematoxylin and eosin.

Statistical Analysis

Values are reported as the mean $\pm$ standard deviation (SD). Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). One-way ANOVA test was performed, and post-hoc multiple comparisons were done using Student Newman-Keuls test. Statistically significant differences were defined as those with a p value less than 0.05.

Results

Depletion of TRF2 inhibits the proliferation of human glioblastoma stem cells

Perhaps because of its critical role in telomere maintenance (de Lange, 2005), mutations of TRF2 have not been identified as a cause of cancer. To determine whether TRF2 mutations are associated with glioblastomas, we mined The Cancer Genome Atlas (TCGA) database and found that the *Terf2* gene is not mutated in any GBM patients (0 out of 235 sequenced) (Supplemental Figure 2A) and is deleted in no more than 2 out of the 559 patients with copy number data, with the 2 possible deletions likely being noise (data not shown). Compared to

the TCGA's normal brain samples, tumors have a significantly lower level of TRF2 (Supplemental Figure 2B). This can be readily explained since TRF2 is important in the stem cell population, which represents a minority of cells within the tumor, while the majority of the cells in the tumor are non-stem cells with lower TRF2 levels, possibly explaining the lower expression overall in the tumor. In addition, the Cahoy et al. (2008) mouse brain dataset indicates that TRF2 expression in neurons is between two and three times the expression levels in astrocytes, oligodendrocytes or oligodendrocyte progenitor cells (Supplemental Figure 2C). We previously found a similar scenario with GLUT3 which is upregulated in tumors compared to glial cells but not compared to the whole brain controls due to strong expression in neurons (Flavahan et al., 2013).

We generated two lentiviral constructs with two different shRNA sequences directed against the human TRF2 mRNA (sh1 and sh2) and a control lentivirus that included a non-targeting RNA sequence (NT) (Supplemental Figure 1A). Expression of the shRNAs was driven by the U6 promoter. GSCs infected with lentiviruses were selected by incubation for 3 days in medium containing puromycin, and cell lysates were subjected to immunoblot analysis using a TRF2 antibody to confirm the degree of TRF2 knockdown. GSC infected with TRF2 sh1 and sh2 lentiviruses exhibited a more than 10-fold reduction of TRF2 levels compared to uninfected cells and cells infected with the NT control lentivirus (Supplemental Figure 1B).

To determine whether depletion of TRF2 affected the growth of GSCs, we utilized functionally validated GSCs that had been isolated from glioblastomas from two different patients (T4302 and T3691) that we previously validated for differences in tumor formation based on CD133 enrichment (Liu et al., 2006; Li et al., 2009). First, at 3 days after infection of GSC with TRF2 sh1, TRF2 sh2 or control lentiviruses, the cells were collected and processed for flow cytometry-based analysis of cell cycle and apoptosis. In GSC isolated from tumors from two different patients (T4302 and T3691), knockdown of TRF2 resulted in a significant increase in the percentage of GSC in G1 phase and a significant decrease in the percentage of GSC in S phase compared to GSC expressing a non-targeting control shRNA and to untransfected GSC (Figure 1). These findings indicate that TRF2 plays an important role in promoting the proliferation of GSC. We also quantified apoptosis using FITC-labeled Annexin V. The percentage of Annexin V and propidium iodide double-positive (apoptotic) cells was determined by FACS analysis. We found that knockdown of TRF2 did not significantly affect the basal level of apoptosis in T4302 and T3691 GSCs (Figure 2).

Next, we performed a tumorsphere formation assay in which dissociated GSCs expressing NT, TRF2 sh1 or TRF2 sh2, and untransfected control cells (UN), were plated at a fixed density (100 cells/well) in 96 well plates and cultured for 7 days. Under these conditions untransfected GSCs and NT GSCs formed approximately 10–12 aggregates (tumorspheres) per well (Figure 3A, B), with an average neurosphere diameter of approximately 135 μ m (Figure 3C). In contrast, the number of tumorspheres formed was significantly reduced by more than 60%, and the average tumorsphere diameter was significantly reduced by more than 70%, in GSCs expressing TRF2 sh1 or TRF2 sh2 (Figure 3A–C).

TRF2 depletion suppresses the proliferation of GSCs

We also evaluated the effects of TRF2 depletion on the proliferation of GSCs using two different assays. In UN and NT GSCs the numbers of cells increased by more than 6-fold during a 5 day culture period, whereas TRF2 sh1 and TRF2 sh2 GSCs increased by only 2-fold during the 5 day period (Figure 4). Depletion of TRF2 with shRNAs resulted in a similar suppression of cell growth in GSCs from both glioblastoma patients. The results of a BrdU incorporation assay confirmed that lentivirus-mediated knockdown of TRF2 significantly reduces the proliferation of GSC (Figure 5).

TRF2 knockdown depletes REST and Olig2, and up-regulates neuronal proteins and p53 in human GSCs

It was previously reported that TRF2 interacts with REST to suppress expression of neuronal genes and thereby maintain NPCs and neuroblastoma cells in a self-renewing state (Zhang et al., 2008). We found that shRNA-mediated depletion of TRF2 resulted in a significant reduction in REST levels in GSCs (Figure 6A, D). Levels of Olig2, a protein involved in the self-renewal of glial progenitor cells (Xue et al., 2009) was previously shown to be expressed at high levels in aggressive gliomas (Ligon et al., 2004; Rhee et al., 2009; Wagoner and Roopra, 2012). We found that Olig2 protein levels were significantly reduced in GSCs in which TRF2 had been depleted by RNA interference (Figure 6A, C). We also measured levels of L1CAM and β 3-tubulin, two proteins encoded by REST target genes that are known to play important roles in the differentiation of neurons and growth of their axons (Gabay et al., 2003; Bibel et al., 2004; Maness and Schachner, 2007; Schäfer and Frotscher, 2012). GSC infected with sh1 or sh2 TRF2 lentiviruses exhibited significantly elevated levels of L1CAM and β 3-tubulin compared to UN GSCs and NT lentivirus-infected GSCs (Figure 6A, E and G). Interestingly, we also found that levels of the tumor suppressor protein p53 were significantly increased in response to TRF2 depletion in GSCs (Figure 6A, F). Thus, targeting TRF2 causes GSCs to up-regulate the expression of proteins involved in cell differentiation, and cell cycle arrest/apoptosis.

TRF2 depletion sensitizes GSCs to temozolomide

Patients with glioblastoma are often treated with the alkylating agent temozolomide, which can extend survival by several months in some patients (Stupp et al., 2005, 2009; Nagasawa et al., 2012; Thomas et al., 2013). We therefore asked whether depletion of TRF2 might increase the sensitivity of GSCs to temozolomide. GSC were infected with NT, TRF2 sh1 and TRF2 sh2 lentiviruses and were then exposed to increasing concentrations of temozolomide for 48 hours. Quantification of cell viability revealed that GSCs infected with either of the TRF2 shRNA lentiviruses were significantly more vulnerable to temozolomide induced death as compared to GSCs infected with NT lentivirus and untransfected GSCs (Figure 7). Similar results were obtained in GSCs isolated from two different patients.

Molecular targeting of TRF2 extends survival in a mouse xenograft glioblastoma model

To determine the potential clinical application of interventions that target TRF2 for glioblastoma treatment, we determined whether infection of human GSCs with TRF2 shRNA lentiviruses would modify the disease course in a xenograft mouse model. As

described previously, when human GSCs are injected into the brains of nude mice they propagate aggressive tumors that spread throughout the brain and lead to death of the animals (Lathia et al., 2011a, 2011b). We found that whereas half of the mice xenografted with NT lentivirus-infected GSC died within 20 days, most of those xenografted with TRF2 sh1 or TRF2 sh2 lentiviruses survived more than 50 days, a statistically significant extension of survival (Figure 8). Similar results were obtained with GSC isolated from tumors from the two different patients (T4302 and T3691) (Figure 8A, B).

Discussion

Our findings demonstrate a critical role for TRF2 in maintaining GSC in a highly proliferative and chemotherapy-resistant state. Lentivirus-mediated expression of short hairpin RNAs with sequences targeted against the TRF2 mRNA resulted in a large reduction in TRF2 protein levels in GSC isolated from brain tumors from two different patients. As a result, the growth of the GSC in tumorsphere cultures was significantly inhibited, as was the proliferation of GSC in dissociated cell cultures. Targeted depletion of TRF2 did not trigger apoptosis of GSC under basal culture conditions, but did significantly increase the sensitivity of the GSC to temozolomide and reduced tumorigenesis in an in vivo mouse xenograft model in which human GSC were injected directly into the brain of mice. As a result of lentivirus-mediated knockdown of TRF2, the survival of mice bearing glioblastoma brain tumors was significantly extended.

There are at least two mechanisms by which TRF2 depletion inhibits the proliferation and reduces the survival of GSC. First, by reducing the amount of TRF2 at telomeres, targeted depletion of TRF2 may activate DNA checkpoint and render cells vulnerable to apoptosis. Previous studies have shown that TRF2 is highly expressed in a range of tumors including breast cancers (Diehl et al., 2011), colorectal carcinomas (Dong et al., 2009) and epithelial cancers (Blanco et al., 2007). Our analysis of the TCGA database indicated that the gene encoding TRF2 (*Terf2*) is not mutated or deleted in GBM patients. It was previously reported that inhibition of TRF2 using a dominant negative mutant can trigger cell cycle arrest and/or apoptosis in several types of tumor cells (Biroccio et al., 2006; Buscemi et al., 2009). We found that GSC express considerable TRF2 and that depletion of TRF2 causes a major reduction in cell growth and increases GSC sensitivity to the DNA alkylating agent temozolomide. Further studies will be required to establish the extent to which destabilization of telomeres contributes to the inhibitory effects of TRF2 shRNA expression on GSC growth and viability. Nevertheless our findings are consistent with recent data showing that chemical and genetic manipulations that damage/shorten telomeres can inhibit glioblastoma cell growth and may trigger apoptosis (Fujita et al., 2010; Marian et al., 2010; Merle et al., 2011).

Consistent with a previous study of human neuroblastoma and pluripotent stem cells (Zhang et al., 2008), we found that depletion of TRF2 results in a reduction of REST levels in human GSC. The decline in REST levels was associated with an increase in the levels of β 3-tubulin and L1CAM, two neuronal proteins that are REST target genes (Kallunki et al., 1997; Zhang et al., 2008). The latter results are consistent with the possibility that depletion of TRF2 inhibits the proliferation of GSC by de-repressing REST target genes resulting in

cell cycle arrest and acquisition of properties of differentiated neurons including the expression of neuronal proteins. Indeed, it was recently reported that overexpression of the transcription factor neurogenin 2 (which induces neuronal differentiation in neural progenitor cells) in CD133⁺ glioblastoma cells inhibits tumorsphere formation and triggers cells death (Guichet et al., 2013), demonstrating that neuronal differentiation signals adversely affect GSC growth.

Our finding that L1CAM levels increase in GSC in response to targeted depletion of TRF2 merits discussion in light of previous studies of L1CAM's functions in tumor cells and neurons. Several previous studies have provided evidence that L1CAM promotes the migration of cancer cells and the invasiveness of tumors, including glioblastomas (Suzuki et al., 2005; Siesser and Maness, 2009; Amoureux et al., 2010). Moreover, knockdown of L1CAM levels can sensitize GSC to radiation (Cheng et al., 2011). On the other hand, L1CAM is upregulated upon differentiation of neural stem cells into neurons and overexpression of L1CAM can enhance neuronal differentiation and axon growth (Cui et al., 2010, 2011; Cherry et al., 2012). Future studies will be required to determine whether L1CAM enhances or antagonizes the inhibitory effects of TRF2 depletion on GSC growth and survival. However, it should be noted that if L1CAM contributes to GSC invasiveness, this action of L1CAM is unable to overcome the adverse effects of TRF2 depletion on the GSC because TRF2 depletion inhibited the growth of GSC in culture and GSC-derived brain tumors in vivo, even as L1CAM levels were increased.

Finally, we found that p53 levels were elevated in GSC in response to targeted depletion of TRF2. This upregulation of p53 might be the result of telomere damage and/or reduced levels of REST because telomere damage can trigger p53 expression (Karlseder et al., 2004; Stagno D'Alcontres et al., 2007; Hayashi et al., 2012) and REST represses p53 expression (Lietz et al., 2001). The increased levels of p53 in GSC infected with TRF2 shRNA lentivirus likely contributes to the increased sensitivity of the TRF2-depleted GSC to temozolomide-induced death because p53 is known to trigger apoptosis of glioblastoma cells (Srivenugopal et al., 2001). On the other hand, some data suggest that DNA methylation status may determine whether p53 confers vulnerability or resistance to temozolomide in glioma cells (Hermisson et al., 2006). However, we found that targeted depletion of TRF2 using lentiviruses that express TRF2 shRNA suppresses tumorigenesis and extends the survival of mice in an animal model of GSC-induced brain tumors. Therefore, upregulation of p53 is associated with a better clinical outcome in this model of GSC-induced brain tumors. Indeed, previous findings suggest that p53 either does not affect TMZ sensitivity or even decreases TMZ sensitivity in glioma and glioblastoma tumor-initiating cells (Blough et al., 2011).

In conclusion our data suggest that targeted depletion of TRF2 inhibits the growth of GSC by a 'double hit' mechanism with one damaging blow occurring at telomeres and the other damaging blow being inflicted upon REST. Our findings provide a rationale developing interventions that inhibit or deplete TRF2 from GSC in the treatment of glioblastoma patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by the Intramural Research Program of the National Institute on Aging (NIH), and by the National Natural Sciences Foundation of China (81272432, 31070948).

References

- Abrajano JJ, Qureshi IA, Gokhan S, Molero AE, Zheng D, Bergman A, Mehler MF. Corepressor for element-1-silencing transcription factor preferentially mediates gene networks underlying neural stem cell fate decisions. *Proc Natl Acad Sci USA*. 2010; 107:16685–16690. [PubMed: 20823235]
- Amoureux MC, Coulibaly B, Chinot O, Loundou A, Metellus P, Rougon G, Figarella-Branger D. Polysialic acid neural cell adhesion molecule (PSA-NCAM) is an adverse prognosis factor in glioblastoma, and regulates olig2 expression in glioma cell lines. *BMC Cancer*. 2010; 10:91. [PubMed: 20219118]
- Ballas N, Grunseich C, Lu DD, Speh JC, Mandel G. REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. *Cell*. 2005; 121:645–657. [PubMed: 15907476]
- Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature*. 2006; 444:756–760. [PubMed: 17051156]
- Bibel M, Richter J, Schrenk K, Tucker KL, Staiger V, Korte M, Goetz M, Barde YA. Differentiation of mouse embryonic stem cells into a defined neuronal lineage. *Nat Neurosci*. 2004; 7:1003–1009. [PubMed: 15332090]
- Biroccio A, Rizzo A, Elli R, Koering CE, Belleville A, Benassi B, Leonetti C, Stevens MF, D'Incalci M, Zupi G, Gilson E. TRF2 inhibition triggers apoptosis and reduces tumorigenicity of human melanoma cells. *Eur J Cancer*. 2006; 42:1881–1888. [PubMed: 16750909]
- Blanco R, Muñoz P, Flores JM, Klatt P, Blasco MA. Telomerase abrogation dramatically accelerates TRF2-induced epithelial carcinogenesis. *Genes Dev*. 2007; 21:206–220. [PubMed: 17234886]
- Blough MD, Beauchamp DC, Westgate MR, Kelly JJ, Cairncross JG. Effect of aberrant p53 function on temozolomide sensitivity of glioma cell lines and brain tumor initiating cells from glioblastoma. *J Neurooncol*. 2011; 102:1–7. [PubMed: 20593219]
- Buscemi G, Zannini L, Fontanella E, Lecis D, Lisanti S, Delia D. The shelterin protein TRF2 inhibits Chk2 activity at telomeres in the absence of DNA damage. *Curr Biol*. 2009; 19:874–879. [PubMed: 19375317]
- Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA, Thompson WJ, Barres BA. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J Neurosci*. 2008; 28:264–278. [PubMed: 18171944]
- Cheng L, Wu Q, Huang Z, Guryanova OA, Huang Q, Shou W, Rich JN, Bao S. L1CAM regulates DNA damage checkpoint response of glioblastoma stem cells through NBS1. *EMBO J*. 2011; 30:800–813. [PubMed: 21297581]
- Cherry JF, Carlson AL, Benarba FL, Sommerfeld SD, Verma D, Loers G, Kohn J, Schachner M, Moghe PV. Oriented, multimeric biointerfaces of the L1 cell adhesion molecule: an approach to enhance neuronal and neural stem cell functions on 2-D and 3-D polymer substrates. *Biointerphases*. 2012; 7(1–4):22. [PubMed: 22589065]
- Chong JA, Tapia-Ramírez J, Kim S, Toledo-Aral JJ, Zheng Y, Boutros MC, Altschuller YM, Frohman MA, Kraner SD, Mandel G. REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. *Cell*. 1995; 80:949–957. [PubMed: 7697725]
- Conti L, Crisafulli L, Caldera V, Tortoreto M, Brilli E, Conforti P, Zunino F, Magrassi L, Schiffer D, Cattaneo E. REST controls self-renewal and tumorigenic competence of human glioblastoma cells. *PLoS One*. 2012; 7(6):e38486. [PubMed: 22701651]

- Coulson JM. Transcriptional regulation: cancer, neurons and the REST. *Curr Biol*. 2005; 15:R665–R668. [PubMed: 16139198]
- Covey MV, Streb JW, Spektor R, Ballas N. REST regulates the pool size of the different neural lineages by restricting the generation of neurons and oligodendrocytes from neural stem/progenitor cells. *Development*. 2012; 139:2878–2890. [PubMed: 22791895]
- Cui YF, Hargus G, Xu JC, Schmid JS, Shen YQ, Glatzel M, Schachner M, Bernreuther C. Embryonic stem cell-derived L1 overexpressing neural aggregates enhance recovery in Parkinsonian mice. *Brain*. 2010; 133:189–204. [PubMed: 19995872]
- Cui YF, Xu JC, Hargus G, Jakovcevski I, Schachner M, Bernreuther C. Embryonic stem cell-derived L1 overexpressing neural aggregates enhance recovery after spinal cord injury in mice. *PLoS One*. 2011; 6(3):e17126. [PubMed: 21445247]
- Dasari VR, Kaur K, Velpula KK, Gujrati M, Fassett D, Klopfenstein JD, Dinh DH, Rao JS. Upregulation of PTEN in glioma cells by cord blood mesenchymal stem cells inhibits migration via downregulation of the PI3K/Akt pathway. *PLoS One*. 2010; 5(4):e10350. (2010). [PubMed: 20436671]
- Diehl MC, Idowu MO, Kimmelshue KN, York TP, Jackson-Cook CK, Turner KC, Holt SE, Elmore LW. Elevated TRF2 in advanced breast cancers with short telomeres. *Breast Cancer Res Treat*. 2011; 127:623–630. [PubMed: 20625812]
- de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev*. 2005; 19:2100–2110. [PubMed: 16166375]
- Dong W, Shen R, Wang Q, Gao Y, Qi X, Jiang H, Yao J, Lin X, Wu Y, Wang L. Sp1 upregulates expression of TRF2 and TRF2 inhibition reduces tumorigenesis in human colorectal carcinoma cells. *Cancer Biol Ther*. 2009; 8:2166–2174. [PubMed: 19783902]
- Flavahan WA, Wu Q, Hitomi M, Rahim N, Kim Y, Sloan AE, Weil RJ, Nakano I, Sarkaria JN, Stringer BW, Day BW, Li M, Lathia JD, Rich JN, Hjelmeland AB. Brain tumor initiating cells adapt to restricted nutrition through preferential glucose uptake. *Nat Neurosci*. 2013; 16:1373–1382. [PubMed: 23995067]
- Fujita K, Horikawa I, Mondal AM, Jenkins LM, Appella E, Vojtesek B, Bourdon JC, Lane DP, Harris CC. Positive feedback between p53 and TRF2 during telomere-damage signalling and cellular senescence. *Nat Cell Biol*. 2010; 12:1205–1212. [PubMed: 21057505]
- Gabay L, Lowell S, Rubin LL, Anderson DJ. Deregulation of dorsoventral patterning by FGF confers trilineage differentiation capacity on CNS stem cells in vitro. *Neuron*. 2003; 40:485–499. [PubMed: 14642274]
- Guichet PO, Bieche I, Teigell M, Serguera C, Rothhut B, Rigau V, Scamps F, Ripoll C, Vacher S, Taviaux S, Chevassus H, Duffau H, Mallet J, Susini A, Joubert D, Bauchet L, Hugnot JP. Cell death and neuronal differentiation of glioblastoma stem-like cells induced by neurogenic transcription factors. *Glia*. 2013; 61:225–239. [PubMed: 23047160]
- Gürsel DB, Berry N, Boockvar JA. The contribution of Notch signaling to glioblastoma via activation of cancer stem cell self-renewal: the role of the endothelial network. *Neurosurgery*. 2012; 70:N19–N21. [PubMed: 22251985]
- Haar CP, Hebbar P, Wallace GC 4th, Das A, Vandergrift WA 3rd, Smith JA, Giglio P, Patel SJ, Ray SK, Banik NL. Drug resistance in glioblastoma: a mini review. *Neurochem Res*. 2012; 37:1192–1200. [PubMed: 22228201]
- Hayashi MT, Cesare AJ, Fitzpatrick JA, Lazzerini-Denchi E, Karlseder J. A telomere-dependent DNA damage checkpoint induced by prolonged mitotic arrest. *Nat Struct Mol Biol*. 2012; 19:387–394. [PubMed: 22407014]
- Hermisson M, Klump A, Wick W, Wischhusen J, Nagel G, Roos W, Kaina B, Weller M. O6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells. *J Neurochem*. 2006; 96:766–776. [PubMed: 16405512]
- Kallunki P, Edelman GM, Jones FS. Tissue-specific expression of the L1 cell adhesion molecule is modulated by the neural restrictive silencer element. *J Cell Biol*. 1997; 138:1343–1354. [PubMed: 9298989]

- Kamal MM, Sathyan P, Singh SK, Zinn PO, Marisetty AL, Liang S, Gumin J, El-Mesallamy HO, Suki D, Colman H, Fuller GN, Lang FF, Majumder S. REST regulates oncogenic properties of glioblastoma stem cells. *Stem Cells*. 2012; 30:405–414. [PubMed: 22228704]
- Karlseder J, Hoke K, Mirzoeva OK, Bakkenist C, Kastan MB, Petrini JH, de Lange T. The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. *PLoS Biol*. 2004; 2(8):E240. [PubMed: 15314656]
- Lathia JD, Gallagher J, Myers JT, Li M, Vasanji A, McLendon RE, Hjelmeland AB, Huang AY, Rich JN. Direct in vivo evidence for tumor propagation by glioblastoma cancer stem cells. *PLoS One*. 2011a; 6(9):e24807. [PubMed: 21961046]
- Lathia JD, Hitomi M, Gallagher J, Gadani SP, Adkins J, Vasanji A, Liu L, Eyler CE, Heddleston JM, Wu Q, Minhas S, Soeda A, Hoeppner DJ, Ravin R, McKay RD, McLendon RE, Corbeil D, Chenn A, Hjelmeland AB, Park DM, Rich JN. Distribution of CD133 reveals glioma stem cells self-renew through symmetric and asymmetric cell divisions. *Cell Death Dis*. 2011b; 1(2):e200. [PubMed: 21881602]
- Li Z, Bao S, Wu Q, Wang H, Eyler C, Sathornsumetee S, Shi Q, Cao Y, Lathia J, McLendon RE, Hjelmeland AB, Rich JN. Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. *Cancer Cell*. 2009; 15:501–513. [PubMed: 19477429]
- Lietz M, Bach K, Thiel G. Biological activity of RE-1 silencing transcription factor (REST) towards distinct transcriptional activators. *Eur J Neurosci*. 2001; 14:1303–1312. [PubMed: 11703459]
- Ligon KL, Alberta JA, Kho AT, Weiss J, Kwaan MR, Nutt CL, Louis DN, Stiles CD, Rowitch DH. The oligodendroglial lineage marker OLIG2 is universally expressed in diffuse gliomas. *J Neuropathol Exp Neurol*. 2004; 63:499–509. [PubMed: 15198128]
- Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, Lu L, Irvin D, Black KL, Yu JS. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer*. 2006; 5:67. [PubMed: 17140455]
- Lottaz C, Beier D, Meyer K, Kumar P, Hermann A, Schwarz J, Junker M, Oefner PJ, Bogdahn U, Wischhusen J, Spang R, Storch A, Beier CP. Transcriptional profiles of CD133+ and CD133- glioblastoma-derived cancer stem cell lines suggest different cells of origin. *Cancer Res*. 2010; 70:2030–2040. [PubMed: 20145155]
- Maness PF, Schachner M. Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration. *Nat Neurosci*. 2007; 10:19–26. [PubMed: 17189949]
- Marian CO, Cho SK, McEllin BM, Maher EA, Hatanpaa KJ, Madden CJ, Mickey BE, Wright WE, Shay JW, Bachoo RM. The telomerase antagonist, imetelstat, efficiently targets glioblastoma tumor-initiating cells leading to decreased proliferation and tumor growth. *Clin Cancer Res*. 2010; 16:154–163. [PubMed: 20048334]
- Merle P, Evrard B, Petitjean A, Lehn JM, Teulade-Fichou MP, Chautard E, De Cian A, Guittat L, Tran PL, Mergny JL, Verrelle P, Tchirkov A. Telomere targeting with a new G4 ligand enhances radiation-induced killing of human glioblastoma cells. *Mol Cancer Ther*. 2011; 10:1784–1795. [PubMed: 21987532]
- Nagasawa DT, Chow F, Yew A, Kim W, Cremer N, Yang I. Temozolomide and other potential agents for the treatment of glioblastoma multiforme. *Neurosurg Clin N Am*. 2012; 23:307–322. [PubMed: 22440874]
- Ning H, Li T, Zhao L, Li T, Li J, Liu J, Liu Z and Fan D. TRF2 promotes multidrug resistance in gastric cancer cells. *Cancer Biol Ther*. 2006; 5:950–956. [PubMed: 16760674]
- Otto SJ, McCorkle SR, Hover J, Conaco C, Han JJ, Impey S, Yochum GS, Dunn JJ, Goodman RH, Mandel G. A new binding motif for the transcriptional repressor REST uncovers large gene networks devoted to neuronal functions. *J Neurosci*. 2007; 27:6729–6739. [PubMed: 17581960]
- Preusser M, de Ribaupierre S, Wöhrer A, Erridge SC, Hegi M, Weller M, Stupp R. Current concepts and management of glioblastoma. *Ann Neurol*. 2011; 70:9–21. [PubMed: 21786296]
- Rhee W, Ray S, Yokoo H, Hoane ME, Lee CC, Mikheev AM, Horner PJ, Rostomily RC. Quantitative analysis of mitotic Olig2 cells in adult human brain and gliomas: implications for glioma histogenesis and biology. *Glia*. 2009; 57:510–523. [PubMed: 18837053]

- Sato A, Sunayama J, Matsuda K, Seino S, Suzuki K, Watanabe E, Tachibana K, Tomiyama A, Kayama T, Kitanaka C. MEK-ERK signaling dictates DNA-repair gene MGMT expression and temozolomide resistance of stem-like glioblastoma cells via the MDM2-p53 axis. *Stem Cells*. 2011; 29:1942–1951. [PubMed: 21957016]
- Schäfer MK, Frotscher M. Role of L1CAM for axon sprouting and branching. *Cell Tissue Res*. 2012; 349:39–48. [PubMed: 22370595]
- Siesser PF, Maness PF. L1 cell adhesion molecules as regulators of tumor cell invasiveness. *Cell Adh Migr*. 2009; 3:275–277. [PubMed: 19483471]
- Silvestre DC, Pineda JR, Hoffschir F, Studler JM, Mouthon MA, Pflumio F, Junier MP, Chneiweiss H, Boussin FD. Alternative lengthening of telomeres in human glioma stem cells. *Stem Cells*. 2011; 29:440–451. [PubMed: 21425407]
- Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB. Identification of a cancer stem cell in human brain tumors. *Cancer Res*. 2003; 63:5821–5828. [PubMed: 14522905]
- Soldati C, Bithell A, Johnston C, Wong KY, Teng SW, Beglopoulos V, Stanton LW, Buckley NJ. Repressor element 1 silencing transcription factor couples loss of pluripotency with neural induction and neural differentiation. *Stem Cells*. 2012; 30:425–434. [PubMed: 22162260]
- Srivenugopal KS, Shou J, Mullapudi SR, Lang FF Jr, Rao JS, Ali-Osman F. Enforced expression of wild-type p53 curtails the transcription of the O(6)-methylguanine-DNA methyltransferase gene in human tumor cells and enhances their sensitivity to alkylating agents. *Clin Cancer Res*. 2001; 7:1398–1409. [PubMed: 11350911]
- Stagno D'Alcontres M, Mendez-Bermudez A, Foxon JL, Royle NJ, Salomoni P. Lack of TRF2 in ALT cells causes PML-dependent p53 activation and loss of telomeric DNA. *J Cell Biol*. 2007; 179:855–867. [PubMed: 18056407]
- Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO. European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005; 352:987–996. [PubMed: 15758009]
- Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtari K, Wesseling P, Villa S, Eisenhauer E, Gorlia T, Weller M, Lacombe D, Cairncross JG, Mirimanoff RO. European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups; National Cancer Institute of Canada Clinical Trials Group. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol*. 2009; 10:459–466. [PubMed: 19269895]
- Sun YM, Greenway DJ, Johnson R, Street M, Belyaev ND, Deuchars J, Bee T, Wilde S, Buckley NJ. Distinct profiles of REST interactions with its target genes at different stages of neuronal development. *Mol Biol Cell*. 2005; 16:5630–5638. [PubMed: 16195345]
- Suzuki T, Izumoto S, Fujimoto Y, Maruno M, Ito Y, Yoshimine T. Clinicopathological study of cellular proliferation and invasion in gliomatosis cerebri: important role of neural cell adhesion molecule L1 in tumour invasion. *J Clin Pathol*. 2005; 58:166–171. [PubMed: 15677537]
- Tamura K, Aoyagi M, Wakimoto H, Ando N, Nariai T, Yamamoto M, Ohno K. Accumulation of CD133-positive glioma cells after high-dose irradiation by Gamma Knife surgery plus external beam radiation. *J Neurosurg*. 2010; 113:310–318. [PubMed: 20205512]
- Thomas RP, Recht L, Nagpal S. Advances in the management of glioblastoma: the role of temozolomide and MGMT testing. *Clin Pharmacol*. 2013; 5:1–9. [PubMed: 23293540]
- Wagoner MP, Roopra A. A REST derived gene signature stratifies glioblastomas into chemotherapy resistant and responsive disease. *BMC Genomics*. 2012; 13:686. [Epub ahead of print]. [PubMed: 23216891]
- Wakimoto H, Kesari S, Farrell CJ, Curry WT Jr, Zaupa C, Aghi M, Kuroda T, Stemmer-Rachamimov A, Shah K, Liu TC, Jeyaretna DS, Debasitis J, Pruszk J, Martuza RL, Rabkin SD. Human glioblastoma-derived cancer stem cells: establishment of invasive glioma models and treatment

- with oncolytic herpes simplex virus vectors. *Cancer Res.* 2009; 69:3472–3481. [PubMed: 19351838]
- Wang J, Wakeman TP, Lathia JD, Hjelmeland AB, Wang XF, White RR, Rich JN, Sullenger BA. Notch promotes radioresistance of glioma stem cells. *Stem Cells.* 2010; 28:17–28. [PubMed: 19921751]
- Xue H, Wu S, Papadeas ST, Spusta S, Swistowska AM, MacArthur CC, Mattson MP, Maragakis NJ, Capecchi MR, Rao MS, Zeng X, Liu Y. A targeted neuroglial reporter line generated by homologous recombination in human embryonic stem cells. *Stem Cells.* 2009; 27:1836–1846. [PubMed: 19544414]
- Yan X, Ma L, Yi D, Yoon JG, Diercks A, Foltz G, Price ND, Hood LE, Tian Q. A CD133-related gene expression signature identifies an aggressive glioblastoma subtype with excessive mutations. *Proc Natl Acad Sci USA.* 2011; 108:1591–1596. [PubMed: 21220328]
- Zhang P, Pazin MJ, Schwartz CM, Becker KG, Wersto RP, Dilley CM, Mattson MP. Nontelomeric TRF2-REST interaction modulates neuronal gene silencing and fate of tumor and stem cells. *Curr Biol.* 2008; 18:1489–1494. [PubMed: 18818083]
- Zheng H, Ying H, Yan H, Kimmelman AC, Hiller DJ, Chen AJ, Perry SR, Tonon G, Chu GC, Ding Z, Stommel JM, Dunn KL, Wiedemeyer R, You MJ, Brennan C, Wang YA, Ligon KL, Wong WH, Chin L, DePinho RA. p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. *Nature.* 2008; 455:1129–1133. [PubMed: 18948956]

Main Points

1. Glioblastoma stem cells (GSCs) isolated from surgical specimens express TRF2 and REST.
2. RNA interference-mediated reduction in TRF2 depletes REST and inhibits GSC proliferation.
3. TRF2 knockdown sensitizes GSCs to temozolomide-induced death.
4. Targeted depletion of TRF2 increases the survival of mice bearing GSC xenografts.

Figure 1. TRF2 depletion induces cell cycle arrest of GSCs

(A, B) T4302 and T3691 GSCs were infected by lentivirus directing expression of either non-targeting shRNA (NT) or TRF2 specific shRNAs (sh1 and sh2), and were selected with 1 μ g/ml puromycin for 3 days. Then cells were fixed in 70% ethanol and labeled with 10 mg/ml PI. Cell cycle distribution was determined by flow cytometry. (A) Data were generated from three independent experiments. * $p < 0.05$ with one-way ANOVA comparison of the control groups to the corresponding TRF2 knockdown groups. (B) Representative FACS plots are displayed.

Figure 2. Loss of TRF2 does not induce apoptosis in GSCs

T4302 and T3691 GSCs were infected and selected as described. Cells were trypsinized and quantified for apoptosis using the Annexin V-FITC Apoptosis Detection kit (Oncogene). (A) The percentage of FITC positive cells was determined by FACS analysis, and dead cells were excluded by propidium iodide (PI) staining. Each data point represents mean $\pm$ SD of three independent experiments. (B) Relative fluorescence in GSCs double-stained with Annexin V-FITC and PI. In each panel, viable Annexin V⁻/PI⁻ cells are in the lower-left region. The Annexin V⁺/PI⁺ fraction (upper right) consisted of apoptotic cells, whereas a small fraction of Annexin V⁺/PI⁻ cells (lower-right) was in early apoptosis. Necrotic cells were Annexin V⁻/PI⁺ (upper-left).

Figure 3. Loss of TRF2 impairs tumorsphere formation by glioblastoma stem cells

GSCs (spheres culture, 12 hours after passage) were infected with the non-targeting control lentivirus (NT) or lentiviruses directing expression of a TRF2 shRNA (sh1, sh2), and were selected with 1 μ g/ml puromycin for 3 days. One hundred GSC cells of each group were then seeded per well in 96-well tissue culture plates in the presence of 1 μ g/ml puromycin. Eight wells were plated for each group. The numbers of tumorspheres in each well were determined 8 days later. * $p < 0.001$ (one-way ANOVA comparison of the control groups to the corresponding TRF2 knockdown groups). (A) Images of neurospheres formed by T4302 cells expressing non-targeting shRNA or TRF2-specific shRNA (sh1, sh2). (B, C) Results of quantification of neurosphere number (B) and average diameter (C). Similar results were demonstrated in T3691 cells. UN: uninfected control.

Figure 4. Depletion of TRF2 inhibits growth of glioblastoma stem cells

GSC isolated from glioblastoma tumors from two different patients (A; T4302 and (B; T3691) were isolated and infected as described. Cells were then plated in 96-well plates in triplicate at 5000 cells per well. Total viable cell numbers were then determined by the Promega Cell Proliferation Assay-MTS assay daily. Data are mean $\pm$ SD from 3 independent experiments. * $p < 0.05$; # $p < 0.001$ with one-way ANOVA comparison of the control groups to the corresponding TRF2 knockdown groups on the same day. As demonstrated by growth curve assays, the total number of GSCs increased roughly six or seven fold over 5 days, whereas these cells depleted of TRF2 increased two or three fold in number over five days.

Figure 5. TRF2 knockdown reduced the proliferation of glioblastoma stem cells

(A) Images of dissociated adherent GSCs in cultures which had been infected by lentivirus and selected by 1 μ g/ml puromycin. The GSCs were then incubated for 2 hours in the presence of BrdU (10 μ M), fixed, and immunostained with antibodies against BrdU (green) and PI (red). Bar=40 μ m. (B) BrdU-positive cells were counted using a fluorescence microscope and expressed as a percentage of total number of cells determined by PI nuclear staining. The BrdU-labeling index of GSCs was significantly reduced from 50% in the

control group to 20% in the TRF2 shRNA group. Each data point represents mean $\pm$ SD of 3 independent experiments. *P<0.01 (one-way ANOVA).

Figure 6. Loss of TRF2 results in degradation of REST and derepression of REST target genes (A) The protein levels of TRF2, Olig2, REST, L1CAM, β-tubulin3 and p53 of each group were determined by immunoblotting. Interfering TRF2 expression with two different shRNAs (sh1 and sh2) results in a decrease in REST protein levels and an increase in REST target genes, L1CAM and β-tubulin3. The GSCs marker Olig2 is decreased and DNA damage marker p53 is increased. (B, C, D, E, F, G) Densitometric analysis of the immunoblots. Ratio for UN (uninfected control) was arbitrarily presented as 1. Data are

mean $\pm$ SD of the ratios from 3 independent experiments. * $p < 0.05$ compared to the control value.

Figure 7. Glioblastoma stem cells are more sensitive to temozolomide following TRF2 knockdown

(A) T4302 and (B) T3691 cells were infected with non-targeting control lentivirus or lentivirus expressing TRF2 shRNA (sh1, sh2). Following puromycin selection, 1×10^4 cells of each group were plated in a 96-well plate and treated with various concentrations of temozolomide for 48 hours. Total viable cell numbers were then determined by the Cell Counting Kit-8 (CCK-8). Each data point represents mean $\pm$ SD of 3 independent experiments. * $P < 0.01$ (one-way ANOVA).

Figure 8. TRF2 knockdown inhibits xenograft tumor formation by glioblastoma stem cells
 T4302 and T3691 cells were infected with non-targeting control lentivirus or lentivirus expressing TRF2 shRNA (sh1, sh2). Following puromycin selection, 5000 cells of each group were intracranially transplanted into the right frontal lobe of immunocompromised mice. Kaplan-Meier survival curves demonstrate increased survival when TRF2 is targeted with shRNA in comparison to a non-targeting control (A: T4302 and B: T3691) (n = 6 mice per group). *p<0.05 (log-rank analysis of survival curves).