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Inhibition of Y-box binding protein-1 slows the growth of glioblastoma multiforme and sensitizes to temozolomide independent O^6-methylguanine-DNA methyltransferase

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Abstract

Glioblastoma multiforme (GBM) is an aggressive type of brain tumor where <3% of newly diagnosed cases in the patients will survive >5 years. In adults, GBM is the most common type of brain tumor. It is rarer in children, where it constitutes ~15% of all brain tumors diagnosed. These tumors are often invasive, making surgical resection difficult. Further, they can be refractory to current therapies such as temozolomide. The current dogma is that temozolomide resistance rests on the expression of O^6-methylguanine-DNA methyltransferase (MGMT) because it cleaves methylated DNA adducts formed by the drug. Our laboratory recently reported that another drug resistance gene known as the Y-box binding protein-1 (YB-1) is highly expressed in primary GBM but not in normal brain tissues based on the evaluation of primary tumors. We therefore questioned whether GBM depend on YB-1 for growth and/or response to temozolomide. Herein, we report that YB-1 inhibition reduced tumor cell invasion and growth in monolayer as well as in soft agar. Moreover, blocking this protein ultimately delayed tumor onset in mice. Importantly, inhibiting YB-1 enhanced temozolomide sensitivity in a manner that was independent of MGMT in models of adult and pediatric GBM. In conclusion, inhibiting YB-1 may be a novel way to improve the treatment of GBM. [Mol Cancer Ther 2009;8(12):3276–84]

Introduction

Glioblastoma multiformes (GBM) are WHO grade 4 astrocytomas that are very aggressive types of brain tumors associated with poor prognosis despite recent therapeutic advancement. This is the case for patients diagnosed with GBM whether they are children or adults. Pediatric GBM (pediatric grade 4 astrocytoma) are rare and deadly brain tumors that account for 8% to 12% of all pediatric brain tumors and are the third leading cause of death in children ages <16 years (1). The 3-year survival of pediatric GBM patients is <20% (2). Current treatment for pediatric GBM is surgical resection followed by radiation therapy and chemotherapy. The most potent antitumor agent against adult and pediatric glioblastomas thus far is temozolomide, an orally administered DNA-methylating agent. Patients receiving temozolomide treatment show enhanced response rates; for example, those on temozolomide treatment have a 10- to 12-month survival rate compared with 4 months without such treatment (3). Temozolomide in combination with radiation also increases survival, but again this is only by a few months rather than years (4). Perhaps there is the potential to enhance the therapeutic benefits of temozolomide by a combinatory drug regimen including molecular targeted therapy to ultimately improve cure rates.

We recently reported that the Y-box binding protein-1 (YB-1), an oncogenic transcription/translation factor, is highly expressed in primary pediatric glioblastoma (2), yet its functional role in these tumors has not been described. YB-1 controls the oncogenome by shuttling between cytoplasm and nucleus. In the cytoplasm, YB-1 functions as a translation factor. On phosphorylation by AKT (5) or RSK (6) at Ser^{102} located in the DNA/RNA-binding domain, YB-1 translocates into nucleus where it regulates transcription (5) by binding to Y-box (sequence motif CTGATTGG) in the promoter regions of growth-promoting genes such as *Her-2* and *EGFR* (7). YB-1 has also been implicated in

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drug resistance by increasing the expression of multidrug resistance gene (mdr1), multidrug resistance–related protein-1 (mrp1), and major vault protein (mvp; ref. 8). Much of what is known about YB-1 was revealed through studies of adult cancers; however, very little is known about the functional role of YB-1 in pediatric malignancies. It has recently been shown that the expression of nuclear YB-1 in glioma models can be used to increase cell killing when cells are exposed to cytotoxic viruses in combination with the chemotherapeutic agent irinotecan or radiation (9, 10). This is because YB-1 facilitates viral replication and therefore can be used for gene therapy approaches. YB-1, also called nuclease-sensitive element binding protein 1 (NSEP1), is among the top genes that are differentially expressed in pediatric glioblastoma, as well as in adult glioblastoma, when compared with control brain tissues (11, 12). YB-1 is detectable in 100% (14 of 14 cases) of primary pediatric glioblastoma tumor tissues based on quantitative reverse transcription-PCR, whereas at the protein level it is detectable in 81% (26 of 32 cases) of tumors at high levels (2, 11). Thus, YB-1 is a fairly common molecular marker in both primary pediatric GBM and adult GBM.

We sought to characterize the role of YB-1 in mediating GBM growth, invasion, and tumorigenesis. Furthermore, we investigated the possibility that inhibiting YB-1 may sensitize pediatric GBM and/or adult GBM to temozolomide.

Material and Methods

Cell Culture

The SF188 and SJG2 pediatric glioblastoma cell lines as well as U251 adult glioblastoma cells were obtained from Dr. Nada Jabado (University of McGill). T98G cells (adult glioblastoma) were purchased from the American Tissue Culture Collection. The cell lines were cultured in MEM/Earle's BSS (Hyclone) and DMEM/high glucose (Hyclone), respectively, supplemented with 10% fetal bovine serum (Invitrogen) and cultured at 37°C in a 5% CO_2 incubator.

Immunocytochemistry for YB-1 and pYB-1^{S102}

SF188 and U251 cells (1.0×10^5) were seeded on glass coverslips, washed with PBS, fixed with 2% formaldehyde for 20 min, rinsed twice with PBS, and then incubated with PBS containing 0.1% Triton X-100 (Sigma) for 30 min. Next, the coverslips were washed with PBS and incubated with rabbit anti–YB-1 or anti–pYB-1^{S102} (Cell Signaling Technology) antibody diluted in buffer containing 10% bovine serum albumin and 2% goat serum for 1 h at room temperature in a humidified container. After washing three times with PBS, glass slides were incubated with Alexa 488 anti-rabbit antibody (Invitrogen, Molecular Probes) for 1 h, washed three times, and then mounted using Vectashield adhesive medium (Vector Laboratories). 4′,6-diamidino-2-phenylindole was used for nuclear staining. Cells were observed by Olympus BX61 fluorescent microscope and photographed using DP71 digital camera.

Short Hairpin RNA and Small Interfering RNA Transfections to Silence YB-1

SF188 wild-type cells were plated in 6-well culture plates (3×10^5 per well) 24 h before transfection. Cells were transfected with either 10 μg shYB-1 or the empty vector and selected in G418 until stable pooled clones were established. This method was used to establish stable shYB-1–expressing cell lines from the SF188 and U251 parental cells. Loss of YB-1 expression was confirmed to be >90% by immunoblotting and quantitative reverse transcription-PCR. For the transient inhibition of YB-1, small interfering RNA (siRNA) was used. In this case, 10 nmol/L control (oligonucleotide sequence UUCUCCGAACGUGUCACGU; Qiagen) or YB-1 siRNA (oligonucleotide sequence CCACGCAAUUACCAGCAAA; Dharmacon) was introduced into the SF188 cells using RNAiMAX (Invitrogen). Following transient transfection, levels of YB-1 were evaluated by immunoblotting and tumor cell growth was assessed after 8 days using MTS assay (Promega) for SF188 cells. Similarly, T98G cells were treated with YB-1 siRNA (5 nmol/L) as described above and tumor cell growth was assessed 1, 2, and 3 days after transfection using MTS assay (Promega).

Immunoblotting

Harvested cells were washed with cold PBS and pelleted by centrifugation at 3,000 rpm for 3 min. After the supernatant was aspirated, the pellets were resuspended in 4-packed-cell volumes of egg lysis buffer and incubated on ice for 20 to 30 min. The protein extracts (10-50 μg) were mixed with 5× sample loading buffer, boiled for 5 min, and detected by immunoblotting using the following antibodies: anti–YB-1 (1:1,500; Abcam), anti–phosphorylated histone 2AX (pH2AX^{S139}; 1:1,000; Abcam), anti–O^6-methylguanine-DNA methyltransferase (MGMT; 1:200; Abcam), anti–pYB-1^{S102} (1:1,000; Cell Signaling Technology), and anti-vinculin (1:1,000; Sigma).

Invasion Assay

Cellular invasion was evaluated by quantifying the number of cells that migrated through a polyethylene terephthalate membrane coated with a layer of Matrigel (13). Briefly, tumor cells (1×10^5 in 100 μL warmed 0.1% fetal bovine serum MEM/Earle's BSS) were seeded on the top of Transwell inserts containing 40 μL diluted Matrigel (Matrigel/serum-free medium = 1:8). The bottom chamber was filled with 600 μL MEM/Earle's BSS, supplemented with 10% fetal bovine serum, as chemoattractants. Cells were cultured for 16 to 27 h at 37°C in a 5% CO_2 incubator. The cells that did not invade were subsequently scraped off the top chamber and the membranes were fixed with 500 μL ice-cold methanol (100%) for 5 min at room temperature. Cells were visualized by staining with Hoechst (0.5 μg/mL in PBS) for 5 min. Following 3 × 5 min PBS washes, membranes were placed on the glass slides with gelvatol on the surface. The cells that had migrated through the membranes were quantified in three randomly chosen visual fields under the microscope.

Soft-Agar Assays

MEM/Earle's BSS medium (2×; Hyclone) was mixed with 1.2% agarose solution in 1:1 ratio. The mixture

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was added into 6-well plates with 1 mL in each well to create the bottom layer. The top layer was made by adding SF188 cells (5×10^4 per well) into a 1:1 mixture of 2× medium and a 0.6% agarose solution. For drug treatment study, the cell layer was composed of tumor cells, 1:1 mixture of 2× medium and 1.2% agarose, and varying concentrations of temozolomide (British Columbia Cancer Agency). The assays were incubated at 37°C in a 5% CO_2 incubator for 28 days. The size and number of colonies were measured under a microscope (Leica DMIL). The experiment was done in triplicate on three different occasions.

Effect of Silencing YB-1 in Xenograft Model

SF188 cells have been shown to be tumorigenic in rats (14); however, it has not been tested in mice. We conducted a pilot study to determine the conditions required for tumor formation in mice. The SF188 control cells were injected s.c. into the lower hind flank of female 6- to 8-week-old BALB/c *nu/nu* mice ($n = 12$). The weight of the mice and the size of the tumors were measured for 30 days and the tumors were harvested at the end of the study. Following this pilot study, SF188 control and shYB-1 cells were injected bilaterally into the lower hind flank of female *nu/nu* mice (left, SF188 control; right, SF188 shYB-1; $n = 8$). The weight of the mice and the size of the tumor on each side were measured using calipers for 28 days. In detail, cells were washed with HBSS (Invitrogen) twice and counted to prepare 1×10^6 cells, which were subsequently mixed with Matrigel (BD Bioscience) in a 1:1 ratio with a total volume of 150 μL. The tumor cells/Matrigel was injected s.c. into the left or right lower hind region of female *nu/nu* mice using a 26-gauge needle. The weight of the mice and the size of the tumor on each side were measured using calipers for 28 days. Mice were terminated with CO_2. Tumors were frozen in liquid nitrogen at the time of harvest. Tumor volumes were calculated from the equation: $(4/3)\pi(a/2) \times (b/2) \times (c/2)$, where

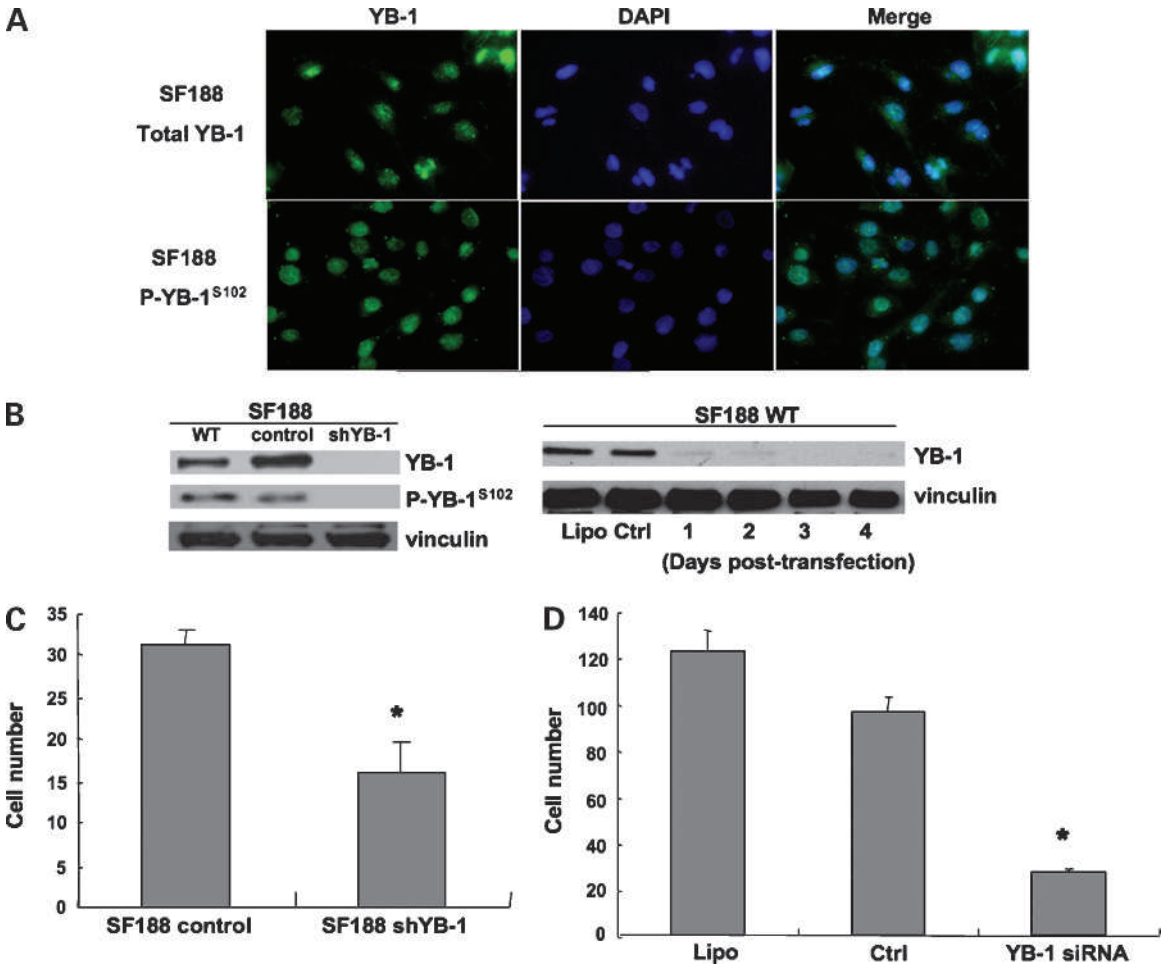

Figure 1. Cellular invasion through Matrigel was inhibited by transient and stable YB-1 knockdown. **A,** YB-1 and pYB-1^{S102} proteins were highly expressed in the nucleus of SF188 wild-type (*WT*) cells. **B,** levels of YB-1 and pYB-1^{S102} were confirmed by immunoblotting. Silencing YB-1 with either shRNA (*right*) or siRNA (*left*) decreased its expression by >90%. Vinculin was measured as a loading control. **C,** SF188 control and shYB-1 cells were assessed using the Matrigel invasion assay. **D,** changes in invasion were confirmed using transient inhibition of YB-1 with siRNA. The cells were treated with siYB-1 for 24 h, and on the following day, the cells were placed into the Matrigel invasion assay. The transient loss of YB-1 caused a marked suppression of invasion. Representative images of invasive cells. *Lipo,* Lipofectamine-treated cells; *Ctrl,* control siRNA-treated cells.

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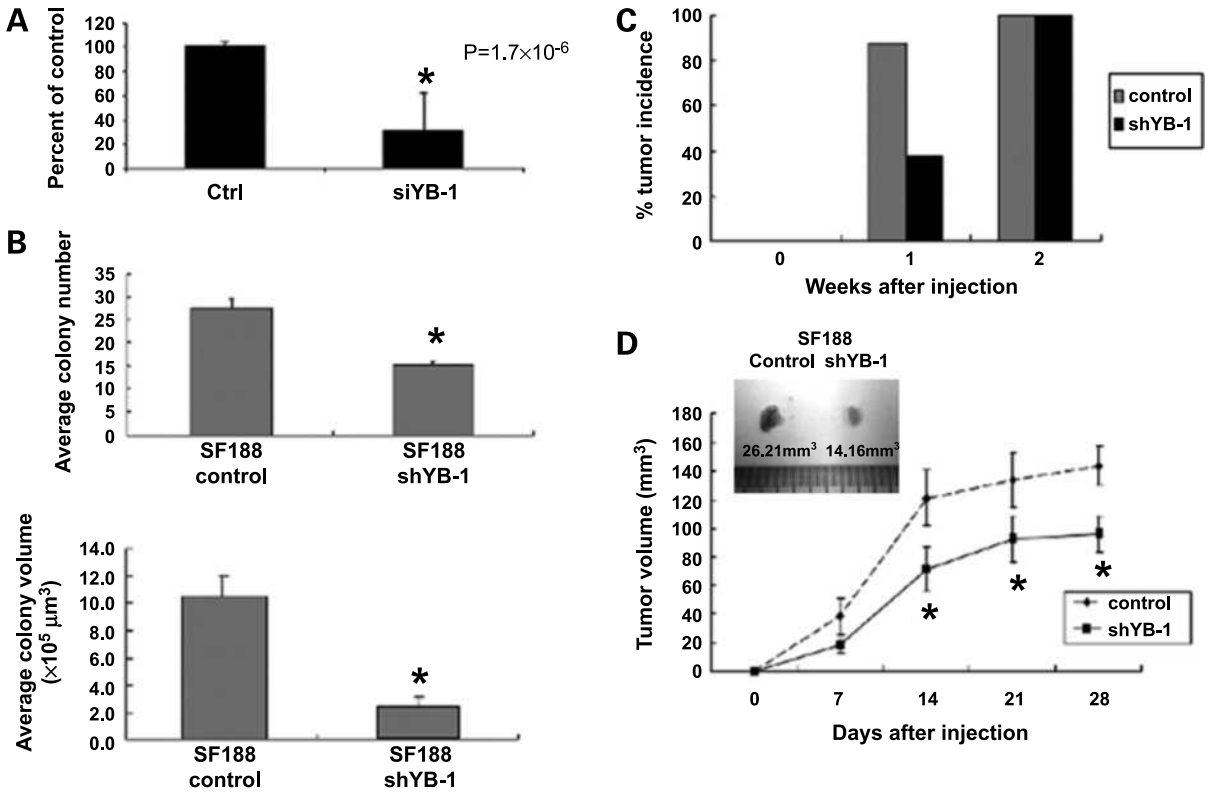

Figure 2. Knockdown of YB-1 reduced tumor cell growth and delayed tumor formation. **A,** cell growth was also assessed in monolayer following YB-1 inhibition using siRNA. The growth of SF188 cells was inhibited by ~70% after 8 d in culture using a MTS assay. Each treatment group was evaluated in replicates of six. **B,** SF188 control and shYB-1 cells were plated in soft agar for 28 d. Colonies ≥70 μm were counted under the microscope. Loss of YB-1 decreased the average number difference of the colonies formed compared with the control. The size of the colonies was also significantly attenuated following loss of YB-1. **C** and **D,** SF188 control and shYB-1 cells were bilaterally injected into the hind flank of female *nu/nu* mice. Tumor incidence was monitored and 83% of the injection sites containing control SF188 cells developed tumors, whereas only 40% of those with shYB-1–containing cells developed lesions after 1 wk. By the second week, all of the mice developed tumors; however, there were significant differences in the tumor volume between the groups (those with shYB-1 were smaller) throughout the time course of 28 d. An example of these differences is illustrated where the control tumor was 26.21 mm^3, whereas the shYB-1–expressing tumor was only 14.16 mm^3. Differences in tumor size were determined using a Student's *t* test ($P < 0.05$).

a, *b*, and *c* were the width, length, and depth of the tumors, respectively. All studies were conducted in accordance with the University of British Columbia Animal Care and Use Guidelines. Differences in tumor size was determined using a Student's *t* test where significance was determined if $P < 0.05$.

Apoptosis Assays

SF188 control and shYB-1 cells were treated with various concentrations of Taxol or temozolomide and incubated for 24 h before being stained with Annexin V (Promega). Staining was done according to the manufacturer's protocol as described previously by us (15). In brief, the harvested cells were washed once with cold PBS followed by 15-min incubation with 1× binding buffer containing Annexin V and 7AAD. Then, 250 μL of 1× binding buffer were added into each sample and flow cytometry analysis was done within 1 h. The experiment was repeated on three separate occasions. Following this, we confirmed that the cells were undergoing apoptosis by evaluating changes in chromatin condensation. SF188 control and shYB-1 cells (3,000 per well) were seeded in a 96-well plate and treated with temozolomide or Taxol with various concentrations for 24 h. After the medium was aspirated, 100 μL PBS containing 2% paraformaldehyde and Hoechst dye (1 μg/mL) was added to each well and the cells were kept at room temperature for 20 min. The plates were analyzed and the images were taken on the ArrayScan VTI Reader (Cellomics).

Analysis of YB-1 Expression in Adult Glioblastoma

Gene Expression Omnibus[6] data were mined to examine YB-1 expression in adult GBM (16). GSE4290 provided the largest data set for a comparison containing 23 samples from epilepsy patients used as nontumor samples and 81 grade 4 adult GBM samples (17). Expression and clustering analyses were done as described previously (12). We used probe sets described previously as segregating nontumor from tumor samples based on known functions in general and alternative splicing, RNA export, RNA degradation, miRNA processing, and nonsense-mediated decay (12). Hierarchical clustering was done with the EBI Profiler tool

[6] www.ncbi.nlm.nih.gov/geo

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using a Euclidean distance measure with the complete linkage algorithm.[7]

Results and Discussion

Characterization of YB-1 in the Pediatric Glioblastoma Cell Line SF188

We characterized the SF188 pediatric glioblastoma cell line to show that it expresses high levels of YB-1 and pYB-1^{S102}, where it is primarily localized to the nucleus (Fig. 1A). It therefore served as a model to silence YB-1 stably and subsequently to study the ramification(s) on tumor growth and sensitivity to chemotherapy. By stably expressing a shYB-1 plasmid, the level of YB-1 inhibition was confirmed to be reduced by >80% (Fig. 1B, *left*). Further, YB-1 was transiently silenced using siRNA and a 4-day time course was conducted. YB-1 was reduced by ~100% one day after transfection and this prominent knockdown was sustainable (Fig. 1B, *right*). Thus, levels of YB-1 were manipulated by either stable or transient inhibition using siRNA for the studies that followed. We chose to use both short hairpin RNA (shRNA) and siRNA approaches with two different targeting sequences against YB-1 to validate our findings. In addition, concerns may arise with the shRNA approach given that after long-term culturing following the loss of YB-1 adaptive bypass mechanisms may develop, which could have introduced artifacts into the models.

Inhibition of YB-1 Suppresses the Tumorigenic Potential of SF188 Cell *In vitro* and *In vivo*

To understand how YB-1 may contribute to pediatric glioblastoma progression, YB-1 was silenced with either shRNA or siRNA and cellular invasion was examined 24 h later. Invasion was reduced by ~50% to 70% whether YB-1 was silenced stably or transiently compared with the control cells (Fig. 1C and D). Together, these results strongly suggest that attenuated YB-1 expression significantly inhibited SF188 cell invasiveness. These data are consistent with reports indicating that YB-1 regulates invasion proteases such as matrix metalloproteinase-2 (18). On further examination, the loss of YB-1 expression significantly inhibited tumor cell growth in monolayer after 8 days (Fig. 2A). Tumor cell growth was decreased by >70% when YB-1 was silenced compared with the cells treated with the scrambled control. Similarly, loss of YB-1 significantly reduced the number and size of colonies that formed in soft agar (Fig. 2B, *top* and *bottom*, respectively). In this model of pediatric glioblastoma, it appeared that YB-1 is needed for optimal tumor cell invasion and growth.

Collectively, these data supported the idea that inhibiting YB-1 may delay tumor growth in mice. Yet, the SF188 model had not been characterized in mice; therefore, a pilot study was conducted. SF188 control cells (1×10^6 per site) were injected into the lower right hind flank of female *nu/nu* mice ($n = 12$ animals). By the first week, 90% of the mice developed palpable tumors. By the end of the second week, all of the mice developed tumors. These data indicated that the SF188 cells were highly aggressive given the short amount of time needed for tumor development. At the end of this 30-day study, all of the mice developed tumors and spontaneous regression was never observed (Supplementary Fig. S1). Once the tumors were collected at the termination of the study, we noted that they were fairly well vascularized and the average tumor size was 27 mm^3 (Supplementary Fig. S1). Next, SF188 control and shYB-1 cells (1×10^6 per site) were bilaterally injected into the hind flank of female *nu/nu* mice. One week after injection, 83% of the mice developed tumors on the side where SF188 control cells were injected, indicating the reproducibility of tumor formation in this model compared with the wild-type SF188 cells described above. In contrast, only 40% of the mice had palpable tumors on another side where shYB-1 cells were injected (Fig. 2C). By the

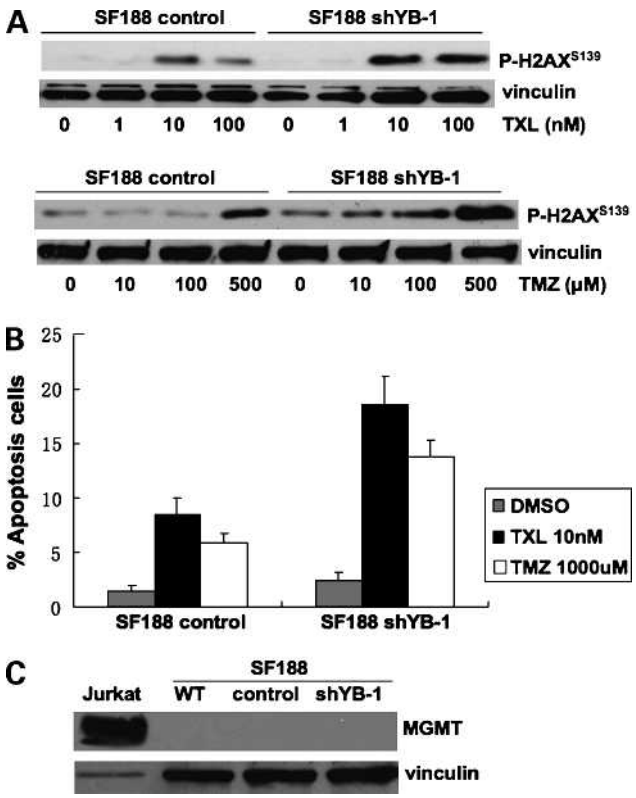

Figure 3. Induction of apoptosis and inhibition of colony formation in soft agar after Taxol and temozolomide treatment. **A,** inhibition of YB-1 sensitized SF188 cells to Taxol (*TXL*; 10 or 100 μmol/L) and temozolomide (*TMZ*; 10, 100, and 500 μmol/L) based on the induction of pH2AX^{S139}. The cells were treated with the agents for 24 h and pH2AX^{S139} was evaluated by immunoblotting where vinculin was included as a sample loading control. **B,** when the cells were treated in the same manner, loss of YB-1 also increased DNA fragmentation based on chromatin condensation compared with the controls when the cells were exposed to Taxol or temozolomide. **C,** sensitization of SF188 cells to temozolomide was independent of MGMT given that these cells do not express this enzyme based on immunoblotting. Jurkat cells were included as a positive control.

[7] www.ebi.ac.uk/expressionprofiler

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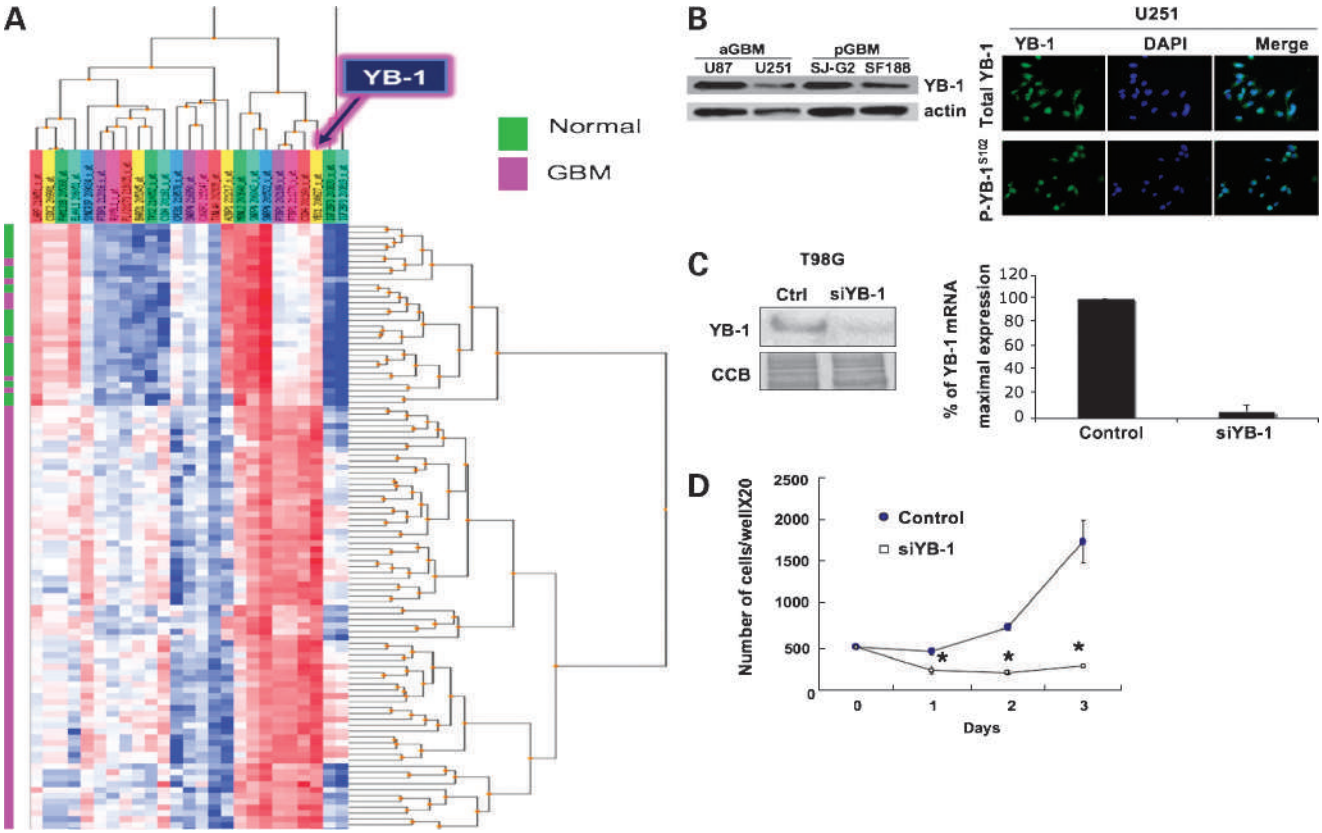

Figure 4. Inhibition of YB-1 in adult glioblastoma increases sensitivity to temozolomide. **A,** hierarchical cluster analysis revealed elevated YB-1 expression association with adult glioblastoma. Shown is the clustering analysis for 21 nontumor samples (*NT*) and 81 adult glioblastoma samples (*GB*). The expression levels are indicated by a gradient from low (*blue*) to high (*red*). **B,** YB-1 protein is also readily detectable in the adult glioblastoma (*aGBM*) cell lines U251 and U87 as well as in the pediatric glioblastoma (*pGBM*) cell lines SJG2 and SF188. Based *in situ* immunofluorescence, YB-1 and pYB-1^{S102} are located in the nucleus of U251. **C,** expression of YB-1 was also found in the adult glioblastoma cell line T98G; therefore, it was silenced with siRNA resulting in >90% loss of protein expression. Levels of mRNA were similarly reduced. Sample loading for the immunoblot was verified using Coomassie blue staining (*CCB*) of the membrane. **D,** following the loss of YB-1, cell growth was assessed 1, 2, and 3 d later. The growth of T98G was significantly reduced at each time point $P < 0.001$.

second week, small tumors developed in this group; however, they grew much slower than the controls (Fig. 2D). The loss of YB-1 sustained growth suppression throughout the ensuing time course (Fig. 2D). Representative images of the tumors at the termination of the study illustrate differences in tumor size where the control was 26.1 mm^3 versus shYB-1–expressing tumor, which was only 14.16 mm^3 (Fig. 2D). Given the data presented here, where pediatric glioblastoma displays YB-1 dependency, we expect that it may also be a viable molecular target for brain tumors.

Inhibition of YB-1 Increased the Sensitivity of SF188 Cells to Temozolomide

Although we have shown that targeting YB-1 alone suppresses tumor growth in preclinical models, we realize that, in the clinical setting, inhibiting this target would likely involve combinations with current chemotherapies such as temozolomide. Initially, we characterized the SF188 cells for sensitivity to temozolomide and noted that it only inhibited growth in monolayer at relatively high doses (500 μmol/L; Supplementary Fig. S2A). Following this, SF188 cells were treated with 100, 500, or 1,000 μmol/L temozolomide for 24 h and apoptosis was assessed by flow cytometry using Annexin V. Overall, the cells were relatively insensitive to temozolomide, as it took 1,000 μmol/L to induce cell death and this was again quite marginal in that only 4% of the cell population underwent apoptosis (Supplementary Fig. S2B). Likewise, temozolomide failed to significantly inhibit SF188 growth in soft agar at doses up to 500 μmol/L (Supplementary Fig. S2C); therefore, we concluded that SF188 cells were not very sensitive to temozolomide. This is in keeping with a previous report indicating that the IC_{50} of temozolomide was 426 ± 216 μmol/L for SF188 cells where cell growth was assessed in monolayer for 96 h (14). We therefore surmised that high levels of YB-1 might play a role in recalcitrance to temozolomide due to its role in mediating pleiotrophic resistance in other cancer models. We questioned whether silencing YB-1 would render cancer cells more sensitive to temozolomide by evaluating apoptosis through the induction of pH2AX^{S139}. Taxol was used as a positive control for the induction of apoptosis (15). We noted a dramatic increase in the pH2AX^{S139} in SF188 shYB-1

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cells compared with the control cells following exposure to temozolomide (Fig. 3A). As an aside, inhibiting YB-1 also sensitized the cells to Taxol (Fig. 3A), which confirms our recent report in a model of breast cancer (15). Similar findings were observed when the cells were treated with Taxol or temozolomide and apoptosis was measured by Annexin V or chromatin condensation (Supplementary Fig. S3A-C; Fig. 3B). Previous studies indicate that temozolomide insensitivity can be attributed to high levels of the DNA repair enzyme MGMT (19, 20). This led us to address whether inhibiting YB-1 causes SF188 cells to become more sensitive to temozolomide because it decreased MGMT levels. Yet, to our surprise, the mechanism of temozolomide sensitization was not related to MGMT because SF188 cells do not express this protein (Fig. 3C). Therefore, we surmise that the high expression of YB-1 is attributing to temozolomide resistance.

Given these intriguing findings, we next addressed whether YB-1 is highly expressed in primary adult glioblastoma. Previous studies indicated the YB-1 was among the top differentially expressed RNA regulatory factors in a comparison of 10 primary adult GBM multiforme and 10 nontumor control brain tissue samples (12). To expand these studies, we performed a similar analysis using data mined from Gene Expression Omnibus where 21 normal and 81 adult GBM were evaluated. Interestingly, in this larger data set, YB-1 expression remained among the top 25 differentially expressed genes (data not shown) and its expression was consistently higher in the tumors compared with normal tissues (Fig. 4A). Expression of YB-1 in normal samples strongly segregated from the tumors. It was noteworthy that only 8 high-grade GBM fell within the node containing the normal samples (Fig. 4A). This indicated that 90% (73 of 81 tumors) of the tumors had distinguishably higher levels of YB-1 compared with the control group. In further support of this finding, the adult glioblastoma cell lines U87 and U251 both express YB-1 as do the pediatric cell lines SJG2 and SF188 (Fig. 4B). In the U251 cell line, YB-1 and pYB-1^{S102} were readily detectable and mainly found in the nucleus (Fig. 4B). The presence of YB-1 was also readily detectable in the adult GBM cell line T98G (Fig. 4C). Therefore, its expression was silenced using siRNA resulting in a 90% decrease in protein and mRNA levels (Fig. 4C). The effect on cell growth was assessed over a time course of 3 days where loss of YB-1 markedly suppressed tumor cell proliferation (Fig. 4D). To complement these studies, YB-1 was stably silenced in U251 cells using short hairpin RNA (Fig. 5A). This model was chosen given its relative insensitivity to temozolomide where the IC_{50} was ~600 μmol/L (21). The stable knockdown of YB-1 led to a ~90% reduction in its protein expression (Fig. 5A). Importantly, inhibiting YB-1 remarkably sensitized the U251 cells to temozolomide and Taxol (Fig. 5B). This was again independent of MGMT expression (Fig. 5C). Finally, we questioned whether YB-1 inhibition would have any effect in GBM cells that express MGMT. We inhibited YB-1 for 48 h and then exposed the cells to temozolomide for an additional 72 h. The loss of YB-1 further suppressed cell growth in the presence of temozolomide (Fig. 6A). This was not because YB-1 inhibited MGMT (Fig. 6B). This reinforces the idea that inhibiting YB-1 sensitizes brain tumor cells to temozolomide in a MGMT-independent manner.

At this time, we do not know how YB-1 is sensitizing GBM to apoptosis. One theory is that temozolomide induces DNA damage and that, when high levels of YB-1 are present, these defects may be repaired. There are indeed several examples in the literature attributing YB-1 to DNA repair (22–25). Thus, by inhibiting YB-1 in conjunction with temozolomide treatment, the DNA damage optimally triggers cell death (Fig. 6C). It is also possible that YB-1 may be regulating other methyltransferases

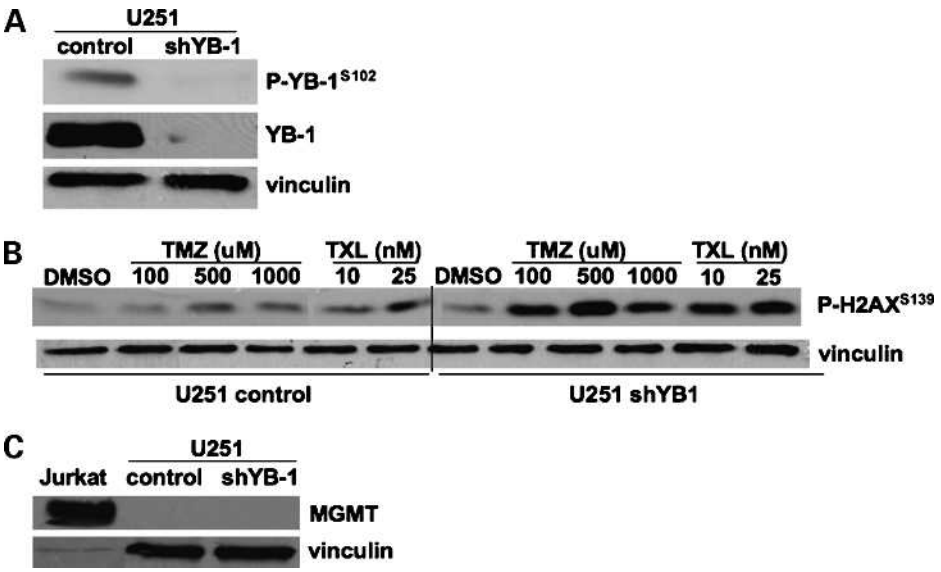

Figure 5. YB-1 inhibition in MGMT-negative adult glioblastoma cells. **A,** a stable shYB-1–expressing U251 cell line was developed where YB-1 and pYB-1^{S102} were decreased by at least 80%. **B,** inhibition of YB-1 sensitizes these cells to temozolomide and Taxol following exposure for 24 h. Apoptosis was monitored by pH2AX^{S139} and equal loading was accounted for using vinculin. **C,** U251 cells failed to express MGMT based on immunoblotting. Jurkat extracts were used as a positive control for MGMT.

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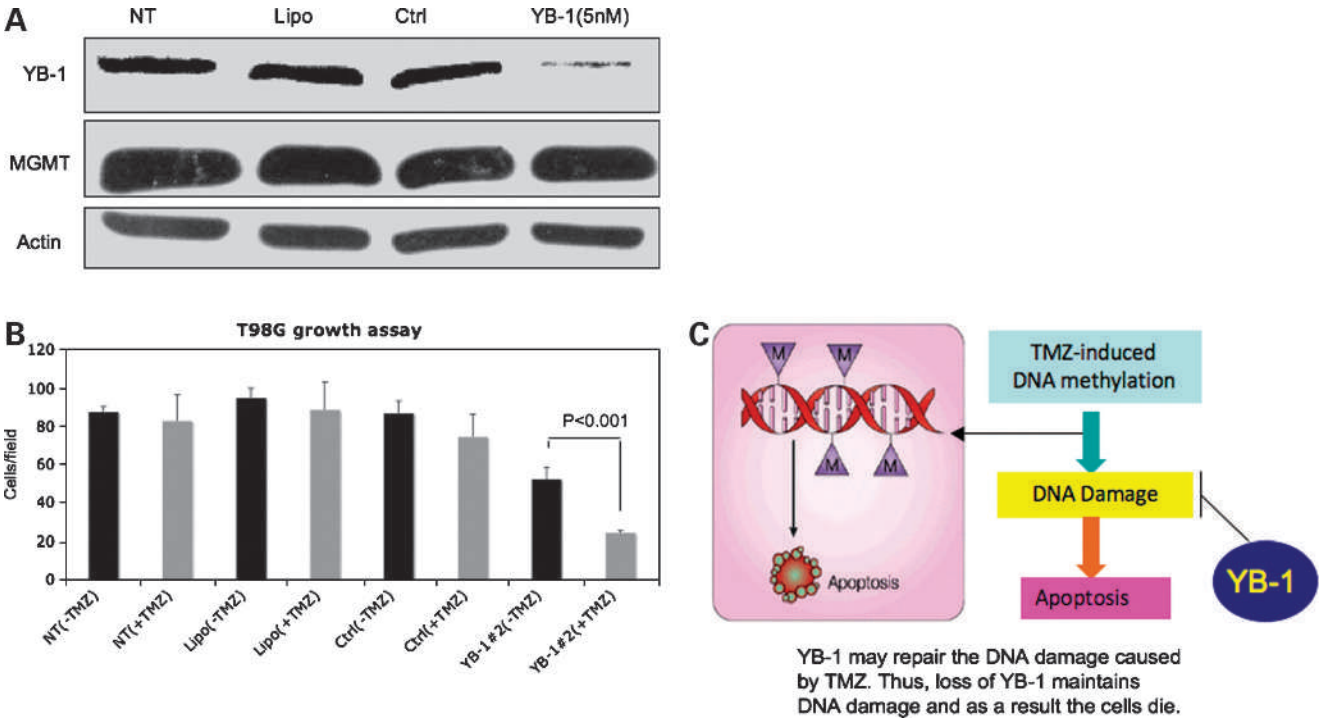

Figure 6. YB-1 inhibition in MGMT-positive adult glioblastoma cells and its interaction with MGMT pathway. **A,** to evaluate the effect of YB-1 downregulation on MGMT expression in MGMT-positive cells, T98G cells were transiently downregulated using YB-1 siRNA. YB-1 expression reduced >90% compared with negative control siRNA-transfected cells. Silencing YB-1 had no effect on MGMT expression. **B,** downregulation of YB-1 improved the effect of temozolomide (100 μmol/L) and significantly inhibited T98G cell growth. **C,** schematic model of antiproliferative effect of inhibiting YB-1 through its repair function on the DNA damage caused by temozolomide. The treatment of brain tumor cells with temozolomide causes DNA methylation, which under normal circumstances would trigger cell death. It is well documented that YB-1 has DNA repair activity; therefore, it is possible that this is why inhibiting this oncogene further sensitizes GBM cells to temozolomide. Alternatively, when GBM cells express MGMT, the DNA damage is reversible because this methyltransferase cleave the DNA adducts formed by temozolomide. Again, inhibition of YB-1 suppresses DNA repair and the cells are further sensitized to temozolomide. *NT,* nontreated.

that cleave adducts caused by temozolomide. This too awaits experimental verification.

YB-1 has been associated previously with drug resistance; examples include cisplatin (26, 27), paclitaxel (28), and 5-flurouracil (29). Thus, it is quite conceivable that cotargeting YB-1 with classic anticancer agents could improve patient care. For example, YB-1–expressing tumors can be used to allow for replication of cytotoxic viruses that enhance the effect of chemotherapy (9). In fact, YB-1 is shown to be involved in the replication of adenovirus type 5, a commonly used vector in gene therapy. Therefore, it is suggested that YB-1 can trigger an oncolytic effect in YB-1 nuclear-positive cancer cells treated with adenoviruses (30). In closing, we conclude that inhibiting YB-1 has the potential to improve the treatment of GBM that arise in children and adults.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Gottardo NG, Gajjar A. Chemotherapy for malignant brain tumors of childhood. J Child Neurol 2008;23:1149–59.

2. Faury D, Nantel A, Dunn SE, et al. Molecular profiling identifies prognostic subgroups of pediatric glioblastoma. J Clin Oncol 2007; 25:1196–208.

3. Friedmann HS, Kerby T, Calvert H. Temozolomide and treatment of malignant glioma. Clin Cancer Res 2000;6:2585–97.

4. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987–96.

5. Sutherland BW, Kucab JE, Wu J, et al. Akt phosphorylates the Y-box binding protein-1 at Ser^{102} located in the cold shock domain and affects the anchorage-independent growth of breast cancer cells. Oncogene 2005;24:4281–92.

6. Stratford AL, Fry CJ, Desilets C, et al. Y-box binding protein-1 serine 102 is a downstream target of p90 ribosomal S6 kinase in basal-like breast cancer cells. Breast Cancer Res 2008;10:1–12.

7. Wu J, Lee C, Yokom D, et al. Disruption of the Y-box binding protein-1 results in suppression of the epidermal growth factor receptor and HER-2. Cancer Res 2006;66:4872–9.

8. Wu J, Stratford AL, Astanehe A, Dunn SE. YB-1 is a transcription/translation factor that orchestrates the oncogenome by hardwiring signal transduction to gene expression. Transl Oncogenomics 2007;2: 1–17.

9. Mantwill K, Kohler-Vargas N, Bernshausen A, et al. Inhibition of the multidrug-resistant phenotype by targeting YB-1 with a conditionally oncolytic adenovirus: implications for combinatorial treatment regimen with chemotherapy agents. Cancer Res 2006;66:7195–202.

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10. Bieler A, Mantwill K, Holzmuller R, et al. Impact of radiation therapy on the oncolytic adenovirus dl520: implications on the treatment of glioblastoma. Radiother Oncol 2008;86:419–27.

11. Haque T, Faury D, Albrecht S, et al. Gene expression profiling from formalin-fixed paraffin-embedded tumors of pediatric glioblastoma. Clin Cancer Res 2007;13:6284–92.

12. Cheung HC, Baggerly KA, Tsavachidis S, et al. Global analysis of aberrant pre-mRNA splicing in glioblastoma using exon expression arrays. BMC Genomics 2008;9:1–16.

13. Dunn SE, Torres JV, Barrett JC. Up-regulation of urokinase type plasminogen activator by insulin-like growth factor-1 depends upon phosphatidyl inositol-3 kinase and Map kinase kinase. Cancer Res 2001;61:1367–74.

14. Ma J, Murphy M, O'Dwyer PJ, Berman E, Reed K, Gallo JM. Biochemical changes associated with a multidrug-resistant phenotype of a human glioma cell line with temozolomide-acquired resistance. Biochem Pharmacol 2002;63:1219–28.

15. Lee C, Dhillon J, Wang M, et al. Targeting YB-1 in Her-2 overexpressing breast cancer cells induces apoptosis via the mTOR/STAT3 pathway and suppresses tumor growth in mice. Cancer Res 2008;68:8661–6.

16. Barrett T, Troup DB, Wilhite SE, et al. NCBI GEO: mining tens of millions of expression profiles—database and tools update. Nucleic Acids Res 2007;35:760–5.

17. Sun L, Hui AM, Su Q, et al. Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain. Cancer Cell 2006;9:287–300.

18. Mertens PR, Alfonso-Jaum MA, Steinmann K, Lovett DH. A synergistic interaction of transcription factors AP2 and YB-1 regulates gelatinase A enhancer-dependent transcription. J Biol Chem 1998;273:32957–65.

19. Hegi ME, Diserens AC, Gorlie T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 2005;352:997–1003.

20. Donson AM, Addo-Yobo SO, Handler MH, Gore L, Foreman NK. MGMT promoter methylation correlates with survival benefit and sensitivity to pediatric glioblastoma. Pediatr Blood Cancer 2007;48:403–7.

21. Hermisson M, Klumpp A, Wick W, et al. O^6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human glioma cells. J Neurooncol 2006;96:766–76.

22. Das S, Chattopadhyay R, Bhakat KK, et al. Stimulation of NEIL2-mediated oxidized base excision repair via YB-1 interaction during oxidative stress. J Biol Chem 2007;282:28474–84.

23. de Souza-Pinto NC, Mason PA, Hashiguchi K, et al. Novel DNA mismatch-repair activity involving YB-1 in human mitochondria. DNA Repair 2009;8:704–19.

24. Kuwano M, Uchiumi T, Hayakawa H, et al. The basic and clinical implication of ABC transporters, Y-box protein-1 (Yb-1) and angiogenesis related factors in human malignancies. Cancer Sci 2003;94:9–14.

25. Gaudreault I, Guay D, Lebel M. YB-1 promotes strand separation *in vitro* of duplex DNA containing either mispaired bases or cisplatin modifications, exhibits endonucleolytic activities and binds several DNA repair proteins. Nucleic Acids Res 2004;32:316–27.

26. Ohga T, Uchiumi T, Makino Y, et al. Direct involvement of the Y-box binding protein YB-1 in genotoxic stress-induced activation of the human multidrug resistance gene-1. J Biol Chem 1998;273:5997–6000.

27. Ohga T, Koike K, Ono M, et al. Role of the human Y box-binding protein YB-1 in cellular sensitivity to the DNA-damaging agents cisplatin, mitomycin C, and ultraviolet light. Cancer Res 1996;56:4224–8.

28. Fujita T, Ito K, Izumi H, et al. Increased nuclear localization of transcription factor Y-box binding protein 1 accompanied by up-regulation of P-glycoprotein in breast cancer pretreated with paclitaxel. Clin Cancer Res 2005;11:8837–44.

29. Stein U, Bergmann S, Scheffer GL, et al. YB-1 facilitates basal and 5-fluorouracil-inducible expression of the human major vault protein (MVP) gene. Oncogene 2005;24:3606–18.

30. Lage H, Surowiak P, Holm PS. YB-1 as a potential target in cancer therapy. Pathologe 2008;29:187–90.

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