

## The Cell Cycle Inhibitors p21<sup>Cip1</sup> and p27<sup>Kip1</sup> Control Proliferation but Enhance DNA Damage Resistance of Glioma Stem Cells



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### Abstract

High-grade gliomas are the most prevalent and lethal primary brain tumors. They display a hierarchical arrangement with a population of self-renewing and highly tumorigenic cells called cancer stem cells. These cells are thought to be responsible for tumor recurrence, which make them main candidates for targeted therapies. Unbridled cell cycle progression may explain the selective sensitivity of some cancer cells to treatments. The members of the Cip/Kip family p21<sup>Cip1</sup> and p27<sup>Kip1</sup> were initially considered as tumor suppressors based on their ability to block proliferation. However, they are currently looked at as proteins with dual roles in cancer: one as tumor suppressor and the other as oncogene. Therefore, the aim of this study was to determine the functions of these cell cycle inhibitors in five patient-derived glioma stem cell-enriched cell lines. We found that these proteins are functional in glioma stem cells. They negatively regulate cell cycle progression both in unstressed conditions and in response to genotoxic stress. In addition, p27<sup>Kip1</sup> is upregulated in nutrient-restricted and differentiating cells, suggesting that this Cip/Kip is a mediator of antimitogenic signals in glioma cells. Importantly, the lack of these proteins impairs cell cycle halt in response to genotoxic agents, rendering cells more vulnerable to DNA damage. For these reasons, these proteins may operate both as tumor suppressors, limiting cell proliferation, and as oncogenes, conferring cell resistance to DNA damage. Thus, deepening our knowledge on the biological functions of these Cip/Kips may shed light on how some cancer cells develop drug resistance.

*Neoplasia* (2017) 19, 519–529

### Introduction

Gliomas are the most common type of primary brain neoplasms, accounting for approximately 30% of central nervous system tumors and 80% of all malignant brain tumors. Within this group, glioblastoma multiforme, a high-grade glioma (World Health Organization grade IV glioma) [1,2], is characterized by elevated intratumoral heterogeneity, diffuse infiltration throughout the brain parenchyma, and resistance to traditional therapies, which inevitably leads to tumor recurrence and the demise of the patient [3]. The high rate of cancer relapse suggests that current therapies do not eradicate all malignant cells. In this regard, a

Abbreviations: CSC, cancer stem cell; DDR, DNA damage response; GSC-ECLs, glioma stem cell-enriched cell line; NR, nutrient restriction.

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Received 18 January 2017; Revised 28 March 2017; Accepted 3 April 2017

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1476-5586

<http://dx.doi.org/10.1016/j.neo.2017.04.001>

subpopulation of tumor cells called cancer stem cells (CSCs) has been identified in gliomas and in many other cancers. These cells are characterized by their capacity of self-renewal and by their enriched tumorigenic potential [4–6]. Furthermore, CSCs are also known for their ability to differentiate into both rapidly proliferating progenitor-like tumor cells and more differentiated tumor cells that define the histological features of the tumor entity [7]. Importantly, CSCs appear to be more resistant towards radio- and chemotherapy than the highly proliferative progenitors that coexist within the tumor. To effectively eradicate CSCs and thus avoid cancer recurrence, it is critical to target their essential functions. Increased resistance of glioblastoma multiforme cells to radiotherapy was suggested to be due to the DNA damage response (DDR), which is preferentially activated in CSCs as compared to non-CSC counterparts [8–10].

The DDR is a network of signaling pathways that is able to sense and repair DNA lesions. The activation of DDR also modulates other cellular processes, including cell cycle checkpoint regulation and programmed cell death. DDR induces cell cycle arrest to allow repair of DNA lesions; however, if too much damage has been sustained, DDR triggers cell death to avoid the generation of deleterious mutations [11]. The progression along the cell cycle requires the activation of different cyclin-dependent kinases (CDKs). Tight CDK regulation involves CDK inhibitors (CKIs) which ensure the correct timing of CDK activation in different phases of the cell cycle. The CKIs include the INK4 family and the Cip/Kip family. In mammals, the Cip/Kip family consists of three proteins, p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, and p57<sup>Kip2</sup>. As a critical negative regulator of the cell cycle, p21<sup>Cip1</sup> binds to and inhibits both CDK/cyclin complexes and PCNA [12,13]. Therefore, based on its capacity to block cell proliferation, p21<sup>Cip1</sup> may act as a tumor suppressor [14,15]. However, evidence has revealed novel functions of p21<sup>Cip1</sup>, such as the control of cell migration, regulation of apoptosis, and maintenance of stem cell pools, among others [13,16–18]. In fact, p21<sup>Cip1</sup> can acquire an antiapoptotic gain of function in the cytoplasm, pointing to a dual role for p21<sup>Cip1</sup> as both a tumor suppressor and an oncogene [19,20]. Also, p21<sup>Cip1</sup> induction is essential for the onset of cell cycle arrest in DDR, giving cells time to repair critical damage [21]. Despite the absence of p21<sup>Cip1</sup> in some cancer types, its overexpression or cytoplasmic localization correlates with poor prognosis in malignant tumors of the skin, pancreas, breast, prostate, ovary, cervix, and brain [18]. Similarly, p27<sup>Kip1</sup> may act as a tumor suppressor through inactivation of cyclin/CDK complexes in the nucleus, but tumorigenic properties of p27<sup>Kip1</sup> have also been proposed, especially when located in cytoplasm [20,22]. Cytosolic p27<sup>Kip1</sup> promotes cell proliferation via interaction with cyclin D/CDK4 [23] and cell migration via inhibition of RhoA/ROCK signaling [24]. In many cancers, p27<sup>Kip1</sup> expression is reduced in the nucleus and exhibits different degrees of cytoplasmic localization [25]. High nuclear and low cytoplasmic expression of p27<sup>Kip1</sup> has been associated with a better prognosis in high-grade astrocytoma [26]. Moreover, an inverse correlation between p27<sup>Kip1</sup> immunoreactivity and the Ki-67 labeling index was observed in patients with malignant gliomas [27].

Although p21<sup>Cip1</sup> and p27<sup>Kip1</sup> were initially considered as tumor suppressors, it rapidly became clear that the situation was not so simple. It appears that the loss of the regulatory mechanisms governing Cip/Kip proteins may lead to the specific loss of its tumor suppressor function while maintaining the oncogenic ones, favoring cancer development. In this context, we wondered whether p21<sup>Cip1</sup> and p27<sup>Kip1</sup> function as tumor suppressors or oncogenes in patient-derived glioma stem cell-enriched cell lines (GSC-ECLs) [28]. To address this issue, we

investigated the role of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in the response of GSC-ECLs to camptothecin (CPT), a potent and specific inhibitor of eukaryotic DNA topoisomerase I that induces DNA double-strand breaks. Initially, we examined the expression pattern of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in five GSC-ECLs. After CPT exposure, we observed a marked increase in the expression levels of these Cip/Kips, which displayed a predominant nuclear localization. Finally, by small interfering RNA (siRNA)-mediated downregulation of both p21<sup>Cip1</sup> and p27<sup>Kip1</sup>, we determined that these CKIs confer protection against CPT in a cell line-dependent manner. This protection may be due, at least in part, to the ability of these inhibitors to halt cell cycle progression. Therefore, the significance of cell cycle regulators in the pathobiology of these tumors is of interest, and the roles of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> need to be elucidated further.

## Materials and Methods

### *Culture of Human Glioma-Derived Cells and Treatments*

Brain tumor-derived cultures were isolated from biopsies and established as previously described [28]. GSC-ECLs were cultured in serum-free medium consisting of neurobasal medium supplemented with B27, N2, 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 2 mM L-glutamine, 2 mM nonessential amino acids, 50 U/ml penicillin/streptomycin (all from Invitrogen, Carlsbad, CA), 20 µg/ml bovine pancreas insulin, and 75 µg/ml low-endotoxin bovine serum albumin (Sigma, St. Louis, MO) and plated onto Geltrex-coated plates (10 µg/ml) (Invitrogen, Carlsbad, CA). Cells were routinely grown to confluence, dissociated using Accutase, and then split 1:2 to 1:3. Medium was replaced every 2 to 3 days. To induce differentiation, cells were cultured for 14 days in the same medium without bFGF and EGF.

To induce genotoxic stress, cells were incubated in 1 µM CPT (Sigma, St. Louis, MO). To generate nutritional stress, cells were incubated in neurobasal medium without supplements.

### *Reverse Transcription Polymerase Chain Reaction (RT-PCR)*

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. cDNA was synthesized from 500 ng of total RNA using MMLV reverse transcriptase (Promega, Madison, WI). Quantitative PCR studies were carried out using SYBR Green-ER™ qPCR SuperMix UDG (Invitrogen, Carlsbad, CA). Primers used were the following: p21<sup>Cip1</sup> forward 5'-ATGACAGATTCTAC CACTC-3', reverse 5'-AAGACACACAACTGAGAC-3'; p27<sup>Kip1</sup> forward 5'-GGCTAACTCTGAGGACAC-3', reverse 5'-TTCTTCT GTTCTGTTGGC-3'; and RPL7 forward 5'-AATGGCGAGGA TGGCAAG-3', reverse 5'-TGACGAAGGCGAAGAAGC-3'. All samples were analyzed using an ABI PRISM 7500 Sequence Detector System (Applied Biosystems, Foster City, CA) and were normalized to RPL7 gene expression.

### *Immunostaining and Fluorescence Microscopy*

Cells were analyzed for *in situ* immunofluorescence. Briefly, cells were rinsed with phosphate-buffered saline (PBS) and fixed in PBS with 4% formaldehyde for 25 minutes. After two washes with PBS with 0.1% bovine serum albumin (PBSA), cells were permeabilized with 0.1% Triton X-100 in PBSA with 10% normal goat serum for 30 minutes, washed twice, and stained with the corresponding primary antibodies. Fluorescent secondary antibodies were used to localize the antigen/primary antibody complexes. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and examined under a Nikon

Eclipse TE2000-S inverted microscope equipped with a 20× E-Plan objective and a super high-pressure mercury lamp. The images were acquired with a Nikon DXN1200F digital camera, which was controlled by the EclipseNet software (version 1.20.0 build 61). The following primary antibodies were used:  $\alpha$ -p21<sup>Cip1</sup> (Cat. 556430 clone SX118) (BD Pharmingen, Becton-Dickinson, San Jose, CA),  $\alpha$ -p27<sup>Kip1</sup> (sc-528) (Santa Cruz Biotechnology, Santa Cruz, CA), and  $\alpha$ -MAP-2 (M1406) (Sigma, St. Louis, MO).

### Western Blotting

Cells were lysed in ice-cold radioimmunoprecipitation assay buffer supplemented with a protease and phosphatase inhibitor mixture, and protein concentration was determined using Bicinchoninic Acid Protein Assay (Pierce, Rockford, IL). Equal amounts of protein were run on 12% polyacrylamide gel electrophoresis and transferred to PVDF-FL membrane (Millipore, Billerica, MA). The membrane was blocked for 1 hour in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) containing 0.1% Tween 20 and then incubated overnight at 4°C in a solution containing Odyssey blocking buffer, 0.05% Tween 20, and the corresponding primary antibodies. The membrane was washed 4 × 5 minutes with Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.1% Tween 20 (TTBS); then incubated for 1 hour in a solution containing Odyssey blocking buffer, 0.2% Tween 20, and IR-Dye secondary antibodies (1:20,000, LI-COR Biosciences, Lincoln, NE); and subsequently washed 4 × 5 minutes in TTBS and 1 × 5 minutes in TBS. Immunocomplexes were visualized using the Odyssey Infrared Imaging System (LI-COR). The following primary antibodies were used:  $\alpha$ -p21<sup>Cip1</sup> (Cat. 556430 clone SX118) (BD Pharmingen, Becton-Dickinson, San Jose, CA),  $\alpha$ -p27<sup>Kip1</sup> (sc-528) (Santa Cruz Biotechnology, Santa Cruz, CA), and  $\alpha$ -actin (sc-1616) (Santa Cruz Biotechnology, Santa Cruz, CA). Antigen/primary antibody complexes were detected with near-infrared fluorescence-labeled IR-Dye 800CW or IR-Dye 680RD secondary antibodies (LI-COR Biosciences, Lincoln, NE).

### Cell Transfection and RNA Interference

Cells were transfected with the corresponding siRNA using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Briefly, 2 × 10<sup>5</sup> cells/well (six-well plate) were transfected with Silencer Select Negative Control #2 (Ambion, cat. 4390846), Silencer Select Validated CDKN1A siRNA (Ambion, siRNA ID: s417), and Silencer Select Validated CDKN1B siRNA (Ambion, siRNA ID: s2838). The concentrations of siRNA used for cell transfections (2.5–5 nM) were selected based on dose-response studies.

### Flow Cytometric Analysis of Cell Viability Using Propidium Iodide (PI)

Single-cell suspensions were obtained by treatment with Accutase (37°C for 5–10 minutes), centrifuged at 200×g for 5 minutes, and resuspended in FACS buffer (2.5 mM CaCl<sub>2</sub>, 140 mM NaCl, and 10 mM HEPES, pH 7.4). Next, 100  $\mu$ l of cellular suspension was incubated with 5  $\mu$ l of PI (1 mg/ml) in PBS for 5 minutes in the dark. Cells were immediately analyzed by flow cytometry. Results were expressed as the percentage of cells that displayed PI fluorescence (nonviable) to the total number of cells processed. Fluorescence intensity was determined by flow cytometry on a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Flow cytometry data were analyzed using BD AccuriC6 software.

### Flow Cytometric Analysis of Bromodeoxyuridine (BrdU) Incorporation and Cell Cycle Distribution

To characterize the distribution of cell populations throughout the cell cycle and the fraction of cells capable of incorporating BrdU, the *BrdU Flow Kit* (BD Biosciences, San Jose, CA) was used. After the corresponding treatments, cells were incubated with BrdU (10  $\mu$ M) for 2 hours. Cultures were then processed following manufacturer's instructions. Fluorescence intensity was determined by flow cytometry on a BD Accuri C6 flow cytometer. Flow cytometry data were analyzed using BD AccuriC6 software.

### Assessment of DNA Fragmentation

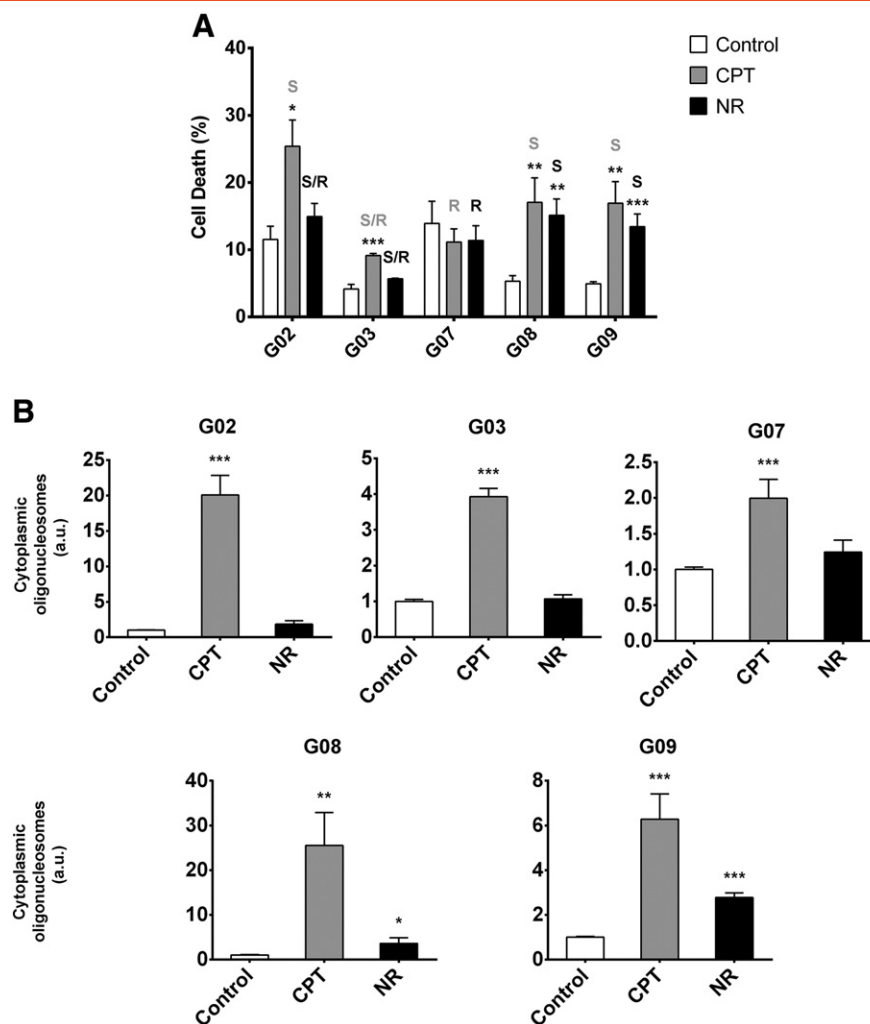
Apoptosis was quantified by direct determination of nucleosomal DNA fragmentation with Cell Death Detection ELISA<sup>Plus</sup> kit (Roche, Mannheim, Germany). This assay uses specific monoclonal antibodies directed against histones and DNA, allowing the determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Briefly, 3 × 10<sup>4</sup> cells were plated on 96-well plates in 150  $\mu$ l of culture medium. Forty-eight hours after CPT (1  $\mu$ M) addition or nutrient restriction, cells were processed according to the manufacturer's manual. The mono- and oligonucleosomes were determined using an anti-histone-biotin antibody and an anti-DNA-peroxidase antibody. The resulting color development, which was proportional to the amount of nucleosomes captured in the antibody sandwich, was measured at 405-nm wavelength using a Benchmark microtiter plate reader (Bio-Rad, Hercules, CA). Results were expressed as fold change, calculated from the ratio of absorbance of treated samples to that of the untreated ones.

## Results

### GSC-ECLs Display Differential Degrees of Susceptibility to Stress Conditions

CSC resistance to chemo- and radiotherapy is clinically important as most current anticancer agents target the tumor bulk but not the CSC population. Thus, to gain insight into the responses achieved by GSC-ECLs upon DNA damage, we sought to compare how five GSC-ECLs (G02, G03, G07, G08, and G09) that were previously established and characterized in our laboratory [28] respond to CPT. To do so, we determined the percentage of cell death of GSC-ECLs after 48 hours of 1- $\mu$ M CPT exposure by PI staining. As indicated in Figure 1, the tested cell lines responded differently to genotoxic stress. We found that CPT exposure led to a considerable increase in cell death in the G02 (16.5 ± 4%), G08 (14.07 ± 3.96%), and G09 (11.99 ± 3.19%) cell lines (Figure 1A, gray bars); however, for the G03 cell line, this increment was only slight (4.98 ± 0.62%). Notably, the G07 cell line was almost totally insensitive to this topoisomerase I inhibitor.

Tumor cells emerge as a result of genetic and epigenetic alterations of signal circuitries promoting cell growth and survival, whereas their expansion relies on nutrient supply. More specifically, cells possessing flexibility in nutrient utilization will be able to survive under nutrient stress. Keeping this in mind, we wondered whether these GSC-ECLs are susceptible to nutritional stress and, if so, if they will respond equally among them or in a cell line-specific manner as occurred with CPT treatment. To find the answer, we cultured these cell lines in basal medium (deprived of supplements) for 48 hours and assessed cell death. Similarly to what occurred under CPT exposure, nutrient limitation led to a significant decrease in the G08 and G09 cell line viability and a slight decrease in the G03 cell line. Again, no significant changes were observed in the G07 cell line. However, whereas the G02 cell line was



**Figure 1.** GSC-ECLs exhibit differential susceptibilities in response to stress conditions. (A) Bar charts show the mean of PI-stained cells 48 hours after CPT or nutrient restriction exposure. Percentage of PI positive cells was determined by flow cytometric analysis. Each bar represents the mean  $\pm$  S.E.M. of three independent experiments. A one-way ANOVA was used to classify cell lines according to their susceptibility to stress conditions. *R*, resistant; *S*, sensitive. (B) Forty-eight hours after CPT exposure or nutrient restriction, cells were harvested, and cytoplasmic DNA oligomers were quantified by immunoassay. Results are presented as DNA oligomers fold induction versus untreated control cells, arbitrarily set as 1. Each bar represents the mean  $\pm$  S.E.M. of three independent experiments performed in triplicate. Student's *t* test was used to compare CPT- or NR-treated samples to untreated controls (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ). *a.u.*, arbitrary units; *NR*, nutrient restriction.

very sensitive to CPT, no major changes in its viability were detected under nutritional stress (Figure 1A, black bars). After carrying out a one-way ANOVA to compare the responses to stress of the different cell lines, we were able to identify resistant cell lines (R), sensitive cell lines (S), and some that could not be classified as either (S/R) (Figure 1A).

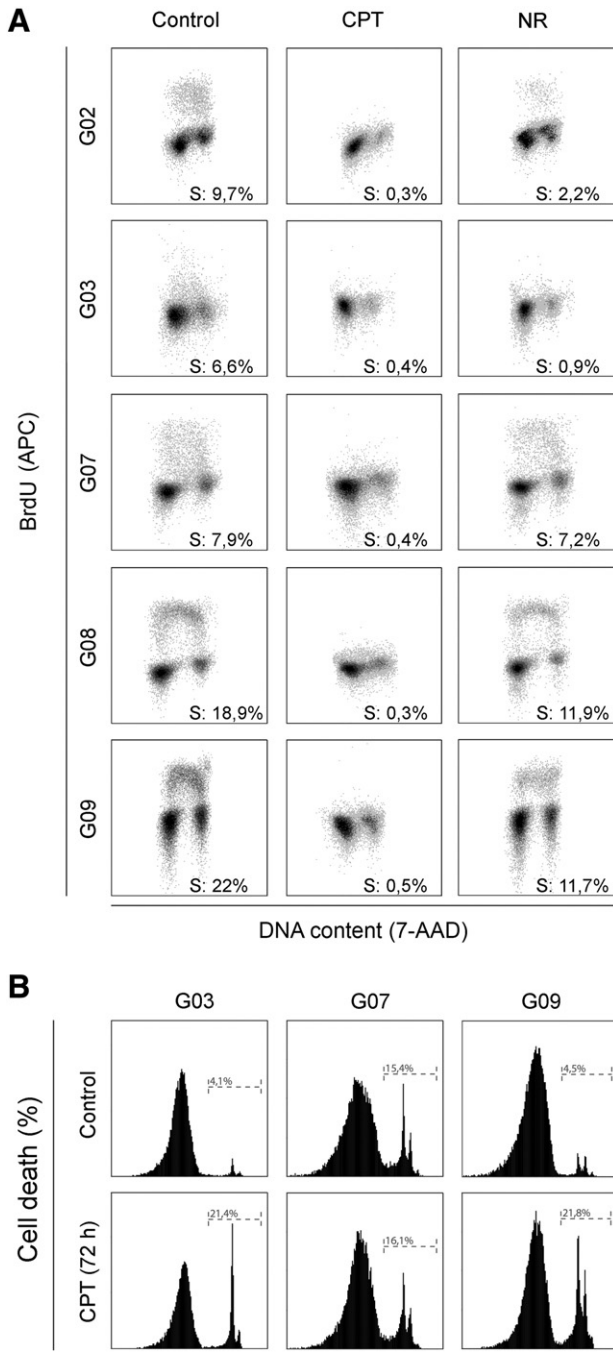
The loss of cell viability was accompanied by morphological changes such as ballooning and cell detachment, which are suggestive of apoptotic processes (data not shown). Thus, to investigate whether the observed decrease in cell viability was due to the induction of apoptosis, we determined cell death levels of stressed and unstressed cells by quantifying cytoplasmic DNA oligomers. Results indicate the presence of significantly higher levels of histone-bound oligonucleosomes in CPT-treated cells compared to nontreated counterparts, ranging from approximately 2- to 25-fold increase in the G07 and G08 cell lines, respectively. Interestingly, nutritional restriction led to an increase in the percentage of DNA oligomers only in some cell lines. Concordant with our previous data obtained by PI

staining, the G08 and G09 cell lines were more susceptible to nutrient and growth factors deprivation, whereas the G02, G03, and G07 cell lines appeared to be relatively insensitive (Figure 1B).

#### Stress Conditions Promote Cell Cycle Arrest in GSC-ECLs

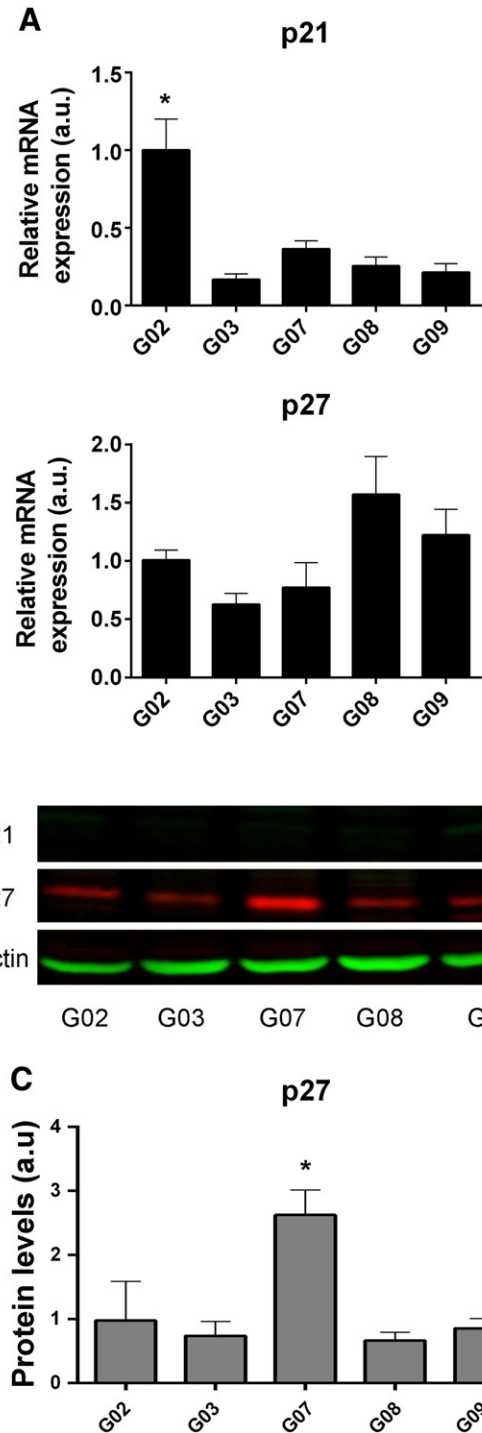
To further elucidate if the loss of cell viability after CPT exposure was accompanied by changes in cell proliferation, we measured BrdU incorporation by flow cytometry. As judged by BrdU labeling, CPT treatment led to an almost complete inhibition of DNA replication. In the case of nutrient restriction, all tested cell lines displayed a partial reduction in their BrdU incorporation (Figure 2A).

Importantly, we found that cells endowed with a higher proliferation rate when unstressed were the ones that were more sensitive to CPT-induced DNA damage (Figures 1 and 2A). This is in accordance with the fact that the cytotoxicity of CPT is highly S phase specific. Thus, we wondered whether the CPT-resistant phenotype exhibited by the G03 and G07 cell lines was associated with their low



**Figure 2.** (A) Stress conditions reduce GSC-ECL proliferation. Cells were subjected to CPT or nutrient restriction (NR), or left untreated for 48 hours. Then, cells were pulse labeled with BrdU for 2 hours prior to harvesting and stained with BrdU-APC conjugate and with 7-amino-actinomycin D (7-AAD) for determination of DNA synthesis and DNA content, respectively. A representative flow cytometry plot is shown for each experimental condition. Dot plots show incorporation of BrdU into DNA (y-axis) against DNA content (7-AAD staining; x-axis). The percentage of cells in S phase was determined by quantifying BrdU<sup>+</sup> events. (B) Representative histograms of PI-stained CPT-treated (1 μM) GSC-ECLs over a 72-hour period or untreated cells. Percentage of PI positive cells was determined by flow cytometric analysis.

proliferating rates. It stands to reason that possibly during the time frame of CPT exposure, a great proportion of the G03 and G07 cell populations did not have enough time to enter S phase and thereby had not been affected by CPT toxicity. To solve this issue, we extended the



**Figure 3.** p21<sup>Cip1</sup> and p27<sup>Kip1</sup> expression in GSC-ECLs. (A) Analysis of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> mRNA expression levels by quantitative RT-PCR in GSC-ECLs. RPL7 expression was used as normalizer. Graphs show mRNA fold change relative to G02 cell line. Bars represent the mean ± S.E.M. of three different experiments performed in triplicate. (B) p21<sup>Cip1</sup> and p27<sup>Kip1</sup> protein levels were analyzed by Western blot. Actin was used as loading control. (C) The intensity of p27<sup>Kip1</sup> and corresponding actin band was evaluated by densitometric analysis. Values were normalized to actin, and intensity is expressed as fold change relative to G02 line expression. A Newman-Keuls test was conducted to detect significant differences between cell lines (\*P < .05).

time of CPT exposure for up to 72 hours and assessed cell death. Importantly, the G03 cell line, which displays an intermediate susceptibility (S/R) at 48 hours, finally was sensitive to CPT at 72 hours posttreatment. On the contrary, the G07 cell line maintained its resistance to genotoxic stress at this time point (Figure 2B).

#### p21<sup>Cip1</sup> and p27<sup>Kip1</sup> Expression in GSC-ECLs

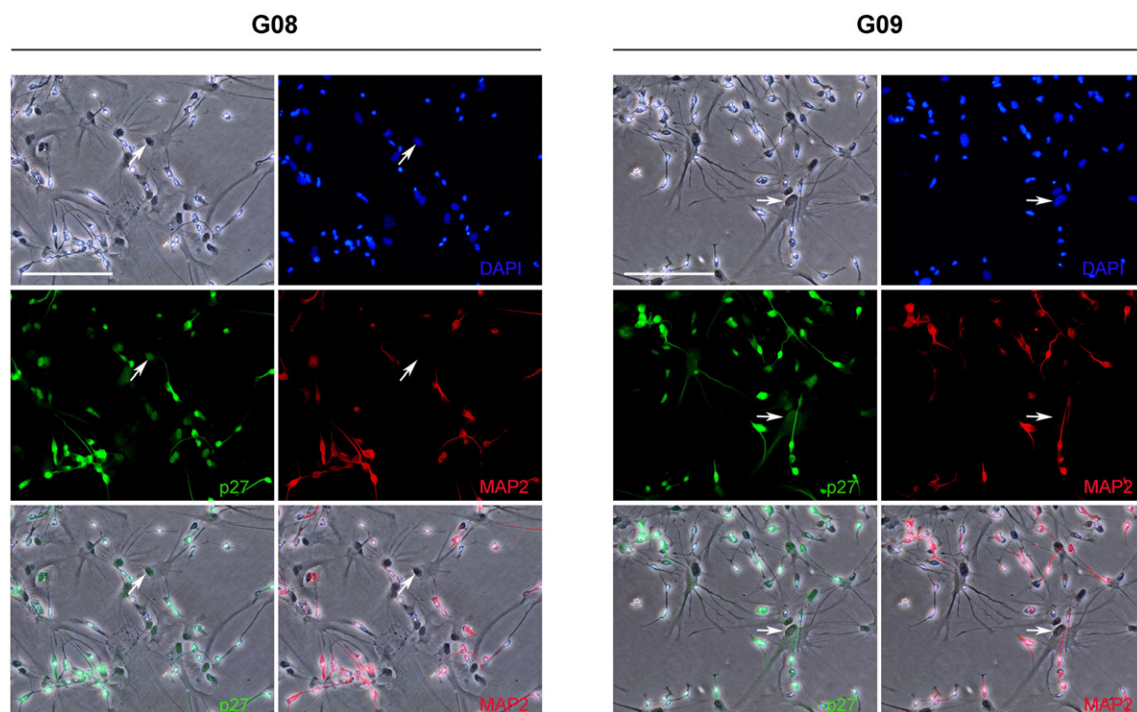
Initially, we examined the expression levels of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in GSC-ECLs. Real-time RT-PCR analysis showed that all tested cell lines express similar levels of p21<sup>Cip1</sup> mRNA except for G02, which expresses higher levels of this transcript (Figure 3A). In parallel, we performed Western blots analysis and observed that p21<sup>Cip1</sup> protein levels were practically below the detection threshold (Figure 3B). Similarly, p27<sup>Kip1</sup> mRNA expression levels did not significantly differ among all studied cell lines (Figure 3A). However, we found that GSC-ECLs expressed easily detectable amounts of p27<sup>Kip1</sup> protein, which was more abundant in the G07 cell line (Figure 3B). Interestingly, we determined that, within these cell lines, there was not a tight correlation between p27<sup>Kip1</sup> mRNA levels and the corresponding protein product. These findings suggest that mechanisms controlling protein concentration (e.g., many steps in transcription and translation as well as degradation) may operate distinctively in each cell line.

CSCs frequently give rise to a differentiated progeny that has lost the ability for self-renewal. Therefore, this would imply that remnant regulatory mechanisms that guide the differentiation process are still present in cancer cells. Therefore, to gain insight into the differentiation process of GSC-ECLs, we assessed by immunofluorescence staining the expression of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in G08 and G09 cell lines 14 days after differentiation onset. Similarly to what we observed in undifferentiated cells, p21<sup>Cip1</sup> immunoreactivity was almost completely

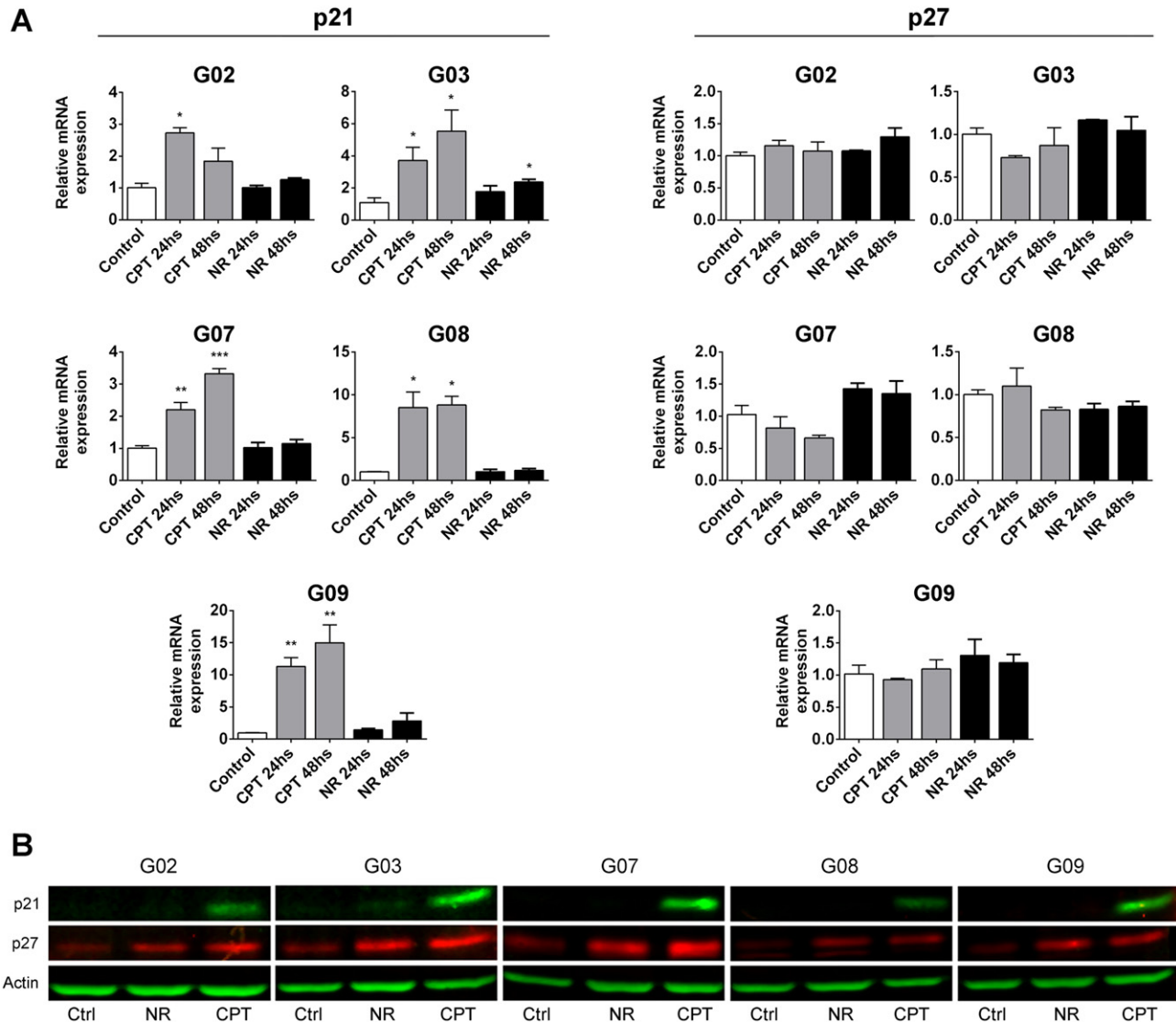
absent in differentiating cells (data not shown). On the other hand, we found that p27<sup>Kip1</sup> was present in nearly all differentiated cells. Importantly, cells exhibiting protruding processes and expressing the neuronal marker MAP2 appeared intensely stained for p27<sup>Kip1</sup> (Figure 4). However, astrocyte-like cells (MAP2<sup>-</sup>) showed lower p27<sup>Kip1</sup> immunoreactivity which could only be detected in the nucleus (Figure 4, white arrows). GSC-ECLs significantly diminish their proliferation rates at day 14 of differentiation [28]. The fact that p27<sup>Kip1</sup> but not p21<sup>Cip1</sup> is present in the nucleus of differentiated cells suggests that this Cip/Kip may be involved in mechanisms that govern CSC transition to a postmitotic state.

#### Stress Conditions Induce the Expression and the Relocalization of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in GSC-ECLs

A better understanding of the molecular mechanisms that impact CSC response and resistance to treatments may be helpful in predicting clinical outcome and developing more effective therapies for different subtypes of high-grade gliomas. In this sense, considerable evidence exists demonstrating the roles of cell cycle mediators in determining a cell's fate upon different forms of stress [16,29,30]. Thus, to investigate possible molecular mechanisms underlying the responses of GSC-ECLs to CPT and to nutrient restriction, we explored changes in the expression levels of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> under these stress conditions. Real-time RT-PCR analysis revealed that p21<sup>Cip1</sup> mRNA expression levels robustly increased at both 24 and 48 hours after CPT addition in all cell lines. Contrarily, with the exception of G03 cell line, no significant changes in p21<sup>Cip1</sup> mRNA expression levels were observed in cells exposed to nutrient restriction. On the other hand, p27<sup>Kip1</sup> mRNA levels did not vary significantly upon treatments (Figure 5A). Next, we performed Western blot analysis to evaluate if there was a



**Figure 4.** Differentiated glioma stem cells express high levels of nuclear p27<sup>Kip1</sup>. Representative phase contrast and immunofluorescent images of G08 and G09 cell lines after 14 days under differentiating conditions (bFGF and EGF withdrawal). Cells were stained with antibodies against MAP2 and p27<sup>Kip1</sup>. Nuclei were counterstained with DAPI. Objective 20 $\times$ ; scale bar: 50  $\mu$ m. White arrows point to less intensely labeled astrocyte-like cell nuclei.



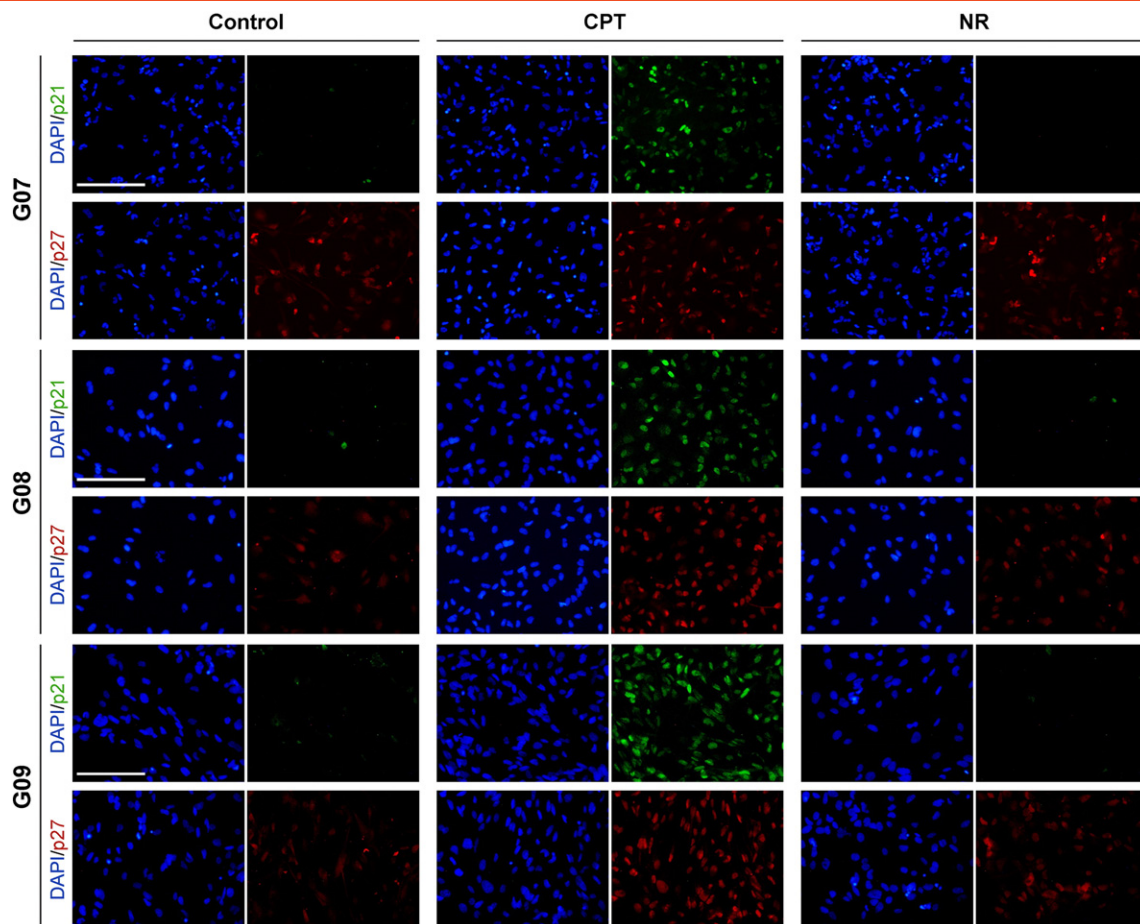
**Figure 5.** p21<sup>Cip1</sup> and p27<sup>Kip1</sup> abundance after CPT exposure or nutrient restriction in GSC-ECLs. (A) Analysis of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> mRNA expression levels by real-time RT-PCR in GSC-ECLs after CPT treatment or nutrient restriction over a 24- or 48-hour period. RPL7 expression was used as normalizer. Graphs show mRNA fold change relative to untreated control cells, arbitrarily set as 1. Bars represent the mean  $\pm$  S.E.M. of three different experiments performed in triplicate. Student's *t* test was used to compare CPT- or NR-treated to untreated samples (\**P*  $\leq$  .05, \*\**P*  $<$  .01, \*\*\**P*  $<$  .001). (B) Western blot analysis of GSC-ECLs under control conditions and after 48 hours of CPT exposure or nutrient restriction. Antibodies against p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, and actin (loading control) were used.

correlation between the mRNA expression and the corresponding protein levels. As indicated in Figure 5B, p21<sup>Cip1</sup> expression was robustly induced only in CPT-treated cells. Conversely, a marked increase in the levels of p27<sup>Kip1</sup> was observed 48 hours after genotoxic stress or nutrient restriction.

An important aspect of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> functions is associated with their cellular localization. Thus, to investigate whether CPT treatment or nutrient restriction modulates the subcellular localization of these Cip/Kips, we assessed p21<sup>Cip1</sup> and p27<sup>Kip1</sup> distribution by fluorescence microscopy. In accordance with Western blot results, immunofluorescence staining revealed a strong induction of p21<sup>Cip1</sup> in CPT-treated cells. Importantly, p21<sup>Cip1</sup> showed a marked nuclear localization. Again, the expression levels of p21<sup>Cip1</sup> were undetectable in unperturbed or nutrient-restricted cells. With regard to p27<sup>Kip1</sup>, both experimental conditions led to an increase in the nuclear abundance of this Cip/Kip, which was more evident in CPT-treated cells (Figure 6).

#### siRNA-Mediated Gene Silencing of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> Sensitizes GSC-ECLs to Genotoxic Stress

Because p21<sup>Cip1</sup> and p27<sup>Kip1</sup> are induced by genotoxic stress and high-grade gliomas display an elevated tumor recurrence after standard treatments, we wondered if these Cip/Kips may contribute to GSC-ECL chemoresistance. To address this issue, we examined the effect elicited by specific siRNAs targeting p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, or both in the cellular response triggered by CPT. To do so, we first transfected G07, G08, and G09 cell lines with the specific siRNAs, and 48 hours posttransfection, we determined the expression levels of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> mRNAs by real-time RT-PCR. In all cases, we found that the levels of the target transcripts decreased significantly (Figure 7A). We also corroborated that the expression levels of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> were not affected when nontargeting (NT) siRNA was used as negative control. Additionally, 24 hours after CPT exposure, we performed immunofluorescence assays to determine the expression levels of



**Figure 6.** p21<sup>Cip1</sup> and p27<sup>Kip1</sup> expression and localization in GSC-ECLs exposed to stress conditions. Immunofluorescence staining of CPT-treated (1  $\mu$ M) or nutrient-restricted cells over a 24-hour period. The figure shows representative photomicrographs of G07, G08, and G09 cells stained with primary antibodies against p21<sup>Cip1</sup> and p27<sup>Kip1</sup>. Nuclei were counterstained with DAPI. Objective 20 $\times$ ; scale bar, 50  $\mu$ m.

p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in siRNA-transfected cell populations. We observed that even in the presence of genotoxic stress, the expression of each Cip/Kip was markedly reduced with respect to that determined in the NT siRNA-transfected counterparts (Figure 7B).

To establish if p21<sup>Cip1</sup> and p27<sup>Kip1</sup> are involved in mechanisms that confer resistance of GSC-ECLs to stress conditions, we measured cell death after silencing these Cip/Kips either individually or simultaneously under basal conditions or when subjected to genotoxic stress. When evaluating the effect of siRNA-mediated gene silencing in unstressed cells, no changes in the extent of cell death were observed. In contrast, when cells were exposed to CPT, we found that only the simultaneous silencing of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> led to a significant increase in cell death in the G08 and G09 cell lines. Strikingly, in the G07 cell line, no changes were observed under these conditions (Figure 7C).

#### *siRNA-Mediated Gene Silencing of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> Increases Proliferation and Impairs the Cell Cycle Arrest After DNA Damage*

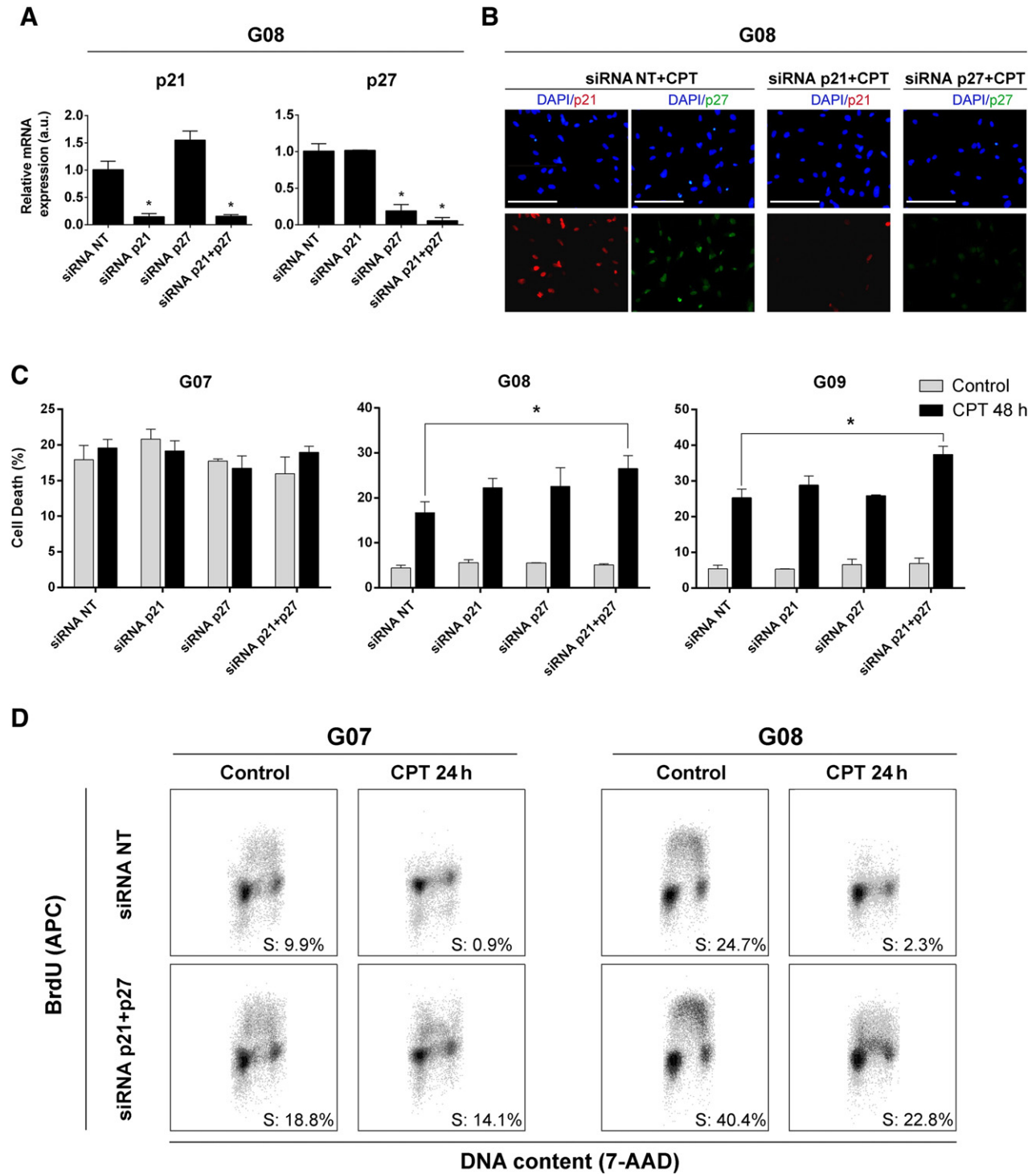
The observed increase in p21<sup>Cip1</sup> and p27<sup>Kip1</sup> and their nuclear localization triggered by CPT suggest that these proteins are necessary to block cell cycle progression of GSC-ECLs exposed to genotoxic stress, thereby preventing cell death. To assess this, we first evaluated G08 cell line viability and cell cycle arrest along the first 24 hours post-CPT addition. We found that CPT led to an almost complete

cell cycle halt without a significant increase in cell death (data not shown). Then, to gain insight into the role of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in cell cycle regulation, we used siRNA to target both Cip/Kips in G07 and G08 cell lines (a resistant and a sensitive cell line, respectively) and determined cell proliferation by BrdU incorporation before and 24 hours after genotoxic insult. Silencing of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in untreated cells led to a marked increment in the percentage of BrdU<sup>+</sup> cells, which implies that these Cip/Kips negatively regulate cell cycle progression in unperturbed cells. Furthermore, in both G07 and G08 cell lines, simultaneous knockdown of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> significantly impaired cell cycle arrest upon CPT exposure (Figure 7D). This finding suggest that, in GSC-ECLs, the DDR induces p21<sup>Cip1</sup> gene expression and stabilizes p27<sup>Kip1</sup> protein product to mediate cell cycle arrest to give time to repair the damaged DNA and prevent excessive cell death.

#### **Discussion**

High-grade gliomas are extremely lethal infiltrative primary brain tumors that cannot be completely removed by surgery. Even with the current standard treatment, involving surgery, radiation, and chemotherapy with temozolamide, the median life expectancy of high-grade gliomas is 15 to 18 months [2]. The almost total recurrence of these tumors is due to an incomplete eradication of tumorigenic cells. To this end, the emergence of the CSC theory has brought consciousness to the





**Figure 7.** p21<sup>Cip1</sup> and p27<sup>Kip1</sup> downregulation affects proliferation and viability of GSC-ECLs. (A) mRNA expression levels of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in NT-, p21<sup>Cip1</sup>-, p27<sup>Kip1</sup>-, or dual-siRNA-transfected G08 cells were analyzed 48 hours posttransfection by real-time RT-PCR. RPL7 expression was used as normalizer. Graph shows mRNA fold change relative to NT-siRNA transfectants, arbitrarily set as 1. (B) Representative immunofluorescent images of NT-, p21<sup>Cip1</sup>- or p27<sup>Kip1</sup>-siRNA transfectants exposed to CPT (1  $\mu$ M, 24 hours) 24 hours posttransfection. Cells were stained with antibodies against p21<sup>Cip1</sup> and p27<sup>Kip1</sup>. Nuclei were counterstained with DAPI. (C) G07, G08, and G09 cells were transfected with NT-, p21<sup>Cip1</sup>-, p27<sup>Kip1</sup>-, or dual-siRNA and exposed to CPT 24 hours posttransfection. Bar charts show the mean of PI-stained cells 48 hours after CPT addition. Each bar represents the mean  $\pm$  S.E.M. of three independent experiments. Student's *t* test was used to compare p21<sup>Cip1</sup>-, p27<sup>Kip1</sup>-, or dual- to NT-siRNA transfectants (\**P* < .05). (D) Twenty-four hours posttransfection with the corresponding siRNA, G08 and G09 cells were left untreated or subjected to CPT for 24 hours. A representative flow cytometry plot is shown for each experimental condition. Dot plots show incorporation of BrdU into DNA against DNA content. The percentage of cells in S phase was determined by quantifying BrdU<sup>+</sup> events.

fact that eradicating CSCs may be critical to overturn treatment resistance. CSCs resist treatments due to several mechanisms. One such mechanism involves the active transport of chemotherapeutic agents to the extracellular space via ABC-type transporters on the cell surface. Other mechanisms include the upregulation of antiapoptotic proteins; the DNA damage checkpoint; and the activation of distinct molecular networks including Notch, NF- $\kappa$ B, EZH2, and PARP signaling [10,31–34]. Besides, gliomas usually display altered cell cycle profiles such as deletions of *CDKN2A* and *RBI* genes and overexpression/amplification of *CDK4* and/or *CDK6* [35–37]. These alterations result in cell cycle checkpoints' failure. When checkpoint arrest control is compromised, initiation of S phase or mitosis may proceed despite cellular damage [30]. Thus, unraveling the role of cell cycle regulators in controlling glioma CSC radio- and chemoresistance is essential for the development of innovative targeted cancer therapies.

Cip/Kips promote cell cycle arrest in response to antiproliferative signals such as DNA damage or growth factor withdrawal [38]. However, these inhibitors also participate in numerous cell cycle-independent functions, such as apoptotic processes, cell migration, and DNA repair [29]. Given their multiple roles, the cellular and molecular context in which they are found will determine whether they function as tumor suppressors or as oncogenes. These considerations, together with the fact that all tested GSC-ECLs harbor homozygous deletion of *CDKN2A/ARF*, prompted us to explore the role of two Cip/Kip family members, p21<sup>Cip1</sup> and p27<sup>Kip1</sup>, in the cellular response to stress conditions. In this scenario, we evaluated the susceptibility of different GSC-ECLs to genotoxic or nutritional stress. Our findings allowed us to classify the tested cell lines after genotoxic exposure or nutrient restriction into groups either sensitive or resistant. Consistent with the fact that the cytotoxicity of CPT is S phase specific, we found that lines displaying higher levels of BrdU incorporation were more sensitive to topoisomerase I inhibition. Also, under nutritional stress, these cell lines responded distinctively. Although the tested GSC-ECLs differ considerably in their resistance to stress conditions and proliferative capacity, they did not show considerable differences with respect to p21<sup>Cip1</sup> and p27<sup>Kip1</sup> expression levels. Moreover, in studied cell lines, these Cip/Kips underwent a similar regulation in response to differentiating conditions. Similarly to what occurs during neurogenesis, a marked increase in p27<sup>Kip1</sup> expression levels was observed [39]. This finding suggests that the role of this Cip/Kip is preserved in glioma CSC differentiation. In this regard, it has been reported that high expression of p27<sup>Kip1</sup> is associated with a better prognosis in malignant gliomas [40]. This correlation could be explained, at least in part, by considering that tumors harboring higher expression of this protein could correspond to more differentiated and therefore less aggressive tumors.

p21<sup>Cip1</sup> and p27<sup>Kip1</sup> are well-known inducers of cell cycle arrest in response to genotoxic and nutritional stress [38]. Thus, the study of how these Cip/Kips are regulated in response to stress conditions in patient-derived GSC-ECLs is relevant. To this end, we determined that p21<sup>Cip1</sup> and p27<sup>Kip1</sup> were induced upon CPT exposure in all tested cell lines. Contrarily, in nutrient-restricted cells, only p27<sup>Kip1</sup> was upregulated. In all cases, a predominant nuclear localization of these Cip/Kips was observed upon stress. The response displayed by p21<sup>Cip1</sup> was expected as this protein is a recognized key player in the DDR [16]. However, to the best of our knowledge, the role of p27<sup>Kip1</sup> in the DDR of glioma CSCs was currently unclear.

Given the molecular complexity and the intertumor heterogeneity present in high-grade gliomas, patient-derived GSC-ECLs raise the

possibility of studying the DDR in a cell line-specific manner, paving the way to the development of tailor-made therapies. Thus, by silencing p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in resistant and sensitive GSC-ECLs, we evaluated the role of these Cip/Kips in cell survival after DNA damage. siRNA-mediated downregulation allowed us to determine that, in two sensitive cell lines (G08 and G09), p21<sup>Cip1</sup> and p27<sup>Kip1</sup> appeared to be relevant to prevent cell death when facing genotoxic stress. Strikingly, in the aforementioned cell lines, only the simultaneous silencing of both Cip/Kips caused an increase in DNA damage-induced cell death. Furthermore, the dual silencing also impaired the ability of glioma stem cells to arrest cell cycle after CPT exposure, which could explain, at least in part, the observed increment in cell death. Therefore, the lack of these proteins may contribute to sensitize glioma CSCs to chemotherapeutic agents. On the other hand, no appreciable changes were detected when silencing p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in the resistant cell line (G07). Nevertheless, as occurred in a sensitive cell line, the simultaneous silencing of both Cip/Kips caused a defective cell cycle halt in DNA-damaged cells. The highly resistant phenotype of this cell line (even in the absence of p21<sup>Cip1</sup> and p27<sup>Kip1</sup>) could be attributed to a disconnection between apoptosis, DDR, and cell cycle checkpoint regulation, rendering cells less vulnerable to genotoxic agents. These results, together with the fact that both Cip/Kips were upregulated and localized in the nucleus after CPT treatment, indicate that these proteins may have functional redundancy in the regulation of cell cycle progression in response to DNA damage in glioma stem cells.

The complex network regulating p21<sup>Cip1</sup> and p27<sup>Kip1</sup> functions warrants caution with regard to its application for cancer therapy. In this sense, our findings suggest that these proteins may be exerting antagonistic functions in glioma stem cells. Although p21<sup>Cip1</sup> and p27<sup>Kip1</sup> confer protection against DNA damage, they also negatively control proliferation of GSC-ECLs. Thus, the downregulation of these proteins may lead to uncontrolled tumor cell proliferation. The results presented herein highlight the importance of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in the cell cycle control and drug resistance of glioma stem cells, providing new insights into the field of glioma biology.

### Conflict of Interest

None.

### Funding

This research was supported by Fundación para la Lucha contra las Enfermedades Neurológicas de la Infancia (FLENI) and did not receive any specific grant from other funding agencies.

### Acknowledgements

We gratefully acknowledge Dr. Andrés Cervio (Neurosurgery Department, FLENI), Dr. Horacio Martinetto (Neuropathology Department, FLENI), and Dr. Blanca Diez (Neurooncology Department, FLENI) for their cooperation. We also would like to thank María Cecilia Donato for her wise advices. V. A. F., M. S. R. V., and S. M. are Graduate Fellows and L. R. is Research Member of CONICET. G. A. V. R. is a postdoctoral Fellow of Instituto Nacional del Cáncer.

### References

- [1] Lathia JD, Mack SC, Mulkearns-Hubert EE, Valentim CL, and Rich JN (2015). Cancer stem cells in glioblastoma. *Genes Dev* **29**, 1203–1217. <http://dx.doi.org/10.1101/gad.261982.115>.
- [2] Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, and Belanger K, et al (2009). 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* **10**, 459–466. [http://dx.doi.org/10.1016/S1470-2045\(09\)70025-7](http://dx.doi.org/10.1016/S1470-2045(09)70025-7).
- [3] Cloughesy TF, Cavenee WK, and Mischel PS (2014). Glioblastoma: from molecular pathology to targeted treatment. *Annu Rev Pathol* **9**, 1–25. <http://dx.doi.org/10.1146/annurev-pathol-011110-130324>.
- [4] Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, and Dirks PB (2003). Identification of a cancer stem cell in human brain tumors. *Cancer Res* **63**, 5821–5828.
- [5] Visvader JE and Lindeman GJ (2008). Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* **8**, 755–768. <http://dx.doi.org/10.1038/nrc2499>.
- [6] Pollard SM, Yoshikawa K, Clarke ID, Danovi D, Stricker S, Russell R, Bayani J, Head R, Lee M, and Bernstein M, et al (2009). Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. *Cell Stem Cell* **4**, 568–580. <http://dx.doi.org/10.1016/j.stem.2009.03.014>.
- [7] Reya T, Morrison SJ, Clarke MF, and Weissman IL (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105–111. <http://dx.doi.org/10.1038/35102167>.
- [8] Eramo A, Ricci-Vitiani L, Zeuner A, Pallini R, Lotti F, Sette G, Pilozi E, Larocca LM, Peschle C, and De Maria R (2006). Chemotherapy resistance of glioblastoma stem cells. *Cell Death Differ* **13**, 1238–1241. <http://dx.doi.org/10.1038/sj.cdd.4401872>.
- [9] Altaner C (2008). Glioblastoma and stem cells. *Neoplasma* **55**, 369–374.
- [10] Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, and Rich JN (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**, 756–760. <http://dx.doi.org/10.1038/nature05236>.
- [11] Ciccia A and Elledge SJ (2010). The DNA damage response: making it safe to play with knives. *Mol Cell* **40**, 179–204. <http://dx.doi.org/10.1016/j.molcel.2010.09.019>.
- [12] Bloom J and Pagano M (2004). To be or not to be ubiquitinated? *Cell Cycle* **3**, 138–140.
- [13] Pei XH and Xiong Y (2005). Biochemical and cellular mechanisms of mammalian CDK inhibitors: a few unresolved issues. *Oncogene* **24**, 2787–2795. <http://dx.doi.org/10.1038/sj.onc.1208611>.
- [14] Martin-Caballero J, Flores JM, Garcia-Palencia P, and Serrano M (2001). Tumor susceptibility of p21(Waf1/Cip1)-deficient mice. *Cancer Res* **61**, 6234–6238.
- [15] Franklin DS, Godfrey VL, O'Brien DA, Deng C, and Xiong Y (2000). Functional collaboration between different cyclin-dependent kinase inhibitors suppresses tumor growth with distinct tissue specificity. *Mol Cell Biol* **20**, 6147–6158.
- [16] Abbas T and Dutta A (2009). p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer* **9**, 400–414. <http://dx.doi.org/10.1038/nrc2657>.
- [17] Coqueret O (2003). New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? *Trends Cell Biol* **13**, 65–70.
- [18] Romanov VS, Pospelov VA, and Pospelova TV (2012). Cyclin-dependent kinase inhibitor p21(Waf1): contemporary view on its role in senescence and oncogenesis. *Biochemistry (Mosc)* **77**, 575–584. <http://dx.doi.org/10.1134/S000629791206003X>.
- [19] Kreis NN, Sanhaji M, Rieger MA, Louwen F, and Yuan J (2014). p21Waf1/Cip1 deficiency causes multiple mitotic defects in tumor cells. *Oncogene* **33**, 5716–5728. <http://dx.doi.org/10.1038/onc.2013.518>.
- [20] Blagosklonny MV (2002). Are p27 and p21 cytoplasmic oncoproteins? *Cell Cycle* **1**, 391–393. <http://dx.doi.org/10.4161/cc.1.6.262>.
- [21] Gartel AL and Tyner AL (1999). Transcriptional regulation of the p21(WAF1/CIP1) gene. *Exp Cell Res* **246**, 280–289. <http://dx.doi.org/10.1006/excr.1998.4319>.
- [22] Sicinski P, Zacharek S, and Kim C (2007). Duality of p27Kip1 function in tumorigenesis. *Genes Dev* **21**, 1703–1706. <http://dx.doi.org/10.1101/gad.1583207>.
- [23] Larrea MD, Liang J, Da Silva T, Hong F, Shao SH, Han K, Dumont D, and Slingerland JM (2008). Phosphorylation of p27Kip1 regulates assembly and activation of cyclin D1-Cdk4. *Mol Cell Biol* **28**, 6462–6472. <http://dx.doi.org/10.1128/MCB.02300-07>.
- [24] Besson A, Gurian-West M, Schmidt A, Hall A, and Roberts JM (2004). p27Kip1 modulates cell migration through the regulation of RhoA activation. *Genes Dev* **18**, 862–876. <http://dx.doi.org/10.1101/gad.1185504>.
- [25] Slingerland J and Pagano M (2000). Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol* **183**, 10–17. [http://dx.doi.org/10.1002/\(SICI\)1097-4652\(200004\)183:1<10::AID-JCP2>3.0.CO;2-I](http://dx.doi.org/10.1002/(SICI)1097-4652(200004)183:1<10::AID-JCP2>3.0.CO;2-I).
- [26] Hidaka T, Hama S, Shrestha P, Saito T, Kajiwara Y, Yamasaki F, Sugiyama K, and Kurisu K (2009). The combination of low cytoplasmic and high nuclear expression of p27 predicts a better prognosis in high-grade astrocytoma. *Anticancer Res* **29**, 597–603.
- [27] Kirla RM, Haapasalo HK, Kalimo H, and Salminen EK (2003). Low expression of p27 indicates a poor prognosis in patients with high-grade astrocytomas. *Cancer* **97**, 644–648. <http://dx.doi.org/10.1002/cncr.11079>.
- [28] Videla Richardson GA, Garcia CP, Roisman A, Slavutsky I, Fernandez Espinosa DD, Romorini L, Miriuka SG, Arakaki N, Martinetto H, and Scassa ME, et al (2016). Specific preferences in lineage choice and phenotypic plasticity of glioma stem cells under BMP4 and Noggin influence. *Brain Pathol* **26**, 43–61. <http://dx.doi.org/10.1111/bpa.12263>.
- [29] Lim S and Kaldis P (2013). Cdks, cyclins and CKIs: roles beyond cell cycle regulation. *Development* **140**, 3079–3093. <http://dx.doi.org/10.1242/dev.091744>.
- [30] Malumbres M and Barbacid M (2009). Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer* **9**, 153–166. <http://dx.doi.org/10.1038/nrc2602>.
- [31] Wang J, Wakeman TP, Lathia JD, Hjelmeland AB, Wang XF, White RR, Rich JN, and Sullenger BA (2010). Notch promotes radioresistance of glioma stem cells. *Stem Cells* **28**, 17–28. <http://dx.doi.org/10.1002/stem.261>.
- [32] Venere M, Hamerlik P, Wu Q, Rasmussen R, Song LA, Vasani A, Tenley N, Flavahan WA, Hjelmeland AB, and Bartek J, et al (2014). Therapeutic targeting of constitutive PARP activation compromises stem cell phenotype and survival of glioblastoma-initiating cells. *Cell Death Differ* **21**, 258–269. <http://dx.doi.org/10.1038/cdd.2013.136>.
- [33] Bhat KP, Balasubramanian V, Vaillant B, Ezhilarasan R, Hummelink K, Hollingsworth F, Wani K, Heathcock L, James JD, and Goodman LD, et al (2013). Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. *Cancer Cell* **24**, 331–346. <http://dx.doi.org/10.1016/j.ccr.2013.08.001>.
- [34] Chalmers AJ (2007). Radioresistant glioma stem cells—therapeutic obstacle or promising target? *DNA Repair* **6**, 1391–1394. <http://dx.doi.org/10.1016/j.dnarep.2007.03.019>.
- [35] Goldhoff P, Clarke J, Smirnov I, Berger MS, Prados MD, James CD, Perry A, and Phillips JJ (2012). Clinical stratification of glioblastoma based on alterations in retinoblastoma tumor suppressor protein (RB1) and association with the proneural subtype. *J Neuropathol Exp Neurol* **71**, 83–89. <http://dx.doi.org/10.1097/NEN.0b013e31823fe8f1>.
- [36] Costello JF, Plass C, Arap W, Chapman VM, Held WA, Berger MS, Su Huang HJ, and Cavenee WK (1997). Cyclin-dependent kinase 6 (CDK6) amplification in human gliomas identified using two-dimensional separation of genomic DNA. *Cancer Res* **57**, 1250–1254.
- [37] He J, Olson JJ, and James CD (1995). Lack of p16INK4 or retinoblastoma protein (pRb), or amplification-associated overexpression of cdk4 is observed in distinct subsets of malignant glial tumors and cell lines. *Cancer Res* **55**, 4833–4836.
- [38] Abukhdeir AM and Park BH (2008). P21 and p27: roles in carcinogenesis and drug resistance. *Expert Rev Mol Med* **10**, e19. <http://dx.doi.org/10.1017/S1462399408000744>.
- [39] Nguyen L, Besson A, Heng JI, Schuurmans C, Teboul L, Parras C, Philpott A, Roberts JM, and Guillemot F (2006). p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. *Genes Dev* **20**, 1511–1524. <http://dx.doi.org/10.1101/gad.377106>.
- [40] Alleyne Jr CH, He J, Yang J, Hunter SB, Cotsonis G, James CD, and Olson JJ (1999). Analysis of cyclin dependent kinase inhibitors in malignant astrocytomas. *Int J Oncol* **14**, 1111–1116.