UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Scienze Cardiologiche, Toraciche e Vascolari

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE MEDICHE, CLINICHE E SPERIMENTALI

INDIRIZZO NEUROSCIENZE

CICLO XXV

THE ROLE OF MITOTIC SLIPPAGE, USP1-REGULATED APOPTOSIS, AND MULTIPLE TREATMENTS IN THE ACTION OF TEMOZOLOMIDE IN GLIOBLASTOMA MULTIFORME

Direttore della Scuola: Ch.mo Prof. Gaetano Thiene

Coordinatore di Indirizzo: Ch.ma Prof.ssa Elena Pegoraro

Supervisore: Ch.ma Prof.ssa Elena Pegoraro

Dottorando: Dott. Alberto Feletti

With the guide and supervision of

Prof. Russell O. Pieper, PhD

The Brain Tumor Research Center

Department of Neurological Surgery

Chairman: Prof. Mitchel S. Berger, M.D.

University of California San Francisco

San Francisco, CA, USA

To my family

"I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

Isaac Newton
(Philosophiae naturalis principia matematica)

Contents

Abbreviation index

ATCC	American Type Culture Collection
APC	Anaphase-promoting complex
ATM/ATR	Ataxia Telangiectasia (Mutated)/Ataxia telangiectasia-Rad3-related
Cdc25	Cell division cycle 25
Cdk1	Cyclin-dependent kinase
Cdt1	Chromatin licensing and DNA replication factor 1
Chk1/2	Checkpoint kinase 1/2
CT	Chemotherapy
DAPI	4'-6-Diamidino-2-phenylindole
DIC	Differential interference contrast
DMEM-HG	Dulbecco's modified Eagle's medium-high glucose
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DSBs	Double-strand breaks
ECL	Enhanced chemiluminescence
FACS	Fluorescence-activated cell sorting
FANCD2	Fanconi complementation group D2
FasR	Fas receptor
FBS	Fetal bovine serum
FITC	Fluorescein-5-isothiocyanate
FUCCI	Fluorescent Ubiquitination-based Cell Cycle Indicator
GBM	Glioblastoma multiforme
HRP	Horseradish peroxidase
mAG2	monomeric Azami Green 2
MGMT	O^6-methylguanine methyltransferase
mKO1	monomeric Kusabira Orange 1
MMC	Mithomycine
MMR	Mismatch Repair
MMS	Methyl methanesulfonate
MTIC	5-(3-methyltriazen-1-yl)imidazole-4-carboxamide

O^6MeG	O^6-methylguanine
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PFA	Paraformaldehyde
PI	Propidium Iodide
Plk1	Polo-like kinase
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homologue
PVDF	Polyvinylidene difluoride
RT	Radiotherapy
SCF	SKP1–CUL1–F-box-protein
siRNA	small interference ribonucleic acid
TBS	Tris-buffered saline
TBST	Tris-buffered saline and Tween 20
TMZ	Temozolomide
UAF1	USP1-associated factor 1
USP1	Ubiquitin specific peptidase 1

Abstract

Background. Temozolomide (TMZ) is a methylating drug that is commonly used in the treatment of glioma. Although many features are still unclear, its general mechanism of action is well described. TMZ induces O^6-methylguanine (O^6MeG) lesions in DNA, which, in the absence of repair by O^6-methylguanine methyltransferase (MGMT), mispair with thymine and start a futile cycle of repair-resynthesis events. The resultant DNA double-strand breaks (DSBs) activate the components of G2 checkpoint, and cells with a 4N DNA content accumulate and remain arrested at the G2/M boundary for several days. Cell death subsequently occurs by senescence, necrosis, or mitotic catastrophe, while apoptosis has been ruled out in many studies. Moreover, the effect of multiple TMZ treatments on G2 arrest and apoptosis induction is not clear. Repair of methylating drug-induced DNA lesions requires monoubiquitination of PCNA and FANCD2. Loss of either protein or inhibition of their monoubiquitination increases drug toxicity. USP1 is a hydrolase that removes monoubiquitin from PCNA and FANCD2, and can potentially play a role in TMZ mechanism of action.

Materials and methods. U87, U251 (TMZ-sensitive, low MGMT), and GBM8 (TMZ-resistant, high MGMT) cell lines were used for experiments. The treatment was scheduled with 100μM TMZ for 3 hours for 1, 2, or 3 consecutive days. Cell cycle progression was studied with both FACS-based analysis and a novel time-lapse microscopic real-time analysis using FUCCI (Fluorescent Ubiquitination-based Cell Cycle Indicator), and apoptosis was measured with FACS-based Annexin V-PI analysis. To address the possible role of USP1 in TMZ action, we examined expression of USP1 at the mRNA levels in expression microarray databases derived from primary GBM. We also used siRNA targeting USP1 to modulate USP1 expression, and studied the effect of USP1 downregulation on TMZ-induced G2 arrest, cell death, and clonogenicity.

Results. Compared to single treatment, multiple TMZ treatments cause a significant reduction of clonogenicity in TMZ-sensitive cells and induce a significant increase of apoptosis, particularly in a late stage. However, multiple treatments don't have any major effect on cell cycle profile. Time-lapse microscopic analysis with FUCCI system showed that TMZ-sensitive glioma cells arrest at the G2 checkpoint for less than 48 hours and, in the presence of an

activated G2 checkpoint, they replicate their DNA without cellular division, re-enter the cell cycle at the next G1 phase, and repeat the cycle, ultimately giving rise to polyploid cells. siRNA-mediated suppression of USP1 had no effect on cell cycle progression or the extent of temozolomide-induced G2 arrest. However, while USP1 knockdown alone had minimal effect on cell death, it increased temozolomide-induced loss of clonagenicity both in TMZ-sensitive and TMZ-resistant cells. Further examination of the mechanism of cell death suggested that while control cells, control cells exposed to TMZ, or USP1-suppressed cells rarely underwent apoptotic cell death, temozolomide-treated cells in which USP1 levels were suppressed underwent high rates of apoptosis.

Conclusions. The present studies show that TMZ can induce apoptosis in TMZ-sensitive glioma cells, which is visible after 3 days but significant after 7 days. Multiple TMZ treatments don't affect cell cycle profile, but significantly increase apoptosis. Moreover, time-lapse studies suggest a novel mechanism of action for TMZ, alternative to the one commonly accepted. These results have significant implications for the development of TMZ resistance. Furthermore, rather than sensitizing cells to DNA damaging agents, USP1 appears to suppress latent apoptotic pathways and to protect cells from temozolomide-induced apoptosis. These results identify a new function for USP1 and suggest that suppression of USP1 and/or USP1 controlled pathway may be a means to enhance the cytotoxic potential of temozolomide and to sensitize TMZ-resistant GBM cells.

Riassunto

Introduzione. La temozolomide (TMZ) è un farmaco alchilante frequentemente utilizzato nella chemioterapia dei gliomi. Nonostante molti aspetti siano ancora enigmatici, il suo meccanismo di azione generale è ben noto. La TMZ induce metilazione della guanina nel DNA (O^6MeG) che, in assenza di riparazione ad opera di O^6-methylguanine methyltransferase (MGMT), si appaia con una timina innescando un ciclo futile di riparazione e risintesi. Ne risultano rotture del DNA a doppio filamento (DSBs) che attivano i componenti del checkpoint in G2, e le cellule con DNA 4N si accumulano e arrestano in G2 per parecchi giorni. Le cellule muoiono poi per senescenza, necrosi, o catastrofe mitotica, mentre l'apoptosi è stata a lungo negata. Inoltre non è chiaro l'effetto di somministrazioni multiple di TMZ sull'arresto in G2/M e sull'induzione di apoptosi. La riparazione delle lesioni al DNA causate dai farmaci alchilanti richiede la monoubiquitinazione di PCNA e FANCD2; la perdita di una delle due proteine o l'inibizione della loro monoubiquitinazione potenzia la tossicità indotta dagli agenti metilanti. USP1 è una idrolasi in grado di rimuovere la monoubiquitina da PCNA e FANCD2, e per questo può essere un regolatore della risposta alla TMZ.

Materiali e metodi. Sono state utilizzate le linee cellulari U87, U251 (TMZ-sensibili, bassi livelli di MGMT) e GBM8 (TMZ-resistenti, alti livelli di MGMT). Il protocollo di trattamento prevede 1, 2 o 3 dosi di TMZ 100μM per 3 ore. La progressione nel ciclo cellulare è stata studiata sia con FACS sia con una nuova tecnica di microscopia time-lapse in tempo reale (FUCCI, Fluorescent Ubiquitination-based Cell Cycle Indicator), mentre l'apoptosi è stata verificata al citofluorimetro con il metodo dell'annessina V-Propidio Ioduro. Per verificare il possibile ruolo di USP1 nell'azione della TMZ, dopo aver esaminato su databases di mRNA microarray l'espressione di USP1 nei glioblastomi, le cellule sono state transfettate con RNA a interferenza contro USP1 o di controllo. È quindi stato studiato l'effetto della soppressione dei livelli di USP1 sull'arresto in G2/M, la morte cellulare e la clonogenicità indotte dalla TMZ.

Risultati. Trattamenti multipli con TMZ riducono la clonogenicità delle cellule di glioma sensibili al farmaco in maniera significativamente superiore rispetto al trattamento singolo, non modificano l'entità dell'arresto in G2, mentre inducono un significativo aumento dell'apoptosi in particolare in fase tardiva. L'analisi in time-lapse con il sistema FUCCI ha

mostrato che le cellule sensibili alla TMZ subiscono un arresto in G2 inferiore alle 48 ore. Inoltre, in presenza di attivazione del checkpoint in G2, replicano il DNA ma non si dividono, rientrando nel ciclo cellulare in G1 e dando origine a cellule poliploidi. La soppressione dei livelli di USP1 da sola ha effetti minimi sulla progressione del ciclo cellulare e sulla morte cellulare sia nelle cellule sensibili che in quelle resistenti alla TMZ. Allo stesso modo, la soppressione dei livelli di USP1 non altera l'entità dell'arresto in G2/M indotto dalla TMZ. Tuttavia il knockdown di USP1 sorprendentemente incrementa la perdita di clonogenicità indotta dalla TMZ sia nelle cellule sensibili che in quelle resistenti. A differenza delle cellule di controllo in cui USP1 è stato soppresso, o di quelle con normale espressione di USP1 e trattate con TMZ, le cellule USP1-knockdown trattate con TMZ subiscono un'alta percentuale di morte per apoptosi.

Conclusioni. I risultati dei nostri studi hanno mostrato che il trattamento delle cellule di glioma con TMZ può indurre apoptosi, e che questa è evidenziabile già dopo 3 giorni, sebbene diventi significativa solo tardivamente. Trattamenti multipli non modificano l'entità dell'arresto in G2, ma aumentano significativamente l'apoptosi. Inoltre gli studi di time-lapse permettono di proporre un nuovo meccanismo di azione per la TMZ, diverso da quello finora comunemente accettato, con significative implicazioni sullo sviluppo della resistenza al farmaco. La deubiquitinasi USP1, piuttosto che impedire l'attivazione di PCNA e FANCD2 e inibire in questo modo la riparazione del danno al DNA indotto dagli agenti metilanti, come indirettamente suggerito da studi precedenti, sembra invece sopprimere vie apoptotiche latenti e proteggere le cellule dall'apoptosi indotta dalla TMZ. La soppressione di USP1 o delle vie controllate da USP1 può rappresentare un modo per incrementare il potenziale citotossico della TMZ e per sensibilizzare GBM prima resistenti.

1. Introduction

Glioblastoma multiforme WHO grade IV (GBM) is one of the most aggressive human malignant diseases and the most frequent primary tumor of the central nervous system. It has an incidence of 4-5/100000 inhabitants per year in Europe and North America [23,56]. GBM has a dismal prognosis, with a survival rate less than 10% two years after diagnosis, and lower than 3% five years after diagnosis [10,17,20].

Currently, there are several treatment options for GBM. Of course surgery is the first-line therapy. It is necessary to assess the correct diagnosis, improve symptoms due to mass effect, enhance radiotherapy (RT) and chemotherapy (CT) effects, and ultimately prolong patients' survival. Moreover, many researchers have indicated that the extent of resection is of prognostic value [83]. However, considering a GBM has an average of 10^{11} cells, a 99% resection of the tumor would reduce the amount of cells to 10^9 cells. Therefore it's evident that surgery cannot be the ultimate treatment for such a disease. RT can potentially further reduce the tumor mass to 10^7 cells. However, the best chances to improve survival are expected from CT agents.

It has been shown that the alkylating drug temozolomide (TMZ) prolongs patients' survival when it is administered during and after RT as part of the first line therapy [89]. TMZ is a methylating prodrug derived from imidazotriazine. It is orally administered and has a 100% biodisponibility [8,66]. The compound is spontaneously hydrolyzed to the active product 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) at physiological pH [22,94]. MTIC methylates DNA at nucleophylic centres creating a variety of DNA adducts, including N^7-methylguanine, O^6-methylguanine, and N^3-methyladenine [22] (Figure 1). All of these adducts have a role in the cytotoxic potential of TMZ. However, the best evidence to date indicates that the mutagenic and toxic effects of TMZ are directly related to methylation of the O^6 position of guanine, and to the cellular response to the lesion [31].

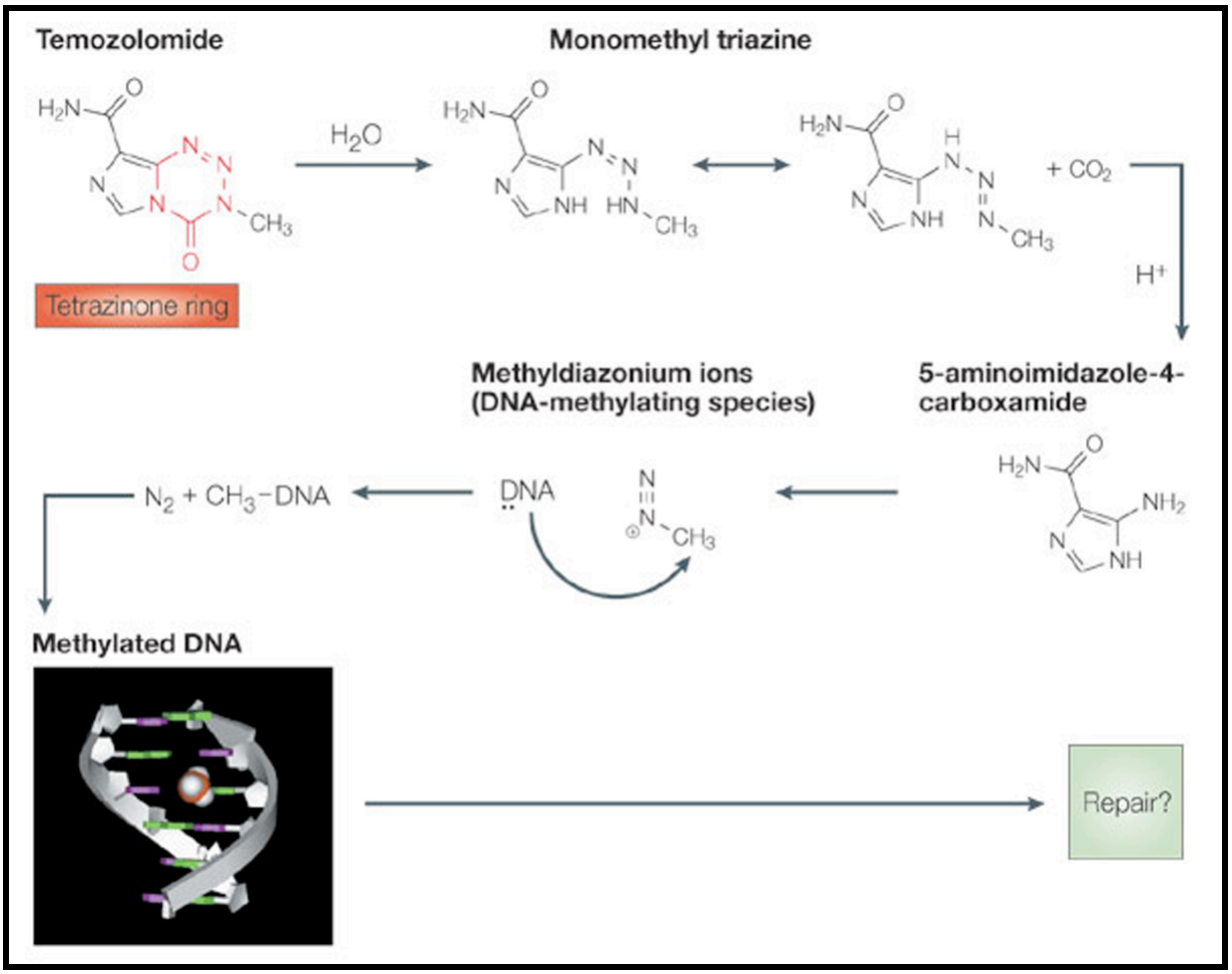


Figure 1. Temozolomide and its chemical reaction with DNA. Temozolomide acts as a prodrug, transporting a methylating agent (the methyldiazonium ion) to guanine bases within the major groove of DNA. The mechanism of activation involves chemical hydrolytic cleavage of the tetrazinone ring at physiological pH to give the unstable monomethyl triazene, which then undergoes further cleavage to liberate the stable 5-aminoimidazole-4-carboxamide and the highly reactive methyldiazonium methylating species. (Modified from Neidle S and Thurston DE, 2005 [65]).

O^6G methylation (O^6MeG), the most relevant TMZ-induced lesion, is repaired by O^6-methylguanine-DNA methyltransferase (MGMT), which directly transfers the methyl group from guanine oxygen to a cysteine residue (Cys145) on the MGMT molecule. This reaction irreversibly inactivates the enzyme [32,35,74-76]. In those cells where MGMT is low or absent, O^6MeG is not repaired, and consequently mispairs with thymine (T) instead of cytosine (C) during DNA replication [51]. Tumor cells with a competent mismatch-repair system (MMR) are able to remove the T residue, but they insert again a T because MMR does not repair the guanine methylation [1,24]. As a consequence, a futile MMR cycle develops [38,50]. This interferes during the following DNA replication cycle leading to double-strand breaks (DSBs) and ultimately to cytotoxic effect [70] (Figure 2). For this reason, an effective

therapy requires low MGMT levels, and a proficient post-replicative MMR system. Cells with high MGMT content or an impaired MMR system show a reduced sensitivity to alkylating agents. MGMT level significantly varies in tumor cells. Brain tumors usually express low MGMT levels [6,15,78,86], likely due to the methylation of MGMT gene promoter [27,28].

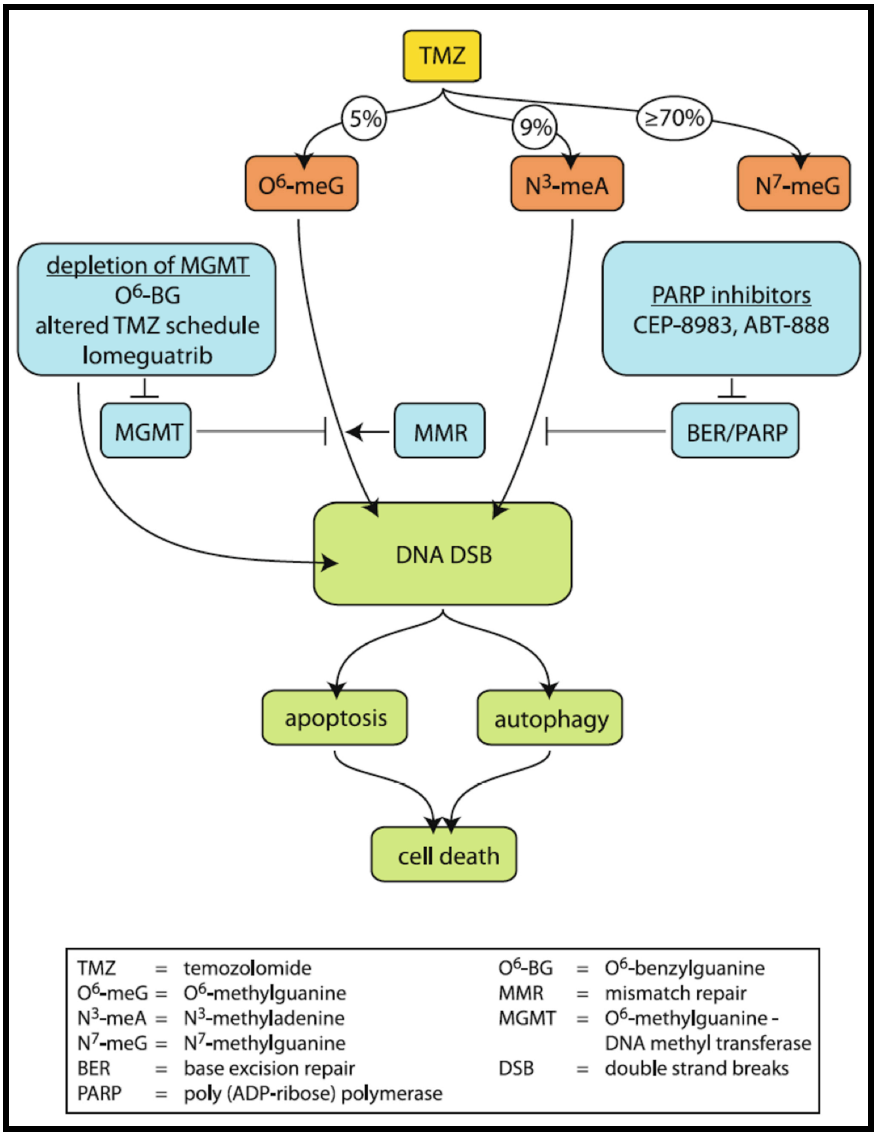


Figure 2. Temozolomide methylates DNA producing about a dozen of adducts. One of them, O⁶MeG, although representing only 5% of all adducts, is the most damaging lesion for the cell. Guanine O⁶-alkylation is repaired by MGMT enzyme. When MGMT is absent, a mismatch between O⁶MeG and thymine activates the Mismatch Repair System (MMR). MMR can act only on the most recently synthesized filament and is unable to remove the methylation on guanine. This causes futile MMR cycles that ultimately lead to DSBs and eventually to cell death.

Although the cellular response to methylating agents mainly depends on DNA repair systems, it has been shown that it's also related to cell cycle G2-checkpoint [41]. TMZ-induced double-

strand breaks (DSBs) trigger Chk1 and Chk2 through the activation of ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) phosphoinositide 3-kinase-related kinases. Chk1 phosphorylate Cdc25C phosphatase, which is therefore inactivated, and consequently Cdc2 remains bound to Cyclin B in an inactive, phosphorylated state [42]. The end result of DNA damage-induced Chk1 activation is G2/M arrest, which is supposed to give cells more time to repair the DNA damages caused by the drug before entry into mitosis [13]. More recently Wee1, which is responsible for Cdc2 phosphorylation, has been identified as a major determinant of the DNA damage-induced G2 arrest in GBM cells [60]. Aurora A and Polo-like kinase 1 (Plk1) have also been implied in promoting entry and progression through mitosis, and participating in a feedback activating loop with Cdk1 [9,57,96].

TMZ ultimately pushes human glioma cells towards different fates. Of course, cells can survive the treatment, and this is particularly true for cells undergoing a prolonged G2 arrest [41]. Conversely, TMZ can induce cell death through necrosis or mitotic catastrophe in a p53-independent manner, or a G2/M arrest followed by senescence [42]. Another study proposed that TMZ can induce autophagy but not apoptosis in glioma cells [49,64], while glioma cells grown as spheroids can undergo apoptosis after treatment with alkylating agents [36]. Most of the published studies excluded apoptosis as a consequence of TMZ treatment in glioma cells. However, a more recent paper showed that human glioma cells could undergo apoptosis in a dose- and time-dependent manner after treatment with methylating agents creating the specific O^6MeG DNA lesion, in a p53-dependent way [79]. It has been proposed that O^6MeG-induced DSBs can activate the ATM/ATR-Chk1 pathway, and stabilize p53. This would induce an increased expression of Fas receptor (FasR), a Caspase 8 activation, and apoptosis induction. Apoptosis in glioma after TMZ treatment can also be induced through a p53-independent pathway, involving Bcl-2 degradation and Caspase 9 activation [79]. The described model implies that DNA replication and cellular proliferation are essential for O^6MeG-induced apoptosis in glioma cells.

Although apoptosis induced by O^6-methylating agents has been extensively described in several experimental systems as rodent cell lines [70], lymphoblastic cells [25,40], and human peripheral lymphocytes [80], it is still enigmatic in brain gliomas, especially in relation with treatment protocols used in clinical practice, that are very often different compared to those used in the laboratory. Despite the well-known effectiveness of O^6-alchylating drugs in glioma

treatment, the median survival for GBM patients is still very low (12-14 months) [21]. For this reason, an improvement of glioma therapy is urgently needed. The possibility to induce apoptosis in gliomas with TMZ is particularly interesting, because it represents a finely controlled cell-death pathway. Moreover, it can avoid the inflammatory response activation and its related side effects.

Therefore, the first aim of our work was to verify the ability of TMZ to induce cell cycle arrest and apoptosis in glioma cell lines, with comparable TMZ dosage that is used on patients and fractionated into multiple treatments, as in clinical practice. Then we tried to better explore the relation between G2 checkpoint and apoptosis. However, fluorescent-activated cell sorting (FACS)-based cell cycle analysis provides just a static view of cell cycle profile, and considers the whole cells population. In order to focus on the single-cell level, and to analyze cell cycle in a dynamic setting, we used the FUCCI (Fluorescent Ubiquitination-based Cell Cycle Indicator) technique, a new set of chimeric proteins, which are able to visualize and track cell cycle progression in cultured cells [81,82]. This method exploits cell cycle-dependent proteolysis of the ubiquitination oscillators, Cdt1 and Geminin, to specifically mark the G1/S/G2/M transition in living cells. Cdt1 and Geminin are substrates of the ubiquitin E3 ligase complexes APC^{Cdh1} and SCF^{Skp2}, respectively [99]. While APC^{Cdh1} is most active during G1 phase, SCF^{Skp2} functions primarily during S, G2, and early M phases [3,55,58,59,91,101]. Consequently, Geminin and Cdt1 are specifically degraded during G1 and S/G2/M, respectively [69]. Therefore, by fusing the red- and green-emitting fluorescent proteins monomeric Kusabira Orange 1 (mKO1) and monomeric Azami Green 2 (mAG2) to Cdt1 and Geminin, respectively, it is possible to achieve striking contrast between the nuclei of cells in G1 phase (red) and those of cells in S/G2/M (green).

With the aim of increasing the cytotoxic response to TMZ, we studied its potential interaction with USP1 (ubiquitin specific peptidase 1). USP1 is an ubiquitin-hydrolase, which is able to remove a single ubiquitin molecule from target-proteins like PCNA (proliferating cell nuclear antigen) and FANCD2 (Fanconi anemia D2 protein) [45,68]. The catalytic activity of USP1 is largely increased upon formation of a complex with USP1-associated factor 1 (UAF1) [98]. PCNA ubiquitination is central to the normal DNA damage response process in eukariotes [4]. Monoubiquitination of PCNA is essential for translesion synthesis that promotes lesion bypass synthesis across the damaged base [48,104]. It has been shown that USP1 down-regulation

following DNA damage is associated with the activation of PCNA- and FANCD2-related DNA-repair systems, with the result of an increased cells survival [62,67,72]. Conversely, a persistent deubiquitination of PCNA can increase the cytotoxic effect of methyl-methane-sulfonate (MMS), an alkylating agent [67]. This effect is particularly interesting, as MMS is able to generate, besides ^{7}MeG and ^{3}MeA, also a small amount of O^{6}MeG, which is the most biologically-relevant lesion induced by TMZ. Because USP1 plays important roles in the two essential DNA damage response pathways, it represents a promising target to improve the efficacy of the commonly used DNA damaging drugs [14].

2. Materials and Methods

2.1 Cell cultures

Human GBM U87 cell line was obtained from the American Type Culture Collection (ATCC), human GBM U251 cell line and primary GBM8 cell line were obtained from the Tissue Core of the Brain Tumor Research Center, University of California, San Francisco. U87 is a PTEN-mutated, p53-wild type cell line, U251 is PTEN-mutated, and p53-wild type, xenograft-derived GBM8 is PTEN-wild type and P53-mutated. All cell lines were growth in DMEM-HG (Dulbecco's modified Eagle's medium-High Glucose, GIBCO®) supplemented with 10% bovine fetal serum and 1% Penicillin/Streptomycin (GIBCO/BRL®). Depending on the experiment, cells have been grown in 100mm dishes, 6-well plates, or 2-well coverglass chambers (Nalgene Nunc®) and incubated at 37°C in 95% humidified air with 5% CO_2. All the experiments were carried on 50-70% confluent cell cultures.

2.1.1 TMZ treatment

TMZ (Sigma, St. Louis, MO, USA) was dissolved into dimethyl-sulfoxyde (DMSO; Sigma, St. Louis, MO, USA), filtered, aliquoted, and stored at -20°C. GBM cell lines were treated with TMZ 100μM for 3 hours. The wells were washed with PBS (phosphate-buffered saline) and filled with new DMEM. In multiple treatment experiment, TMZ was administered in consecutive days. Cells were then trypsinized and harvested at 3 and 7 days after treatment.

2.1.2 Clonogenicity assays

Treated and control cells were plated at different concentrations (2000, 5000, 10000, and 20000 cells/well) in 6-well plates, and incubated at 37°C with a 5% CO_2. Fourteen days later, cells were stained with Methylen Blue (Sigma). Colonies of more than 50 cells were counted. Each experiment had been repeated at least 3 times.

2.1.3 siRNA transfection

Cells were plated into 6-well plates in order to obtain 60-70% confluency after 24 hours. Then they were transfected with either USP1-siRNA 500nm (Thermo Scientific Dharmacon ON-

TARGETplus SMARTpool) or with Control-siRNA 200nM (Thermo Scientific Dharmacon Accell) following the protocol. DharmaFECT 4 Transfection Reagent (Thermo Scientific Dharmacon) was used. Cells were then treated with TMZ after a 72-hour incubation at 37°C and 5% CO_2.

2.1.4 Creation of FUCCI-encoding glioma cells

In order to clone FUCCI (Fluorescent Ubiquitination-based Cell Cycle Indicator; MBL International Corporation, Woburn, MA, USA) plasmids (a fusion protein of a fragment of Cdt1 with monomeric Kusabira-Orange 2, mKO2-hCdt1, and a fusion protein of a fragment of Geminin with monomeric Azami-Green 1, mAG1-hGem), DH5a competent cells were first transformed and selected with kanamycin. Amplified plasmids were then purified using the QIAfilter Plasmid Midi kit (Qiagen, Valencia, CA, USA). The plasmids presence was verified with electrophoresis after digestion with XhoI restriction enzyme.

Glioma cells were plated to obtain 60-70% confluency after 24 hours. To develop cells expressing both vectors, glioma cells were serially infected with plasmid constructs encoding each protein (400ng/ml plasmid in 10cm plate + Fugene 6 Transfection Reagent). Cells were incubated at 37°C and 5% CO_2 for 48 hours. Transfected cells were selected in 200μg/ml G418 (Neomycin) for 5 days. In order to select only the double transfected cells, cells were subjected to FACS sorting (Fluorescence-activated cell sorting, FACS Aria III, Becton Dickinson, San Jose, California). Cells expressing Geminin (red) were first sorted, plated, and regrown for 1 week after which cells expressing Cdt1 (green) were similarly FACS sorted. The two detection channels we used for the FUCCI dyes were FITC (488nm laser with 530/30nm filter) and DS Red (561nm laser with 670/30nm filter). The identity of the double-sorted cells was then verified with Spinning Disk Confocal Microscopy and time-lapse imaging, and the G1 and G2 specificity of the constructs was confirmed by measuring the DNA content of transfected cells with the FACS Aria II Flow Cytometer.

2.2 Cytometry

Cytometry was used to study cell cycle arrest, apoptosis, and to select FUCCI-transfected glioma cells.

2.2.1 Cell cycle analysis

TMZ-treated and control cells were harvested after trypsinization, along with supernatant. After 3 PBS washing cycles and centrifugation at 800 rpm for 5 minutes, cells were fixed in ethanol 70% (vol/vol) and stored at -20°C. Cells were then washed with PBS and incubated in PBS with Propidium Iodide (PI) 40μg/ml and RNase A 200μg/ml for 15 minutes in dark at room temperature. Samples were processed with FACS Calibur and data analyzed with CellQuest Pro software. At least 10^4 cells were collected for each sample, and in triplicate. G1, S, and G2 populations were analyzed with ModFit LT software (Verity Software House, Topsham, ME, USA).

2.2.2 Apoptosis analysis

Apoptosis after TMZ treatment was measured using Annexin V-Propidium Iodide technique. TMZ treated and control cells were harvested after trypsinization along with supernatant. After 3 washing cycles in PBS and centrifugation at 800 rpm for 5 minutes, cells were tested with ApopNexin FITC kit (Millipore, Billerica, MA, USA) following the provided protocol. FACS Calibur (Becton Dickinson, San Jose, California) was used for cell detection. At least 10^4 cells were collected for each sample, and in triplicate. Vital, apoptotic, and necrotic cells were analyzed with FlowJo 8.8.6 software (Ashland, OR, USA).

Annexin V is a protein, which has a very high affinity for membranes containing the negatively charged phospholipid phosphatidylserine (PS). PS is exposed on the outer membrane at the very beginning of apoptosis, before DNA fragmentation. For this reason, Annexin V staining is considered the method of choice to detect apoptosis at an early stage. PS translocation to the outer cellular surface does not happen only during apoptosis, but also during necrosis. In the early stage of apoptosis, the cellular membrane is still intact; however, the membrane is disrupted during necrosis. Therefore, because this process is caused by early permeabilization of the cell membrane (lysis), necrosis is easily detected in vitro by exposure to a DNA binding dye such as PI. The exclusion of such a hydrophilic dye indicates cell viability. Cells in the metabolically active, early and middle stages of apoptosis do exclude the DNA binding dye, and are stained only by Annexin V-FITC.

2.2.3 Cell sorting

Hoechst 33342 solution (56μl from a stock of 1mg/ml; AnaSpec Inc., San Jose, CA, USA) was added onto FUCCI-marked glioma cells in a 10cm plate. After a 30-minute incubation, cells were harvested and analyzed with a FACS Aria III Cytometer. The detection channels used for FUCCI probes are FITC (488nm laser with 530/30nm filter), and DS Red (405nm laser with a 450/25nm filter). Hoechst 33342 was excited with UV radiation (405nm laser with a 450/25nm filter). An 85μm nozzle at 35PSI was used.

2.3 Imaging of cultured cells

FUCCI-transfected and selected cells were plated into Lab-Tek Chambered Coverglass with phenol red-free DMEM, 10% FBS, and 1% Penicillin/Streptomycin. A Spinning Disk Confocal Microscope, equipped with incubator, motorized cart, autofocus, and an AxioCam high-resolution monochromatic camera was used for time-lapse imaging. The microscope registered images sequences on 3 channels (FITC, DsRed, and DIC) at 16 bit every 15 minutes for 4 days. A PlanAPO 20X (NA 0,4) objective was used. All the images were acquired with AxioVision 3.1 software.

2.4 Immunoblotting

GBM cells were washed with PBS and lysed in buffer containing 20nmol/L Tris-HCl pH 7.4, 100nmol/L NaCl, 300mmol/L sucrose, 6mmol/L $MgCl^2$, 1mmol/L EGTA, 0.5% Triton X-100, 1X PhosStop (Roche), and protease inhibitor cocktail (Roche) for one hour on roller in cold room. The concentration of the lysates was measured using Protein Assay reagent (Bio-Rad Laboratories) and 50μg of proteins were subjected to SDS-PAGE electrophoresis and transfer onto Immuno-Blot PVDF membranes (Bio-Rad Laboratories). The membranes were blocked in 1X TBS containing 5% milk and 0.05% Tween-20 for 1 hour, incubated with primary antibody diluted in blocking buffer overnight at 4C°, washed with 1X TBS-T for three times, and incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnologies) diluted in blocking buffer for 1 hour. Protein expressions were detected by using ECL reagents (Amersham Pharmacia Biotech). Primary antibodies were obtained from Santa Cruz (beta-

actin and Geminin), Dharmacon (USP1), or Cell signaling (Cdt1, Cdc2, pCdc2, Cdc25c, pCdc25c, Chk1, pChk1, Chk2, pChk2, p53, Wee1, pWee1).

2.5 Immunocytochemistry

Cells were plated in Lab-Tek 2 Chamber Slide (Thermo Scientific), treated with TMZ for 3 hours, and analyzed for Phospho-Histone H2AX expression after 2 days. Cells were fixed with 4% PFA for 15 minutes at room temperature, washed 3 times, and permeabilized with 0.2% Triton for 5 minutes at room temperature. After washing them three times, cells were blocked with 10% FBS in PBS for 1 hour, incubated with Anti-gamma H2A.X phospho S139 (Abcam) 1:500 in 10% FBS + 0.1% Triton in PBS for 1 hour at room temperature, washed 3 times, and incubated with 488 anti-mouse secondary antibody (Invitrogen) 1:500 in 10% FBS + 0.1% Triton in PBS for 30 min at room temperature in dark. After 3 washes, the chamber was removed, and mounting media containing DAPI was added onto the cells. Slides were analyzed by Zeiss AMZ AxioObserver fluorescent microscope.

2.6 Statistical analysis

Kolmogorov-Smirnov's non-parametric test and t Student's test were used for averages comparison.

3. Results

3.1 Multiple TMZ treatments reduce glioma cells clonogenicity

In order to determine whether different protocols of TMZ administration (1, 2, or 3 doses in 3 consecutive days, 3 hours 100μM TMZ per treatment) have different effects on the ability of glioma cells to form colonies and proliferate, we performed clonogenicity studies with TMZ-sensitive U251 and U87 glioma cell lines. A single TMZ treatment can induce a 10 times reduction of colonies compared to controls. Colonies are further reduced of 80% after a second dose, and undergo a further reduction of 40% after the third administration (Figure 3).

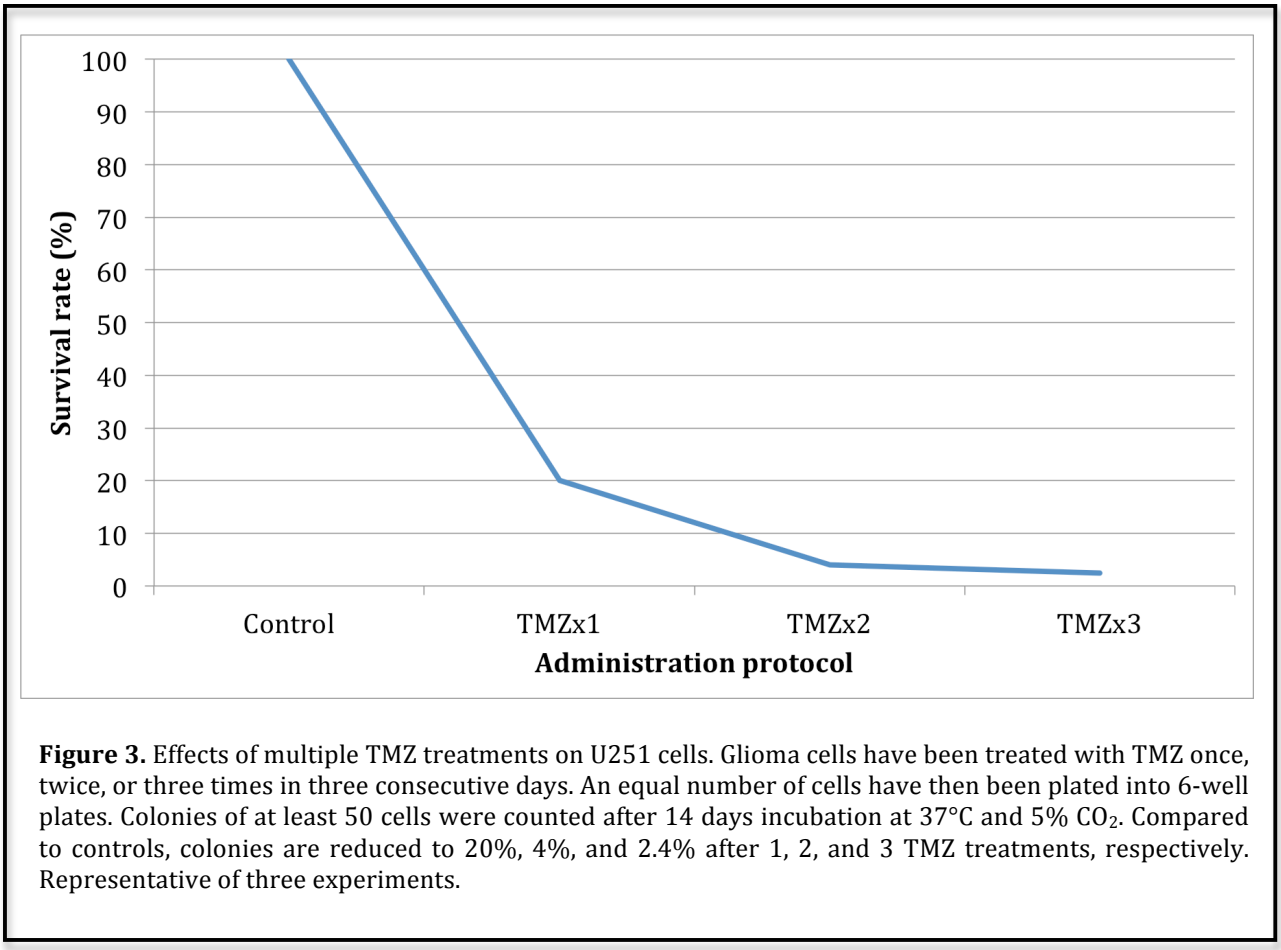


Figure 3. Effects of multiple TMZ treatments on U251 cells. Glioma cells have been treated with TMZ once, twice, or three times in three consecutive days. An equal number of cells have then been plated into 6-well plates. Colonies of at least 50 cells were counted after 14 days incubation at 37°C and 5% CO_2. Compared to controls, colonies are reduced to 20%, 4%, and 2.4% after 1, 2, and 3 TMZ treatments, respectively. Representative of three experiments.

3.2 Multiple TMZ treatments don't significantly increase the number of G2/M arrested cells in TMZ-sensitive cells compared to single treatment

We first confirmed G2/M arrest after a single TMZ treatment in U87 and U251 cells, and no arrest in TMZ-resistant GBM8 cells. Afterwards we focused on the effects of multiple TMZ treatments on cells undergo G2 arrest. Therefore, we designed a study comparing 3 different treatment protocols (1, 2, or 3 TMZ administrations in 3 consecutive days). Cells were initially exposed to 100μM TMZ. TMZ was removed after 3 hours; cells were gently washed with PBS, and incubated in fresh medium. Cells were harvested and analyzed 3 days after the last TMZ treatment, as G2/M arrest is generally evident at this time (Figure 4).

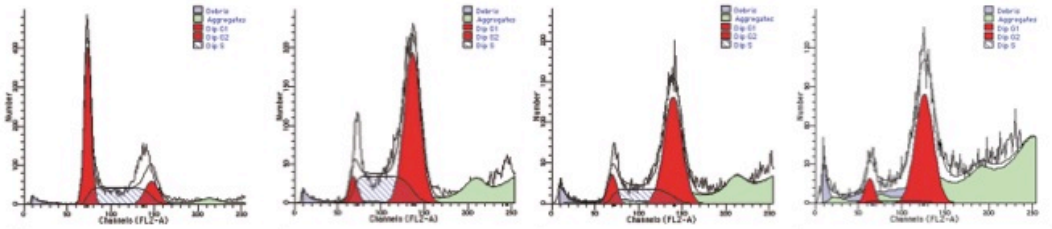
Day	1	2	3	4	5	6	7	8	9	10
Day post-TMZ				1	2	3	4	5	6	7
Harvesting						X				X
TMZx1			X							
TMZx2		X	X							
TMZx3	X	X	X							

Figure 4. GBM cell lines were plated in 6-well plates. After 24-hour incubation, TMZ 100μM was administered for 3 hours daily, for 1, 2, or 3 consecutive days. Cells were harvested at day 3 and 7 after the last TMZ treatment for cytometry testing.

A fluorescence-activated cell sorting-based cell cycle analysis with a FACS Calibur showed that TMZ induces a G2/M arrest in U87 and U251 cell lines, but has no effect on cell cycle in GBM8 cells (Figure 5). In TMZ-sensitive cell lines, the increase of G2/M arrest after multiple drug administrations is not significant (Figure 5).

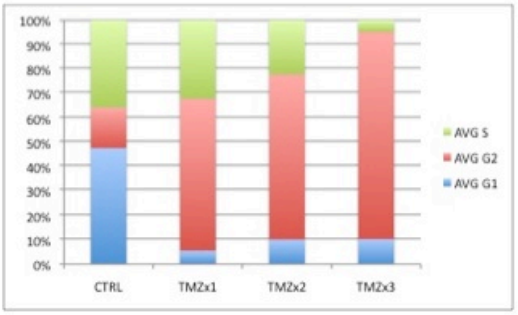
I U87 – Day3

A

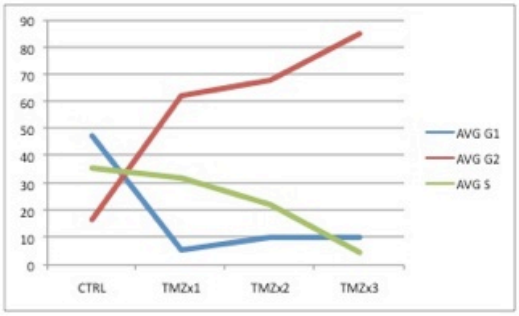


G1	47.56	5.56	10.12	10.22
G2	16.76	62.30	67.68	85.11
S	35.67	32.12	22.19	4.66

B

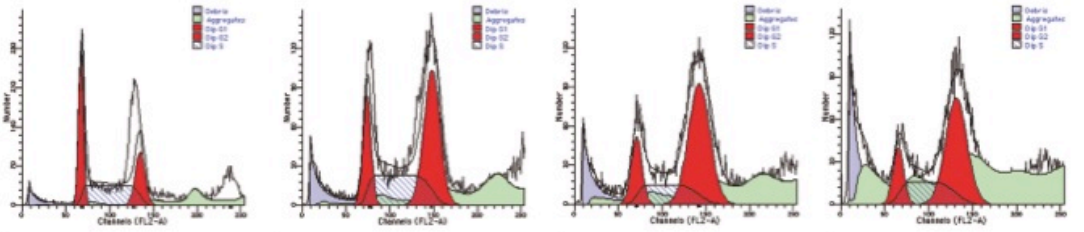


C



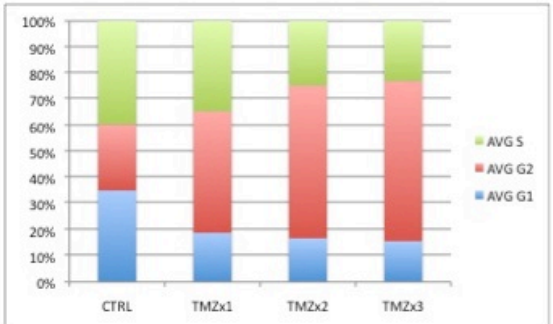
II U251 – Day3

A

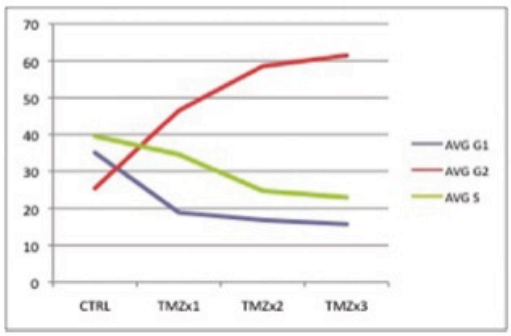


G1	35.07	18.84	16.77	15.64
G2	25.38	46.54	58.54	61.44
S	39.54	34.61	24.68	22.91

B



C



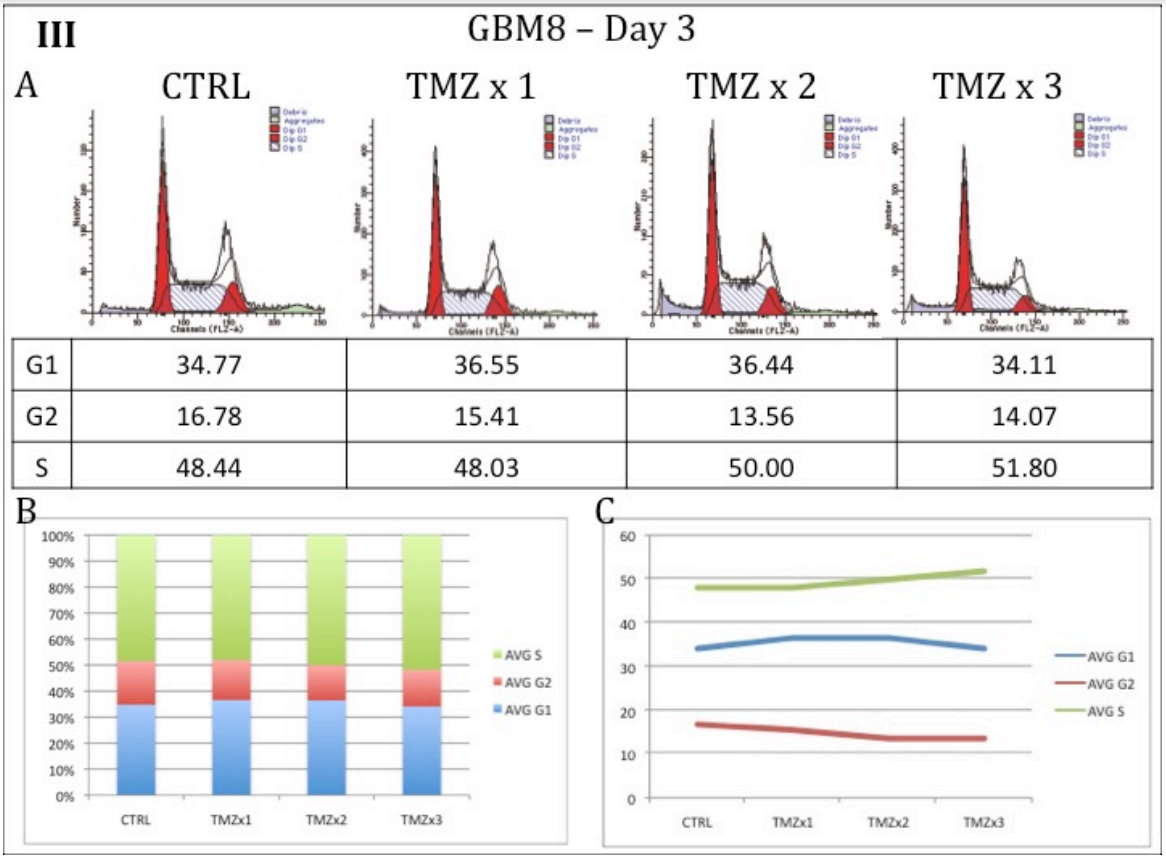


Figure 5 (pages 15 and 16). Effect of multiple TMZ treatments on cell cycle progression. Panels I, II, and III are referred to U87, U251, and GBM8 cells, respectively, 3 days after treatment. G1 and G2 peaks after cytometry processing are shown in quadrants A. Quadrants B and C graphically show cell cycle progression in control and TMZ-treated cells. Representative of three experiments.

3.3 TMZ induces apoptosis 3 days after treatment

Apoptosis was studied with the Annexin V-PI method. This technique allows visualizing cells during early and middle stages of apoptosis.

Only a small amount of cells are apoptotic three days after TMZ treatment. Slight variations of response among cell lines may occur. Moreover, the differences between different administration protocols are statistically significant. Multiple TMZ treatments significantly increase apoptosis compared to controls ($p=0.001$) and single-treatment ($p=0.001$) in TMZ-sensitive cell lines (Figure 6). U251 is the most sensitive cell line, with a 17.3% apoptotic cells after three TMZ administrations. Not surprisingly, TMZ-resistant GBM8 cell line does not undergo apoptosis.

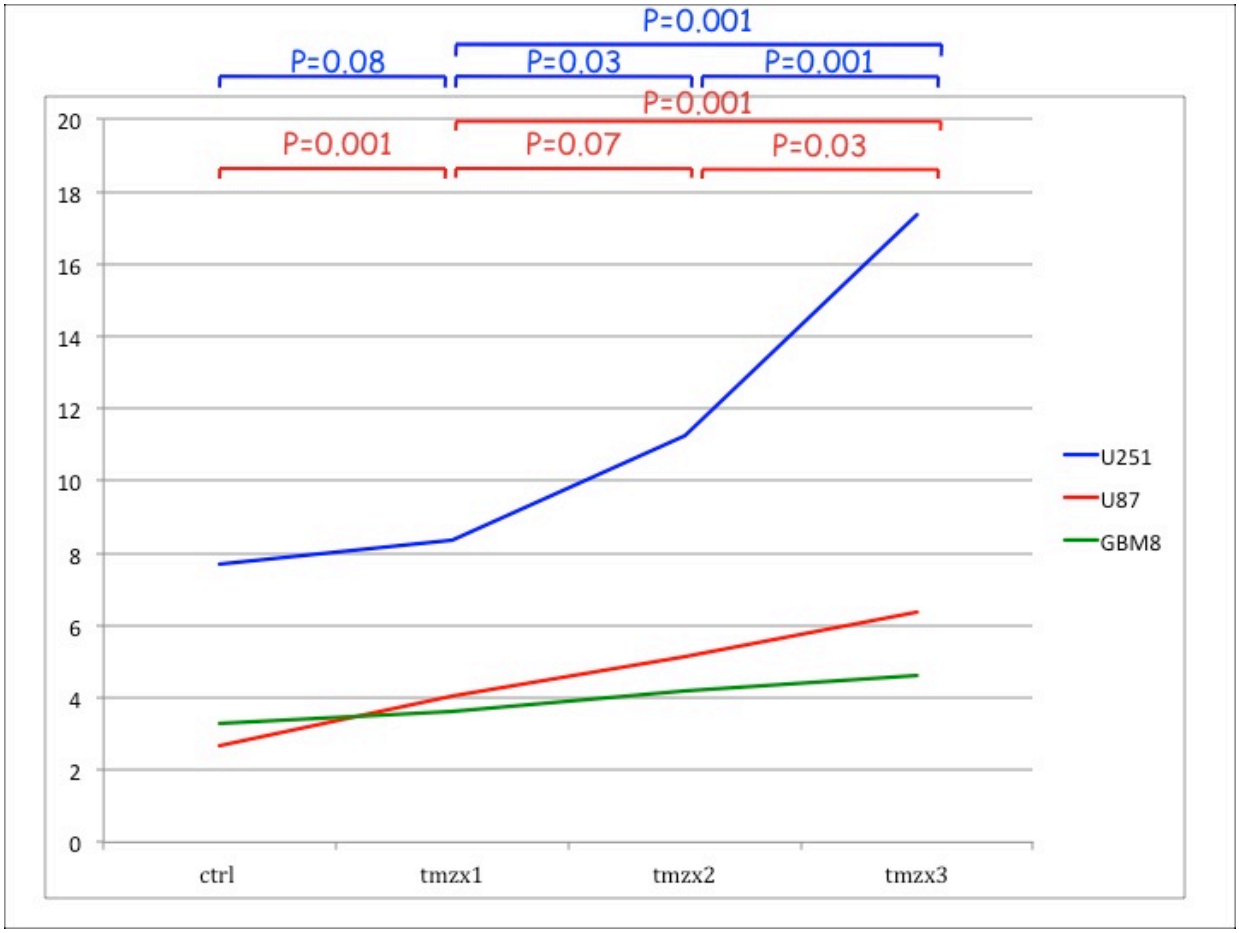


Figure 6. Rate of apoptotic cells 3 days after the last TMZ treatment. Representative of three experiments.

3.4 Apoptosis significantly increases 7 days after TMZ treatment, and with multiple treatments

Apoptosis significantly increases 7 days after TMZ treatment, with obvious variations depending on the different cell lines. Particularly, after three TMZ treatments, apoptotic cells are 15.4% in U87 and as much as 30.7% in U251. Differences between the administration protocols are statistically significant ($p<0.001$) (Figure 7).

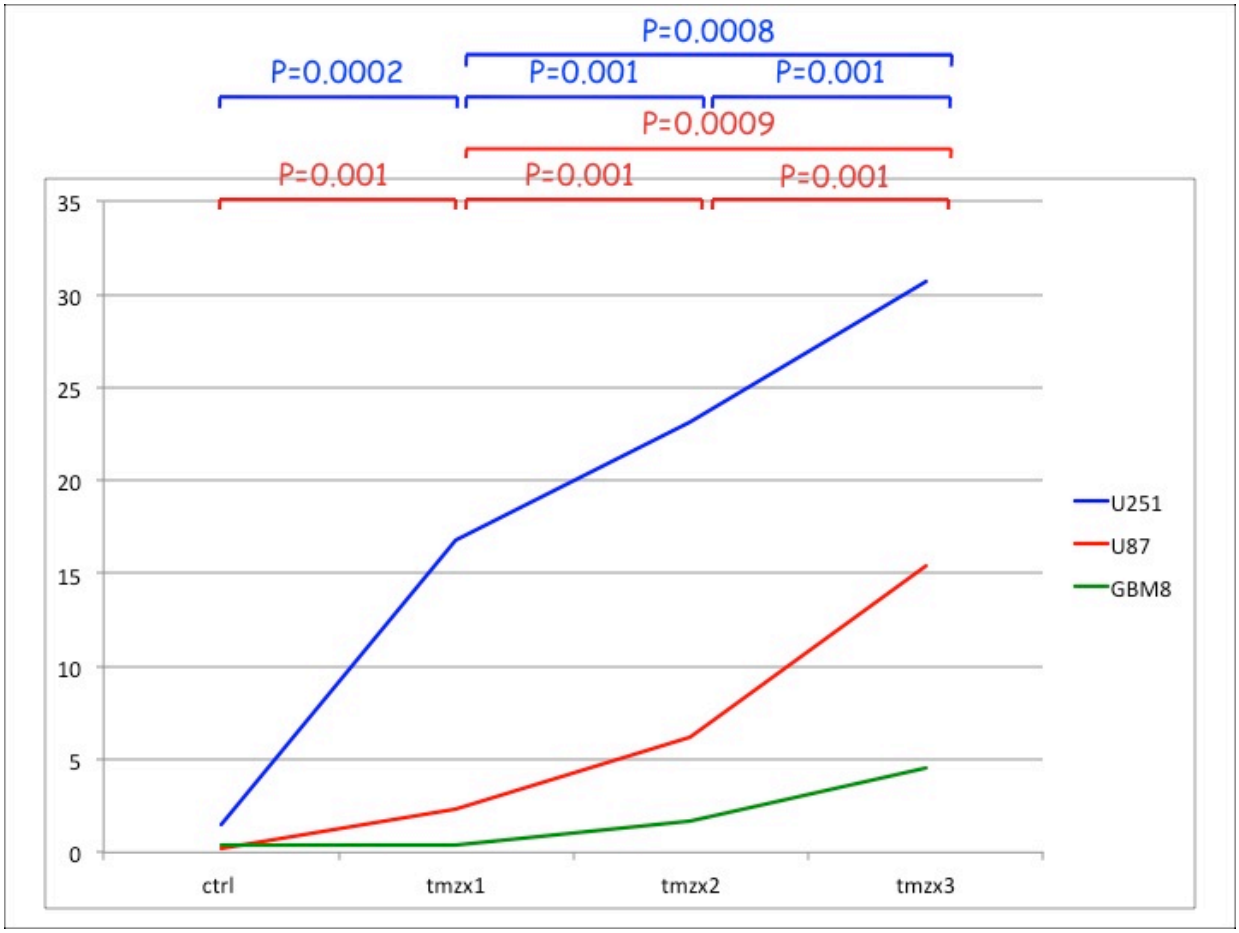


Figure 7. Rate of apoptotic cells 7 days after the last TMZ treatment. Representative of three experiments.

3.5 Transfection with FUCCI does not change biological characteristics of U87 cells

It is interesting that the number of apoptotic cells increased when undergoing multiple TMZ treatments, although the cell cycle profiles were similar among the 3 different treatment protocols. Therefore, in order to address this question, we decided to use FUCCI probes to study the cell cycle progression in TMZ treated cells at the single-cell level. U87 cells were co-transfected with both FUCCI plasmids and double sorted, as already explained. The G1- and G2-specificity of the constructs was confirmed with fluorescence-activated cell sorting-based DNA quantification and immunoblotting (Figure 8). We confirmed that the two fluorescent replication-licensing factors used, Cdt1 and Geminin, are only present during G1 and S/G2/M phases of the cell cycle, respectively (Figure 8). Therefore, in U87FUCCI cells the color of nuclei correlates with the phase of the cell cycle.

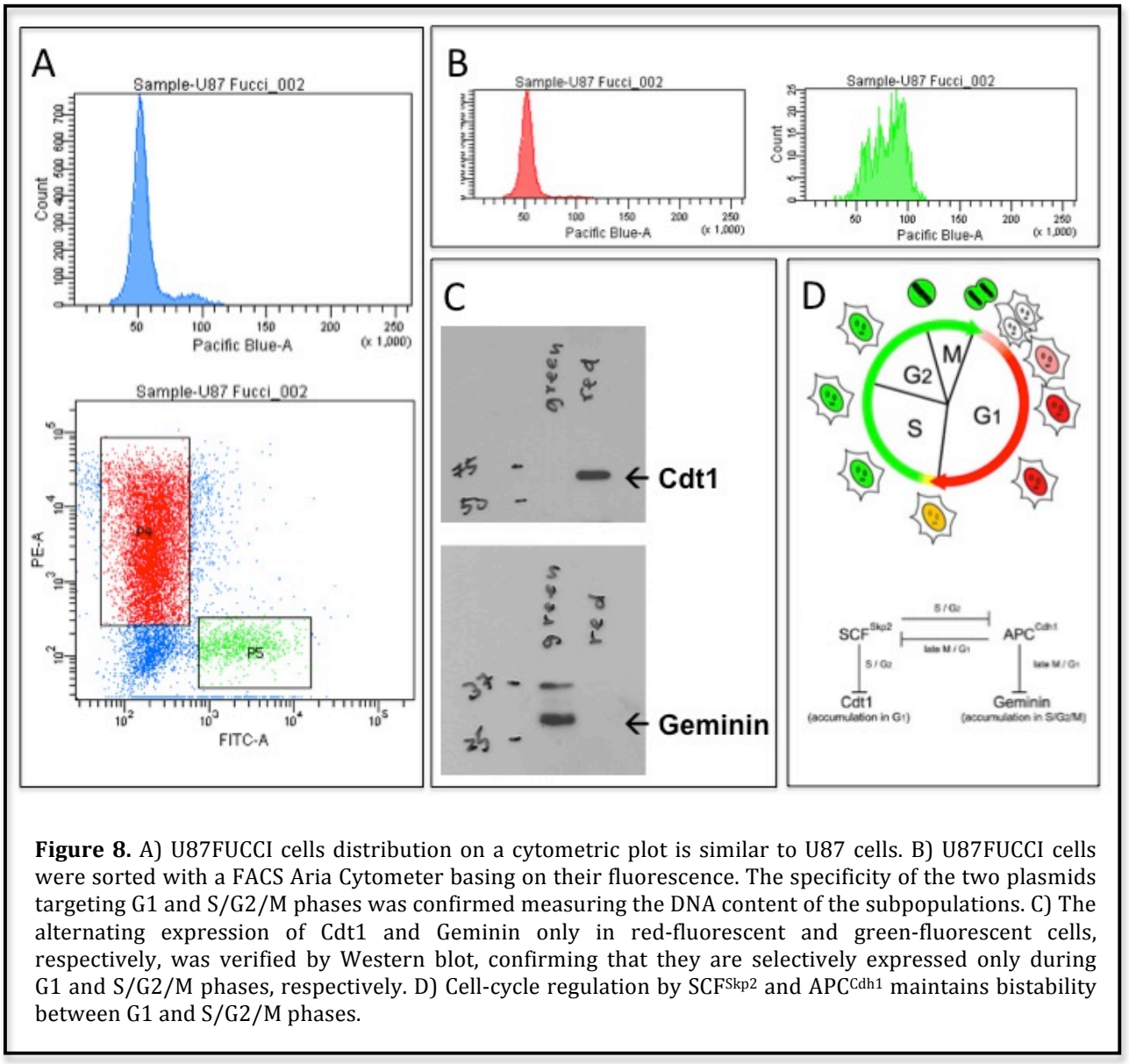


Figure 8. A) U87FUCCI cells distribution on a cytometric plot is similar to U87 cells. B) U87FUCCI cells were sorted with a FACS Aria Cytometer basing on their fluorescence. The specificity of the two plasmids targeting G1 and S/G2/M phases was confirmed measuring the DNA content of the subpopulations. C) The alternating expression of Cdt1 and Geminin only in red-fluorescent and green-fluorescent cells, respectively, was verified by Western blot, confirming that they are selectively expressed only during G1 and S/G2/M phases, respectively. D) Cell-cycle regulation by SCF^{Skp2} and APC^{Cdh1} maintains bistability between G1 and S/G2/M phases.

Then, we verified that the characteristics of FUCCI transfected U87 cells are similar to wild-type U87 cells in terms of response to TMZ. Similarly to U87 cells, 2 days after TMZ exposure U87FUCCI cells undergo a G2-arrest, which lasts at least until day 5, as shown with fluorescence-activated cell sorting-based cell cycle analysis (Figure 9A). We also showed phosphorylation of H2AX histone 2 days after TMZ treatment, confirming that TMZ causes DSBs in U87FUCCI cells (Figure 9B). We also confirmed that TMZ-induced activation of MMR promoted induction of Chk1, Chk2, and phosphorylation and inactivation of the Chk1 kinase substrate Cdc25C. This event is temporally associated with phosphorylation of Cdc2 and with the onset of TMZ-induced G2-M arrest [41,42]. Consistently, we found a reduction of the inactive phosphorylated Wee1 and the induction of p53, which have been associated with G2-

M checkpoint activation [43] (Figure 9C). The expected effect of TMZ on U87FUCCI cells proliferation was also proved with a proliferation assay (Figure 9D).

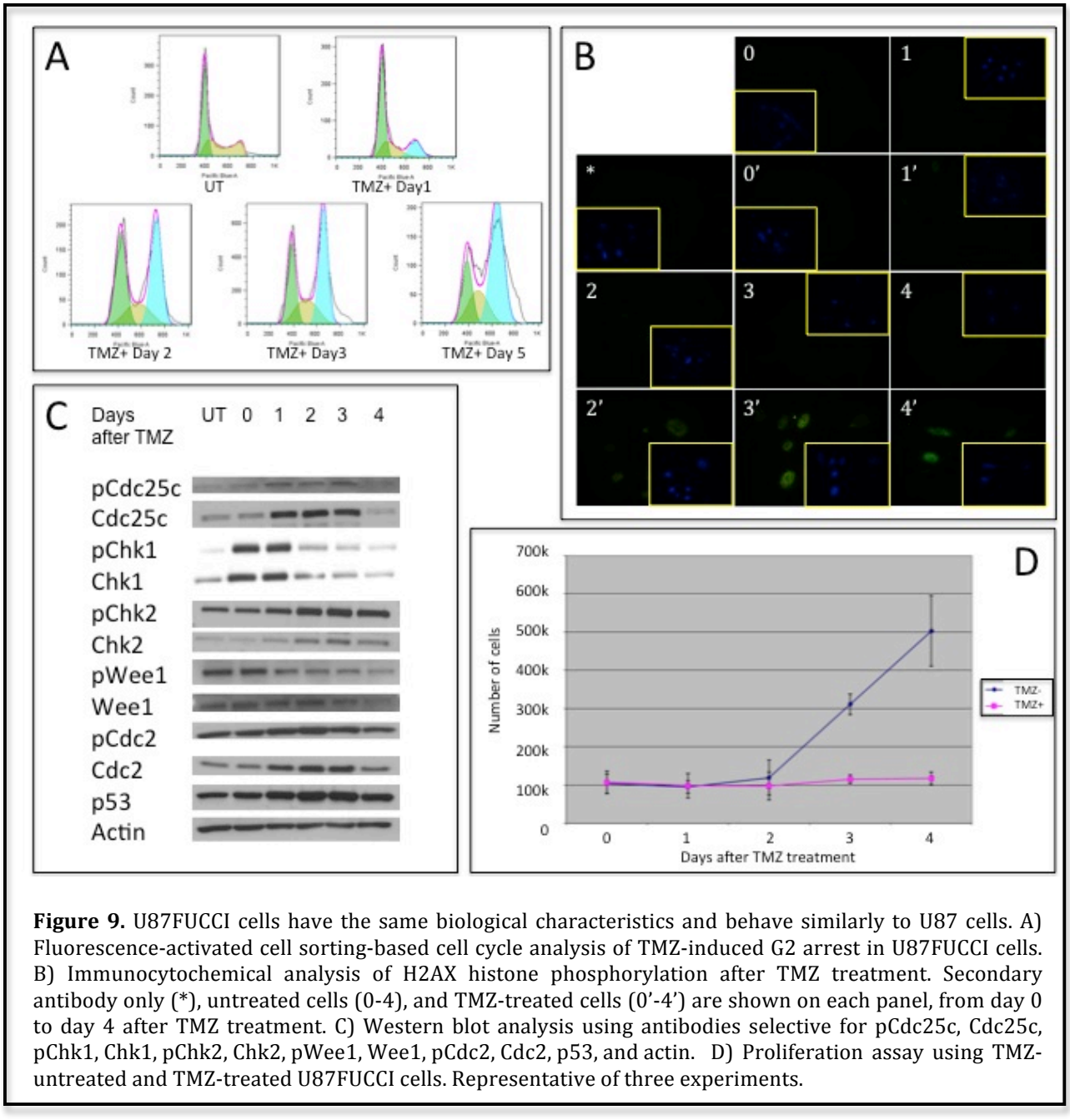
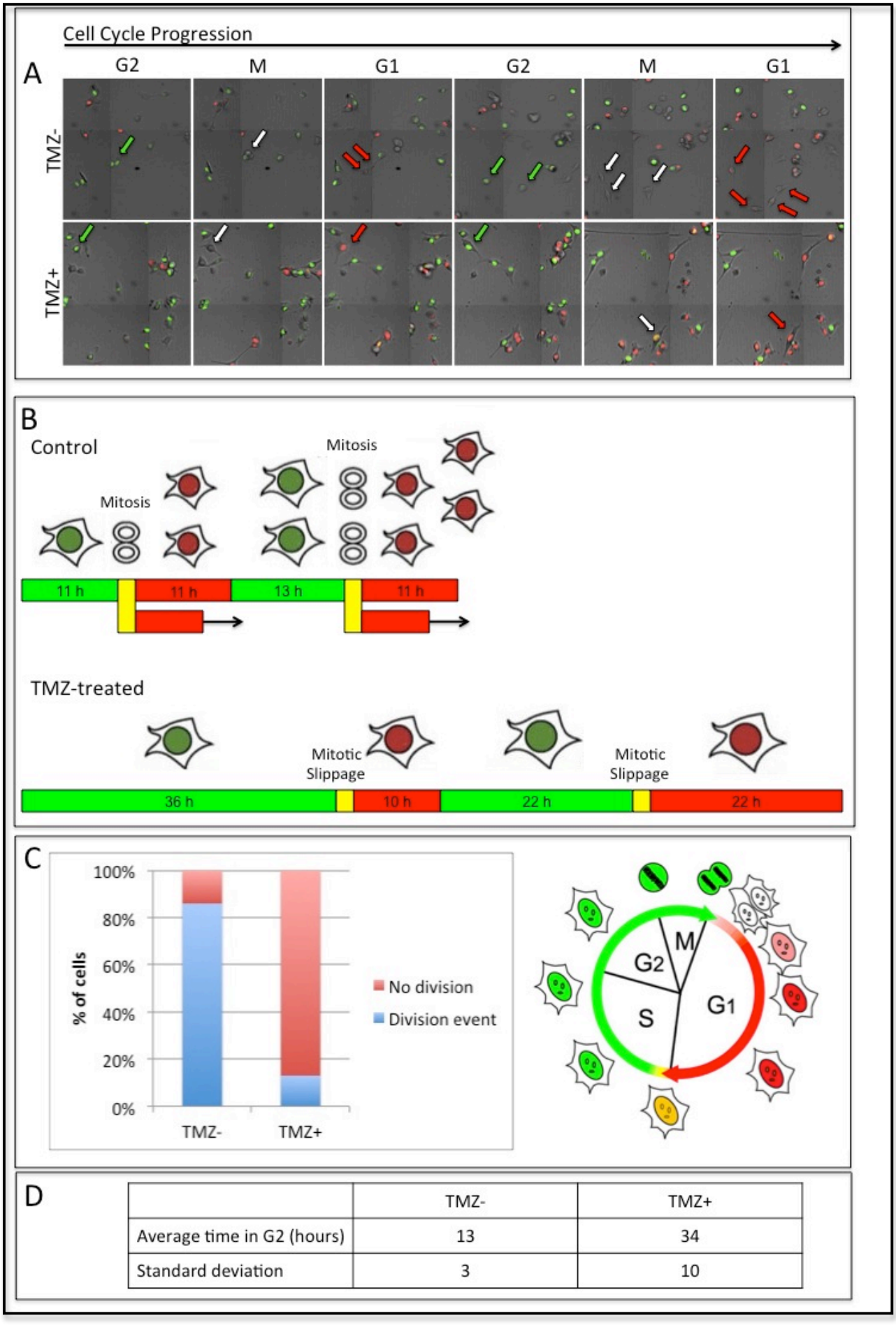


Figure 9. U87FUCCI cells have the same biological characteristics and behave similarly to U87 cells. A) Fluorescence-activated cell sorting-based cell cycle analysis of TMZ-induced G2 arrest in U87FUCCI cells. B) Immunocytochemical analysis of H2AX histone phosphorylation after TMZ treatment. Secondary antibody only (*), untreated cells (0-4), and TMZ-treated cells (0'-4') are shown on each panel, from day 0 to day 4 after TMZ treatment. C) Western blot analysis using antibodies selective for pCdc25c, Cdc25c, pChk1, Chk1, pChk2, Chk2, pWee1, Wee1, pCdc2, Cdc2, p53, and actin. D) Proliferation assay using TMZ-untreated and TMZ-treated U87FUCCI cells. Representative of three experiments.

3.6 TMZ induces mitotic slippage and G2-delay, not G2-arrest, in U87 cells

Transfected cells were then tracked at the microscope with time-lapse imaging after TMZ treatment. We anticipated green cells to divide during mitosis and turn red, as they progress from G2 through M and into G1 phases. This was exactly what we observed tracking TMZ-untreated cells (Figure 10A top).



D

	TMZ-	TMZ+
Average time in G2 (hours)	13	34
Standard deviation	3	10

Figure 10 (previous page). A) Time-lapse imaging of TMZ-untreated and TMZ-treated U87FUCCI cells. Green arrows point out green-fluorescent G2-phase cells; red arrows indicate red-fluorescent G1-phase cells; white arrows show cells during mitosis (M), which can appear either non-fluorescent or yellow. B) Graphic representation of timing of cell cycle progression for cells visualized in panel A. C) Percentage of cells that didn't divide in M phase in control cells and TMZ-treated cells (mitotic slippage; 14% and 87%, respectively). We considered all cells that were traceable during the 90-hour experiment. We excluded divisions in the first 24 hours, as TMZ has no detectable effects during the first day after treatment. D) Table showing the average time (±standard deviations) spent in green (G2) phase by TMZ-untreated and TMZ-treated cells.

About 86% of control cells progressed through the cell cycle as expected (Figure 10C). Conversely, 87% of TMZ-treated cells progressed from green to red without dividing (Figure 10A bottom, and Figure 10C). Although those cells were still cycling, as confirmed by the color change during time-lapse, they failed entering mitosis. Then, we focused on G2 phase, and we found that most of U87 cells don't permanently arrest after TMZ treatment. However, the time spent during G2 phase is longer after TMZ treatment compared with untreated cells (average G2-time 34±10 hours and 13±3 hours, respectively. Figure 10B and 10D).

3.7 USP1 knockdown increases TMZ sensitivity in GBM cells

In order to verify the role of USP1 in glioma cells response to TMZ, a clonogenicity assay was performed plating different amounts of U251 cells in 6-well plates, and in triplicate. Cells were treated with either a control or an anti-USP1 siRNA (Figure 11).

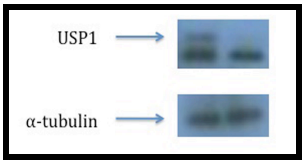


Figure 11. USP1 knockdown using siRNA. All the experiments were performed using cells with at least 80% USP1 knockdown.

In this way we showed that TMZ sensitivity is increased in USP1-knockdown cells. Particularly, cells with low or absent USP1 have a 70% reduction in proliferation and colony formation ability (Figure 12).

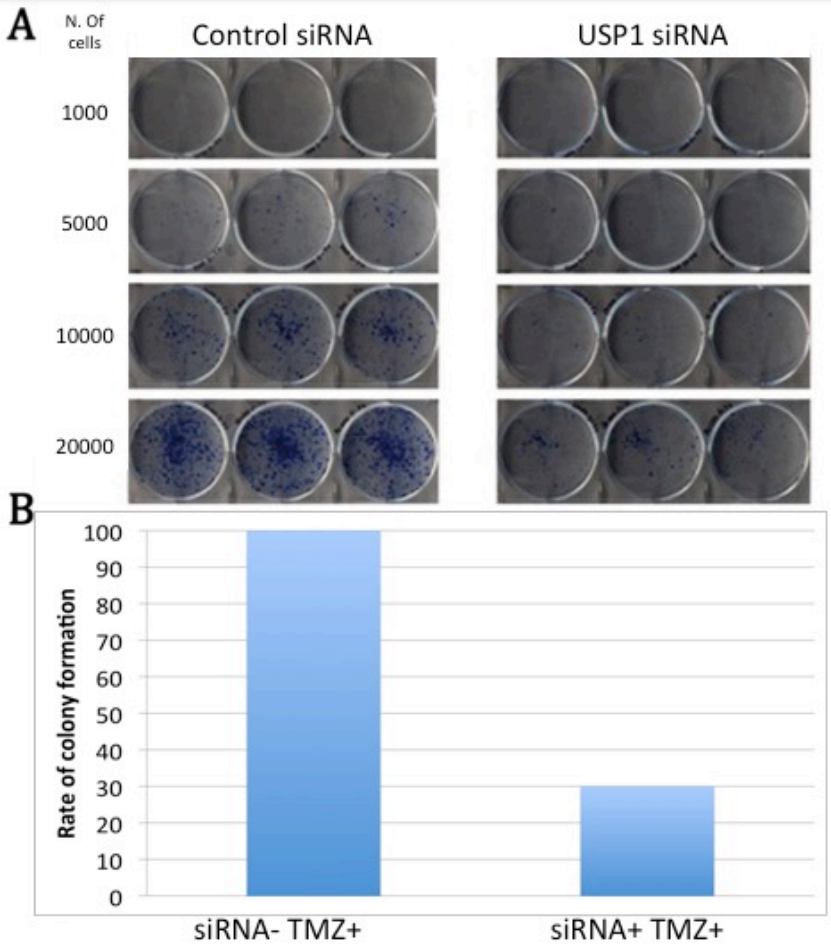


Figure 12. Clonogenicity assay using U251 cells treated with both TMZ and siRNA against USP1. A) Colonies of U251 cells treated with either scramble or USP1 siRNA, after TMZ exposure. B) USP1 increases the resistance to TMZ in glioma cells. Compared to controls (cells treated with scramble siRNA and TMZ), cells with a low amount of USP1 undergo a clonogenicity reduction of about 70%. Representative of three experiments.

A similar study was performed using GBM8 cells, which had been shown to be TMZ resistant in terms of both G2/M arrest and apoptosis induction. Surprisingly, USP1 knock down increases TMZ effects also in GBM8 cells, reducing their proliferation ability and decreasing colony formation capability (Figure 13).

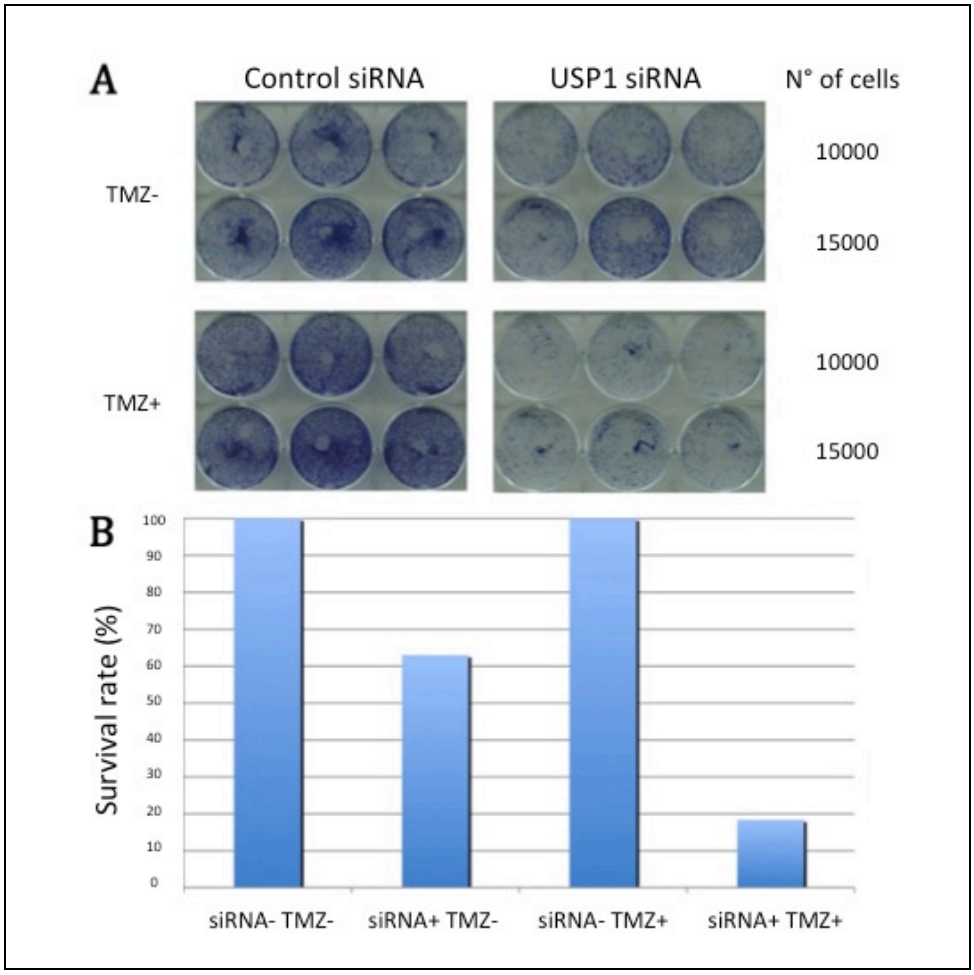


Figure 13. A) Clonogenicity assay after TMZ treatment using GBM8 cells exposed to either scramble or USP1-targeting siRNA. B) Histogram showing that USP1 knockdown alone causes a 30% reduction of clonogenicity compared to controls. USP1 knockdown enhances TMZ effect, with a clonogenicity decrease of more than 80%, despite TMZ resistance. Representative of three experiments.

3.8 USP1 knockdown, alone or in combination with TMZ, has no effect on cell cycle

In order to verify whether the clonogenicity reduction induced by TMZ in USP1 knockdown cells depends on cell cycle, we performed a fluorescence-activated cell sorting-based cell cycle analysis. We showed not only that USP1 knockdown has no effect on cell cycle progression, but also that it does not significantly affect the TMZ-related G2 arrest (Figure 14).

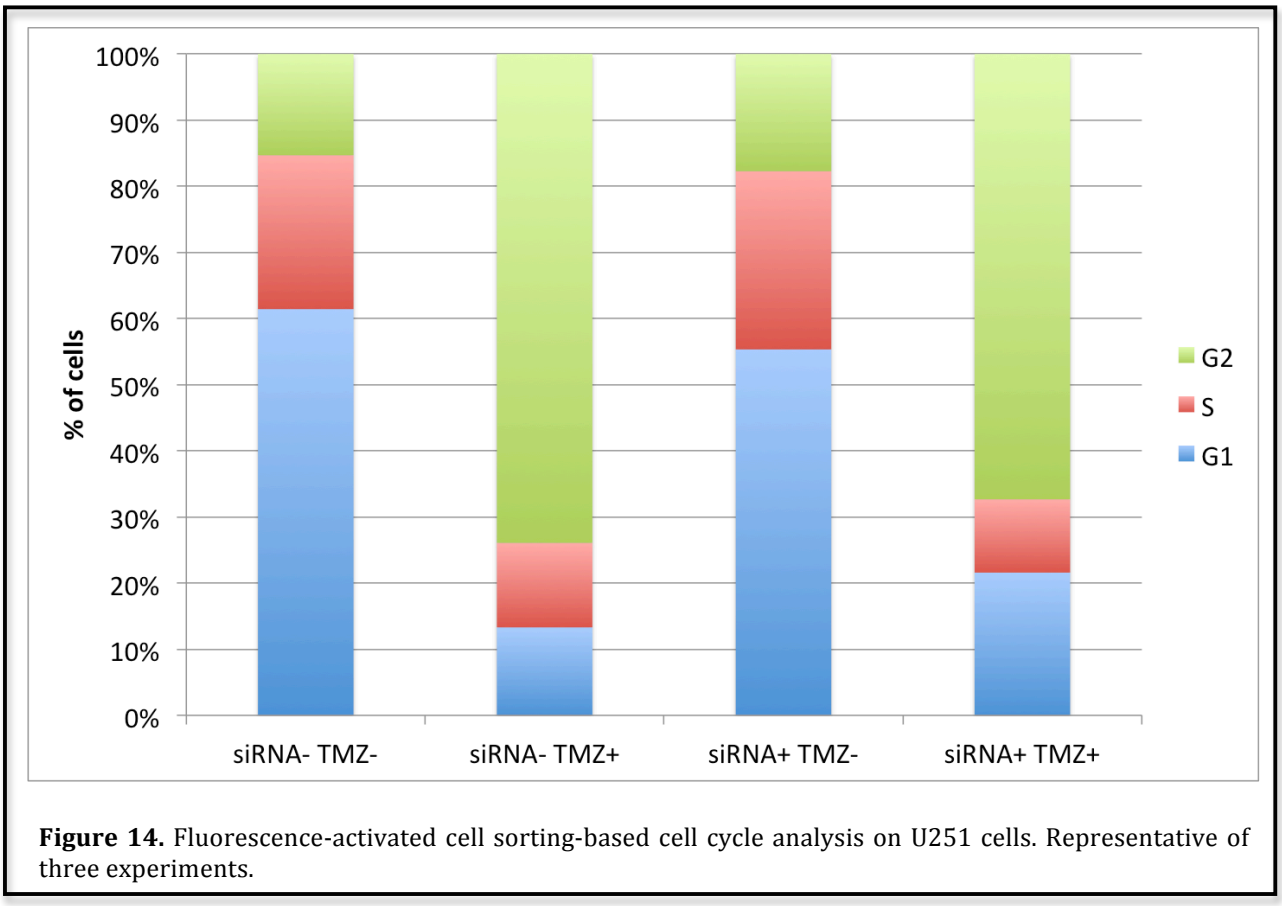


Figure 14. Fluorescence-activated cell sorting-based cell cycle analysis on U251 cells. Representative of three experiments.

3.9 USP1 knockdown enhances TMZ pro-apoptotic effect

We then verified the possible action of USP1 on TMZ-induced apoptosis. To this purpose, cells exposed to either scramble siRNA or siRNA targeting USP1 were treated with a single 100μM TMZ dose for 3 hours, and tested 7 days later with Annexin V-PI technique.

In TMZ-sensitive U251 cells, while USP1 suppression alone determines only a modest increase of apoptosis (15.9%; controls 2.3%; p=0.07), it significantly enhances the proapoptotic effect of TMZ (37.7%; controls 4.6%; p=0.004) (Figure 15).

The same effect is visible also in TMZ-resistant GBM8 cell line. TMZ-induced apoptosis is significantly higher in USP1-knockdown cells (30.7%) than USP1-knock down TMZ-untreated cells (p=0.001), USP1-wild type TMZ-treated cells (p=0.0008), and USP1-wild type TMZ-untreated cells (p=0.0004) (Figure 16).

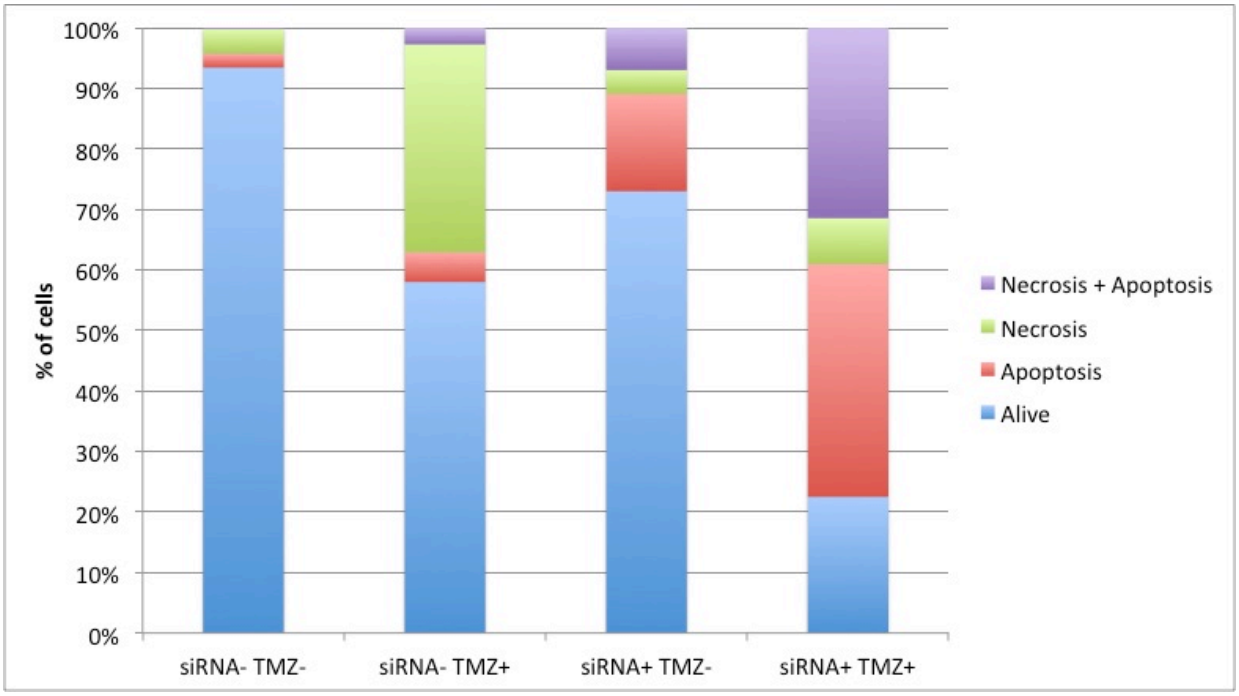


Figure 15. Fluorescence-activated cell sorting-based apoptosis analysis on U251 cells (Annexin V-PI method). Representative of three experiments.

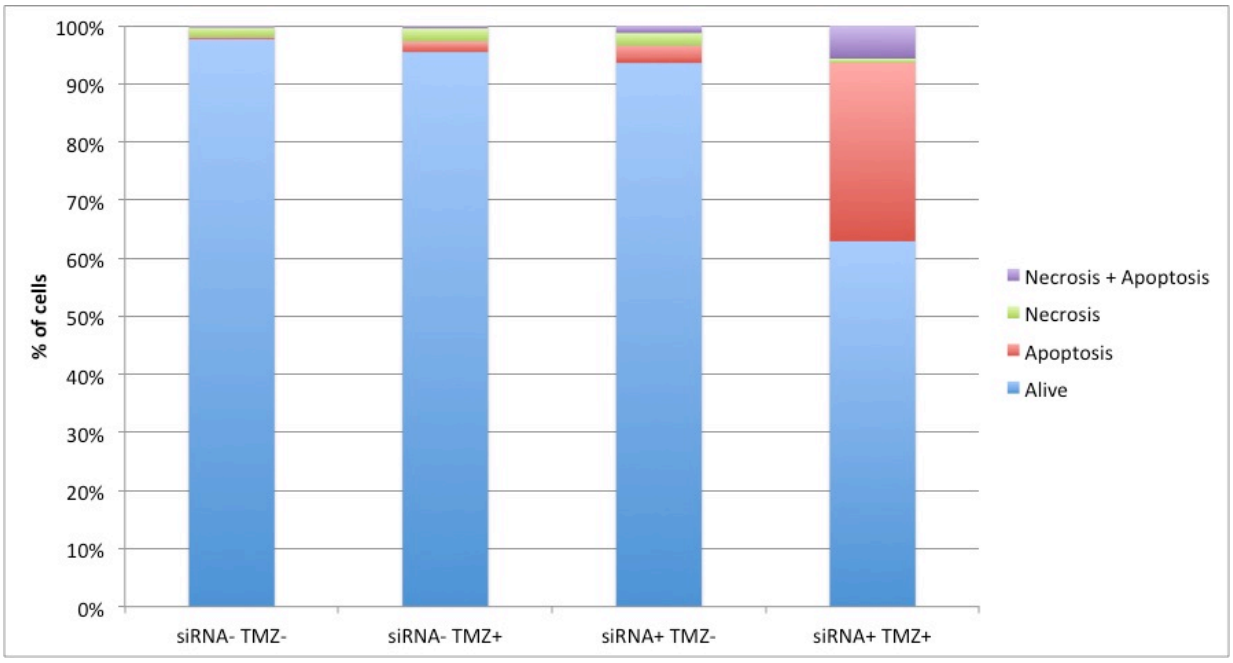


Figure 16. Fluorescence-activated cell sorting-based apoptosis analysis in GBM8 cells (Annexin V-PI method). TMZ or USP1 knockdown have no effect alone (1.8% and 2.9% apoptosis, respectively; controls 0.3%). Representative of three experiments.

4. Discussion and future directions

Patients affected by glioblastoma multiforme have a dismal prognosis. Besides surgery and RT, standard treatments currently include methylating agents as TMZ, and chloroethylating agents like carmustine and nimustine. However, TMZ is currently much more used than chloroethylating agents because of its reduced side effects. Despite the fact that TMZ is the most used chemotherapeutic agent currently used in clinical practice for the treatment of GBM, and its molecular pathways have been well described, the resistance developed by tumor cells still limits the great potentials of this drug. Therefore, this commonly used O^6G-methylating drug requires a deeper insight into the mechanisms through which it carries its genotoxic effect on glioma cells, so that the treatments for future patients can be improved. The current protocol for TMZ is a daily dose of 150 to 200mg per square meter of body-surface area for 5 days of every 28-day cycle [89]. TMZ has demonstrated schedule-dependent antitumor activity in xenografts derived from glioma [30,77,87]. However, there is lack of studies focusing on the molecular reasons of the effectiveness of a multiple-treatment schedule for TMZ. Therefore, we first focused on different treatment schedules with single agent TMZ, in order to study the effects on cell cycle progression and cell death.

Initially, we confirmed that TMZ increases the percentage of cells in G2/M phase of the cell cycle in TMZ-sensitive U87 and U251 cell lines, but not in TMZ-resistant GBM8 cell line, as expected. GBM8 resistance is due to MGMT expression. The G2 checkpoint is regulated by a number of kinases and phosphatases, which are finely balanced [57]. The G2 checkpoint activation after DNA damage induced by TMZ is a well-defined process (Figure 17).

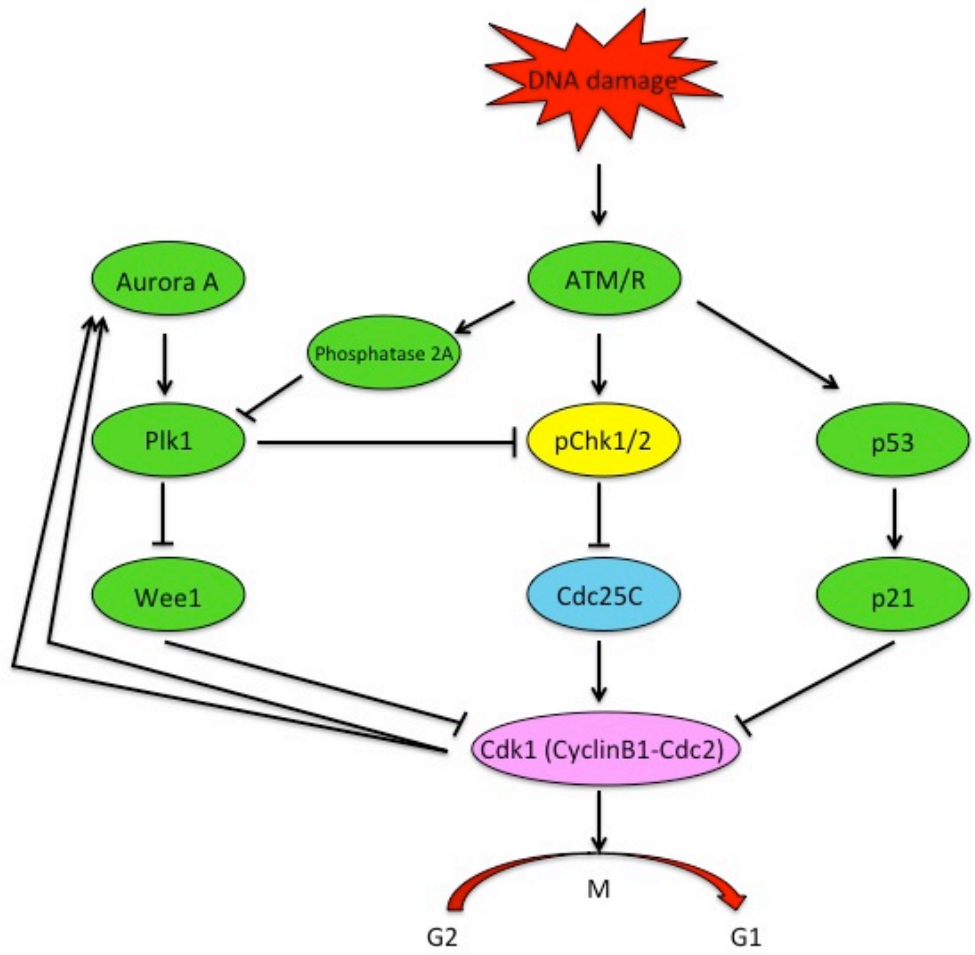


Figure 17. The final activation of G2 checkpoint is controlled by Cdk1 (cyclin-dependent kinase). When Cdk1 is phosphorylated, it is unable to promote mitosis. Cdk1 phosphorylation depends on proteins of Cdc25 phosphatase family. In their active, non-phosphorylated form, Cdc25A, Cdc25B, and Cdc25C dephosphorylate and activate Cdk1. Cdc25 proteins are controlled by Chk1 and Chk2. When Chk1 and Chk2 are activated by DNA damage, they phosphorylate and inactivate Cdc25 proteins.

After determining that colony formation significantly decreases with the number of TMZ doses, we initially explored the possibility of an increased G2 arrest to explain our results. However, the modest increase of G2/M arrest obtained with multiple TMZ doses is not statistically significant. Indeed, the percentage of G2-arrested cells is very high (over 80%) after the first dose of TMZ. Cell cycle arrest occurring after DNA damage is a defense mechanism that allows the cell enough time to repair it [5]. However, the cell fate is extremely variable during and after arrest. The cells can actually survive after the DNA damage. This phenomenon often occurs in glioma cells particularly after a prolonged G2/M arrest. Conversely, the cells can also undergo mitotic catastrophe, necrosis, senescence, or autophagy, which ultimately lead to cell death.

Apoptosis as a consequence of methylating drugs in gliomas has always been denied. In 2007, a German group was able to detect apoptosis in glioma cells with the cytometric method of sub-G1 peak [79]. They claimed apoptosis is a late effect of TMZ, which is visible only at least 4 days after drug administration. Apoptosis represents the ideal model for drug-induced cell death, as its finely controlled steps can avoid any inflammatory response. For these reasons, we focused on the apoptotic response of glioma cells following different TMZ administration protocols, and the relation between apoptosis and cell cycle.

We confirmed that apoptosis is possible in sensitive glioma cell lines after TMZ administration. Different to previously published papers, we showed that a limited amount of apoptotic cells are visible even 3 days after treatment. This difference can be due to the fact that Annexin V-PI is a more sensitive method than sub-G1 peak to detect early apoptosis.

Moreover, multiple TMZ treatments significantly enhance apoptosis, especially after the third administration. However, the amount of cells undergoing apoptosis is greater 7 days after the third administration. Also the differences between the administration schedules are statistically more evident at a late stage. Of course, response can vary in different cell lines, with an apoptosis rate ranging from 15% in U87 cells to more than 30% in U251 cells at day 7. Therefore, apoptotic response after TMZ significantly increases in a time- and schedule-dependent manner, and is most clear in a late phase.

It has been shown that O^6MeG can activate an apoptotic pathway only in cells that are actively cycling and proliferating [79]. Surprisingly, cells that are mostly arrested after the first TMZ treatment can undergo apoptosis, and apoptosis rate increases in relation with the number of treatments, although cell cycle is not significantly affected by administration schedule. In order to further explore these contrasting findings, and to find the relation between cell cycle and apoptosis, we moved from a population-study to a single cell-level. FUCCI probes allow tracking single live cells, visualizing cell cycle phases over time. The system is based on the fact that Cdt1 and Geminin, which are replication-promoting factors, are only present during specific phases of the cell cycle. Geminin is linked to a green-fluorescent protein and stains S/G2/M cells. Cdt1 is linked to a red-fluorescent protein and stains G1 cells (Figure 8).

As a preliminary study, the ability of the FUCCI system to accurately monitor cell cycle progression in glioma cells was first examined. U87 cells transfected with constructs encoding

mKO2-hCdt1 or mAG1-hGem were first sorted for cells expressing red fluorescence (Cdt1), after which the cells were grown for 24 hours and resorted for cells expressing green fluorescence (Geminin). In this way we created a population containing and expressing both constructs. FACS-based analysis of the mixed population revealed a high degree of correlation between red cells with 2N DNA content and green cells with 4N DNA content, consistent with the idea that Cdt1 is a marker of cells in the G1 phase of the cell cycle, and Geminin is a marker of cells in the G2 phase.

U87FUCCI cells were then exposed to TMZ and monitored for response. Similarly to U87 cells, U87FUCCI cells exhibited H2AX foci beginning 2 days after TMZ exposure, and also activated the G2 checkpoint, increasing phosphorylation of Chk1, cdc25C, cdc2, Wee1, and p53. In these cells, the DNA damage and the supposed G2/M arrest lasted for at least 4 days following TMZ treatment, and this observation is consistent with the inability to resolve TMZ-induced DNA damage or to inhibit the checkpoint activation. Consistent with previous observations, the cells also underwent what appeared to be a G2 arrest with cells accumulating with 4N DNA beginning 2 days after TMZ exposure and remaining arrested through the 4 days of the study [41-43].

In parallel studies, control and TMZ-treated cells pre-selected for expression of both FUCCI constructs were grown in control environment chambers and followed by time-lapse fluorescence microscopy for cell cycle progression. In control cells, the initial unsorted, unsynchronized population was a mixture of red and green cells consistent with distribution across all phases of the cell cycle. Following individual cells over the 90 hours of the experiment revealed that the cells toggled between red and green expression with the S/G2/M (green) phases of the cell cycle lasting an average of 12-14 hours and the G1 (red) phase lasting an average of 20 hours. Conversely, TMZ-sensitive glioma cells do not undergo prolonged G2 arrest but rather arrest at the G2 checkpoint for less than 48 hrs. Furthermore, these cells do not bypass the checkpoint and die during mitosis, but rather in the presence of an activated G2 checkpoint, skip mitosis entirely, re-enter the cell cycle at the next G1 phase, replicate their DNA, and repeat the cycle, ultimately giving rise to TMZ-resistant polyploid cells. Actually mitosis is a delicate phase of the cell cycle. Cells skipping mitosis might possibly avoid cell death and become TMZ-resistant. These findings are consistent with FACS-based cell cycle data showing a G2 arrest, as FACS offers just a static picture of the treated cells,

which are not arrested, but delayed in G2 phase. Of course, these results need to be confirmed in more TMZ-sensitive and TMZ-resistant cell lines. Moreover, it would be interesting verifying if cells undergoing mitotic slippage can still form colonies. This would suggest that mitotic failure is not only a resistance mechanism for the single cell, but also a prerequisite for regrowth.

The G2 checkpoint is part of a defense system used by the cells to resist from genotoxic insults. In the presence of DSBs and stalled replication forks, the ataxia-telangiectasia mutated (ATM) and the ataxia-telangiectasia Rad3-related (ATR) activate checkpoints transmitting signals to Chk1 and Chk2 [11,37,90,95]. ATM and ATR transmit signals to major downstream targets, such as Chk1 and Chk2 [44]. These kinases negatively regulate Cdc25 phosphatase family proteins that dephosphorylate Cdks involved in the cell cycle transition [7]. This can lead to failure of the cell to enter mitosis and proceed to G1 phase of the cell cycle (Figure 17).

Mitotic slippage has already been described as one of the three causes of tetraploidization during tumorigenesis, along with cell fusion and failure to complete cytokinesis [18,33]. Tetraploidization has been proposed as an intermediate step toward aneuploidy in human cancer [88]. Aneuploidy is expected to allow tumor cells to sustain a higher incidence of mutations, thereby increasing the probability of adaptive changes [93]. For this reason, we hypothesize that TMZ-induced aneuploidy could represent a mechanism of resistance for tumor cells treated with methylating agents. It has been shown that permanently damaged telomeres can induce bypass of mitosis and endoreduplication [19]. In a recent paper, Hyun et al showed that DNA damage induces ATM/Chk1 pathway-dependent re-replication without cell division in HeLa cells [46]. As TMZ activates the same pathway, we hypothesize that ATM/Chk1 pathway can be involved to explain our findings as well. G2 checkpoint is also regulated by Wee1 [26,52,54,71,97,100]. G2 checkpoint abrogation by Wee1 inhibition has been shown to result in mitotic catastrophe due to unrepaired DSBs in glioblastoma [60]. All these molecules are involved in promoting mitosis and are affected by TMZ. Therefore, future studies should address the possible role of such pathways in TMZ-induced mitotic slippage in GBM. A better comprehension of the involved mechanisms can potentially offer new tools to improve treatment of glioblastoma. For example, it would be worth verifying whether pushing cells into mitosis might increase the cytotoxic potential of TMZ.

Besides opening new perspectives to explain the molecular effects of TMZ in glioma, mitotic failure could also be an intriguing way to find an explanation for increased apoptosis after multiple TMZ treatments. Apoptosis has been described as an abortive entry into cell cycle, given the morphological similarities between apoptosis and mitosis [85]. A number of studies have associated molecules that are clearly linked to cell proliferation, such as Myc or E2F-1, with apoptosis [29,84]. Moreover, apoptosis has been described as a possible consequence of mitotic failure [12,16,47,103]. New studies with FUCCI-expressing cells treated with multiple TMZ doses might help clarifying this phenomenon.

DNA repair in response to methylating agents such as temozolomide has recently been shown to require monoubiquitination of PCNA and FANCD2, and loss of either protein, or inhibition of their monoubiquitination, enhances methylating agent-induced toxicity [67,72]. Monoubiquitination of both proteins is reversed by the deubiquitanse USP1, suggesting that USP1 may be a key controller of temozolomide sensitivity [39]. Actually, ubiquitination not only marks the proteins directed to degradation, but it can also lead them to specific subcellular localizations and promote protein-protein interactions [45].

Because USP1 has the potential to deubiquitinate PCNA and FANCD2 and to suppress DNA repair, we anticipated that USP1 levels in GBM might be lower than in normal controls. Analysis of expression array data from three separate databases however suggested that USP1 mRNA levels were consistently elevated in a large percentage of GBM [34,73,92]. Consistent with this observation, USP1 mRNA and protein levels were high in most GBM cell lines examined. siRNA-mediated suppression of USP1 had no effect on cell cycle progression or the extent of temozolomide-induced G2 arrest, suggesting that the extent of temozolomide-induced DNA damage in USP1-deficient cells was comparable to that in control cells. Surprisingly, while USP1 knockdown alone had no effect on cell death, it increased temozolomide-induced loss of clonogenicity both in cells that were sensitive or resistant to temozolomide. Further examination of the mechanism of cell death suggested that while control cells, control cells exposed to TMZ, or USP1-suppressed cells rarely underwent apoptotic cell death, temozolomide-treated cells in which USP1 levels were suppressed underwent high rates of apoptosis. These preliminary results raise the question about the molecular pathways involved by USP1 in the prevention of apoptosis. Further studies will have to first clarify the role of MGMT and caspases cascade.

The deubiquitinase USP1 has been indirectly suggested to inhibit the function of PCNA and FANCD2, to inhibit the repair of methylating agent-induced DNA damage, and to sensitize cells to DNA damage [48] (Figure 18).

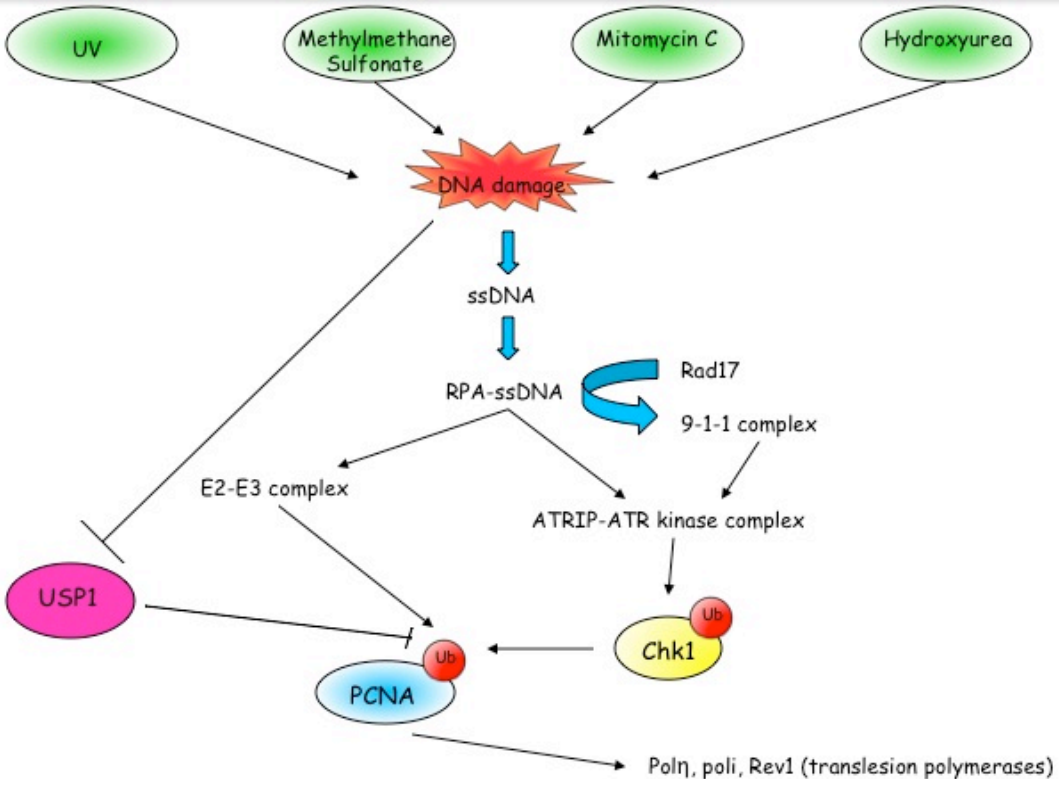


Figure 18. PCNA pathway regulation. PCNA is an structural and functional component, which is essential for the stability of DNA replication fork. Monoubiquitinated PCNA mediates DNA translesional synthesis. After DNA damage, Rad6 and Rad18 (E2-E3 complex) allow PCNA monoubiquitination. Monoubiquitinated PCNA increases its affinity for translesion polymerases, and the cell can therefore bypass DNA lesion and replicate. PCNA ubiquitination is controlled by USP1 in human cells.

When PCNA is not monoubiquitinated, cells are more sensitive to UV radiations and methylmethane sulfonate (MMS) [67]. As the levels of PCNA monoubiquitination are inversely related to USP1 levels after UV-induced DNA damage, USP1 expression has been associated to a reduced survival of cells after genotoxic damage [67]. Conversely, after treatment with MMS and mitomycin (MMC), PCNA monoubiquitination has not been related to a reduction of USP1, which rather increases at a later stage [67]. Two hypotheses have been proposed to explain these results. Monoubiquitinated PCNA could somehow sequester and ultimately allow the removal of USP1. Otherwise, different types of DNA damage could differently modulate USP1 activation acting on UAF1 molecule [67]. Basing on these divergent observations, it is not possible to assign a clear role to USP1 regarding the PCNA-mediated cellular response after

genotoxic damage. Another study showed that USP1 suppression is well tolerated in human cells, and gives resistance to DNA damage, increasing monoubiquitinated FANCD2 levels [68]. The FA pathway (Fanconi Anemia pathway) has actually a crucial role for the repair of secondary lesions, as DNA DSBs and replication arrest, produced when the primary lesion O^6MeG is processed by MMR system [2,53,63,102] (Figure 19). However, the same authors report that the impossibility to deubiquitinate FANCD2 may lead to homologue recombination without DNA damage, and ultimately to chromosome instability. Recently, Oestergaard et al showed that in chicken cells the persistent monoubiquitination of FANCD2 due to USP1 knockdown causes a hypersensitivity to DNA crosslinking. Therefore, the authors propose for USP1 a function of activator, and not inhibitor, of FA pathway. In this model, USP1 would limit the accumulation of FANCD2 on chromatine only after DNA damage [72]. New studies are needed to understand the role of USP1 also in the regulation of FANCD2.

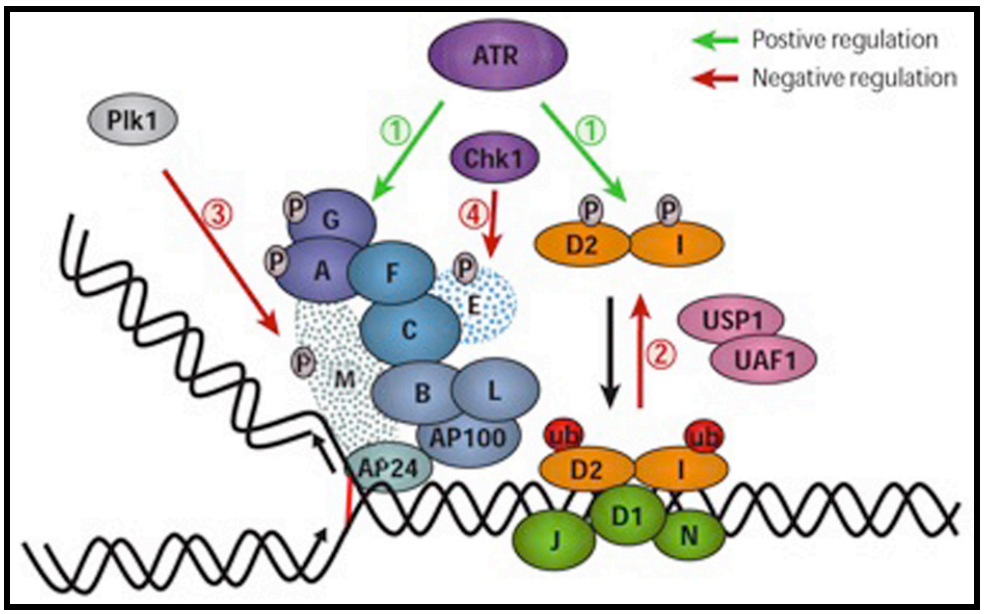


Figure 19. FA pathway regulation. Green arrows are activating, red arrows are inhibiting stimuli. 1) ATR activates the pathway by phosphorylating FANCI, FANCD2, FANCA, and probably FANCG. 2) USP1, associated with its activator UAF1, removes monoubiquitin from FANCD2 and FANCI, therefore inhibiting the pathway. 3) During mitosis, Plk1 phosphorylates FANCM, therefore creating a link site for ubiquitin ligase βTRCP. FANCM is then monoubiquitinated and is degraded by proteasome. 4) Chk1-dependent phosphorylation of FANCE causes its degradation (Moldovan GL, D'Andrea AD, 2009 [61]).

In human cells, USP1 knockdown has been associated with a higher survival after DNA damage. The present studies, however, show that primary GBM and GBM cell lines have high levels of USP1, and that rather than sensitizing cells to DNA damaging agents, USP1 appears to suppress latent apoptotic pathways and to protect cells from temozolomide-induced

apoptosis. These results identify a new function for USP1 and suggest that suppression of USP1 and/or USP1 controlled pathway may be a means to enhance the cytotoxic potential of temozolomide and to sensitize previously resistant GBM.

References

1) Acharya S, Wilson T, Gradia S, Kane MF, Guerrette S, Marsischky GT, Kolodner R, Fishel R. hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. Proc Natl Acad Sci U S A 1996;93:13629-34.

2) Andreassen PR, D'Andrea AD, Taniguchi T. ATR couples FANCD2 monoubiquitination to the DNA-damage response. Genes Dev 2004;18:1958-1963.

3) Bashir T, Pagano M. Don't skip the G1 phase: how APC/CCdh1 keeps SCFSKP2 in check. Cell Cycle. 2004 Jul;3(7):850-2.

4) Bergink S, Jentsch S. Principles of ubiquitin and SUMO modifications in DNA repair. Nature 2009;458:461-7.

5) Bignami M, O'Driscoll M, Aquilina G, Karran P. Unmasking a killer: DNA O(6)-methylguanine and the cytotoxicity of methylating agents. Mutat Res 2000;462:71-82.

6) Bobola MS, Berger MS, Ellenbogen RG, Roberts TS, Geyer JR, Silber JR. O6-Methylguanine-DNA methyltransferase in pediatric primary brain tumors: relation to patient and tumor characteristics. Clin Cancer Res 2001;7:613-619.

7) Boutros R, Dozier C, Ducommun B. The when and wheres of CDC25 phosphatases. Curr Opin Cell Biol 2006;18:185-91.

8) Brada M, Judson I, Beale P, Moore S, Reidenberg P, Statkevich P, Dugan M, Batra V, Cutler D. Phase I dose-escalation and pharmacokinetic study of temozolomide (SCH 52365) for refractory or relapsing malignancies. Br J Cancer 1999;81:1022-30.

9) Bu Y, Yang Z, Li Q, Song F. Silencing of polo-like kinase (Plk) 1 via siRNA causes inhibition of growth and induction of apoptosis in human esophageal cancer cells. Oncology 2008;74:198-206.

10) Buckner JC. Factors influencing survival in high-grade gliomas. Semin Oncol 2003;30(Suppl 19):10-14.

11) Caporali S, Falcinelli S, Starace G, Russo MT, Bonmassar E, Jiricny J, D'Atri S. DNA damage induced by temozolomide signals to both ATM and ATR: role of the mismatch repair system. Mol Pharmacol 2004;66:478-91.

12) Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. Oncogene 2004;23:2825-37.

13) Chalmers AJ, Ruff EM, Martindale C, Lovegrove N, Short SC. Cytotoxic effects of temozolomide and radiation are additive- and schedule-dependent. Int J Radiat Oncol Biol Phys 2009;75:1511-9.

14) Chen J, Dexheimer TS, Ai Y, Liang Q, Villamil MA, Inglese J, Maloney DJ, Jadhav A, Simeonov A, Zhuang Z. Selective and cell-active inhibitors of the USP1/ UAF1 deubiquitinase complex reverse cisplatin resistance in non-small cell lung cancer cells. Chem Biol 2011;18:1390-400.

15) Chen JM, Zhang YP, Wang C, Sun Y, Fujimoto J, Ikenaga M. O6-methylguanine-DNA methyltransferase activity in human tumors. Carcinogenesis 1992;13:1503-1507.

16) Chu R, Terrano DT, Chambers TC. Cdk1/cyclin B plays a key role in mitotic arrest-induced apoptosis by phosphorylation of Mcl-1, promoting its degradation and freeing Bak from sequestration. Biochem Pharmacol 2012;83:199-206.

17) Curran WJ Jr, Scott CB, Horton J, Nelson JS, Weinstein AS, Fischbach AJ, Chang CH, Rotman M, Asbell SO, Krisch RE, et al. Recursive partitioning analysis of prognostic factors in three Radiation Therapy Oncology Group malignant glioma trials. J Natl Cancer Inst 1993;85:704-710.

18) Davoli T, de Lange T. The causes and consequences of polyploidy in normal development and cancer. Annu Rev Cell Dev Biol 2011;27:585-610.

19) Davoli T, Denchi EL, de Lange T. Persistent telomere damage induces bypass of mitosis and tetraploidy. Cell 2010;141:81-93.

20) De Angelis LM. Brain tumors. N Engl J Med 2001;344:114-123.

21) De Angelis LM, Loeffler JS, Adam N. Mamelak AN. Primary and Metastatic Brain Tumors. In Pazdur R, Coia LR, Hoskins WJ, and Wagman LD. Cancer Management: A Multidisciplinary Approach, 2007; 10th Edition.

22) Denny BJ, Wheelhouse RT, Stevens MF, Tsang LL, Slack JA. NMR and molecular modeling investigation of the mechanism of activation of the antitumor drug temozolomide and its interaction with DNA. Biochemistry 1994;33:9045-9051.

23) Dresemann G. Temozolomide in malignant glioma. Onco Targets Ther 2010;3:139-46.

24) Duckett DR, Drummond JT, Murchie AI, Reardon JT, Sancar A, Lilley DM, Modrich P. Human MutSalpha recognizes damaged DNA base pairs containing O6-methylguanine, O4-methylthymine, or the cisplatin-d(GpG) adduct. Proc Natl Acad Sci U S A 1996;93:6443-7.

25) Dunkern T, Roos W, Kaina B. Apoptosis induced by MNNG in human TK6 lymphoblastoid cells is p53 and Fas/CD95/Apo-1 related. Mutat Res 2003;544:167-172.

26) Elder RT, Yu M, Chen M, Zhu X, Yanagida M, Zhao Y. HIV-1 Vpr induces cell cycle G2 arrest in fission yeast (Schizosaccharomyces pombe) through a pathway involving regulatory and catalytic subunits of PP2A and acting on both Wee1 and Cdc25. Virology 2001;287:359-70.

27) Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 1999;59:793-797.

28) Esteller M, Risques RA, Toyota M, Capella G, Moreno V, Peinado MA, Baylin SB, Herman JG. Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res 2001;61:4689-4692.

29) Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC. Induction of apoptosis in fibroblasts by c-myc protein. Cell 1992;69:119-28.

30) Friedman HS, Dolan ME, Pegg AE, Marcelli S, Keir S, Catino JJ, Bigner DD, Schold SC Jr. Activity of temozolomide in the treatment of central nervous system tumor xenografts. Cancer Res 1995;55:2853-7.

31) Friedman HS, Kerby T, Calvert H. Temozolomide and treatment of malignant glioma. Clin Cancer Res 2000;6:2585-97.

32) Friedman HS, McLendon RE, Kerby T, Dugan M, Bigner SH, Henry AJ, Ashley DM, Krischer J, Lovell S, Rasheed K, Marchev F, Seman AJ, Cokgor I, Rich J, Stewart E, Colvin OM, Provenzale JM, Bigner DD, Haglund MM, Friedman AH, Modrich PL. DNA mismatch repair and O6-

alkylguanine-DNA alkyltransferase analysis and response to Temodal in newly diagnosed malignant glioma. J Clin Oncol 1998;16:3851-7.

33) Ganem NJ, Storchova Z, Pellman D. Tetraploidy, aneuploidy and cancer. Curr Opin Genet Dev 2007;17:157-62.

34) Gene Expression Omnibus: http://www.ncbi.nlm.nih.gov/geo/

35) Gerson SL. Clinical relevance of MGMT in the treatment of cancer. J Clin Oncol 2002;20:2388-99.

36) Günther W, Pawlak E, Damasceno R, Arnold H, Terzis AJ. Temozolomide induces apoptosis and senescence in glioma cells cultured as multicellular spheroids. Br J Cancer 2003;88:463-469.

37) Guo Z, Kumagai A, Wang SX, Dunphy WG. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in Xenopus egg extracts. Genes Dev 2000;14:2745-56.

38) Hampson R, Humbert O, Macpherson P, Aquilina G, Karran P. Mismatch repair defects and O6-methylguanine-DNA methyltransferase expression in acquired resistance to methylating agents in human cells. J Biol Chem 1997;272:28596-28606.

39) Hibbert RG, Sixma TK. Intrinsic flexibility of ubiquitin on proliferating cell nuclear antigen (PCNA) in translesion synthesis. J Biol Chem 2012;287:39216-23.

40) Hickman MJ, Samson LD. Apoptotic signaling in response to a single type of DNA lesion, O(6)-methylguanine. Mol Cell 2004;14:105-116.

41) Hirose Y, Berger MS, Pieper RO. p53 effects both the duration of G2/M arrest and the fate of temozolomide-treated human glioblastoma cells. Cancer Res 2001;61:1957-1963.

42) Hirose Y, Berger MS, Pieper RO. Abrogation of the Chk1-mediated G(2) checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells. Cancer Res 2001;61:5843-5849.

43) Hirose Y, Kreklau EL, Erickson LC, Berger MS, Pieper RO. Delayed repletion of O6-methylguanine-DNA methyltransferase resulting in failure to protect the human glioblastoma cell line SF767 from temozolomide-induced cytotoxicity. J Neurosurg 2003;98:591-8.

44) Houtgraaf JH, Versmissen J, van der Giessen WJ. A concise review of DNA damage checkpoints and repair in mammalian cells. Cardiovasc Revasc Med 2006;7:165-72.

45) Huang TT, D'Andrea AD. Regulation of DNA repair by ubiquitylation. Nat Rev Mol Cell Biol 2006;7:323-334.

46) Hyun SY, Rosen EM, Jang YJ. Novel DNA damage checkpoint in mitosis: Mitotic DNA damage induces re-replication without cell division in various cancer cells. Biochem Biophys Res Commun 2012;423:593-9.

47) Joshi S, Braithwaite AW, Robinson PJ, Chircop M. Dynamin inhibitors induce caspase-mediated apoptosis following cytokinesis failure in human cancer cells and this is blocked by Bcl-2 overexpression. Mol Cancer 2011;10:78.

48) Kannouche PL, Wing J, Lehmann AR. Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. Mol Cell 2004;14:491-500.

49) Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 2004;11:448-457.

50) Karran P, Bignami M. DNA damage tolerance, mismatch repair and genome instability. Bioessays 1994;16:833-839.

51) Karran P, Marinus MG. Mismatch correction at O6-methylguanine residues in E. coli DNA. Nature 1982;296:868-9.

52) Katayama K, Fujita N, Tsuruo T. Akt/protein kinase B-dependent phosphorylation and inactivation of WEE1Hu promote cell cycle progression at G2/M transition. Mol Cell Biol 2005;25:5725-37.

53) Kennedy RD, D'Andrea AD. The Fanconi Anemia/BRCA pathway: new faces in the crowd. Genes Dev 2005;19:2925-2940.

54) Kim SY, Ferrell JE Jr. Substrate competition as a source of ultrasensitivity in the inactivation of Wee1. Cell 2007;128:1133-45.

55) Kondo T, Kobayashi M, Tanaka J, Yokoyama A, Suzuki S, Kato N, Onozawa M, Chiba K, Hashino S, Imamura M, Minami Y, Minamino N, Asaka M. Rapid degradation of Cdt1 upon UV-induced DNA damage is mediated by SCFSkp2 complex. J Biol Chem. 2004 Jun 25;279(26):27315-9.

56) Lantos PL, VandenBerg SR, Kleihues P. Tumours of the Nervous System. In: Greenfield's Neuropathology, Graham DI, Lantod PL (eds), 6th ed. Arnold: London. 1996; pp. 583-879.

57) Lee HJ, Hwang HI, Jang YJ. Mitotic DNA damage response: Polo-like kinase-1 is dephosphorylated through ATM-Chk1 pathway. Cell Cycle 2010;9:2389-98.

58) Li M, Zhang P. The function of APC/CCdh1 in cell cycle and beyond. Cell Div. 2009 Jan 19;4:2.

59) Li X, Zhao Q, Liao R, Sun P, Wu X. The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. J Biol Chem. 2003 Aug 15;278(33):30854-8.

60) Mir SE, De Witt Hamer PC, Krawczyk PM, Balaj L, Claes A, Niers JM, Van Tilborg AA, Zwinderman AH, Geerts D, Kaspers GJ, Peter Vandertop W, Cloos J, Tannous BA, Wesseling P, Aten JA, Noske DP, Van Noorden CJ, Würdinger T. In silico analysis of kinase expression identifies WEE1 as a gatekeeper against mitotic catastrophe in glioblastoma. Cancer Cell 2010;18:244-57.

61) Moldovan GL, D'Andrea AD. How the fanconi anemia pathway guards the genome. Annu Rev Genet 2009;43:223-249.

62) Murai J, Yang K, Dejsuphong D, Hirota K, Takeda S, D'Andrea AD. The USP1/UAF1 complex promotes double-strand break repair through homologous recombination. Mol Cell Biol 2011;31:2462-9.

63) Nakanishi K, Yang YG, Pierce AJ, Taniguchi T, Digweed M, D'Andrea AD, Wang ZQ, Jasin M. Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci U S A 2005;102:1110-1115.

64) Natsumeda M, Aoki H, Miyahara H, Yajima N, Uzuka T, Toyoshima Y, Kakita A, Takahashi H, Fujii Y. Induction of autophagy in temozolomide treated malignant gliomas. Neuropathology. 2011 Oct;31(5):486-93.

65) Neidle S, Thurston DE. Chemical approaches to the discovery and development of cancer therapies. Nat Rev Cancer 2005;5:285-296.

66) Newlands ES, Blackledge GR, Slack JA, Rustin GJ, Smith DB, Stuart NS, Quarterman CP, Hoffman R, Stevens MF, Brampton MH, et al. Phase I trial of temozolomide (CCRG 81045: M&B 39831: NSC 362856). Br J Cancer 1992;65:287-91.

67) Niimi A, Brown S, Sabbioneda S, Kannouche PL, Scott A, Yasui A, Green CM, Lehmann AR. Regulation of proliferating cell nuclear antigen ubiquitination in mammalian cells. Proc Natl Acad Sci U S A 2008;105:16125-16130.

68) Nijman SM, Huang TT, Dirac AM, Brummelkamp TR, Kerkhoven RM, D'Andrea AD, Bernards R. The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. Mol Cell 2005;17:331-339.

69) Nishitani H, Lygerou Z, Nishimoto T. Proteolysis of DNA replication licensing factor Cdt1 in S-phase is performed independently of geminin through its N-terminal region. J Biol Chem 2004;279:30807-16.

70) Ochs K, Kaina B. Apoptosis induced by DNA damage O6-methylguanine is Bcl-2 and caspase-9/3 regulated and Fas/caspase-8 independent. Cancer Res 2000;60:5815-5824.

71) O'Connell MJ, Raleigh JM, Verkade HM, Nurse P. Chk1 is a wee1 kinase in the G2 DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation. EMBO J 1997;16:545-54.

72) Oestergaard VH, Langevin F, Kuiken HJ, Pace P, Niedzwiedz W, Simpson LJ, Ohzeki M, Takata M, Sale JE, Patel KJ. Deubiquitination of FANCD2 is required for DNA crosslink repair. Mol Cell 2007;28:798-809.

73) Oncomine: https://www.oncomine.org/resource/login.html

74) Paz MF, Yaya-Tur R, Rojas-Marcos I, Reynes G, Pollan M, Aguirre-Cruz L, García-Lopez JL, Piquer J, Safont MJ, Balaña C, Sanchez-Cespedes M, García-Villanueva M, Arribas L, Esteller M. CpG island hypermethylation of the DNA repair enzyme methyltransferase predicts response to temozolomide in primary gliomas. Clin Cancer Res 2004;10:4933-8.

75) Pegg AE. Repair of O6-alkylguanine by alkyltransferases. Mutat Res 2000;462:83-100.

76) Pieper RO. Understanding and manipulating O6-methylguanine-DNA methyltransferase expression. Pharmacol Ther 1997;74:285-97.

77) Plowman J, Waud WR, Koutsoukos AD, Rubinstein LV, Moore TD, Grever MR. Preclinical antitumor activity of temozolomide in mice: efficacy against human brain tumor xenografts and synergism with 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res 1994;54:3793-9.

78) Preuss I, Eberhagen I, Haas S, Eibl RH, Kaufmann M, von Minckwitz G, Kaina B. O6-methylguanine-DNA methyltransferase activity in breast and brain tumors. Int J Cancer 1995;61:321-326.

79) Roos WP, Batista LF, Naumann SC, Wick W, Weller M, Menck CF, Kaina B. Apoptosis in malignant glioma cells triggered by the temozolomide-induced DNA lesion O6-methylguanine. Oncogene 2007;26:186-197.

80) Roos W, Baumgartner M, Kaina B. Apoptosis triggered by DNA damage O6-methylguanine in human lymphocytes requires DNA replication and is mediated by p53 and Fas/CD95/Apo-1. Oncogene 2004;23:359-367.

81) Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi S, Fukami K, Miyata T, Miyoshi H, Imamura T, Ogawa M, Masai H, Miyawaki A. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. Cell 2008;132:487-498.

82) Sakaue-Sawano A, Ohtawa K, Hama H, Kawano M, Ogawa M, Miyawaki A. Tracing the silhouette of individual cells in S/G2/M phases with fluorescence. Chem Biol 2008;15:1243-1248.

83) Sanai N, Berger MS. Glioma extent of resection and its impact on patient outcome. Neurosurgery 2008;62:753-64.

84) Shan B, Lee WH. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol Cell Biol 1994;14:8166-73.

85) Shi L, Nishioka WK, Th'ng J, Bradbury EM, Litchfield DW, Greenberg AH. Premature p34cdc2 activation required for apoptosis. Science 1994;263:1143-5.

86) Silber JR, Blank A, Bobola MS, Ghatan S, Kolstoe DD, Berger MS. O6-methylguanine-DNA methyltransferase-deficient phenotype in human gliomas: frequency and time to tumor progression after alkylating agent-based chemotherapy. Clin Cancer Res 1999;5:807-814.

87) Stevens MF, Hickman JA, Langdon SP, Chubb D, Vickers L, Stone R, Baig G, Goddard C, Gibson NW, Slack JA, et al. Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (CCRG 81045; M & B 39831), a novel drug with potential as an alternative to dacarbazine. Cancer Res 1987;47:5846-52.

88) Storchova Z, Kuffer C. The consequences of tetraploidy and aneuploidy. J Cell Sci;121(Pt 23):3859-66.

89) Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987-996.

90) Suzuki K, Kodama S, Watanabe M. Recruitment of ATM protein to double strand DNA irradiated with ionizing radiation. J Biol Chem 1999;274:25571-5.

91) Tada S. Cdt1 and geminin: role during cell cycle progression and DNA damage in higher eukaryotes. Front Biosci. 2007 Jan 1;12:1629-41.

92) The Stanford Tissue Microarray Database TMAD: http://tma.stanford.edu

93) Thompson DA, Desai MM, Murray AW. Ploidy controls the success of mutators and nature of mutations during budding yeast evolution. Curr Biol 2006;16:1581-90.

94) Tsang LL, Quarterman CP, Gescher A, Slack JA. Comparison of the cytotoxicity in vitro of temozolomide and dacarbazine, prodrugs of 3-methyl-(triazen-1-yl)imidazole-4-carboxamide. Cancer Chemother Pharmacol 1991;27:342-346.

95) Unsal-Kaçmaz K, Makhov AM, Griffith JD, Sancar A. Preferential binding of ATR protein to UV-damaged DNA. Proc Natl Acad Sci U S A 2002;99:6673-8.

96) Van Horn RD, Chu S, Fan L, Yin T, Du J, Beckmann R, Mader M, Zhu G, Toth J, Blanchard K, Ye XS. Cdk1 activity is required for mitotic activation of aurora A during G2/M transition of human cells. J Biol Chem 2010;285:21849-57.

97) van Vugt MA, Brás A, Medema RH. Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells. Mol Cell 2004;15:799-811.

98) Villamil MA, Chen J, Liang Q, Zhuang Z. A noncanonical cysteine protease USP1 is activated through active site modulation by USP1-associated factor 1. Biochemistry 2012;51:2829-39.

99) Vodermaier HC. APC/C and SCF: controlling each other and the cell cycle. Curr Biol 2004;14:R787-96.

100) Watanabe N, Arai H, Nishihara Y, Taniguchi M, Watanabe N, Hunter T, Osada H. M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. Proc Natl Acad Sci U S A 2004;101:4419-24.

101) Xouri G, Dimaki M, Bastiaens PI, Lygerou Z. Cdt1 interactions in the licensing process: a model for dynamic spatiotemporal control of licensing. Cell Cycle. 2007 Jul 1;6(13):1549-52.

102) Yamamoto K, Ishiai M, Matsushita N, Arakawa H, Lamerdin JE, Buerstedde JM, Tanimoto M, Harada M, Thompson LH, Takata M. Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. Mol Cell Biol 2003;23:5421-5430.

103) Zhu LH, Bi W, Liu XD, Li JF, Wu YY, Du BY, Tan YH. Induction of apoptosis by evodiamine involves both activation of mitotic arrest and mitotic slippage. Oncol Rep 2011;26:1447-55.

104) Zhuang Z, Johnson RE, Haracska L, Prakash L, Prakash S, Benkovic SJ. Regulation of polymerase exchange between Poleta and Poldelta by monoubiquitination of PCNA and the movement of DNA polymerase holoenzyme. Proc Natl Acad Sci U S A 2008;105:5361-6.