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TARGETED DNA REPAIR GENE EXPRESSION STUDY IN GLIOBLASTOMA PATIENT BIOPSIES: CLINICAL IMPACT AND CHARACTERIZATION OF THE NEIL3 GLYCOSYLASE, A NOVEL CANDIDATE TARGET FOR THERAPY

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AFFIDAVIT

I hereby confirm that the PhD Thesis entitled "TARGETED DNA REPAIR GENE EXPRESSION STUDY IN GLIOBLASTOMA PATIENT BIOPSIES: CLINICAL IMPACT AND CHARACTERIZATION OF THE NEIL3 GLYCOSYLASE, A NOVEL CANDIDATE TARGET FOR THERAPY" has been written independently and without any other sources than cited.

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Luxembourg, the 12th February 2019

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More than just a scientific adventure, the process of undertaking a PhD is for me the culmination of a long and tortuous odyssey through various education systems from preliminary school to lycée and up to Bachelor's and finally Master's degree. It was not easy

as over the years I failed some courses and I even had one misstep during my lycée years (1 in a total of 19 cumulated years of education, still not bad overall I think ^^). Science was always my go-to favourite subject and as I finish my PhD I also complete one of my life goals. The others are still a bit too far-fetched as working on the ISS as a scientist always attracted me. Who does not want to be 408km over Earth and flying around mending to the culture of algae or bacteria in a pressurized tin box all while orbiting at a speed of 7.66 km/s? Well life goals are not only there for you to accomplish them but also to dream about them in order to keep you excited.

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Thank you all and know that even if you had a minimal appearance during this journey, you helped me grow as a person and be who I am today. Now enough chatter, I wish you a pleasant reading of my thesis!

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

α-KG	α-ketoglutarate
2-HG	2-hydroxyglutarate
8-oxoG	7,8-dihydro-8-oxo-2'-deoxyguanonsine or 8-oxoGuanine
9-1-1	Rad9-Hus1-Rad1 complex
ALT	Alternative lengthening of telomeres
AP site	Apurinic/apyrimidinic site
BBB	Blood brain barrier
BER	Base excision repair
bp	Base pair
BIR	Break-induced repair
CCNU	Lomustine
CNS	Central nervous system
CpG	5'-cytosine-phosphate-3'-guanine
CTC	Circulating tumor cell
CTD	C-terminal domain
DAPI	4',6-diamidino-2-phenylindole
DB	DNA-binding domain
DDR	DNA damage response
d-loop	Displacement loop
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dRP	Deoxyribose phosphate
DSB	Double-stranded DNA break
dsDNA	Double-stranded DNA
FACS	Fluorescence-activated cell sorter
FISH	Fluorescent in situ hybridization
G4	Guanine quadruplex
GBM	Glioblastoma
G-CIMP	Glioma CpG island methylator phenotype
GD	Glycosylase domain
GFP	Green fluorescent protein
Gh	Guanidinohydantoin
GSC	Glioma stem-like cell
H2TH	Helix-2 turn-helix domain
HDAC	Histone deacetylase
HR	Homologous recombination
i	inhibitor
ICL	DNA interstrand cross-link
IF	Immunofluorescence
IR	Ionizing radiation
kb	kilobase
KPS	Karnofsky performance status
lncRNA	Long non-coding RNA
LP-BER	Long-patch BER
MEF	Mouse embryonic fibroblast
MMR	Mismatch repair
MRN	Mre11/RAD50/Nbs1 complex
MTIC	5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide
N1-meA	N1-methyladenine
N3-meA	N3-methyadenine
N3-meC	N3-methylcytosine
N7-meG	N7-methylguanine
NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining
NLS	Nucleotide localization sequence
nt	nucleotide
O6-BG	O6-benzylguanine
O6-meG	O6-methylguanine
OS	Overall survival
PCR	Polymerase chain reaction
PD	Population doubling
PFS	Progression free survival
ROS	Reactive oxygen species
RT	Radiation therapy
RTK	Receptor tyrosine kinase
seDSB	Single-ended double-strand break
Sh	Spiroiminohydantoin
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SP-BER	Short-patch BER
SSA	Single-strand annealing
SSB	Single-stranded DNA break
ssDNA	Single-stranded DNA
TBHP	Tert-butyl hydroxyperoxide
TCGA	The Cancer Genome Atlas
TERRA	Telomeric repeat-containing RNA
Tg	Thymine glycol
TIF	Telomere dysfunction-induced foci
t-loop	Telomere loop
TME	Tumor microenvironment
TMM	Telomere maintenance mechanism
TMZ	Temozolomide
TRAP	Telomerase repeated amplification protocol
TPE	Telomere position effect
TRF	Telomere restriction fragment
WB	Western Blot
WHO	World Health Organization

SUMMARY

Despite surgical resection and the combination of ionizing radiation and chemotherapy with temozolomide (TMZ), a DNA methylating agent introduced in 2005, glioblastoma (GBM) remains a lethal disease associated with poor prognosis, treatment resistance and inevitable relapse. Resistance to chemoradiation is mediated in great part by complex and redundant DNA repair mechanisms.

The main aim of this project was to propose novel molecular targets and strategies in the fight against GBM, based on the identification and characterization of the DNA repair machineries that i) are altered during glioblastomagenesis and ii) mediate chemoresistance and tumor relapse. To this end, we have embarked on the expression analysis of a selection of genes encompassing the major DNA repair pathways and cell cycle-related factors, in a clinically-relevant cohort of paired primary and recurrent biopsies from GBM patients.

In the first part of this thesis, we report the generation and validation of a DNA repair and cell cycle gene signature that clustered GBM specimens in two major groups displaying an inverse expression profile of the signature and a third, less defined group. Specific analysis of the tumor pairs revealed that GBM recurrences frequently displayed a gene expression profile different from that of the matched primary biopsy, indicating that tumor progression is associated with significant deregulation of DNA repair and cell cycle pathways. Furthermore, the gene signature expression pattern observed at relapse was linked to progression-free survival. Finally, our signature exposed therapeutic group-specific vulnerabilities to inhibitors of the DNA damage response and/or genotoxicants, as well as specific alterations in key core GBM pathways. Thus, our gene signature bears clinical relevance, with the prospect of better patient stratification and personalized therapeutic strategies.

In the second part of this thesis, we have exploited our gene expression dataset to uncover DNA repair genes specifically deregulated in GBM. We report the upregulation of NEIL3 encoding a member of the NEIL DNA glycosylase family, and the downregulation of NEIL1 and NEIL2, the other members of this family. In view of the documented role of NEIL3 in promoting repair of oxidative DNA damage at telomeres, we investigated the impact of depleting NEIL3 in GBM cell lines. We found that NEIL3 knockdown resulted in telomere shortening, downregulation of the shelterin factor TRF1 and deregulation of chromosome-specific telomeric repeat-containing RNAs (TERRAs). In parallel, we observed an increase in telomere dysfunction-induced foci (TIFs) after NEIL3 loss, suggesting the activation of the DNA damage response at telomeres. Finally, NEIL3 depletion was associated with increased

sensitivity to oxidative DNA damage as well as TMZ. Hence, we propose that NEIL3 could represent an attractive therapeutic target for improved treatment of GBM.

CHAPTER 1

INTRODUCTION

1. Glioblastoma

1.1. Description and standard of care

Glioblastoma (GBM) is a grade IV astrocytoma (World Health Organization, WHO) that accounts for about 15% of all primary brain tumors and 50% of all malignant central nervous system (CNS) tumors (Ostrom et al., 2014). In adults, the yearly incidence of GBM is estimated at 3.2 per 100.000 persons with a median age at occurrence of 64 years (Ostrom et al., 2014). In contrast, pediatric GBM (highest incidence between ages 15-19) account for about 3-15% of primary CNS tumors (Das and Kumar 2017). GBMs are associated with very poor patient prognosis with reported median overall survival (OS) in adults of about 15 months (Stupp et al., 2005) and in pediatric cases ranging from 13 to 72 months (Song et al., 2010).

GBMs arise from astrocytes, mainly as de novo primary tumors (about 95% of the cases) without any measurable malignant precursor. Additionally, lower-grade astrocytomas (WHO grade II or III) can develop to secondary GBMs (5% of patients). Diagnosis of CNS tumors was originally based solely on the neuropathological analysis of a patient biopsy. In recent years, the WHO classification has been revised to integrate genotypic and phenotypic parameters (Louis et al., 2016), leading to a more robust and improved diagnosis of brain cancers (Figure 1). Moreover, DNA methylation profiling emerged as a promising and complementary tool for routine diagnosis and classification of CNS tumors in the clinic (Capper et al., 2018).

The current standard management of GBM consists of maximal surgical resection, followed by radiotherapy (RT) with concomitant and adjuvant chemotherapy with the DNA alkylating agent temozolomide (TMZ). This standard of care was first introduced in 2005 following the seminal study by Stupp et al. (2005) (Figure 2). Prior to this date, alkylating agents including TMZ were mainly used as rescue therapy in patients with recurrent tumors (Gallego 2015; Weller et al., 2013). TMZ is an oral methylating agent capable of crossing the blood brain barrier (Patel et al., 2003). The survival benefit achieved by the use of TMZ in combination with RT is very limited with a median overall survival increase of about 2.5 months compared to RT alone (Stupp et al., 2005). In addition, as will be discussed later, the efficiency of TMZ is limited to a patient population distinguished by the epigenetic silencing

of the MGMT gene, coding for the enzyme catalyzing the removal of the most cytotoxic lesion induced by TMZ, O6-methylguanine (O6-meG).

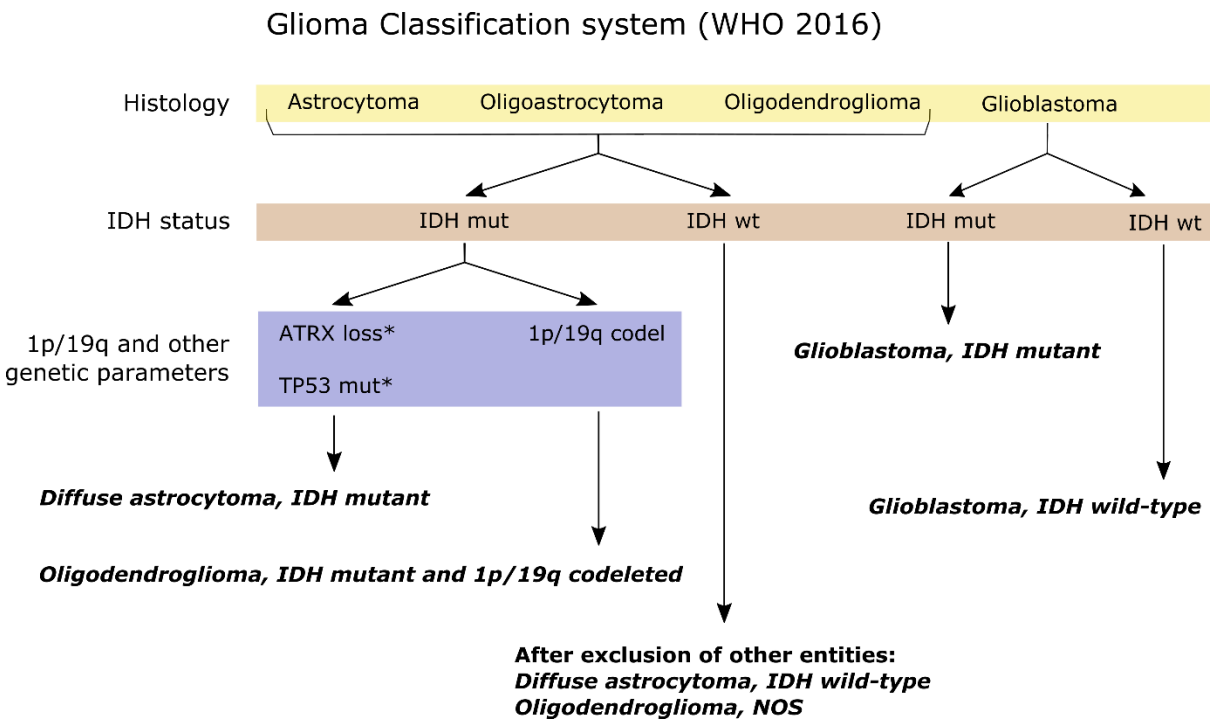

Figure 1. Current updated algorithm for the classification of diffuse gliomas. The current diagnosis of central nervous system tumors is based on histological and genetic features. The flow of analysis to determine tumor type and grade is not always top-down, and molecular signatures can sometimes outweigh histological features in order to achieve an integrated diagnosis. *Characteristic not required for diagnosis; NOS, not otherwise specified (adapted from Louis et al., 2016).

Thus, despite an aggressive treatment combination, the median survival in newly diagnosed patient does not exceed 15 months (Stupp et al., 2005) and the estimated 5-year survival rate is only about 5% (American Cancer Society 2017). Favorable prognostic factors include younger age at diagnosis, higher performance status (e.g. the commonly used Karnofsky performance status, (Lamborn et al., 2004)), tumor location and extent of tumor resection (Thakkar et al., 2014). Elderly patients (> 60 years old) are often excluded from multimodal therapies because of higher risk of treatment intolerance. Nevertheless, TMZ monotherapy or hypofractionated radiotherapy (i.e. large doses in several sessions) have been proposed as tolerable alternatives in older patients (Zarnett et al., 2015). Presently, as no gold standard treatment for recurrent GBM has been established, treatment strategies are frequently proposed in the context of a clinical trial using novel therapeutic drugs (Weller et al., 2013). For lack of better alternatives, and despite its inefficacy in treating patients with a

tumor harbouring an unsilenced MGMT gene, TMZ-based chemotherapy is still presently used in the clinic for newly diagnosed patients.

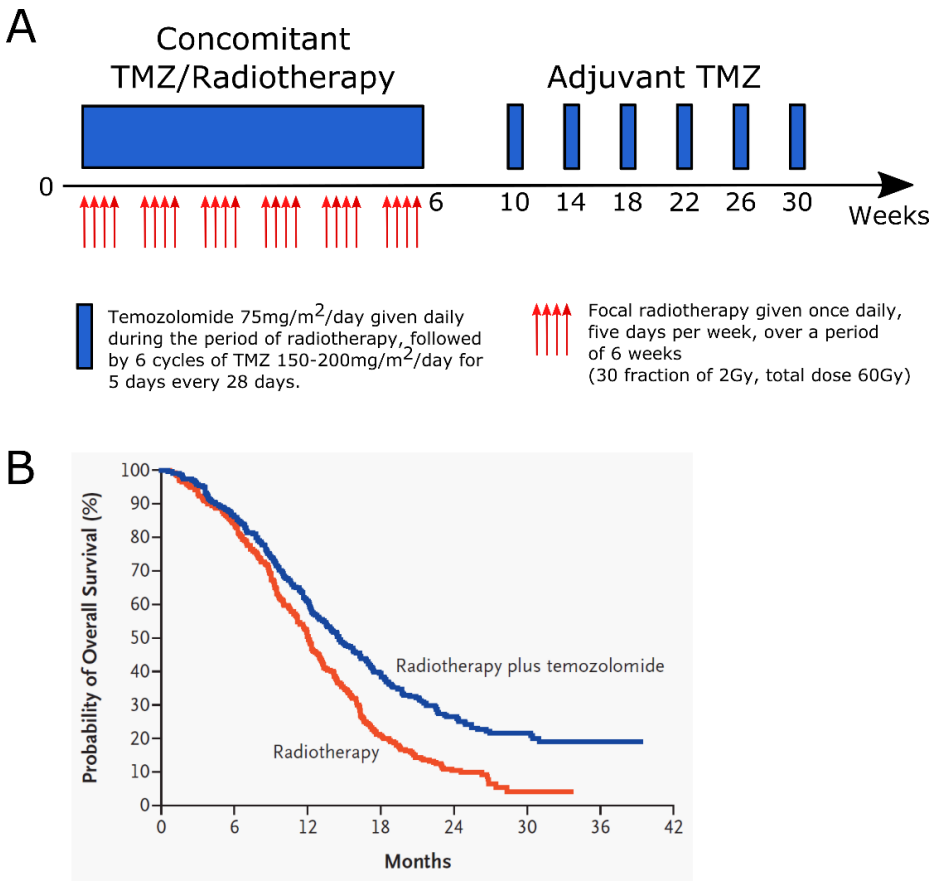

Figure 2. Glioblastoma standard-of-care treatment strategy and impact on patient prognosis. A. Treatment scheme adopted following the seminal study by Stupp et al in 2005: standard radiotherapy plus daily concomitant TMZ, followed by adjuvant chemotherapy. **B.** Overall survival of GBM patients treated with radiotherapy alone or in combination with TMZ demonstrating a significant survival benefit (Stupp et al., 2005). To note, the specific impact of MGMT methylation on TMZ treatment efficacy is illustrated later (Fig. 9).

1.2. Key genetic drivers of glioblastomagenesis and patient stratification

GBM tumors exhibit an important inter- and intratumor heterogeneity both at the cellular and molecular level which translates into the inability of basic histopathological features to clearly define patient outcome. Moreover, incomplete knowledge of driving alterations and suboptimal disease classification also hinder therapy development for GBM patients. In an effort to identify putative GBM tumor drivers, whole genome analyses conducted by the Cancer Genome Atlas (TCGA) research network have generated an extensive list of gene expression alterations (i.e., deregulated genes, CpG island methylation patterns) and genomic

abnormalities (i.e., mutations, copy number alterations) associated with glioblastomagenesis (Brennan et al., 2013; Cancer Genome Atlas Research 2008; Noushmehr et al., 2010). These include alterations of the tyrosine kinase receptor (RTKs) signalling pathway (observed in 90% of GBMs) manifested by the deregulation of specific RTKs and downstream factors. In particular, the EGFR receptor is found amplified in about 50% of GBMs, generally leading to overexpression of the protein (Furnari et al., 2015). Moreover, 50% of EGFR amplified tumors overexpressed a truncated protein called EGFR variant III (EGFRvIII) which is constitutively active (Gan et al., 2013). Amplification of PDGFRA is also observed in 10% of GBM tumors and is associated to poor prognosis in recurring patients (Phillips et al., 2013). In addition, the genes encoding negative regulators of the RAS and AKT downstream signalling pathway, NF1 and PTEN, are frequently inactivated or deleted in GBM (Brennan et al., 2013). Likewise, inhibition of the apoptotic pathway by inactivating TP53 mutations is detected in about 30% of primary GBMs. Deregulation of the retinoblastoma pathway (RB) with most commonly inactivation or deletion of the CDKN2A/B locus (80% of tumors) and amplification of CDK4 and CDK6 (16% of tumors) are also observed (Brennan et al., 2013) (Figure 3A). Lastly, a subset of tumors has been identified, that are characterized by a similar profile of gene promoter methylation (called the glioma-CpG island methylator phenotype (G-CIMP)) and are associated with somatic IDH1 mutations (Noushmehr et al., 2010). Although not frequently observed in primary GBM (<5% of patients), the mutation status of the isocitrate dehydrogenase genes IDH1/2 (hereby referred to as IDH) is a critical factor in low-grade gliomas and secondary GBM (70-80% of cases) which has been included as the primary determinant of the novel WHO glioma classification (Louis et al., 2016). The IDH mutations occur early in gliomagenesis and confer neo-enzymatic activity. Specifically, mutant IDH catalyzes the production of the oncometabolite 2-hydroxyglutarate (2-HG) from α-ketoglutarate (α-KG) (Cohen et al., 2013) (details in section 2.2). The presence of these alterations in GBM patients were found to be related with patient prognosis (Noushmehr et al., 2010; Ruano et al., 2009; Verhaak et al., 2010).

Importantly, the integration of these key alterations into a multidimensional genomic data study led to a robust gene expression-based molecular classification of GBM (Verhaak et al., 2010). Originally, the patterns of somatic mutations and DNA copy number alterations described above were considered to define 4 subgroups; namely the classical, mesenchymal,

proneural and neural GBM subtypes. Interestingly, such a classification led to the observation that the survival advantage of treated patients varied according to the subtype (Verhaak et al., 2010) with the best prognosis seen in the proneural type and a clear treatment effect observed in both classical and mesenchymal subtypes. Importantly, the neural subtype, whose expression pattern was closely related to normal brain, was rapidly disproved by the community as a result of glial cell contamination in the considered patient biopsies (Verhaak et al., 2010). Nevertheless, this first milestone study demonstrated the relevance of stratifying patients based on molecular profiles.

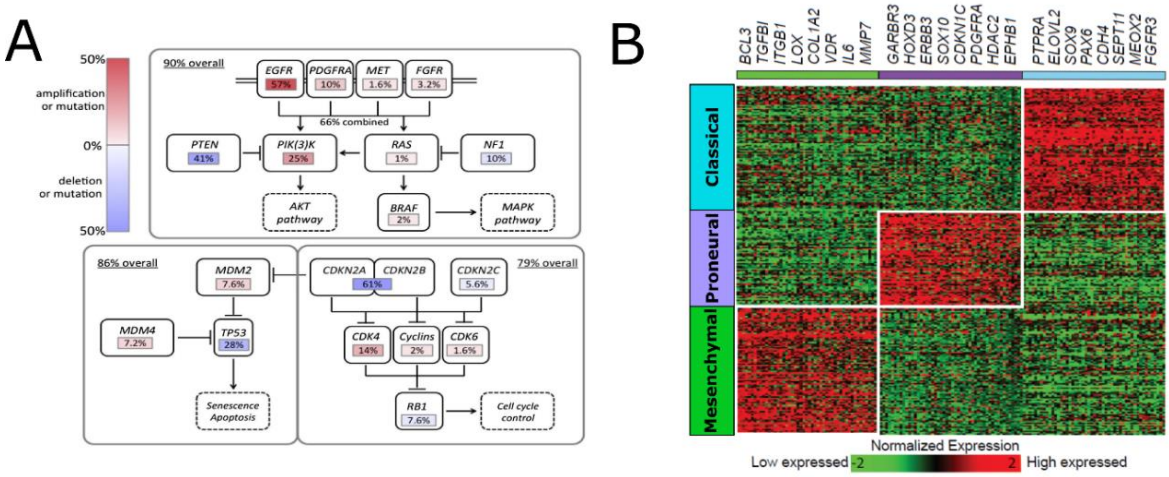

Figure 3. Defining gene expression and molecular alterations in GBM. A. Overall alteration rates in the canonical PI3K/MAPK (upper panel), p53 signaling (bottom left panel) and Rb (bottom right panel) regulatory pathways. The color scale indicates the frequency and the type of genetic alteration, with activating mutation/amplification and inactivating mutation/deletion indicated in red and blue, respectively (adapted from Brennan et al., 2013) **B.** Heatmap of the updated Verhaak GBM classifiers obtained in an integrative multiomics approach in order to filter out the non-tumor gene signature. This approach resulted in the removal of the neural subtype which was thought to mostly consist of glial cells. Representative genes of the signature are shown for each subtype. Mesenchymal-type GBMs are shown in green, proneural in purple and classical in light blue. Expression intensity is represented as a green (low expressed) to red (high expressed) color scale (adapted from Wang et al., 2017).

A similar study in pediatric GBM uncovered somatic driver mutations in the replication-independent histone variant H3.3 gene (H3F3A) as well as in the H3.3 histone chaperones ATRX and DAXX (Schwartzentruber et al., 2012). As a result, a novel classification was proposed that stratified GBMs across the entire age spectrum (adult and pediatric) into 6 biologically-relevant subgroups based on DNA methylation clusters and carried clinical implications, with mutated H3F3A at position K27 associated to worst survival (Sturm et al., 2012) (Figure 3B).

In the recent years, the constant optimization of multi-omics analysis and the rise of single cell transcriptomics have revolutionized the search for genetic biomarkers in cancer. In line with these advances, the GBM expression subgroups were further refined by eliminating the influence of the tumor-associated infiltrating cells on the gene signature, which further confirmed the exclusion of the neural subtype (Wang et al., 2017) (Figure 3C). Moreover, further characterization of the intra-tumoral heterogenenity using spatially distinct tumor fragments and single tumor cell transcriptomics revealed patterns of spatial and patient-specific tumor evolution (Sottoriva et al., 2013) and dynamic transcriptional program transitions (Patel et al., 2014). In addition, a recent study describing the use of uncommon multifocal (several spatially distinct tumor entities in the same patient) GBMs in combination with a multi-omic approach, identified common genetic alterations in different tumor foci from the same patient. This observation suggests a monoclonal origin of multifocal GBMs, that in parallel gain distinct aberrations throughout their development, translating into a marked genetic heterogeneity in the patient's tumor (Abou-El-Ardat et al., 2017). Finally, further developments illustrated by the multi-omics analysis of a comprehensive TCGA cohort containing adult gliomas of different grades (grade II, III and IV) have identified molecular correlations that provide insights into the progression from low- to high-grade disease, as well as 6 clinically relevant subsets based on the correlation of methylation and gene expression profiles (Ceccarelli et al., 2016).

Despite these efforts, the mechanisms driving GBM development and response to treatment remain unclear and the implementation of successful GBM therapies still awaits efficient patient stratification and the identification of innovative subgroup-specific treatments.

1.3.Treatment resistance and tumor relapse

Several intrinsic or acquired mechanisms drive fast tumor repopulation/regrowth after resection and limit treatment efficacy, thus ultimately leading to tumor recurrence and patient death.

The brain parenchyma is protected from the peripheral blood circulation by the blood–brain barrier (BBB) (Chow and Gu 2015). Although the BBB offers protection against the toxicity of many xenobiotics and pathogens, it imposes a challenge on researchers and

clinicians who have to design/select drug compounds capable of penetrating into this shielded environment in order to reach the tumor. Additionally, the tumor microenvironment (TME) plays a crucial role in tumorigenesis, fostering tumor initiation, progression and invasion and also promoting resistance against cell death signals, the host immune response and therapeutics. The GBM TME is composed of highly proliferative cancer cells, resident or infiltrating immune cells, stromal and vascular endothelial cells. These different cell populations create distinct and heterogeneous niches within the tumor (Figure 4). Furthermore, GBMs show extensive neo-angiogenesis leading to vascular abnormalities due to elevated levels of vascular endothelial growth factor (VEGF) resulting in leaky blood vessels that fail to deliver constant oxygen delivery within the tumor (Wesseling et al., 1997). As a result, glioblastomas show extensive regions of hypoxia which translates in a decrease in

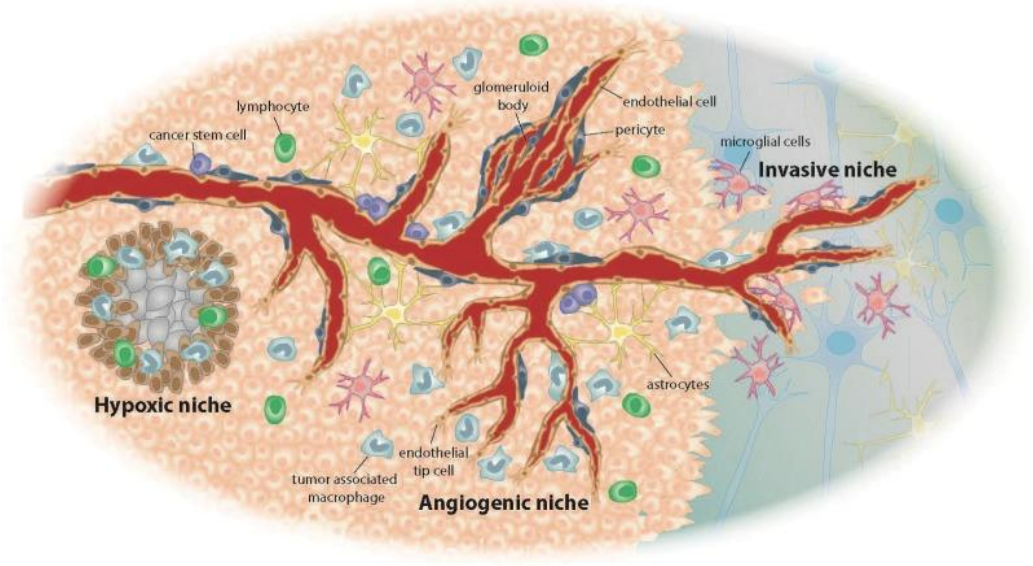

Figure 4. Representation of the different tumor microenvironment (TME) niches present in GBM. Glioblastoma cells are embedded in a heterogeneous TME which is not only composed of diverse stromal cells, including endothelial cells, the various infiltrating and resident immune cells, and other glial cell types, but also distinct regions whose anatomical and functional traits are partly driven by the GBM cells themselves. These niches actively regulate metabolic needs, immune surveillance, survival and invasion, and have also been proposed to play a crucial role in glioma stem cell maintenance. See text for details (De Vleeschouwer and Bergers, 2017).

oxygen molecules, thus limiting the potency of ionizing radiation (IR) to generate oxidative damage by producing reactive oxygen species and free radicals (Rockwell et al., 2009). These conditions also attract immune cells such as macrophages that display proangiogenic and immunosuppressive properties thus helping tumor expansion (Glass and Synowitz 2014). Additionally, GBM cells also have the propensity to invade the neighbouring normal brain tissue either as single cells or by taking advantage of adjacent blood vessels to reach normal brain parenchyma (Cuddapah et al., 2014). Thus, the infiltrative nature of GBM makes

complete tumor resection impossible as cells having escaped the tumor bulk to invade the brain parenchyma will not be removed. All these niches undergo dynamic spatio-temporal alterations during tumor growth creating protected tumor microenvironments that sustain the aggressive growth of GBM during progression as well as response to therapeutic agents (Hambardzumyan and Bergers 2015).

Furthermore, a subpopulation of tumor propagating cells with stem-like cell properties, named glioma stem-like cells (GSCs), have been proposed to affect gliomagenesis, tumor recurrence and treatment resistance (Lathia et al., 2015). GSCs have been shown to promote the angiogenic tumor niche by expressing high levels of VEGF (Bao et al., 2006b) In addition, GSCs were also found to be enriched in perinecrotic tumor areas (near the hypoxic niche) (Seidel et al., 2010) where their stem cell properties are enhanced through upregulation of the hypoxia-induced factor HIF-1α (allowing survival in this more hostile environment) (Soeda et al., 2009). It has also been demonstrated that GSCs are inherently more resistant to chemoradiation (Bao et al., 2006a; Chen et al., 2012), in part because of their ability to find shelter in specific brain regions that have been reported to afford protection against DNA damage (Roos et al., 2017). Additionally, these cells have been proposed to initiate tumor cell repopulation after resection of the tumor mass (Singh et al., 2003). In this regard, a recent study has uncovered the presence of GBM-derived circulating tumor cells (CTCs) in a xenografted mouse model. These cells presented GSCs properties and were able to return to the brain where they induced tumor relapse (Liu et al., 2018). Finally, GSCs can be isolated from patient material and propagated as neurospheres (Azari et al., 2011) which possess the ability to recapitulate the phenotypes of the original tumor when xenotransplanted in mice (Galli et al., 2004) and thus represent an important tool for functional studies.

2. DNA repair mechanisms and their role in GBM resistance

The development of resistance to chemoradiation is promoted in great part by complex DNA repair machineries that remove DNA lesions. Here, following an introduction to the DNA damage response, we will briefly introduce the salient features of the major DNA repair pathways that affect the resistance of GBM cells to chemoradiation.

2.1.The DNA damage response and the DNA repair paradox

Genomic DNA is constantly challenged with damages that arise from endogenous sources, for instance from normal cellular processes such as the misincorporation of bases by DNA polymerases during replication, oxidative DNA damage due to the generation of reactive oxygen species (ROS) by cellular metabolism and spontaneous hydrolysis of bases (Lindahl 1993; Marnett and Plastaras 2001; Tubbs and Nussenzweig 2017). Moreover, DNA damage can result from exogenous insults like chemical mutagens, ionizing radiations (IR) and genotoxic compounds present naturally in the environment or administered, for instance as part of cancer therapy (Ciccia and Elledge 2010).

In order to prevent the potentially lethal consequences of DNA damage, complex signalling and repair mechanisms have evolved to tackle the diverse threats to genome integrity. The major DNA repair pathways include direct repair (dedicated enzyme), base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), interstrand DNA cross-link (ICL) repair and finally, double-strand break (DSB) repair, itself consisting of two distinct major sub-pathways, namely homologous recombination (HR) and non-homologous end-joining (NHEJ) (Figure 5).

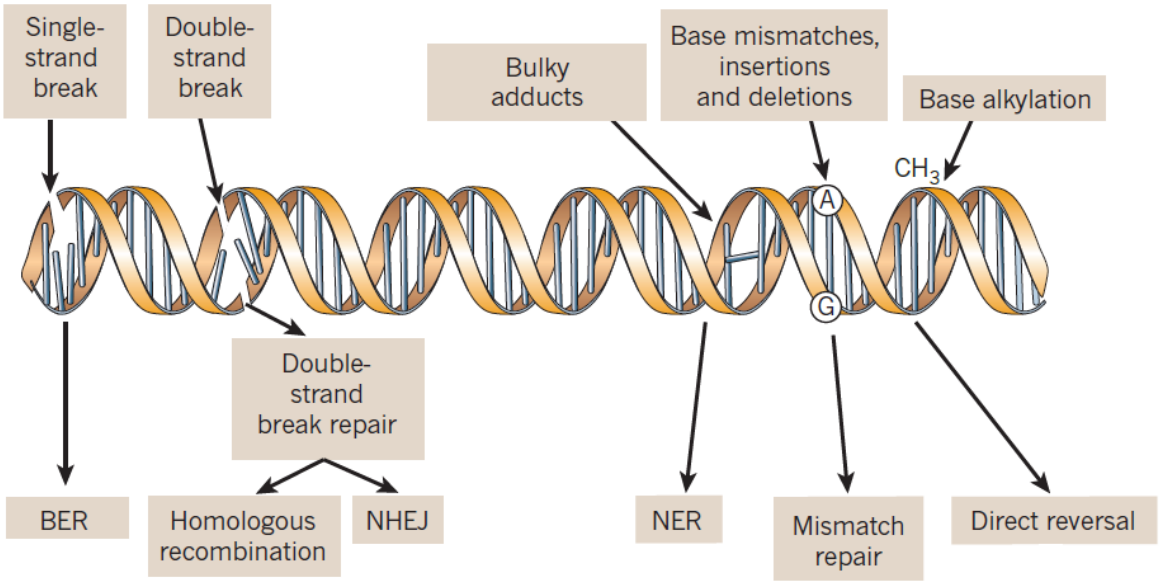

Figure 5. Schematic illustration of the types of DNA lesions incurred by DNA and the major DNA repair pathways that remove these lesions and maintain genomic stability. See text for details (adapted from Lord et al., 2012). A, adenine; G, guanine; CH_3, methyl group.

Specific checkpoint signalling pathways are activated upon genotoxic stress to block cell cycle progression and allow DNA repair to occur, or when DNA repair cannot be achieved,

trigger apoptosis (Haber 2015). The checkpoint signalling pathways are part of the DNA damage response (DDR) that orchestrates a sequence of events leading to the mobilization of the DNA repair machineries, access to the lesion site via remodelling of the local chromatin structure, DNA repair and restoration of an intact chromatin structure (Dabin et al., 2016; Soria et al., 2012). Central to the activation of the DDR are the ATM (Ataxia Telangiectasia mutated) and ATR (Ataxia Telangiectasia and Rad3-related) kinases which respond to different types of DNA lesions (Marechal and Zou 2013). Specifically, ATM is recruited to DSB sites through the MRN complex (MRE11-RAD50-NBS1) while ATR activation is promoted by recognition of ssDNA present at replication fork collapse by the 9-1-1 sensor (RAD9-RAD1-HUS1) (Awasthi et al., 2015). Downstream effectors will be mobilized upon activation by phosphorylation of both factors (Figure 6). Specifically, targets of ATM include p53 and the effector kinase CHK2, resulting in the control of G1 to S cell cycle progression, whereas ATR primarily acts through CHK1 and controls DNA replication (intra-S checkpoint) and the G2/M checkpoint (Marechal and Zou 2013). One of the most important substrate of ATM and ATR is H2AX, a variant of histone H2A that is present in about 10-15% of the nucleosomes. Phosphorylation of H2AX on serine 139 (a modification known as γ-H2AX) by ATM or ATR induces chromatin structure alteration at the DSB site that act as scaffold for the recruitment of the DSB machinery (Kinner et al., 2008; Podhorecka et al., 2010). Notably, γ-H2AX can be observed as foci that persist until DNA repair has been achieved. The quantification of γ-H2AX levels is a sensitive marker for DSBs and has become crucial in research and clinical studies (Redon et al., 2011). Interestingly, ATM- and ATR-dependent signalling overlaps have been demonstrated at DNA double strand breaks (Jazayeri et al., 2006), illustrating the complexity and redundancy of the DDR. The cooperation of all these signalling factors compose the DDR and is crucial for the maintenance of genome integrity. Finally, in addition to ATM, a third kinase, the DNA-dependent protein kinase (DNA-PK) plays a particular role during the G1 phase of the cell cycle in sensing DSB, phosphorylating □-H2AX and promoting the repair of DSBs by end-joining (Blackford and Jackson 2017; Burma and Chen 2004).

DNA lesions that accumulate due to defective or overwhelmed DNA repair can lead to increased mutational burden and/or genomic instability, which are driving forces in tumorigenesis (Curtin 2012; Hanahan and Weinberg 2011; Lengauer et al., 1998). Examples include dominant gain of function mutations in oncogenes and loss of tumor suppressor genes (including DNA repair genes) (Hanahan and Weinberg 2000). Analysis of the mutational landscape of different cancer genomes illustrates how variations in cancer risk result from tissue-specific vulnerabilities to DNA damage caused by specific defects in DNA repair pathways (Tubbs and Nussenzweig 2017).

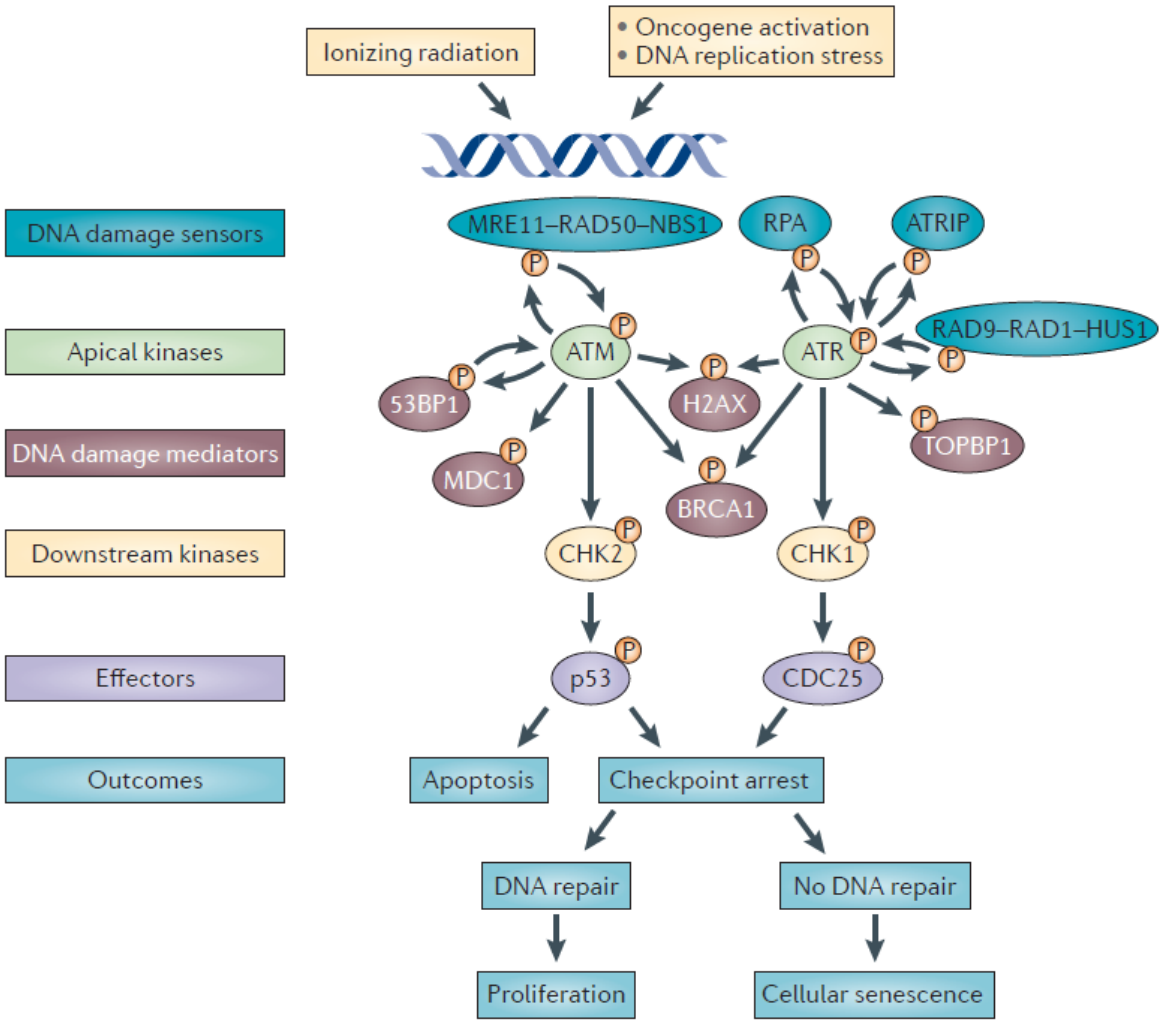

Figure 6. Description of the DNA damage response signalling pathway. The DNA damage response (DDR) pathway is composed of two main DNA damage sensors: the MRE11–RAD50–NBS1 (MRN) complex is able to detect DNA double-strand breaks (DSBs) and the RAD9–RAD1–HUS1 (9-1-1) complex recognizes exposed regions of single-stranded DNA (ssDNA). Upon recognition of the lesion, these sensors recruit the ataxia-telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3-related (ATR) kinases, respectively. In turn, these kinases will phosphorylate (P) the histone variant H2AX on Ser139 (a modification known as γ-H2AX) and trigger the recruitment of several mediators of DNA repair factors and the execution of essential cellular programs including transient cell cycle arrest followed by repair of DNA damage and resumption of proliferation, or when the DNA repair machineries are overwhelmed or DNA repair cannot occur, cellular senescence or cell death. See text for details (Sulli et al., 2012).

Yet, although DNA damage repair is paramount to prevent genetic instability and tumorigenesis, it is also required to counter the accumulation of endogenous damage occurring during DNA replication in highly-proliferative cancer cells (Hanahan and Weinberg 2011; Hoeijmakers 2001) and constitutes the major defence wall against genotoxic anti-cancer agents (Bartkova et al., 2005; Sawicka et al., 2004). This apparent paradox is explained by the notion that cancer cells defective in a given DNA repair pathway become "addicted" to other, partially redundant pathway(s) for survival/response to DNA damage (Nickoloff et al., 2017). Documented examples of DNA repair gene addiction include breast and ovarian cancers harbouring mutations in the tumor suppressor genes BRCA1/2, thus displaying a defect in the recombinational repair of DNA double-strand breaks and synthetic lethality with inhibition of the single-strand DNA break repair factor PARP1 (Turk and Wisinski 2018). These observations have fostered classical therapeutic strategies relying on mainstays of cancer therapies such as IR and genotoxicants to overwhelm highly proliferative tumor cells and trigger cell death (Chabner and Roberts 2005). They have also led to more recent strategies targeting components of the DNA damage response (DDR), including modulation of cell cycle, mitotic progression and genetic stability (Dominguez-Brauer et al., 2015; Lord et al., 2015), which have emerged as an important therapeutic approach against many cancers. Unfortunately, the multiplicity of DNA repair mechanisms, combined with inherent or acquired DNA lesion-tolerance mechanisms hamper the efficacy of the current genotoxic treatment strategies against cancer cells. Thus, identifying and characterizing novel therapeutic targets for DNA-repair based therapeutic strategies clearly remains a crucial challenge.

2.2. Repair of chemoradiation-induced DNA lesions in GBM cells

As discussed previously, the first-line treatment for GBM is composed of chemoradiation; specifically, the concomitant use of IR + TMZ.

The impact of IR is mediated in part by the formation of ROS induced by water radiolysis that will ultimately create oxidative stress in tumor cells and affect cellular components like DNA, proteins and lipids. Guanines have a lower redox potential than other nucleobases (Xie et al., 2007) and are a frequent target for different reactive species, resulting in several oxidation products including 7,8-dihydro-8-oxo-2-deoxyguanosine (8-oxoG), the most

ubiquitous and best characterized product formed in DNA (Markkanen 2017). Because they are rich in guanine, telomeric repeats are particularly prone to ROS-induced oxidative base lesions (Cadet et al., 2008; Dizdaroglu and Jaruga 2012), which interferes with telomere homeostasis, a crucial hallmark of cancer (Hanahan and Weinberg 2011), whereas oxidative base damage can also promote mutation and DNA strand breaks elsewhere in the genome (Cooke et al., 2003; Ensminger et al., 2014; Jena 2012). Base excision repair (BER) is the predominant pathway for the repair of oxidative DNA base damage (Krokan and Bjoras 2013). However, the most deleterious DNA lesions inflicted by IR are DSBs, which can be repaired by two major mechanisms, homologous recombination (HR) and non-homologous end joining (NHEJ).

On the other hand, TMZ is a mono-alkylating agent that forms methyl adducts at the *N*- and *O*- atoms in DNA bases via a S_N-1 (first-order nucleophilic substitution) mechanism. Interestingly, TMZ is a prodrug whose conversion into its active form, the methyldiazonium cation, is mediated by spontaneous hydrolysis at physiological pH (Ramirez et al., 2013) (Figure 7A).

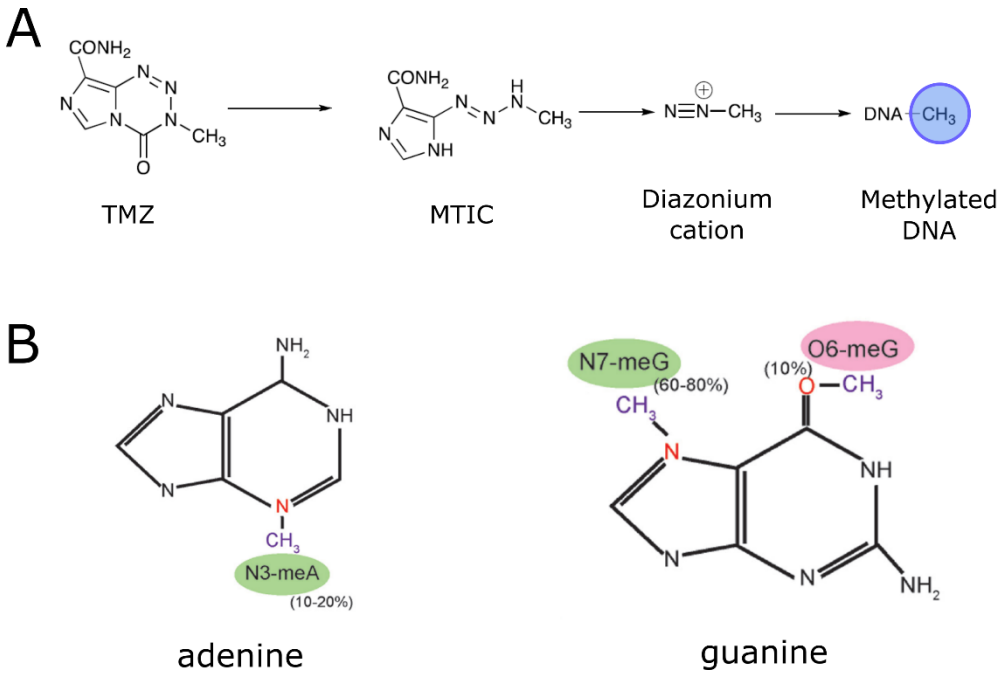

Figure 7. Structure of TMZ and its activated products, and major methylation sites induced by TMZ. A. At physiological pH, TMZ is spontaneously hydrolysed to MTIC (5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide) and the powerful alkylating agent, methyldiazonium cation (adapted from Ramirez et al., 2013). **B.** Representation of the major DNA lesions induced by TMZ and respective methylation frequencies on adenine and guanine DNA bases (adapted from Yoshimoto et al., 2012).

The major lesions induced by TMZ are N7-methylguanine (N7-meG, 60-80%), N3-methyladenine (N3-meA, 10-20%) and O6-methylguanine (O6-meG, 5-10%) (Figure 7B) (Fu et al., 2012; Yoshimoto et al., 2012). N7-meG and N3-meA are repaired primarily by base excision repair (BER) (Bobola et al., 2012), with nucleotide excision repair (NER) proposed to also contribute to the repair of N7-meA and to a lesser extent of N3-meA (Plosky et al., 2002; Smith and Engelward 2000). In contrast, the most cytotoxic lesion induced by TMZ, O^6-meG, is removed by a direct repair mechanism catalysed by the O^6-methylguanine-DNA methyltransferase (MGMT), in a reaction where the methyl group is transferred from the damaged guanine to a cysteine residue present in the active site (C145) of MGMT (Sharma et al., 2009). This reaction is suicidal as the methyl-linked MGMT reaction is irreversible, promoting ubiquitination of the protein and subsequent degradation by the proteasome (Ramirez et al., 2013). When O6-meG is not repaired (for instance in tumors presenting a methylated MGMT gene promoter), it is mispaired with thymine during DNA replication which can eventually cause G:C to A:T transitions hence increase mutation rates (Warren et al., 2006). The O6-meG:T mismatch is recognized by the mismatch repair (MMR) machinery (Kyrtopoulos et al., 1997). However, MMR cannot remove O6-meG, and its action leads instead to single-stranded DNA (ssDNA) gaps that are converted into DSBs during the next rounds of replication, when encountered by the replication fork (Fan et al., 2013; Gupta et al., 2018). TMZ can also induce minor DNA lesions like the N1-methyladenine (N1-meA) and the N3-methylcytosine (N3-meC) that are repaired through direct removal catalysed by the alpha-ketoglutarate (α-KG) and Fe(II) dependent DNA demethylase ALKBH2/3 (Fu et al., 2012; Wyatt and Pittman 2006) (described in Figure 9A right panel). Notably, up-regulation of ALKBH2 confers resistance to TMZ in GBM cells (Johannessen et al., 2013). Also relevant to GBM is the observation that the oncometabolite 2-hydroxyglutarate (2-HG) generated in IDH mutated tumor cells, is able to act as a competitive inhibitor of α-KG, thus impairing the repair activity of the ALKBH2/3 enzymes (Wang et al., 2015). Finally, in addition to causing base methylation, TMZ, also induces oxidative DNA damage (Lopes et al., 2013; Ozben 2007). An overview of the repair pathways responsible for the removal of TMZ- and IR-induced DNA lesion is provided in Figure 8.

2.3. The DNA repair pathways: their clinical impact in GBM patients and therapeutic targeting potential

2.3.1. Repair of the O6-methylguanine lesion by MGMT and clinical relevance of the MGMT promoter methylation status in GBM

As discussed in the previous chapter, MGMT is the dedicated enzyme removing the most cytotoxic lesion induced by TMZ, the O6-meG adduct, using a suicidal enzymatic reaction that leads to degradation of the enzyme (Figure 9A left panel). Therefore, the levels of MGMT protein are a crucial determinant in the response of GBM cells to TMZ. Importantly,

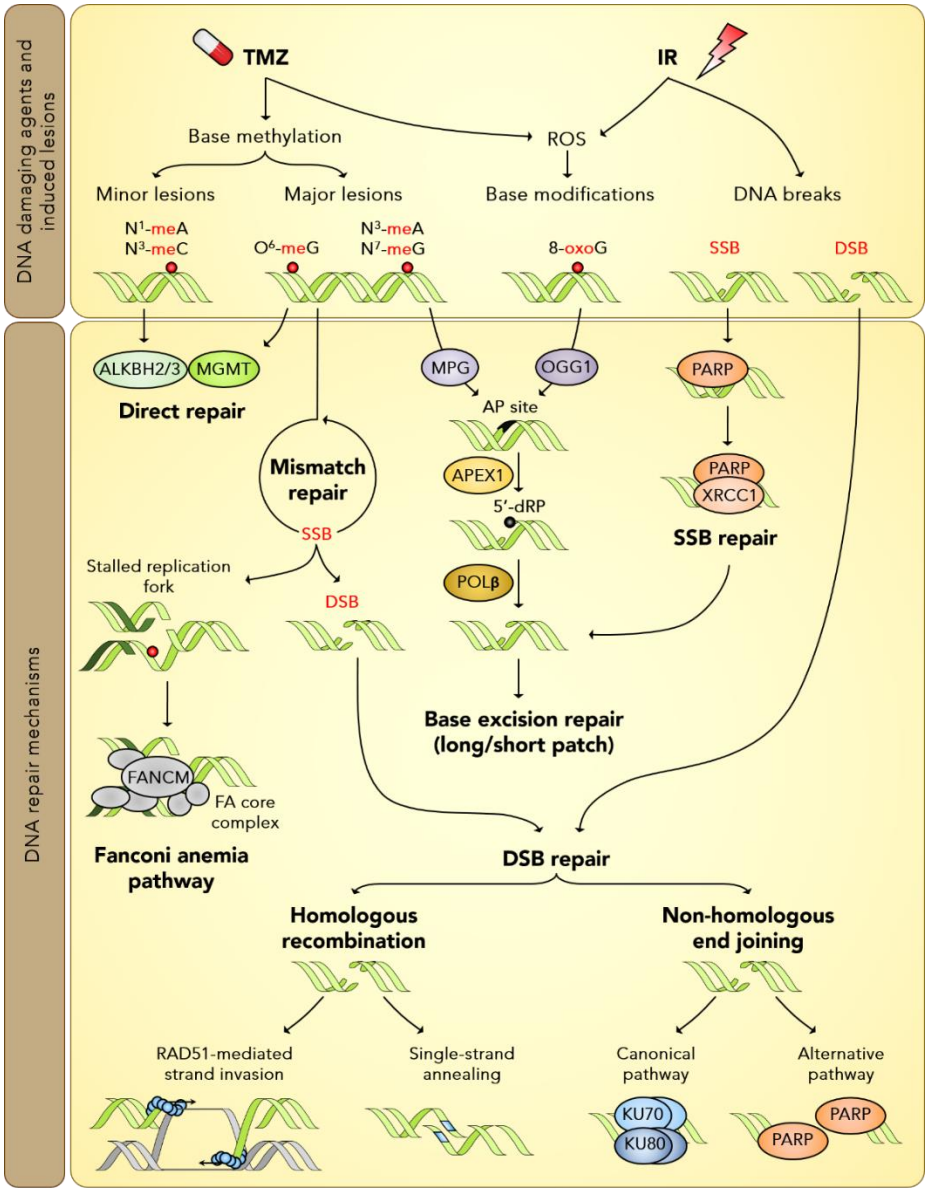

Figure 8. Schematic overview of the DNA repair pathways involved in the removal of IR- and TMZ-induced lesions. The top panel represents a non-exhaustive summary of the main DNA lesions induced by both TMZ and IR and the bottom panel describes the various DNA repair pathways involved in the repair of the mentioned lesions and their crosstalk. See text and Chapter 3 for more details on the different repair processes (Erasimus et al., 2016).

epigenetic silencing of MGMT through promoter CpG island methylation is observed in about 45% of GBMs (Hegi et al., 2005). In this study, MGMT promoter methylation was recognized as a significant predictive marker for GBM patient response to TMZ, as nearly 50% of patients presenting a methylated MGMT promoter were still alive 2 years after concomitant radio- and TMZ-based chemotherapy, compared to only 14% of patients with an unmethylated MGMT promoter (Hegi et al., 2005) (Figure 9B). This observation is related to the fact that promoter methylation negatively regulates MGMT protein expression which directly sensitizes the tumor cells to TMZ therapy (Friedman et al., 1998; Younis et al., 2016). The properties of MGMT and its impact on alkylated DNA damage repair and chemoresistance have led to substantial efforts in GBMs and other solid tumors to deplete the MGMT pool using pseudosubstrates like O6-benzylguanine (O6-BG). Unfortunately, patient treated with the combination of TMZ and O6-BG were faced with severe myelosuppression toxicity during clinical trials (Quinn et al., 2009; Ranson et al., 2006). Several approaches have attempted to overcome this drawback, including direct local administration of O6-BG (Koch et al., 2007) or the use of folate conjugates in order to target the tumor cells specifically (Javanmard et al., 2007). Other approaches rely on the observation that the expression of a O6-BG-resistant MGMT gene construct (MGMT-P140K) in hematopoietic cells provided significant protection against toxicity from O6-BG treatment (Kramer et al., 2006).

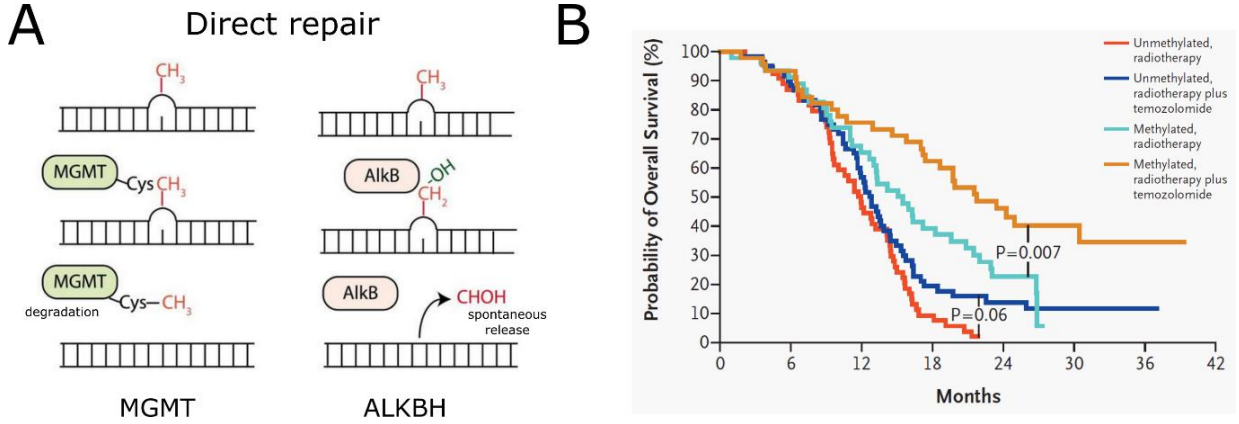

Figure 9. A. Direct repair of methylated DNA bases by MGMT and the ALKBH2/3 enzymes. MGMT removes the O6-meG adduct via a suicide reaction that transfers the methyl group on its catalytic site. The resulting modified enzyme is thought to be targeted to ubiquitination-mediated degradation by the proteasome. ALKBH2 and ALKBH3 are Fe(II)/α-ketoglutarate-dependent dioxygenases that repair N1-meA and N3-meC in DNA by hydroxylation of the alkyl group thereby releasing formaldehyde (Fu et al., 2012). Of note, these enzymes are inhibited by 2-hydroxyglutarate produced by mutant IDH (Wang et al., 2015). **B. Impact of MGMT promoter methylation status on GBM patient prognosis.** Shown are Kaplan-Meier curves illustrating the effect of patient treatment (RT alone or RT+TMZ) on overall survival in relation to the MGMT promoter methylation status (methylated, unmethylated) of the tumor (Hegi et al., 2005).

Thus a clinical trial has been undertaken in which newly diagnosed MGMT-positive patients were transplanted with modified hematopoietic CD34+ cells expressing a MGMT-P140K which conferred chemoprotection to O6-BG/TMZ combination treatment (Adair et al., 2012).

2.3.2. Mismatch repair (MMR)

Mis-incorporations of bases during DNA replication in the newly synthesized strand are recognized and repaired by the mismatch repair (MMR) pathway thereby preventing mutations in dividing cells. The multi-step process is described in detail in Figure 10.

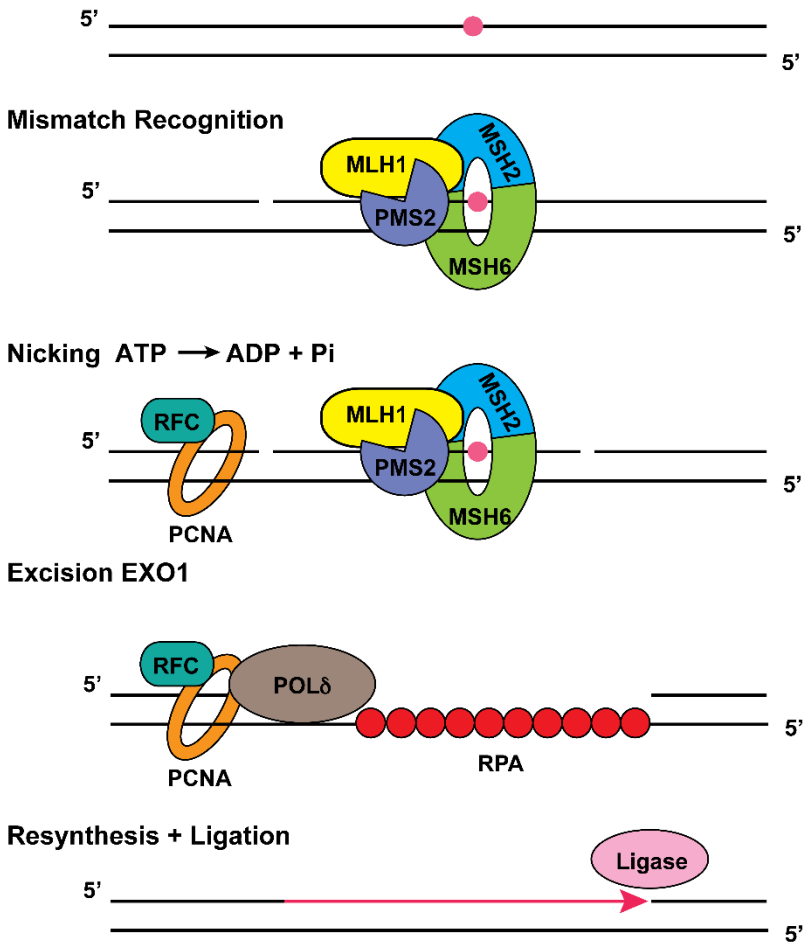

Figure 10. Representation of the mismatch repair pathway. First, the DNA base mismatches can be recognized by different MutS complexes. Specifically, MutSα (composed of MSH2 and MSH6) recognizes simple base-base mismatches and small nucleotide insertions/deletions (1-2 bp) whereas MutSβ (composed of MSH2 and MSH3 (not represented here)) detects larger insertion/deletion loops (2-10 nucleotides). If a DNA nick is present at the 5'-end of the lesion, the replication clamp loader (RFC) and the proliferating cell nuclear antigen (PCNA) are loaded on the DNA. The MutLα complex (MLH1/PMS2) is then recruited at the site of the mismatch. The exonuclease 1 (EXO1, not depicted) will then excise the DNA surrounding and including the mismatch. Finally, replication protein A (RPA) protects the resulting single-strand DNA before filling of the gap by DNA polymerase delta (POLδ) takes place. Finally, DNA ligase 1 (LIG1) seals the nick (adapted from Hsieh and Yamane, 2008).

As seen before, active MMR contributes to the toxicity of O6-meG left unrepaired by MGMT, by inducing mutagenesis as well as potentially lethal DSBs. Importantly, The mutagenic impact of TMZ as a driving mechanism in tumor progression and the selection of TMZ-resistant clones through defective MMR is well documented in gliomas (van Thuijl et al., 2015; Xie et al., 2016), as well as GBM (Cancer Genome Atlas Research 2008; Yip et al., 2009). In fact, MMR dysfunction leads to TMZ tolerance (Humbert et al., 1999), promoting cell survival at the expense of increased mutations rates (Hunter et al., 2006). Ultimately, the question arises if TMZ treatment is profitable in patients presenting low levels of MGMT coupled with deficient MMR. Nevertheless, novel treatment strategies in MMR deficient cells have emerged based on synthetic lethality by inhibition of either POLβ or POLγ leading to accumulation of oxidative DNA damage (Martin et al., 2010).

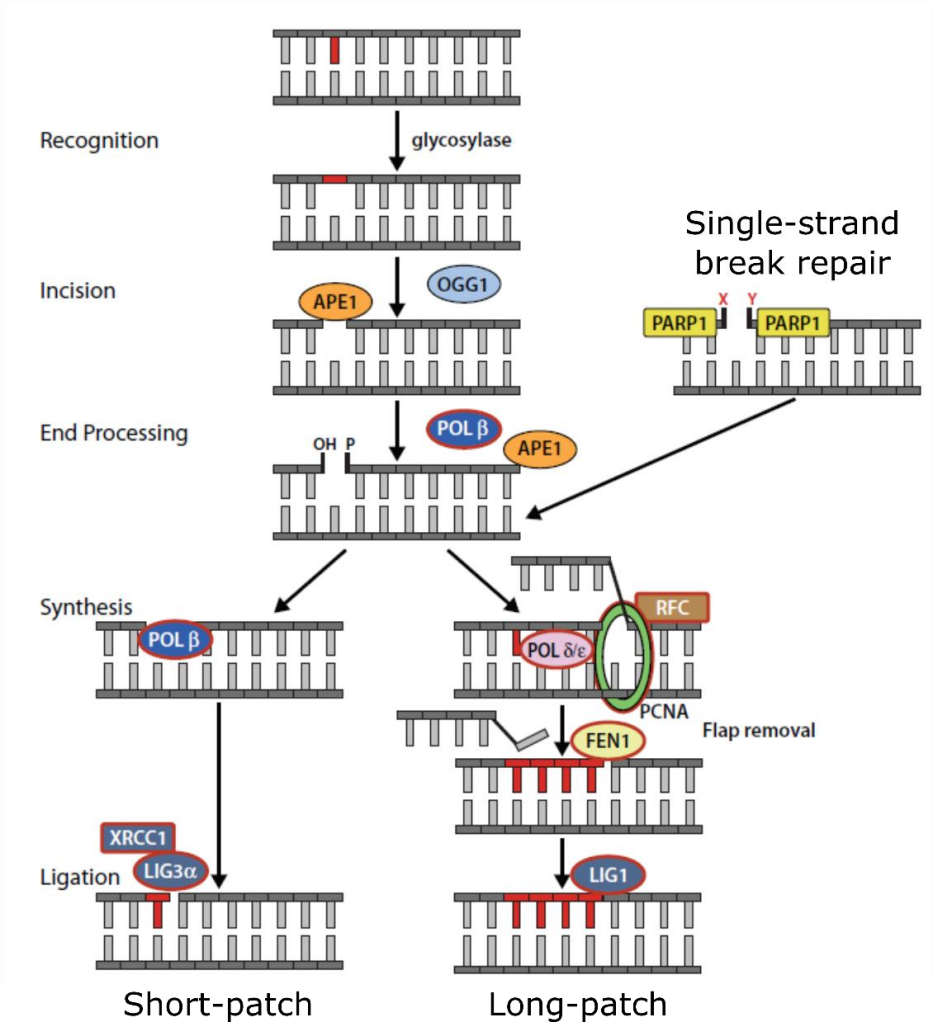

Figure 11. Description of the base excision and single-strand break repair mechanisms. See text for details (adapted from Akbari et al., 2015).

2.3.3. Base excision repair (BER)

The BER pathway operates to remove simple base lesions that do not significantly distort the DNA helix such as base alterations (e.g. oxidation, methylation) and SSBs (Kunz et al., 2009; Lindahl and Wood 1999). In mammalian cells, BER-mediated repair is subdivided in 'short-patch' BER (SP-BER, single nucleotide) and 'long-patch' BER (LP-BER, several nucleotides) that are characterized by the size of the repair patch and the enzymes involved (Fortini et al., 1998; Frosina et al., 1996). BER enzymes are also involved in the repair of ssDNA breaks. The three processes are represented in Figure 11.

During BER, the damaged base is removed and subsequently replaced with an undamaged one. Repair is initiated by the recognition and removal of the modified DNA base, through cleavage of the N-glycosidic bond, by a DNA glycosylase, to generate an apurinic/apyrimidinic site (AP-site). To note, spontaneous hydrolysis of the glycosidic bond of a nucleotide occurs at high frequency under normal aerobic conditions resulting in the generation of AP sites (Lindahl 1993). In mammalian cells, 11 different DNA glycosylases grouped in 4 structurally distinct categories have been identified: the helix-hairpin-helix (HnH) family, the 3-methyl-purine glycosylase (MPG), the uracil DNA glycosylases (UDGs) and the endonuclease VII-like (NEIL) family (Jacobs and Schar 2012). This structural diversity reflects the substrate specificity of each enzyme as they all share the same common principle of action and only differ by the type of lesions and/or DNA context in which they operate. Moreover, further distinction can be made with mono-functional enzymes that only present a glycosylase activity, compared to bi-functional glycosylases that also possess an AP lyase activity allowing to cut the DNA phosphodiester bond, creating a SSB without the need for an AP endonuclease (Jacobs and Schar 2012). The different DNA glycosylase families, their substrate specificities and mode of action is summarized in Table 1. Some of these DNA glycosylases, including NEIL3 – a central player of the present thesis, remain poorly characterized (see later).

AP sites are then incised at the 5' end of the sugar moiety by the AP-endonuclease 1 (APE1), generating a single nucleotide gap intermediate flanked by a 3'-OH and a 5'-deoxyribose phosphate (dRP) end that blocks further processing of the site. In SP-BER, the 5'-dRP residue is trimmed by the DNA polymerase β (Sobol et al., 1996) before gap filling synthesis and subsequent ligation of the repair intermediate by the XRCC1-DNA ligase 3α

complex (Tomkinson et al., 2001) in order to restore the original sequence. Alternatively, the AP site can be processed by the LP-BER sub-pathway, which is dependent on the proliferating nuclear antigen (PCNA), and relies on the combination of the DNA polymerases β/δ and ε which will fill the nick by strand displacement synthesis resulting in a flap of 2-10 nucleotides (Fortini et al., 1998; Frosina et al., 1996). Cleavage of the displaced DNA by the flap endonuclease 1 (FEN1) then takes place before sealing of the nick by DNA ligase 1. The choice between SP- and LP-BER is thought to be dependent on the specificity of the initiating DNA glycosylase (Fortini et al., 1999) and availability of BER factors, thus displaying important cell-type specificity (Bauer et al., 2011; Tichy et al., 2011).

Additionally, BER is also involved in the repair of SSBs (SSBR) in a sequence of events that involves recognition of the damaged termini by the poly(ADP-ribose) polymerase I (PARP1) factor (Caldecott 2008; Liu et al., 2017) and lesion processing by downstream proteins such as the polynucleotide kinase (PNK) that converts potentially improper 5' or 3' margins of the DNA break into 5'-phosphate and 3'-hydroxyl ends for further processing by SP-BER or LP-BER (Caldecott 2014). Thus, the SSBR mechanism is considered a related form of BER.

Direct modification of DNA bases such as methylation and oxidation are among the prominent lesions induced by the concomitant use of TMZ and IR. BER is the supported pathway for the repair of oxidized bases (detailed in section 3.3.1.) and the two major TMZ-induced lesions, 7-meG which does not affect base pairing (Karran et al., 1982) and the more cytotoxic 3-meA adduct that blocks both replication and the transcription machineries (Larson et al., 1985). Of note, BER is a double-edged sword in the management of methylated base damage, since SSBs generated as repair intermediate during the repair process can induce DSBs if encountered by the replication fork (Ensminger et al., 2014) (detailed in chapter 2.3.4.2.). Thus, these authors showed that MPG-defective cells which fail to remove *N*-methylpurines from DNA and do not initiate BER, display strongly reduced levels of methylation-induced DSBs and chromosomal aberrations compared with wild-type cells. Interestingly, higher expression of the DNA glycosylase MPG has been shown to mediate TMZ resistance in GBM cells and associated to poor patient outcome (Agnihotri et al., 2014; Agnihotri et al., 2012; Liu et al., 2012). Although these and other authors have suggested that targeting BER factors might help potentiate TMZ cytotoxicity in GBM (Bobola et al., 2012),

inhibitors of DNA glycosylases are thus far restricted to small molecules targeting OGG1 (Tahara et al., 2018) and NEIL1 (Jacobs et al., 2013), and their effect is still under investigation. Other BER targets include PARP1 for which several direct inhibitors (PARPi) have been developed (Michels et al., 2014).

Table 1. Summary of the different subgroups of the mammalian DNA glycosylases and their main substrate specificity.

Structural families	Name		Substrates	DNA context
Helix-hairpin-helix (HnH)	OGG1	8-oxoG DNA glycosylase 1	8-oxoG, FaPy	Opposite C and dsDNA
	MYH	MutY homolog	8-oxoG	Opposite A and dsDNA
Uracil DNA glycosylase (UDG)	UNG	Uracil-N glycosylase	U, 5-FU	
	SMUG1	Single-strand specific monofunctional uracil DNA glycosylase 1		ss and dsDNA
	MBD4	Methyl-binding domain glycosylase 4	T, U, 5-FU	opposite G and dsDNA
	TDG	Thymine DNA glycosylase	5-hmU, 5-fC	
Methyl-purine glycosylase	MPG	3-methyl-purine glycosylase	3-meA, 7-meG, 3-meG	ss and dsDNA
Endonuclease VIII-like (NEIL) glycosylase	NTHL1	Endonuclease III-like 1	Tg, 5-hC, 5-hU	dsDNA
	NEIL1	Endonuclease VIII-like glycosylase 1	Tg, FapyG, Gh, Sh	ss, bubble and G4 DNA
	NEIL2	Endonuclease VIII-like glycosylase 2	5-hU, 8-oxoG	ss and bubble DNA
	NEIL3	Endonuclease VIII-like glycosylase 3	Gh, Sh, Tg	ds telomeric DNA and G4 structures

*5-fC, 5-formylcytosine; 5-FU, 5-fluorouracil; C, cytosine; ds, double strand; FaPy, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidinel; G4, G-quadruplex; Gh, guanidinohydantoin; h, hydroxyl; me, methyl; sh, spiroiminohydantoin; ss, single strand; T, thymine; Tg, thymine glycol; U, uracil. * M, monofunctonal; B, bifunctional. Adapted from (Jacobs and Schaer 2012, Dou et al., 2003 and Zhou et al., 2013).*

Their mode of action is based on the formation of persistent PARP1-SSB complexes that lead to replication fork collapse and the formation of DSBs requiring HR-mediated repair. Hence, PARPi are particularly effective in the context of synthetic lethality in tumors with defective HR components like BRCA1 or BRCA2, which are unable to repair DSBs induced by PARP inhibition (Lord and Ashworth 2017). Targeting of PARP1 in GBM appears appealing as several studies have highlighted the radio- (Lesueur et al., 2018) and TMZ chemotherapy (Carruthers and Chalmers 2012) sensitizing effect of different PARPi molecules on GBM cells *in vitro* and in preclinical settings (Jannetti et al., 2018). In addition, it has been suggested that the expression levels of both MPG and POLβ modulate the cytotoxic effect of TMZ in glioma cells, in combination with the inhibition of BER through PARPi and methoxyamine (Tang et al., 2011). To date, phase I clinical trials combining standard-of care chemoradiotherapy and

PARPi (olaparib, veliparib) are under investigation in both newly diagnosed (Fulton et al., 2018) and recurrent GBM patients (Robins et al., 2016). Unfortunately, the trial initiated by Robins et al did not unveil any significant improvement in the progression free survival (PFS) of recurrent GBM patients. Although patient stratification may facilitate the implementation of such trials and reveal PARPi-driven survival benefits, it should be noted that synthetic lethality with PARP inhibition can be mediated by various defects in a plethora of DNA repair pathways (Michels et al., 2014) and it is currently impossible to predict sensitivity to PARPi based on gene expression (McGrail et al., 2017). Other BER factors like APE1 and POLβ have been shown to be promising targets in tumor treatment strategies. Firstly, the use of molecules like lucanthone or methoxyamine, which bind to the abasic site thus preventing their processing by APE1, have been shown to potentiate TMZ cytoxicity *in vitro* (Abbotts and Madhusudan 2010; Luo and Kelley 2004). Interestingly, methoxyamine is currently tested in a phase II clinical trial in combination with TMZ in recurrent GBM patients (NCT02395692). Although POLβ is overexpressed in several human tumors (Albertella et al., 2005) and was proposed as a promising target for sensitization of tumor cells to TMZ, none of the small molecule inhibitors developed so far have demonstrated high specificity against this enzyme (Goellner et al., 2011).

2.3.4. Double-strand break repair (DSBR)

Double strand breaks (DSBs) are the most threatening forms of DNA lesions; if misrepaired or unrepaired, they can lead to chromosomal rearrangements, chromosomal loss, carcinogenesis or cell death. In order to preserve genome integrity, cells rely on two major pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR). Importantly, the activity of HR and NHEJ is cell cycle dependent. HR is activated during the S/G2 phase when a homologous duplex (a sister chromatid) is present which can serve as template for repair. In contrast, NHEJ acts through most of the cell cycle but is most prominent in the G1 phase. In addition, HR is considered an error free mechanism whereas NHEJ, which simply apposes, processes and ligates the broken DNA ends, is an error-prone mechanism.

The following paragraphs detail NHEJ and HR, emphasizing those factors that have been shown to play a role in the response of GBM cells to TMZ. Figure 12 illustrates these pathways as well as additional DSBR pathways such as the micro-homology mediated sub-pathway of NHEJ (alt-EJ or alt-NHEJ) and the SSA pathway, whose involvement in GBM remains undocumented.

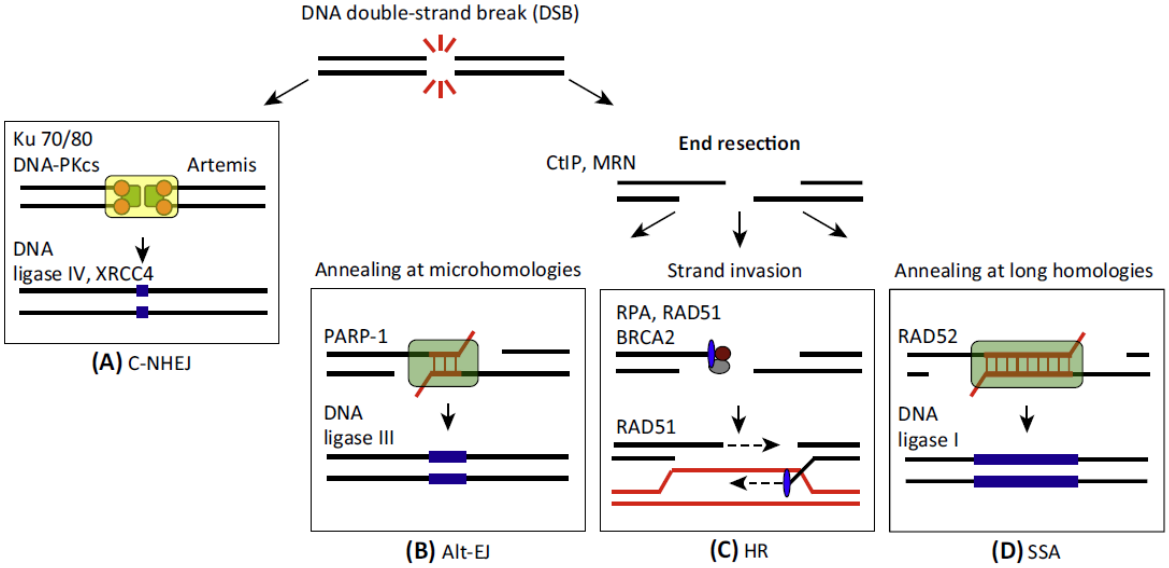

Figure 12. Overview of the major DNA double-strand break repair pathways. The extent of DNA end resection, incurred by the broken ends, which is under complex regulation, is a crucial parameter in determining the choice of DSBR pathway. **A.** In the absence of resection, repair through error-prone c-NHEJ will be favoured. **B-D.** End resection of the broken ends generates 3'OH-ended ssDNA tails that can be engaged by alt-NHEJ (**B**) or HR leading to strand invasion of a homologous, intact duplex DNA (**C**), or expose regions of complementary DNA allowing repair by SSA (**D**). See text for details (adapted from Ceccaldi et al., 2015).

2.3.4.1. Non-homologous end-joining (NHEJ)

DSBR mediated by NHEJ is based on the juxtaposition and direct ligation of the two DSB ends (Chiruvella et al., 2013). The repair process can be subdivided in the canonical NHEJ (c-NHEJ) and the alternative NHEJ (alt-NHEJ) sub-pathways (Figure 12A and B) that are both error-prone, frequently resulting in small genomic rearrangements at the break site or chromosomal translocations when DNA ends from different parts of the genome are joined (Lieber 2010). For instance, NHEJ plays a central role in mediating chromosome end-to-end fusions at telomeres with compromised structural integrity (details in section 3.3).

Classical NHEJ (c-NHEJ) operates during the whole cell cycle. The core c-NHEJ machinery encompasses the DNA-dependent protein kinase (DNA-PK) complex formed of the DNA end-binding Ku70/80 heterodimer and the DNA-PK catalytic subunit (DNA-PKcs). The

repair process is divided into several sequential steps starting with the recognition of the DNA ends by the Ku70/80 heterodimer which protects the DNA ends from degradation, juxtaposes them, activates DNA-PKcs and serve as a scaffold for the recruitment of downstream NHEJ factors (Gottlieb and Jackson 1993; Hammel et al., 2010; Mari et al., 2006). This step is followed by DNA end processing in order to create ligatable ends and finally ligation of both DNA extremities by DNA ligase IV/XRCC4. Interestingly, inhibition of DNA-PKcs enhances TMZ therapy (Lan et al., 2016) and radiosensitizes GBM cells both *in vitro* and *in vivo* (Timme et al., 2018). Moreover, the DNA-PK specific inhibitor CC-115 has been tested in a phase I trial (Munster et al., 2017) and is currently being tested in combination with TMZ in a GBM-specific phase II trial (NCT02977780). Other factors such as Artemis are involved in NHEJ, for instance for processing of the DNA ends when they are not clean (i.e. when they are generated by IR), to facilitate DNA ligation (Chang and Lieber 2016).

On the other hand, alt-NHEJ is mostly active during the S/G2 phase of the cell cycle and is based on DNA end-joining facilitated by micro-homologies fortuitously exposed in the two DNA ends. The enzymatic mechanisms of alt-NHEJ are less well defined than for c-NHEJ. This pathway is independent of the c-NHEJ factors like DNA-PK, relying instead on the SSB sensor PARP1 (Audebert et al., 2004; Della-Maria et al., 2011), which can also recognize DSBs (Audebert et al., 2006; Yang et al., 2018). Interestingly alt-NHEJ is suppressed by the Ku70/80 heterodimer, implying a competition between c- and alt-NHEJ for binding DSBs (Wang et al., 2006). Also involved in alt-NHEJ are DNA ligase III (Lig3) and DNA ligase I (Lig1) (Audebert et al., 2004). As PARP1 is required in alt-NHEJ, PARPi are thought to be an attractive target for therapy in cancer associated with increased alt-NHEJ capacity. PARPi under investigation in GBM are described in section 2.3.3. Of note, alt-NHEJ pathway components have also been proposed as therapeutic targets in high-risk neuroblastoma (Newman et al., 2015). In this context, specific DNA ligases such as Lig3α appear potentially attractive in the targeting of alt-NHEJ proficient tumors and are currently under investigation (Tomkinson et al., 2013).

2.3.4.2. Homologous recombination (HR)

HR is the pathway of choice for the error-free repair of DSBs as it utilizes a homologous, intact DNA duplex as template for repair of the broken DNA (Figure 12C).

The process is more prevalent during DNA replication at the S/G2 phase, since identical sister chromatids are already available as template for repair (Beucher et al., 2009). Essential HR steps include DNA end resection to provide 3'-ended ssDNA strands on which the RAD51 recombinase will assemble filaments that initiate DNA strand invasion. DNA end resection is a highly regulated process that only occurs in the S and G2 phases of the cell cycle and involves several factors including the MRN complex (endonuclease activity), exonuclease 1 (EXO1), and the helicase Bloom syndrome protein (BLM). This process generates 3'-OH ending single-stranded DNA tails that are initially bound by the heterotrimer ssDNA binding protein RPA. The RAD51 recombinase will then assemble filaments on RPA-coated ssDNA, in the presence of mediators such as BRCA2 and PALB2, and these "presynaptic" filaments initiate the search for homologous DNA on the sister chromatid and engages strand exchange. This step, called synapsis, leads to the formation of a displacement (D)-loop that can be subjected to different subpathways, with different genetic outcomes (Li and Heyer 2008). In one such mechanism, the D-loop is extended during elongation of the invading strand by DNA synthesis and captured by the second DNA end. Further DNA synthesis based on the complementary information present on the sister chromatid, followed by ligation, ensure error-free DNA repair. The two duplexes are connected by four-way DNA structures called Holliday junctions. Holliday junction resolution is mediated by two different nucleases, the MUS81/EME1 complex or GEN1, which process the structure differently with the possibility to induce DNA crossovers (Matos and West 2014). The repaired sequences are then ligated, restoring the two DNA strands (Wright et al., 2018). Additionally, a complementary repair process, named single-strand annealing (SSA), is also involved in DSBR but is independent of strand invasion and instead uses resected DNA ends to anneal exposed complementary sequences in order to complete repair (Bhargava et al., 2016) (see Figure 12D).

Inhibition of the HR in cancer cells is enticing but the intricacy of the pathways involved and the complexity of its regulation mechanisms limit the therapeutic potential of HR targeting. Nevertheless, novel strategies emerge that exploit the synthetic lethality relationship between BRCA mutated tumor cells (HR-deficient) and their propensity to be more sensitive to PARPi as discussed in section 2.3.3. To date, very few direct inhibitors of HR factors have been developed. Interestingly, several RAD51 inhibitors have been shown to induce radiosensitization (Budke et al., 2012; King et al., 2017) and enhance the sensitivity of

GBM cells to the alkylating drug CCNU (Berte et al., 2016). Moreover, the use of histone deacetylase inhibitors (HDACi) like valproic acid or vorinostat was shown to radiosensitize tumor cells by indirect inhibition of the DSB repair machinery and are under clinical trials in different cancers (Groselj et al., 2013) including GBM (Kamrava et al., 2008).

It is important to note that the collision of the DNA replication fork with single-stranded DNA (ssDNA) gaps, for instance those generated during MMR processing of O6-meG left unrepaired by MGMT or during BER, gives rise to single-ended DSBs (seDSBs) as opposed to the classical two-ended DSBs generated by IR (Li and Heyer 2008). The repair of seDSBs also involves the HR and NHEJ machineries, including a mechanism of recombination-dependent DNA replication called break-induced replication (BIR) (Anand et al., 2013; Sotiriou et al., 2016).

In summary, the repair of both endogenous and exogenous DNA lesions involves multiple, often redundant DNA damage pathways that are under the control of complex regulatory mechanisms. In this context, it is important to note that several genetic and epigenetic alterations described in GBM have been shown to directly influence DNA repair, thus impacting treatment efficacy. For instance, IDH1 mutation and EGFRvIII have been shown to promote TMZ and radio-resistance by promoting RAD51-mediated homologous recombination (Ohba et al., 2014) and double strand break repair initiation by DNA-PKcs (Mukherjee et al., 2009), respectively. On the other hand, alterations seen mainly in pediatric GBMs such as the specific G34 mutation in H3.3 (Schwartzentruber et al., 2012) and its histone chaperone ATRX (Conte et al., 2012), sensitized GBM cells to DNA damage and thus are associated to better survival.

The complexity of these processes represents a crucial challenge in the development of efficient therapeutic strategies and the care of GBM patients. DNA repair inhibition in combination with genotoxicants is still a fairly new approach in many cancers, thus identification of DDR factors involved in the response of GBM cells to the current therapy remains a crucial step towards the improvement of the standard-of-care for newly diagnosed and recurred GBM patients.

3. Telomere homeostasis and the immortal phenotype of cancer cells

Telomeres, the physical ends of linear eukaryotic chromosomes, are constituted of duplex repetitive, non-coding TTAGGG sequences (between 9-15 kb in size) that terminate with a single-stranded, G-rich overhang (about 50 to 300 nt in size) (de Lange 2005). Telomeres act as disposable DNA buffers, which are truncated after each cell division, to circumvent the end replication problem (Wynford-Thomas and Kipling 1997), thereby preventing senescence and cell death that occur if telomere length falls below a critical threshold (the so-called Hayflick limit) (Shay and Wright 2005), and protecting upstream protein-coding regions.

Responsible for the maintenance of proper telomere length is the nucleoprotein reverse transcriptase called telomerase, composed of a catalytic subunit (TERT) and a RNA component (TERC) which serves as template for the synthesis of TTAGGG repeats (Blackburn 2005). Active in normal stem cells, telomerase undergoes strong negative regulation in all other cell types (Cifuentes-Rojas and Shippen 2012; Wright et al., 1996). However, reactivation of a telomere maintenance mechanism (TMM) is a crucial step during tumorigenesis in order to ensure telomere homeostasis and enable limitless replicative potential (Hanahan and Weinberg 2011). Importantly, telomerase reactivation is the TMM observed in 90% of cancers (Shay and Wright 2011) while telomerase-negative cancer cells use a recombination-based mechanism called Alternative Lengthening of Telomeres (ALT) to maintain telomere length (Reddel 2003).

3.1.Telomere structure, function and maintenance

Telomeres are bound by a protein complex named shelterin formed of TRF1, TRF2, POT1, TPP1, TIN2 and RAP1 (de Lange 2005) (Figure 13A). These proteins protect chromosome ends from DNA degradation and from being recognized as DNA ends by the DSB repair machinery, thus preventing end-to-end fusions (de Lange 2005). TRF1 and TRF2 are tightly bound to the duplex telomeric DNA (Hanaoka et al., 2005), whereas the TPP1-POT1 heterodimer binds the 3’ single-stranded overhang (Wang and Lei 2011). RAP1 can also bind the telomeric sequence and interacts with TRF2 (Li et al., 2000) whereas TIN2 is the cornerstone of the complex as it interconnects TRF1/2 and TPP1/POT1 into a single entity (Ye et al., 2004). Finally, TRF2 can mediate the invasion of the 3’ single-stranded overhang into duplex telomere sequences, leading to a displacement (D)-loop which allows telomere looping into a lariat structure called

t-loop (telomere-loop) that protects the 3'-end (Griffith et al., 1999) (Figure 13B). Importantly, the t-loop prevents the activation of the ATM-dependent DNA damage signalling and end-joining by the NHEJ machinery. In addition, POT1 bound to the t-loop prevents ATR-dependent DNA damage signalling and, together with Rap1, negatively regulates the action of the HR machinery at telomeres (Schmutz and de Lange 2016). Although crucial for telomere capping, TRF1 and TRF2 also promote safe and efficient processing of telomeric DNA during replication (Sfeir et al., 2009; Ye et al., 2010) by orchestrating the dissolution of the t-loop mediated by the RTEL1 helicase (Sarek et al., 2015).

Notably, telomeres are rich in guanine and it has been shown that consecutives runs of guanine, preferentially in the 3' single-stranded overhang of telomeres can fold into a four-stranded DNA structure known as G-quadruplex (G4) (Biffi et al., 2013). Importantly, G4 in telomeric DNA (Williamson 1994) inhibit the telomerase activity (Wang et al., 2011) (Figure

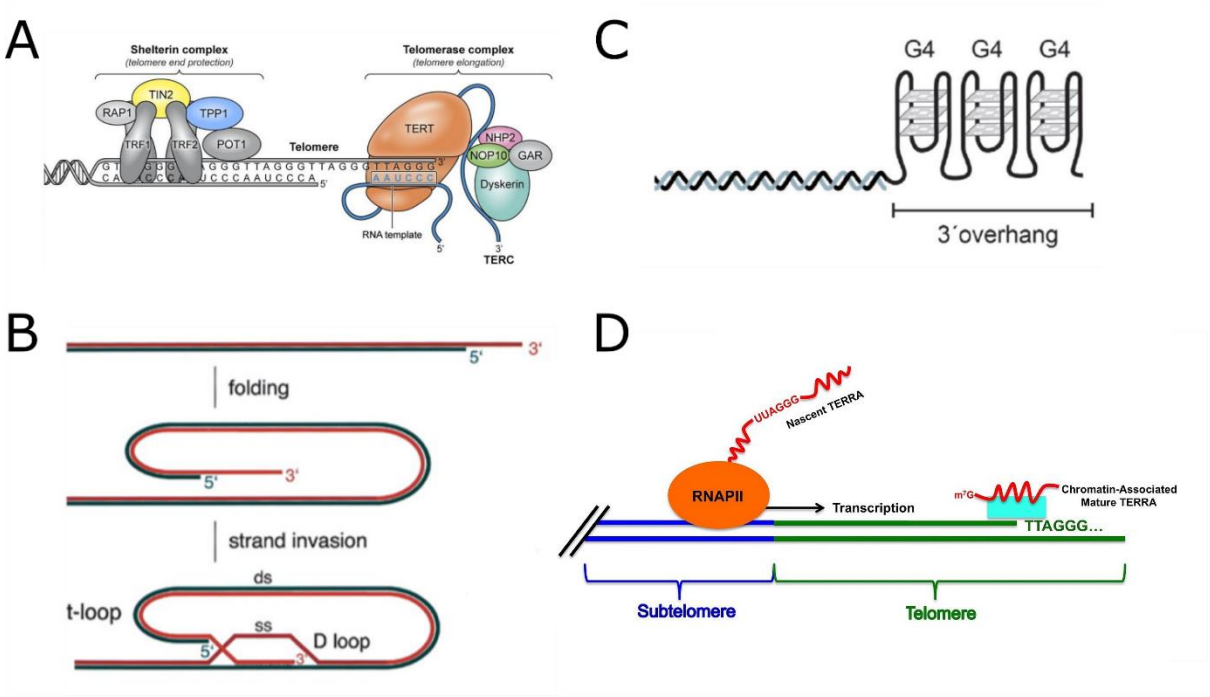

Figure 13. Salient features of human telomeres. A. Schematic description of telomeres, the shelterin complex and telomerase. Shown are the various proteins composing the shelterin complex that protects telomeres, as well as the 4 scaffold proteins (dyskerin, NOP10, NHP2, and GAR) associated with telomerase, itself composed of an RNA template (TERC) and a reverse transcriptase TERT subunit (adapted from Townsley et al., 2014). **B.** In a reaction mediated by TRF2, the G-rich 3'-overhang invades the telomeric duplex DNA to create a displacement (D)-loop that is stabilized by POT1, and the telomere folds back on itself forming a lariat structure known as the T-loop (adapted from de Lange et 2005). **C.** The 3' G-rich overhang at the telomeres can form G-quadruplex structures involved in telomere end protection and regulation (adapted from Rhodes et al., 2015). **D.** Telomeres are transcribed into long non-coding RNAs called TERRAs which originate from the subtelomeric region. TERRA transcripts can be redirect and associated to the telomeres and regulate telomere homeostasis. See text for details.

13C). As will be seen below, G-quadruplex structures also influence the repair of oxidative DNA damage.

For a long time, it was thought that telomeres were transcriptionally inactive due the presence of specific “silencing” histone modifications (like H3K9me3) typically found in heterochromatic regions (such as the pericentromeric regions), as well as the presence of methylated CpG islands in subtelomeric regions (Schoeftner and Blasco 2010). Nevertheless, long non-coding (lnc) telomeric repeat-containing RNAs (TERRAs) were found to be transcribed from telomeres throughout eukaryotes (Azzalin et al., 2007; Cusanelli and Chartrand 2015; Schoeftner and Blasco 2008) (Figure 13D). The transcription of TERRA molecules originates in the subtelomeric region. Thus, TERRAs consist of chromosome end-specific subtelomeric sequences with telomeric repeats at their 3’ ends. These lncRNAs play several critical roles in telomere biology, including the regulation of telomerase activity and heterochromatin formation at chromosome ends (Deng et al., 2009). The emerging evidence indicates that a considerable fraction of the TERRAs are mostly present in non-chromatin-associated RNA pools (Porro et al., 2010) and that they preferentially associate with their telomere of origin forming RNA:DNA hybrids known as R-loops (Balk et al., 2013; Cusanelli and Chartrand 2015). When bound to telomeres, TERRAs are thought to either affect telomere homeostasis, negatively by direct inhibition of the telomerase (Redon et al., 2010) or positively by promoting the lengthening of shortened telomeres they are associated to (Cusanelli et al., 2013). Moreover, TERRA expression has been proposed to participate in telomere protection by preventing activation of the DDR at the telomeres during replication. Interestingly, deregulation of TERRAs was shown to induce widespread telomere dysfunction (Lopez de Silanes et al., 2014). The function and regulation of TERRAs in cells remains a subject of intense investigation, especially in the context of cancer where TERRAs represent an appealing target for novel treatment strategies.

3.2. Reactivation of a telomere maintenance mechanism (TMM) in cancer: specificities of glial tumors

Reactivation of the telomerase is generally mediated by activating somatic mutation of the TERT gene promoter (TERTp) or amplification of both subunits of telomerase TERT/TERC (Gaspar et al., 2018). Notably, ectopic expression of the telomerase catalytic subunit alone is sufficient to immortalize normal human cells (Bodnar et al., 1998). In contrast, the modalities

and molecular details underlying the establishment of the ALT pathway remain largely unknown (Brosnan-Cashman et al., 2018; Nabetani and Ishikawa 2011), although its prevalence in specific cancer types such as sarcomas and low-grade gliomas is associated to mutations in the H3.3 chaperones ATRX and DAXX (Amorim et al., 2016; Brosnan-Cashman et al., 2018; Liau et al., 2015). These two different TMMs are thought to be mutually exclusive and their representation throughout all tumor types is very diverse.

Compared to other high-grade solid tumors, GBM patients present the highest prevalence of TERTp mutations (about 80% of cases) (Boldrini et al., 2006; Killela et al., 2013). Intriguingly, reactivation of telomerase is predominant in adult GBMs (80%) and less so in low-grade gliomas and pediatric GBMs where ALT is more frequently observed (44%) (Killela et al., 2013; Sturm et al., 2014). Recent studies have examined the prognostic value of telomerase-associated parameters like telomerase activity, telomere length and expression of its catalytic subunit, in relation to GBM. Expression of hTERT in GBM patients was found to be associated with higher telomerase activity and adverse prognosis (Boldrini et al., 2006). Additionally, in a cohort of GBM patients harboring TERTp mutations, poor prognosis was confined to the patients who also displayed a specific SNP (rs2853669) in the promoter region of hTERT (Simon et al., 2015; Spiegl-Kreinecker et al., 2015). Since overexpression of hTERT in normal human fibroblasts cells enhances their DNA repair capacity (Shin et al., 2004), it is possible that the increased hTERT expression observed in GBM tumors impacts their resistance to genotoxicants and thus patient survival. In line with this notion, several studies have demonstrated that targeting telomerase either by RNAi-mediated depletion of hTERT (Masutomi et al., 2005), direct inhibition using the antagonist imetelstat in GSCs (Marian et al., 2010)or in a murine orthotopic mouse model (Ferrandon et al., 2015), or indirect restriction of telomerase activity by stabilization of G-quadruplexes using G4-specific ligands (Merle et al., 2011), enhanced the sensitivity of GBM cells *in vitro* and *in vivo* to IR. Furthermore, a similar observation was made in an ALT-positive preclinical model of GBM treated with G4 ligands (Jeitany et al., 2015). These findings demonstrate that the reactivation of a TMM is not only important for telomere homeostasis but can also indirectly influence other cellular pathways important for the fitness of tumor cells.

3.3. Impact of DNA damage at the telomeres and its repair

In addition to maintaining proper telomere length, ensuring telomere integrity by protecting against DNA lesions has evolved as a critical task in cancer cells. Telomeres are notably prone to oxidative stress (Kawanishi and Oikawa 2004) and represent preferential sites of persistent DNA damage (Coluzzi et al., 2014; Hewitt et al., 2012). Given the reactivity of guanine toward oxidative stress, their abundance in telomere repeats and their propensity to generate G4 structures that modify their oxidation potential compared to duplex DNA (Fleming and Burrows 2013), protection at the telomeres is particularly required against oxidative guanine damages. Thus, 8-oxoG, the major lesion induced by oxidative stress on DNA, present in the telomere sequence or within the dNTP pool, regulates telomerase activity (Fouquerel et al., 2016) and disrupts the shelterin complex (Opresko et al., 2005). In addition, the low redox potential of 8-oxoG makes it a target for further oxidation into DNA modifications like the hydantoins guanidinohydantoin (Gh) and spiroiminohydantoin (Sh) (Luo et al., 2000; Luo et al., 2001) which have been shown *in vitro* to destabilize DNA replication substrates (i.e. primer extension substrates) to a much greater extent than 8-oxo-G (Kornyushyna et al., 2002). Furthermore, compared to 8-oxoG, these lesions are more mutagenic (Henderson et al., 2003) and have been shown to impede transcription by blocking RNA polymerase II (Kolbanovskiy et al., 2017). Whereas 8-oxoG is efficiently removed by BER when present in duplex DNA (David et al., 2007), it is poorly repaired when present in single-stranded or G4 contexts and causes significant distortion of the G4 structure (Jia et al., 2015; Zhou et al., 2015). Likewise, Gh and Sh lesions were not a substrate for BER DNA glycosylases when present in G4 structures formed at specific gene promoters (i.e., MYC, VEGF) (Fleming et al., 2017).

However, recent studies using telomere G4-related oligonucleotides with an additional repeat, as well as similar substrates mimicking G4 structures forming at gene promoters, have uncovered a novel mechanism whereby a fifth G-track located a few nucleotides distant from the guanine tracks forming the G4 structure could act as a spare tire, facilitating extrusion of the damaged track to preserve intact G4 folding (in case of 8-oxoG lesions) (An et al., 2016) or facilitate BER (in case of Gh and Sh lesions) (Fleming et al., 2015).

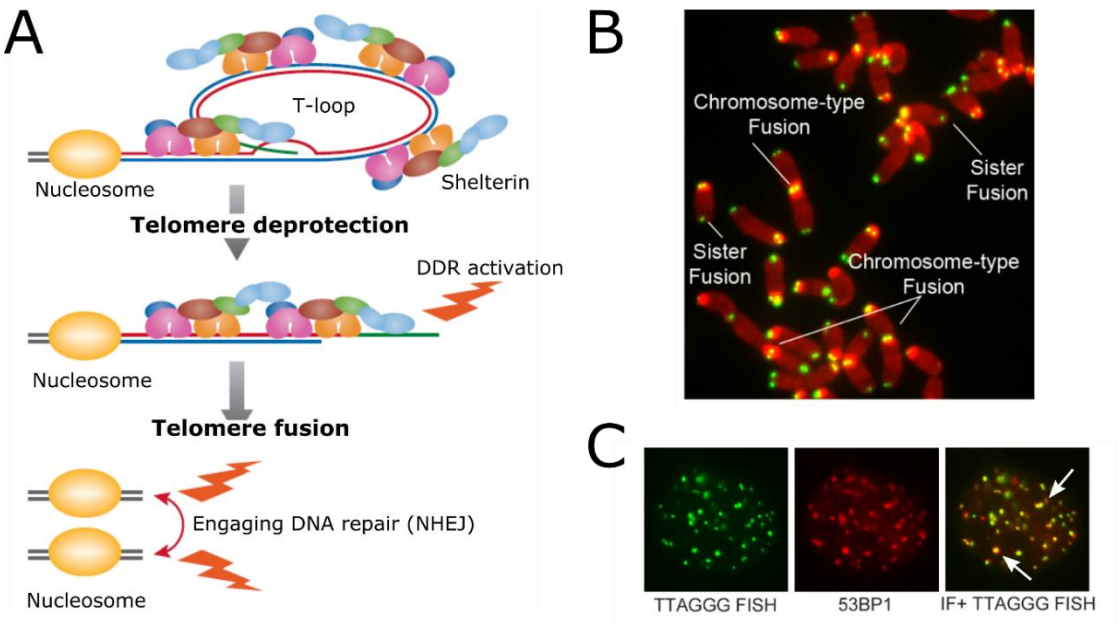

Figure 14. Telomere dysfunction, activation of the DDR and end-to-end fusion. A. "Healthy" telomeres are fully protected from DNA damage signalling and the action of NHEJ through proper capping by the shelterin complex. Progressive telomere shortening (e.g., following DNA replication) or loss of telomere integrity activates the DDR resulting in the formation of TIFs. Depending on the extent of deprotection, such telomeres may still able to prevent NHEJ. However, exacerbation of telomere deprotection ultimately triggers not only DNA damage signalling but also NHEJ-mediated telomere fusions. NHEJ, non-homologous end joining (adapted from Price 2012). **B.** Representative image of telomere fusions in metaphase spreads of TIN2-deficient MEFs. Green, C-rich telomere PNA probe; red, DAPI DNA stain (adapted from Frescas and de Lange, 2014). **C.** Example of telomere dysfunction induced foci (TIF) by co-localization (white arrows) of 53BP1 (immunofluorescence, red) and telomeres (FISH, green) in TIN2-deficient MEFs (adapted from Frescas and de Lange, 2014).

Under normal conditions, DNA repair needs to be harnessed at telomeres to prevent activation of the DDR resulting in end-to-end fusions. This role is devoted in part to the shelterin component TRF2 which can repress ATM-mediated DNA damage signalling (Karlseder et al., 2004) as well as NHEJ at telomeres (Bae and Baumann 2007). However, telomere uncapping can result from critical shortening of telomeres or loss of telomere-protective factors such as TRF2 (Takai et al., 2003) (see Table 2). Due to their resemblance with damaged DNA, uncapped telomeres might become associated with DNA damage response factors such as 53BP1, phosphorylated H2AX and ATM, and the co-localization of these factors at the chromosome ends is termed telomere dysfunction induced foci (TIF) (Takai et al., 2003). To note, unlike genomic breaks, deprotected telomeres do not induce direct DDR-dependent proliferation arrest signalling but instead are passed on to the daughter cells to induce p53-dependent growth arrest in stable diploid G1 phase cells to

ensure proper repair before genome instability can occur (Cesare et al., 2013). Severe loss of telomere integrity results not only in TIFs but also in the activation of the DSBR machinery, notably c-NHEJ, at telomeres leading to chromosome end-to-end fusions (Smogorzewska et al., 2002) (Figure 14).

Interestingly, sister chromatid fusions might promote tumorigenesis by initiating gene amplification processes frequently observed in human tumors (Lo et al., 2002). Recent findings also indicate that both TERRA downregulation and their unplanned accumulation at telomeres is associated with activation of DDR at the chromosome ends (Deng et al., 2009; Lopez de Silanes et al., 2010). Moreover, TERRA transcripts promote structural changes in the chromatin of uncapped telomeres (Porro et al., 2014) thus participating actively in the DDR response at dysfunctional chromosome ends.

Table 2. Representative gene alterations impacting telomere homeostasis.

Gene	Alteration		Global effect	Impact at telomeres	References
NTHL1	Deletion		Defective repair of oxidative base lesions	Telomere fragility in mice and length attrition in telomerase negative cells	Vallabhaneni et al., 2013
OGG1	Deletion (S. cerevisae)	Increase in oxidized guanine levels	Telomere lengthening and reduction of telomere bound proteins	Lu et al., 2010	
	Deletion (M. musculus)		Telomere shortening in mouse embryonic fibroblasts in normoxia conditions	Wang et al., 2010	
POT1	Deletion	NA	DDR activation and aberrant HR	Wu et al., 2006	
TERRA	Upregulation	NA	In the absence of active TMM induces telomere shortening	Maicher et al., 2012 Yu et al., 2014	
	Accumulation	NA	In the absence of telomerase and presence of HR induces telomere lengthening		
TERC	Deletion (M. musculus)	Abnormalities in mutiple organs	Shortened telomeres after several generations of terc$^{-/-}$ intercrosses	Argilla et al., 2004	
TRF2	Deletion	Induction of apoptosis or cell senescence	Telomere uncapping Activation of DDR inducing TIFs and telomere end-to-end fusions	Karlseder et al., 1999 ; Takai et al., 2003	

DDR, DNA damage response; HR, homologous recombination; NA, not available; TIF, telomere dysfunction-induced foci; TMM, telomere maintenance mechanism.

3.3.1. The NEIL3 DNA glycosylase: its function in the repair of telomeric DNA damage and tumor development

As stated before, BER is the predominant pathway for the repair of oxidative DNA base damage (Krokan and Bjoras 2013). To date, five mammalian DNA glycosylases are specialized in the repair of oxidative DNA damage: the 8-oxoguanine-DNA glycosylase (OGG1) which acts mostly on purines (8-oxoG lesions), the endonuclease three homolog 1 (NTHL1) that processes mainly pyrimidine lesions (5-hydroxy lesions) and finally the three members of the Nei Endonuclease VIII-Like (NEIL) family, NEIL1, NEIL2 and NEIL3. The substrate specificities and the DNA context in which these DNA glycosylases operate, is detailed in Table 1.

In contrast to OGG1 and NTHL1, the NEIL DNA glycosylase family is specialized in the removal of the more complex oxidative DNA lesions in specific DNA contexts. Specifically, NEIL1 is preferentially involved in the removal of oxidized purines such as Gh and Sh in duplex DNA (Krishnamurthy et al., 2008; Zhao et al., 2010) as well as in the replication-dependent repair of oxidized pyrimidines (5-hydroxyuracil, formamidopyrimidines) in single-stranded, bubble, and forked DNA structures (Dou et al., 2003). Meanwhile, NEIL2 primarily intervenes in the transcription-coupled repair of oxidized pyrimidines, removing lesions present in single-stranded and bubble DNA structures (Dou et al., 2003). Contrasting with NEIL1 and NEIL2 which show very similar substrate specificity, NEIL3 displays distinct properties that translate into a preference for DNA damage present in specific DNA regions, as detailed in the next section.

3.3.1.1. NEIL3 structure and activity towards oxidized guanine lesions

The NEIL3 gene sequence encodes a protein of 605 amino acids that is composed of an N-terminal Nei-like glycosylase domain (GD), a DNA-binding domain (DB) of the helix-two-turn-helix type (H2TH), two zinc finger domains, as well as a nuclear localization sequence (NLS) that allows its translocation into the nucleus. What distinguishes NEIL3 from its family members is a long intrinsically disordered C terminal domain (CTD) (Liu et al., 2010; Liu et al., 2013) (Figure 15A). The biochemical characterization of NEIL3 glycosylase domain revealed a distinct preference for the removal of oxidative DNA lesions like Gh and Sh (Krokeide et al., 2013). Furthermore, NEIL3 preferentially removed Gh and Sh guanine lesions in G4

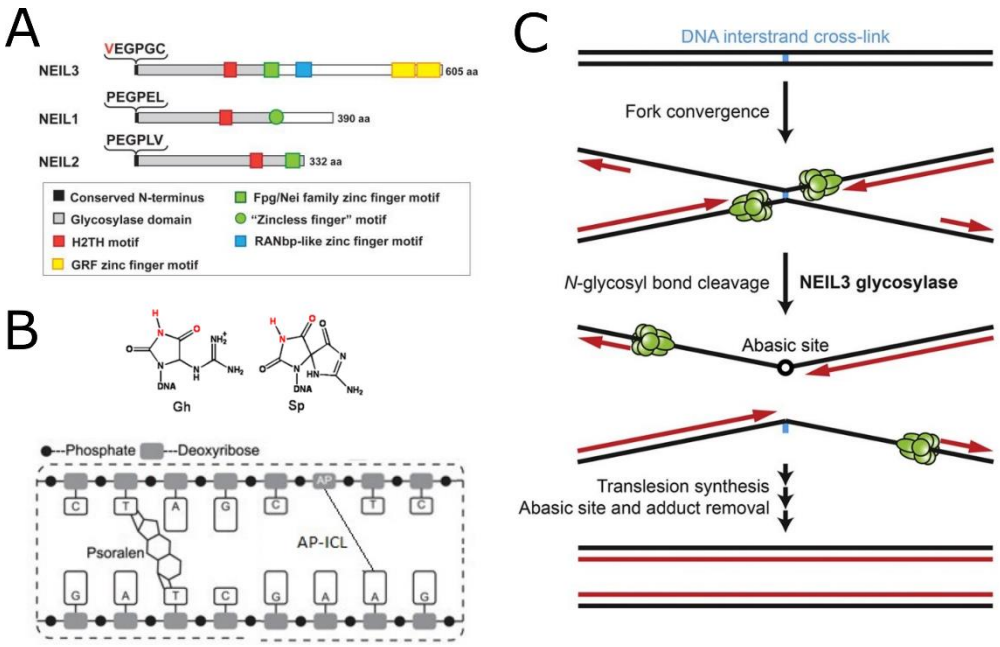

Figure 15. Structure of NEIL3, its substrates and role in the unhooking of ICLs. A. Schematic overview of the structural features of NEIL3 and the other NEIL glycosylase family members (NEIL1/2). The three enzymes present a similar N-terminal sequence with the presence of a large glycosylase domain (grey), a conserved N-terminus (black), a helix-2-turn-helix DNA binding motif (red), a Fpg/Nei family specific DNA binding Zinc finger motif present in NEIL1 and NEIL3 (green square) while NEIL2 displays a zincless finger motif (green sphere). Compared to its family members, NEIL3 has a large disordered C-terminal sequence that harbors two more zinc finger motifs (blue and yellow) whose functions are not characterized to date (adapted from Liu et al., 2013). **B.** Summary of the DNA lesions recognized and processed by NEIL3. Top panel: oxidized guanine lesions guanindinohydantoin (Gh) and spiroiminohydantoin (Sh). Bottom panel: Exogenous psoralen-induced inter-strand crosslink (ICL) and endogenous abasic site ICL generated after spontaneous depurination or the action of DNA glycosylases during the BER-mediated repair of a damaged base (adapted from Semlow et al., 2016). **C.** NEIL3 is involved in the replication-coupled unhooking of ICLs. The process involves cleavage of the N-glycosyl bond at the lesion site, releasing an abasic site on one strand and the crosslinked bases on the other strand. The replication fork is then free to resume replication and the remaining lesions are subsequently repaired (adapted from Semlow et al., 2016).

quadruplex DNA structures present at the telomeres and in promoter regions (Zhou et al., 2015; Zhou et al., 2013). Intriguingly, in the same study, NEIL1 was also found to be involved in the removal of oxidized lesion in G4 structures but showed a preference for Gh lesions compared to NEIL3 which excised Tg, Gh and Sh with higher efficiency in the telomere sequence context (Zhou et al., 2013). Importantly, a recent study unveiled that NEIL3 could be directly mobilized to the telomeres through its disordered CTD domain (Zhou et al., 2017). Specifically, the authors made the important observation that the shelterin subunit TRF1 was able to actively recruit the enzyme to the telomeres by direct protein-protein interaction in order to initiate BER-mediated repair of telomere damage during the S phase of the cell cycle (Zhou et al., 2017). Moreover, siRNA-mediated knockdown of NEIL3 in human colorectal carcinoma (HCT116) cells induced dysfunctional telomeres with an increase in telomere loss and sister chromatid fusions, thus correlating the *in vitro* functional studies and supporting a

direct role of NEIL3 in telomere homeostasis *in vivo*. It must be noted that the DNA glycosylases NTHL1 and OGG1 have also been indirectly implicated in the protection of telomere integrity. Intriguingly, OGG1 loss in S. cerevisae induced telomere lengthening through increased oxidative DNA damage (Lu and Liu 2010), meanwhile OGG1 deletion in mouse embryonic fibroblasts (MEFs) had opposite effects in normoxia and hypoxia conditions with the observation of telomere shortening and lengthening (Wang et al., 2010), respectively. Moreover, NTHL1 knockout mouse cells displayed telomere fragility due to defective repair of oxidative base lesions and telomere shortening (Vallabhaneni et al., 2013), further implying the peculiar role of BER-mediated repair of oxidative telomere DNA damage. Alterations in several representative DNA repair and telomere factors, and their impact on telomere homeostasis are summarized in Table 2.

Finally, a BER-independent function has also been identified for NEIL3. Indeed, NEIL3 was described as a primary unhooking mechanism for specific DNA interstrand cross-links (ICLs) associated with psoralen and abasic sites (Figure 15B), through its DNA glycosylase activity (Semlow et al., 2016) (Figure 15C). For instance, NEIL3 was found to incise psoralen-induced ICLs in four-stranded DNA structures (Martin et al., 2017).

3.3.1.2. NEIL3 expression signature and function in normal and tumor cells

High expression of NEIL3 seems to be confined to cells with a high proliferative potential, such as neural and hematopoeitic stem cells (Hildrestrand et al., 2009; Reis and Hermanson 2012), while it appears to be repressed in non-dividing cells (Neurauter et al., 2012). In newborn mice, expression of Neil3 was specifically observed in subregions of the brain where neurogenesis takes place, such as the subventircular zone, and decreased during development (Hildrestrand et al., 2009). This study was further correlated with the observation that homozygous depletion of the gene induced defects in adult progenitor cell populations (Regnell et al., 2012). Although the *in vivo* function of NEIL3 remains unclear, it was shown to regulate neurogenesis after a hypoxic-ischemic event in mice (Sejersted et al., 2011) and promote cardiac fibroblast proliferation after myocardial infarction (Olsen et al., 2017). Moreover, a recent study demonstrated that triple negative Neil DNA glycosylase deficient mice (Neil1$^{-/-}$, Neil2$^{-/-}$ and Neil3$^{-/-}$) showed no increased propensity to cancer or

higher mutation frequencies and that MEFs did not accumulate oxidative DNA damage and neither telomere length was affected (Rolseth et al., 2017).

In addition, NEIL3 was shown to be significantly upregulated in a panel of 16 different tumor tissues (Hildrestrand et al., 2009). Particularly, analysis of the TCGA database revealed the upregulation of NEIL3 and concomitant downregulation of NEIL1 and NEIL2 in a subset of different cancer types. Deregulation of the whole NEIL glycosylase family was further associated with increased somatic mutation load (Shinmura et al., 2016). Of note, this study covered 13 cancer types but not GBM. However, a study on GBM patients in the Han Chinese population found that a specific single nucleotide polymorphism in NEIL3 (rs12645561) was associated with the occurrence of GBM (Jin et al., 2013). Furthermore, NEIL3 knockdown was shown to reduce cell proliferation and increase sensitivity to the oxidative stress inducer paraquat and the DNA crosslinking agent cisplatin in mouse embryonic fibroblasts (Rolseth et al., 2013) and to ATR inhibitors in GBM cells (Klattenhoff et al., 2017).

Although the available evidence pinpoints NEIL3 as a potential target in cancer cells, its role in telomere homeostasis, specifically in relation to glioma development and chemoresistance, remains vastly unexplored.

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CHAPTER 2

SCOPES & AIMS

Today still, most anti-cancer treatments rely heavily on genotoxicants or IR to induce tumor cell death through accumulation of DNA damage. Because tumor cells respond to these insults by engaging the DNA damage response and executing complex DNA repair pathways, targeting components of these mechanisms has emerged as an important therapeutic approach against many cancers. At the same time, predicting tumor response to DNA damaging agents remains a challenge that is exacerbated by the multiplicity and intricacy of the DNA repair pathways. In addition to MGMT, complex DNA repair pathways and DDR components contribute to resistance to TMZ and IR in GBM, thus mediating its poor outcome. The management of newly diagnosed GBM patients has not changed since the introduction of TMZ in 2005 and no novel approach tested in the context of clinical trials has yet demonstrated encouraging benefits.

As exposed in Chapter 1, DNA repair factors are under intense scrutiny in order to identify novel targets for the sensitization of GBM cells to the current therapy or the development of altogether new treatment strategies. As an approach to identify novel DNA repair and DDR factors involved in the response of GBM cells to chemoradiation, shRNA/siRNA-based *in vitro* screens have revealed several hitlists of potential targets that may translate to the clinic, whilst also contributing to our understanding of DNA repair in GBM (Agnihotri et al., 2014; Luo et al., 2009; Svilar et al., 2012). Nevertheless, despite these advantages, RNA interference screens are inherently limited by the nature/properties of the cells they are using, which adapt to the *in vitro* environment and may not fully recapitulate the complexity of GBM observed in patients.

The heterogeneity of GBM tumors adds to the complexity of this disease and increasing studies have aimed to stratify patients into clinically-relevant prognostic subgroups based on specific molecular alteration profiles (Verhaak et al., 2010; Brennan et al., 2013; Schwarzentruber et al., 2013; Wang et al., 2017). Such studies have mainly focused on whole transcriptomic/genetic data generated in primary GBM specimens. Although various molecular alterations were discovered and associated to patient prognosis, methylation of the MGMT promoter remains the only marker proven to effectively predict TMZ efficacy in the clinic (Hegi et al., 2005). Thus, although these studies have shed light into the complexity of GBM, it may be argued that they have failed to grasp the determinants behind chemo- and radioresistance in part because of the huge span of genes they are analysing. In addition, the use of cohorts of paired GBM specimens (i.e., biopsies from the same patient obtained at the time of primary tumor operation and at recurrence) to explore DNA repair and the impact of chemoradiation has not been considered. Indeed, although the use of paired biopsies is gaining momentum, their

availability, especially specimens predating the introduction of TMZ in the clinic, is still limited in part by the speed of progress in the biobanking of reliable and clinically annotated specimens. Yet, such paired biopsies offer the possibility to examine, through comparison of primary and matched recurrent samples, the impact of chemoradiation on crucial parameters such as tumor phenotype, activation of the DDR, mobilization of DNA repair factors, and changes in the expression of DNA repair/DDR factors.

In this context, the general aim of the project was to undertake a novel approach to understand how DNA repair mechanisms contribute to the resistance of GBM tumor cells to chemoradiation therapy and propose improved strategies to undermine DNA repair or exploit specific vulnerabilities associated with GBM cells. Specifically, we decided to focus on a cohort of paired GBM samples from patients treated with RT or RT plus TMZ with the aim of exploring alterations in DNA repair and cell cycle gene expression in primary GBMs and their recurrences. By doing this, we were hoping to combine the benefits of using GBM specimens and a targeted gene expression analysis focusing only on the major factors in the DNA damage response and -related cell cycle genes. In addition, the uniqueness of our patient cohort, would allow different comparisons to be made, in order to unravel the DNA repair makeup of tumor cells during glioblastomagenesis and examine the possible effect of TMZ and IR on the DDR and DNA repair.

Consequently, my research project was divided in two axes with specific aims:

1) Analyze the expression of selected components of the DDR, DNA repair and cell cycle machineries in a cohort of paired primary and recurrent GBM biopsies in order to uncover and characterize gene expression alterations associated with chemoradiation. This research led to the proposal of a novel clinically applicable gene signature that exposes specific vulnerabilities against genotoxicants and inhibitors of the cell cycle and DDR, with the prospect of personalized therapeutic strategies (Chapter 4)
2) Exploit the gene expression data obtained in (1) to identify key molecular players of the DNA damage response in GBM for further functional characterization using patient-derived cell lines. This research led to the identification of the NEIL3 DNA glycosylase as a novel factor involved in tumor development, response to TMZ and telomere integrity in GBM cells (Chapter 5)

CHAPTER 3

RESULTS

DNA repair mechanisms and their clinical impact in glioblastoma

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RATIONALE

This first part of the result section represents a comprehensive review of the DNA repair mechanisms driving chemoradiation resistance and tumor relapse in GBM, including DNA damage response-related biomarkers. In particular, we introduce the most prominent cellular systems as well as the animal models currently available for the study of DNA repair in the context of glioblastoma. Moreover, we summarize recent progress in the knowledge of the major pathways and factors involved in the removal of IR- and TMZ-induced DNA lesions. In line with these observations, we introduce current therapeutic strategies relying on DNA repair inhibitors tested *in vitro* or in clinical trials, and present the main challenges in drug delivery to the brain as well as the strategies developed to circumvent this major hurdle. Finally, we review the novel genetic and epigenetic alterations that were found to shape the DNA repair makeup of GBM in both adult and pediatric patients, and discuss their potential implications for personalized therapy.

Personal contributions: I am first co-first author of this review and my main significant contributions were to address and investigate novel DNA-damage response-related biomarkers of GBM in the literature (Chapter 6) and the design, including the conception/drawing, of the Figure representing the main pathways involved in the repair of IR- and TMZ-induced DNA lesions (Figure 1), as well as the depiction of the molecular basis of small molecule inhibitors (Figure 4). Finally, I was involved in the establishment and description of the list of alterations affecting DNA repair and chromatin factors in both adult and pediatric GBMs (Table 2).

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Review

DNA repair mechanisms and their clinical impact in glioblastoma

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ABSTRACT

Despite surgical resection and genotoxic treatment with ionizing radiation and the DNA alkylating agent temozolomide, glioblastoma remains one of the most lethal cancers, due in great part to the action of DNA repair mechanisms that drive resistance and tumor relapse. Understanding the molecular details of these mechanisms and identifying potential pharmacological targets have emerged as vital tasks to improve treatment. In this review, we introduce the various cellular systems and animal models that are used in studies of DNA repair in glioblastoma. We summarize recent progress in our knowledge of the pathways and factors involved in the removal of DNA lesions induced by ionizing radiation and temozolomide. We introduce the therapeutic strategies relying on DNA repair inhibitors that are currently being tested in vitro or in clinical trials, and present the challenges raised by drug delivery across the blood brain barrier as well as new opportunities in this field. Finally, we review the genetic and epigenetic alterations that help shape the DNA repair makeup of glioblastoma cells, and discuss their potential therapeutic impact and implications for personalized therapy.

Contents

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1. Introduction

Glioblastoma (GBM, grade IV glioma)[2] represent the most frequent and aggressive malignant primary brain tumors in humans. GBMs encompass secondary GBMs (about 5% of the cases) which develop from lower-grade diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III), and primary GBMs (about 95% of the cases) which arise rapidly de novo without clinical or histological evidence of a less malignant precursor. GBMs display very poor prognosis and lack therapeutic options, further complicated by the presence of the blood brain barrier (BBB). Current management of GBMs usually consists of surgical resection, followed by radiotherapy (RT) with concomitant and adjuvant chemotherapy with the DNA alkylating agent temozolomide (TMZ) [1], both treatments inducing DNA damage. Although TMZ displays antitumor activity and limited toxicity, its survival benefit remains unsatisfactory – a mere 2.5 months [1], due to rapid occurrence of resistance and tumor relapse.

GBMs are characterized by an important intertumor and intratumor heterogeneity both at the cellular and genomic levels [2,3]. Significant progress has been made in our characterization of the molecular alterations found in GBM biopsies, leading to a comprehensive landscape of somatic genomic alterations in glioblastoma and refining the list of putative GBM driver genes [4]. Adult and pediatric GBM subtypes with different presentation and/or progression courses as well as therapeutic responses have been proposed based on molecular analyses [5]. Likewise, advances in surgical sample collection, integrated genomic analyses and single-cell technology have shed new light on the (regional) intratumor heterogeneity within GBM patients, revealing patterns of cancer evolution at the single-patient level [6,7]. Although what drives such dynamic heterogeneity at the cellular level remains unclear ([8], and references therein), it may hold important keys to understanding the response of GBM cells to genotoxics and tumor recurrences [9,10].

Sophisticated cellular mechanisms have evolved to detect, signal and repair the various DNA lesions inflicted in our chromosomes by endogenous or exogenous genotoxics. The robustness of the DNA repair mechanisms composing this DNA damage response (DDR) is ensured in part by the inherent redundancy of the many pathways that can remove a specific lesion and also by the fact that DNA repair pathways function in complex networks, with DNA-repair intermediates produced by a given pathway oftentimes forming substrates for another pathway [11]. In non-neoplastic cells, accurate DNA repair is essential to prevent genetic instability, a driving force in tumorigenesis. Thus, cancer cells often display genetic or epigenetic alterations that affect DNA repair factors [12]. Paradoxically, DNA repair mechanisms are also paramount to the removal of lesions induced by genotoxic anti-cancer agents, and a crucial factor contributing to the development of resistance and tumor relapse. Furthermore, tumorigenic cells experience greater dependence on residual DNA repair mechanisms that help them cope with exacerbated DNA damage resulting from increased cellular metabolism (i.e., oxidative DNA damage) as well as replication and/or mitotic stress [13], whereas conditions imposed by the tumor microenvironment (e.g., hypoxia) can profoundly alter the expression and function of DNA repair genes. Understanding the molecular details underlying the response of tumor cells to genotoxics and deciphering the exploitable cancer-specific genetic alterations in the DNA damage response are recognized as crucial steps in the development of strategies that will improve cancer management. In recent years, several therapeutic strategies and DNA repair inhibitors have been elaborated to take advantage of defective DNA repair or saturate altogether the DNA repair capacities in cancer cells [12,14].

The main DNA lesions induced by ionizing radiation (IR) and TMZ, together with the major factors involved in their repair are recapitulated in Fig. 1. The most severe DNA lesion inflicted by IR are double-strand breaks (DSBs), which are repaired by two mechanisms each composed of several pathways – homologous recombination (HR) and non-homologous end joining (NHEJ) [15,22]. Other forms of DNA damage induced by IR include base damage and single-strand breaks (SSBs), which are repaired, respectively by base excision repair (BER) and SSB repair. The major lesions induced by TMZ are N^7-methylguanine (N^7-meG) and N^3-methyladenine (N^3-meA), which are primarily repaired by BER, and O^6-methylguanine (O^6-meG), a highly cytotoxic lesion which is removed by the O^6-methylguanine-DNA methyltransferase (MGMT) product of the MGMT gene [16]. MGMT is a notable exception to the multi-protein DNA repair pathways, as it works single-handedly in a suicidal reaction that transfers the alkyl group from guanine to an internal cysteine residue. Loss of MGMT by promoter hypermethylation is commonly observed in colorectal carcinomas, gliomas, non-small cell lung carcinomas, lymphomas and head and neck carcinomas [29]: in colorectal cancer, such inactivation is an early event associated with mutagenic consequences [30]. MGMT promoter CpG methylation is observed in about 45% of patients presenting with GBM and is associated with increased sensitivity to TMZ and prolonged survival [31]. In the absence of MGMT, unrepaired O^6-meG can pair with cytosine or thymine leading to O^6-meG/T mispairs that are recognized by the mismatch repair (MMR) machinery. However, MMR action leaves the O^6-meG intact, introducing instead SSBs that are converted to potentially lethal DSBs at replication forks. TMZ-induced mutational inactivation of MMR genes has been observed in recurrent GBM tumors [9,32], consistent with the notion that loss of MMR contributes to resistance to TMZ. Repair of DSBs generated as a result of MMR activity involves the DSB repair machineries as well as proteins belonging the Fanconi Anemia (FA) pathway involved in the recombinational repair of perturbed or broken replication forks [33,34].

2. Scopes of this review

Various reviews have addressed genetic, biochemical and molecular aspects of DNA repair and therapy in glioblastoma [35–37]. Here, we focus on recent developments in the following fields of primary GBM research: i) the experimental and preclinical models used in studies of DNA repair in GBM, ii) the regulation/inhibition of MGMT expression and activity, iii) novel insights into the DNA damage response and DNA repair inhibitors for the treatment of GBM, and iv) DDR-relevant genetic and epigenetic alterations identified in primary GBMs.

Throughout this review, we will also emphasize how strategies driven by RNA interference have contributed to the identification

[2] The abbreviations used in this review are defined in Table 1.

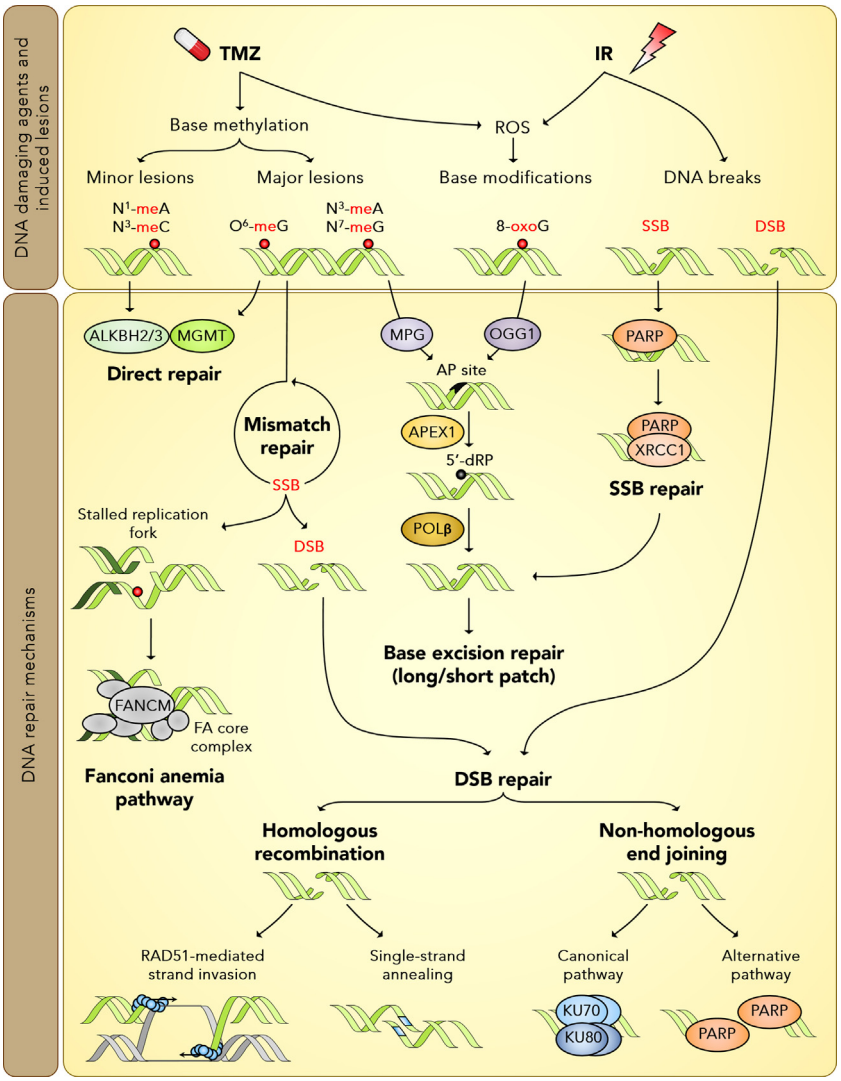

Fig. 1. DNA repair pathways involved in the removal of IR- and TMZ-induced lesions. Schematic overview the lesions induced in DNA by IR and TMZ, as well as the repair mechanisms that have been implicated in their removal in GBM. Only those factors that are discussed in the review have been indicated. The reader is referred to Ciccia [15] for the mechanistic details of each pathway and the molecular composition of the various DNA repair machineries. *Removal of TMZ-induced alkylated bases:* Direct reversal of O^6-meG is mediated by MGMT. In the absence of MGMT, O^6-meG can direct misincorporation of T during DNA synthesis, resulting in mismatch repair (MMR)-driven futile cycles that lead to the generation of DNA single-strand breaks (SSBs), double-strand breaks (DSBs) and replication-associated DSBs. Removal of N^7-meG and N^3-meA, which is mediated by Base Excision Repair (BER), is initiated by the DNA glycosylase MPG. Repair of the resulting apurinic/apyrimidinic (AP) site can involve the replacement of either a single nucleotide (short-patch BER) or several nucleotides (long-patch BER). TMZ can also generate N^1-methyladenine (N^1-meA) and N^3-methylcytosine (N^3-meC) [16,17]. These lesions may be removed by the oxidative demethylases ALKBH2 and ALKBH3 which are capable of directly reversing N^1-meA and N^3-meC in DNA [18,19]. ALKBH2 was found to confer resistance to TMZ in GBM cells [20]. *Repair of oxidative DNA damage:* TMZ- and IR-induced oxidative DNA damage includes oxidized bases (such as 8-Oxo-7,8-dihydroguanine (8-oxoG)) that are recognized and excised by a variety of DNA glycosylases that initiate BER [21]. *SSB repair:* SSBs generated by IR and Reactive Oxygen Species (ROS) or during processing of TMZ-induced DNA lesions are recognized and bound by poly(ADP-ribose) polymerase family members such as PARP1, leading to the recruitment of the scaffold protein XRCC1. Repair is then achieved through short- or long-patch BER repair. *DSB repair:* Repair of DSBs can be achieved by two major pathways, homologous recombination (HR) and non-homologous end joining (NHEJ) DNA repair, each composed of several sub-pathways [15,22]. HR pathways act upon 3′-extended single-stranded DNAs produced by DSB resection. Homology-directed strand invasion, mediated by RAD51 filaments, provides a major mechanism for the recombinational repair of DSBs in GBM cells. When resection exposes complementary sequences (blue segments), repair can occur via RAD52-mediated single-strand annealing. Although this mechanism is involved in the repair of IR-induced DSBs, its relevance in the context of TMZ-induced lesions has thus far not been reported. Recombinational repair of replication-associated DSBs involves the Fanconi Anemia (FA) pathway and HR factors [23]. Recognition of DSBs by either KU70/KU80 or PARP leads respectively, to canonical NHEJ and alternative NHEJ (A-NHEJ, itself composed of sub-pathways), a backup pathway that has been described in GBM cells [24,25]. Finally, translesion synthesis polymerases (not represented) that allow bypass of TMZ-induced lesions during DNA replication have been implicated as a mechanism to tolerate TMZ-induced DNA lesions [26–28].

and characterization of the DNA repair factors and molecular mechanisms underlying the cellular response of GBMs to IR and TMZ (hereby referred to as chemoradiation).[3]

3. Experimental and pre-clinical models for the study of DNA repair in GBM

3.1. Cellular models

Established GBM cell lines (such as U87, U251 etc.) and low-passage primary cell lines grown in monolayer cultures in serum–complemented media have commonly served as in vitro models of GBM. More recently, GBM cell cultures have also been established from tumor-derived single-cell suspensions grown in serum-free medium containing EGF and FGF (neurobasal medium). Under these conditions, the cells generate spheres and display stem cell properties, including self-renewal and the ability to differentiate into multiple cell types resembling central nervous system (CNS) cell lineages [41] and are often referred to as GBM stem cells (GSCs). GSCs are sometimes isolated based on the expression of specific markers (such as CD133, CD44 and A2B5) although expression of these markers in patients is highly heterogeneous [7] and their relevance as stem-cell markers is controversial. It remains to be determined whether GSCs represent a genetically defined, stable subpopulation of cells or an adaptive cellular state responding to microenvironmental changes. Yet, several studies suggest that GCSs are more resistant to DNA damage than their non-GSCs counterparts [10,42] and are able to mediate tumor cell repopulation during recurrence [43–45]. GSCs can be maintained in adherent cultures when the serum-free culture system is adjusted [46], which greatly facilitates their experimental study and in particular ensures the uniform distribution of genotoxics during cell cytotoxicity experiments or high-throughput genetic screens.

3.2. In vivo animal models

Transgenic and knockout mice for DNA repair functions have provided invaluable knowledge on spontaneous and genotoxic-induced carcinogenesis, as well as the deleterious consequences of defective DNA repair [47,48]. Mouse models have revealed the importance of DSB repair mechanisms during brain development and in neurological diseases [49]. Chemically-induced rodent models have been used to investigate glioblastomagenesis whereas genetically-engineered mouse models (GEMMs) harboring selected GBM driver mutations offer unique contexts to investigate GBM development driven by specific alterations and test potential therapeutics [47,50]. Unlike medulloblastoma [51], the impact of selective DNA repair gene knockout on tumorigenesis and DNA repair in GEMMs of GBM still awaits to be analyzed (see Section 6.2.1 for a description of the epigenetic factor SETD2).

Xenograft models developed from human GBM-derived cells provide another preclinical model to investigate glioblastomagenesis and test therapeutic strategies using genotoxics. Although a great number of publications report observations made on tumors grown from subcutaneous implants of GBM-derived cells, stereotactic intracranial implantation in immunodeficient rodents provides clinically more relevant and experimentally reproducible models. Notable in the context of treatment with genotoxics is that the BBB and also DNA repair-relevant features of the tumor microenvironment (including tumor-stroma interactions and the impact of hypoxia) are recapitulated in these models. Xenograft models can be generated from established GBM cell lines or, more relevantly, obtained from patient-derived xenografts (PDX) where human GBM tissue is transplanted in the animal following limited in vitro processing [47].

Currently, most animal experiments designed to assess the response of GBM cells to genotoxics expose the animal to the DNA-damaging agent for some time during tumor development, without prior tumor resection. The time selected for genotoxic treatment is usually chosen based on tumor mass analysis by in vivo imaging (e.g., magnetic resonance imaging or bioluminescence analysis) or predetermined time course of tumor appearence [7]. In such cases, the entire tumor mass is challenged with the drug (Fig. 2, panel A). Genotoxic treatment can also be carried out in vitro, prior to implantation of the surviving cells in the animal (Fig. 2, panel B). This latter strategy has been used, for instance, to investigate the effect of combined treatment with the PARP inhibitor olaparib and IR on tumor formation by GSCs [52]. One idea behind this approach is that the in vitro treatment might lead to the selection of a subpopulation of surviving cells with increased DNA repair capacity, that may reflect in part the features/properties of the invasive cells that have migrated away from the tumor mass at the time of resection and chemoradiation. Although neither strategy accurately reflects the clinical situation with regard to tumor recurrence, the development of surgical resection models for GBM is still in its infancy [53]. Thus, attempts to assess the effects of genotoxics in animals that have been subjected to tumor micro-resection, and possibly also IR, (Fig. 2, panel C) have to our knowledge not been reported.

4. Novel insights into the regulation of MGMT expression and its inhibition

MGMT provides the main mechanism for removal of the cytotoxic O^6-MeG lesion. Identifying the factors that orchestrate MGMT expression as well as therapeutic means to debilitate this line of defense against TMZ is thus the object of intense efforts. The main features of MGMT regulation and inhibition are recapitulated in Fig. 3.

A thorough review on the regulation of MGMT expression, including its epigenetic silencing and value/use as a biomarker and clinical target, has recently been published [36]. Here, we focus on the molecular progress in this field as well as aspects that are most relevant to genotoxic chemotherapy.

4.1. Transcriptional regulation of MGMT

The tumor suppressor gene TP53 encodes a transcriptional activator that orchestrates fundamental cellular responses to a variety of stress signals including DNA damage [65]. TP53 mutations and loss of heterozygozity are frequently observed in GBM [66], as is the case with the amplification or overexpression of its negative regulators MDM2 and MDM4 [67]. Overexpression of p53 has been proposed to negatively regulate MGMT transcription by sequestering Sp1 transcription factor [63], thereby sensitizing tumor cells to alkylating agents in vitro. In glioma cells, p53 overexpression can be induced by interferon beta (IFN-β), which was shown to sensitize cells to TMZ [68]. However, IFN-β has also been reported to induce sensitization of GSCs to TMZ, independent

[3] The chloroethylnitrosourea alkylating agents 1,2-bis[2-chloroethyl]-1-nitrosourea (BCNU, carmustine) and 1-[2-chloro-ethyl]-3-cyclohexyl-1-nitrosourea (CCNU, lomustine) are also part of the arsenal against GBM although, since the advent of TMZ they have been more generally administered as second line agents, when recurrence occurs. Removal of DNA lesions induced by these compounds involves most of the mechanisms mediating repair of TMZ-induced lesions, including MGMT. However, as bi-functional agents, BCNU and CCNU are also able to induce interstrand crosslinks [16] whose removal also involves factors from the FA and nucleotide excision repair pathways [38,39]. Other genotoxics that have been considered for the treatment of GBM, such as the DNA topoisomerase inhibitor irinotecan [40] are not addressed here.

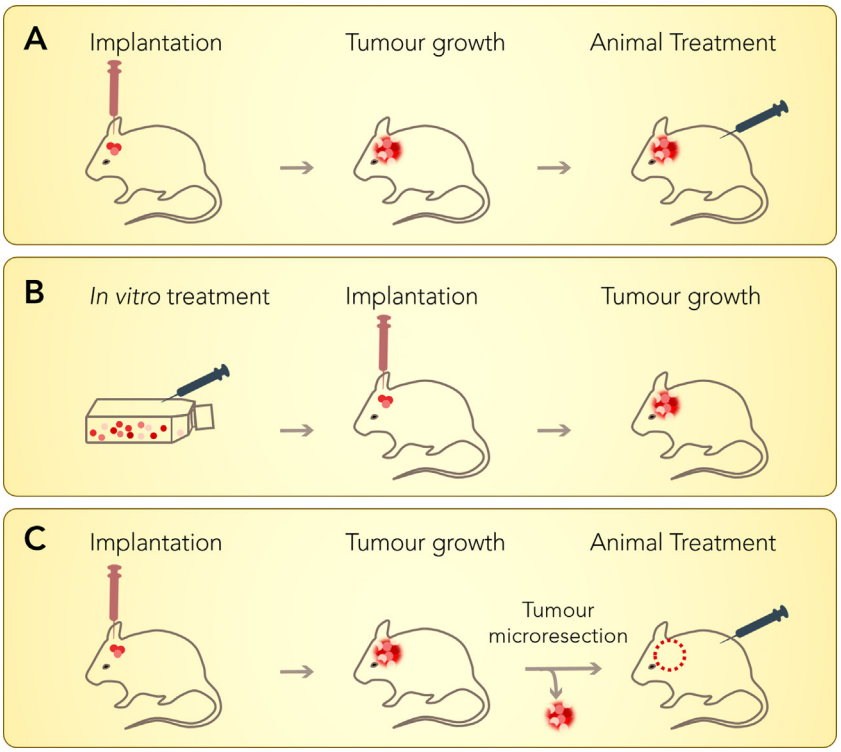

Fig. 2. Assessing GBM tumor response to genotoxics using orthotopic xenograft models. The response of GBM tumors (derived from established cell lines/GSCs/patient-derived GBM cells) to genotoxics can be tested in various xenograft settings. A. Xenotransplantation followed by treatment is the most commonly used orthotopic model. In this case, tumor growth can be monitored by MRI or bioluminescence analysis, followed by treatment and its evaluation. B. Cells are treated in vitro and the surviving cell population is implanted, followed by monitoring of tumor formation. C. Same as in A., except that tumor formation is followed by surgical resection, leaving resection margins as well as infiltrating cells that have migrated from the tumor mass. The animal is then treated and tumor recurrences are monitored. This model best reflects the current clinical situation in the setting of GBM.

of MGMT and p53 status [69]. Although uncertainties remain about the underlying mechanisms, Motomura et al. [70] have proposed IFN-β as a good candidate for adjuvant GBM therapy, especially for patients with an unmethylated MGMT promoter, based on a retrospective study of 68 newly diagnosed primary GBM patients showing that treatment with IFN-β increased the median survival time from 12.5 (TMZ alone) to 17.2 months (TMZ + IFN-β).

A long list of microRNAs (miRs) has been shown to target MGMT for downregulation (Fig. 3). Some miRs modulate TMZ sensitivity in vitro [59,64,71–73] and have been proposed to predict either the response to chemoradiation in GBM patients [73,74] or patient outcome in different GBM subtypes [75].

MGMT expression is induced by c-MYB and appears to be positively regulated by the transcription factor ZEB1 (zinc finger E-box binding homeobox 1), which operates through inhibition of miR-200c, a negative regulator of c-MYB [59]. Thus, ZEB1 was found to modulate TMZ chemoresistance in GBM cells and the authors have suggested that it could be a target for future therapeutic approaches.

4.2. MGMT inhibition: pseudosubstrates and genetic therapy

MGMT operates by stoichiometrically transfering the methyl group of O^6-MeG to a cysteine residue within its active site. This reaction is irreversible as it is followed by the ubiquitination and degradation of MGMT. Such a suicide reaction has been exploited in therapeutic strategies aiming at decreasing the pool of MGMT molecules in MGMT-positive patients (Fig. 4, panel A). However, attempts to sensitize GBMs and other solid tumors to TMZ through pharmacological depletion of MGMT levels using the pseudosubstrates O^6-benzylguanine (O^6-BG) or O^6-(4-bromothenyl) guanine (O^6-BTG, also known as lomeguatrib or PaTrin-2) have been faced with severe myelosuppression toxicity, forcing the use of decreased doses of TMZ which proved inefficient in clinical trials [76,77]. A number of approaches have been considered to overcome these adverse effects whilst targeting tumor cells more efficiently, including local administration of O^6-BG [78] or the use of glucose- [79] or folate-conjugates [80] of O^6-BG and O^6-BTG that target tumor cells through highly-expressed transporters or receptors. However, these early efforts have not been fruitful.

More recently, expression of the O^6-BG-resistant MGMT (P140K) mutant by hematopoietic cells was shown to provide significant protection against toxicity from O^6-BG/alkylator chemotherapy [81], leading to a prospective phase I/II clinical trial in which newly diagnosed MGMT-positive GBM patients were transplanted with autologous MGMT(P140K) gene–modified hematopoietic $CD34^+$ cells [82]. Gene therapy increased tolerance to the O^6-BG/TMZ combination as well as patient survival, supporting further development of chemoprotective gene therapy in GBM patients treated with O^6-BG and TMZ [83].

Other molecules that might in the future be considered to sensitize MGMT-positive patients to TMZ, include the direct inhibitor disulfiram [61,84], as well as various epigenetic drugs that sensitize MGMT-positive GBM cells to TMZ by down-regulating MGMT gene expression. Examples include the histone deacetylase inhibitors levetiracetam and valproic acid [85], and the DNA methylation inhibitor decitabine [56].

5. Inhibition of IR- and TMZ-induced DNA damage repair in GBM

5.1. DNA damage signaling and its inhibition

Various forms of DNA damage activate key sensor kinases such as ATM and ATR, triggering the phosphorylation of downstream

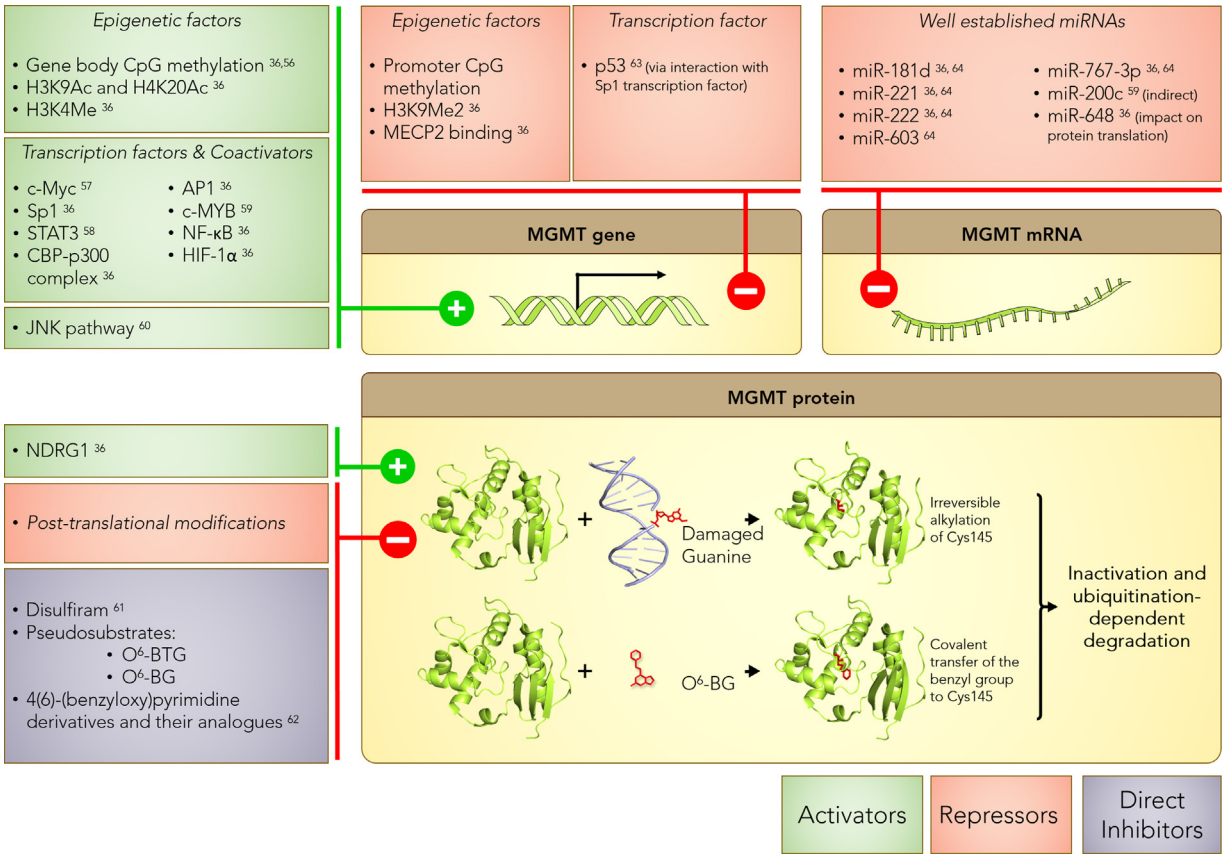

Fig. 3. MGMT regulation and inhibition. Depicted are the activating factors (green boxes) and repressing factors (peach boxes) of MGMT expression as well as direct inhibitors that affect MGMT activity (violet boxes). Various epigenetic mechanisms have been shown to modulate MGMT expression, including CpG methylation of MGMT promoter and gene body, histone H3K4 methylation and H3K9 dimethylation, acetylation of histones H3K9 and H4K20, and binding by the methyl-CpG binding protein MeCP2. MGMT Expression can also be controlled by a variety of transcription factors, as well as numerous miRs. Note that miR-648 interferes with MGMT protein translation whereas miR200 impacts MGMT through targeting of cMYB (see Section 4.1). At the post-translational level, MGMT can be stabilized by binding of the NDRG1 protein. Following binding of a methylated guanine or O^6-BG, MGMT becomes a substrate for ubiquitin conjugation, leading to its subsequent degradation by the proteasome. Direct inhibitors of MGMT include the pseudosubstrates O^6-BG and O^6-BTG, as well as disulfiram. PDB structure references: 1EH6 (native MGMT), 1EH7 (O^6-MeG-bound MGMT) and 1EH8 (O^6-BG-bound MGMT) [54]; 1T38 (O^6-MeG-DNA) [55]. This figure is inspired from [36,57,58,60,62].

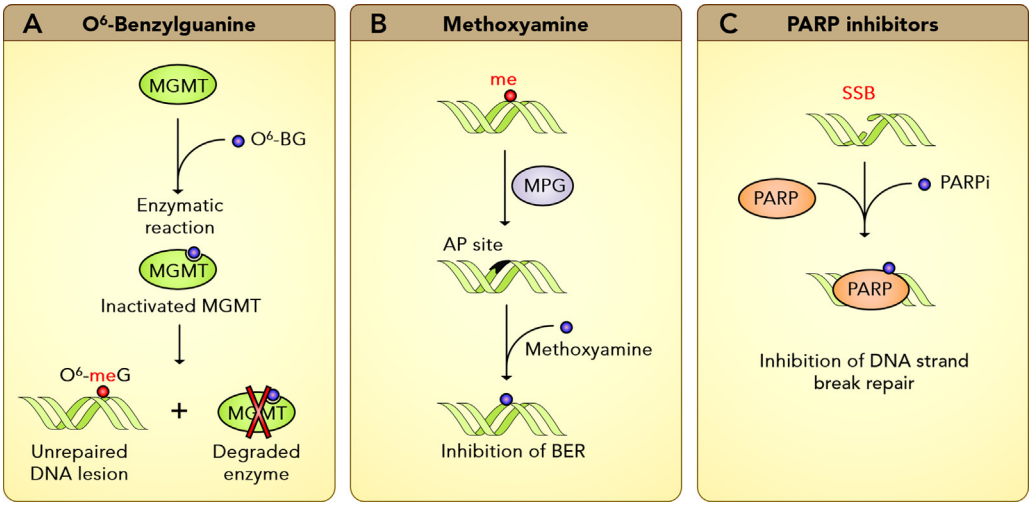

Fig. 4. Molecular basis of DNA repair inhibition by O6-benzylguanine, methoxyamine and PARP inhibitors. See text for details.

targets involved in cell-cycle arrest, DNA repair and apoptosis. ATM and ATR are activated, respectively, by DSBs and single-stranded DNA (ssDNA) structures. Key targets of ATM include p53 and the effector kinase CHK2, resulting in the control of the G1/S and intra-S checkpoints, whereas ATR acts primarily through CHK1 and controls the intra-S and G2/M checkpoints. However, there are overlaps between ATM- and ATR-dependent signaling [86].

Based on the characterization of genetically-engineered mouse models, Squatrito et al. [87] provided evidence that the ATM/CHK2/p53 pathway operates as a tumor suppressor in the brain. Chk2-/- mice exhibited increased GBM resistance to IR and failed to activate DNA-damage-induced cell-cycle checkpoints. The authors also showed that crucial DDR components were constitutively activated in primary GBM biopsies from patients not previously exposed to chemoradiation. Robust activation of the ATM/CHK2/p53 cascade in GBM specimens was also observed by Bartkova et al. [88] who identified oxidative DNA damage and replication stress as the underlying sources of endogenous genotoxic stress. Constitutive activation of the DDR has led to the suggestion that its inhibition might have potential application in the management of GBM. Thus, in contrast with the observations made in the Chk2 null glioma mouse model [87], dual inhibition of CHK1 and CHK2 [43] or ATM [89] sensitized GSCs to IR in vitro. Likewise, the ATM inhibitor KU-60019 has been described as an efficient radiosensitizer of GBM cells both in vitro [90] and, when administered intratumorally by convection-enhanced delivery or osmotic pump, in orthotopic GBM mouse models [91]. Importantly, the authors showed that p53 mutant GBM cells were much more sensitive to KU-60019 radiosensitization than their isogenic wild-type counterparts. The IR-induced synthetic lethality between p53 and ATM likely reflects the inability of DNA damage-ridden cells to execute functional cell cycle checkpoints, leading to mitotic catastrophe [92]. Other molecules that may show promise include AZ32, an orally-bioavailable, BBB-penetrant ATM inhibitor which has been reported to radiosensitize GBM cells in xenograft models [93].

The stimuli that activate ATM and ATR can also be generated during processing of the major lesions elicited by TMZ. BER-mediated removal of N^7-meG and N^3-meA – a process that is independent of MGMT and MMR, leads to the early phosphorylation/activation of CHK1 driven by ssDNA repair intermediates [94]. Although ssDNA and DSB stimuli are not produced when the O^6-MeG lesion is processed by MGMT, its handling by MMR in the absence of MGMT results in SSBs and DSBs that trigger the DDR and late activation of CHK1 and CHK2 [94,95]. Eich et al. [95] have shown that both ATM and ATR contribute to the resistance of GBM cells to TMZ in vitro; however, cells depleted of ATR by small interfering RNAs (siRNA) showed a more pronounced sensitivity to the drug compared to ATM-depleted cells, and knockdown of ATR, but not ATM, abolished phosphorylation of the DSB marker H2AX, as well as CHK1 and CHK2.

Litman-Flynn et al. [96] have shown that GBM cells relying on the alternative lengthening of telomere (ALT) pathway to overcome cell senescence (about 11% of adult GBM and 44% of pediatric GBM [97], see Section 6.1) were hypersensitive to ATR inhibitors. Such inhibitors appeared highly specific for ALT cells and may therefore offer a useful treatment approach in ALT-positive GBMs.

Aghinotri et al. [98] recently uncovered a novel function for ATM as an important regulator of BER in pediatric GBM, through modulation of MPG (3-methylpurine-DNA glycosylase, also known as alkylpurine–DNA-*N*-glycosylase (APNG)) glycosylase activity. The authors demonstrated direct phosphorylation of MPG by activated ATM, and showed that combined depletion of ATM and MPG resulted in increased TMZ-induced cytotoxicity in vitro and prolonged survival in mice with intracranial GBM xenografts. It was therefore suggested that ATM inhibition, in addition to preventing DSB repair, may sensitize cancer cells to TMZ through inhibition of BER, and that the combination of ATM and BER inhibitors may improve GBM treatment. The nature of the signals triggering ATM-mediated phosphorylation of MPG is currently unknown.

In summary, ATM and ATR appear to be attractive targets in combination with chemoradiation treatment.

5.2. Strategies to inhibit base excision repair

In 2012, Agnihotri et al. reported a positive correlation between the expression levels of the BER factor MPG and the TMZ IC50 in adult GBM cells, where MPG was found to promote TMZ resistance [99]. Later, the authors identified a similar correlation in pediatric GBM lines and also uncovered MPG and the apurinic/apyrimidinic (AP) endonuclease 1 (APEX1) amongst TMZ sensitizers in a siRNA screen with TMZ-resistant pediatric GBM cell lines [98]. Targeting MPG together with non-BER genes, or multiple targeting of BER genes in pediatric cells caused additive sensitivity to TMZ, whereas re-introduction of MPG in TMZ-sensitive cells conferred resistance to TMZ in orthotopic mouse models of pediatric and adult GBM [98].

MPG catalyzes the cleavage of N^7-meG and N^3-meA, generating abasic sites that are substrates for APEX1, and the MPG-APEX1 axis in GBM cells could serve as target for therapeutic strategies with methoxyamine (Fig. 4, panel B). This small molecule inhibitor binds the abasic sites generated by MPG, thereby preventing further processing. Compared to treatment with either molecule alone, the combination of methoxyamine plus TMZ dramatically increased survival of mice injected intracranially with SJG2 cells, as well as in an orthotopic PDX mouse model generated from pediatric GBM primary cells [98,99].

Other strategies targeting BER to sensitize GBM cells to TMZ include inhibition of DNA polymerase β whose lyase activity is required for excising 5′-deoxyribose phosphate (5′dRP) residues generated by APEX1 (Fig. 1) [100].

5.3. DSB repair pathways and their inhibition

Little or no sensitization towards IR was observed when the RAD51 recombinase was inhibited in LN-229 cells, whereas inhibition of the NHEJ factor DNA-dependent protein kinase (DNA-PK) resulted in increased sensitivity to IR irrespective of RAD51 knockdown [101]. Hypersensitization of GBM cells to IR was also observed following depletion of 53BP1, a positive regulator of NHEJ, in U87, U251 and T98G cells [102]. However, targeting HR in T98G [103] and GSCs [104] resulted in sensitization to IR, suggesting that HR can also contribute to IR-induced DSB repair in GBM.

The importance of the major DSB repair pathways in the response of GBM cells to alkylating agents was addressed by Quiros et al. [101], who described the impact of inhibiting HR or NHEJ on the response of LN-229 to TMZ. RNA interference (RNAi)-mediated depletion of the HR factors RAD51 or BRCA2 specifically sensitized GBM cells to TMZ in MGMT-negative backgrounds, underlining O^6-meG as the crucial lesion leading to DSBs in the absence of MGMT. That RAD51 knockdown resulted in increased sensitivity to TMZ in the absence of MGMT was confirmed by Short el al [103]. In contrast, hampering NHEJ through pharmacological inhibition of DNA-PK did not sensitize LN-229 cells significantly to TMZ [101]. Likewise, depletion of 53BP1 had no significant effect on the response of U87, U251 and T98G cells to TMZ [102]. It should be mentioned, however, that the NHEJ factors LIG4 and XRCC4 were identified as TMZ-sensitizers in pediatric GBM cell lines [98] whilst siRNA-mediated depletion of LIG4 sensitized A172 cells to TMZ [105]. Several alternative NHEJ pathways (A-NHEJ) have been described [22]. LIG4 and XRCC4 mediate the ultimate step of the

canonical NHEJ pathway. It is therefore possible that NHEJ intermediates are repairable by several alternative routes until the final ligation step which, once engaged through canonical NHEJ, can only be carried out by LIG4/XRCC4.

Recently, gene expression analysis using The Cancer Genome Atlas (TCGA) database by Rivera et al. [106] showed that components of the meiotic HR machinery are expressed in GBM. In addition, increased levels of the meiotic recombinase DMC1 were found in a battery of GBM cell lines compared to non-neoplastic brain. In vitro, siRNA-mediated depletion of DMC1 resulted in increased genomic instability and replication stress, and decreased proliferation in the absence of exogenous genotoxic stress, whereas it inhibited IR-induced activation of the DDR and radiosensitized GBM cells. Intracranial implantation of DMC1-depleted U87 in mice decreased tumor growth and prolonged survival. Pharmacological inhibition of DMC1 has hitherto not been reported.

5.4. Endogenous and chemoradiation-induced oxidative DNA damage in GBM: extra burden of DNA damage

Elevated rates of reactive oxygen species (ROS) production are a hallmark of most cancer cells where they are associated with tumor development and progression [107]. In GBM, increased ROS levels, DNA damage and genetic instability were observed in cells overexpressing EGFRvIII, a common oncogenic variant of EGFR amongst GBM patients [108], leading to a DNA repair pathway addiction phenotype that was exploited by Nitta et al. [109]. In this textbook illustration of the framework of non-oncogene addiction [110], the authors carried out a siRNA screen of 240 DDR genes, identifying BER genes involved in oxidative DNA damage repair, as well as poly(ADP-ribose) polymerase 1 (PARP1) (see Section 5.5) amongst those gene silencings that sensitized U87MG cells overexpressing EGFRvIII, but not the parental U87MG cell line, to IR. Of note, overexpression of EGFRvIII has also been linked to increased radioresistance in U87 cells, through the upregulation of DNA-PK [111].

In addition to causing base alkylation, TMZ, like IR, also induces oxidative DNA damage [112,113] which further mobilizes the BER machinery. Thus, Svilar et al. [114] showed that TMZ induced the production of ROS in GBM and validated the involvement of oxidative DNA glycosylases that do not recognize alkylated bases, but instead act upon oxidative DNA lesions, in a siRNA screen targeting “druggable” targets (5520 genes) for depletions conferring TMZ hypersensibility in T98G cells.

It therefore appears that BER and DSB repair mechanisms play a crucial role in the repair of oxidative DNA damage from endogenous and exogenous sources, which could be exploited in therapeutic approaches.

5.5. Strategies using PARP inhibitors

PARP1 is a sensor of SSBs involved in several forms of DNA repair (Fig. 1) [115]. Small molecule PARP inhibitors (PARPi), administered as monotherapies or in combination with genotoxic chemotherapeutics, have showed encouraging results against tumors harboring DNA repair defects, in which they induce synthetic lethality (a thorough review of the use of PARP inhibitors in the treatment of cancer is provided by [116]). Well characterized examples include the sensitivity of BRCA1 and BRCA2 deficient breast cancer cells defective in homologous recombination, to inhibition of PARP via small molecule inhibitors [117].

PARPi are thought to mediate their cytotoxic effects by trapping PARP1 enzymes (as well as the other PARP family member PARP2) on SSBs formed by endogenous cellular metabolism or as intermediates of crucial DNA repair pathways (Fig. 4, panel C). In addition, they prevent auto-PARylation (an activity required for dissociation of PARP from DNA and completion of repair) as well as PARylation of chromatin proteins that mediate the recruitment of DNA repair factors. Thus, by blocking pathways such as SSB repair, BER and A-NHEJ, persistent PARP-SSB complexes ultimately lead to replication collapse and the formation of DSBs that require HR for accurate repair.

The therapeutic potential of PARPi is not confined to cells deficient in HR. Thus, synthetic lethality was uncovered between deficiency in XRCC1 (involved in SSB repair, BER and A-NHEJ, Fig. 1) and PARP inhibition [118,119]. More recently, Horton et al. [120] showed that Polβ-/- and Xrcc1-/- mouse fibroblasts were hypersensitive to PARPi. In these defective cells, endogenous DNA damage led to unrepaired BER intermediates sufficient to trigger increase in PARP binding sites in the presence of PARPi, leading to replication fork disruption and DSB. The authors therefore suggested that BER deficiency could represent a therapeutic opportunity for PARPi single-agent therapy.

Several reports suggest that PARPi may also synergize with genotoxic therapeutics, including IR and TMZ, two treatments which bear direct relevance to GBM. Thus, Quiros et al. [101], presented evidence that MGMT-deficient LN-229 cells were hypersensitive to TMZ-induced DNA damage following treatment with the PARPi olaparib, which was exacerbated upon depletion of RAD51 recombinase. Dungey et al. [121] reported that olaparib sensitized the GBM cell lines T98G, UC373-MG, UVW and U87-MG to IR in vitro. A similar observation was made by Russo et al. [122] with U251 cells and the PARPi E7016. In both cases, treatment with PARPi was found to inhibit the repair of IR-induced DSBs. In addition, compared to IR plus TMZ, the trimodal combination of IR, TMZ and E7016 resulted in delayed tumor growth of U251 subcutaneous xenografts [122]. The sensitizing effect of another PARPi, ABT-888, in combination with IR and TMZ was also observed in various GBM cell lines [123].

Understanding the determinants of PARPi sensitivity and the mechanisms of resistance to PARPi is crucial to identify the cancer patient populations that may benefit from treatment with PARPi. Resistance to PARPi can occur via i) secondary mutations restoring BRCA1/2 open reading frame, ii) overexpression of the RAD51 recombinase and/or loss of PARP1 expression, iii) upregulation of drug efflux pumps, and iv) loss of the DNA repair factor 53BP1 [124]. In a recent study, Venere et al. [52] provided additional support for GBM clinical trials with PARPi, showing that, compared with non-GSCs, GSCs exhibited higher levels of ROS, increased oxidative base damage and SSBs, and exacerbated dependence on PARP1 activity, which could be exploited in vitro and in vivo. Thus, inhibition of PARP sensitized GSCs to IR, hampered growth, self-renewal and DNA repair, and inhibited tumor initiation in orthotopic xenotransplantation experiments [52]. The sensitivity of GSCs to PARPi was also reported by Majuelos et al. [125] using olaparib. In addition, the authors tested the effect of PARPi in U87 (PTEN-deficient) and LN229 (PTEN-proficient) GBM cells. PTEN-deficient cells were more sensitive to olaparib than the proficient cells, and this sensitivity was not increased in combination with IR or TMZ. Microarray analysis of U87 cells following treatment with PARPi revealed the down-regulation of HR factors, which was corroborated by the observation that HR is impaired in PTEN-deficient cells treated with PARPi.

Taken together, these studies suggest that conditions of increased ROS levels (e.g., cells expressing EGFRvIII), as well as defective BER or HR may provide contexts in which GBM patients might be amenable to PARPi-based therapeutic strategies. Single-agent and combination clinical trials involving PARPi have been reviewed [116]. A phase I clinical trial is underway to test the combination of olaparib and TMZ in patients with relapsed GBM (clinicalTrials.gov identifier: NCT01390571).

Table 1
List of abbreviations.

Abbreviation	Definition
2-HG	2-hydroxyglutarate
ALT	alternative lengthening of telomeres
A-NHEJ	alternative non-homologous end-joining
AP site	apurinic/apyrimidinic site
BBB	blood brain barrier
BCNU	bis-chloroethylnitrosourea, carmustine
BER	base excision repair
CCNU	1-(-2-chloroethyl)-3-cyclohexyl-1-nitrosourea, lomustine
CED	convection-enhanced delivery
CNS	central nervous system
CRISPR	clustered regularly-interspaced short palindromic repeats
DDR	DNA damage response
DSB	double-strand break
FA	fanconi anemia
GBM	glioblastoma
G-CIMP	glioma CpG island methylator phenotype
GEMM	genetically-engineered mouse model
GSC	glioma stem cell
HDAC	histone deacetylase
HR	homologous recombination
IR	ionizing radiation
MMR	mismatch repair
N^1-meA	N^1-methyladenine
N^3-meA	N^3-methyladenine
N^3-meC	N^3-methylcytosine
N^7-meG	N^7-methylguanine
NHEJ	non-homologous end-joining
O^6-BG	O^6-benzylguanine
O^6-BTG	O^6-bromothenylguanine, lomeguatrib
O^6-meG	O^6-methylguanine
PARPi	PARP inhibitor
PDB	protein database
PDX	patient derived xenograft
PRC2	polycomb repressive complex 2
RNAi	RNA interference
ROS	reactive oxygen species
RT	radiotherapy
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SSB	single strand break
ssDNA	single-stranded DNA
TALEN	transcription activator-like effector nuclease
TCGA	The Cancer Genome Atlas
TMZ	temozolomide
α-KG	α-ketoglutarate

6. Novel DNA-damage response-related biomarkers of GBM

Table 2 summarizes the DDR- and DNA repair-relevant features of well-established molecular alterations that have been used in clinical diagnosis and prognosis of adult and pediatric GBMs, as well as recently-described alterations that impact the DDR, some of which are proving valuable as markers to improve the classification of gliomas and/or as therapeutic targets [155,156]. Here, we focus on telomere-maintenance mechanisms and DNA repair changes associated with epigenetic alterations in pediatric and adult GBM. Specifically, we address alterations affecting histone H3 variants, the histone chaperones ATRX and DAXX, the histone methyltransferases EZH2 and SETD2, and also isocitrate dehydrogenase IDH1 (including its impact on the glioma-CpG island methylator phenotype (G-CIMP)).

6.1. Telomere-maintenance mechanisms

Telomeres are nucleoprotein structures that protect the ends of chromosomes and prevent their recognition as DSBs. Although maintenance of telomeres requires the taming of DSB repair pathways (NHEJ and HR), other DNA repair factors collaborate with telomere-specific factors and epigenetic mechanisms to protect chromosome ends [157]. Central to the synthesis of telomeric DNA sequences is telomerase, a specialized ribonucleoprotein reverse transcriptase. In most cells, telomerase activity decreases as cells differentiate and telomeres gradually shorten after each round of cell division, resulting in DNA instability, cellular senescence and ultimately cell death. In order to maintain viable telomere length, tumor cells rely either on the re-expression of telomerase or a telomerase-independent, HR-dependent mechanism called alternative lengthening of telomeres (ALT). Reactivation of telomerase is largely limited to adult GBMs (80% vs 3–11% in pediatric GBM), whereas, a reverse pattern is observed for ALT (pediatric GBMs (44%), adult GBMs (11%)) [158].

In recent years, several studies have examined the prognostic value of telomerase-associated parameters like telomere length, telomerase activity and expression of its catalytic subunit, hTERT, in relation to GBM. Expression of hTERT is associated with high telomerase activity and adverse prognosis in GBM patients [146]. Induction of hTERT expression through mutations in the promoter core region of hTERT is responsible for the reactivation of the telomerase in various cancer types including GBM. In a cohort of GBM patients, where 80% were found to carry hTERT promoter mutation, a bad prognostic value was confined to those patients who also harbored the G-allele of a SNP in the promoter of hTERT (rs2853669) [149,159].

Overexpression of hTERT in normal fibroblasts led to enhancement of DNA repair capacity [148]. Whether a similar situation occurs in GBM tumors might explain why increased hTERT expression confers resistance to genotoxics and poorer patient survival. In line with the observation that RNAi-mediated depletion of hTERT resulted in impaired DNA damage response and sensitivity to IR [160], direct inhibition of telomerase activity in GSCs using the antagonist imetelstat [162], or treatment of telomerase-reactivated GBM cells with quadruplex-selective ligands that stabilize G-quadruplex structures present in telomeric DNA [161,162], inhibited cellular proliferation and sensitized cells to IR in vitro. A similar observation was made with ALT-positive GBM cells [163], although these cells were naturally more resistant to IR than GSCs with reactivated telomerase [164].

6.2. DNA repair changes associated with epigenetic alterations in pediatric and adult GBMs

6.2.1. Driver mutations in histone H3 variants and histone chaperones

Recent analyses have underlined the unique molecular features of pediatric GBMs [5,165,166]. Specifically, driver mutations in the H3F3A gene encoding the histone variant H3.3 as well as in the H3.3 histone chaperones ATRX and DAXX were uncovered at high frequency in pediatric GBMs [136,137]. The point mutations in H3.3 resulted in the amino acid substitutions K27M or G34R/V, which were mutually exclusive and heterozygously expressed. K27M substitutions were also observed in the HIST1H3B gene encoding H3.1. Residues K27 and K36 (close to H3G34) in H3/H3.3 are critical sites for post-translational modifications, and the epigenetic impact of the observed mutations was seen at the level of gene expression and chromatin dynamics. Thus, K27M and G34R/V mutations showed distinct gene expression profiles and DNA methylation patterns. Importantly, nearly all tumors bearing G34R/V mutations also exhibited mutations in ATRX/DAXX and displayed the ALT-phenotype [136,167], in line with the documented requirement of these chaperones for the deposition of H3.3 at normal telomeres [168,169]. The H3.3K27M mutation resulted in reduced levels of H3K27me3, a repressive mark deposited by EZH2, the catalytic subunit of polycomb repressive complex 2 (PRC2). In

Table 2
Established and newly described alterations affecting DNA repair and chromatin factors in primary adult and pediatric GBMs.

Gene	Function	Alteration type*	General effect	Impact on DDR	Drug**	Ref.
Established markers in primary adult GBMs						
MGMT	Removal of O^6-meG in DNA	CpG-island promoter methylation	Increased overall survival in patients presenting alteration	Reduced O^6-meG removal; increased response to TMZ treatment	O^6-BG	[4,31,126]
IDH1	TCA cycle enzyme	Gain-of-function mutation: R132H (±5%)	Alteration of metabolic, DNA methylation and gene expression profiles	Increased RAD51-mediated HR; 2-HG mediated inhibition of ALKBH2/3	AGI-5198	[4,19,126–128]
EGFR	Cell surface tyrosine kinase receptor	Focal amplification (±50%) as well as activating, intragenic deletions (i.e: EGFRvIII variant)	Tumor growth stimulation by activation of downstream RAS/MAP, mTOR and PI3K/Akt signaling pathways	EGFRvIII variant is associated to increased ROS production and DNA damage, as well as activation of DNA-PKCs and NHEJ-mediated DSB repair	Erlotinib Cetuximab	[4,109,111,126]
PDGFRA	Cell surface tyrosine kinase receptor	Focal amplification (11%) and activating, intragenic deletion (Δe8-9)	Stimulation of tumor growth	***	Sorafenib Imatinib	[4,126,129]
PTEN	Tumor suppressor, inhibitor of oncogenic Akt signaling pathway	Focal deletion (10%), mutation (30%)	Re-activation of Akt pathway signaling	Impaired DNA repair in GBM cells; compromised HR in normal astrocytes	/	[4,126,130–132]
TP53	Tumor suppressor, DDR factor involved in cell cycle regulation and apoptosis modulation	Mutation and LOH	Loss of cell cycle checkpoint control, tumor proliferation	See Section 4.1	/	[4,126,133]
CDKN2A/B	Tumor suppressor, negative regulator of cell cycle, regulator of p53	Focal/Homozygous deletion (60%), promoter hypermethylation	Dysregulation of cell cycle control	***	/	[4,126]
Established alterations in primary pediatric GBMs						
H3F3A/B	Histone variant H3.3	Mutations (K27M, G34V/R, K36M)	Epigenetic changes and alteration of gene expression profiles	See Section 6.2.1	/	[134–136]
HIST1H3B	Histone variant H3.1	Mutation (K27M)	Epigenetic changes and alteration of gene expression profiles	See Section 6.2.1	/	[137]
ATRX	Histone H3.3 chaperone	Mutation	Decreased H3.3 deposition at telomeres, epigenetic changes, ALT-phenotype	Increased sensitivity to DNA damaging agents; increased DNA damage	/	[136,138]
SETD2	H3K36 specific tri-methyltransferase	Mutation (15%)	Defective H3K36 tri-methylation	Defective HR-mediated DSB repair; impaired MMR	/	[139–142]
Recently described alterations in primary GBMs						
EZH2	Mono-, di- and tri-methylation of H3K27	Up-regulation	Epigenetic changes and alteration of gene expression profiles; tumor proliferation	Inhibition associated with increased expression of HR genes, including RAD51	GSK126 DZNeP	[143–145]
hTERT	Catalytic subunit of telomerase	Overexpression through activating mutation in promoter core region (80%)	Cellular transformation, immortalization	Improved DNA repair capacity	GRN163L Telomestatin	[146–149]
ID1	Transcription factor	High expression (50%)	Increased tumor growth	Deficient DDR; downregulation of LIG4 and ATM	Cannabidiol	[150,151]
PGRN	Secreted growth factor	Overexpression	Tumor progression	Increased DDR and resistance to TMZ	/	[152]
XRCC6BP1	DNA –dependent protein kinase	Amplification (14%)	Increased proliferation	Improved DSB repair	/	[153,154]

Abbreviations: ATRX, α-thalassemia/mental retardation syndrome X-linked; CDKN2A/B, Cyclin-dependent kinase inhibitor 2A/B; EGFR, Epidermal growth factor receptor; EZH2, Enhancer of zeste homolog 2; H3F3A/B, H3.3 histone; HIST1H3B, histone cluster 1, H3b; hTERT, human telomerase reverse transcriptase; ID1, Inhibitor of DNA binding 1; IDH1, Isocitrate dehydrogenase 1; MGMT, O6-methylguanine-DNA methyltransferase; PDGFRA, platelet-derived growth factor receptor; PGRN, Progranulin; PTEN, phosphatase and tensin homolog; SETD2, SET domain containing 2; TP53, tumor protein p53; XRCC6BP1, XRCC6 binding protein 1; 2-HG, 2-hydroxyglutarate; α-KG, ketoglutarate.

* Incidence when available, unless quoted in the text.

** Example of drug.

*** These important markers have been included although the association between the indicated alterations and DNA repair has not been reported in GBM.

addition, mutant H3.3K27M was shown to interact with EZH2 and inhibit PRC2 activity [170].

The oncogenic mechanisms associated with the H3.3K27M and H3.3G34R/V mutations in pediatric GBMs have been reviewed by Yuen [166]. Remarkably, a recent report by Gallo et al. [171] identified mixed lineage leukemia 5 (MLL5)-mediated repression of H3F3B, the second gene encoding human H3.3, as a mechanism that phenocopies the DNA methylation profiles of pediatric GBMs with H3.3 mutations, in adult GSCs. Moreover, the authors demonstrated that MLL5 action impacts chromatin structure, thus modulating gene expression to maintain the tumorigenic and self-renewal properties of adult GSCs.

Whether H3.3 mutations or expression alterations result in altered DNA repair capacities in pediatric GBM remains to be investigated. However, studies of the phenotypes associated with EZH2 depletion suggest a strong connection between epigenetic remodeling and DNA repair in pediatric as well as adult GBM. EZH2 is overexpressed in adult and pediatric GBM [172]. This observation led de Vries et al. [143] to target EZH2 via inducible short hairpin RNAs (shRNA) in mouse-derived GSCs. Prolonged EZH2

depletion led to global reformatting of gene expression profiles yielding highly proliferative and undifferentiated tumors and resulting in increased tumor progression. Importantly, gene ontology analysis identified DNA Repair as a major enriched term in EZH2-depleted GBMs where key HR factors including RAD51 were upregulated. In vitro and in vivo experiments indicated that enhanced DNA repair resulting from EZH2 inhibition decreased the sensitivity of GBM cells to TMZ, highlighting a crucial role for RAD51-mediated DSB repair in this process. The authors suggest that concomitant inhibition of EZH2 and HR (e.g., RAD51) might potentiate TMZ toxicity in GBM [143].

Although they may operate more indirectly, the H3.3G34R/V mutations, like H3K27M, could also affect DNA damage response activities in GBM. Indeed, point mutations in H3.3G34 decrease the levels of H3K36me3 on the same and nearby nucleosomes, and the evidence suggests that the G34R/V mutant histones disrupt the activity of SETD2, the only methyltransferase that mediates trimethylation of K36me2 in H3 and H3.3 [166]. H3.3 variant histones are enriched at telomeres and pericentromeres. Thus, it remains to be seen how H3.3G34R/V-induced local perturbations of H3K36me3 and H3.3K36me3 might affect DNA repair and genetic instability. However, the documented role of H3K36me3, a mark associated with transcription elongation in active genes, as an important modulator of MMR [141] and HR-mediated DNA repair [142,173] suggests that depletion of SETD2 may impact the response of GBM cells to genotoxics. Importantly, point mutations in SETD2 have been identified in pediatric and adult GBMs [174].

6.2.2. IDH1 and the glioma-CpG island methylator phenotype (G-CIMP)

Mutations in isocitrate dehydrogenase (IDH) 1 and 2 (metabolic enzymes which convert isocitrate into α-ketoglutarate (α-KG, also called 2-oxoglutarate)) occur predominantly as driver mutations in low grade gliomas and secondary high grade gliomas, although IDH1 mutations have been observed in 6% of GBM [4,175–177]. IDH1 mutations are associated with longer patient survival and improved response to TMZ. The most frequent mutation in IDH1 is R132H, a gain-of-function mutation that confers the ability to convert α-KG into 2-hydroxyglutarate (2-HG). Because 2-HG inhibits Fe(II)- and 2-oxoglutarate-dependent oxygenases such as histone lysine demethylases [178] and DNA demethylases [179], its accumulation in IDH1R132H tumors has the potential to generate global epigenetic defects and alter gene expression [180]. The R132H mutation in IDH1 is closely associated with the glioma-CpG island methylator phenotype (G-CIMP) defined as the concerted hypermethylation of a large number of loci [181]. Although it is more prevalent among low- and intermediate-grade gliomas, this phenotype appears to be also enriched in a subset of primary GBMs belonging to the proneural subtype (one of 4 GBM subtypes – proneural, neural, classical, mesenchymal – proposed by Verhaak et al. [66]) where it is associated with a younger age at diagnosis and better survival [181]. Expression of a G-CIMP phenotype, which could be obtained by the sole introduction of exogenous IDH1R132H into primary astrocytes was suggested to result from 2-HG-mediated inhibition of DNA demethylating enzymes such as TET2, and probably also from DNA-methylation promoting alterations in H3K9me2 and H3K27me3 [182]. Recently, a refined classification of gliomas based on G-CIMP status, DNA methylation profiles and IDH mutation has been proposed [183].

The impact of mutant IDH1 on the cellular response to TMZ was investigated by Ohba et al. [127], using MGMT-deficient human astrocytes immortalized by expression of virally-encoded E6, E7 and hTERT, and infected with lentiviral constructs encoding wild-type or mutant IDH1 (which in both cases led to cellular transformation). Compared with wild-type cells, IDH1R132H cells displayed more efficient processing of TMZ-induced DSB lesions and increased survival. Furthermore, mutant IDH1-driven transformation resulted in enhanced RAD51-mediated HR, and siRNA-mediated depletion of RAD51 reduced the TMZ resistance conferred by overexpression of mutant IDH1. Thus inhibitors of HR may offer an efficient therapeutic options in IDH1 mutant GBM tumors.

In addition to an indirect effect resulting from the impact of global epigenetic and gene expression alterations, mutations in IDH1 have recently been shown to also exert a direct effect on alkylated DNA repair enzymes in GBM cells. Thus, Wang et al. [19] found that 2-HG produced by IDH1 mutants inhibited, in vitro, purified recombinant forms of the Fe(II)- and α-KG-dependent DNA demethylases ALKBH2 and ALKBH3, the major enzymes involved in the removal of N^1-meA and N^3-meC (Fig. 1). Furthermore, characterization of GBM cells engineered to express wild-type or R132H mutant IDH1, together with manipulation of heterozygous $IDH1^{+/R132C}$ fibrosarcoma cells and ALKBH2/3-overexpression experiments indicated that mutant IDH1 sensitize cells to DNA alkylating agents through 2-HG-mediated inhibition of ALKBH2 and ALKBH3. The clinical relevance of these findings are supported by a previous report that ALKBH2 confers resistance to TMZ in GBM cells [20].

IDH1-mediated oxidative decarboxylation of isocitrate to α-KG is accompanied by production of NADPH from NADP+. IDH1 mutant cells are thought to display lower production of NADPH, an important player in the cellular defense against oxidative damage, which was proposed to sensitize GBM to chemoradiation [184]. Shi et al. [185] showed that the decreased intracellular NADPH levels elicited by overexpression of the IDH1R132H mutation were associated with glutathione (GSH) depletion and ROS generation. However, conflicting data have been obtained when the impact of IDH1R132H overexpression on the response of GBM cells to genotoxics was tested. Thus, one study found that, compared with control cells and cells overexpressing wild-type IDH1, overexpression of IDH1R132H sensitized U87 and U251 cells to TMZ [186]. Another study found that overexpression of IDH1R132H conferred sensitivity to IR, but not TMZ in U87 and U373, whereas overexpression of wild-type IDH1 increased resistance to TMZ compared to control cells [141]. It should be noted that IDH1-overexpressing cell lines most likely do not recapitulate the endogenous IDH1 mutant status of glioma cells (see Section 7.2).

Although MGMT promoter methylation is more prevalent in G-CIMP GBMs than in non-G-CIMP GBMs (79% vs 46%, respectively), MGMT promoter methylation correlated with patient response only in the classical subgroup but not in the proneural subgroup [4]. Furthermore, analysis of hypermethylated and downregulated genes within proneural G-CIMP positive tumors [181], did not show significant enrichment in DNA damage signaling and DNA repair. These observations suggest that the impact of the G-CIMP phenotype on DNA repair in GBMs might be limited.

7. Discussion

7.1. Integrative strategies that must be considered for genotoxic-based therapeutic management of GBM

Elucidating the DNA repair pathways and factors activated by GBM cells in response to IR and TMZ-induced lesions, identifying selective DNA repair inhibitors that can be used as monotherapy or in combination with chemoradiation, and defining the (epi)genetic features that shape the DNA-repair makeup of GBM cells, are crucial steps to tackle the development of resistance currently seen in GBM patients whilst tailoring treatment to the patient's tumor.

Significant progress has been made in our understanding of the molecular details of the DNA repair mechanisms that operate in GBM, leading to the discovery of novel, druggable DNA repair axes,

including the ATM-BER axis reported by Agnihotri et al. [98]. Such studies are expanding the list of pathways that could be targeted by a given inhibitor. In addition, several studies have helped refine the molecular settings in which specific DNA repair inhibitors, such as PARPi, can be used. Most relevant in this respect is the fact that GBM cells have to cope with pervasive oxidative DNA damage resulting from ROS production, rendering them more dependent upon the BER machinery.

GBM driver mutations have recently been described in genes encoding (e.g., H3.3, ATRX, DAXX) or affecting (e.g., IDH1) epigenetic factors, raising the issue of whether drugs capable of reprogramming abnormal epigenomes may compose part of the therapeutic arsenal against glioblastoma [187]. Although driver mutations in histone H3 variants have been discovered in pediatric GBMs, the evidence suggests that at least some of their phenotypic consequences might also be recapitulated in adult GBMs, through MLL5-mediated repression of H3.3 expression. Moreover, it is notable that the resulting amino-acid changes in H3.3 mutants affect post-translational modifications that can also be impacted by alterations in epigenetic writers such as EZH2 and SETD2, as such alterations are also found in adult GBMs. Thus, future lessons learned from the characterization of pediatric GBMs might, to some extent, serve in the management of adult GBMs as well. The changes in gene expression and chromatin dynamics induced by such mutations/alterations appear to impact the DNA repair capacity and response of GBM cells to chemoradiation. Based on the documented effect of histone deacetylase (HDAC) inhibitors [188,189] and DNA methyltransferase inhibitors [190,191] on DNA repair, it is tempting to speculate that epigenetic drugs may help reverse the adverse effects (e.g., resistance to genotoxics) associated with altered DNA repair in GBM, in part by impacting the expression of DNA repair factors or perturbing DNA repair-associated chromatin modifications. Moreover, HDAC inhibitors have been shown to trigger not only widespread changes in histone acetylation but also, by mechanisms that remain obscure, the production of ROS [188], thus imposing an extra burden for DNA repair that might be exploited in therapeutic strategies.

How specific GBM driver mutations/alterations affecting other processes than epigenetics impact DNA repair remains a matter of investigation that would greatly benefit from the characterization of DNA repair genes and pathways in GEMMs. Likewise, knocking down selected DNA repair genes in these models may help elucidate the contribution of specific DNA repair defects (for instance, caused by downregulation of DNA repair factors) to glioblastomagenesis and response to genotoxics.

7.2. Considerations on the cellular and animal models used to study DNA repair in GBM

The great variety of GBM-derived cells currently used both in vitro and in preclinical studies with animal models raises issues that are of paramount importance in DNA repair and chemotherapy. Indeed, preservation of the genetic and epigenetic alterations of the original tumor is crucial to maintain the "DNA repair makeup" of the tumor cell and study its impact on DNA repair and cellular response to genotoxics. Although they commonly serve as in vitro model, established GBM cell lines (such as U87, U251, T98 etc.) suffer the limitation of having incurred genetic alterations, changes in DNA ploidy, clonal selection and novel gene–gene interactions during adaptation to and prolonged passages in monolayer cultures. The inability of these models to preserve genetic and epigenetic features of the original tumor is also reflected in their failure to recapitulate the pathohistological GBM phenotypes in xenografts [47,192]. Thus, tumor invasion, infiltration, necrosis, and vascular proliferation in these models are usually not comparable to a GBM in patients. The questionable pertinence of such cellular models for the study of DNA damage also stems from the conflictual results to which they can give rise, as illustrated, e.g., by two studies of the response to TMZ conferred by mutant IDH1 expressed in U87 cells ([141,186], see Section 6.2.2). Although the observed discrepancies might be due to inter-laboratory variations within U87 lines, it may be argued that U87 cells, which were established from an adult GBM, do not represent an adequate model to investigate the impact of mutant IDH1 since, unlike the astrocyte-derived model generated by Ohba [127], expression of the IDH1 mutation played no role in their genesis. Thus, extrapolating conclusions from studies involving established GBM cell lines must be done with caution. Organotypic spheroids formed from patient-derived GBM biopsies better preserve the original tissue architecture, the genomic profile and DNA ploidy of the parental tumor [7,193]. Importantly, such spheroids do not undergo passaging and selection in vitro and, instead, can be maintained by serial transplantation in vivo, which maintains the original genetic and phenotypic heterogeneity of the biospy [194]. Similarly, GSC sphere cultures partially reiterate the molecular features of the original tumor and we have recently shown that such cell lines establish similar histological phenotypes as organotypic spheroid-based xenografts [194]. Nevertheless, GSC cultures also undergo in vitro selection and changes in DNA ploidy [7].

GBM cell lines are often preferred tools because they can be engineered to express a transgene or subjected to RNA interference-mediated gene silencing using shRNAs. In the case of PDXs based on organotypic spheroids, efficient gene silencing is challenging but can be obtained with lentiviral vectors without selection, which minimizes the risks of genetic drift. In principle, xenograft models should also be amenable to gene knockout through genome-editing technologies such as TALEN (Transcription Activator-Like Effector Nucleases) or CRISPR (Clustered, Regularly Interspaced, Short Palindromic Repeats), although the cellular manipulations and time constraints associated with these techniques will lead to selection processes and possibly genetic drift.

7.3. Non-coding RNAs in the response of GBM tumors to chemoradiation

Targeting of miRs regulating DNA repair is associated with a therapeutic potential [195]. With the exception of the miRs that affect MGMT expression, this review has not addressed the roles played by miRs in glioblastomagenesis and response to genotoxics. Other non-coding RNAs known as long non-coding RNAs have emerged as essential elements involved in GBM development and progression [196]. The roles of non-coding RNAs in DNA repair as well as their potential as biomarkers and potential therapeutic targets in the management of GBM remains largely to be elucidated.

7.4. Improving the delivery of chemotherapeutic agents to the brain

The need for chemotherapeutic agents that cross the BBB efficiently and maintain an effective steady state concentration remains a serious challenge in the management of GBM. With the exception of TMZ, most of the anti-cancer agents that have been tested for the management of GBM fail to cross the BBB efficiently [197]. Due to a short half-life in plasma, TMZ itself must be administered in high systemic doses to achieve therapeutic levels in the brain, leading to undesired side-effects, whereas the TMZ/O^6-BG combination results in hematopoietic toxicity.

Local intracerebral administration of drugs or macromolecules has been regarded as desirable in order to bypass the obstacle of the BBB and eliminate the potential undesired toxicity. Delivery

technologies like drug-impregnated wafers (such as BCNU-loaded polymers (Gliadel® Wafer) [198]), Ommaya reservoir, or convection-enhanced delivery (CED) systems, make it possible to use drugs that do not necessarily cross the BBB or whose adverse systemic effects are too unsafe for the patient [199]. CED, for instance, delivers drugs, macromolecules or nanoparticules through one to several catheters placed stereotactically directly within the resection cavity. Its safety was established in a study investigating the delivery of a MGMT-siRNA/cationic liposome complex in normal rat and pig brains treated or not with TMZ [200]. Animal studies have also shown that CED-mediated distribution of O^6-BG via nanoparticles, in parallel with TMZ treatment resulted in a 3-fold increase in median overall survival compared with TMZ-only control animals [201].

Although such delivery techniques should greatly widen the pipeline of genotoxic agents and DNA inhibitors that could be used to treat GBM, the invasive nature of these procedures remains an obstacle. Thus, the design of novel anti-cancer agents, including DNA repair inhibitors, with better CNS bioavailability and the ability to be administered intravenously or orally remains a crucial challenge in the management of GBM.

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CHAPTER 4

RESULTS

A DNA repair and cell-cycle gene expression signature in primary and recurrent glioblastoma: prognostic value and clinical implications

Gobin M, Nazarov P, Warta R, Timmer M, Reifenberger G, Felsberg J, Vallar L, Chalmers AJ, Herold-Mende CC, Goldbrunner R, Niclou SP and Van Dyck E.

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RATIONALE

The manuscript comprises the first part of my thesis project aims which consisted in investigating changes in the expression of DNA repair and cell cycle genes in a cohort of paired biopsy samples (primary and recurrent tumor of the same patient) from patients treated with RT or RT+TMZ as a step to unravel the impact of chemoradiation on the DDR and identify potential targets for improving GBM therapy. In this manuscript, we identify and describe a 27-gene signature that classified our patient cohort into two main groups which displayed inverse expression profiles. Interestingly, the tumor at relapse frequently display a gene expression profile different from that of its matched primary biopsy. Moreover, within the two main groups, the expression pattern at relapse was associated to progression-free survival. Finally, we demonstrate experimental evidence of group-specific vulnerabilities against genotoxicants and inhibitors of the cell cycle and DDR, with the prospect of personalized therapeutic strategies based on patient profiling using our gene signature.

Personal contributions: I was directly involved in the extraction and quality control of the RNA obtained from patient biopsies in order to provide optimal starting material for the targeted gene expression analysis using the nCounter mRNA counting technology. Even though the bioinformatical was not done directly by me, I provided feedback and ideas for the analysis in order to obtain biologically relevant results. In addition, I carried out analyses based on the genomic data, sample stratification, clinical data (e.g., survival parameters) to establish crucial associations revealed in the manuscript. I also carried out the initial characterization of the group-specific GBM cell lines received from our collaborators and performed *in vitro* proof of concept cytotoxicity assays using these cell lines. Apart from the heatmaps and immunohistochemical pictures, all the presented figures were designed and generated by hand. Finally, I was actively involved in the redaction of the manuscript.

Translational Science

Cancer Research

A DNA Repair and Cell-Cycle Gene Expression Signature in Primary and Recurrent Glioblastoma: Prognostic Value and Clinical Implications

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Abstract

Inevitable tumor recurrence and a poor median survival are frustrating reminders of the inefficacy of our current standard of care for patients with newly diagnosed glioblastoma (GBM), which includes surgery followed by radiotherapy and chemotherapy with the DNA alkylating agent temozolomide. Because resistance to genotoxic damage is achieved mainly through execution of the DNA damage response (DDR) and DNA repair pathways, knowledge of the changes in DNA repair and cell-cycle gene expression that occur during tumor development might help identify new targets and improve treatment. Here, we performed a gene expression analysis targeting components of the DNA repair and cell-cycle machineries in cohorts of paired tumor samples (i.e., biopsies from the same patient obtained at the time of primary tumor operation and at recurrence) from patients treated with radiotherapy or radiotherapy plus temozolomide. We identified and validated a 27-gene signature that resulted in the classification of GBM specimens into three groups, two of which displayed inverse expression profiles. Each group contained primary and recurrent samples, and the tumor at relapse frequently displayed a gene expression profile different from that of the matched primary biopsy. Within the groups that exhibited opposing gene expression profiles, the expression pattern of the gene signature at relapse was linked to progression-free survival. We provide experimental evidence that our signature exposes group-specific vulnerabilities against genotoxicants and inhibitors of the cell cycle and DDR, with the prospect of personalized therapeutic strategies.

Significance: Findings suggest that classification of GBM tumors based on a DNA repair and cell-cycle gene expression signature exposes vulnerabilities to standard-of-care therapies and offers the potential for personalized therapeutic strategies.

Introduction

Despite surgical resection and genotoxic treatment with ionizing radiation (IR) and the DNA alkylating agent temozolomide, glioblastoma (GBM) remains one of the most lethal cancers. Although occasional long-term survivors are reported (1), patients with GBM have a poor median survival (<1 year; refs. 2, 3), and all patients ultimately succumb due to treatment resistance and tumor relapse. Resistance to chemoradiation is promoted by complex DNA repair mechanisms, including O^6-methylguanine-DNA methyltransferase (MGMT), which mediates the direct removal of O^6-methylguanine (O^6-meG), the most cytotoxic lesion induced by temozolomide. In the absence of MGMT, processing of O^6-meG by the mismatch repair (MMR) pathway ultimately leads to perturbations of the replication fork and double-stranded DNA breaks (DSB) that require complex machineries for their repair. The other lesions induced by temozolomide are repaired mainly through base excision repair (BER) or direct removal mechanisms catalyzed by the DNA demethylases ALKBH2/3 (reviewed in ref. 4).

Concurrent and maintenance temozolomide was introduced to first-line treatment for GBMs in 2005 (2). Prior to this date, temozolomide and other alkylating agents were mainly used as second-line therapy in patients with recurrent tumors (5, 6). In the clinic, two populations of patients can be distinguished on the basis of methylation of the MGMT gene promoter-associated CpG island. Although patients with MGMT promoter–unmethylated GBM do not benefit from temozolomide, epigenetic silencing of MGMT (observed in about 40% of patients with GBM) confers a small but significant survival benefit (2.5 months) in patients exposed to temozolomide and IR compared with patients treated with IR only (7). However, improved therapeutic strategies are clearly needed in all cases.

Targeting components of the DNA damage response (DDR), including modulation of cell cycle and mitotic progression and genetic stability (8, 9), has emerged as an important therapeutic

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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approach against many cancers. As a step toward improved strategies to undermine DNA repair or exploit specific vulnerabilities associated with GBM cells, we quantified the mRNA expression levels of a selection of genes covering the major DNA repair pathways, as well as important regulatory proteins and cell-cycle control genes, in GBM specimens and control, nontumor tissues. To this end, we exploited a cohort of paired GBM samples (i.e., matched primary and recurrent tumor from the same patients) that would allow us to address treatment-induced changes in gene expression, including biopsies obtained from patients treated with radiotherapy only (pre-2005) or with radiotherapy plus temozolomide (post-2005) so that the impact of temozolomide could be investigated.

Materials and Methods

Study cohort and validation datasets

The Köln cohort is described in Table 1. The expression data (Illumina microarray) pertaining to the 27 DNA repair and cell-cycle gene signature together with the relevant clinical data of the Heidelberg cohort (46 GBM pairs) are presented in Supplementary Table S1. Ethical guidelines were followed for patient sample collection and all samples were anonymized. Written informed consents were received from the patients, and the project was approved by local ethical committees in Cologne (Köln cohort, Application No. 03-170) or Heidelberg (Heidelberg cohort, Application No. 207/2005). Research was conducted according

Table 1. Clinical and relevant molecular features of the Köln cohort

Patient features			
Male		32	
Female		13	
Mean age at diagnosis (y)		56.7 ± 10	
Median		58 (30–73)	
<50		12	
50–70		30	
>70		3	
Clinical features		**Initial**	**Recurrence**
Karnofsky performance score	>90%	3	1
	70%–90%	28	22
	<70%	3	8
	NA	9	4
Resection	Gross total	16	13
	Partial	10	18
	NA	10	12
Tumor location[a]	Frontal	9	5
	Temporal	12	9
	Parietal	4	3
	Occipital	2	3
	Multiple	15	14
	NA	1	1
Histopathologic features			
GBM (grade 4)		78	
Tumor content		>80%	
Ki-67 (mitotic index)		<10%	24
		10%–30%	26
		>30%	9
		NA	19
IDH1 mutation status		Negative	78 (100%)
Patient biopsies		**TMZ-naïve**	**TMZ-treated**
Unique samples	Primary	11	32
	Recurrence	10	25
Sample pairs[b]		9	24
MGMT promoter status[c] (patients)			
Unmethylated		20	
Methylated		20	
NA		3	
PFS (months)			
Mean		14.2	
Median		9.8 (3.5–79)	
OS (months)			
Mean		21.8	
Median		18.1 (4.6–87.9)	

Abbreviation: TMZ, temozolomide.

[a]In all paired biopsies (33 samples) of cohort, the recurrence is found at the same location as the primary tumor.

[b]Primary and recurrent samples from same patient.

[c]MGMT status was assessed in primary samples.

to the principles expressed in the Declaration of Helsinki. The Wang cohort is composed of 61 GBM pairs from three individual RNA-seq datasets (Korean_SMC, HF_MDA, TCGA_GBM) analyzed by RNA sequencing and described in (10), and which are publicly available in the GlioVis database (http://recur.bioinfo.cnio.es).

Biopsy procedure and characterization

All patients with GBM of the Köln cohort underwent surgical resection using standard craniotomy. The extent of resection was evaluated through pre- and postoperative MRI. Snap-frozen biopsies were used for analysis only if examination of their formalin-fixed paraffin-embedded counterpart by 2 independent neurohistopathologists revealed >80% tumor cells. MGMT promoter methylation and IDH1 mutation status were assessed by DNA pyro-sequencing as described previously (11, 12). IHC stainings for Ki-67, PTTG1, AURKA, AURKB, and CENPA were performed on representative tissue sections from selected cases of primary and recurrent tumors from G1 and G3 groups. IHC was performed on an automated immunostainer (DAKO) using the UltraVision Quanto horseradish peroxidase detection system with 3,3′-diaminobenzidine tetrahydrochloride as chromogen (Thermo Fisher Scientific) and pretreatment of the sections by heating them in citrate buffer pH 6.0. The following primary mouse mAbs were used: anti-Ki67 (clone MIB-1, DAKO), anti-PTTG1 (clone DCS-280, MBL Co., 1:50), anti-AURKA (clone JLM28, Leica Biosystems, 1:50), anti-AURKB (clone mAbcam 3609, Abcam, 1:30), and anti-CENPA (clone 3-19, MBL Co., 1:100). The expression of Ki-67 was estimated using the following categories: <5%, 5%–10%, 10%–20%, 20%–30%, 30%–40%, 40%–50%, and >50% labeled nuclei.

RNA preparation and gene expression analysis

Total RNA was extracted using the miRNeasy RNA Isolation Kit (Qiagen) according to the manufacturer's instructions. Samples with RNA integrity number (RIN) >7 and $A_{260/280}$ 1.8–2.2 were sent to the VIB Nucleomics Core Facility for gene expression analysis using the direct multiplexed nCounter technology (NanoString; ref. 13) analysis and 154 gene probes (including 2 control, housekeeping genes) designed *in silico*. Gene expression data are available in the ArrayExpress database (https://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6425.

Characterization of the GBM patient–derived spheroid cell lines

GBM patient–derived spheroid cell lines from G1 and G3 group tumors were obtained from the Heidelberg cohort, at the Department of Neurosurgery (University Clinic Heidelberg, Heidelberg, Germany). The cell lines were controlled for potential cross-contamination using short tandem repeat DNA typing and tested free of *Mycoplasma*. Cells were cultured in serum-free CSC medium (DMEM F-12 (Biowest L0093-500), supplemented with 20% BIT-100 (Provitro 204/3100), 1 U/mL Heparin (Sigma H3149-25KU), 4 mmol/L Ultraglutamine (Lonza BE17-605E/U1), 100U Pen-Strep (Lonza DE17-603E), 20 ng/mL EGF (Provitro 1325950500), and 20 ng/mL FGF (Miltenyi Biotec 130-093-841) in a humidified 37°C incubator under normal conditions (5% CO_2).

The G1 or G3 status of the two cell lines was ascertained by qRT-PCR analysis of the following genes from the signature: CCNA2, CDC25, EME1, and TOP2A. To this end, RNA was prepared using TRIzol reagent (Ambion 15596018) followed by retrotranscription using the iScript cDNA Synthesis Kit protocol (Bio-rad 1708891). cDNA was then subjected to qPCR reaction in a ViiA7 Real-time PCR system (Applied Biosystems), using the FastSYBR green mix (Applied Biosystems 4385612) and the following primers: CCNA2 (Forward (F): AAGACGAGACGGGTTGC, Reverse (R): GGCTGTTTACTGTTTGCTTTCC), CDC25C (F: GACACCCAGAAGAGAATAATCATC, R: CGACACCTCAGCAACTCAG), EME1 (F: CTCCATGATACCCCAGAGAGG, R: CCTGGACCTTCTGACTCGG, TOP2A (F: ACAAGACATCAAAGTGAAGTAAAGCC, R: GCAGACTCAAAACACAGACAAAGC). The housekeeping genes used for normalization were GAPDH (F: CATGAGAAGTATGACAACAGCCT, R: AGTCCTTCCACGATACCAAAGT) and Ezrin (F: TGCCCCACGTCTGAGAATC, R: CGGCGCATATACAACTCATGG). Comparison of the relative expression of each gene in the G1 and G3 cell line was carried out using the $2^{(-\Delta\Delta Ct)}$ method. Western blot analysis of Ki-67 expression levels in total cell extracts was carried out using the following mAbs: anti-Ki67 (clone MIB-1, DAKO, 1:100), anti-GAPDH (clone 5174S, Cell Signaling Technology, 1:1,000).

For cytotoxicity assays, 4,000 cells/well were seeded in triplicates in a 96-well plate format (Greiner 655101) followed by incubation with the selected drugs for 48 or 72 hours. Cytotoxicity was assayed using the WST-1 reagent (Roche 11644807001) and absorbances were determined using the Clariostar reader (BMG Labtech).

Statistical analysis

Differential gene expression analysis. Differentially expressed genes were identified following raw RNA counts normalization, using the *DESeq2* package (raw RNA counts; ref. 14) or *limma* package (15). Significant genes were identified using FDR and fold change. Functional annotation of the genes was carried out using the *topGO* R package. *P* values in the functional enrichement analysis were corrected for multiple hypothesis testing by FDR.

Gene signature establishment and patient clustering. A thorough coexpression analysis was used to assess a gene expression pattern able to segregate the Köln cohort. First, we identified a cluster of genes with highly correlated profiles as determined by calculation of the coefficients of determination (R^2) observed between log-transformed gene profiles. Second, this cluster of 52 highly correlated genes was considered for the classification of the clinical samples using nonnegative matrix factorization (NMF; ref. 16). After 1,000-fold NMF iterations with different initial estimations, the samples that clustered together in 90% of the runs were assigned to two independent groups (G1 or G3), whereas samples showing less concordance were assigned to an "undefined" G2 group. Refinement of the original 52-gene signature was achieved using the method developed by Heinäniemi and colleagues (17). In this analysis, gene pairs are randomly assigned, tested for specificity to our signature, and sorted according to their potential to participate in the patient clustering determination. This resulted in a refined signature with 27 genes which have the same capacity to segregate our cohort in 2 distinct groups as the initial 52-gene signature, as determined by NMF analysis. The robustness of the patient grouping obtained with both signatures was assessed by leave-one-out cross validation (LOOCV) cross-validation and 5-fold cross validation. For the

validation of the signature in the Heidelberg, Wang and Murat cohorts, and The Cancer Genome Atlas (TCGA) GBM RNA expression dataset, NMF was used to group GBM specimens based on the expression of the 27 gene signature, as described above.

Univariable Cox regression analysis. Univariable Cox proportional hazards models were built using the R package *survival* (18), to investigate the potential impact of the following clinical factors on survival: gender, age, MGMT status and Karnofsky Performance Scale (KPS). Of these, age and KPS were considered as continuous variables, while others as categorical.

Signature reproducibility performance. Correlation between the gene expression levels pertaining to the 27 gene signature among the Köln cohort and the validation datasets (Heidelberg and Wang cohorts) was assessed by calculating the logFC for each gene, followed by linear regression analysis.

Results

To investigate DNA repair and cell-cycle control gene expression in GBM, we used nCounter technology, a robust and sensitive method allowing multiplexed analysis of a panel of selected genes (19), to analyze RNA extracted from the Köln cohort (Table 1) composed of samples from paired GBM biopsies ($n = 66$, of which 18 from temozolomide-naïve patients and 48 from temozolomide-treated patients) as well as unpaired biopsies ($n = 12$) and control, tumor-adjacent tissues ($n = 9$). All patients received radiotherapy and harbored wild-type IDH1, as determined by DNA sequencing. Because of the nCounter gene expression format, we focused on 154 genes encompassing the major DNA repair pathways: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), nonhomologous end joining (NHEJ), direct repair enzymes (e.g., MGMT), Fanconi anemia (FA), as well as a selection of genes encoding effectors and regulators of cell cycle, DNA replication, DDR, centromere, and centrosome dynamics (Supplementary Table S2). These genes were chosen based on the available literature on the response of GBM cells to chemoradiation and in particular to temozolomide (4).

Crucial alterations in DNA repair and cell-cycle gene expression distinguish subsets of GBM tumors

In preliminary comparisons of tumor samples with control, tumor-adjacent tissues, detailed in Supplementary Data S1, we identified lists of differentially expressed genes (DEG) associated with primary (72 genes) and recurrent GBM tumors (71 genes). These lists displayed extensive overlap, with 67 genes in common that exhibited comparable fold change direction and intensities. Accordingly, no DEGs were identified upon direct comparison of primary and recurrent GBM samples. Notably, no temozolomide-associated changes in gene expression were observed when temozolomide-naïve and temozolomide-treated recurrent biopsies were compared.

Failure to identify temozolomide-associated gene expression changes using global DEG analysis of unstratified specimens prompted us to examine whether stratification would help uncover such genes in a subset of patients. To address this issue and with the aim of relating our findings to tumor recurrence, we first subjected the gene expression data from the paired biopsies (66 specimens) to a coexpression analysis and obtained a set of 52 genes associated with highly correlated profiles (Supplementary Fig. S1A). We then used this set of genes for the stratification of our specimens using NMF. The resulting heatmap (Supplementary Fig. S1B) revealed that this 52-gene signature segregated the biopsies into 2 well-defined groups (hereafter called G1 and G3) that displayed an inverse expression pattern of the gene signature, leaving 10 samples with a more neutral profile in a separate group called G2. Our 52-gene signature was robust, as evaluated by 2 forms of cross-validation [mean sample misclassification error: 0.0606 (LOOVC), 0.087 (5-fold cross-validation)].

The signature contained 2 subsets of genes [component A ($n = 27$) and B ($n = 25$) of Supplementary Fig. S1C] characterized by inverse expression in G1 and G3. Furthermore, component A was found to contain all the cell cycle–associated genes in the signature.

A 27 DNA repair and cell-cycle gene signature in primary and recurrent GBM

Inspired by Heinäniemi and colleagues (17), we refined the 52-gene signature by analyzing the predictive power of all gene pairs able to reproduce the initial classification. We identified a subset of 27 genes within which each gene pair displayed high prediction ability [mean misclassification error: 0.0758 (LOOCV), 0.086 (5-fold cross-validation)] and, interestingly, highly positive coexpression (Fig. 1A). Notably, 21 of those 27 genes belonged to component A (Supplementary Fig. S1C) of the original signature. The resulting heatmap generated by NMF clustering is presented in Fig. 1B, again revealing G1 and G3 groups presenting an inverse gene expression pattern, and a third less-defined group (G2), as observed with the original signature (Supplementary Fig. S1B).

All 3 groups contained both primary and recurrent samples. Indeed, stratification into 3 groups was also obtained when primary and recurrent biopsies were considered separately, and when an independent cohort of primary GBMs (20) was examined (see below). However, whereas some patients (13/33) had both their primary and recurrent biopsies in the same group, in 20 of 33 patients, the recurrence was traced to a group distinct from that of the primary tumor (Fig. 1C), a notion hereafter referred to as group migration. These observations indicate that relapse was often associated with significant alterations in DNA repair and cell-cycle gene expression. The degree of alteration culminated in patients whose paired biopsies showed inverse gene expression of the signature (Fig. 1B). Similar group migrations were observed with the original 52-gene signature (Supplementary Fig. S1D).

Description of the 27-gene signature

Our 27-gene signature contains important effectors and regulators of the cell cycle, centromere and centrosome dynamics, chromosome segregation, and mitosis (AURKA, AURKB, CCNA2, CCNB1, CDC25C, CDC6, CDK1, CENPA, CENPF, MKI67, PCLAF, PLK1, PTTG1, TOP2A), as well as genes encoding crucial HR factors such as the RAD51 recombinase, the chromatin remodelers RAD54B and RAD54L, and enzymes involved in Holliday junction resolution (EME1/MUS81 complex) and/or NER (ERCC3(XPB), ERCC4(XPF)). Also in the signature were genes encoding the DNA glycosylase NEIL3, Fanconi Anemia factors (FANCD2, UBE2T), the ubiquitin protein ligase UBE3B and 2 specialized DNA polymerases, POLM, and POLQ, involved in NHEJ pathways of DSB repair. None of these genes have been

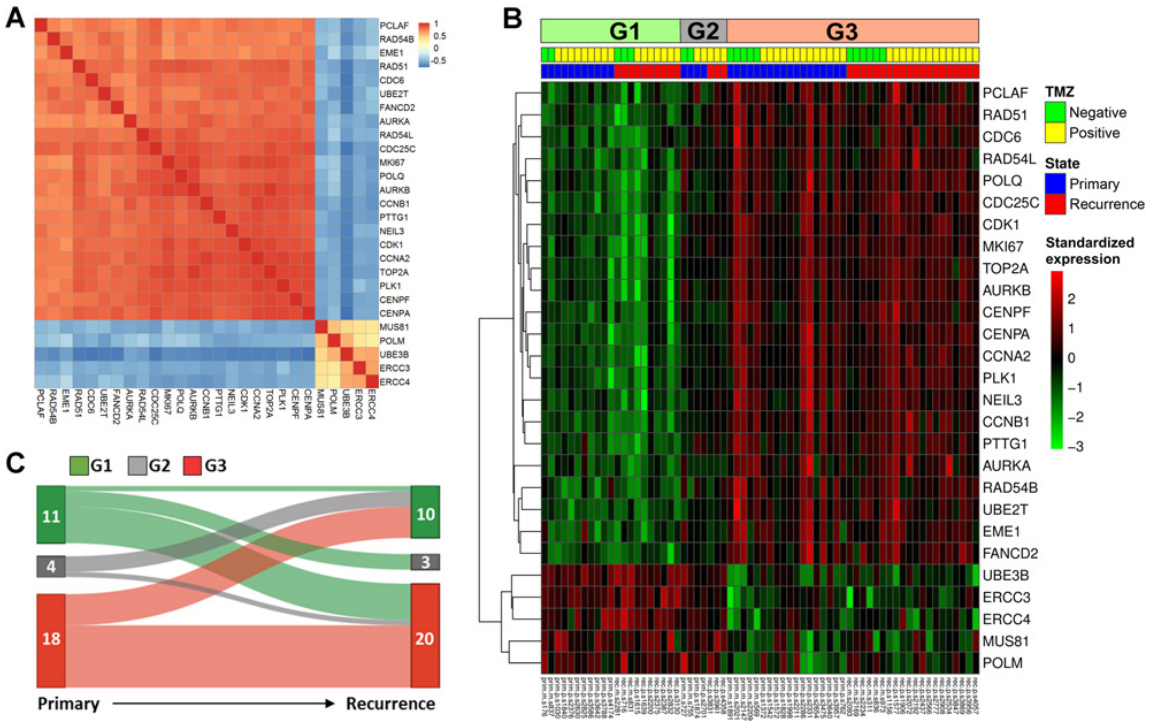

Figure 1.

A 27 gene signature of DNA repair and cell-cycle expression in primary and recurrent GBM. **A,** Coexpression heatmap of the 27 genes identified following gene pair-combination analysis. **B,** Expression heatmap of the 66 GBM specimens from the Köln cohort obtained with the 27-gene signature. Genes were sorted by hierarchical clustering, as shown on the left of the heatmap. Standardized expression values are depicted using a red (high) to green (low) color key. Neon green and yellow squares mark temozolomide-naïve and -positive patients, and blue and red squares denote primary and recurrent samples, respectively. NMF clustering groups are shown in light green (G1), gray (G2), and light red (G3). **C,** Sankey diagram illustrating the changes in gene expression groups (G1, green; G2, grey; G3, red) observed at relapse among the tumor pairs stratified by the 27-gene signature. The total number of samples in each group is indicated.

associated to sites of frequent copy number alterations (21) or found to be significantly mutated (22) in GBM.

Given the inverse gene expression profile displayed by the G1 and G3 groups and the large number of genes involved in cell cycle and mitosis regulation in our signature, including MKI67 encoding the proliferation marker Ki-67, we further investigated the expression of this marker at the protein level. IHC staining of Ki-67 revealed that 95% of the G1 samples exhibited a low Ki-67 index (<10%), whereas 72% of G3 samples displayed a high Ki-67 index (>30%; Fig. 2), in line with the gene expression data and suggesting that the net effect of the observed upregulation of the cell-cycle genes in G3 was increased proliferation. Additional immunostainings for PTTG1, AURKA, AURKB, and CENP-A were carried out using selected primary and recurrent tumors from the G1 and G3 groups (Fig. 2), globally validating the expression of these genes at the protein level.

Validation of the 27 DNA repair and cell-cycle gene signature

We next sought to validate our signature by challenging two datasets: the Heidelberg cohort (biopsy pairs $n = 46$) and a heterogenous collection described in ref. 10, hereby referred to as the Wang cohort (biopsy pairs $n = 61$).

NMF clustering of the Heidelberg cohort generated 3 groups that exhibited profiles reminiscent of the G1, G2, and G3 groups (Supplementary Fig. S2A) and displayed significant group migration (Supplementary Fig. S2B). Similar observations were made with the Wang dataset (Supplementary Fig. S2C and S2D). Furthermore, although the heatmaps generated by NMF suggested that, when considered individually, not all the genes of our signature performed equally well in the validation datasets, the behavior of the gene signature was concordant among all datasets, as revealed by linear regression analysis (P values: 0.0019 for Köln and Heidelberg; 6.1×10^{-6}, for Köln and Wang; Supplementary Fig. S2E and S2F). Thus, our 27-gene signature allowed classification of GBM tumors based on DNA repair and cell-cycle gene expression, and performed equally well with datasets generated by different RNA analysis platforms.

We next asked whether the stratification afforded by our signature now allowed temozolomide-associated changes to be uncovered in specific subsets of samples. Comparable numbers of recurrences from temozolomide-naïve and temozolomide-treated patients were found within each group (Fig. 1B), suggesting that the clustering was independent of temozolomide treatment. Moreover, within-group comparisons of the recurrences from temozolomide-naïve and temozolomide-treated patients

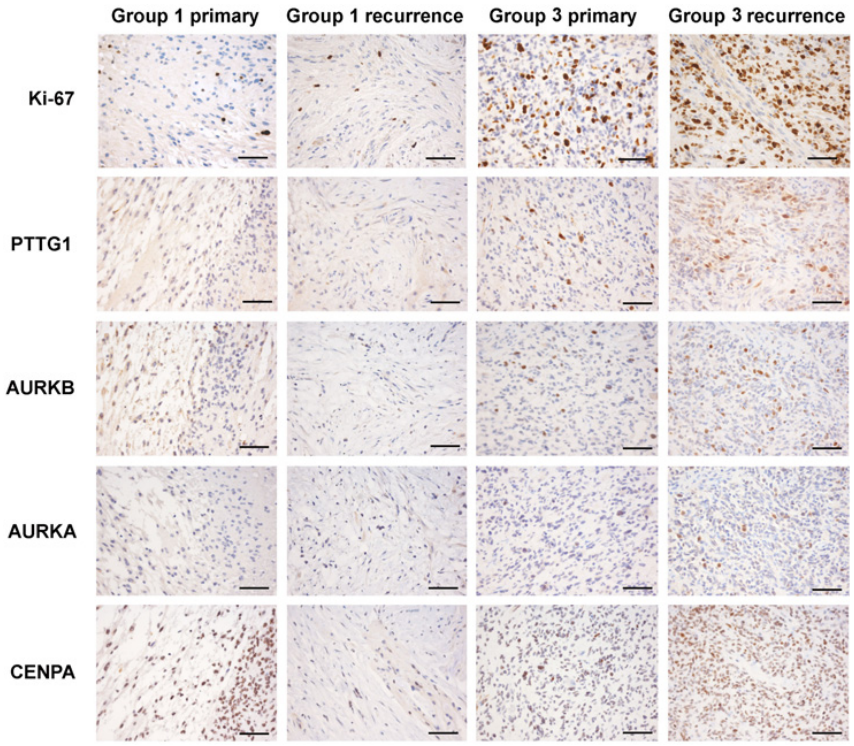

Figure 2.
Immunostaining for Ki-67, PTTG1, AURKA, AURKB, and CENPA in sections of G1- and G3-group tumors. Shown are selected primary and recurrent GBM specimens representative of the G1 and G3 groups from the Köln cohort. Note the high and low nuclear Ki-67 positivity in G3 and G1 tumors, respectively. In addition, little or no expression of PTTG1, AURKA, AURKB, and CENPA is detected in G1 tumors as compared with their clear detection in G3 tumors. All sections are counterstained with hemalum. Brown staining indicates immunopositivity. Magnification of each picture was ×400 (scale bars, 50 μm).

did not uncover DEGs among the whole gene set, indicating that the initial failure to observe temozolomide-associated genes in our cohort was not due to lack of patient stratification. Finally, when we considered the 31 of 33 patients with known MGMT methylation status of the primary tumor, we found no statistically significant differences in the representation of MGMT-methylated and -unmethylated specimens among the 3 groups. The lack of annotations on MGMT promoter methylation status in the Heidelberg and Wang cohorts led us to ascertain this observation using the cohort of primary GBM specimens with well-defined MGMT status described by Murat and colleagues (20). NMF clustering of the 84 samples of this cohort using our 27-gene signature again identified 3 groups, including well-defined G1 and G3 groups (Supplementary Fig. S3A). In these 2 groups, the relative distribution of specimens with methylated and unmethylated MGMT promoter was identical (Supplementary Fig. S3A), indicating that the clustering was independent of MGMT promoter methylation status.

Specific alterations of DNA repair and cell-cycle gene expression at relapse correlate with prognosis

We next examined progression-free survival (PFS) and overall survival (OS) in the 3 groups. When only primary tumors were considered, analysis of these endpoints showed no significant differences between the groups, neither in the paired GBM cohorts, nor in two independent cohorts of primary GBMs (Murat and colleagues; Supplementary Fig. S3B; ref. 20), and the TCGA GBM RNA expression dataset (Supplementary Fig. S4). In contrast, when only the recurrent biopsies of the Köln cohort were analyzed, we found a significant difference in PFS between patients with G1 and G3 biopsies: PFS was longer among patients with a G3 recurrence (12.3 months), compared with those with a G1 recurrence (6.05 months, Fig. 3A). A similar trend was observed with OS, although not statistically significant (inset in Fig. 3A). Importantly, univariable Cox analysis of the Köln dataset interrogating age, gender, MGMT promoter status, KPS, and grouping identified the recurrence association with a specific group as the sole parameter influencing PFS ($P = 0.019$; Supplementary Table S3).

Group migration analysis in the Köln dataset revealed that all transitions were possible (Fig. 1C), and this was corroborated in the other datasets (Supplementary Fig. S2B and S2E). To further explore the impact of group migration on the survival parameters, we assembled patients of the Köln cohort based on the migration behavior of their recurrence, thus creating 4 categories (A–D) corresponding, respectively, to those patients with altered recurrence in G1 (A), G2 (B), G3 (C) or with unaltered recurrence (D). Pairwise analyses revealed that the worst PFS and OS were associated with category A (i.e, progression to G1; Fig. 3B). These findings were reinforced by the observation that both PFS (Fig. 3C) and OS (Fig. 3D) were significantly worse in patients with G1 recurrences (rG1) originating from G3 primaries (pG3; defined as pG3-rG1, red line) than in patients with G3 recurrences originating from either G1 or G3 primaries (pG1-rG3, black line and pG3-rG3, green line), suggesting that these patients contributed the major determinant behind the poor prognosis associated with category A. Taken together, our data

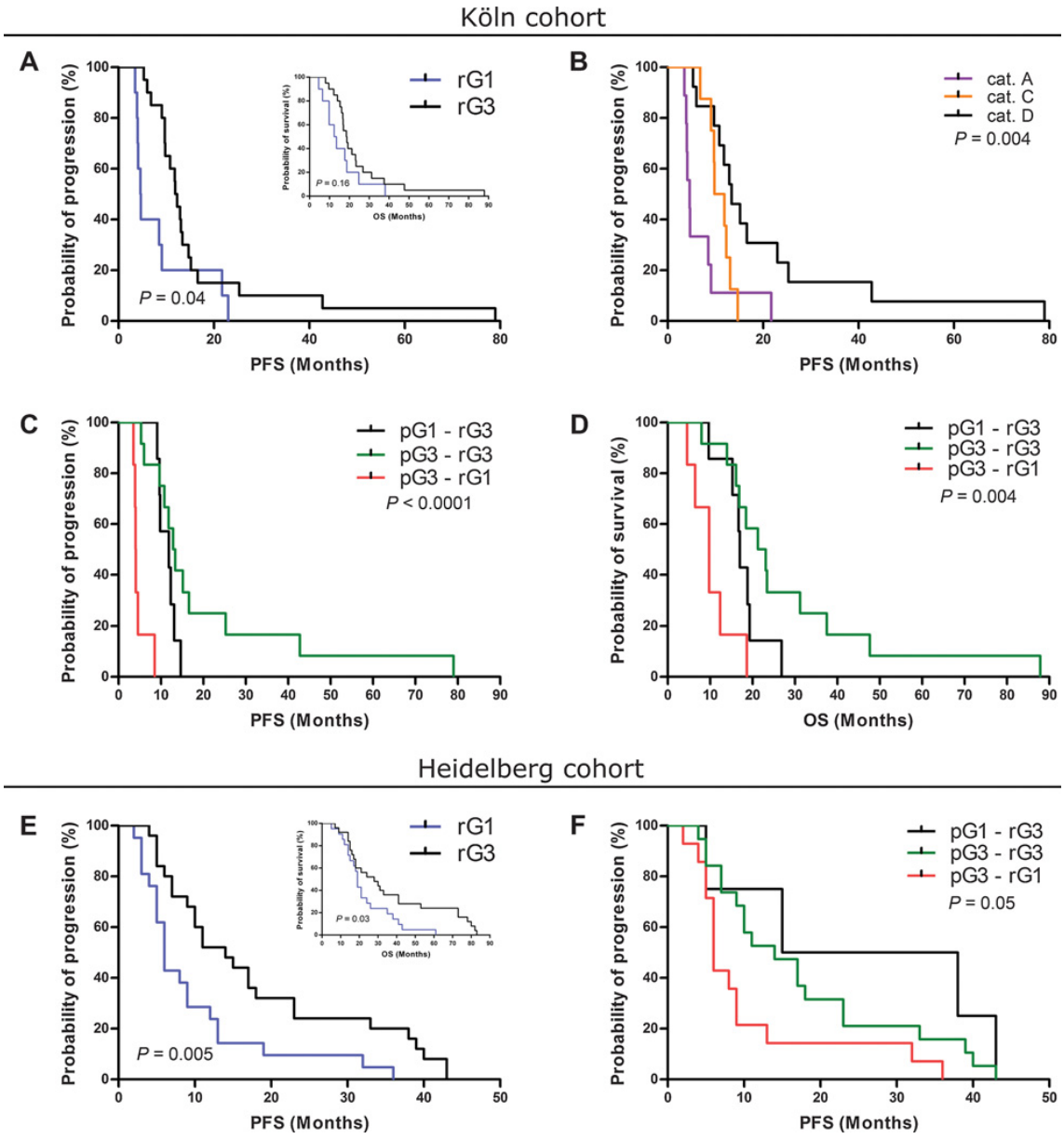

Figure 3.

Prognostic values associated with the 27 DNA repair and cell-cycle gene signature in the Köln (**A–D**) and Heidelberg (**E** and **F**) cohorts. **A,** Kaplan–Meier plots of the PFS and OS (inset) associated with patients displaying a recurrent GBM tumor in the G1 (rG1, blue line) or G3 (rG3, black line) expression group. **B,** PFS associated with the following migration categories: cat. A (migration to G1, purple line), cat. C (migration to G3, orange line) and cat. D (no migration, black line). **C** and **D,** Kaplan–Meier plots of the PFS (**C**) and OS (**D**) associated with the indicated comparisons. **E,** Kaplan–Meier plots of the PFS and OS (inset) associated with patients displaying a recurrent GBM tumor in G1 (rG1, blue line) or G3 (rG3, black line). **F,** Kaplan–Meier plots of the OS associated with the indicated comparisons.

suggest that alterations of the DNA repair and cell-cycle gene expression signature at relapse are associated with significant changes in prognosis, with a transition to G1 translating into the poorest prognosis. Finally, the G1 and G3 groups presented similar proportions of recurrent tumors from temozolomide-treated and -naïve patients (Fig. 1B), indicating that the observed difference in survival between G1 and G3 was not related to temozolomide treatment.

That a G1-type expression pattern at relapse was associated with worse survival parameters was also observed in the Heidelberg cohort where statistically significant differences in PFS and OS were seen both when all recurrences were considered (Fig. 3E) and when group migration was highlighted (Fig. 3F).

Having shown that a recurrent G1 tumor was associated with worse prognosis (Fig. 3C, D, and F), we next examined whether it was the migration toward the G1 group or the mere presence in G1 that was associated with poor prognosis, by comparing pG1-rG1 and pG2/G3-rG1 patients. As only one patient of the Köln cohort belonged to the first category, we focused on the Heidelberg cohort. Although pG1-rG1 patients ($n = 3$) had worse prognosis than pG2/G3-rG1 patients ($n = 14$; PFS: 6 vs. 15.5 months; OS: 17 vs. 25.4 months), the differences were not statistically significant.

No association could be established between gene expression at relapse and survival parameters in the Wang cohort. Based on published work (23), we considered differences in the age distribution as a potential explanation for this finding. Indeed, while there was no statistical difference in age between the Köln cohort (median age: 58 years, range: 30–73) and the Heidelberg cohort (59 years, range: 32–81; $P = 0.73$, Wilcoxon test), the difference was significant between the Köln and Wang cohorts (median age: 52 years, range: 29–74; $P = 1.2 \times 10^{-5}$, Wilcoxon test), with the age distribution in the Wang cohort being shifted towards younger patients. In addition, unlike the Köln and Heidelberg cohorts, Cox regression analysis indicated that age had an impact on survival of patients in the Wang cohort ($P = 1.04 \times 10^{-6}$). Age is a strong prognostic factor among patients with GBM, with younger age correlating with improved survival (24). Thus, although the contribution of other clinical factors cannot be excluded, our observations suggest that our failure to validate the prognostic impact of our signature in the Wang cohort may in part be due to the increased proportion of younger patients in this cohort, which obscures the impact of our patient stratification.

GBM transcriptional subtypes associated with the DNA repair and cell-cycle gene signature

Recent refinement of the gene expression subtypes associated with GBMs has led to three subtypes: proneural, classical, and mesenchymal (10). Having validated our signature using cohorts from this study, we used its annotations to probe the association between our groups and the transcriptional subtypes. Notably, when all the biopsies were considered (i.e., primary and recurrent specimens), we observed specific enrichment of mesenchymal-, classical- and proneural-type tumors in group G1 (49%, $P = 0.06$), G2 (52%, $P = 0.01$), and G3 (42%, $P = 0.01$), respectively (Supplementary Fig. S2D).

Biological processes associated with the G1 and G3 groups

We next investigated the biological processes that distinguished the G1 and G3 groups displaying inverse expression profiles of our gene signature, using the RNA expression dataset from the Wang cohort whose stratification is shown in Supplementary Fig. S2. We identified 2061 DEGs between the two groups (FDR < 0.001; $|\log FC| > 1$), of which 253 were upregulated in G3 and 1808 in G1 (Supplementary Fig. S5A; Supplementary Table S4). Gene Ontology (GO) enrichment analysis (Supplementary Fig. S5B) indicated that GO terms corresponding to biological processes related to cell cycle and cell division, chromosome organization and segregation, DNA replication and repair, and chromatin assembly and dynamics predominated among the DEGs upregulated in the G3 group. These results are in full agreement with the initial distinction of the G1 and G3 groups obtained with our gene signature. In contrast, the list of biological processes significantly enriched among the DEGs upregulated in the G1 group included terms related to vesicle-mediated transport, apoptosis and autophagy, cellular adhesion, response to (chemical) stimulus, and response to stress. These results are consistent with the notion that G3 group tumors develop cellular programmes to sustain high proliferation, in contrast to G1 tumors which engage critical survival cellular processes.

Mutations and copy number alterations underlying the groups identified by the DNA repair and cell-cycle gene signature

As a step to determine whether specific genetic aberrations were associated with tumor development and/or progression within the groups identified by our signature, we took advantage of the availability of exome sequencing and copy number data for a subset of paired tumors of the Wang cohort characterized by Kim and colleagues (25). We generated an oncoprint where the grouping assigned by NMF based on our signature was superimposed on the panel of genetic aberrations related to frequently amplified, deleted and mutated gene components of the major signaling pathways involved in the pathogenesis of GBM, previously determined for these samples (Fig. 4A). Notable differences between the G1 and G3 group tumors are schematically summarized in Fig. 4B. Unlike G3 tumors, all G1 tumors displayed deletion of PTEN and also a high frequency of EGFR and PDGFRA amplification, strongly implicating growth factor tyrosine kinase receptor pathways and the PI3K/PTEN/AKT pathway in the etiology of these tumors (26). In contrast, G3 tumors were characterized by an increased number of cases with RB1 deletion, as well as a high frequency of mutations in TP53, PTEN, and NF1, suggesting that PI3K/PTEN/AKT, RB/CDKN2A-16, and TP53 pathways contributed to the development of G3 tumors. Tumors from the G2 groups presented a composite pattern, for instance displaying increased frequency of TP53 mutations compared with G1 tumors, but also higher frequency of PTEN deletion compared to G3 tumors (Fig. 4A). Finally, when group migrations were considered in this small cohort, the majority of cases did not associate with mutations/alterations in the genes characterized by Kim and colleagues (25). In addition, no group migration involving pG3-rG1 was observed in this cohort. However, we noted that one case of pG1-rG3 migration involved transitions in the clonal status of TP53 and PTEN mutations, from subclonal in the initial G1 tumor to clonal in the recurrent G3 tumor.

Experimental validation of the therapeutic vulnerabilities exposed by the DNA repair and cell-cycle gene signatures

We next wanted to know whether our 52- and 27-gene signatures exposed group-specific vulnerabilities against clinically relevant genotoxics agents as well as inhibitors of the DDR and perturbators of the cell cycle. Indeed, increased expression of TOP2A in the G3 group suggested that G3 tumors might be more sensitive to topoisomerase II inhibitors such as etoposide (27). Likewise, the downregulation of NER genes ERCC3/XPB and ERCC4/XPF in this group suggested that agents such as cisplatin that cause interstrand crosslinks requiring NER for their repair (28, 29), might be more efficient against G3 tumors. We also considered inhibition of RAD51, involved in the recombinational repair of DSBs (30), which was upregulated

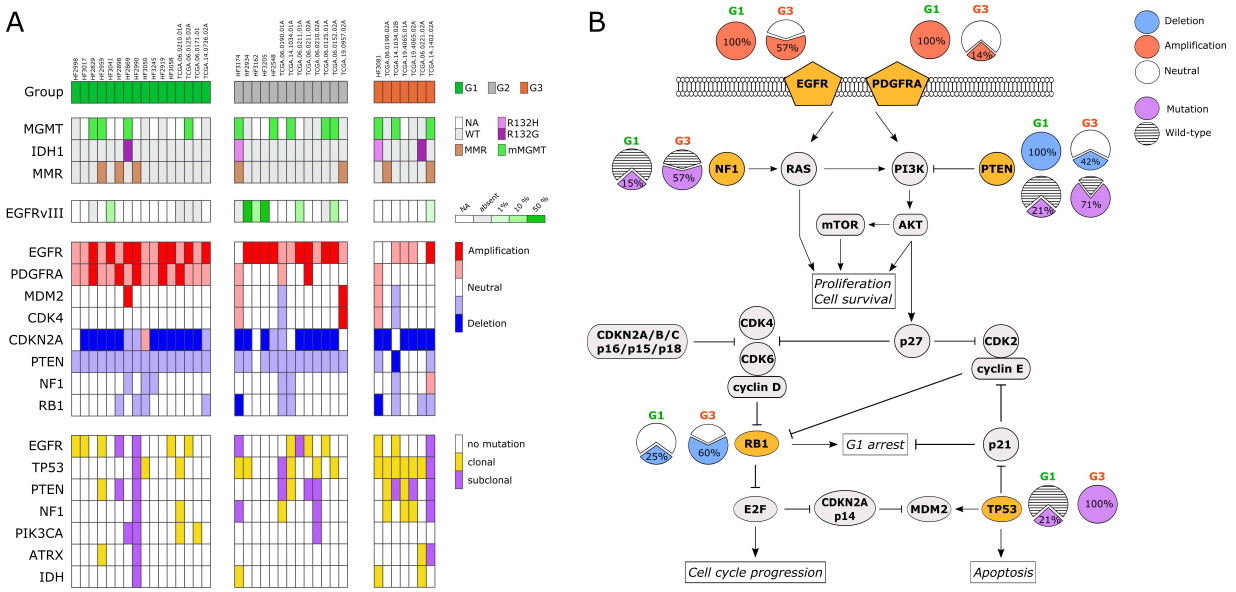

Figure 4.

A, Mutations and copy number alterations associated with the GBM groups identified by the DNA repair and cell-cycle gene signature. **A,** Oncoprint showing the group status (G1, G2, G3) of the analyzed samples (first panel) and cooccurrence with the indicated mutations and copy-number alterations, as determined by Kim et al. (25). Second panel: clinical features [MGMT promoter methylation (mMGMT), mutated IDH1 (R132H or R132G), MMR mutations]; third panel: EGFRvIII mutation; fourth panel: copy number alterations in the indicated genes; bottom panel: clonal/subclonal somatic mutations. **B,** Schematic summary of the aberrations that distinguish G1 and G3 group tumors and their involvment in the GBM oncogenic pathways. Genes harboring significant aberrations in G1 and G3 tumors are highlighted in yellow, with the percentage of mutations or copy number alterations found in each group specified in a pie chart; mutated and wild-type genes are depicted in purple and striped, respectively; copy number alterations (deletion, amplification, neutral) affecting these genes are presented in blue, red, and white pie charts, respectively. Representation of the oncogenic pathways is based on Crespo et al. (26).

in group G3 compared to G1, as well as inhibitors of mitotic kinases, given the strong expression of AURKA, AURKB, CDK1 and PLK1 in G3.

Having successfully established two cell lines, NCH741f and NCH481, from clinical biopsies obtained from G1- and G3-group patients, respectively, of the Heidelberg cohort, we first verified their G1 and G3 status by RT-qPCR analysis of selected genes from the 27-gene signature, including CCNA2 and CDC25C, which together form a gene pair with high predictive ability (mean misclassification error: LOOCV = 0.07576; 5-fold cross validation = 0.09774 ± 0.0015; Fig. 5A, left). We also performed Western blot analysis of Ki-67 showing increased Ki-67 levels in the G3 cell line compared with the G1 cell line (Fig. 5A, middle), consistent with the calculated doubling times for these cell lines (NCH481: ca. 57 hours; NCH741f: ca. 104 hours). Importantly, these cell lines were obtained and maintained in serum-free medium conditions, which is crucial to preserve the biological status and behavior of GBM cells, including their response to therapeutics (31). Under these conditions, the cells grew as spheroids (Fig. 5A, right).

We next treated the 2 cell lines with the DNA-damaging agents etoposide and cisplatin, as well as inhibitors of RAD51 (i.e., RI-1) and mitotic kinases [i.e., anti-AURKA (alisertib) and pan-AURK (tozasertib)] to which we expected cells from the G3 group to be more sensitive compared with G1 cells. Given that inhibitors of the DNA repair factor PARP (PARPi; ref. 9) are currently in clinical trials against GBM (32), we also tested the PARPi olaparib. In this case, however, we could not predict differences in sensitivity to the PARPi among G1- and G3-group tumor cells based on the expression of our gene signature components because synthetic lethality with PARP inhibition can be mediated by various defects in a plethora of DNA repair pathways (33, 34). The data from WST-1 cytotoxicity assays illustrated in Fig. 5B, show the statistically significant increase in sensitivity of G3-group derived cells relative to G1-group cells, to all tested compounds with the exception of olaparib, for which no significant differences were observed, in line with our gene expression data.

Taken together, these data provide experimental evidence to suggest that analysis of GBM biopsies using our DNA repair and cell-cycle gene signature may help identify novel therapeutic strategies against subsets of GBM tumors.

Discussion

Previous studies sought to identify molecular profiles specific to resistance to chemoradiotherapy by interrogating clinical outcome data and gene expression data generated from primary GBM specimens (i.e., collected before treatment; ref. 20). In this study, we focused on a cohort of paired GBM samples from patients treated with radiotherapy or radiotherapy plus temozolomide with the aim of exploring alterations in DNA repair and cell-cycle gene expression in primary GBMs and their recurrences. Although the use of paired biopsies is gaining momentum, their availability is still limited by the speed of progress in the biobanking of reliable and clinically annotated specimens. For GBM, paired biopsies predating the introduction of temozolomide in the clinic represent a scarce resource.

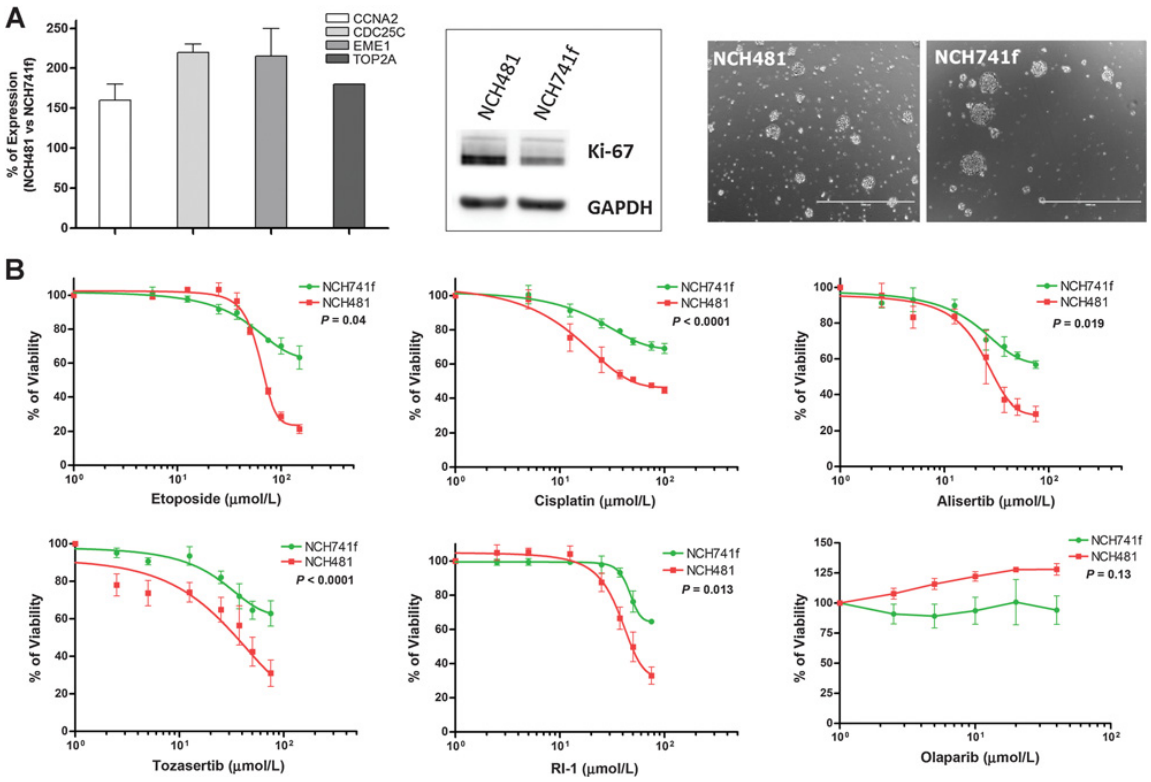

Figure 5.

In vitro validation of the therapeutic vulnerabilities exposed by profiling of GBM tumors using the DNA repair and cell-cycle gene signature. **A,** Characterization of the G1 (NCH741f) and G3 (NCH481) GBM patient-derived cell lines. Left, plot of the expression levels obtained by quantitative RT-PCR of the indicated genes confirming their expected upregulation in the G3 group relative to the G1 group ($P < 0.05$). Expression data were normalized against the GAPDH and ezrin housekeeping genes. Middle, Western blot analysis of Ki-67 in total cell extracts. Right, optical microscopy images illustrating the two spheroid cultures (scale bar, 1 mm). **B,** Effect of etoposide and cisplatin, as well as the indicated inhibitors [RI-1 (RAD51i); alisertib (AURKAi); tozasertib (pan-AURKi)] on the viability of NCH741f (green line) and NCH481 cells (red line), as assessed using the WST-1 reagent. Differences in sensitivity between the two cell lines were analyzed by direct *t* test comparisons and their significance are indicated by *P* values.

Our analysis did not reveal temozolomide-related alterations in the studied genes, or expression patterns predictive of response to temozolomide. This observation suggests that the dynamics of mRNA expression in response to temozolomide treatment cannot be assessed in clinical samples collected at the time of second surgery, most likely due to the delay between the cycles of temozolomide administration, tumor relapse, and sampling of the recurrent biopsy. Experiments to investigate this question would benefit from patient-derived xenograft models where time courses of gene expression in response to treatment can be carried out, as underlined by studies in ovarian (35) or breast (36) cancers. The mutagenic impact of temozolomide as a driving mechanism in tumor progression and the selection of temozolomide-resistant clones through defective MMR is well documented in gliomas (37–39) and GBM (40, 41). Future work should focus on the mutational status of the MMR genes and other mutations induced by temozolomide in our cohorts.

Our 27-gene signature clustered primary and recurrent GBMs according to DNA repair and cell-cycle gene expression. Performance comparison with other signatures is hampered by the current paucity of studies on paired biospies. Thus, a 412-gene classifier has been described that segregated paired GBM specimens into 2 groups, revealing cases of group migration; however, no association between patient grouping and survival was reported (42). Although our gene signature could not be used to predict relapse-free survival based on the profiling of primary GBM samples, its expression pattern at the time of recurrence correlated with prognosis. As our signature contains 14 cell cycle genes that display high positive coexpression, it is striking that decreased expression of these genes at relapse in G1 was associated with shorter PFS. It is possible that the high Ki-67 proliferation index seen in the G3 recurrent tumors is associated with exacerbated genetic and/or chromosomal instability, leading to increased mitotic catastrophe and cell death. Increased chromosomal instability has been described in recurrent GBM (25, 43). In addition, deregulation of CENPA and CENPF, present in our signature, could contribute to the observed phenotype, because misregulation of centromere and kinetochore function leading to chromosomal instability contribute to tumor development and response to chemoradiation (44). Alterations in the division

mode and differentiation status of the tumor cells and their subpopulations may also affect chromosomal instability and proliferation in ways that remain to be explored. As no standard second-line treatment of GBM has yet been determined, patients with recurrent GBM often receive complex modalities (5, 6). Because the survival outcome of these patients may benefit from second-line therapy, in depth analysis of these modalities may help determine the extent to which the improved OS of patients with a G3-group recurrence reflects the impact of DNA repair and cell-cycle gene expression on the tumor cell response to second-line treatment. Such an analysis was not possible in our cohorts, due to the lack of documented patient records and the large panel of drugs that were considered as second-line treatment, resulting in multiple subgroups of patients whose small size precluded any statistically significant analysis.

It is notable that the G1 group from the Wang cohort was enriched in mesenchymal-type specimens, since this subtype has been shown to be associated with worse PFS in recurrent GBM (ref. 45 and references therein). Furthermore, analysis of key molecular alterations that underlie GBM in a small subset of tumors from this cohort suggests that there are distinct molecular drivers behind the groups identified by our signature. However, extensive molecular characterization of the paired biopsies of our cohorts, for example, by large-scale next-generation sequencing approaches, will be required to understand the full impact of chemoradiation on genomic alterations and reveal the molecular drivers behind the groups detected in our studies and their associated prognosis. Such comprehensive analyses are currently pursued in large collaborative efforts such as the one undertaken by the Glioma Longitudinal Analysis (GLASS) consortium (46).

Our gene signature exposes vulnerabilities against specific DDR inhibitors, perturbators of mitosis, and/or genotoxic agents, including several mainstream chemotherapeutics (etoposide, cisplatin, carboplatin) previously considered, either alone (47–49) or in combination (50, 51) against GBM. It is tempting to speculate that the heterogeneity of the cohorts used in these studies contributed to the limited efficiency attributed to these compounds and that future studies might benefit from patient's tumor stratification based on our signature. Potential modalities also involve the targeting of crucial components of the DNA damage response including RAD51 (9), which our data suggest could prove an attractive target to undermine HR-mediated repair of replication fork-associated DSBs in highly proliferative cells (e.g., in G3-group tumors) or to sensitize these cells to IR and genotoxic agents known to induce DSBs. The identification of patients that are likely to benefit from PARP inhibition remains a challenge (33, 34), and we could not predict differences in sensitivity to the PARPi olaparib used as a single agent based on the expression of our gene signature components. Future experiments will need to consider PARPi in combination with DNA-damaging agents or other DDRi. Our data also lend support to the use of mitosis perturbators against GBM. Indeed, our signature contains crucial regulators of this process, including PTTG1/securin and the mitotic kinases AURKA, AURKB, CDK1 and PLK1, which have gained recent attention as therapeutical targets in GBM. Thus, *in vitro* and xenograft animal model studies have advocated the use of AURK inhibitors in combination with radiotherapy and temozolomide against GBM cells (52–55). Our data suggest that, when used as single agents, mitotic kinase inhibitors may be particularly effective against G3-group tumors. Finally, although our experimental data, obtained with genotoxicants and inhibitors used in monotherapy formats, suggest that G3-group tumor cells are particularly amenable to DNA repair–based strategies, future experiments using combination therapy may unveil modalities targeting specifically G1-group cells. How our gene signature may contribute to precision medicine for GBM is summarized in Supplementary Fig. S6. We are aware that our stratification strategy still leaves aside a considerable subset of patients (e.g., from the G2 group). However, in view of the fact that GBM remains an incurable disease, our results may carry significant clinical prospects in the fight against GBM. The modalities discussed here in the framework of our gene signature might be applicable for the management of both primary and recurring GBM.

Disclosure of Potential Conflicts of Interest

G. Reifenberger reports receiving commercial research grants from Merck and Roche, has received speakers bureau honoraria from Abbvie, and is a consultant/advisory board member for Abbvie. A.J. Chalmers reports receiving commercial research grants from AstraZeneca and Vertex Pharmaceuticals, has erceived speakers bureau honoraria Tesaro and Bayer, and is a consultant/advisory board member for Hox Therapeutics and AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Supplementary data 1

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This document details the differential gene expression analysis carried out using the Köln dataset.

No TMZ-related changes in the expression profile of DNA repair and cell cycle genes at relapse

We first used DESeq to identify differentially-expressed genes (DEGs) that might be informative of glioblastomagenesis, tumor progression and response to TMZ, using distinct pairwise comparisons. As expected since all patients were TMZ- naive at the time of first presentation, no DEGs were found when primary biopsies from TMZ-naive and TMZ-treated patients were compared (FDR<0.05 and [FC]>1.5) (data not shown). Notably, a similar observation was made when recurrent biopsies from both groups (TMZ-naive and TMZ-treated) were compared (data not shown), suggesting that any impact of TMZ on DNA repair and cell cycle gene expression was not detectable at the time of relapse.

Given these observations, we decided to create 2 groups containing, respectively, all primary and all recurrent tumors (whether from TMZ-naive or -treated patients) for further analyses. We first identified tumor development/progression-relevant DEGs by comparing primary or recurrent tumors with control, tumor-adjacent tissue. Seventy-two DEGs were identified for the comparison of primary tumors vs control samples (PvsC) (Figure SD1A), and 71 for that of recurrences vs controls (RvsC), respectively (data not shown). Notably, 67 genes were common to both lists and displayed comparable fold-change directions (up or down) and intensities, as indicated by correlation analysis (Figure SD1B). Functional annotation of these DEGs using WebGestalt[1] revealed that the main DNA repair pathways represented by the down-regulated genes corresponded to NER, BER and MMR, whereas the up-regulated genes were related to components of the cell cycle, as well as the repair of DNA double-strand breaks (DSBs) by homologous recombination (Table SD1). Finally, when primary and recurrent tumors were compared directly, no DEGs were identified (data not shown), suggesting that in the absence of patient stratification, no difference can be observed at the mRNA expression level between primary and recurrent biopsies.

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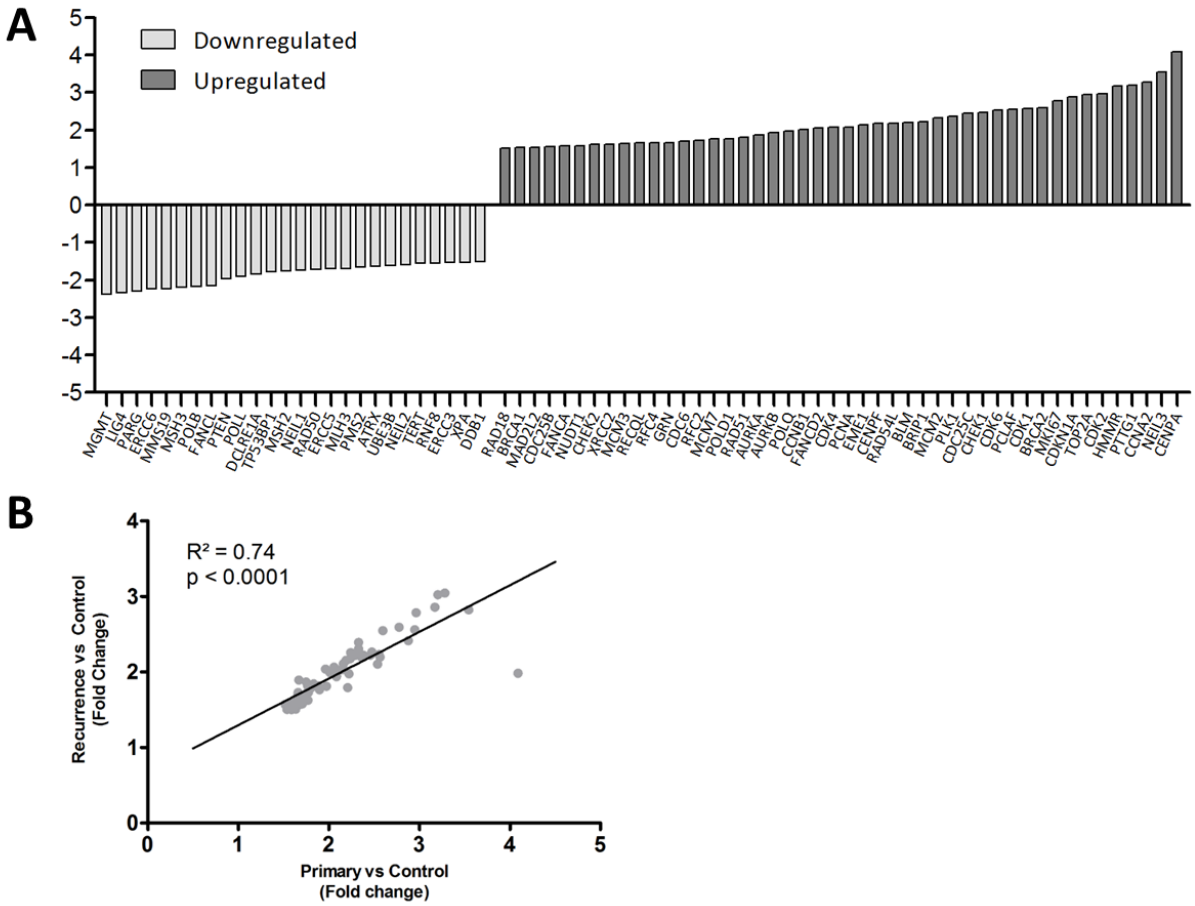
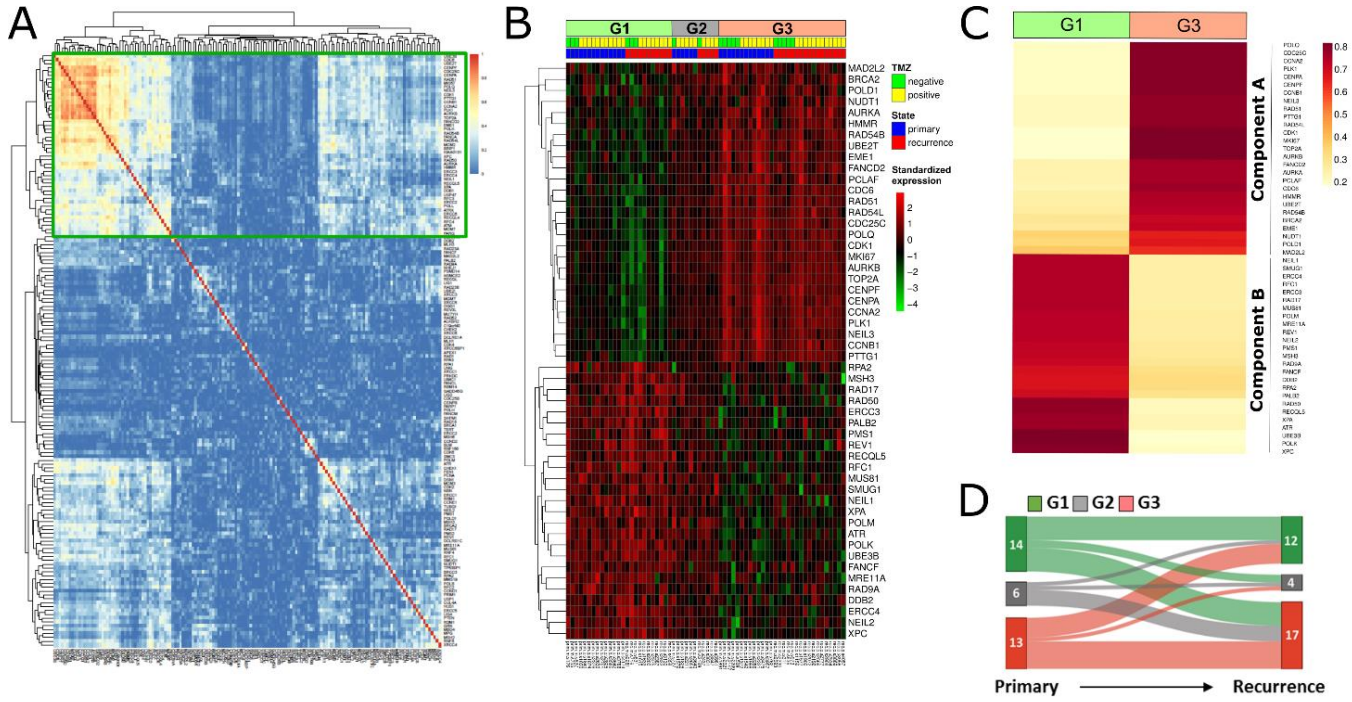


Figure SD1. Differentially expressed genes associated with primary GBM. A. Bar plot of the deregulation scores observed for the 72 DEGs identified in the comparison of combined primary biopsies with control, tumor-adjacent tissues (treshold: FDR < 0.05; [FC]>1.5). Up- and down-regulated genes are indicated in dark and light gray, respectively, with the corresponding fold change presented in Y-axis. **B.** Scatter plot displaying the fold change correlation between the comparisons of primary tumors versus controls (PvsC) and recurrent tumors versus controls (RvsC), revealing a high significant ($p < 0.0001$) R^2 value (0.74).

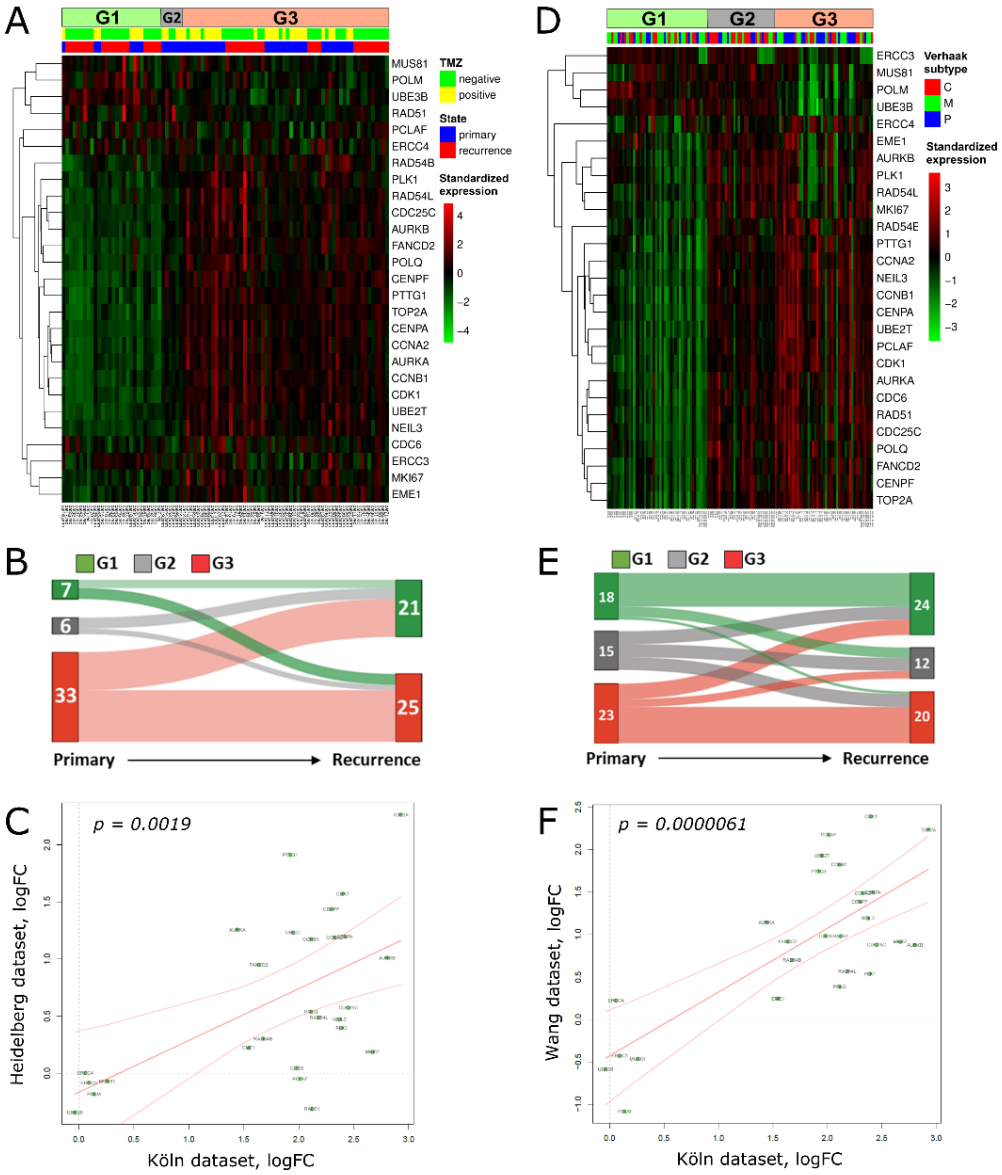
Table SD1.

Pathway	Number [1]	Total [2]	Genes
Down-regulated genes			
Nucleotide Excision Repair	6	13	DDB1, ERCC3, ERCC5, ERCC6, MMS19, XPA*
Mismatch Repair	4	7	MLH3, MSH2, MSH3, PMS2
Base Excision Repair	4	9	NEIL1, NEIL2, POLB, POLL
Up-regulated genes			
Cell cycle, DNA replication, centrosome biology and related stress response	28	33	AURKA, AURKB*, CCNA2, CCNB1, CDC25B, CDC25C, CDC6, CDK1, CDK2, CDK4, CDK6, CDKN1A, CHEK1, CHEK2, HMMR, PCLAF, MAD2L2*, MCM2, MCM3, MCM7, MKI67, PCNA, PLK1, PTTG1, RAD18, RFC2*, RFC4, TOP2A
Homologous Recombination	9	16	BLM, BRCA1, BRCA2, BRIP1, EME1, POLD1, RAD51, RAD54L, XRCC2
Fanconi Anemia	7	12	FAAP24**, FANCA, FANCD2, BRCA1, BRCA2, XRCC2, RAD51

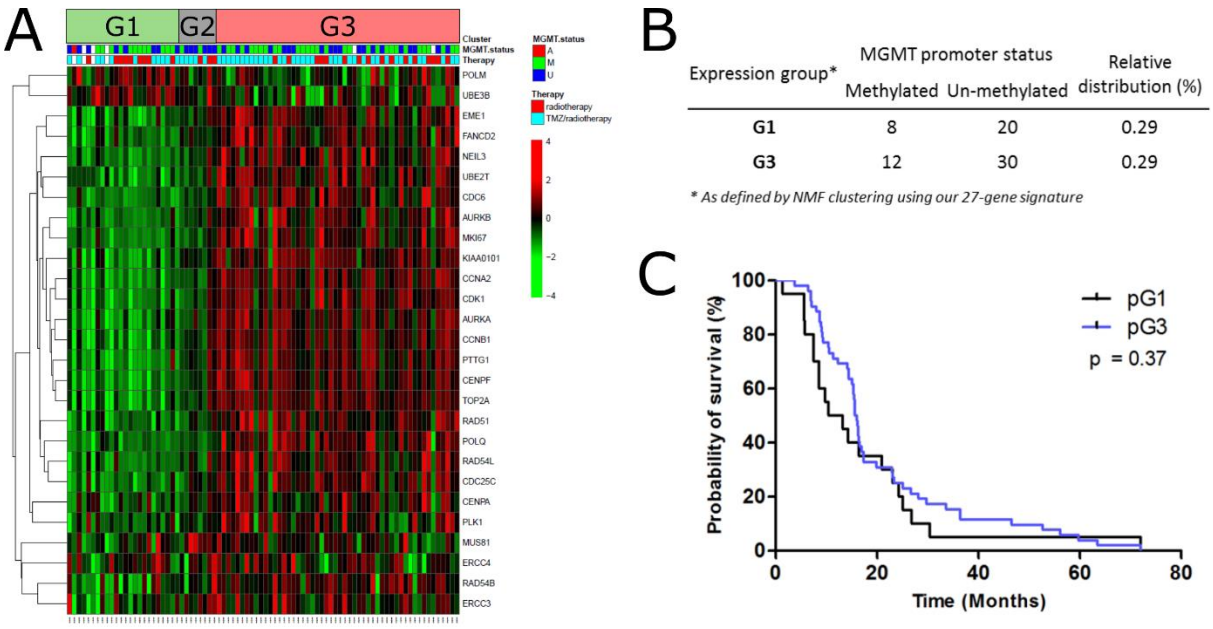
[1]: number of DEGs; [2]: total number of genes from the indicated pathway in the gene set; * and **: DEGs found solely in the comparison of primary GBMs with controls, and recurrent GBMs with controls, respectively.



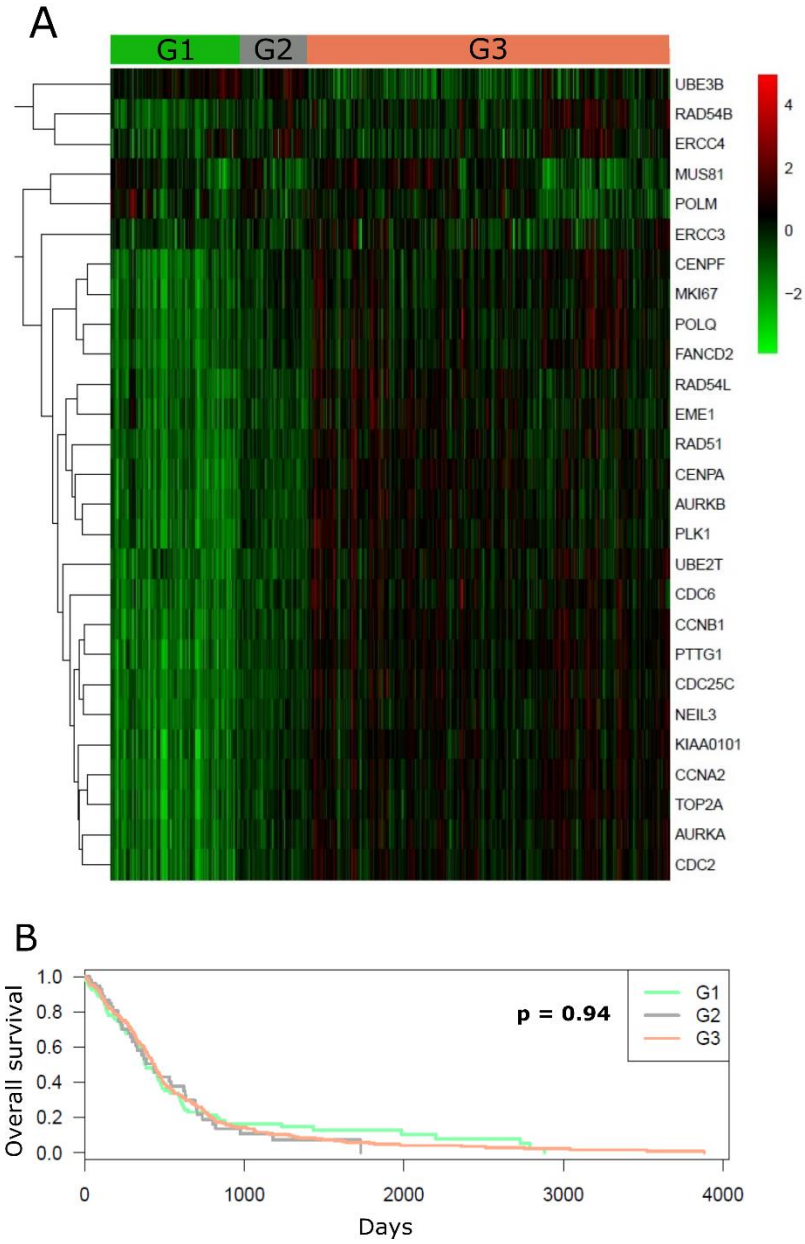
Supplementary Figure S1. A. Gene expression correlation heatmap of the 156 genes included in the study showing the 52 high correlated genes (upper left corner) that were considered for NMF analysis. Correlation values are indicated by a red (high) to blue (low) color key. **B.** Heatmap showing the gene expression pattern and patient segregation proposed by the NMF analysis. Standardized expression values are depicted using a red (high) to green (low) color key. Neon green and yellow squares marks TMZ-negative and -positive patients, whereas blue and red squares denote primary and recurrent samples, respectively. NMF clustering is shown in light green (G1), grey (G2) and light red (G3). **C.** Heatmap illustrating the 2 components forming the 52-gene signature as defined by NMF analysis. **D.** Sankey diagram illustrating expression group transition (G1, green; G2, grey; G3, red) between primary and recurrent tumor pairs as stratified by the 27-gene signature. Numbers refer to the total number of samples in each group.



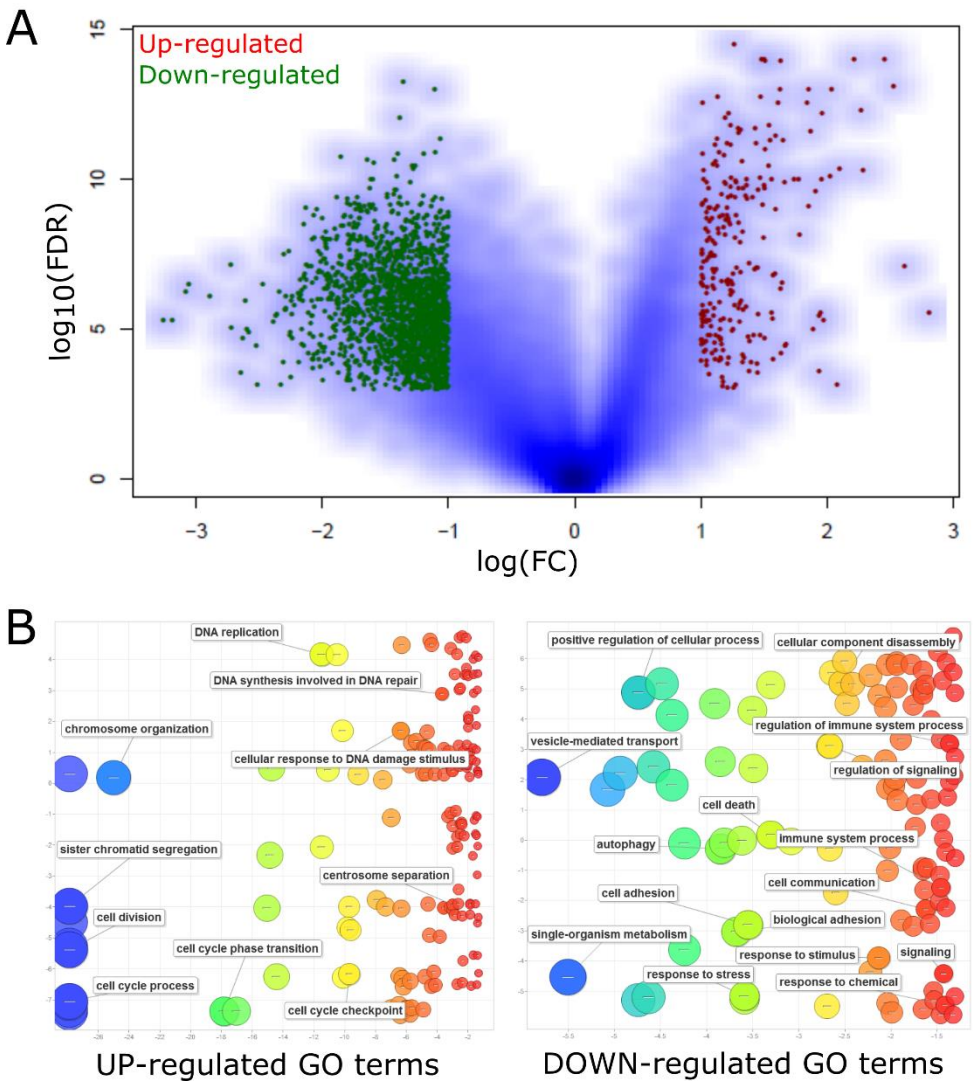
Supplementary Figure S2. Validation of the 27 DNA repair and cell cycle gene signature. Representative validation heatmaps of the 27-gene signature in two independent cohorts of paired GBM specimens (**A.** Heidelberg; **D.** Wang) and their associated intergroup migration plots (**B.** Heidelberg; **E.** Wang). The neural (N), mesenchymal (M) and classical (C) transcriptional subtypes of the samples in the Wang cohort are superimposed to the heatmap in **D**. **C** and **F**. Linear regression analysis of the 27-gene signature performance across the Köln, Heidelberg and Wang datasets. Shown are scatter plots of the logFCs in G3 compared to G1 in the comparison of the Köln with and Heidelberg (**C**) or Wang (**F**) datasets. The consistency of the observed LogFCs is illustrated by the significant linear dependencies in both comparisons. The estimated linear models are shown in thick red lines, with thin lines depicting the 95% confidence interval of the linear model.



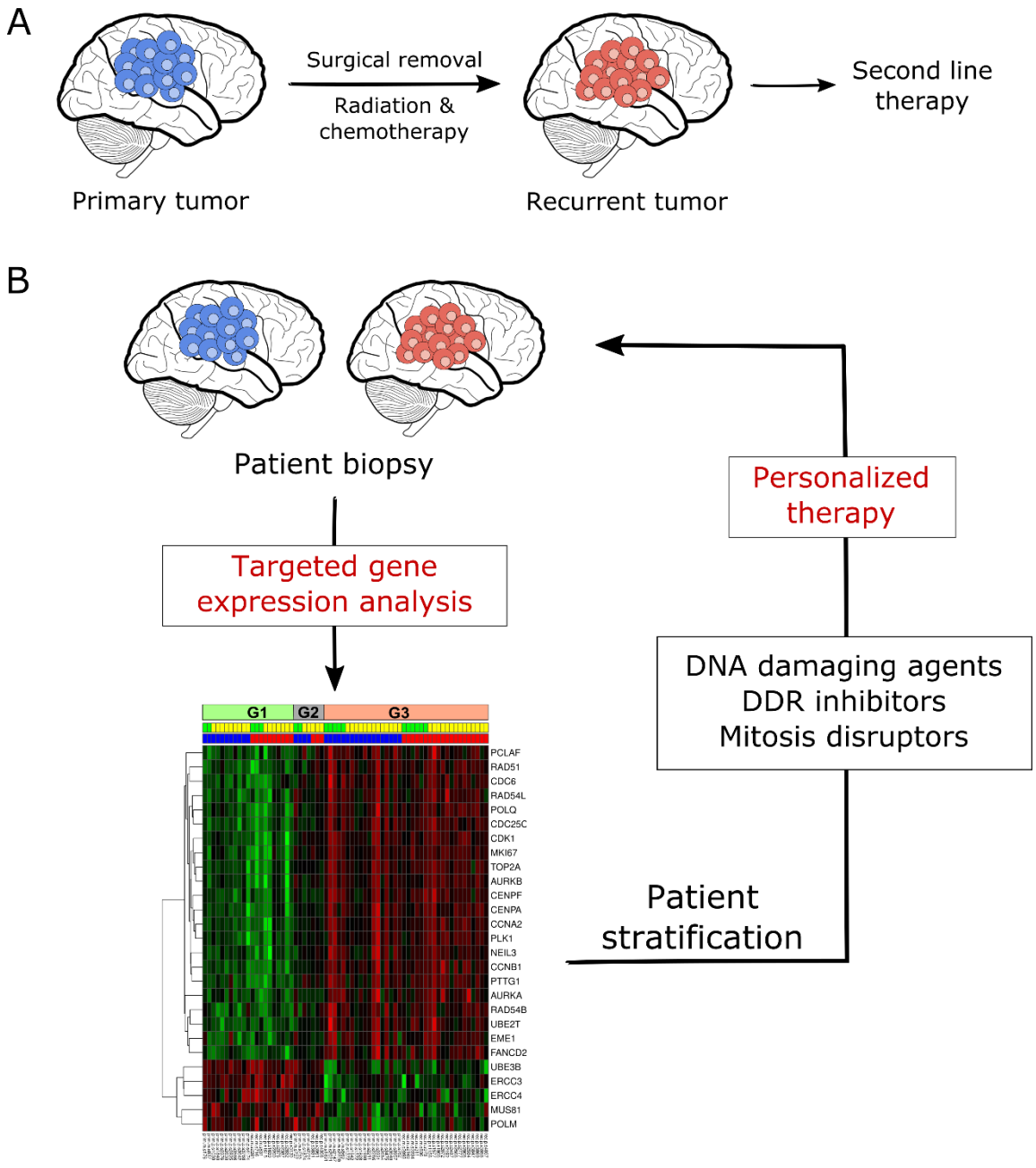
Supplementary Figure S3. A. Heatmap showing the gene expression pattern and patient segregation proposed by the NMF analysis on the Murat et al.[18] cohort of primary GBMs. Standardized expression values are depicted using a red (high) to green (low) color key. MGMT promoter methylation status is depicted in red (not available), neon green (methylated), and blue (unmethylated) squares, whereas patient therapy in light blue (TMZ + radiotherapy) and red (radiotherapy alone) squares. NMF clustering is shown in light green (G1), grey (G2) and light red (G3) on top of the heatmap. **B.** Distribution of the MGMT promoter methylation status (methylated vs un-methylated) amongst G1- and G3-group tumors within the Murat et al.[18] cohort. **C.** Kaplan-Meier plots of the overall survival associated with G1- and G3-group tumors.



Supplementary Figure S4. A. Heatmap showing the gene expression pattern and patient segregation proposed by the NMF analysis in the TCGA primary GBM RNA expression dataset (Affymetrix HuEx expression data, 440 patients). Standardized expression values are depicted using a red (high) to green (low) color key. NMF clustering is shown in light green (G1), grey (G2) and light red (G3) on top of the heatmap. **B.** Kaplan-Meier plots of the overall survival associated with G1-, G2- and G3-group tumors.



Supplementary Figure S5. A. Volcano plot of the DEGs obtained in the comparison of G1 and G3 samples in the Wang cohort. Each dot represents a gene, with green and red dots indicating genes significantly down- and upregulated in the G3 group compared to the G1 group, respectively ([logFC]>1 and FDR < 0.001). The X-axis specifies the log(Fold Change) values and Y-axis the $\log_{10}$(False discovery rate). **B.** Representative gene ontology (GO) terms associated with the genes found upregulated (left panel) or downregulated (right panel) in the G3 group, as plotted by REVIGO analysis. Only GO terms with p-value < 0.05 were plotted. The bubble color (blue, very significant; red, less significant) indicates the p-value of each GO term whereas bubble size denotes its frequency in the GO database.



Supplementary Figure S6. DNA repair- and cell-cycle-based strategies for personalized management of GBM. **A**. Our current management of primary and recurrent GBM suffers from lack of adequate patient stratification and limited therapeutic options. **B**. Illustration of the clinical perspectives for personalized therapy offered by transcription profiling of the identified DNA repair and cell cycle gene signature in primary and recurrent GBM specimens.

Supplementary Table S1. Summary of the studied genes, their related cellular pathways and the deregulated genes in the comparison of tumor to control tissue.

Gene	Definition (Genecards)	UP (P vs C)	UP (R vs C)	DOWN (P vs C)	DOWN (R vs C)
ALKBH2	AlkB Homolog 2, Alpha-Ketoglutarate Dependent Dioxygenase				
APEX1	Apurinic/Apyrimidinic Endo-deoxyribonuclease 1				
ATM	Ataxia Telangiectasia Mutated, ATM Serine/Threonine kinase				
ATR	Ataxia Telangiectasia And Rad3-Related Protein, ATR Serine/Threonine kinase				
ATRX	Alpha Thalassemia/Mental Retardation Syndrome X-Linked, Chromatin remodeler			yes	yes
AURKA	Aurora Kinase A	yes	yes		
AURKB	Aurora Kinase B	yes			
BLM	Bloom Syndrome RecQ Like Helicase	yes	yes		
BRCA1	Breast Cancer Type 1 Susceptibility Protein	yes	yes		
BRCA2	Breast Cancer Type 2 Susceptibility Protein	yes	yes		
BRCC3	BRCA1/BRCA2-Containing Complex Subunit 3				
BRIP1	BRCA1 Interacting Protein C-Terminal Helicase 1	yes	yes		
CCNA2	Cyclin A2	yes	yes		
CCNB1	Cyclin B1	yes	yes		
CCND1	Cyclin D1				
CCND2	Cyclin D2				
CCNE1	Cyclin E1				
CDC25B	Cell Division Cycle 25B	yes	yes		
CDC25C	Cell Division Cycle 25C	yes	yes		
CDC6	Cell Division Cycle 6	yes	yes		
CDK1	Cyclin dependent kinase 1	yes	yes		
CDK2	Cyclin dependent kinase 2	yes	yes		
CDK4	Cyclin dependent kinase 4	yes	yes		
CDK6	Cyclin dependent kinase 6	yes	yes		
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A	yes	yes		
CENPA	Histone H3-Like Centromeric Protein A	yes	yes		
CENPB	Centromere Protein B				
CENPF	Centromere Protein F	yes	yes		
CHEK1	Checkpoint Kinase 1	yes	yes		
CHEK2	Checkpoint Kinase 2	yes	yes		
CUL4A	Cullin 4A				
DCLRE1A	DNA Cross-Link Repair 1A			yes	yes
DCLRE1C	DNA Cross-Link Repair 1C, Artemis				
DDB1	Damage Specific DNA Binding Protein 1			yes	yes
DDB2	Damage Specific DNA Binding Protein 2		yes		
DSN1	DSN1 Homolog, MIS12 Kinetochore Complex Component		yes		
EME1	Essential Meiotic Structure-Specific Endonuclease 1	yes	yes		
ERCC1	Excision Repair Cross-Complementation Group 1				
ERCC2	ERCC Excision Repair 2, TFIIH Core Complex Helicase Subunit				
ERCC3	ERCC Excision Repair 3, TFIIH Core Complex Helicase Subunit			yes	yes
ERCC4	ERCC Excision Repair 4, Endonuclease Catalytic Subunit				
ERCC5	ERCC Excision Repair 5, Endonuclease			yes	yes
ERCC6	ERCC Excision Repair 6, Chromatin Remodeling Factor			yes	yes
FAAP24	Fanconi Anemia Core Complex Associated Protein 24		yes		
FANCA	Fanconi Anemia Complementation Group A	yes	yes		
FANCD2	Fanconi Anemia Complementation Group D2	yes	yes		
FANCF	Fanconi Anemia Complementation Group F				
FANCL	Fanconi Anemia Complementation Group L			yes	yes
FANCM	Fanconi Anemia Complementation Group M				
FEN1	Flap Structure-Specific Endonuclease 1				
GADD45G	Growth Arrest And DNA Damage Inducible Gamma				
GRN	Granulin Precursor	yes	yes		
HMMR	Hyaluronan Mediated Motility Receptor	yes	yes		
HUS1	HUS1 Checkpoint Clamp Component				
PCLAF	PCNA Clamp Associated Factor	yes	yes		
LIG1	DNA Ligase 1				
LIG3	DNA Ligase 3				
LIG4	DNA Ligase 4			yes	yes
MAD2L2	Mitotic Arrest Deficient-Like Protein 2	yes			
MBD4	Methyl-CpG Binding Domain 4, DNA Glycosylase				
MCM2	Minichromosome Maintenance Complex Component 2	yes	yes		
MCM3	Minichromosome Maintenance Complex Component 3	yes	yes		
MCM7	Minichromosome Maintenance Complex Component 7	yes	yes		
MGMT	O-6-Methylguanine-DNA Methyltransferase			yes	yes
MKI67	Marker Of Proliferation Ki-67	yes	yes		
MLH1	MutL Homolog 1				
MLH3	MutL Homolog 3			yes	yes
MMS19	MMS19 Homolog, Cytosolic Iron-Sulfur Assembly Component			yes	yes
MPG	N-Methylpurine DNA Glycosylase				
MRE11A	Meiotic Recombination 11 Homolog, Double Strand Break Repair Nuclease				
MSH2	MutS Homolog 2			yes	yes
MSH3	MutS Homolog 3			yes	yes
MSH6	MutS Homolog 6				
MUS81	SLX3 Structure-Specific Endonuclease Subunit Homolog				
MUTYH	MutY DNA Glycosylase homolog				

NBN	Nijmegen Breakage Syndrome 1				
NEIL1	Nei Like DNA Glycosylase 1			yes	yes
NEIL2	Nei Like DNA Glycosylase 2			yes	yes
NEIL3	Nei Like DNA Glycosylase 3	yes	yes		
NHEJ1	Non-Homologous End Joining Factor 1				
NSMCE2	Non-Structural Maintenance Of Chromosomes Element 2 Homolog				
NUDT1	Nudix Hydrolase 1	yes	yes		
OGG1	8-Oxoguanine DNA Glycosylase				
PALB2	Partner And Localizer Of BRCA2				
PARG	Poly(ADP-Ribose) Glycohydrolase			yes	yes
PARP1	Poly(ADP-Ribose) Polymerase 1				
PCNA	Proliferating Cell Nuclear Antigen	yes	yes		
PLK1	Polo Like Kinase 1	yes	yes		
PMS1	PMS1 Homolog 1, Mismatch Repair System Component				
PMS2	PMS1 Homolog 2, Mismatch Repair System Component			yes	yes
POLB	DNA Polymerase Beta			yes	yes
POLD1	DNA Polymerase Delta 1, Catalytic Subunit	yes	yes		
POLH	DNA Polymerase Eta				
POLK	DNA Polymerase Kappa				
POLL	DNA Polymerase Lambda			yes	yes
POLM	DNA Polymerase Mu		yes		
POLQ	DNA Polymerase Theta	yes	yes		
PRIM1	Primase (DNA) Subunit 1				
PRKDC	Protein Kinase, DNA-Activated, Catalytic Polypeptide				
PSMD14	Proteasome 26S Subunit, Non-ATPase 14				
PTEN	Phosphatase And Tensin Homolog			yes	yes
PTTG1	Pituitary Tumor-Transforming 1	yes	yes		
RAD1	Rad1-Like DNA Damage Checkpoint Protein				
RAD17	RAD17 Checkpoint Clamp Loader Component				
RAD18	RAD18, E3 Ubiquitin Protein Ligase	yes	yes		
RAD23A	RAD23 Homolog A, Nucleotide Excision Repair Protein				
RAD23B	RAD23 Homolog B, Nucleotide Excision Repair Protein				
RAD50	RAD50 Double Strand Break Repair Protein			yes	yes
RAD51	RAD51 Recombinase	yes	yes		
RAD52	RAD52 Homolog, DNA Repair Protein				
RAD54B	RAD54 Homolog B				
RAD54L	RAD54-Like	yes	yes		
RAD9A	RAD9 Checkpoint Clamp Component A				
RBM14	RNA Binding Motif Protein 14				
RDM1	RAD52 Motif Containing 1				
RECQL	RecQ Like Helicase	yes	yes		
RECQL4	RecQ Like Helicase 4				
RECQL5	RecQ Like Helicase 5				
REV1	REV1, DNA Directed Polymerase				
REV3L	REV3 Like, DNA Directed Polymerase Zeta Catalytic Subunit				
RFC1	Replication Factor C Subunit 1				
RFC2	Replication Factor C Subunit 2	yes			
RFC3	Replication Factor C Subunit 3				
RFC4	Replication Factor C Subunit 4	yes	yes		
RNF168	Ring Finger Protein 168				
RNF4	Ring Finger Protein 4				
RNF8	Ring Finger Protein 8, E3 Ubiquitin Protein Ligase			yes	yes
RPA1	Replication Protein A1				
RPA2	Replication Protein A2				
RPA3	Replication Protein A3				
RRM1	Ribonucleotide Reductase Catalytic Subunit M1				
SEM1	SEM1, 26S Proteasome Complex Subunit				
SMC3	Structural Maintenance Of Chromosomes 3				
SMUG1	Single-Strand-Selective Monofunctional Uracil-DNA Glycosylase 1				
TERT	Telomerase Reverse Transcriptase			yes	
TOP2A	Topoisomerase (DNA) II Alpha	yes	yes		
TP53BP1	Tumor Protein P53 Binding Protein 1			yes	yes
TUBG1	Tubulin Gamma 1				
UBE2I	Ubiquitin Conjugating Enzyme E2 I				
UBE2T	Ubiquitin Conjugating Enzyme E2 T				
UBE3B	Ubiquitin Protein Ligase E3B			yes	yes
UIMC1	Ubiquitin Interaction Motif Containing 1				
UNG	Uracil DNA Glycosylase				
USP1	Ubiquitin Specific Peptidase 1				
USP47	Ubiquitin Specific Peptidase 47				
XPA	Xeroderma Pigmentosum Group A-Complementing Protein			yes	
XPC	Xeroderma Pigmentosum, Complementation Group C				
XRCC1	X-Ray Repair Cross Complementing 1				
XRCC2	X-Ray Repair Cross Complementing 2	yes	yes		
XRCC3	X-Ray Repair Cross Complementing 3				
XRCC4	X-Ray Repair Cross Complementing 4				
XRCC5	X-Ray Repair Cross Complementing 5				
XRCC6	X-Ray Repair Cross Complementing 6				
XRCC6BP1	XRCC6-Binding Protein 1				

Supplementary Table S2. Univariate Cox regression analysis of factors influencing PFS and OS in the Köln patient cohort.

Variable	Category	p-value	
		PFS	**OS**
Age	Continuous	0.612	0.457
Gender	Male Female	0.435	0.255
KPS	Continous	0.587	0.169
MGMT promoter status	Un-methylated Methylated	0.527	0.918
Groups (signature specific)	G1 G3	**0.019**	0.132

KPS, Karnofsky performance score; PFS, progression free survival; OS, overall survival

CHAPTER 5

RESULTS

Identification of a novel DNA repair target in GBM: Impact of NEIL3 depletion on telomere homeostasis and resistance to TMZ

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In preparation

RATIONALE

The results described in this section are based on the identification of key DNA repair and cell cycle factors significantly deregulated in GBM biopsies. Specifically, we uncovered the dysregulation of all three members of the NEIL glycosylase family (NEIL1-3), with NEIL3 being highly upregulated whereas NEIL1 and NEIL2 were downregulated. Based on these observations and the fact that NEIL3 has been shown to have a role in the repair of oxidized DNA damage at the telomeres but had hitherto not been described in the context of GBM, NEIL3 was chosen as candidate gene for further characterization *in vitro*. Our results indicate that shRNA-mediated depletion of NEIL3 sensitizes GBM cells to DNA damaging agents (TMZ and tert-butyl hydroxyperoxide (TBHP)). Importantly, loss of NEIL3 resulted in telomere erosion, the downregulation of the shelterin component TRF1 and the deregulation of long non-coding telomeric repeat-containing RNAs (TERRAs). Finally, NEIL3-depletion resulted in an increase in telomere dysfunction induced foci (TIFs), indicating that the DNA damage response was activated at telomeres upon loss of NEIL3. We propose that NEIL3 may represent an attractive therapeutic target for improving GBM treatment. The data is presented in the form of a manuscript.

Personal contributions: The analysis of the deregulated gene lists generated from our gene expression analysis and the selection of the candidate gene was done in great part by me. Moreover, I sat up all the experiments and produced, as well as analysed, the majority of the data presented in the manuscript. For this project, I was helped by a Master student (8-month traineeship) which was under my direct supervision. Finally, I was actively involved in the redaction of the manuscript.

INTRODUCTION

Glioblastoma (GBM) is the most frequent, aggressive and lethal tumor of the central nervous system. Despite a treatment modality composed of maximal surgical resection followed by ionizing radiation (IR) and chemotherapy with the alkylating agent temozolomide (TMZ) (1), newly diagnosed patients face inevitable tumor relapse driven by DNA repair-mediated chemoresistance mechanisms and poor prognosis. In addition to methyl base damage, single stranded DNA breaks (SSBs) and double stranded DNA breaks (DSBs), IR and TMZ induce oxidative DNA damage (2), thus exacerbating the damage load associated with replication stress in rapidly growing tumor cells and contributing to the constitutive activation of the DNA damage response (DDR) observed in GBM cells (3, 4).

Telomeres, the physical ends of linear eukaryotic chromosomes, are constituted of repetitive, non-coding TTAGGG sequences bound by a protein complex named shelterin formed of TRF1, TRF2, POT1, TPP1, TIN2 and RAP1 (5). This complex protects chromosome ends from DNA degradation and from being recognized as DNA ends by the DSB repair machinery, thus preventing chromosomal end-to-end fusions. Telomeres also act as disposable DNA buffers, which are truncated after each cell division, to circumvent the end replication problem (6), thereby protecting upstream protein-coding regions. Responsible for telomere elongation and the maintenance of proper telomere length is a nucleoprotein reverse transcriptase called telomerase, composed of a catalytic subunit (TERT) and a RNA component (TERC) which serves as template for the synthesis of TTAGGG repeats (7, 8). Active in normal stem cells, telomerase undergoes strong negative regulation in all other cell types (9, 10). However, the activation of a telomere maintenance mechanism (TMM) through telomerase reactivation or alternative lengthening of telomeres (ALT) is a crucial step to

ensure telomere homeostasis and enable limitless replicative potential during tumorigenesis (11). In GBM tumors specifically, deregulation of telomere-associated parameters such as activating TERT promoter mutations (12) or the occurrence of ALT (13), have been shown to predict patient prognosis (14, 15) and have gained interest as potential therapeutic targets (16). Ensuring telomere homeostasis by protecting against DNA lesions has evolved as a critical task in cancer cells. Telomeres in particular are notably prone to oxidative stress (17, 18) and represent preferential sites of persistent DNA damage (19). Given the reactivity of guanines towards oxidative stress, the richness in guanine of telomeres and their potential to assemble into G-quadruplex structures that modify the pathway of guanine oxidation compared to duplex DNA (20), protection is particularly required against oxidative guanine damage. Thus, 8-oxoG hinders telomerase activity (21) and disrupts the shelterin complex (22). Although not frequently observed, misincorporation of A opposite 8-oxoG can occur if left unrepaired which leads to mutagenesis in the telomeric sequence after replication (23). Likewise, oxidized dNTPs represent a serious threat since insertion of 8-oxoGTP opposite A during replication or by telomerase during telomere elongation (21) can generate GTAGGG and TGAGGG sequence variants (23), which perturb the binding of the shelterin complex to the telomeres. Besides, studies in budding yeast have shown that telomere sequence mutations, introduced via nucleotide substitutions in the RNA template of telomerase, led to telomere shortening progressing to cellular senescence (24) or unsegregated chromosomes due to delayed sister chromatid separation (25). Further oxidation of 8-oxoG to guanidinohydantoin (Gh) and spiroiminohydantoin (Sh) (26, 27) has also been shown to cause base mispairing mutagenesis (28).

Base excision repair (BER) is the predominant pathway for the repair of oxidative DNA base damage (29). In human cells, five DNA glycosylases (OGG1, NTH1, NEIL1, NEIL2 and NEIL3) initiate BER via excision of the damaged base (30, 31). OGG1 and NTH1 remove specific oxidative base lesions including 8-oxoG and thymine glycol (Tg) from duplex DNA, respectively (32, 33), while the NEIL glycosylase family, composed of NEIL1, NEIL2 and NEIL3, recognizes a much wider array of substrates present in different DNA contexts (34). Compared to its family members, NEIL3 presents unique structural features such as a long intrinsically disordered C-terminal domain (35, 36) and exhibits a preference for oxidized purines present in the telomeric sequence (37). Notably, the C-terminal domain of NEIL3 mediates its direct recruitment to telomeres by the shelterin subunit TRF1 during S-phase. This association with telomeres, which increases upon oxidative stress, allows the mobilization of downstream BER components such as the AP endonuclease APE1, PCNA and FEN1, and subsequent repair of telomeric DNA (38). In the same study, siRNA-mediated knockdown of NEIL3 in colon carcinoma cells (HCT116) induced a 2-fold increase in telomere loss and sister chromatid fusions as well as extra telomeric signals, further implying a direct role of NEIL3 in telomere homeostasis. Intriguingly, recent studies have unveiled a role for NEIL3, independent of other BER components, in the excision and unhooking of both psoralen and AP site-induced DNA cross-links (39, 40). In addition, the evidence also suggests that NEIL glycosylases may have gene regulatory functions, including at the epigenetic level through active DNA demethylation (41, 42). Although the *in vivo* function of NEIL3 remains unclear, it has been shown to regulate proliferation of neural stem cells (43-45), in line with the observation that mouse Neil3 mRNA expression levels were particularly high at embryonic days E12-13, at the onset of neurogenesis, and the observation that during embryogenesis and in newborn mice, Neil3

expression was specifically apparent in brain areas such as the subventricular zone, known to host neural stem and progenitor cells (46). In addition, NEIL3 mRNA levels were found to be higher in a panel of 18 tumor tissues (gliomas were not included) compared to their normal counterparts (46). Notably, analysis of the Cancer Genome atlas (TCGA) database revealed the upregulation of NEIL3 and concomitant downregulation of NEIL1 and NEIL2 in a subset of 13 different carcinomas where these deregulations were associated with an increased somatic mutation load (47). Interestingly, NEIL3 knockdown was shown to reduce cell proliferation and increase sensitivity to the oxidative toxicant paraquat and the DNA crosslinking agent cisplatin in mouse embryonic fibroblasts (48) and to ATR inhibitors in GBM cells (49). Although the available evidence pinpoints NEIL3 as a potential target in cancer cells, its role in telomere homeostasis, specifically in relation to glioma development and chemoresistance, remains vastly unexplored.

In a previous analysis of DNA repair and cell cycle gene expression in clinical GBM biopsies, we identified NEIL3 as being highly upregulated compared to non-tumor, adjacent tissue (50). Intriguingly, both NEIL1 and NEIL2 were downregulated in these tumor samples compared to controls, corroborating the findings of Shinmura et al (47) for other cancer types (carcinomas) in the TCGA dataset. Our observations prompted us to characterize the function of NEIL3 in telomere dynamics and response to TMZ in GBM cells. Our results indicate that NEIL3 inhibition sensitized GBM cells to DNA damaging agents and interfered with telomere stability. Ultimately, these observations suggest that NEIL3 could present a novel therapeutic opportunity for GBM patients.

MATERIALS & METHODS

Targeted gene expression analysis

Analysis of DNA repair and cell cycle genes was conducted on a paired (primary and recurrent tumor from the same patient) GBM biopsy cohort. Differentially regulated genes were assessed by comparison of primary and recurrent patient biopsies to control, tumor adjacent samples. The study cohort and analysis are described in Gobin et al (50) and the complete gene expression data can be accessed under accession number E-MTAB-6425 (ArrayExpress).

Cell culture and shRNA-mediated gene knockdown

The adherent U87 glioblastoma and U2OS osteosarcoma cell lines were cultured in DMEM serum-containing medium (DMEM High Glucose (Biowest, L0106-500), 10% FBS (Gibco, 10500-064), 4mM Ultraglutamine (Lonza, BE17-605E/U1), 100U Pen-Strep (Lonza, De17-603E)). The NCH644 spheroid glioblastoma stem-like cell line was cultured in NSC non-serum containing medium (Neurobasal medium (Life technologies, 21103049), 2% B-27 (ThermoFisher, 123587-010), 1U/mL Heparin (Sigma-Aldrich, H3149-25KU), 4mM Ultraglutamine (Lonza, BE17-605E/U1), 100U Pen-Strep (Lonza, De17-603E), 20ng/mL EGF (Provitro, 1325950500), 20ng/mL FGF (Miltenyi, 130-093-841)). Cells were subcultured every 48-72h or when they reached 75% confluence.

NEIL3 knockdown was achieved by shRNA-mediated RNA interference using 2 individual sequences: shNEIL3-2 (Dharmacon, V2LHS_156511) and shNEIL3-3 (Dharmacon, V3LHS_327509)). The transduced cells were kept under selective pressure with 1µg of puromycin (pGIPZ-based shRNA plasmid).

Proliferation assay

On Day 0, $6x10^5$ NCH644 shControl or shNEIL3 cells were seeded in 5mL of medium in a T25 flask. After 5 days, cells were harvested and counted before being reseeded in fresh medium ($6x10^5$ cells, same amount as Day 0). This was done for 5 consecutive times for a total of 25 days. Each time, cells were counted and at the end, the cumulative population doublings were calculated using: PD(t) = PD(t-1)+(log(Nt/N(T-1)) / log(2), where N(t) is the number of cells counted at time (t) and N(t-1) the number of cells counted at time (t-1). Triplicate results were plotted against time in order to generate proliferation curves.

Real-time quantitative PCR

Total mRNA extracted by TRIzol was retrotranscribed using the iScript cDNA synthesis kit (Biorad, 1708890) in a reaction composed of 1µg of RNA, 1x iScript mastermix (kit) and 1µL of iScript RT enzyme (kit). The sample was then incubated in a thermocycler using the following protocol: 5min at 25°C, 20min at 46°C, 1min at 95°C. Finally, the cDNA samples were diluted to a total volume of 100µL before quantitative PCR in a reaction (final volume 5µL) consisting of 1x FASTSybr Green mix, 1µL of cDNA (from total RNA RT or TERRA specific RT) and 250nM of gene specific forward and reverse primer. All the reactions were done in triplicate in a VIIA7 Real-time PCR system (Applied Biosystems) using the FastSybr protocol. Relative expression values were calculated using the 2^{-ddCT} equation and a detailed description of the primers used is provided in Supplementary Table S1.

Telomere Restriction Fragment (TRF)

The classical TRF southern blot method followed by non-radioactive labelling using the TeloTAGGG kit (Sigma, 12209136001) was used to assess average telomere length. First,

genomic DNA was extracted from a total of $5x10^6$ cells using the DNeasy Blood & tissue kit (Qiagen, 69504) and digested with HinfI and RsaI. The fragments were separated on a 0.8% agarose gel electrophoresis for 6h at 75V followed by 10min of depurination (0.25M HCl), 2x15min of alkaline denaturation (0.5M NaOH, 1.5M NaCl) and 2x15min of neutralization (0.5M Tris-HCl, 3M NaCl, pH 7.5) before transfer by capillarity on a Hybond N nitrocellulose membrane (Amersham, RPN2020N). The stack was composed of a large whatman paper, gel, membrane, 2 whatman papers, topped by a pile of absorbent paper and weights to facilitate uniform transfer. After overnight transfer, the DNA was fixed to the membrane by UV crosslinking at 120mJ for 2min (UVP CL-1000L) and placed in a hybridization tube. The membrane was pre-hybridized in DIG Easy Hyb (kit) for 30min at 42°C before hybridization with the telomere probe (1:5000 dilution in DIG Easy Hyb) for 3h at 42°C in a hybridization oven under constant rotation (rotisserie). Several washes of 10min were performed with stringent wash buffer 1 (SSC 2x, 0.1% SDS), stringent wash buffer 2 (SSC 0.2x, 0.1% SDS) and wash buffer 1x (kit). The membrane was then incubated in freshly prepared blocking buffer (1x maleic acid (kit), 1x blocking buffer (kit)) for 30min at RT before being subjected to anti-DIG working solution (1:10000 anti-DIG AP antibody diluted in blocking buffer) for 30min at RT under rotation (tube roller). After a last washing step with wash buffer 1x for 2x15min at RT, the membrane was incubated in detection buffer 1x (kit) for 5min before revelation of the signal with 2mL of substrate solution (kit). The chemiluminescent signal was collected using an imaging device (GE Healthcare, Imagequant LAS 4000) and the resulting image was analysed to estimate the telomere length.

lncRNA TERRA expression

The telomeric transcripts TERRA were quantified using an adapted protocol described in Feretzaki and Lingner (51). First, column extracted RNA (free of gDNA contamination) from three biological replicates was retrotranscribed using the SuperScript III Reverse Transcriptase kit (Invitrogen, 18080044) in two separate reactions representing the housekeeping genes (GAPDH and GUSB) and TERRAs, respectively. The samples were first denatured for 5min at 65^{o}C in a reaction containing 2pmol of gene specific oligonucleotides (GAPHD, GCCCAATACGACCAAATCC; GUSB, AATACAGATAGGCAGGGCGTTCG; TERRA, CCCTAACCCTAACCCTAACCCTAACCCTAA), 0.5mM dNTPs mix (Invitrogen, 18427-013) and either 1.5µg (housekeeping genes) or 3µg (TERRAs) of RNA. The reaction volume contained 1x First strand buffer (kit), 5mM DTT (kit), 20U RNase inhibitor (kit) and 200U SuperScript III RT (kit) and the extension step was carried out at 55^{o}C for 60min followed by 70^{o}C for 15min in a PCR thermocycler. Finally, the cDNA was diluted to a final volume of 40µL before quantification by classical qPCR in a reaction (final volume of 5µL) containing 1x FASTSybr Green mix (Applied Biosystems, 4385612), 1µL of cDNA (housekeeping genes or TERRA) and 50nM of specific forward and reverse primer. All the reactions were done in triplicate in a VIIA7 Real-time PCR system (Applied Biosystems) using the FastSybr protocol and the relative expression was calculated using the 2^{-ddCT} method. The primer pairs used for quantification are detailed in Supplementary Table S1.

Real-time Quantitative Telomeric repeat amplification protocol (qTRAP)

The protocol was adapted from Hou et al (52). Briefly, total proteins from 5x10^{5} cells were extracted in non-denaturing conditions using CHAPS buffer (5mg/mL CHAPS, 1mM $MgCl_2$, 1mM EGTA, 10% glycerol, 5mM Tris-HCl pH 7.5, 1 tablet/10mL phosphatase (Roche, 04906837001) and protease inhibitor (Roche, 04693132001), 1µL/mL RNase inhibitor

(Invitrogen, 10777019)) and telomerase activity was measured in triplicates using various amounts protein concentrations (range: from 0.01 to 1µg) in a qPCR reaction (final volume of 10µL) containing 1x Fast SYBR Green Mastermix (Applied Biosystems, 4385612), 625µM dNTP mix, 800nM TS primer (AATCCGTCGAGCAGAGTT), 250nM ACX primer (GCGCGGCTTACCCTTACCCTTACCCTAACC). The samples were first incubated for 20min at 25°C to allow elongation of the TS primer by telomerase before standard qPCR in a VIIA7 qPCR system using the FastSybr protocol. The Ct values were then plotted for each protein concentration and telomerase activity was visualized by linear regression curve fitting.

Spheroid formation assay

A total of $1.5x10^5$ cells were collected and run through a BD FACS Aria cell sorter. First, cell doublets and dead cells were filtered out and 1, 5 or 10 cells were then seeded automatically in individual wells containing 100µL of culture medium (NSC). The plates were incubated at 37°C for 3 weeks until first visible spheroids could be seen. At this stage, the spheroid-containing wells in each plate were counted and an average % of spheroid formation capacity was calculated.

Unicolor FACS competition assay

The assay was based on Burgess et al (53). A 1:1 mix ratio of U87 GFP-negative (wild-type) and GFP-positive (harbouring shControl or shNEIL3) cells were plated under either control or 50µM TMZ treatment conditions. After 7 days of incubation at 37°C, cells were collected and counted by BD FACS Canto. Doublets and dead cells were left out prior to GFP-specific gating. A total of $2x10^5$ events were recorded for each condition and the % of GFP-negative and -positive population in each sample was assessed in triplicates.

Cytotoxicity/Viability assay

Sensitivity to temozolomide (TMZ) and tert-butyl hydroxyperoxide (TBHP) was assessed in adherent cells using the MTT Cell proliferation kit (Sigma, 11465007001) and in spheroid cells with the WST1 Cell proliferation reagent (Sigma, 11644807001). Either 1500 cells (U87, adherent) or 4000 cells/well (NCH644, spheroid) were seeded in triplicates in a 96-well plate (Greiner, 655101) followed by incubation with escalating drug concentrations for 24h, 48h or 72h at 37^{o}C in a total volume of 100µL. Adherent cells were then incubated with 10% (of total volume) of MTT reagent for 4h followed by solubilization overnight using 100µL of buffer (kit), whilst spheroid cells were incubated with 10% (of total volume) of WST1 reagent for 6h. Absorbance values (550nm: MTT; 450nm: WST and >630nm: reference wavelength) were determined using the Clariostar reader (BMG Labtech).

Telomere-specific fluorescent in situ hybridization (Telo-FISH)

A total of 750.000 cells were seeded and treated with 0.5µg/mL of colcemide (Karyomax, Gibco 15212012) for 24h before collection for preparation of metaphase spreads. The cell pellet was resuspended with 7mL of pre-warmed hypotonic solution (0.54% KCl) and incubated for 20min at 37^{o}C. The swollen cells were then fixed with 1mL of cold fixation buffer (3:1 mix of Ethanol 100% / acetic acid) and washed 2 times before being dropped onto a slide from a distance of 2m in order for the nuclei to blow up and spread the condensed chromosomes. The slides were then fixed with 4% PFA for 2min and treated with pepsin solution (0.001% pepsin, 100µM citric acid) before being fixed a second time with 4% PFA for 2min. The nuclei were carefully washed after each step with PBS 1x for 3x5min. The slides were then dehydrated in an ethanol sequence (70%, 90% and 100%) for 1min and air dried, before being subjected to 60µL of hybridization mix containing 50nmole of Cy3-PNA C-rich

telomere probe (Eurogentec, PN-TC050-005) diluted in blocking buffer (70% formamide, 5mM Tris pH 7.2, 10% blocking buffer (Roche, 11096176001)). The PNA probe and DNA was then denatured by placing the slide on a hot plate at 85^{o}C for 3min before 2h of incubation in the dark at RT. Lastly, the slide was washed for 2x15min with wash 1 (50% formamide, 10mM Tris pH 7.2, 0.1% BSA) followed by 2x5min of wash 2 (10mM Tris pH 7.2, 150mM NaCl, 1.6% Tween-20). Nuclei were counterstained with DAPI and visualized by confocal microscopy (Zeiss LSM 880) at 630x magnification.

Immunofluorescence-coupled fluorescent in situ hybridization (IF-FISH)

Cells mounted on a slide were first permeabilized in 0.5% TBS-Triton for 10min then fixed with 4% PFA for 10min at RT before being immunolabeled with γ-H2AX primary antibody (1:1000, Abcam ab22551) followed by Alexa 488 secondary antibody (1:500, Invitrogen A28180). The slides were then directly dehydrated for 1min in ethanol series (75%, 80% and 100%) followed by air drying before hybridization at RT for 2h with 50nmole of Cy3-PNA C-rich telomere probe (Eurogentec, PN-TC050-005) diluted in blocking buffer (70% formamide, 5mM Tris pH 7.2, 10% blocking buffer (Roche, 11096176001)). The slides were then washed for 2x15min with wash 1 (50% formamide, 10mM Tris pH 7.2, 0.1% BSA) followed by 2x5min of wash 2 (10mM Tris pH 7.2, 150mM NaCl, 1.6% Tween-20). Nuclei were counterstained with DAPI and visualized by confocal microscopy (Zeiss LSM 880) at 630x magnification.

RESULTS

Deregulation of the NEIL glycosylase family in patient-derived GBM biopsies

For this study, we took advantage of a targeted gene expression analysis encompassing 156 genes of the DNA repair and cell cycle pathways conducted on GBM patient-derived biopsies. The dataset was generated in the laboratory and is described in detail in (50). Briefly, we first identified DEGs by comparing primary or recurrent tumors with control, tumor-adjacent tissue. Seventy-two DEGs were identified for the comparison of primary tumors vs control samples (Supplementary Figure S1), and 71 for that of recurrences vs controls, respectively (data not shown). Notably, there was an extensive overlap between both lists, with 67 common genes displaying comparable fold-change directions and intensities. In the interest of finding potential novel targets for treatment strategies in newly diagnosed GBM, we focused on genes that were highly upregulated in the comparison of primary vs control tissue, hence suggesting a notable role in gliomagenesis and/or potential addiction in GBM cells. Intriguingly, we observed specific deregulation of the NEIL glycosylase family, with NEIL1 and NEIL2 being downregulated and NEIL3 highly upregulated (Supplementary Figure S1, genes highlighted in green). This observation was comforted by a study demonstrating reduced NEIL1 and NEIL2 expression whilst elevated NEIL3 expression levels in a subset of different cancer types (47). Despite NEIL3 being a well-characterized DNA glycosylase and known to be highly upregulated in different cancers, its function in tumorigenesis and specifically in GBM remains obscure.

RNA interference-mediated knockdown of NEIL3 in glioblastoma patient-derived cell lines

Since NEIL3 was found to be upregulated in GBM patient biopsies, we decided to examine the role of NEIL3 by gene knockdown in GBM patient derived cell lines. To this end, we had access to the well characterized U87 adherent cell line and the NCH644 patient-derived spheroid cell line. Notably, NCH644 presents GBM stem-like cell properties and is able to recapitulate essential features of the original tumor when xenografted into immunodeficient mice (54). Knockdown of NEIL3 in the two cell lines was achieved by stable lentiviral integration of vectors expressing NEIL3-targeting shRNAs. Two independent shRNA constructs were used: shNEIL3-2 and shNEIl3-3. Reduced NEIL3 expression at the mRNA (Fig. 1A) and protein level (Fig. 1B) was observed in NCH644. Similar reduction of mRNA levels were observed in U87 cells (data not shown). Notably, shNEIL3-3 showed a consistently higher knockdown efficiency than shNEIL3-2 in both cell lines. Furthermore, NEIL3 depletion had no apparent effect on cell proliferation (Fig. 1C) and spheroid formation capacity (Fig. 1D) in NCH644. In summary, we were able to efficiently reduce NEIL3 mRNA expression by RNA interference in both GBM cell lines (±80% in NCH644 and ±60% in U87, respectively) which in addition did not affect the stem-like cell properties of NCH644.

Reduced NEIL3 expression sensitizes MGMT-negative and MGMT-positive GBM cells to the methylating agent TMZ and oxidative stress inducer TBHP

Since NEIL3 was also found to be strongly upregulated in patient samples that underwent standard chemoradiation therapy (recurrent GBM specimen), we hypothesized a direct role of this DNA glycosylase in the resistance mechanism to TMZ and radio-therapy induced oxidative DNA damage. As stated in the Chapter 1, the expression of MGMT is a major determinant behind the resistance of GBM tumors to TMZ. Although U87 and NCH644 cells are clearly not isogenic, we thus took advantage of their different MGMT expression status

(U87: MGMT-negative; NCH644: MGMT-positive) to examine the effect of NEIL3 knockdown on treatment sensitivity in clinically relevant conditions.

First, we observed increased sensitivity of both U87 shNEIL3 cell lines to escalating TMZ doses as assessed by standard cell viability analysis (Fig. 2A). This observation was reinforced by a cell growth competition assay in which U87 cells (GFP-negative) were mixed together in a 1:1 ratio with either U87 shControl or shNEIL3 cells (GFP-positive due to the presence of the GFP gene on the pGIPZ lentiviral vector expressing the shRNAs). The cell mixture was then treated or not with TMZ and after several days, the two populations were counted and discriminated by GFP fluorescence in a flow cytometer. In the non-treated condition, the population ratio remained unchanged in all the considered cell line combinations (Fig. 2B). In contrast, we observed a significant decrease of both shNEIL3 GFP-positive populations when TMZ was added (Fig. 2B).

In order to analyse the impact of NEIL3 depletion in MGMT-positive cells, we also subjected NCH644 to TMZ (48h) and TBHP (24h), a potent oxidative stress inducer in order to simulate oxidative DNA damage. In both cases, a significant increase in sensitivity was observed in the NCH644 shNEIL3-3 cell line (Fig. 2C and D). Although not significant, a similar trend could be seen in shNEIL3-2 cells. The differences in sensitivity caused by the 2 shRNAs targeting NEIL3 is currently unknown. Notably, TBHP seemed to have a greater effect on cell survival than TMZ. This could be due to the fact that TBHP induces overall oxidative stress compared to TMZ, which mainly methylates DNA. Despite a significant difference, the impact of NEIL3 depletion on TMZ or TBHP sensitivity was relatively limited in NCH644 and U87 shNEIL3 cell lines.

Loss of NEIL3 impacts telomere length homeostasis and induces telomere dysfunction foci

Given the documented role of NEIL3 in removing telomeric DNA damage (55), we set out to analyse its role in telomere biology in GBM cells.

To this end, we evaluated the average telomere length in both GBM cell lines using the TRF southern blot approach. NCH644 cells were found to be telomerase-positive, as illustrated by the size range (<15 kb) and homogeneous mean telomere length distribution observed by TRF analysis (Fig. 3A) (56). Surprisingly, we observed that NCH644 depleted cells presented significant reduced mean telomere length (Fig. 3A) compared to the control cell line. The reduction in length reached ~20% and ~25% in shNEIL3-2 and shNEIL3-3 cells, respectively (Fig. 3B). Notably, this telomere erosion phenotype was visible as early as 7 cell passages after introduction of the shNEIL3 plasmids and further passaging of the cells did not increase the extent of erosion nor reverse telomere length, suggesting the existence in NCH644 cells of a mechanism capable of maintaining the telomeres at a viable length but unable to restore their original size. In contrast to NCH644, no erosion was detected upon depletion of NEIL3 in U87 (Supplementary Fig. S2A). This observation could be explained by the fact that U87 displays shorter telomeres compared to NCH644 (3.5 vs 6.5kB, respectively, Fig 3A and Supplementary Fig. S2A) which could hinder an observable impact on telomere length in these cells. We also analysed the effect of TMZ and TBHP treatment on NCH644 and U87 telomeres. When applied for 72h, the genotoxic treatment did not exacerbate the telomere length deficit observed in NCH644 and did not induce any telomere loss in U87 (Supplementary Fig. S2B). Considering that the telomere deficit phenotype was exclusive to NCH644, we decided to focus our efforts on this cell line.

Telomere dysfunction (e.g., associated with telomere shortening and/or the dissociation of telomere-binding proteins) was shown to induce a DNA damage response in

mammalian cells, characterized by the presence of telomere dysfunction-induced foci (TIFs) containing telomere-associated DNA damage factors (57). Furthermore, severe loss of telomere integrity can also trigger DSB repair mechanisms involving NHEJ, resulting in telomere fusions and chromosomal instability. Therefore, we analysed the co-localization of telomeres (using a telomeric probe FISH) and γ-H2AX (by IF) in control and NEIL3-depleted NCH644 cells. As presented in Figure 3C, TIFs were almost exclusively observed in the NEIL3-depleted cells (white arrows). Indeed quantification indicated a 5-7 fold increase in the number of TIFs in NEIL3-depleted cells compared to control (Fig. 3D).

Finally, to examine whether TIFs were associated with increased genetic instability in NEIL3-depleted cells, we examined metaphase spreads from control and NEIL3-depleted cells. Although careful analysis of several metaphase spreads remains to be done, our preliminary data indicate the increased occurrence of telomere abnormalities in NEIL3-depleted cells, in particular extra-chromosomal signals and loss of telomere signal (Fig. 3E). The overall telomere signal intensity was also more heterogeneous after NEIL3 depletion. However, we did not detect chromosome end-to-end fusions in our NEIL3-depleted cell lines, suggesting that telomere uncapping associated with loss of NEIL3 did not lead to dramatic rearrangements mediated by NHEJ. Moreover, the lack of deleterious telomere abnormalities (i.e. fusions) could explain why NEIL3 depletion did not impact the proliferation of NCH644.

Knockdown of NEIL3 disrupts telomere stability and alters the expression of the lncRNAs TERRA

The observation that NEIL3 depletion induced such drastic telomere length attrition in NCH644 cells prompted us to analyze the integrity of the telomerase and shelterin complex. To this end, we quantified the expression of key components of the shelterin complex (TERF1,

TERF2, POT1 and TINF2) and the telomerase catalytic subunit hTERT by RT-qPCR in control and NEIL3-depleted cells. Interestingly, we observed significant upregulation of the transcripts encoding the enzymatic subunit of telomerase (hTERT) and the shelterin subunits POT1 and TIN2 (Fig. 4A). On the other hand, TRF2 was unaltered in NEIL3-depleted cells whereas TRF1 mRNA levels were significantly downregulated (Fig. 4A), and this observation was confirmed at the protein level (Fig. 4B and Supplementary Fig. S3).

These observations suggest that NEIL3 depletion affects the expression of components of the shelterin complex, leading in particular to the downregulation of TRF1. We put distinct emphasis on TRF1 in view of a recent study demonstrating that recruitment of NEIL3 to the telomeres was associated to direct protein-protein interaction with TRF1 (38). Moreover, upregulation of both TIN2 and POT1 further imply the hypothesis that NEIL3 depletion-mediated downregulation of TRF1 affects the stability of the shelterin complex. In addition, these results suggest that telomere erosion is potentially stabilized by upregulation of the telomerase catalytic subunit.

As NCH644 cells are telomerase-positive and since hTERT mRNA levels were upregulated in NEIL3-depleted cells, we next tested the possibility that the initial telomere erosion observed in these cells is counterbalanced by enhanced telomerase activity. For this reason, we determined telomerase activity in cell extracts prepared from control and NEIL3-depleted cells using the qTRAP assay. A control protein extract from U2OS cells (a telomerase negative ALT cell line) was added to each experiment in order to determine the background activity obtained in the absence of telomerase. As presented in Fig. 4C, telomerase activity is detected in NCH644 cell extracts, confirming that these cells rely on telomerase activity for elongation of their telomeres. Strikingly, we did not observe any difference in telomerase

activity in NEIL3-depleted cells when compared to control cells (Fig. 4C), indicating that the resistance against further telomere erosion observed after a few passages following loss of NEIL3 is not mediated by increased activation of telomerase.

Long non-coding, chromosome-specific telomeric RNA sequences named TERRA play a crucial role in telomere length sensing and regulation (58). Particularly, upon telomere shortening, TERRA molecules were shown to accumulate into nuclear foci which promoted nucleation of telomerase on short telomeres, thus forming TERRA-telomerase clusters which promoted telomere elongation (59). In order to analyse if these lncRNAs were affected by NEIL3 depletion, we quantified the expression of TERRAs originating from specific chromosome ends (2q, 10q, 13q 15q, 17p and XpYp) by RT-qPCR (Fig. 4D). Compared to control cells, we observed significant upregulation (±50%) of 2q- and 10q-TERRA and downregulation of 13q- and 17p-TERRA (±50% and ±70%, respectively) in both NEIL3 depleted cell lines (Fig. 4D). These observations could indicate a possible mechanism by which NCH644 shNEIL3 cells limit telomere dysfunction.

All these observations demonstrate that NEIL3 depletion gives rise to dysfunctional telomeres by affecting the shelterin complex and that telomerase activity in these cells is possibly sufficient to counteract NEIL3-mediated telomere shortening. Lastly, the altered expression levels of chromosome-specific TERRAs are further indications of the telomeric dysfunction caused by NEIL3 knockdown.

DISCUSSION

The safeguarding of telomere integrity and length homeostasis is a critical factor in rapidly dividing cells (i.e. tumor cells). Besides the protection of the chromosome ends by the shelterin complex, cells have to maintain intact telomeric repeats which are otherwise prone to undergo oxidative DNA damage. The repair of such insults is predominantly achieved by BER with specific DNA glycosylases recognizing and removing distinct lesions present at the telomeres.

Compared to its family members, NEIL3 was found to be highly upregulated in a DNA repair and cell cycle targeted gene expression analysis comparing primary and recurrent GBM biopsies to control, tumor adjacent tissue. This first observation was corroborated by a recent *in silico* study analysing the expression of the three NEIL family members in different types of carcinomas from the TCGA (47), indicating a peculiar role of this enzyme in GBM. In this study, we focused on the role of the NEIL3 DNA glycosylase in GBM cells by examining the phenotypes associated with RNAi-mediated depletion of NEIL3.

Notably, knockdown of NEIL3 in the telomerase-positive NCH644 cell line induced telomere shortening and the deregulation of key factors of the shelterin complex and TERRAs. Several DNA glycosylases involved in the repair of oxidative lesions by BER have been shown to preserve telomere integrity, including OGG1 (60) and NTHL1 (61). However, disruption of Ogg1 in yeast and mice led to telomere elongation (60), whereas Nth1 deficiency in mice resulted in increased telomere fragility due to defective repair of oxidative base lesions and telomere shortening (61). These observations suggest that the various DNA glycosylases that operate at telomeres exert specific functions that impact telomere integrity differently.

Moreover, in a recent study, NEIL3-deficient HCT116 cells presented increased telomere dysfunction and pinpointed TRF1 as the primary factor recruiting NEIL3 to the telomeres (38). Intriguingly, we observed specific downregulation of TRF1 both at the mRNA and protein level in NEIL3 depleted cells (NCH644), while the expression of other shelterin factors were found to be upregulated (TIN2 and POT1) or unchanged (TRF2). However, whether the deregulation of TRF1 is associated with its dissociation from telomeres remains to be determined. Nevertheless, in view of these observations, it is tempting to suggest an increase in oxidative DNA damage at the telomeres, as a result of NEIL3 loss, which in turn possibly disturbs the binding of TRF1 on the telomeric sequence. Since TRF1 directly binds the telomeric sequence to promote the recruitment of the other members of the shelterin complex, loss of TRF1 would impact proper assembly of the complex at the chromosome ends, leading to telomere de-protection and erosion, as suggested by the 5-7 fold increase in telomere dysfunction-induced foci (TIFs) observed in these cells. In addition, a recent study in GBM, demonstrated that genetic or chemical depletion of TRF1 led to increased DNA damage located at the telomeres as well as impaired tumor initiation and progression in xenografted mice (62). Thus, it will be crucial to verify that the decreased TRF1 levels are indeed associated with its loss at telomeres, by specific experiments such as ChIP analysis looking at telomere occupancy by TRF1 in NEIL3 depleted cells. In line with our observation that both TIN2 and POT1 were upregulated in shNEIL3 cells, it was shown that TRF1 loss from telomeres increased the association of TIN2 with TRF2 (63) and overexpression of POT1 was demonstrated to protect against loss of telomeric ssDNA in TRF2 negative cells (64). This further implies that TRF1 downregulation could be associated with loss of its binding at the telomeres and that the upregulation of the TIN2/POT1 shelterin factors potentially helps

445 stabilizing the dysfunctional telomeres. Again it will be important to examine the association
446 of TRF2 and POT1 at telomeres in NEIL3-depleted cells.

447 The erosion of telomeres observed upon loss of NEIL3 seemed to reach a stable limit,
448 suggesting that mechanisms are at hand to prevent further erosion and potentially
449 deleterious effects on chromosomal stability. Telomerase is the primary responsible for
450 telomere lengthening in cells and we observed that TERT mRNA levels were upregulated in
451 these cells, suggesting the possibility that increased telomerase activity counterbalances the
452 dysfunction of telomeres in the absence of NEIL3. However, our data indicate that the activity
453 of the telomerase is not affected by NEIL3 depletion, suggesting that the level of telomerase
454 activity in these cells is sufficient to counter telomere erosion when telomere length falls
455 below a certain threshold, or that other mechanisms contribute to prevent further telomere
456 length deficit. It is notable that in a telomerase null background, mouse $Nth1^{-/-}$ bone marrow
457 cells underwent severe telomere loss at some chromosome ends, indicating that NTHL1 and
458 TERT cooperate in maintaining telomere function in replicating cells (61). On the other hand,
459 the expression of TERT has been shown to increase resistance to chemotherapeutics and pro-
460 apoptotic signals (65), modulate chromatin structure and response to DNA damage (66), and
461 enhance the DNA repair capacities in TERT overexpressing fibroblasts (67), indicating several
462 telomere-independent functions of telomerase. It would therefore be interesting to examine
463 whether telomere loss is exacerbated in GBM cells carrying the double depletion of TERT and
464 NEIL3, as well as in ALT-positive cells carrying the NEIL3 depletion.

465 The expression levels of the lncRNAs TERRA were deregulated upon loss of NEIL3, with
466 up- or down-regulation observed for specific telomeres. A role for TERRA in telomere
467 regulation has been proposed and it has been demonstrated that telomere shortening

induces TERRA expression (68). Because TERRAs, like the shelterin complex, have also been shown to help recruit telomerase enzymes to short telomeres (69), it is possible that the upregulation of TERRAs facilitates its recruitment, hence preventing further telomere erosion in NCH644 shNEIL3 cells. It remains to be seen whether the upregulation of TERRA seen at specific telomeres reflects an exacerbated erosion of these telomeres. Importantly, TERRAs have also been shown to recruit chromatin remodelling factors to the telomeres (70), suggesting another mechanism whereby telomere erosion might be counterbalanced in NEIL3-depleted cells. Finally, as downregulation of TERRA was also observed (in 13p and 17q), it is possible that certain telomeres are long enough and hence do not need high TERRA expression levels. Knowledge of the specific length of all telomeres in NCH644 cells and how each telomere is affected by NEIL3 depletion will be required to test this hypothesis. Finally, as ALT represents an important telomere maintenance mechanism in GBM cells, particularly in pediatric GBM, it will be crucial to extend these studies and examine the impact of depleting NEIL3 on the mechanisms that operate to maintain ALT in GBM cells.

Interestingly, NEIL3 knockdown also sensitized both U87 and NCH644 cell lines, which are MGMT promoter methylation negative and positive cells respectively, to TMZ and TBHP. Of note, NEIL3 was uncovered independently during an in vitro shRNA screen targeting the DDR by Hélène Erasimus, a previous PhD student in the lab, as a novel candidate target for the sensitization of NCH644 cells to TMZ. Although the telomere length deficit was not observed in U87 cells, probably as a result of shorter base telomere length compared to NCH644, further experiments are needed to assess the impact of shorter telomeres on the sensitization of both cell lines to TMZ and TBHP. Nevertheless, our observations highlight NEIL3 as an attractive target in improving GBM standard-of-care in both all GBM patient populations,

491 including MGMT methylation-negative patients which do not profit from TMZ chemotherapy.
492 In this regard, a high-throughput drug screen revealed that small purine analogs and several
493 drugs (ellipticine, aurintricarboxylic acid, CGP-74514A) were potent inhibitors of the
494 combined glycosylase/AP lyase activity of NEIL1 in mouse embryonic fibroblasts (71),
495 suggesting that these compounds could also potentially inhibit NEIL3 activity. Genetic
496 depletion of NEIL3 was found to compromise HR-mediated repair of DSBs at the replication
497 fork and sensitize GBM cells to ATR inhibitors (49), further implying the potential clinical
498 applications of NEIL3 targeting in GBM. Hence, the BER-independent functions of NEIL3 such
499 as the management of ICLs blocking the replication fork, also seem important in cancer cells
500 and further experiments would need to clarify and assess if this function is important in the
501 context of GBM. Finally, since they lack the C-terminal domain mediating interaction with
502 TRF1, it may be expected that NEIL1 and NEIL2 overexpression will not rescue the telomere-
503 dysfunction phenotype associated with NEIL3-depletion. It remains to be learned, however,
504 whether such an overexpression would rescue the increased sensitivity to TMZ and TBHP
505 displayed by NEIL3-depleted cells. Such experiments may help identify to which extent the
506 telomeric defects induced by loss of NEIL3 contribute to the sensitivity of NEIL3-defective cells
507 to these genotoxicants.

508 Telomeres can also directly alter gene expression by silencing nearby genes through
509 spreading of telomeric heterochromatin. This reversible process, known as telomere position
510 effect (TPE), is well characterized in yeast and it has been shown that the effect can extend a
511 few kb towards the subtelomeres (72) or over long distances by looping of the telomeric
512 sequence (73) by spreading of telomeric heterochromatin marks such as tri-methylation of
513 the H3 histone at position K9. The effect was also observed in human fibroblasts on genes

514 located near telomeres (74), and shortening of telomeres was found to elicit a loss of TPE
515 (75). Moreover, the presence of the heterochromatic mark H3K9me3 at telomeres has been
516 shown to regulate TERRA levels (76). Hence, telomere length deficit in NEIL3 knockdown
517 NCH644 cells could result in the deregulation of genes repressed by the TPE and impact the
518 observed sensitivity to TMZ and TBHP. This reasoning can also be extended to the
519 deregulation of chromosome-specific TERRAs observed in this study. Thus, the effect of NEIL3
520 depletion on TPE should be investigated using an engineered cell system based on a luciferase
521 reporter stably integrated in the genome of HeLa cells either at a telomeric position or an
522 internal chromosomal position (control cell line) (77) and validated in the context of NCH644
523 shNEIL3 cell lines using either whole transcriptomic assays to assess global gene expression
524 changes or quantification of genes known to be regulated by TPE.

525 In summary, our observations underline NEIL3 as an attractive candidate target in GBM.
526 However, our knowledge on the telomere dysfunction phenotype induced by NEIL3 depletion
527 illustrates just one of the aspects associated with this DNA glycosylase; further experiments
528 will be needed to extend our insights into the broader aspects of GBM biology and resistance
529 to standard-of-care.

530

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FIGURES

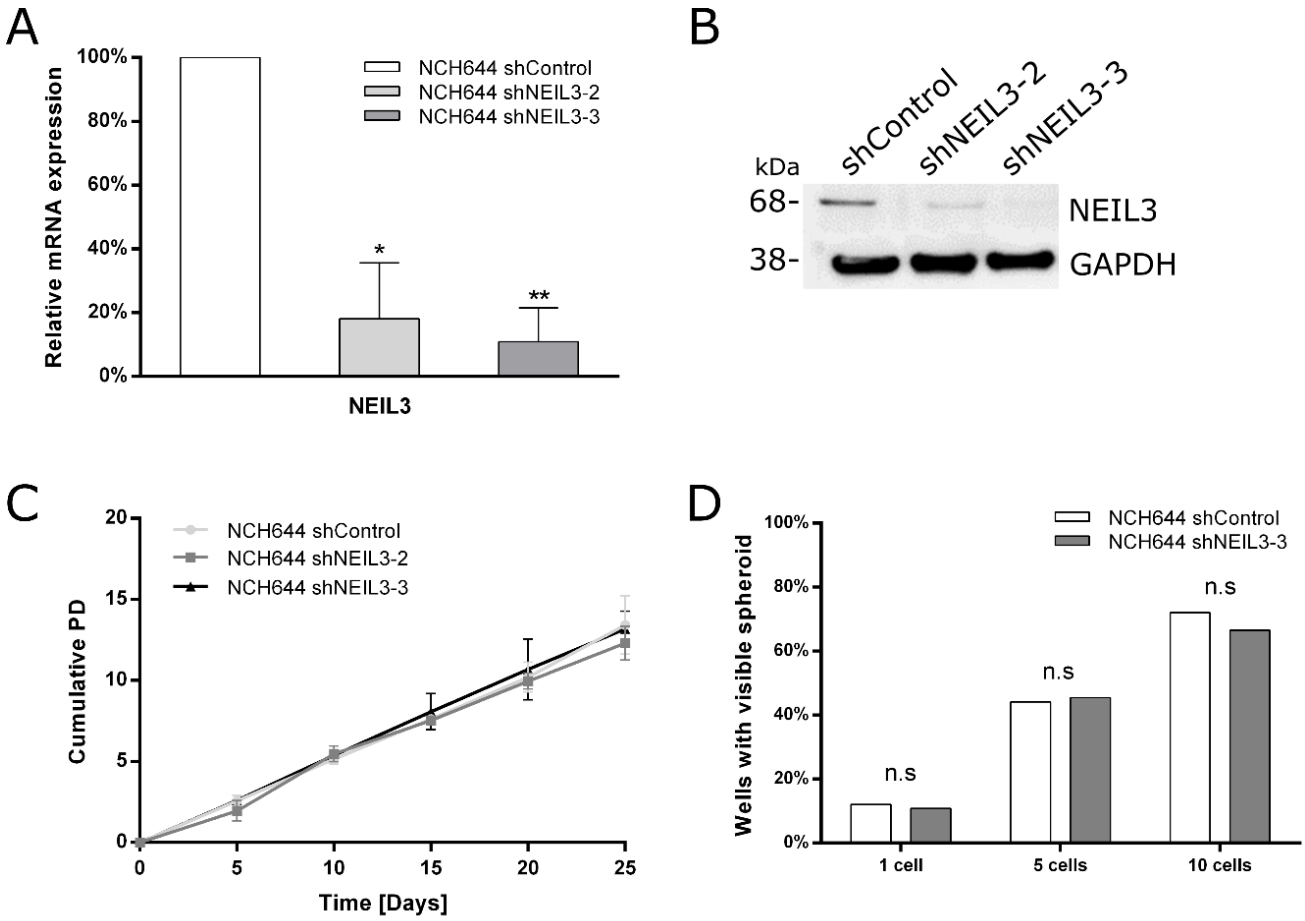
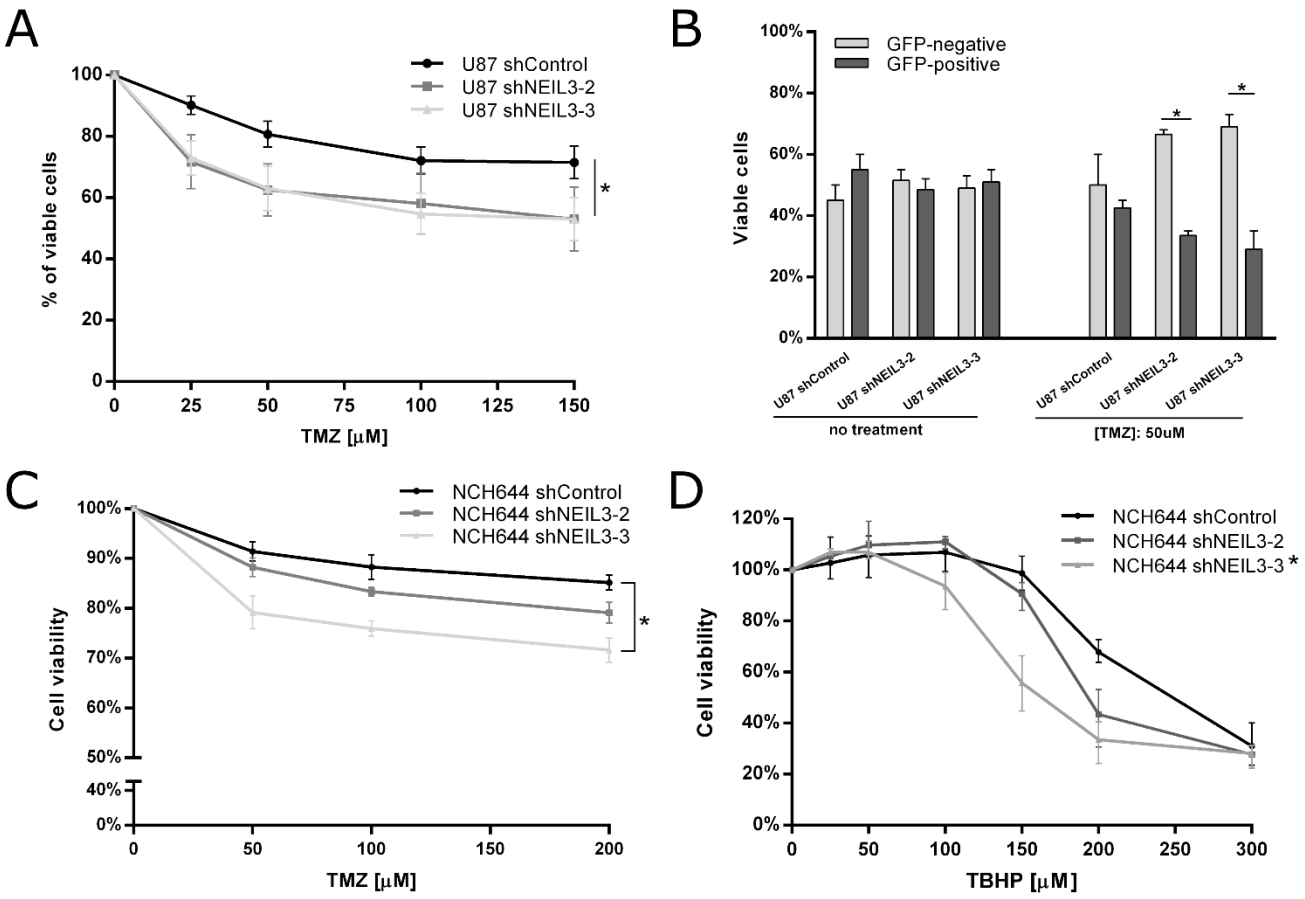


Figure 1. Knockdown of the NEIL3 DNA glycosylase in NCH644 GBM stem-like cells. A. Relative mRNA expression of NEIL3 in control and NEIL3-depleted NCH644 cells as assessed by RT-qPCR. The expression values were normalized against GAPDH and compared to shControl. Significance was determined by t-test (*, p-value < 0.05; **, p-value < 0.01). **B.** Western blot analysis of NEIL3 in total cell extracts confirming the mRNA expression levels. **C.** Effect of NEIL3 knockdown on cell proliferation as assessed by cumulative population doubling time over 25 days. **D.** Impact of NEIL3 on spheroid formation capacity of 1, 5 or 10 cells seeded in individual wells and incubated for 3 weeks. Visible spheroid were then counted and the percentage of spheroid-containing wells was calculated. Significance was assessed by t-test.



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751 **Figure 2. Effect of TMZ and TBHP treatment in U87 and NCH644 shNEIL3 cell lines. A.** Viability
752 of U87 shNEIL3 compared to shControl cells after 72h TMZ treatment as assessed by the MTT
753 reagent. **B.** Cell growth competition assay in U87 shNEIL3 cells. A 1:1 ratio of U87 (GFP-
754 negative) and U87 shRNA cells (GFP-positive) were mixed together and treated (50µM) or not
755 with TMZ. Viable cells were counted by FACS using a GFP gating strategy to discriminate the
756 two populations. The percentage of viable cells was assessed in each condition. **C** and **D.** Effect
757 of 48h of TMZ (**C**) and 24h of TBHP (**D**) treatment on the viability of NCH644 shNEIL3 compared
758 to shControl cells as assessed by WST1 reagent. In all experiments, differences in sensitivity
759 between the cell lines was analyzed by direct t-test comparison and the significance is
760 indicated by * (p-value <0.05).

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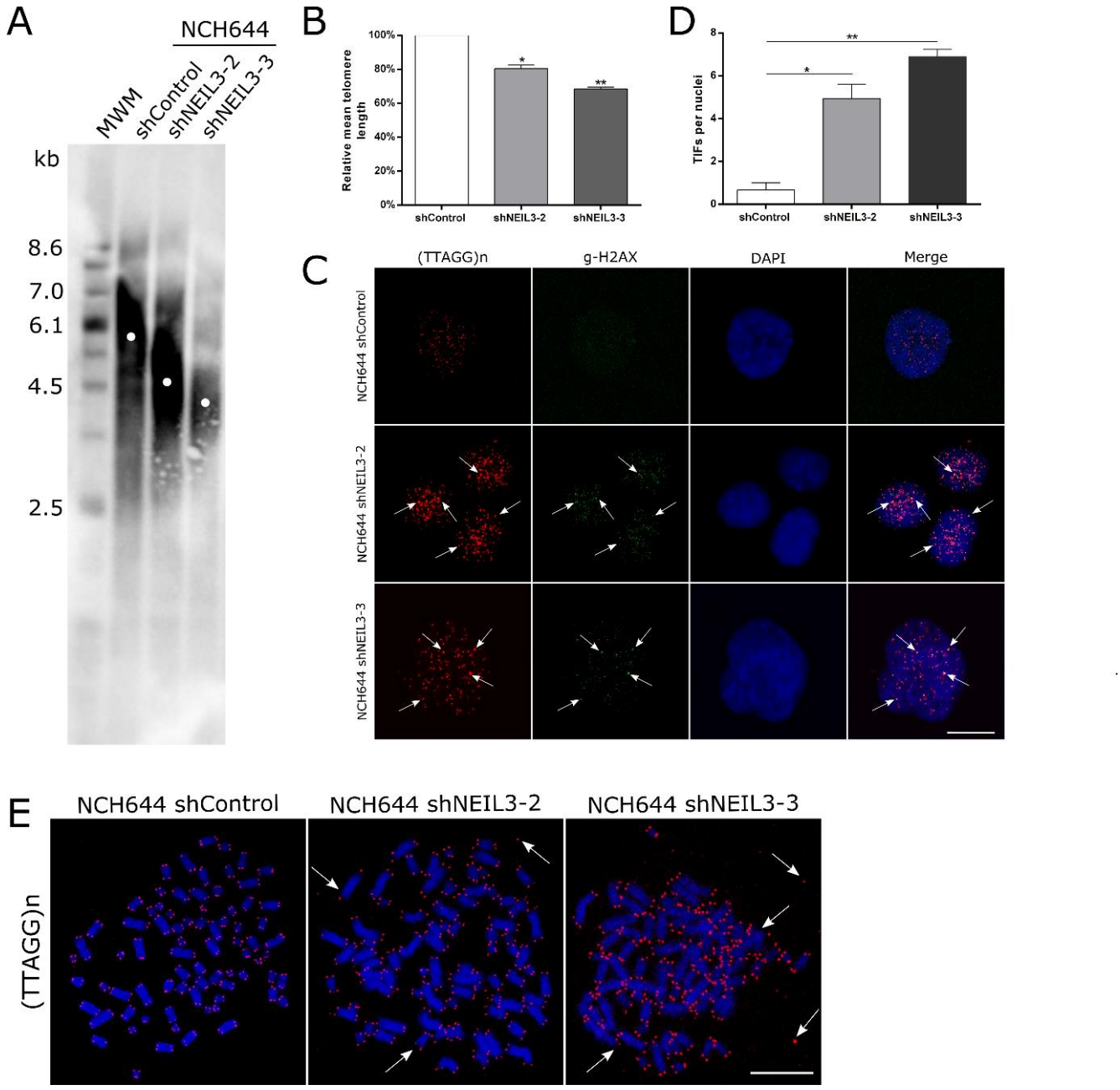

Figure 3. Impact of NEIL3 depletion on telomeres in NCH644 cells. A. Representative TRF Southern blot for evaluation of telomere length in NCH644 shNEIL3. Molecular weight is presented in kb and white dots indicate mean telomere length in each cell line. **B.** Relative mean telomere length comparing shNEIL3 to shControl cells. Significance was calculated by t-test and displayed as * (p-value < 0.05) and ** (p-value < 0.01). **C.** Representative images of telomere dysfunction-induced foci (TIFs) in NCH644 shNEIL3 cells. Co-localization of γ-H2AX foci (immunofluorescence) and telomeres (FISH) are indicated by white arrows. Scale bar represents 5µm. **D.** Quantification of TIFs per nucleus in NCH644 shNEIL3 cells. Significant

771 differences were calculated by t-test comparisons and are represented by * (p-value < 0.05)
772 or ** (p-value <0.01). **E**. Visualization of telomeres using fluorescent in situ hybridization
773 (FISH) in NCH644 shNEIL3 depleted and control cells. White arrows indicate loss of signal or
774 extra-telomeric signals. Telomere abnormalities were not quantified due to insufficient
775 biological replicates. Scale bar represents 5μm.

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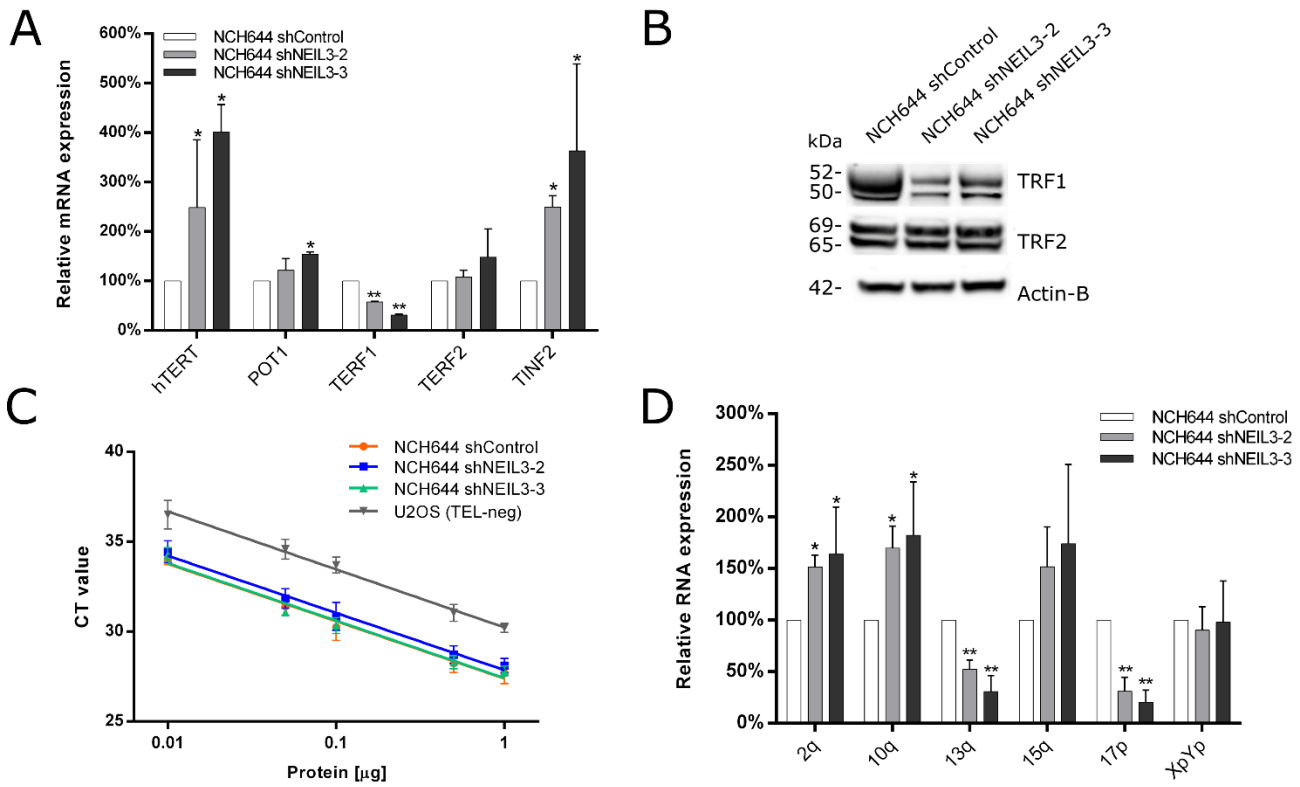

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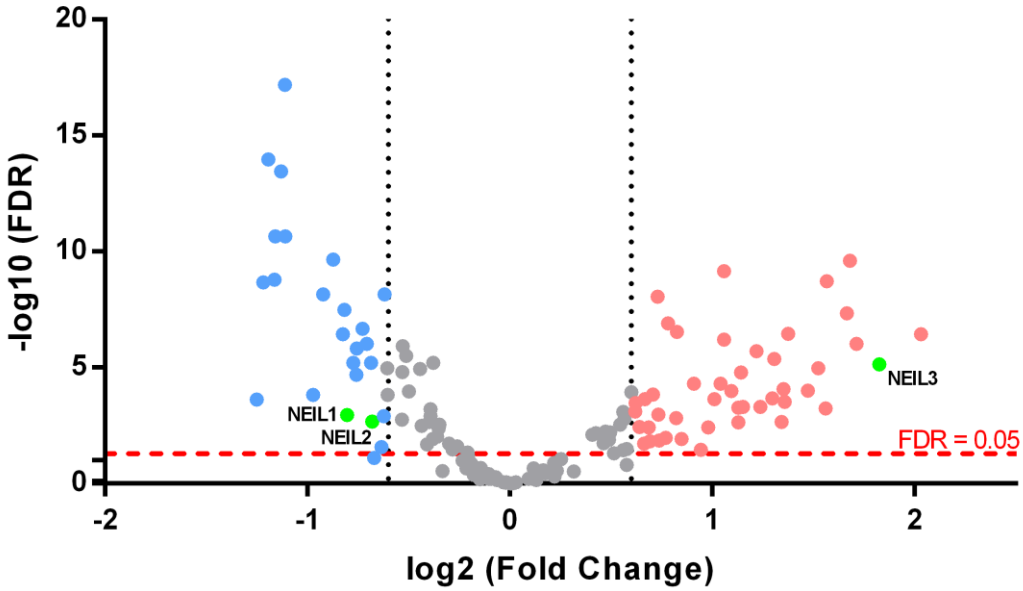
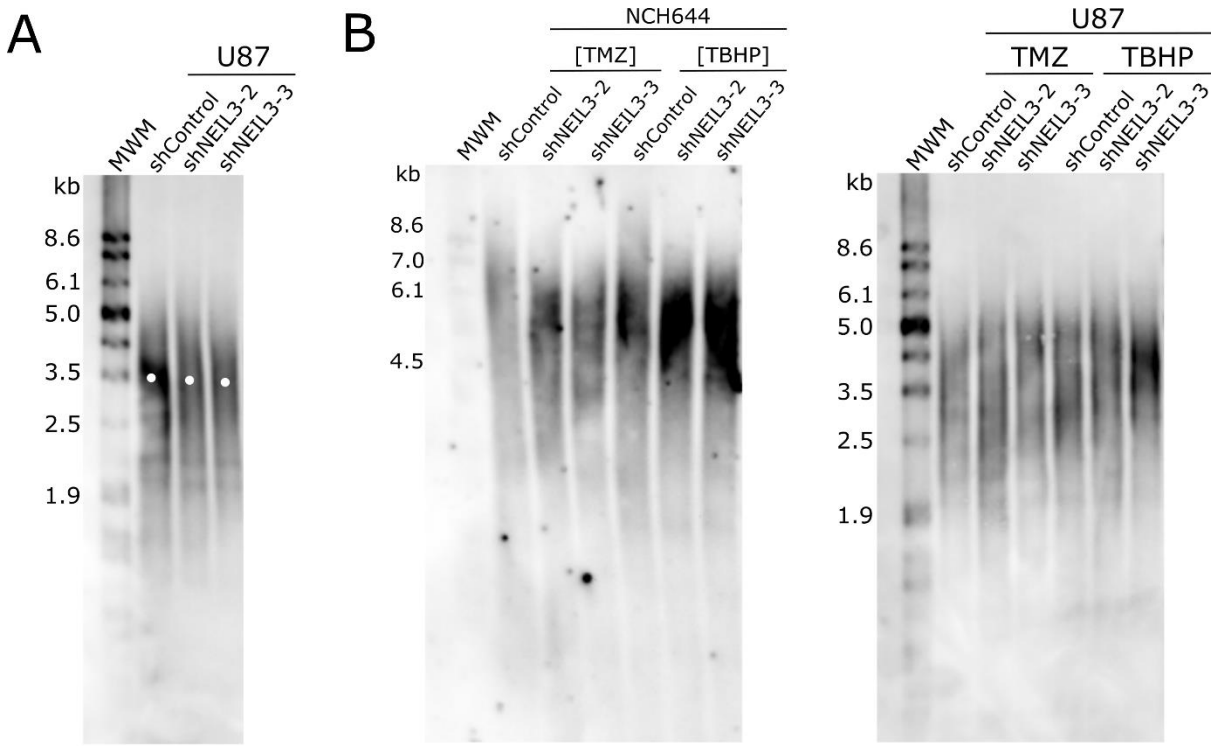
778 **Figure 4. Knockdown of NEIL3 deregulates the expression of shelterin components and**
779 **chromosome-specific TERRAs. A** Relative mRNA expression of the telomerase enzymatic
780 subunit (hTERT) and shelterin complex factors (POT1, TERF1, TERF2 and TINF2) in NCH644
781 shNEIL3 compared to shControl cells as assessed by RT-qPCR. Expression values were
782 normalized using GAPDH and ezrin. **B.** Representative WB of TRF1 and TRF2 in NCH644
783 shControl and shNEIL3 cells. Note that TRF1 is observed as a doublet with the lower band
784 considered to represent a spliced form of TRF1, lacking 20 amino acids (78). TRF2 is also
785 observed as a doublet but the difference between these two forms is not known (79). Actin-
786 β was used as loading control and the molecular weight of each protein is indicated in kDa on
787 the left of the blot. **C.** Quantification of telomerase activity by qTRAP. Triplicate Ct values at
788 each protein concentration was plotted for all cell lines and linear regression analysis was
789 performed. The U2OS telomerase-negative ALT cell line was used as negative control of
790 telomerase activity. **D.** Relative RNA expression of different chromosome-specific TERRAs (2q,
791 10q, 13q, 15q 17p and XpYp) in NCH644 shNEIL3 compared to shControl cells as assessed by
792 RT-qPCR. Expression values were normalized using GAPDH and GUSB. In all the experiments,
793 significant differences are represented with * (p value < 0.05) and ** (p-value < 0.01).

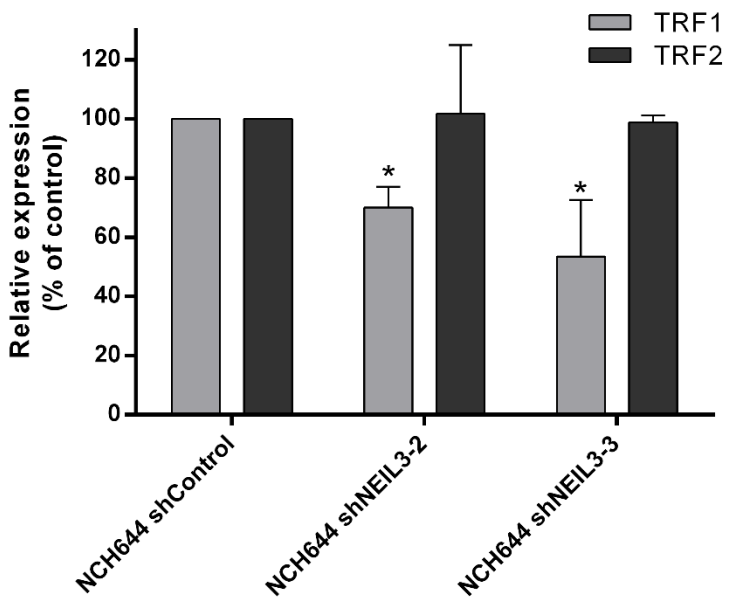
SUPPLEMENTARY FIGURES



Supplementary Figure S1. Volcano plot of DNA repair and DDR gene expression deregulation in primary GBM biopsies compared to control tissue. Upregulated genes are depicted in red and downregulated genes are shown in blue. Members of the NEIL glycosylase family are highlighted in green. FDR threshold was set to 0.05 (red dotted line in Y-axis) and log2(FC) > 0.6 (black dotted line in X-axis) (data taken from 50).



Supplementary Figure S2. Representative visualization of the mean telomere length in U87 and the effect of TMZ and TBHP treatment on both NCH644 and U87 shNEIL3 cells. Representative TRF Southern blots of U87 telomeres upon NEIL3 depletion (**A**) and (**B**) effect of TMZ/TBHP (72h) treatment on the telomere length of NCH644 (left panel) and U87 (right panel).



Supplementary Figure S3. Quantification of the protein levels of TRF1 and TRF2 in NCH644 shNEIL3 cells. Shown are the relative protein levels of TRF1 and TRF2 in NCH644 NEIL3 depleted when compared to control cells.

Supplementary Table S1. Description of the qPCR primer pairs used in the study.

mRNA target	Sequence (5'->3')	Size (bp)	Tm (°C)	Orientation	Design
Ezrin	TGCCCCACGTCTGAGAATC	19	59	forward	qPrimerDepot
	CGGCGCATATACAACTCATGG	21	59	reverse	
GAPDH	CATGAGAAGTATGACAACAGCCT	23	58	forward	qPrimerDepot
	AGTCCTTCCACGATACCAAAGT	22	59	reverse	
hTERT	CCGATTGTGAACATGGACTACG	22	59	forward	qPrimerDepot
	CACGCTGAACAGTGCCTTC	19	59	reverse	
NEIL3	GCCTGGAGTAGGGAACATCA	20	58	forward	qPrimerDepot
	GAGCAAGTCCTGCTTTACGG	20	59	reverse	
POT1	AGCCTTACGTGTTTGGGCATC	21	61	forward	qPrimerDepot
	GCATTGGCTGAACATCACACAA	22	60	reverse	
TERF1	AACAGCGCAGAGGCTATTATTC	22	58	forward	qPrimerDepot
	CCAAGGGTGTAATTCGTTCATCA	23	59	reverse	
TERF2	CAGTGTCTGTCGCGGATTGAA	21	60	forward	qPrimerDepot
	CATTGATAGCTGATTCCAGTGGT	23	58	reverse	
TINF2	GTGGAACATTTTCCGCGAGTA	21	58	forward	qPrimerDepot
	GCCCATACAAAGGCGTTCG	19	59	reverse	
GAPDH (qTERRA)	AGCCACATCGCTCAGACAC	19	57	forward	Feretzaki and Lingner, 2017
	GCCCAATACGACCAAATCC	19	52	reverse	
GUSB (qTERRA)	CAGCGTGGAGCAAGACAGTGG	21	63	forward	Feretzaki and Lingner, 2017
	AATACAGATAGGCAGGGCGTTCG	23	62	reverse	
2q-TERRA	AAAGCGGGAAACGAAAAGC	19	57	forward	Feretzaki and Lingner, 2017
	GCCTTGCCTTGGGAGAATCT	20	60	reverse	
10q-TERRA	ATGCACACATGACACCCTAAA	21	57	forward	Feretzaki and Lingner, 2017
	TACCCGAACCTGAACCCTAA	20	57	reverse	
13q-TERRA	CTGCCTGCCTTTGGGATAA	19	57	forward	Feretzaki and Lingner, 2017
	AAACCGTTCTAACTGGTCTCTG	22	58	reverse	
15q-TERRA	GCAAATGCAGCAGTCCTAATG	21	58	forward	Feretzaki and Lingner, 2017
	GACCCTGACCCTAACCCTAA	20	57	reverse	
17p-TERRA	CTTATCCACTTCTGTCCCAAGG	22	58	forward	Feretzaki and Lingner, 2017
	CCCAAAGTACACAAAGCAATCC	22	58	reverse	
XpYp-TERRA	AAGAACGAAGCTTCCACAGTAT	22	57	forward	Feretzaki and Lingner, 2017
	GGTGGGAGCAGATTAGAGAATAAA	24	58	reverse	

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CHAPTER 6

CONCLUSIONS & PERSPECTIVES

The purpose of this thesis was to address the DNA repair mechanisms that mediate resistance to the current first line genotoxic treatment for GBM (RT + TMZ). Particularly, we investigated the expression of key factors of the DNA damage response and cell cycle in clinically relevant paired GBM patient biopsies. The generated gene expression dataset set the foundation for the identification of a DNA repair and cell cycle based gene profiling of GBM patients with the prospect of personalized therapy and also uncovered a promising novel target gene in the fight against glioblastoma. The data presented in the previous chapters underline the benefit of a targeted gene expression study using paired biopsies in GBM and also raise a number of exciting points that would deserve further investigation.

In **Chapter 3**, we provided an overview of the DNA repair mechanisms driving chemoradiation resistance and tumor relapse in GBM, including novel DNA damage response-related biomarkers gaining interest in the community. Recent progress in the knowledge of the major pathways involved in the removal of IR- and TMZ-induced DNA lesions were put into perspective with the current therapeutic strategies relying on DNA repair inhibitors tested *in vitro* or in clinical trials. In line with these studies, we presented cellular systems as well as animal models most suitable for the study of DNA repair in the context of glioblastoma. Finally, we discussed the potential for personalized therapy of novel genetic and epigenetic alterations that were found to affect the DNA damage response of GBM in both adult and pediatric patients. Emerging topics in GBM not discussed in this review but worth considering include circulating tumor cells (CTCs) which are known to disseminate via the bloodstream and are involved in tumor spreading and recurrence (Chistiakov and Chekhonin 2018). In GBM specifically, detection of CTCs in the blood of patients was achievable based on the quantification of telomerase activity (Macarthur et al., 2014), a feature of a majority of adult GBMs extensively discussed in Chapter 3. As pointed out by Chistiakov and Chekhonin (2018), "The identification of glioblastoma CTCs might have a promising clinical potential for early tumor diagnosis and prognosis". Furthermore, it may also be argued that knowledge of the DNA repair status of these cells may help dictate novel therapeutic strategies. Indeed, in breast cancer, CTCs were found to be more resistant to chemotherapy compared to primary tumor cells (Gong et al., 2015). Moreover, inhibition of the checkpoint kinases Chk1 and Chk2 resensitized these cells to cisplatin both *in vitro* and in xenografted mice. Lastly, the identification and isolation of CTCs in the blood of cancer patients and the possibility to generate so-called CTC derived explants, which recapitulate the molecular traits as well as the patient's response to chemotherapy in mouse models (Lallo et al., 2017), present themselves

as valuable tools for preclinical studies to tailor treatment strategies directly adapted to the patient. This non-invasive procedure is particularly interesting in tumors, such as GBM, for which biopsy extraction is very delicate.

In **Chapter 4**, we proposed a novel, clinically relevant GBM patient stratification strategy based on a DNA repair and cell cycle gene signature. Current efforts to identify molecular profiles specific to chemoradiotherapy resistance focused on whole transcriptomic expression data generated from GBM specimens collected before treatment (Murat et al., 2008). In this study, we performed a DNA repair and cell cycle targeted gene expression analysis in a cohort of paired tumor samples from patients treated with RT or the combination of RT and TMZ. The availability of paired GBM specimens, specifically predating the introduction of TMZ in the clinic, still represent a limited resource but their use is gaining momentum with the constant improvement of biobanking protocols in close partnership with clinicians. Ours is one of the first studies to demonstrate the benefit of using paired GBM specimens. Specifically, it led to the identification of a 27-gene signature that resulted in the classification of our GBM specimens in two major groups displaying inversed expression profiles associated to patient prognosis and a third, less defined group. Analysis of the gene components of this signature revealed possible group-specific vulnerabilities against DNA damaging agents as well as DDRi and cell cycle inhibitors, which have emerged as important therapeutic tools against many cancers. It is unfortunate that our patient profiling strategy still leaves aside a considerable amount of patients (G2 group). In addition, although our preliminary experiments with G1 and G3 patient-derived cell lines indicated that significant differences in sensitivity were observed, none of the tested compounds appeared to completely eradicate one population or the other when applied as mono-treatments. It is clear that combinations will need to be tested to identify effective G1- and G3-specific treatments.

Yet, our analysis of the mutations and chromosome alterations underlying these two groups suggest that G1 and G3 GBMs are driven by distinct pathways frequently altered in GBM. Targeting these pathways thus represents an attractive therapeutic approach. Most notably, we demonstrated that G1 tumors frequently amplified the EGFR and PDGFRA receptors, which have been the subject of a considerable amount of advanced clinical trials in recurring GBM patients, with the use of specific inhibitors such as erlotinib (EGFR) and dasatinib (PGFRA) in combination with TMZ, that unfortunately have not shown any significant survival benefit to date (Lassman et al., 2015; van den Bent et al., 2009). Additionally, the increased frequency of PTEN deletion, which has been shown to drive cell

growth by activation of the AKT/mTOR pathway (Miller et al., 2011), in G1 samples, advocates the use of inhibitors of this particular pathway such as temsirolimus (mTOR inhibitor) (Wick et al., 2016) in the context of PTEN-deficient GBM tumors. Finally, deregulation of the RB pathway by deletion of RB1 or CDKN2A, is a common feature seen in GBM (Wiedemeyer et al., 2010). In this regard, as we observed increased RB1 deletion rates in G3 patients, the use of CDK4 or CDK6 inhibitors could be proposed as therapeutic options for these patients. In combination with RT, the CDK4 inhibitor palbociclib has been shown to provide a significant survival advantage in preclinical settings (Whittaker et al., 2017). To conclude, patient profiling based on our DDR gene signature also highlights the potential application of G1 or G3 specific treatment approaches targeting pathways other than the DNA repair/cell cycle and ultimately novel synthetic lethality approaches combining multiple pathway inhibitors could be considered.

As exposed beforehand, GBM patients are in dire need of novel therapeutic strategies since the IR and TMZ combination treatment, introduced in 2005, is still currently applied in the clinic for lack of better alternatives. In cancer, the chromosome ends play a vital role in their proliferation potential and thus targeting their integrity is gaining interest in the community. Telomere maintenance plays an important role in glioma predisposition, initiation and prognosis (Walsh et al., 2015). In glioblastoma specifically, the picture is quite complex, with adult GBMs mainly reactivating telomerase whereas ALT is observed in a significant portion of pediatric GBMs. In **Chapter 5**, we identified NEIL3 as a potential candidate target gene in GBM based on the impact of its depletion on telomere homeostasis and sensitivity to TMZ and oxidative DNA lesions. Notably, we observed telomere shortening in conjunction with deregulation of shelterin components such as TRF1, POT1 and TIN2 as well as chromosome-end specific TERRAs, upon knockdown of NEIL3 in GBM cells. Interestingly, NEIL3 was shown to be directly recruited by TRF1 to promote BER-mediated repair of oxidative guanine lesions at the telomeres (Zhou et al., 2017). Moreover, expression of NEIL3 in mouse cells was found to provide resistance to ciplatinum agents that cause DNA adducts and crosslinks (Rolseth et al., 2013), in line with the recent demonstration of its involvement in the unhooking of psoralen- or abasic site-induced inter-strand crosslinks (ICLs), thus suggesting an additional, BER-independent role of this enzyme in the support of genomic stability (Martin et al., 2017; Semlow et al., 2016). Thus, the targeting of NEIL3 in cancer cells could provide means to induce telomere-associated DNA damage and overall genetic instability. Thus it is tempting to propose treatment strategies based on the use of genotoxic

agents such as TMZ or cisplatin in combination with small molecule inhibitors targeting NEIL3, which are under investigation as a few molecules were already found to specifically target NEIL1 and NEIL2 (Jacobs et al., 2013). In this context, it is important to note that a shRNA screen targeting the DDR conducted in the laboratory, identified NEIL3 as a TMZ sensitizer in NCH644 GBM cells but not in the human neural stem cell line hNSC100, indicating that NEIL3 inhibition in combination with genotoxicants could provide means to target the tumor cells without generating additional detrimental side effects.

In addition, therapeutic strategies aiming to destabilize telomere homeostasis are currently under intense scrutiny in GBM, in great part through the use of G quadruplex ligands capable of hindering the binding and activity of telomerase (Lagah et al., 2014; Zhou et al., 2016), resulting in anti-proliferative effects mediated by telomere instability. To note, drug-induced resistance to telomerase activity inhibitors in telomerase-positive cells may occur through activation of the alternative lengthening of telomeres (ALT) mechanism (Hu et al., 2012). Interestingly, the G4 ligand telomestatin has been shown to effectively cause telomere dysfunction and trigger the DDR in various ALT cells (Temime-Smaali et al., 2009). Additionally, the G4 stabilizing compound TMPyP4 was found to suppress the proliferation of ALT-positive cells (Kim et al., 2003). The fact that GBMs present up to 25% of ALT tumors (Hakin-Smith et al., 2003) further encourages the study and testing of G-quadruplex stabilizing drugs in this particular context. Combining the anti-proliferative activity of such G4 ligands, in both telomerase-positive and ALT cells, with the inhibition of telomeric DNA damage repair via NEIL3 targeting could provide novel means of treatment in GBM patients in the future.

Finally, despite extensive biochemical characterization of the enzymatic activity of NEIL3, its ubiquitous cellular function, apart from its role in promoting proliferation of neural and cardiac stem cells (Olsen et al., 2017; Sejersted et al., 2011), remains unsettled. Hence, the functional characterization achieved in GBM cells contributes to provide a novel and innovative candidate in the fight against this deadly disease. The observations presented and discussed in this manuscript are crucial preliminary results that pave the way to more in-depth analyses of the role of NEIL3 in telomere homeostasis in the context of GBM.

In conclusion, our original aims have been fulfilled with the generation of valuable DNA repair and cell cycle gene expression data which, after analysis, contributed to a clinically relevant gene signature for GBM patient profiling with the prospect of personalized treatment strategies, and the identification of a novel, promising candidate target gene for innovative therapeutic approaches.

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