proceedings

MDPI

Type of the paper: Proceedings

BRAT1 depletion impairs DNA damage repair in glioblastoma cell lines †

Johanna Ertl[1], Ömer Güllülü[2], Stephanie Hehlgans[2], Franz Rödel[2, 3, 4], Donat Kögel[1, 3], Benedikt Linder[1, *]

[1] Experimental Neurosurgery, Department of Neurosurgery, Neuroscience Center, Goethe University Hospital, 60528 Frankfurt am Main, Germany; ertl.j95@gmail.com (J. E.), koegel@em.uni-frankfurt.de (D. K.), linder@med.uni-frankfurt.de (B. L.)

[2] Radiotherapy and Oncology, Goethe University Hospital, 60590 Frankfurt am Main, Germany; Oemer.Guelluelue@kgu.de (Ö. G.), stephanie.hehlgans@kgu.de (S. H.), franz.roedel@kgu.de (F. R.)

[3] German Cancer Consortium (DKTK), Partner Site Frankfurt, 60590 Frankfurt am Main, Germany and German Cancer Research Center DKFZ, 69120 Heidelberg, Germany

[4] Frankfurt Cancer Institute, Goethe University, 60590 Frankfurt am Main, Germany

* Correspondence: linder@med.uni-frankfurt.de; Tel.: +49 69 6301 6930

† Presented at the 1st International Electronic Conference on Cancers: Exploiting Cancer Vulnerability by Targeting the DNA Damage Response, 01/02/2021 - 14/02/2021

Received: date; Accepted: date; Published: date

Abstract: Glioblastoma (GBM) is the most malignant primary brain tumor in adults. This is partly due to the presence of so-called glioma stem-like cells (GSCs). Previous work from us showed that after combination treatment of GSCs with Arsenic Trioxide and Gossypol, the protein breast cancer type 1 susceptibility protein (BRCA1)-associated Ataxia telangiectasia mutated (ATM)-activator 1 (BRAT1) was one of the most downregulated proteins. This protein is largely undescribed, but it has been shown to interact with ATM, BRCA1 and DNA-dependent kinase, catalytic subunit (DNA-PKcs) during DNA damage repair. Here, we aim to analyze the radio-sensitizing effect of BRAT1 depletion on differentiated GBM cells and GSCs. We show that BRAT1 1) is needed for effective DNA repair after irradiation, 2) knockdown sensitizes GBM cells to radiation treatment and 3) plays a role in BRCA1 recruitment to DNA damage sites. Future experiments will lead to a deeper understanding of the mechanism of enhanced radio-resistance in GSCs.

Keywords: Glioblastoma; Radio-Resistance; cancer stem cells; DNA damage repair; BRAT1; BRCA1; ATM

1. Introduction

Diffuse gliomas are malignant tumors originating from glial cells or their precursors in the central nervous system. Phenotypically and genotypically, a distinction is made between diffuse or anaplastic astrocytomas (WHO grade II and III), WHO grade II and III oligodendrogliomas, grade IV glioblastomas (GBM) and diffuse gliomas of the childhood. These are then further divided into different subgroups according to their genotypic characteristics [1–3]. Among those cancers, GBMs represent the most malignant tumor type, with a median survival rate of only 15 months despite maximally possible treatment [4,5]. This is primarily caused by early relapses due to diffuse tumor cells migrating as single cells or small spheres into the neuropil (network of intertwined neurons and glia cells). Therefore, patients cannot be completely cured by surgical resection of these tumors [6]. GBMs are thus usually treated with a combination of radio- and chemotherapy [7], despite being highly resistant to it [8]. More precisely, GBMs are first surgically removed, followed by chemoradiation with the DNA alkylating drug temozolomide [8]. These two therapies primarily aim

to cause DNA damage in the tumor cells, more specifically, DNA double- and single-strand breaks (DSBs and SSBs).

A DNA DSB thus formed is recognized by the Mre11/Rad50/Nbs1 (MRN) complex. The two proteins Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia mutated and Rad3-related (ATR) are activated by phosphorylation, which in turn leads to phosphorylation of many other downstream proteins that contribute to cell cycle arrest, DNA repair and apoptosis. Notable examples include the histone-variant H2AX, which in the phosphorylated state represents a marker for the DNA DSB and checkpoint kinases 1 and 2 (CHK1 and CHK2), which cause cell cycle arrest [9,10]. DNA DSBs are mainly repaired via at least two different pathways: homologous recombination (HR) and non-homologous end joining (NHEJ) [11]. The first requires the sister chromatid as a template, so that any DNA sequence that may have been lost can be repaired accurately. Therefore, HR can only take place after DNA replication during the late S and G2 phase. NHEJ, on the other hand, recombines the break ends, which can lead to possible errors. However, recent reports revealed that the nascent RNA template is used to conduct the process as error-free as possible [12]. This can take place without a template and hence in all phases of the cell cycle [9]. Nevertheless, it has been shown that a subgroup of GBM cells, glioma stem-like cells (GSCs), are particularly resistant to radiotherapy due to activating mutations in the DNA damage response (DDR), which allows them to survive in a quiescent state [10,13]. In principle, there are two different and opposing models of the origin of cancer: the cancer stem cell model and the clonal evolution model. According to the cancer stem cell model, a tumor arises from and/or harbors cancer cells with a stem cell-like phenotype (cancer stem-like cells, CSCs), which in turn originate from mutations of embryonic stem cells or progenitor cells or from de-differentiated cancer cells. Tumors can therefore be replenished via asymmetric division of the CSCs. This cell mass then makes up most of the tumor. Thus, the tumor is considered a heterogeneous mass [14]. According to the model of clonal evolution, a tumor can arise from sequential mutations of a normal differentiated cell. This gives the cell the ability to divide in a non-controlled manner. Accumulating mutations give rise to subclones, which then also contribute to the enlargement of the tumor. In this model, tumor heterogeneity arises from intraclonal genetic and epigenetic variations, as well as microenvironmental influences [15]. Recent research showed that tumor heterogeneity is more likely to result from non-hierarchical reversible state changes. It is hypothesized that most cancer cells can obtain this stem-like phenotype and de-differentiate into CSCs, but can also become differentiated cancer cells again [16].

According to the cancer stem cell model, the goal of a novel therapy is to eliminate this type of cells from the tumor population to reduce the probability of a relapse. For this, we have recently shown that inhibiting the Hedgehog- and NOTCH-pathways in GSCs with Arsenic Trioxide (ATO) in combination with the anticancer agent (-)-Gossypol (Gos) reduces not only stemness markers/properties, but also decreases expression of DNA repair proteins. Two of these are the proteins breast cancer type 1 susceptibility protein (BRCA1)-associated ATM activator 1 (BRAT1) and ATM [17]. BRAT1 is ubiquitously expressed and interacts with BRCA1 and ATM, being involved in DDR, cell proliferation and mitochondrial function. Moreover, it is required for protein stability of phosphatidylinositol 3-kinase-related kinases (PIKK), such as ATM and DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), as well as for neuronal growth as it is related to lethal neonatal rigidity and multifocal seizure syndrome [18–20].

This study aims to analyze the role of BRAT1 in radio-sensitization in GSCs and fetal calf serum (FCS)-grown (i.e. differentiated) highly radio-resistant GBM cells. We have so far shown that BRAT1 is required for efficient DNA repair after irradiation but does not affect proliferation. Furthermore, initial results gave an indication that BRAT1-depleted GBM cells showed sensitization to radiation-induced cell death and that BRAT1 is required to recruit BRCA1 to the DNA damage site. Further experiments are focusing on analyzing the downstream effects of BRAT1 loss, e.g. by finding additional novel BRAT1 interaction partners.

2. Results

2.1. BRAT1 expression is increased in GBM

In previous work, we already showed that BRAT1 and ATM, two DNA repair proteins, are significantly decreased in GSCs after treatment with ATO/(-)-Gos [17]. Subsequent work revealed that BRAT1 is overexpressed in GBM compared to healthy tissues (Figure 1A). It furthermore correlates negatively with patient survival according to the Gravendeel-dataset [21] analyzed via the GlioVis portal [22] (Figure 1B).

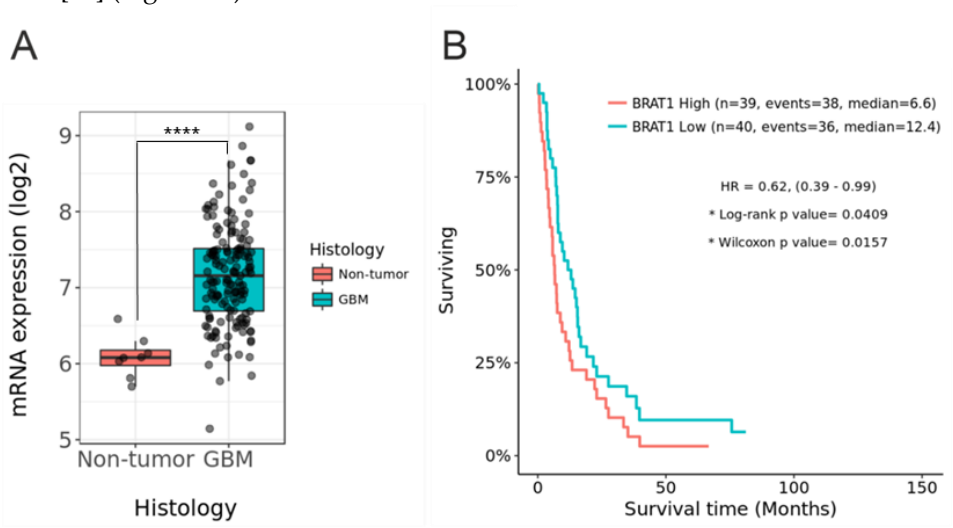

Figure 1. BRAT1 expression is increased in GBM. (**A**) Increased levels of breast cancer type 1 susceptibility protein (BRCA1)-associated Ataxia telangiectasia mutated (ATM) activator 1 (BRAT1) compared to healthy tissue. Analysis of Gravendeel-dataset [21] using the GlioVis portal [22]. Pairwise t test between group levels with corrections for multiple testing (p-values with Bonferroni-correction). Error bars are SEM. **** $p < 0.0001$ (**B**) Higher BRAT1 levels are associated with decreased GBM patient survival.

Our *in silico* analysis of publicly available datasets (Figure 1) revealed that BRAT1 is upregulated in GBMs compared to healthy brain tissue (Figure 1A) and this upregulation is associated with a poor patient survival (Figure 1B). Because of these findings and our own observations, we next aimed to elucidate the role of BRAT1 in the regulation of radio-resistance of GSCs. Thus, we established stable BRAT1-knockdown GBM cells and GSCs using lentiviral shRNA.

2.2. Validation of BRAT1-knockdown

The study aims to investigate the potential radio-sensitizing effects of BRAT1 in highly radio-resistant GBM cells and GSCs. For this, stable BRAT1-knockdown (KD) NCH644 and U251 cells were developed using four different lentiviral shRNA constructs A, B, C and D (Figure 2). The different shBRAT1 knockdown cell lines showed a clear reduction in BRAT1-expression in both cell lines compared to shCtrl cells (Figure 2A). Densitometric quantification of the corresponding bands in the western blots further confirmed this observation. In NCH644 cells, the remaining BRAT1-expression was less than 2% in shBRAT1 cells A, C, and D, and approximately 5% in shBRAT1 KD B. Residual BRAT1 expression was slightly higher in U251 cells. Here, it was approximately 10% for shBRAT1 A and D, 20% for C, and 23% for B (Figure 2B).

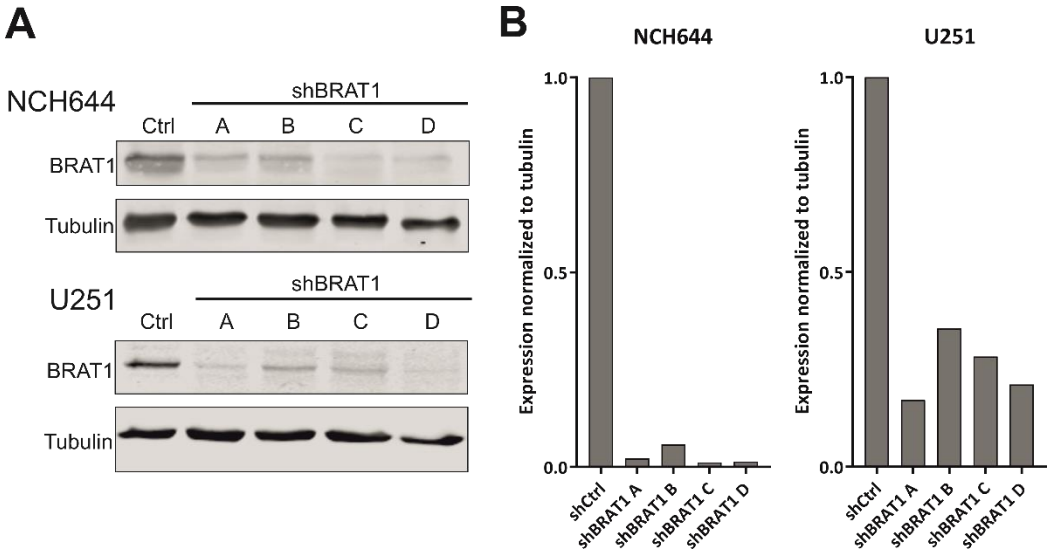

Figure 2. Validation of stable shRNA BRAT1-knockdown NCH644 GSCs and U251 GBM cells. (**A**) Western blot analysis of BRAT1 in stable shRNA-mediated BRAT1-knockdown cells showing reduction of BRAT1 in all different knockdown clones (A-D) in both U251 and NCH644 cells. Tubulin as housekeeping protein. (**B**) Quantitative analysis of the BRAT1-western blots visualizes knockdown-efficiency in all shRNA-mediated BRAT1-knockdown cell lines. Remaining BRAT1-expression was normalized to Tubulin and BRAT1-expression of shCtrl cells.

After confirmation of an efficient knockdown using multiple shRNA sequences, we decided to further work with BRAT1-knockdown cell lines C and D in NCH644 and KD A, C and D in U251 cells. Since BRAT1 is thought to regulate cell proliferation [18], we asked whether an attenuation of BRAT1 affects the proliferation of untreated GBM cells. However, a colorimetric MTT cell viability assay revealed similar proliferation kinetics for both shCtrl and BRAT1-depleted GBM cells (data not shown).

2.3. BRAT1 is essential for an efficient and timely DNA repair

Since BRAT1 expression was previously shown to be increased in radio-resistant GBM cells and GSCs [17] we next examined the DNA repair proficiency in BRAT1 knockdown cells using γH2AX and 53BP1 foci assays. For this, the cells were irradiated with either 10 and 20 Gy (U251 GBM cells) or 8 and 10 Gy (NCH644 GSC cells). At 1h and 24h after irradiation, the cells were fixed and immunostained against γH2AX and 53BP1 to visualize and quantify DNA DSBs. It was then analyzed whether BRAT1-depleted cells show differences in DSB numbers compared to the control cells 1h and 24h after irradiation.

The higher the irradiation dose, the more DNA DSBs were generated. 1h after irradiation, both shCtrl and BRAT1-KD cells showed similar numbers of DNA DSBs of around 30 foci/cell after irradiation with 10 Gy and 50 foci/cell after 20 Gy irradiation in GBM cells (Figure 3A), However, 24h after irradiation, the control GBM cells had repaired most of their DNA DSBs, namely 30 (10 Gy) and 50 γH2AX foci/cell (20 Gy) and 6 (10 Gy) and 19 γH2AX foci/cell remained on average (20 Gy), whereas the BRAT1-KD GBM cells continued to show significantly higher DNA DSB numbers (shBRAT1 A: 10 Gy 1h: 40 γH2AX foci/cell, 10 Gy 24h: 25 γH2AX foci/cell average in GBM cells). This observation occurred in both GBM (Figure 3A) and GSC cells (Figure 3B) and γH2AX and 53BP1 foci numbers were comparable. In addition, GSC cells exhibited significantly increased DNA DSB numbers in BRAT1-KD cells compared to shCtrl cells 1h after irradiation (8 Gy 1h: shCtrl: ca. 32 γH2AX foci/cell, shBRAT1 A: ca. 40 γH2AX foci/cell), which indicates a delayed DDR and general sensitization to irradiation (Figure 3B).

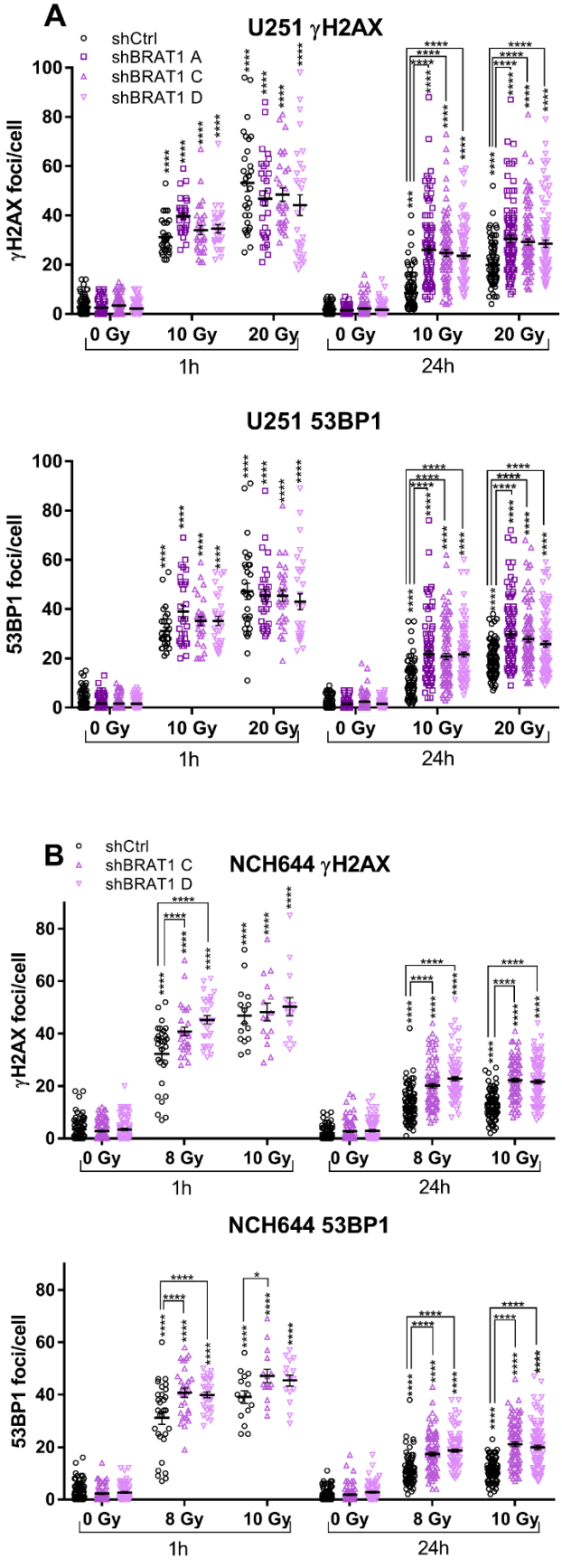

Figure 3. BRAT1 is essential for an efficient and timely DNA repair. (**A**) γH2AX- and 53BP1 foci assays 1h and 24h after irradiation with 10 Gy or 20 Gy in U251 cells. (**B**) γH2AX- and 53BP1 foci assays 1h and 24h after irradiation with 8 Gy or 10 Gy in NCH644 cells. At 1h after irradiation γH2AX- or 53BP1-

positive foci were counted in 15 nuclei per condition; in non-irradiated cells and 24h after irradiation, foci in 50 cells were counted per condition. The experiment was performed n=2 times in biological replicates. Error bars are SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ against respective non-irradiated cells or as indicated; Two-Way ANOVA with Tukey's multiple comparisons test.

These results demonstrated that BRAT1 is required for efficient DNA repair, because BRAT1-knockdown cells, in contrast to shCtrl cells, showed significantly increased residual DNA damage at 24h after irradiation (Figure 3).

Based on the high numbers of remaining DNA DSBs in BRAT1-deficient GBM cells, we hypothesized that their colony formation ability might be impaired after irradiation due to DNA damage-induced growth arrest and/or cell death induction. To test this hypothesis, the U251 GBM cells were irradiated with different doses and 10 days later, they were fixed, stained with crystal violet and subsequently counted this approach revealed that the number of the colonies larger than 50 cells decreased in all cell lines with increasing irradiation dose, while there was no difference in colony formation between shCtrl and BRAT1-KD GBM cells (data not shown).

2.4. Downregulation of BRAT1 results in elevated cell death after irradiation

One of the main questions we aimed to address is whether the downregulation of BRAT1 sensitizes GBM cells to irradiation. To answer this, cells were irradiated with 10 or 20 Gy, and 72h later cells were subjected to Annexin V/Propidium iodide staining (Figure 4). The analysis given in Figure 4 shows that the cell death increased significantly with increasing irradiation dose. After irradiation with 10 Gy, the percentage of dead shCtrl and shBRAT1 A cells was about 30%, that of shBRAT1 cells C and D about 40%. After irradiation with 20 Gy, this percentage increased to about 50% for shCtrl and shBRAT1 A cells, but was already about 60% for shBRAT1 cells C and D.

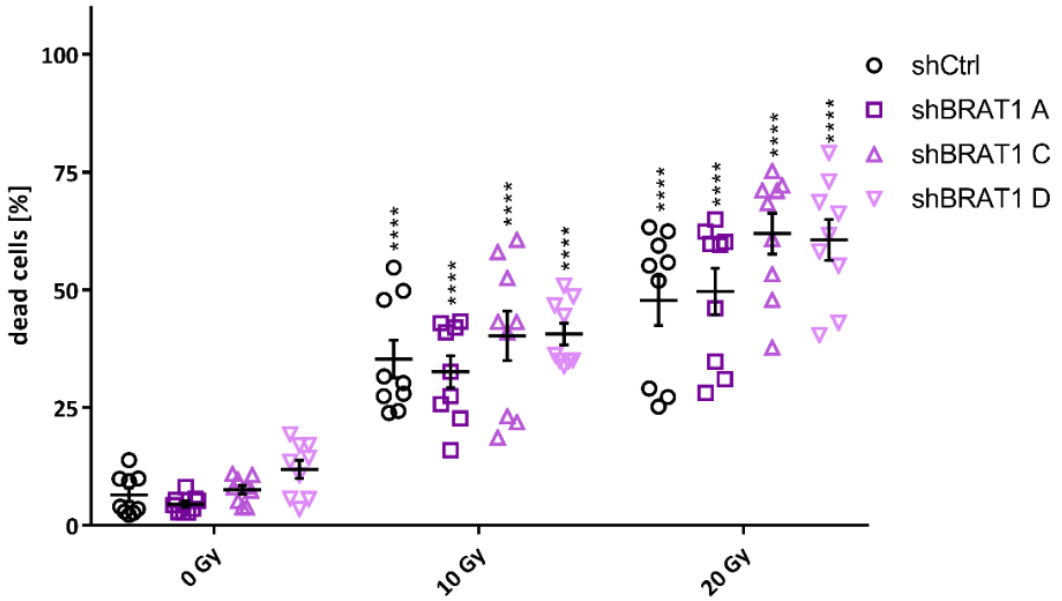

Figure 4. Downregulation of BRAT1 results in elevated cell death after irradiation. Scatterplots of cell death analysis of U251 GBM shCtrl or BRAT1-KD cells 72h after irradiation with 10 or 20 Gy using Annexin V/Propidium iodide staining. The experiment was performed n=3 times, each in triplicates, in biological replicates. Error bars are SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ against respective non-irradiated cells or as indicated; Two-Way ANOVA with Tukey's multiple comparisons test.

The cell death analysis revealed a slightly increased susceptibility towards irradiation-induced cell death in the U251 BRAT1-KD cells C and D compared to control cells, but not in A KD cells.

2.5. BRAT1 is necessary for BRCA1-recruitment to DNA damage sites

Although very little is known about the molecular function of BRAT1, it has been reported to interact with BRCA1 [23]. BRCA1 is a tumor suppressor protein involved in initial stages of the DDR. After irradiation-induced DNA damage, it is phosphorylated by pATM and locates to the DNA damage site, where it initiates HR [24]. To further investigate the nature of BRAT1-BRCA1 interaction, an immunofluorescence staining of GSCs and GBM cells was performed after irradiation with 8 Gy, or 10 Gy, respectively. BRCA1, as well as a DNA DSB marker (53BP1) were detected to visualize the recruitment of BRCA1 to the DSB (Figure 5). Without irradiation, no or few 53BP1 foci were evident in the different cells and BRCA1 displayed diffuse distribution throughout the nucleus and in the perinuclear region. At 1h after irradiation, all cells showed 53BP1 foci. BRCA1-positive nuclear dots, some of which colocalized with 53BP1, were formed in the control cells. In contrast, BRCA1 remained diffusely distributed in and around the nucleus in both GBM and GSC BRAT1-KD cells. At 24h after irradiation, 53BP1 foci continued to appear in all cells (Figure 5). In both U251 cell lines (shCtrl and shBRAT1 A) and NCH644 shBRAT1 C cells, BRCA1 was diffusely distributed throughout the nucleus and in the perinuclear region. However, it is noticeable that BRCA1 positive foci were still detectable in the NCH644 shCtrl cells 24h after irradiation, which was not the case in the other cells (Figure 5). Additionally, it appears that detectable signals of 53BP1 and BRCA1 colocalized in only a few cases, providing the possibility that distinct types of foci with predominant recruitment of 53BP1 or BRCA1 were formed.

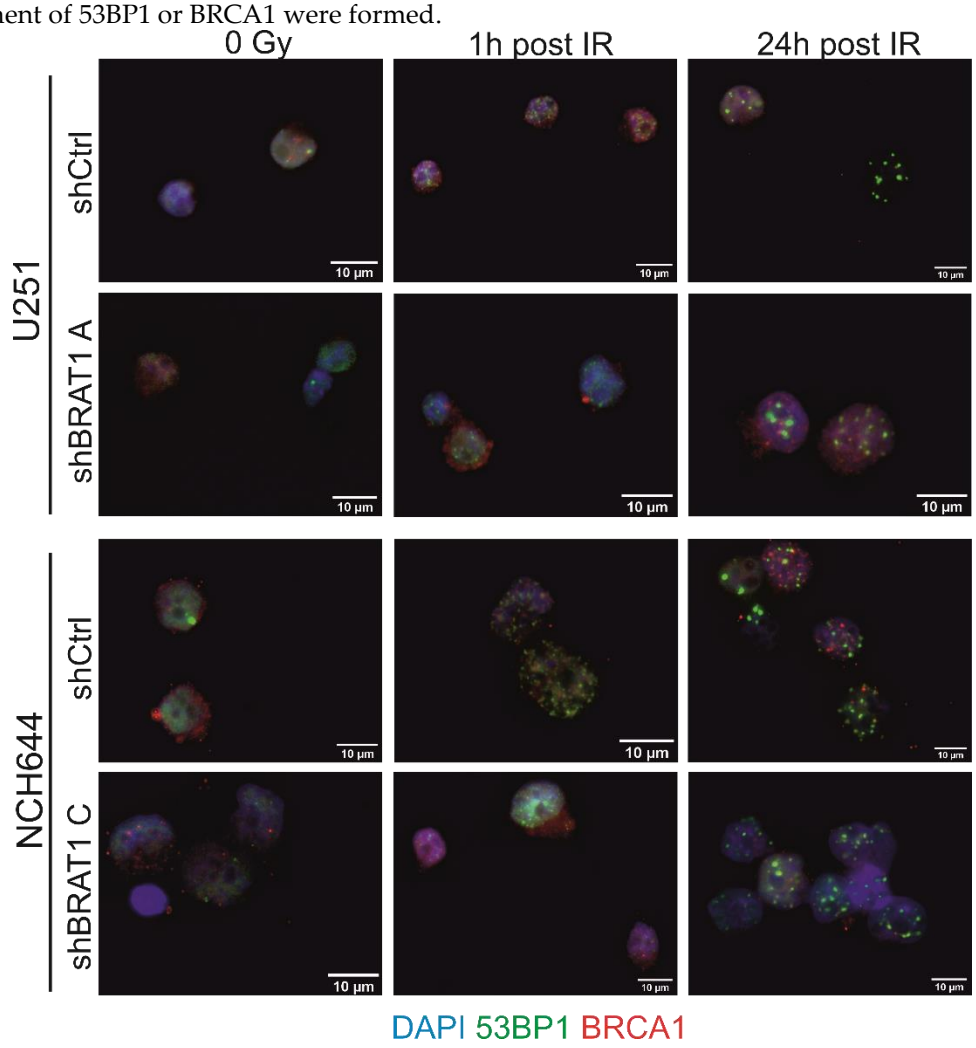

Figure 5. BRAT1 is necessary for BRCA1-recruitment to DNA damage sites. Representative immunofluorescence microscopy images of GBM and GSC cells after irradiation with 8 Gy (NCH644) or 10 Gy (U251) and subsequent fixation after 1h or 24h. DAPI in blue, BRCA1 in red, 53BP1 in green. At least 3 images were taken with at least 10 cells in total of each shCtrl and all shBRAT1-KD cells. One representative BRAT1 knockdown cell line is shown.

It was found that after irradiation, in both cell lines BRCA1 remained diffusely distributed throughout the nucleus and in the perinuclear region, which was not the case in control cells. This indicates that BRAT1 is essential for the recruitment of BRCA1 to the DNA DSB.

3. Discussion

The most malignant primary brain tumor in adults, glioblastoma, is usually treated with combined chemoradiation following surgery according to current standards, despite their radio- and chemoresistance [8]. One of the reasons for the robust radio-resistance in this tumor entity could be the existence of GSCs in the tumors. These cells can self-renew infinitely, upregulate DNA damage repair and escape radio- and chemotherapy by surviving in a quiescent state [10,13,25]. Our previous proteomics approach revealed that BRAT1 is one of the most decreased proteins after combined differentiation-inducing treatment of GSCs with ATO and Gos. Similarly this treatment induced DNA damage and reduced the expression of key components of the DDR [17]. This guided us to the major hypothesis of the current study stating that BRAT1 might play an essential role in resistance to DNA damage and radiation therapy. Therefore, an shRNA mediated BRAT1 depletion was used to investigate various aspects of the cellular radiation response. Since it is reported that BRAT1 plays a role in cell proliferation [18], we first performed an MTT cell viability assay, but it became clear that BRAT1 knockdown does not affect the proliferation of untreated GBM cells. Then, residual DNA damage was analyzed, as BRAT1 has been described to interact with ATM and BRCA1, two fundamental proteins in early DDR [18]. Because it is proposed that BRAT1 1) is needed for ATM phosphorylation at Ser1981, 2) is needed for the function of BRCA1 and 3) interacts with DNA-PKcs [23,26,27], we hypothesized that DNA DSBs introduced by IR may not be repaired adequately in cells depleted of BRAT1 and tested this in GBM cells and GSCs for the first time. Indeed, in GBM cells, shCtrl and BRAT1-KD cells showed equal levels of DSBs 1h after irradiation with 10 or 20 Gy. However, 24h after irradiation, almost all irradiation-induced DNA DSBs were repaired in the control cells, but still significantly elevated levels of DSBs were visible in BRAT1-KD cells, indicating a defective DNA repair. This was also the case in GSCs, whereas we additionally observed that already 1h after irradiation with 8 or 10 Gy significantly more DSBs were visible in the cells with depleted BRAT1. This suggests a general sensitization to irradiation in GSCs. Nonetheless, additional GSC lines are needed to solidify this hypothesis. Taken together, BRAT1 is important for effective DNA repair. Subsequently, we performed a colony formation assay to answer the question whether the colony formation ability is impaired in irradiated GBM cells lacking BRAT1 since they showed strongly elevated DNA DSB levels even 24h after irradiation. Unexpectedly, BRAT1 depleted GBM cells did not show significant differences compared to the control GBM cells. Next, Annexin V/propidium iodide staining followed by flow cytometric analysis was used to determine whether BRAT1 depletion leads to increased cell death after irradiation. Surprisingly, our cell death experiments showed that BRAT1-KD only moderately sensitizes GBM cells towards cell death induction. One possible explanation could be that the remaining amount of BRAT1 present in the cells is still sufficient in providing a survival benefit or that additional repair modalities can be exploited by the cells. On the other hand, given that we observe such a profound incapability of the cells to repair DNA damage, as shown using the foci assay, we suggest that multiple consecutive radiation treatments might result in greater differences in cell death.

Aglipay et al. previously reported that BRAT1 interacts with BRCA1 [23]. We therefore hypothesized that BRAT1-KD might prevent BRCA1 from binding to DNA DSBs. To test this hypothesis, an immunofluorescence staining was performed including 53BP1 (DNA DSB marker) and BRCA1. It was shown that in GBM cells and GSCs with BRAT1, BRCA1 accumulated around the DSB after irradiation and partly colocalized with the foci, i.e. was recruited to the DSB. In contrast, without BRAT1, BRCA1 remained diffusely distributed in and around the nucleus even after irradiation. This demonstrates that BRAT1 is responsible for the recruitment of BRCA1 to the DNA DSB. In addition to that, we frequently observed a strong perinuclear BRCA1-signal in BRAT1-KD cells, suggesting a novel function for BRAT1 in regulating the cytosolic to nuclear transport of BRCA1 upon DNA damage induction.

In summary, BRAT1 is essential for an efficient DNA repair, but does not affect GBM cell proliferation and colony formation, in contrast to previous findings in U2OS and HeLa cells [20], pointing towards cell type specific functions. Moreover, there are hints that GBM cells depleted of BRAT1 have a reduced radio-resistance because they show a slightly elevated cell death after irradiation. However, further investigations need to clarify this conclusively. We could also show that BRAT1 is responsible for BRCA1 recruitment to the DNA DSB. Future experiments will aim to further determine and characterize potential interactors and to analyze downstream effects of BRAT1 loss. In that way, we hope to gain insights of the mechanism of radio-resistance, also to better predict the effectiveness of radiotherapies on an individual basis.

4. Materials and Methods

4.1. Analysis of TCGA datasets

Analysis of the Gravendeel dataset [21] was performed and plots were exported via the GlioVis portal [22]. Differences of BRAT1 expression were compared between GBMs and healthy tissues based on histology and the Kaplan-Meier curve was derived from the Gravendeel datasets of all GBM subtypes combined with a median cut-off together with confidence intervals.

4.2. Cells and cell culture

Experiments were performed with NCH644 human glioblastoma stem-like cells [28] and U251 MG glioblastoma cells [29]. NCH644 cells were cultured in Neurobasal medium (Gibco, Darmstadt, Germany) supplemented with 1x B27 supplement, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 1x GlutaMAX (all from Gibco), 20 ng/mL epidermal growth factor, 20 ng/mL fibroblast growth factor (both from Peprotech, Hamburg, Germany) and 2 µg/mL Puromycin dihydrochloride (sc-108071B, Lot # L2214, Santa Cruz Biotechnology, Inc., Heidelberg, Germany). U251 MG cells were cultured in Dulbecco's modified Eagle's medium with GlutaMAX and heat-inactivated 10% fetal calf serum, 100 U/mL Penicillin, 100 µg/mL Streptomycin (all from Gibco) and 1 µg/mL Puromycin dihydrochloride (sc-108071B, Lot # L2214, Santa Cruz Biotechnology, Inc.). Both cell lines were cultured at 37°C and 5% CO_2 and were passaged twice a week in a ratio of 1:10 or 1:20, respectively.

4.2.1. Establishment of stable shRNA BRAT1 knockdown cells

Stable shRNA-mediated BRAT1-KD cells (pLKO.1-puro_shBRAT1_A, pLKO.1-puro_shBRAT1 _B, pLKO.1-puro_shBRAT1_C, pLKO.1-puro_shBRAT1_D; MISSION®, Sigma-Aldrich, Darmstadt, Germany) or control cells expressing non-mammalian targeting control shRNA (pLKO.1-Puro_shCtrl; MISSION® SHC002, Sigma-Aldrich) were generated using HEK293T cells. Shortly, 150,000 of HEK293T cells were seeded per well into 6-well plates the day before transfection with 2 µg plasmid DNA (pLKO.1-puro), 1.5 µg gag/pol plasmid (psPAX2, addgene #12260) and 0.5 µg VSV-G envelope plasmid (pMD2.G, addgene #12259) in 57 µL Opti-MEM (Invitrogen, Frankfurt am Main, Germany) and 6 µL FuGENE HD (Promega, Walldorf, Germany) transfection reagent. 6h later, medium was changed, and the viral supernatant was collected after additional 16h and 40h, pooled and filtered through a 0.45 µm filter, followed by dilution of the supernatant with fresh medium in a ratio of 1:1 and addition of 3 µg/mL hexadimethrine bromide (polybrene, Sigma-Aldrich). 5 µg/mL Puromycin were supplied and maintained to select for positively transduced cells. psPAX2 and pMD2.G were gifts from Didier Trono (Addgene plasmid # 12260 ; http//n2t.net/addgene:12260 ; RRID:Addgene_12260; Addgene plasmid # 12259 ; http//n2t.net/addgene:12259 ; RRID:Addgene_12259).

4.3. Irradiation and Antibodies

Cells were irradiated with single doses of 2, 4, 6, 8, 10 or 20 Gy using a linear accelerator (Elekta Synergy, Elekta, Crawley, UK). 6 MV photon energy, 100 cm focus to isocenter distance and a dose rate of 6 Gy/min were used.

The following primary antibodies and dilutions were used: rabbit-anti-BRCA1 1:200 (sc-6954, Santa Cruz Biotechnology, Inc.), mouse-anti-γH2AXSer139 1:1,000 (#05-636, clone JBW301, Merck Millipore, Darmstadt, Germany) and rabbit-anti-53BP1 1:1,000 (NB#100-304, Novus Biologicals, Wiesbaden, Germany) for immunofluorescence; rabbit-anti-BRAT1 1:250 (HPA029455 Sigma) and mouse-anti-Tubulin 1:5,000 (T6199, Sigma-Aldrich) for western blot.

The following secondary antibodies with corresponding dilutions were used: F(ab')2-goat-anti-rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 1:500 (A-11070, Thermo Fisher, Frankfurt am Main, Germany); F(ab')2-goat-anti-mouse IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 594 1:500 (A-11020, Thermo Fisher) for immunofluorescence; IRDye 800CW goat-anti-rabbit 1:1,000 (926-32211) and IRDye 680RD goat-anti-mouse 1:1,000 (926-68070; both LI-COR Biosciences, Bad Homburg, Germany) for western blot.

4.4. SDS PAGE and Western Blot

Cell lysis, SDS-PAGE and Western Blotting were performed as previously described [30]. In short, membranes were blocked for 1h at room temperature with 5% BSA/TBS-Tween-20 (TBS-T), following an incubation with the primary antibodies as mentioned above diluted in 5%BSA/TBS-T at 4°C overnight. The above described secondary antibodies, diluted in 5% BSA/TBS-T, were incubated for 1h at room temperature and signals were detected using a LI-COR Odyssey reader (LI-COR Biosciences).

4.5. Immunofluorescence Microscopy

8,000 U251 or NCH644 cells per well were seeded on 8-well chamber slides (Falcon, Corning, Amsterdam, New York, USA). In case of NCH644 cells, the slides were coated with 10 μg/mL Laminin (Sigma, L2020) at 4°C overnight beforehand. One day after seeding, the cells were irradiated as indicated. After different time points, they were fixed using 4% Formaldehyde/0,25% Triton X-100/ PBS, the above-mentioned primary antibodies were diluted in 5% BSA/PBS and incubated at 4°C overnight. Secondary antibodies were used as mentioned above, diluted in 5% BSA/PBS and incubated for 1h at room temperature in the dark. After this, the cells were either mounted on ProLong Gold antifade reagent with DAPI (Invitrogen) or Fluoroshield with DAPI histology mounting medium (Sigma). Slides were then stored at 4°C until analysis using a Nikon Eclipse TE2000-S inverted fluorescence microscope operated by the software NIS Elements AR version 4.2 (both Nikon Instruments Europe B.V., Amsterdam, Netherlands) at 60x magnification.

In the foci assay, the foci of 50 cells per condition were counted and in the analysis of BRCA1 immunofluorescence, at least 3 images of at least 10 cells per condition were obtained.

4.6. Flowcytometric analysis

Cell death was analyzed using an Annexin V/Propidium Iodide assay. For this, 30,000 U251 cells were seeded in 24-well plates and irradiated as indicated. 72h after irradiation, cells were harvested using Trypsin and resuspended in FACS-buffer (10 mM HEPES/NaOH pH7.4, 140 mM NaCl, 5 mM $CaCl_2$) containing Annexin V-APC (BD Biosciences, Heidelberg, Germany) and Propidium Iodide (Merck, Darmstadt, Germany). Afterwards, the cells were analyzed by a BD Accuri FACS (BD Biosciences).

4.7. Statistics

All statistical analyses (two-way ANOVA and Tukey's multiple comparisons test) were performed with GraphPad Prism 7 (GraphPad Software, La Jolla, California, USA).

5. Conclusions

Radio-resistance is a major problem in the treatment of glioblastomas. Among other factors, a subpopulation of cells, the glioma stem-like cells, are considered responsible for this. Based on our previous work we identified BRAT1 as a potentially important protein for DDR. So far, in this study,

we were able to show that BRAT1 is required for effective DNA repair and recruitment of BRCA1 to the DNA damage site. However, it has no effect on cell proliferation and colony formation. Furthermore, our findings reveal that BRAT1 loss causes GBM cells to be more susceptible to irradiation-induced cell death. Further investigations would particularly focus on exploring possible interactors and their co-operative impact on BRAT1-dependent mediation of radio-resistance in GSCs and GBMs. Thus, this study will pave the way on better understanding and assessing the effectiveness of radiotherapies on an individual basis.

Author Contributions: Conceptualization, B. L.; methodology, B. L., J. E, S. H. and Ö. G.; validation, B. L., D. K., S. H., Ö. G., and F. R.; formal analysis, J. E. and BL; investigation, B. L. and J. E.; resources, B. L., D. K. and F. R.; data curation, J. E. and B. L.; writing—original draft preparation, J. E.; writing—review and editing, D. K., B. L., S. H., Ö. G. and F. R.; visualization, J. E.; supervision, B. L. and D. K..; project administration, B. L.; funding acquisition B. L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors would like to thank Hildegard König for her ongoing excellent technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Louis, D.N.; Perry, A.; Reifenberger, G.; Deimling, A. von; Figarella-Branger, D.; Cavenee, W.K.; Ohgaki, H.; Wiestler, O.D.; Kleihues, P.; Ellison, D.W. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol.* **2016**, *131*, 803–820, doi:10.1007/s00401-016-1545-1.
2. Verhaak, R.G.W.; Hoadley, K.A.; Purdom, E.; Wang, V.; Qi, Y.; Wilkerson, M.D.; Miller, C.R.; Ding, L.; Golub, T.; Mesirov, J.P.; et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* **2010**, *17*, 98–110, doi:10.1016/j.ccr.2009.12.020.
3. Olar, A.; Aldape, K.D. Using the molecular classification of glioblastoma to inform personalized treatment. *J. Pathol.* **2014**, *232*, 165–177, doi:10.1002/path.4282.
4. Naumann, U.; Harter, P.; Rubel, J.; Ilina, E.; Blank, A.-E.; Esteban, H.; Mittelbronn, M. Glioma cell migration and invasion as potential target for novel treatment strategies. *Translational Neuroscience* **2013**, *4*, doi:10.2478/s13380-013-0126-1.
5. Tran, B.; Rosenthal, M.A. Survival comparison between glioblastoma multiforme and other incurable cancers. *J. Clin. Neurosci.* **2010**, *17*, 417–421, doi:10.1016/j.jocn.2009.09.004.
6. an Claes; Idema, A.J.; Wesseling, P. Diffuse glioma growth: a guerilla war. *Acta Neuropathol.* **2007**, *114*, 443–458, doi:10.1007/s00401-007-0293-7.
7. Davis, M.E. Glioblastoma: Overview of Disease and Treatment. *Clin. J. Oncol. Nurs.* **2016**, *20*, S2-8, doi:10.1188/16.CJON.S1.2-8.
8. Becker, K.P.; Yu, J. Status quo--standard-of-care medical and radiation therapy for glioblastoma. *Cancer J.* **2012**, *18*, 12–19, doi:10.1097/PPO.0b013e318244d7eb.
9. Annovazzi, L.; Mellai, M.; Schiffer, D. Chemotherapeutic Drugs: DNA Damage and Repair in Glioblastoma. *Cancers (Basel)* **2017**, *9*, doi:10.3390/cancers9060057.
10. Bao, S.; Wu, Q.; McLendon, R.E.; Hao, Y.; Shi, Q.; Hjelmeland, A.B.; Dewhirst, M.W.; Bigner, D.D.; Rich, J.N. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **2006**, *444*, 756–760, doi:10.1038/nature05236.
11. Erasimus, H.; Gobin, M.; Niclou, S.; van Dyck, E. DNA repair mechanisms and their clinical impact in glioblastoma. *Mutat. Res. Rev. Mutat. Res.* **2016**, *769*, 19–35, doi:10.1016/j.mrrev.2016.05.005.
12. Chakraborty, A.; Tapryal, N.; Venkova, T.; Horikoshi, N.; Pandita, R.K.; Sarker, A.H.; Sarkar, P.S.; Pandita, T.K.; Hazra, T.K. Classical non-homologous end-joining pathway utilizes nascent RNA for error-free double-strand break repair of transcribed genes. *Nat Commun* **2016**, *7*, 13049, doi:10.1038/ncomms13049.
13. Chen, J.; Li, Y.; Yu, T.-S.; McKay, R.M.; Burns, D.K.; Kernie, S.G.; Parada, L.F. A restricted cell population propagates glioblastoma growth after chemotherapy. *Nature* **2012**, *488*, 522–526, doi:10.1038/nature11287.
14. Bradshaw, A.; Wickremsekera, A.; Tan, S.T.; Peng, L.; Davis, P.F.; Itinteang, T. Cancer Stem Cell Hierarchy in Glioblastoma Multiforme. *Frontiers in Surgery* **2016**, *3*, 21, doi:10.3389/fsurg.2016.00021.

15. Adams, J.M.; Strasser, A. Is tumor growth sustained by rare cancer stem cells or dominant clones? *Cancer Res* **2008**, *68*, 4018–4021, doi:10.1158/0008-5472.CAN-07-6334.
16. Dirkse, A.; Golebiewska, A.; Buder, T.; Nazarov, P.V.; Muller, A.; Poovathingal, S.; Brons, N.H.C.; Leite, S.; Sauvageot, N.; Sarkisjan, D.; et al. Stem cell-associated heterogeneity in Glioblastoma results from intrinsic tumor plasticity shaped by the microenvironment. *Nat Commun* **2019**, *10*, 1787, doi:10.1038/s41467-019-09853-z.
17. Linder, B.; Wehle, A.; Hehlgans, S.; Bonn, F.; Dikic, I.; Rödel, F.; Seifert, V.; Kögel, D. Arsenic Trioxide and (-)-Gossypol Synergistically Target Glioma Stem-Like Cells via Inhibition of Hedgehog and Notch Signaling. *Cancers (Basel)* **2019**, *11*, doi:10.3390/cancers11030350.
18. Jean AF; Ouchi T. BRAT1 in Brain Function. *Journal of Neuro-Oncology and Neuroscience* **2016**, *1*.
19. So, E.Y.; Ouchi, T. The Potential Role of BRCA1-Associated ATM Activator-1 (BRAT1) in Regulation of mTOR. *2373-9436* **2013**, *1*, 3.
20. So, E.Y.; Ouchi, T. BRAT1 deficiency causes increased glucose metabolism and mitochondrial malfunction. *BMC Cancer* **2014**, *14*, 548, doi:10.1186/1471-2407-14-548.
21. Gravendeel, L.A.M.; Kouwenhoven, M.C.M.; Gevaert, O.; Rooi, J.J. de; Stubbs, A.P.; Duijm, J.E.; Daemen, A.; Bleeker, F.E.; Bralten, L.B.C.; Kloosterhof, N.K.; et al. Intrinsic gene expression profiles of gliomas are a better predictor of survival than histology. *Cancer Res* **2009**, *69*, 9065–9072, doi:10.1158/0008-5472.CAN-09-2307.
22. Bowman, R.L.; Wang, Q.; Carro, A.; Verhaak, R.G.; Squatrito, M. GlioVis data portal for visualization and analysis of brain tumor expression datasets. *Neuro-oncology* **2017**, *19*, doi:10.1093/neuonc/now247.
23. Aglipay, J.A.; Martin, S.A.; Tawara, H.; Lee, S.W.; Ouchi, T. ATM activation by ionizing radiation requires BRCA1-associated BAAT1. *J. Biol. Chem.* **2006**, 9710–9718, doi:10.1074/jbc.M510332200.
24. Yoshida, K.; Miki, Y. Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer science* **2004**, *95*, doi:10.1111/j.1349-7006.2004.tb02195.x.
25. Schulz, A.; Meyer, F.; Dubrovska, A.; Borgmann, K. Cancer Stem Cells and Radioresistance: DNA Repair and Beyond. *Cancers (Basel)* **2019**, *11*, 862, doi:10.3390/cancers11060862.
26. Ouchi, M.; Ouchi, T. Regulation of ATM/DNA-PKcs Phosphorylation by BRCA1-Associated BAAT1. *Genes Cancer* **2010**, *1*, 1211–1214, doi:10.1177/1947601911404222.
27. So, E.Y.; Ouchi, T. Functional interaction of BRCA1/ATM-associated BAAT1 with the DNA-PK catalytic subunit. *Exp. Ther. Med.* **2011**, *2*, 443–447, doi:10.3892/etm.2011.232.
28. Campos, B.; Wan, F.; Farhadi, M.; Ernst, A.; Zeppernick, F.; Tagscherer, K.E.; Ahmadi, R.; Lohr, J.; Dictus, C.; Gdynia, G.; et al. Differentiation therapy exerts antitumor effects on stem-like glioma cells. *Clin. Cancer Res.* **2010**, *16*, 2715–2728, doi:10.1158/1078-0432.CCR-09-1800.
29. Vaheri, A.; Ruoslahti, E.; Westermark, B.; Ponten, J. A common cell-type specific surface antigen in cultured human glial cells and fibroblasts: loss in malignant cells. *J. Exp. Med.* **1976**, *143*, 64–72, doi:10.1084/jem.143.1.64.
30. Antonietti, P.; Linder, B.; Hehlgans, S.; Mildenberger, I.C.; Burger, M.C.; Fulda, S.; Steinbach, J.P.; Gessler, F.; Rödel, F.; Mittelbronn, M.; et al. Interference with the HSF1/HSP70/BAG3 Pathway Primes Glioma Cells to Matrix Detachment and BH3 Mimetic-Induced Apoptosis. *Mol. Cancer Ther.* **2017**, *16*, 156–168, doi:10.1158/1535-7163.MCT-16-0262.