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ATRX INACTIVATION AND IDH1-R132H DRIVE PREFERENTIAL SENSITIVITY TO PROTON VS. X-RAY RADIOTHERAPY IN GLIOMA STEM CELLS

Ángel Adrián García

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ATRX INACTIVATION AND IDH1R132H DRIVE PREFERENTIAL SENSITIVITY TO PROTON VS. X-RAY RADIOTHERAPY IN GLIOMA STEM CELLS

by

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ATRX INACTIVATION AND IDH1$^{R132H}$ DRIVE PREFERENTIAL SENSITIVITY TO PROTON VS. X-RAY RADIOTHERAPY IN GLIOMA STEM CELLS

A Thesis

Presented to the Faculty of

The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
Master of Science

by
Ángel Adrián Garcés, B.S.
Houston, Texas
December 2021
Dedicated to the loving memory of

Sandra Whaley Bishnoi, PhD (1974-2018)
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graduated from Rice in 2018 and dedicated my MS at MD Anderson Cancer Center, the hospital where she received chemotherapy, to her memory. Thank you for everything Dr. Bishnoi, I strive to continue your mission of mentoring and inspiring the next generation of physicians and scientists throughout my academic career.
ATRX INACTIVATION AND IDH1R132H DRIVE PREFERENTIAL SENSITIVITY TO PROTON VS. X-RAY RADIOTHERAPY IN GLIOMA STEM CELLS

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Background: Glioma Stem Cells (GSCs) are self-renewable, treatment resistant cells in the glioma tumor mass known to promote tumor development. In contrast to traditional photon-based radiation therapy (XRT), proton radiation therapy (PRT) may induce more complex DNA damage and therefore might have the potential to eliminate GSCs. Although previous studies have individually linked IDH mutations, specifically IDH1R132H, and ATRX inactivating mutations to improved patient outcomes and suppressed DNA damage repair compared to their respective wild-types, the mechanisms by which these two genetic alterations interact in GSCs treated with PRT compared to XRT are currently unknown. We hypothesize that ATRX\(^{\text{Loss}}\) and IDH1\(^{R132H}\) both drive preferential sensitivity to PRT compared to XRT.

Methods: Isogenic human GSC lines TS543-ATRX\(^{WT}\), TS543-ATRX\(^{Loss}\), MGG18-IDH1\(^{WT}\), and MGG18-IDH1\(^{R132H}\) were subjected to either XRT or PRT. Human GSC lines TS603-ATRX\(^{WT}\)/IDH1\(^{R132H}\) and GS522-ATRX\(^{Loss}/\text{IDH1}^{R132H}\) were subjected to a combination of Ivosidenib, a reversible selective IDH1\(^{R132}\) inhibitor, and either XRT or PRT. Extreme limiting dilution analysis (ELDA) was used to calculate the active cell frequency, a measure of GSC self-renewal. Post-radiation GSC viability was quantified using the CellTiterGlo 3D assay at 14 days after XRT or PRT.\(^{1,2}\) The primary
mechanisms of radiation-induced cell death were determined using the RealTime-Glo Annexin V apoptosis and necrosis assay at 0-72 hours after irradiation.

**Results:** Using isogenic TS543 GSCs, ATRX\textsuperscript{Loss} diminished cell viability and self-renewal primarily by inducing cell death via apoptosis and secondary necrosis compared to ATRX\textsuperscript{WT}. Isogenic MGG18-IDH1\textsuperscript{R132H} GSCs treated with PRT consistently exhibited increased apoptotic and necrotic cell death compared to XRT. MGG18-IDH1\textsuperscript{WT} demonstrated increased apoptotic cell death after PRT compared to XRT. Finally, combining Ivosidenib with either XRT or PRT diminished survival by upregulating apoptotic and necrotic cell death in TS603-ATRX\textsuperscript{WT}/IDH1\textsuperscript{R132H} GSCs. However, the opposite effects were observed in GS522-ATRX\textsuperscript{Loss}/IDH1\textsuperscript{R132H} GSCs.

**Conclusions:** PRT was more effective than XRT in inducing GSC death across several cell lines. ATRX inactivation increased the efficacy of PRT via apoptotic and necrotic cell death. IDH1\textsuperscript{R132H} does not significantly improve radiation induced cell death in ATRX\textsuperscript{WT} GSCs. Combining IDH1\textsuperscript{R132H} inhibitors with PRT in ATRX\textsuperscript{WT}/IDH1\textsuperscript{R132H} GSCs may represent a novel treatment strategy to overcome radioresistance.
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Introduction

Clinical Standard of Care

Glioblastoma Multiforme

Glioblastoma Multiforme (GBM-Grade IV Glioma) is the most prevalent and aggressive glioma in the USA to date, with a median survival of only 15 months.\textsuperscript{3-5} Although the exact symptoms experienced by a patient with GBM depends greatly on tumor’s size and location, many patients exhibit seizures as well as headaches and neurological deficits caused by a heightened intracranial pressure.\textsuperscript{6} Found predominately in non-Hispanic white males, there are few established risk factors that are linked to GBM development apart from ionizing radiation exposure. The high median age of diagnosis (65 years old) and difficulty in performing complete tumor resection are negative prognostic markers contributing to the uncurable nature of GBM.\textsuperscript{7}

The diagnostic criteria set forth by the World Health Organization (WHO) in 2016 classifies gliomas from least to most malignant (grade I-IV) using histopathology and molecular biomarkers.\textsuperscript{8} Although 90\% of GBM tumors develop spontaneously with no precursor lesions as primary GBM in elderly adult patients, young adult patients more often exhibit malignant transformation of a low grade glioma (LGG-WHO Grade II) to a high-grade glioma (HGG-Grade III) or secondary GBM. Patients harboring secondary GBM tumors exhibit substantially increased median survival (5-10 years) compared to patients with primary GBM (1-2 years).\textsuperscript{9,10} The current standard of care for glioblastoma involves surgical removal followed by chemotherapy, especially with the DNA alkylating agent temozolomide (TMZ), and radiation therapy.\textsuperscript{3,11,12}
Low-Grade Gliomas

Although treatments for LGGs are not as standardized as GBM, maximal safe surgical resection, chemotherapy, and radiation therapy are still frequently utilized in the clinic.\textsuperscript{13} Diffuse grade II and III gliomas can be subclassified into oligodendrogliomas (ATRX wild-type, IDH mutant, chromosomal 1p/19q co-deletion) and astrocytomas (ATRX inactivation, IDH mutant, chromosomal 1p/19q intact) by using a combination of molecular and histopathologic analytical approaches (Figure 1).\textsuperscript{11} Patients with LGGs commonly experience uncontrolled seizures and can be further identified as “low-risk (age<40 years old AND gross total resection)” or “high-risk (age>40 years old OR subtotal resection)” depending on age and surgical approach. The specific surgical technique used to extract the tumor is decided upon based on how close the tumor is to areas in the brain that control important motor or sensory functions.\textsuperscript{14}

The importance of resecting as much tumor mass possible cannot be understated as Smith et. al (2008) demonstrated that the five-year patient survival rate drops substantially from 97% to 76% if <90% of the tumor mass is removed.\textsuperscript{15} While the probability of tumor recurrence is significantly elevated for high-risk patients, especially those with wtIDH tumors, the decision on whether or not to pursue post-operative chemoradiotherapy remains unclear. This decision is especially difficult in low-risk patients with a potential 5-year recurrence rate of 52% who might receive more benefit from routine MRI surveillance.\textsuperscript{16} Therefore, it is important to develop new therapeutic modalities based on a tumor’s molecular profile to diminish the risk of both tumor recurrence and malignant transformation, which is seen in upwards of 72% of all LGG cases within 3-5 years of diagnosis (Figure 2).\textsuperscript{17}
Figure 1: Histopathological and genetic profiles of low-grade gliomas. Representative hematoxylin and eosin (H&E) photomicrographs of (A) astrocytomas and (B) oligodendrogliomas. Astrocytomas are characterized by extended cell shape as well as inconsistently shaped, hyperchromatic nuclei. Oligodendrogliomas exhibit round shaped nuclei with perinuclear haloes. IDH: Isocitrate Dehydrogenase. ATRX: alpha-thalassemia X-linked mutant retardation syndrome. 1p/19q co-deletion: whole arm loss of chromosomes 1p/19q.

Figure 2: Low-grade glioma to glioblastoma multiforme malignant transformation. LGG malignant transformation is defined starting from grade II glioma. Grade I gliomas (most common: pilocytic astrocytoma) are not included since they are generally considered benign and are frequently cured with surgery alone. Grade I gliomas are primarily found only in pediatric patients, while grade II-IV gliomas are found primarily in adult patients.\textsuperscript{18}

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Molecular Diagnostic Tools

*Isocitrate Dehydrogenase Mutations*

Isocitrate Dehydrogenase (IDH) mutations represent a viable therapeutic target for impairing malignant transformation due to their high prevalence in LGGs and secondary GBMs. However, these mutations are rarely found in primary GBMs. The most common IDH mutations present in LGGs associated with tumorigenesis are heterozygous missense mutations at arginine 132 of the IDH1 isoform (IDH1<sup>R132</sup>), at arginine 140 of the IDH2 isoform (IDH2<sup>R140</sup>), or at arginine 172 of the IDH2 isoform (IDH2<sup>R172</sup>). IDH1 functions in the cytosol while IDH2 functions in the mitochondria. However, the mutation of IDH1 arginine 132 to histidine (IDH1<sup>R132H</sup>) is by far the most prevalent in clinical care representing 70-80% of low-grade glioma (LGG) and secondary GBM tumor cases.

IDH1<sup>R132H</sup> is often the first genetic alteration to occur in patients, presenting in approximately 80% of grade II-III gliomas. An analysis of 486 patients conducted by Tom et al. (2019) discovered that patients harboring IDH1<sup>R132H</sup>/chromosomal 1p19q<sup>codeletion</sup> (oligodendrogliomas) exhibit higher overall survival and longer time to malignant transformation compared to patients with IDH1<sup>R132H</sup>/chromosomal 1p19q<sup>intact</sup> and IDH<sup>WT</sup> tumors. The repeated association of IDH mutations with favorable clinical prognoses has motivated many studies into investigating the biomolecular changes exhibited by cells harboring IDH mutations. A leading hypothesis by which IDH1<sup>R132H</sup> specifically drives improved patient survival and therapeutic response is due to the neomorphic production of 2-Hydroxyglutarate (2-HG) from alpha-ketoglutarate (α-KG) in the cytosol compared to IDH1<sup>WT</sup> (*Figure 3*). The accumulation of supraphysiological
2-HG concentrations inside cells with IDH1<sup>R132H</sup> serves as a critical biomarker and competitive inhibitor of α-ketoglutarate dependent dioxygenases, which are known to regulate the tumor epigenome, cell differentiation, angiogenesis, and hypoxic stress.<sup>26-28</sup> The inhibition of α-ketoglutarate dependent dioxygenases due to heightened concentrations of 2-HG results in a variety of intracellular changes that are correlated with improved patient survival (Figure 4).<sup>29</sup>
Figure 3: IDH1\textsuperscript{R132H} induces production of (R)-2-Hydroxyglutarate

(A) IDH1\textsuperscript{WT} converts isocitrate and NADP\textsuperscript{+} into α-ketoglutarate, NADPH, and CO\textsubscript{2}.

(B) IDH1\textsuperscript{R132H} reduces the α-ketoglutarate created by IDH1\textsuperscript{WT} into the oncometabolite (R)-2-Hydroxyglutarate (2-HG).

This figure was created by combining the ideas presented in Figure 2 of “Molenaar RJ, Maciejewski JP, Wilmink JW, et al: Wild-type and mutated IDH1/2 enzymes and therapy responses. Oncogene 37:1949-1960, 2018” and Figure 3 of “Tommasini-Ghelfi S, Murnan K, Kouri FM, et al: Cancer-associated mutation and beyond: The emerging biology of isocitrate dehydrogenases in human disease. Sci Adv 5:eaaw4543, 2019.” Both articles are licensed under the Creative Commons Attribution Non-Commercial-ShareAlike 4.0 International License.
Figure 4: The effects of (R)-2-Hydroxyglutarate in IDH mutant gliomas.
The oncometabolite (R)-2-Hydroxyglutarate (2-HG) acts as a competitive inhibitor of α-ketoglutarate dependent dioxygenases, which promotes changes in reactive oxygen species production, dsDNA damage response, epigenetic states, and angiogenesis.

Since 2-HG is known to enter the nucleus from the cytosol, changes in the epigenetic landscape of IDH1<sup>R132H</sup> tumors compared to IDH1<sup>WT</sup> tumors are currently a highly active area of research. An analysis of GBM samples from The Cancer Genome Atlas (TCGA) conducted by Noushmehr et al (2010) first identified a connection between IDH1<sup>R132H</sup> and the glioma-CpG island methylator phenotype (G-CIMP). This phenotype (G-CIMP+) was found predominately in proneural GBMs and was associated with a longer survival time after diagnosis compared to G-CIMP negative (G-CIMP-) tumors. The absence of the G-CIMP phenotype was found mainly in mesenchymal GBM cells. A follow-up study conducted by de Souza et al (2018) further classified IDH mutant, G-CIMP+ tumors into G-CIMP high and G-CIMP low. G-CIMP high tumors exhibited significantly higher levels of DNA methylation and improved survival outcomes compared to patients with G-CIMP low tumors, despite the fact that both subtypes harbored IDH mutations. These findings establish the potential value of DNA hypermethylation due to histone demethylase inhibition by 2-HG as a future prognostic marker for patients with LGG or secondary GBM.

Although the mechanisms by which cytosolic 2-HG produced by IDH1<sup>R132H</sup> promotes favorable patient outcomes are still under investigation, several reports have presented evidence to suggest that IDH1<sup>R132H</sup> promotes favorable outcomes to radiation therapy. Sulkowski et al (2017) demonstrated that 2-HG directly contributes to the creation of dsDNA lesions by ionizing radiation and is implicated in impairing dsDNA damage repair via Homologous Recombination (HR) in HCT116 and HeLa cells. Further studies demonstrated that <em>in vitro</em> radiosensitivity in IDH1<sup>R132H</sup> human astrocytes, glioma stem cells, and cholangiocarcinoma is further enhanced in the presence of poly(ADP-
ribose) polymerase (PARP) inhibitors, such as Olaparib and Veliparib. In addition to the increased dsDNA damage and impaired dsDNA lesion repair exhibited in irradiated IDH1<sup>R132H</sup> cells, Yin et al (2019) demonstrated that IDH1<sup>R132H</sup> suppresses the expression of TIGAR (TP53-induced glycolysis and apoptosis regulator) due to the presence of the heterochromatin associated epigenetic alteration H3K9me3 at TIGAR promoters. TIGAR is a critical gene that impairs the production of fructose-2,6-bisphosphate, which suppresses glycolysis as well as reactive oxygen species (ROS) production. Yu et al (2020) built upon these results by demonstrating that isogenic IDH1<sup>R132H</sup> U87 glioblastoma cells naturally exhibit increased ROS production without the addition of external agents compared to IDH1<sup>WT</sup> U87 glioblastoma cells. In the subpopulation of highly chemoradioresistant human glioma stem cells, IDH1<sup>R132H</sup> is known to decrease migration and invasion while simultaneously increasing apoptosis and differentiation. These findings implicate IDH1<sup>R132H</sup> as a highly indispensable factor in ensuring that radiation therapy can effectively induce dsDNA damage and cancer cell death.

In addition to radiosensitivity, several studies have linked IDH mutations to improved chemotherapeutic sensitivity. Specifically, IDH1<sup>R132H</sup> has been implicated in promoting glioma cell death after treatment with the DNA alkylating agent temozolomide (TMZ), which is considered part of the standard of care for glioblastoma, through a variety of mechanisms. One highly studied mechanism of IDH1<sup>R132H</sup> driven TMZ sensitivity is the suppression of NADPH and GSH production, possibly due to the decreased expression of the antioxidant Nrf2 in U87 and U251 IDH1<sup>R132H</sup> glioblastoma cells. TMZ sensitivity is further enhanced when administered in combination with PARP inhibitors, as demonstrated by increased γH2AX foci Lu et al (2017) in U87
IDH1<sup>R132H</sup> glioblastoma cells. Sule et al (2021) further built upon these findings by establishing that U87- IDH1<sup>R132H</sup> and T229-IDH1<sup>R132H</sup> glioblastoma cells as well as HCT116-IDH1<sup>R132H</sup> colon cancer cells treated with a combination of PARP inhibitors and ATR inhibitors experienced decreased clonogenic survival, suppressed HR, premature mitotic entry, and increased apoptosis. Although several studies have established an association between IDH1<sup>R132H</sup> and improved chemotherapeutic sensitivity, the use of IDH1<sup>R132H</sup> itself as a therapeutic modality has not been explored until recently. Promising preclinical data has demonstrated the efficacy of IDH1<sup>R132H</sup> peptide vaccines through their ability to bind onto neoepitopes on MHC class II and induce CD4+ Th cell IFN-γ production. This data led to the development of a Phase I clinical trial (Platten et al 2021, ClinicalTrials.gov identifier NCT02454634) that demonstrated vaccine induced T and B cell immunogenicity in 33 patients with either grade III or IV IDH1<sup>R132H</sup> astrocytomas. Although several studies have investigated the biomolecular mechanisms by which IDH mutations independently confer positive patient prognosis, the interactions between IDH mutations and other LGG genetic alterations are still under active investigation.
Alpha-Thalassemia X-linked Mutant Retardation Syndrome Mutations

Inactivating mutations of the N-terminal chromatin binding ATRX-DNMT3-DNMT3L (ADD) domain in the ATRX protein (alpha-thalassemia X-linked mutant retardation syndrome; ATRX\textsuperscript{Loss}) represents another important genetic alteration and potential therapeutic target specific to patients with astrocytomas.\textsuperscript{44} Since ATRX\textsuperscript{Loss} commonly presents alongside IDH mutations in tumors with intact 1p/19q chromosomes, the World Health Organization modified the diagnosis of astrocytomas to include these genetic alterations alongside established histopathological guidelines.\textsuperscript{11,45} ATRX\textsuperscript{Loss} is predominately found in young adult patients with grade II astrocytomas (median age=30 years old) and grade III anaplastic astrocytomas (median age=35 years old). ATRX\textsuperscript{Loss} has frequently been associated with improved progression-free and overall survival compared to tumors with wild-type ATRX expression (ATRX\textsuperscript{WT}).\textsuperscript{44-46} This also holds true for adult patients with ATRX\textsuperscript{Loss} GBM and pediatric patients with ATRX\textsuperscript{Loss} high-grade gliomas (HGGs).\textsuperscript{47,48}

ATRX itself is a ATP dependent SWI/SNF2 chromatin remodeling protein that maintains genome stability by depositing histone H3.3 at telomeres and pericentromeric heterochromatin when combined with its transcriptional co-factor DAXX (Death-Domain Associated Protein; See Figure 5).\textsuperscript{46} In gliomas, ATRX\textsuperscript{Loss} has been linked to promoting the development of the alternative lengthening of telomeres (ALT) phenotype. This is a homologous recombination (HR) based mechanism for telomere maintenance that does not require telomerase. The main function of ALT is to help cancer cells proliferate indefinitely to evade apoptosis or senescence.\textsuperscript{49} ALT+ gliomas are characterized by the presence of tumor suppressing promyelocytic leukemia nuclear bodies (PML-NBs),
ultrabright telomeric DNA foci, heterogeneous telomere length due to inter/intra-chromosomal telomere exchange, and extrachromosomal DNA C-circles.\cite{46,50} Mechanistically, Li et al (2019) established that the ATRX-DAXX-H3.3 complex is required to protect chromosomal telomere structure and to maintain normal telomere DNA replication. Loss of ATRX or DAXX induces delayed telomere replication stress and dysfunction that cannot be fixed by telomerase, thereby resulting in a dependence on the ALT mechanism to maintain telomere length and promote cell immortalization (Figure 6).\cite{51}
Figure 5: Intracellular characteristics of ATRX\textsuperscript{WT} and ATRX\textsuperscript{Loss} cells

ATRX has the capability to function both dependently and independently of its transcriptional co-factor DAXX to promote chromatin stability and telomere repair.

Figure 6: ATRX<sup>Loss</sup> promotes telomere maintenance via ALT.

Although ATRX intact cells can repair telomere length through the conventional telomerase pathway, ATRX<sup>Loss</sup> cells lose the ability to recruit H3.3 and localize to telomere ends, thereby requiring the use of the alternative lengthening of telomeres (ALT) pathway.

Alongside its role in promoting the development of the ALT phenotype, several reports have established a connection between ATRX\textsuperscript{Loss} and radiation induced cytotoxicity via the creation of dsDNA breaks. Koschmann et al (2016) discovered that mouse GBM cells harboring shp53/NRAS/shATRX exhibited significantly diminished cell proliferation rates \textit{in vitro} compared to mouse GBM cells with shp53/NRAS/intact ATRX\textsuperscript{WT} due to heightened dsDNA damage from exposure to increasing doses of ionizing radiation. The authors present strong evidence to suggest that ATRX\textsuperscript{Loss} significantly impairs the successful resolution of dsDNA lesions via NHEJ. No significant changes in HR mediated DNA damage repair were attributed to ATRX\textsuperscript{Loss}\textsuperscript{48}.

On the contrary, Juhász and colleagues (2018) established that ATRX is indispensable to radiation-induced dsDNA lesion repair due to its function downstream of RAD54 by complexing with PCNA and RFC-1 to form double Holliday junctions and promote sister chromatid exchange.\textsuperscript{52} Follow up studies confirmed that the repair of two-ended dsDNA breaks via HR-DSBR (Double-Strand Break Repair) is predominately driven by ATRX while repair via HR-SDSA (Synthesis-Dependent Single Strand Annealing) is predominately driven by RECQ5. Despite the risks of carcinogenesis posed by loss of heterozygosity associated with the formation of crossover products, ATRX-driven HR repair is the dominant pathway to repair two-ended dsDNA breaks created by ionizing radiation in HeLa, U2Os, and 82-6 cell lines. Inhibiting access to either one or both of these modes of HR repair via siRNA knockdown (siATRX or siRECQ5) results in enhanced dsDNA breaks as quantified by γH2AX foci.\textsuperscript{53}

Another mechanism by which ATRX\textsuperscript{Loss} drives radiosensitivity is through the formation of DNA G-quadruplexes (G4) secondary structures and chromosome breaks, as
demonstrated by Wang et al (2019) in isogenic normal human astrocyte (NHA) and human glioma stem cells (GSC). ATRX\textsuperscript{WT} is known to protect cells from genomic instability by binding and resolving G4 quadruplexes through the deposition of H3.3 at G-rich sites as well as protecting stalled replication forks from collapsing and causing DNA damage.\textsuperscript{46,54,55} Wang et al (2019) report that although ATRX\textsuperscript{Loss} alone was insufficient to induce apoptotic cell death, isogenic NHAs and GSCs harboring ATRX\textsuperscript{Loss} exhibited dramatically lower cell viability, significant apoptotic cell death, higher phosphorylated CHK1 expression, and higher counts of γH2AX/53BP1 dsDNA damage foci when treated with G4 stabilization compounds (CX-3543, Pyridostatin, or CX-5461) compared to ATRX\textsuperscript{WT} cells. This effect was further pronounced when combining G4 stabilization agents with ionizing radiation, which demonstrates the important role of ATRX\textsuperscript{WT} in protecting cells from radiation induced dsDNA damage.\textsuperscript{55,56} These findings suggest that combining G4 stabilization agents with ionizing radiation might prove to be an effective new treatment option for patients with astrocytomas.

Due to the prevalent use of isogenic cell lines to study the functions of ATRX\textsuperscript{Loss} and IDH mutations independently \textit{in vitro}, the combined effects of IDH mutations and ATRX\textsuperscript{Loss} in the same cell are still not completely understood. ATRX\textsuperscript{Loss} and IDH\textsuperscript{R132H} are known to work together in driving the development of the ALT\textsuperscript{+} phenotype and gliomagenesis \textit{in vitro}.\textsuperscript{57} Additionally, ATRX\textsuperscript{Loss} has previously been implicated in promoting transwell migration in IDH\textsuperscript{R132H} GSCs compared to ATRX\textsuperscript{WT}, possibly due to the heightened expression of the GNA13 protein.\textsuperscript{58} These findings implicate a specific biomolecular mechanism that can be targeted to ensure effective gross total surgical resection of astrocytomas. Although ATRX\textsuperscript{Loss} also drives the formation of DNA G4
quadruplexes in isogenic GSCs, IDH1\textsuperscript{R132H} does not significantly enhance G4 development when expressed alongside ATRX\textsuperscript{Loss} in the same cell.\textsuperscript{55} Human astrocytes and glioblastoma cells treated with increasing concentrations of known PARP inhibitor Olaparib exhibited a similar trend with regards to clonogenic survival and phosphorylated CHK1 (Ser-345) expression, a measure of DNA replication stress due to its affinity for ssDNA breaks with ATR and RPA proteins, when comparing cells with ATRX\textsuperscript{Loss} or IDH1\textsuperscript{R132H} alone compared to ATRX\textsuperscript{Loss} and IDH1\textsuperscript{R132H}. PARP inhibitors appear to synergize well with ATR inhibitors to impair cell cycle arrest in S phase and allow the repair of ssDNA breaks in isogenic ATRX\textsuperscript{Loss} and IDH1\textsuperscript{R132H} glioblastoma cells.\textsuperscript{41,59}

Although this synergistic effect has not yet been explored in glioma cells harboring both ATRX\textsuperscript{Loss} and IDH1\textsuperscript{R132H}, these results demonstrate promising evidence to suggest that G4 stabilization compounds might synergize well with ATR and PARP inhibitors to further promote the efficacy of radiation induced DNA damage and cytotoxicity in low-grade glioma cells.
Glioma Stem Cells

Although IDH mutations and ATRX\textsuperscript{Loss} serve as valuable molecular markers in glioma diagnosis, the development of new therapeutic modalities that enhance patient survival is further complicated due to the intratumoral genetic, epigenetic, and phenotypic heterogeneity present in LGG and GBM.\textsuperscript{60,61} One of the key drivers of tumor heterogeneity are the glioma stem cells (GSC) in the tumor mass, which are heavily implicated in post-treatment tumor recurrence and diminished patient survival.

The cancer stem cell (CSC) hypothesis states that tumors develop from self-renewable, undifferentiated cells, similar to how normal stem cells differentiate into a diverse array of cell types in the human body with a set lifetime.\textsuperscript{62} Lapidot et al (1994) were the first to identify CSCs using acute myeloid leukemia as their model system.\textsuperscript{63} Since then, CSCs have been found in various types of solid tumors, including glioblastoma, breast, and pancreatic cancers.\textsuperscript{64} The discovery of glioma and glioblastoma tumor initiating stem cells in particular by Singh et al in 2003 has spurred the development of many studies working to understand the mechanisms by which GSCs are involved in tumor formation and how they impact therapeutic sensitivity.\textsuperscript{64} Although convincing evidence for the existence of GSCs has been presented by several research groups, the identification of one specific biomarker to easily find GSCs in the tumor mass is still an active area of investigation. Several cell surface markers are known to be upregulated in GSCs, such as CD133, CD15, LICAM, and SOX2.\textsuperscript{65,66} However, these cell surface markers are not ubiquitous enough in all GSCs to be designated as biomarkers. Proneural GSCs exhibit heightened CD133 expression and grow as neurospheres in culture while mesenchymal GSCs express lower levels of CD133 on
their cell surfaces and grow semi-adherantly in culture.\textsuperscript{67,68} The identification of GSCs becomes further complicated due to their ability to dynamically change their cell surface receptors in response to the upregulation of neurodevelopmental transcription factors, such as POU3F2, SOX2, SALL2, and OLIG2 as identified by Suva et al (2014).\textsuperscript{69,70} The function of a GSC also depends greatly on its specific microenvironmental “niche,” the most common of which are the perivascular tumor niche, the hypoxic niche, and the invasive niche in GBM.\textsuperscript{71,72} The heavily dynamic nature of GSCs has led to some to question the CSC hypothesis altogether and suggest that instead of operating as a distinct cell subpopulation, these cells are merely adapting to microenvironmental cues to evade cytotoxicity and promote tumor survival.\textsuperscript{73} Despite the genetic and phenotypic plasticity exhibited by GSCs, many new treatments are focused on modulating the “stemness” of GSCs to ensure their elimination and inability to promote secondary tumor formation.\textsuperscript{72}

GSCs are able to differentiate into several different cell types within the tumor microenvironment, such as stromal cells, vasculature, fibroblasts, and tumor associated macrophages.\textsuperscript{65,72} GSCs also contribute to tumor chemoradioresistance due to their ability to indefinitely self-renew as well as transition back to an undifferentiated state after differentiating into a tumor cell as a means to avoid elimination by cytotoxic therapies. Dahan et al (2014) proved that glioblastoma cells exhibit plasticity and can de-differentiate into a cancer stem-cell like state when exposed to ionizing radiation, even at subtoxic doses, via the upregulation of the anti-apoptotic protein survivin.\textsuperscript{74} Additionally, Bao et al (2006) demonstrated that human glioma xenograft treated with ionizing radiation caused a shift towards an increase of CD133+ GSCs both \textit{in vitro} and \textit{in vivo}. CD133+ GSCs exhibited higher post-radiation clonogenic surviving fraction, a decrease
in cleaved caspase-3, and increased expression of key DNA damage repair checkpoint proteins, including pATM, pRAD17, pChk1, and pChk2, thereby promoting the development of a radioresistant phenotype.\textsuperscript{75} Balbous et al (2016) built upon these findings and demonstrated the efficacy of reversing the radioresistant phenotype of GSCs through the combination of RAD51 inhibitors with ionizing radiation.\textsuperscript{76} The radioresistance of GSCs can also be understood through the suppression of reactive oxygen species (ROS) scavenger production generally exhibited by CSCs, thereby leading to decreased intracellular oxidative stress.\textsuperscript{77-79}

With regards to chemotherapeutic resistance, Temozolomide treated GSCs created from the de-differentiation of GBM cells (induced GSCs, or iGSCs) exhibited higher proliferation rates and survival rates than the original GBM cells due to the activation of the NOTCH1 and Wnt/β-Catenin pathways as well as the expression of hypoxia-inducible factors (HIF) 1α and 2α.\textsuperscript{80,81} The unique ability of GSCs to indefinitely self-renew, differentiate, and de-differentiate results in the generation of clones with various levels of chemoradioresistance scattered throughout the tumor.\textsuperscript{82,83} These characteristics ultimately work to promote gliomagenesis and tumor diffusion throughout the brain, thereby increasing the difficulty of surgically resecting the entirety of the tumor mass from the patient (Figure 7). Therefore, the elimination of the entire GSC population to prevent unfavorable patient prognoses cannot be understated. For these reasons, I selected isogenic glioma stem cells as the model system for my \textit{in vitro} studies to identify the mechanisms by which ATR\textsuperscript{X}Loss and IDH mutations drive radiation sensitivity.
Figure 7: A mechanistic summary of cancer stem cell driven tumor recurrence.
Cancer Stem Cells (CSC) possess the innate ability to self-renew and differentiate into any type of cell within the tumor. The chemoradioresistant and immunosuppressive nature of CSCs make their elimination from the tumor quite difficult, which may result in secondary tumor formation. Figure created using Biorender.com.
Radiation Therapy: Protons vs. X-Rays

A highly promising therapeutic modality for targeting and effectively treating the entirety of the glioma mass and GSC subpopulation is proton radiation therapy (PRT). First introduced in the 1950s, PRT may preferentially target tumor cells while sparing healthy tissues compared to conventional X-ray therapy (XRT). X-rays deposit a significant entry dose before reaching the tumor and a significant exit dose after leaving the tumor. However, protons deposit a comparatively lower entry dose before coming to a halt at the Bragg peak, defined as the depth at which a proton of a given energy maximizes its dose deposition. Protons have the added benefit of not depositing an exit dose, thereby reducing the risk of off-target radiation toxicity.

In addition to its favorable dose-depth profile, PRT is known to induce more clustered and complex lethal dsDNA breaks (DSBs) compared to conventional XRT. This is indicated by the enlarged size and prevalence of gH2AX foci in vitro. The resolution of these clustered DSBs relies heavily on proteins involved in the homologous recombination (HR) DNA damage repair pathway, especially RAD54, compared to non-homologous end joining (NHEJ). However, despite these biological advantages, each mono-energetic proton beam creates a “pristine” Bragg peak that is too thin to deliver the maximum dose to the entirety of the tumor. To solve this problem, a “Spread-Out” Bragg peak is created by delivering several proton beams of various energies together to create an area with uniform dose distribution. This ensures that the entirety of the tumor receives an equal dose while also minimizing off-target radiation toxicity to sensitive areas around the tumor (Figure 8).
The density of ionization events responsible for DSBs is directly proportional to Linear Energy Transfer (LET), which refers to the energy lost along the path length of a proton beam. The LET of a proton beam directly impacts its Relative Biological Effectiveness (RBE), defined as a ratio of XRT to PRT dose that achieves a given biological effect. Specifically, Carter et al (2018) demonstrated that high LET protons induce higher levels of single stranded and double stranded DNA damage as well as ubiquitination on H2B lysine 120 compared to low LET protons in HeLa and oropharyngeal squamous cell carcinoma lines. Motivated by previous results demonstrating the efficacy of high LET protons in inducing cancer cell death, Ma et al (2020) created a modified proton SOBP with a higher weight placed on low-energy, high LET proton beams without impacting the dose delivered. This new “downslope” dose-depth profile successfully delivered a higher LET, which resulted in an increased number of persistent γH2AX and 53BP1 dsDNA damage foci as well as decreased clonogenic survival in H460 lung cancer cells. These results demonstrate the importance of optimizing future treatment plans to emphasize the delivery of high LET protons for maximum therapeutic efficacy.

Although PRT offers benefits over conventional XRT, the diffuse nature of LGGs presents difficulties in eliminating the entirety of the tumor mass and GSC population. Alan Mitteer et al (2015) demonstrated that PRT induced DSBs and GSC toxicity is dependent upon the successful generation of ROS. However, the understanding of how key genetic alterations used in the diagnosis of low-grade gliomas, specifically IDH mutations and ATRX inactivation, impact the cell death mechanisms that govern the efficacy of XRT vs. PRT induced GSC toxicity remain unknown.
Figure 8: Traditional spread-out Bragg peak (SOBP) for proton radiotherapy. 
(A) The percent dose-depth curve for a proton SOBP delivered using 16 discrete energies at The University of Texas MD Anderson Cancer Center. Higher energy beams are traditionally weighted more heavily than lower energy beams when creating a traditional SOBP. (B) The normalized dose-depth curve demonstrates the rising LET towards the SOBP distal fall off point.

Source: Ma D, Bronk L, Kerr M, et al: Exploring the advantages of intensity-modulated proton therapy: experimental validation of biological effects using two different beam intensity-modulation patterns. Scientific Reports 10, 2020. “This is an open access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.”
Hypothesis and Specific Aims

Specific Aim 1: To determine the independent effects of ATRX<sup>Loss</sup> and IDH1<sup>R132H</sup> on the viability and self-renewal of irradiated human glioma stem cells.

*Aim 1A:* To investigate the efficacy of ATRX<sup>WT</sup> and ATRX<sup>Loss</sup> to impair cell viability and self-renewal after treatment with XRT vs. PRT in human glioma stem cells.

*Aim 1B:* To investigate the efficacy of IDH1<sup>WT</sup> and IDH1<sup>R132H</sup> to impair cell viability and self-renewal after treatment with XRT vs. PRT in human glioma stem cells.

Specific Aim 2: To identify the primary post-XRT and post-PRT cell death mechanisms in ATRX<sup>Loss</sup> isogenic human glioma stem cells.

*Aim 2A:* To quantify the extent of apoptotic and necroptotic cell death in ATRX<sup>WT</sup> and ATRX<sup>Loss</sup> human glioma stem cells treated with XRT.

*Aim 2B:* To quantify the extent of apoptotic and necroptotic cell death in ATRX<sup>WT</sup> and ATRX<sup>Loss</sup> human glioma stem cells treated with PRT.

Specific Aim 3: To elucidate the efficacy of combining IDH1<sup>R132H</sup> inhibitors with radiation therapy in ATRX<sup>WT</sup> and ATRX<sup>Loss</sup> human glioma stem cells.

*Aim 3A:* To investigate the effects of combining radiation therapy (XRT or PRT) with Ivosidenib, a selective IDH1<sup>R132H</sup> inhibitor, on cell viability, apoptosis, and necrosis in ATRX<sup>WT</sup>/IDH1<sup>R132H</sup> human GSCs.

*Aim 3B:* To investigate the effects of combining radiation therapy (XRT or PRT) with Ivosidenib, a selective IDH1<sup>R132H</sup> inhibitor, on cell viability, apoptosis, and necrosis in ATRX<sup>Loss</sup>/IDH1<sup>R132H</sup> human GSCs.
Central Hypothesis

I hypothesized that ATRX inactivation and IDH mutations (specifically IDH1<sup>R132H</sup>) work together to stimulate apoptotic and necrotic cell death in GSCs more effectively after PRT compared to XRT.
Materials and Methods

Glioma Stem Cell Culture

TS543 (Glioblastoma) and TS603 (WHO grade III anaplastic oligodendroglioma) GSCs were isolated by Drs. Cameron W. Brennan and Ingo Mellinghoff at Memorial Sloan Kettering Cancer Center. These cells were gifted to A Garcés by Dr. Jason Huse at MD Anderson Cancer Center in Houston, TX. TS543-ATRX\textsuperscript{WT}, TS543-ATRX\textsuperscript{Loss}, and TS603 GSCs were cultured in suspension as neurospheres in DMEM/F12 media (Gibco 10-090-CV) with 2% B27 supplement without Retinoic Acid (Life Technologies Corp., 12587-010), 1% penicillin-streptomycin-amphotericin B antibiotic (Cellgro #30-004-C1), 50 \(\mu\)g/mL plasmocin anti-mycoplasma prophylactic (InvivoGen ant-mpp), 2 \(\mu\)g/mL Heparin (StemCell Technologies 07980), 20 ng/mL human recombinant Epidermal Growth Factor (EGF; StemCell Technologies 78006.1), and 20 ng/mL human recombinant basic Fibroblast Growth Factor (bFGF; Stemcell Technologies 78003). TS543-ATRX\textsuperscript{Loss} was created using an shRNA construct against ATRX (sh590) and selected for using 1 \(\mu\)g/mL puromycin (StemCell Technologies 73342) at least 24 hours prior to irradiation. TS543-ATRX\textsuperscript{Loss} grew primarily in an adherent manner while TS543-ATRX\textsuperscript{WT} primarily grew as neurospheres in suspension. TS603 endogenously expresses the heterozygous IDH1\textsuperscript{R132H} mutation and ATRX\textsuperscript{WT}\textsuperscript{.96,97} The cells were passaged and received fresh media every 3-5 days. After thawing from -80°C, the cells were cultured a maximum of four passages.

MGG18 cell culture was performed as described previously in Tateishi et al (2015).\textsuperscript{98} MGG18 is an IDH1\textsuperscript{WT} patient derived GSC line generously donated by Dr. Daniel P. Cahill at Massachusetts General Hospital. MGG18-IDH1\textsuperscript{WT} and MGG18-
IDH1<sup>R132H</sup> were cultured in suspension as neurospheres in neurobasal media with 2% B27 supplement without Retinoic Acid (Life Technologies Corp., 12587-010), 0.5% N2 supplement (Gibco/Invitrogen #17502-048), 0.5% penicillin-streptomycin-amphotericin B antibiotic (Cellgro #30-004-C1), 3 mM L-Glutamine (Cellgro #25-005-C1), 50 µg/mL plasmocin anti-mycoplasma prophylactic (InvivoGen ant-mpp), 20 ng/mL human recombinant EGF (R&D Systems #236-EG-200, Minneapolis, MN), and 20 ng/mL human recombinant bFGF (Peptrotech #100-18B, Rocky Hill, NJ). IDH1<sup>R132H</sup> was activated using 1 µg/mL doxycycline (StemCell Technologies 72742) at least 24 hours prior to irradiation. The cells were passaged and received fresh media every 3-5 days. After thawing from -80°C, the cells were cultured a maximum of four passages.

GS 5-22 is an ATRX<sup>Loss</sup>/IDH1<sup>R132H</sup> GSC line derived from a patient with secondary GBM and were graciously donated by Dr. Eric Sulman while at MD Anderson Cancer Center in Houston, TX. GS 5-22 was cultured in suspension as neurospheres in DMEM/F12 media (Gibco 10-090-CV) with 2% B27 supplement without Retinoic Acid (Life Technologies Corp., 12587-010), 1% penicillin-streptomycin-amphotericin B antibiotic (Cellgro #30-004-C1), 50 µg/mL plasmocin anti-mycoplasma prophylactic (InvivoGen ant-mpp), 2 µg/mL Heparin (StemCell Technologies 07980), 20 ng/mL human recombinant Epidermal Growth Factor (EGF; StemCell Technologies 78006.1), and 20 ng/mL human recombinant basic Fibroblast Growth Factor (bFGF; Stemcell Technologies 78003). The cells were passaged and received fresh media every 3-5 days. After thawing from -80°C, the cells were cultured a maximum of four passages.
**X-ray and Proton Irradiations**

Based on the methods presented in Ma et al (2020), all X-ray irradiations were conducted with a 6 MV linear accelerator (Truebeam, Varian Medical Systems, Palo Alto, CA) available at the MD Anderson Proton Therapy Center (PTC) in Houston, TX. GSCs were seeded in 96 well ultra-low attachment plates and irradiated at a solid water equivalent depth of 10 cm using a 30 cm x 30 cm field size. Proton irradiations were conducted using a 100 MeV proton passive scattered beam with a water equivalent range of 4.3 cm and 1 cm spread-out Bragg peak (SOBP) width at the MD Anderson PTC. GSCs were seeded in 96 well ultra-low attachment plates and irradiated at a solid water equivalent depth of 3.8 cm, which is approximately at the center of the SOBP (Figure 9). All X-ray and proton irradiations were conducted on the same day. All cells experienced a 10-minute transportation time to the PTC and were incubated at 37°C and 5% CO₂ at the PTC while waiting for irradiation.

*Figure 9: Custom proton SOBP created for all GSC irradiations.*

The proton SOBP was constructed based on the ~300 nm spheroid diameter measured prior to irradiation. The original SOBP was created by Mr. Conor McFadden. Dosimetry was conducted by Mr. Conor McFadden and Dr. David Flint. Created in Biorender.
Scanning Electron Microscopy

Scanning Electron Microscopy was performed by Mr. Kenneth Dunner at the MD Anderson High Resolution Electron Microscopy Facility. The exact protocol reported below can be found at this link and in Bu et al (2007). At 72 hours post-irradiation, TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} were suspended in 500-1000 µL of Accutase for 8 minutes, washed twice in PBS, and then fixed in 4% paraformaldehyde. Fixed samples were first washed with 0.1 M cacodylate buffer (pH 7.3) and post fixed using 1% cacodylate buffered osmium tetroxide. Subsequently, the samples were washed with 0.1M cacodylate buffer and then distilled water. The samples were then treated with Millipore-filtered 1% aqueous tannic acid, washed in distilled water, treated with Millipore-filtered 1% aqueous uranyl acetate, and then rinsed thoroughly with distilled water. Sample dehydration was conducted with increasing concentrations of ethanol followed by transfer to increasing concentrations of hexamethyldisilazane (HMDS). After air drying overnight, the samples were mounted onto double-stick carbon tabs (Ted Pella, Inc., Redding, CA) and coated under vacuum using a Balzer MED 010 evaporator (Technotrade International, Manchester, NH) with platinum alloy for a thickness of 25 nm, then immediately flash carbon coated under vacuum. The samples were transferred to a desiccator for examination in a JSM-5900 scanning electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 10 kV. Funding was made available for this facility through the NIH CCSG grant P30CA016672.
Apoptosis and Necrosis Assay

All GSCs were seeded at 2000 cells/well in 96 well ultra-low attachment plates and allowed to grow for 1-2 days to form spheroids prior to irradiation. 100, 1000, or 10000 nM Ivosidenib (MedChemExpress HY-18767) was suspended in media containing 0.1% DMSO 1-2 hours prior to irradiation. The extent of both apoptosis and necrosis was quantified using the RealTime-Glo™ Annexin V Apoptosis and Necrosis assay starting at approximately 1 hour after irradiation. Luminescence (early apoptosis) and florescence (secondary necrosis) measurements were taken using the PerkinElmer Victor X3 multimode plate reader (Part Number 2030-0050) every 8 hours for 48 hours and at 72 hours post-irradiation (Figure 10). To multiplex with the CellTiter-Glo® 3D cell viability assay, the media was refreshed after 72 hours (Figure 11-12). All data was normalized to the initial measurements collected at either t=0 hours or 0 Gy and presented as a percent fold change. Results represent an average of at least two biological replicates with 16 wells seeded for each individual cell line or treatment per experiment.

**Figure 10: Annexin V Apoptosis Necrosis Assay Biomolecular Mechanism.**

The luminescence-based apoptosis assay measures the levels of cell surface phosphotidylserine (PS) when the Annexin V LgBit and SmBit are in close enough proximity to bind their complimentary units of NanoBiT® Luciferase. The florescence-based necrosis assay relies on a cell-impermeable dye that binds to intracellular DNA only when the cell membrane structure is compromised, a hallmark of necrotic cell death. © 2021 Promega Corporation. Reprinted with permission from Promega Corporation.
Cell Viability Assay

GSCs were seeded and irradiated in the same manner as detailed in “Apoptosis and Necrosis Assay.” Cell viability was measured at 14-16 days post-irradiation using the CellTiter-Glo® 3D (CTG3D) assay. All plates equilibrated to room temperature for at least 30 minutes prior adding the CTG3D reagent. GSCs were pipetted up and down 10 times to induce cell lysis immediately after adding the CTG3D reagent. The samples were left in a sterile environment at room temperature for 25 minutes in the dark before reading the luminescence viability signal using the PerkinElmer Victor X3 multimode plate reader (Part Number 2030-0050). Raw luminescence data was converted into ATP concentration by generating an ATP standard curve (Promega E6011). All ATP data was normalized to the 0 Gy negative control and presented as a percent fold change from 0 Gy. Results represent an average of at least two biological replicates with 16 wells seeded for each individual cell line or treatment per experiment.

To identify the primary cell death mechanisms activated post-XRT and post-PRT, we treated TS543-ATRXWT and TS543-ATRXLoss GSCs 1-2 hours prior to irradiation with either 0.05% DMSO, 10 ng/mL TNF-α (Cell Signaling Technologies 8902SC), 10 ng/mL TNF-α+10000 nM Z-VAD(OMe)-FMK (Irreversible, cell permeable pan-caspase inhibitor; Cell Signaling Technologies 60332), 10 ng/mL TNF-α+20000 nM Nec1s (RIPK1 inhibitor; BioVision 2535), or 10 ng/mL TNF-α+10 nM GSK2593074A76 (RIPK1/RIPK3 inhibitor; MedChemExpress HY-122909). GSCs subject to cell death analysis were only treated once with one of the above drug combinations. However, the media was still refreshed every 3-5 days prior to adding the CTG3D reagent.
Figure 11: CellTiterGlo 3D Viability Assay Biomolecular Mechanism.

(A) The luciferase reaction when combined with ATP of lysed cells and oxygen creates a luminescent signal that is used to calculate the original intracellular ATP concentration, an indicator of the number of viable cells in culture. (B) A representative ATP standard curve fit to a linear quadratic model to account for the curvilinear relationship between the number of cells seeded and luminescent signal often found in 3D cell culture. © 2021 Promega Corporation. Reprinted with permission from Promega Corporation.
Figure 12: CellTiterGlo 3D and Annexin V multiplex workflow.
The combination of the non-lytic Annexin V assay and lytic CellTiterGlo 3D assay required a fresh media change after 72 hours to ensure that Annexin V probe induced cytotoxicity did not occur. Annexin V timepoints included 0, 8, 16, 24, 32, 40, 48, and 72 hours after irradiation. Media changes were conducted by removing and replacing 50% of a well’s volume with fresh media. Intracellular ATP concentrations for each well was calculated using luminescence values generated using the luciferase based CellTiterGlo 3D reagent and an ATP standard curve. L: Luminescence. F: Fluorescence.
Limiting Dilution Analysis

All GSCs were seeded at 20, 10, 5, and 1 cell/well via serial dilution at 24 hours prior to XRT or PRT in a 96 well ultra-low attachment (ULA) plate. For LDA experiments only, X-ray irradiations were conducted with a 320 KV, 12.5 mA beam using the XRAD 320 at the MD Anderson Sheikh Zayed Building. Samples were placed at a depth of 51 cm from the focus and irradiated using a field size of 20 cm x 20 cm. Proton irradiations were conducted at the MD Anderson PTC. GSCs were imaged using the Biotek Cytation 5 cell-imaging multimode reader equipped with Gen5 software at 4 days post-irradiation. Spheroids were defined as structures with >5 cells and diameter >20 nm. The log fraction of non-responding wells refers to the number of wells seeded in which spheroids did not form after irradiation; this was quantified using Extreme Limiting Dilution Analysis (ELDA-See Figure 13). The online ELDA platform can be found at https://bioinf.wehi.edu.au/software/elda/. Results represent an average of at least 2 biological replicates with 12 wells seeded at a given cell density per experiment.

Figure 13: Extreme Limiting Dilution Analysis (ELDA) workflow.
GSCs were treated with Accutase prior to seeding into 96 well ULA plates to ensure the formation of a single cell suspension. Irradiation occurred within 24 hours of seeding and the resulting spheroids were manually counted after 4 days. NSFF: Neurosphere Formation Frequency. Created in Biorender.
Results

Effects of X-Rays and Protons on ATRX\textsuperscript{Loss} isogenic glioma stem cells

To investigate how the ATRX status of a glioma stem cell (GSC) impacts its ability to self-renew after radiation therapy, we conducted extreme limiting dilution analysis (ELDA) on TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} GSCs treated with either XRT or PRT as reported by Hu and Smyth (2009).\textsuperscript{101} The active cell frequency, denoted here as the neurosphere formation frequency (NSFF), was calculated using a maximum likelihood estimation. The log-log transformation fits of each condition were then compared using a likelihood ratio test to determine whether an observed difference is statistically significant.

We first demonstrated that the self-renewal capacity as measured by NSFF of both TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} follows an inverse relationship with the radiation dose administered (Figure 14, Tables 1-2, 5-6). Since the validity of ELDA data output requires that the single hit hypothesis is met, a likelihood ratio test of all the TS543-ATRX\textsuperscript{WT} data inputs in Table 1 and all the TS543-ATRX\textsuperscript{Loss} data inputs in Table 4 was conducted as well as a score test for heterogeneity to ensure that the results were consistent across different biological replicates (Table 3 and 6). The data across all radiation doses for both TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} did not reject the null hypotheses for either of these tests, indicating that the NSFF calculated by ELDA can be trusted. To further understand and compare the effects of ATRX\textsuperscript{Loss} and ATRX\textsuperscript{WT} on GSC post-radiation self-renewal, we seeded isogenic TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} GSCs 24 hours prior to treatment with either 6 Gy XRT or 6 Gy PRT and manually quantified spheroid growth after four days (Figure 15; Tables 7-12). Similar to
the data presented in Figure 14, the null hypotheses for the single-hit hypotheses and
score tests for heterogeneity were not rejected for both TS543-ATRXWT (Table 9) and
TS543-ATRXLoss (Table 12). We determined that ATRXLoss significantly impairs GSC
self-renewal after both XRT and PRT. After comparing the ELDA calculated NSFF for
both cell lines, TS543-ATRXLoss treated with 6 Gy PRT exhibited a 1.81-fold decrease in
NSFF compared to TS543-ATRXWT. However, this same fold NSFF decrease was only
1.54 when comparing TS543-ATRXLoss and TS543-ATRXWT GSCs irradiated with 6 Gy
XRT. These results demonstrate the effectiveness of PRT over XRT in enhancing
ATRXLoss driven reduction of post-radiation GSC self-renewal.
Figure 14: TS543 self-renewal capacity is negatively correlated with XRT dose.
ELDA plot generated after treatment with 4, 6, or 8 Gy of ionizing XRT for (A) TS543-ATRXWT and (B) TS543-ATRXLoss isogenic GSCs. The solid line fits the complementary log-log transformation of the Poisson probability formula using a generalized linear model approach. The dotted lines indicate the upper and lower one-sided 95% confidence intervals of this fit. The NSFF was calculated using maximum likelihood estimation. To test whether two conditions with different NSFFs were statistically different from each other, their log-log transformation fits were compared using a likelihood ratio test. **p<0.01. The ELDA methodology was developed by Hu and Smyth (2009).101
Table 1: ELDA input data for TS543-ATRXWT at 4, 6, and 8 Gy XRT.
The raw data of positive cultures, indicating successful neurosphere formation, for TS543-ATRXWT GSCs treated with 4, 6, and 8 Gy XRT.

Table 2: ELDA estimates of NSFF with 95% confidence intervals for TS543-ATRXWT at 4, 6, and 8 Gy XRT.
Neurosphere formation frequency (NSFF) refers to the active cell frequency estimated by ELDA with the upper and lower 95% confidence intervals. The fold reduction represents a ratio of the NSFF calculated for TS543-ATRXLoss compared to TS543-ATRXWT GSCs at a specific radiation dose.

Table 3: ELDA calculated adherence to the single-hit model and heterogeneity for TS543-ATRXWT GSCs irradiated at 4, 6, and 8 Gy XRT.
Since LDA accuracy relies on the validity of the Poisson single-hit model, a likelihood ratio test for whether the data follows a single-hit model was calculated. A p-value less than 0.05 indicates adherence to the single-hit model. The score test for heterogeneity determines whether assays conducted on different days exhibit a similar NSFF. A p-value less than 0.05 indicates that different assay replicates are not heterogeneous. The slope of the solid line also provides information on these two parameters. Slopes greater than 1 could be indicative of a multi-hit model while slopes less than 1 could indicate assay heterogeneity or impeded ability of a sample to form neurospheres due to interference.
Table 4: ELDA input data for TS543-ATRX\(^{\text{Loss}}\) at 4, 6, and 8 Gy XRT.
The raw data of positive cultures, indicating successful neurosphere formation, for TS543-ATRX\(^{\text{Loss}}\) GSCs treated with 4, 6, and 8 Gy XRT.

Table 5: ELDA estimates of NSFF with 95% confidence intervals for TS543-ATRX\(^{\text{Loss}}\) at 4, 6, and 8 Gy XRT.
Neurosphere formation frequency (NSFF) refers to the active cell frequency estimated by ELDA with the upper and lower 95% confidence intervals. The fold reduction represents a ratio of the NSFF calculated for TS543-ATRX\(^{\text{Loss}}\) compared to TS543-ATRX\(^{\text{WT}}\) GSCs at a specific radiation dose.

Table 6: ELDA calculated adherence to the single-hit model and heterogeneity for TS543-ATRX\(^{\text{Loss}}\) GSCs irradiated at 4, 6, and 8 Gy XRT.
Since LDA accuracy relies on the validity of the Poisson single-hit model, a likelihood ratio test for whether the data follows a single-hit model was calculated. A p-value less than 0.05 indicates adherence to the single-hit model. The score test for heterogeneity determines whether assays conducted on different days exhibit a similar NSFF. A p-value less than 0.05 indicates that different assay replicates are not heterogeneous. The slope of the solid line also provides information on these two parameters. Slopes greater than 1 could be indicative of a multi-hit model while slopes less than 1 could indicate assay heterogeneity or impeded ability of a sample to form neurospheres due to interference.
Figure 15: ATRX_{Loss} impairs GSC self-renewal after both XRT and PRT. Post-radiation neurosphere self-renewal frequency (NSFF) was measured using Extreme Limiting Dilution Analysis (ELDA) in TS543-ATRX^{WT} and TS543-ATRX^{Loss} human GSCs treated with (A) 6 Gy X-ray radiotherapy (XRT) or (B) 6 Gy Proton radiotherapy (PRT). The solid line fits the complementary log-log transformation of the Poisson probability formula using a generalized linear model approach. The dotted lines indicate the upper and lower one-sided 95% confidence intervals of this fit. The NSFF was calculated using maximum likelihood estimation. To test whether our two cell lines exhibited significantly different NSFFs after a given dose of radiation, a chi-square test of independence was performed. **p<0.01. The ELDA methodology was developed by Hu and Smyth (2009).^{101}
Table 7: ELDA input data for TS543-ATRX$^{WT}$ and TS543-ATRX$^{Loss}$ at 6 Gy XRT.
The raw data of positive cultures, indicating successful neurosphere formation, for
TS543-ATRX$^{WT}$ and TS543-ATRX$^{Loss}$ GSCs treated with 6 Gy XRT.

<table>
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Table 8: ELDA estimates of NSFF with 95% confidence intervals for TS543-ATRX$^{WT}$ and TS543-ATRX$^{Loss}$ at 6 Gy XRT.
Neurosphere formation frequency (NSFF) refers to the active cell frequency estimated by
ELDA with the upper and lower 95% confidence intervals. The fold reduction represents
a ratio of the NSFF calculated for TS543-ATRX$^{Loss}$ GSCs treated with 6 Gy XRT
compared to TS543-ATRX$^{WT}$ treated with 6 Gy XRT.

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<thead>
<tr>
<th>Group</th>
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<th>NSFF Estimate</th>
<th>Upper 95% CI</th>
<th>Fold Reduction Relative to ATRX$^{WT}$</th>
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Table 9: ELDA calculated adherence to the single-hit model and heterogeneity for
TS543-ATRX$^{WT}$ and TS543-ATRX$^{Loss}$ at 6 Gy XRT.
Since LDA accuracy relies on the validity of the Poisson single-hit model, a likelihood
ratio test for whether the data follows a single-hit model was calculated. A p-value less
than 0.05 indicates adherence to the single-hit model. The score test for heterogeneity
determines whether assays conducted on different days exhibit a similar NSFF. A p-value
less than 0.05 indicates that different assay replicates are not heterogeneous. The slope of
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could be indicative of a multi-hit model while slopes less than 1 could indicate assay
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Table 10: ELDA input data for TS543-ATRX<sup>WT</sup> and TS543-ATRX<sup>Loss</sup> at 6 Gy PRT.
The raw data of positive cultures, indicating successful neurosphere formation, for TS543-ATRX<sup>WT</sup> and TS543-ATRX<sup>Loss</sup> GSCs treated with 6 Gy PRT.

<table>
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<th>Dose (cells/well)</th>
<th>Tested (number of wells)</th>
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Table 11: ELDA estimates of NSFF with 95% confidence intervals for TS543-ATRX<sup>WT</sup> and TS543-ATRX<sup>Loss</sup> at 6 Gy PRT.
Neurosphere formation frequency (NSFF) refers to the active cell frequency estimated by ELDA with the upper and lower 95% confidence intervals. The fold reduction represents a ratio of the NSFF calculated for TS543-ATRX<sup>Loss</sup> GSCs treated with 6 Gy PRT compared to TS543-ATRX<sup>WT</sup> treated with 6 Gy PRT.

<table>
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<tr>
<th>Group</th>
<th>Lower 95% CI</th>
<th>NSFF Estimate</th>
<th>Upper 95% CI</th>
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Table 12: ELDA calculated adherence to the single-hit model and heterogeneity for TS543-ATRX<sup>WT</sup> and TS543-ATRX<sup>Loss</sup> at 6 Gy PRT.
Since LDA accuracy relies on the validity of the Poisson single-hit model, a likelihood ratio test for whether the data follows a single-hit model was calculated. A p-value less than 0.05 indicates adherence to the single-hit model. The score test for heterogeneity determines whether assays conducted on different days exhibit a similar NSFF. A p-value less than 0.05 indicates that different assay replicates are not heterogeneous. The slope of the solid line also provides information on these two parameters. Slopes greater than 1 could be indicative of a multi-hit model while slopes less than 1 could indicate assay heterogeneity or impeded ability of a sample to form neurospheres due to interference.

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Previous studies have implicated ATRX inactivation in reduced glioblastoma cell survival after treatment with ionizing radiation therapy. Therefore, we subjected TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} GSCs to a range of XRT and PRT doses (4, 6, and 8 Gy) and examined cell viability by quantifying the concentration of intracellular ATP present at 14 days post-radiation using the CellTiterGlo 3D assay. When comparing raw post-XRT and post-PRT ATP concentrations (Figure 16A), PRT significantly reduces GSC viability regardless of ATRX mutational status. To account for the differences in growth kinetics between TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} GSCs, the data was normalized with respect to the 0 Gy condition for each individual cell line (Figure 16B). This normalization allowed for comparison of the changes in cell viability exhibited in TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} with increasing doses of radiation. In addition to corroborating the PRT vs. XRT trends presented in Figure 16A, we discovered that ATRX\textsuperscript{Loss} also contributes to decreasing post-radiation GSC viability to a higher extent than ATRX\textsuperscript{WT}. These results hold true even when cells were treated with 0.05% DMSO prior to irradiation (Figure 18). To determine the correct statistical tests for analyzing the data, cumulative frequency plots of the data were fitted to a cumulative Gaussian curve and further analyzed for normality using the Shapiro-Wilk test (Figure 17 and 19, Tables 13 and 14). Since the data was not consistently normal, we chose to use the non-parametric Kolmogorov-Smirnoff test to calculate statistical significance between different conditions. Our results provide further evidence to suggest that PRT is more effective than XRT in eliminating GSCs.
Figure 16: ATRX\textsuperscript{Loss} enhances GSC sensitivity to PRT over XRT.
Cell viability was measured using the luminescence based CellTiterGlo 3D assay at 14 days post-irradiation. (A) Intracellular ATP concentration was calculated using a curvilinear ATP standard curve as detailed in Figure 11 and (B) normalized relative to the 0 Gy negative control. Since the data do not consistently exhibit normality across doses and cell lines, a non-parametric approach was used to demonstrate statistical significance. The cumulative distributions of each condition were compared using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals in black.
Figure 17: Cumulative frequency plots of raw ATP data for TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{loss} treated with 0, 4, 6, or 8 Gy of XRT and PRT.

The raw ATP data presented in Figure 16 was fit to the cumulative Gaussian function using a non-weighted least squares regression model in Graphpad Prism 9. Although the data appears normal for the combination of all radiation doses for each cell line upon visual inspection, further analysis was conducted using the Shapiro-Wilk test, whose results are shown in Table 13.

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<th>Shapiro-Wilk P Value</th>
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Table 13: Shapiro-Wilk Test for normality of raw ATP data for TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{loss} treated with 0, 4, 6, or 8 Gy of XRT and PRT.

The Shapiro-Wilk test for normality was chosen due to its high average empirical power compared to other tests at both small and larger sample sizes of symmetric and asymmetric distributions.\textsuperscript{103} W and P values for each condition were calculated in Graphpad Prism 9.
Figure 18: ATRX<sup>Loxx</sup> contributes to enhanced GSC sensitivity to PRT over XRT in the presence of 0.05% DMSO.

Cell viability was measured using the luminescence based CellTiterGlo 3D assay at 14 days post-irradiation. (A) Intracellular ATP concentration was calculated using a curvilinear ATP standard curve as detailed in Figure 11 and (B) normalized relative to the 0 Gy negative control. Since the data do not consistently exhibit normality across doses and cell lines, a non-parametric approach was used to demonstrate statistical significance. The cumulative distributions of each condition were compared using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals in black.
Figure 19: Cumulative frequency plots of raw ATP data for TS543-ATRX<sup>WT</sup> and TS543-ATRX<sup>1-loss</sup> treated with 0, 4, 6, or 8 Gy of XRT and PRT.

The raw ATP data presented in Figure 18 was fit to the cumulative Gaussian function using a non-weighted least squares regression model in Graphpad Prism 9. Although the data appears to violate normality upon visual inspection, further analysis was conducted using the Shapiro-Wilk test, whose results are shown in Table 14.

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<th>Shapiro-Wilk P Value</th>
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<td>TS543-ATRX&lt;sup&gt;WT&lt;/sup&gt; PRT (0.05% DMSO)</td>
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<td>TS543-ATRX&lt;sup&gt;1-loss&lt;/sup&gt; PRT (0.05% DMSO)</td>
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<td>0.9588</td>
<td>0.2716</td>
<td>Yes</td>
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</table>

Table 14: Shapiro-Wilk Test for normality of raw ATP data for TS543-ATRX<sup>WT</sup> and TS543-ATRX<sup>1-loss</sup> treated with DMSO and 0, 4, 6, or 8 Gy of XRT and PRT.

The Shapiro-Wilk test for normality was chosen due to its high average empirical power compared to other tests at both small and larger sample sizes of symmetric and asymmetric distributions. W and P values for each condition were calculated in Graphpad Prism 9.
**ATRXLoss drives necroptosis after proton radiotherapy**

Radiation therapy is known to induce glioma cell death via apoptosis at low doses and via necroptosis at high doses.\textsuperscript{104,105} To determine the primary mechanisms of cell death driven by ATRX\textsuperscript{WT} and ATRX\textsuperscript{Loss} in human GSCs, we first imaged TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} via scanning electron microscopy (SEM) at 72 hours after treatment with 0 Gy, 6 Gy XRT, or 6 Gy PRT (Figure 20). ATRX\textsuperscript{Loss} GSCs displayed more extensive membrane blebbing, swelling, and decomposition compared to ATRX\textsuperscript{WT} GSCs when irradiated with either 6 Gy XRT or PRT. all of which are consistent with necrotic cell death. These traditionally recognized hallmarks of necrotic cell death were more prevalent after PRT compared to XRT.\textsuperscript{106}

We used the RealTime-Glo Annexin V assay to further quantify the mechanisms of cell death present in irradiated TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} GSCs. The extent of apoptotic cell death was assessed using a Luciferase based luminescent probe to quantify the extent of cell surface phosphatidylserine. The extent of secondary necrosis was quantified using a florescent, membrane-impermeable dye that binds nuclear DNA only by entering through holes in the cell membrane. To account for the baseline level of cell death in the cell line used for each experiment, we presented the data for each condition normalized to 0 Gy. PRT induced significantly higher apoptotic (Figure 21A) and necrotic (Figure 21B) cell death compared to XRT in both TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} GSCs. Irradiated TS543-ATRX\textsuperscript{Loss} did not exhibit a significant increase in apoptotic cell death compared to TS543-ATRX\textsuperscript{WT}. To account for the baseline signal at the start of the experiment, we present the raw luminescence (Figure 22A; apoptosis) and florescence (Figure 23A; necrosis) from t=0 hours to t=72 hours after irradiation.
with 8 Gy XRT or 8 Gy PRT as well as the normalized data with respect to t=0 hours (Figure 22B and 23B). The raw data demonstrates a robust enhancement of apoptotic and necrotic cell death in TS543-ATRX<sub>Loss</sub> compared to TS543-ATRX<sub>WT</sub>. Although this trend is recapitulated in the normalized apoptosis data, there are no significant differences between any of the conditions in the normalized necrosis data. This could be due to the data’s high standard deviation; therefore, presenting the median and interquartile range might hold better predictive value of the data’s central tendency in this case. Nevertheless, our data demonstrates the potential benefits of PRT over XRT in inducing apoptotic and necrotic cell death to eliminate ATRX<sub>Loss</sub> GSCs.
Figure 20: Scanning Electron Microscopy (SEM) images of irradiated TS543-ATRX<sup>WT</sup> and TS543-ATRX<sup>Loss</sup>.

Representative SEM images of TS543-ATRX<sup>WT</sup> and TS543-ATRX<sup>Loss</sup> irradiated with 0 Gy, 6 Gy XRT, or 6 Gy PRT. Magnification of the 0 Gy images was 10000x. Magnification of the 6 Gy XRT and 6 Gy PRT images was 8000x. The red arrows indicate membrane swelling and permeabilization, two indicators of necrotic cell death.
Figure 21: ATRX\textsuperscript{Loss} enhances radiation-induced necrotic cell death over apoptotic cell death compared to ATRX\textsuperscript{WT} in human GSCs.

TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} GSCs were subject to 2, 4, 6, and 8 Gy XRT or PRT. Cell death was measured using the RealTime-Glo Annexin V (A) luminescence-based apoptosis assay at 8 hours post-irradiation and (B) fluorescent-based necrosis assay at 72 hours post-irradiation according to the workflow detailed in Figure 12. All measurements for each cell line were normalized to their respective 0 Gy negative controls. For this figure only, XRT was conducted using the XRAD 320 at MD Anderson Sheikh Zayed Building. P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1\%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95\% confidence intervals in black.
Figure 22: Proton radiotherapy significantly enhances apoptotic cell death in both ATRX\textsuperscript{WT} and ATRX\textsuperscript{Loss} GSCs compared to X-ray radiotherapy. TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} GSCs were subject to 8 Gy XRT or PRT. (A) Raw RealTime-Glo Annexin V luminescent output measurements were taken at t=0, 12, 24, 48, and 72 hours after irradiation. (B) All measurements for each condition were normalized to the luminescence measured at t=0 hours. P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals in black.
Figure 23: ATRX\textsuperscript{Loss} significantly enhances radiation induced necrotic cell death over ATRX\textsuperscript{WT} in TS543 GSCs.

TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} GSCs were subject to 8 Gy XRT or PRT. (A) Raw RealTime-Glo Annexin V fluorescent output measurements were taken at t=0, 12, 24, 48, and 72 hours after irradiation. (B) All measurements for each condition were normalized to the luminescence measured at t=0 hours. P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals in black.
The formation of the RIP1/RIP3 necrosome is required for necroptotic cell death. However, cells can be rescued from necroptosis by pharmacologically inhibiting RIP1 or RIP3, especially with the selective RIP1 inhibitor Nec-1s.104,107 To further characterize the mechanisms by which ATRX\textsuperscript{Loss} promotes cell death, we measured post-radiation cell viability in TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} GSCs when combined with TNF-\(\alpha\) and irreversible chemical inhibitors known to impair apoptosis (Z-VAD(OMe)-FMK; pan-caspase inhibitor) and necroptosis (RIP1 inhibitor Nec1s; RIP1/RIP3 inhibitor GSK2593074A). GSCs were treated with TNF-\(\alpha\) before radiation to stimulate extrinsic necroptosis. Z-VAD(OMe)-FMK was administered to more accurately simulate GBM therapeutic resistance due to GBM upregulation of anti-apoptotic proteins (Bcl-w, Bcl-2, and Bcl-xL) and downregulation of pro-apoptotic proteins (Bax, Bak, Bok, and NOXA).108 Although TS543-ATRX\textsuperscript{WT} GSCs are highly XRT resistant, their viability increases substantially when pre-treated with RIPK1/RIPK3 inhibitor GSK2593074A (Figure 24A). This trend disappeared after normalization of the data to its 0 Gy (Figure 24B), possibly due to TS543-ATRX\textsuperscript{WT} radioresistance. On the other hand, TS543-ATRX\textsuperscript{Loss} exhibits heavy resistance to all pharmacological inhibitors added (Figure 25A), but a dependency on the necroptotic cell death pathway as demonstrated by the 0 Gy normalized data (Figure 25B). These trends are recapitulated when TS543-ATRX\textsuperscript{WT} (Figure 26) and TS543-ATRX\textsuperscript{Loss} (Figure 27) are treated with PRT. As demonstrated in Figure 16, TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} are heavily sensitized to PRT over XRT, even in the presence of the RIPK1/RIPK3 inhibitor GSK2593074A (Figure 28). Our data demonstrate that ATRX\textsuperscript{WT} GSCs heavily rely on the formation of the RIPK1/RIPK3 necrosome for radiation induced cell death.
Figure 24: ATRX<sup>WT</sup> drives post-XRT cell death via apoptosis and necroptosis. TS543-ATRX<sup>WT</sup> was treated with combinations of 0.05% DMSO, 10 ng/mL TNF-α to activate extrinsic apoptosis, 10 μM Z-VAD(OMe)-FMK (pan-caspase inhibitor), 20 μM Necrostatin-1s (RIPK1 inhibitor), and 10 nM GSK074 (RIPK1/RIPK3 inhibitor) prior to treatment with 0, 4, and 8 Gy XRT. Raw ATP values are presented in (A) while normalized ATP values relative to each condition’s respective 0 Gy control are presented in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Figure 25: ATRX\textsuperscript{Loss} drives post-XRT cell death primarily via necroptosis.

TS543-ATRX\textsuperscript{Loss} was treated with combinations of 0.05% DMSO, 10 ng/mL TNF-\(\alpha\) to activate extrinsic apoptosis, 10 \(\mu\)M Z-VAD(OMe)-FMK (pan-caspase inhibitor), 20 \(\mu\)M Necrostatin-1s (RIPK1 inhibitor), and 10 nM GSK074 (RIPK1/RIPK3 inhibitor) prior to treatment with 0, 4, and 8 Gy XRT. Raw ATP values are presented in (A) while normalized ATP values relative to each condition’s respective 0 Gy control are presented in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Figure 26: ATRX<sup>WT</sup> drives post-XRT cell death via apoptosis and necroptosis.

TS543-ATRX<sup>WT</sup> was treated with combinations of 0.05% DMSO, 10 ng/mL TNF-α to activate extrinsic apoptosis, 10 uM Z-VAD(OMe)-FMK (pan-caspase inhibitor), 20 uM Necrostatin-1s (RIPK1 inhibitor), and 10 nM GSK074 (RIPK1/RIPK3 inhibitor) prior to treatment with 0, 4, and 8 Gy XRT. Raw ATP values are presented in (A) while normalized ATP values relative to each condition’s respective 0 Gy control are presented in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Figure 27: ATRX<sup>Loss</sup> does not significantly drive PRT induced necroptosis. TS543-ATRX<sup>Loss</sup> was treated with combinations of 0.05% DMSO, 10 ng/mL TNF-α to activate extrinsic apoptosis, 10 μM Z-VAD(OMe)-FMK (pan-caspase inhibitor), 20 μM Necrostatin-1s (RIPK1 inhibitor), and 10 nM GSK074 (RIPK1/RIPK3 inhibitor) prior to treatment with 0, 4, and 8 Gy XRT. Raw ATP values are presented in (A) while normalized ATP values relative to each condition’s respective 0 Gy control are presented in (B). P-values were calculated using the Kolumogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Figure 28: Proton radiotherapy significantly decreases GSC viability independent of ATRX status compared to ionizing X-ray radiotherapy. Data for irradiated TS543-ATRX WT and TS543-ATRX Loss GSCs treated with either 10 ng/mL TNF-α or 10 ng/mL TNF-α combined with 10 μM Z-VAD(OMe)-FMK (pan-caspase inhibitor) and 10 nM GSK074 (RIPK1/RIPK3 inhibitor).
Effects of X-rays and Protons on IDH1R132H isogenic glioma stem cells

Considering the prevalence of IDH1R132H in patients with low-grade gliomas and secondary glioblastomas, we investigated how IDH1R132H affects the sensitization of human GSCs to PRT compared to XRT using the isogenic doxycycline activated cell lines MGG18-IDH1WT and MGG18-IDH1R132H. Following the same ELDA methodology as described above for TS543 GSCs, we quantified an inverse relationship between NSFF and radiation dose for MGG18-IDH1WT and MGG18-IDH1R132H after treatment with XRT (Figure 29, Tables 15-20) and PRT (Figure 30, Tables 21-26). MGG18-IDH1R132H exhibited a 2.12-fold decrease in NSFF compared to MGG18-IDH1WT irradiated with 6 Gy XRT. MGG18-IDH1R132H exhibited a 2.05-fold decrease compared to MGG18-IDH1WT irradiated with 6 Gy PRT (Figure 31, Tables 27-32). These results suggest that PRT is equally as effective as XRT in impairing self-renewal in the MGG18 GSC line.

In addition to its established presence as a positive prognostic marker for patients with low-grade gliomas and glioblastomas, IDH1R132H is known to promote radiation-induced GBM cell death via the production of the oncometabolite 2-Hydroxyglutarate (2-HG). To investigate the effects of IDH1R132H on GSC radiosensitivity, we exposed MGG18-IDH1WT and MGG18-IDH1R132H with 0, 4, 6, and 8 Gy XRT or PRT and quantified the intracellular ATP concentration using the CellTiterGlo 3D assay as described previously at 14 days post-irradiation. In the raw ATP data, MGG18-IDH1R132H treated with PRT demonstrates lower cell viability than MGG18-IDH1WT GSCs (Figure 32A). However, this might be misleading since the MGG18-IDH1R132H 0 Gy negative controls independently demonstrated differences in viability. After normalizing each condition to its 0 Gy control, no statistically significant differences in
post-XRT or post-PRT cell viability between MGG18-IDH1\textsuperscript{WT} and MGG18-IDH1\textsuperscript{R132H} were observed except at 8 Gy PRT, at which MGG18-IDH1\textsuperscript{R132H} exhibited higher viability than MGG18-IDH1\textsuperscript{WT} (Figure 32B). Our data also demonstrates that while MGG18-IDH1\textsuperscript{R132H} GSCs do not exhibit noticeable differences in viability when comparing XRT to PRT, MGG18-IDH1\textsuperscript{WT} GSCs are more sensitized to PRT over XRT at 8 Gy. The cumulative frequency plots and Shapiro-Wilk test results led us to analyze statistical significance using the same non-parametric Kolmogorov-Smirnoff approach that was conducted for the isogenic TS543-ATRX\textsuperscript{WT} and TS543-ATRX\textsuperscript{Loss} cell viability data (Figure 33, Table 33). A closer analysis of the cell death mechanisms activated from 0-72 hours after irradiation with 8 Gy XRT compared to 8 Gy PRT revealed a heightened increase in apoptotic cell death in both MGG18-IDH1\textsuperscript{WT} and MGG18-IDH1\textsuperscript{R132H} treated with 8 Gy PRT (Figure 34A). However, only MGG18-IDH1\textsuperscript{R132H} exhibited statistically significant increases in necrotic cell death after 8 Gy PRT compared to 8 Gy XRT (Figure 34B).
Figure 29: MGG18 self-renewal is inversely related to XRT dose delivered. ELDA plot generated after treatment with 2, 4, 6, or 8 Gy of ionizing XRT for (A) MGG18-IDH1WT and (B) MGG18-IDH1R132H isogenic GSCs.
Table 15: ELDA input data for MGG18-IDH1WT at 2, 4, 6, and 8 Gy XRT. The raw data of positive cultures, indicating successful neurosphere formation, for MGG18-IDH1WT GSCs treated with 2, 4, 6, and 8 Gy XRT.

<table>
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Table 16: ELDA estimates of NSFF with 95% confidence intervals for MGG18-IDH1WT at 2, 4, 6, and 8 Gy XRT. Neurosphere formation frequency refers to the active cell frequency estimated by ELDA with the upper and lower 95% confidence intervals. The fold reduction represents a ratio of the NSFF calculated for MGG18-IDH1R132H compared to MGG18-IDH1WT GSCs at a given radiation dose.

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<tr>
<th>Group</th>
<th>Lower 95% CI</th>
<th>NSFF Estimate</th>
<th>Upper 95% CI</th>
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Table 17: ELDA calculated adherence to the single-hit model and heterogeneity for MGG18-IDH1WT GSCs irradiated at 2, 4, 6, and 8 Gy XRT. A likelihood ratio test with \( p < 0.05 \) indicates the probability that the single-hit model is accurate. A score test with \( p < 0.05 \) likely indicates that assay replicates completed at different times are not heterogeneous.

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<th>Goodness of Fit Test</th>
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Table 18: ELDA input data for MGG18-IDH1<sup>R132H</sup> at 2, 4, 6, and 8 Gy XRT.
The raw data of positive cultures, indicating successful neurosphere formation, for MGG18-IDH1<sup>R132H</sup> GSCs treated with 2, 4, 6, and 8 Gy XRT.

<table>
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<th>Dose (cells/well)</th>
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<td>MGG18-IDH1-R132H-6Gy-XRT</td>
</tr>
<tr>
<td>20</td>
<td>48</td>
<td>29</td>
<td>MGG18-IDH1-R132H-6Gy-XRT</td>
</tr>
<tr>
<td>1</td>
<td>36</td>
<td>1</td>
<td>MGG18-IDH1-R132H-8Gy-XRT</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>7</td>
<td>MGG18-IDH1-R132H-8Gy-XRT</td>
</tr>
<tr>
<td>10</td>
<td>36</td>
<td>13</td>
<td>MGG18-IDH1-R132H-8Gy-XRT</td>
</tr>
<tr>
<td>20</td>
<td>36</td>
<td>23</td>
<td>MGG18-IDH1-R132H-8Gy-XRT</td>
</tr>
</tbody>
</table>

Table 19: ELDA estimates of NSFF with 95% confidence intervals for MGG18-IDH1<sup>R132H</sup> at 2, 4, 6, and 8 Gy XRT.
Neurosphere formation frequency refers to the active cell frequency estimated by ELDA with the upper and lower 95% confidence intervals. The fold reduction represents a ratio of the NSFF calculated for MGG18-IDH1<sup>R132H</sup> compared to MGG18-IDH1<sup>WT</sup> GSCs at a given radiation dose.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lower 95% CI</th>
<th>NSFF Estimate</th>
<th>Upper 95% CI</th>
<th>Fold Reduction Relative to IDH1&lt;sup&gt;WT&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH1&lt;sup&gt;R132H&lt;/sup&gt; 2 Gy XRT</td>
<td>1/14.40</td>
<td>1/11.60</td>
<td>1/9.43</td>
<td>2.02</td>
</tr>
<tr>
<td>IDH1&lt;sup&gt;R132H&lt;/sup&gt; 4 Gy XRT</td>
<td>1/23.10</td>
<td>1/18.60</td>
<td>1/15.04</td>
<td>2.21</td>
</tr>
<tr>
<td>IDH1&lt;sup&gt;R132H&lt;/sup&gt; 6 Gy XRT</td>
<td>1/29.90</td>
<td>1/23.10</td>
<td>1/17.82</td>
<td>2.11</td>
</tr>
<tr>
<td>IDH1&lt;sup&gt;R132H&lt;/sup&gt; 8 Gy XRT</td>
<td>1/29.40</td>
<td>1/21.90</td>
<td>1/16.36</td>
<td>1.84</td>
</tr>
</tbody>
</table>

Table 20: ELDA calculated adherence to the single-hit model and heterogeneity for MGG18-IDH1<sup>R132H</sup> GSCs irradiated at 2, 4, 6, and 8 Gy XRT.
A likelihood ratio test with p<0.05 indicates the probability that the single-hit model is accurate. A score test with p<0.05 likely indicates that assay replicates completed at different times are not heterogeneous.

<table>
<thead>
<tr>
<th>Goodness of Fit Test</th>
<th>χ² Value</th>
<th>Degrees of Freedom</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Likelihood ratio test of single-hit model</td>
<td>0.0477</td>
<td>1</td>
<td>0.827</td>
</tr>
<tr>
<td>Score test of heterogeneity</td>
<td>0.37</td>
<td>1</td>
<td>0.543</td>
</tr>
</tbody>
</table>
Figure 30: MGG18 self-renewal is inversely related to PRT dose delivered.
ELDA plot generated after treatment with 2, 4, or 6 Gy PRT for (A) MGG18-IDH1\textsuperscript{WT} and (B) MGG18-IDH1\textsuperscript{R132H} isogenic GSCs.
Table 21: ELDA input data for MGG18-IDH1<sup>WT</sup> at 2, 4, and 6 Gy PRT.
The raw data of positive cultures, indicating successful neurosphere formation, for MGG18-IDH1<sup>WT</sup> GSCs treated with 2, 4, or 6 Gy PRT.

<table>
<thead>
<tr>
<th>Dose (cells/well)</th>
<th>Tested (number of wells)</th>
<th>Positive Cultures</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>7</td>
<td>MGG18-wtIDH1-2Gy-PRT</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>8</td>
<td>MGG18-wtIDH1-2Gy-PRT</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>11</td>
<td>MGG18-wtIDH1-2Gy-PRT</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>12</td>
<td>MGG18-wtIDH1-2Gy-PRT</td>
</tr>
<tr>
<td>1</td>
<td>36</td>
<td>4</td>
<td>MGG18-wtIDH1-4Gy-PRT</td>
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<tr>
<td>5</td>
<td>36</td>
<td>16</td>
<td>MGG18-wtIDH1-4Gy-PRT</td>
</tr>
<tr>
<td>10</td>
<td>36</td>
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<td>MGG18-wtIDH1-4Gy-PRT</td>
</tr>
<tr>
<td>20</td>
<td>36</td>
<td>36</td>
<td>MGG18-wtIDH1-4Gy-PRT</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>1</td>
<td>MGG18-wtIDH1-6Gy-PRT</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>11</td>
<td>MGG18-wtIDH1-6Gy-PRT</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>20</td>
<td>MGG18-wtIDH1-6Gy-PRT</td>
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<tr>
<td>20</td>
<td>24</td>
<td>23</td>
<td>MGG18-wtIDH1-6Gy-PRT</td>
</tr>
</tbody>
</table>

Table 22: ELDA estimates of NSFF with 95% confidence intervals for MGG18-IDH1<sup>WT</sup> at 2, 4, and 6 Gy PRT.
Neurosphere formation frequency refers to the active cell frequency estimated by ELDA with the upper and lower 95% confidence intervals. The fold reduction represents a ratio of the NSFF calculated for MGG18-IDH1<sup>R132H</sup> compared to MGG18-IDH1<sup>WT</sup> GSCs at a given radiation dose.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lower 95% CI</th>
<th>NSFF Estimate</th>
<th>Upper 95% CI</th>
<th>Fold Reduction Relative to IDH1&lt;sup&gt;WT&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH1&lt;sup&gt;WT&lt;/sup&gt; 2 Gy PRT</td>
<td>1/5.63</td>
<td>1/3.72</td>
<td>1/2.53</td>
<td>1.00</td>
</tr>
<tr>
<td>IDH1&lt;sup&gt;WT&lt;/sup&gt; 4 Gy PRT</td>
<td>1/8.72</td>
<td>1/6.93</td>
<td>1/5.53</td>
<td>1.00</td>
</tr>
<tr>
<td>IDH1&lt;sup&gt;WT&lt;/sup&gt; 6 Gy PRT</td>
<td>1/9.89</td>
<td>1/7.45</td>
<td>1/5.65</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 23: ELDA calculated adherence to the single-hit model and heterogeneity for MGG18-IDH1<sup>WT</sup> GSCs irradiated at 2, 4, and 6 Gy PRT.
A likelihood ratio test with p<0.05 indicates the probability that the single-hit model is accurate. A score test with p<0.05 likely indicates that assay replicates completed at different times are not heterogeneous.

<table>
<thead>
<tr>
<th>Goodness of Fit Test</th>
<th>χ² Value</th>
<th>Degrees of Freedom</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Likelihood ratio test of single-hit model</td>
<td>1.29</td>
<td>1</td>
<td>0.256</td>
</tr>
<tr>
<td>Score test of heterogeneity</td>
<td>2.5</td>
<td>1</td>
<td>0.114</td>
</tr>
</tbody>
</table>
Table 24: ELDA input data for MGG18-IDH1<sup>R132H</sup> at 2, 4, and 6 Gy PRT.

The raw data of positive cultures, indicating successful neurosphere formation, for MGG18-IDH1<sup>R132H</sup> GSCs treated with 2, 4, and 6 Gy PRT.

<table>
<thead>
<tr>
<th>Dose (cells/well)</th>
<th>Tested (number of wells)</th>
<th>Positive Cultures</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>4</td>
<td>MGG18-IDH1-R132H-2Gy-PRT</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>6</td>
<td>MGG18-IDH1-R132H-2Gy-PRT</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>10</td>
<td>MGG18-IDH1-R132H-2Gy-PRT</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>12</td>
<td>MGG18-IDH1-R132H-2Gy-PRT</td>
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<tr>
<td>1</td>
<td>36</td>
<td>0</td>
<td>MGG18-IDH1-R132H-4Gy-PRT</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>14</td>
<td>MGG18-IDH1-R132H-4Gy-PRT</td>
</tr>
<tr>
<td>10</td>
<td>36</td>
<td>27</td>
<td>MGG18-IDH1-R132H-4Gy-PRT</td>
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<td>MGG18-IDH1-R132H-4Gy-PRT</td>
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<tr>
<td>1</td>
<td>24</td>
<td>2</td>
<td>MGG18-IDH1-R132H-6Gy-PRT</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>4</td>
<td>MGG18-IDH1-R132H-6Gy-PRT</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>11</td>
<td>MGG18-IDH1-R132H-6Gy-PRT</td>
</tr>
<tr>
<td>20</td>
<td>24</td>
<td>20</td>
<td>MGG18-IDH1-R132H-6Gy-PRT</td>
</tr>
</tbody>
</table>

Table 25: ELDA estimates of NSFF with 95% confidence intervals for MGG18-IDH1<sup>R132H</sup> at 2, 4, and 6 Gy PRT.

Neurosphere formation frequency refers to the active cell frequency estimated by ELDA with the upper and lower 95% confidence intervals. The fold reduction represents a ratio of the NSFF calculated for MGG18-IDH1<sup>R132H</sup> compared to MGG18-IDH1<sup>WT</sup> GSCs at a given radiation dose.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lower 95% CI</th>
<th>NSFF Estimate</th>
<th>Upper 95% CI</th>
<th>Fold Reduction Relative to IDH1&lt;sup&gt;WT&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH1&lt;sup&gt;WT&lt;/sup&gt; 2 Gy PRT</td>
<td>1/8.41</td>
<td>1/5.63</td>
<td>1/3.84</td>
<td>1.51</td>
</tr>
<tr>
<td>IDH1&lt;sup&gt;WT&lt;/sup&gt; 4 Gy PRT</td>
<td>1/13.81</td>
<td>1/10.85</td>
<td>1/8.54</td>
<td>1.57</td>
</tr>
<tr>
<td>IDH1&lt;sup&gt;WT&lt;/sup&gt; 6 Gy PRT</td>
<td>1/21.11</td>
<td>1/15.30</td>
<td>1/11.14</td>
<td>2.05</td>
</tr>
</tbody>
</table>

Table 26: ELDA calculated adherence to the single-hit model and heterogeneity for MGG18-IDH1<sup>R132H</sup> GSCs irradiated at 2, 4, and 6 Gy PRT.

A likelihood ratio test with p<0.05 indicates the probability that the single-hit model is accurate. A score test with p<0.05 likely indicates that assay replicates completed at different times are not heterogeneous.
Figure 31: IDH1\textsuperscript{R132H} impairs GSC self-renewal after both XRT and PRT.
Post-radiation neurosphere self-renewal was measured using Extreme Limiting Dilution Analysis (ELDA) in MGG18-IDH1\textsuperscript{WT} and MGG18-IDH1\textsuperscript{R132H} human GSCs treated with (A) 6 Gy X-ray radiotherapy (XRT) and (B) 6 Gy Proton radiotherapy (PRT).
**Table 27: ELDA input data for MGG18-IDH1<sup>WT</sup> and MGG18-IDH1<sup>R132H</sup> at 6 Gy XRT.**

The raw data of positive cultures, indicating successful neurosphere formation, for MGG18-IDH1<sup>WT</sup> and MGG18-IDH1<sup>R132H</sup> GSCs treated with 6 Gy XRT.

<table>
<thead>
<tr>
<th>Dose (cells/well)</th>
<th>Tested (number of wells)</th>
<th>Positive Cultures</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>1</td>
<td>MGG18-wtIDH1-6Gy-PRT</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>11</td>
<td>MGG18-wtIDH1-6Gy-PRT</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>20</td>
<td>MGG18-wtIDH1-6Gy-PRT</td>
</tr>
<tr>
<td>20</td>
<td>24</td>
<td>23</td>
<td>MGG18-wtIDH1-6Gy-PRT</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>2</td>
<td>MGG18-IDH1-R132H-6Gy-PRT</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>4</td>
<td>MGG18-IDH1-R132H-6Gy-PRT</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>11</td>
<td>MGG18-IDH1-R132H-6Gy-PRT</td>
</tr>
<tr>
<td>20</td>
<td>24</td>
<td>20</td>
<td>MGG18-IDH1-R132H-6Gy-PRT</td>
</tr>
</tbody>
</table>

**Table 28: ELDA estimates of NSFF with 95% confidence intervals for MGG18-IDH1<sup>WT</sup> and MGG18-IDH1<sup>R132H</sup> at 6 Gy XRT.**

Neurosphere formation frequency refers to the active cell frequency estimated by ELDA with the upper and lower 95% confidence intervals. The fold reduction represents a ratio of the NSFF calculated for MGG18-IDH1<sup>R132H</sup> GSCs treated with 6 Gy XRT compared to MGG18-IDH1<sup>WT</sup> treated with 6 Gy XRT.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lower 95% CI</th>
<th>NSFF Estimate</th>
<th>Upper 95% CI</th>
<th>Fold Reduction Relative to IDH1&lt;sup&gt;WT&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH1&lt;sup&gt;WT&lt;/sup&gt; 6 Gy XRT</td>
<td>1/13.90</td>
<td>1/10.90</td>
<td>1/8.60</td>
<td>1.00</td>
</tr>
<tr>
<td>IDH1&lt;sup&gt;R132H&lt;/sup&gt; 6 Gy XRT</td>
<td>1/29.90</td>
<td>1/23.1</td>
<td>1/17.8</td>
<td>2.12</td>
</tr>
</tbody>
</table>

**Table 29: ELDA calculated adherence to the single-hit model and heterogeneity for TS543-ATRX<sup>WT</sup> and TS543-ATRX<sup>loss</sup> at 6 Gy XRT.**

Since LDA accuracy relies on the validity of the Poisson single-hit model, a likelihood ratio test for whether the data follows a single-hit model was calculated. A p-value less than 0.05 indicates adherence to the single-hit model. The score test for heterogeneity determines whether assays conducted on different days exhibit a similar NSFF. A p-value less than 0.05 indicates that different assay replicates are not heterogeneous. The slope of the solid line also provides information on these two parameters. Slopes greater than 1 could be indicative of a multi-hit model while slopes less than 1 could indicate assay heterogeneity or impeded ability of a sample to form neurospheres due to interference.

<table>
<thead>
<tr>
<th>Goodness of Fit Test</th>
<th>χ² Value</th>
<th>Degrees of Freedom</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Likelihood ratio test of single-hit model</td>
<td>0.462</td>
<td>1</td>
<td>0.497</td>
</tr>
<tr>
<td>Score test of heterogeneity</td>
<td>0.0692</td>
<td>1</td>
<td>0.793</td>
</tr>
</tbody>
</table>
Table 30: ELDA input data for MGG18-IDH1<sup>WT</sup> and MGG18-IDH1<sup>R132H</sup> at 6 Gy PRT.
The raw data of positive cultures, indicating successful neurosphere formation, for MGG18-IDH1<sup>WT</sup> and MGG18-IDH1<sup>R132H</sup> GSCs treated with 6 Gy PRT.

Table 31: ELDA estimates of NSFF with 95% confidence intervals for MGG18-IDH1<sup>WT</sup> and MGG18-IDH1<sup>R132H</sup> at 6 Gy PRT.
Neurosphere formation frequency refers to the active cell frequency estimated by ELDA with the upper and lower 95% confidence intervals. The fold reduction represents a ratio of the NSFF calculated for MGG18-IDH1<sup>R132H</sup> GSCs treated with 6 Gy XRT compared to MGG18-IDH1<sup>WT</sup> treated with 6 Gy PRT.

Table 32: ELDA calculated adherence to the single-hit model and heterogeneity for MGG18-IDH1<sup>WT</sup> and MGG18-IDH1<sup>R132H</sup> at 6 Gy PRT.
Since LDA accuracy relies on the validity of the Poisson single-hit model, a likelihood ratio test for whether the data follows a single-hit model was calculated. A p-value less than 0.05 indicates adherence to the single-hit model. The score test for heterogeneity determines whether assays conducted on different days exhibit a similar NSFF. A p-value less than 0.05 indicates that different assay replicates are not heterogeneous. The slope of the solid line also provides information on these two parameters. Slopes greater than 1 could be indicative of a multi-hit model while slopes less than 1 could indicate assay heterogeneity or impeded ability of a sample to form neurospheres due to interference.
Figure 32: IDH1R132H does not enhance GSC sensitivity to PRT over XRT.

Cell viability was measured using the luminescence based CellTiterGlo 3D assay at 14 days post-irradiation. (A) Intracellular ATP concentration was calculated using a curvilinear ATP standard curve as detailed in Figure 11 and (B) normalized relative to the 0 Gy negative control. Since the data do not consistently exhibit normality across doses and cell lines, a non-parametric approach was used to demonstrate statistical significance. The cumulative distributions of each condition were compared using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals in black.
Figure 33: Cumulative frequency plots of raw intracellular ATP data for MGG18-IDH1\WT and MGG18-IDH1\RI32H treated with 0, 4, 6, or 8 Gy of XRT and PRT. The raw ATP data presented in Figure 32 was fit to the cumulative Gaussian function using a non-weighted least squares regression model in Graphpad Prism 9. Although the data appears to violate normality upon visual inspection, further analysis was conducted using the Shapiro-Wilk test, whose results are shown in Table 33.

Table 33: Shapiro-Wilk Test for normality of raw ATP data for MGG18-IDH1\WT and MGG18-IDH1\RI32H treated with 0, 4, 6, or 8 Gy of XRT and PRT. The Shapiro-Wilk test for normality was chosen due to its high average empirical power compared to other tests at both small and larger sample sizes of symmetric and asymmetric distributions.\(^{103}\) W and P values for each condition were calculated in Graphpad Prism 9.
Figure 34: Protons increase apoptotic cell death in both IDH1^{WT} and IDH1^{R132H} GSCs, but only necrotic cell death in IDH1^{R132H} GSCs compared to X-rays. MGG18-IDH1^{WT} and MGG18-IDH1^{R132H} GSCs were subject to 8 Gy XRT or PRT. RealTime-Glo Annexin V (A) luminescence (apoptosis) and (B) fluorescence (necrosis) output measurements were taken at t=0, 12, 24, 48, and 72 hours after irradiation. All measurements for each condition were normalized to the luminescence measured at t=0 hours. P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
**IDH1\textsuperscript{R132H} drives radiosensitivity in the context of ATRX\textsuperscript{Loss}**

Ivosidenib is a newly developed chemical inhibitor selective for arginine 132 of cytosolic IDH1 that significantly reduces the production of 2-HG. This inhibitor was first shown to impair the growth of AML blast cells in vitro as well as promoted their differentiation.\textsuperscript{109} As a result of promising preclinical data, Ivosidenib is currently in two independent phase I clinical trials for patients with low-grade gliomas.\textsuperscript{109-111} Due to the combined presence of IDH1\textsuperscript{R132H} and ATRX inactivating mutations in patients with astrocytoma,\textsuperscript{11} we investigated how IDH1\textsuperscript{R132H} impacts the cell death mechanisms that drive radiosensitivity in ATRX\textsuperscript{Loss} GSCs that we previously discovered by combining Ivosidenib with radiation therapy. To determine the effects of this combination therapy on cell viability, we first treated the IDH1\textsuperscript{R132H}/ATRX\textsuperscript{Loss} GS522 GSC line with Ivosidenib 1-2 hours prior to 4-10 Gy XRT or PRT. Following previously described methods, we quantified the intracellular ATP concentration at 14 days post-irradiation using the CellTiterGlo 3D assay. Inhibiting IDH1\textsuperscript{R132H} with Ivosidenib prior to XRT does not demonstrate consistent statistically significant effects on GS522 post-radiation viability (Figure 35). Although the effects are similar for GS522s treated with Ivosidenib and PRT, these cells exhibit slight radioresistance at higher PRT doses, especially at 8 Gy (Figure 36). Consistent with isogenic MGG18 and TS543 GSCs, GS522 exhibits profound sensitivity to PRT over XRT in both the raw ATP and 0 Gy normalized data (Figure 37). Using the RealTime-Glo Annexin V apoptosis and necrosis assay protocol detailed previously, we discovered that combining Ivosidenib with either 6 Gy XRT (Figures 38-39) or 6 Gy PRT (Figures 40-41) impairs both apoptotic and necrotic cell death in an Ivosidenib dose-dependent manner.
Figure 35: Combining IDH1<sup>R132H</sup> inhibitors with XRT does not significantly reduce viability in ATRX<sup>Loss/IDH1<sup>R132H</sup></sup> GSCs.

GS522 GSCs were subject to treatment with Ivosidenib (IDH1<sup>R132H</sup> inhibitor) 2 hours prior to treatment with 4, 6, 8, or 10 Gy XRT. Cell viability was measured at 14 days post-irradiation using the CellTiterGlo 3D assay. The raw intracellular ATP concentrations measured are displayed in (A) while the normalized data relative to 0 Gy are displayed in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals in black.
Figure 36: Combining IDH1<sup>R132H</sup> inhibitors with PRT slightly increases post-radiation viability in ATRX<sup>Loss</sup>/IDH1<sup>R132H</sup> GSCs.

GS522 GSCs were subject to treatment with Ivosidenib (IDH1<sup>R132H</sup> inhibitor) 2 hours prior to treatment with 4, 6, 8, or 10 Gy PRT. Cell viability was measured at 14 days post-irradiation using the CellTiterGlo 3D assay. The raw intracellular ATP concentrations measured are displayed in (A) while the normalized data relative to 0 Gy are displayed in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals in black.
Figure 37: Proton radiotherapy significantly decreases the viability of GS522 (ATRX\textsuperscript{1.05V/IDH1\textsuperscript{R132H}}) GSCs compared to ionizing X-ray radiotherapy. Cell viability was measured at 14 days after irradiation with 0, 4, 6, 8, or 10 Gy XRT or PRT using the CellTiterGlo 3D assay. The raw intracellular ATP concentrations measured are displayed in (A) while the normalized data relative to 0 Gy are displayed in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Figure 38: Combining IDH1^{R132H} inhibitors with XRT significantly decreases apoptotic cell death in ATRX^{Loss}/IDH1^{R132H} GSCs.

GS522 GSCs were subject to treatment with Ivosidenib (IDH1^{R132H} inhibitor) 2 hours prior to treatment with 6 Gy XRT. Apoptotic cell death was measured using the Annexin V luminescence assay with measurements taken at t=0, 8, 16, 24, 32, 40, 48, and 72 hours post irradiation. The raw intracellular ATP concentrations collected are presented in (A) while the data normalized to t=0 hours for each Ivosidenib concentration is presented in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Combining IDH1R132H inhibitors with XRT significantly decreases necrotic cell death in ATRX Loss/IDH1R132H GSCs. GS522 GSCs were subject to treatment with Ivosidenib (IDH1 R132H inhibitor) 2 hours prior to treatment with 6 Gy XRT. Necrotic cell death was measured using the Annexin V necrosis assay with measurements taken at t=0, 8, 16, 24, 32, 40, 48, and 72 hours post irradiation. The raw intracellular ATP concentrations collected are presented in (A) while the data normalized to t=0 hours for each Ivosidenib concentration is presented in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Figure 40: Combining IDH1\textsuperscript{R132H} inhibitors with PRT significantly decreases apoptotic cell death in ATRX\textsuperscript{Loss}/IDH1\textsuperscript{R132H} GSCs.

GS522 GSCs were subject to treatment with Ivosidenib (IDH1\textsuperscript{R132H} inhibitor) 2 hours prior to treatment with 6 Gy PRT. Apoptotic cell death was measured using the Annexin V luminescence assay with measurements taken at t=0, 8, 16, 24, 32, 40, 48, and 72 hours post irradiation. The raw intracellular ATP concentrations collected are presented in (A) while the data normalized to t=0 hours for each Ivosidenib concentration is presented in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Figure 41: Combining IDH1<sup>R132H</sup> inhibitors with PRT significantly decreases necrotic cell death in ATRX<sup>Loss</sup>/IDH1<sup>R132H</sup> GSCs.

GS522 GSCs were subject to treatment with Ivosidenib (IDH1<sup>R132H</sup> inhibitor) 2 hours prior to treatment with 6 Gy PRT. Necrotic cell death was measured using the Annexin V necrosis assay with measurements taken at t=0, 8, 16, 24, 32, 40, 48, and 72 hours post irradiation. The raw intracellular ATP concentrations collected are presented in (A) while the data normalized to t=0 hours for each Ivosidenib concentration is presented in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
**IDH1<sup>R132H</sup> drives radioresistance in the context of wild-type ATRX**

In addition to identifying molecular markers for diagnosing astrocytomas, the World Health Organization (WHO) designated oligodendrogliomas as ATRX<sup>WT</sup>/IDH1<sup>R132H</sup> tumors in 2016. We treated TS603s, a ATRX<sup>WT</sup>/IDH1<sup>R132H</sup> human GSC line, with combined Ivosidenib and radiation therapy to determine how the intact ATRX protein and IDH1<sup>R132H</sup> impact GSC radiosensitivity. When pre-treating cells 1-2 hours prior to radiation therapy, Ivosidenib improves TS603 sensitivity to both XRT (Figure 42) and PRT (Figure 43) in a dose-dependent manner, as indicated by both raw and normalized ATP data relative to 0 Gy obtained using the CellTiterGlo 3D viability assay. To quantify the cell death mechanisms present in TS603 GSCs treated with radiation therapy and Ivosidenib, we used the RealTime-Glo Annexin V apoptosis and necrosis assay to measure apoptotic and necrotic cell death from 0-72 hours after irradiation. After analysis of the raw data, we discovered that combining Ivosidenib with 8 Gy XRT suppresses apoptotic and necrotic cell death in TS603 GSCs (Figures 44A and 45A). To account for the differences in luminescent and florescent signal at the beginning of the experiment, we normalized the data for each Ivosidenib concentration to 0 hours (Figures 44B and 45B). Normalizing the data demonstrates that Ivosidenib increases apoptotic and necrotic cell death in TS603s subject to 8 Gy XRT. Although similar trends were found in TS603 GSCs treated with combined 8 Gy PRT and Ivosidenib, they were not found to be statistically significant (Figures 46 and 47). Our results suggest that IDH1<sup>R132H</sup> promotes radiosensitivity in ATRX<sup>Loss</sup> GSCs, but radioresistance in ATRX<sup>WT</sup> GSCs. However, confirming these results by using limiting dilution analysis (ELDA) will be critical in solidifying the validity of these results.
Figure 42: Combining IDH1<sup>R132H</sup> inhibitors with XRT significantly reduces viability in ATRX<sup>WT</sup>/IDH1<sup>R132H</sup> GSCs.

TS603 GSCs were subject to treatment with Ivosidenib (IDH1<sup>R132H</sup> inhibitor) 2 hours prior to treatment with 4, 6, 8, or 10 Gy XRT. Cell viability was measured at 14 days post-irradiation using the CellTiterGlo 3D assay. The raw intracellular ATP concentrations measured are displayed in (A) while the normalized data relative to 0 Gy are displayed in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals in black.
Figure 43: Combining IDH1\textsuperscript{R132H} inhibitors with PRT significantly decreases post-radiation viability in ATRX\textsuperscript{WT}/IDH1\textsuperscript{R132H} GSCs.

TS603 GSCs were subject to treatment with Ivosidenib (IDH1\textsuperscript{R132H} inhibitor) 2 hours prior to treatment with 4, 6, 8, or 10 Gy PRT. Cell viability was measured at 14 days post-irradiation using the CellTiterGlo 3D assay. The raw intracellular ATP concentrations measured are displayed in (A) while the normalized data relative to 0 Gy are displayed in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals in black.
Figure 44: Combining IDH1<sup>R132H</sup> inhibitors with XRT significantly increases apoptotic cell death in ATRX<sup>WT</sup>/IDH1<sup>R132H</sup> GSCs.

TS603 GSCs were subject to treatment with Ivosidenib (IDH1<sup>R132H</sup> inhibitor) 2 hours prior to treatment with 8 Gy XRT. Apoptotic cell death was measured using the Annexin V luminescence assay with measurements taken at t=0, 8, 16, 24, 32, 40, 48, and 72 hours post irradiation. The raw intracellular ATP concentrations collected are presented in (A) while the data normalized to t=0 hours for each Ivosidenib concentration is presented in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Figure 45: Combining IDH1<sup>R132H</sup> inhibitors with XRT significantly increases necrotic cell death in ATRX<sup>WT</sup>/IDH1<sup>R132H</sup> GSCs.

TS603 GSCs were subject to treatment with Ivosidenib (IDH1<sup>R132H</sup> inhibitor) 2 hours prior to treatment with 8 Gy XRT. Necrotic cell death was measured using the Annexin V necrosis assay with measurements taken at t=0, 8, 16, 24, 32, 40, 48, and 72 hours post irradiation. The raw intracellular ATP concentrations collected are presented in (A) while the data normalized to t=0 hours for each Ivosidenib concentration is presented in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Figure 46: Combining IDH1<sup>R132H</sup> inhibitors with PRT slightly, but not significantly increases apoptotic cell death in ATRX<sup>WT</sup>/IDH1<sup>R132H</sup> GSCs.

TS603 GSCs were subject to treatment with Ivosidenib (IDH1<sup>R132H</sup> inhibitor) 2 hours prior to treatment with 8 Gy PRT. Apoptotic cell death was measured using the Annexin V luminescence assay with measurements taken at t=0, 8, 16, 24, 32, 40, 48, and 72 hours post irradiation. The raw intracellular ATP concentrations collected are presented in (A) while the data normalized to t=0 hours for each Ivosidenib concentration is presented in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Figure 47: Combining IDH1<sup>R132H</sup> inhibitors with PRT does not significantly impact necrotic cell death in ATRX<sup>WT</sup>/IDH1<sup>R132H</sup> GSCs.

TS603 GSCs were subject to treatment with Ivosidenib (IDH1<sup>R132H</sup> inhibitor) 2 hours prior to treatment with 8 Gy XRT. Necrotic cell death was measured using the Annexin V necrosis assay with measurements taken at t=0, 8, 16, 24, 32, 40, 48, and 72 hours post irradiation. The raw intracellular ATP concentrations collected are presented in (A) while the data normalized to t=0 hours for each Ivosidenib concentration is presented in (B). P-values were calculated using the Kolmogorov-Smirnov test and Holm-Sidak’s test for multiple comparisons. Outliers were removed using ROUT (Q=1%) in Graphpad Prism 9. The mean is reported along with its upper and lower 95% confidence intervals.
Discussion

We demonstrated that ATRX\textsuperscript{Loss} impairs GSC self-renewal and viability by increasing radiation induced apoptotic and necrotic cell death compared to ATRX\textsuperscript{WT}. The radiosensitization driven by ATRX inactivation was profoundly increased in GSCs treated with PRT compared to XRT. This is consistent with results presented by Alan Mitteer et al (2015) that demonstrate enhanced GSC toxicity after PRT compared to XRT due to the elevated reactive oxygen species production in the cell.\textsuperscript{95} With respect to ATRX\textsuperscript{Loss} in GSCs, we corroborate previous work conducted by Koschmann et al (2016) that identified ATRX\textsuperscript{Loss} sensitized GBM cells to ionizing radiation by impairing DSB repair activity via NHEJ, as demonstrated by decreased DNA-PKCs phosphorylation.\textsuperscript{112} The results of our cell viability assays are also in line with the consensus that PRT exhibits a higher relative biological effectiveness (RBE) when compared to XRT, especially with regards to cell death.\textsuperscript{113,114} Although clinical PRT is assigned a consistent RBE of 1.1 by radiation oncologists, the RBE of PRT is variable.\textsuperscript{115,116} The distal dose fall off regions of the spread-out Bragg peak (SOBP) are especially well known to exhibit higher RBE values than the middle of the SOBP. Our irradiations were conducted in the middle of the SOBP to ensure that the entire GSC spheroid received a uniform dose and for its clinical relevance since most of the tumor volume is located within the SOBP. However, the exact RBE at each point of the SOBP is dependent on the LET and other biological factors, including tumor genotype, endpoint, and tumor microenvironment.\textsuperscript{115} Our results demonstrate that ATRX\textsuperscript{Loss} enhances the cytotoxicity of radiation therapy in GSCs to a higher extent after PRT compared to XRT. This suggests that PRT might serve
as a more effective treatment modality to eliminate GSCs and impair secondary tumor recurrence in patients with ATRX\textsuperscript{Loss} tumors.

Although previous studies suggest that ATRX\textsuperscript{Loss} gliomas might exhibit radioresistance due to their heightened proliferation and ability to maintain telomere length via the alternative lengthening of telomeres (ALT) phenotype\textsuperscript{50,117}, we demonstrated that ATRX\textsuperscript{Loss} drives radiosensitization via PRT induced necrosis. Our work corroborates that of Eid et. al (2015), who proved that ATRX\textsuperscript{Loss} increases the proportion of necrotic cells in vitro via flow cytometry using 8-MG-BA glioma as a model system\textsuperscript{118}. Although RIPK3 has been previously shown by Lee et al. (2013) to phosphorylate the ATRX transcriptional co-activator DAXX at Ser-668, thereby allowing DAXX to leave the nucleus in rat retinal ganglion cells, we are the first to establish a connection between ATRX\textsuperscript{WT} and RIPK1/RIPK3 mediated necroptosis in GSCs\textsuperscript{119}. This gives rise to the possibility that the ATRX-DAXX complex phosphorylates RIPK3 in the nucleus; however, further investigation is required to confirm this proposed mechanism.

Alongside our results demonstrating ATRX\textsuperscript{Loss} driven radiosensitivity in isogenic human GSCs, we provide evidence to support the conclusion that PRT significantly enhances apoptosis and necrosis in isogenic IDH1\textsuperscript{R132H} GSCs compared to XRT. Although IDH1\textsuperscript{R132H} suppresses post-radiation GSC self-renewal after XRT and PRT, we did not exhibit any significant differences in cell viability between IDH1\textsuperscript{WT} and IDH1\textsuperscript{R132H} after radiation therapy. These results are in contrast to previous reports identifying IDH1\textsuperscript{R132H} as a key radiosensitizing agent in glioblastoma\textsuperscript{120-122}. However, a consensus on whether IDH1\textsuperscript{R132H} promotes sensitivity or resistance to radiation therapy has not yet been conclusively defined. Several groups have demonstrated that IDH1\textsuperscript{R132H}...
isogenically induces radiosensitization in different cancer cell lines (HCT116 and U87MG) by upregulating the production of dsDNA damage response proteins gH2AX and 53BP1 as well as by suppressing 3D spheroid growth. These results corroborate the clinical findings that patients with IDH mutant gliomas tend to exhibit higher median survival than those with wtIDH tumors. The enhanced chemoradioresistance of glioma stem cells might contribute to the non-significant differences in cell viability that we observed. Verifying the presence of the IDH1R132H experimentally via western blot or by quantifying the intracellular concentration of 2-HG before and after radiation therapy for the isogenic MGG18 GSC line could provide valuable insight into these experimental findings.

Finally, we established that IDH1R132H promotes radiosensitivity in ATRX Loss GSCs and promotes radioresistance in ATRXWT GSCs, as demonstrated in experiments combining radiation therapy with the selective IDH1R132H inhibitor Ivosidenib. Although IDH1R132H inhibitors are currently seeing success in clinical trials when administered independently, our results demonstrate that combining them with radiation therapy in real time could be non-advantageous and possibly dangerous for patients with astrocytomas (ATRX Loss and IDH mutant). We corroborated the findings presented by Molenaar et al (2015), who demonstrated that combining AGI-5198 (another selective IDH1R132H inhibitor) with ionizing radiation in U251 glioblastoma cells increases clonogenic survival and decreases ROS levels in vitro. Although many studies have focused on investigating the role of IDH1R132H alone, there is a recent increase in research efforts dedicated to identifying the role of other key genetic alterations that commonly present alongside IDH1R132H in both low-grade gliomas and glioblastomas. Nuñez et al
(2019) discovered that IDH1R132H promotes radioresistance in the context of ATRX\textsuperscript{Loss} and TP53 inactivation by suppressing gH2AX and 53BP1 recruitment to dsDNA breaks as well as upregulating the expression of homologous recombination specific proteins (ATM, RAD51, and BRCA1) for dsDNA break (DSB) repair.\textsuperscript{102} A follow up study conducted by Kadiyala et al (2021) supported this conclusion by demonstrating that combining AGI-5198 (IDH1R132H inhibitor) with ionizing radiation in ATRX\textsuperscript{Loss}/IDH1R132H glioma neurospheres reduced clonogenic survival and increased the extracellular secretion of tumor damage associated molecular pattern molecules (DAMPs) HMGB1, ATP, and calretinin.\textsuperscript{125} These results directly contradict previous findings that IDH1R132H promotes patient survival by enhancing glioma dsDNA damage caused by radiation therapy. Our results build upon the discoveries of Molenaar et al (2015) by identifying an alternative mechanism for IDH1R132H inhibitor induced radioresistance that incorporates the ATRX status of the cell. It will be interesting to investigate the connections, if any, between IDH1R132H and subsequent 2-HG production on RIPK1/RIPK3 mediated necroptosis. Taking into consideration the presence of ATRX status of a patient’s tumor, we argue that combining IDH1R132H inhibitors with radiation might serve beneficial for patients with oligodendrogliomas.
Conclusion

In this study, we identified the potential for increased effectiveness of PRT vs. XRT in overcoming the chemoradioresistant nature of GSCs in several human cell lines. Using isogenic GSCs, we demonstrated that ATRX\textsuperscript{Loss} promotes radiosensitivity primarily via the RIPK1/RIPK3 mediated necroptosis cell death pathway. We also provide evidence to support the conclusion that the role of IDH1\textsuperscript{R132H} with regards to radiosensitivity depends heavily on a GSC’s ATRX status. Specifically, IDH1\textsuperscript{R132H} promotes radiosensitivity and suppressed apoptotic/necrotic cell death in the context of ATRX\textsuperscript{Loss}; the exact opposite was found in GSCs containing IDH1\textsuperscript{R132H} and ATRX\textsuperscript{WT}. Although our results suggest that PRT alone might be the best treatment option for patients with astrocytomas, the combination of IDH1\textsuperscript{R132H} inhibitors with PRT could potentially serve as a highly effective novel treatment strategy to eliminate GSCs in patients with oligodendrogliomas and overcome ATRX\textsuperscript{WT} driven radioresistance.
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Ángel Adrián Garcés was born on November 27, 1995 to Pedro Garcés and Maria del Pilar Loya. After graduating with an International Baccalaureate diploma from Allen High School (Allen, TX) in June 2014, he completed his Bachelor’s of Science (B.S.) in Inorganic Chemistry with a Distinction in Research at Rice University in May 2018. Ángel matriculated into the Master’s of Biomedical Sciences (M.S.) program at The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences in August 2018 and joined the lab of David Grosshans, M.D., Ph.D. in March 2019. Since Ángel’s long-term goal is to become a physician-scientist, he will pursue combined M.D./Ph.D. training at the University of Wisconsin School of Medicine and Public Health Medical Scientist Training Program starting in August 2022.

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