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An Idh1 Mutation Prevalent In Glioma Confers Deficient Dna Repair And Sensitivity To Parp Inhibition

Nathaniel David Robinson
Yale University

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An IDH1 mutation prevalent in glioma confers deficient DNA repair and sensitivity to PARP inhibition

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Nathaniel Robinson
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Abstract

AN IDH1 MUTATION PREVALENT IN GLIOMA CONFERS DEFICIENT DNA REPAIR AND SENSITIVITY TO PARP INHIBITION.

Nathaniel D. Robinson, Parker Sulkowski, Gregory A. Breuer, Ranjini K. Sundaram, Karin R. Purshouse, Nathan R. Fons, Peter M. Glazer, Ranjit S. Bindra. Department of Therapeutic Radiology, Yale University, School of Medicine, New Haven, CT.

High-grade gliomas (HGGs) are devastating malignancies of the central nervous system, and few treatment options are available for these tumors. In the most malignant form of the disease, glioblastoma multiforme (GBM), over 90% of patients will succumb to their tumor within 5 years after standard of care treatment, consisting of surgery, radiation therapy, and temozolomide chemotherapy. It is now clear that gliomas are molecularly heterogeneous entities, with mutations in tumor suppressors and oncogenes defining many distinct sub-types with important therapy implications. However, almost all HGGs are treated with a limited array of initial therapies, regardless of these molecular differences. Isocitrate dehydrogenase 1 (IDH1), a gene recently found to be mutated in many gliomas, is involved in the conversion of isocitrate to 2-oxoglutarate in cells. The IDH1 R132H mutant enzyme converts 2-oxoglutarate to the oncometabolite (R)-2-hydroxyglutarate (2HG), which leads to profound metabolic alterations in tumor cells. In addition, recent studies indicate that mutations in IDH1 may also induce altered DSB repair, differential sensitivities to chemo-radiotherapy, and substantial changes in chromatin modifications. Here, we present the creation of a novel HeLa cell line harboring an engineered IDH1 R132H mutation at the endogenous gene locus using CRISPR-Cas9 gene editing. We validated the cell line using a variety of biochemical and
functional assays. In particular, we demonstrated that our mutant cell clones secrete high levels of 2HG, and confirmed that the levels of this oncometabolite can be suppressed with small molecule inhibitors of mutant IDH1. We then performed a focused drug screen using select small molecule inhibitors of DNA repair, leveraging our observation that IDH1 mutant cells are more sensitive to radiation. We report that IDH1-R132h confers increased sensitivity to BMN-673, a PARP inhibitor known to preferentially kill cells with decreased homologous recombination (HR) functionality. We also demonstrate synergy between BMN-673 and the platinating agent, cisplatin, that is enriched by the IDH1-R132H mutation. Finally, preliminary gene expression analysis does not identify any significant decreased expression in a panel of DNA repair-related genes, suggesting that some alternative mechanism may be responsible for the drug sensitivity effect we see. Taken together, these findings suggest that IDH1 mutant tumors may be sensitive to PARP inhibition, representing a new treatment strategy for a devastating disease.
Acknowledgements

This paper is the product of a great deal of time and effort on the part of many. I wish to thank the Principle Investigator of this work and my mentor, Ranjit Bindra. There is no idea in this paper that was not either from his own mind or at least directly shaped by his insight. He has also provided endless support as a resource and mentor, and for all of these reasons and many more I owe him a great deal of gratitude. I am also grateful for the support of my department chair, Peter Glazer, who has also contributed his vast scientific expertise to this project.

Within the lab, I owe tremendous gratitude to Gregory Breuer, Nathan Fons, and Ranjini Sundaram, all of whom have provided their scientific opinion and technical expertise. They, along with Karin Purshouse, deserve special mention and thanks for their combined efforts in generating the IDH1 mutant cell line upon which this thesis is based. Of additional note, this paper is part of a larger work that has been directed by Parker Sulkowski in conjunction with Christopher Corso, both members of the Bindra and Glazer labs. My work has been generously funded by the Howard Hughes Medical Research Fellowship, which provided me not only with a living stipend but also tremendous opportunities for travel, networking, and scientific and personal growth.

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Introduction

Mutations in isocitrate dehydrogenase-1 (IDH1) occur in a wide variety of tumors, especially those of the central nervous system (CNS). Active site mutations, usually at Arg 132 to His, occur in 12% of primary glioblastoma multiforme (GBM), the most advanced form of the disease, and are present in the majority of secondary gliomas that have progressed from lower-grade tumors. In addition to CNS tumors, mutations in IDH1 and 2 are present in subsets of many cancer types, including hematologic malignancies such as acute myeloid leukemia (AML) and T-cell lymphoma, as well as other neoplasms like chondrosarcoma and intrahepatic cholangiocarcinoma.

IDH1 plays a critical role in cellular metabolism and is closely related to cellular respiration and the tricyclic acid cycle. Normally, the enzyme catalyzes the conversion of isocitrate to α-ketoglutarate (αKG), producing nicotinamide adenine dinucleotide phosphate (NADPH) in the process. However, mutant IDH1 (mIDH1), most often R132H, instead converts αKG to 2-hydroxyglutarate (2HG). IDH1 is located in the cytosolic compartment, as opposed to the closely related IDH2 and IDH3 (Fig. 1). This elevated 2HG is a hallmark of IDH1-mutant tumors, and is believed to confer many of the relevant tumorigenic effects upon them. IDH1-R132H has pleomorphic effects spanning a wide range of cellular activities. Given its close relationship to the tricyclic acid cycle, it is perhaps not surprising that it alters metabolic flux through several pathways, including pyruvate metabolism through pyruvate carboxylase. Indeed, significant alterations in gene expression of key metabolic pathways are seen in gliomas with IDH mutations. However, there are many other effects of 2HG that are currently under active investigation.
Intriguingly, IDH1-R132H inhibits TET2, a member of the TET family of tumor suppressors that are believed to regulate gene expression by demethylating DNA. Recent work by Turcan et al. in an IDH1-R132H-overexpressing astrocyte model has shown that the R132H mutation is sufficient to establish the “hypermethylator” phenotype characteristic of gliomas. 2HG is thought to be an oncometabolite, and while the mechanism of action has not been entirely elucidated, its effects on the cell are becoming increasingly well characterized. In addition to increasing DNA methylation, it has been shown that at high concentrations 2HG can inhibit the JmjC histone demethylases, several of which are tumor suppressors. 2HG thus has many broad and far-reaching effects on the cell, ranging from metabolic alterations to profound changes in DNA methylation. It is likely that 2HG induces many more changes that in turn promote oncogenesis.

An overview of gliomas and glioblastoma, and current treatment strategies

The IDH1-R132H mutation is extremely common, and occurs in 80-90% of secondary gliomas. Management of gliomas, especially high-grade gliomas (HGG) and GBM, is a challenging and complex medical process. The typical prognosis for glioma is quite poor. For even low-grade gliomas, the 10-year overall survival in adults is 43%; it is 14% in the elderly. Patients with GBM, or WHO Grade IV glioma, fare much worse, with a median survival time of only 15 months.

Overwhelmingly, gliomas have been treated with a combination of radiation therapy and temozolomide (TMZ), an alkylating agent, as well as surgery when appropriate. Recent
clinical trials have demonstrated important, though ultimately limited, successes, including extending progression-free survival without prolonging overall survival\textsuperscript{14}. IDH1-mutant tumors are more sensitive to alkylators such as TMZ and cisplatin (CDDP), and demonstrate increased reactive oxygen species in response to chemotherapy\textsuperscript{15}. Unfortunately, despite our increasingly more detailed understanding of how HGGs behave, clinicians are left with a dearth of therapeutic options. Genetic testing of tumors does play a role in the care of these patients; those with tumors that harbor the IDH1-R132H mutation as well as increased methylation at the O(6)-methylguanine-methyltransferase (MGMT) gene locus tend to have a better prognosis\textsuperscript{16}. However, though important for prognosis, tumor sequencing has yet to translate to dictating therapy.

Because IDH1-R132H is so prevalent, IDH1-mutant glioma is an intriguing target for the development of novel therapies. As part of this effort, several groups have developed inhibitors specific to IDH1-R132H on the presupposition that the mutated form of the enzyme is a tumor driver for glioma and other neoplasms\textsuperscript{17}. However, the mutation confers several characteristics, especially in glioma, that suggest this may not be the case. Recent work has shown that IDH1-R132H gliomas have decreased NAD\textsuperscript{+} and are exquisitely sensitive to nicotinamide phosphoribosyltransferase (NAMPT) inhibitors, which block a key NAD\textsuperscript{+} salvage pathway\textsuperscript{18}. Another group studying AML, in which mutant IDH1 or 2 occurs approximately 15\% of the time, found the mutation to be synthetic lethal with the anti-apoptotic gene BCL-2\textsuperscript{19}. 
Figure 1. Schematic of the tricyclic acid cycle and the relationship to IDH1. Mutant IDH1 (mIDH1) converts $\alpha$-ketoglutarate to 2-hydroxyglutarate, an oncometabolic with many far-reaching effects on the cell. It increased oxidative stress and causes epigenetic changes. Together, along with other unknown mechanisms, these changes promote tumorigenesis.
These investigations, as well as other observations of IDH1-mutant gliomas, suggest that inhibiting mIDH1 may not be the best strategy for treatment\textsuperscript{20}. Indeed, the metabolic and methylation changes induced by mIDH1 and 2HG may provide a therapeutic handle for treatment, and may also represent an opportunity to develop targeted therapies. IDH1-R132H gliomas tend to progress more slowly than IDH1-WT tumors, which argues in favor of non-oncogenic addiction rather than oncogenic tumorigenesis, as one would predict an oncogene to induce rapid and uncontrollable cell division and growth\textsuperscript{21,22}. Additionally, some recent studies have suggested that IDH1-R132H actually attenuates the DNA damage response\textsuperscript{23-25}. Because impaired DNA repair in tumors, notably BRCA-deficient tumors which are sensitive to PARP inhibitors, can serve as actionable pharmacologic targets, this finding warrants further investigation\textsuperscript{26-28}.

\textit{DNA repair, and implications for cancer therapeutics}

The disruption of the balance between DNA damage and repair is a key characteristic of many cancers\textsuperscript{29}. Given previous reports that mIDH1 can blunt the DNA damage response, this may serve as an important future therapeutic target for IDH1-mutant gliomas and other cancers. DNA damage can take on many forms, including single-stranded breaks, crosslinking, base mismatch, and double-stranded breaks. The repair mechanism of choice is dictated by which type of damage is present. Single-stranded breaks are repaired via base excision repair (BER), while crosslinks are repaired via the nucleotide excision repair (NER) pathway. Defects in NER lead to a cancer predisposition, as well as a variety of other diseases including ultraviolet radiation-
sensitivity syndrome\textsuperscript{30}. Base-pair mismatches are repaired via the mismatch repair (MMR) pathway.

The final variety of DNA damage is the double-stranded break. The dominant repair pathway is homologous recombination (HR), which occurs most often in S- and G2-phase when the sister chromatid is available as a template\textsuperscript{31}. When the sister template is not available, the double-stranded break is repaired via non-homologous end joining (NHEJ). Because there is no reference sequence available to copy from, NHEJ is inherently error-prone as it introduces insertions and deletions, commonly referred to as indels. These indels lead to genomic instability that can eventually cause oncogenic gain-of-function or loss of tumor-suppressor gene expression, which can eventually result in transformation from a healthy cell to a cancer cell\textsuperscript{29}. Together, these DNA repair pathways form a redundant system, such that a cell with a defect in one can generally compensate by up-regulating genes involved in other pathways.

Knowledge of DNA damage and repair has long been leveraged for the treatment of various neoplasms. Many of the oldest treatment modalities function primarily through causing DNA damage, albeit in an untargeted manner. For example, radiation therapy causes both single-stranded and double-stranded breaks. Alkylators, such as temozolomide, nitrosoureas, and cisplatin, cause base damage, DNA crosslinks, and bulky adducts, while antimetabolites such as 5-fluorouracil and folate analogues result in replication lesions\textsuperscript{32}. These non-targeted treatment strategies are predicated on the principle that a cancer cell tends to divide more rapidly than a normal cell, so that increased DNA damage would be more lethal to a rapidly dividing cell.
In addition to these conventional chemotherapeutic options, a variety of targeted therapies focused specifically on DNA damage and repair are currently under laboratory and clinical investigation. The error-prone NHEJ repair pathway has been exploited in tumors that have deficiencies in DNA repair. Currently, the best example is the strategy of poly-(ADP-ribose)-polymerase (PARP) inhibition in BRCA-deficient tumors, which causes increased errors due to NHEJ-based repair of double-stranded breaks that accumulate and become lethal. PARP inhibitors are often used in combination with conventional chemotherapy, often with alkylators such as temozolomide or platinum-containing agents like carboplatin. Investigators have thus complemented broad chemotherapy with novel strategies to focus strong cytotoxic agents while potentiating targeted, rationally designed small molecules.

An important principle in understanding DNA repair as a therapeutic target is the concept of synthetic lethality. A synthetic lethal gene pair is any set of genes in which mutation or inactivation of one or the other leads to a viable cell, while inactivating both is lethal. There have been many synthetic lethal interactions identified in the human genome, but the most relevant example for cancer care is the previously mentioned interaction between BRCA and PARP. BRCA-deficient tumors, such as certain breast and ovarian cancers, have a defective HR pathway. However, they remain viable because the cell has many redundant DNA repair mechanisms. Pharmacologic inhibition of PARP, which functions in the BER pathway, is not lethal in normal cells with intact HR, but it results in cell death in BRCA-deficient cancer cells. This shunts double-stranded break repair towards error-prone NHEJ, which results in the accumulation of errors which in turn causes apoptosis. Additionally, PARP inhibition causes stalled replication forks which
also leads to cell cycle arrest (Fig. 2). In this case, \textit{BRCA} and \textit{PARP} are considered synthetic lethal, and the therapeutic strategy leverages this relationship to specifically target BRCA-deficient cancer cells. Defective DNA repair in cancer cells is therefore an important target for novel therapeutics, as they can be targeted while limiting toxicity to normal cells using synthetic lethal strategies.
**Figure 2.** Representation of the effect of PARP inhibitors on healthy cells with intact DNA repair pathways and on BRCA-deficient cells. This is an example of the clinical application of synthetic lethal gene pairs.
Statement of Purpose and Hypothesis

The purpose of this work, broadly, is to identify new areas of study for the treatment of IDH1-mutant glioma. This devastating disease is in dire need of new therapeutics, as the standard of care has remained largely unchanged over the last ten years. There is a wealth of both clinical and laboratory data in IDH1-mutant tumors. One key finding has been that IDH1-R132H confers increased sensitivity to radiotherapy and alkylating agents, which suggests a deficient response to DNA damage. We hypothesize that mIDH1 does not function as an oncogene in the classic sense, but rather that the production of 2HG promotes the transformation of a cell into a cancer cell through some alternative mechanism. Additionally, we seek to determine if these tumors are susceptible to small molecule inhibitors of the DNA damage response, specifically inhibitors of DNA repair pathways. Given that ionizing radiation causes an increase in double-stranded DNA breaks, we further suggest that homologous recombination may be a targetable pathway in IDH1-mutant tumors, and that drugging these cells appropriately may potentiate the effect of other DNA damaging agents.
Methods

Unless otherwise mentioned, all experiments were carried out by Nathaniel Robinson under the guidance of Ranjit Bindra.

Chemicals. (2R)-octyl-α-hydroxyglutarate (Cayman), (2S)-octyl-α-hydroxyglutarate (Cayman), AGI-5198 (Selleckchem), octyl-α-ketoglutarate (Cayman), BMN-673 (Selleckchem), cisplatin (CDDP; Tocris), olaparib (Selleckchem), TH287 (Sigma), TCS2312 (Tocris), BEZ-235 (Selleckchem), KU5593 (Selleckchem), AZD7762 (Tocris), MK-1775 (Selleckchem), NU-7441 (Selleckchem), VE822 (Selleckchem), Methyl methanesulfonate (MMS; Sigma), temozolomide (TMZ; Sigma), lomustine (CCNU; Selleckchem), irinotecan (Selleckchem), mitomycin C (MMC; Cayman), razoxane (Santa Cruz Biotech), aphidicolin (Sigma).

Cell lines and culture conditions. HeLa cells were cultured in high glucose Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS; Clontech Laboratories and Thermo Fisher Scientific). HCT116 parental and HCT116 IDH1 R132H/+ colon cancer cell lines (Horizon Discovery HD 104-013) were cultured in McCoy’s 5A Medium containing 10% FBS. Immortalized human astrocytes were a gift from T. Chan and cultured as previously described in DMEM +10% FBS. Breast cancer cell line MCF10A was cultured in DMEM:F12 media with 10% FBS (Horizon Discover HD 101-013). All cells were maintained at 37°C with 5% CO2.

CRISPR-Cas9 gene editing. CRISPR-Cas9 gene editing of HeLa cells was performed by Karen Purshouse, Nathan Fons, Ranjini Sundaram, and Gregory Breuer. Validation experiments and figures presented in this manuscript were performed by Nathaniel
Robinson. IDH1 R132H/+ mutant HeLa cells were generated using the CRISPR-Cas9 gene editing system\textsuperscript{34}. Guide RNAs were designed to the IDH1 locus using the MIT-Broad design tool (http://crispr.mit.edu/), and single-stranded donor DNA containing the requisite mutation to convert Arg-132 to His plus a silent Bcl1 restriction endonuclease site was synthesized. These were co-transfected along with a separate plasmid containing the Cas9 cDNA using the Amaxa Nucleofector system (Lonza). Targeted cleavage was confirmed by T7 endonuclease assay. The protocol for this assay can be found in Ran et al., 2013. Following limiting dilution, single-cell colonies were screened for the heterozygous mutation by high-resolution melt analysis utilizing the silent Bcl1 restriction endonuclease site. This screening was performed by Nathan Fons in our lab. TOPO clones were generated using the TOPO-TA Cloning Kit per manufacturer’s protocol (Cat. #450071; Thermo Fisher Scientific). Sanger sequencing of both endogenous DNA as well as TOPO clones confirmed the presence of the heterozygous mutation.

**Western blotting.** Cells were lysed in AZ lysis buffer (50 mM Tris, 250 mM NaCl, 1% Igepal, 0.1% SDS, 5 mM EDTA, 10 mM Na2P2O7, 10 mM NaF) supplemented with Protease Inhibitor Cocktail (Roche) on ice for 20 min. Cellular debris was cleared by centrifugation and lysate protein concentration was quantified using the DC Protein Assay (Bio-Rad). Lysate containing 80 µg protein was subjected to SDS-PAGE in a Mini-PROTEAN TGX 4- 20% gradient gel (Bio-Rad) and then transferred to nitrocellulose or PVDF membrane. The following primary antibodies were used for western blot analysis: rabbit monoclonal anti-IDH1 (D2H1, Cell Signaling), rabbit polyclonal anti- IDH1-R132H (H09, Dianova), mouse monoclonal pan Actin Antibody
(ACTN05(C4)), ThermoFischer, mouse monoclonal anti DYKDDDDK Tag (9A3, Cell Signaling), rabbit polyclonal anti-SMC1 (Bethyl), mouse monoclonal anti-Vinculin (SPM227, Abcam). Membranes were imaged on a GelDoc imaging system (BioRad).

**HGDH-mediated enzyme assay.** Protocol as previously published\(^\text{35}\). For intracellular measurements, cells were lysed at \(10^6\) cells/mL of Cell Lysis Buffer (Cat. #9083, Cell Signaling) and various amounts added to the reaction mix to ensure measurements were taken within the dynamic range of the assay. Cell lysate from cells in culture was added to 75 µL of reaction mix containing 100 mM HEPES at pH 8.0, 1.0 U/mL Diaphorase, 5 µM resazurin, 100 µM NAD+, and 1.0 µg/mL HGDH in 96-well plates. The reactions were well-mixed and incubated at room temperature in the dark for 30 minutes. For extracellular measurements, 25 µL of conditioned media was added to 75 µL of reaction mix and incubated in the dark for 30 minutes. Following incubation, the plates were read on a plate reader in fluorescence mode (BioTek), with excitation of 510 nm and emission of 590 nm. Data is presented as the mean of 3 biological replicates ± SEM.

**NAD+ assay.** NAD+ levels were quantified using the NAD/NADH Quantification Kit per manufacturer’s protocol (Cat. #MAK037, Sigma). Cells were treated in triplicate with indicated concentrations of BMN-673 of FK866 or (2R)-octyl-2-HG. Data is presented as the mean of 3 biological replicates ± SEM.

**Annexin V apoptosis assay.** Cells were prepared using the APC Annexin V Apoptosis Detection Kit With PI protocol (Cat. #640930, BioLegend). Data are presented as the mean of 3 biological replicates ± SEM.
**Clonogenic survival assay.** Cells in culture were irradiated at varying doses of ionizing radiation. Four to six hours after irradiation, they were trypsinized, washed, counted, and seeded in 6-well plates in triplicate at 3-fold dilutions ranging from 9000 to 37 cells per well. Depending on colony size, these plates were kept in the incubator for 10 to 14 days. Following incubation, colonies were washed in PBS, stained with crystal violet, and counted and quantified. For drug treatments, cells were counted and diluted in media containing various concentration of drug. They were then immediately seeded in 6-well plates in triplicate at 3-fold dilutions, ranging from 9000 to 37 cells per well. These plates were kept in the incubator for 10 to 14 days, following which they were washed in PBS, stained with crystal violet, and manually counted. Data is presented as the mean ± SD. For the one clonogenic survival assay on HCT116 cells in response to radiation, cells were seeded in 96 well plates following irradiation. After one week, they were fixed in 70% EtOH, stained with DAPI, and imaged on a Cytation 3 automated imaging system (Biotek). Colonies were counted using a custom image analysis pipeline on CellProfiler (http://cellprofiler.org/).

**Cell cycle analysis.** Cells were fixed in triplicate with ice cold 70% ethanol for 2 h, pelleted and washed with PBS, then suspended in 500 µl of PI/RNAse Staining buffer (BD Biosciences) or 1:10000 DAPI in PBS and analyzed by flow cytometry. Data was analyzed using FlowJo software and presented as a representative plot.

**Short-term, high-throughput growth delay assays.** Cells were plated in 96-well black-walled plates (Costar) at a concentration of 2500 cells per well and allowed to adhere at room temperature for 60 min prior to return to the incubator. To avoid edge effects, the perimeter wells and the inter-well spaces were filled with sterile PBS. For growth delay
assays containing (2R)-octyl-2-HG, cells were cultured with indicated concentration for
10 days prior to plating. After 24 h, the media was changed and indicated drugs dissolved
in either DMSO or DMF (cisplatin only) were added in quadruplicate at varying
concentrations. For synergy experiments, cells were replica-plated and drugs added in
single wells at the indicated concentrations. At 96 h after the addition of drugs, cells were
washed in PBS, fixed in 70% ethanol, and stained with Hoechst at 1 µg/mL. The plates
were then imaged on a Cytation 3 automated imager (BioTek), and cells were counted
using CellProfiler (http://cellprofiler.org/). For synergy experiments, experiments were
analyzed for synergistic interactions by the Loewe synergy and antagonism method using
Combenefit (http://www.cruk.cam.ac.uk/research-groups/jodrell-group/combenefit)37.

Neutral comet assay. Comet assay was performed by Parker Sulkowski per the
manufacturer’s protocol (Trevigen). Cells were trypsinized, washed in PBS and
suspended in LM agarose (Trevigen).Neutral electrophoresis was conducted at 21 V for
1 hour. Images were collected using an EVOS FL microscope (Advanced Microscopy
Group) and analyzed using Open Comet software38. Data are presented as the mean of 3
biological replicates ± SEM, with greater than 100 cells analyzed per replicate.

Cell viability assays. Adherent cells were seeded at a density of 2500 cells per well, and
suspension cells at a density of 5000 cells per well in solid white 96 well plates (Costar)
and incubated under indicated conditions in sextuplicate. Cell viability was assayed using
the Cell Titer Glo Kit (Promega) per manufacturer’s protocol and data is presented as
mean ± SEM.

LC-MS analysis. LC-MS was performed by Maureen Kachman at the University of
Michigan Metabolomics Resource Core. LC-MS analysis was performed on an Agilent
system consisting of a 1290 UPLC module coupled with a 6490 QqQ mass spectrometer (Agilent Technologies, CA, USA.) Metabolites were separated on an Acquity HSS-T3, 1.8 µm, 2.1 x 50 mm column (Waters Corp, MA, USA), held at 40°C, using 2 mM ammonium formate in water, adjusted to pH 3.3 with formic acid as mobile phase A, and acetonitrile containing 0.1% formic acid as mobile phase B. The flow rate was 0.2 mL/min and 2HG D- and L- isomers were separated with an isocratic elution (99%A and 1%B) for 6 minutes. The mass spectrometer was operated in ESI- mode, monitoring transitions 363.2 -> 147.2 and 368.3 ->151.2 for 2HG and 13C5-2HG respectively, with a dwell time of 1000, the collision energy 8, and cell accelerator voltage 4. A standard curve with 6 points from 0 to 20 µM of a mixture of D- and L-2HG, was created and derivatized in the same manner as the samples, along with the individual D- and L-2HG standards, for quantification and retention time confirmation purposes. Data were normalized to the internal standard prior to quantification with the standard curve, and data were further normalized to the protein levels of the cell pellets for final analysis. Data is presented as the mean of three biological replicates ± SD.

**Gene microarray analysis.** Samples were prepared for microarray analysis by Ranjini Sundaram. We utilized the Affymetrix GeneChip Microarray (Affymetrix). One sample each of wild-type and IDH1-R132H mutant was sent of the following cell line pairs: HeLa, HCT116 colon cancer cell line, human astrocyte, and MCF10A breast cancer cell line. Analysis of both raw microarray data and gene set enrichment analysis was performed by Gregory Breuer. Data were normalized to internal reference, and then expression values in the mutant line was normalized to its corresponding wild-type line to generate a fractional expression value for each probe. Analysis was performed either as
individual cell line pairs with no statistical analysis, or else pooled into wild-type and IDH1-mutant pools for which a t-statistic was generated. The raw data was then filtered to a list of 233 genes based on the BROCA gene set with some modifications \(^{39,40}\). Gene set enrichment analysis was performed using pooled raw expression data, and analyzed using the Gene Set Enrichment Analysis online toolkit provided by the Broad Institute (http://software.broadinstitute.org/gsea).

**Statistical analysis.** Data are presented as means ± SEM or SD as indicated, and compared using Student’s t test. All tests were two-sided. Statistical analyses were carried out using GraphPad Prism software. A P value of less than 0.05 was considered statistically significant.
Results

Generation and validation of IDH1-mutant HeLa cells

HeLa cells containing an endogenous IDH1-R132H mutant locus were generated using the CRISPR-Cas9 gene editing system\textsuperscript{34,41}. A schematic of the strategy used in our laboratory is provided in Figure 3A. A single amino acid substitution was induced approximately 30 base pairs upstream of a nearby protospacer adjacent motif (PAM) site, converting the CGT (Arg) codon to a CAT (His) codon (Fig. 3B). We validated that we can achieve targeted cleavage at the IDH1 locus by the Cas9 enzyme using a T7 endonuclease assay. This assay involves transfection with a Cas9 cDNA on a plasmid expression vector either alone or with an additional guide RNA (gRNA). Targeted cleavage should produce insertions and deletions of varying lengths. After a PCR at the target locus, a hybridization step produces mismatched DNA dimers that can then be cleaved by the T7 endonuclease. A DNA gel electrophoresis demonstrates that we achieve Cas9-mediated cleavage at the IDH1 locus only in the presence of the gRNA (Fig. 3C).

In addition to the Arg to His modification, a silent BclI restriction endonuclease site was added to assist in clone screening and validation. This modification allows for recognition by the BclI restriction enzyme while preserving the amino acid coding sequence. A restriction fragment length polymorphism (RFLP) analysis shows that a PCR amplification product incubated with BclI results in two predictable smaller fragments, in addition to the full-length wild-type amplicon, only in the mutant cell line (Fig. 3D).
Figure 3. CRISPR-Cas9 gene editing strategy for the generation of IDH1-R132H mutant HeLa cells. A, schematic overview of CRISPR-Cas9 system. B, targeted IDH1 locus. Note Arg 132 to His induced mutation, as well as silent Bcl1 restriction endonuclease site introduced by CRISPR donor DNA. C, T7 endonuclease assay demonstrating appropriate cleavage in cells transfected with both Cas9 cDNA and gRNA targeted to the IDH1 locus. D, restriction fragment length polymorphism analysis of CRISPR-generated clone produces digest products when incubated with Bcl1 restriction endonuclease. Full-length DNA fragment indicates preservation of wild-type loci in addition to engineer mutant locus.
Once a mutant clone was identified via high-resolution melt analysis (data not shown) and RFLP analysis, Sanger sequencing was used to confirm that the mutation is present in the putative mutant clone (Fig. 4A). Also seen is the silent Bcl1 restriction site. Note that double peaks indicate that the mutations are not present on all alleles, which is a critical element of the model cell line as wild-type IDH1 is likely essential to the phenotype\textsuperscript{5}. One important element of validation is ensuring that the Cas9 cDNA has not been incorporated into the genome. Because the CRISPR-Cas9 system induces multiple dsDNA breaks, there is a chance for random insertion of the gene itself. We confirmed that this was not the case by Western blot analysis, using a cell line that stably expresses Cas9-FLAG as a control (Fig. 4B). Finally, we validated and optimized an IDH1-R132H-specific antibody against a commercially available R132H/+ HCT116 cell line, using the wild-type isogenic line as a control (Fig. 4C). This same antibody was used to confirm expression IDH1-R132H in our mutant HeLa cell line (Fig. 4D). The pan-IDH1 antibody detects both wild-type and mutant protein; note that total IDH1 levels remain consistent between wild-type and mutant lines for both HCT116 and HeLa cell line pairs.
**Figure 4.** Confirmation of IDH1-R132H HeLa cell clone. 

**A,** Sanger sequence of mutant clone demonstrates double peaks at codon 132, as well as thirteen bases downstream. Arrows indicate positions of R132H mutation and Bcl1 restriction site placement. 

**B,** Western blot of wild-type and IDH1-R132H mutant HeLa cells as well as a control line with stable expression of Cas9-Flag, probed for Flag, Cas9, and actin loading control. 

**C,** Confirmatory Western blot for IDH1 on HCT116 wild-type and IDH1-R132H/+ heterozygous cell lines. 

**D,** Western blot of wild-type and IDH1-R132H HeLa cells for total IDH1 and IDH1-R132H, with SMC1 loading control.
IDH1-mutant cells produce 2HG by a colorimetric enzyme-mediated assay and LC/MS

Because expression of 2HG is a hallmark of all IDH1-mutant tumors, it is essential that our model systems phenocopy this feature. We utilized an enzyme-based assay to detect and measure 2HG in both the intracellular and extracellular compartments of cultured cells\(^3\). This assay is schematized in Figure 5A. Briefly, exogenous HGDH and diaphorase enzymes, as well as excess NAD\(^+\), are provided to either a cell lysate or conditioned media from cultured cells. Diaphorase converts rasazurin, which is blue, to resorufin, pink or light red, in the presence of high levels of NADH. The output is easily read on a fluorescence plate reader. Doping the reaction mixture with known quantities of 2HG demonstrates a dynamic range of roughly three orders of magnitude, and provides a standard curve (Fig. 5B).

The HGDH enzyme is highly specific for D-2HG, the enantiomer of 2-HG produced by IDH1-R132H. Intracellular measurements of wild-type and R132H-mutant HeLa and HCT116 cells demonstrate a significantly increased intracellular concentration of 2-HG (Fig. 5C). This difference is recapitulated in extracellular concentrations (data not shown), suggesting that the molecule passes through the cell membrane under excess intracellular load. However, 2HG is highly membrane-impermeable when added to the media of cells in culture\(^9\). Fortunately, a membrane-permeable form which contains an octyl group, octyl-2HG, is commercially available. When added to the media of cultured HeLa cells, octyl-2HG is readily taken up by wild-type cells, and is measurable in the intracellular compartment at levels comparable to mutant cells producing endogenous
2HG (Fig. 5D). Finally, we used liquid chromatography-mass spectrometry (LC-MS) to confirm these findings. LC-MS analysis demonstrated a more than 100-fold difference in intracellular 2HG concentration that was consistent between both HeLa and HCT116 cell line pairs (Fig. 5E). LC-MS is significantly more sensitive than the enzyme-based assay, which likely accounts for the difference in relative concentration seen between the two assays.

Recent years have seen many efforts to develop mutant IDH1-specific inhibitors in the hopes of blocking the generation of 2HG. One such small molecule, AGI-5198, has previously been reported to be highly specific for the mutant form of the enzyme, and can significantly repress the amount of 2HG in a mutant cell\textsuperscript{17}. We found that even a 96-hour short-term exposure to AGI-5198 in enough to significantly repress the concentration of 2HG in IDH1 mutant cells at a range of concentrations, while having no effect on wild-type cells (Fig. 5F). Taken together, these data confirm that both our engineered HeLa cell and commercially available HCT116 IDH1 mutant cell lines can serve as functional and biologically relevant independent cell models for further analysis.
Figure 5. The IDH1-R132H mutation increases 2HG in HeLa cells and HCT116 cells. **A**, schematic of the enzyme-mediated 2HG assay. Resorufin is a fluorescent reporter that emits at approximately 590 nm. **B**, dose curve using the aforementioned assay with known concentrations of 2HG. **C**, intracellular measurement of 2HG, expressed as molar count of 2HG per 1M cells in WT and IDH1-R132H HeLa and HCT116 cell lines. **D**, intracellular measurements exogenous octyl-2HG expressed as molar count per 1MG cells in WT HeLa cells co-incubated with octyl-2HG. **E**, LC-MS analysis of D-2HG in WT and IDH1-R132H HeLa and HCT116 cell lines. **F**, extracellular measurement of resorufin emission in WT and IDH1-R132H HeLa cells after co-incubation with AGI-5198 at the indicated concentrations.
IDH1-R132H confers a growth delay phenotype and increased radiosensitivity

We next sought to further characterize the growth characteristics of IDH1-mutant cells. A previous report demonstrated that IDH1 decreases proliferation of glioma cell lines both in vitro and in vivo\(^\text{21}\). To confirm that this is also true in our cell lines, we used a short-term, 96-hour growth delay assay. Cells were seeded at identical concentrations in 96-well plates at time 0, then sequentially fixed, stained, and imaged every 24 hours after for four days. These images were then run through an automated imaging pipeline to generate an absolute cell count. We find that the IDH1-mutant HeLa cells grow significantly more slowly than the isogenic wild-type cells (Fig. 6A). This finding is recapitulated in the HCT116 cell line pair (Fig. 6B), suggesting that the IDH1-R132H mutation affects the rate of cell growth. Next, we performed flow cytometry on DAPI-stained cells to determine if this observation is merely a clonal effect on cell cycle\(^\text{42}\). This assay shows that the IDH1-R132H mutation does not affect cell cycle (Fig. 6C), and instead points to some alternative mechanism of growth delay. Additionally, it was possible that the mutation is merely causing increased cell turnover and death, so we performed flow cytometry and used Annexin-V staining as a measure of apoptosis. We found that there is no significant difference in the number of cells undergoing apoptosis between wild-type and mutant cells in both the HeLa and HCT116 cell lines (Fig. 6D).
Figure 6. Growth characteristics of IDH1-R132H mutant cell lines. A, 96-hour growth curves of WT and IDH1-R132H Hela and B HCT116 cells. C, flow cytometry plots of cell cycle in WT and IDH1-R132H HeLa cells stained with DAPI. D, annexin-V apoptosis assay of WT and IDH1-R132H HeLa and HCT116 cells. P values are non-significant. E, clonogenic survival assay in response to indicated doses of ionizing radiation (IR) in WT and IDH1-R132H HeLa and F HCT116 cells. G, neutral comet assay 24 hours after 5 Gy of IR in WT and IDH1-R132H HeLa cells.
A known observation from the clinic is that IDH1-mutant gliomas are more susceptible to radiotherapy\textsuperscript{13,43}. Based on this characteristic of IDH1-mutant tumors, we hypothesized that the IDH1-R132H mutation would confer increased radiosensitivity in cultured cells. To test this, we used a clonogenic survival assay, which is a well-characterized technique for assessing the effect of radiation exposure on cells\textsuperscript{44}. Cultured cells are exposed to varying doses of radiation, and then plated at a wide range of concentrations. Once cell colonies reach greater than 50 cells in size as determined by direct visual inspection, cells are fixed and stained with Crystal Violet, dried, and then counted manually. This technique demonstrates that IDH1-mutant HeLa cells are significantly more radiosensitive than wild-type cells at radiation doses as low as 2 Gy (Fig. 6E). This finding was recapitulated, though less significantly, in the HCT116 cell line pair (Fig. 6F).

Because radiation exposure causes increased double-stranded DNA breaks, we hypothesized that the increased radiosensitivity seen in the clonogenic survival assay was due to deficient dsDNA break repair mechanisms. This would manifest itself as a diminished DNA damage response and thus increased double-stranded breaks after exposure to radiation. We quantified this using single cell gel electrophoresis, also known as a comet assay. 24 hours after exposure to 5 Gy of ionizing radiation, we saw increased comet tail moment in the IDH1-R132H HeLa cells as compared to wild-type (Fig. 6G). The comet tail moment is a proxy measurement for the number of double-stranded DNA breaks in a cell. This suggests that the IDH1-R132H mutant cells have a deficient response to double-stranded DNA breaks.
A focused screen of DNA repair inhibitors reveals sensitivity of IDH1 mutants to the PARP inhibitor BMN-673

Given our findings that IDH1-R132H inhibits double-stranded DNA break repair, we hypothesized that this could be leveraged for potential targeted therapies of IDH1-mutant tumors. We developed an imaging-based automated high-throughput screening platform to rapidly test multiple drugs in a highly consistent manner. The screening platform is based on the short term growth-delay assay and is schematized in Figure 7A. Prior to screening, we sought to establish the ideal seeding density of HeLa cells, which were chosen to serve as the primary cell line for screening because they grow reliably and adhere well to 96-well plates. To do this, we performed 96-hour growth delay assays with cells plated at 1.25k, 2.5k, 5k, and 10k seeding densities (data not shown). We determined that a seeding density of 2.5k cells per well was ideal for the 96-hour time point, as the cells grew well without reaching confluence during that time.

After 24 hours in culture, cells were treated with one of up to ten different drugs across a range of concentrations to establish a dose response curve. We developed and validated a high-throughput image analysis pipeline built in CellProfiler, an open source and modular image analysis program designed for applications in molecular and cellular biology. This software reliably detects individual cells in both sparse and nearly confluent wells.
Figure 7. Overview of short-term growth delay assay drug screen. A, workflow for screening 96-well plates. Cells are plated at t=0 and given 24 hours to adhere and recover. Drugs were then added and the cells were incubated for 96 hours. They were then fixed, stained, and imaged. Images were run through automated image processing pipelines to establish dose-response curves for each agent. B, summary of 10 selected DNA repair inhibitors, their target, and IC\textsubscript{50} in WT and IDH1-R132H HeLa cells.
The drugs making up our focused DNA repair inhibition panel, as well as a summary of relative IC$_{50}$ in the HeLa cell line pair, are given in Figure 7B. For the panel, we chose a broad variety of small molecules that would each target a specific node of DNA repair pathways. One drug, BMN-673, showed a nearly ten-fold difference in IC$_{50}$ between wild-type and IDH1-mutant HeLa cells in our initial screen. BMN-673 is a highly potent PARP inhibitor, originally developed to treat BRCA-deficient tumors that have an impaired DNA damage response, specifically in the homologous recombination (HR) pathway. This increased sensitivity to PARP inhibition was consistent with the increased radiosensitivity also seen in the IDH1-mutant cells, so we chose to further characterize the effect of BMN-673.

This differential cell kill in IDH1-R132H versus wild-type HeLa was recapitulated in several repeat short-term growth delay assays (Fig. 8A). An even more exaggerated differential was seen in the HCT116 cell line pair (Fig. 8B). To validate these findings in the short-term growth delay, we chose an orthogonal long-term colony forming assay that does not depend on our automated imaging pipeline. Similar to the clonogenic survival assay, we plated cells and exposed them to varying concentrations of BMN-673 for 24 hours, and then re-seeded them at a wide range of concentrations. Once colonies reached sufficient size of >50 cells per colony, they were stained and manually counted. The colony-forming assays recapitulated the short-term growth delay assay with a nearly ten-fold increased sensitivity of IDH1-R132H cells compared to wild-type cells in both HeLa (Fig. 8C) and HCT116 (Fig. 8D) cell line pairs.

BMN-673 is a novel and highly potent PARP inhibitor. To ensure that increased cell killing seen in the IDH1-mutant cells is due to this mechanism of PARP inhibition and
not some off target effect, we next screened the cell line pairs against a series of additional, less potent PARP inhibitors. As expected, we found a modest but significant increased sensitivity of IDH1-mutant cells to a panel of PARP inhibitors including rucaparib, olaparib, ABT888, and MK4827 (Fig. 8E).

Finally, we sought to establish if this effect is mediated by 2HG or through some other IDH1-R132H-dependent mechanism. To test this, we incubated wild-type HCT116 cells in varied concentrations of octyl-2HG for one week, then treated those cells with BMN-673. We found that octyl-2HG can sensitize wild-type cells to the PARP inhibitor, phenocopying the IDH1-mutant cell line in an octyl-2HG dose-dependent manner (Fig. 8F). The most robust sensitization occurs at a BMN-673 dose of 300 nM (Fig. 8G). This experiment confirms that sensitivity to PARP inhibition in the IDH1 mutant can be attributed to the increased intracellular concentration of 2HG.
Figure 8. IDH1-R132H confers sensitivity to the PARP inhibitor BMN-673. **A,** short-term growth delay assay dose-response to the indicated concentration of BMN-673 in WT and IDH1-R132H HeLa and **B** HCT116 cells. **C,** long-term colony forming assay dose-response to the indicated concentration of BMN-673 in WT and IDH1-R132H HeLa and **D** HCT116 cells.
Figure 8 (continued). E, short-term growth delay assay dose-response to the indicated concentration of the PARP inhibitors rucaparib, olaparib, ABT888, and MK4827 in WT and IDH1-R132H HeLa cells. F, short-term growth delay assay dose-response to the indicated concentration of BMN-673 in WT HCT116 cells, pre-treated with the indicated concentration of octyl-(R)-2HG. G, normalized response to the indicated concentration of octyl-(R)-2HG of WT HCT116 cells treated with either DMSO or 300 nM BMN-673.
**IDH1-R132H confers modest sensitivity to cisplatin, which in turn has a synergistic relationship with BMN-673**

Given the dual findings of increased DNA damage in response to radiation, as well as increased sensitivity to PARP inhibition in the setting of increased 2HG, we hypothesized that IDH1 mutants might be more sensitive to DNA damaging agents. Indeed, some data suggests that 2-HG sensitizes IDH1-mutant cells specifically to DNA alkylators. We thus performed short-term growth delay assays on HeLa cells treated with varying concentrations of multiple DNA damaging agents (Fig. 9). We saw that the IDH1 mutant cell line was modestly more sensitive to cisplatin, a platinum-based alkylating agent known to cross-link purine bases thus causing increased DNA damage. It is known that PARP inhibition can sensitize cells to cisplatin in the setting of tumors with diminished a diminished DNA damage response, so we hypothesized that these two agents may work synergistically to treat IDH1-mutant cells.
Figure 9. Short-term growth delay assay in WT and IDH1-R132H HeLa cells dose-response to the indicated DNA damaging agents.
To test this, we performed synergy assays based on a short-term growth delay assay. Varying doses of BMN-673 were crossed with varying doses of cisplatin, with care taken to ensure that the IC$_{50}$ of both agents was incorporated. We used the open source software Combenefit to assess for a potential synergistic relationship between the two. We found a specific synergistic relationship between BMN-673 and cisplatin in both wild-type and IDH1-mutant cells (Fig. 10A). In these figures, blue indicates greater synergy, and a larger area indicates a more robust effect. Importantly, this relationship was stronger in the mutant cells, as seen by a much larger area of dark blue. Absolute percentage of cell survival is consistent with this exaggerated effect in the IDH1 mutants (Fig. 10B).

Indeed, at one specific drug combination dose, nearly 90% of IDH1-mutant cells were killed compared to less than half of wild-type cells (Fig. 10C). Single-agent IC$_{50}$ curves generated by Combenefit are in line with what we have observed under the standard growth delay assay conditions (Fig. 10D). These data suggest that a known synergistic interaction between PARP inhibitors and cisplatin can be potentiated by the IDH1-R132H mutation.
Figure 10. Synergistic relationship between BMN-673 and Cisplatin. A, synergy plots between BMN-673 and cisplatin in WT and IDH1-R132H mutant HeLa cells. Blue squares indicated increased synergy, with larger areas showing a more robust effect.
Figure 10 (continued). B, absolute cell survival for synergy matrices between BMN-673 and cisplatin in WT and IDH1-R132H mutant HeLa cells. Numbers indicate percent cell survival compared to DMSO control. C, fractional cell survival at a select combination dose of 4 nM BMN-673 and 1.1 µM cisplatin (CDDP) in WT and IDH1-R132H mutant HeLa cells. D, single-agent dose response curves of BMN-673 and cisplatin in WT and IDH1-R132H mutant HeLa cells.
Gene Microarray analysis reveal modest expression changes in a panel of genes related to DNA repair

To begin exploring the potential mechanistic link between increased 2-HG and sensitivity to PARP inhibition, we performed a gene microarray (Affymetrix) analysis to determine if the phenotype seen could be ascribed to specific gene expression differences. To do this, we generated cDNA libraries from RNA from both wild-type and IDH1-R132H clones of both HeLa cells and HCT116 cells. To improve statistical power, we also analyzed human astrocytes containing a stably overexpressing copy of either IDH1-WT or IDH1-R132H that have been previous published, as well as both IDH1-WT and IDH1-R132H MCF10A breast cancer cells. Raw values for each probe were normalized to the wild-type expression value of each pair to generate a relative fractional expression value of mutant line compared to wild-type line for each cell line pair. We filtered this data to a focused list of genes correlated with increased cancer risk as well as genes relevant to DNA repair.

We arbitrarily set a threshold of pooled IDH1-mutant expression to be <50% of the wild-type pooled expression as a positive hit for further validation. However, no genes in this set satisfied this requirement; 32 genes had a pooled expression level in the IDH1 mutant lines of less than 90% compared to wild-type (Fig. 11A). We did, however, see many genes with modestly reduced expression in the mutant lines versus wild-type that are directly related to DNA repair, including ALKBH2, RPA1, ERCC1, and MRE11A. ERCC1 plays a role in nucleotide excision repair (NER). Of note, reduced ERCC1 expression is correlated with increased sensitivity to PARP inhibitors and cisplatin in lung cancer and is associated with a better prognosis. MRE11A is part of the HR
pathway for double-stranded break repair, and decreased expression is associated with a better outcome in colorectal cancer\textsuperscript{49}. Given the modest reduction in gene expression of these and other DNA repair genes, however, this is an unlikely explanation for the observed phenotype.

Because we did not see strong gene expression differences in individual genes, we next used the Gene Set Enrichment Analysis applet developed by the Broad Institute to investigate if the phenotype seen could be attribute to broad changes in gene expression across a panel of related genes that correlate with DNA repair\textsuperscript{50}. This technique utilizes gene sets that are highly correlated with 50 well-validated cellular processes as a proxy for understanding the relative activity of large networks of genes, rather than individual expression levels. To perform the analysis, we pooled raw gene expression microarray data and found relative enrichment of wild-type over mutant probes in gene sets correlated with the following cellular processes: KRAS signaling, hypoxia, allograft rejection, bile acid metabolism, Wnt-beta catenin signaling, IL-2 signaling, MTOR signaling, heme metabolism, DNA repair, protein secretion, adipogenesis, cholesterol homeostasis, oxidative phosphorylation, fatty acid metabolism, JAK-STAT signaling, and glycolysis (Fig. 11B). This figure depicts genes ordered from the largest positive difference to the largest negative distance; a sharper, larger positive peak enrichment score thus indicates greater gene set enrichment in wild-type versus mutant expression data. The DNA repair gene set was ranked the twelfth most enriched of the 50 gene sets analyzed. However, it is unclear how significant this difference is, and if the PARP inhibitor sensitivity we observe in the IDH1 mutants can be attributed to it.
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**Figure 11.** Gene expression analysis of pooled data from WT and IDH1-R132H mutant HeLa, HCT116, human astrocyte, and MCF10A cell line pairs. **A,** pooled expression data of the 32 genes most down-regulated in the IDH1-R132H mutant cell lines, from a pre-selected gene panel of cancer- and DNA repair-relevant genes. **B,** gene set enrichment plots of the top twelve most highly enriched genetic hallmark pathways. Larger, steeper positive peaks define a higher enrichment score, which indicates increased gene expression in WT versus IDH1-R132H cell lines.
Discussion

We report here a novel HeLa cell model with an engineered endogenous IDH1-R132H mutation. This cell line expresses the protein at physiologic levels, generates high levels of intra- and extra-cellular 2-hydroxyglutarate, and exhibits many characteristics consistent of IDH1-mutant gliomas including increased radiosensitivity. It is easy to work with and is suitable for cell-based screening assays. The HeLa cell is a well-studied model system, and we believe this isogenic cell line pair will serve as a useful reagent for future study of IDH1 mutations by our group and others.

This is also the first report that IDH1-R132H confers a defective response to double-stranded DNA breaks. Importantly, this feature confers increased sensitivity to a panel of PARP inhibitors, especially the highly-potent PARP inhibitor BMN-673. However, the mechanistic link between increased 2HG and decreased DNA damage response remains an interesting and important question. Gene microarray and gene set enrichment analysis have demonstrated modest changes at specific genes responsible for DNA repair, meaning it is unlikely that the mechanism of action is occurring solely at the gene expression level. Published data suggest that 2HG may actually interact directly with epigenetic modifiers that utilize the structurally similar small molecule 2-oxoglutarate (2OG) as a cofactor. Specifically, 2HG has been shown inhibit the activity of several of the JmjC histone demethylases, including KDM2A, KDM4A, KDM4C, and KDM7A. This family of demethylases has recently been implicated as a potential therapeutic target, and are known to have an oncosuppressive effect. Inhibition of these enzymes could thus be oncogenic, and could serve as a potential mechanism for how IDH1 mutations confer tumorigenesis. Further, 2HG has been crystalized in the αKG binding
pocket of histone demethylases, suggesting it can function as a competitive inhibitor of molecules which share a similar structure\textsuperscript{52}. Ongoing work in our laboratory suggests that 2HG may indeed function as a direct inhibitor of epigenetic modifiers, which could serve as the key mechanistic link between these observations (data not published).

The treatment of gliomas, especially GBM, remains a great challenge, and our study suggests a potential benefit from a novel treatment strategy including PARP inhibitors. BMN-673, also known as talazoparib, is currently in clinical trials for the treatment of BRCA-deficient late-stage ovarian cancers, having passed Phase I trials for safety and tolerance\textsuperscript{53}. Utilizing a small molecule that has already been well-studied in animals and humans alike could dramatically shorten the lead-time from bench to bedside, opening up the possibility of a clinical trial for IDH1-mutant gliomas in the near future. There remains much work to be done, however; at present, the penetration of BMN-673 to the CNS is unknown. A recent study of rucaparib, another PARP inhibitor, showed poor penetration across the blood-brain barrier\textsuperscript{54}. This represents only one of many challenges facing not just treatment of glioma, but any drug development campaign. An important next step, currently underway in our laboratory, is investigating the \textit{in vivo} efficacy of BMN-673 in a mouse xenograft model\textsuperscript{55}. Demonstrating a robust effect in the mouse model would be a critical step towards future clinical trials.

There have been many efforts to develop inhibitors of IDH1-R132H in an attempt to suppress the generation of 2HG. The hypothesis, presumably, is that 2HG is in and of itself oncogenic, and thus reducing its production is a viable therapeutic option. Interestingly, our data suggest that this strategy would be ineffective at best, and, at worst, would actually negate the therapeutic advantage we have identified. Our proposed
model is consistent with a synthetic lethal treatment strategy, wherein a cancer cell exhibits a defective cellular process that can be exploited by targeting an additional pathway. In this case, IDH1-R132H results in massively elevated 2HG, which in turn promotes increased DNA damage and/or diminished DNA repair. This makes the IDH1-mutant cell susceptible to agents that specifically impair the DNA damage response, which we see when we expose the cells to the PARP inhibitor BMN-673. We are thus proposing that IDH1-R132H causes a phenotype similar to BRCA-null mutations, and therefore IDH1-mutant tumors can be treated with similar targeted strategies.

The PARP inhibitor olaparib is known to act as a radiosensitizer even at low doses. Given that radiotherapy is already part of the standard of care for gliomas, it is easy to imagine a future clinical trial adding a PARP inhibitor to existing treatment plans. Further, the synergistic relationship between BMN-673 and cisplatin is even more encouraging, as it suggests we may be able to enrich cytotoxic specificity for IDH1-mutant tumors. There may also be additional benefits when treating cells with other alkylating agents such as temozolomide. Additionally, a studied synergistic interaction between PARP inhibitors and NAMPT inhibitors is particularly promising in IDH-mutant tumors, especially given recent findings that the IDH1-R132H mutation confers extreme sensitivity to NAMPT inhibition. Other groups are investigating the role for immunotherapy and vaccines in treating gliomas. There are thus many potential therapeutic strategies that could leverage our novel findings.
Conclusions

We conclude with the new observation that the IDH1-R132H confers sensitivity to PARP inhibitors, and that this works through increased intracellular 2HG. Further, this is associated with increased double-stranded DNA damage in response to ionizing radiation, which suggests a defect in DNA repair via homologous recombination. While gene expression investigations show no specific genetic lesion, gene set enrichment analysis show broad downregulation of genetic programs related to DNA repair in a panel of IDH1 mutant cell lines.

Our work, in combination with that of many other groups, makes a significant contribution to the molecular understanding of IDH1 mutations, which are relevant to wide variety of tumors including gliomas. This early work in the laboratory has important and direct implications to the clinic as we begin to see a shift in the treatment of CNS malignancies towards targeted therapies. Tumor sequencing is already being used as a prognostic aid to predict response to radiation and chemotherapy, and we will soon be able to offer treatment options tailored to individual genetic subtypes. The finding that 2HG confers sensitivity to PARP inhibitors is one of many recent advances in the basic and translational research of glioma, and suggests that we may indeed be close to prolonging survival in this disease as we progress towards its eventual and inevitable cure.
References