Title

Using Patient-Derived Gliomaspheres to Molecularly Characterize and Dissect Distinctive Traits of Isocitrate Dehydrogenase I Mutant Gliomas for Therapeutic Benefit

Author

Garrett, Matthew Clark

Publication Date

2016

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Using Patient-Derived Gliomaspheres to Molecularly Characterize and Dissect Distinctive Traits of Isocitrate Dehydrogenase 1 Mutant Gliomas for Therapeutic Benefit

A dissertation submitted in partial satisfaction of the requirements for degree Doctor of Philosophy in Neuroscience

by

Matthew Clark Garrett

2016

ABSTRACT OF THE DISSERTATION

Using Patient-Derived Gliomaspheres to Molecularly Characterize and Dissect Distinctive Traits of IDH1 Mutant Gliomas for Therapeutic Benefit

by

Matthew Clark Garrett

Doctor of Philosophy in Neurocience

University of California, Los Angeles 2016

Professor Harley Kornblum, Chair

Glioblastoma (GBM) is the most common and malignant primary brain tumor. Despite maximal therapy prognosis remains poor. In the preceding decade, histological methods of categorization have begun to give way to molecular and genetic methods. Most prominently, the IDH1 mutation, when found in glioblastoma correlates with a better prognosis than those glioblastomas in which the mutation is not found. It is not known why this correlation exists. The mutant IDH1 enzyme is able to reduce alpha-ketoglutarate to create extremely high levels of 2-hydroxyglutarate but the tumorigenic significance of this reaction is unknown.

In this dissertation we use an extensive collection of IDH1mutant and IDH1wildtype patient-derived gliomaspheres to dissect genetic and metabolic differences

that distinguish the IDH1mutant subgroup. We found that the IDH1mutant gliomas were readily identifiable by their slower growth and distinctive expression profile. However, pharmacologic and genetic inhibition of the IDH1mutant enzyme did not change this signature in fact it did not seem to have any effect on these cells other than to decrease the levels of 2-hydroxyglutarate.

The expression profile of IDH1mutant cells showed a large set of down-regulated genes in comparison to IDH1wildtype cells. One of the few genes to be up-regulated was Olig2 which upon further study we revealed to be essential for growth and a regulator of other important genes such as Tet2. Further, Olig2 expression may be targetable by the histone deacetylase inhibitor (HDACi) class of drugs in particular valproic acid.

When we subjected the expression profiles of IDH1mutant glioma cells to metabolic analysis we found that IDH1mutant cells were enriched for pathways involved in DNA repair following radiation while IDH1wildtype glioma cells were enriched for pathways involved in de novo DNA synthesis. Following these results we confirmed that IDH1mutant cells do show better recovery following radiation and IDH1wildtype cells show a greater utilization and dependence on the de novo pathway for DNA synthesis.

Historically, glioblastoma has been defined based on histologic criteria and while two glioblastoma samples may contain two mutually exclusive lists of mutations they would be considered minor variants within the larger fairly homogenous class. It is now becoming clear constitute a distinct group of brain tumors with different characteristics, prognosis and response to therapy and thus need to studied separately from IDH1wildtype glioblastomas.

The dissertation of Matthew Clark Garrett is approved.

Albert Lai

Heather Christofk

Linda Liau

Harley Kornblum, Committee Chair

University of California, Los Angeles

2016

Dedication

This work is dedicated to my fiancée Julie Gutowski for her encouragement and mouse cartoons and to my parents Don and Frances Garrett for their support and passing on their sense of discipline and passion for solving puzzles.

Table of Contents

Figures and Tables

Chapter 2

Chapter 3

Chapter 3 (continued)

Acknowledgements

This work was constructed with the guidance, support, scientific acumen and creative intervention of Dr. Harley Kornblum. It was a great privilege to have him as a mentor and to collaborate on this work with him.

I would like to acknowledge the insights and training provided by my thesis committee members: Dr. Linda Liau, Dr. Heather Christofk, and Dr. Albert Lai.

Finally I would like to thank those scientists who have paved the way and those who continue to sustain the light of empirical tested, scientific knowledge and understanding into the future.

Chapter 2 is a version of a work in preparation for publication:

Olig2 provides novel target in IDH1mutant tumors. Matthew Garrett, Rosemarie Tsoa, Jantzen Sperry, Jack Mottehedeh, Ascia Eskin, Giovanni Coppola, Ya-Shin Shih, Robert Prins, Albert Lai, Arthur Chou, Linda Liau, Timothy Cloughesy, William Yong, Hong Wu, Stanley Nelson and Harley Kornblum.

Chapter 3 is a version of a work in preparation for publication:

Metabolic characterization of IDH1mutant and IDH1wildtype gliomaspheres to uncover cell-type specific vulnerabilities. Matthew Garrett, Jantzen Sperry, Dan Braas, Weihong Yan, Thuc Le, Giovanni Coppola, Caius Radu, Thomas Graeber, Frank Pajonk, Heather Christofk, Harley Kornblum.

I would like to acknowledge the contribution of these collaborators

Rosemarie Tsoa: Olig2 CRISPR virus, ChIP-qPCR assays

Jantzen Sperry: Radiation, dT experiments

Kirsten Ludwig: Radiation treatments

Dan Braas: LC-MS and data analysis

Weihong Yan: Bio-informatic analysis of KEGG modules

Thuc Le: Nucleotide LC-MS and data analysis

Caius Radu: Nucleotide LC-MS and data analysis

Thomas Graeber: Bio-informatic analysis of KEGG modules

Ascia Eskin: Microarray expression analysis

Giovanni Coppola: Microarray expression analysis

Jonathan Nakashima: Luciferase bio-imaging

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED TWO PAGES.**

NAME	POSITION TITLE
Garrett, Matthew C	Neurosurgery Resident
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Yale University	BS	05/02	Biochemistry
Columbia University	MD	05/09	Medicine
University of California, Los Angeles (UCLA)	Internship	06/10	General Surgery
University of California, Los Angeles (UCLA)	Residency		Neurosurgery

A. Personal Statement

I am currently a fourth year resident in the neurosurgery program at UCLA Medical Center about to complete my Ph.D. from the STAR program. I am interested in becoming an academic neurosurgeon specializing in adult brain tumors. In addition to my future clinical practice I wish to use the tissue specimens that I collect in a research setting to further characterize the genetics and molecular pathways of these tumors to develop novel therapeutics.

Positions and Employment

2002-03 Laboratory Technician, Memorial Sloan-Kettering Cancer Center
2007-08 Doris Duke Research Fellow, Department of Neurosurgery, Columbia University

Medical Board Certifications

2011 Board Certification, California Medical Board

Honors

2002 Graduated BS with distinction Yale University
2007 Doris Duke Fellowship Recipient

B. Selected Peer-reviewed Publications (selected from 26 peer-reviewed publications)

Garrett MC Komotar RJ, Starke RM, Otten ML, Solomon RA, Connolly ES A Theoretical Model for Quantifying the Operative Benefit of Unruptured Aneurysms by Location, Age, and Size, Future Neurology 2010.

Garrett MC, Komotar RJ, Starke RM, Doshi D, Otten ML, Connolly ES. Elevated troponin levels are predictive of mortality in surgical intracebral hemorrhage patients. Neurocritical Care 2010 Apr;12(2): 199-203.

Garrett MC, Otten ML, Starke RM, Komotar RJ, Magotti P, Lambris JD, Rynkowski MA, Connolly ES. Synergistic neuroprotective effects of C3a and C5a receptor blockade following intracerebral hemorrhage. Brain Res. 2009 Nov 17;1298:171-7.

Garrett MC, Komotar RJ, Starke RM, Merkow MB, Otten ML, Connolly ES, Predictors of seizure onset after intracerebral hemorrhage and the role of long-term antiepileptic therapy. J Crit Care 2009 Sep;24(3);335-9

Garrett MC, Komotar RJ, Starke RM, Merkow MB, Otten ML, Sciacca RR, Connolly ES. The efficacy of direct extracranial-intracranial bypass in the treatment of symptomatic hemodynamic failure secondary to athero-occlusive disease: a systematic review. Clin Neurol Neurosurg. 2009 May;111(4) 319-26.

Komotar RJ, Otten ML, Garrett MC, Anderson RC, Treatment of early childhood medulloblastoma by postoperative chemotherapy alone-a critical review. Clin Med Oncol. 2009 Mar 4;3:13-4.

Garrett MC, Komotar RJ, Starke RM, Merkow MB, Otten ML, Connolly ES. Radiographic and clinical predictors of hemodynamic insufficiency in patients with athero-occlusive disease. J Stroke Cerebrovasc Dis. 2008 Nov-Dec;17(6):340-3.

Rynkowski MA, Kim GH, Garrett MC, Zacharia BE, Otten ML, Sosunov SA, Komotar RJ, Hassid BG, Ducruet AF, Lambris JD, Connolly ES. C3a receptor antagonist attenuates brain injury after intracerebral hemorrhage. J Cereb Blood Flow Meta. 2009 Jan;29(1):98-107.

Garrett MC, McCullough-Hicks ME, Kim GH, Komotar RJ, Kellner CP, Hahn DK, Otten ML, Rynkowski MA, Merkow MB, Starke RM, Connolly ES. Plasma acrolein levels and their association with delayed ischemic neurological deficits following aneurysmal subarachnoid haemorrhage: a pilot study. Br. J Neurosurg. 2008 Aug;22(4):546-9.

Garrett MC, Komotar RJ, Merkow MB, Starke RM, Otten ML, Connolly ES. The extracranial-intracranial bypass trial: implications for future investigations. Neurosurg Focus. 2008;24(2): E4

Chapter 1

Introduction

Glioblastoma

Glioblastoma is the most common malignant primary brain tumor with a dismal prognosis. Standard of care involves surgical resection, chemotherapy and radiation, however these tumors invariably recur and are almost universally fatal. [1] While surgical resection may appear on MRI to be curative, closer inspection at the histologic level finds tumor cells diffusely spread far beyond the central tumor mass. Famously, in an attempt at a cure, a neurosurgeon removed an entire hemisphere only to have the tumor recur on the other hemisphere. [2] While surgical resection is helpful in debulking the majority of the tumor cells and providing some symptomatic relief due to compression of adjacent brain structures, better therapy is clearly needed to address the diffuse microscopic disease surgery leaves behind. [3] Following surgery most patients are treated with radiation and temozolomide, a chemotherapy agent designed to alkylate or methylate DNA causing deleterious point mutations that often lead to apoptosis and cell death. Initial trials of the drug showed a statistically significant but clinically modest benefit. [4] However, one negative drawback is that when the tumor cells recur they have often picked up additional mutations associated with the temozolomide therapy. [5]

Gliomasphere as a Model for studying Glioma

In vitro models are necessary to dissect the molecular and biochemical processes that occur inside the cells of living tissue. The first in vitro models of glioblastoma were grown as adherent cultures in serum. These cells maintained many of the mutations from the original tumor as well as the ability to recreate a tumor when transplanted into immune-deficient mice. However, the rate of successful conversion from tumor to cell line was low and the tumors created by xenotransplant were more circumscribed than their parent tumors. In 1992, Reynolds et al. [6] published a serum-free method of culturing neural stem cells. When this culturing method was applied to tumor tissue a new in vitro tumor cell was discovered. [7] These floating in vitro glioma cells have since been named "gliomaspheres." The authors noted that these new cells derived from tumor tissue had numerous stem cell properties such as the expression of stem cell markers e.g. CD133, the ability to self-renew into floating spheres and when deprived of growth factor or subjected to serum treatment to "differentiate" into multiple different mature lineages e.g. oligodendrocyte, astrocyte, and neuron. These cells were also highly tumorigenic in mouse xenograft models.

Moving one step further, Singh et al. showed that the cultures themselves held some heterogeneity. [8] The authors found that in one particular culture, those cells that expressed the cell surface marker CD133 were able to proliferate faster, make spheres and induce tumors in mice whereas the cells in the culture that did not express this marker could not. This discovery led to several new hypotheses about the nature of glioblastoma. One new hypothesis was that the origin of glioblastoma was a transformed neural stem cell and the tumor mass was similar to a model of neural differentiation and was composed of a mixture

of differentiated and undifferentiated cell types all derived from the original tumorigenic stem cell that provided the bulk of growth and cell division. Another hypothesis was that the cell of origin was a non-stem cell but during the tumorigenic process the cell de-differentiated to take on more stem cell traits. Regardless of the cancer's cell of origin, these observations highlighted the heterogeneous nature of glioblastoma tissue and indicated that certain cell populations within the tumor may be more tumorigenic and thus more important therapeutically. It has since become clear that this trait is not present in all cultures. Many gliomasphere cultures have CD133 negative cells that are able to induce tumors in vivo. [9]

Despite numerous attempts, no group has yet found a universal stem cell marker that unequivocally labels the tumorigenic fraction in every tumor. However, one trait that does seem to hold for the majority of cultures is that once a gliomasphere culture is exposed to serum, the normally floating culture becomes adherent, non-tumorigenic and will not reverse to its former state by re-introduction to the serum-free neurosphere media. A similar phenomenon is observed when neural precursor cells are exposed to serum. In a now classic paper, Yamanaka et al. [10] found four factors that when co-expressed in a fibroblast would revert that cell to a pluripotent stem cell (iPSC). Similarly, Suva et al. [11] found that there were four transcription factors (Pou3f2, Sox2, Sall2, and Olig2) that when introduced to a serum-treated cell could revert that adherent non-tumorigenic cell back to a floating, sphere-forming tumorigenic cell. The authors showed that each of these factors was essential and if one were missing the resulting cell would not be tumorigenic. However, the Olig2 factor could be functionally replaced by RCOR2, a gene closely linked to histone deacetylation (HDAC) and the resulting cell would again be tumorigenic. It is still unclear whether this finding is due to Olig2 and HDAC having redundant functions or whether HDACs activate

Olig2. Finally, adding in vivo relevance to the gliomasphere model, when sorting a freshly resected glioblastoma there was heterogenous expression of each of those four factors, but those few cells that expressed all four factors also expressed CD133.

The cancer stem cell field remains divided on the clinical and scientific importance of many of the traits seen in gliomaspheres. Many hypothesize that the culture itself is heterogeneous with some cells having unlimited potential for cell division and some cells having more limited potential. Many scientists perform "clonal assays" where cells are plated at low density to see what fraction of cells in a given culture have the capacity to create a sphere derived from a single cell. [12] Whether or not these tests have validity, the gliomasphere culture system remains the current preferred method of growing gliomasphere cells in vitro and provides several advantages. One such advantage is it allows the growth and propogation of glioma cells harboring the IDH1 mutation. Although it is possible to create gliomasphere lines from these tumors, the cells are fragile and difficult to maintain and thus there are relatively few IDH1mutant lines available. As such no group has been able to fully characterize IDH1mutant gliomaspheres. To address this short-coming we provide a full characterization of the expression and metabolic profile of these cells in chapters 2 and 3.

TCGA

In the era before sequencing was widely available the first attempts to categorize and assess glioblastomas were based on their histologic characteristics. Variable levels of cellularity, mitotic activity, vascular proliferation and necrosis were used to assign tumors a grade from II to IV. [13] Additionally pathologists noted that many tumors were composed of

cells that resembled astrocytes while other tumors contained cells that resembled oligodendrocytes and other tumors contained a mixture of both cell types. In the decades that followed the creation of the WHO criteria, histologic characteristics have given way to more sophisticated molecular analyses. A large collection of tumor tissue was subjected to expression, mutation and methylation analysis. [14 15] It was from these initial studies that the IDH1mutation was discovered [16] and that the group of tumors classified as Grade IV glioblastomas were sub-classified into four groups based on their expression patterns: Mesenchymal, Proneural, Neural and Classical. The names were given based on similarities to established cell types and pathways. The proneural subtype was named based on similarity to neural progenitor cells (NPCs) and was characterized by mutations in IDH1 and PDGFRa. Neural gliomas more closely resembled differentiated neural cells. Mesenchymal in contrast had a signature that was less neural and more de-differentiated. This class was characterized by a high frequency of NF1 mutations. Classical gliomas were noted to have increased EGFR signalling. These datasets also revealed that the IDH1mutant group also had a distinctive methylation pattern. The term "hyper-methylated" was used to describe this phenomenon based on the fact that the IDH1mutant tumors had more total methylated sites. However, this term is somewhat misleading as IDH1mutant tumors are really differentially methylated with many sites being more hyper-methylated in IDH1wildtype tumors and less methylated in IDH1mutant tumors. A more accurate term that just defines the signature of hyper-methylated sites frequently seen in IDH1mutant tumors is glioma-CpG island methylator phenotype (g-CIMP). At first, these divisions seemed academic with classical, mesenchymal, and neural tumors showing a similar prognosis. However, there was an improved prognosis in the proneural group particularly in tumors harboring the IDH1

mutation. Interestingly, when the standard temozolomide regimen was compared to a more "aggressive" temozolomide regimen, the mesenchymal, classical and neural groups saw a survival benefit with the more "aggressive" regimen. In contrast, the proneural group showed no benefit suggesting two possibilities. The first, proneural tumors are not sensitive to temozolomide. The second and less likely possibility is that proneural tumors are highly sensitive to temozolomide and a maximal biological effect is reached at the standard dosage.

Since that time, new evidence has cast doubt on the validity of this tumor grouping system. Single cell-RNA sequencing of GBM cells has found that within a single tumor, there are cells that would fall into each of the TCGA categories. [17] Interestingly this heterogeneity was lost when the glioma tissue was converted into a gliomasphere culture and each cell in a given gliomasphere culture was always a member of the same group. Additionally Laks et al. [18] found that in terms of TCGA class, there was only a weak correlation between a parent tumor and its derived gliomasphere culture. Additionally they noted that no gliomasphere line was classified into the neural category. Taken together it raises the concern that the group assignments made in the TCGA may say less about the tumor as a whole and may be more a result of sampling error. Additionally it may be possible that the neural group is the result of contamination by normal tissue. There was however, an exception in both of these studies. IDH1mutant tumors were composed of 100% Proneural cells and their resulting cell cultures were also always Proneural.

The role of IDH1mutation in gliomas

The IDH1 enzyme catalyzes the conversion of alpha-ketoglutarate to isocitrate in the cytosol. The most common IDH1 mutation is a single point mutation from arginine to histidine in the active site at codon 132. [16] This mutation enables the enzyme to catalyze a new reaction, namely to reduce alpha-ketoglutarate into 2-hydroxyglutarate and consume NADPH to NADP+.[19] Following the discovery of the IDH1 mutation many investigators sought to determine what new malignant traits this mutation would bestow upon a cell. Unexpectedly it appeared that in general the addition of the IDH1mutation led to slower growth in most brain tumor models. [20] This was a perplexing result and was initially hypothesized to be the reason why IDH1mutant tumors had a better prognosis compared to those glioblastomas without the IDH1 mutation. However this theory was inconsistent with the idea of cellular evolution. Why would a mutation that slows growth be selected over neighboring cells without that mutation and a presumably faster rate of growth?

One possibility is that the IDH1mutation enables cells to resist death or anti-growth signals in their microenvironment. This theory is supported by the discovery that the 2-HG molecule could inhibit the function of alpha-ketoglutarate-dependent enzymes by outcompeting alpha-ketoglutarate. [21] This led to the hypothesis that the IDH1mutation might give a cell the ability to resist environmental influences and prevent differentiation from a progenitor cell to a more differentiated and less prolific cell type. In several cellular contexts notably fat cells[22], chrondrocytes[23] and liver cells[24], over-expressing the IDH1mutation in precursor/stem cells prevent those cells from differentiating. In each of these previous studies there was a key mediator gene that was essential for differentiation. During differentiation, this gene was activated by the demethylation of a key histone mark in the promoter or enhancer region. In the presence of the IDH1mutation or high levels of 2-HG,

this histone demethylation was prevented and the cell failed to differentiate and instead maintained its proliferative potential. However, trying to show that the IDH1mutation has the ability to block differentiation in neural cells has been more elusive. Over-expressing the IDH1 mutatation in a mouse sub-ventricular zone (SVZ) stem cell culture changed the default differentiation from a GFAP-positive astrocyte to a TUJ1 positive neuron, however it did not prevent differentiation or lead to increased growth. [22]

A second possibility is that the effect of the IDH1mutation is slow but over time can gradually convert the epigenetic state of a cell to a more malignant phenotype. The most popular model of this theory is that the IDH1 mutant enzyme impairs the ability of the Tet enzymes to demethylate DNA. In this case, the activity of the DNA methyltransferase (DNMT) enzyme family is unopposed and leads to a gradual increase of methylation throughout the genome. The methylation of CpG islands, particularly in key regulatory regions, tends to decrease expression of those genes. Over time more and more tumor suppressor genes would become repressed until the cell becomes tumorigenic. The strongest evidence for this theory comes from Turcan et al. In this study, over-expression of the IDH1mutant enzyme in an astrocyte line led to the gradual induction of expression of nestin and a small increase in growth over many passages. This long-term IDH1mutant expression was also associated with increased DNA and histone methylation. [25]

A third possibility is that the IDH1 mutation predisposes to further mutations. When studying patients with IDH1mutant tumors it was noted that following resection when the tumor eventually grew back, the tumors had often acquired a new set of mutations. Mutations that were present in the first tumor were no longer in the second tumor. The only mutations that were always present were IDH1 and p53. [5] This result implies that the IDH1

mutation is likely the initial mutation in gliomagenesis and is sufficient to generate enough mutations for tumorigenesis multiple times throughout a patient's life. However, first p53 must be rendered non-functional. It is not clear how the IDH1mutation leads to further mutations. One possibility is the methylation and down-regulation of DNA repair machinery in particular MGMT. IDH1 mutation and MGMT methylation are correlated although there are IDH1mutant tumors with unmethylated MGMT. [26] [27] As additional evidence while MGMT methylation is an independent predictor of a positive response to temozolomide in IDH1wildtype glioma cells, it is not a predictor of chemotherapy response in IDH1mutant cells implying that either MGMT itself or the MGMT pathway may be non-functional in IDH1mutant cells. [28] Another possibility is that the IDH1 mutation may lead to higher levels of endogenous reactive oxygen species (ROS) predisposing to DNA damage. While presumably the IDH1mutant enzyme would lead to an alteration in the NADPH/NADP equilibrium it is not obvious a priori what effect this would have on total endogenous ROS levels. Consequently, the question of whether the addition of the IDH1mutation to cells causes an increase or a decrease in ROS levels is still a matter of debate with different studies showing conflicting results. [29,30] However, the more relevant questions are whether endogenous IDH1mutant cells themselves have high or low ROS levels and how these cells respond to the addition of exogenous ROS from therapies such as radiation. We will answer this question in Chapter 3.

Attempts to study the effects of IDH1 mutation in vivo have been hampered by the fact murine knock-in models of the IDH1mutant gene under the Nestin promoter are embryonic lethal. However, it is notable that when those neural cells are recovered from the non-viable embryos they do not possess any deficit in differentiation.[29] The only other piece

of in vivo evidence comes from the study of human patients with a condition called "2-Hydroxyglutaric aciduria." [31] These patients have an inborn mutation that leads to high levels of 2-HG systemically. As opposed to a mutation that leads to the generation of 2-HG these patients have a mutation in the enzyme that breaks down 2-HG. Notably there are two enantiomers of the 2-HG molecule. The IDH1 mutation generates only D-2HG. There are multiple subtypes of 2-Hydroxyglutaric Aciduria that produce D, S or both enantiomers. Oddly the patients with high levels of S-2HG tend to show the most severe neural and cognitive deficits as well as a predisposition to develop glioblastoma. However, given the differences in the metabolic pathways involved it is unclear how much relevance this syndrome has to IDH1mutant glioblastomas.

The Link between Tet2 and IDH1 in Leukemia

The IDH1 mutation is also found in other cancers, most notably acute myeloid leukemia(AML). In contrast to the opaque mechanism by which the IDH1mutation leads to brain cancer, the mechanism in leukemia has been more clearly defined. Sequencing studies of large sets of leukemia cells have found that IDH1, IDH2 and Tet2 mutations are both common and mutually exclusive. AML patients that harbor mutations in either the IDH1, IDH2 or Tet2 genes have hyper-methylated tumors and the over-expression of the IDH1mutant gene in an IDH1WT AML cell causes that cell to become hyper-methylated.[32] Over-expression of the IDH1mutant enzyme in erythroleukemia line TF-1 led to those cells being able to grow without growth factor and being able to resist the differentiating effects of

erythropoietin. This effect can be pheno-copied by knocking down the Tet2 gene. [33] Knocking in the IDH1mutant gene into hematopoietic stem cells leads to an expansion of immature cell types [34] and knocking out the Tet2 gene leads to mice developing spontaneous leukemia.[35] In contrast to the Turcan study, which required over forty passages, the effects of IDH1 mutation in leukemia cells took only 10 days. It appears from these studies that Tet2 inhibition is a common and sufficient event to initiate tumorigenesis in leukemia cells and that the IDH1mutant enzyme is sufficient to induce Tet2 dysfunction.

There is a significant amount of research trying to make a connection between IDH1 mutations and Tet2 function in brain cancer. However, in contrast to leukemia the connection is far less clear. Tet2 mutations are relatively rare in glioblastoma. Tet2 knock-out mice do not develop brain tumors.[29] While all IDH1mutant tumors are hyper-methylated, there are many hyper-methylated tumors in TCGA that do not have an IDH1 mutation. [14] Further, it appears that the methylation pattern seen in IDH1mutant tumors is important for growth based upon studies where the cells were pharmacologically demethylated with decitabine. However, pharmacological demethylation also slows the growth of IDH1wildtype gliomas. [36] [37]

Small Molecule IDH1mutant inhibitor

Following the discovery of the IDH1mutation there was a great deal of interest in developing small molecule inhibitors that might be able to block the ability of the IDH1mutant enzyme to produce 2-hydroxyglutarate. Eventually such a compound was

found [38] (Xcessbio C227) and tested on IDH1mutant cells of all tumor types. The compound is highly effective at reducing 2-HG levels in a wide array of IDH1mutant models however the biological effects seemed to vary by cell type. In the case of the leukemia model TF-1, the inhibitor prevented the changes seen following IDH1mutant expression. [39] The c227 inhibitor was able to induce differentiation and decrease growth. Consistent with these findings, early clinical trials with the similar inhibitors in AML are also promising. [40] After taking the IDH1mutant inhibitor, patients with IDH1mutant acute myeloid leukemia show a progressive decrease in the number of immature tumor-type myeloid cells with a corresponding increase in mature differentiated cells. Most encouraging, unlike traditional chemotherapy, there is no myelosuppression seen across the other myeloid lineages.

However, in the case of IDH1mutant glioma models, the results were more mixed. The first attempt to treat an IDH1mutant glioma with the inhibitor met with some success. In the treated mice the tumors were somewhat smaller and had increased expression of GFAP which might be associated with differentiation. [41] However, later attempts to repeat this data have failed. In one of the more thorough studies, Tateishi et al. treated IDH1mutant cells with the c227 inhibitor for over a year and found no difference on either DNA methylation or histone modification. Worse yet, there was a slight increase in growth with the addition of the c227 inhibitor.[42]

One difficulty with attempting to disprove the efficacy of the c227 inhibitor in brain tumor models is the issue of time. While Turcan et al. [25] was able to demonstrate an increase in methylation with the addition of the IDH1mutant enzyme, the effect required the cells to undergo 40 passages and presumably several hundred cell divisions. Even then the majority of the newly methylated sites were only partially methylated. Any study that fails to find an

effect of adding the IDH1mutant enzyme can be criticized for not giving the cells enough time no matter how much time was given. One possibility for the disagreement between the two studies is that while the IDH1 mutation is able to induce methylation, once the methylation is induced, it is irreversible. It is also difficult to translate these results to a clinical context where the vast majority of cells in the brain are post-mitotic. We present our results of the c227 IDH1mutant inhibitor on our IDH1mutant cells in Chapters 2 and 3.

The role of Olig2 in IDH1mutant gliomas

Olig2 is one of the 125 transcription factors that are defined by a canonical basic helix-loop helix (bHLH) motif. [43] The protein was first discovered and studied in the context of in situ hybridization and fate-mapping experiments aimed at determining the origin of oligodendrocytes in the central nervous system. [44] However more recent studies have discovered that Olig2 plays an important role in reactive gliosis, motor neurons and radial glia. [45] [46] The most interesting role for Olig2 is in the developing radial glial and neural stem cells where Olig2 can both promote growth and self- as well as repress growth and promote differentiation depending on the context. [47] [48] In contrast to the majority of neurogenic developmental bHLH transcription factors e.g. MASH1, MATH, NGN1 and NGN2 that are transiently expressed in various stages of progenitor development, Olig2 is constitutively expressed throughout neural development even to fully formed, differentiated neurons. [47] The constitutive expression and changing roles has led to the question of whether Olig2's function is regulated at the post-translational level via phosphorylation as opposed to the transcriptional level. In a study by Sun et al, the investigators showed that the proliferative function of Olig2 was dependent on the phosphorylation state of three serine

residues (Ser10, Ser13, and Ser14). [49] This pro-growth role in neural progenitor cells along with the observation that Olig2 is frequently expressed in diffuse gliomas [50 51] led to interest in whether the phosphorylation state of Olig2 determined the growth pattern of Olig2 positive gliomas. Supporting this notion a murine model of glioma using a phosphomimetic mutant version of Olig2 (aspartate or glutamate being substituted for serine) were more tumorigenic and a phosphonull mutant of Olig2 (glycine/alanine being substituted for serine) was less tumorigenic. [49]

Following the TCGA classification of glioblastoma there was interest in genetically defining differences between the groups and identifying a small set of genes that seemed to be most driving the expression pattern that defined each class. The four genes that most defined the Mesenchymal group were "YKL40, SERPINE1, TIMP1, and TGFBI." In contrast the genes that most defined the Proneural group were "DLL3, Olig2, ASCL1, and NCAM1."[52 14 15] While the Olig2 gene is expressed in a majority of glioblastomas, [50] we will show in Chapter 2 that it is particularly highly expressed in IDH1mutant tumors. Despite being found in GBM, oligodendrocyte precursors, neural precursor cells and motor neurons, its role in development as well as tumorigenesis is still being defined. The most clearly defined role of Olig2 is in the inhibition of p53 function via post-translational modification or inhibition of p21 function. [53] Interestingly, the role of Olig2 seems to be identical in both neural stem cells as well as malignant glioma. However, while most glioblastomas have an intact p53, IDH1mutant tumors are frequently p53 deficient. [5] It's unclear whether Olig2 has a pro-growth role in the setting of p53 deficiency.

Effect of the IDH1mutation on Metabolism

The fact that IDH1mutant tumors carry a better prognosis than those without IDH1mutations has led to the hypothesis that this new enzyme may have deleterious effects on cellular metabolism. To investigate this hypothesis, investigators have over-expressed the IDH1mutant gene and then performed mass spectroscopy to look at the differences between parental and transformed lines. All the studies to date have found that the addition of the IDH1mutant enzyme places a metabolic burden that makes the cell less fit and less resilient. The first studies focused on the fact that the IDH1mutant enzyme was converting large amounts of alpha-ketoglutarate into 2-HG making the assumption that alpha-ketoglutarate was derived largely from glutamine. Taking these facts together it was hypothesized that the IDH1 mutation led to cells becoming glutamine deficient. Seltzer et al. confirmed this by showing that the addition of the IDH1mutant enzyme made cells more vulnerable to glutaminase inhibition. [54] Another set of studies focused on the fact that the IDH1mutant enzyme consumes one molecule of NADPH and produces a molecule of NADP+ and, therefore, might have an effect on the level of reactive oxygen species (ROS). Results on this topic have been mixed. Attempts to knock-in the IDH1mutation under a Nestin neural stem cell driver was embryonic lethal but the salvaged cells had lower levels of ROS. [29] In contrast, over-expressing the IDH1mutant enzyme in U87 cells increased ROS levels and made the cells more vulnerable to radiation. [30]

More recently it was discovered that IDH1 has an important role in reductive carboxylation, which is the ability of the cell to convert glutamine to citrate without going through the TCA cycle. This allows the cell to participate in lipogenesis and membrane synthesis in a hypoxic environment. The presumption would be that with a mutation in the

IDH1 enzyme, the native function of the enzyme would be diminished. Again results have been mixed with Grassian et al. reporting that overexpression of the IDH1mutant gene inhibited the ability of cells to perform reductive carboxylation under hypoxia [55] while Reitman et al. using the same cell line found that the IDH1mutation actually facilitated the ability of the cell to convert glutamine into fatty acids (palmitate) under hypoxic conditions.[56]

All of these studies suffer from two methodological problems. The first is that the metabolic effect of the IDH1mutant enzyme depends on the baseline metabolic background of the host cell. There is increasing evidence that the IDH1mutation is likely one of the first mutations to occur in gliomagenesis [5] and thus over many rounds of cell growth and selection, the cells have time to adjust and adapt to whatever metabolic effects the IDH1mutation may have. If the IDH1mutation were as detrimental to cellular function as the previous studies suggest, the IDH1mutation would be deleted along with the other genes that impede accelerated cellular growth e.g. PTEN, p53, NF1. The second is that these studies fail to answer the more practical question of what is the metabolic effect of blocking the IDH1mutant enzyme in a glioma cell that already has it. In order to address these short-comings and more definitively answer the question of what metabolic differences exist between IDH1mutant and IDH1wildtype cells, we perform a full metabolic analysis on our collection of patient-derived gliomaspheres in chapter 3.

Chapter 2

Olig2 as a novel target in IDH1mutant tumors

Background: Large scale sequencing of tumor banks has revealed a subset of tumors that have a mutation in the isocitrate dehydrogenase (IDH1) enzyme that leads to very high levels of a compound, 2-hydroxyglutarate (2-HG), which is normally found at vanishingly small levels. While initial studies provided evidence that the 2-HG molecule may be an "oncometabolite," more recent studies using pharmacological inhibition of IDH1mutant enzyme have yielded mixed results. Thus we sought to identify a better molecular target to inhibit growth in IDH1mutant tumors.

Methods We propagated 74 patient-derived gliomasphere lines (7 bearing IDH1mutations) and performed expression analysis using U133 Plus 2.0 microarrays. Using both this dataset and the TCGA dataset we identified a list of 6 genes that were differentially over-expressed in IDH1mutant tumors. We used a CRISPR-Cas9 system to knock-down Olig2 expression and determine its effect on growth. We also tested the ability of valproic acid, an HDAC to knock-down olig2 expression and reduce growth in IDH1mutant tumor cells.

Results In this study, we show that IDH1mutant gliomaspheres are a good model for IDH1mutant tumors. In addition to 2-HG production and relatively slower growth, IDH1mutant gliomaspheres also phenocopy the expression and methylation patterns of in vivo IDH1mutant tumors. We identified Olig2 as one of the few molecular targets that is up-regulated in

IDH1mutant tumors for evaluation. CRISPR knock-down of Olig2 effectively slowed growth in IDH1mutant lines in vitro. Expression analysis after Olig2 knock-down identified Tet2 as a repressive target of Olig2. ChIP pull-down confirmed direct binding of Olig2 to the Tet2 promoter. Finally we show that the HDAC inhibitor valproic acid may be an effective therapy at reducing Olig2 expression and slowing growth in some IDH1mutant tumors.

Conclusions Recent success with IDH1mutant inhibitors in leukemia has not translated to brain tumors. Different molecular targets are needed, however there are very few genes which are consistently up-regulated in IDH1mutant tumors. In this study we present Olig2, a gene consistently and highly up-regulated in IDH1mutant tumors that is necessary for growth and at least in some tumors can be targeted by HDAC inhibitors.

Key Words IDH1 mutation, 2-hydroxyglutarate, TET2, Olig2, Glioma, Glioblastoma

Abbreviations IDH1 = Isocitrate Dehydrogenase 1; 2-HG = 2-Hydroxyglutarate; Tet2 = Ten Eleven Translocation Gene 2

Since the discovery of the IDH1mutation in glioma there have been numerous studies to discover the mechanism by which this mutant enzyme leads to tumorigenesis[22,25,57] and whether IDH1mutant inhibition might reverse this process and restore normal function. IDHa mutant (IDH1mut) overexpression models have been employed in a variety of cell

types with each cell type seeming to display a different phenotype. However, there is a common theme amongst these studies. In each study the underlying mechanism is that D-2HG causes dysfunction in α-KG-dependent dioxygenase enzymes by competing with α-KG for a co-factor binding pocket[21].

The discovery that the Tet family of demethylases can be inhibited by 2-HG, coupled with the observation that IDH1mutant gliomas have a hyper-methylation phenotype highlighted the possibility that the IDH1 mutation was working through Tet inhibition. Recently it was discovered that hyper-methylation of CTCF sites in IDH1mutant tumors may lead to disruption of chromosome topology and enhancer structure with subsequent oncogene expression [58]. However, while some studies have been able to successfully induce methylation changes via induced IDH1mutant expression [59] [25], no study has been able to show that reduction of 2-HG can reverse this process. This question is of high clinical importance since the discovery of a pharmacological IDH1mutant inhibitor (c227)[38] that can reduce 2-HG to near wildtype levels. However, while early reports from clinical trials in lymphoma are promising[40], consistent results in glioma have been more elusive with some studies showing the c227 inhibitor may be beneficial[41] while others showing that IDH1mutant inhibition has at best no effect and may possibly increase growth and give IDH1mutant cells a metabolic growth advantage[42]. Even more discouraging, in this latter study, even after a year of continuous IDH1mutant inhibition there were no changes in either DNA methylation or histone modification.

With hope fading that the c227 or related inhibitors will ever be useful as monotherapy for IDH-mutant glioma we endeavored to find a new genetic target specific to IDH1mutant tumors that could be exploited for therapeutic benefit. We used patient-derived

gliomaspheres as our model system. After verifying that many of the defining traits of IDH1mutant tumors are mirrored in their corresponding gliomaspheres, we employed an expression analysis for candidate genes.

Methods

Collection of in vitro cultures.

High grade glioma samples are collected under institutional review board-approved protocols and graded by neuropathologists. On the day of resection samples are taken directly from the operating room and digested in papain. Acellular debris is removed and the remained cells are incubated in DMEM/F12 supplemented with B27, penicillin/ampicillin, heparin, EGF and bFGF for several days until spheres begin to form. Frozen stocks are made at passage 5 to maintain cells at low passage.

Expression Analysis

RNA was purified and was hybridized to U133 Plus 2.0 Arrays at the UCLA DNA Microarray Facility. Standard wash, stain protocols, and scanning were used. The CEL files generated were normalized using RMA from Bioconductor, relative to other Affymetrix microarrays of the same platform using the Celsius Database [60]. RMA normalized data was imported into dChip for differential expression analysis[61]. Samples were clustered on the 5000 most variable genes. The 7 IDH1 mutant neurosopheres were compared with 67 neurospheres without the IDH1 mutation. A fold change and t-test analysis was used to measure significance

Reduced Bisulfite Sequencing

RRBS was done following the protocol published by Chou et al[62]. Briefly, DNA was isolated from IDH1mutant and IDH1WT gliomaspheres digested with the restriction enzyme MspI to enrich for fragments containing CpG islands, and end-repaired using methylated cytosine. After adapter ligation, the DNA was size-fractionated by gel electrophoresis, and DNA fragments between 100 and 400 base pairs in length were isolated to minimize large fragments with poor sequencing coverage. Isolated DNA fragments were then bisulfite treated, amplified, mixed with unmodified PhiX DNA (a bacterial genome inserted for quality control and to assess mapping), and sequenced on an Illumina Genome Analyzer IIx. The Novoalign software package (www.novocraft.com) was used to align the sequence data. Aligned sequence data were then sorted using the SAMTools software package and stored in SAM format for further analysis.

Methylation status was determined at individual CpG sites, and the results were compiled to show the level of methylation at individual CpG islands. CpG islands were mapped by previously published definitions [63].

2-Hydroxyglutarate Measurement

2-hydroxyglutarate levels were determined using the protocol described in Balss et al. [64] This assay is based on the conversion of D-2HG to alpha-ketoglutarate (a-KG) in the presence of the enzyme (D)-2-hydroxyglutarate dehydrogenase (HGDH) and nicotinamide adenine dinucleotide (NAD+). The enzyme HGDH was a gift from Dr von Deimling's laboratory. In brief, cell pellets were harvested in Cell Lysis Buffer (Cell Signaling) and then the lysate was divided into two parts, one for protein content determination by Pierce BCA protein assay kit (Thermo Scientific), and another for D2HG assay. The lysate for D2HG

assay was firstly deproteinized by adding 3ml of Proteinase K (Qiagen) and incubating 3 hrs at 37C. Then 25 ml of lysate was reacted with 75ml of assay solution at room temperature for 30 minutes. Each sample was performed in triplicate. The assay buffer contains, 100mM NAD+ (Applichem, Darmstadt, Germany), 0.1mg HGDH, 5mM resazurin (Applichem) and 0.01 U/ml diaphorase (MP Biomedical, Irvine CA, USA) in 100mM HEPES pH 8.0. Fluorometric detection as carried out in Wallace Victor2 1420 Multilable HTS Counter (Perkin Elmer) with Em 540nm/Ex610nm. D2HG content was calculated using a standard curve and plotted as D2HG pmole/mg protein.

Olig2 ChIP

ChIP-qPCR was performed by preparing fixed nuclei from IDH1mutant gliomaspheres and then sonicating the DNA. Protein A beads (Life Technologies 10001D) were initially incubated with 1ug Olig2 antibody (Millipore AB9610) then washed and incubated with the sonicated DNA. Protein-DNA complexes were then reverse cross-linked and the protein was digested away. The amount of resulting DNA was assessed using qPCR. Primers were designed that encompassed a likely target from the Tet2 protomter site (Forward ATGGCTGCCCTTTAGGATTT Reverse: GTTTCGCGGCATAAGAGAAG). Results were compared to bead alone. Following Olig2 CRISPR knock-down, expression of Tet2 was assessed using qPCR and the following primers (Tet2 mRNA: Forward GGAGCATTAGAAGGGGGAAG, Reverse GGATGCTTGATTTTCCCTGA).

CRISPR-Cas9

pgRNA-humanized vector was purchased from Addgene (plasmid#44248). This vector is under the control of the Murine U6 promoter. Three guide RNA sequences targeting the transcribed region were designed following the instructions from Lentiviral CRISPR Toolbox on the Zhang Lab website (**http://www.genome-engineering.org/crispr**), confirmed by direct sequencing and packaged into lentiviruses. The CRISPR plasmid was obtained from Addgene (Plasmid#52961). An endogenous IDH1mutant line is infected with all four viruses and after four days the knockdown of Olig2 expression is confirmed via Western blot.

Results

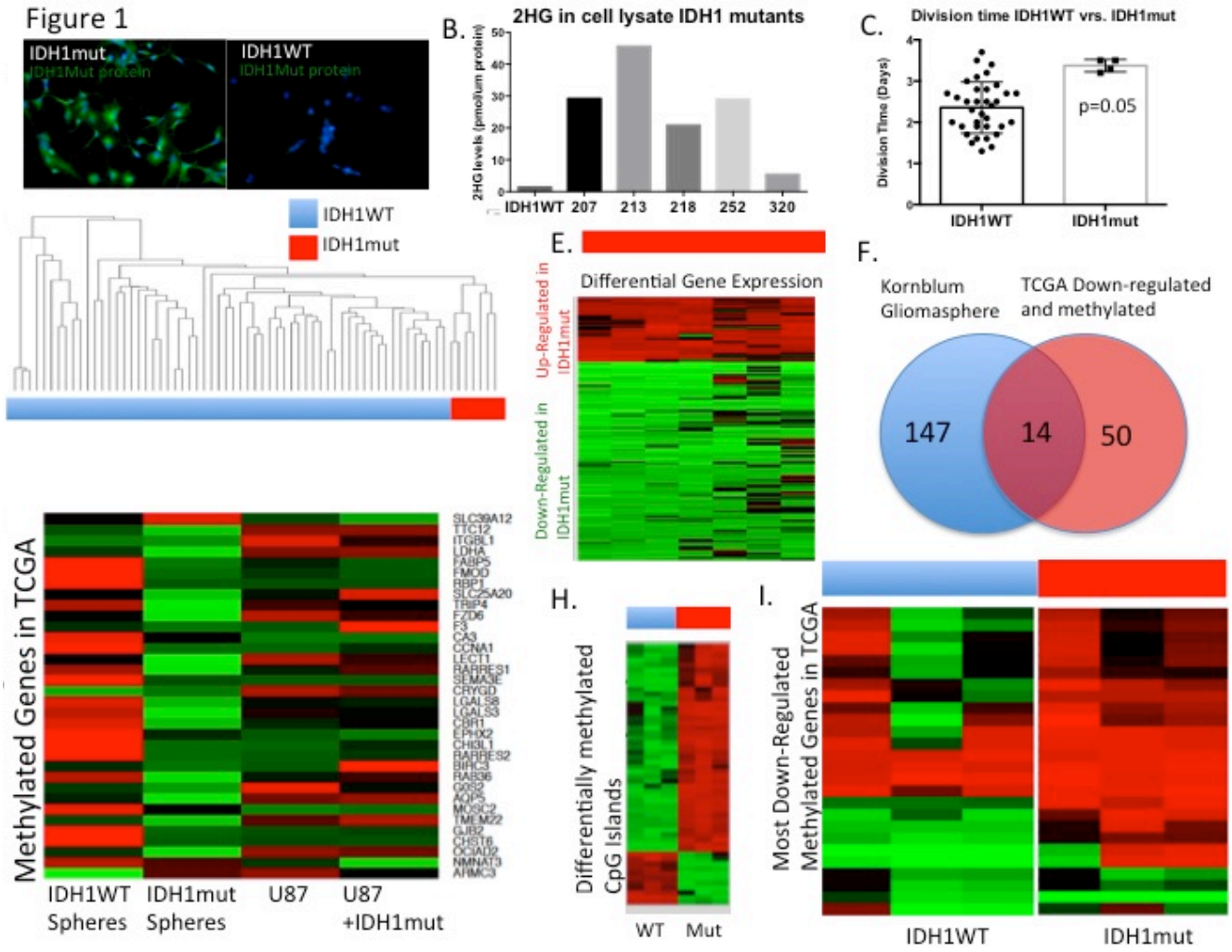
Gliomaspheres as a model for IDH1mutant tumors

Endogenous patient-derived IDH1mutant gliomaspheres have been historically difficult to generate and thus their validity as good models has never been formally assessed. Thus we first set out to verify that IDH1mutant gliomaspheres retained many of the most significant traits seen in in vivo IDH1mutant tumors. Immunohistochemistry using a specific IDH1mutant antibody (Dianova H09) revealed that all cells in the culture retained expression of the IDH1mutant enzyme (Figure 1a). All lines produced 2-HG, although at varying concentrations. Consistent with previous observations[65], our one homozygous IDH1mutant line (HK 320) produced the lowest level of 2-hydroxyglutarate (Figure 1b). Consistent with the improved survival data in IDH1mutant gliomas[66] a cohort of 39 gliomaspheres (4 IDH1mut) showed that IDH1mutant gliomaspheres had a significantly longer division time (Figure 1c) than IDH1WT gliomaspheres. Unbiased clustering was performed on expression data from all seventy-four gliomasphere lines (including 7 IDH1mutant lines) and found that

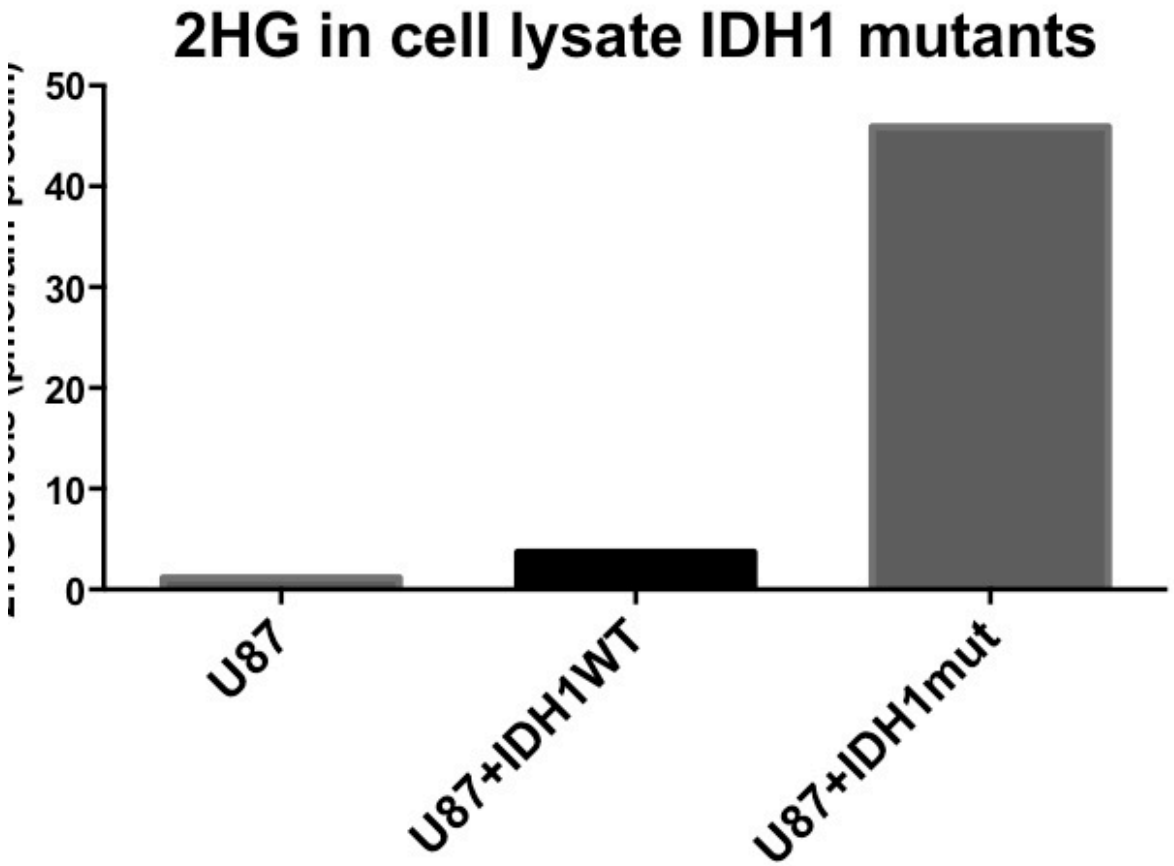
IDH1mutant gliomaspheres constituted a group distinct from IDH1WT gliomaspheres (Figure 1d).

We examined gene expression differences between IDH WT and mutant cells and noted that the IDH1mutant gliomaspheres contained a large set of very down-regulated genes compared to IDH1WT gliomaspheres (Figure 1e).

Figure 1


IDH1mut
IDH1Mut protein
IDH1WT
IDH1Mut protein
B.
2HG in cell lysate IDH1 mutants
2HG levels (pmol/um protein)
IDH1WT 207 213 218 252 320
C.
Division time IDH1WT vrs. IDH1mut
Division Time (Days)
p=0.05
IDH1WT
IDH1mut
IDH1WT
IDH1mut
E.
Differential Gene Expression
Up-Regulated in IDH1mut
Down-Regulated in IDH1mut
F.
Kornblum Gliomasphere
TCGA Down-regulated and methylated
147
14
50
Methylated Genes in TCGA
SLC39A12
TTC12
ITGBL1
LDHA
FABP5
FMOD
RBP1
SLC25A20
TRIP4
FZD6
F3
CA3
CCNA1
LECT1
RARRES1
SEMA3E
CRYGD
LGALS8
LGALS3
CBR1
EPHX2
CHI3L1
RARRES2
BIRC3
RAB36
GOS2
AQP5
MCSC2
TMEM22
GJB2
CHST6
OCIAD2
NMNAT3
AHMC3
IDH1WT Spheres
IDH1mut Spheres
U87
U87 +IDH1mut
H.
Differentially methylated CpG Islands
WT
Mut
I.
Most Down-Regulated Methylated Genes in TCGA
IDH1WT
IDH1mut

igure 2


2HG in cell lysate IDH1 mutants
50
40
30
20
10
0
U87
U87+IDH1WT
U87+IDH1mut

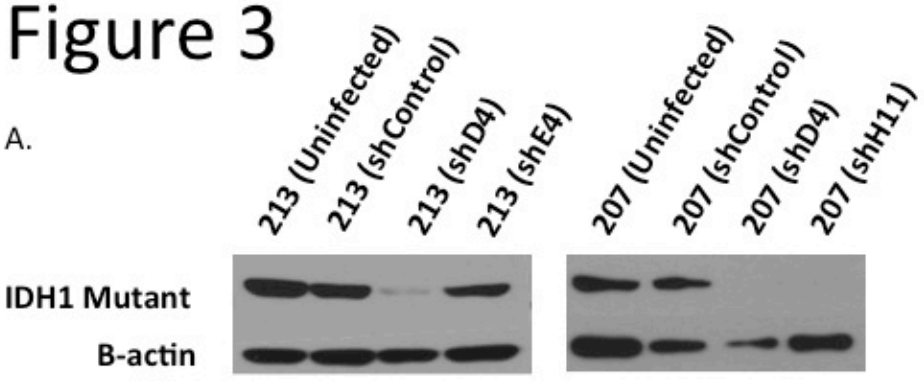
We next wanted to see if the down-regulated genes seen in our IDH1mutant gliomaspheres were the same genes seen in the IDH1mutant tumors from the TCGA data set (Cancer Genome Atlas). We identified 147 genes in our gliomasphere dataset that were down-regulated at least four-fold and $p<0.0005$. We then compared this list to a published list [67] of 50 of the most down-regulated and methylated genes found in TCGA. Significantly, fourteen genes were in both lists ($p<0.01$) (Figure 1F). We then wanted to determine if over-expression of the IDH1mutant gene could induce this expression pattern. We took the established glioma model U87 and over-expressed the IDH1mutant gene in three different cultures. We were able to verify the model by measuring high levels of 2-HG production in the transformed U87 lines (Figure 2). We obtained microarray expression data on these U87 lines and compared them to U87 control cultures. We selected a subset of 32 of the most down-regulated methylated genes from the Nousmehr 50 gene list as our comparison group. When comparing endogenous IDH1mutant and IDH1WT gliomaspheres we saw significant gene down-regulation in the IDH1mutant group. However we did not see this difference in the U87-IDH1mutant condition (Figure 1G). Finally we performed reduced representation bisulfite sequencing (RRBS) to determine if IDH1mutant gliomaspheres had higher levels of methylation than corresponding IDH1WT gliomaspheres. We found that IDH1mutant gliomaspheres had more global methylation and when we looked specifically at those 32 Noushmehr genes we found that our IDH1mutant gliomaspheres had more methylation in those genes than corresponding IDH1WT gliomaspheres (Figure 1H and 1I).

IDH1mutant expression signature and growth is independent of 2-hydroxyglutarate

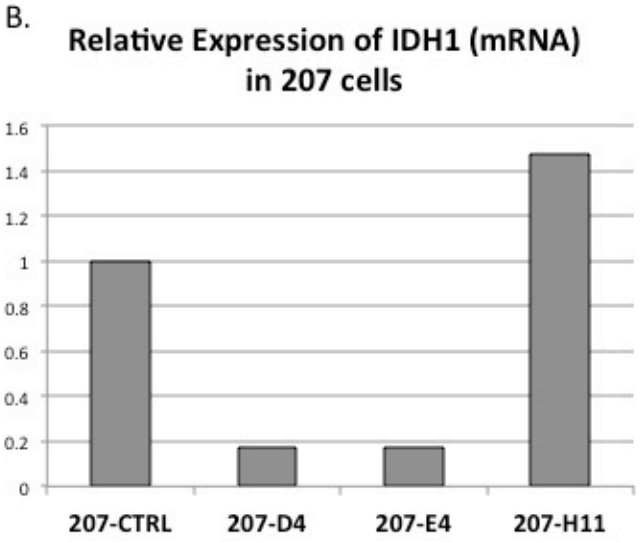
Convinced that our gliomaspheres retained an expression and methylation signature similar to that of in vivo IDH1mutant tumors, we then sought to determine if 2-HG depletion could change this expression pattern. We again identified the list of differentially expressed genes between our IDH1mutant (n=7) and IDH1 WT(n=67) gliomaspheres (Figure 1e). We designed several siRNA lentiviral constructs against the IDH1 protein (Figure 3a). We found the D4 construct to be the most consistently effective. Notably, the siRNA construct was not able to distinguish IDH1mutant from IDH1wildtype transcript and thus knocked down total IDH1 protein in both IDH1mut (Figure 3b) and IDH1wildtype (Figure 3c) cultures. Then we utilized a commercially available small molecule inhibitor of the IDH1mutant enzyme (Xcessbio c227). When we measured the resulting depletion of 2-HG we found that the c227 was much more effective at reducing the levels of 2-HG (Figure 4a). We then allowed these cells to grow for 8 weeks before analyzing their transcriptome via microarray. When looking at the previously described list of differentially expressed genes we saw no significant change following long-term 2-HG depletion (Figure 4b). We then searched more broadly for any gene that consistently changed more than two-fold across the various cell lines. In the set of lines exposed to c227 there were no genes identified. In the set of lines infected with siRNA against IDH1 the only gene that changed was IDH1. We then tested the effect of 2-HG depletion on growth by plating 200,000cells and allowing the cells to grow for two weeks. Again we found no difference in growth. (Figure 4c)

Figure 3

A.


213 (Uninfected)
213 (shControl)
213 (shD4)
213 (shE4)
207 (Uninfected)
207 (shControl)
207 (shD4)
207 (shH11)
IDH1 Mutant
B-actin

B.


Relative Expression of IDH1 (mRNA) in 207 cells
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
207-CTRL
207-D4
207-E4
207-H11

C.

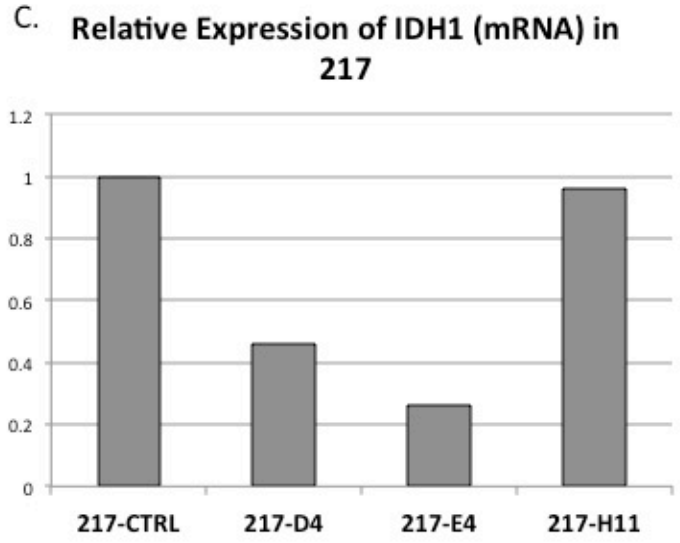
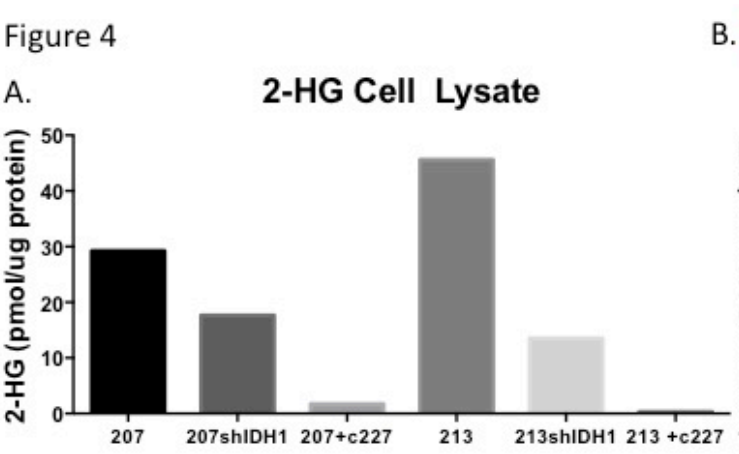
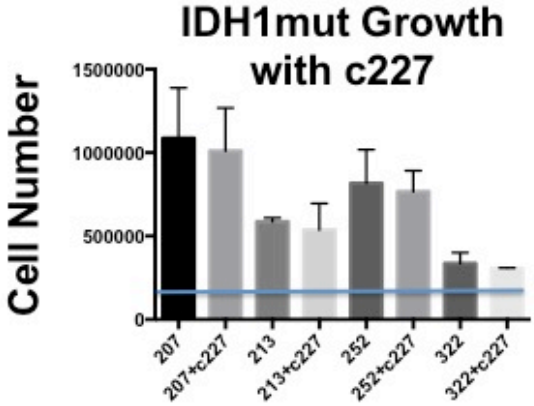
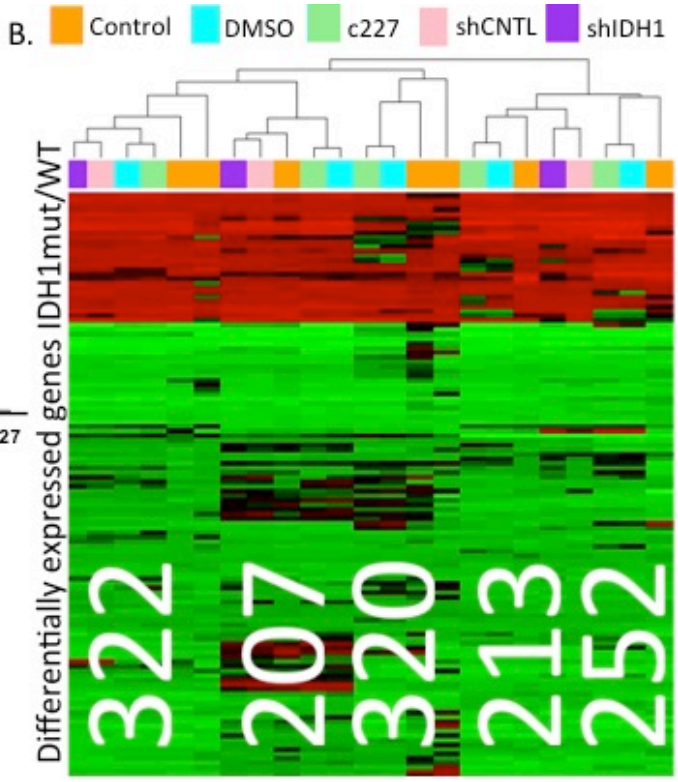

Relative Expression of IDH1 (mRNA) in 217
1.2
1
0.8
0.6
0.4
0.2
0
217-CTRL
217-D4
217-E4
217-H11

Figure 4


A.
2-HG Cell Lysate
2-HG (pmol/ug protein)
50
40
30
20
10
0
207
207shIDH1
207+c227
213
213shIDH1
213 +c227

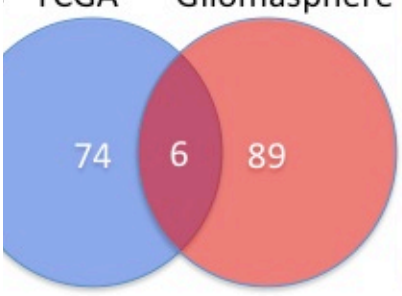

IDH1mut Growth with c227
Cell Number
1500000
1000000
500000
0
207
207+c227
213
213+c227
252
252+c227
322
322+c227


B.
Control
DMSO
c227
shCNTL
shIDH1
Differentially expressed genes IDH1mut/WT
322
207
320
213
252

Olig2 as a novel target

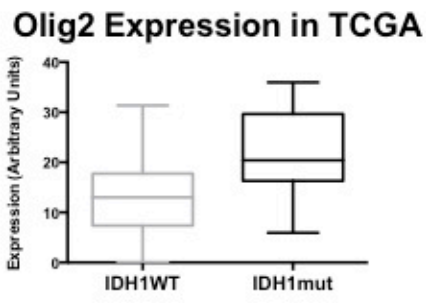
Given that 2-HG depletion seemed to be an ineffective strategy for growth arrest we tried to find another genetic target, specific to IDH1mutant glioma cells that might be exploited for therapy. This was complicated by the fact that IDH1mutant gliomas are characterized by widespread down-regulation of numerous genes. However, when comparing all the IDH1mutant genes that were up-regulated in both TCGA as well as our gliomaspheres we identified six possible targets (Figure 5a). Notably, Olig2 was one of the six possible targets. This gene was chosen for further study based upon the previous discussion regarding the ability of Olig2 to regulate the fate choices between self-renewal and differentiation in neural stem cells as well as the role of Olig2 in being able to restore tumorigenicity in serum-treated gliomaspheres. We found Olig2 to be highly expressed in the TCGA dataset as well as gliomaspheres (Figure 5b, 5c). Notably in sections from IDH1mutant tumors nearly 100% of tumor cells stained for Olig2 (Figure 5d). Using a CRISPR-Cas9 system targeted against Olig2, we determined that Olig2 knockdown was able to slow growth in one of the IDH1mutant cell lines. (Figure 5e, 5f).

gure 5

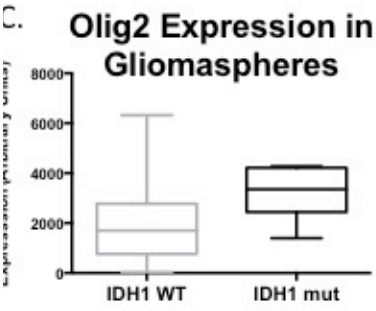


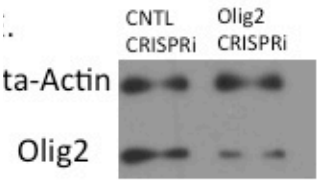
Gene Name
GALANT13
KCNH8
OLIG2
RANBP17
SH3GL2
TOX3

B.

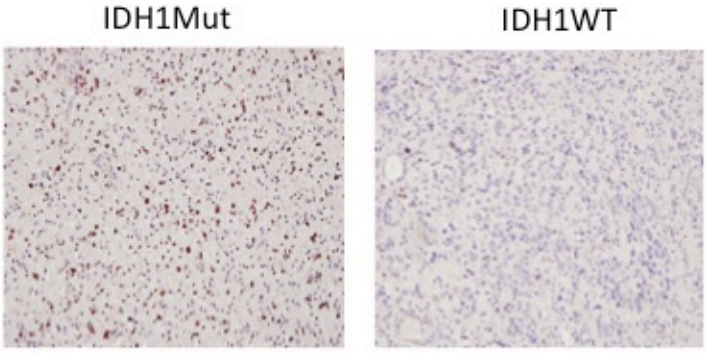


C.

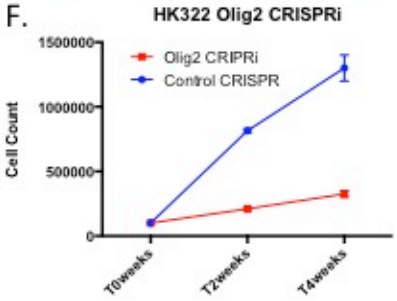




D.



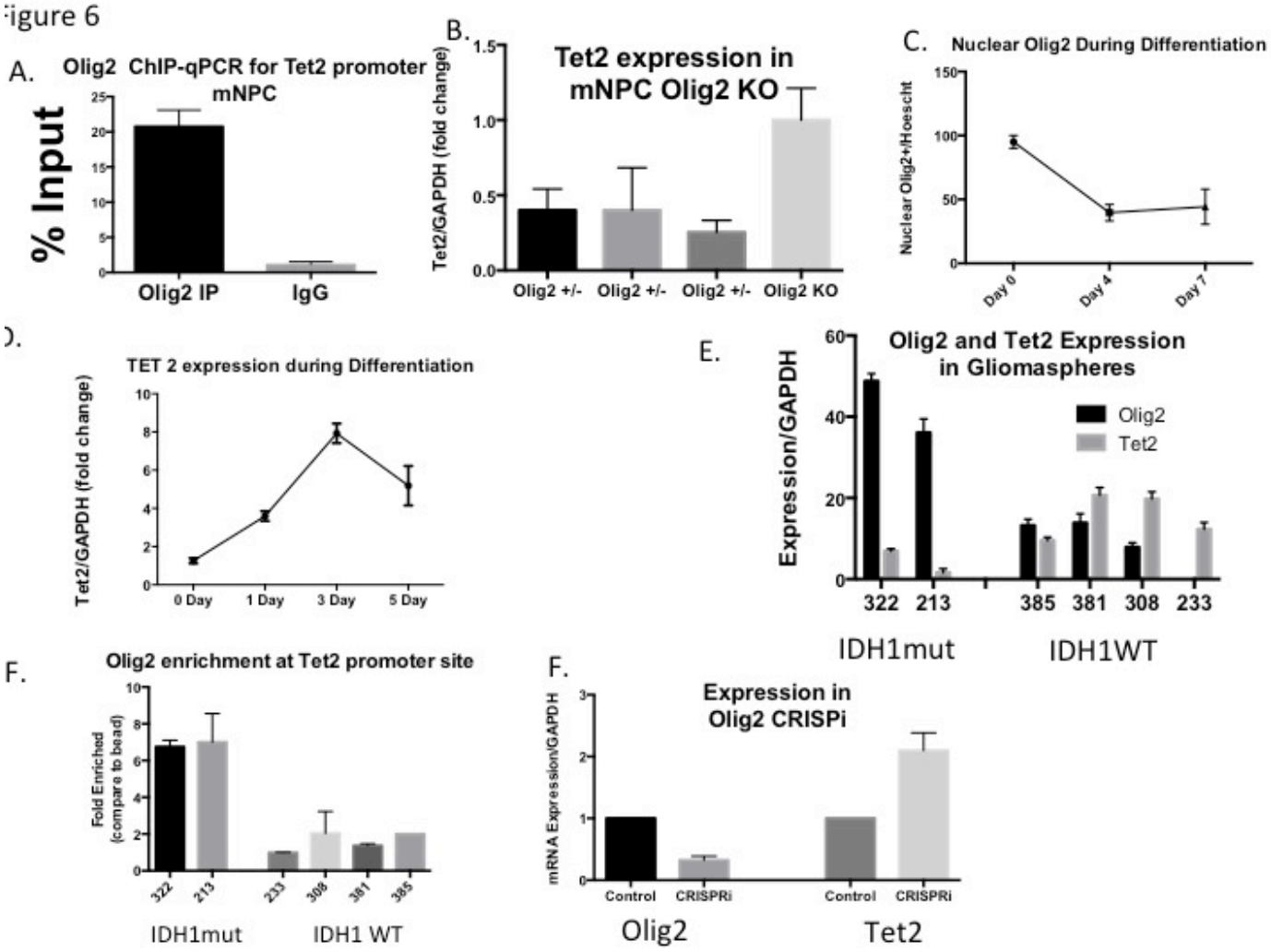
F.



Olig2 regulates Tet2

After discovering that Olig2 is a central driver of growth in IDH1mutant cells, we sought to discover particular targets that Olig2 might regulate. In working with murine neural stem cells discovered that Olig2 regulates Tet2 in murine neural precursor cells during differentiation. In the neural precursor state (mNPC) Olig2 binds the Tet2 promoter preventing expression. During differentiation, Olig2 leaves the nucleus and Tet2 expression increases (Figure 4a-d). Given the similarity in expression patterns between IDH1mutant tumors and neural precursor cells and our observation that IDH1mutant cells have low Tet2 expression (Figure 4E), we sought to find out if Olig2 was directly repressing Tet2 in IDH1mutant tumors as well. We prepared fragmented DNA from a panel of IDH1mutant (HK213 and HK322) and IDH1wildtype (HK 233, HK308, HK381, HK385) lines. We precipitated the DNA fragments using an Olig2 antibody and then used qPCR to test for the presence of the Tet2 promoter. We found that the Tet2 promoter in IDH1mutant gliomaspheres had high levels of Olig2 binding (Figure 4e). In contrast there was minimal Olig2 binding to the Tet2 promoter in IDH1wildtype cells. When he knocked down Olig2 in an IDH1mutant gliomasphere line (HK322) we saw a corresponding increase in the expression of Tet2 (Figure 4f).

Figure 6


A.
Olig2 ChIP-qPCR for Tet2 promoter mNPC
% Input
Olig2 IP
IgG
B.
Tet2 expression in mNPC Olig2 KO
Tet2/GAPDH (fold change)
Olig2 +/-
Olig2 +/-
Olig2 +/-
Olig2 KO
C.
Nuclear Olig2 During Differentiation
Nuclear Olig2+/Hoescht
Day 0
Day 4
Day 7
D.
TET 2 expression during Differentiation
Tet2/GAPDH (fold change)
0 Day
1 Day
3 Day
5 Day
E.
Olig2 and Tet2 Expression in Gliomaspheres
Expression/GAPDH
Olig2
Tet2
322
213
385
381
308
233
IDH1mut
IDH1WT
F.
Olig2 enrichment at Tet2 promoter site
Fold Enriched (compare to bead)
322
213
233
308
381
385
IDH1mut
IDH1 WT
F.
Expression in Olig2 CRISPi
mRNA Expression/GAPDH
Control
CRISPRi
Control
CRISPRi
Olig2
Tet2

Olig2 is targetable by HDAC inhibitors

Transcription factors are difficult to target pharmacologically. In order to discover potential drug targets we searched the literature for compounds that were able to reduce Olig2 expression. We found two studies that used histone de-acetylase inhibitors (HDACi) to reduce olig2 expression and induce differentiation in oligodendrocyte progenitors[68,69]. Using this as background we tested the effect of valproic acid (VPA) on reducing Olig2 expression and slowing growth in three IDH1mutant lines. The effect was mixed with two lines (HK 252 and HK 322) showing decreased Olig2 expression and decreased growth in response to valproic acid (Figure 7a-b) and one line (HK 213) showing no response in either Olig2 expression or growth(Figure 8). We further hypothesized that the high Olig2 expression may be partially responsible for the signature of gene down-regulation that we observed from the expression arrays. We tested the response to either VPA treatment or Olig2 knockdown on one of the most differentially down-regulated and methylated genes in IDH1mutant tumors, CHI3L1(Figure 9a) and found that it increased both in response to VPA as well as Olig2 CRISPRi (Figure 9b-c). However, when we checked the regulatory methylation island we found that the gene was still nearly 100% methylated even after CRISPi Olig2 and VPA treatment. Finally we tested to see if VPA and c227 had any synergistic properties in regards to cell growth, however, once again c227 did not seem to have any consistent effect on growth (Figure 10).

Figure 7

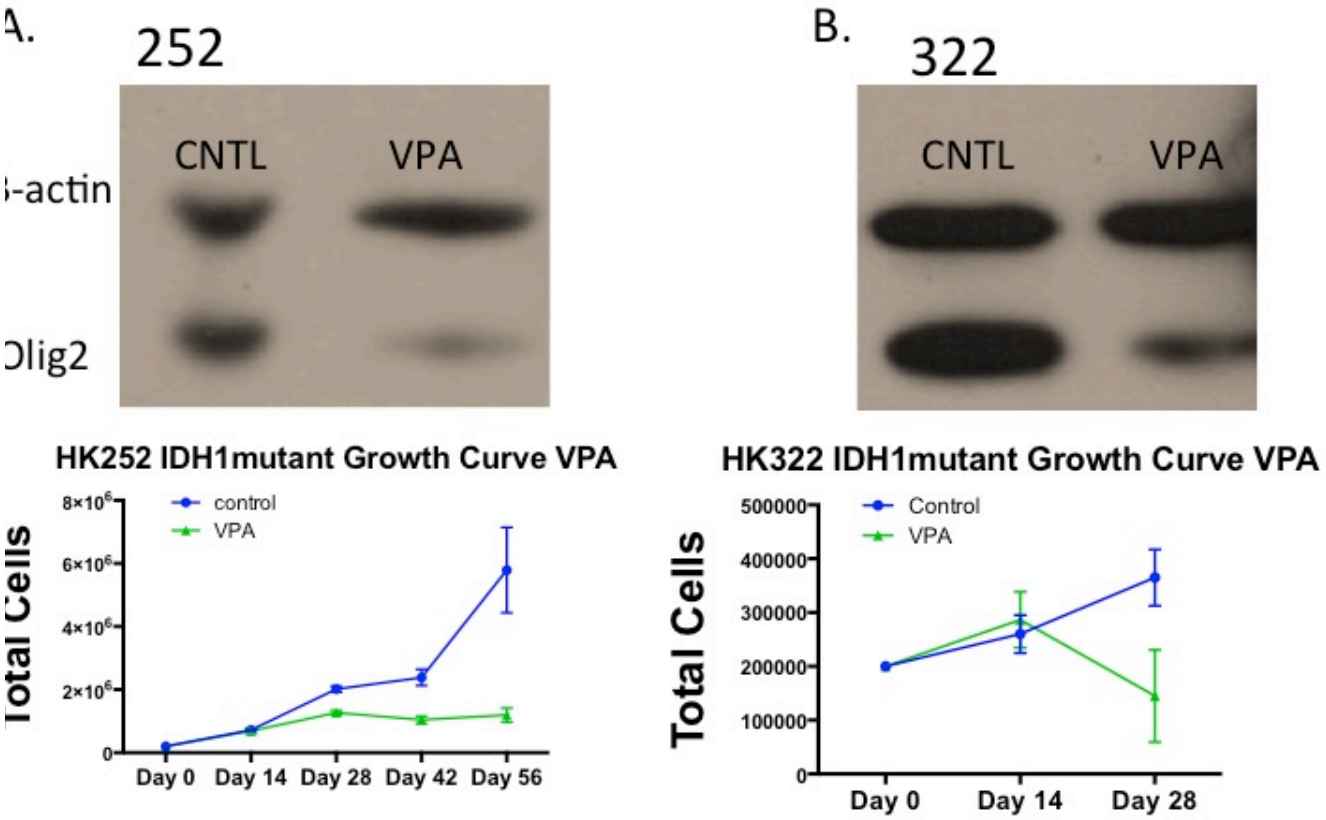
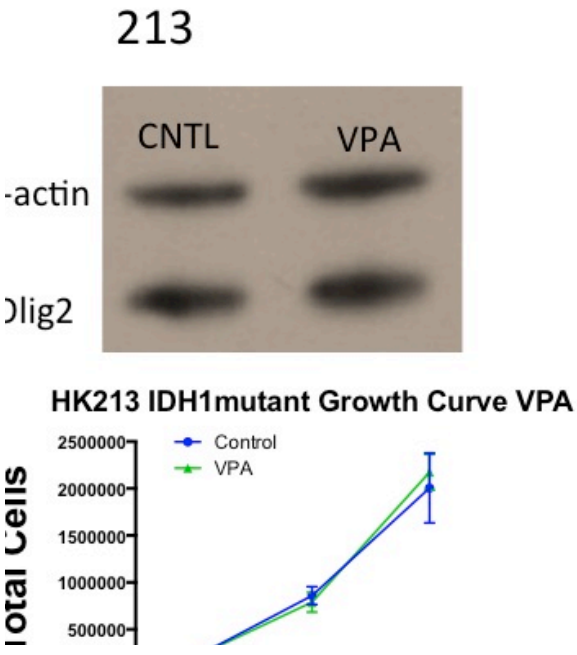
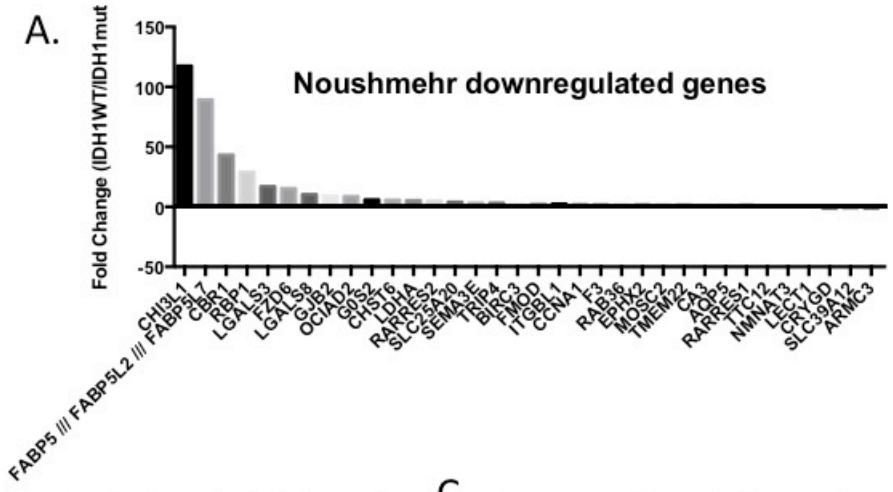
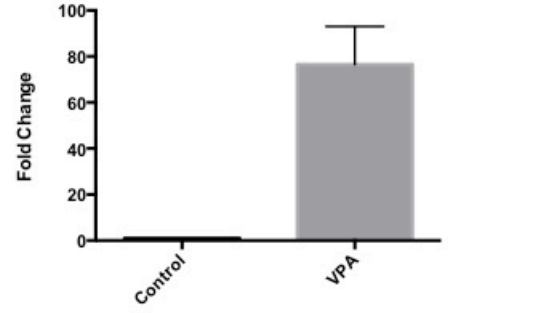


Figure 8



igure 9


A.
Noushmehr downregulated genes
Fold Change (IDH1WT/IDH1mut
150
100
50
0
-50
CHI3L1
FABP5 /// FABP5L2 /// FABP5L7
CBR1
RBP1
LGALS3
FZD6
LGALS8
GJB2
OCIAD2
G0S2
CHST6
LDHA
RARRES2
SLC25A20
SEMA3E
TRIP4
BIRC3
FMOD
ITGBL1
CCNA1
F3
RAB36
EPHX2
MOSC2
TMEM22
CA3
AQP5
RARRES1
TTC12
NMNAT3
LECT1
CRYGD
SLC39A12
ARMC3


Expression of CHI3L1 after Olig2 CRISPRi (HK322)
Fold Change
100
80
60
40
20
0
Control
VPA

C.

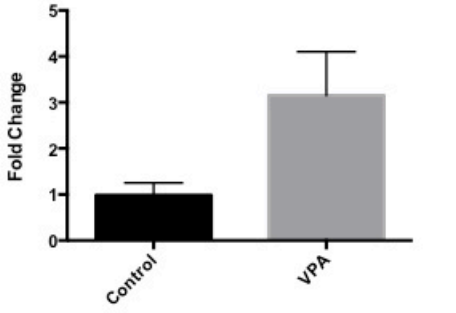
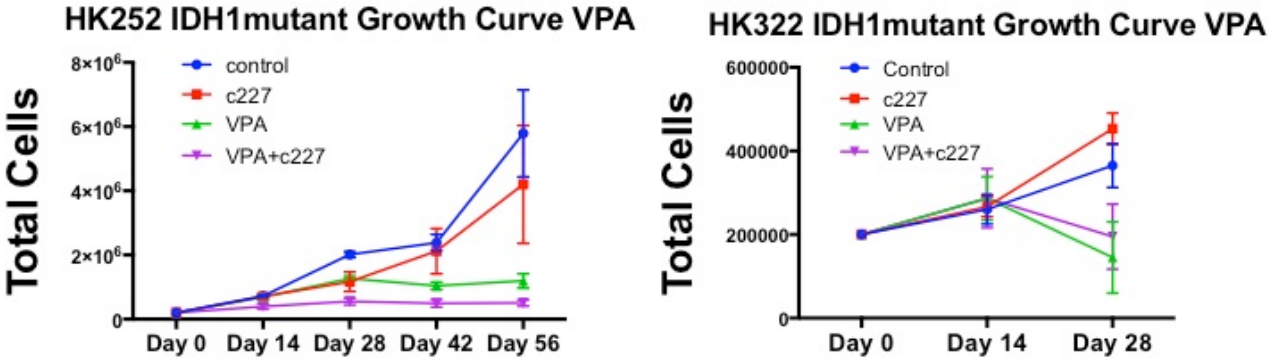

Expression of CHI3L1 after VPA (HK322)
Fold Change
5
4
3
2
1
0
Control
VPA

Figure 10


HK252 IDH1mutant Growth Curve VPA
Total Cells
8×10^6
6×10^6
4×10^6
2×10^6
0
control
c227
VPA
VPA+c227
Day 0
Day 14
Day 28
Day 42
Day 56
HK322 IDH1mutant Growth Curve VPA
Total Cells
600000
400000
200000
0
Control
c227
VPA
VPA+c227
Day 0
Day 14
Day 28

Discussion

The study of IDH1mutant gliomas has been hampered by the difficulty in establishing and maintaining IDH1mutant gliomaspheres in vitro. Many studies have relied on over-expression of the IDH1mutant gene on an IDH1wildtype background. The results of these studies have shed light on the potential mechanisms by which the IDH1mutant enzyme could lead to tumorigenesis. However, it has never been shown how well these over-expression models recapitulate the traits and behavior of endogenous IDH1mutant cells. In this study we show that endogenous IDH1mutant gliomaspheres capture many of the genetic and epigenetic traits of in vivo IDH1mutant tumors. In contrast, over-expressing the IDH1mutant gene was unable to phenocopy the expression pattern seen in in vivo tumors. More discouraging, genetic and pharmacological IDH1mutant inhibition was unable to alter the expression signature nor able to alter growth. Thus we found a new genetic target that seems to both drive growth and be targetable by the HDAC inhibitor, valproic acid.

Following the initial discovery of existence of the IDH1mutation in glioma[16] as well as a host of other cancer types there was a presumption that, like most oncogenes, the addition of the mutation would bestow upon a cell an oncogenic property such as increased growth or resistance to cell death. Similarly, it was presumed that this property would be preserved across tissue types. However, on the contrary, in most contexts the addition of the IDH1mutation actually decreases growth and makes cells more susceptible to cell death.[30] These results raise the question of whether the IDH1mutation is essential for maintaining increased growth or whether its role is limited to tumor initiation. To that end, some studies have found that the IDH1mutant enzyme or high levels of 2-hydroxyglutarate can block

normal differentiation in some cells by interfering with the histone modifications of critical differentiation genes.[22,24] However, there has been no evidence that this phenomenon occurs in neural stem cells and we have observed no deficit in differentiation when the IDH1mutation is over-expressed in mouse embryonic neural stem cells.

The idea that the IDH1mutation functioned by inhibiting Tet2 was strengthened by the finding that IDH1mutation and Tet2 mutation were both common and mutually exclusive in AML.[32] Interestingly, Tet2 mutations are relatively rare in glioblastomas. One would presume that if Tet2 were a critical tumor suppressor there would be a high frequency of mutation to allow for tumorigenesis. As a possible explanation for the rarity of Tet2 mutations, we observed that IDH1mutant tumors repress Tet2 transcriptionally via Olig2. This may also explain why targeting 2-HG production does not lead to any demethylation [42] or change in expression in these tumors.

With the finding that targeting IDH1 may be an ineffective strategy in these tumors, other targets are clearly needed. However, the widespread genetic down-regulation of IDH1mutant tumors makes it difficult to find specific genetic targets. Indeed we only found 6 genes that were up-regulated in both IDH1mutant tumors and gliomaspheres. Of these 6 genes we decided to focus on Olig2 given its well-researched role in gliomagenesis as well as neural development. [53,70] Suva et al. found that Olig2 was one of four necessary transcription factors to restore tumorigenicity to a serum-treated gliomasphere. Additionally this study found that the Olig2 transcription factor could be replaced by over-expression of a histone deacytelase gene (HDAC) suggesting that the role of Olig2 and HDAC genes may be heavily overlapping. This finding along with others gave rise to the hypothesis that HDAC inhibitors could block Olig2 function. Of note, HDAC inhibitors have been used in glioblastoma

clinical trials with mixed results.[71] [72] [73] [74] However, the data from this study provides a possible explanation for the mixed results and indicates that valproic acid anti-tumor efficacy is not universal to all glioma cells and may be limited to Olig2-driven cells.

While this study may generate some enthusiasm for the idea of valproic acid as a possible therapy to target Olig2 in these tumors there are some important caveats. First, it has proved difficult to directly inhibit Olig2 expression in IDH1mutant lines and thus we only present evidence of the effect of Olig2 knock-down in one line. Olig2 knockdown correlates with an approximate two-fold increase in Tet2 expression and we observed a previously methylated and repressed gene upregulate however there was no corresponding change in CpG island methylation state suggesting other regulatory mechanisms may be at work. While valproic acid may provide tested and relatively safe pharmacologic option to target Oligs it should be noted that,valproic acid was only effective against two of the three lines tested and the amount of Olig2 knock-down was only moderate. It is also unclear how much of the observed growth phenotype can be attributed to Olig2, rather it is highly likely that the growth phenotype seen in those two lines may be due to off-target effects.

In conclusion, while some traits are always lost when a cell is moved from a natural tissue environment to an artificial media environment, in the case of endogenous IDH1mutant gliomaspheres it appears many of the most important traits are retained. More importantly, these traits cannot be reproduced by over-expression of the IDH1mutant enzyme alone. While targeting the IDH1mutant enzyme itself seems to be an ineffective strategy, our genetic screen has revealed an essential and targetable gene in Olig2.

Chapter 3

Metabolic characterization of IDH1mutant and IDH1wildtype gliomaspheres to uncover cell-type specific vulnerabilities

Background: Large scale sequencing of tumor banks has revealed a subset of tumors that have a mutation in the isocitrate dehydrogenase (IDH1) enzyme, which bestows a novel function of reducing alpha-ketoglutarate into 2-hydroxyglutarate (2-HG). There has been considerable interest in defining metabolic differences of IDH1mutant tumors to exploit in therapy. However, most studies are limited by over-expressing the mutant IDH1 gene on an IDH1WT background. In this study we attempt to define metabolic differences between a cohort of patient-derived IDH1mutant and IDH1WT gliomaspheres to design patient-specific therapy.

Methods We propagated 59 patient-derived gliomasphere lines (7 bearing IDH1mutations) and performed expression analysis using U133 Plus 2.0 microarrays. Using both this dataset and the TCGA dataset we performed a KEGG analysis to define the pathways that were differentially enriched in IDH1mutant and IDH1WT cells. We used NOVA and LCMS with labeled glucose and glutamine to determine differences in metabolite uptake and utilization. We then used inhibitors of de novo synthesis and Xray radiation treatment to test the predictions made by our expression analysis.

Results Expression analysis showed IDH1WT cells to be enriched for pathways involving de novo DNA synthesis while IDH1mutant cells were enriched for pathways involving DNA repair after radiation. Using LC-MS we were able to define labeling patterns between IDH1WT and IDH1mutant cells particularly in regards to glucose utilization in nucleotide precursors. More direct tracing experiments revealed IDH1WT cells to utilize more of the de novo pathway to synthesize nucleotides and consequently to be more sensitive to inhibitors of de novo synthesis. IDH1mutant cells were found to have better growth after radiation. When examining the effect of IDH1mutant overexpression on an IDH1WT line we observed depletion of glutamine/TCA cycle intermediates and increase in ROS levels.

Key Words IDH1 mutation, 2-hydroxyglutarate, Metabolism, De Novo Synthesis, Glioma, Glioblastoma

Abbreviations IDH1 = Isocitrate Dehydrogenase 1; 2-HG = 2-Hydroxyglutarate; LCMS = Liquid Chromatography Mass Spectroscopy

Introduction

Most oncogenes and tumor suppressors directly impact cellular metabolism and conversely there are many examples of mutations in metabolic genes that become tumorigenic [75]. A point mutation in the IDH1 gene was initially identified through exome sequencing of colon tumor and glioblastoma multiforme samples [16 76], however in contrast to most metabolic mutations which involve a loss of function, this mutation was found to bestow a new enzymatic function of reducing alpha-ketoglutarate(a-KG) to 2-

hydroxyglutarate (2-HG) [19]. In the presence of the IDH1mutation, the 2-HG molecule normally found at vanishingly small levels can increase to millimolar amounts. Understandably there has been considerable interest in what role this potential new "oncometabolite" might have on potential cells. Given the structural similarity of the 2-HG molecule to a-KG it was suspected that 2-HG may be a competitive inhibitor that blocked access to a-KG dependent enzymes that regulate cell epigenetics [25] [24].

However, apart from the question of the effect on epigenetics, there has been considerable interest in what effect the IDH1mutation has on the cell itself and what the discovery of an IDH1 mutation can tell us about that glioma. These questions are important for two reasons. The first reason comes from the rationale that if the IDH1mutant enzyme changes the metabolic state of the cell, this may perhaps make the cell more or less vulnerable to certain types of therapy. For example, some studies have found that the IDH1mutation makes cells more vulnerable to radiation [30] or NAD+ depletion [42]. This issue has become even more clinically relevant with the discovery that the presence of the IDH1mutation can be diagnosed via imaging even prior to surgery [77]. The second reason comes from the observation that IDH1 mutant tumors have a better prognosis than IDH1 wild-type tumors and that there is now available a pharmacological inhibitor of the IDH1 mutant enzyme that blocks 2-HG formation[38]. If it is the case that the IDH1mutation is actually a metabolic burden to the cell then use of this inhibitor may actually aid the tumor cell and accelerate growth. Studies using this inhibitor in in vivo xenograft models have led to mixed results with some showing slowed growth [41] while others accelerated growth [42].

Attempts to focus on isolating metabolic differences between IDH1mutant and IDH1wildtype glioblastomas have historically suffered from an unproven assumption that the

metabolic differences between IDH1mutant and IDH1wildtype tumors can be largely attributed to the presence or absence of the IDH1mutation itself[55,78]. However, more recent evidence suggests that the IDH1mutation may be one of the initial mutations to occur in those gliomas [5,79] and large scale bioinformatics analyses of mutational, expression and epigenetic datasets reveals that IDH1mutant and IDH1wildtype tumors are different on a very fundamental level [80] and may have different cells of origin and different paths of tumorigenesis. Unfortunately, attempts to study cells derived from endogenous IDH1mutant tumors have been hampered by the difficulty involved in establishing and maintaining such lines.

To address this issue we have performed a metabolic analysis on a cohort of patient-derived IDH1mutant and IDH1wildtype tumor cells to determine differences between these groups that may potentially be exploitable for therapy. Initially, we used KEGG GSEA expression analysis on our collection of 59 gliomaspheres (7 IDH1mutant) as well as the TCGA dataset to determine which metabolic pathways may be differentially enriched in one group or the other. We then follow up the predictions made by this expression analysis and determine important differences between groups and propose hypotheses for patient-specific therapy. Finally, once we have identified these metabolic differences we determine how much of these differences can be attributed to the IDH1mutation itself by creating an "artificial" IDH1mutant by over-expressing the IDH1mutation in an IDH1WT cell and inhibiting the IDH1mutant enzyme in an IDH1mutant cell.

Methods

Collection of in vitro cultures.

High grade glioma samples are collected under institutional review board-approved protocols and graded by neuropathologists. On the day of resection samples are taken directly from the operating room and digested in papain. Acellular debris is removed and the remained cells are incubated in DMEM/F12 supplemented with B27, penicillin/ampicillin, heparin, EGF and bFGF for several days until spheres begin to form. Frozen stocks are made at passage 5 to maintain cells at low passage.

Gene set enrichment analysis

RNA was purified from 59 patient-derived gliomasphere cultures and hybridized to Affymetrix U133 Plus 2.0 arrays. For KEGG-based analysis, we collapsed gene expression probes based on enzyme activity (Enzyme Commission numbers[EC]) rather than on gene identity to avoid unequal representation of equivalent enzymatic function within pathways-thus emphasizing potential flux through the network. The metric used for gene ranking was the signal to noise ratio (SNR) between the IDH1mutant and IDH1WT samples. The metric was calculated for all candidate probesets of each gene or enzymatic activity and the probeset with maximum absolute metric value was retained. Probeset annotation was based on UniGene build #201 and UniGene intentifiers were mapped to each EC using the gene names provided by KEGG. Pathways with fewer than three or greater than 500 nodes represented by the data were excluded from the analysis. This resulted in 167 KEGG modules in the TCGA dataset and 186 modules in the gliomasphere data set.

LC-MS

Cells were cultured for 24 hr and rinsed with PBS, and either unlabeled media, 50% 13C-glucose labeled media or 50% 13C-glutamine labeled media was added. After 24 hour culture cells were rinsed with ice-cold 150mM NH4AcO (pH 7.3), followed by addition of 400ul cold methanol and 400ul cold water. Cells were transferred to an Eppendorf tube, and 10nmol norvaline as well as 400ul chloroform were added to each sample. For the metabolite extraction, samples were vortexed for 5 min on ice and spun down, and the aqueous layer was transferred into a glass vial and dried. Metabolites were resuspended in 70% CAN and a 5-ul sample was loaded onto a Phenomenex Luna 3u NH2 100A (150 x 2.0 mm) column. The chromatographic separation was performed on an UltiMate 3000 RSLC (Thermo Scientific) with mobile phases A(5 mM NH4AcO pH 9.9) and B(ACN) and a flow rate of 300 ul/min. The gradient ran from 15% A to 95% A over 18 min, 9 min isocratic at 95% A, and re-equilibration for 7 min. Metabolite detection was achied with a Thermo Scientific Q Exactive mass spectrometer run in polarity switching mode (+3.0 kV/-2.25kV). TraceFinder 3.1 (Thermo Scientific) was used to quatify metabolites as the area under the curve using retention time and accurate mass measurements (≤3ppm). Relative amounts of metabolites were calculated by summing up all isotopomers of a given metabolite and normalized to the internal standard and cell number.

Nucleotide Tracing

Cell lines were grown in neurosphere media supplemented with full labeled C14 glucose as well as labeled nucleotide precursors (dT, dC, dA, dG). The cells were allowed to grow for 48 hours at which point the cells were harvested and lysed. The DNA was extracted

and analyzed on LC-MS to determine the percent contribution from glucose (de novo) versus salvage pathway. Next cells were grown in neurosphere media and subjected to 1mM dT treatment and allowed to grow for 48 hours. At this point the cells were harvested and stained with PI and subjected to flow cytometry analysis to determine cell cycle distribution. Another cohort of cells were allowed to grow for 14 days before being counted to determine the effect of dT on growth.

ROS Measurement

HK 308 was infected with a lentivirus containing the IDH1mutant gene. Over-expression of the IDH1mutant protein was confirmed by western blot and 2-HG measurement. IDH1mutant and IDH1wildtype cells were allowed to grow in neurosphere media. They were then collected, stained with DCFDA and run analyzed on flow cytometry under the GFP filter. The total ROS level was the integration of the area under the curve.

Radiation

Cell lines were plated at 200,000 cells in 3ml of neurosphere media. They were then subjected to one dose of radiation and allowed to recover for several days. When the control group was ready to be passaged all samples from that cell line were passaged, counted and compared to the control (no radiation group).

Results

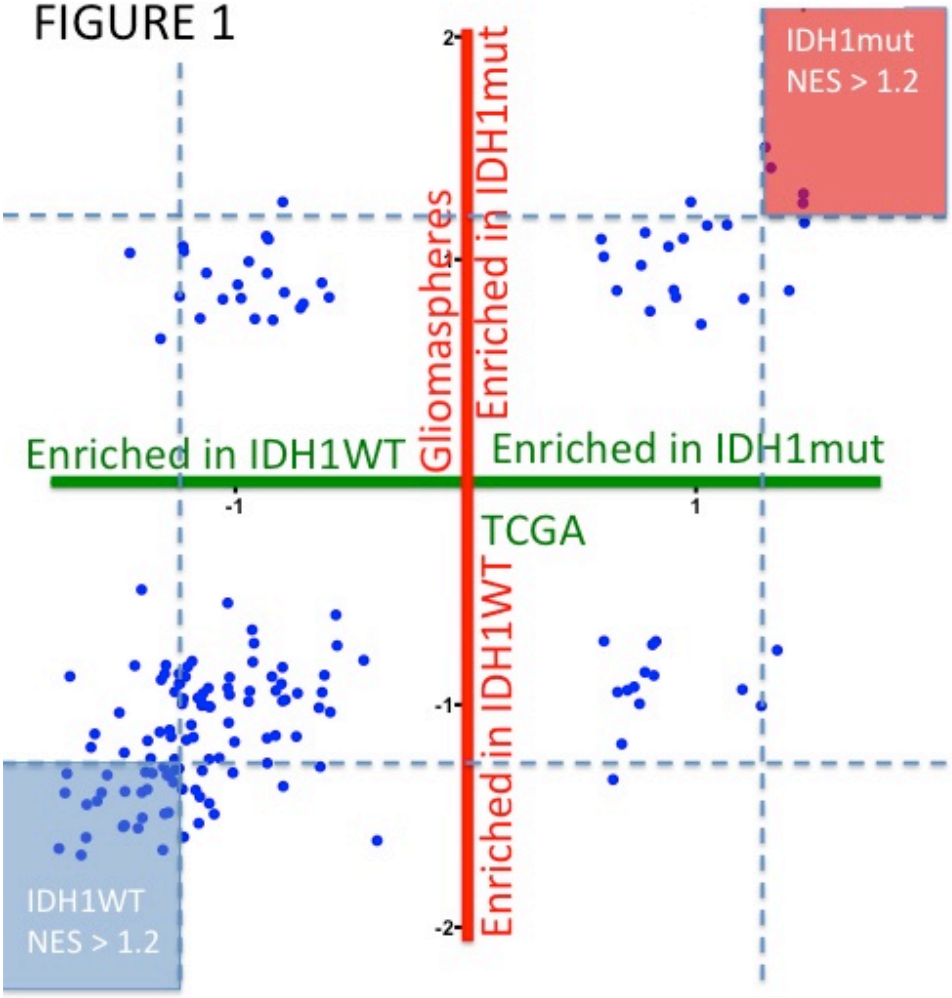
KEGG GSEA analysis

Expression data from fifty-nine gliomasphere lines (52 IDH1WT and 7 IDH1mutant) was subjected to a GSEA analysis using only KEGG gene modules. A similar comparative analysis was performed on IDH1mutant and IDH1WT samples in the TCGA dataset. Each

KEGG module was assigned a normalized enrichment score (NES) for each dataset and then plotted (Figure 1). We noted a positive correlation between the gliomasphere and TCGA dataset giving confidence that our in vitro cells were a good model for in vivo tumors.

There were fewer modules enriched in the IDH1mutant group in both the TCGA (37/167 gene set modules) as well as our gliomasphere data set (50/186 gene set modules). In order to identify some potential target metabolic pathways we used a cut-off enrichment value of 1.2. Even with this liberal cut-off we only identified four modules that were enriched in IDH1mutant cells in both data sets. Of these four modules the "Homologous Recombination" and "Nucleotide Base Excision Repair" modules were selected for further study due to the clinical relevance in terms of response to radiation. In contrast there were 35 modules that were enriched in IDH1WT cells (Figure 2). The "Pentose Phosphate Pathway" and "Amino Sugar and Nucleotide Sugar Metabolism" were selected for further study to determine if IDH1WT cells are in fact more dependent on the de novo pathway of nucleotide synthesis. The genes enriched for each pathways are shown in Figures 3-6.

FIGURE 1



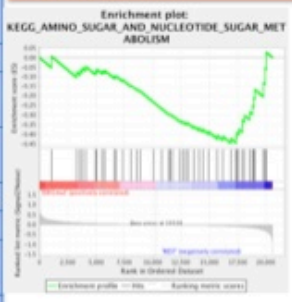
Enriched in IDH1mut
HOMOLOGOUS RECOMBINATION
DNA REPLICATION
BASE EXCISION REPAIR
CELL CYCLE

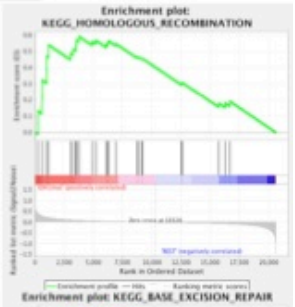
Enriched in IDH1WT
GLYCOSAMINOGLYCAN DEGRADATION
ANTIGEN PROCESSING AND PRESENTATION
LYSOSOME
STARCH AND SUCROSE METABOLISM
ARACHIDONIC ACID METABOLISM
TOLL LIKE RECEPTOR SIGNALING PATHWAY
HEMATOPOIETIC CELL LINEAGE
CYTOKINE CYTOKINE RECEPTOR INTERACTION
AMINO SUGAR AND NUCLEOTIDE SUGAR METABOLISM
PENTOSE PHOSPHATE PATHWAY

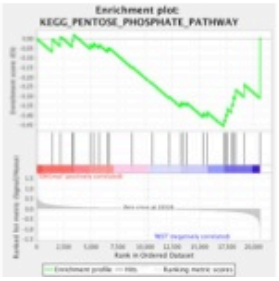
Figure 2

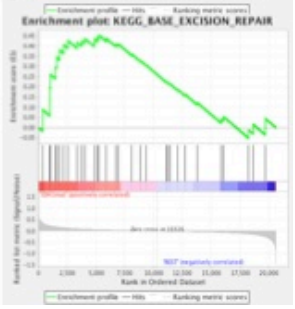
Enriched in IDH1WT	
GLYCOSAMINOGLYCAN DEGRADATION	APOPTOSIS
ANTIGEN PROCESSING AND PRESENTATION	NATURAL KILLER CELL MEDIATED CYTOTOXICITY
LYSOSOME	FOCAL ADHESION
STARCH AND SUCROSE METABOLISM	B CELL RECEPTOR SIGNALING PATHWAY
ARACHIDONIC ACID METABOLISM	NOD LIKE RECEPTOR SIGNALING PATHWAY
TOLL LIKE RECEPTOR SIGNALING PATHWAY	CELL ADHESION MOLECULES CAMS
HEMATOPOIETIC CELL LINEAGE	TYPE I DIABETES MELLITUS
CYTOKINE CYTOKINE RECEPTOR INTERACTION	HYPERTROPHIC CARDIOMYOPATHY HCM
AMINO SUGAR AND NUCLEOTIDE SUGAR METABOLISM	GLUTATHIONE METABOLISM
PENTOSE PHOSPHATE PATHWAY	ARGININE AND PROLINE METABOLISM
LEISHMANIA INFECTION	VIRAL MYOCARDITIS
REGULATION OF ACTIN CYTOSKELETON	INSULIN SIGNALING PATHWAY
COMPLEMENT AND COAGULATION CASCADES	MAPK SIGNALING PATHWAY
LEUKOCYTE TRANSENDOTHELIAL MIGRATION	BLADDER CANCER
ETHER LIPID METABOLISM	CHEMOKINE SIGNALING PATHWAY
RIG I LIKE RECEPTOR SIGNALING PATHWAY	INTESTINAL IMMUNE NETWORK FOR IGA PRODUCTION
JAK STAT SIGNALING PATHWAY	NICOTINATE AND NICOTINAMIDE METABOLISM
ECM RECEPTOR INTERACTION	

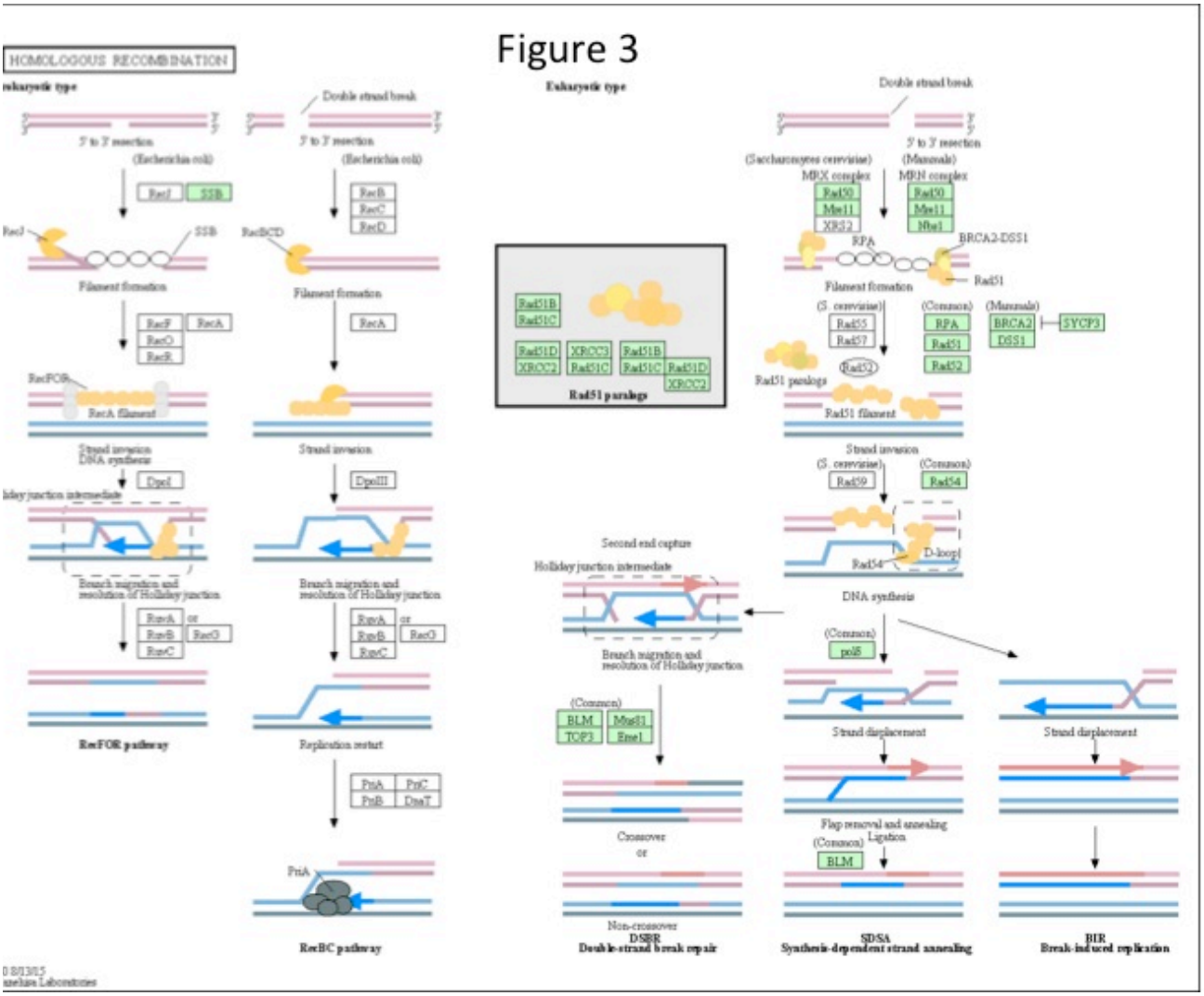
Enriched in IDH1mut
HOMOLOGOUS RECOMBINATION
DNA REPLICATION
BASE EXCISION REPAIR
CELL CYCLE










HOMOLOGOUS RECOMBINATION
Eukaryotic type
Double strand break
5' to 3' resection
(Escherichia coli)
RecJ
SSB
RecB
RecC
RecD
RecBCD
Filament formation
RecF
RecO
RecR
RecA
RecFOR
RecA filament
Strand invasion
DNA synthesis
DpoI
DpoIII
Holliday junction intermediate
Branch migration and resolution of Holliday junction
RuvA
RuvB
RuvC
or
RecG
RecO
Replication restart
PriA
PriB
PriC
DnaT
RecFOR pathway
RecBC pathway
Figure 3
Eukaryotic type
(Saccharomyces cerevisiae)
(Mammals)
MRX complex
MRN complex
Rad50
Mre11
XRS2
Nbs1
RPA
BRCA2-DSS1
Rad51
(S. cerevisiae)
(Common)
(Mammals)
Rad55
Rad57
Rad52
BRCA2
DSS1
SYCP3
Rad51 paralogs
Rad51 filament
Rad59
Rad54
D-loop
DNA synthesis
Rad51B
Rad51C
Rad51D
XRCC3
XRCC2
Rad51 paralogs
Second end capture
Holliday junction intermediate
Branch migration and resolution of Holliday junction
BLM
TOP3
Mus81
Eme1
Crossover
or
Non-crossover
DSBR
Double-strand break repair
pol δ
Strand displacement
Flap removal and annealing
Ligation
SDSA
Synthesis-dependent strand annealing
BIR
Break-induced replication

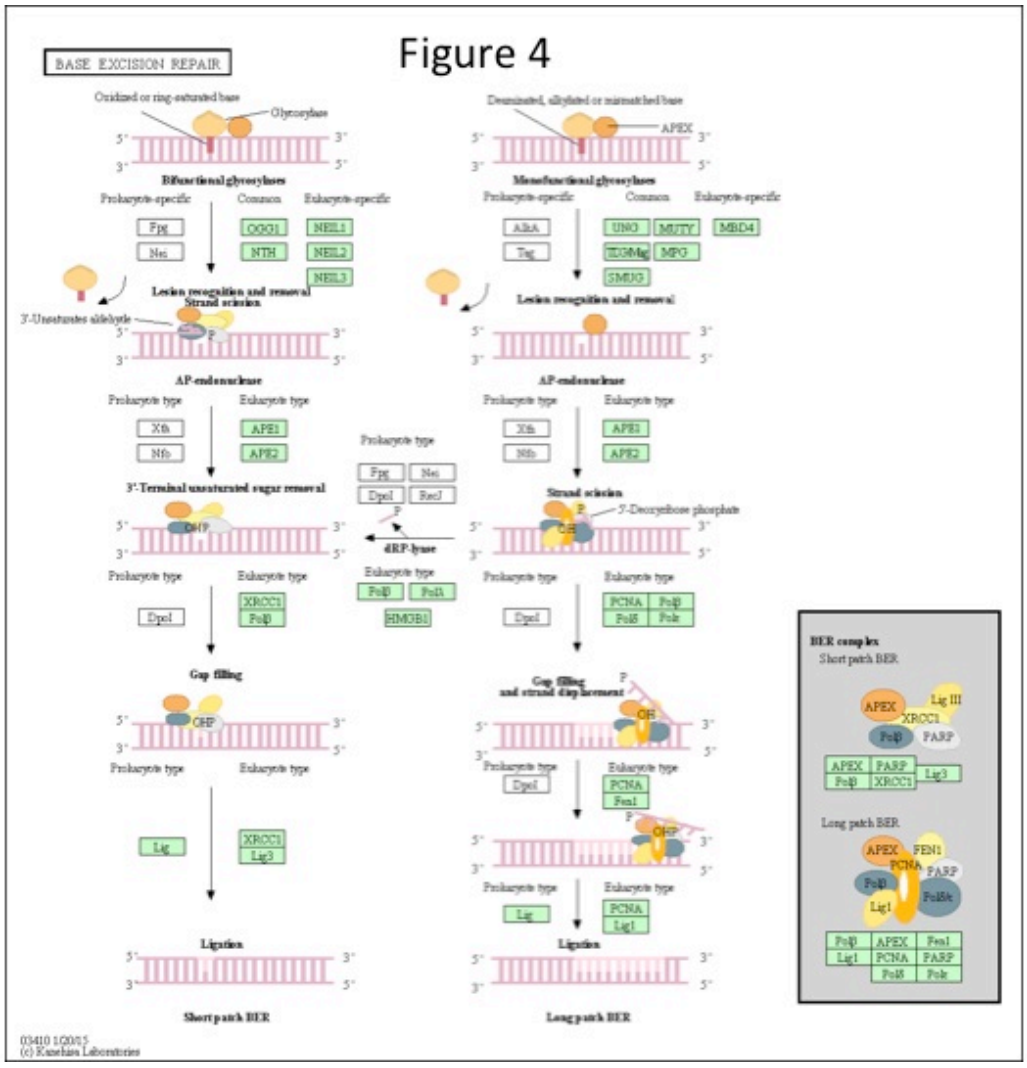


Figure 4
BASE EXCISION REPAIR
Oxidized or ring-saturated base
Glycosylase
Bifunctional glycosylases
Prokaryote-specific
Common
Eukaryote-specific
Fpg
Nei
OGG1
NTH
NEIL1
NEIL2
NEIL3
3'-Unsaturated aldehyde
Lesion recognition and removal
Strand scission
AP-endonuclease
Prokaryote type
Eukaryote type
Xth
Nfo
APE1
APE2
3'-Terminal unsaturated sugar removal
DPUP
DpoI
XRCC1
Polβ
Gap filling
OHP
Lig
Ligation
Short patch BER
Deaminated, alkylated or mismatched base
APEX
Monofunctional glycosylases
AlkA
Tag
UNG
MUTY
MBD4
hUDGMg
MPG
SMUG
PCNA
Polδ
Polε
Strand scission
5'-Deoxyribose phosphate
dRP-lyase
Polβ
Pol λ
HMGB1
Gap filling and strand displacement
Fen1
Lig1
Long patch BER
BER complex
Short patch BER
Lig III
PARP
Lig3
Long patch BER
FEN1
Polδε
(c) Kaneka Laboratories

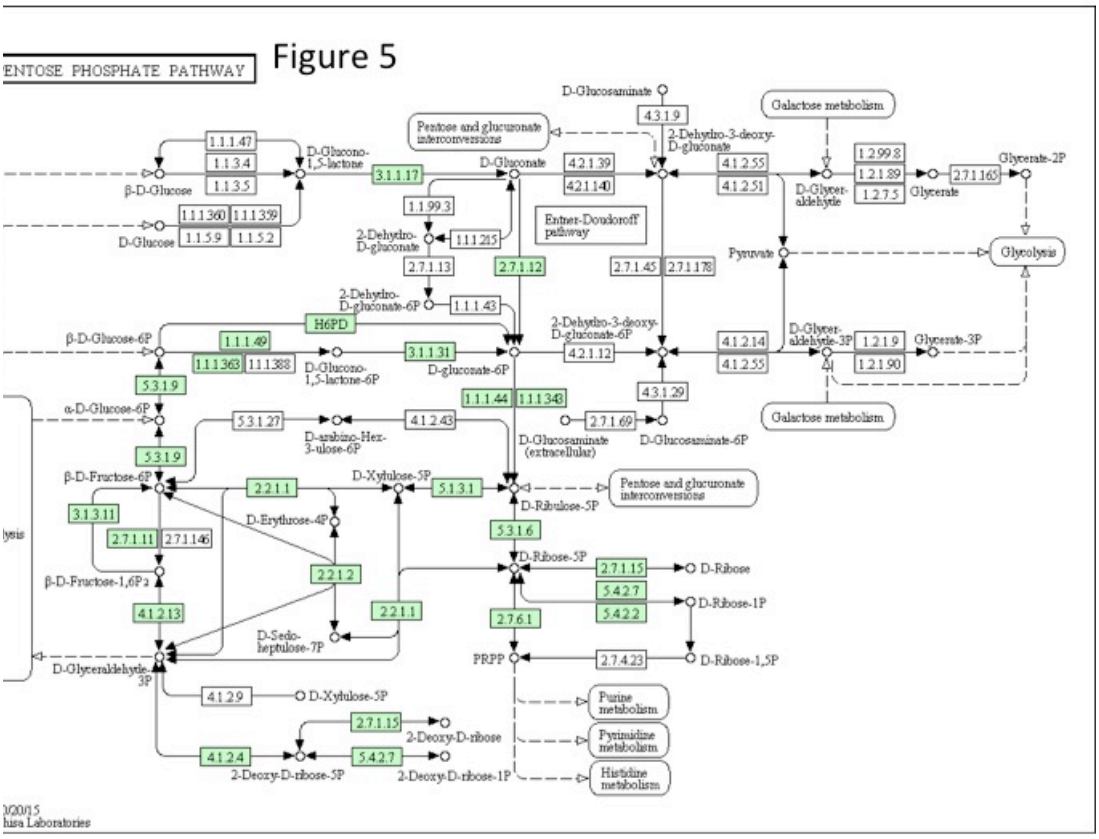
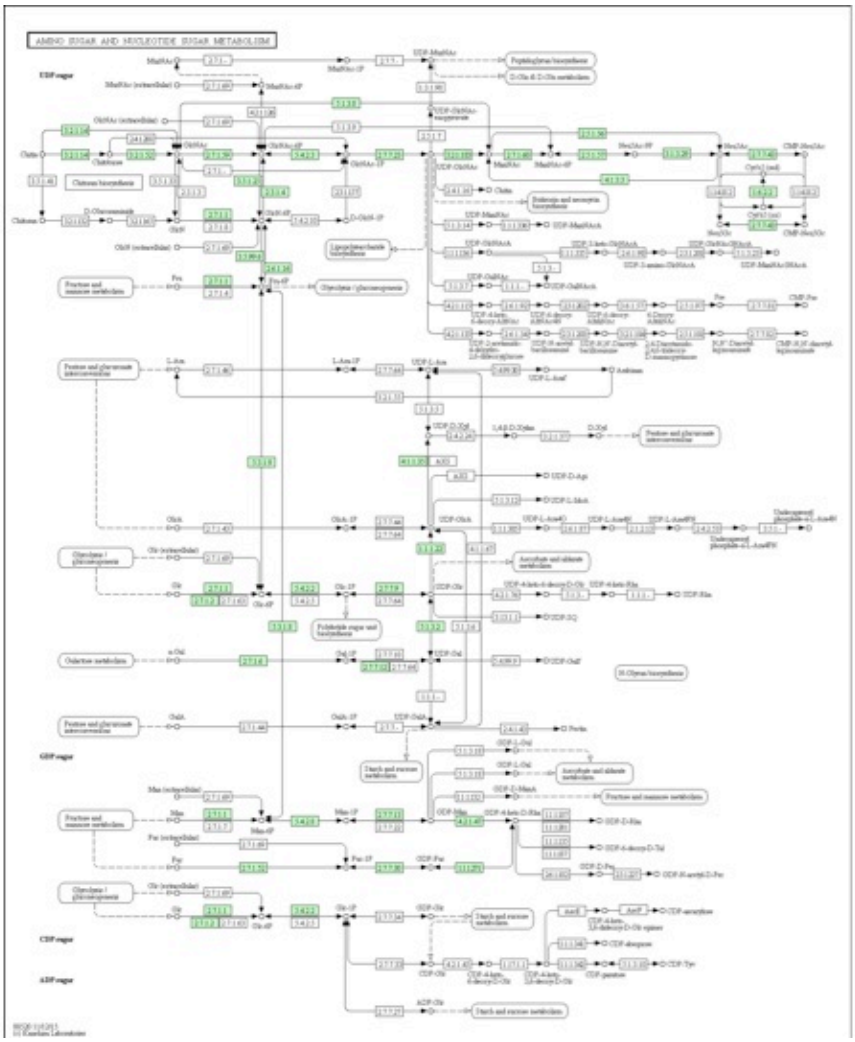

ENTOSE PHOSPHATE PATHWAY
Figure 5
D-Glucosaminate
Galactose metabolism
Pentose and glucuronate interconversions
2-Dehydro-3-deoxy-D-gluconate
Glycerate-2P
β-D-Glucose
D-Glucono-1,5-lactone
D-Gluconate
D-Glucose
2-Dehydro-D-gluconate
Entner-Doudoroff pathway
D-Glyceraldehyde
Glycerate
Pyruvate
Glycolysis
2-Dehydro-D-gluconate-6P
H6PD
β-D-Glucose-6P
2-Dehydro-3-deoxy-D-gluconate-6P
D-Glyceraldehyde-3P
Glycerate-3P
D-Glucono-1,5-lactone-6P
D-gluconate-6P
α-D-Glucose-6P
D-arabino-Hex-3-ulose-6P
D-Glucosaminate (extracellular)
D-Glucosaminate-6P
β-D-Fructose-6P
D-Xylulose-5P
Pentose and glucuronate interconversions
D-Ribulose-5P
D-Erythrose-4P
D-Ribose-5P
D-Ribose
β-D-Fructose-1,6P2
D-Ribose-1P
D-Sedo-heptulose-7P
PRPP
D-Ribose-1,5P
D-Glyceraldehyde-3P
D-Xylulose-5P
Purine metabolism
Pyrimidine metabolism
2-Deoxy-D-ribose
Histidine metabolism
2-Deoxy-D-ribose-5P
2-Deoxy-D-ribose-1P

Figure 6



Metabolic Profile

To follow-up the results from the expression analysis and to investigate any further metabolic differences between the groups, a cohort of 18 IDH1WT and 5 IDH1mut lines were subjected to a panel of metabolic measures including: glucose uptake, glutamine uptake and lactate production. Glucose uptake was significantly higher in IDH1WT cells although there was no significant difference in the lactate to glucose ratio with both cohorts being highly glycolytic. Interestingly the net glutamine uptake for all cells tested was near zero (Figure 7a-c).

To further define the different utilization of these metabolites we performed LC-MS on 3 IDH1WT (HK157, HK301, and HK308) and 3 IDH1mut (HK213, HK252, and HK322) lines with both fully labeled C13 Glucose and fully labeled C13 Glutamine. We then performed principle component analysis (PCA) to see if the collection of samples naturally partitioned into groups. Consistent with the previously seen differences in glucose uptake, IDH1mutant and IDH1WT samples partitioned into separate groups when accessing for glucose labeling (Figure 7d). Applying PCA to the samples according to glutamine labeling or total metabolite amount did not distinguish the samples into distinct groups.

With the result that glucose labeling could distinguish IDH1mutant and IDH1WT samples we performed a t-test to determine which metabolites were statistically different between the groups. Out of 159 metabolites measured we identified 28 metabolites that were significantly different. Of these 28 metabolites 9 were nucleotide precursors and all of these

nucleotide precursors showed higher glucose labeling in the IDH1WT group(Figure 7e). The complete set of differentially labeled metabolites is also shown.(Figure 8) This result is consistent with our prediction from the expression analysis that IDH1WT cells are more dependent on de novo nucleotide synthesis.

FIGURE 7

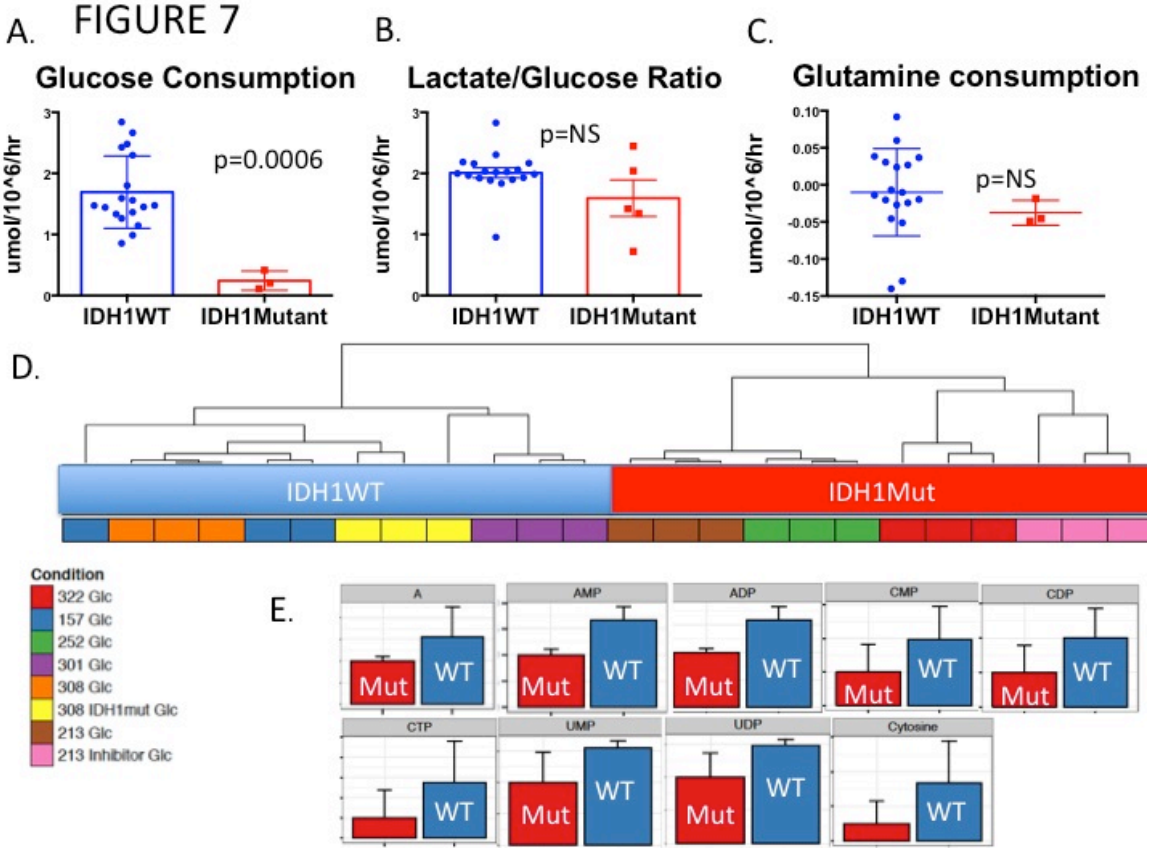
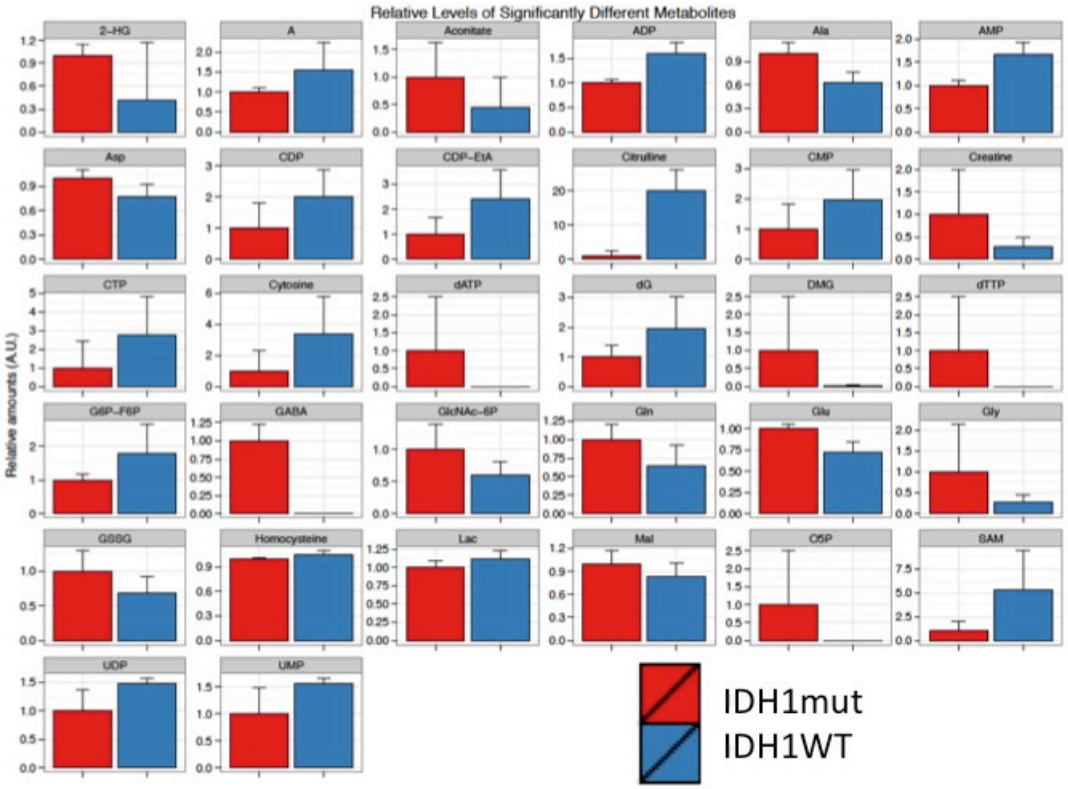

A.
Glucose Consumption
umol/10^6/hr
p=0.0006
IDH1WT
IDH1Mutant
B.
Lactate/Glucose Ratio
umol/10^6/hr
p=NS
IDH1WT
IDH1Mutant
C.
Glutamine consumption
umol/10^6/hr
p=NS
IDH1WT
IDH1Mutant
D.
IDH1WT
IDH1Mut
Condition
322 Glc
157 Glc
252 Glc
301 Glc
308 Glc
308 IDH1mut Glc
213 Glc
213 Inhibitor Glc
E.
A
AMP
ADP
CMP
CDP
CTP
UMP
UDP
Cytosine
Mut
WT

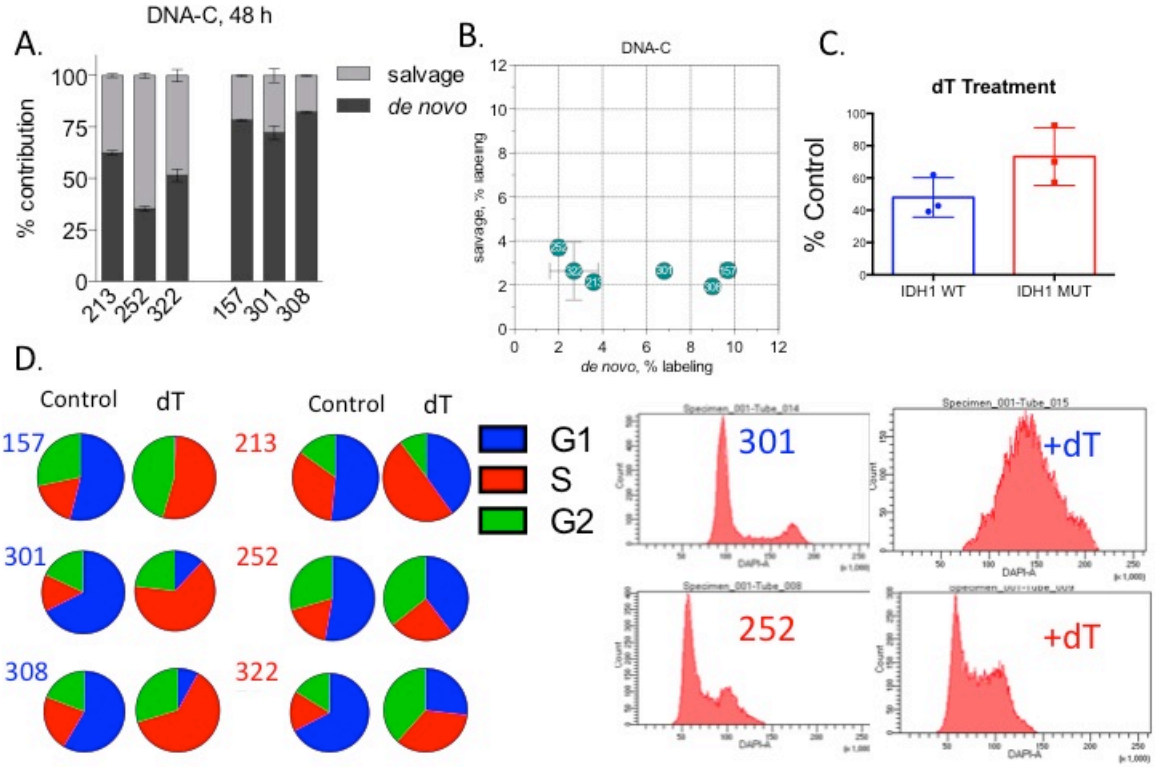
Figure 8


Relative Levels of Significantly Different Metabolites
Relative amounts (A.U.)
2-HG
A
Aconitate
ADP
Ala
AMP
Asp
CDP
CDP-EtA
Citrulline
CMP
Creatine
CTP
Cytosine
dATP
dG
DMG
dTTP
G6P-F6P
GABA
GlcNAc-6P
Gln
Glu
Gly
GSSG
Homocysteine
Lac
Mal
O5P
SAM
UDP
UMP
IDH1mut
IDH1WT

De Novo versus Salvage Nucleotide Synthesis

Using the findings from the expression and LC-MS data we looked more directly at the difference in nucleotide synthesis between IDH1WT and IDH1mut cells. Again using LC-MS we grew the same cohort of three IDH1WT lines and three IDH1mut lines in media with labeled glucose and labeled dT, dC, dA, dG, and dU for 48 hours. After the incubation period, DNA was extracted, digested to single nucleotides and then run on LC-MS to determine for each nucleotide of cytosine whether it was derived from the de novo or salvage pathway. While all samples utilized both pathways, the three IDH1WT samples used primarily de novo synthesis while the IDH1mutant samples used both pathways relatively equally (Figure 9a-b). To see if this difference could be exploited we utilized high levels of dT, a specific inhibitor of the de novo pathway to see if there would be a differential response between the two groups (Figure 9c). Predicting that this inhibitor would have an effect on the ability of cells to pass through S phase, we treated cells for four days (~1 division time) with dT and then performed a cell cycle analysis using propidium iodide. All cell lines saw an increase in the number of cells in S phase however in the IDH1mutant samples, cells were able to pass through S phase and proceed with cell division. In contrast, at the end of the four day treatment period almost all IDH1wildtype cells were found in S phase (Figure 9d). Consistent with this observation, IDH1wildtype cells also showed a more severe growth restriction in the presence of dT compared to IDH1mutant cells.

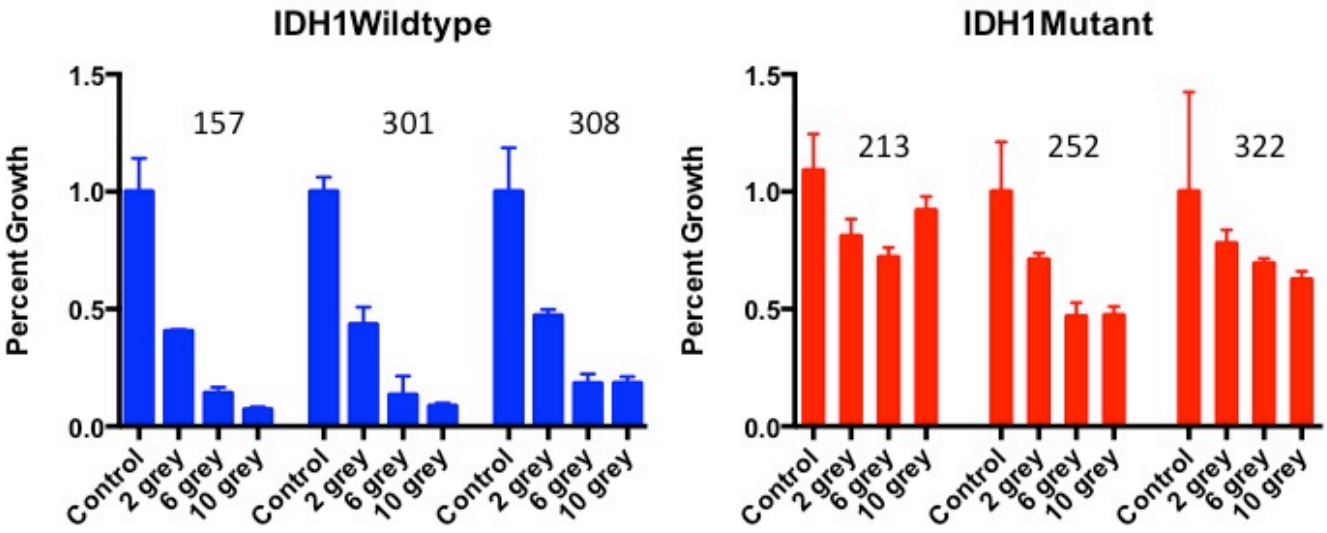
FIGURE 9


A.
DNA-C, 48 h
% contribution
salvage
de novo
213
252
322
157
301
308
B.
DNA-C
salvage, % labeling
de novo, % labeling
C.
dT Treatment
% Control
IDH1 WT
IDH1 MUT
D.
Control
dT
157
213
301
252
308
322
G1
S
G2
301
+dT
252
+dT

DNA repair in response to radiation

Having discovered that GSEA expression analysis had accurately predicted that IDH1wildtype cells did in fact utilize more de novo nucleotide synthesis we next turned to the modules that were found to be enriched in IDH1mutant cells namely the "homologous recombination module." Looking specifically at which genes were enriched, we noted that there were many genes thought to be involved in DNA repair after radiation, e.g. the Rad51 family. Thus we sought to determine if there was a difference in the ability to recover after radiation. We plated cells at equal density, exposed them to radiation and allowed them to grow. Notably, the IDH1WT cells showed a more severe deficit in growth compared to the IDH1mutant cells which were able to grow at near control levels(Figure 10).

FIGURE 10


IDH1Wildtype
IDH1Mutant
Percent Growth
1.5
1.0
0.5
0.0
157
301
308
213
252
322
Control
2 grey
6 grey
10 grey

IDH1mutant overexpression as a model for IDH1mutant cells

We next sought to determine whether these differences were due to the presence of the IDH1mutant protein and how well IDH1mutant overexpression models captured the phenotype seen in the endogenous IDH1mutant lines. We over-expressed the IDH1mutant protein in an IDH1WT background (HK308+IDH1mut) and pharmacologically inhibited the IDH1mutant protein in an endogenous IDH1mutant cell (HK213+c227) and used LC-MS to confirm 2-HG production and inhibition respectively (Figure 11a). Once we confirmed the appropriate effects on 2-HG production, we looked for differences in glucose and glutamine consumption. However, the addition or inhibition of the IDH1mutant enzyme did not seem to make a difference (Figure 11b-c). This was a surprising result given that the expression of the IDH1mutant enzyme leads to production of high levels of 2-hydroxyglutarate and presumed consumption of alpha-ketoglutarate. To investigate this further, we used LC-MS and labeled glucose and labeled glutamine tracing to determine how the cell makes 2-HG. Both endogenous IDH1mutant as our over-expression model primarily use glutamine to make 2-HG (Figure 11d). However, given that we did not observe an increase in the amount of glutamine consumption with the addition of the IDH1mutant gene we hypothesized that the cell may be depleted of glutamine. Consistent with this prediction we saw lower levels of glutamine as well as all TCA cycle intermediates when the IDH1mutant gene was overexpressed (Figure 11e). However, this did not accurately reflect the differences between endogenous IDH1mutant and IDH1wildtype cells which had roughly comparable levels of glutamine and TCA cycle intermediates, nor did we see an increased in TCA intermediates

repletion when the c227 inhibitor was used on an IDH1mutant line(Figure 12). Looking more broadly at all metabolites when these new samples were subjected to the same PCA analysis as described above we found that the HK-308 +IDH1mut clustered with the IDH1WT group and the HK213+c227 clustered with the IDH1mutant group(Figure 2D).

Finally, we looked at the effect of the IDH1mutant enzyme on ROS levels. Interestingly, studies have reported mixed results regarding the effect of the IDH1mutation on ROS with different results in different cell types[29,73]. We found that the endogenous IDH1mutant lines had significantly higher ROS levels than the IDH1WT lines and this appears to be due to the IDH1mutation itself because when we over-expressed the IDH1mutant enzyme in HK308, the ROS levels increased significantly (Figure 11f). However, pharmacological inhibition of the IDH1mutant protein had only a modest effect at lowering ROS.

FIGURE 11

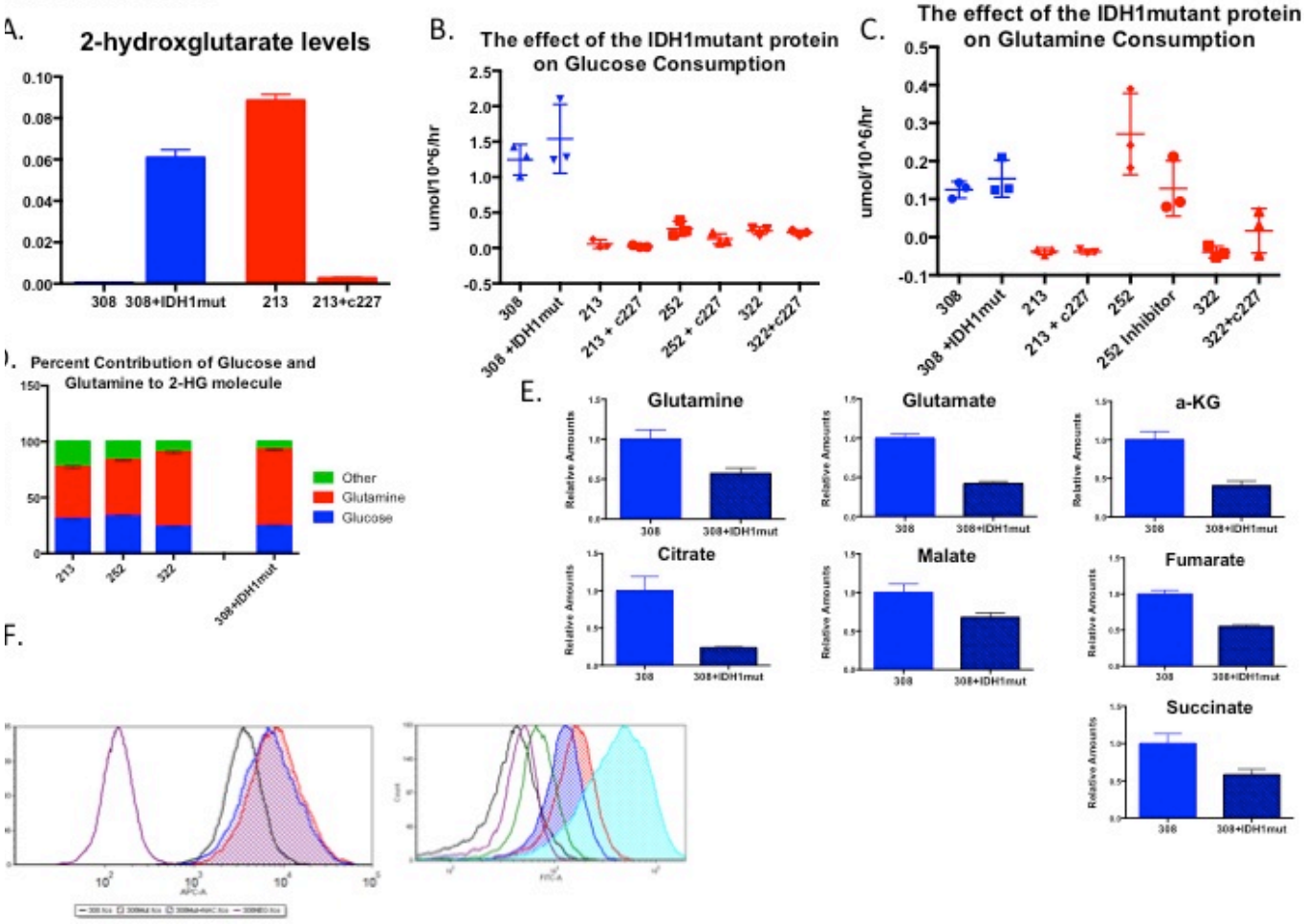
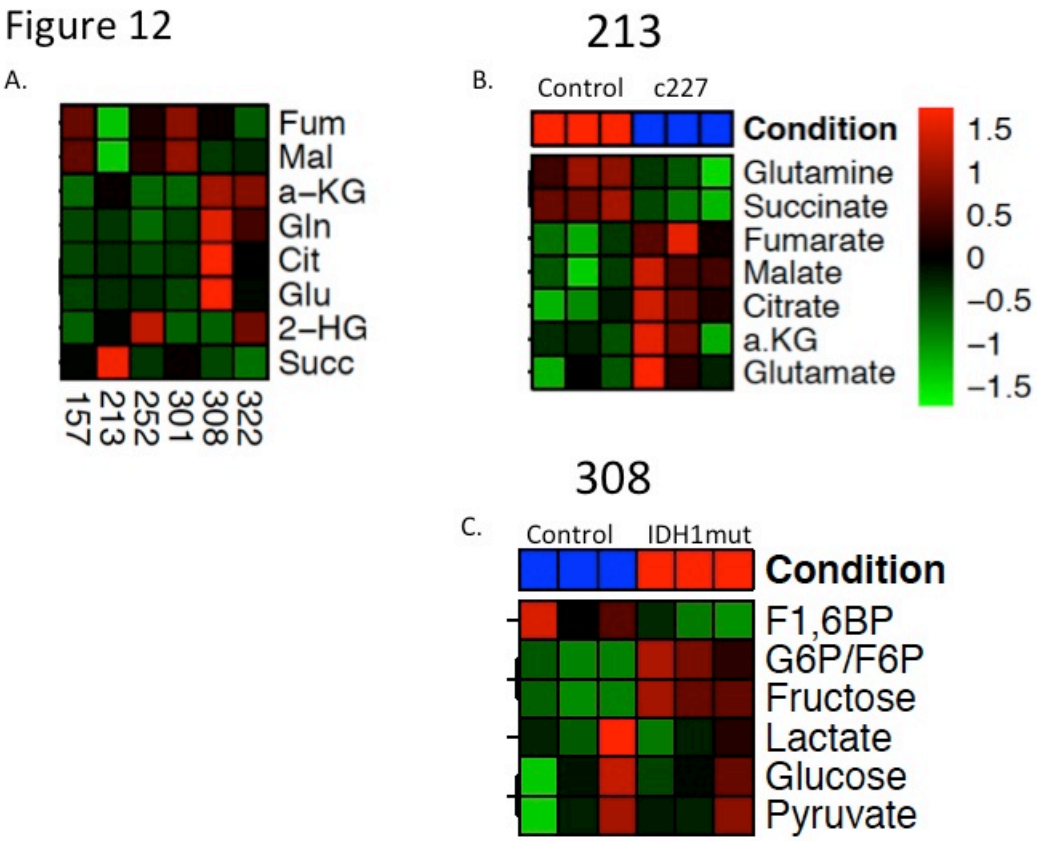

A.
2-hydroxglutarate levels
308
308+IDH1mut
213
213+c227
B.
The effect of the IDH1mutant protein on Glucose Consumption
umol/10^6/hr
308
308 +IDH1mut
213
213 + c227
252
252 + c227
322
322+c227
C.
The effect of the IDH1mutant protein on Glutamine Consumption
umol/10^6/hr
308
308 +IDH1mut
213
213 + c227
252
252 Inhibitor
322
322+c227
D.
Percent Contribution of Glucose and Glutamine to 2-HG molecule
Other
Glutamine
Glucose
213
252
322
308+IDH1mut
E.
Glutamine
Glutamate
a-KG
Citrate
Malate
Fumarate
Succinate
Relative Amounts
308
308+IDH1mut
F.

Figure 12


A.
Fum
Mal
a–KG
Gln
Cit
Glu
2–HG
Succ
157
213
252
301
308
322
213
B.
Control
c227
Condition
Glutamine
Succinate
Fumarate
Malate
Citrate
a.KG
Glutamate
1.5
1
0.5
0
–0.5
–1
–1.5
308
C.
Control
IDH1mut
Condition
F1,6BP
G6P/F6P
Fructose
Lactate
Glucose
Pyruvate

Discussion

With evidence mounting that IDH1mutant gliomas may constitute a distinct subclass of gliomas that follow an independent path of tumorigenesis[81] and mixed evidence on the efficacy of direct IDH1 inhibition on cell growth[41 42] we endeavored to characterize metabolic differences between IDH1mutant and IDH1wildtype gliomas. We used expression analysis from our collection of patient-derived gliomaspheres as well as TCGA expression data to isolate differentially expressed metabolic pathways for further testing. One difficulty in performing expression analysis is that the transcriptome of IDH1mutant cells is characterized by primarily down-regulated genes. As a result, very few modules were found to be enriched in IDH1mutant gliomas either in vivo (TCGA) or in vitro (gliomaspheres). Even when we lowered our enrichment cut-off to 1.2 only four modules were significant in both the TCGA dataset as well as our gliomasphere dataset. In contrast of the 135 modules tested 35 were significantly enriched in IDH1WT glioma cells using the same cut-off. Giving confidence to our in vitro model, there was a significant correlation between the modules enriched in our gliomaspheres and the TCGA database. Importantly, there were no modules that were enriched in opposite directions (e.g. enriched in IDH1WT in one data set and IDH1mut in the other). In addition to validating our in vitro gliomasphere system this initial screen also identified several pathways for further study. In the IDH1wildtype group we saw significant enrichment in two pathways involved in de novo nucleotide synthesis (Pentose shunt pathway, nucleotide synthesis). In the IDH1mut group we saw two pathways that indicated enhanced repair following radiation (Homologous Recombination and

Nucleotide Excision). Consistent with these predictions we observed that the main difference between IDH1mutant and IDH1WT cells in terms of metabolite uptake and utilization was that IDH1WT cells took up far more glucose/min than IDH1mut cells and tended to allocate that glucose differentially towards nucleotide precursors. We then used labeled nucleotide precursors and labeled glucose to more directly show IDH1wildtype cells preferentially use de novo synthesis over salvage. This also made them more vulnerable to a de novo pathway inhibitor (dT).

In contrast, IDH1mutant cells seem to have enriched DNA repair pathways. In addition to temozolamide, radiation is the current standard of care for all glioblastomas following diagnosis and surgical resection. The superior prognosis of IDH1mutant gliomas following resection, chemotherapy and radiation led many to believe that this subclass of tumor was preferentially sensitive to those therapies. Supporting this hypothesis, Li et al. [30] showed that the IDH1mutatation itself when introduced to a glioma cell line could lead to higher ROS levels following radiation and increased cell death. Consistent with this study we also found that IDH1mutant glioma cells do have higher levels of ROS than IDH1wildtype cells and that over-expression of the IDH1mutant enzyme in an IDH1WT glioma cell can increase the ROS levels to IDH1mut levels. However, contrary to that study we found that IDH1mutant cells are less vulnerable to radiation than IDH1WT cells. Its important to note that there could be additional reasons for this, including the slower division time may give IDH1mutant cells more time to repair double-stranded breaks.

One fallacy in the literature has been the assumption that the differences between IDH1mutant and IDH1wildtype cells depend on the presence of the IDH1mutant protein itself. However, in our analysis we found that there are profound metabolic differences

between IDH1 mutant and IDH1wildtype cells that are independent of the IDH1mutant protein. Additionally, the artificial addition of the IDH1mutant enzyme leads to experimental artifacts that are not seen in the endogenous IDH1mutant cells. More important therapeutically there has been concern that the improved prognosis of IDH1mutant tumors may be due to the presence of a functioning IDH1 mutant enzyme and that if the enzyme were pharmacologically inhibited the cell might take on the more malignant phenotype of an IDH1wildtype glioma cell. However, the findings of this study indicate that pharmacologically inhibiting the IDH1mutant enzyme did not significantly change the cell's metabolic profile. The fact that the addition of the IDH1mutant enzyme on a IDH1WT background did not change the profile suggests different cells of origin between IDH1mut and IDH1WT glioma cells. However, it should be noted that the HK308 cells were infected with an IDH1mutant lentivirus and then allowed to recover and adjust for two weeks. Its possible that if given longer this cell may eventually adopt a metabolic profile more similar to IDH1mutant glioma cells.

In conclusion, as traditional histologic diagnosis gives way to more sophisticated molecular subclasses it will become increasingly important to characterize these subclasses and define their metabolic vulnerabilities. In this study we utilized an unbiased expression analysis of a large dataset of gliomaspheres to guide our investigation into the metabolic differences between IDH1mutant and IDH1WT glioma cells. In addition to shedding light on how to treat these two different varieties of glioma, this study also serves as a model of how to use large collections of patient-derived tumor cells to determine therapy for particular tumor subclasses.

Chapter 4

Summary and Perspectives

Cancer research has focused on the central question of what makes a cancer cell different then all of the normal cells in the surrounding tissue. The answer to that question will lead to therapies that will selectively kill or arrest the growing cancer cell and leave the surrounding tissue unharmed. While cancer cells may take on many new traits in terms of cell survival, replication, metabolism, invasion and migration, it is generally assumed that the fundamental and instigating difference between a cancer cell and a normal cell is a mutation in one or more base pairs of the cell's DNA. Often the same mutations tend to appear in multiple tumors within the same tissue type or other tissue types. It is assumed that these mutations are modular and independent and are accrued in a largely random order. Each additional mutation gives that cell a slight survival or growth advantage over its neighbor and thus will eventually be selected to become increasingly more prevalent in the tumor. However, work from Yamanaka et al. in induced pluripotent stem cells showed that a cell's identity was defined or could be re-defined by only a handful of transcription factors. [10] Supporting this notion, Suva et al. [11] showed that a patient-derived glioblastoma culture could be similarly converted between malignant and non-malignant states by a collection of four transcription factors. Of note, one of these factors was Olig2. High Olig2 expression is one of the few genetic traits that define the IDH1mutant subglass of gliomas. Using this as a rationale we designed a study to show that Olig2 was essential for growth in IDH1mutant cells. This is of critical importance for while a given mutation in the DNA sequence may be

irreversible, transcription factors can be turned on or off in response to environmental stimuli. Historically, transcription factors have been difficult to target pharmacologically, however in conferring with colleagues we found a class of drugs, histone deacytelase inhibitors (HDACi) and one member of that class in particular, valproic acid, that did seem to effectively decrease Olig2 expression as well as decrease growth of IDH1mutant cells. HDACi and valproic acid came to therapeutic attention earlier after some sub-group analysis from large clinical trials showed that the patients who received valproic acid had longer survival. More direct randomized studies had mixed results. Data from this thesis may explain this observation. Valproic acid seems to have no effect on Olig2 negative tumor cells and the majority of glioblastomas are IDH1wildtype with variable levels of Olig2 expression. A focused trial on the effect of valproic acid on IDH1mutant tumors would be fairly low-risk study to perform and may offer more promising results.

Following in this theme, the third chapter of this thesis casts light on a fallacy that has likely been plaguing many large glioblastoma clinical trials. This fallacy is that there is a common cell of origin for all gliomablastomas and that as this cell acquires mutations in a relatively random order as it progresses to from a low grade tumor to a higher grade tumor. However, the results of these studies indicate that IDH1mutant gliomas are an entirely distinct class of tumors from IDH1wildtype gliomas and should not be included in the same clinical trials for two reasons. The first and most basic reason is that IDH1mutant tumors tend to have a better prognosis such that if the two cohorts have an unequal distribution of IDH1mutant tumors the results will be skewed in one direction or the other. The second reason is subtler. In performing genetic and metabolic analyses comparing IDH1mutant and IDH1wildtype glioma cells it becomes clear that IDH1wildtype glioblastoma cells have taken

on many of the malignant traits seen in a variety of cancers e.g. aerobic glycolysis, up-regulation of stem cell and dedifferentiation markers, Ras pathway, AKT pathway, DNA synthesis pathways. IDH1mutant gliomablastoma cells do not seem to possess any of those traits. As a result, any therapy directed towards exploiting cancer-specific traits while sparing normal tissue is likely destined to fail against IDH1mutant cells as they are surprisingly similar in many respects to normal tissue. However, in this obstacle there is also an opportunity. For while their similarity to normal tissue makes it difficult to target them specifically, the fact that they do not display malignant and deranged behavior means it may be possible to revert these cells to a normal non-malignant phenotype.

1 Lacroix, M. *et al.* A multivariate analysis of 416 patients with glioblastoma multiforme: prognosis, extent of resection, and survival. *Journal of neurosurgery* **95**, 190-198, doi:10.3171/jns.2001.95.2.0190 (2001).

2 Dandy, W. Removal of right cerebral hemisphere for certain tumors with hemiplegia. *JAMA* **90**, 823-825 (1928).

3 Stummer, W., van den Bent, M. J. & Westphal, M. Cytoreductive surgery of glioblastoma as the key to successful adjuvant therapies: new arguments in an old discussion. *Acta neurochirurgica* **153**, 1211-1218, doi:10.1007/s00701-011-1001-x (2011).

4 Stupp, R. *et al.* Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *The Lancet. Oncology* **10**, 459-466, doi:10.1016/S1470-2045(09)70025-7 (2009).

5 Johnson, B. E. *et al.* Mutational analysis reveals the origin and therapy-driven evolution of recurrent glioma. *Science* **343**, 189-193, doi:10.1126/science.1239947 (2014).

6 Reynolds, B. A. & Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707-1710 (1992).

7 Galli, R. *et al.* Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer research* **64**, 7011-7021, doi:10.1158/0008-5472.CAN-04-1364 (2004).

8 Singh, S. K. *et al.* Identification of human brain tumour initiating cells. *Nature* **432**, 396-401, doi:10.1038/nature03128 (2004).

9 Ogden, A. T. *et al.* Identification of A2B5+CD133- tumor-initiating cells in adult human gliomas. *Neurosurgery* **62**, 505-514; discussion 514-505, doi:10.1227/01.neu.0000316019.28421.95 (2008).

10 Yamanaka, S. & Takahashi, K. [Induction of pluripotent stem cells from mouse fibroblast cultures]. *Tanpakushitsu kakusan koso. Protein, nucleic acid, enzyme* **51**, 2346-2351 (2006).

11 Suva, M. L. *et al.* Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. *Cell* **157**, 580-594, doi:10.1016/j.cell.2014.02.030 (2014).

12 Nakano, I. *et al.* Siomycin A targets brain tumor stem cells partially through a MELK-mediated pathway. *Neuro-oncology* **13**, 622-634, doi:10.1093/neuonc/nor023 (2011).

13 Kernohan, J. W., Mabon, R. F. & et al. A simplified classification of the gliomas. *Proceedings of the staff meetings. Mayo Clinic* **24**, 71-75 (1949).

14 Verhaak, R. G. *et al.* Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer cell* **17**, 98-110, doi:10.1016/j.ccr.2009.12.020 (2010).

15 Phillips, H. S. *et al.* Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer cell* **9**, 157-173, doi:10.1016/j.ccr.2006.02.019 (2006).

16 Parsons, D. W. *et al.* An integrated genomic analysis of human glioblastoma multiforme. *Science* **321**, 1807-1812, doi:10.1126/science.1164382 (2008).

17 Patel, A. P. *et al.* Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* **344**, 1396-1401, doi:10.1126/science.1254257 (2014).

18 Laks, D. R. *et al.* Large-scale assessment of the gliomasphere model system. *Neuro-oncology*, doi:10.1093/neuonc/now045 (2016).

19 Dang, L. *et al.* Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* **462**, 739-744, doi:10.1038/nature08617 (2009).

20 Shi, J. *et al.* An IDH1 mutation inhibits growth of glioma cells via GSH depletion and ROS generation. *Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology* **35**, 839-845, doi:10.1007/s10072-013-1607-2 (2014).

21 Xu, W. *et al.* Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer cell* **19**, 17-30, doi:10.1016/j.ccr.2010.12.014 (2011).

22 Lu, C. *et al.* IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature* **483**, 474-478, doi:10.1038/nature10860 (2012).

23 Suijker, J. *et al.* Inhibition of mutant IDH1 decreases D-2-HG levels without affecting tumorigenic properties of chondrosarcoma cell lines. *Oncotarget* **6**, 12505-12519, doi:10.18632/oncotarget.3723 (2015).

24 Saha, S. K. *et al.* Mutant IDH inhibits HNF-4alpha to block hepatocyte differentiation and promote biliary cancer. *Nature* **513**, 110-114, doi:10.1038/nature13441 (2014).

25 Turcan, S. *et al.* IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature* **483**, 479-483, doi:10.1038/nature10866 (2012).

26 Tuononen, K. *et al.* The hypermethylation of the O6-methylguanine-DNA methyltransferase gene promoter in gliomas--correlation with array comparative genome hybridization results and IDH1 mutation. *Genes, chromosomes & cancer* **51**, 20-29, doi:10.1002/gcc.20927 (2012).

27 Leu, S., von Felten, S., Frank, S., Boulay, J. L. & Mariani, L. IDH mutation is associated with higher risk of malignant transformation in low-grade glioma. *Journal of neuro-oncology*, doi:10.1007/s11060-015-2048-y (2016).

28 Wick, W. *et al.* Prognostic or predictive value of MGMT promoter methylation in gliomas depends on IDH1 mutation. *Neurology* **81**, 1515-1522, doi:10.1212/WNL.0b013e3182a95680 (2013).

29 Sasaki, M. *et al.* D-2-hydroxyglutarate produced by mutant IDH1 perturbs collagen maturation and basement membrane function. *Genes & development* **26**, 2038-2049, doi:10.1101/gad.198200.112 (2012).

30 Li, S. *et al.* Overexpression of isocitrate dehydrogenase mutant proteins renders glioma cells more sensitive to radiation. *Neuro-oncology* **15**, 57-68, doi:10.1093/neuonc/nos261 (2013).

31 Patay, Z. *et al.* Successive distinct high-grade gliomas in L-2-hydroxyglutaric aciduria. *Journal of inherited metabolic disease* **38**, 273-277, doi:10.1007/s10545-014-9782-8 (2015).

32 Figueroa, M. E. *et al.* Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer cell* **18**, 553-567, doi:10.1016/j.ccr.2010.11.015 (2010).

33 Losman, J. A. *et al.* (R)-2-hydroxyglutarate is sufficient to promote leukemogenesis and its effects are reversible. *Science* **339**, 1621-1625, doi:10.1126/science.1231677 (2013).

34 Sasaki, M. *et al.* IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics. *Nature* **488**, 656-659, doi:10.1038/nature11323 (2012).

35 Dawlaty, M. M. *et al.* Combined deficiency of Tet1 and Tet2 causes epigenetic abnormalities but is compatible with postnatal development. *Developmental cell* **24**, 310-323, doi:10.1016/j.devcel.2012.12.015 (2013).

36 Borodovsky, A. *et al.* 5-azacytidine reduces methylation, promotes differentiation and induces tumor regression in a patient-derived IDH1 mutant glioma xenograft. *Oncotarget* **4**, 1737-1747, doi:10.18632/oncotarget.1408 (2013).

37 Turcan, S. *et al.* Efficient induction of differentiation and growth inhibition in IDH1 mutant glioma cells by the DNMT Inhibitor Decitabine. *Oncotarget* **4**, 1729-1736, doi:10.18632/oncotarget.1412 (2013).

38 Deng, G. *et al.* Selective inhibition of mutant isocitrate dehydrogenase 1 (IDH1) via disruption of a metal binding network by an allosteric small molecule. *The Journal of biological chemistry* **290**, 762-774, doi:10.1074/jbc.M114.608497 (2015).

39 Wang, F. *et al.* Targeted inhibition of mutant IDH2 in leukemia cells induces cellular differentiation. *Science* **340**, 622-626, doi:10.1126/science.1234769 (2013).

40 IDH1 inhibitor shows promising early results. *Cancer discovery* **5**, 4, doi:10.1158/2159-8290.CD-NB2014-178 (2015).

41 Rohle, D. *et al.* An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells. *Science* **340**, 626-630, doi:10.1126/science.1236062 (2013).

42 Tateishi, K. *et al.* Extreme Vulnerability of IDH1 Mutant Cancers to NAD+ Depletion. *Cancer cell* **28**, 773-784, doi:10.1016/j.ccell.2015.11.006 (2015).

43 Ledent, V., Paquet, O. & Vervoort, M. Phylogenetic analysis of the human basic helix-loop-helix proteins. *Genome biology* **3**, RESEARCH0030 (2002).

44 Lu, Q. R. *et al.* Sonic hedgehog--regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. *Neuron* **25**, 317-329 (2000).

45 Lu, Q. R. *et al.* Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. *Cell* **109**, 75-86 (2002).

46 Buffo, A. *et al.* Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 18183-18188, doi:10.1073/pnas.0506535102 (2005).

47 Kageyama, R. & Nakanishi, S. Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. *Current opinion in genetics & development* **7**, 659-665 (1997).

48 Lee, J. E. Basic helix-loop-helix genes in neural development. *Current opinion in neurobiology* **7**, 13-20 (1997).

49 Sun, Y. *et al.* Phosphorylation state of Olig2 regulates proliferation of neural progenitors. *Neuron* **69**, 906-917, doi:10.1016/j.neuron.2011.02.005 (2011).

50 Ligon, K. L. *et al.* The oligodendroglial lineage marker OLIG2 is universally expressed in diffuse gliomas. *Journal of neuropathology and experimental neurology* **63**, 499-509 (2004).

51 Bouvier, C. *et al.* Shared oligodendrocyte lineage gene expression in gliomas and oligodendrocyte progenitor cells. *Journal of neurosurgery* **99**, 344-350, doi:10.3171/jns.2003.99.2.0344 (2003).

52 Bhat, K. P. *et al.* Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. *Cancer cell* **24**, 331-346, doi:10.1016/j.ccr.2013.08.001 (2013).

53 Ligon, K. L. *et al.* Olig2-regulated lineage-restricted pathway controls replication competence in neural stem cells and malignant glioma. *Neuron* **53**, 503-517, doi:10.1016/j.neuron.2007.01.009 (2007).

54 Seltzer, M. J. *et al.* Inhibition of glutaminase preferentially slows growth of glioma cells with mutant IDH1. *Cancer research* **70**, 8981-8987, doi:10.1158/0008-5472.CAN-10-1666 (2010).

55 Grassian, A. R. *et al.* IDH1 mutations alter citric acid cycle metabolism and increase dependence on oxidative mitochondrial metabolism. *Cancer research* **74**, 3317-3331, doi:10.1158/0008-5472.CAN-14-0772-T (2014).

56 Reitman, Z. J. *et al.* Cancer-associated isocitrate dehydrogenase 1 (IDH1) R132H mutation and d-2-hydroxyglutarate stimulate glutamine metabolism under hypoxia. *The Journal of biological chemistry* **289**, 23318-23328, doi:10.1074/jbc.M114.575183 (2014).

57 Goyal, L. *et al.* Prognosis and Clinicopathologic Features of Patients With Advanced Stage Isocitrate Dehydrogenase (IDH) Mutant and IDH Wild-Type Intrahepatic Cholangiocarcinoma. *The oncologist* **20**, 1019-1027, doi:10.1634/theoncologist.2015-0210 (2015).

58 Flavahan, W. A. *et al.* Insulator dysfunction and oncogene activation in IDH mutant gliomas. *Nature* **529**, 110-114, doi:10.1038/nature16490 (2016).

59 Li, S. *et al.* Tumor-suppressive miR148a is silenced by CpG island hypermethylation in IDH1-mutant gliomas. *Clinical cancer research : an official journal of the American Association for Cancer Research* **20**, 5808-5822, doi:10.1158/1078-0432.CCR-14-0234 (2014).

60 Day, A., Carlson, M. R., Dong, J., O'Connor, B. D. & Nelson, S. F. Celsius: a community resource for Affymetrix microarray data. *Genome biology* **8**, R112, doi:10.1186/gb-2007-8-6-r112 (2007).

61 Li, C. & Wong, W. H. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 31-36, doi:10.1073/pnas.011404098 (2001).

62 Chou, A. P. *et al.* Identification of retinol binding protein 1 promoter hypermethylation in isocitrate dehydrogenase 1 and 2 mutant gliomas. *Journal of the National Cancer Institute* **104**, 1458-1469, doi:10.1093/jnci/djs357 (2012).

63 Gardiner-Garden, M. & Frommer, M. CpG islands in vertebrate genomes. *Journal of molecular biology* **196**, 261-282 (1987).

64 Balss, J. *et al.* Enzymatic assay for quantitative analysis of (D)-2-hydroxyglutarate. *Acta neuropathologica* **124**, 883-891, doi:10.1007/s00401-012-1060-y (2012).

65 Jin, G. *et al.* Disruption of wild-type IDH1 suppresses D-2-hydroxyglutarate production in IDH1-mutated gliomas. *Cancer research* **73**, 496-501, doi:10.1158/0008-5472.CAN-12-2852 (2013).

66 Cheng, H. B. *et al.* IDH1 mutation is associated with improved overall survival in patients with glioblastoma: a meta-analysis. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* **34**, 3555-3559, doi:10.1007/s13277-013-0934-5 (2013).

67 Noushmehr, H. *et al.* Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer cell* **17**, 510-522, doi:10.1016/j.ccr.2010.03.017 (2010).

68 Wu, Y. *et al.* Regulation of glioblastoma multiforme stem-like cells by inhibitor of DNA binding proteins and oligodendroglial lineage-associated transcription factors. *Cancer science* **103**, 1028-1037, doi:10.1111/j.1349-7006.2012.02260.x (2012).

69 Siebzehnrubl, F. A. *et al.* Histone deacetylase inhibitors increase neuronal differentiation in adult forebrain precursor cells. *Experimental brain research* **176**, 672-678, doi:10.1007/s00221-006-0831-x (2007).

70 Mehta, S. *et al.* The central nervous system-restricted transcription factor Olig2 opposes p53 responses to genotoxic damage in neural progenitors and malignant glioma. *Cancer cell* **19**, 359-371, doi:10.1016/j.ccr.2011.01.035 (2011).

71 Kerkhof, M. *et al.* Effect of valproic acid on seizure control and on survival in patients with glioblastoma multiforme. *Neuro-oncology* **15**, 961-967, doi:10.1093/neuonc/not057 (2013).

72 Weller, M. *et al.* Prolonged survival with valproic acid use in the EORTC/NCIC temozolomide trial for glioblastoma. *Neurology* **77**, 1156-1164, doi:10.1212/WNL.0b013e31822f02e1 (2011).

73 Barker, C. A., Bishop, A. J., Chang, M., Beal, K. & Chan, T. A. Valproic acid use during radiation therapy for glioblastoma associated with improved survival. *International journal of radiation oncology, biology, physics* **86**, 504-509, doi:10.1016/j.ijrobp.2013.02.012 (2013).

74 Berendsen, S. *et al.* Prognostic relevance of epilepsy at presentation in glioblastoma patients. *Neuro-oncology*, doi:10.1093/neuonc/nov238 (2015).

75 King, A., Selak, M. A. & Gottlieb, E. Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. *Oncogene* **25**, 4675-4682, doi:10.1038/sj.onc.1209594 (2006).

76 Sjoblom, T. *et al.* The consensus coding sequences of human breast and colorectal cancers. *Science* **314**, 268-274, doi:10.1126/science.1133427 (2006).

77 Pope, W. B. *et al.* Non-invasive detection of 2-hydroxyglutarate and other metabolites in IDH1 mutant glioma patients using magnetic resonance spectroscopy. *Journal of neuro-oncology* **107**, 197-205, doi:10.1007/s11060-011-0737-8 (2012).

78 Izquierdo-Garcia, J. L. *et al.* Glioma cells with the IDH1 mutation modulate metabolic fractional flux through pyruvate carboxylase. *PloS one* **9**, e108289, doi:10.1371/journal.pone.0108289 (2014).

79 Watanabe, T., Nobusawa, S., Kleihues, P. & Ohgaki, H. IDH1 mutations are early events in the development of astrocytomas and oligodendrogliomas. *The American journal of pathology* **174**, 1149-1153, doi:10.2353/ajpath.2009.080958 (2009).

80 Eckel-Passow, J. E. *et al.* Glioma Groups Based on 1p/19q, IDH, and TERT Promoter Mutations in Tumors. *The New England journal of medicine* **372**, 2499-2508, doi:10.1056/NEJMoa1407279 (2015).

81 Cancer Genome Atlas Research, N. *et al.* Comprehensive, Integrative Genomic Analysis of Diffuse Lower-Grade Gliomas. *The New England journal of medicine* **372**, 2481-2498, doi:10.1056/NEJMoa1402121 (2015).