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PhD Thesis

Mikkel Staberg

Therapeutic Potential of Combination Therapy in the Treatment of Glioblastoma

Supervisors
Hans Skovgaard Poulsen, MD, DMSc
Petra Hamerlik, PhD

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Mikkel Staberg, MSc.

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Thesis title

Therapeutic potential of combination therapy in the treatment of glioblastoma

Author

Mikkel Staberg, MSc

Department

Department of Radiation Biology, Department of Oncology, Rigshospitalet, Copenhagen, Denmark

Principal supervisor

Chief physician Hans Skovgaard Poulsen, MD, DMSc, Department of Radiation Biology, Department of Oncology, Rigshospitalet, Copenhagen, Denmark

Co-supervisor

Petra Hamerlik, PhD, Brain Tumor Biology, Danish Cancer Society Research Center, Copenhagen, Denmark

Assessment committee

Professor Julia Sidenius Johansen (Chairman), Institute for Clinical Medicine, Herlev Hospital, University of Copenhagen, Denmark

Professor Anne Kiltie, Department of Oncology, University of Oxford, England

Professor Michael Robert Horsman, Institute for Clinical Medicine, Department for Experimental Clinical Oncology, University of Aarhus, Denmark

PREFACE

The present PhD thesis "Therapeutic potential of combination therapy in the treatment of glioblastoma " is submitted in order to achieve the PhD degree at the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, December 3rd, 2016.

The work presented in the thesis was carried out at the Department of Radiation Biology, Department of Oncology, Rigshospitalet, Copenhagen, Denmark.

The result section of this thesis consists of one paper published in *Cancer Cell International* (Study I), one paper published in *Cellular Oncology* (Study II), and one manuscript prepared for submission (Study III).

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LIST OF SELECTED ABBREVIATIONS

BBB	blood brain barrier
BER	base excision repair
bHLH	basic helix-loop-helix
CCNU	lomustine
CNS	central nervous system
CSC	cancer stem cell
DDR	DNA-damage response
DNA-PK	DNA-dependent protein phosphokinase
DSB	DNA double-strand break
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EZH2	enhancer of zeste homolog 2
FDA	U.S. food and drug administration
FGF	fibroblast growth factor
GBM	glioblastoma
GSC	glioma stem cell
GSI	gamma secretase inhibitor
HAT	histone acetyltransferase
HDAC	histone deacetylase
HES	hairy/enhancer of split
HIF-1	hypoxia-inducible factor 1
HMT	histone methyltransferase
HR	homologous recombination
IDH	isocitrate dehydrogenase
JmjC	jumonjii C family
KDM	histone demethylase
NICD	notch intracellular domain
NF1	neurofibromin 1
NHEJ	non-homologous end joining

NSC	neural stem cell
MGMT	O^6-methylguanine-DNA methyltransferase
MRN	MRE11-Rad50-NBS1 complex
OS	median overall survival
PDGFRA	platelet-derived growth factor alpha
PFS	median progression-free-survival
PRC	polycomp-repressive complex
PTEN	phosphatase and tensin homolog
PTM	post-translational modification
RTK	receptor tyrosine kinase
siRNA	small interfering RNA
TGF-α	transforming growth factor alpha
TMZ	temozolomide
TSA	trichostatin A
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VPA	valproic acid
VP-16	etoposide
WHO	World Health Organization

The following three studies form the basis of the thesis:

I. *Staberg M, Michaelsen SR, Olsen LS, Nedergaard MK, Villingshøj M, Stockhausen MT, Hamerlik P, and Poulsen HS. Combined EGFR- and notch inhibition display additive inhibitory effect on glioblastoma cell viability and glioblastoma-induced endothelial cell sprouting in vitro. Cancer Cell Int. 2016 Apr 26; 16:34*

II. *Staberg M, Michaelsen SR, Rasmussen RD, Villingshøj M, Poulsen HS, and Hamerlik P. Inhibition of histone deacetylases sensitizes glioblastoma cells to lomustine. Cellular Oncology, 2016, Oct 20.*

III. *Staberg M, Michaelsen SR, Villingshøj M, Poulsen HS, and Hamerlik P. Lysine-specific histone demethylase KDM2B regulates chemoresistance and maintenance of glioblastoma cells. In preparation for submission.*

SUMMARY

Glioblastoma is the most malignant brain tumor in adults. Median survival is only about 15 months despite aggressive treatment, consisting of surgery followed by radio- and chemotherapy, stressing the need for new therapies. Development of glioblastoma is thought to be a result of both genetic and epigenetic alterations, ultimately leading to oncogenic transformation of normal glia cells. Several features are suggested to give rise to the poor prognosis of glioblastoma including treatment resistance, a high degree of abnormal blood vessels, and high heterogeneity, both within the single tumor and from patient to patient. Thus, investigations are needed to identify the genetic-molecular alterations that glioblastoma tumors depend on in order to overcome treatment and regrow after initial surgery.

The findings presented in this thesis illustrate the promising potential of combinational treatments in the management of glioblastoma. The work shows that glioblastoma display aberrant activation of epigenetic modulating enzymes, such as histone deacetylases and histone demethylases, maintaining glioblastoma cell viability. Upon inhibition by treatment with epigenetic inhibitors, this results in induced apoptosis of glioblastoma cells, an effect that is even more pronounced when combined with traditional chemotherapeutic agents.

The EGFR and Notch pathways are shown to be of great importance for glioblastoma cell survival and for the formation of new blood vessels, a process known as angiogenesis. Results presented herein, demonstrate a potential combinational treatment strategy by simultaneous targeting of the EGFR and Notch signaling pathways. Combined inhibition of Notch and EGFR was shown to result in additive inhibition of tumor cell viability and tumor-induced endothelial angiogenesis.

Overall, the presented data suggests that targeting redundant signaling pathways can overcome required or initial treatment resistance, thus leading to improved tumor cell elimination. We hypothesize that future therapies will likely be a result of combination therapies for glioblastoma patients based on their molecular tumor profile, resulting in enhanced therapeutic benefit.

DANSK RESUMÉ

Glioblastom er den mest ondartede hjernekræftsygdom hos voksne. Selv når der anvendes en særlig aggressiv behandling, bestående af operation efterfulgt af stråle- og kemoterapi, er gennemsnitoverlevelsen kun ca. 15 måneder, hvilket understreger nødvendigheden af nye behandlingsmuligheder. Glioblastom menes at opstå som følge af både genetiske og epigenetiske ændringer førende til transformering af normale gliaceller. Forskellige karakteristika er ansvarlige for den dårlige prognose af glioblastom patienter, såsom behandlingsresistens, en øget mængde af abnorme blodkar, samt en meget heterogen tumor, både i selve tumoren og fra patient til patient. Derfor er det nødvendigt, at identificere de molekylære ændringer som tumorceller benytter sig af for at kunne modstå behandlingen, samt gendanne tumoren efter den oprindelige operation.

Resultater præsenteret i denne tese illustrerer det lovende potentiale ved at benytte kombinationsbehandlinger som et led i kampen mod glioblastom. Resultaterne demonstrerer, at glioblastom udviser dereguleret aktivering af epigenetiske enzymer, såsom histon-deacetylaser og histon-demetylaser, der er involveret i opretholdelsen og overlevelsen af glioblastom tumorceller. Vi finder, at når man hæmmer disse enzymer med epigenetiske hæmmere, fører det til induceret celledød, og denne effekt kan yderligere forøges ved kombination med traditionel kemoterapi.

Både EGFR og Notch signalvejene har vist sig at være vigtige for overlevelsen af glioblastom tumorceller, samt dannelsen af nye blodkar, en proces kendt som angiogenese. Resultater heri, demonstrerer en mulig ny kombinationsbehandling, hvor EGFR og Notch signalvejene hæmmes simultant. Vi viser, at når EGFR og Notch hæmmes på samme tid, resulterer det i øget tumorcelledød samt hæmning af endothelcelle-medieret angiogenese, induceret af tumorcellerne.

Disse resultater indikerer, at behandlinger der rammer fælles signalveje, kan anvendes til at overkomme behandlingsresistens, samt fører til øget eliminering af hele tumoren. Vi mener derfor, at fremtidige behandlinger af glioblastom patienter vil være et resultat af kombinationsbehandlinger baseret på den molekylære tumorprofil, hvilket i sidste ende vil øge den terapeutiske effekt.

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1. INTRODUCTION

1.1. CANCER AND HALLMARKS OF CANCER

Cancer is one of the most common diseases worldwide with around 8.2 million cancer deaths in 2012 [1]. The disease is a consequence of abnormal cell divisions and can lead to formation of solid tumors and spreading to surrounding tissue. Cancer arises through accumulation of mutations and/or altered transcription in genes involved in regulation of cell growth, which can result from both inherited genetic alterations and environmental factors.

Malignant tumors can arise from different types of tissue, but they follow a common set of characteristics in their physiological behavior known as the hallmarks of cancer. Originally, in 2000, Hanahan and Weinberg proposed six hallmarks necessary for tumor formation and growth and defined as: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (Figure 1) [2]. Recently, these original hallmarks were updated with deregulating cellular energetics and avoiding immune destruction as emerging hallmarks [3]. Further, they described two tumor enabling characteristics for acquisition of the hallmarks. Genomic instability generating increased mutation frequency and tumor-promoting inflammation driven by some of the cells in the immune system (Figure 1). Taken together, this illustrates the complex biology and heterogeneity involved in tumor formation and recognition of these concepts will eventually identify new ways to improve the treatment of human cancer.

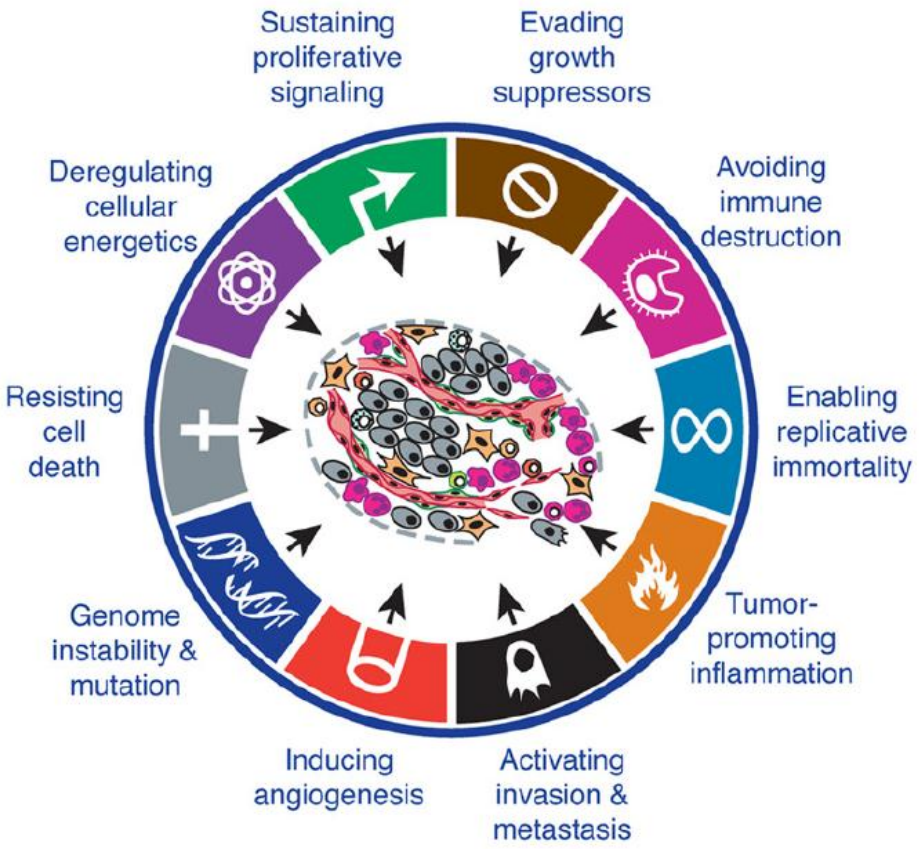

Figure 1: Several processes have been attributed the onset of cancer including the 10 hallmarks suggested by Hanahan and Weinberg. Adapted from [3].

1.2. GLIOMAS

1.2.1. INCIDENCE

Glioma comprises all tumors believed to be of glial cell origin and accounts for almost 80% of primary malignant brain tumors. Of all the malignant gliomas, glioblastoma (GBM) is the most common and most aggressive brain tumor disease in adults and the annual incidence of new GBM cases is around 3-4 cases per 100,000 individuals in western countries [4]. For the remaining glioma subgroups, the annual incidence ranges from 0-1 cases per 100,000

individuals and the frequency of new brain tumor cases revealed a minor but significant increase (0.9%) in the years from 1985 to 1994 [5].

1.2.2. CLASSIFICATION, GRADING AND SUBTYPES

Gliomas are classified as astrocytomas or oligodendrogliomas according to the World Health Organization (WHO) classification and characterized by their resemblance to astrocyte or oligodendrocyte cells [6,7]. In addition, gliomas are separated into grades (I-IV) by the WHO grading scale dependent on their histological and genetic profile (Figure 2) [8]. Histological gradings are defined on the basis of main features including nuclear atypia, (proliferative) mitosis activity, microvascular proliferation as well as necrosis [9]. The grade I and II are determined as low-grade, whereas grade III and IV characterizes the high-grade gliomas [7].

GBM belongs to the grade IV malignant gliomas and is identified by histologic features including increased mitotic activity, necrosis and vascular proliferation [7,9]. GBM can occur as either primary or secondary GBMs where primary GBM is the most common type (>90%) and emerge as a *de novo* lesion without any prior diagnosed lower grade tumor. The secondary GBM type occurs from progressed lower grade tumors (grade II/III) and is often identified as a recurrence in younger patients [8]. Oligodendroglial tumors commonly display 1p/19q chromosome co-deletion and mutations of IDH1 or IDH2 *(*isocitrate dehydrogenase 1 and 2). Conversely, TP53 mutation or chromosome 17p13 loss is commonly seen in astrocytic tumors. Finally, most primary GBMs display PTEN mutations and amplification of EGFR (Figure 2) [10].

In the recent years, a lot of effort has been made in defining GBMs in subtypes based on their genetic and molecular profiles, with the aim to correlate these to prognosis and response to treatment. Based on high-throughput microarray and DNA-sequencing data, distinctive molecular differences were found in the

primary and secondary GBM groups, which led to characterization of three major subtypes; proneural, mesenchymal and classical [11-14]. The proneural subtype is characterized by alterations in PDGFRA, IDH and TP53, belonging to the secondary GBMs, and is present in younger patients and associated with better outcome. The mesenchymal group is common in older patients, characterized by lost/mutated NF1 and associated with worse prognosis. The majority of GBMs belong to the classical subtype displaying Notch pathway activation, PTEN loss and EGFR amplification [11-13]. In addition, a paper by Sturm et al. described the identification of six epigenetic GBM subgroups based on their global DNA methylation pattern associated with specific molecular alterations and defined clinical parameters [15]. Overall, the subtyping of GBMs by defining their molecular profiles may help in understanding the pathology of GBM, and additionally lay the foundation for personalized treatment in a clinical setting.

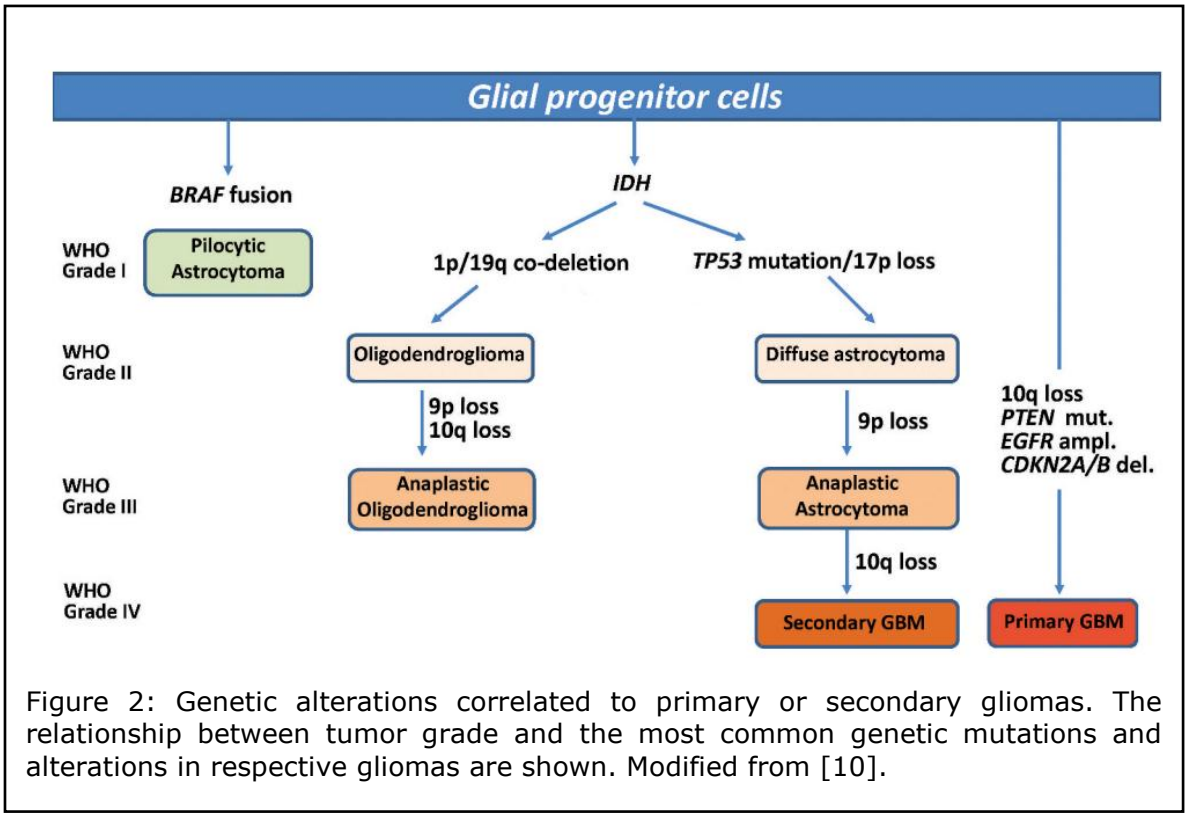

Figure 2: Genetic alterations correlated to primary or secondary gliomas. The relationship between tumor grade and the most common genetic mutations and alterations in respective gliomas are shown. Modified from [10].

1.2.3. ANGIOGENESIS

A hallmark in GBM is robust angiogenesis. For GBM tumors to survive and grow, they rely on formation of blood vessels in order to obtain oxygen and nutrients. In angiogenesis, new blood vessels are formed from already existing vessels. One of the key proteins driving angiogenesis is vascular endothelial growth factor A (VEGF), commonly upregulated in GBM compared to lower-grade gliomas [16]. VEGF binds VEGF-receptor 2 (VEGFR2) on endothelial cells, stimulating cellular pathways associated with proliferation, migration and reduced apoptosis, ultimately leading to enhanced angiogenesis [17]. Hence, the treatment with compounds targeting VEGF or VEGFR2 has been comprehensively investigated in several pre-clinical and clinical trials (reviewed in [18]). Bevacizumab, a humanized monoclonal antibody, targeting VEGF is currently the only drug targeting angiogenesis that has been approved by the U.S. food and drug administration (FDA) in the treatment of recurrent GBM [19,20]. It is supposed to exert its anti-angiogenic effect by binding VEGF, thereby abrogating activation of VEGF-receptors on endothelial cells.

1.2.4. TREATMENT

Despite the identification of distinct molecular GBM subtypes, the standard treatment for GBM consists of surgery combined with radiotherapy and chemotherapy, in the form of the alkylating agent temozolomide [21]. Epigenetic silencing of the MGMT (O^6-methylguanine-DNA methyltransferase) gene predicts longer survival in patients receiving temozolomide [22]. Although it is not currently used for treatment stratification of GBM patients, it illustrates a need for further exploration of individualized treatments for specific GBM subgroups. Even with aggressive therapy, almost all patients will experience tumor relapse that is highly resistant to additional treatment, demonstrating post-recurrence survival rates of only 6-9 months [23]. For

recurrent GBM, no standard treatment has been established but possible therapies include re-challenge with temozolomide, other alkylating agents (lomustine, carmustine, carboplatin) or bevacizumab [24]. In Denmark, the majority of recurrent GBM patients are currently treated with combined lomustine (CCNU) and bevacizumab therapy. This is based on results presented in a recent phase II trial, indicating prolonged median progression-free-survival (PFS) and median overall survival (OS) when combining CCNU and bevacizumab compared to either drug alone in recurrent GBM patients [25]. However, this combination regimen is still up for debate based on preliminary results from the EORTC 26101 phase III trial, displaying increased PFS but similar OS, when comparing the combination regimen with each single-line treatment, in recurrent GBM patients [26].

In addition to bevacizumab, several other specifically targeting therapeutic modalities, such as kinase inhibitors and antibodies, have been tested against various targets in GBM. Since EGFR is amplified and overexpressed in about half of all GBM, various approaches have been tried in order to inhibit EGFR and associated growth factor pathways. Gefitinib (Iressa), a small-molecule inhibitor and cetuximab, a monoclonal antibody, both targeting EGFR, have demonstrated some effect in a subset of GBM patients but without consistent improvement in PFS and OS [27-30]. Immunotherapy is emerging as a new therapeutic approach in the treatment of GBM. Hence, an immunotherapy vaccine (rindopepimut) targeting the mutated EGFR variant, EGFRvIII, has been tested in newly diagnosed GBM patients, but failed to show survival benefit in a recent phase III trial [31]. Another approach involves the modulation of immune checkpoint blockade by PD-1 inhibitors and several clinical trials are under way in GBM (reviewed in [32]).

In summary, several targeted therapies have been investigated in GBM, but so far no treatment has shown superiority to the standard treatment comprising surgery combined with radiotherapy and chemotherapy. However, the most

effective treatment may be a result of a treatment regimen tailored to the molecular phenotype of a patient's tumor, and comprise a combination of chemotherapy and cytostatic agents.

1.3. CANCER STEM CELLS

Solid tumors consist of heterogeneous cancer cells together with a mixture of vascular elements, stromal components and inflammatory cells [2]. Cancer arises through a series of mutations and molecular alterations, ultimately resulting in a cell with unlimited and uncontrolled proliferation potential [2]. In general, two hypothetical models can explain this transformation. The stochastic model, hypothesizing that every cell in a tumor has the same chance of acquiring tumorigenic potential and is determined through some stochastically varying intrinsic factors [33]. Conversely, the hierarchical model suggests that there exists a rare subset of cells (i.e. cancer stem cells; CSCs) in the tumor with capacity to proliferate, and ability to generate new tumors that consists of both CSCs (self-renewal) and terminally differentiated cells (the bulk tumor) [33]. Thus, the latter model can be seen as a cancer stem cell theory and illustrates a need to eliminate all CSCs in order to ultimately terminate the growth of a tumor and preventing the risk of relapse [34]. So far, the presence of CSCs has been identified in solid tumors including breast [35], colon [36], pancreas [37], and brain cancer [38]. In addition to their tumorigenic capacity, the CSCs have shown to be highly resistant to radiation and chemotherapy compared to the bulk tumor cells, underscoring their role in tumor recurrence and poor outcomes [39,40].

1.3.1. NORMAL NEURAL STEM CELLS

Normal neural stem cells (NSCs) are multipotent cells located in the adult brain and with capacity of self-renewal, proliferation and differentiation into the three lineages: neurons, astrocytes and oligodendrocytes (reviewed in [41]). The population of NSCs, primarily present in the subventricular zone [42], is sustained though asymmetric cell division giving rise to one daughter cell (self-renewal) and a more differentiated progenitor cell [43]. In addition, NSCs can give rise to terminally differentiated cells through generation of the proliferating transit-amplifying progenitor cells [44]. When grown in culture in defined serum-free media (with growth factors), NSCs grow as non-adherent cell aggregates termed neurospheres [45] expressing stem cell markers including the intermediate filament Nestin [46] and the surface glycoprotein CD133 [47,48].

1.3.2. GLIOMA STEM CELLS

Several studies have now shown the presence of CSCs in brain tumors, designated glioma stem cells (GSCs), that display similar characteristics to NSCs, including self-renewal, proliferation and with capacity to form tumors [38,49,50]. Additionally, they display a hierarchical organization capable of giving rise to more differentiated progeny [51]. GSCs are thought to arise from transformed stem- or progenitor cells, or already differentiated cells acquiring stemness characteristics through reprogramming or dedifferentiation as a result of tumorigenic alterations [52-54]. In GBM, there is a high degree of tumor cell plasticity, illustrating capacity of interconversion between GSCs and non-GSCs [55,56].

GSCs obtained from GBM patient tissue and grown as neurospheres (also called tumorspheres) typically express stem cell markers including CD15, CD44, Nestin, Nanog, Oct4 and Sox2, and the intensively investigated CD133,

demonstrating their resemblance to NSCs [57-61]. CD15 (SSEA-1) is associated with embryonic stem cells in the developing brain and was found to be enriched in GSCs correlated with a high tumorigenic capacity compared to CD15-negative cells [57,62]. The transmembrane glycoprotein, termed CD44, acting as a adhesion molecule, and normally found on embryonic epithelia during development, was found to be expressed in all GBM cell lines and tumors tested [63,64]. Additionally, depletion of CD44 by a monoclonal antibody abrogated tumor progression suggesting a role in tumorigenesis [65]. CD44 have been found to be co-expressed with Sox2, Nestin, and Olig2 further supporting its potential as a marker for stem- and/or progenitor cells [66]. Recent investigations demonstrated increased Nanog, Oct4 and Sox2 expression in high-grade gliomas compared to low-grade gliomas and data indicate a synergistic collaboration between Nanog, Oct4 and Sox2 regulating pluripotency and self-renewal in stem cells [58,67-69]. Remarkably, merely one hundred CD133-postive tumor cells displayed capacity to initiate tumors in immunodeficient mice, while 100,000 CD133-negative tumor cells failed to form tumors [38,70]. In contrast, other studies have shown that also CD133-negative glioma tumor cells harbor tumor-initiating potential [71]. These mixed results suggest that markers should not be the sole measure of stemness, but should be used in conjugation with functional assays demonstrating neurosphere and tumor formation. Despite controversies suggesting that no real markers has been established identifying GSCs [72], CD133 is still the most prominent marker used for identification and enrichment of GSCs.

1.3.2.1. GLIOMA STEM CELLS AND ANGIOGENESIS

Blood vessels in GBM are generally disorganized, twisted and with a low pericyte coverage, making the vessels leaky resulting in a chaotic blood flow and hypoxia. Hypoxic regions of a tumor are suggested to promote and

maintain tumor cells in an immature state, correlating with a more malignant tumor phenotype [73,74]. Consistent with this, hypoxia stimulates the formation of neurospheres *in vitro* of both GSCs and non-stem cells and the expression of several stem cell markers (CD133, Sox2, Oct4, and Nestin) was reported to be upregulated during hypoxic conditions [73-77]. Additionally, in a study by Bao et al. they showed that CD133-positive GSCs compared to their non-stem counterparts (CD133-negative) expressed elevated levels of VEGF and induced more vascular and necrotic tumors in mice xenografts [78]. VEGFR2 is mainly expressed by endothelial cells and binds VEGF leading to modulation of vascularization. However, various studies indicate that tumor-secreted VEGF, in addition to paracrine stimulation of endothelial cells, also can stimulate GBM cells themselves through an autocrine mechanism, thereby contributing to tumor resistance [79-81]. In agreement with this, VEGFR2 was shown to be preferentially expressed in CD133-positive GSCs, and upon depletion of VEGFR2, this resulted in reduced tumor formation *in vivo* [82].

In summary, these data indicate that the hypoxic niche may maintain a GSC phenotype and that GSCs can sustain their own vascular niche trough expression of angiogenic factors such as VEGF.

1.3.2.2. THERAPEUTIC RESISTANCE OF GLIOMA STEM CELLS

In GBM, one main cause for treatment failure is tumor resistance to radiotherapy and chemotherapy. Recently, studies have demonstrated that treatment with radiotherapy and chemotherapy results in enrichment of tumor cells expressing CD133, suggesting that conventional treatments enhance the GSC population [39,83]. GSCs demonstrate an enhanced capacity of activating DNA repair pathways upon DNA damage induced by chemotherapy and radiation, suggesting better recovery than non-GSCs [39,84]. The Notch pathway has also been shown to contribute to treatment resistance, and upon inhibition of Notch, this sensitized GBM cells to radiotherapy [85]. Expression

of the DNA repair enzyme MGMT, capable of removing DNA adducts upon treatment with alkylating agents, was found to be upregulated in GSCs making them resistant to temozolomide [86]. Additionally, a drug resistance gene, termed BCPR1, was found to be highly expressed in GSCs contributing to temozolomide resistance [40]. Besides the aberrant activation of DNA damage repair pathways, additional mechanisms contributing to GSC treatment resistance has been suggested, including reduced proliferation, expression of drug efflux transporter proteins, low apoptotic rate and a quiescent phenotype [87-91].

Collectively, several lines of evidence points to the presence of GSCs that is responsible for resistance to conventional drugs and tumor recurrence (Figure 3). Hypothesizing that GSCs gives rise to GBM tumors, sustaining a high genetic and molecular heterogeneity, and maintaining bulk tumor growth, the elimination of GSCs could eventually result in long-term cures. Thus, by targeting the GSC population in combination with current therapies targeting the bulk of the tumor, this could lead to complete tumor elimination and enhance long-term survival in GBM patients.

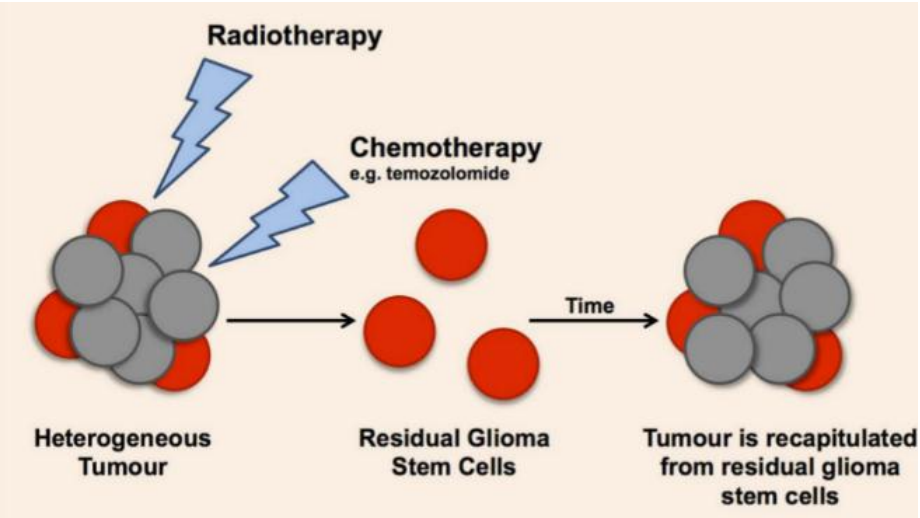

Figure 3: Resistance of glioma stem cells in GBM. Conventional therapies comprising radiotherapy and chemotherapy targets highly proliferative tumor bulk cells. The glioma stem cell (GSC) population, which is quiescent and therapy-resistant, will eventually recapitulate the tumor leading to recurrence. Adopted from [88].

1.4. SIGNALING IN GLIOBLASTOMA

Tumors of malignant gliomas are believed to be derived from transformed glial cells and display a highly heterogeneous cellular and molecular profile, which results from the accumulation of genetic and epigenetic alterations [92,93]. However, in GBM, some common genetic alterations have been identified in a number of pathways: The RAS-MAPK-, the PI3K-Akt-, the p53-, and the RB-pathway [94,95]. Additionally, features such as amplification and/or overexpression of EGFR, overexpression of the Notch signaling pathway, and dysregulation of histone modifying enzymes are common in GBM and will be further reviewed in the following sections.

1.4.1. NOTCH

Notch signaling is involved in the development of tissue and organs including the vasculature, and has been shown to be involved in several cancers including GBM [96-98]. Upon formation of the central nervous system (CNS), Notch expression has been associated with undifferentiated cells and the expression is reduced in adults [99]. Consistent with this, the Notch signaling pathway is suggested to promote self-renewal and abrogate cellular differentiation, thereby contributing to the maintenance of stem- and progenitor cells [100,101].

1.4.1.1. NOTCH SIGNALING IN GBM

Four Notch receptors have been identified (Notch 1-4), which are activated by Notch ligands (Delta-like (Dll) 1, 3-4 and Jagged 1-2) [102].

Upon activation of Notch receptors by their ligands, located on a neighboring cell, the receptor-ligand complex undergoes at least two subsequent proteolytic cleavages, ultimately leading to formation of a Notch intracellular

domain (NICD) [103-105]. One such protease, responsible for the cleavage of the trans-membrane Notch domain, is the multi-subunit protease complex known as the γ-secretase complex [106]. When Notch is active, the NICD is released into the cytoplasm thus translocating into the nucleus forming a transcriptional activation complex with the DNA binding protein CSL, thereby initiating transcription of Notch target genes [107]. Some of the best characterized transcriptional targets of Notch are members of the Hairy/enhancer of split (Hes 1-7) and Hey (Hey 1-2 and HeyL) family of basic helix-loop-helix (bHLH) transcriptional repressors [108,109].

A number of studies have indicated overexpression of Notch receptors and their ligands in GBM and GBM-derived cell cultures [110-112]. Commonly, aberrant activation of Notch molecules is associated with an immature phenotype indicating a role in GSCs. As an example, Notch receptors and the downstream target Hey, was found to be upregulated in CD133-positive glioma cells [113]. In addition, Notch has been shown to activate transcription of Nestin in gliomas [114], whereas inhibition of the Notch pathway lead to depletion of Nestin- and CD133-positive GBM neurosphere cells [115,116].

Notch signaling has also shown to be involved in tumor angiogenesis and is correlated with an aggressive clinical behavior in tumors expressing high levels of Notch ligands [117,118]. A study by Funahashi et al., demonstrated that inhibition of the Notch pathway, induced by a Notch-1 decoy, impaired neo-angiogenesis and tumor growth *in vivo* [119]. Further, they showed that the decoy also lead to inhibited VEGF-induced angiogenesis in skin, illustrating a role for Notch receptor function in response to induction of angiogenesis [119]. Consistent with this, inhibition of Notch ligand Dll-4 repressed tumor growth, due to formation of poorly perfused non-functional tumor vessels [120,121].

Taken together, this indicates that the Notch pathway has a prominent role in tumorigenesis through stimulation of tumor angiogenesis, tumor maintenance and induction of tumor cell stemness. Hence, one approach to inhibit Notch

signaling is by the use of gamma-secretase inhibitors (GSIs). The anti-cancer effects of GSIs have been investigated in several pre-clinical studies and have been shown to reduce glioma tumor growth and additionally sensitize tumor cells to radiation and chemotherapy [85,122,123]. Despite the encouraging pre-clinical results, treatment with GSIs can lead to side effects such as gastrointestinal toxicity due to accumulation of secretory goblet cells in the intestine [124,125]. Nevertheless, this can be avoided by optimized drug administration, and clinical trials are now running testing GSIs as mono-therapy or in combinational treatment regimens in patients with primary or recurrent GBM (http://www.clinicaltrials.gov and [98]).

1.4.2. EGFR

The epidermal growth factor receptor is one of the most investigated RTKs and is involved in regulation of cell survival, angiogenesis, proliferation, differentiation and migration [126]. It belongs to the ErbB/HER family of RTKs consisting of four closely related members (EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4), and the EGFR have been extensively studied for its role in GBM [127]. Additionally, a common EGFR mutation (EGFRvIII) results in constitutively active downstream signaling and has been shown to contribute to stemness and resistance to radiation in GBM [128,129].

1.4.2.1. EGFR SIGNALING IN GBM

Activation of EGFR is mediated through binding of ligands (e.g. EGF: epidermal growth factor and TGF-α: transforming growth factor alpha) and stimulates activation of downstream signaling pathways (PI3K-Akt and RAS-MAPK) involved in cell growth and proliferation [130]. Both NSCs and GSCs have shown to sustain proliferation *in vitro* through stimulation with EGF and other

EGFR-ligands [131,132] and abrogation of EGFR signaling have shown to induce differentiation of GBM neurospheres [133].

Both the EGFR and EGFRvIII variant have been shown to correlate with a GSC phenotype responsible for self-renewal and tumorigenesis [128,134,135]. Additionally, the EGFRvIII variant has been shown to be co-expressed with CD133 indicating its potential as a marker of the GSC population [128,136].

The EGFR signaling pathway is a major contributor to tumor angiogenesis in GBM. Activation of EGFR stimulates expression of VEGF via the PI3K-Akt pathway and regulates expression of a transcription factor, termed hypoxia-inducible factor-1 (HIF-1), important in response to hypoxic conditions [137,138]. In line with this, treatment with inhibitors targeting EGFR, have shown capacity to block tumor angiogenesis through downregulation of VEGF and HIF-1 expression [139,140].

Despite the high prevalence of EGFR amplifications and numerous clinical trials targeting EGFR, by using tyrosine-kinase inhibitors (TKIs) or monoclonal antibodies, alone or in combination with other drugs, no clear survival benefit has been demonstrated in GBM [29,141-144]. Several aspects contributing to the disappointing clinical results using TKIs against EGFR has been suggested, including activation of redundant signaling pathways, inadequate tissue penetration, inadequate target inhibition and cellular heterogeneity [14,145,146]. Thus, one way of improving the efficacy of EGFR inhibitors could be through increased dosing, the use of new generation inhibitors, or given the heterogeneity of the tumor, the use of combinatorial treatment regimens (reviewed in [126]).

1.4.3. NOTCH AND EGFR CROSSTALK

Over the years, a number of studies have indicated the existence of a signaling interplay between the EGFR and Notch pathways in cancer [147-149]. Consistent with this, Notch molecules were found to correlate with GBMs displaying EGFR gene amplification and mutation [11,150]. One of the most noteworthy studies, by Purow et al., demonstrated that Notch-1 regulates the transcriptional activity of EGFR through a p53-dependent mechanism [149]. Furthermore, data have demonstrated that oncogenic Ras signaling activates Notch, and that Notch-1 is required for maintenance of Ras-transformed fibroblasts [151]. Another study indicated that Notch controls PI3K-Akt activity through suppression of PTEN expression [152]. In addition, data suggests that Notch-1 contribute to acquired resistance to gefitinib (EGFR TKI) or trastuzumab (ErbB2 antibody) in lung- and breast cancer, respectively [153,154].

Considering the important roles of Notch and EGFR signaling in angiogenesis, maintenance and survival of GBM cells, one possible approach to overcome redundant signaling and acquired drug resistance, is the simultaneous use of EGFR and Notch inhibitors. As an example, this treatment approach has shown enhanced anti-tumorigenic and anti-angiogenic effects compared to either mono-therapy in basal-like breast cancer, and in head and neck squamous cell carcinoma [155,156].

1.4.4. EPIGENETICS

Epigenetics are defined as changes in gene expression independent of changes in the DNA sequence and can be passed onto the next cell generation. The regulation of gene expression is controlled by several mechanisms including DNA methylation, small interfering RNAs (siRNAs), histone variants and histone post-translational modifications (PTMs) [157]. Gene transcription is dependent

on accessibility of transcription factors to the DNA and is modulated in part by the degree of chromatin condensation regulated by epigenetic marks. Thus, gene transcription can either be turned on or off dependent on the combinations of different epigenetic marks, which ultimately results in different cellular phenotypes of cells harboring the same genome [158]. In human cancer, DNA methylation and histone PTMs are often dysregulated leading to aberrant gene transcription, and loss of histone acetylation has been identified as a common hallmark in cancer [159].

1.4.5. CHROMATIN AND HISTONE MODIFICATIONS

Chromatin is composed of DNA molecules and histone proteins efficiently packed into complexes allowing for large amounts of DNA to be stored in the nucleus of the cell. The nucleosome particle represents the primary structural unit of chromatin and consists of 147 base pairs of DNA wrapped around core histone octamers. The histone octamer is composed of two copies of each histone protein; H2A, H2B, H3 and H4 [160]. Each histone protein has an N-terminal tail extruding from the nucleosome core, where histone modifications regulates the interaction between the DNA and histone proteins [161]. So far, identified PTMs of the histone tails comprise acetylation, methylation, phosphorylation and ubiquitylation together with other less studied modifications [162]. The epigenetic regulators are divided into groups dependent on their functions and termed epigenetic writers, epigenetic erasers, and epigenetic readers as illustrated in Figure 4 [163]. The epigenetic writers facilitate epigenetic marks on DNA or histones, which in turn are removed by the epigenetic erasers. The epigenetic readers are effector proteins that recognize specific epigenetic marks. Chromatin can be either loosely condensed (euchromatin) representing generally active transcription or highly condensed (heterochromatin) constituting repressed transcription [164]. Thus, the chromatin occurs as central dynamic components accountable for

regulating gene transcription and other DNA-based cellular processes through balanced regulation determined by the histone modifying enzymes.

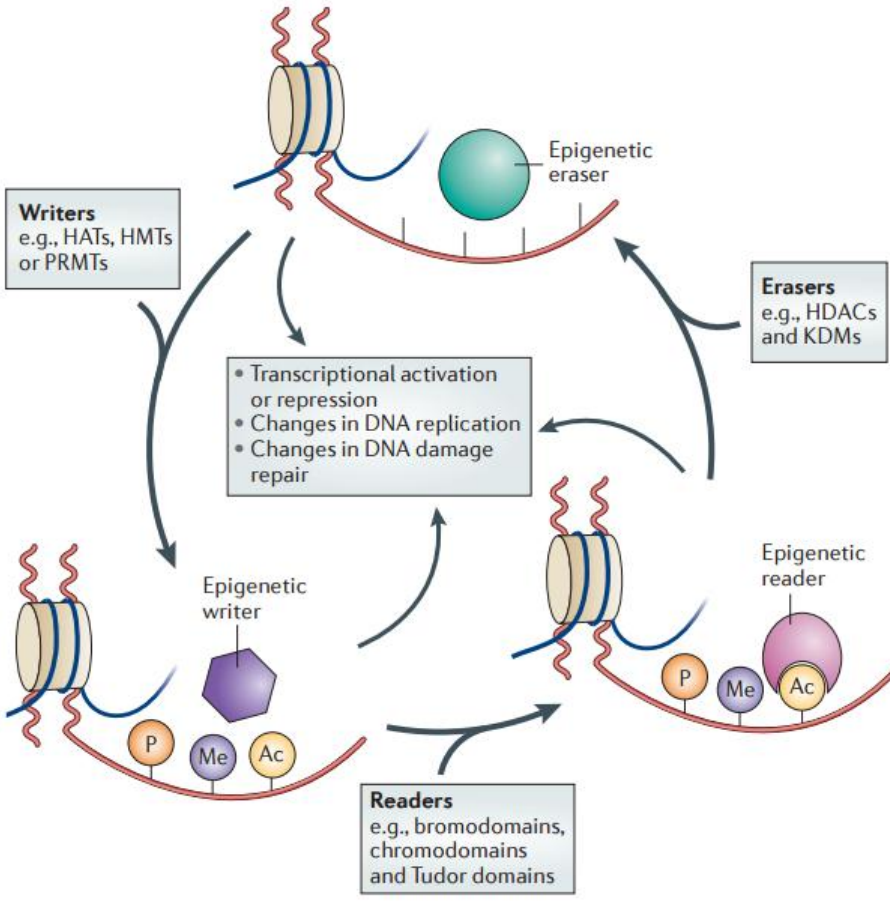

Figure 4: Epigenetic modifying proteins. The epigenetic machinery is a dynamic entity regulated by various epigenetic modifiers. Epigenetic writers such as histone acetyltransferases (HATs) and histone methyltransferases (HMTs) facilitate epigenetic marks on histone tails, whereas epigenetic erasers such as histone deacetylases (HDACs) and histone demethylases (KDMs) remove these marks. The epigenetic readers such as bromodomain-containing proteins recognizes the epigenetic marks and recruits various molecules involved in gene transcription, DNA replication, DNA damage, and chromatin remodeling. Adopted from [163].

1.4.5.1. HISTONE ACETYLATION

The level of acetylation is regulated by the histone acetyltransferases (HATs) facilitating acetylation and the histone deacetylases (HDACs) removing acetyl groups from the histone tails (Figure 4). Upon acetylation, this leads to removal of positive charges, subsequently reducing binding between histones and DNA, leading to increased DNA accessibility for transcription factors and subsequent transcriptional activity [165]. In addition, HDACs have also been shown to interact with and regulate several non-histone proteins (e.g. transcription factors and transcriptional corepressors) regulating cellular processes including cell cycle progression, differentiation and apoptosis [166,167].

The classical HDACs can be divided into three classes: class I HDACs (HDAC1, 2, 3 and 8), class II HDACs (HDAC4, 5, 6, 7, 9, 10), and class IV (HDAC11) and are dependent of zinc ions for enzymatic activity [168-170]. Furthermore, a class III HDACs exist characterized by their dependence on NAD+ as energy source and therefore constitute a distinct group of HDACs [171]. Altered HDAC expression has been identified in several tumors, generally displaying higher expression of class I HDACs and lower expression of class II HDACs compared to normal tissue, respectively [172]. Thus, it is suggested that overexpression of HDACs leading to repressed transcription of tumor-suppressor genes could be a common feature in tumor initiation and progression. One example is the $p21^{WAF1}$ protein, inhibiting cell cycle progression, which has been shown to be epigenetically inactivated by hypoacetylation of the promoter region [173]. Given the often aberrant expression of HDACs, the use of histone deacetylase inhibitors (HDACi) was identified as possible anti-cancer agents. Several HDACi have been identified showing anti-cancer activity including induction of cell-cycle arrest, apoptosis, differentiation, and inhibition of angiogenesis [174,175]. In addition to a direct effect on gene transcription, histone acetylation also regulates other cellular processes including DNA repair and replication [157,176]. As a result, HDACi treatment has been shown to inhibit

DNA repair events, leading to sensitization of cancer cells to chemo- and radiotherapy through increased DNA damage [177,178]. Further, it has been proposed that chromatin relaxation, induced by HDACi, renders the DNA more accessible, thereby potentiating the effect of DNA-damaging drugs [179,180].

Currently, a number of HDACi have been FDA-approved for treatment of refractory cutaneous T-cell lymphoma [181] and are further being tested in clinical studies in solid tumors (reviewed in [182]). Despite their promising anti-cancer potential, and their ability to cross the blood brain barrier (BBB), the treatment of GBM with HDACi as monotherapy has only showed modest response rates [183]. Thus, several clinical trials are now underway combining HDACi with other cytotoxic agents in GBM (https://clinicaltrials.gov/).

1.4.5.2. HISTONE METHYLATION

In contrast to histone acetylation, methylation of histones does not substantially change the amino acid charge, but leads to activation/repression of gene transcription dependent on which residues are modified. Histone methyltransferases (HMTs) catalyze the addition of methyl groups on lysine and arginine residues, whereas this process is counteracted by lysine-specific histone demethylases (KDMs) removing methylation groups (Figure 4). Two families of KDMs have been identified: The LSD family and the Jumonji C (JmjC) family identified by their catalytic JmjC domain [184]. The LSD family is capable of removing mono- or di-methyl groups, whereas the JmjC-family furthermore can remove tri-methyl groups [185]. As an example, methylation at histone H3 lysine 4 (H3K4), H3K36, and H3K79 is commonly associated with gene activation, while methylation at H3K9, H3K27, and H4K20 has been related to gene silencing [186]. Overall, the balance between methyltransferases and demethylases define the histone methylation pattern, subsequently activating or repressing individual genes. Hence, KDM proteins have been shown to be involved in various cellular processes including DNA

replication, DNA damage response, cell differentiation and self-renewal of embryonic stem cells [187-189].

It is believed that deregulated expression of KDMs is involved in the development of cancer [190]. LSD1 (KDM1A) has been shown to be overexpressed in several cancers (bladder-, colorectal-, and prostate cancer) including GBM [191-194]. A member of the JmjC-familiy, KDM2B, is expressed in embryonic stem cells, where it maintains stem cell status and upon differentiation the expression levels declines [195,196]. In addition, KDM2B protects cells from replicative senescence, oxidative stress and ROS-induced DNA damage through regulation of antioxidant genes [195,197,198]. Recently, KDM2B was found to be overexpressed in breast- and pancreatic cancer, and identified as an oncogene sustaining tumor growth [199,200]. Further, depletion of KDM2B promoted differentiation of cancer cells and lead to loss of stemness [199,200]. Consistent with this, a study reported that another member of the JmjC familiy, the KDM5B, was expressed in slow-growing tumor-maintaining melanoma cells, illustrating the possible role of KDMs in the maintenance of the cancer stem cell subpopulation [201].

KDMs have also been correlated to chemoresistance. In a recent study by Ramadoss et al., they found that KDM3A was involved in stemness and chemoresistance of ovarian cancer [202]. The authors demonstrated that depletion of KDM3A downregulated stem cell markers Sox2 and Nanog, and further abrogated growth of cisplatin-resistant ovarian tumors in mice [202]. Similar results were found in breast cancer, demonstrating KDM3A to play a role in invasion, apoptosis and resistance to chemotherapy [203].

In the light of the often dysregulated KDMs in cancer, and their contribution to stemness and treatment resistance, the use of small-molecule inhibitors targeting KDMs could be a new weapon in the treatment of cancer. A number of KDM inhibitors (KDMi) have already been developed and shown efficacy in preclinical studies (reviewed in [190]). For example, the KDMi JIB-04 was

found to selectively inhibit the JmjC-familiy, leading to inhibited tumor growth of both lung- and breast cancer cells *in vitro* and *in vivo* [204].

1.5. DNA DAMAGE RESPONSE

In order for cells to maintain the integrity of the DNA, cells have evolved a multifaceted system termed the DNA-damage response (DDR) that detects various types of DNA damage and subsequently activates a network of signaling pathways ultimately repairing the DNA [205,206]. Upon severe DNA damage, the DDR machinery can induce cells to undergo apoptosis or cellular senescence, thereby eliminating the risk of a mutated genetically unstable cell, which eventually could transform into cancer [207,208]. DNA-damaging agents can induce various DNA damage lesions including intrastrand crosslinks, interstrand crosslinks, DNA-protein crosslinks, single-strand breaks (SSBs), and double-strand breaks (DSBs) [209]. Several DNA repair pathways exist to resolve specific type of lesions, including base excision repair (BER), mismatch repair, nucleotide excision repair, non-homologous end joining (NHEJ), and homologous recombination (HR), where the latter two are the main pathways responsible for repair of DSBs [205,210]. Upon DSBs, the MRE11-Rad50-NBS1 (MRN) complex senses and binds to the site of damage, and subsequently recruits and activates the ATM kinase [211,212]. ATM signal downstream activating several other proteins including Chk2, BRCA1, and p53, ultimately resulting in cell cycle arrest and DNA damage repair [213]. At sites of DSBs, ATM or DNA-dependent protein phosphokinase (DNA-PK) can phosphorylate H2AX leading to its active form γH2AX. Collectively, γH2AX, DNA-PK and the MRN complex attracts and activates other proteins involved in DSB repair including BRCA1, BRCA2, CtIP and 53BP1 [214]. In response to SSB or DNA replication fork collapse, the ATR kinase is recruited to sites of DNA damage, activating the Chk1 protein, which is involved in S and G2/M cell cycle checkpoints [215,216].

NHEJ is a DNA repair process used throughout the cell cycle, but predominantly in G1- and early S-phase, and is mediated by the joining of the two broken DNA ends, which is highly prone to errors (Figure 5) [217]. Several proteins are involved in NHEJ where key factors include the Ku70/Ku80 complex binding to exposed breakpoints, the DNA-PK exposing the DNA ends to nucleases, and the DNA ligase IV complex joining the repaired DNA ends [218-220].

In contrast, HR is an error-free repair pathway maintaining genomic stability, and is dependent on a non-damaged DNA strand as a template for repair and acts in late S- and G2-phase of the cell cycle (Figure 5) [221]. HR relies on nuclease-mediated resection of damaged DNA ends following polymerization of new DNA, and finally resolution and ligation of the two strands [222,223]. Important factors involved in HR comprise the MRN complex, BRCA1, RPA, BRCA2, and Rad51 [224-227].

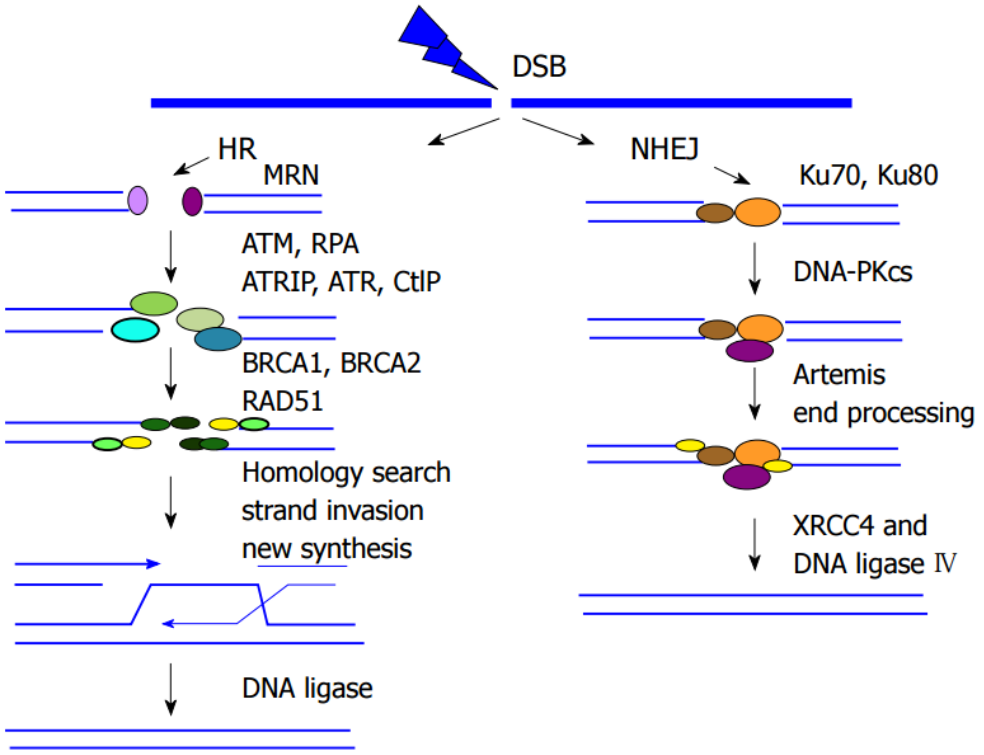

Figure 5: Simplified overview of the DNA repair mechanisms homologous recombination (HR) and non-homologous end joining (NHEJ) activated upon DNA double-strand breaks (DSBs). In HR, the MRN complex identifies DSBs and subsequently activates the ATM resulting in formation of single-strand DNA overhangs. Following, these overhangs are processed by proteins RPA, ATR and coated with RAD51, leading to homology sequence search and strand invasion. The process ends with DNA synthesis, and ligation of the two DNA strands. In NHEJ, the Ku70/Ku80 proteins sense DSBs and recruits DNA-PKs, which in turn activates the effector complex (XRCC4/ligase IV) ligating the broken DNA ends. Adopted from [206].

Both HR and NHEJ constitute critical DSB repair pathways, and as such, dysregulation of HR and NHEJ have been shown to be involved in gliomagenesis [228,229]. Overexpression of several DNA repair proteins including Rad51, BRCA1, NBS1, Chk1 and Chk2 has been identified in various tumors [230-233], and high tumor levels of DNA-PK were found to correlate with poor survival in GBM [234]. In addition, dysregulation of several of the above-mentioned DNA repair enzymes (DNA-PK, Rad51, BRCA1) have shown to contribute to chemotherapy and radiotherapy resistance [233,235,236].

Considering that chemo- and radiotherapy relies on induction of DNA damage in tumor cells, ultimately leading to apoptosis, aberrant expression of DNA repair enzymes can indicate either resistance or favorable response to therapies that induce the corresponding type of DNA damage [237]. Thus, several inhibitors abrogating the DNA damage response are now under clinical trials (reviewed in [209]) and have been considered as promising targets for radio- and chemosensitization [238-240].

1.5.1. DNA DAMAGE RESPONSE AND HISTONE MODFICATIONS

Histone modifications including acetylation and methylation are involved in DNA damage repair. As an example, HDAC1 and HDAC2 were found to be recruited to DNA-damage sites leading to hypoacetylation of chromatin, and upon depletion of HDAC1/2, this resulted in hypersensitive cells to DNA-damaging agents and impaired NHEJ [241]. In contrast, HDAC9 or HDAC10 depletion was found to impair the HR pathway [242]. Consistently, treatment with HDACi, including TSA and vorinostat, was shown to downregulate DNA-PK and key NHEJ-proteins Ku70, Ku80 in melanoma and non-small lung carcinoma cancer cells [178,243,244].

In addition, KDMs have also been shown to be involved in the DNA damage response. KDM4D was shown to be recruited to DNA-damage sites promoting DSB repair by activation of ATM, and upon KDM4D depletion, this sensitized cells to ionizing radiation [245]. Consistent with this, human LSD1 was shown to play a direct role in the DNA-damage response pathway leading to moderate hypersensitivity to γ-irradiation upon LSD1 knockdown [246].

Taken together, this suggests that various histone modifying enzymes are involved in sensing and activating the DNA-damage response and take part in distinct DNA repair pathways (HR and NHEJ). Thus, the sensitizing effect of

inhibitors of HDACs and KDMs may be explained by abrogated DNA damage signaling and repair, potentiating the effect of conventional treatments.

2. AIM OF THE THESIS

Objectives

Current therapies only extend survival of GBM patients with few months. A number of studies have highlighted the glioma stem cells as contributors to tumor formation, treatment resistance and recurrence. Both, the Notch and EGFR signaling pathways are aberrantly expressed in GBM, where they are believed to sustain the GBM cells by contributing to self-renewal, proliferation, angiogenesis and migration. Additionally, emerging evidence indicates that aberrant expression of epigenetic modifying enzymes, such as the histone deacetylases and histone demethylases, are involved in tumorigenesis and therapeutic resistance in GBM.

Thus, the objective of this thesis was to investigate molecular factors involved in GBM maintenance, and explore combined treatment regimens for increased GBM cell elimination.

Specific aims:

1. Investigate the EGFR and Notch pathways' contribution to GBM maintenance and the therapeutic potential of combined Notch and EGFR inhibition.

2. Characterize the expression of histone deacetylases in GBM and investigate the sensitizing effect of HDAC-inhibition to lomustine.

3. Elucidate the functional relevance of histone demethylase KDM2B in GBM and its therapeutic potential.

3. SUMMARY OF RESULTS

3.1. STUDY I

In study I, we showed that GBM cell cultures express EGFR and Notch. We hypothesized that Notch and EGFR expression is associated with an angiogenic phenotype. Thus, we wanted to investigate the effect of EGFR and Notch inhibition on GBM-induced endothelial sprouting and GBM cell maintenance. Inhibition of EGFR (by Iressa) and Notch (by DAPT) abrogated GBM-induced endothelial sprouting, and reduced VEGF-secretion in GBM cells. When combining Iressa and DAPT, this resulted in additive inhibition of GBM-induced endothelial cell sprouting. Moreover, we showed that combined treatment with Iressa and DAPT further inhibited GBM cell viability as well as downstream pro-survival signaling pathways. Overall, this indicates that both EGFR and Notch are involved in GBM maintenance and GBM-induced angiogenesis. Thus, a combined treatment approach, targeting both of these pathways, could prove favorable over single-line treatment in a clinical setting.

3.2. STUDY II

Study II was aimed at investigating the effect of a pan-histone deacetylase inhibitor, Trichostatin A (TSA), on GBM survival and maintenance. First, we demonstrated upregulated expression of histone deacetylases in both GBM patient tumor samples and cell cultures, and found the expression of HDAC1 and HDAC3 to correlate with increasing histological grading of gliomas. Treatment with TSA resulted in increased formation of γH2AX foci, indicating impaired double-strand break repair, and further inhibited cell viability and induced apoptosis in GBM cells. Next, we investigated the effect of TSA on the capacity to sensitize cells to lomustine chemotherapy. Upon pre-treatment with TSA before lomustine, this potentiated the induction of γH2AX foci and induced

cell cycle arrest in the G1 or G2/M cell cycle phase. Additionally, the combined treatment even further decreased cell viability and enhanced induction of apoptosis compared to either agent alone. In summary, the results presented in study II suggest that specific HDACs are aberrantly expressed in GBM, and that GBM cells can be sensitized to standard treatments by HDACi. This illustrates a rationale for further investigations on combination therapies using HDACi as sensitizers in GBM.

3.3. STUDY III

In study III, we aimed at interrogating the role of histone demethylase KDM2B in GBM maintenance and survival. KDM2B was found to be differentially expressed in GBM patients and cell cultures compared to non-neoplastic tissue. Upon KDM2B depletion, using siRNA-mediated knockdown, this resulted in reduced cell viability and induction of apoptosis. Further, the knockdown of KDM2B sensitized cells to lomustine and etoposide chemotherapy. In order to evaluate KDM2B as a therapeutic target in a clinical setting, we employed GSK-J4, a histone demethylase inhibitor that displays inhibition of KDM2B enzyme activity. We showed that GSK-J4 reduced KDM2B expression, inhibited cell viability and induced apoptosis in a dose-dependent manner. Furthermore, we confirmed that combined treatment with GSK-J4 and either lomustine or etoposide chemotherapy induced synergistic inhibition of cell viability *in vitro*. Finally, preliminary results demonstrated that GSK-J4 reduced the population of CD133-positive GBM cells and reduced the expression of the stem cell marker Sox2, indicating that GSK-J4 targets the glioma stem cell population. In summary, the results in study III demonstrate the notion, that aberrant expression of histone demethylases may sustain GBM maintenance. Thus, targeting of KDM2B in combination with standard chemotherapy may target both cancer stem and non-stem GBM cells.

4. STUDY I

Combined EGFR- and notch inhibition display additive inhibitory effect on glioblastoma cell viability and glioblastoma-induced endothelial cell sprouting in vitro

Mikkel Staberg, Signe Regner Michaelsen, Louise Stobbe Olsen, Mette Kjølhede Nedergaard, Mette Villingshøj, Marie-Thérése Stockhausen, Petra Hamerlik, and Hans Skovgaard Poulsen

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Combined EGFR- and notch inhibition display additive inhibitory effect on glioblastoma cell viability and glioblastoma-induced endothelial cell sprouting in vitro

Mikkel Staberg[1*†], Signe Regner Michaelsen[1†], Louise Stobbe Olsen[1], Mette Kjølhede Nedergaard[2], Mette Villingshøj[1], Marie-Thérése Stockhausen[1], Petra Hamerlik[1,3] and Hans Skovgaard Poulsen[1]

Abstract

Background: For Glioblastoma (GBM) patients, a number of anti-neoplastic strategies using specifically targeting drugs have been tested; however, the effects on survival have been limited. One explanation could be treatment resistance due to redundant signaling pathways, which substantiates the need for combination therapies. In GBM, both the epidermal growth factor receptor (EGFR) and the notch signaling pathways are often deregulated and linked to cellular growth, invasion and angiogenesis. Several studies have confirmed cross-talk and co-dependence of these pathways. Therefore, this study aimed at testing a combination treatment strategy using inhibitors targeting the notch and EGFR pathways.

Methods: For evaluation of cell viability a standard MTT assay was used. Western blotting (WB) and Q-RT-PCR were employed in order to assess the protein- and mRNA expression levels, respectively. In order to determine angiogenic processes, we used an endothelial spheroid sprouting assay. For assessment of secreted VEGF from GBM cells we performed a VEGF-quantikine ELISA.

Results: GBM cells were confirmed to express EGFR and Notch and to have the capacity to induce endothelial cell sprouting. Inhibition of EGFR and Notch signaling was achieved using either Iressa (gefitinib) or the gamma-secretase inhibitor DAPT. Our data showed that DAPT combined with Iressa treatment displayed increased inhibitory effect on cell viability and abrogated expression and activation of major pro-survival pathways. Similarly, the combinational treatment significantly increased abrogation of GBM-induced endothelial cell sprouting suggesting reduced GBM angiogenesis.

Conclusion: This study finds that simultaneous targeting of notch and EGFR signaling leads to enhanced inhibitory effects on GBM-induced angiogenesis and cell viability, thereby stressing the importance of further evaluation of this targeting approach in a clinical setting.

Keywords: Glioblastoma, Angiogenesis, Endothelial spheroid sprouting, Notch, EGFR, DAPT, Iressa, Gamma-secretase inhibitor, Tyrosine-kinase inhibitor

*Correspondence: mikkel.staberg@regionh.dk
†Mikkel Staberg and Signe Regner Michaelsen contributed equally to this work
[1] Department of Radiation Biology, The Finsen Center, Copenhagen University Hospital, Copenhagen, Denmark
Full list of author information is available at the end of the article

Background

Glioblastoma (GBM) is a devastating tumor of the brain and current therapies have only a palliative effect. GBM tumors are proliferative and infiltrative with a prominent angiogenic phenotype [1]. Thus, therapies targeting angiogenesis have become interesting in the treatment of GBM and the humanized antibody bevacizumab targeting vascular endothelial growth factor A (VEGF) are approved for patients with recurrent GBM [2]. However, as the effect of this and other anti-angiogenic therapies tested in GBM are very limited [3], new alternative strategies for targeting GBM in general and angiogenesis in particular are needed.

GBM is often associated with mutation and amplification of the epidermal growth factor receptor (EGFR) gene and consequently, the importance of EGFR signaling for tumor development and maintenance has gained much attention [4]. Overexpression of EGFR has been correlated to the malignant phenotype of GBM and the most common EGFR mutation in GBM EGFRvIII leads to constitutive active signaling [5–8]. Activation of EGFR downstream signaling pathways leads to increased proliferation and tumorigenesis, and stimulates angiogenesis via upregulation of pro-angiogenic molecules in the tumor cells [9, 10]. In line with this finding, anti-EGFR therapies have been shown to reduce the production of the pro-angiogenic factor VEGF and reduce vascular formation [11, 12]. Similarly to EGFR, the Notch pathway has also gained attention as a potential target in GBM. The notch gene family consists of four transmembrane receptors (notch1–4) and their ligands (jagged1–2 and Dll1, Dll2 and Dll4) [13]. Ligand binding to the receptor results in two successive proteolytic cleavages which activate downstream signaling resulting in transcription of downstream targets such as Hes1 and Hey1 [14]. The Notch pathway has been linked to a number of GBM specific processes including cellular responses to hypoxia, angiogenesis and tumor growth [15, 16]. Thus, the Notch pathway represents a highly interesting therapeutic target.

Increasing evidence points to a cross-talk between the Notch and EGFR pathway [17, 18]. In line with this, GBM tumors with EGFR amplification display overexpression of notch-regulated genes [19] and it has been shown that notch signaling can induce EGFR upregulation through a P53-dependent mechanism in GBM [20]. It is also believed that the interplay between notch and EGFR is involved in the genesis and maintenance of tumor cells in various cancers including GBM [18, 21]. Thus, this study aimed at investigating the functional interplay between EGFR and notch signaling and elucidating its role in GBM cell maintenance and GBM-induced endothelial cell (EC) sprouting as a surrogate marker for angiogenesis-like processes.

This was done by evaluating the effect of mono- or combined therapy using the tyrosine-kinase inhibitor iressa (TKI; targeting EGFR) and the gamma-secretase inhibitor DAPT (GSI; targeting notch signaling). In the present study, we have used two primary GBM cell cultures with confirmed notch and EGFR expression. Both iressa as well as DAPT single-agent treatment abrogated EGFR and notch signaling, respectively, leading to reduced cell viability, and decreased VEGF expression and GBM-induced EC sprouting. Upon combinational treatment with both iressa and DAPT, the inhibitory effect on cell viability and EC sprouting was even more pronounced. Our data indicate that the cross-talk between EGFR and Notch signaling pathways are crucial for GBM maintenance and vascular phenotype.

Methods

Cell cultures

GBM cell cultures used in this study were CPH036 (p6) and CPH047 (p3m1). These were established from patient tumor tissue derived from initial surgery before any other treatment and have previously been described in regard to EGFR status and expression of markers related to stemness and the neuronal lineages [22]. We further analyzed the IDH status of the cell cultures by dideoxy sequencing of *IDH1* codon 132 and *IDH2* codon 140 and 172. Both cell lines were found to be *IDH1/2* wild-type (unmutated). Cells were cultured as floating neurospheres in Neurobasal®-A media (NB media) supplemented with N2, B27, bFGF (10 ng/ml), EGF (10 ng/ml), L-glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml) (all from Invitrogen) and incubated in cell culture flasks (NUNC) in a humidified chamber with 5 % CO_2 at 37 °C. Spheres were dissociated at every experiment and at new passage to obtain single cells. Endothelial cells (EC) used in this study represents primary human dermal microvascular endothelial cells (HMVEC) from Lonza. EC were incubated in endothelial growth medium-2 (EGM-2) added EGM-2 microvascular (MV) supplements (VEGF, EGF, bFGF, long R3 insulin-like growth factor (R^3-IGF-1), ascorbic acid, hydrocortisone, GA-1000 and 5 % fetal calf serum (FCS); all from Lonza. Cells were incubated at 5 % CO_2 at 37 °C and passaged at sub-confluence.

Reagents

Drugs used in experiments were DAPT (*N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester) obtained from Merck Millipore and iressa (Gefitinib) from tocris bioscience. All drugs were dissolved in DMSO which was also used for treatment controls. Recombinant human $VEGF_{165}$ from Miltenyi Biotec was used to induce a pro-angiogenic response.

Western blotting

Protein lysates for western blotting (WB) were prepared and obtained from cell pellets by sonication in ice-cold modified RIPA buffer [50 mM Tris–HCl (pH 7.4), 1 % NP40, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM EDTA] supplemented with protease and phosphatase inhibitor mixture II and III (calbiochem). Determination of protein concentrations was done by the BCA protein assay (pierce). WB was performed by separation of protein lysates on NuPage 4–12 % Bis–Tris gels following electroblotting onto nitrocellulose membranes using the Novex NuPAGE SDS-PAGE gel system (invitrogen). Membranes were blocked in 5 % non-fat dry-milk in wash buffer for one hour at room temperature following incubation with primary antibodies overnight at 4 °C. Primary antibodies used are displayed in Additional file 1: Figure S4. The following day membranes were washed and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature and developed using the SuperSignal West Dura Extended Duration Substrate (pierce biotechnology) and the biospectrum imaging system (UVP).

Quantitative real-time PCR (Q-RT-PCR)

Total RNA was purified from GBM cell pellets as previously described [23]. In short, RNA was obtained by using the RNeasy Mini kit and QIAshredder and submitted to a DNase treatment (all from Qiagen). For cDNA synthesis and Q-RT-PCR reactions the SuperscriptTM III platinum® two step qRT-PCR kit with SYBR® Green (Invitrogen) was used. Gene expression levels were quantified according to the comparative Ct method and normalized to expression of the three housekeeping genes TOP1, EIF4A2, and CYC1 (primerdesign). Primers used in Q-RT-PCR reactions for amplification of target genes are displayed in Additional file 1: Figure S5.

Cell viability assay

Cells were plated at concentrations of 2.5–3.5 $\times$ 10^4 cells per well in 96-well plates and incubated for 7 days with either 100 µl of growth medium or medium containing indicated treatments or control. Cell viability was measured using a 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (sigma) by the addition of 20 µl of MTT solution (5 mg/ml, dissolved in sterile water) to each well and incubation for 4 h before the addition of 100 µl of solubilization buffer (10 % sodium dodecyl sulfate, 0.01 M HCl). Absorbance at 570 nm was measured the next day using Synergy2 microplate reader with Gen5 software.

VEGF enzyme-linked immunosorbent assay (ELISA)

One million cells were grown for 14 days in 10 ml of culture media added vehicle or inhibitors for 14 days. Conditioned media was collected and VEGF (-A) levels were quantified using the Human VEGF Quantikine ELISA kit (R&D Systems) following manufacturer's instructions. Quantification was done by measuring the absorbance at 450 nm with 570 as a reference using the Synergy2 microplate reader.

Spheroid sprouting assay

A spheroid sprouting assay was employed in order to assess the angiogenic-like sprouting process in response to pro-angiogenic stimulus and principally performed as described previously [24]. Cell spheroid formation was obtained by seeding 2000 HMVEC (EC) in each well in non-adherent round-bottomed 96-well plates in growth medium (EGM-2MV) containing 0.3 % methylcellulose (Sigma-Aldrich) and incubated at 37 °C and 5 % CO_2 for 24 h. Next, the single spheroids from the wells were collected and embedded into collagen gels, consisting of a collagen solution (1 mg/ml rat tail collagen from BD Biosciences) with 0.2 M NaOH, 1 $\times$ Medium 199 (Sigma-Aldrich), 0.6 % methylcellulose, (Sigma-Aldrich) in HMVEC basal medium (EBM-2), in 4-well plates. For each well containing spheres, these were stimulated with 50 % FCS in EBM-2 medium added the experimental factor. In the cases where this was conditioned media from GBM cells, the media was collected from 1 $\times$ 10^6 cells grown for 14 days with or without the indicated treatments and subsequently up-concentrated around 10 times by centrifugation at max speed using Amicon Ultra Centrifugal Filter Units (Merck Millipore). Spheres were incubated for 16 h and spheroid sprouting was visualized by using an Eclipse TS100 phase-contrast microscope, Digital Sight imaging system and the NIS Elements F3.2 software (all from Nikon). Quantification of spheroid sprouting (number of sprouts and total sprout length per sphere) was determined using ImageJ software.

Statistics

Statistics were performed using a one-way Analysis of Variance test (ANOVA) to compare multiple data groups, followed by Tukeys post hoc test, for comparison of multiple samples or by an un-paired two-tailed student's t test when comparing two samples. The software used for the above statistics and creation of figures was Graphpad Prism 6.0. The effect of combination therapy was done in SAS version 9.4 (SAS Institute, Denmark) by general linear modeling and analysis of the response levels was done on the log scale. Tests for additivity were made by comparing the sum of the two treatment effects on the log scale with the combination treatment and the hypothesis of additive effect were rejected if the comparison demonstrated significant interaction i.e. evidence of synergistic

or sub-additive effect. A p value < 0.05 was considered statistically significant.

Results

Characterization of GBM cell cultures for EGFR- and notch signaling pathway component expression

Q-RT-PCR and western blotting (WB) were employed in order to determine the expression levels of EGFR and EGFRvIII and Notch family molecules in two primary GBM cell cultures (CPH036 and CPH047). Both mRNA and protein analysis found that the two cultures were positive for EGFR, whereas only CPH047 displayed expression of the mutated EGFR variant, EGFRvIII, (Fig. 1a, b). Further, mRNA analysis found that both cell cultures express notch receptors 1–3 and their notch receptor ligands jagged-1, jagged-2, Dll-1 and Dll-4 (Fig. 1c) and the expression was confirmed when examining a selection of these molecules at the protein level (Fig. 1d). Protein expression of the notch downstream effector protein, Hes-1, confirmed active Notch signaling in both cultures (Fig. 1d).

Iressa and DAPT abrogates downstream survival pathway signaling through the EGFR- and notch pathways and reduces cell viability in vitro

Following verification that the GBM cells expressed components of the EGFR- and notch signaling pathways, we wanted to investigate the effect of EGFR and Notch inhibition. We used the EGFR inhibitor iressa, and the notch inhibitor DAPT for investigating the effect of EGFR and notch signaling abrogation on the downstream survival kinases Akt and Erk. In CPH036 cells, monotherapy with iressa (5 μM) inhibited EGFR phosphorylation (pY1086) but had no effect on phosphorylation of the downstream effector proteins Akt (p-Akt) and Erk (p-Erk) as seen in Fig. 2a. DAPT (5 μM) mono-therapy had minor effect on p-EGFR and displayed inhibition of p-Akt but without effect on p-Erk. Upon combined Iressa and DAPT treatment this resulted in both inhibition of p-Akt and p-Erk in CPH036 cells. In CPH047 cells monotherapy with either Iressa or DAPT reduced p-Akt and p-Erk levels to some degree and upon combined treatment this effect was even more pronounced (Fig. 2a). Furthermore, as seen in Fig. 2b, mono-therapy, with Iressa or DAPT, decreased Hes-1 expression in CPH047 cells whereas only DAPT could inhibit Hes-1 expression in CPH036 cells. Upon combinational treatment with Iressa and DAPT an additive downregulation of Hes-1 expression was seen in CPH047 cells, whereas no direct additive effect could be seen in the CPH036 cells.

Following confirmation that the inhibitors abrogated downstream signaling through survival pathways Akt and Erk, we examined the effect of Iressa and DAPT on cell viability in vitro. As seen in Fig. 2c single-agent treatment of the CPH036 cells, with either DAPT or Iressa, was able to significantly decrease cell viability compared to control. Upon co-administration of both drugs, this effect was even further potentiated and confirmed to be additive, as a test for additivity was not rejected ($p = 0.56$ for 0.5 μM iressa + 5 μM DAPT and $p = 0.50$ for 2 μM Iressa + 20 μM DAPT). In CPH047 cells, higher concentrations (20 μM DAPT or 2 μM iressa) of each inhibitor were needed to significantly inhibit cell viability and upon combined treatment this inhibitory effect was further enhanced and again confirmed to be additive ($p = 0.98$) (Fig. 2d). In conclusion, combinational therapy with Iressa and DAPT display pronounced inhibitory effect as compared to mono-therapy in GBM cells on both downstream signaling of the EGFR- and notch pathway and cell viability.

Capacity of GBM cell cultures to secrete and express VEGF and to induce endothelial cell sprouting

VEGF is a well-known inducer of angiogenesis in GBM [25]. This prompted us to investigate the level of VEGF expression and secretion in CPH036 and CPH047 cells. We found that both cell cultures were positive for VEGF mRNA expression and protein secretion (Fig. 3a) together with other pro-angiogenic factors (Additional file 1: Figure S1). The expression of VEGF receptors (VEGFR-1 and -2) could not be detected in the GBM cells (Additional file 1: Figure S2), suggesting only paracrine effects of VEGF upon secretion from the tumor cells. Knowing that the GBM cells secrete VEGF into the culture media we assessed whether conditioned media from CPH036 and CPH047 was sufficient in inducing angiogenic-like processes in EC. We performed a cell sprouting assay which measures the cells ability to migrate, proliferate and form tube-like structures, all processes required in angiogenesis. Upon exposure of EC to conditioned media obtained from either CPH036 or CPH047 cells this clearly induced sprouting as displayed in Fig. 3b. The relative number of sprouts per sphere and relative total sprout length per sphere was quantified to be significantly increased compared to control (NB unconditioned media) as shown in Fig. 3c, d. These data implied that the examined GBM cells have the capacity of inducing angiogenesis-like processes of EC in vitro possibly through secretion of VEGF.

Iressa and DAPT abrogates GBM-induced endothelial cell sprouting and reduces VEGF expression and secretion by GBM cells

Following confirmation that the GBM cells displayed capacity to induce EC sprouting, we investigated how this ability was affected by EGFR and Notch inhibition.

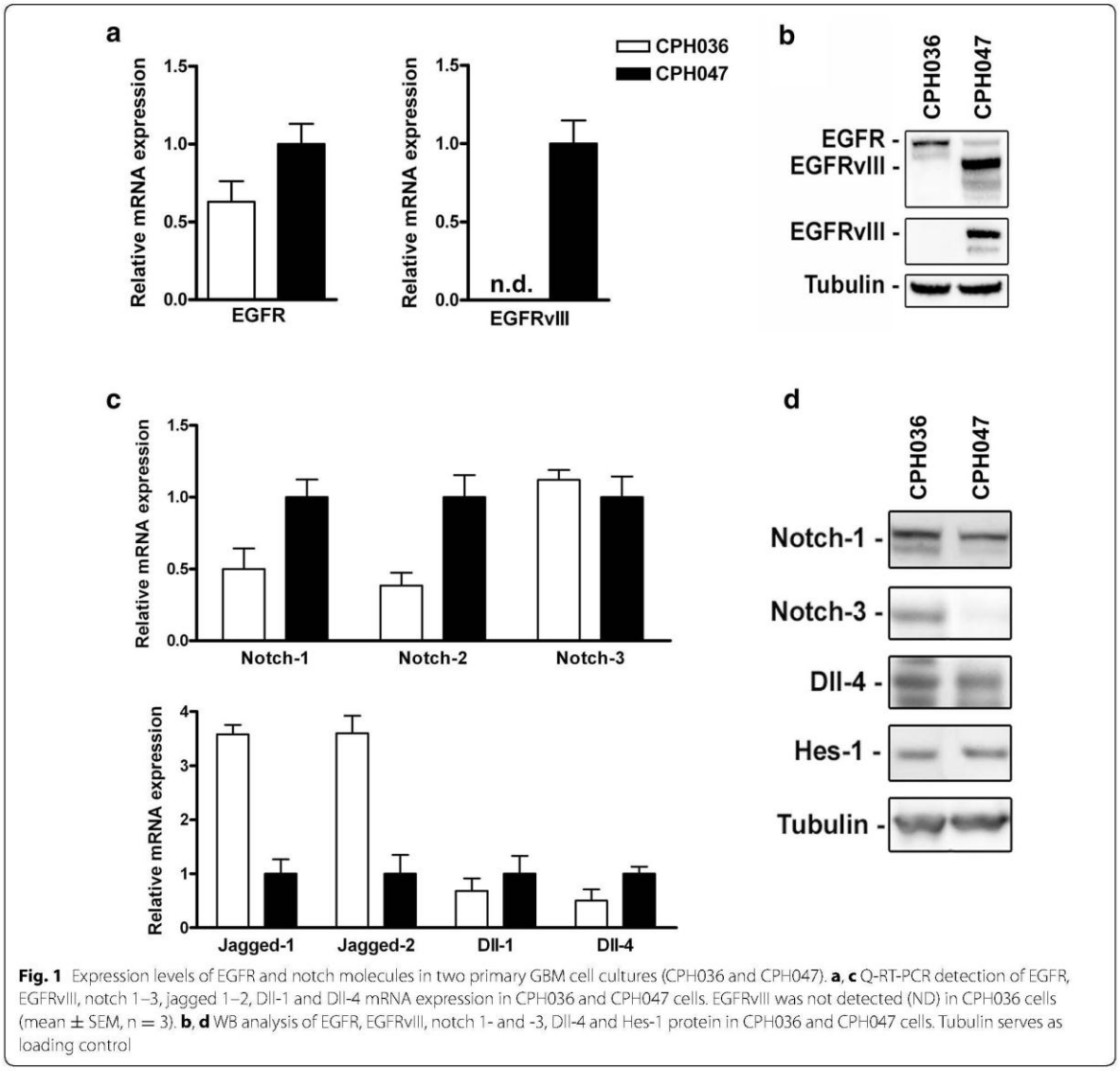

Fig. 1 Expression levels of EGFR and notch molecules in two primary GBM cell cultures (CPH036 and CPH047). **a**, **c** Q-RT-PCR detection of EGFR, EGFRvIII, notch 1–3, jagged 1–2, Dll-1 and Dll-4 mRNA expression in CPH036 and CPH047 cells. EGFRvIII was not detected (ND) in CPH036 cells (mean ± SEM, n = 3). **b**, **d** WB analysis of EGFR, EGFRvIII, notch 1- and -3, Dll-4 and Hes-1 protein in CPH036 and CPH047 cells. Tubulin serves as loading control

EC were subjected to conditioned media collected from GBM cells receiving either no treatment (DMSO) or treatment with 5 μM iressa and 5 μM DAPT alone or in combination (5 μM DAPT +5 μM iressa). We found that mono-therapy with Iressa or DAPT of CPH036 and CPH047 cells significantly reduced the capacity of GBM-induced EC spheroid sprouting (Fig. 4a–c, e). Upon treatment with combined DAPT and iressa an increased co-inhibitory effect of quantified EC sprouting could be seen compared to mono-therapy (Fig. 4c, e). In CPH036 cells, the co-inhibitory effect was confirmed to be additive for both the number ($p = 0.62$) and length ($p = 0.59$) of spouts. For the CPH047 cells the length of spouts was borderline non-significant ($p = 0.080$), demonstrating a trend towards additivity. Conversely, the co-inhibitory effect for the number of sprouts in CPH047 cells showed a significant interaction ($p = 0.046$), but with an effect that was less (76 % reduction) than would be expected if additive (83 % reduction), suggesting that the combination was sub-additive. Further, the inhibitory effect of DAPT and Iressa on EC sprouting could be confirmed not to be a result of non-metabolized inhibitor leftovers inducing EC death since conditioned media from GBM cells treated with DAPT, Iressa or a combination had no

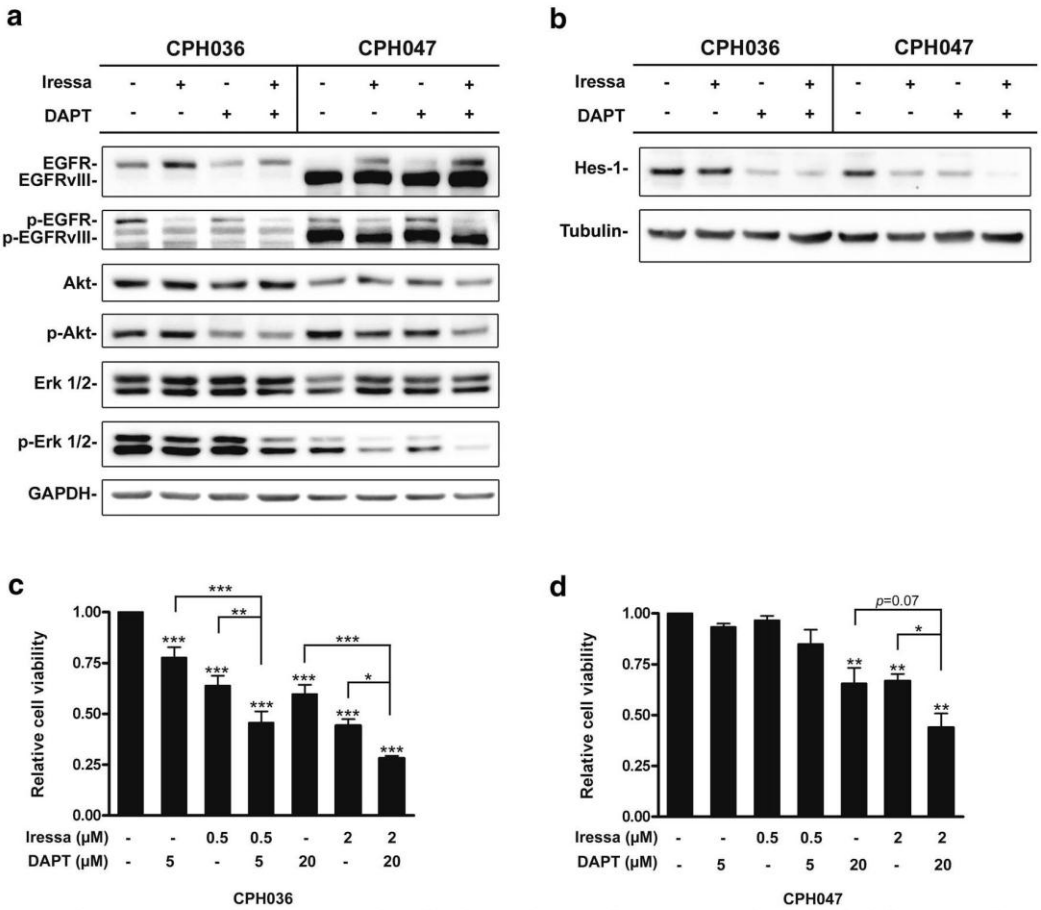

Fig. 2 Iressa and DAPT treatment inhibits the EGFR- and notch pathways abrogating downstream signaling and cell viability in vitro. **a**, **b** WB analysis of CPH36 and CPH047 cells treated with 5 μM iressa, 5 μM DAPT or a combination for 24 h. WB was investigated for expression of EGFR, EGFRvIII, Akt, p-Akt, Erk, p-Erk and Hes-1. GAPDH and tubulin serves as loading controls. **c**, **d** Cell viability of CPH036 or CPH047 cells following 7 days of treatment with iressa, DAPT or a combination in the indicated doses (mean ± SEM, n = 5). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

effect on EC proliferation (Additional file 1: Figure S3). To examine whether the effect of inhibited GBM-induced EC sprouting could be due to the effect of Iressa and DAPT on VEGF expression and secretion by the GBM cells, we measured VEGF secretion following treatment. Iressa treatment resulted in almost complete inhibition of VEGF expression and secretion in CPH036 and CPH047 cells (Fig. 4d, f), while DAPT treatment was able to partially abrogate VEGF expression and secretion, however with less potency, compared to iressa. Upon combined treatment no additive effect could be observed as a result of almost complete inhibition of VEGF secretion and expression by Iressa treatment (Fig. 4d, f). Summed, both iressa and DAPT display capacity to inhibit GBM-induced cell sprouting in EC and upon combinational treatment this effect is even further enhanced. Further, the results indicate that this effect, at least partly, could be a result of inhibition of VEGF expression.

Discussion

EGFR and notch are both involved in regulation of GBM cancer cells by promoting their survival, therapeutic resistance and pro-angiogenic signaling [13, 26, 27]. Thus, there is a rationale for treatment with inhibitors targeting both the EGFR and Notch signaling axis in GBM. The main focus of this study was to investigate the effect of simultaneous EGFR and notch abrogation on GBM cell maintenance and EC sprouting.

Aberrant expression of components of the EGFR and notch pathway has in GBM been confirmed previously [27,

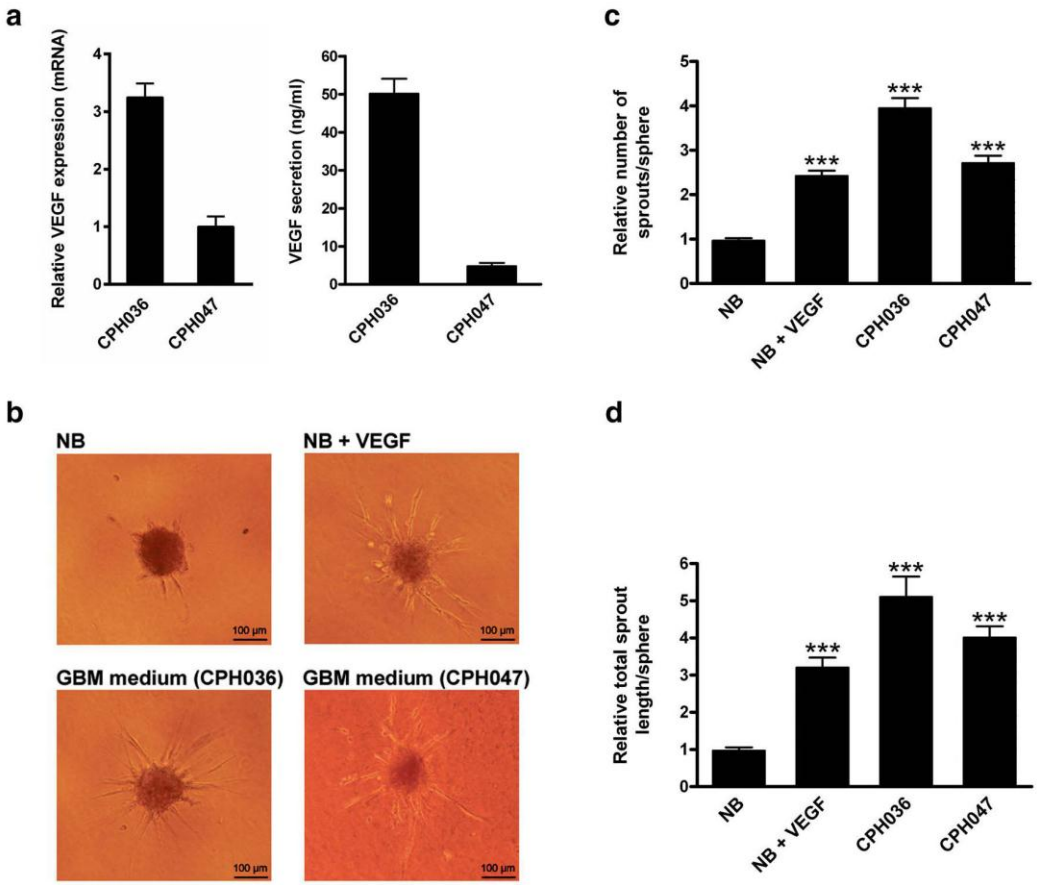

Fig. 3 EC cell sprouting stimulation by GBM-conditioned cell culture media. CPH036 and CPH047 cells were grown for 14 days and the conditioned media was collected and concentrated. EC were grown in presence of 20 ng/ml VEGF or conditioned media from CPH036 or CPH047 cells. **a** Relative VEGF expression analyzed by Q-RT-PCR, and VEGF secretion (ng/ml) analyzed by ELISA of CPH036 and CPH047 cells (mean ± SEM, n = 6 for Q-RT-PCR and n = 3 for ELISA). **b** Representative pictures showing sprouting of EC upon stimulation with VEGF or conditioned media from either CPH036 or CPH047 cells. **c**, **d** EC sprouting quantified as number of sprouts and total length of sprouts per sphere, respectively, and displayed relative to NB unconditioned media (mean ± SEM, n = 3 measuring an average of 10 spheres per condition in each experiment). *Scale bar* shows 100 µm. ***$p < 0.001$ vs. NB medium

28]. In accordance with this, we identified heterogeneous expression of EGFR/EGFRvIII, notch ligands and notch receptors in our GBM cell cultures. EGFR and Notch are important regulators of angiogenesis and abrogation of either of these pathways results in reduced angiogenesis in GBM [15, 29]. In the examined GBM cells, we confirmed endogenous expression and secretion of the key pro-angiogenic cytokine VEGF [25], for which increased expression has been correlated with increased glioma malignancy and poor prognosis [30, 31]. Furthermore, we confirmed that the examined GBM cells were able to induce EC sprouting (Fig. 3b–d) indicating that these cells had the capacity to induce neo-angiogenesis of surrounding EC by the secretion of pro-angiogenic factors.

Upon abrogation of Notch and EGFR signaling by DAPT or Iressa treatment, respectively, this inhibited the expression and secretion of VEGF in our GBM cells (Fig. 4d, f). This supports that VEGF-induced angiogenesis is dependent of active signaling through the notch and EGFR pathways as also shown by others [32–34]. Recently, Wang et al. [29] showed that combined treatment with the anti-EGFR antibody Cetuximab together with DAPT displayed downregulation of VEGF in Head Neck Squamous Cell Carcinoma [29], which is in line with our observations. VEGF is generally considered to be a positive upstream regulator of Notch with Notch acting as an upstream regulator of VEGFRs [35]. Moreover, our data demonstrate that Notch regulates VEGF

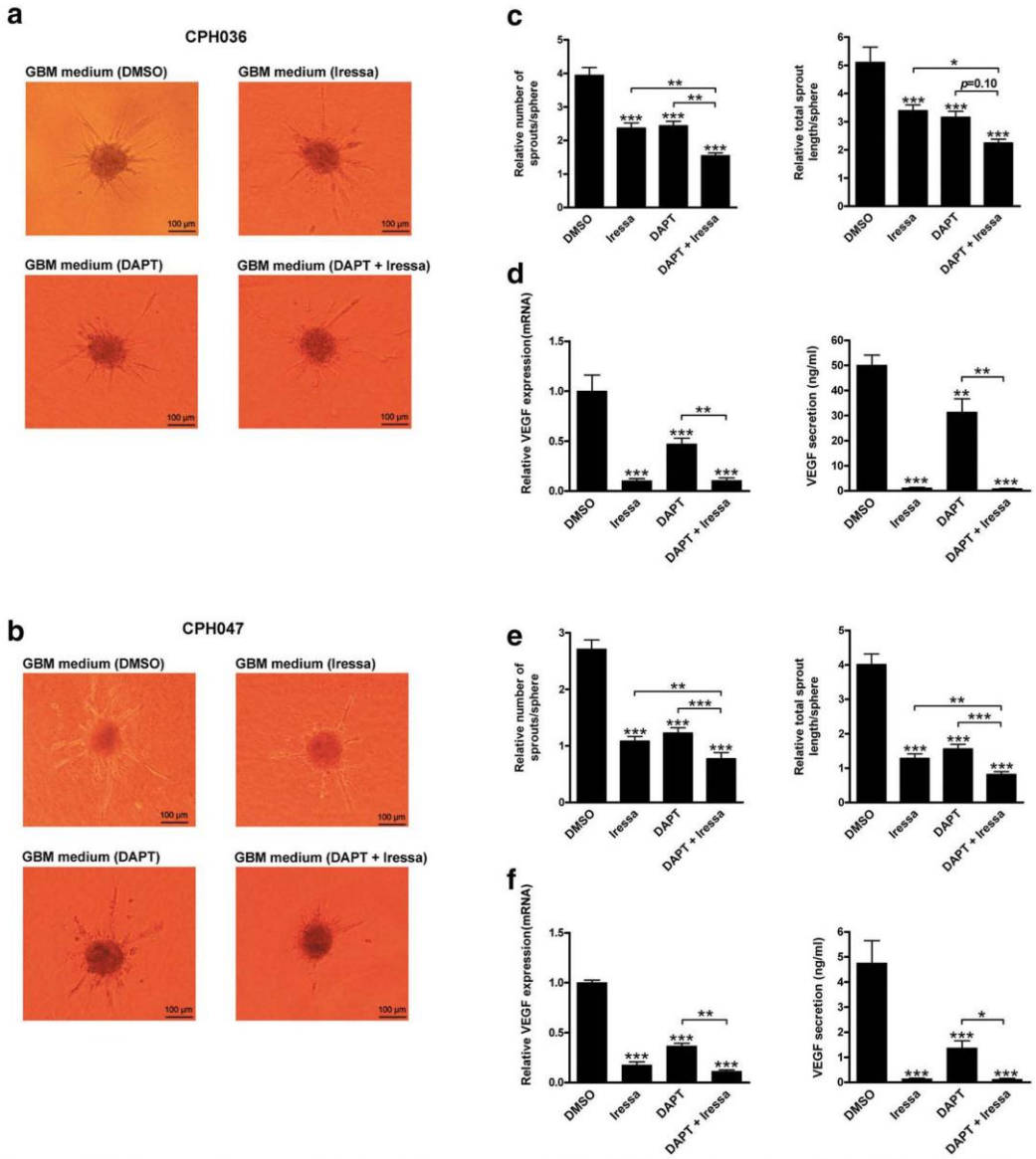

Fig. 4 Iressa and DAPT treatment abrogates GBM-induced EC sprouting, and inhibits VEGF expression and secretion in GBM cells. CPH036 and CPH047 cells were treated with 5 µM Iressa, 5 µM DAPT or a combination for 14 days. The conditioned GBM media was concentrated and subjected to EC for sprouting analysis. **a**, **b** Representative pictures showing sprouting of EC upon administration of conditioned media obtained from CPH036 or CPH047 cells treated with indicated inhibitors. **c**, **e** Relative EC sprouting quantified as total number of sprouts and total length of sprouts per sphere upon stimulation with conditioned inhibitor-treated media from either CPH036 or CPH047 cells, respectively (mean ± SEM, n = 3 measuring an average of 10 spheres per condition in each experiment). **d**, **f** VEGF mRNA levels in inhibitor-treated CPH036 and CPH047 cells were analyzed by Q-RT-PCR and normalized to the mean gene expression of the control. VEGF secretion levels (ng/ml) analyzed by ELISA from inhibitor-treated CPH036 and CPH047 cells as measured by VEGF-ELISA (mean ± SEM, n = 3). *Scale bar* shows 100 µm. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

expression, indicating the existence of a positive feedback-loop regulatory mechanism.

Studies have shown that treatment with small molecule inhibitors targeting EGFR or Notch is able to inhibit GBM angiogenesis in vitro [36, 37]. We observed that treatment with DAPT plus iressa was not sufficient to fully block EC sprouting (Fig. 4c, e) despite almost complete inhibition of VEGF secretion upon combined treatment (Fig. 4d, f)

suggesting that other angiogenic stimulators are involved in GBM-induced EC sprouting. Factors including angiogenin, PDGF-AA, IGFBP-3 are known to be implicated in angiogenesis [38–40] and were confirmed to be present in the GBM-conditioned media at comparable levels to VEGF (Additional file 1: Figure S1) which could explain additional stimulation of EC sprouting.

Aberrant EGFR and notch signaling regulate cell viability and therapeutic resistance of GBM cells [17, 27, 41]. Both, EGFR and notch regulated signaling are in GBM linked to the RAS-RAF-MEK-ERK and the PI3K-Akt-mTOR signaling pathways [42, 43]. Interestingly, it has been shown that Notch signaling is dependent on mTOR in lung and kidney tumor cells [44], indicating the existence of a positive feedback loop between Notch and EGFR signaling. Our results show that the inhibition of EGFR signaling results in decreased Hes-1 levels supporting that EGFR signaling stimulate activity of the Notch pathway. Further, we found upon combined treatment targeting both Notch and EGFR an increased inhibition of GBM cell viability compared to mono-therapy alone. This was probably a result of more effective inhibition of the pro-survival pathways Akt and Erk which we observed upon combination therapy (Fig. 2a). Cenciarelli et al. [28] showed that co-treatment with GSI-X and AG1478 (targeting Notch and EGFR, respectively) displayed synergistic anti-proliferative effects in GBM in vitro [28]. Taken together, our data and those of others indicate redundant signaling between EGFR and Notch, which indicate a need for further preclinical and clinical evaluation of simultaneous inhibition of Notch and EGFR which are upstream of key pro-survival regulatory pathways as the RAS-RAF-MEK-ERK and PI3K-Akt-mTOR.

Over the last years, a number of pharmacological studies have been conducted testing either EGFR or Notch pathway inhibitors in patients with various cancer types including GBM (http://www.clinicaltrial.gov). For GBM patients a number of different EGFR targeting drugs have been tested in the clinic, but overall results have been disappointing with non or very limited clinical benefits [45]. So far, only one study has been reported for the use of a notch-specific inhibitor in glioma patients. In this study, the Merck-developed GSI termed MK-0752 was tested in various advanced solid tumors and the results indicated some clinical benefits especially in glioma patients [46]. Still, the anti-tumor activity was not impressive with most patients obtaining stable disease as best response [46]. Data from a currently ongoing phase II trial, treating patients with recurrent or progressive GBM with another GSI (RO4929097), are yet to be published, but will further shed light on the effect of single-agent treatment with Notch inhibitors. Overall, the results from recent and/or ongoing clinical trials evaluating EGFR- and notch-specific inhibitors as mono-therapies imply certain clinical limitations of this approach.

In this study, we find that a combined treatment strategy that targets both EGFR and notch signaling pathways results in enhanced inhibitory effect on cell viability and EC sprouting, compared to either of the mono-therapies, supporting the important role of notch/EGFR signaling cross-talk in GBM. Taken together, this fact and the above mentioned clinical studies support the rationale for combined treatment strategy employing both EGFR and notch inhibitors.

Additional file

Additional file 1: Figure S1. Evaluation of angiogenic factors secreted by GBM cells. **Figure S2.** Expression of VEGF receptors (VEGFR-1 and VEGFR-2) in GBM cell cultures CPH036 and CPH047 and in endothelial cells (HMVEC). **Figure S3.** Effect of conditioned cell media from GBM cell cultures treated with inhibitors on endothelial cell proliferation. **Figure S4.** Overview of primary antibodies used for western blotting. **Figure S5.** Overview of primer sets used for Q-RT-PCR.

Abbreviations

GBM: glioblastoma; EGFR: epidermal growth factor receptor; VEGF: vascular endothelial growth factor A; TKI: tyrosine-kinase inhibitor; GSI: gamma-secretase inhibitor; EC: endothelial cell.

Authors' contributions

MTS and HSP designed the study. MS, SRM, LSO and MV all participated in the experimental work. PH and MKN assisted in data interpretation and revision of the manuscript. MS and SRM drafted the manuscript. All authors read and approved the final manuscript.

Author details

[1] Department of Radiation Biology, The Finsen Center, Copenhagen University Hospital, Copenhagen, Denmark. [2] Department of Clinical Physiology, Nuclear Medicine & PET and Cluster for Molecular Imaging, Copenhagen University Hospital and University of Copenhagen, Copenhagen, Denmark. [3] Brain Tumor Biology Group, Danish Cancer Society Research Center, Copenhagen, Denmark.

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Competing interests

The authors declare that they have no competing interests.

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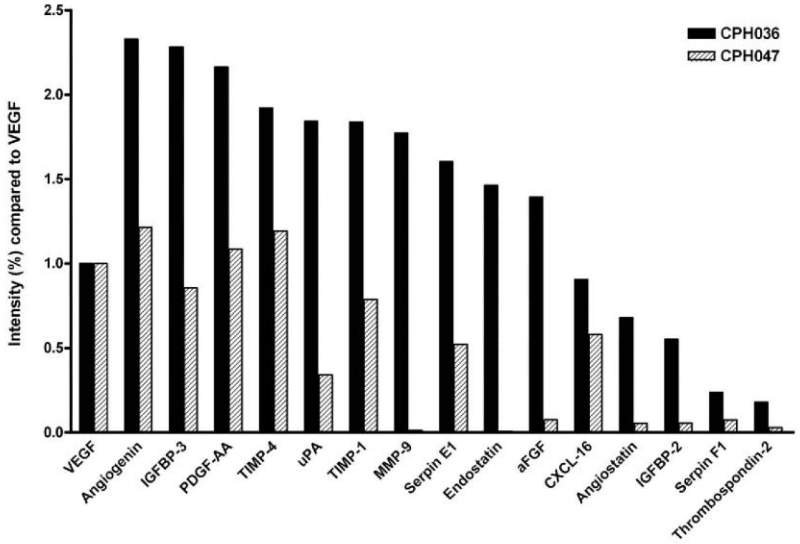
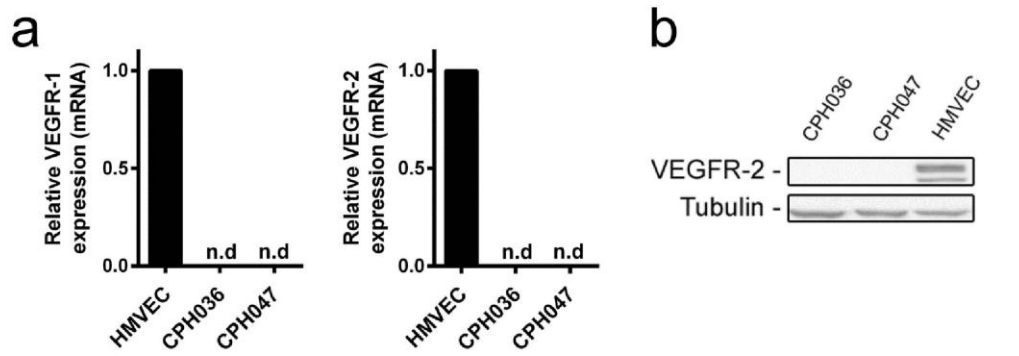
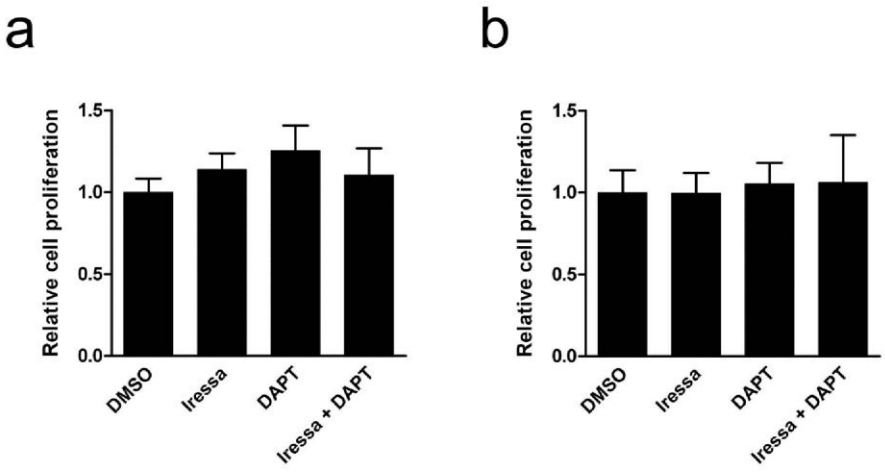


Supplementary Figure 1. Evaluation of angiogenic factors secreted by GBM cells. Relative secretion of angiogenic factors set relative to VEGF secretion. One million GBM cells (CPH036 and CPH047) were incubated for 14 days and the conditioned cell media was harvested, concentrated around 10 times and subjected to the Proteome Profiler human angiogenesis array kit (R&D systems) used in accordance with the manufacturer's instructions. Signal was detected utilizing the Super-Signal West Dura extended Duration Substrate (Pierce Biotechnology) in the UVP Biospectrum AC imaging system which also was used for quantification of relative protein expression. Mean of one experiment performed in duplicate is shown.



Supplementary Figure 2. Expression of VEGF receptors (VEGFR-1 and VEGFR-2) in GBM cell cultures CPH036 and CPH047 and in endothelial cells (HMVEC). (a) Expression levels of VEGFR-1 and VEGFR-2 evaluated by Q-RT-PCR. (b) Expression of VEGFR-2 identified by western blotting using primary antibody against VEGFR-2 and tubulin.



Supplementary Figure 3. Effect of conditioned cell media from GBM cell cultures treated with inhibitors on endothelial cell proliferation. Conditioned media was obtained from $1x10^6$ cells of either CPH036 (a) or CPH047 (b) cells treated for 14 days with DMSO, 5µM Iressa, 5µM DAPT or a combination. HMVEC (EC) cells were plated in 96-well plates (2,000 cells in 0.1 mL EC media) and the following day the media was changed to EC media added 10% of conditioned media from the GBM cells that had been concentrated around 10 times. Cells were incubated for 20 hrs and level of proliferation was examined by BrdU assay using the Cell Proliferation ELISA, BrdU kit (Roche A/S) using a 20 hrs BrdU incubation step and otherwise following the instructions by the manufacturer. Quantification was done by measuring the absorbance at 370nm with 492nm as a reference using Synergy2 microplate reader with Gen5. Data are shown as mean ± SEM obtained from three independent experiments. Significant difference was tested with a one-way ANOVA with a Tukey post-hoc test.

Dilution	Antibody	Manufacturer
1:1000	Rabbit anti-Akt	Cell Signaling, #9272
1:1000	Rabbit anti-pAkt	Cell Signaling, #9271
1:1000	Rabbit anti-Erk 1/2	Cell Signaling, #9102
1:1000	Mouse anti-pErk 1/2	Cell Signaling, #9106
1:20.000	Sheep anti-EGFR	Fitzgerald, #20-ES04
1:1000	Rabbit anti-pEGFR	Invitrogen, #44-790G
1:50	Mouse anti-EGFRvIII	Duke University, L8A4
1:100	Goat anti-Notch 1	Santa Cruz, #SC-23304
1:1000	Rabbit anti-Notch 3	Cell Signaling, #5276
1:1000	Rabbit anti-Dll 4	Abcam, #ab7280
1:2000	Rabbit anti-Hes 1	Toray Industries inc, Japan
1:1000	Rabbit anti-VEGFR-2	Cell signaling, #55b11
1:1000	Rabbit anti-Tubulin	Cell Signaling, #2125

Supplementary Figure 4. Overview of primary antibodies used for western blotting. Antibodies are listed with dilution, manufacturer (university) and catalog number.

Target gene	Forward primer (5' - 3')	Reverse primer (5' - 3')
EGFR	GGC ATA GGA ATT TTC GTA GTA CAT	TCC TTG GGA ATT TGG AAA TT
EGFRvIII	ATG CGA CCC TCC GGG ACG	ATC TGT CAC ATA ATT ACC T
Dll-1	GCC GAC AAG AAT GGC TTC	CCG GCC TTT TTC TTT CAG
Dll-4	GGT CAG ACC TGG TTA TTG G	CGA AAG ACA GAT AGG CTG
Hes-1	AGC GGG CGC AGA TGA C	CGT TCA TGC ACT CGC TGA A
Notch-1	CTT CCC CTA CGG CCG CGA	CAG GTA GAC GAT GGA GCC GCG GA
Notch-2	GCC TGT ATG TGC CCT GTG CAC C	AGC CTC CAT TGC GGT TGG CAC
Notch-3	CTG GCT GAC AGC TCG GTC ACG C	AGT GGC AGT GGC AGC TGC ATA G
Jagged-1	ATG GGG AGT GTG ATA CCA	GAG ACT GGA AGA CCG ACA
Jagged-2	CGG CCA CCT GGA CAA TAA	CAA CCG TCT CCA CCT TGA
VEGF	CCT TGC TGC TCT ACC TCC AC	ATC TGC ATG GTG ATG TTG GA
VEGFR-1	GGC TCT GTG GAA AGT TCA GC	GTG ACC AAC ATG GAG TCG TG
VEGFR-2	GTG ACC AAC ATG GAG TCG TG	TGC TTC ACA GAA GAC CAT GC

Supplementary Figure 5. Overview of primer sets used for Q-RT-PCR. All primers were obtained from DNA Technology A/S.

5. STUDY II

Inhibition of histone deacetylases sensitizes glioblastoma cells to lomustine

Mikkel Staberg, Signe Regner Michaelsen, Rikke Darling Rasmussen, Mette Villingshøj, Hans Skovgaard Poulsen, and Petra Hamerlik

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ORIGINAL PAPER

Inhibition of histone deacetylases sensitizes glioblastoma cells to lomustine

Mikkel Staberg[1] · Signe Regner Michaelsen[1] · Rikke Darling Rasmussen[2] · Mette Villingshøj[1] · Hans Skovgaard Poulsen[1] · Petra Hamerlik[2]

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Abstract
Purpose Glioblastoma (GBM) ranks among the deadliest solid cancers worldwide and its prognosis has remained dismal, despite the use of aggressive chemo-irradiation treatment regimens. Limited drug delivery into the brain parenchyma and frequent resistance to currently available therapies are problems that call for a prompt development of novel therapeutic strategies. While only displaying modest efficacies as mono-therapy in pre-clinical settings, histone deacetylase inhibitors (HDACi) have shown promising sensitizing effects to a number of cytotoxic agents. Here, we sought to investigate the sensitizing effect of the HDACi trichostatin A (TSA) to the alkylating agent lomustine (CCNU), which is used in the clinic for the treatment of GBM.
Methods Twelve primary GBM cell cultures grown as neurospheres were used in this study, as well as one established GBM-derived cell line (U87 MG). Histone deacetylase (HDAC) expression levels were determined using quantitative real-time PCR and Western blotting. The efficacy of either CCNU alone or its combination with TSA was assessed using various assays, i.e., cell viability assays (MTT), cell cycle assays (flow cytometry, FACS), double-strand DNA break (DSB) quantification assays (microscopy/immunofluorescence) and expression profiling assays of proteins involved in apoptosis and cell stress (Western blotting and protein array).
Results We found that the HDAC1, 3 and 6 expression levels were significantly increased in GBM samples compared to non-neoplastic brain control samples. Additionally, we found that pre-treatment of GBM cells with TSA resulted in an enhancement of their sensitivity to CCNU, possibly via the accumulation of DSBs, decreased cell proliferation and viability rates, and an increased apoptotic rate.
Conclusion From our data we conclude that the combined administration of TSA and CCNU eradicates GBM cells with a higher efficacy than either drug alone, thereby opening a novel avenue for the treatment of GBM.

Keywords Glioblastoma · Epigenetics · Trichostatin A · HDACi · Lomustine · Sensitization

Abbreviations

GBM	glioblastoma
TSA	trichostatin A
HDACi	histone deacetylase inhibitor
CCNU	lomustine
DSB	double-stranded break

1 Introduction

Glioblastoma (GBM) is the most prevalent and aggressive brain tumor in adults with an average survival rate of approximately 15 months, despite maximal therapeutic intervention [1]. A high degree of heterogeneity, a robust vasculature, as well as enduring resistance to

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✉ Mikkel Staberg
mikkel.staberg@regionh.dk

✉ Petra Hamerlik
pkn@cancer.dk

[1] Department of Radiation Biology, The Finsen Center, Rigshospitalet, Copenhagen, Denmark

[2] Brain Tumor Biology Group, Danish Cancer Society Research Center, Copenhagen, Denmark

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current therapies are among the most prevalent hallmarks of GBM [2].

DNA methylation and histone modifications are common epigenetic changes that play central roles in many cellular processes, including proliferation, survival, angiogenesis, invasion and differentiation [3–6]. Functional interplays between histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate the chromatin state (relaxation and condensation, respectively) and its accessibility to DNA repair proteins upon induction of DNA damage [3, 7]. Epigenetic deregulation has long been suggested to play a role in gliomagenesis [8]. Class I HDACs have been found to be associated with the occurrence of de-differentiated, locally advanced and strongly proliferating tumors [9, 10], whereas class II HDACs have been found to be directly involved in tumorigenesis [11]. In the past, several HDAC inhibitors (HDACi) have been developed and US FDA-approved, such as vorinostat and romidepsin for the treatment of refractory cutaneous T-cell lymphoma [12]. Trichostatin A (TSA) is a pan-HDACi targeting class I/II HDACs and has been shown to sensitize GBM cells to radiation by inducing cell cycle arrest and differentiation [13–15].

GBMs are genetically highly unstable and exhibit a constitutive activation of DNA damage response pathways essential for their survival [16]. Based on this notion, agents preventing the repair of DNA damage in conjunction with chemo-irradiation have been developed for the treatment of GBM patients. One of these agents, lomustine (CCNU) is a highly lipid-soluble DNA alkylator that crosses the blood–brain barrier and has been shown to target proliferating GBM cells both in vitro and in vivo [17, 18]. It was also found, however, that CCNU treatment alone yielded only a modest improvement in GBM patient survival [19–21]. Thus, we hypothesized that pre-treatment of GBM cells with a HDACi (TSA) would result in chromatin restructuring and, thus, a limited access of the DNA repair machinery to DNA damage induced by CCNU, thereby increasing its therapeutic efficacy.

Here, we show that GBM cells exhibit elevated HDAC1, HDAC3 and HDAC6 expression levels compared to non-neoplastic brain tissues. Combined treatment of GBM cells with TSA and CCNU proved more efficient in impairing cell cycle progression and cell viability compared to either drug alone, which may at least in part be due to a reduced DNA repair capacity and a higher apoptotic rate. Our data indicate that the therapeutic efficacy of CCNU may be improved by co-administration of TSA, which warrants further preclinical evaluation of this combinational approach in the management of GBM.

2 Materials and methods

2.1 Primary cell cultures and reagents

GBM cell lines 017 (CPH017p4), 036 (CPH036p6), 047 (CPH047p3m1) and 048 (CPH048p6) have been described before [22]. GBM cell lines 4121 (GBM03), IN84 (GBM02), Xeus, T91, T115, IN326, 1587 and 1966 were obtained from The Danish Cancer Society Research Center and have also been described before [23, 24]. The GBM-derived cell line U87 MG (HTB-14) was purchased from the ATCC (VA, USA). For the in vitro studies GBM cells were maintained in Neurobasal®-A medium supplemented with N2, B27, bFGF (10 ng/ml), EGF (10 ng/ml), L-glutamine, penicillin (50 U/ml) and streptomycin (50 µg/ml) (Invitrogen, Taastrup, Denmark), and incubated in cell culture flasks in an incubator with 5 % CO_2 at 37 °C. Twice a week fresh medium was added and spheres were dissociated at every passage. For the experiments, cells were dissociated, counted using a NucleoCounter® NC-200 (Chemometec, Allerod, Denmark), seeded in media with supplements and treated with trichostatin A (TSA), lomustine (CCNU) or a combination of these agents (Sigma-Aldrich, CA, USA). As a control, cells were treated with equal concentrations of DMSO.

2.2 Patient tissues

Tumor tissues were obtained from GBM patients during initial surgery at Rigshospitalet, Denmark with written consent. The tumor tissues were used according to the Declaration of Helsinki and the Danish legislation. Tumors were diagnosed as GBM according to the WHO 2000/2007 guidelines, and the use of patient-derived tissues was approved by the Danish Data Protection Agency (2006-41-6979) and the Scientific Ethical Committee for Copenhagen and Frederiksberg (KF-01-327718). At the Danish Cancer Society Research Center tissues for the generation of cell cultures were obtained in agreement with the Danish Ethical Committee guidelines and in all cases informed consent was obtained from the patients the day before surgery.

2.3 In silico analysis

For survival and HDAC expression analyses in normal brain tissues compared to gliomas (WHO grade II, III and IV) the Repository for Molecular Brain Neoplasia Data (Rembrandt) data set was used, available at the GlioVis website (http://gliovis.bioinfo.cnio.es/).

2.4 Western blotting

For the inhibitor studies, cells were pre-treated with TSA for 6 h followed by treatment with CCNU for an additional 24 h and harvested. Whole cell protein lysates were prepared from cell pellets by sonication in ice-cold modified RIPA buffer (50 mM Tris–HCl [pH 7.4], 1 % NP40, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with protease and phosphatase inhibitor mixtures II and III (Millipore, Copenhagen, Denmark). Tissue protein lysates were extracted in the same buffer using a TissueLyser (Qiagen) and protein concentrations were determined using a BCA assay (Pierce Biotechnology, Rockford, IL). Next, the proteins were separated in 4–12 % NuPAGE Bis-Tris gels (Invitrogen) and electro-blotted onto nitrocellulose membranes (Invitrogen). Subsequently, the membranes were blocked for 1 h at room temperature (RT) and incubated with primary antibodies in 5 % non-fat milk overnight (ON) at 4 °C followed by incubation with horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h at RT. Finally, the blots were developed using a SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology) after which the protein bands were captured using a Biospectrum Imaging System (UVP, Upland, CA, USA). The primary antibodies used are listed in Supplementary Table S1.

2.5 MTT assay

Cell viabilities were measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Sigma-Aldrich). To this end, GBM cells were dissociated and seeded at a density of 2×10^4 cells per well in 96-well plates and incubated ON. After this, the cells were pre-treated with TSA for 24 h followed by the addition of CCNU and incubated for another 72 h. After this treatment, 20 µl (5 mg/ml) MTT solution was added to each well and incubated for 4 h followed by the addition of 100 µl solubilization buffer (10 % SDS, 0.03 M HCl). The next day the absorbance was measured at 570 nm with 690 nm as background reference using a Synergy2 microplate reader in conjunction with Gen5, Microplate Data Collection and Analysis Software (Biotek, Winooski, VT, USA). Each experiment was performed in three replicate wells and repeated independently at least three times.

2.6 Quantitative real-time PCR

Total RNA was extracted from GBM spheres and cell pellets using a QIAshredder and RNeasy Mini kit (Qiagen). Total RNA from tumor tissue samples was extracted using TRIzol®Reagent (Gibco) and a Qiagen TissueLyser prior to purification using a RNeasy Mini kit. For cDNA synthesis and quantitative real-time PCR (qRT-PCR) amplifications a SuperscriptTM III Platinum® Two Step qRT-PCR kit with SYBR® Green (Invitrogen) was used. Gene expression levels were quantified using a comparative Ct method and normalized to the expression of three genes (*TOP1*, *EIF4A2* and *CYC1*) included in the human geNorm housekeeping gene selection kit (Primerdesign, Southampton, UK). The primers used for qRT-PCR were: HDAC3 forward: 5′-TAG ACA AGG ACT GAG ATT GCC-3′; HDAC3 reverse: 5′-GTG TTA GGG AGC CAG AGC C-3′; HDAC1 forward: 5′-GGT CCA AAT GCA GGC GAT TCC T-3′; HDAC1 reverse: 5′-TCG GAG AAC TCT TCC TCA CAG G-3′; HDAC4 forward: 5′-AGG TGA AGC AGG AGC CCA TTG A-3′, HDAC4 reverse: 5′-GGT AGT TCC TCA GCT GGT GGA T-3′, HDAC6 forward: 5′-GCC TCA ATC ACT GAG ACC ATC C-3′; HDAC6 reverse: 5′-GGT GCC TTC TTG GTG ACC AAC T-3′ (TAG Copenhagen A/S, Denmark). The gene expression levels were related to those in normal brain (NB) obtained from Clontech (Takara), USA, cat. # 80151 and Ambion, USA, cat. # 7962 or normal human astrocytes (NHA) obtained from Lonza, Switzerland, cat. # CC-2565.

2.7 Immunofluorescence imaging

GBM cells (2×10^5 per condition) were seeded on coverslips pre-coated with Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Thermo Fisher Scientific). The next day, the cells were pre-treated with TSA for 6 h followed by treatment with CCNU for another 24 h. Next, the cells were washed in PBS, fixed in 4 % paraformaldehyde and immunostained using an anti-γH2AX Ser139 antibody (1:1000, Millipore # 05–636). Secondary detection was carried out using an Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) and the nuclei of the cells were counterstained with DAPI. Imaging was performed on a Zeiss LSM 700 Confocal Microscope and quantification was performed using a ScanR screening station (Olympus) by acquiring 100 non-overlapping images for each treatment condition. A minimum of 1000 cells was assessed and processed using the ScanR analysis software tool (Olympus). Each experiment was repeated independently at least two times.

2.8 Cell cycle analysis

For cell cycle analysis, GBM cells (2×10^5 per well) were seeded in 6-well plates and incubated ON. The next day, the cells were pre-treated with TSA for 6 h, followed by treatment with CCNU for another 24 h. During the last 20–60 min of

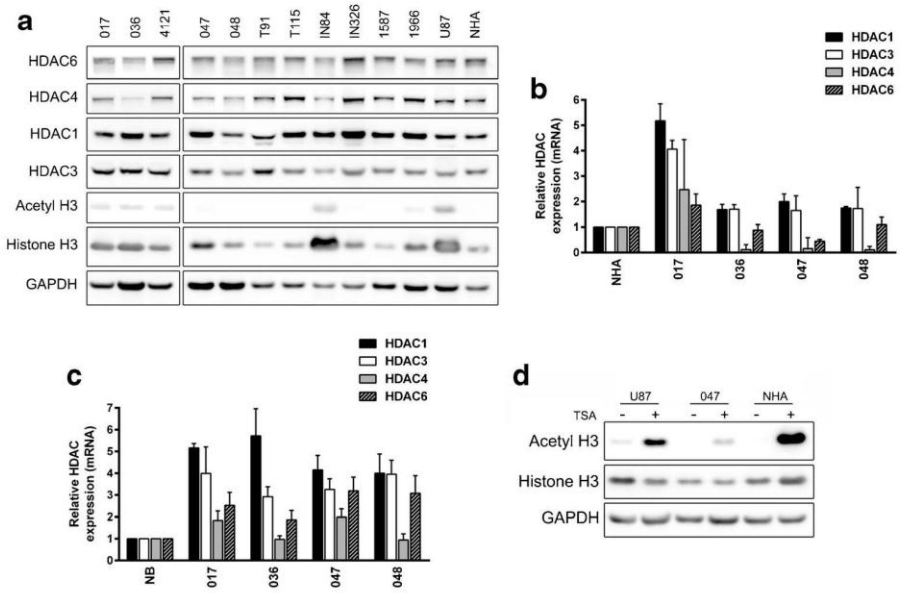
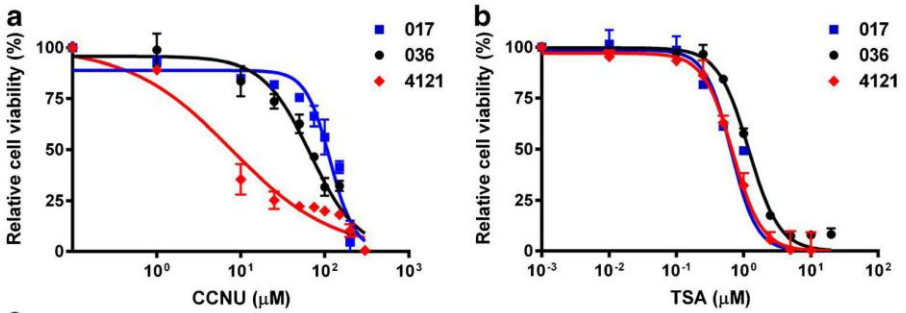


Fig. 1 Increased HDAC expression in GBM. **a** GBM cell panel and normal human astrocytes (NHA) assessed for HDAC1, HDAC3, HDAC4, HDAC6, acetylated histone H3 and total histone H3 expression by Western blotting. **b** Patient-derived cell cultures and (**c**) patient tissues assessed for HDAC1, HDAC3, HDAC4 and HDAC6 expression relative to NHA or normal brain (NB), respectively, by qRT-PCR. **d** U87, 047 and NHA cells treated with 0.5 μM TSA for 24 h assessed for acetylated histone H3 and total histone H3 expression using Western blotting. GAPDH was used as a loading control for the Western blots

this treatment, the cells were pulse-labelled with 10 μM 5-ethynyl-2′-deoxyuridine (EdU), after which the cells were collected and fixed in 4 % paraformaldehyde. Next, the cells were subjected to flow cytometry staining using a Click-iT EdU Alexa Flour 647 Flow Cytometry Assay Kit (Invitrogen) following the manufacturer's instructions. Flow images were acquired on a FACS Verse Cell Sorter (BD Biosciences) and the data were analyzed using FlowJo software. Single cells were analyzed by gating out doublets and quantified as cells in the G1, S, or G2/M phase of the cell cycle. For each condition, 10.000 events were acquired and each experiment was repeated independently at least three times.

2.9 Mitotic index analysis

In order to assess cells arrested in mitosis we performed flow cytometry of cells positive for phospho-histone H3



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Cell lines	GI_{50} CCNU (μM)	GI_{50} TSA (μM)
017	119±19	0.65±0.1
036	67±4	1.19±0.01
4121	8±1.5	0.69±0.06

Fig. 2 Differential inhibitory effects of TSA and CCNU on GBM cell viability. GBM cells (017, 036, 4121) were treated with increasing concentrations of **a** CCNU and **b** TSA for 72 h after which cell viabilities were assessed by MTT assay. **c** GI_{50} values calculated for each cell line treated with CCNU or TSA as indicated. Data are presented as mean ± SEM ($n = 2$)

Ser10 staining (i.e., cells in mitosis). GBM cells (2×10^5 per well) were seeded in 6-well plates and incubated ON. The next day, the cells were pre-treated with TSA for 6 h, followed by treatment with CCNU for another 24 h. Twelve hours before the end of this treatment, the cells were administered either nocadazole or DMSO. Next, the cells were collected, fixed in 4 % paraformaldehyde, stained for p-histone H3 Ser10 (Cell Signaling, #3377) and counterstained with Hoechst. Finally, images were acquired on a FACS Verse Cell Sorter (BD Biosciences) and the data were analyzed using FlowJo software.

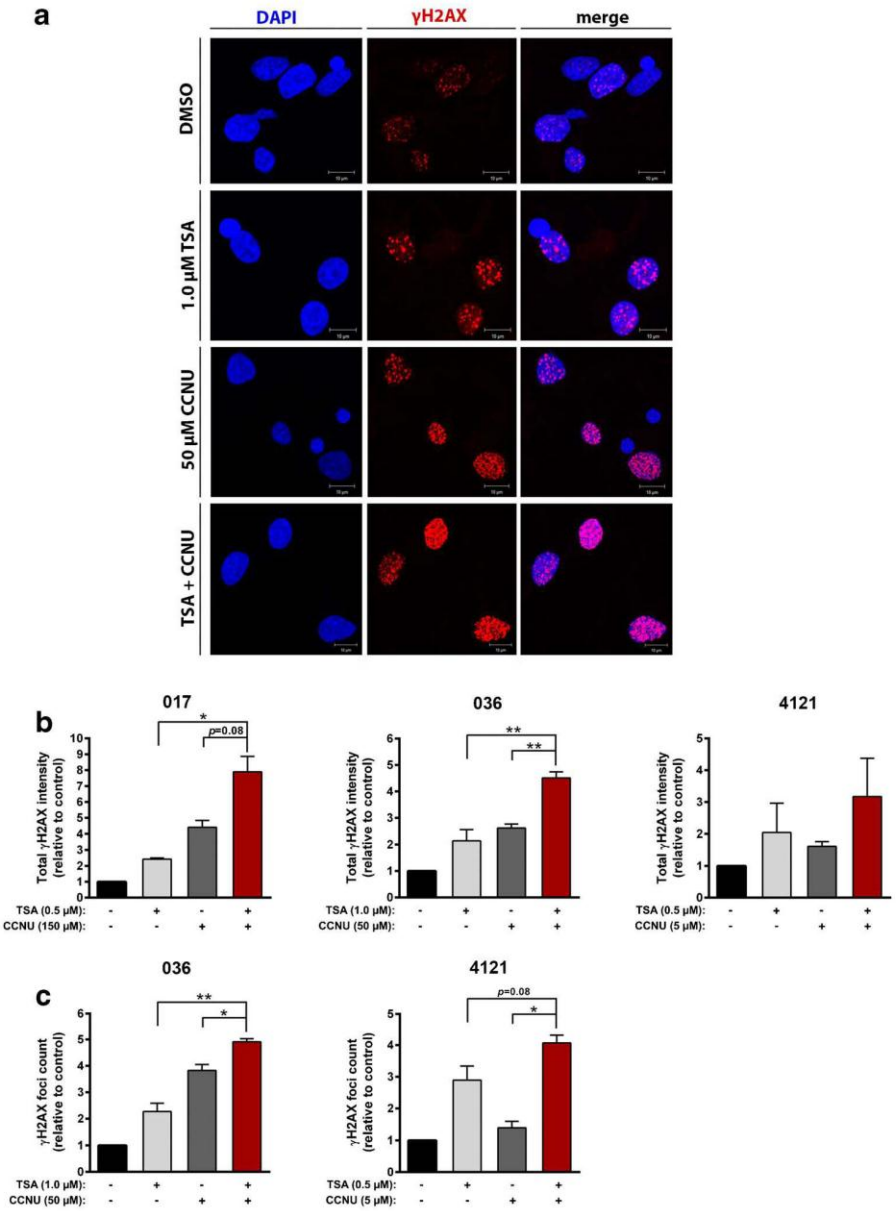
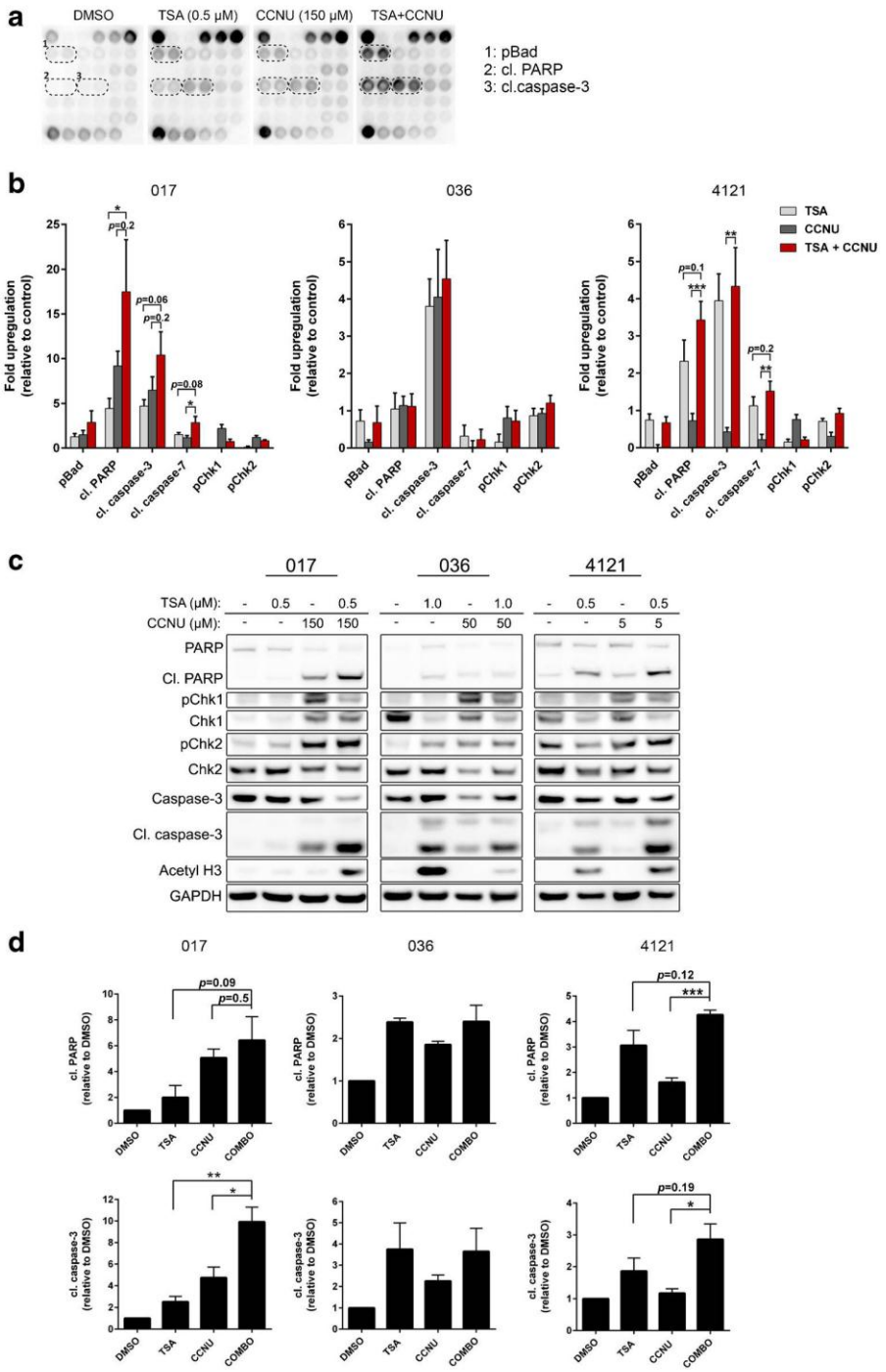


Fig. 3 TSA and CCNU treatment results in enhanced induction of DNA double stranded breaks. GBM cells (017, 036, 4121) were treated with the indicated GI_{50}-derived concentrations of CCNU, TSA or its combination and subjected to immunofluorescence analyses. The cells were stained with an anti-γH2AX Ser139 antibody and counterstained with DAPI. **a** Representative images showing γH2AX staining in 036 cells treated with DMSO (control), TSA, CCNU or its combination. **b** Quantified total γH2AX intensities and **c** γH2AX foci counts obtained from 100 non-overlapping images in GBM cells treated with TSA, CCNU or its combination, relative to control (DMSO; *black bar*) treated cells. The γH2AX foci count could not be quantified in 017 cells due to both intense and diffuse signals in these cells. Data are presented as mean ± SEM ($n = 2$ for 017 and $n = 3$ for 036 and 4121 cells). * $p < 0.05$, ** $p < 0.01$



2.10 Cell stress and apoptosis protein array

GBM cells (0.5–1.0 x 10^6 per condition) were pre-treated with TSA for 6 h, followed by treatment with CCNU for another 24 h. Next, the cells were harvested and lysed in a PathScan Sandwich ELISA Lysis Buffer (Cell Signaling Technology, MA, USA). The protein concentrations of the cell lysates were determined using a BCA assay. Subsequently, the cell lysates were transferred to a Pathscan Stress and Apoptosis Signaling Antibody Array Kit (Cell Signaling Technology) for the evaluation of 18 proteins involved in cell stress

◀ **Fig. 4** TSA and CCNU treatment results in apoptosis through the activation of pro-apoptotic proteins. GBM cells (017, 036, 4121) were treated with CCNU, TSA or its combination, harvested and submitted to protein array and Western blot analyses. **a** Representative image showing the levels of 18 phosphorylated proteins involved in cell stress and apoptosis, evaluated by array hybridization in 017 cells. Three major pro-apoptotic proteins (pBad, cleaved PARP and cleaved caspase-3) are marked 1, 2 and 3. **b** Quantitative analysis of the protein arrays showing fold differences in protein levels in cells treated with TSA, CCNU or its combination. The concentrations used of TSA and CCNU correspond to the designated concentrations in the Western blots under c. The quantified values were normalized to internal α-tubulin levels and set relative to the control (DMSO). Data are presented as mean ± SEM ($n = 2$ for 036 and $n = 3$ for 017 and 4121 cells). **c** GBM cell lysates submitted to Western blotting using anti-pChk1 (Ser345), anti-total Chk1, anti-pChk2 (T68), anti-total Chk2, anti-cleaved caspase-3, anti-total caspase-3, anti-cleaved/anti-total PARP and anti-acetylated histone H3 antibodies. GAPDH serves as loading control. **d** Semi-quantification of densitometry of cleaved PARP and cleaved caspase-3 levels deduced from Western blotting. The quantified expression levels were normalized to internal GAPDH levels and set relative to control (DMSO) levels. Data are presented as mean ± SEM ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

and apoptosis. In short, cell lysates were diluted to 0.4–0.8 μg/μl and applied to a nitrocellulose-coated glass slide with primary antibodies and incubated for 2 h. Next, the slide was washed and incubated with a detection antibody cocktail for 1 h. Subsequently, the slide was washed and incubated 30 min with a HRP-conjugated secondary antibody after which a LumiGLO/Peroxide solution (Cell Signaling Technology) was added. Finally, the slide was developed using a Biospectrum Imaging System (UVP) and the signal intensities of each spot (protein) were measured using an UVP system, normalized to intern α-tubulin levels and set relative to control (DMSO) treatment. Each experiment was performed on duplicate spots and repeated independently at least two times.

2.11 Statistics

Data are expressed as mean ± standard deviation (SD) or standard error of mean (SEM). Statistical analyses were performed using a two-tailed Student's t-test. The software used for statistics and the generation of figures was Graphpad Prism 6.0 for windows (GraphPad, San Diego, CA, USA).

3 Results

3.1 HDAC 1, 3 and 6 are overexpressed in GBM

First, we sought to assess the expression of representative members of class I (HDAC1 and HDAC3) and class II (HDAC4 and HDAC6) HDACs in both GBM cells and non-neoplastic brain control cells (normal human astrocytes, NHA). We found that the expression of HDAC1, 3 and 6 were slightly and variably increased in GBM cells compared to NHA cells at both the protein (Fig. 1a) and the mRNA (Fig. 1b) level. In contrast, we found that the expression of HDAC4 was not increased in most of the GBM cells tested. Next, we set out to confirm our findings using a panel of primary GBM tissue samples, and found that the expression levels of HDAC1 and HDAC3 were markedly increased compared to those in normal brain tissues (Supplementary Fig. S1). These findings were substantiated by qRT-PCR-based expression analyses of HDAC1, 3, 4 and 6 in matched normal patient-derived tissue samples (Fig. 1c). Due to the limited sample size, we decided to subsequently validate our findings in silico using the REMBRANDT glioma dataset available through a GlioVis online application. Again, we found an increased HDAC1, 3 and 6 expression in GBM samples compared to non-neoplastic brain samples (Supplementary Fig. S2a). In addition, we found that the HDAC1 and 3 expression levels correlated with WHO tumor grades, with highest expression levels in GBM samples (Supplementary Fig. S2b). Additional Kaplan-Meier survival curve analyses revealed that HDAC3 expression is associated with a poor survival of GBM patients (Supplementary Fig. S2c).

3.2 GBM cells display differential sensitivities to TSA and CCNU

TSA, an inhibitor of class I and class II HDACs, has previously been shown to induce cell cycle arrest and apoptosis in GBM cells in vitro [13, 25]. To confirm its potency, we validated the induction of histone H3 acetylation by 0.5 μM TSA in both GBM (U87, 047) and NHA cells (Fig. 1d). Subsequently, we evaluated the sensitivity of three preselected GBM cell lines (017; 036; 4121) to TSA and CCNU alone. We found that the 017, 036 and 4121 cells exhibited different sensitivities to CCNU, with GI_{50} values of 8, 67 and 119 μM, respectively (Fig. 2a,c). Interestingly, we found that the GBM cell lines exhibited similar sensitivities to TSA, with GI_{50} values ranging from 0.65 to 1.19 μM (Fig. 2b,c). Moreover, we confirmed that a TSA concentration corresponding to the observed GI_{50} values (0.5–1.0 μM) was sufficient to induce acetylation of histone H3 and cleavage of caspase-3 in our model cell lines (Supplementary Fig. S3).

3.3 Combined TSA-CCNU treatment results in enhanced induction of DNA damage and apoptosis, and a reduction in survival of GBM cells

Targeting HDACs has previously been shown to impair the capacity of cancer cells to repair DNA damage incurred by exogenous stimuli [23, 26]. Here, we evaluated the capacity of GBM cells to repair DNA damage using γH2AX (foci count

and total intensity measurement) as a surrogate marker for DSBs [27]. GBM cells were treated with either TSA, CCNU or its combination (TSA+CCNU) at concentrations corresponding to their GI_{50} values. We found that single-agent treatment with either TSA or CCNU led to increased γH2AX foci counts and intensities compared to the respective untreated baseline levels (Fig. 3 and Supplementary Fig. S4). Importantly, we found that this effect was enhanced upon combined treatment with both TSA and CCNU (Fig. 3 and Supplementary Fig. S4). Additional results from a 18 protein array screen (Fig. 4a,b and Supplementary Table S2) revealed that the treatment effects on DSBs correlated with significant inductions of pro-apoptotic proteins, including phosphorylated Bad (pBad), cleaved PARP (cl.PARP) and cleaved caspase-3 (cl.Caspase-3). These results were further substantiated by independent Western blotting experiments conducted in the 017, 036 and 4121 cell lines (Fig. 4c,d) as well as in three other GBM cell lines (Supplementary Fig. S5). Despite the above mentioned combinatory effect on DNA damage induction, we failed to observe any enhanced activation of the Chk1 or Chk2 kinases upon TSA-CCNU treatment (Fig. 4c and Supplementary Fig. S5). Importantly, we found that the induction of apoptosis was associated with a decreased cell viability (Fig. 5a) for which a clear TSA-CCNU combinatory effect was observed compared to either drug alone. Subsequent cell cycle analyses (Fig. 5b, Supplementary Fig. S6 and S7) revealed that both TSA and CCNU treatment led to a reduction in actively proliferating cells (EdU-positive, S phase cells), which was further enhanced upon a combined TSA-CCNU administration. Taken together, we conclude that our results show an enhanced effect of a combined TSA and CCNU treatment on the induction of DNA damage and apoptosis, as well as on the reduction of survival of GBM cells, probably caused by an impaired capacity to repair DNA damage leading to an increased apoptotic rate.

4 Discussion

GBM is among the deadliest of solid tumors for which currently available post-operative therapies (chemo-irradiation) offer only palliation [28]. Therefore, there is an urgent need for novel (targeted) therapies, which significantly improve GBM patient survival and abrogate commonly observed recurrences. Despite promising results from in vitro studies, mono-HDAC inhibitor (HDACi) therapies have only exhibited limited efficacies in clinical settings, and pre-clinical studies have encouraged the use of HDACi in combination with other anti-cancer drugs. HDACs have been found to be upregulated in various solid tumors and to play key roles in oncogenesis [10, 12]. Additionally, it has been found that in a number of malignancies such as prostate [29], colorectal [30], breast [31], lung [32], liver [33] and gastric [34] cancer, the expression of individual HDACs is inversely correlated with disease-free and overall survival rates, and is associated with a poor prognosis [12].

Based on aberrant activation of DNA damage responses in malignant gliomas [16, 23, 35] and marked resistances to DNA damaging therapies, it has been suggested that HDACi may be used as sensitizers to DNA damaging agents in GBM [36–38]. Moreover, the HDACi TSA has been found to sensitize GBM cells to ionizing radiation [15]. Hence, we hypothesized that TSA treatment may sensitize GBM cells to the alkylating agent CCNU by chromatin remodeling [39] leading to an impaired accessibility to DNA repair proteins.

A number of previous studies reported elevated expression levels of HDAC4 and HDAC6 in brain tumors [40, 41]. Here, we found that HDAC1, 3 and 6 are upregulated in GBM cells and in primary patient tissues compared to non-neoplastic brain controls at both the mRNA and protein levels. Despite the overall higher HDAC expression levels, we observed hyper-acetylation of histone H3 in GBM cells compared to normal human astrocytes. This phenomenon has previously also been observed by others [23, 41] and has been attributed to a significantly higher transcriptional activity commonly observed in GBM cells [41]. Our in silico analyses were consistent with our experimental findings and revealed increased expression levels of HDAC1, 3 and 6 in GBMs compared to non-neoplastic brain tissues and an inverse correlation of HDAC3 mRNA expression with GBM patient survival. Overall, these data indicate that both class I and class II HDACs are commonly overexpressed in GBMs and play important roles in the pathogenesis of these tumors.

We found that our GBM cell cultures displayed variable degrees of sensitivity to CCNU treatment, with GI_{50} values ranging from 8 to 119 μM, while the sensitivity to TSA treatment was rather consistent (GI_{50}; 0.5 to 1.0 μM). The differential sensitivity of GBM cells to CCNU may be attributed to cell-specific resistance mechanisms such as high expression levels of multidrug resistance proteins and/or slow cell cycle kinetics. Indeed, our unpublished results revealed significantly longer cell doubling times in 017 and 036 cells compared to 4121 cells (data not shown).

TSA has previously been shown to induce apoptosis through caspase-3 activation and induction of the pro-apoptotic protein Bad [42, 43]. Our findings indicate that both TSA and CCNU alone can upregulate the expression of several intrinsic pro-apoptotic proteins (including Bad, PARP, caspase-3 and caspase-7) and that upon TSA-CCNU combination treatment this effect is enhanced, suggesting a more profound induction of apoptosis. Increased apoptosis was found to be associated with decreased cell viability. Intriguingly, besides a reduction in S phase cells (commonly observed upon combination treatment in all GBM cell lines used in our study), we did not observe a uniform response with respect to G1 or G2/M cell cycle arrest. These different

responses in cell cycle dynamics and checkpoint activation may be attributed to inherent cell-specific characteristics such as p53 status (wild-type versus mutated) or expression of other cell cycle regulating proteins (i.e., cyclins, cyclin-dependent kinases etc.). To assess whether GBM cells arrest at the G1 or G2/M checkpoint upon CCNU and TSA treatment, we employed FACS analysis in conjunction with p-histone H3Ser10, a mitotic index (MI) marker, after nocodazole (NOC) treatment, which normally blocks cells in the G2/M phase. We did, however, not observe any increase in MI upon NOC treatment neither in single-agent nor in combo-agent treated GBM cells. This indicates that the cells either arrest at G1 (017 cells) and do not enter the S phase or, alternatively, die via apoptosis induction at the G2/M phase prior to cell division (036 and 4121 cells). Previously, it has been shown that both TSA and CCNU treatment can reduce the number of actively proliferating cells, a phenotype often accompanied by G2/M cell cycle phase arrest [25, 44, 45]. Cornago et al. reported that HDACi can impair G2 checkpoint activation and, thereby, promote premature entry of GBM cells into mitosis (i.e., before DNA damage is repaired), resulting in cell death via a process called mitotic catastrophe [46].

Depletion of HDAC1-3 has been shown to sensitize tumor cells to DNA damaging agents as a result of a reduction in DNA damage repair capacity [47, 48]. HDACi leads to hyperacetylation of histones resulting in loose, transcriptionally active chromatin, making the DNA more prone to damage by additional agents such as CCNU. Indeed, we found that the

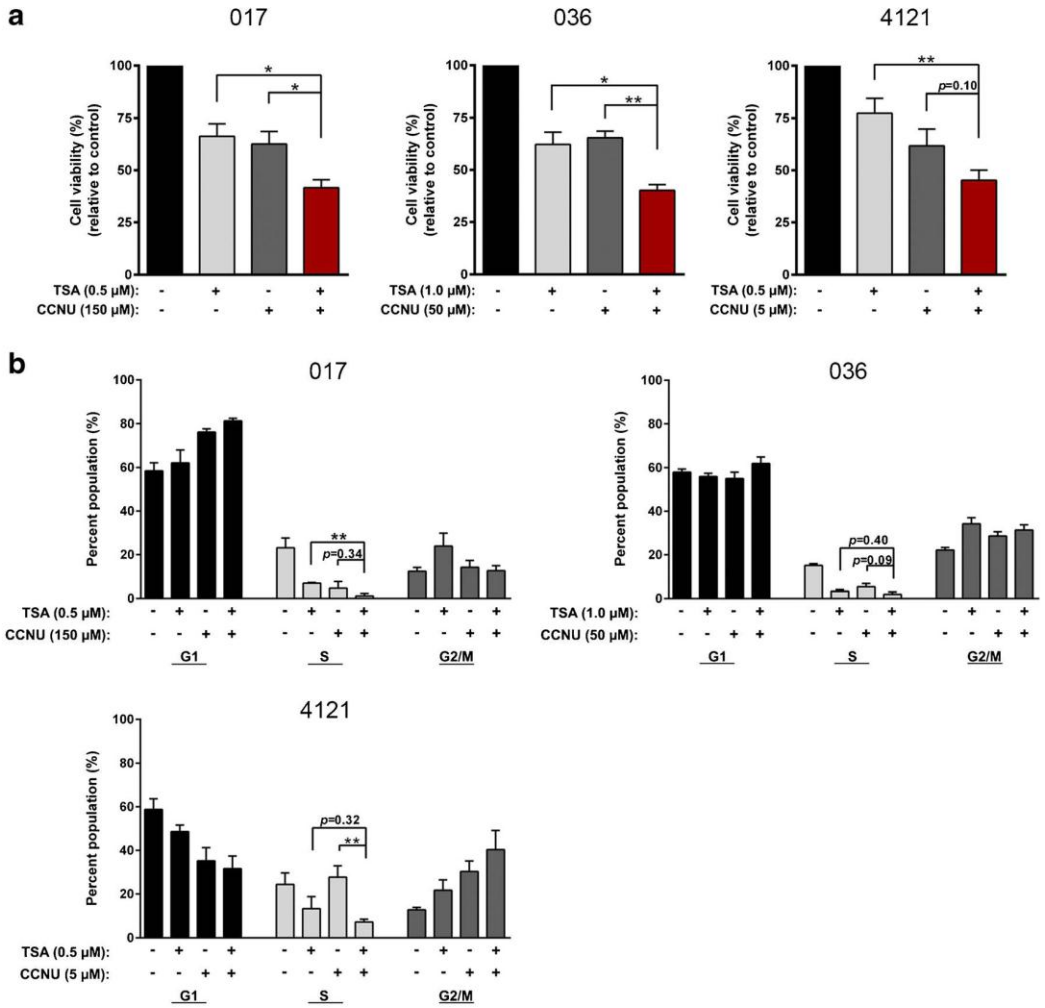


Fig. 5 TSA and CCNU treatment results in reduced cell viability and impairs cell cycle progression. **a** GBM cells (017, 036, 4121) were pre-treated with TSA for 24 h followed by administration of CCNU for another 72 h after which cell viabilities were measured by MTT assay. The results are shown relative (%) to control (DMSO; *black bar*) treated cells and presented as mean ± SEM ($n = 3$). **b** GBM cells (017, 036, 4121) were pre-treated with TSA for 6 h followed by administration of CCNU for another 24 h, fixed and submitted to FACS-based cell cycle analyses. Quantified cell cycle distributions (% G1, S, G2/M) are presented as mean ± SEM ($n \geq 3$), * $p < 0.05$, ** $p < 0.01$

co-administration of TSA and CCNU resulted in increased γH2AX foci formation and intensity compared to either of the drugs alone. The combined treatment in 017 cells resulted in intense and diffuse γH2AX signals, indicative of apoptosis [49, 50]. Our findings are consistent with previously published data showing enhanced γH2AX induction in erythroleukemic cells when TSA treatment was combined with ionizing radiation [36]. Moreover, it has been reported that the HDACi SAHA can cause a delay in DNA damage repair after radiation treatment [51] and sensitize GBM cells to PARP inhibition in GBM [23], breast cancer [52], prostate cancer [53] and ovarian cancer [54].

Currently, no HDACi has been US FDA approved for the treatment of GBM as mono-therapy, but several clinical trials are underway investigating HDACi in combination with other cytotoxic drugs such as temozolomide, bevacizumab and/or radiation therapy in GBM (https://clinicaltrials.gov). Considering the number of ongoing clinical trials and the finding that transformed cells are more sensitive to HDACi treatment than normal cells [55], there is a rationale for a combinational targeted approach using HDACi.

In summary, we found a higher efficacy of a combined treatment of GBM cells with TSA and CCNU compared to either of the drugs alone. Our data further indicate that this efficacy is due to an impaired DNA repair capacity, which subsequently leads to cell death via apoptosis. We conclude that our findings and those of others [13, 23, 26, 36, 38, 43, 46] warrant a further pre-clinical investigation and a thorough evaluation of combined HDACi applications for the treatment of GBM.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

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Supplementary data and figures

Inhibition of histone deacetylases sensitizes glioblastoma cells to lomustine

Mikkel Staberg[1], Signe Regner Michaelsen[1], Rikke Darling Rasmussen[2], Mette Villingshøj[1], Hans Skovgaard Poulsen[1] and Petra Hamerlik[1,2]

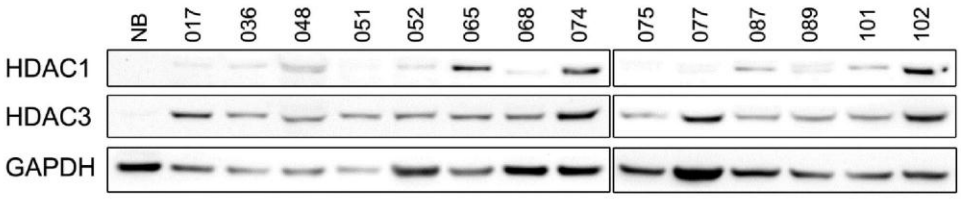
[1]Department of Radiation Biology; The Finsen Center; Rigshospitalet, Copenhagen, Denmark

[2]Brain Tumor Biology Group, Danish Cancer Society Research Center, Copenhagen, Denmark

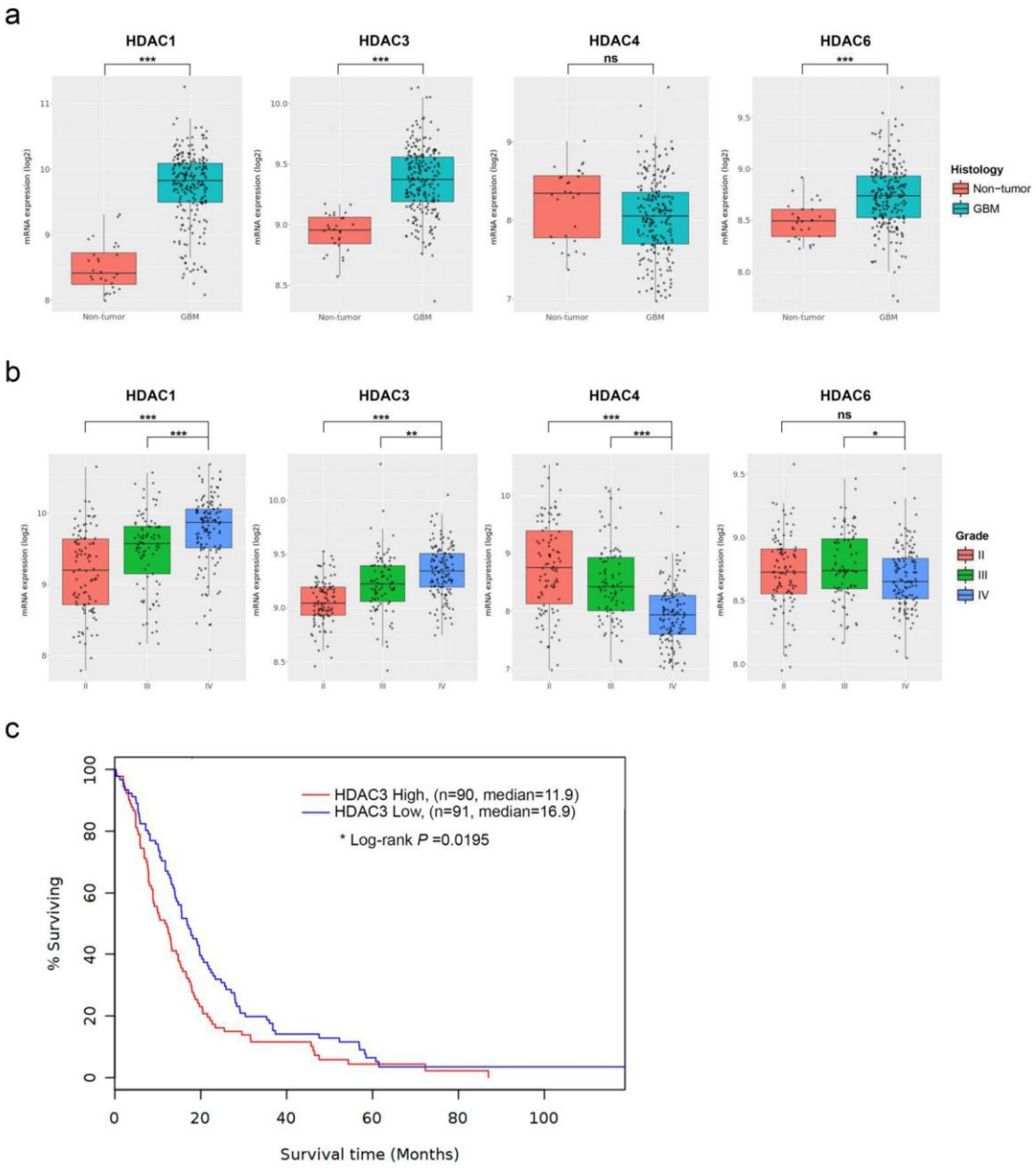
Correspondence should be addressed to:

Mikkel Staberg, mikkel.staberg@regionh.dk, phone: +45 35 45 63 28, fax: +45 35456301 or

Petra Hamerlik, pkn@cancer.dk, phone: +45 35 25 74 05, fax: +45 35257721

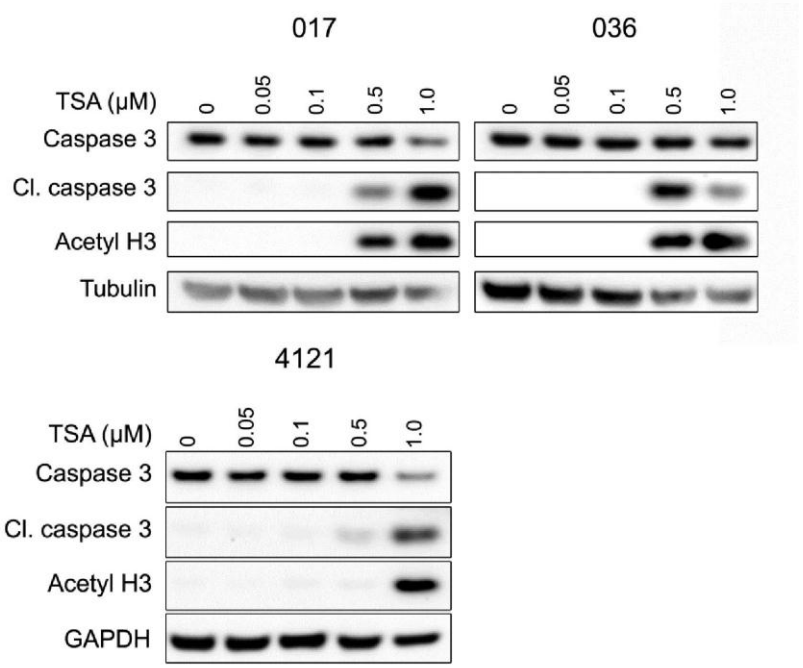


Supplementary Figure S1. Expression of HDAC1 and HDAC3 in a panel of GBM patients. Tumor tissue from patients were submitted to WB analysis with primary antibodies against HDAC1, HDAC3 or GAPDH. NB represents normal brain lysate (Novus Biologicals, #NB820-59177).

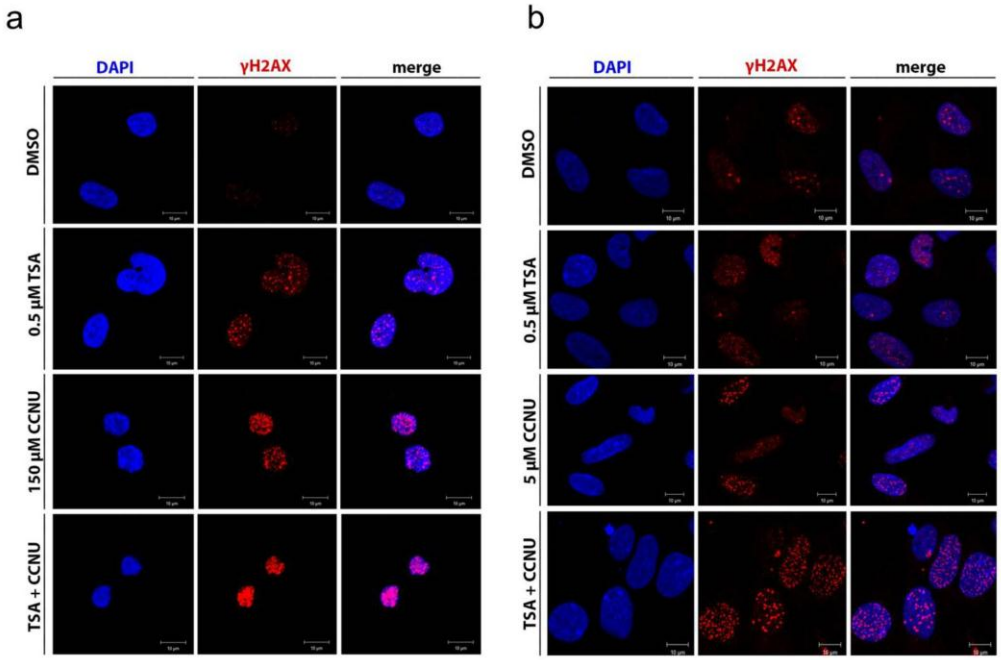


Supplementary Figure S2. *In silico* analysis obtained from the GlioVis website (http://gliovis.bioinfo.cnio.es/) using the Rembrandt data set. HDAC expression levels (HDAC1, HDAC3, HDAC4 and HDAC6) in GBM (grade IV) compared to (a) non-tumor tissue or (b) WHO

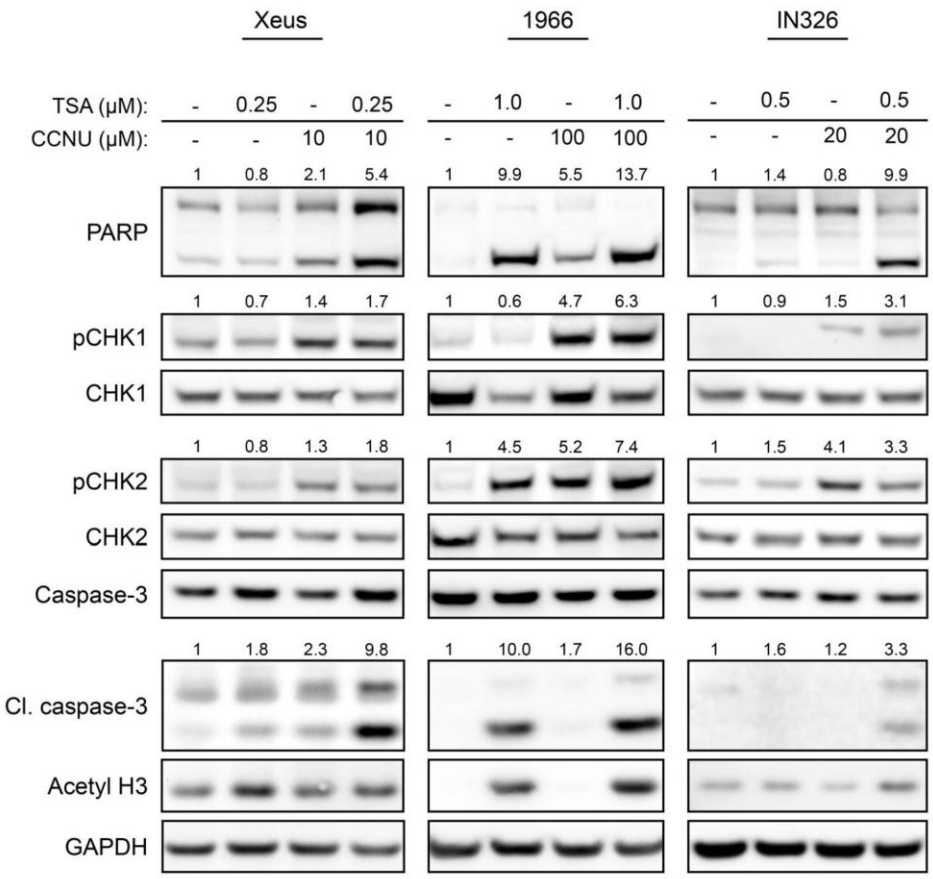
grade II and III brain tumors. (c) Kaplan-Meier survival curve displaying inversely correlation to HDAC3 expression (n=181 patients).



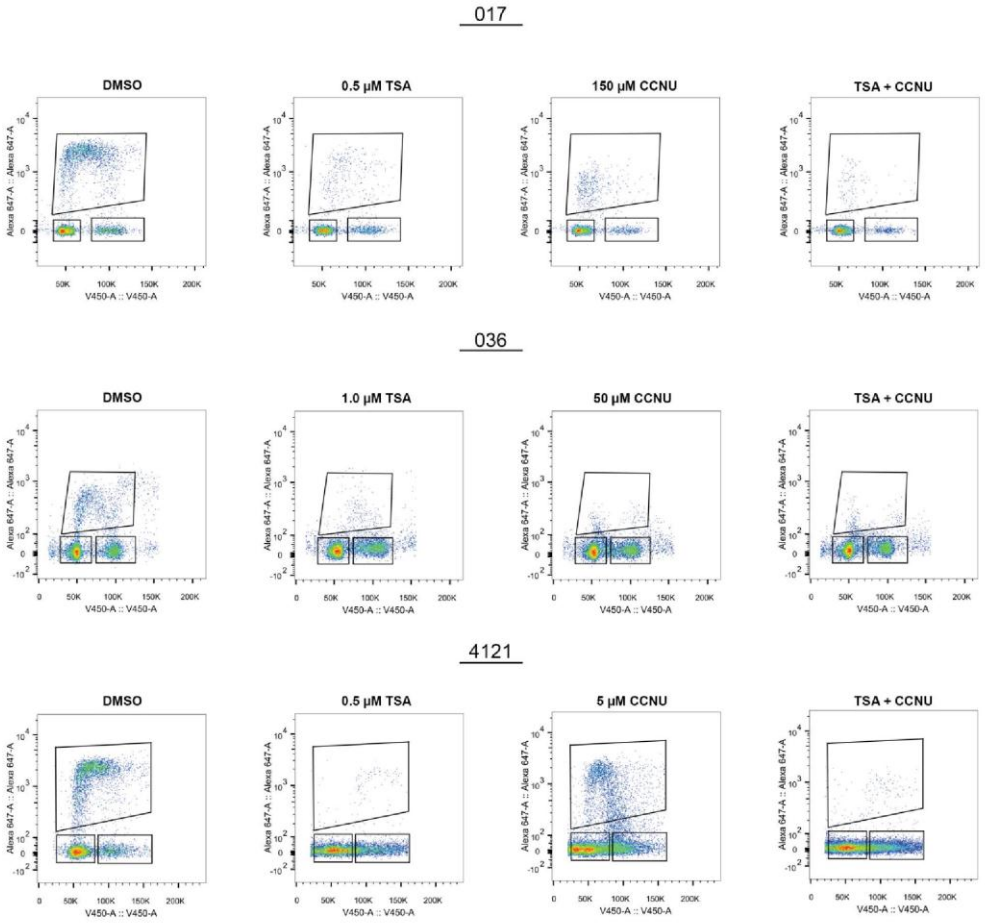
Supplementary Figure S3. TSA acetylates histone H3 and leads to cleavage of caspase-3. GBM cells (017, 036 and 4121) were treated with increasing doses of TSA for 48 hours. Cell lysates were submitted to WB with primary antibodies against caspase-3, cleaved caspase-3, acetyl histone H3, tubulin or GAPDH.



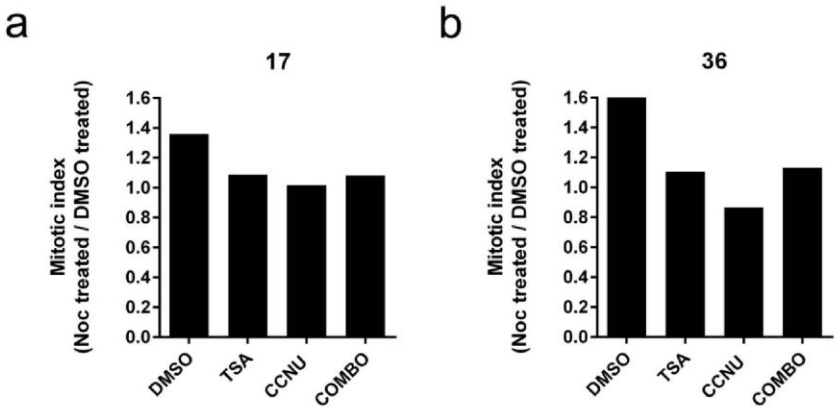
Supplementary Figure S4. TSA and CCNU treatment leads to induction of γH2AX. GBM cells were treated with TSA, CCNU or a combination and submitted to immunofluorescence analysis against γH2AX staining. DAPI serves as counterstaining. Representative pictures of (a) 017 or (b) 4121 GBM cells treated with TSA, CCNU or a combination inducing γH2AX upregulation.



Supplementary Figure S5. TSA and CCNU treatment leads to induction of pro-apoptotic proteins. GBM cells (Xeus, 1966 and IN326) were pretreated with TSA for 6 hours following administration of CCNU for additional 24 hours and subjected to WB against pChk1 (S345), total Chk1, pChk2 (T68), total Chk2, cleaved caspase-3, total caspase-3, cleaved/total PARP and acetylated histone H3. GAPDH serves as loading control. Numbers above bands indicate semi-quantification of respective bands analyzed using the VisionWorks®LS software (UVP, UK). The quantified expression levels were normalized to internal GAPDH levels and set relative to control (DMSO).



Supplementary Figure S6. Flow cytometric analysis of GBM cells. GBM cells (017, 036, 4121) were pre-treated with TSA for 6 h followed by administration of CCNU for another 24 h. Cells were pulse labelled with EdU 20-60 min. before harvesting and fixation in 4% paraformaldehyde. Fixed cells were submitted to the Click-iT EdU Alexa Flour 647 Flow Cytometry Assay Kit (Invitrogen) and analyzed using a FACS Verse Cell Sorter (BD Biosciences). Figure display representative FACS plots for cells treated with DMSO, TSA, CCNU or a combination. The dot plot represents EdU incorporation (y-axis) vs DNA content determined by hoechst staining (x-axis). Cells in S-phase were estimated by quantifying cells positive for EdU.



Supplementary Figure S7. Analysis of mitotic index in GBM cells. (a) 017 and (b) 036 cells were pre-treated with TSA for 6 h followed by administration of CCNU for another 24 h. Twelve hours before end of incubation cells were administered nocadazole or DMSO. Cells were fixed, stained with p-histone H3 Ser10 antibody (Cell Signaling #3377) and counterstained with hoechst. The number of cells positive for p-histone H3 Ser10 was analyzed using a FACS Verse Cell Sorter (BD Biosciences). The mitotic index was calculated by dividing nocadazole-treated cells by DMSO-treated cells applying the populations positive for p-histone H3 Ser10.

Dilution	Antibody	Manufacturer
1:1000	Rabbit anti-acetyl histone H3	Cell signaling, #9671
1:1000	Rabbit anti-cleaved caspase-3	Cell signaling, #9664
1:200	Mouse anti-caspase-3	Santa Cruz, #sc-7272
1:400	Rabbit anti-pCHK2	R&D Systems, #AF-1626
1:400	Mouse anti-CHK2	Danish Cancer Center Society
1:10000	Rabbit anti-GAPDH	Santa Cruz, #sc-25778
1:1000	Rabbit anti-HDAC1	Abcam, #ab-109411
1:500	Rabbit anti-HDAC3	Novus, #nb100-1669
1:1000	Rabbit anti-tubulin	Cell signaling, #2125
1:1000	Rabbit anti-PARP	Cell signaling, #9542
1:250	Rabbit anti-pCHK1	Cell signaling, #2348
1:500	Mouse anti-CHK1	Santa Cruz, #sc-8408
1:1000	Rabbit anti-HDAC4	Cell signaling, #7628
1:1000	Rabbit anti-HDAC6	Cell signaling, #7558
1:1000	Rabbit anti-histone H3	Novus, #nb500-171
1:1000	Mouse anti-HDAC1	Cell signaling, #5356

Supplementary Table S1. Overview of primary antibodies used for western blotting (WB). Antibodies are listed with dilution, manufacturer and catalog number.

	017			036			4121		
Protein	**TSA**	**CCNU**	**TSA+CCNU**	**TSA**	**CCNU**	**TSA+CCNU**	**TSA**	**CCNU**	**TSA+CCNU**
P44/42 MAPK (ERK1/2)	0.46±0.92	1.01±1.32	0.43±0.75	-0.10±0.49	0.34±0.43	0.46±0.51	0.80±0.87	-0.03±0.20	0.38±0.53
Akt Ser473	1.17±0.64	0.94±0.76	1.09±0.99	0.29±0.52	0.43±0.12	0.05±0.25	0.78±0.67	0.04±0.25	0.40±0.54
Bad Ser136	1.26±0.89	1.49±1.17	2.87±3.18	0.72±0.59	0.15±0.12	0.68±0.89	0.74±0.41	0.01±0.16	0.67±0.39
HSP27 Ser82	0.57±0.51	1.56±1.67	0.78±0.69	-0.31±0.15	0.36±0.21	0.03±0.42	-0.03±0.19	0.09±0.28	0.22±0.13
Smad2 Ser465/467	1.61±1.04	0.00±0.51	0.15±0.41	0.53±0.48	0.00±0.19	0.32±0.47	0.58±0.38	-0.07±0.32	0.12±0.42
p53 Ser15	-0.97±0.61	-0.81±0.34	-0.67±0.26	-0.20±1.04	13.02±3.04	1.57±2.4	0.98±1.71	19.03±21.84	4.24±5.60
p38 MAPK Thr180/Tyr182	-0.078±0.13	-0.29±0.26	-0.53±0.15	0.03±0.35	-0.27±0.15	-0.22±0.29	0.34±0.08	-0.03±0.14	-0.08±0.26
SAPK/JNK Thr183/Tyr185	0.103±0.40	1.87±1.40	0.84±0.80	0.30±0.46	0.16±0.31	0.25±0.37	0.41±0.28	0.09±0.25	0.23±0.38
PARP Asp214	4.42±2.78	9.16±4.05	17.47±14.29	1.05±0.85	1.14±0.50	1.12±0.67	2.32±1.38	0.72±0.48	3.42±1.22
Caspase-3 Asp175	4.70±1.78	6.47±3.58	10.39±6.35	3.8±1.47	4.05±2.56	4.54±2.07	3.94±1.76	0.42±0.29	4.33±2.55
Caspase-7 Asp198	1.50±0.53	1.14±0.60	2.85±1.66	0.32±0.58	0.02±0.34	0.22±0.55	1.12±0.59	0.21±0.34	1.52±0.62
IkBα Total	-0.41±0.10	0.47±0.56	-0.31±0.33	-0.15±0.06	0.00±0.04	-0.30±0.12	0.13±0.16	-0.03±0.17	-0.07±0.10
Chk1 Ser345	-0.15±0.12	2.20±1.04	0.72±0.62	0.15±0.42	0.80±0.61	0.72±0.57	0.15±0.18	0.75±0.32	0.21±0.16
Chk2 Thr68	0.10±0.23	1.18±0.51	0.82±0.24	0.86±0.39	0.93±0.25	1.21±0.41	0.71±0.19	0.30±0.26	0.92±0.32
IkBα Ser32/36	2.04±1.84	0.58±1.34	1.38±2.51	1.94±0.94	1.56±1.38	1.95±1.71	1.57±1.15	0.42±0.87	1.15±1.45
eIF2α Ser51	0.21±0.74	0.57±1.36	-0.19±0.94	0.73±0.49	0.28±0.43	0.22±0.54	1.35±0.73	0.12±0.43	0.53±0.65
TAK1 Ser412	0.38±0.59	1.36±1.35	0.49±0.70	0.20±0.45	0.00±0.35	0.26±0.51	0.36±0.27	0.00±0.27	0.03±0.36
Survivin Total	0.09±0.36	0.03±0.22	0.08±0.34	-0.59±0.10	-0.61±0.17	-0.54±0.06	0.00±0.12	-0.16±0.12	-0.32±0.10

Supplementary Table S2. TSA and CCNU treatment induces apoptosis through upregulation of pro-apoptotic proteins. GBM cells were pretreated with TSA for 6 hours following administration of CCNU for additional 24 hours and subjected to Pathscan Stress and Apoptosis Array Kit (Cell signaling). Table display the relative fold change of 18 phosphorylated proteins involved in cell stress and apoptosis compared to DMSO. Data were normalized to internally tubulin levels and set relative to control (DMSO). Results presented as mean ± s.d.

6. STUDY III

Lysine-specific histone demethylase KDM2B regulates chemoresistance and maintenance of glioblastoma cells

Mikkel Staberg, Signe Regner Michaelsen, Mette Villingshøj, Hans Skovgaard Poulsen, and Petra Hamerlik

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Lysine-specific histone demethylase KDM2B regulates chemoresistance and maintenance of glioblastoma cells

Mikkel Staberg[1], Signe Regner Michaelsen[1], Mette Villingshøj[1], Hans Skovgaard Poulsen[1] and Petra Hamerlik[2#]

[1]Department of Radiation Biology; The Finsen Center; Rigshospitalet; Copenhagen, Denmark

[2]Brain Tumor Biology Group, Danish Cancer Society Research Center, Copenhagen, Denmark

#) Corresponding author:

Petra Hamerlik, pkn@cancer.dk, phone: +45 35 25 74 05, fax: +45 35 25 77 21

Key words: glioblastoma, epigenetics, histone-demethylase, chemoresistance, sensitization

Abbreviations: GBM, glioblastoma; GSC, glioma stem cell; KDM, lysine-specific histone demethylase; CCNU, lomustine; VP-16, etoposide; CI, combination index.

Abstract

Glioblastoma (GBM) is the most deadly and malignant of all brain tumors in adults partly due to acquired resistance to conventional drugs leading to recurrence. A population of glioma stem cells (GSCs) with capacity of self-renewal and tumor formation is believed to contribute to therapeutic resistance and relapse. Emerging evidence indicate that aberrant expression of epigenetic enzymes, resulting in altered gene transcription, may be responsible for stemness and drug resistance in GBM. In this study, we demonstrate that the histone demethylase KDM2B promotes GBM cell maintenance and chemoresistance, possibly through repression of the cyclin-dependent kinase inhibitor p21 and maintained EZH2 expression. KDM2B inhibition by siRNA-mediated knockdown induced apoptosis through cleaved PARP and caspase-3, reduced EZH2 expression, and sensitized cells to the conventional chemotherapies lomustine and etoposide. Consistently, a histone demethylase inhibitor, GSK-J4, decreased KDM2B expression, and inhibited cell viability and tumor-sphere formation. Combined administration of GSK-J4 and lomustine or etoposide displayed synergistic inhibition of cell viability. In summary, our results indicate a novel mechanism by which KDM2B promotes GSC maintenance and chemoresistance, proposing KDM2B as a novel therapeutic target for elimination of resistant GBM cell clones.

Introduction

Glioblastoma (GBM) is a devastating disease with dismal prognosis. Current treatment options consist of maximal surgical resection combined with radiotherapy and chemotherapy. Despite this aggressive treatment, patients will eventually experience relapse, leading to an average survival rate of about 15 months [1]. This is attributed high intratumoral heterogeneity and treatment resistance, why new therapies are needed. It is generally believed that a subpopulation of tumor cells exists, termed glioma stem cells (GSCs), with stem cell potential including self-renewal and tumor-sphere growth, and that display high resistance to conventional treatments [2-4]. In the last few years, emerging evidence have identified the dysregulation of transcription factors and histone-modifying enzymes to be involved in tumorigenesis [5,6]. In GBM, the high heterogeneity and altered signalling pathways are suggested to occur, in part, by a deregulated epigenetic machinery leading to changes in DNA methylation and chromatin marks, thus resulting in aberrant gene expression [7,8]. Thus, cancer therapies aiming at modulating key epigenetic enzymes have shown their potential as anti-cancer agents, and histone deacetylase inhibitors (HDACi) are now FDA approved in the treatment of cutaneous T-cell lymphoma [9]. Additionally, in the recent years it has been demonstrated that enzymes, such as lysine methyltransferases (KMTs) and demethylases (KDMs), through changes in the histone methylation profile, are involved in the regulation of gene expression. Thus, histone methylation regulates several cellular processes including transcription, replication, cell cycle process, and DNA repair [10]. Emerging evidence have indicated mutation or overexpression of several KDMs in various cancers suggesting that the targeting of KDMs represents an attractive therapeutic approach [11]. The lysine-specific histone demethylase KDM2B, also known as Fbxl10 or Jhdm1b, belongs to the Jumanji C (JmjC) domain family, and removes methyl groups from histone H3K36 [11-13], normally correlated to gene activation [14]. Recently, it has been demonstrated that KDM2B is involved in the regulation of cell growth, migration and angiogenesis [13,15]. Additionally, it was found that KDM2B is overexpressed in leukemia and pancreatic cancer where it is required for cell proliferation and cell maintenance [16,17]. However, the function of KDM2B in glioma has so far not been investigated. Thus, in this study, we sought to examine the role of KDM2B in GBM. We find that KDM2B is differentially expressed among GBM patient tissue and GBM cell lines compared to non-neoplastic tissue. Abrogation of KDM2B by either small-molecule inhibition (by GSK-J4) or siRNA-mediated knockdown reduced viability of established patient-derived primary GBM cells through induction of apoptosis, and sensitized these cells to chemotherapy (etoposide and lomustine). Further, GSK-J4

treatment inhibited the tumor-sphere forming potential, and this effect was potentiated upon co-administration with lomustine. Finally, preliminary results indicated that GSK-J4 treatment reduces Sox2 expression, and the fraction of CD133-positive glioblastoma cells, suggesting preferential targeting of the GSCs. In summary, our results demonstrate that glioblastoma cell growth and maintenance is at least partly dependent on KDM2B. Further, we find that combined therapy targeting KDM2B together with conventional chemotherapy may display enhanced capacity for elimination of glioblastoma cancer cells.

Materials and methods

Primary cell cultures and glioma patient tissue

Primary GBM cell lines were derived from brain tumor patients and obtained from the Danish Cancer Society Research Center. Cells were grown and maintained in Neurobasal®-A media (NB) (Invitrogen, #10888-022) supplemented with B27 (#12587-010), bFGF (10 ng/ml, #13256-029), EGF (10 ng/ml, #PHG0311), Glutamax, penicillin (50 U/ml), and streptomycin (50 µg/ml, #15070-063) (all from Invitrogen, Taastrup, Denmark) with 5% CO_2 at 37 °C. The 4121 and 1587 GBM cells grow as tumor neurospheres whereas the T115 cell line grow adherently. Glioma patient tissue was collected at initial surgery at Rigshospitalet, Denmark, with obtained written consent from patients, and approved by the Danish Data Protection Agency (2006-41-6979) and the Scientific Ethical Committee for Copenhagen and Frederiksberg (KF-01-327718). Establishment of glioma cell cultures (performed at the Danish Cancer Society Research Center), from primary human brain tumor patients, were approved by the Danish Ethical Committee guidelines with obtained informed consents from patients 24h prior to surgery. For experiments, cells were dissociated and counted using a NucleoCounter® NC-200 (ChemoMetec, Denmark). Cells were seeded in supplemented NB media and treated with lomustine (CCNU, #L5918), GSK-J4 (#SML0701) from Sigma-Aldrich, or etoposide (VP-16).

siRNA transfections

For siRNA transfection experiments, constructs targeting KDM2B (KDM2B-1 and KDM2B-2; #1299001) and siCTRL (si-negative control duplex, #462001) were obtained from Thermo Fisher Scientific. Cells were transfected with 50 nM of siKDM2B or siCTRL using Lipofectamine® RNAiMAX (#1377150) and incubated for six hours before plating for individual assays. Knockdown was confirmed by qRT-PCR (after 48 h) and by Western blotting (after 72 h).

Western blotting

Cells were lysed in whole-cell lysis buffer (50 mM Tric-HCl, 10% glycerol, 2% SDS) or modified RIPA buffer (50 mM Tris-HCl, 1% NP40, 0.25% Na-deoxycholate, 150 mM NaCl, 1mM EDTA)

supplemented with protease and phosphatase inhibitors and protein concentrations were estimated by BCA protein assay (Pierce Biotechnology, Rockford, IL). Protein samples were separated on 4–12% NuPAGE Bis-Tris gels (NP0336BOX) (Invitrogen) and electroblotted onto nitrocellulose membranes (Invitrogen, LC2000). The membranes were blocked for 1 h at room temperature (RT) and incubated with primary antibodies in 5% non-fat milk overnight (ON) at 4 °C followed by horseradish peroxidase (HRP) conjugated secondary antibodies for 1 hour at RT. Blots were developed using either the SuperSignal West Dura Extended Duration Substrate (#34075) or the SuperSignal West Femto Maximum Sensitivity Substrate (#34095) from ThermoFischer and developed with the Biospectrum Imaging System (UVP, Upland, CA, USA). Primary antibodies used are shown in Table S1.

MTT assay

For estimation of cell viability a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Sigma M-5655) was employed. GBM cells were plated at a density of 1 x 10^4 cells per well in 96-well plates and incubated ON. Subsequently, cells were treated with GSK-J4, CCNU, VP-16 or their combinations at designated concentrations and further incubated for 72 h. At end of incubation, 20 µl (5 mg/ml) MTT solution was administered and incubated for four hours followed by addition of 100 µl solubilization buffer (10% SDS, 0.03 M HCl). The following day, absorbance was measured at 570 nm with 690 nm as a background reference using a Synergy2 microplate reader with Gen5, Microplate Data Collection and Analysis Software (Biotek, Winooski, VT, USA). Each experiment was performed in duplicate wells and independently at least three times.

Quantitative real-time PCR

Total RNA was purified from GBM patient tissue and cell pellets using the the QIAshredder (79654) and RNeasy Mini kit (#74104) from Qiagen. Glioma tumor tissue was extracted using TRIzol®Reagent (Gibco) and a Qiagen TissueLyser before purificaion. Synthesis of cDNA and Q-RT-PCR reactions was performed using the SuperscriptTM III Platinum® Two Step qRT-PCR kit with SYBR® Green (Invitrogen, #11735-032). Gene expression levels were determined applying the comparative Ct method and normalized to expression of three house-keeping genes (TOP1, EIF4A2 and CYC1) included in the human geNorm house-keeping gene selection kit (Primerdesign). Primers used for estimation of mRNA levels were: KDM2B forward; 5'- CAT GGA GTG CTC CAT CTG CAA TG-3', KDM2B reverse; 5'- ACT TCG GAC ACT CCC AGC

AGT T-3'. Sox2 forward; 5'- GGC AGC TAC AGC ATG ATG C-3', Sox2 reverse; 5'- TCG GAC TTG ACC ACC GAA C-3'. Primers were obtained from DNA Technology A/S.

Immunofluorescence imaging

For immunoflouroscence staining, GBM cells were plated on pre-coated (Geltrex; Thermo Fisher Scientific) coverslips and incubated for 48 hours, fixed in 4% PFA and immunostained for anti-KDM2B (Novus #H00084678-M09). Nuclei of the cells were counter-stained with DAPI. Pictures were acquired on a Zeiss LSM 700 Confocal Microscope.

Flow cytometry analysis

Transfected GBM cells were plated and incubated for 72 hours. At end of incubation, cells were pulse-labeled with 10 µM (5-ethyl-2'-deoxyuridine; EdU) for 20 min, and fixed in 4% PFA. Cell samples were submitted to the Click-iT EdU Alexa Flour 647 Flow Cytometry Assay Kit (Invitrogen) following manufacturer's instructions in order to determine cells in S-phase. For estimation of CD133-positive populations, cells were plated and left ON. Following, cells were treated with indicated concentrations of GSK-J4 and incubated for 72 hours. Cells were harvested and incubated with an FITC-conjugated monoclonal antibody against CD133 (CD133/2; Miltenyi Biotec). Dead cells were excluded through 7-AAD staining, and isotype controls were used to establish proper gating. Sample acquisition was done on the FACS Verse Cell Sorter (BD Biosciences) and data were analyzed using FlowJo software. At least 10.000 events were acquired for each condition.

Limiting dilution assay

Cells were dissociated, counted and plated in 96-well plates at cell densities ranging from 1 to 50 cells/well (16 replicate wells per condition). The following day, cells were treated with indicated concentrations of either GSK-J4, CCNU, combinations thereof or equal amounts of DMSO. The formation of tumor-spheres were evaluated after 10 days of incubation and each well was analyzed for presence or absence of at least one tumor-sphere. The calculation of estimated stem cell frequency in each condition was done by employing the extreme limiting dilution analysis [18].

Combination Index calculations

In order to assess the efficacy for combinational treatments on cell viability (MTT), the free available Compusyn software (www.combosyn.com) for calculation of a combination index (CI) was used. From this, a CI > 1.1 indicates antagonism, a CI of 0.9-1.1 indicates additivity, and a CI < 0.9 indicates synergy [19]. The CI values calculated were obtained from at least three independent experiments and presented as mean ± standard error of the mean.

Statistics

Data are presented as mean ± standard deviation (SD) or standard error of mean (SEM). A two-tailed Student's t-test was used to test for statistical significance. Graphpad Prism 6.0 (GraphPad, San Diego, CA, USA) was used for statistical calculations and for creation of figures.

Results

KDM2B expression in GBM

Recent evidence indicates that KDM2B acts as an oncogene sustaining cancer growth of leukaemia, breast- and pancreatic cancer [17,20,21]. Thus, we sought to evaluate the expression of KDM2B in GBM. First, in a panel of GBM cell cultures, analyzed by Western blotting, we demonstrated increased KDM2B expression in 5 of the 8 cell cultures tested compared to normal human astrocytes (NHA) (Fig 1A). The expression of KDM2B was further evaluated by qRT-PCR showing higher KDM2B mRNA levels in all but the 017 GBM cell lines compared to NHA (Fig. 1B). We choose three GBM cell lines representative for the differential expression of KDM2B for further studies. The 4121 and 1587 cell lines growing as tumor-spheres expressing high KDM2B, and the T115 characterized by adherent growth and displaying low KMD2B expression. The subcellular distribution of KDM2B was evaluated in the cultures by immunofluorescence of cells grown adhesively on coated coverslips. As seen in Fig. 1C, KDM2B expression was both evident in the nucleus and cytoplasm in both GBM cell lines and NHA cells. In order to investigate KDM2B in a clinical setting, we performed qRT-PCR in primary GBM tissue samples compared to normal brain. The degree of KDM2B expression varied for the individual patient tissue, with increased expression observed in 50% of tumors as compared to normal brain (Fig. 1D). In summary, these data indicate that KDM2B expression is heterogeneous expressed in both GBM patient and cell cultures, and overall is higher in GBM compared to non-neoplastic brain cells and tissue.

KDM2B knockdown inhibits cell viability in vitro

Next, we wanted to investigate the effect of KDM2B knockdown in GBM cells. Cells were transiently transfected with two independent siRNA constructs targeting KDM2B or control-siRNA (siCTRL). As shown in Fig. 2A and B, KDM2B mRNA and protein levels were significantly downregulated in 4121, 1587 and T115 cells transfected with siKDM2B-1 and siKDM2B-2 compared to siCTRL transfected cells. The inhibition of KDM2B upon transfection was confirmed by observed induction of histone H3K36me2 in 4121 cells, indicating abrogated histone demethylase activity (supplementary Fig. S2A). In order to examine the role of KDM2B on cell viability, we investigated the siRNA-mediated KDM2B knockdown by MTT assay. The results demonstrated that KDM2B knockdown inhibited GBM cell viability (Fig. 2C), with the most

prominent effect seen in 4121 and 1587 cells expressing high levels of KDM2B (Fig. 1). In addition, KDM2B depletion reduced the percentage of proliferating cells (S-phase) as shown in Fig. 2D. In summary, these results suggest that KDM2B regulates GBM cell growth.

KDM2B knockdown induce apoptosis and sensitizes GBM cells to chemotherapy

KDM2B has been shown to be involved in the DNA damage repair response [22] and several other histone demethylases have been correlated to treatment resistance in various cancers [23,24]. Thus, we speculated whether KDM2B contributes to GBM chemoresistance. Previously, KDM2B was shown to regulate histone methyltransferase EZH2, important in tumor cell proliferation, chemoresistance, migration and invasiveness [25,26]. We found, that upon siRNA-mediated depletion of KDM2B, EZH2 expression decreases, a change associated with p21 induction, and PARP and caspase-3 cleavage (Fig. 3A). Furthermore, we assessed GBM cell viability 72 h post transfection with siKDM2B constructs combined with increasing doses of either lomustine (CCNU) or etoposide (VP-16) (Fig. 3B). Our data demonstrated that knockdown of KDM2B significantly enhanced the effect of chemotherapy on cell viability in 4121, 1587 and T115 GBM cells relative to siCTRL transfected cells (Fig 3B). In addition, this was further supported by WB analysis showing increased induction of PARP and caspase-3 cleavage in siKDM2B-1 transfected cells exposed to same concentrations of lomustine compared to siCTRL transfected cells (Fig. 3C). Taken together, our data indicate that KDM2B is associated with resistance to chemotherapy, and that GBM cells can be sensitized by targeting KDM2B.

Pharmalogical inhibition of KDM2B reduce GBM cell viability and tumor-sphere formation in vitro

As our results demonstrated that KDM2B is involved in GBM cell survival, and that knockdown of KDM2B sensitized GBM cells to chemotherapy, we wished to test the effect of chemical inhibition of KDM2B. A small-molecule inhibitor GSK-J4 has been shown to inhibit KDM2B enzyme activity as well as other KDM variants [27]. As shown in Fig. 4A, treatment with increasing concentration of GSK-J4 inhibited cell viability in a dose-dependent manner, displaying highest sensitivity in the KDM2B high-expressing cultures 4121 and 1587. We also found that increasing concentrations of GSK-J4 reduced the expression of KDM2B, and induced expression of p21,

cleaved PARP, and cleaved caspase-3 (Fig. 4B). The increase in H3K36me2 confirmed inhibition of KDMs. EZH2 has been suggested to promote and maintain a GSC phenotype [28]. We found decreased expression of EZH2 upon GSK-J4 treatment (Fig. 4B). Thus, we wanted to address the effect of GSK-J4 on the tumor-sphere-forming potential in 4121 and 1587 cells by applying a limiting dilution assay. GBM cells were plated at varying cell densities, treated with increasing concentrations of GSK-J4, and evaluated after 10 days for formation of tumor-spheres. As shown in Fig. 4C, GSK-J4 abrogated tumor-sphere formation in a dose-dependent manner, and reduced the estimated stem cell frequency in both cell lines (Fig. 4D). Taken together, this indicate anti-tumor effects of GSK-J4 on cell viability and clonogenicity in GBM.

GSK-J4 combined with chemotherapy display enhanced inhibition of cell viability and tumor-sphere formation in vitro

Our data indicate that siRNA-mediated knockdown of KDM2B sensitizes GBM cells to chemotherapy (Fig 3). Thus, we wanted to elucidate the effect of combined treatment with GSK-J4 and CCNU or VP-16 on cell viability. As shown in Fig. 5A, low-dose (LD) combinations of GSK-J4 and CCNU displayed a minor increase in inhibition of cell viability compared to either mono-therapy, which was confirmed by CI calculations indicating sub-additivity (Fig. 5C). However, when applying high-dose (HD) combinations of GSK-J4 and CCNU, the 4121 and 1587 GBM cells displayed synergistic inhibition (CIs < 0.9) of cell viability, whereas the T115 cell lines exhibited additivity (Fig. 5A and C). Similarly, when treating GBM cells with LD combinations of GSK-J4 and VP-16, this resulted in minor additive inhibition of cell viability (Fig. 5B). In contrast, administration of HD combinations of GSK-J4 and VP-16 displayed significant synergistic inhibition of cell viability compared to either mono-therapy (Fig. 5B and C). In continuation, we tested the effect of combining GSK-J4 and CCNU on tumor-sphere formation in the 4121 and 1587 cell lines. In both cell lines, mono-therapy with either GSK-J4 or CCNU showed some effect abrogating tumor-sphere formation and this effect was further potentiated upon combined administration, which was also evident by reduced calculated stem cell frequencies (Fig. 5D and E). Taken together, we demonstrate that a combined treatment approach using GSK-J4 together with conventional chemotherapy display synergistic inhibition of GBM cell viability and colony formation *in vitro*.

Discussion

Currently, patients with glioblastoma have a poor prognosis due to relapse and acquired treatment resistance of the recurrent tumor. GBM is a multi-step disease suggested to be a result of both genetic and epigenetic alterations [29,30]. Emerging evidence indicates that epigenetic dysregulation of histone deacetylases and histone demethylases play fundamental roles in the onset and maintenance of GBM [7]. In this study, we tested the hypothesis that the lysine-specific histone demethylase KDM2B is involved in GBM maintenance and treatment resistance. Here, we show that KDM2B is differentially expressed in GBM, and siRNA-mediated or chemical KDM2B inhibition results in decreased cell viability and induced apoptosis. Further, we show that depletion of KDM2B abrogates resistance to conventional chemotherapy, suggesting KDM2B may be a therapeutic target in GBM.

A number of studies indicate that overexpression or mutations of histone methyltransferases and/or histone demethylases are involved in the development of various human cancers [31-34]. The histone H3K27 methyltransferase, EZH2, has been shown to be overexpressed in glioma leading to induction of STAT3 and dedicated a role in GSC self-renewal and GBM malignancy [35-37]. Additionally, several histone demethylases including KDM1, KDM5A and KDM5B have shown to be upregulated in glioma, sustaining cell growth, and for KDM5A mediating resistance to temozolomide [24,38,39]. GBM cell cultures were analyzed for KDM2B expression demonstrating differential KDM2B levels across all cell lines. Highest expression was seen in the 4121 and 1587 cells displaying 2-3 fold upregulation compared to NHA. This result was consistent with qRT-PCR analysis in GBM patient tissue, displaying increased expression of KDM2B compared to normal brain in about 50% of the samples (Fig. 1D). These data indicate that KDM2B is upregulated at least in a subset of gliomas. When the expression of KDM2B was depleted using siRNA-mediated knockdown, this inhibited cell viability (Fig. 2), with the most prominent effect seen in the cell lines expressing high amounts of KDM2B (4121 and 1587). This is consistent with other studies showing decreased cell growth *in vitro* and *in vivo* in pancreatic- and breast cancer upon KDM2B depletion, confirming the requirement for KDM2B in tumor growth [17,20]. Recent evidence suggest that KDM2B is involved in regulation of the apoptotic machinery, demonstrated by de-repression of proapoptic proteins upon KDM2B silencing [40]. In line with this, our data show that KDM2B knockdown upregulated pro-apoptotic proteins cleaved PARP and cleaved caspase-3, and induced expression of the cell cycle inhibitor p21 (Fig. 3). Indeed, several KDMs including LSD1, KDM5B

and KDM2B have shown to be involved in p21 regulation, demonstrating that KDM2B via p21 regulates cell cycle progress and senescence [41-43]. Previously, KDM2B has been shown to be a regulator of EZH2 via demethylation of H3K36me2, leading to repression of tumorsupressors miRNA *let-7b* and *miR-101* [44,45]. Silencing of KDM2B has shown to increase levels of *let-7b* thereby downregulating EZH2 leading to reduced cells in S-phase. This indicates that KDM2B, via changes in histone methylation, regulates EZH2-mediated cell proliferation [45]. Consistent with this, our results show that both siRNA-mediated and pharmalogical inhibition of KDM2B leads to reduced expression of EZH2, induced p21 and inhibited cell viability (Fig. 2, 3 and 4). A common issue in cancer is the rapid emergence of resistant clones after initial therapy leading to recurrence. Epigenetic changes has been suggested to contribute to treatment resistance [46]. Recently, KDM3A was shown to contribute to chemoresistance in ovarian cancer, and KDM5A to confer resistance to temozolomide in GBM [23,24]. Thus, we speculated if KDM2B might also contribute to chemoresistance in GBM. We found that knockdown of KDM2B sensitized cells to CCNU and VP-16, indicating that KDM2B may be a therapeutic target in GBM. To further explore this, we investigated the effect of histone demethylase inhibitor GSK-J4 on GBM cell growth, and found that GSK-J4 inhibited cell viability. Further, GSK-J4 reduced KDM2B and EZH2 expression, and induced expression of p21 and pro-apoptotic proteins in a dose-dependent manner, showing comparable effects as seen in KDM2B knockdown cells (Fig. 3A versus Fig. 4B). Additionally, we demonstrated that GSK-J4 decreased tumor-sphere formation, at concentrations not inducing cell death (2.5 μM, Fig. 4A, C), and upon combined treatment with CCNU and VP-16, this potentiated the inhibitory effect on tumor-sphere formation and cell viability *in vitro*. In a recent study, GSK-J4 was shown to be a promising candidate drug in the treatment of pediatric brainstem glioma harbouring an oncogenic K27M mutation in histone H3.3 [47]. The authors demonstrate that GSK-J4 display potent anti-cancer activity and lead to reduced cell viability, colony formation, and tumor growth in H3K27 mutated gliomas. In addition, GSK-J4 have shown promising anti-cancer effects in ovarian cancer and non-small cell lung cancer [48,49]. However, it should be noted that GSK-J4 also show affinity for other KDMs. Hence, it cannot be excluded that the observed anti-neoplastic effects may be in part mediated through inhibition of other KDMs important for GBM maintenance. Despite of this, based on our results, GSK-J4 may prove to be a new epigenetic drug used for treatment of various cancers including GBM.

Conclusion

In summary, we find that KDM2B was upregulated in a subset of GBMs compared to non-neoplastic tissue. Further, our data indicated that KDM2B has an important function in promoting cell viability through EZH2- and p21-dependent regulations. Finally, depletion of KDM2B by either knockdown or small-molecule inhibitor treatment resulted in increased apoptosis, reduced tumor-sphere formation and sensitized GBM cells to chemotherapy. Thus, we hypothesize that KDM2B may be a novel therapeutic target in the treatment of GBM, which warrants further investigations.

Future directions based on preliminary findings

Collective our results suggest that the observed attenuated GBM cell growth upon KDM2B inhibition could be due to targeting of the glioma stem cells (GSCs). Similar to normal neural stem cells, the GSCs display expression of various stem cell markers, such as CD133 and Sox2 [50-52]. In line with this, KDM2B is suggested to maintain stem cell self-renewal, and upon knockdown, this induced early differentiation [20,53]. Additionally, KDM2B is highly expressed in mouse embryonic stem cells, and is directly regulated by Oct4 and Sox2 [53]. Consistently, *in silico* analysis demonstrated a positive correlation between KDM2B versus CD133 or Sox2 expression in GBM (supplementary Fig. S1). To further investigate if KDM2B inhibition reduces the GSC population, we analyzed KDM2B-depleted GBM cells for expression of Sox2. Knockdown of KDM2B showed a trend towards decreased Sox2 expression in all cell lines tested (supplementary Fig. S2B and C). In continuation, we tested whether GSK-J4 targets the GSC population. GSK-J4 showed capacity to reduce Sox2 expression in the cell lines expressing high KDM2B (4121, 1587 and T140 cells) evaluated by either WB or qPCR (supplementary Fig. S3A and B). Further, GBM cells were plated and treated with increasing concentrations of GSK-J4 for 72 hours, after which cells were stained with an anti-CD133-FITC antibody, and analyzed by flow cytometry. As shown in supplementary Fig. S4, in 1587 cells, GSK-J4 showed dose-dependently depletion of CD133+ cells reducing the population from 4% (DMSO) to 1.5% at 5 µM GSK-J4. This was also the trend for the 4121 cell line but no clear difference could be seen between DMSO (2.43% CD133+) and 5 µM GSK-J4 (2.30% CD133+) treatment. These preliminary results, presented in the supplementary data, indicate that GSK-J4 might target the GSC population. This is in line with a recent study, showing GSK-J4 to target ovarian cancer stem cells demonstrated by reduced expression of stem

cell markers including CD133, Sox2, and Nanog [48]. We will further explore this hypothesis, by uncovering the effects of GSK-J4 on the GSC population. Future experiments will aim at determine expression of other stem cell markers in KDM2B inhibited cells (by siRNA or GSK-J4). In addition, the effect of combined treatment with GSK-J4 and VP-16 will be evaluated in tumor-sphere assays. Finally, as GSK-J4 has shown capacity to abrogate tumor growth *in vivo* [47,48], we hope to validate the inhibitory effects of GSK-J4 and/or siRNA-mediated KDM2B knockdown on intracranial glioma tumor growth.

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Figure legends

Fig. 1 Expression of KDM2B in GBM

(**A**) Expression of KDM2B was assessed in GBM cell lines compared to normal human astrocytes (NHA) by Western blotting. GAPDH serves as loading control. (**B**) The same GBM cell panel was investigated for KDM2B mRNA expression compared to NHA by qRT-PCR. (**C**) Three selected GBM cell lines (4121, 1587 and T115) and NHA were stained for expression of KDM2B by immunofluorescence staining, counterstained with DAPI, and images were visualized by confocal microscopy. (**D**) GBM patient samples were assessed for KDM2B mRNA expression compared to normal brain samples (Brain 1: Clontech #80151, Brain 2: Ambion #7962).

Fig. 2 Loss of KDM2B inhibits GBM cell viability

KDM2B expression levels were evaluated by (**A**) qRT-PCR (n=4) and (**B**) Western blotting (48 and 72 hours after plating, respectively) following transfection with siRNA negative control (siCTRL) or two independent siRNA constructs targeting KDM2B (siKDM2B-1 and siKMD2B-2). (**C**) Growth of transfected GBM cells were assessed over time by applying a MTT assay. Data are normalized to day 1 (24 hours after plating), (n=3). (**D**) Percentage of cells in S-phase upon knockdown of KDM2B was assessed 72 hours post siRNA transfection using EdU-based cell cycle analysis, (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3 KDM2B depletion induces apoptosis and sensitizes GBM cells to chemotherapy

(**A**) Western blot analysis using anti-cleaved/total PARP, anti-EZH2, anti-p21, and anti-cleaved caspase-3 antibodies in siCTRL or siKDM2B GBM cells. GAPDH serves as loading control. (**B**) Transfected GBM cells were plated and incubated ON following administration of either CCNU or VI-16 at indicated concentrations and incubated for additional 72 hours after which cell viablity was measured by MTT assay. The results are shown relative to siRNA negative control (siCTRL, 0 μM) treated cells and presented as mean ± SEM (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (**C**) Western blot analysis using anti-cleaved/total PARP, and anti-cleaved caspase-3 antibodies in transfected (siCTRL or siKDM2B-1) GBM cells treated with indicated concentrations of CCNU for 48 hours.

Fig. 4 GSK-J4 treatment induces apoptosis, and inhibits tumor-sphere formation and cell viability *in vitro*

(**A**) GBM and NHA cells were treated with increasing concentrations of a histone demethylase inhibitor, GSK-J4, for 72 hours after which cell viability was assessed by MTT assay (**B**) GBM cells were treated with increasing concentrations of GSK-J4 for 48 hours, harvested, and submitted to Western blotting using anti-KDM2B, anti-cleaved/total PARP, anti-EZH2, anti-p21, anti-H3K36me2 and anti-cleaved caspase-3 antibodies. GAPDH serves as loading control. (**C**) *In vitro* limiting dilution tumor-sphere forming assay. GBM cells were plated in 96-well plates at various seeding densities (1-50 cells per well, 16 wells per condition) and treated with 0 μM (DMSO, black lines), 1 μM (red lines), 2.5 μM (green lines) and 4 or 5 μM (blue lines) of GSK-J4 for 1587 and 4121, respectively. After ten days, each well was assessed for the presence or absence of tumor-spheres. (**D**) Estimated stem cell frequency for each treatment condition.

Fig. 5 Combined treatment with GSK-J4 and chemotherapy display synergistic inhibition of cell viability and colony formation *in vitro*

(**A**) GBM cells were treated with low-dose (LD) or high-dose (HD) single-therapy or a combination of GSK-J4 and CCNU and cell viability was assessed after 72 hours by MTT assay. (**B**) GBM cells were treated with LD or HD single-therapy or a combination of GSK-J4 and VI-16 and cell viability was assessed after 72 hours by MTT assay. Data are presented as mean ± SEM ($n \geq 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (**C**) In order to assess for synergism in the applied combination regimens (**A** and **B**), combination index (CI) values were calculated using the CompuSyn software. CI values < 0.9 indicate synergy, 0.9-1.1 additivity, and > 1.1 antagonism. CI values were calculated from at least three independent experiments and presented as mean ± SEM. (**D**) *In vitro* limiting dilution tumor-sphere forming assay. GBM cells were plated in 96-well plates at various seeding densities (1-50 cells per well, 16 wells per condition) and treated with 0 µM (DMSO, black lines), 2.5 µM GSK-J4 (red lines), 5 or 25 µM CCNU (green lines) for 4121 and 1587, respectively, or their combinations thereof (blue lines). After ten days, each well was assessed for the presence or absence of tumor-spheres. (**E**) Estimated stem cell frequency for each treatment condition.

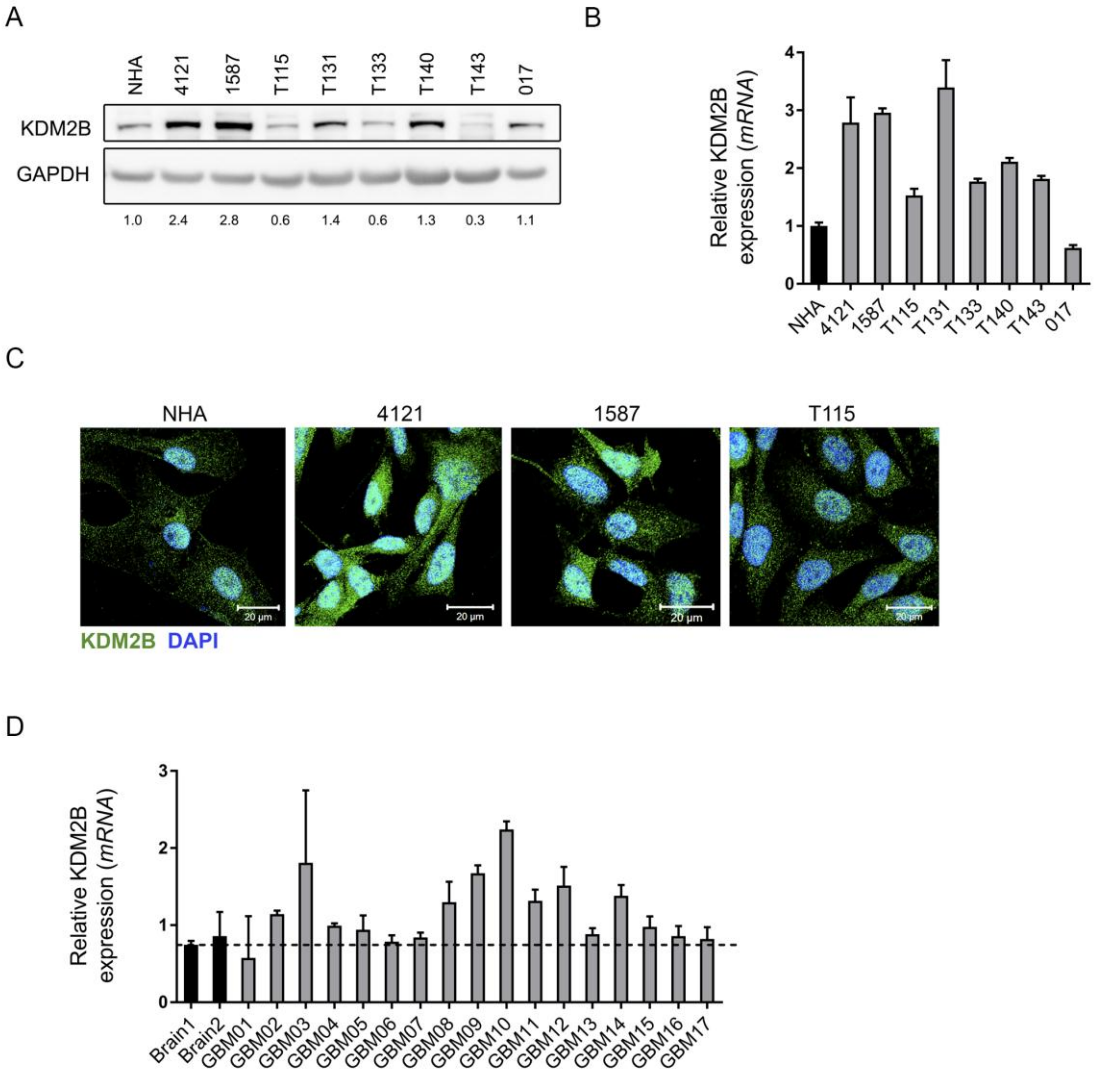
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Fig. 1

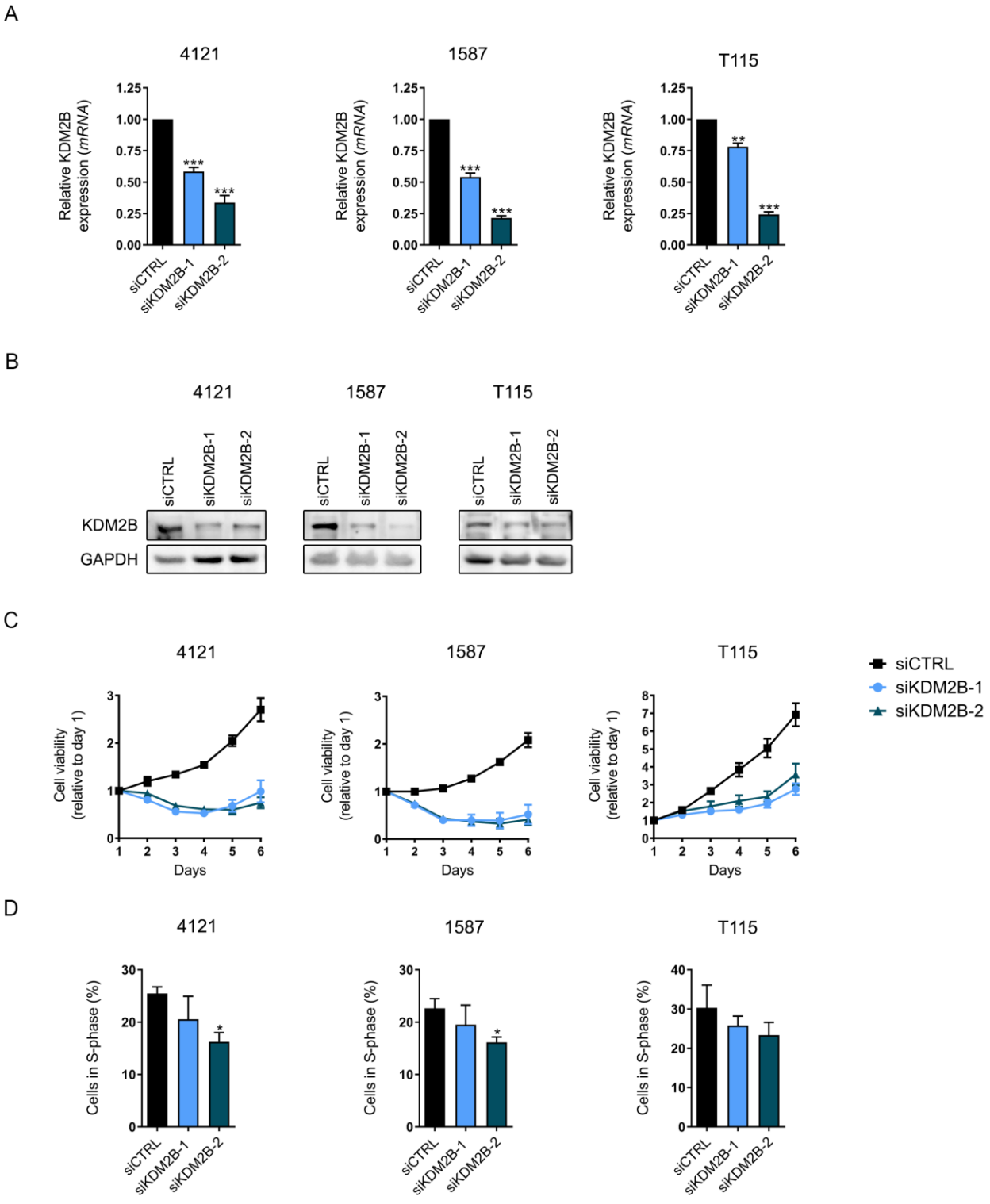

A
4121
1587
T115
Relative KDM2B expression (mRNA)
siCTRL
siKDM2B-1
siKDM2B-2
B
KDM2B
GAPDH
C
Cell viability (relative to day 1)
Days
siCTRL
siKDM2B-1
siKDM2B-2
D
Cells in S-phase (%)

Fig. 2

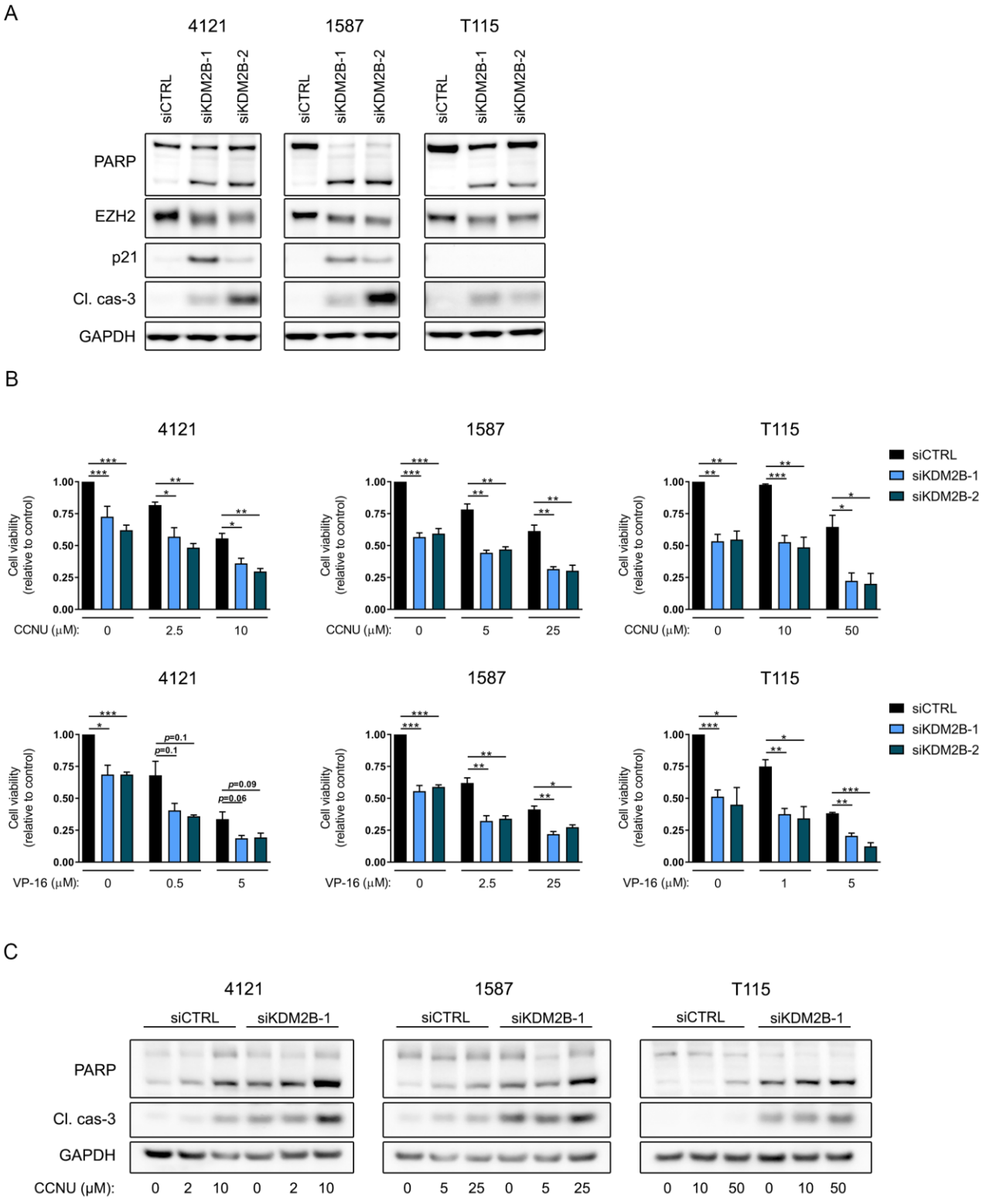
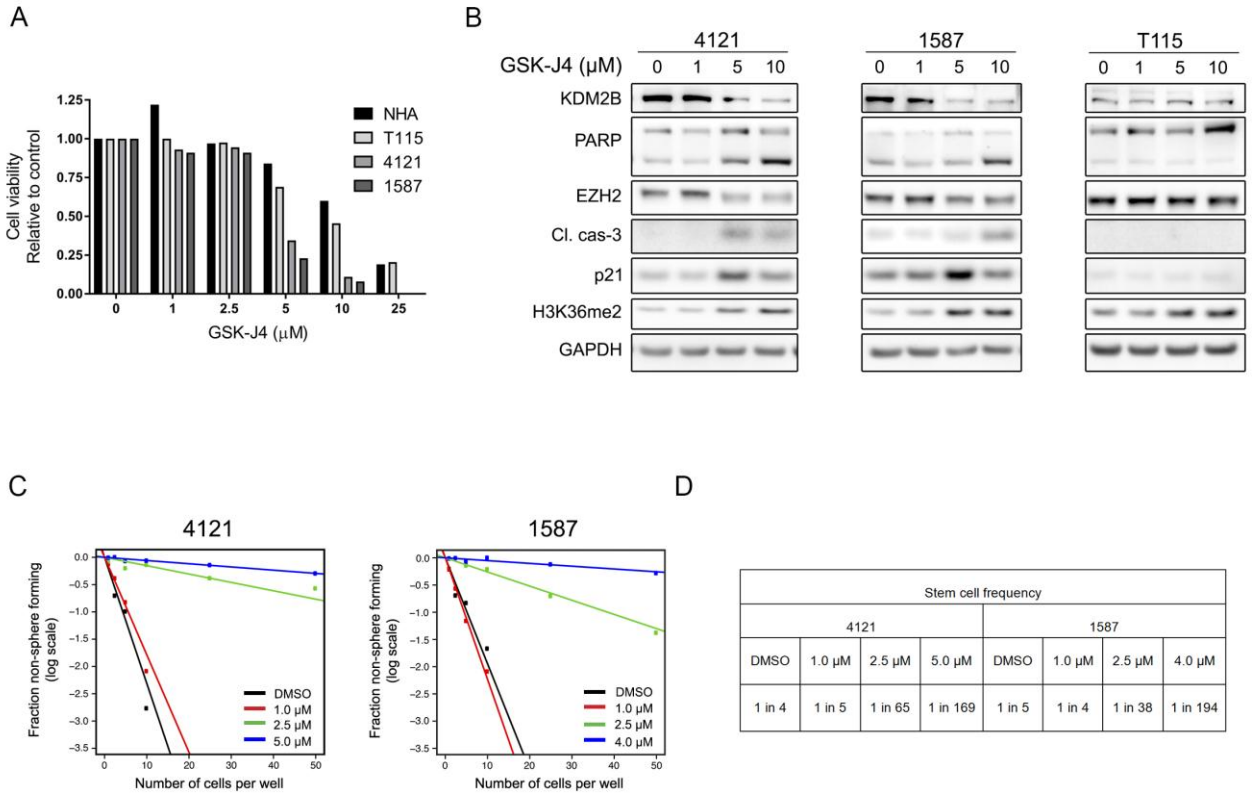

A
4121
1587
T115
siCTRL
siKDM2B-1
siKDM2B-2
PARP
EZH2
p21
Cl. cas-3
GAPDH
B
Cell viability (relative to control)
CCNU (μM):
VP-16 (μM):
C
CCNU (μM): 0 2 10 0 2 10
0 5 25 0 5 25
0 10 50 0 10 50

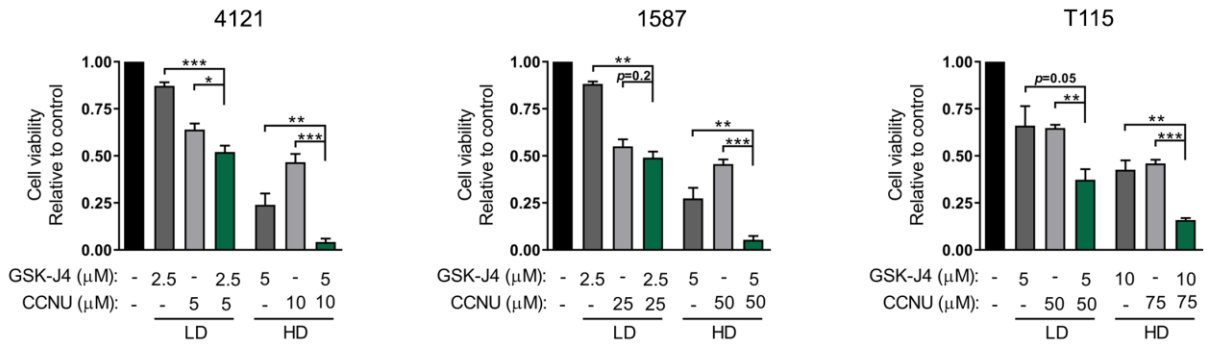
Fig. 3



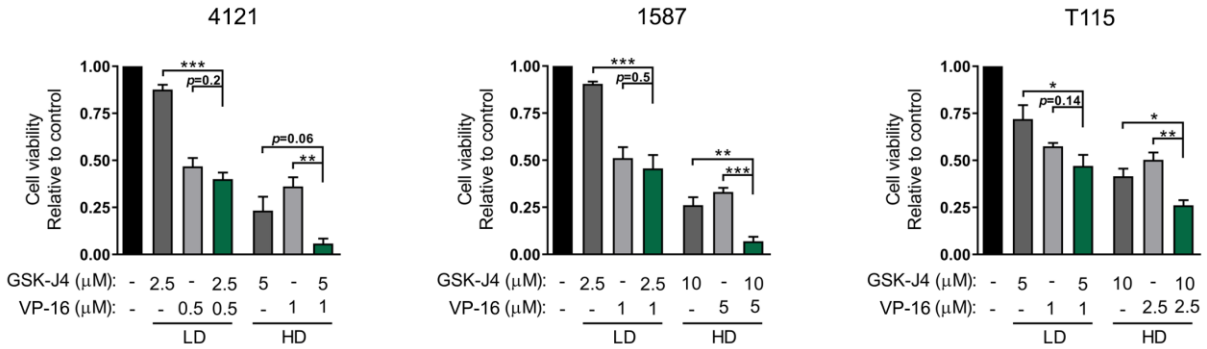
Stem cell frequency							
4121				1587			
DMSO	1.0 μM	2.5 μM	5.0 μM	DMSO	1.0 μM	2.5 μM	4.0 μM
1 in 4	1 in 5	1 in 65	1 in 169	1 in 5	1 in 4	1 in 38	1 in 194

Fig. 4

A



B



C

4121

GSK-J4 (μM)	CCNU (μM)	CI
2.5	5	1.2±0.03
5	10	0.76±0.09
	VP-16 (μM)	
2.5	0.5	1.2±0.02
5	1	0.68±0.13

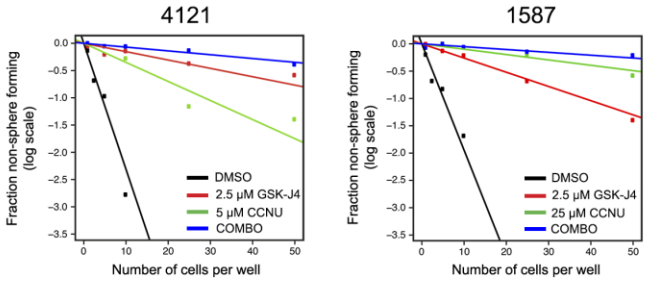
1587

GSK-J4 (μM)	CCNU (μM)	CI
2.5	25	1.1±0.06
5	50	0.47±0.11
	VP-16 (μM)	
2.5	1	1.1±0.08
5	5	0.61±0.10

T115

GSK-J4 (μM)	CCNU (μM)	CI
5	50	1.1±0.06
10	75	1.0±0.03
	VP-16 (μM)	
5	1	1.0±0.04
10	2.5	0.89±0.04

D



E

Stem cell frequency							
4121				1587			
DMSO	2.5 μM GSK-J4	5 μM CCNU	COMBO	DMSO	2.5 μM GSK-J4	25 μM CCNU	COMBO
1 in 4	1 in 65	1 in 28	1 in 143	1 in 5	1 in 38	1 in 101	1 in 192

Fig. 5

Supplementary data and figures

Lysine-specific histone demethylase KDM2B regulates chemoresistance and maintenance of glioblastoma cells

Mikkel Staberg[1], Signe Regner Michaelsen[1], Mette Villingshøj[1], Hans Skovgaard Poulsen[1] and Petra Hamerlik[2]

[1]Department of Radiation Biology; The Finsen Center; Rigshospitalet, Copenhagen, Denmark

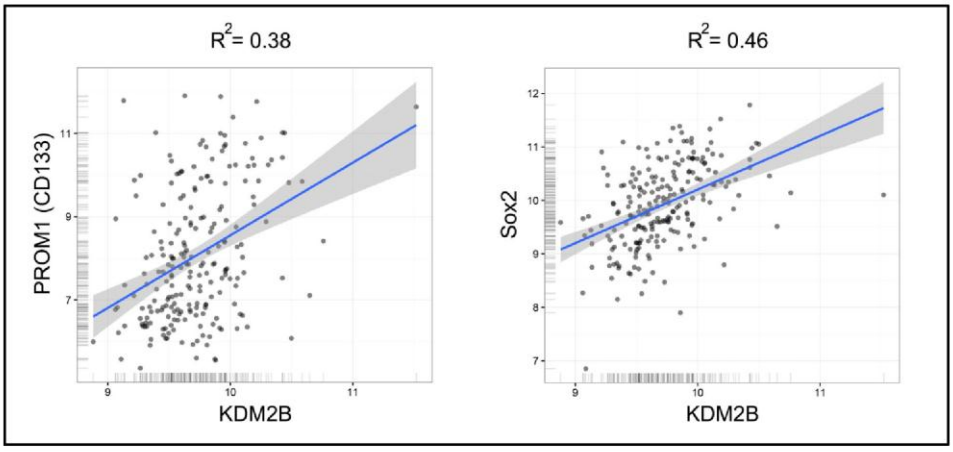
[2]Brain Tumor Biology Group, Danish Cancer Society Research Center, Copenhagen, Denmark

Correspondence should be addressed to:

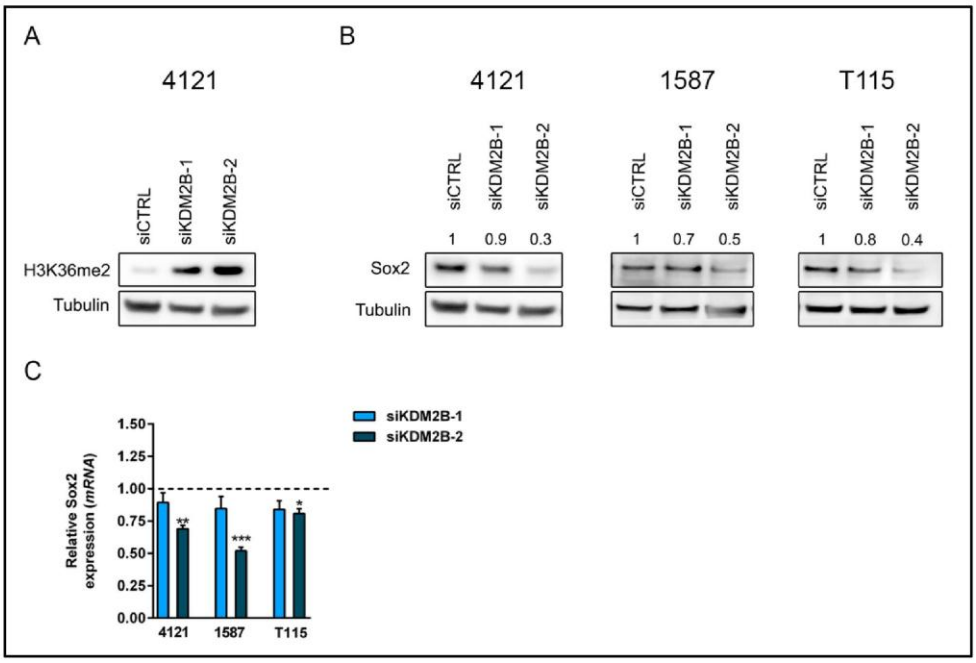
Petra Hamerlik, pkn@cancer.dk, phone: +45 35 25 74 05, fax: +45 35257721

Dilution	Antibody	Manufacturer
1:1000	Rabbit anti-cleaved caspase-3	Cell signaling, #9664
1:10000	Rabbit anti-GAPDH	Santa Cruz, #sc-25778
1:1000	Mouse anti-Sox2	Millipore, #MAB4343
1:500	Mouse anti-KDM2B	Novus, #H0084678-M09
1:1000	Rabbit anti-tubulin	Cell signaling, #2125
1:1000	Rabbit anti-PARP	Cell signaling, #9542
1:1000	Mouse anti-EZH2	BD Biosciences, #612666
1:1000	Mouse anti-p21 (Waf1/Cip1)	Cell signaling, #2946
1:1000	Rabbit anti-H3K36me2	Cell signaling, #2901

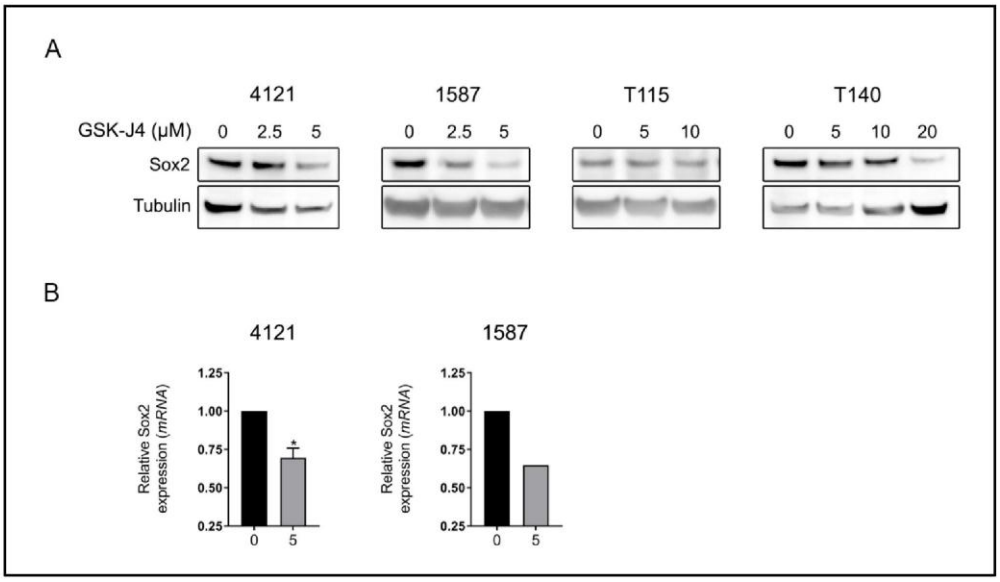
Supplementary Table 1. Overview of primary antibodies used for Western blotting (WB). Antibodies are listed with dilution, manufacturer and catalog number.



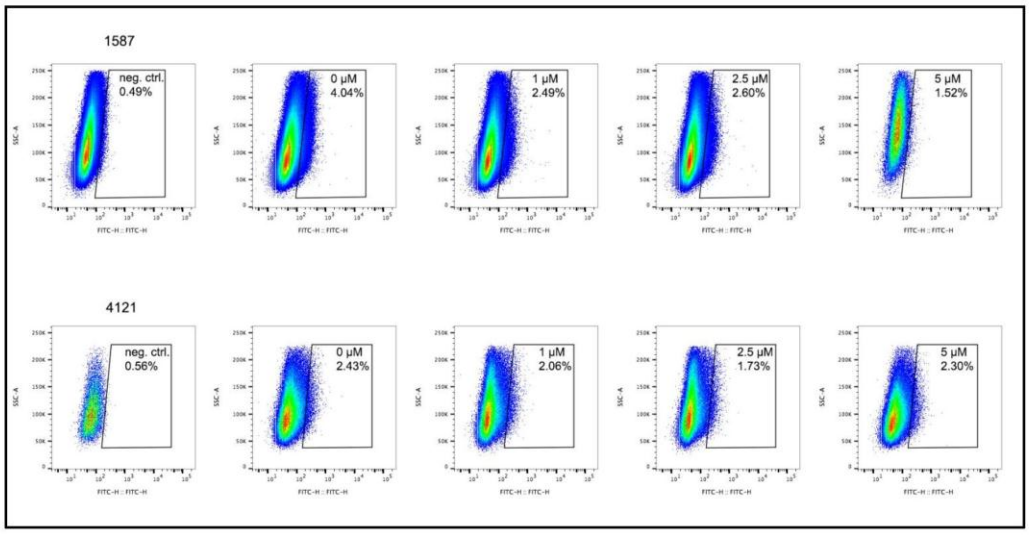
Supplementary Figure S1. KDM2B expression display positive correlation with stem cell markers PROM1 (CD133) and Sox2 in glioblastoma. The analysis was done using the Rembrandt data set obtained at the GlioVis website (http://gliovis.bioinfo.cnio.es/).



Supplementary Figure S2. Expression of Sox2 upon KDM2B depletion. GBM cell lines 4121, 1587 and T115 were transfected with siKDM2B or siCTRL constructs. (**A**) Di-methylation of histone H3 at lysine 36 (H3K36me2) in transfected 4121 cells analyzed by WB. (**B**) Expression levels of Sox2 were analyzed by WB or (**C**) by qRT-PCR, 72 or 48 hours post siRNA transfection, respectively. Bar graphs displayed as mean ± SEM (n=3). Dashed line represents standardized Sox2 mRNA expression in siCTRL-cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Supplementary Figure S3. GSK-J4 reduces Sox2 expression in GBM cells. (**A**) GBM cell lines 4121, 1587, T115 and T140 were treated with increasing concentrations of GSK-J4 for 72 hours, harvested and analyzed by WB for Sox2 expression. (**B**) GBM cells 4121 and 1587 were treated with 0 or 5 μM GSK-J4 for 48 hours, harvested and analyzed for Sox2 mRNA levels by qRT-PCR (n=2 for 4121, n=1 for 1587). Bar graphs displayed as mean ± SEM for 4121. * $p < 0.05$.



Supplementary Figure S4. GSK-J4 reduces CD133-positive GBM cells *in vitro*. GBM cells (4121 and 1587) were plated and treated with increasing concentrations of GSK-J4 for 72 hours. After incubation, cells were stained with an anti-CD133-FITC antibody (Miltenyi Biotec #293C3). Dead cells were excluded using 7-AAD staining. Samples were sorted and acquired on a FACS Verse Cell Sorter (BD Biosciences) and analyzed using FlowJo software. One independent experiment is shown.

7. DISCUSSION

GBM patient survival remains poor and current treatments are only palliative. Thus, there is a demand for new treatment options in the management of GBM. Over the years, several new targets have been identified in GBM, but despite a targeted treatment approach, very little improvement has been achieved in patient survival. This indicates that additional molecular mechanisms and/or subpopulations exists that sustains tumor growth and recurrence after therapeutic intervention. It is generally accepted that a GSCs contributes to tumor angiogenesis, resistance and repopulation after initial treatment [88]. Radiotherapy and chemotherapy exhibit cytotoxic effects through their capacity of inducing DNA damage. Since GSCs display aberrant expression of DNA repair mechanisms [90], the modulation of the response to DNA damage may show advantageous as potential therapeutic targets. Thus, a lot of effort has been made in identifying drugs, which preferably target GSCs and that in combination with conventional treatments or targeted agents lead to enhanced tumor elimination.

The studies comprised in this thesis reveal aberrant expression of several molecules involved in GBM maintenance, regulation of apoptosis, DNA repair and angiogenesis, and aimed at investigating their therapeutic potential. Additionally, the data presented illustrate the potential of applying combinational treatment regimens, which may improve targeting and elimination of GSCs as well as non-GSCs in GBM.

The different GBM models

The GBM cells used throughout the three studies are all obtained from patient tumors, and maintained under the same growth conditions as used for normal

NSCs. These cell culture conditions have been suggested to preserve patient tumor characteristics better than culturing in serum-containing media [131]. Additionally, this way of culturing conserves an undifferentiated phenotype capably of forming tumorspheres, and able to give rise to new tumors, which demonstrates their capacity of self-renewal [247]. Further, the tumorspheres display expression of stem cell markers, such as CD133 and Sox2, overall defining them as GSCs [38,58]. Thus, considering the role of GSCs in tumor initiation, angiogenesis, and treatment resistance, the tumorsphere cultures used in this study present a way of identifying potential targets and uncover mechanisms involved in GSC maintenance.

The studies presented in this thesis are based on results from *in vitro* experiments, which offer a fast and cheap screening of new potential targets, but with some disadvantages. For example, *in vitro* experiments do not take tumor complexity into account. This is illustrated in study I where we used an endothelial sprouting assay as a surrogate marker of angiogenesis. Angiogenesis is a multi-step process that relies on modulation of the surrounding stroma, including degradation of the basement membrane, followed by proliferation and migration of endothelial cells, ultimately forming a new blood vessel [16]. As such, the *in vitro* experiments do not show the full picture since no microenvironment is present, and it will be necessary to investigate the presented combination modality in an *in vivo* setting. Subcutaneous xenografts were previously commonly used as cancer models, but studies have shown that orthotopic tumors better resemble the human counterpart [248]. Thus, the use of intracranial GBM tumor models mimics the real situation better than *in vitro* assays in term of validation of target inhibition and the impact on angiogenesis. On the other hand, intracranial *in vivo* studies are laborious, expensive and still represents some issues such as different pharmacokinetics, and altered molecular profile of the tumor compared to the human counterpart [249]. In addition to tumor complexity, the orthotopic model, in contrast to both *in vitro* and subcutaneous models,

offers a way to study drug delivery to site of the tumor. Normally, the BBB is impenetrable to large molecules such as antibodies and to lesser extent small-molecule drugs [250]. However, in GBM, the dysregulated and leaky vasculature does allow for some transport of drugs into the brain parenchyma. Despite of this, dependent of the drug administered, an optimal dose must be identified showing efficient target inhibition of tumor cells, but without severe patient toxicity. Several new approaches have been tried in order to improve delivery to the tumor, and hence some of them will be discussed here. One approach is the use of nanoparticles aiding in the crossing of the BBB through endothelial endocytosis, and at the same time protecting the loaded drug from degradation (reviewed in [251]). The use of biodegradable wafers, loaded with anti-cancer agents and placed in the tumor-resection cavity, is another method for drug delivery. As shown for the chemotherapeutic drug carmustine, this method display advantages over systemic administration due to higher local concentrations, reduced toxicity, and continuous drug delivery (reviewed in [252]). In line with this, the use of convection-enhanced delivery has also been highly debated. This method relies on implantation of a catheter that can deliver a continuous drug flow into the CNS, thereby bypassing the BBB and increasing drug distribution in the brain parenchyma (reviewed in [253]).

Study I discussion

The data presented in study I indicate aberrant expression of both EGFR/EGFRvIII and Notch in GBM cells. This is consistent with a study, by Brennan et al., showing that Notch pathway components are highly represented in an EGFR core group obtained from glioma patient samples [150]. We did not observe complete inhibition of endothelial sprouting upon inhibition of EGFR and Notch, implying activation of alternative angiogenic pathways. Other agents might be needed, together with EGFR and Notch pathway inhibitors, to fully inhibit GBM-induced angiogenesis. Consistent with

this, Dll4-Notch signaling has been shown to mediate tumor resistance to bevacizumab treatment *in vivo*. Interestingly, this resistance was abolished upon treatment with a GSI abrogating Dll4-Notch signaling and combined treatment with bevacizumab displayed synergistic efficacy [254]. Recently, a phase I study including 20 patients with advanced solid tumors treated with a combination of cediranib (a VEGFR inhibitor) and RO4929097 (a GSI) indicated some anti-tumor effects [255]. The results demonstrated one patient obtaining partial response and 11 patients with stable disease [255]. Furthermore, in a recent phase I study, investigating RO4929097 in combination with bevacizumab in 12 patients with recurrent gliomas, one patient obtained complete response and a second partial response [256]. This suggests that combination regimens using anti-angiogenic agents with Notch pathway inhibitors warrants further investigations. However, several alternative pro-angiogenic factors have been suggested to contribute to anti-angiogenic resistance in glioma, including bFGF, Tie-2, and SDF-1α [257]. This may indicate that inhibition of the VEGF-VEGFR axis is insufficient to completely abrogate angiogenesis, even when using a multi-targeted approach with Notch and EGFR inhibitors. Thus, it will be necessary to evaluate the effect of Notch/EGFR inhibition on other pro-angiogenic molecules.

Both EGFR and Notch molecules have been suggested to sustain an undifferentiated population of GBM cells [112,134]. Hence, considering the importance of EGFR and Notch in the pathogenesis of human cancers including GBM, agents targeting EGFR and Notch are still attractive, despite previous inconsistent clinical results in various cancers [142,144,255,258]. We find that both EGFR and Notch signal through a common signaling pathway as also described by others [259]. Despite the promising data on the anti-cancer effects of DAPT, this GSI have shown gastrointestinal toxicity. Thus, one approach to reduce side effects is by employing improved GSIs with less off-targets effects. The previously mentioned GSI RO4929097 has been shown to be well tolerated, and shown some efficacy in various cancer types either as

mono-therapy or in combination regimens [260-262]. An exploratory phase 0/I study recently published, investigated RO4929097 combined with temozolomide and radiotherapy in newly diagnosed GBM or anaplastic astrocytomas [263]. Interestingly, RO4929097 was present in the brain of patients at low micromolar concentrations, comparable to IC50 values in pre-clinical models, indicating efficient target inhibition. This was further confirmed by downregulation of Notch target genes only in patients with good overall survival (> 14 months). Data also showed that RO4929097 treatment of tumor explants, obtained from patients before initiation of treatment, resulted in depletion of CD133-positive cells, highlighting the contribution of Notch in GSCs. Nonetheless, the study demonstrated ongoing neo-angiogenesis despite confirmed Notch inhibition, suggesting activation of angiogenesis through a Notch-independent mechanism. Hence, this rationale for concomitant use of other anti-angiogenic agents as also discussed previously.

In order to identify patients that will benefit from targeted therapies against EGFR and Notch, clinical studies should be designed for selection of patients. As this has not been routinely done in most studies [28,142,256], this could explain only modest effects observed so far. Recently, it was indicated that assessment of rearrangements, activation and expression levels of the Notch pathway predicts GSI sensitivity, and is correlated to clinical outcome in triple negative breast cancer [264]. Furthermore, it was found that GSCs, belonging to the proneural subtype and demonstrating high Notch pathway activity, was more sensitive to GSI treatment, which may allow for future selection of GBM patients that will benefit from GSI treatment [265]. In a prospective phase II trial in patients with recurrent GBM, individuals with EGFR amplification lacking EGFRvIII expression displayed better PFS and OS following treatment with cetuximab as compared to the patient cohort [30]. This is consistent with a study showing glioma patients that demonstrated high EGFR expression and low Akt to respond better to erlotinib than patients with low EGFR and high Akt [266]. However, other studies show no correlation between EGFR amplification

and benefit from antibodies targeting EGFR; nimotuzumab [267] or cetuximab [268], suggesting that other biomarkers are needed to fully predict efficacy of anti-EGFR therapy.

Taken together, the results presented, by us and several other groups described in this thesis, display an important cross-talk between EGFR and Notch regulating VEGF expression, which is involved in GBM maintenance and angiogenesis. By targeting the GSC population, as a result of therapies targeting EGFR and Notch, this may enhance GSC elimination and abrogate neo-angiogenesis through simultaneous inhibition of redundant signaling pathways. Further, this combination may lead to sensitization to conventional therapies and/or other anti-angiogenic therapies, overall displaying increased treatment efficacy. Hence, in a clinical setting, it could be speculated by applying a three-agent treatment strategy, simultaneously targeting EGFR, Notch, and VEGF/VEGFRs, this might reduce tumor resistance and tumor angiogenesis and improve patient outcome.

Study II discussion

In contrast to genetic mutations, epigenetic changes represent a dynamic process regulated by intra- and extracellular clues, leading to altered transcriptional activity and a dynamic heterogeneous tumor cell population. Consequently, exposure to a drug can favor survival of cancer cells that adapts through expression of drug transporters, DNA-repair molecules, and repression of pro-apoptotic proteins, leading to ineffective treatment and selection of resistant tumor clones. Several of these resistance mechanisms have been identified in the cancer stem cells, displaying a high degree of treatment resistance through mechanisms such as deregulated apoptosis, increased drug efflux, enhanced DNA repair or cell quiescence [269,270]. A common hallmark in GBM is treatment resistance leading to relapse underscoring the need to

identify molecules important for the resistant clones. Indeed, growing evidence indicate the GSCs as main contributors to resistance in GBM [271].

HDAC-inhibitors have shown promising potential for their anti-cancer effects and display little toxicity to normal cells [272]. A recent study demonstrated two HDACi, TSA and valproic acid (VPA), to have similar anti-cancer effects reducing cell proliferation and expression of stem cell markers in patient-derived GSCs [273].

Our data presented in study II showed aberrant expression of HDACs in GBM. Thus, we evaluated the effect of applying TSA in order to sensitize GBM cells to CCNU. Our results showed that TSA combined with CCNU displayed enhanced anti-neoplastic effects. In addition to reduced cell viability and induced cell cycle arrest, we observed increased induction of apoptosis and γH2AX foci formation upon combined treatment with TSA and CCNU compared to either of the drugs alone. These results suggest that HDACi attenuates DNA repair mechanisms upon DSBs. Consistent with this, several HDACi including vorinostat, TSA, and VPA were shown to reduce levels of DNA repair proteins Rad50, Rad51 Ku70, Ku80 and DNA-PK, resulting in prolonged γH2AX expression and sensitization of cancer cells to radiotherapy (reviewed in [274]).

HDACs are believed to be involved in oncogenesis, supported by correlative data indicating perturbed function and/or expression of HDACs in a variety of cancers and often correlated with poor prognosis [181,275]. Consistent with our data, a recent study using qRT-PCR analysis showed significant increase of HDAC1, HDAC3 and HDAC6 expression in GBM, whereas HDAC4 did not reach statistical significance, when compared to non-tumoral tissue [276]. In continuation, HDAC1 and HDAC3 expression inversely correlated with survival, whereas HDAC4, HDAC5, HDAC6, and HDAC11 expression levels were positively correlated with survival in all glioma patients [276]. This indicate that the class I HDACs (including HDAC1 and HDAC3) may be a major

contributor to glioma tumor aggressiveness suggesting that applying HDACi preferentially targeting class I HDACs might be advantageous in a clinical setting.

Study III discussion

In study III, we identified the histone demethylase KDM2B as a novel regulator of GBM viability and maintenance, and to be correlated with resistance to chemotherapy. In mouse embryonic stem cells, KDM2B has been shown to be directly regulated by Oct4 and Sox2, and correlated to an undifferentiated phenotype [196]. In addition, emerging evidence indicate that KDM2B acts as an oncogene maintaining cell proliferation in various cancers, and is correlated to an immature phenotype regulating self-renewal [199,200,277]. The polycomp-repressive complexes 1 and 2 (PRC1 and PRC2) consists of several proteins regulating embryonic development, and are involved in transcriptional activation/repression through interaction with the chromatin [278]. Additionally, polycomb proteins target tumor suppressor genes, and have shown to silence tumor suppressors p14 (ARF), p15 (INK4B), and p16 (INK4A), thus promoting cell proliferation and self-renewal [279-281]. EZH2, a part of the PRC2 complex, have been shown to repress apoptosis in cancer cells [282] and to be important for GSC maintenance [283]. We found that upon depletion of KDM2B, this inhibited GBM cell viability and upregulated pro-apoptotic proteins. Further, our results indicated that KDM2B inhibition reduced EZH2 and Sox2 levels. This suggests that KDM2B depletion abrogates activation of polycomp proteins including EZH2, leading to reduced self-renewal and increased apoptosis as also shown by others [199,200]. In line with this, our preliminary data also indicated that when GBM cells were treated with the pan-KDMi GSK-J4, this depleted the population of CD133-positive cells and reduced Sox2 expression. Taken together, this implies that KDM2B are required for GSC maintenance.

Recently, KDM2B silencing showed attenuated GBM tumor growth *in vivo and* sensitized GBM cells to the pro-apoptotic ligand TRAIL through enhanced apoptosis [284]. Consistent with this, our data show that KDM2B depletion sensitized GBM cells to lomustine and etoposide, suggesting KDM2B to be involved in treatment resistance. Indeed, the histone demethylase KDM5A has been demonstrated to be an important factor in temozolomide resistance in GBM [285]. Thus, these findings indicate that histone modifying enzymes, such as KDM2B, are involved in epigenetic regulation of apoptosis and resistance in GBM, which may lay the foundation for new epigenetic therapies. Consistently, our work showed that GSK-J4 treatment enhanced the therapeutic effect of lomustine and etoposide. Previous pre-clinical studies have shown promising anti-cancer effects of GSK-J4 in ovarian cancer, non-small cell lung cancer and pediatric brain stem glioma [286-288]. Furthermore, combining GSK-J4 with the diabetic drug metformin enhanced the anti-cancer effects in non-small cell lung cancer [287]. In summary, this indicates a potential of using KDMi combined with other cytotoxic or targeted agents.

Epigenetic drugs in clinical studies

Several clinical trials are recruiting or already on the way exploring the effect of epigenetic drugs in combination with other cytotoxic agents in GBM. A recent phase II study investigated concurrent radiation therapy, temozolomide, and VPA in 37 newly diagnosed GBM patients [289]. Notably, this study displayed a median OS and PFS of 29.6 months and 10.5 months, respectively, indicating that the addition of VPA to standard treatments may improve patient outcome when compared to historical data.

It will be necessary to evaluate whether epigenetic drugs are able to reach their target in order to demonstrate that pre-clinical results can be translated into a clinical setting. In a phase I-II study, evaluating the combination of

vorinostat, paclitaxel and bevacizumab in metastatic breast cancer, paired biopsies were analyzed before and after vorinostat administration [290]. The study showed that vorinostat induced hyperacetylation of both histone and non-histone proteins, resulting in induced Hsp70, p27, p21 and downregulated cyclin-dependent kinase-4 [290]. Similarly, in a phase II study investigating vorinostat as monotherapy in recurrent GBM patients, treatment with vorinostat was found to induce acetylation of histones and upregulation of E-cadherin, p21 and p27 in post-treatment surgical samples [183]. Several pre-clinical studies have shown comparable results indicating that the orally administration of HDACi in clinical trials is able to cross the BBB and can effectively reach and carry out its effects on the target tumor [291-293].

One possible explanation for the mixed results of HDACi in clinical trials may be the different dosing's used. Consistent with this, recurrent GBM patients receiving high dose versus low dose vorinostat displayed significantly better OS in a phase I trial [294]. Moreover, the order of HDACi administration has been shown to be of great importance, demonstrating that pre-treatment with HDACi before addition of cytotoxic agents or radiotherapy display the greatest effect in pre-clinical studies [177,180,295]. Another issue that needs to be addressed is the identification of patient subgroups that will benefit from HDACi treatment. Several studies have now identified the protein HR23B as a determinant for response towards HDACi treatment in various cancers [296-298]. HR23B is involved in the shuttling of ubiquitinated cargo proteins to the proteasome, and in HDACi-treated cells, HR23B contributes to inhibition of proteasome activity [296]. Interestingly, in medulloblastoma, the most malignant brain tumor in children, HR23B was identified as a predictive marker for sensitivity to HDACi indicating that this marker may also be used in stratification of GBM patients to HDACi treatment [299].

So far, no clinical trials that would evaluate histone demethylase inhibitors in cancer treatment have been completed. However, currently, two phase I

studies are recruiting patients for investigating the pharmacokinetics and safety of GSK2879552, a KDM1A inhibitor, in acute myeloid leukemia and small cell lung cancer (https://clinicaltrials.gov/). In the light of the oncogenic role of KDM1A in cancer [193,300], and the promising pre-clinical studies of KDM1A inhibitors as anti-cancer drugs [301,302], the results of these clinical trials will suggest whether targeting of histone demethylases warrants further investigations in other cancers. In addition, a set of inhibitors against various KDMs, including KDM2B, has been patented by Celgene Corporation (Quanticel Pharmaceuticals), and expected to go into clinical trials in 2016 [303].

HDACs have shown to interact with other histone modifying enzymes such as KDMs [185,304-306], and global loss of H4K16 acetylation together with loss of trimethylation at histone H4K20 has been identified in primary tumors [159,307]. As such, combination strategies that rely on targeting multiple epigenetic enzymes might display improved anti-cancer effects. Indeed, in GBM this approach has shown promising *in vitro* and *in vivo* anti-cancer effects combining tranylcypromine and vorinostat targeting KDM1A and HDACs, respectively [308].

We believe that the enhanced anti-cancer effects seen upon combining epigenetic drugs with conventional chemotherapy may be explained by a number of issues. Initially, inhibitors of HDACs and KDMs delays repair of DSBs through inhibition of DNA repair molecules (indicated in study II), thus making the cancer cells more vulnerable to DNA-damaging agents. Secondly, the unwinding of chromatin, mainly mediated by HDACi inducing hyper-acetylation, may increase the availability of DNA-damaging drugs to the DNA. Another explanation can be attributed the change in gene transcription, leading to altered expression of drug efflux proteins, tumorsupressor genes, stem cell genes and proteins involved in apoptosis (study II and III). Finally, inhibition of both HDACs and KDMs have been shown to induce differentiation of cancer

stem cells [199,309], which in turn give rise to terminal differentiated cancer cells that are more easily eliminated by conventional chemotherapy.

Taken together, our data and the presented literature indicate that inhibitors targeting epigenetic modifiers (HDACs and KDMs) display prominent anti-cancer effects, thereby opening a new avenue for their use in the treatment of GBM. Thus, new treatment regimens consisting of cytotoxic and targeted anti-cancer drugs must demonstrate improved efficacy in order to avoid non-responsiveness and resistance. Nevertheless, future investigations are needed in order to find predictive biomarkers that can identify patient subgroups that might specifically benefit from such a combined treatment.

8. CONCLUSION AND FUTURE PERSPECTIVES

Overall, the results presented in this thesis suggest the use of a combined treatment approach, targeting key cellular processes involved in cancer maintenance including angiogenesis, proliferation, and DNA damage repair that may lead to increased efficacy in the management of GBM. Several lines of evidence, covered in this thesis, have now identified a population of cancer cells in GBM with stem cell potential that are responsible for treatment resistance and recurrence in GBM. Thus, it is necessary to identify new treatment regimens that targets the cancer stem cell population and eliminates the bulk tumor concurrently in order to improve outcome and reduce the risk of relapse. In addition, when using treatment regimens that can abrogate activation of compensatory survival pathways, this may display enhanced therapeutic effect through elimination of cancer cells that otherwise would survive treatment. Finally, it will be necessary to identify biomarkers that can predict sensitivity and be used to stratify patients for specific treatments.

Angiogenesis and a highly proliferative nature are common hallmarks in GBM and both the EGFR and Notch signaling pathways are important contributors for these processes. We found increased inhibition of cell survival and tumor-induced endothelial sprouting when applying a combination therapy regimen against Notch and EGFR. However, we still observed some endothelial cell sprouting despite attenuated VEGF expression upon Notch and EGFR inhibition. Thus, combined targeting of EGFR and Notch should be used in combination with other anti-angiogenic drugs, which may display increased treatment efficacy as a result of even further inhibition of angiogenesis and cancer cell proliferation. It would be advantageous to test such a treatment regimen in an *in vivo* intracranial model, where it would be possible to more closely evaluate the anti-angiogenic effects. In addition, using GSIs demonstrating less toxicity and better efficacy should be evaluated. The GSI RO4929097 has shown some

efficacy in clinical trials but further development of this drug has been halted by the company, illustrating a need for development of novel GSIs.

Epigenetic modifying enzymes such as HDACs and KDMs have been shown to be implicated in tumorigenesis and maintenance. Thus, several agents targeting the epigenetic machinery have been developed. Even though HDACi as monotherapy have displayed some anti-cancer activity, a growing number of studies points to better and more specific anti-cancer effects when given in combination with other drugs. HDACs and KDMs can be divided into various subclasses and display both oncogenic or tumorsupressor functions. Indeed, our data and that of others indicated that HDAC1 and HDAC3 were correlated to glioma malignancy, whereas other HDACs may display a survival advantage [276]. Since most epigenetic drugs display inhibition of multiple targets, it will be necessary to develop inhibitors that only target defined epigenetic molecules involved in tumorigenesis, thus reducing side-effects and the risk of inhibiting tumorsupressor proteins. As discussed previously, the HR23B protein indicates HDACi sensitivity in some cancers. Thus, by analyzing GBM cell cultures for HR23B expression correlated to HDACi sensitivity, it can be established whether this marker may be suitable for patient enrichment in future clinical trials using HDACi. Given our results displaying enhanced therapeutic effect using HDACi and lomustine in combination, this treatment regimen warrants further investigations in an *in vivo* setting in recurrent GBM.

Several KDMs have been correlated to tumorigenesis. Our data indicated that KDM2B maintains GBM cell viability and chemoresistance. The KDMi GSK-J4 was able to inhibit GBM cell viability, and sensitize these cells to chemotherapy. Taken together, this shows that KDM2B is involved in GBM maintenance. However, future investigations are needed to fully elucidate the functional role of KDM2B in GBM, and the potential therapeutic effect of targeting KDM2B in a clinical setting.

Given our findings, we have illustrated the potential of combining several anti-cancer agents in GBM. The design of anti-cancer therapies targeting the whole tumor, together with agents inducing drug sensitivity through changes in the epigenetic state, will open a possibility to attack the heterogeneous tumor from multiple angles. Hence, this will reduce the risk of resistant cell clones to escape and repopulate the tumor.

9. REFERENCE LIST

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GRADUATE SCHOOL OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

DECLARATION OF CO-AUTHORSHIP

Information on PhD student:	
Name of PhD student	Mikkel Staberg
E-mail	mikkel_staberg@hotmail.com
Date of birth	24-.02-1984
Work place	Department of Radiation Biology, Copenhagen University Hosptal, Rigshospitalet, Denmark
Principal supervisor	Hans Skovgaard Poulsen

Title of PhD thesis:
Therapeutic potential of combination therapy in the treatment of glioblastoma

This declaration concerns the following article:
Combined EGFR- and notch inhibition display additive inhibitory effect on glioblastoma cell viability and glioblastoma-induced endothelial cell sprouting in vitro

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	(A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	A
2. Planning of the experiments and methodology design, including selection of methods and method development	B
3. Involvement in the experimental work	B
4. Presentation, interpretation and discussion in a journal article format of obtained data	B

**Benchmark scale of the PhD student's contribution to the article*		
A. refers to:	*Has contributed to the co-operation*	*0-33 %*
B. refers to:	*Has contributed considerably to the co-operation*	*34-66 %*
C. refers to:	*Has predominantly executed the work independently*	*67-100 %*

Signature of the co-authors:			
Date:	Name:	Title:	Signature:
24-11-2016	PETRA HAMERLIK	PhD	

Signature of the PhD student and the principal supervisor:	
Date: 22.11.2016	Date: 28/11/2016
PhD student:	Principal supervisor:

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GRADUATE SCHOOL OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

DECLARATION OF CO-AUTHORSHIP

Information on PhD student:	
Name of PhD student	Mikkel Staberg
E-mail	mikkel_staberg@hotmail.com
Date of birth	24-02-1984
Work place	Department of Radiation Biology, Copenhagen University Hosptal, Rigshospitalet, Denmark
Principal supervisor	Hans Skovgaard Poulsen

Title of PhD thesis:
Therapeutic potential of combination therapy in the treatment of glioblastoma

This declaration concerns the following article:
Inhibition of histone deacetylases sensitizes glioblastoma cells to lomustine

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	(A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	C
2. Planning of the experiments and methodology design, including selection of methods and method development	C
3. Involvement in the experimental work	C
4. Presentation, interpretation and discussion in a journal article format of obtained data	C

**Benchmark scale of the PhD student's contribution to the article*		
A. refers to:	*Has contributed to the co-operation*	*0-33 %*
B. refers to:	*Has contributed considerably to the co-operation*	*34-66 %*
C. refers to:	*Has predominantly executed the work independently*	*67-100 %*

Signature of the co-authors:			
Date:	Name:	Title:	Signature:
22.11.16	Signe Michaelsen	MSc	Signe Alice
22.11.16	Hette Villingshøj	Technician, MPH	Hette Villingshøj
24.11.16	PETRA HAMERLIK	PhD	Hamerlik

Signature of the PhD student and the principal supervisor:	
Date: 22.11.2016 PhD student:	Date: 28/11/2016 Principal supervisor:

GRADUATE SCHOOL OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

DECLARATION OF CO-AUTHORSHIP

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Name of PhD student	Mikkel Staberg
E-mail	mikkel_staberg@hotmail.com
Date of birth	24-02-1984
Work place	Department of Radiation Biology, Copenhagen University Hosptal, Rigshospitalet, Denmark
Principal supervisor	Hans Skovgaard Poulsen

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This declaration concerns the following article:
Lysine-specific histone demethylase KDM2B regulates chemoresistance and maintenance of glioblastoma cells

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	(A,B,C)
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Signature of the co-authors:			
Date:	Name:	Title:	Signature:
22.11.16	Signe Michaelsen	MSc	Signe Michaelsen
22.11.16	Mette Villingshøj	Technician, MPH	Mette Villingshøj
24.11.16	PETRA HAMERLIK	PhD	Petra Hamerlik

Signature of the PhD student and the principal supervisor:	
Date: 22.11.2016	Date: 25/11/2016
PhD student:	Principal supervisor:

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