UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterizing EGFRvIII-independent glioblastoma maintenance

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in

Biomedical Sciences

by

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The Dissertation of Tiffany Elizabeth Taylor is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

This dissertation is dedicated to my family, friends and teachers, who have been my greatest sources of support, encouragement, and accountability through every endeavor of my life.

My Lord and my God, I am, because You are and Your grace towards me did not prove vain.

EPIGRAPH

Therefore do not cast away your confidence, which has great reward. For you have need of endurance, so that after you have done the will of God, you may receive the promise.

Hebrews 10:35-36 (NKJV)

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LIST OF ABBREVIATIONS

GBM	Glioblastoma
WHO	World Health Organization
CNS	central nervous system
BCNU	carmustine
CCNU	lomustine
TMZ	temozolomide
MGMT	O-6 methylguanine DNA methyltransferase
EGFR	epidermal growth factor receptor
ERBB	erythroblastic leukemia viral oncogene
HER	human epidermal growth factor receptor
RTK	receptor tyrosine kinase
TK	tyrosine kinase
PI3K	phosphatidylinositol 3-kinase
Akt	protein kinase B
MAPK	mitogen-activated protein kinase
Cbl	Casitas B-lineage lymphoma
CR	cysteine rich
BBB	blood brain barrier
CTD	carboxyl terminus domain
GERT	radiotherapy, TMZ, and cetuximab
MAb	monoclonal antibody
MMAF	monomethyl auristatin F

DC	dendritic cell
CTL	cytotoxic T lymphocyte
NSCLC	non-small cell lung carcinoma
RISC	RNA-induced silencing complex
DexAMs	dendritic polyamine complexes
ATP	adenosine triphosphate
Bcl2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma extra large
uPAS	urokinase plasminogen activator system
BH3	Bcl-2 homology domain 3
HDAC	histone deacetylase
GSC	glioma stem cell
ABC	ATP-binding cassette
ABCB1	ABC subfamily B, member 1
ABCG2	ABC subfamily G, member 2
NSC	normal stem cell
BMX	bone marrow tyrosine kinase gene in chromosome X
STAT3	signal transducer and activator of transcription 3
POSTN	periostin
αvβ3	integrin alpha v beta 3
TAM	tumor-associated macrophage
TGFβ	transforming growth factor beta
IL-6	interleukin 6
T_{regs}	regulatory T cells

JAK	Janus kinase
ID3	inhibitor of differentiation 3
PDGFRα/β	platelet-derived growth factor receptor alpha/beta
IDH1	isocitrate dehydrogenase 1
NF1	neurofibromatosis type 1
TNF	tumor necrosis factor
CHI3L1	chitinase 3-like 1
MET	hepatocyte growth factor receptor
TP53	tumor protein p53
EMT	epithelial to mesenchymal transition
SCLC	small cell lung cancer
IGF1R	insulin-like growth factor receptor 1
VEGF/R	vascular endothelial growth factor/receptor
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
PTEN	phosphatase and tensin homolog deleted on chromosome 10
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
GTP	guanosine-5'-triphosphate
MEK	MAPK/ERK Kinase
DOX	doxycycline
DSB	double strand break

HDR	homology-directed recombinational repair
NHEJ	non-homologous end joining
HR	homologous recombination
DNA-PKCs	DNA-dependent protein kinase, catalytic subunit
53BP1	p53 binding protein 1
ATM	ataxia telangiectasia mutated kinase
Ink4a/arf	alternate reading frame protein product of the CDKN2A locus
γH2A.X	gamma H2A histone family, member X
DMSO	dimethyl sulfoxide
ROS	reactive oxygen species
SSB	single strand break
NFκB	nuclear factor kappa B
KLHDC8A	Kelch domain-containing 8a
ORF1	open reading frame 1
BTB	bric a brac, tramtrack, and Broad-complex domain
POZ	portions of zing finger proteins domain
BACK	BTB and C-terminal Kelch domain
CTLH	CRA C-terminal to Lish domain
HGNC	Hugo Gene Nomenclature Committee
BBK	BTB-Back-Kelch
SCF	Skp1-Cullin-F-box
IPP	actin-binding protein IPP
V(D) J	variable, diversity, joining recombination

NRP/B	nuclear-restricted protein/brain
ENC-1	ectodermal-neural cortex
pRb	Retinoblastoma protein
Keap1	Kelch-like ECH-associated protein 1
Nrf2	nuclear factor (erythroid 2-related) factor 2
NADPH	Nicotinamide adenine dinucleotide phosphate
cHL	classical Hodgkin's lymphoma
EBV	Epstein-Barr Virus
UTR	untranslated region
SNP	single nucleotide polymorphism
NLS	nuclear localization sequence
ARE	antioxidant response element
qPCR	quantitative polymerase chain reaction
PDX	patient-derived xenograft
LC-MS/MS	liquid chromatography-mass spectrometry
BirA*	*E. coli* DNA binding biotin ligase
TRiC/CCT	TCP-1 Ring Complex/ Chaperonin-containing TCP-1
EV	empty vector
INQ	intranuclear quality control compartment
FITC	fluorescein isothiocyanate
TRITC	tetramethylrhodamine
JUNQ	juxtanuclear quality control compartment
HSF	heat shock factor

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ABSTRACT OF THE DISSERTATION

Characterizing EGFRvIII-independent glioblastoma maintenance

by

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Doctor of Philosophy in Biomedical Sciences

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Amplification and mutation of the epidermal growth factor receptor (EGFR) gene are common genetic hallmarks of glioblastoma (GBM). The most common mutation is an in-frame deletion of exons 2-7, resulting in a constitutively active variant of the receptor, EGFRvIII. Indeed, these molecules are proven drivers of gliomagenesis; yet, therapies that are directed against them, such as small molecule tyrosine kinase inhibitors (TKIs), have been ineffective due to both upfront and

acquired drug resistance. In a genetic model of tetracycline-regulated EGFRvIII expression, it was confirmed that this receptor is essential for the maintenance of glioma growth in vivo. However, similar to a clinical situation of acquired drug resistance, some tumors eventually regained aggressive growth, and these breakthrough tumors persisted despite sustained suppression of EGFRvIII. In this study, we establish that tumor recurrence in the absence of EGFRvIII is in part, afforded by the ability to overcome oxidative DNA damage. From the approach of characterizing the phenotypes that are indicative of therapeutic sensitivity, we found that these phenotypes are typically overturned upon relapse. Specifically, we established for the first time that an increase in DNA damage burden reflects sensitivity to EGFRvIII inhibition in GBM, and that populations that overcome the need for EGFRvIII receptor function, recover from this DNA damage. Interestingly, blocking EGFRvIII alone was sufficient to induce significant DNA damage and we determined that an increase in endogenous reactive oxygen species (ROS) activity contributed to this phenotype. Likewise, we also observed that the resistant populations displayed significantly lower ROS levels.

These results suggested that an adaptive response mechanism might have been activated to neutralize ROS, therefore affording escape. A key genetic change that was common in multiple resistant models was up-regulation of KLHDC8A. This gene has already been shown to be required for the in vivo maintenance of some EGFRvIII-independent populations, but a functional role beyond this was unknown. Here, we show for the first time that loss of KLHDC8A restores sensitivity to EGFRvIII inhibition, by causing an increase in ROS activity above the toxic threshold. Consequently, this resulted in increased DNA oxidation and persistent DNA damage

in the form of double strand breaks. Thus, we have established a novel link between KLHDC8A and ROS homeostasis. Furthermore, our work demonstrates that the characterization of sensitive phenotypes might uncover novel mechanisms that are essential for progressive escape from EGFRvIII blockade, and may also define novel therapeutic windows, whereby greater tumor response to standard of care may be achievable in GBM.

Chapter 1

Introduction to Glioblastoma and the Epidermal Growth Factor Receptor (EGFR)

1.1 Introduction

Each year approximately 29 out of 100,000 adults (aged 20 years or older) are diagnosed with primary brain tumors in the United States, and about one-third of these tumors are malignant (1-3). These malignant tumors represent ~2% of all cancers diagnosed each year and are the cause of 2% of all deaths from cancer in the United States, with a predicted death toll of ~17,000 Americans in 2016 (1,3). Malignant gliomas are among the deadliest of human cancers because they are highly invasive and neurologically destructive (4). The median survival of patients with the most common and most aggressive of these, grade IV glioblastoma (GBM), is 12-15 months, with a 5-year survival rate that remains at less than 5%, despite the use of intensive treatment modalities (4).

The World Health Organization (WHO) classification of Tumors of the Central Nervous System (CNS) distinguishes gliomas based on their histological appearances, where the grade indicates the level of malignancy (5-7). Specific histological features that distinguish GBMs include necrotic areas surrounded by anaplastic cells (pseudopalisading necrosis) and the presence of hyperplastic blood vessels that facilitate microvascular proliferation (6,8). These features contribute to the therapeutically intractable nature and rapid lethality associated with this tumor grade (4,6).

GBMs account for 60-70% of the malignant gliomas diagnosed in American adults between ages 46-74, and are more frequently diagnosed in men than in women (1). Two major subclasses of GBM (primary and secondary) have been established based on the clinical properties and the chromosomal and genetic aberrations that are unique to each (4,9). Primary GBM arises *de novo* from normal

glial cells, or their precursors, and commonly occurs in patients above the age of 45 (2). In contrast, secondary GBM arises from the progressive transformation of lower grade tumors, and are generally seen in younger patients (4). Primary GBM is believed to account for 95% of all GBMs, while only 5% are believed to occur secondarily, and so with consideration for the predominant clinical situation, our work addresses primary GBM cases (10).

1.2 Standard of Care

Standard treatment for almost all GBM cases begins with surgery, with the goal of achieving gross total resection of the tumor to alleviate GBM symptoms and to facilitate treatment of any residual tumor (11). Yet, even with the recent technological developments in surgical techniques, the vast majority of patients are not cured by surgical resection. Tumors often infiltrate the normal brain parenchyma, and this invasive nature necessitates the use of adjuvant radiotherapy that is either combined with or is subsequently followed by chemotherapy (4). This regimen was established following results from pivotal clinical trials conducted in the 1970s and 1980s to evaluate the benefits of adjuvant therapy, where it was demonstrated that patients with malignant glioma treated after surgery with radiotherapy, or radiotherapy in combination with chemotherapy, displayed an increase in overall survival (12,13). Radiotherapy involves the administration of usually 50 to 60 Gy of irradiation to the whole brain following surgery, and for several decades, nitrosoureas, particularly carmustine (BCNU) and lomustine (CCNU), were the most common chemotherapeutics used alone or in combination with radiotherapy (11,14,15).

In 1999, a novel chemotherapy drug, temozolomide (TMZ), was approved for the treatment of GBM in combination with radiotherapy, and soon became a candidate for the standard treatment of other solid cancers (16-18). Temozolomide is an alkylating agent and its principle mechanism of action is to induce abnormal methylation of DNA bases, such as the formation of O6-methylguanine in DNA (16). This DNA adduct is resistant to successful excision by DNA mismatch repair enzymes, which leads to the production of single and double-strand breaks in the DNA and subsequent activation of cell killing pathways (16,19). Clinical studies later

demonstrated that the median survival in newly diagnosed GBM patients treated with radiation plus TMZ was 14.6 months as opposed to 12.1 months for the radiation only group (20,21). Furthermore, a determinant of the survival advantage observed in GBM patients was found to be the methylation status of a DNA repair gene, O-6 methylguanine DNA methyltransferase (MGMT), in which GBMs containing a methylated MGMT promoter, which silences this gene, was associated with the most favorable prognosis (22,23).

The clinical benefit acquired through the addition of TMZ to the standard treatment strategy for GBM signified a therapeutic breakthrough that had not been experienced in the field in over two decades. Yet, despite such an incredible improvement to the adjuvant therapy regimen, the overall survival rate for GBM patients has remained relatively the same as it was more than fifteen years ago. Tumors inevitably recur, and once they do, life expectancy drastically diminishes and only limited therapeutic options are available.

Major advances in molecular and cell biology have afforded the development of novel second and third line therapies for cancer patients in the realm of targeted therapy. So far, targeted therapy has been used effectively against breast tumors driven by growth factor receptor dysregulation, and in the case of GBM, therapies directed against a number of growth factors and their respective receptors are in various stages of clinical development (24-33). The most common and most clinically advanced of these therapies target the epidermal growth factor receptor gene (EGFR) and a mutant form of this receptor, EGFRvIII. The following sections will explore these two molecules and the current status of the major targeted therapies that have been developed against them to treat GBM (**Table 1.1**).

1.3 The Epidermal Growth Factor Receptor (EGFR)

EGFR (also referred to as ERBB1 or HER1) is a member of the HER superfamily of receptor tyrosine kinases (RTKs) together with ERBB2, ERBB3, and ERBB4 (34). The structure of each member comprises: a ligand-binding ectodomain containing two cysteine-rich regions; a single transmembrane region; and a cytoplasmic tyrosine kinase (TK) domain (**Figure 1.1**). Binding of a cognate ligand to the ligand-binding site of HER receptors induces receptor homo- or heterodimerization, resulting in a conformational change that activates the intracellular TK domain. This results in autophosphorylation of the cytoplasmic tail of the receptor, induction of downstream signaling (through the phosphatidylinositol 3-kinase (PI3K)/Akt and the ras-raf-mitogen-activated protein kinase (MAPK) pathways, among others), and transcription of genes controlling pleiotropic cellular responses (34).

The most common ligands for HER receptors are members of the EGF family of growth factors (i.e., epidermal growth factor, heparin binding EGF-like growth factor, amphiregulin, epiregulin, epigen, betacellulin, and transforming growth factor α) (35). Interestingly, there is no known ligand for ERBB2, which is believed to undergo ligand-independent activation (34). HER receptors are localized at the surfaces of many types of epithelial, mesenchymal, and neuronal cells such that signal transduction from these receptors into the intracellular compartment actuates several cellular processes including cell differentiation, metabolism, proliferation, and survival (36).

EGFR was the first receptor to be sequenced and discovered to possess tyrosine kinase activity (36). In 1984, it was revealed that the sequence of EGFR was

closely related to that of a known oncoprotein, the *erbB* tyrosine kinase, previously revealed to be associated with the onset of erythroleukemia (37). Since then, EGFR has been shown to be frequently overexpressed or hyperactivated in a number of tumors (9). Indeed, amplification of EGFR is the most common genetic aberration associated with primary GBM, with a frequency of about 50% in newly diagnosed cases (4). The downstream signaling effects of such aberrations lead to impaired apoptosis, enhanced proliferation, angiogenesis, necrosis, metastatic spread and treatment refractoriness, suggesting a causative relationship between receptor dysregulation and the pathobiology of many cancers (38).

1.4 EGFR Mutations in GBM

Other mechanisms besides gene amplification are implicated in EGFR dysregulation in primary GBM, such as intrinsic alterations of the receptor structure as a result of mutation (i.e., missense and deletions). EGFR is among the most frequently mutated genes in GBM and recent RNA sequencing efforts have afforded comprehensive characterization of these alterations, which vary in frequency, type and structure (39). A number of EGFR point mutations: R108K, A289V/D/T, and G598V among others, have been identified and are predominantly found within the extracellular domain of the receptor (39). These missense mutations are encountered at an allelic frequency of about 14% in GBM and are predicted to promote an active EGFR conformation, thereby affording constitutive receptor activity (39,40).

Deletion mutations in EGFR have also been described, and the variant diversity that arises from such somatic alterations contributes to the RTK heterogeneity that is inherent to primary GBM. An extensive truncation mutation in the amino-terminal domain of EGFR gives rise to the type I variant (EGFRvI), while deletion of exons 14-15 produces the type II variant (EGFRvII) (40). The clinical relevance of EGFRvI is still undefined, but more recent re-characterization of the EGFRvII variant through single-cell genomic analysis and other functional studies revealed that it is indeed oncogenic, overturning previous claims of its irrelevance in gliomagenesis (41). The cytoplasmic tail mutants, EGFRvIV (exons 25-27 deletion) and EGFRvV (exons 25-28 deletion) are the least frequently encountered variants, but are exclusive to GBM. Additionally, these deletion mutations confer protection from ubiquitin-mediated degradation, likely due to the absence of the Tyrosine (Y) 1045 residue (found in exon 27) that is engaged by Casitas-B-lineage proteins (Cbl)

to promote receptor turnover (42).

The most common EGFR mutant encountered in about 30% of primary GBMs is EGFRvIII (also referred to as de2-7EGFR or ΔEGFR). It is naturally co-expressed with amplified wild-type EGFR (wtEGFR) in about 50% of GBMs, but can also be present independently of this aberration (43-45). EGFRvIII arises through an in-frame deletion of 801 base pairs that encode amino acids 6-273 (exons 2-7) of the extracellular domain of EGFR (40,46). Additionally, this truncation produces a novel glycine residue insertion and confers ligand-independent constitutive, but low, tyrosine kinase activity (46) (**Figure 1.1**). Disrupted ligand binding capacity and low level signaling, especially reduced phosphorylation at Y1045, afford evasion from Cbl-mediated receptor internalization, rendering increased stability of EGFRvIII at the cell surface and amplified mitogenic effects (42,47).

EGFRvIII is tumor-specific, as it has not been found in normal tissues, and its overexpression is associated with enhanced tumorigenicity, resistance to radio- and chemotherapy, and the maintenance of intratumoral heterogeneity (42,46,48-51). Additionally, a number of its oncogenic properties are shared with wtEGFR such as promoting proliferation, angiogenesis, tumor invasion and reducing apoptosis (52-54). EGFRvIII is heterogeneously expressed among cells within a given tumor, being detected at both very high and very low protein levels in GBM (55). At the DNA level, it is also found to be amplified in the form of double minutes, which are circularized double-stranded DNA fragments located outside of chromosomes (56). Such intratumoral heterogeneity supports the notion that different genetic subdomains within GBM tumors purposefully exist, perhaps, to contribute to the tumor's overall fitness and to cultivate a drug-resistant tumor microenvironment.

Indeed, previous work from our lab has demonstrated that inter-clonal cooperation between populations of tumor cells is a predominant mechanism by which GBM aggressive growth is maintained (50). Mutant EGFRvIII was shown to drive this process through the “impartation” of its own intrinsic tumorigenic abilities, via a cytokine circuit, to the less aggressive neighboring cells, the majority of which expressed amplified wtEGFR (50). In the case of EGFRvIII on double minutes, the heterogeneous nature of its expression here is entirely distinct from the mechanism that is observed in the absence of EGFR-specific treatments. Surprisingly, the tumor’s ability to rapidly eliminate EGFRvIII from double minutes affords an advantage to evade therapies that target oncogenes residing on extrachromosomal DNA (56). Thus, it appears that the dynamics of EGFRvIII expression within GBM tumors is regulated such that its loss on extrachromosomal DNA upon exposure to drug and its concentrated expression in only a subpopulation of cells are both optimal conditions for growth and survival of the tumor mass. Nevertheless, the presence of EGFRvIII confers sensitivity to EGFR-targeting agents, suggesting that specifically targeting this molecule is still a rational therapeutic strategy (57,58).

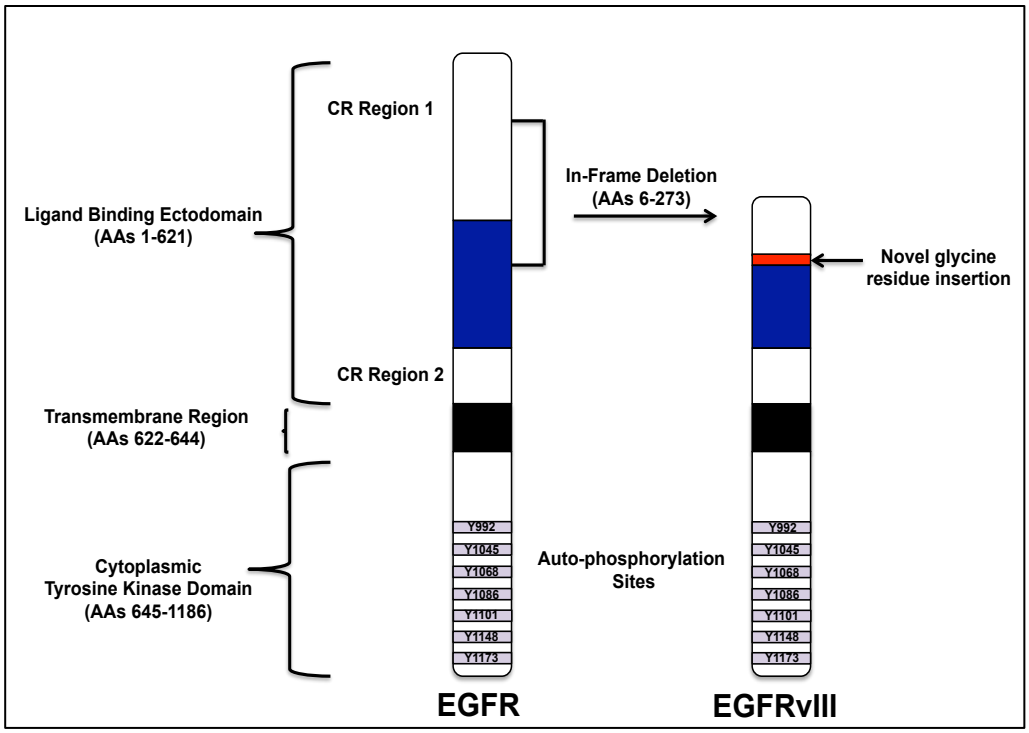

Figure 1.1. Schematic of wild-type EGFR and mutant EGFRvIII protein structures. EGFR is composed of three distinct domains: a ligand-binding extracellular domain containing two cysteine-rich (CR) regions, a transmembrane domain, and a cytoplasmic tyrosine kinase catalytic domain. Mutant EGFRvIII retains the intact intracellular architecture of EGFR, but much of the extracellular region is absent due to the 801 base pair in-frame deletion. Additionally, a distinguishable novel glycine residue exists in this mutant**.**

1.5 EGFR-Targeting Monoclonal Antibodies

Cancer immunotherapy seeks to manipulate a person's own immune system to recognize and specifically destroy tumor cells, using target-specific antigenic proteins and peptides (59). Although tumor immunotherapy has shown some success in the treatment of renal cell carcinoma, melanoma, and hematologic cancers, the application of this approach to glioma presents more of a challenge (59). Limitations include hindrance in the drug's ability to cross the blood-brain barrier (BBB) and induction of potential autoimmunity, which may lead to severe and undesired effects, such as central nervous system toxicity (59,60).

In the early 1900s, Paul Erhlich was the first to propose the process of using monoclonal antibodies to target tumors, and later advances in antibody technology afforded the production of human monoclonal antibodies (60,61). One approach to inhibit EGFR-mediated signaling is to disrupt receptor-activating ligand binding (60). Blocking a ligand from binding to its cognate receptor could normalize growth rates, induce apoptosis, and increase tumor susceptibility to chemotherapeutic agents. Monoclonal antibodies, both unconjugated and conjugated, directed towards wtEGFR and EGFRvIII have been developed for therapeutic use in GBM. The most developed of the unconjugated antibodies is cetuximab (Erbitux®; Merck KGaA), which functions to prevent EGFR-mediated signal transduction by interfering with ligand binding and EGFR extracellular dimerization (60). Additionally, cetuximab is believed to trigger EGFR receptor internalization and destruction (62).

Mouse xenograft (intracranial and subcutaneous) studies demonstrated that treatment with cetuximab decreases tumor proliferation and increases cell death and overall survival (63). Yet, other pre-clinical data suggested that treatment with

cetuximab alone had minimal impact on glioma tumorigenicity; but was synergistic with cytostatic drugs and radiotherapy (62,64). Based on the latter data, the efficacy of combining radiotherapy, TMZ and cetuximab, together known as GERT, in treating patients with primary GBM is being clinically evaluated (65). More recently though, cetuximab alone was shown to be effective against GBM tumors harboring a newly identified exon 27 deletion mutation in the carboxyl-terminus domain of EGFR referred to as EGFR-CTD (66). Particularly, cetuximab impaired tumorigenicity and prolonged the survival of xenograft mice harboring the oncogenic EGFR-CTD deletion mutant (66).

Antibodies are large in molecular weight; but despite this fact, these data suggest that cetuximab can traverse the blood-brain barrier. Thus, while cetuximab alone has on occasion displayed limited clinical effectiveness among GBM patients, it may be a more promising therapeutic for patients with GBMs specifically harboring the EGFR-CTD deletion mutation. Furthermore, it appears that patient stratification will be required to confidently establish the determinants of drug efficacy. Other unconjugated monoclonal antibodies in clinical development to treat GBM include panitumumab (Vectibix®; Amgen**)** and nimotuzumab (Theraloc®; YM BioSciences Inc.), which all function similarly to cetuximab (9,60). So far in glioma clinical trials, panitumumab does not effectively cross the blood brain barrier and nimotuzumab is associated with nervous system toxicity (67). Therefore, the fate of these therapies as treatments for GBM is uncertain.

The specific binding properties of antibodies can be exploited to deliver the toxic properties of either toxins or radioisotopes to target sites, and are referred to as conjugated antibodies. ^{125}I-MAb 425 is one of the most advanced of the radioisotope-

conjugated monoclonal anti-EGFR antibodies. Various phase II clinical trials have reported that ^{125}I-MAb 425, either administered alone or concomitant with radiotherapy or temozolomide, significantly improves median survival in GBM patients (68-70). The ligand-conjugate toxin composed of EGF and diphtheria toxin, DAB389EGF, represents another means to specifically transport toxins to the site of EGFR, and involves the use of cognate ligands as vectors. Results from studies assessing DAB389EGF are encouraging, in which significant tumor regression in subcutaneous glioblastoma xenografts was observed (71). Nonetheless, it still remains to be determined if the performance of these agents in the clinical setting will lead to long-lasting, desirable outcomes for patients.

Some monoclonal antibodies have been engineered to specifically target EGFRvIII. One such antibody is mAb806, which attenuates receptor autophosphorylation by binding to the short cysteine loop of the extracellular domain that is always exposed in EGFRvIII, but may also weakly target amplified wtEGFR, which transiently exposes this epitope during the switch from the inactive to the ligand-activated conformation (72-74). Surprisingly, it does not bind wtEGFR expressed on normal cells, representing a promising feature of this therapy, since many unwanted side effects may be alleviated (75). Pre-clinical data showed that mAb806 strongly inhibits the growth of tumor xenografts that express EGFRvIII and as expected, more weakly those that express wtEGFR (76,77).

To enable clinical development, a humanized version of mAb806 designated ABT-806 (Abbott) was generated (78). Pre-clinical characterization of ABT-806 demonstrated that its antitumor activity is superior to cetuximab against EGFRvIII-expressing tumors, and in the case of wtEGFR-expressing tumors, it is equal in

potency, but with less toxicity (78). These results prompted a Phase I clinical trial with ABT-806, and so far, it is well tolerated and displays excellent bio-distribution and specificity for its target in patients with advanced solid tumors, including glioblastoma (78,79). To date, it is also being clinically evaluated as a monomethyl auristatin F (MMAF) conjugated version called ABT-414, where the conjugate drug is a microtubule depolymerization agent (80). Therefore, both mitotic arrest and selective killing of tumor cells expressing high levels of wild type and mutant forms of EGFR can be achieved. ABT-414 was shown to be effective against recurrent or unresectable GBMs and a phase II clinical trial evaluating its efficacy against newly diagnosed GBMs when combined with TMZ and radiotherapy is currently underway. Of note though, GBMs that expressed low levels of these receptors responded poorly to ABT-414, indicating that EGFR amplification status is key in predicting the best clinical response (80).

1.6 EGFR-Targeting Vaccines

Antitumor vaccines have also been developed with the promise of precisely eradicating tumor cells while limiting toxicity. To date, vaccines in clinical development for the treatment of glioma consist of different combinations of dendritic cells (DCs), peptides, adjuvants, and even autologous tumors (59). The most encouraging of these vaccines are dendritic cell and peptide-based; other combinations have resulted in either no significant improvement over standard therapy or the induction of several adverse events (59). The EGFRvIII–specific vaccines are directed against the novel glycine epitope at the fusion junction that arises as a consequence of the in frame deletion of exons 2-7 from the extracellular domain of wtEGFR (81). The proposed mechanism of action for achieving tumor regression with these therapies begins with capture of the antigenic peptide by the antigen-presenting cell. The peptide is then relocated into the most proximal lymph nodes, where it is presented to circulating cytotoxic T-lymphocytes (CTLs). The CTLs are then activated upon recognition of the antigenic peptide via its T-cell receptor, and finally infiltrate the tumor to eliminate the respective cancer cells (82,83).

Tumorigenicity studies in rodents show that intracerebral treatment with an EGFRvIII synthetic peptide conjugated to keyhole limpet hemocyanin (rindopepimut/PEP-3-KLH/CDX-110®, Celldex Therapeutics) reduces tumor size and increases overall survival (84). Prior to its development, major clinical responses were rarely obtained from previous peptide-based vaccine studies, but rindopepimut in combination with TMZ has been shown to improve both progression free survival (14.2 months vs 7.3 months) and median survival (26 months vs. 15.2 months) in GBM patients (9,82). Additionally, a vaccine comprised of DCs pulsed with

rindopepimut has been tested in the clinic and was shown to increase overall median survival time (22.8 months vs. 15.2 months) without severe adverse events (85). These results confirmed both the safety of rindopepimut and its ability to induce EGFRvIII-specific immune responses in patients with newly diagnosed primary GBM.

A vaccine that specifically recognizes wtEGFR has also been designed, in which a wtEGFR binding peptide is conjugated with a lytic-type peptide containing cationic-rich amino acids that kills the cancer cell by disintegration of the cell membrane (86). This vaccine was designed to serve as an adjuvant for either monoclonal antibodies or tyrosine kinase inhibitors to more effectively eradicate the cancer cells that are intractable to signaling inhibition, a characteristic that renders them resistant to these single-based therapy approaches. This EGFR-lytic peptide can destroy cancer cells as quickly as ten minutes after exposure and exerts strong cytotoxic activity on TKI-resistant glioma cells (86). Though this therapy has not been tested specifically in a glioblastoma xenograft model, it was also shown to be effective in suppressing the growth of breast, pancreatic, and oesophageal xenografts (86,87). Furthermore, other evidence has demonstrated that with just a single amino acid replacement in the lytic peptide sequence, specifically a histidine to an arginine, its anticancer activity is enhanced (88). Taken together, these data suggest a potential value for this therapy specifically against wtEGFR-expressing GBMs.

These pre-clinical and early clinical results are very encouraging; yet, a number of obstacles regarding the efficacy of epitope-specific vaccines remain. For instance, the likelihood that targeting a single heterogeneously expressed tumor antigen could potentially select for the survival and outgrowth of antigen-negative cells. In fact, in a multicenter phase II clinical trial to assess the immunogenicity of the

rindopepimut vaccine alone, tumor recurrence following a significant period of progression-free survival was observed (89). Indeed, 82% of the relapse tumors were completely EGFRvIII-negative, demonstrating that this vaccine can effectively eliminate EGFRvIII-expressing cells, but as a monotherapy, it lacks the long-term efficacy that is needed to eradicate this disease (89,90). Thus, overcoming the inherent heterogeneity associated with GBM demands that emerging therapeutic strategies meet criteria that go beyond antigen-specificity.

1.7 EGFR-Targeting RNA

Interference with transcription or translation is another mechanism through which receptor inhibition may be achieved, and some methods that have been developed over the last decade include antisense RNA, RNA interference (RNAi), and ribozymes (91). Antisense oligonucleotides, such as OGX-011, are already in advanced clinical development for the treatment of NSCLC and prostate cancer, and thus far, the clinical responses are very promising (92-94). These RNAs hybridize to the sense mRNA of the target, resulting in inhibition of translation and protein synthesis.

In an orthotopic xenograft model of human glioblastoma, intratumoral injection of a plasmid or viral vector expressing EGFRvIII-targeted antisense RNA was shown to cause a significant decrease in tumor growth compared with controls (95). In RNA interference methods, the suppression of homologous genes by small interfering RNAs (siRNAs) leads to sequence-specific target mRNA degradation. EGFR-specific siRNAs are directed against the TK domain, and were shown to cause 90% knockdown of EGFR mRNA in U251 glioma cells (96). Furthermore, siRNA mediated-knockdown of EGFR resulted in G2/M arrest and reduced proliferation (96). These findings were confirmed in an intracranial xenograft model, where treatment with EGFR-specific siRNAs increased overall survival by almost 90% (96).

One caveat of this approach is the overall safety of siRNAs as therapeutics, given that in other *in vivo* studies, a vast number of mice fatalities were observed, due to oversaturation of RNAi pathways (91). A strategy in place to overcome this obstacle is to use the lowest possible concentration of siRNAs that provides therapeutic efficacy by designing exogenous siRNAs with increasing length (91). This

would introduce them into the RNAi pathway upstream of the RNA-induced silencing complex (RISC) directly at the step of Dicer cleavage, resulting in enhanced RNAi activity at lower concentrations (91). Another strategy in place is the use of cyclodextrin-modified dendritic polyamine complexes (DexAMs) as a vehicle for translocating siRNAs (97). So far, DexAMs have been shown to deliver EGFRvIII siRNAs efficiently and selectively to glioblastoma cells with minimal toxicity (97). Furthermore, co-delivery of EGFRvIII-siRNA and the EGFR tyrosine kinase inhibitor erlotinib was found to significantly inhibit cell proliferation and induce apoptosis in glioblastoma cells (97).

A third method of RNA-based interference that has been explored involves the use of anti-EGFRvIII hairpin ribozymes. Ribozymes catalytically cleave certain RNA substrates in a sequence-specific manner, in which cleavage is mediated by a catalytic core (9). In pre-clinical *in vitro* studies, treatment with anti-EGFRvIII hairpin ribozymes was shown to reduce EGFRvIII mRNA by 90% and inhibit anchorage-independent growth of U87MG-EGFRvIII glioma cells (98). These encouraging pre-clinical outcomes along with the success of RNA-based approaches in other cancers suggest that upon optimization and refining, these therapies should be further explored in clinical trials for the treatment of GBM.

1.8 EGFR-Targeting Tyrosine Kinase Inhibitors

Small molecule tyrosine kinase inhibitors (TKIs) are the most clinically advanced of the EGFR-targeted therapies, and both reversible and irreversible inhibitors are in clinical trials. Some of the reversible inhibitors include erlotinib (Tarceva®/OSI774; Genentech/Roche/OSI), gefitinib (Iressa®/ZD1839; AstraZeneca), lapatanib (Tykerb®; GlaxoSmithKline) and PKI166 (Novartis), and the irreversible inhibitors include canertinib (CI1033; Pfizer/Warner-Lambert) and pelitinib (EKB-569; Wyest-Ayerst) (27). Mechanistically, these TKIs compete with ATP for binding to the tyrosine kinase domain of EGFR (27). The irreversible and reversible nature of these inhibitors lead to the ablation of both phosphorylation of the receptor and downstream signaling (9). Though a number of these inhibitors have been developed, gefitinib erlotinib, and lapatanib represent the most explored TKI inhibitors in the clinical setting for the treatment of GBM.

In pre-clinical *in vitro* studies, erlotinib was shown to inhibit anchorage-independent growth of glioblastoma cell lines (99). More importantly, this inhibition was shown to correlate with suppressed induction of EGFR mRNA (99). Additionally, long-term exposure to erlotinib was found to down-regulate the expression of both EGFRvIII and molecular effectors of tumor invasion in transformed glioblastoma cell lines (100). The efficacy of erlotinib; however, is more characterized in other cancer cell types, where it has been shown to inhibit cell-cycle progression by inducing G_1/S phase arrest (101,102). Moreover, erlotinib is able to induce apoptosis in colon and pancreatic cancer cell lines by stimulating DNA fragmentation and decreasing the expression of the anti-apoptotic Bcl-2 family members, respectively (101,102). Data

from *in vivo* studies demonstrate that erlotinib also displays anti-angiogenic activity by suppressing vessel formation in pancreatic tumor xenografts (102).

Erlotinib was first clinically tested for the treatment of advanced and metastatic NSCLC, and was shown to significantly improve median survival in patients with these tumors by 42.5% in a phase III randomized trial (9). Yet in a more recent phase II clinical trial for GBM therapy, erlotinib was well tolerated, but only demonstrated a modest effect over placebo (103). These results underscore the notion that differences in tissue-specific biology and/or signaling networks coupled to EGFR greatly influence TKI efficacy. Nevertheless, in the same study testing the efficacy of cetuximab against GBM tumors harboring novel EGFR mutations, erlotinib was also shown to be very effective against the EGFR CTD mutant-expressing GBMs (66).

In the case of gefitinib, its antitumor activity is independent of the expression level of EGFR, but is heavily impacted by its ability to inhibit anti-apoptotic signals (57). In ways similar to erlotinib, it is effective at inhibiting the *in vitro* growth of a variety of human cancer cell (104). Additionally, treatment with gefitinib inhibits the survival and proliferative capacity of adenocarcinoma cancer cells and induces G_0/G_1 arrest in pancreatic cancer cells (102). In the *in vivo* context, gefitinib has been shown to hinder the growth of human breast and ovarian tumor xenografts (104).

Despite its success in other cancer cell types, gefitinib has been less effective against glioblastoma. Similar to erlotinib, the clinical efficacy of gefitinib fails to go beyond the pre-clinical studies, in which in one phase II clinical trial, gefitinib was well tolerated and displayed anti-tumor activity, but the median overall survival time in GBM patients was only 38.4 weeks from treatment initiation (105). A more recent phase II trial revealed that gefitinib reaches high concentrations in tumor tissue and

efficiently dephosphorylates its target; however, more dominant regulatory circuits that promote sustained downstream signal transduction independent of EGFR phosphorylation nullify these effects (30). Gefitinib and erlotinib appear to work best against tumors expressing EGFR with mutations in exons 19 and 21 of the TK domain, but to date, such EGFR mutants have not been found in GBM (9).

Lapatanib is a third tyrosine kinase inhibitor to be explored as a treatment option for GBM. It is currently approved for use in the treatment of HER2-positive breast cancer cells and has generated encouraging results when used as a combination therapy with capecitabine (106). This success led to a phase I/II clinical trial to assess response rate, and pharmacokinetics of lapatinib in patients with recurrent GBM (107). The results from this study revealed that lapatanib alone displays no significant antitumor activity in GBM patients. Yet, not all hope in lapatanib has been lost, as it is currently being evaluated in the context of newly diagnosed GBM, as part of a combination therapy with TMZ and radiation. Perhaps this approach will match the efficacy achieved in breast cancer, but again even these results will not negate the fact that these agents as monotherapies are not durable in GBM. Additionally, though the molecular weights of small molecule inhibitors are within the size limit for molecules that are allowed to cross the BBB, recent studies have shown that plasma concentrations of gefitinib and erlotinib following therapy were only 6-11% of the starting dose (9,58). Thus, insufficient delivery of drug to the target site may be another cause of the disappointing clinical responses to these TKIs.

Table 1.1. EGFR/EGFRvIII-targeted therapies in clinical development for the treatment of GBM. Included here are the major anti-EGFR agents: monoclonal antibodies, tumor-antigen specific peptide vaccines, and small molecule tyrosine kinase inhibitors, along with their current status in clinical trials.

Therapy	Substance	Brand Name	Target(s)	Company/Institution	Status in Clinical Trials
Monoclonal Antibodies	Cetuximab	*Erbitux*	EGFR	Merck KGaA	Phase I/II (as GERT) ongoing
	Panitumumab	*Vectibix*	EGFR	Amgen	Phase II (w/ Irinotecan) terminated
	Nimotuzumab	*Theraloc*	EGFR	YM BioSciences Inc.	Phase III (w/ radiation + TMZ) completed
	^{125}I-MAb 425		EGFR	Fox Chase Cancer Center	Phase II completed
	mAb 806-MMAF	*ABT-414*	EGFR/EGFRvIII	AbbVie	Phase II (w/radiation +TMZ) ongoing
Vaccines	PEP-3-KLH	*Rindopepimut/ CDX-110*	EGFRvIII	Celldex Therapeutics	Phase III (w/ GM-CSF + TMZ) ongoing
Tyrosine Kinase Inhibitors	Geftinib	*Iressa*	EGFR	AstraZeneca Pharmaceuticals	Phase II completed
	Erlotinib	*Tarceva*	EGFR/EGFRvIII	Genentech Inc.	Phase II completed
	Lapatinib	*Tykerb*	EGFR/HER2	GlaxoSmithKline	Phase II ongoing

1.9 Summary and Key Questions

Both wtEGFR and EGFRvIII are bonafide oncogenes that are prevalent in primary GBM, making them attractive targets for therapeutic strategies. Indeed, EGFR overexpression is a poor prognostic factor, correlating with decreased overall survival in GBM patients, and the presence of EGFRvIII also confers a less favorable prognosis in these patients (45,108). Yet, the clinical outcome for GBM patients treated with the many anti-EGFR agents remains poor due to both upfront and acquired resistance. Thus, while knowledge of inherent genetic alterations is pertinent in determining rational therapeutic targets, the monotherapies that have emerged from this knowledge are inadequate for generating a durable response in GBM patients.

GBM heterogeneity supports the likelihood that there are secondary factors that become relevant once the primary oncogenic event has been silenced. The identities of the key factors involved in tumor recurrence following EGFR/EGFRvIII inhibition in GBM have not been thoroughly established. Lack of such knowledge is a critical issue, because without it, the development of efficacious combination therapies will be limited. In this dissertation, we will explore the adeptness of GBMs at overcoming blockade of EGFRvIII receptor function and the predominant “escape” routes that should be considered when exploring strategies to combat therapeutic resistance.

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Chapter 2

Overview of Established Mechanisms of Resistance to EGFR inhibition

2.1 Introduction

Despite advances in our current knowledge of glioma biology and genetics, this disease remains largely incurable. For the reasons discussed in the previous chapter, many of the EGFR-targeting agents hold great potential, as their specific nature is a majorly desirable therapeutic component. However, the reality is that successful treatment of malignant gliomas with these agents alone has proven to be unachievable (1,2). Mechanisms causing resistance to EGFR tyrosine kinase inhibitors are well established and have been implicated in a number of solid tumors. Some of the documented ways include the acquisition of secondary EGFR point mutations, co-activation and/or amplification of other RTKs, heterogeneity, and up-regulation of drug efflux pumps (2). Many of these have been established in non-small cell lung carcinoma (NSCLC) and breast cancer; however, a comprehensive understanding of the mechanisms of therapeutic resistance that are specific to gliomas is undefined (2,3).

Malignant brain tumors are typified by resistance to apoptosis and diffuse invasion into the normal brain parenchyma (4). Once tumor cells disseminate into the normal brain regions, they are protected by an intact BBB, which can significantly limit the efficacy of therapeutic agents that cannot traverse this barrier (5). Furthermore, despite being robustly angiogenic, the tortuous vasculature in malignant brain tumors critically limits drug penetration (5). It is clear that GBMs are recalcitrant by nature with demonstrated ability to escape the need for receptor function when challenged with receptor-targeted therapeutics (6). In this chapter, we will explore some of the established mechanisms of resistance to EGFR inhibition in GBM and other solid tumors that are essential to this “escaper” phenotype.

2.2 Cross-Resistance

A primary mechanism by which some EGFR TKIs (i.e., gefitinib, erlotinib) exert their antitumor effects is through induction of apoptosis (6). During invasion, brain tumor cells often arrest in mitosis, rendering them refractory to radiotherapy and chemotherapy, where the mechanism of antitumor activity is to trigger DNA damage-induced apoptosis in proliferating cells (5). In essence, brain tumor cells that have developed a way to evade chemotherapy and radiotherapy-induced cell death may also be intractable to these TKIs or vice versa, as a result of cross-resistance. Cross-resistance is defined as tolerance to a normally toxic agent, resulting from exposure to a similarly acting substance.

Oncogenic EGFRvIII has been demonstrated to confer resistance to both radiotherapy and chemotherapy by promoting the induction of the anti-apoptotic protein, B-cell lymphoma-extra large (Bcl-xL) (7). Additionally, in a U373MG glioma model harboring tetracycline-regulatable EGFRvIII, Mukasa *et al*. established that despite sustained repression of EGFRvIII, some GBM tumors eventually recur (8). Indeed, these recurrent tumors called “Escapers” are significantly less apoptotic and display restored Bcl-xL protein expression (8). Furthermore, they are resistant to EGFR TKI’s even when EGFRvIII is re-expressed, reflecting a completely EGFRvIII-independent phenotype. This resistance manifests as failure to up-regulate the pro-apoptotic protein BIM (**Figure 2.1**), due to the urokinase plasminogen activator system (uPAS), a mechanism of resistance that we recently established in different glioma models (9).

In NSCLC, it’s been shown that disruption of *BIM* function can also arise from a deletion polymorphism in intron 2 of the gene, which leads to preferential splicing of

exon 3 over exon 4 (10,11). The resulting gene product lacks the Bcl-2 homology domain 3 (BH3) (located in exon 4), which is indispensable for the pro-apoptotic function of *BIM*, and this deficiency is sufficient to confer inherent resistance to EGFR TKI's. Fortunately, this resistance can be circumvented with treatment cocktails, consisting of EGFR TKI's plus either a BH3 mimetic drug or a histone deacetylase (HDAC) inhibitor (11,12). However, in GBM, this polymorphism has not been detected.

These results suggest that EGFR-targeting monotherapies that solely function through the induction of pro-apoptotic signaling may not reach maximum therapeutic efficacy in GBM (8,13). GBMs that initially exhibit a predominant anti-apoptotic phenotype by way of EGFRvIII receptor function appear to regain this property even in the absence of this gene. These tumors robustly respond, ensuring that a compensatory pathway to dampen cell killing activation is already in place even before exposure to EGFR TKI's. In the case of acquired resistance, this property is likely afforded by adjuvant therapy following surgical resection. Thus, achieving durable drug efficacy with alternative agents may require mechanisms of antitumor activity that are entirely different from the components of the standard of care regimen.

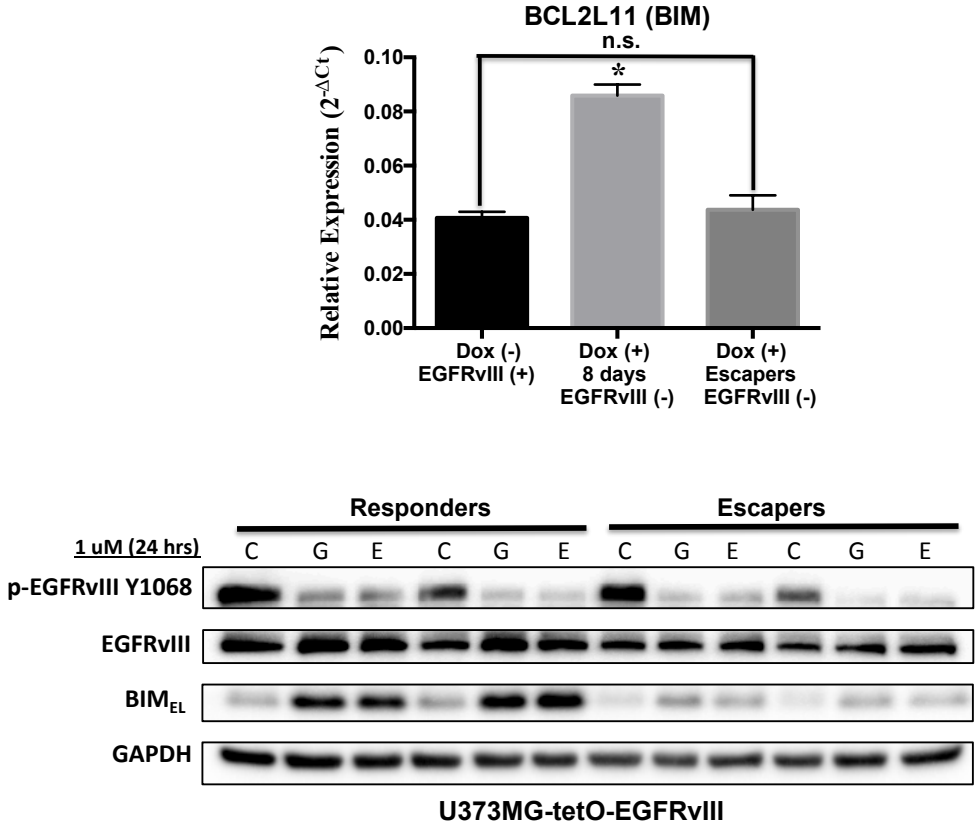

Figure 2.1. Failure to up-regulate the pro-apoptotic protein BIM is an established mechanism of resistance in glioma. When EGFRvIII is inhibited, either genetically or pharmacologically, BIM up-regulation is not observed in "Escapers" at both the mRNA and protein levels. In contrast, the EGFRvIII-dependent populations significantly induce BIM upon EGFRvIII inhibition, suggestive of pro-apoptotic signaling and response. n.s-not significant; C-DMSO control ; G-gefitinib; E-erlotinib

2.3 Glioma Stem Cells

Genetic pathways that are involved in the development of malignant gliomas are now reasonably well characterized; however, the cellular origins of these tumors are poorly understood (14-16). There is growing evidence that glioma stem cells are major contributors to resistance to standard treatments. Several studies have demonstrated that malignant brain tumors that are enriched with cancer stem cells (GSCs) are more resistant to radiotherapy and chemotherapy due to their ability to alter both the DNA damage checkpoint and DNA repair pathways (5,17).

Many conventional therapies specifically target rapidly proliferating cells, while sparing the quiescent, tumor cell compartment. This approach emerged from the notion that proliferation is the main problem to address for successful cancer treatment (18). Indeed, rapidly proliferating cells make up the bulk of the tumor; however, if the GSCs that are left behind can repopulate the tumor, then the question that inevitably arises is: Do the current anti-cancer therapies account for the tumor in its entirety?

Cancer stem cells are thought to be the source of tumor relapse due to their intrinsic and acquired properties, such as increased genetic stability, decreased oxidative stress, and enhanced ATP-binding cassette (ABC) transporter activity (19). In the latter case, activation of these transporters would lead to increased efflux of anti-EGFR agents from the tumor, resulting in decreased intracellular concentrations of drug. Indeed in NSCLC, binding of the EGFR TKI lapatanib to the substrate binding sites of ABC subfamily B, member 1 (ABCB1) and ABC subfamily G, member 2 (ABCG2), actually induces the overexpression of these ABC transporters (20).

The genetic stability of cancer stem cells would render them unperturbed by the pro-apoptotic signals generated by EGFR TKIs. The challenge therefore remains in designing novel therapies that specifically target the GSC population or GSC-associated factors to eradicate these sources of tumor maintenance. Delineating the properties that are most essential to their survival is required, but more importantly, understanding the biological differences between normal stem cells and GSCs is required to develop selective therapies that would spare normal brain cells.

In a study designed to test the efficacy of bortezomib, a protease inhibitor, in treating multiple low- and high-grade GSC cultures, it was revealed that bortezomib can reduce GSC populations by 80% with minimal effects on normal stem cell (NSC) populations (21). Additionally, erlotinib was shown to produce similar results as bortezomib, while TMZ and cisplatin were more toxic to NSCs and less effective against GSCs (21). Thus, it appears that combining newer, promising agents with TKIs, rather than with older chemotherapeutic agents, could result in more durable responses in GBM patients. Furthermore, this could potentially reduce the likelihood of cross-resistance, since the removal of older chemotherapeutics would also eliminate tumor pre-exposure to agents where induction of apoptosis is the predominant cytotoxic property.

The bone marrow tyrosine kinase gene in chromosome X protein (BMX) has also been implicated in maintaining self-renewal and tumorigenic potential of GSC populations by activating the signal transducer and activator of transcription 3 (STAT3) (22). BMX silencing leads to potent inhibition of STAT3 activation and inhibits growth of GSC-derived intracranial tumors (22). Additionally, GSCs can preferentially secrete factors such as periostin (POSTN) to promote the malignant

growth of GBMs (23). Periostin functions as a chemoattractant to recruit monocyte derived macrophages from the peripheral blood into the brain via integrin alpha V Beta 3 ($\alpha_v\beta_3$) signaling (23). Once in the brain, they are transformed into the tumor-supportive M2 subtype of tumor associated macrophages (TAMs) in part by periostin local function (23). Silencing POSTN reduces the recruitment of the M2 subtype of TAMs, leading to inhibition of GBM tumor growth and massive apoptosis in vivo (23).

Interestingly, periostin has been implicated as a driver of emergence from tumor dormancy and activation of metastatic outgrowth in breast cancer (24). Brain tumor cells do not typically metastasize to distant organs; however, the dormancy period observed in breast cancer could be similar to the stasis period observed following EGFRvIII inhibition in our U373MG-tetO-EGFRvIII model (8). The "Escapers" that emerged in our model would then reflect a departure from tumor dormancy. Indeed, in an shRNA *in vivo* screening method to triage putative Escaper-associated genes identified by microarray analysis (8), we found that POSTN supports EGFRvIII-independent tumor maintenance (**Figures 2.2 and 2.3**). Thus, it appears that the expression of a GSC-associated factor confers resistance to EGFR inhibition, and identifying additional factors that may preserve this quiescent population to then ultimately afford escape, is clearly warranted to overcome the contribution of GSCs to therapeutic resistance.

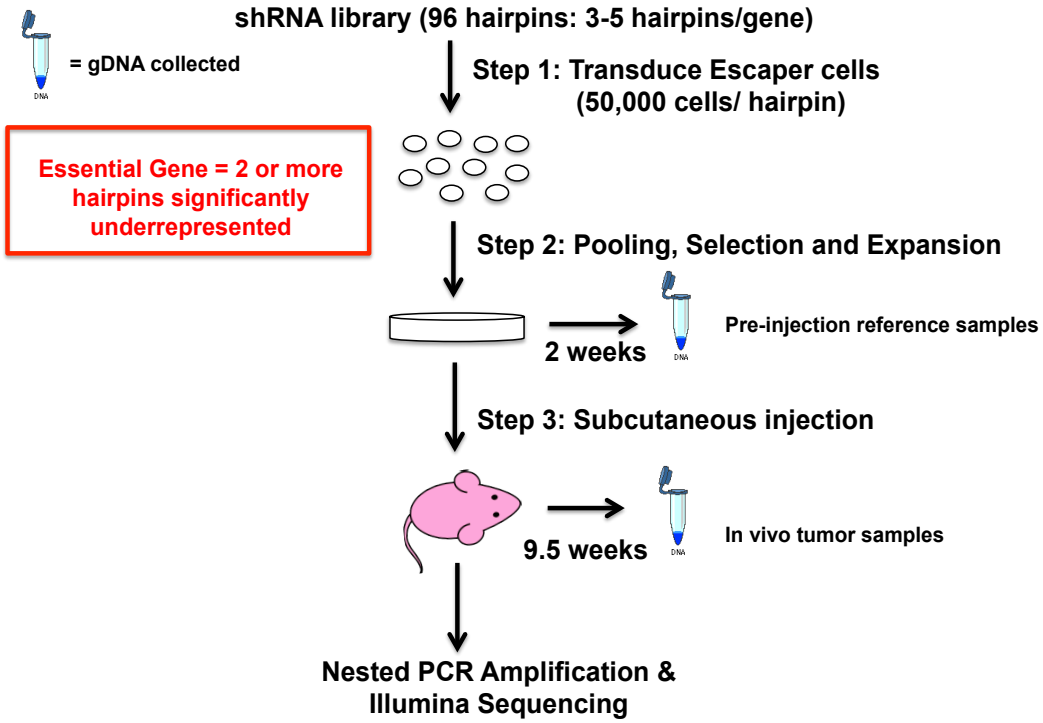

Figure 2.2. In vivo shRNA screen to identify genes that are essential to EGFRvIII-independent tumor maintenance. An outline of the experimental procedure that was used, from the point of transducing the cells with shRNA lentiviral vectors to sample collection and sequencing. The shRNAs used were specific to the 19 "escaper"-associated genes already identified by microarray analysis in Mukasa *et al*., and then 6 internal control genes to substantiate methodology. Hairpin representation values, identified by sequence barcodes, in the resulting *in vivo* tumor samples were subtracted from respective representation values in the pre-injection reference samples to conclude if a gene was essential or not. gDNA-genomic DNA

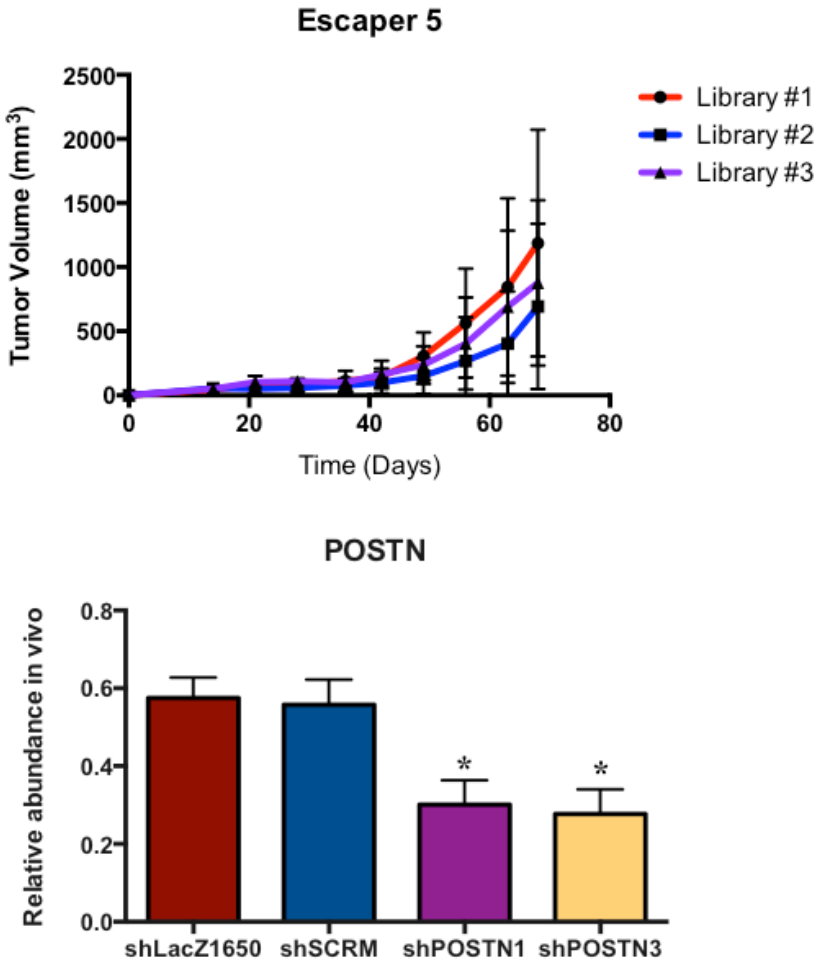

Figure 2.3. Periostin is an essential gene in EGFRvIII-independence tumor maintenance. Three independent hairpin libraries were created to carry out the experiment in triplicate. No significant difference was observed in the growth rate of Escaper 5 tumors resulting from cells transduced with the three libraries. Cells transduced with shRNAs specific to POSTN were significantly underrepresented in the resulting in vivo tumor samples. Relative abundance value reflects the average from the three experiments.

2.4 Tumor Microenvironment

Malignant glioma patients are known to be profoundly immunosuppressed. Thus, even if EGFR-specific monoclonal antibodies or peptide vaccines generate systemic immune responses, they could be negated in the tumor microenvironment by a variety of immunosuppressive growth factors and cytokines (25). In contrast to EGFR TKIs, the mechanisms of resistance to these therapies in any cancer are poorly defined (26). Cytokines, such as the transforming growth factor beta (TGFβ) and interleukin-6 (IL-6), have been shown to promote tumor escape from immunosurveillance, and specifically high plasma levels of TGFβ correlate with a negative prognosis in a variety of cancers (27).

TGFβ confers the transformation of vascular endothelial cells to the pro-angiogenic phenotype that is associated with GBM. Additionally, the production of TGFβ can lead to the accumulation of $CD3^+$, $CD4^+$, $CD25^+$, $FOXP3^+$ T_{Reg} cells. Regulatory T cells (T_{Regs}) are a specialized subpopulation of T cells that function to actively suppress the immune system in order to maintain system homeostasis and to prevent pathological self-reactivity. T_{Regs} are enriched in the peripheral blood of GBM and also in the tumor microenvironment, and though a prognostic role of T_{Regs} present in glioma has not been thoroughly evaluated, T_{Regs} are associated with poor clinical outcome in other systemic tumors (25). In a syngeneic murine model of glioma, depletion of T_{Regs} resulted in complete tumor rejection and enhanced survival (28). Thus, T_{Regs} can antagonize EGFR-specific immunotherapy agents by promoting immunosuppression.

Aside from its involvement in GSC maintenance via BMX, STAT3 also mediates TGFβ production, which has been linked to potent inhibition of both innate

and acquired immune responses (25). Specifically, STAT3 induces immune tolerance via T_{Reg} activity and inhibiting STAT3 impedes T_{Reg} function (29). STAT3 is overexpressed in GBM, and its elevated activity correlates with decreased overall survival in patients (29). Based on these findings, inhibitors of Janus kinase (JAK)/STAT activation have been explored as a potential strategy to overcome T_{Reg}-mediated immunosuppression (25). Presumably, if STAT3 coordinates the activity of T_{Regs} with other cell populations in the tumor microenvironment (e.g. tumor stem cells), then such an approach is expected to be efficacious.

STAT3 is a protumorigenic factor in EGFRvIII-expressing GBMs, and a predicted nuclear interaction with EGFRvIII that alters the binding of STAT3 to DNA has been proposed as the mechanism (30). Currently, GC1008, a TGFβ-specific antibody, and WP1066, a STAT3 inhibitor, are both in clinical development for the treatment of various cancers. Given the role of these factors in immunosuppression, either agent in combination with EGFR-specific immunotherapy agents could potentially be more efficacious in the treatment of malignant glioma.

The secretion of IL-6 by stromal cells into the tumor microenvironment can also promote tumor survival and block apoptosis, thereby resulting in therapeutic resistance (31). These effects are mediated through the IL6R/JAK1/STAT3 pathway and via Bcl-xL function (31). In the former case, signaling through IL6R/JAK1/STAT3 confers *de novo* resistance to irreversible EGFR TKI's in NSCLCs harboring a *T790M* secondary mutation (32). Additionally, TGFβ-dependent IL-6 secretion confers resistance to the EGFR TKI erlotinib in lung tumor cells (33). In GBM, Inda *et al*. demonstrated that EGFRvIII-expressing cells secrete IL-6 in order to activate the wtEGFR-expressing cells through a paracrine cytokine signaling circuit (4). The

secretion of IL-6 was also correlated with enhanced tumorigenic growth of U87 glioma cells (4). An additional mechanism whereby EGFR mediates the production of IL-6 via the inhibitor of differentiation 3 (ID3) was shown to promote tumor cell heterogeneity in GSC populations. These populations as discussed above, are major sources of therapeutic resistance due to their dominant impact on the tumor microenvironment in order to maintain their “stemness” (34). IL-6 has been implicated in drug resistance and survival signaling through both the Bcl-xL and STAT3 pathways in prostate cancer (35). Interestingly, these effects were significantly attenuated by transfection with anti-sense Bcl-xL olignonucleotides, further supporting the notion that RNA-based therapies should be more closely considered as a therapeutic option in GBM therapy (35). Taken together, IL-6 secretion into the microenvironment reflects a common mechanism of acquired resistance to EGFR inhibition and strategies to overcome its effects are clearly warranted.

Stromal cells (e.g., endothelial cells) can also act independently in immunosuppression-mediated resistance. Work from Ricci-Vitiani *et al*. demonstrated that a large proportion of endothelial cells are found to harbor the same mutations as tumor cells (36). Thus, it is presumed that GBM-associated endothelial cells arise from tumor stem cells, and the stem cells that survive therapeutic insult acquire resistance through cell-cell interactions with such stromal cells in the microenvironment. Likewise, others have shown that extracellular matrix proteins as well as hypoxic conditions can also impart radioresistance to the stem cells (17). These findings suggest that conquering immunosuppression-mediated resistance requires targeting both the antigen-expressing tumor cells and the immunosuppressive activities afforded by the tumor microenvironment.

2.5 Histological Heterogeneity

Glioblastomas are considerably heterogeneous, and this intratumoral diversity is becoming increasingly appreciated as a predominant mechanism underlying anti-EGFR therapeutic resistance and tumor recurrence (37). Within these tumors lie mixed cytological subtypes, regional differences in gene expression, and varying representation of key genetic mutations and chromosomal alterations (4). Indeed, the WHO classification of CNS tumors has been restructured in terms of nomenclature to account for this variability (38). Furthermore, concerted efforts at providing a global description of the genetic abnormalities that are present in GBMs resulted in a gene-expression based classification of these tumors into four molecular subtypes: Proneural, Neural, Classical and Mesenchymal (39-41).

The Proneural subtype is associated with two major features: alterations of platelet-derived growth factor receptor alpha gene (*PDGFRA*) and mutations in isocitrate dehydrogenase 1 (*IDH1*) (39,42). Most of the known secondary GBMs are found in this class; consistent with the finding that younger age is also associated with this subtype. Additionally, global transcriptomic analysis revealed that the Proneural tumors resemble oligodendrocytes and express several genes involved in development (39). The Neural subgroup is characterized by expression of neuron markers and genes associated with biological processes such as neuron projection and axon and synaptic transmission. Interestingly, the expression patterns found within this class are most similar to normal brain, and its gene signature can only be concluded as reflecting a cell with a differentiated phenotype (39). Its association with the genetic signatures of multiple cell types (i.e., neural, astrocytes, oligodendrocytes) further demonstrates this genetic ambiguity.

The Mesenchymal subtype is predominantly made up of GBMs harboring aberrations in the neurofibromatosis type 1 gene (*NF1*) and is most reminiscent of astroglial cells (39). Genes associated with inflammation and the tumor necrosis factor (TNF) superfamily are highly expressed in this subtype, which correlates with the high percentage of necrosis that is evident in these tumors (39). Other distinguishable genetic abnormalities found in this subtype include high expression of the mesenchymal markers, chitinase 3-like 1 protein (CHI3L1) and hepatocyte growth factor receptor gene (MET), events from which the group name is derived (41).

The Classical gene signature is strongly associated with astrocytes and is distinguished as the subtype containing the most common genomic aberrations encountered in GBM. The majority of EGFR-amplified or mutated GBMs are found within this subtype, manifesting as polysomy and amplification of chromosome 7 (43). Loss of chromosome 10 (monosomy 10) is another highly frequent event in this subtype, but the overwhelming percentage of EGFR-altered tumors suggests that there is a focused preference for this genomic alteration in GBMs (39,40). This is confirmed by the frequent lack in additional common abnormalities in *TP53, NF1, PDGFRA* or *IDH1*, giving credence to the wave of “EGFR-driven” therapeutic development in GBM (39).

More than one subtype may be found within the same tumor mass, reflecting spatial heterogeneity and another layer of complexity that would challenge patient stratification and treatment simply based on molecular features found within a single tumor specimen (44). Tumor recurrence is observed across all subtypes, but a difference in the degree of clinical response to aggressive therapy by subtype has been observed, ranging from the greatest reduction in mortality seen in the Classical

and Mesenchymal subtypes down to no altered survival benefit in the Proneural subtype (39). In essence, some tumors may be inherently resistant to EGFR-targeted therapy simply because the bulk of the tumor is not majorly dependent on its receptor function.

In NSCLC, histological transformation has even been observed and is attributed to EGFR TKI resistance. Specifically, an epithelial to mesenchymal transition (EMT) and in rare cases, a conversion to small cell lung carcinoma (SCLC) can occur at the time of the development of resistance (12,45). EMT is characterized by loss of cell polarity and cell-cell adhesion, extensive actin cytoskeletal remodeling, and the acquisition of mesenchymal components (46). This process promotes tumor invasion, migration, and progression, and is mediated by a diverse list of cytokines, chemokines and growth factors. Co-targeting the relevant molecular pathways and EGFR can reverse EMT-mediated resistance (47). In the context of the NSCLC conversion to SCLC, the original EGFR activating mutation persists, suggesting that this transformation does not arise from *de novo* clones, but rather emerges from pre-existing cancer cells (48). Cytotoxic chemotherapy and radiation, which is the standard of care for SCLC, is an effective treatment against this switch and in some cases actually primes the tumor to redevelop EGFR TKI susceptibility (45). GBMs; however, do not display evidence of phenotypic transformation upon tumor recurrence (39).

2.6 Compensatory RTK Pathways

Receptor tyrosine kinase (RTK) coactivation or crosstalk is a process in cancer cells defined by synchronous activation of two or more RTKs that supports network robustness and increases the diversity of signaling outcomes, using only a limited repertoire of intracellular signaling factors (49). This process reflects another form of intratumoral heterogeneity that may contribute to the maintenance of individual cell growth within GBMs. Additionally, it is presumed to be an acquired mechanism to reduce dependency on a single RTK, and thus plays a critical role in tumor response to targeted therapeutics (49,50). Indeed, other RTKs, such as the insulin-like growth factor 1 receptor (IGF1R), MET, and PDGFRα/β are altered in GBM, and their ability to compensate for EGFR has been implicated in the persistent activation of downstream survival signaling, even in the presence of EGFR inhibitors (50-53).

As mentioned in chapter 1, EGFR is a member of the HER family, which consists of other structurally similar members prone to form homo- and heterodimers with one another and display functional redundancy. In NSCLC, both HER2 and HER3 are involved in resistance to EGFR TKI's (12). HER2-mediated resistance is afforded by genetic alterations of the gene, while HER3 active heterodimers trigger pro-survival signaling via the PI3K/Akt pathway (54). Interestingly, GSC resistance to EGFR inhibition is mediated by sustained activation of AKT and mitogen activated protein kinase (MAPK) signaling pathways by way of HER2 and HER3 (55). Afatanib, a HER2 specific irreversible inhibitor, is effective against HER2-overexpressing/mutated gastric cancers, but has shown limited activity as a single agent against recurrent GBMs (56). So far, HER3-targeting monoclonal antibodies

look promising based on preclinical studies in others cancers (i.e., head and neck, breast and lung), but have not been evaluated in GBM (12,57).

c-Met is a transmembrane RTK that is frequently amplified in human cancers and in the past was established as the second most common mechanism of resistance to EGFR-TKI's in NSCLC (12). *c-Met* amplified clones exist even before EGFR TKI treatment, suggesting that they are selected out by treatment (58). Interestingly, c-Met can also promote HER3-PI3K/AKT signaling through the stabilization of HER3 phosphorylation (59). This mechanism of action occurs even in the presence of gefitinib, reflecting a bonafide EGFR kinase-independent bypass pathway (60). In GBM, the ability for c-Met to compensate for EGFR is demonstrated in that treatment with erlotinib alone had no discernible effect on inhibiting the activation of the PI3K/AKT pathway in U87MG-EGFRvIII cells. However, when used in combination with the MET inhibitor SU11274, downstream signaling as measured by activated Akt and S6 ribosomal protein was significantly inhibited and anchorage-independent growth of these cells was also reduced (50,61). Similarly, activation of c-Met expression in response to EGFR inhibition leads to the survival of GBM tumor cells in a mouse model of glioblastoma and inhibiting MET reverses this phenotype (62).

IGF1R signaling through PI3K is another common compensatory pathway that mediates resistance to EGFR TKI's (63). Concomitant inhibition of both EGFR and IGF1R is required to effectively abort PI3K/Akt signaling in resistant cells and restore sensitivity to gefitinib (64). Aside from the PI3K/Akt pathway, IGF1R signal transduction also regulates the function of cyclin-dependent kinase inhibitor 1B (p27), by altering its activation and subcellular localization (65). Thus, IGF1R-p27-mediated

cell proliferation and motility may represent another pathway that cancer cells utilize to circumvent EGFR inhibition.

The vascular endothelial growth factor and receptors (VEGF/VEGFRs) are the most common pro-angiogenic factors in GBM. Angiogenic signaling mediated by these molecules induces tumor cell proliferation and migration and suppresses apoptosis and drug penetration. In several human cancers, acquired resistance to EGFR inhibition is associated with increased secretion of VEGF (66). Additionally, the VEGF/VEGFR2 signaling pathway can promote the secretion of more VEGF through a feed-forward loop mechanism involving the PI3K/ mammalian target of rapamycin (mTOR) pathway (12). On the basis of the highly angiogenic nature of aggressive cancers, VEGFR targeting inhibitors have been evaluated for clinical use in many cancers, including GBM. So far, as monotherapies, durable responses have not been observed; however, dual inhibition of EGFR and VEGFR produces antitumor effects in several human cancers (66). This was observed even in the cases of acquired resistance to EGFR TKI's, validating VEGFR as a legitimate EGFR substitute.

PDGFRβ, which is usually suppressed by EGFRvIII through Akt/mTOR complex 1 (mTORC1) signaling, becomes transcriptionally de-repressed upon EGFR inhibition to restore GBM growth and survival, reflecting another established mechanism of resistance (67). Combined abrogation of EGFRvIII and PDGFRβ is then required to block glioma in vivo growth. Alternatively, coactivation of EGFR and PDGFRα occurs in GBM (50). Blocking PDGFRα alone results in only modest antitumor activity, yet PDGFRα inhibition is still required to completely eradicate survival signaling through the PI3K/Akt pathway in GBM. Thus, in multiple cancer types, the signaling outputs from RTKs converge and activate similar signaling

pathways such as PI3K/Akt. This suggests that oncogene “switching” afforded by RTK coactivation within the same tumor, may be a principal mechanism to achieve chemoresistance.

2.7 Aberrant Downstream Pathways

Deletions or decreased function in tumor suppressor genes may also lead to therapeutic resistance due to aberrant signaling. Persistent activation of the PI3K/Akt/mTOR pathway can also be promoted by loss of the phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) gene, occurring in around 40-50% of GBMs (68). Specifically, the *PTEN* gene encodes a dual specificity lipid and protein phosphatase that dephosphorylates PIP_3 to impede signal flux through the PI3K pathway. Co-expression of EGFRvIII and wild type PTEN has been shown to correlate with tumor sensitivity to EGFR TKIs; however, there is considerable variability in patient response despite the presence of this genetic signature (6,69). In fact, Fenton *et al*. demonstrated that PTEN that is intact, but is phosphorylated at tyrosine 240, is frequently encountered in GBM and confers resistance to EGFR inhibitors (70). Hence, PTEN function may prove to be a more superior determinant compared to simply its expression status.

PTEN-deficient glioblastoma cell lines can also employ the autophagic process as a survival pathway of escape (71-73). Specifically, overexpression and accumulation of αB-crystallin following erlotinib treatment was observed in DBTRG-05 and U87 glioma cells (71). This molecule is a heat shock protein that can impair caspase activation, and thus block apoptosis. Inhibiting either autophagy or autophagosome maturation was shown to increase the death-inducing activity of both erlotinib and an mTOR inhibitor, PI-103 (71,72).

Activating mutation and/or amplification of the *PIK3CA* gene, which encodes the p110α catalytic subunit of PI3K, has also been linked to resistance to EGFR inhibition (69). These aberrations confer increased PI3K activity that is unchecked by

PTEN regulation, resulting in cellular transformation and amplified mitogenic effects via Akt (74). Interestingly, *PIK3CA* mutations are mutually exclusive to the PTEN deletions, but are collectively the cause of mutational activation of the PI3K/Akt pathway that is encountered in about 50% of glioblastomas (75). Taken together, the antitumor activity of many EGFR inhibitors can ultimately be negated due to defects in components of the targeted pathway, suggesting that cooperation between TKIs and inhibitors of predictive compensatory mechanisms is required to achieve a more robust anti-tumor effect.

2.8 Secondary Mutations

Finally, the acquisition of secondary mutations in oncogenic drivers is highly associated with the onset of acquired resistance to anti-EGFR therapies. Currently, more than 90% of the known EGFR secondary mutations occur in exons 19-21 (12). In NSCLC, the most common mechanism of resistance is the emergence of *T790M*, a point mutation located in exon 20 of the tyrosine kinase domain of EGFR. This mutation is present in ~50-60% of resistance cases, and occurs at what is defined as a "gatekeeper" residue, which is important for regulating inhibitor specificity within the ATP binding pocket (12). A major consequence of this mutation is enhanced affinity for ATP, which would effectively compete against TKI's, whose target region is also the ATP binding pocket. Interestingly, tumors harboring the *T790M* mutation display extremely slow growth rates, which results in a better prognosis compared to recurrent tumors that lack this aberration (76).

Despite this prognostic advantage, the *T790M* mutation is still considered highly oncogenic, possessing enhanced phosphorylating activity and conferring resistance to both first-generation EGFR inhibitors (i.e., erlotinib, gefitinib) and second-generation EGFR inhibitors (i.e., afatanib) (12,77). For these reasons, T790M-specific third-generation covalent EGFR inhibitors, osimertinib and rociletinib, have been developed. So far, these agents have displayed significant efficacy in mouse models and in the clinic, are well tolerated and have led to response rates in about 60% of patients harboring the *T790M* mutation (77). Nevertheless, patients develop resistance once again even on this treatment plan, suggesting that the development of some sort of drug resistance to targeted therapy is inevitable. Other less frequent secondary mutations identified in the context of acquired resistance in

NSCLC include *D761Y, L747S* and *T854A* (12). These mutations can coexist with *T790M*, but the structural basis underlying their individual contributions to resistance is still unknown.

Preexisting minor clones with the *T790M* mutation have also been identified within treatment-naïve tumors, reflecting an association between this aberration and inherent resistance to EGFR inhibition (76). In this context, it is believed that treatment with TKI's introduces a selective pressure that supports the survival of these clones, which overall manifests as acquired resistance. To this end, newer evidence has established that the *T790M* recurrent tumor consists of both preexisting and *de novo* drug-tolerant cells (78). This suggests that cancer cells that persist following initial therapy may serve as an important reservoir that affords the emergence of acquired resistance in the clinic.

Other secondary mutations that impact therapeutic response to anti-EGFR therapies involve the *KRAS* gene. KRAS is a part of the RAS protein family along with HRAS and NRAS, and these proteins are GTPases that act as molecular mediators for a number of critical signaling processes. Thus, mutations that occur within these molecules promote oncogenic activity, manifesting as constitutive activation of these proteins and uncontrollable cell proliferation and survival (79). The association between *KRAS* mutations and acquired resistance to anti-EGFR therapies has been established in the context of colorectal cancers, specifically in response to the anti-EGFR monoclonal antibody cetuximab (79). The most common mutations found are *G13D* and *G12R*, and both are sufficient to confer resistance to cetuximab. Combinatorial therapy that antagonizes both the EGFR and MEK pathways is then required to overcome this resistance (79).

Analogous to the patterns underlying the emergence of the *T790M* mutation in NSCLC, it is known that drug resistance as a result of *KRAS* mutant alleles is due to both preexistent *KRAS* mutant and amplified clones and *de novo* evolution promoted by ongoing mutagenesis (79). Interestingly, these mutant alleles could be detected in the plasma of patients as early as 10 months prior to radiographic evidence of disease progression, reflecting biomarkers for early detection of risk for this specific mechanism of resistance (79). Yet, as often as secondary mutations have been linked to resistance to EGFR inhibition in other solid tumors, no such mutations have been implicated in GBM.

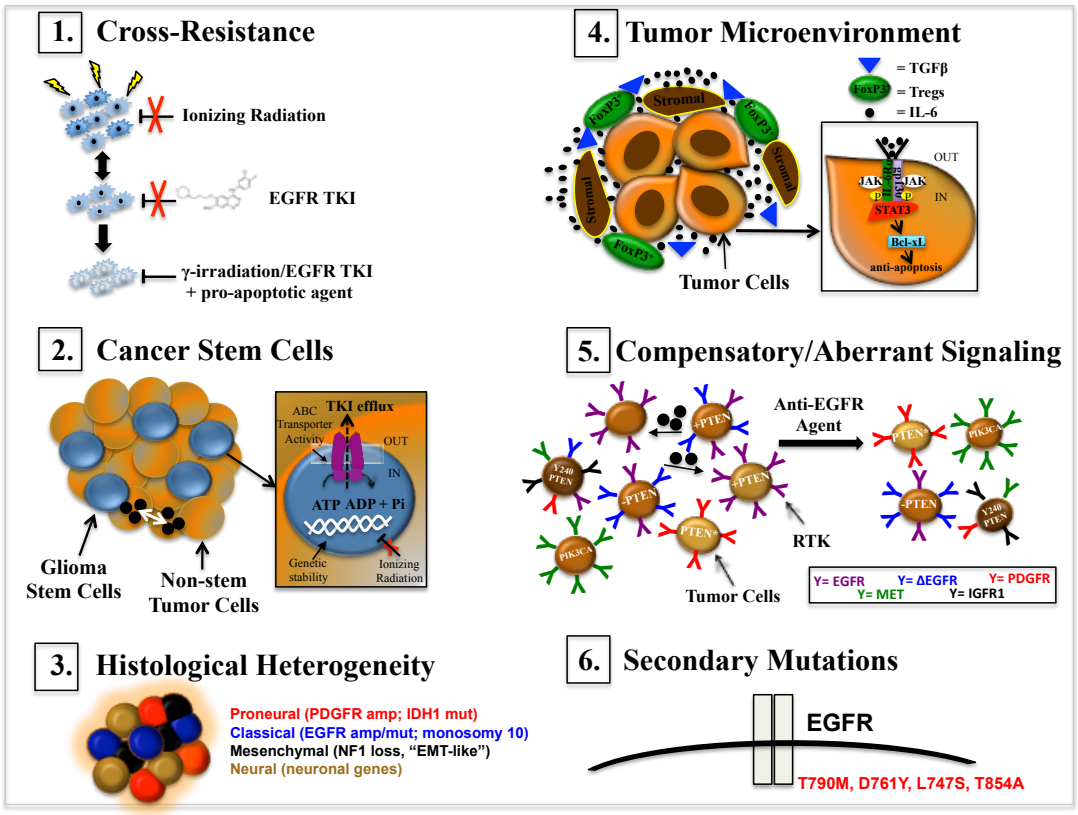

Figure 2.4. Summary of the predominant mechanisms of resistance to EGFR inhibition. (1) Brain tumor cells that are intractable to DNA damage-induced apoptosis may also tolerate apoptotic cues driven by TKI-mediated inhibition of EGFR. Combinatorial therapy using agents that can antagonize anti-apoptotic activity within cancer cells may overcome cross-resistance. (2) Efflux of EGFR TKIs and increased genetic stability support to the maintenance of CSC populations and tumor relapse. Additionally, cell-cell communication between the glioma stem cells and the non-stem tumor cells affords escape from tumor dormancy. (3) More than one molecular subtype may be represented in a given GBM, reflecting both histological and spatial heterogeneity that confer resistance. (4) Enhanced immunosuppression mediated by circulating growth factors, cytokines and suppressor T cells can inhibit the systemic immune responses generated by anti-EGFR immunotherapies. Additionally, circulating IL-6 in the tumor microenvironment can facilitate resistance via the JAK/STAT3/Bcl-xL pathway. (5) Compensatory/Aberrant signaling within GBM tumors may drive resistance to EGFR monotherapies due to: RTK co-activation, PTEN deletion/mutations/modifications (Y240 PTEN), PIK3CA mutations and cell-cell cross talk via secreted factors. (6) Secondary mutations in EGFR that alter the binding affinity of small molecule inhibitors commonly promote therapeutic resistance.

2.9 Discussion

The successful application of EGFR-targeted therapy for the treatment of glioblastoma has proven to be very challenging. Malignant brain tumors require a very complex signaling network that is not driven by EGFR alone, and this complexity dictates tumor sensitivity to EGFR-targeted therapies. In comparison with the progress made to uncover mechanisms of therapeutic resistance in other cancers, the GBM field is still in the budding stage. A deeper understanding of the intricate inter-relationships that underlie the pathobiology of this disease is required to achieve stable therapeutic responses to targeted agents.

It is clear that GBM tumors possess a number of intrinsic variables that would render monotherapies inept; yet, lack in the knowledge of a comprehensive role for these variables has immobilized the design of more effective treatments. Thus, there is still a great need to identify more determinants of resistance as a basis for novel therapeutic strategies that are specific to how GBMs recur. In this dissertation, we characterize both EGFRvIII-dependent and EGFRvIII-independent glioma populations. Such an approach yielded a previously unidentified mechanism of resistance to EGFR blockade in GBM, which emerged from a therapeutic response phenotype that had to be overcome for re-initiation of tumor growth. It is the goal of this thesis to highlight an important association between characterizing a therapeutic response to predict mechanisms of resistance, and linking this information to development of a tailored combination therapy based on identified mechanisms.

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dissertation author is the first author of this review article. Additional data presented in chapter 2 have not been published previously and will not be submitted for publication. This work was supported by the NIH F31 NS076343 to (T.E. Taylor) and the NIH R01 NS080939 to (F. B. Furnari).

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Chapter 3

Overcoming oxidative DNA damage supports escape from dependence on EGFRvIII

3.1 Introduction

Mutant EGFRvIII arises from of an in-frame deletion of 801 base pairs from the extracellular domain of EGFR and possesses ligand-independent constitutive tyrosine kinase activity (1). The ligand-independent nature of EGFRvIII causes defective endocytosis-mediated receptor internalization, resulting in increased stability of the receptor on the cell surface and amplified mitogenic effects (2). EGFRvIII has been detected in as many as 60% of GBMs at the protein level and in various other cancers including breast, lung, prostate, and non-small cell lung carcinoma; however, it has not been found in normal tissues (3). Furthermore, overexpression of EGFRvIII is a negative prognostic factor in GBM (2). Based on these findings, EGFRvIII has been pursued as a tumor-specific therapeutic target for GBM, but only in recent years has its requirement for the maintenance of GBM growth been established.

In 2010, Mukasa *et al*. demonstrated that EGFRvIII is crucial for the maintenance of glioma growth in vivo (4). In this work, U373MG GBM cells were engineered with doxycycline (DOX)-repressible EGFRvIII, and then engrafted subcutaneously into nude mice. In vivo tumorigenicity was only associated with EGFRvIII expression (-DOX), validating this model's dependence on EGFRvIII for tumorigenesis (4). However, when EGFRvIII expression was silenced (+DOX), a significant decline in tumor growth and subsequent cytostasis was observed, reflecting a classic example of oncogene addiction (4). The concept of "oncogenic addiction" was established by Weinstein in the early 2000s, and describes the reliance of tumor cells on continued expression of an oncogene or activated oncogenic signaling for the maintenance of tumorigenicity (5). As a result of this dependence, inactivation of the oncogene or oncoprotein impairs their growth and survival (6). Determinants for

targeted therapy development include demonstrated need of a specific molecule for carcinogenesis and tumor maintenance; therefore, the above results substantiated EGFRvIII as a rational therapeutic target.

Yet, despite sustained repression of EGFRvIII expression, some tumors eventually regained aggressive growth over the course of the experiment (4). The kinetics of this re-growth is analogous to the clinical situation where GBMs are initially sensitive to loss of receptor function, but eventually are able to overcome this loss. Therapeutic response to acute silencing of EGFRvIII (8 days DOX treatment) manifests as a significant reduction in proliferation and a slight increase in apoptosis (4). However, once the re-initiation of tumor growth in the absence of EGFRvIII expression occurs, both the decrease in proliferative capacity and the increase in apoptosis are reversed (4). In parallel with the latter phenotype, the expression of the anti-apoptotic molecule Bcl-xL is diminished at the protein level upon acute silencing of EGFRvIII, but is completely restored in the relapsed tumors (4). These data suggests that in our model and perhaps in GBM patients, overcoming the phenotypes associated with sensitivity to EGFR inhibition is required for escape.

In other solid tumors, response to EGFR inhibition also results in an accumulation of DNA damage in the form of double strand breaks (DSBs), and compromised DNA repair (7,8). For example, in breast cancer cells, treatment with erlotinib alone has been shown to lead to an accumulation of DSBs and suppression of the homology-directed recombinational repair (HDR) pathway (7). To this end, another study conducted in a variety of cancer types, demonstrated that erlotinib as part of a combinatorial therapy with arsenic trioxide leads to inhibition of EGFR-mediated DNA DSB repair and high levels of DNA damage (8). The phenotype in the

latter study was found to be secondary to erlotinib-mediated cell cycle arrest, whereas the results in the breast cancer study reflected a novel mechanism of action for erlotinib, as changes in the cell cycle were not observed (7,8).

DNA double strand breaks are the most lethal DNA lesions a cell can sustain because if left unrepaired, cell death, loss of genetic information and other genetic aberrations, such as chromosomal translocations can occur (9). In eukaryotes, two distinct repair pathways function to restore genomic integrity following DSB formation: non-homologous end-joining repair (NHEJ) and homology-directed recombinational repair (HDR) (10). The most common form of HDR is called homologous recombination (HR) and it requires an identical or very similar large stretch of DNA to serve as a template for repair, reflecting a high-fidelity repair process (11). NHEJ repair involves the rejoining of the broken ends of the fragmented DNA, and consequently, may be more error-prone (10). The decision to employ one repair pathway over the other is tightly regulated by the cell cycle; HR requires a sister chromatid to serve as the genetic template for repair, and so is restricted to the late S/G2 phases of the cell cycle (11). Alternatively, NHEJ is active in all stages of the cell cycle.

A number of reports linking unrepaired DNA DSBs and cancer development exist, but on the contrary, the hijacking of DNA repair pathways to eliminate DNA damage has also been implicated in therapeutic resistance (12-15). Additionally, as mentioned in the previous chapter, the mechanism of action for many cytotoxic chemotherapeutic agents is to inflict DNA damage in order to induce programmed cell death. Thus, DNA damage is causatively linked to tumorigenesis, is used for the treatment of cancer, but can also be circumvented by cancer cells for their survival.

In mammalian cells, both EGFR and mutant EGFRvIII have been established as major regulators in the repair of radiation-induced DNA damage. DNA DSB repair capacity is enhanced by activated EGFR, and is reduced upon EGFR inhibition (16). MAPK signaling was established as the predominant downstream pathway that is engaged by EGFR to specifically facilitate its role in NHEJ repair (14). Previous reports established the involvement of EGFRvIII in radioresistance, and Mukherjee *et al*., extended these findings by uncovering its ability to activate a key DNA repair enzyme, the DNA-dependent protein kinase, catalytic subunit (DNA-PKCs) (13,17). These results confirmed that similar to EGFR, NHEJ repair is also employed by EGFRvIII to circumvent radiation-induced DNA damage, since DNA-PKCs activity is blocked during HR (11).

Considering the above results and observations, blocking EGFRvIII in GBM should lead to an impairment in DNA repair, but this has not been directly tested, especially in a sans radiation setting. Thus, we sought to determine if a persistent DNA damage phenotype upon EGFR inhibition occurs in the context of GBM. Furthermore, we investigated if escape from dependence on EGFRvIII receptor function involves the exploitation of such damage or necessitates adaptive mechanisms to overcome it.

Results

3.2 Doxycycline diet sufficiently silences EGFRvIII and decreases in vivo tumor growth

In the previous study conducted by Mukasa *et al.*, doxycycline was administered via drinking water supplemented with sucrose, and although this is an effective method of regulation, superior approaches have been developed since this study. A number of doxycycline-containing diets are now commercially available with the following advantages over water delivery: protection from light via packaging, longer stability requiring only one change per week, and a reduced risk of dehydration, precluding the need for sucrose. In this study, we employed the diet delivery method to genetically silence EGFRvIII in vivo, and on the basis of the established response phenotypes observed upon acute silencing (8 days DOX treatment), we decided to assess the effectiveness of this approach at this time point.

Tumor-derived U373MG-tetO-EGFRvIII cells that are still dependent on EGFRvIII receptor function were engrafted subcutaneously into nude mice, and after tumors reached an approximate size of 500 mm^3, half of the mice were switched to the doxycycline diet for 8 days. We observed a significant decrease in tumor growth in the group of mice switched to the doxycycline diet, reminiscent of the growth decrease observed with water delivery (**Figure 3.1A**). Additionally, we observed a complete silencing of EGFRvIII mRNA and protein expression (**Figure 3.1B**) upon doxycycline administration. These results demonstrated that the doxycycline diet approach is just as effective as the water delivery method and validated that 8 days of treatment is sufficient for in vivo blockade of EGFRvIII.

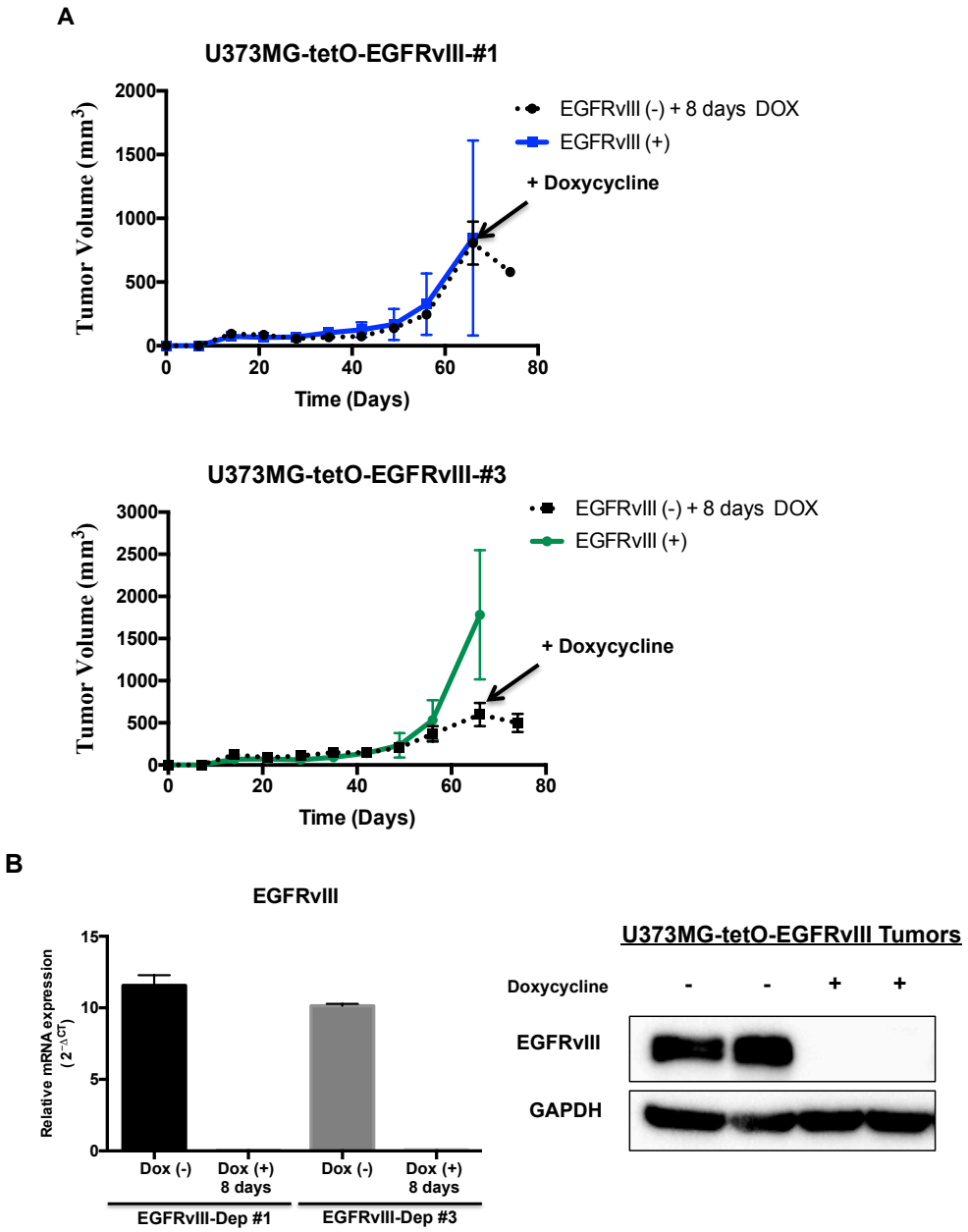


Figure 3.1. Doxycycline diet effectively silences EGFRvIII expression and glioma growth. (A) Growth of subcutaneous tumors from each population during the course of the experiment. Tumor Volume = ½ length x width^2 in unit of mm^3 (+Doxycycline) indicates the start of doxycycline administration. n= 4 mice per group (B) qPCR analysis of EGFRvIII mRNA expression and Western blot analysis of EGFRvIII protein in tumor lysates for each condition.

3.3 Blockade of EGFRvIII alone increases the DNA damage burden in GBM

Although previous reports in other solid tumors have demonstrated that potent EGFR inhibition leads to impaired DNA DSB repair, this phenotype has not been documented in the context of GBM. Additionally, the previous findings suggest that the most appreciative impact on DNA damage and subsequent repair occurs in the presence of an exogenous physical agent, such as ionizing radiation (IR). In the case of the U373MG-tetO-EGFRvIII GBM model, mice were not exposed to any exogenous insult, but given the established role of both EGFR and EGFRvIII in the context of genome stability, we tested the hypothesis that in GBM, blockade of EGFRvIII alone yields a phenotype reflective of attenuated DNA DSB repair.

p53 binding protein 1 (53BP1) is an important regulator of chromatin based DSB signaling (11). It was first described as a binding partner for the tumor suppressor protein p53, from which its name was given (11,18). Functionally, 53BP1 acts as a molecular scaffold to recruit other DSB response proteins to damaged chromatin, and amplifies the activity of the ataxia telangiectasia mutated kinase (ATM) to promote checkpoint signaling in response to DNA damage (19). Additionally, it is known to specifically promote NHEJ repair over HR by antagonizing the resection extension step required for HR to take place (20).

In response to DNA damage in the form of DSBs, 53BP1 rapidly accumulates on chromatin surrounding the DSB sites and its localization persists until the DNA has been repaired (11). On the basis of these known functions for 53BP1, we conducted immunohistochemistry on the tumor samples that were generated in the in vivo experiment described in **section 3.2** to assess 53BP1 positive nuclei, as a marker for

DNA DSBs. We observed a very low percentage of 53BP1 positive nuclei in the EGFRvIII-positive tumors (-DOX), but upon acute silencing of EGFRvIII, there was a significant increase in detectable 53BP1 nuclear staining (**Figure 3.2**). Given that proficient DNA repair is reflected by the clearance of 53BP1 nuclear foci (13), our results indicate that in the absence of EGFRvIII alone, DNA damage repair is attenuated in GBM. Additionally, the persistence in 53BP1 positive nuclei was observed in two different EGFRvIII-dependent populations, suggesting that this response phenotype may be a common phenomenon.

We next examined if this response phenotype also applies to the context of pharmacological inhibition of EGFRvIII, and used EGFRvIII expressing, PTEN wild-type primary *Ink4a/Arf*$^{-/-}$ murine astrocytes to address this question. This model reflects cooperation between Ink4a/Arf deficiency and EGFRvIII signaling that promotes the formation of highly invasive intracranial tumors in mice that are similar to human GBMs (21). Additionally, these cells display sensitivity to the EGFR TKI's (22). To assess the DNA damage in this setting, we chose to examine the accumulation of phosphorylated γH2A.X foci by immunofluorescence staining. ATM-mediated phosphorylation of γH2A.X at serine 139 is the first step in cellular detection of DSBs, thus γH2A.X foci formation has been established as a reliable indicator of DNA damage-induced DSBs (11).

In our control cells (DMSO-treated), we observed very few cells containing greater than eight γH2A.X foci, which is the measure we used as our positive index (**Figure 3.3**). However, when treated with 5μM of the EGFR TKI gefitinib, we observed a 3.9-fold increase (25% versus 6.5%, $p<0.001$) in γH2A.X foci formation at 48 hours post-treatment (**Figure 3.3**). These findings are consistent with the antitumor activity

of erlotinib previously described in breast cancer (7), suggesting that EGFR TKI's may act similarly to intercept DNA damage repair. Additionally, these data imply that the emerging phenotypes from our genetic model are clinically relevant and to our knowledge demonstrate for the first time, that EGFRvIII inhibition alone leads to an increase in the persistence of DNA DSBs in GBM.

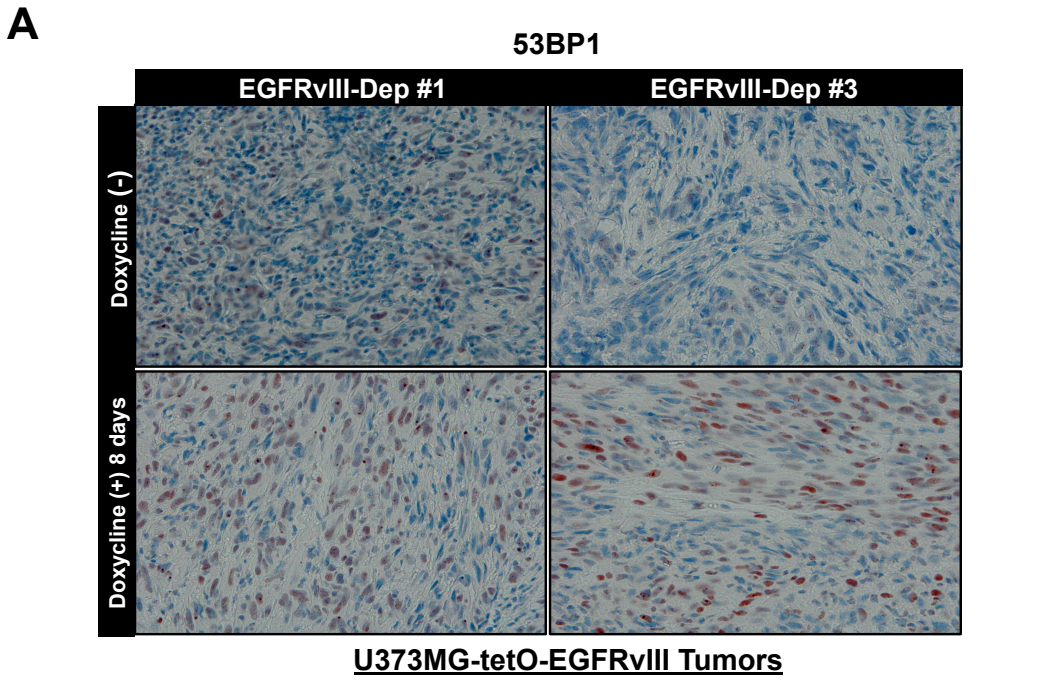


Figure 3.2. An increase in the persistence of unrepaired DNA double strand breaks is associated with loss of EGFRvIII alone. (A) Representative immunohistochemical staining for the surrogate DNA double strand break marker, 53BP1, in resected tumors (B) Quantification of positively stained nuclei using microscopy cell counting software. EGFRvIII-Dep #1: (3.3-fold increase) and EGFRvIII-Dep #3 (13.2-fold increase) Data are means $\pm$ SEM **p<0.01 **** p<0.0001

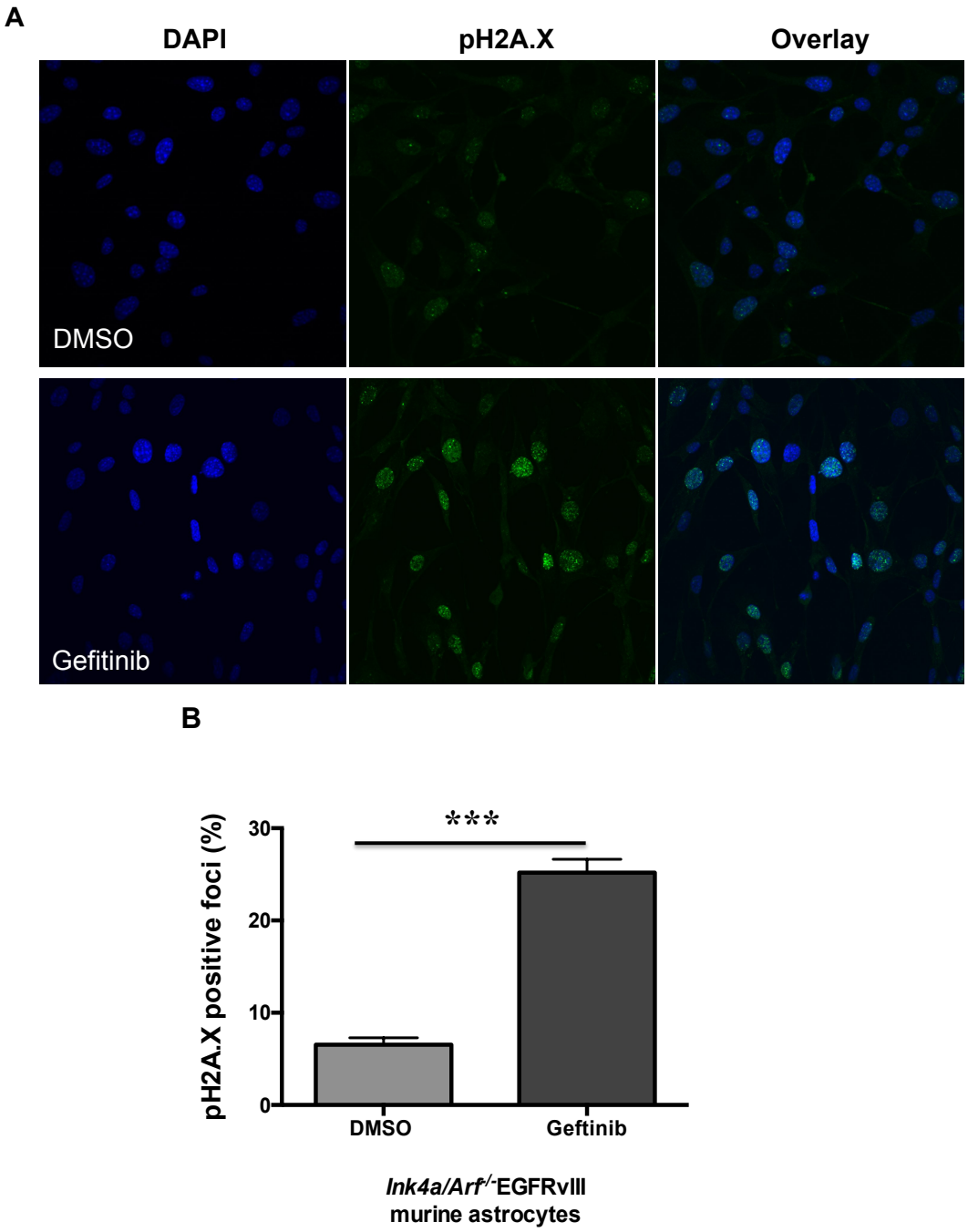


Figure 3.3. Gefitinib-mediated EGFRvIII inhibition increases p-H2AX foci in GBM. (A) Representative immunofluorescence staining for p-H2AX foci formation upon treatment with 5 μM gefitinib for 48 hours. (B) Foci Quantification: shown is the % of positive cells containing > 8 foci. Data are means ± SEM ***p<0.001

3.4 Increased ROS activity is observed with EGFRvIII inhibition

Based on the previous results, we next sought to determine the endogenous source of the DNA damage that we observe with blockade of EGFRvIII. It is known that endogenous DNA damage caused by reactive oxygen species (ROS) is the most frequently occurring damage (23). Reactive oxygen species in the most basic definition are chemically reactive chemical species, containing oxygen. Examples of ROS include superoxide, peroxides, hydroxyl radical, and singlet oxygen (24). Within most mammalian cells, ROS are primarily generated in the mitochondria as natural byproducts of oxidative metabolism (24). Premature electron leakage from the complexes I, II, and III of the electron transport chain can lead to the partial reduction of oxygen and the emergence of superoxide, which is the proximal ROS (23). In this manner, other highly reactive free radicals are derived, that are at leisure to attack DNA, proteins and lipids (23-25).

In the case of DNA, these chemical attacks result in lesions referred to as adducts. These DNA adducts can impair base pairing, block DNA replication and transcription, and promote the formation of DNA single-strand breaks (SSBs) (26). Moreover, when two SSBs emerge in close proximity or when the DNA-replication machinery encounters a SSB or other lesions, the very lethal DNA DSBs are formed. The potential toxic effects of ROS are usually prevented by inherent antioxidant defenses like superoxide dismutase, catalases and glutathione; however, when there is an imbalance between the production of ROS and the neutralizing ability of antioxidants, a state of oxidative stress ensues (27).

Oxidative stress can be defined as the cumulative accumulation of intracellular ROS, which can be both deleterious and beneficial to the cells (27). ROS participate in the intracellular signaling cascades that are commonly activated to maintain the oncogenic phenotype of cancer cells (25). For instance, during hypoxia, which refers to low oxygen conditions within the cell, mitochondrial ROS production is required for activation of the nuclear factor kappa B (NFκ-B) transcription factor to promote cell survival signaling (28). Alternatively, ROS can induce cellular senescence and apoptosis, and therefore function as anti-tumorigenic species. Indeed, the DNA-damaging effects imposed by many cancer therapeutics are mediated through the production of ROS (29). Furthermore, the overgeneration of ROS can cause sensitivity to hypoxia and subsequent cell death in cancer cells (28).

The established link between ROS activity and subsequent DNA damage, and the propensity of these species to antagonize tumorigenic potential, prompted us to interrogate if higher intracellular ROS levels, in the absence of EGFRvIII, was associated with the increase in DNA damage burden. We examined ROS activity by Flow Cytometry in our U373MG-tet-O-EGFRvIII populations in the presence and absence of doxycycline treatment, using two established ROS indicators. MitoSOX Red is a mitochondrial-localized, superoxide indicator and CellROX Orange is a cytosolic localized, general oxidative stress indicator. These two cell permeant dyes are non-fluorescent in their reduced states and become fluorescent upon oxidation by reactive oxygen species. We found that upon silencing of EGFRvIII, ROS activity is significantly elevated in both the cytosolic (8-fold increase) and mitochondrial (13-fold increase) cellular compartments in EGFRvIII-dependent populations (**Figure 3.4**).

We next evaluated if the elevated ROS levels could be rescued by antioxidant treatment. We employed two antioxidants: EUK-134, a dual superoxide dismutase/catalase mimetic to scavenge ROS within mitochondria and Trolox, a vitamin E analogue to scavenge ROS within the cytosol. EGFRvIII-negative cells (+DOX) were treated with each antioxidant for 24 hours prior to labeling with the respective ROS indicator. Indeed, we observed a 50% decrease in mitochondrial ROS activity in the presence of EUK-134 (50 μM) and a 45% decrease in cytosolic ROS activity in the presence of Trolox (100 μM) (**Figure 3.4**). In the ROS activity experiments, doxycycline was administered for 6 days, which corresponds to 3 days of EGFRvIII silencing, and the 53BP1 in vivo experiment reflects 5 days of EGFRvIII silencing. Therefore, our data seems to be consistent with the predicted order of events, where the overgeneration of ROS would occur prior to the corresponding DNA damage.

Similar to our interest in the clinical relevance of the DNA damage phenotype seen in the genetic model, we also examined ROS activity upon pharmacological inhibition of EGFRvIII. EGFRvIII-positive U373MG glioma cells (-DOX) were treated with either DMSO or 5μM of lapatanib and *Ink4a/Arf*$^{-/-}$ EGFRvIII murine astrocytes were treated with either DMSO or 5μM of gefitinib for 48 hours. Lapatanib was superior against EGFR tyrosine kinase activity (**Figure 3.5**). Cells were then labeled with the two ROS indicators. Mitochondrial ROS activity was significantly increased with lapatanib treatment in the U373MG glioma model (3-fold) and also with gefitinib treatment in the murine astrocytes (13-fold) (**Figure 3.5**). However, in both models, no significant difference in cytosolic ROS activity was observed (**Figure 3.5**). Taken

together, our data suggest that EGFRvIII regulates ROS activity levels in GBM, and in part, its receptor activity is required in the context of mitochondrial ROS.

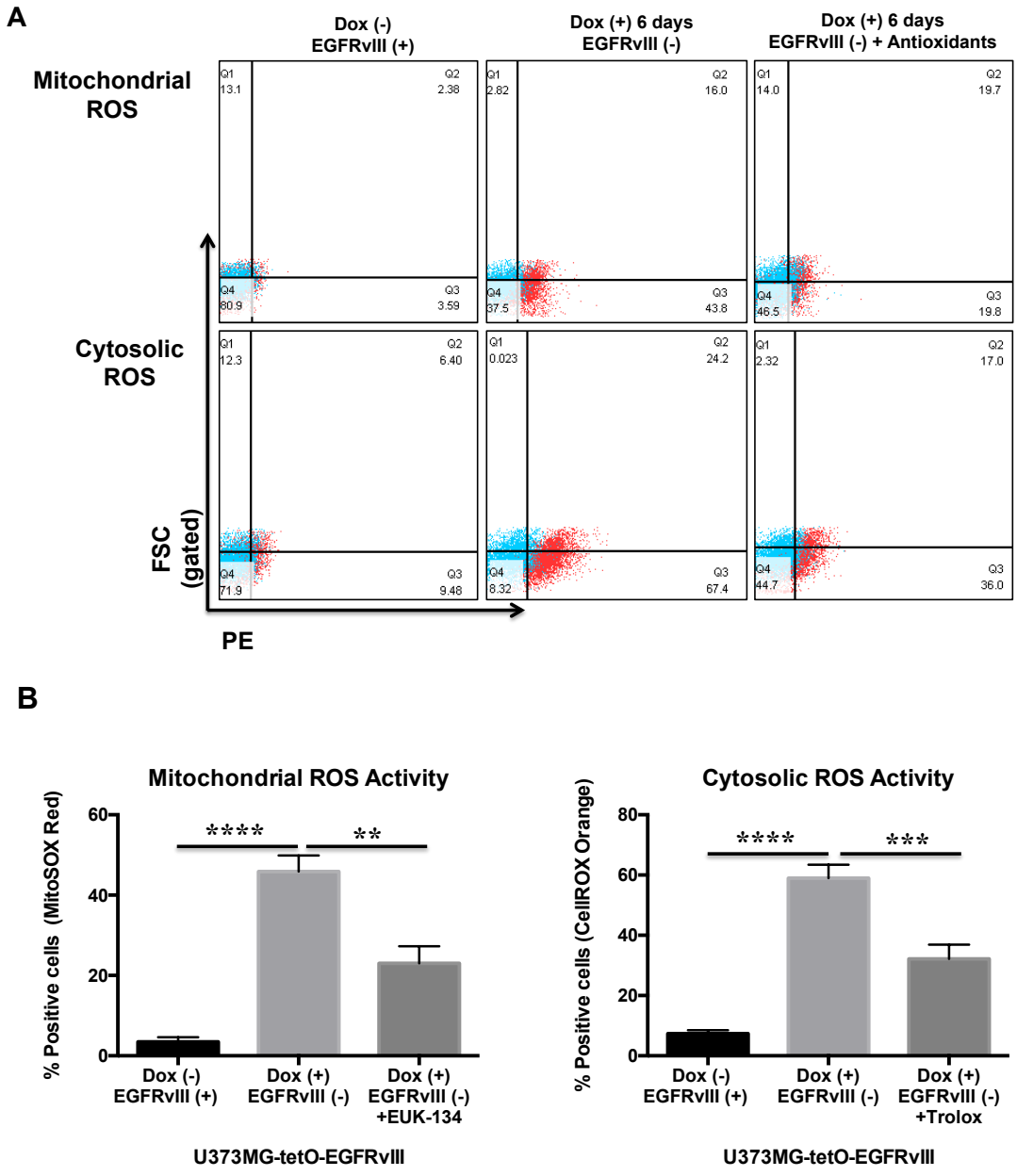
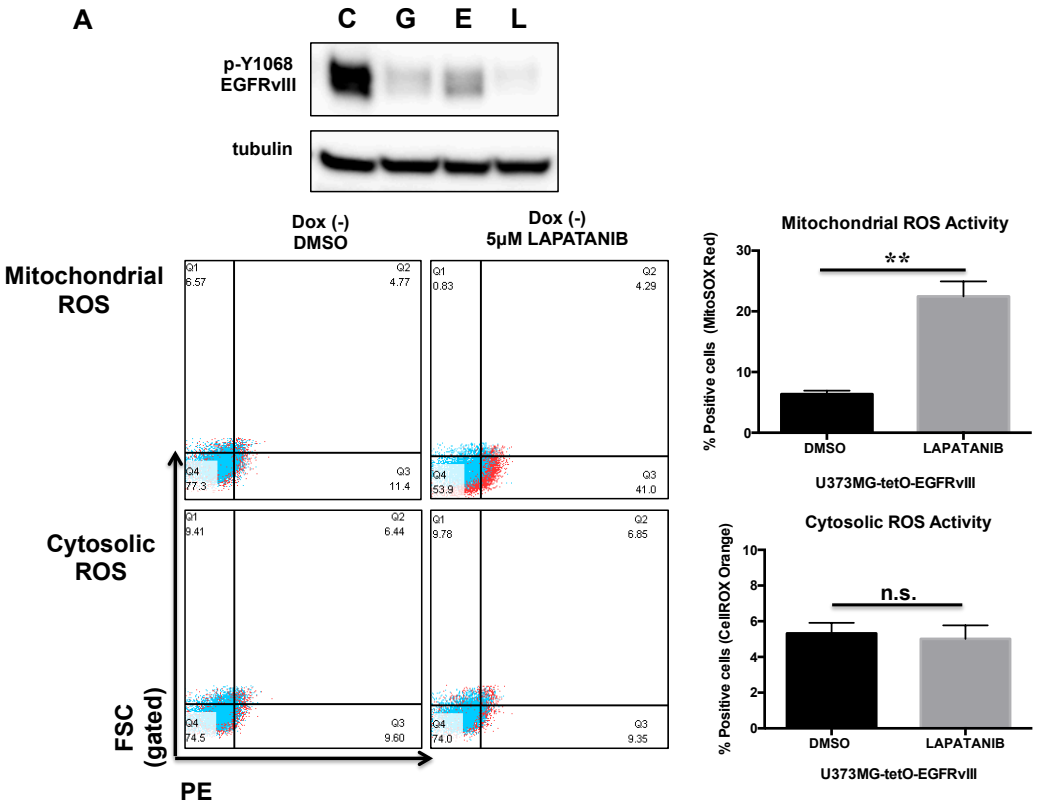
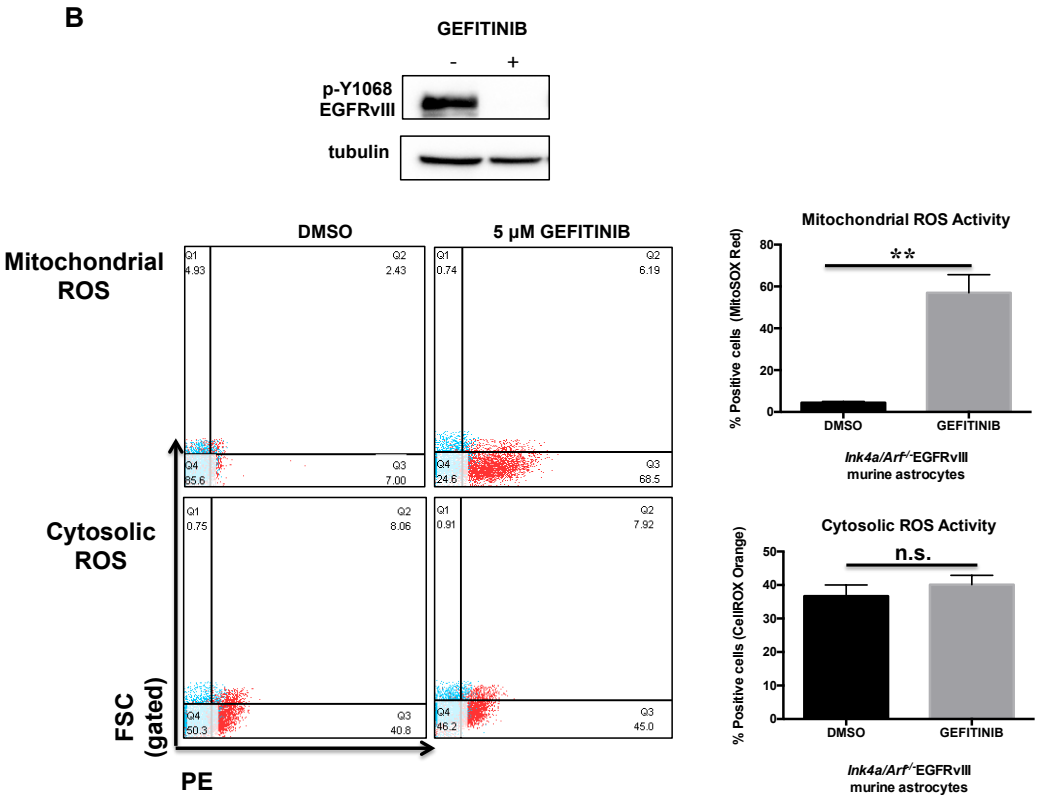


Figure 3.4. EGFRvIII silencing leads to elevated ROS activity in GBM. (A) Representative Flow Cytometry plots depicting ROS activity as measured by the PE-positive population (Q3) for each condition. Blue=unlabeled cells; Red=labeled cells (B) Mean percentage of positive cells after background subtraction in three independent experiments. MitoSOX Red is a superoxide indicator; CellROX Orange is a general oxidative stress indicator. EUK-134 (50 μM); Trolox (100 μM). Data are means ± SEM ****p<0.0001, ***p<0.001, **p<0.01

Figure 3.5. Pharmacological inhibition of EGFRvIII is also associated with elevated ROS activity in GBM. Representative Western Blot analysis, confirming inhibition of EGFRvIII kinase activity by respective TKI's, Flow Cytometry plots depicting ROS activity as measured by the PE-positive population (Q3) in each cell compartment and the mean percentage of positive cells after background subtraction in (A) U373MG-tet-O-EGFRvIII cells and (B) *Ink4a/Arf*$^{-/-}$ EGFRvIII murine astrocytes. Lapatanib and Gefitinib treatment: 5μM for 48 hours. C= DMSO, G=gefitinib, E=erlotinib, L=lapatanib Data are means $\pm$ SEM **p<0.01


A
C G E L
p-Y1068 EGFRvIII
tubulin
Dox (-) DMSO
Dox (-) 5μM LAPATANIB
Mitochondrial ROS
Cytosolic ROS
FSC (gated)
PE
Q1 6.57
Q2 4.77
Q4 77.3
Q3 11.4
Q1 0.83
Q2 4.29
Q4 53.9
Q3 41.0
Q1 9.41
Q2 6.44
Q4 74.5
Q3 9.60
Q1 9.78
Q2 6.85
Q4 74.0
Q3 9.35
Mitochondrial ROS Activity
% Positive cells (MitoSOX Red)
**
DMSO
LAPATANIB
U373MG-tetO-EGFRvIII
Cytosolic ROS Activity
% Positive cells (CellROX Orange)
n.s.
DMSO
LAPATANIB
U373MG-tetO-EGFRvIII


B
GEFITINIB
- +
p-Y1068 EGFRvIII
tubulin
DMSO
5 μM GEFITINIB
Mitochondrial ROS
Cytosolic ROS
FSC (gated)
PE
Q1 4.93
Q2 2.43
Q4 85.6
Q3 7.00
Q1 0.74
Q2 6.19
Q4 24.6
Q3 68.5
Q1 0.75
Q2 8.06
Q4 50.3
Q3 40.8
Q1 0.91
Q2 7.92
Q4 46.2
Q3 45.0
Mitochondrial ROS Activity
% Positive cells (MitoSOX Red)
**
DMSO
GEFITINIB
Ink4a/Arf-/-EGFRvIII murine astrocytes
Cytosolic ROS Activity
% Positive cells (CellROX Orange)
n.s.
DMSO
GEFITINIB
Ink4a/Arf-/-EGFRvIII murine astrocytes

ROS-induced DNA damage is predominantly executed in the form of DNA oxidation, although DNA can undergo other DNA-damaging modifications, such as methylation, depurination and deamination (30). Out of the four nitrogen bases found in DNA, guanine is the most susceptible to DNA oxidation because of its low redox potential (31). The conversion of guanine to 8-oxoguanine is the most common transformation that occurs, arising from ROS-induced hydroxylation and cleavage reactions of the purine ring (32). The 8-oxoguanine DNA lesion is also one of the most characterized, making it a reliable biomarker to indicate the extent of oxidative DNA damage (30).

Based on this information, we next assessed oxidative stress-induced DNA damage by immunohistochemistry, using 8-oxoguanine as our index. This experiment was conducted on in vivo tissue from the U373MG-tetO-EGFRvIII model and also *Ink4a/Arf*$^{-/-}$ EGFRvIII murine astrocytes in vivo samples from our previously published work, where in vivo growth in the presence or absence of gefitinib was measured (22). In the U373MG-tet-O-EGFRvIII model, we observed different intensity patterns for 8-oxoguanine staining between our EGFRvIII-positive populations, but overall, saw a significant increase in DNA oxidation upon EGFRvIII blockade (**Figure 3.6**). In the *Ink4a/Arf*$^{-/-}$ EGFRvIII murine astrocytes, the results were similar, whereby an increase in the detection of 8-oxoguanine was associated with sensitivity to gefitinib treatment (**Figure 3.7**). These results demonstrate that in part, ROS is involved in promoting the DNA damaging effects seen with EGFRvIII inhibition.

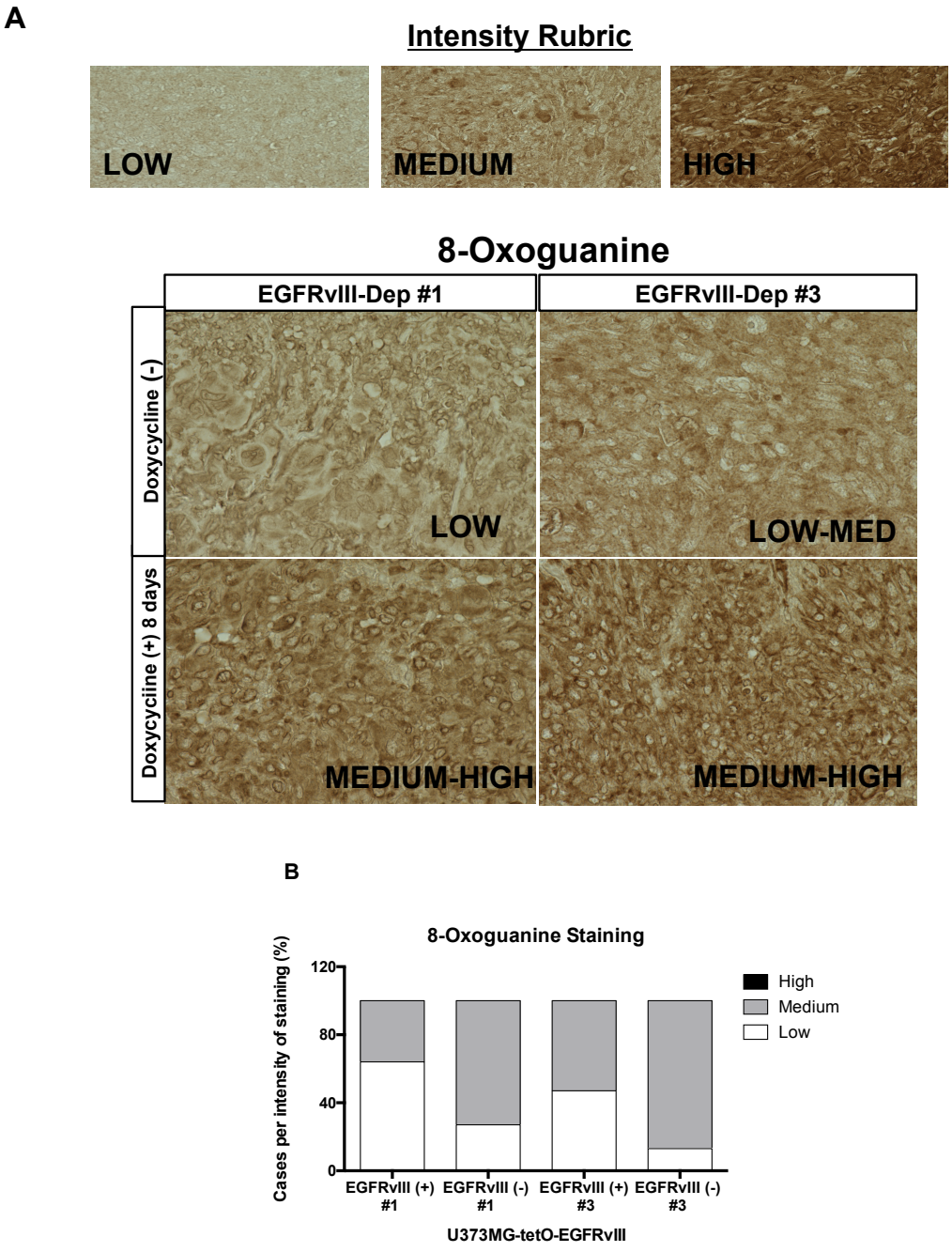


Figure 3.6 Increased DNA oxidation correlates with increased DNA damage burden upon EGFRvIII silencing. (A) Representative immunohistochemical staining for the biomarker of DNA oxidation, 8-oxoguanine, in tumors for each condition (B) Quantification of staining as determined by intensity rubric. Data reflect the individual cases per group and the respective extent of oxidative stress, represented as percentages. EGFRvIII-Dep #1: (-DOX) low-64%; medium-36%; high-0% vs (+DOX) low-27%; medium-73%; high-0% and EGFRvIII-Dep #3: (-DOX) low-47%; medium-53%; high-0% vs (+DOX) low-13%; medium-87%; high-0%

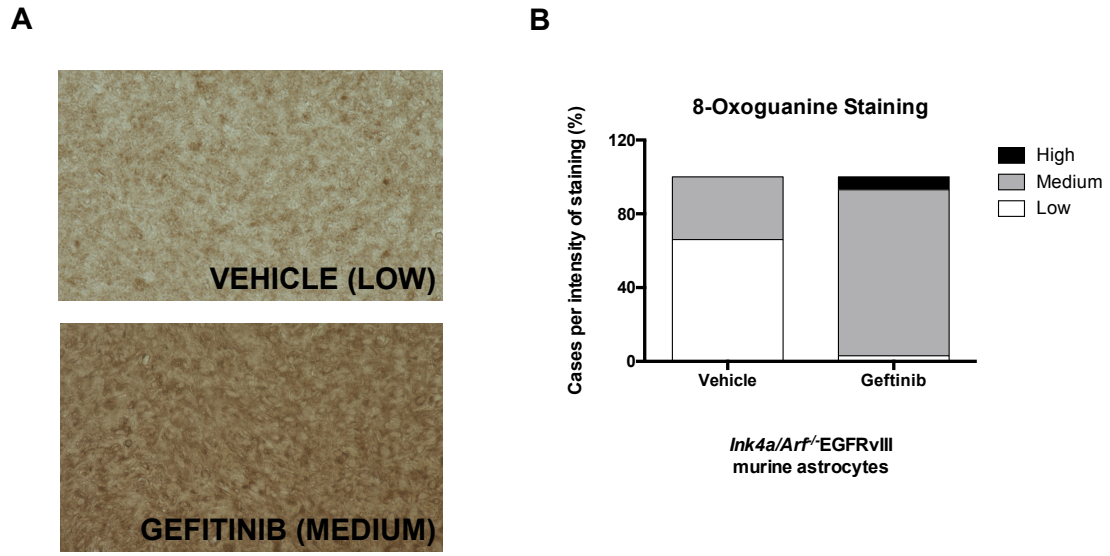


Figure 3.7. Increased DNA oxidation is associated with pharmacological inhibition of EGFRvIII. (A) Representative immunohistochemical staining for the 8-oxoguanine, in resected tumors for each condition (B) Quantification of staining as determined by intensity rubric (Figure 3.6). Data reflect the individual cases per group and the respective extent of oxidative stress, represented as percentages. Vehicle: low-66%; medium-34%; high-0% vs Gefitinib: low-3%; medium-90%; high-7%

3.5 EGFRvIII-independence is associated with overcoming therapeutic sensitivity

We noticed a pattern in our genetic model, whereby many of the phenotypes that were indicative of sensitivity to EGFRvIII inhibition, were reversed upon escape from EGFRvIII receptor function. Thus, we hypothesized that EGFRvIII-independent populations display a reversal in the percentage of DNA DSBs, ROS activity, and DNA oxidative damage. To test this hypothesis, we assessed EGFRvIII-independent relapse tumors and cultured cells for evidence of decreased ROS activity and oxidative DNA damage.

We first wanted to establish that a decrease in the persistence of unrepaired DNA DSBs was associated with overcoming EGFRvIII inhibition in our genetic model. Similar to the approach used for the EGFRvIII-dependent populations, we conducted immunohistochemical staining fro 53BP1 positive nuclei on tumor tissue from two EGFRvIII-independent populations (Escapers). The Escapers were chronically treated with the doxycycline diet, meaning that EGFRvIII is always silenced in this context. Just as we suspected, we observed a significant decrease in 53BP1 positive nuclei (~2-fold) in both populations (**Figure 3.8**).

Next, we examined ROS activity in cell lines that were generated from these Escaper tumors by Flow Cytometry using our established ROS indicators. Indeed, we observed a significant decrease in both mitochondrial (Esc 0: 2-fold; Esc 1: 3.6-fold) and cytosolic ROS activity (Esc 0: 2.2-fold; Esc1: 3.6-fold) (**Figure 3.9**). Consistent with these results, we also saw a decrease in the extent of oxidative DNA damage in Escaper tumor tissue stained for 8-oxoguanine (**Figure 3.10**).

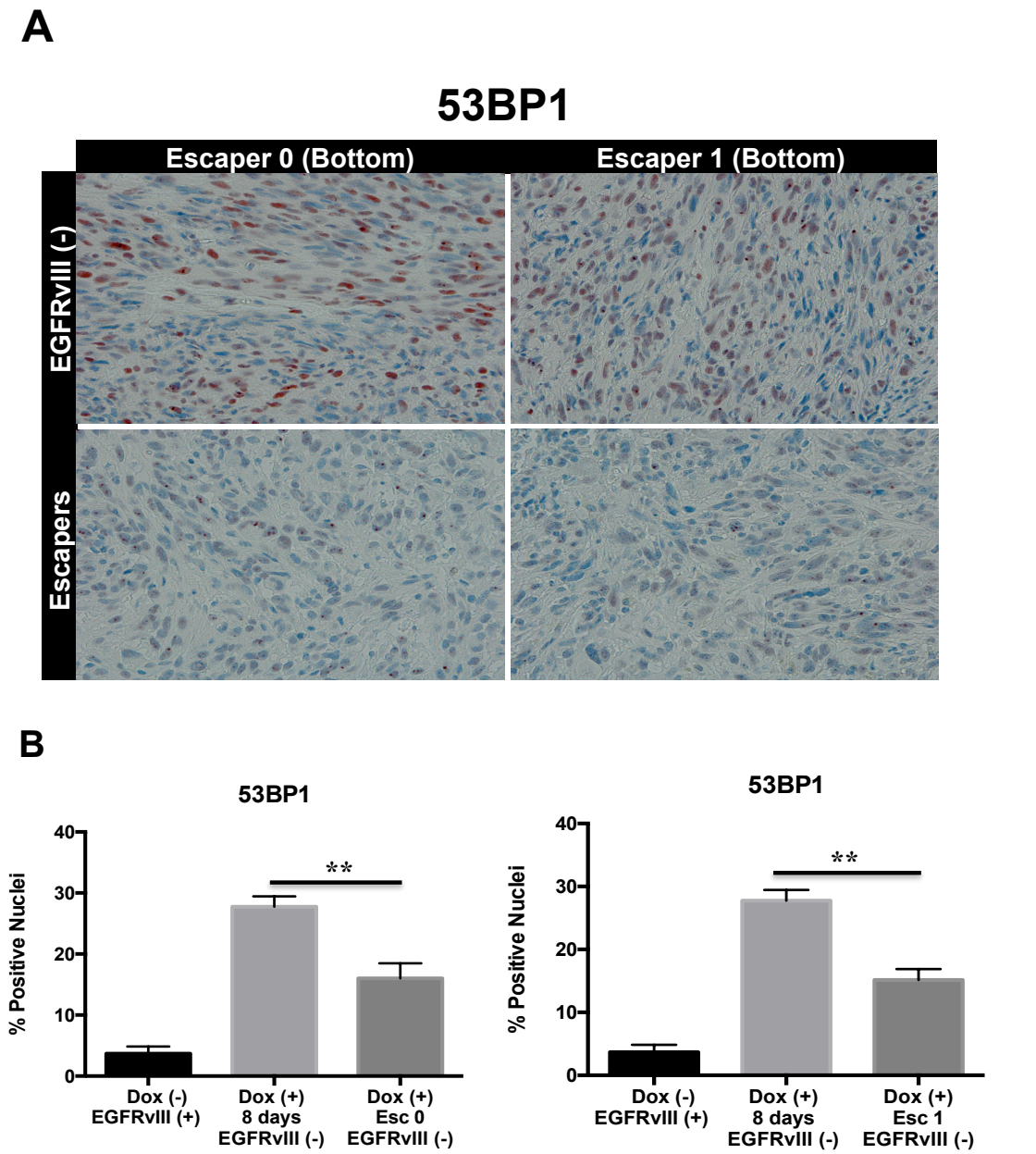


Figure 3.8. Overcoming EGFRvIII blockade is reflected by a significant reduction in the persistence of DNA DSBs. (A) Representative immunohistochemical staining for the surrogate DNA double strand break marker, 53BP1, in resected tumors (B) Quantification of positively stained nuclei using microscopy cell counting software. Esc 0: (45% reduction) and Esc 1 (48% reduction) Data are means $\pm$ SEM **p<0.01

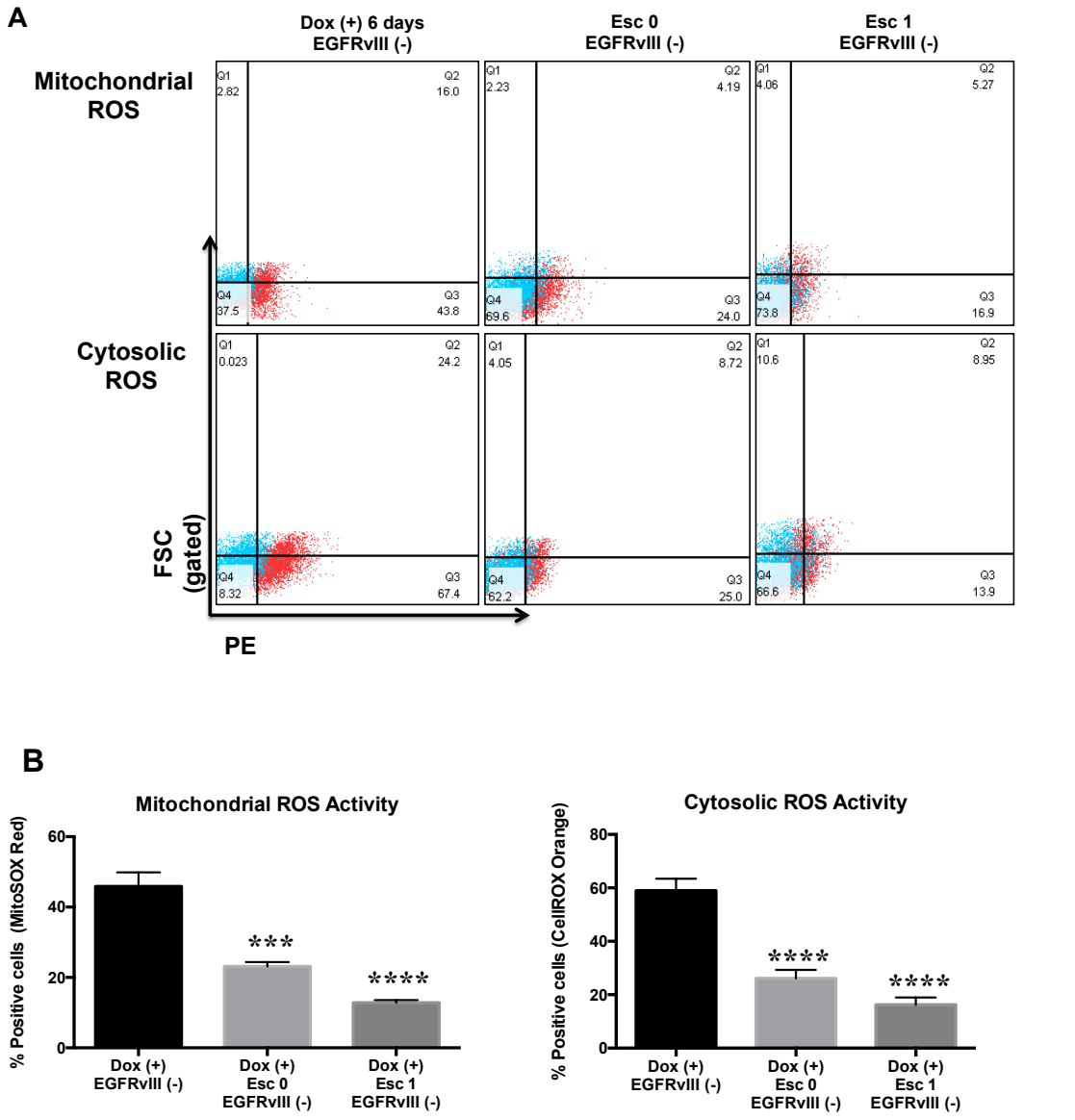


Figure 3.9. Escaper populations display significantly less ROS activity. (A) Representative Flow Cytometry plots depicting ROS activity as measured by the PE-positive population (Q3) for each condition. Blue=unlabeled cells; Red=labeled cells (B) Mean percentage of positive cells after background subtraction in three independent experiments. Data are means $\pm$ SEM ****p<0.0001 ***p<0.001

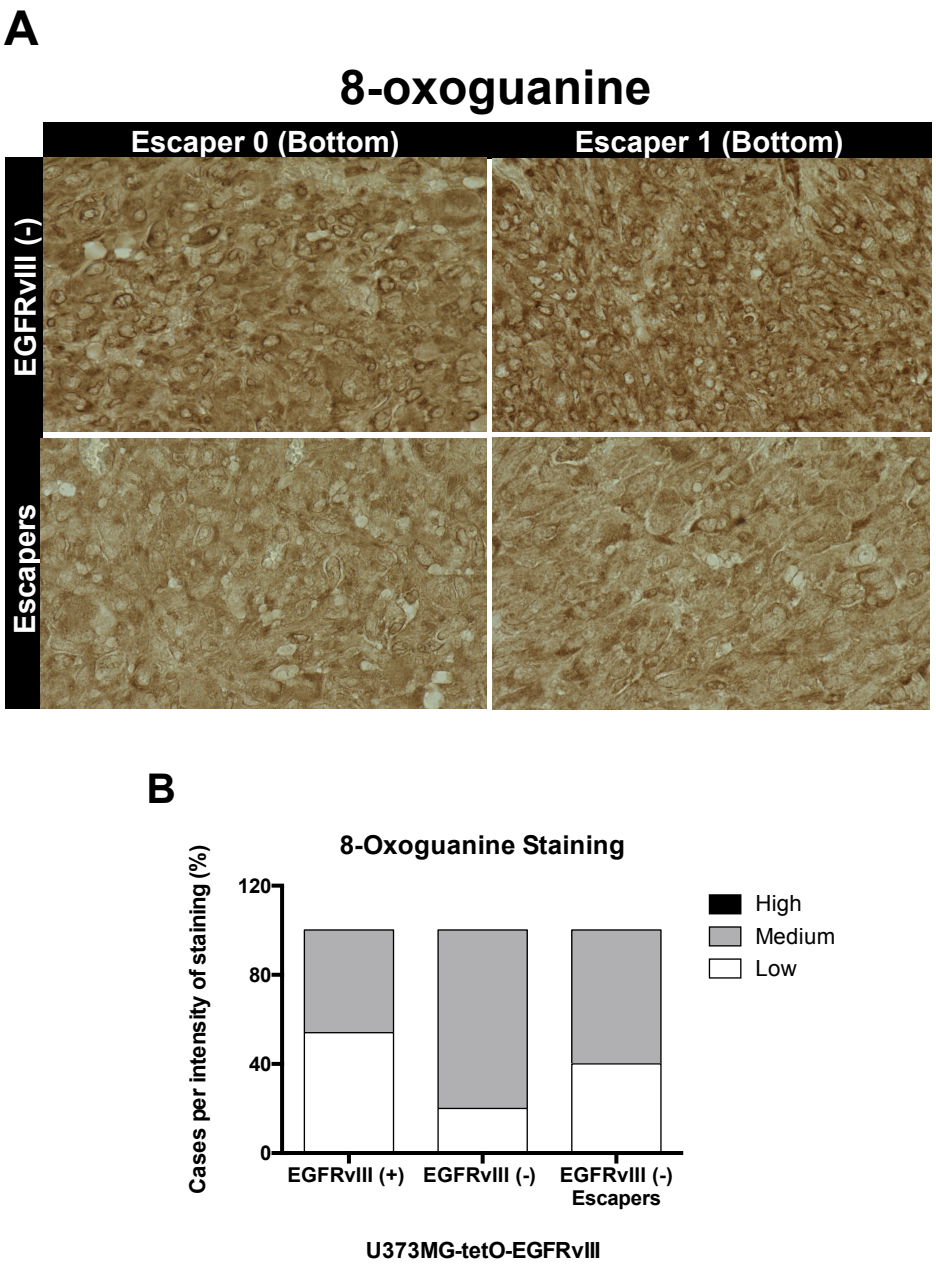


Figure 3.10 A decrease in DNA oxidation accompanies diminished reliance on EGFRvIII (A) Representative 8-oxoguanine immunohistochemical staining in Escaper tumors (B) Quantification of staining as determined by intensity rubric (Figure 3.6). Data reflect the individual cases and their respective extent of oxidative stress, represented as percentages. EGFRvIII (+): low-54%; medium-46%; high-0% vs EGFRvIII (-): low-20%; medium-80%; high-0% vs EGFRvIII (-) Escapers: low-40%; medium-60%; high-0%

These data prompted us to examine the possible ROS levels in EGFR TKI-resistant *Ink4a/Arf*$^{-/-}$ EGFRvIII murine astrocytes that emerged following chronic treatment with gefitinib in vivo (22). Cell lines generated from these tumors are continually cultured in growth media containing 2μM of gefitinib. For this experiment, we challenged the gefitinib resistant cells by treating with 5μM of gefitinib for 48 hours prior to labeling with the ROS indicators. Similar to our Escaper populations, we observed a significant decrease in mitochondrial ROS activity in all four gefitinib resistant populations compared to the sensitive cells (control population) (**Figure 3.11**). These data further demonstrate that the phenotypes associated with genetic escape and pharmacological escape are analogous.

Lastly, we analyzed the extent of oxidative DNA damage in this model by immunohistochemistry. As suspected, we also saw a significant decrease in 8-oxoguanine staining in this context (**Figure 3.12**). Taken together, these data suggest that escape from cytostasis involves recovery from DNA damage following oxidative stress. Furthermore, our findings demonstrate that another phenotype that is indicative of therapeutic sensitivity is also overturned in order for GBMs to overcome reliance on EGFRvIII.

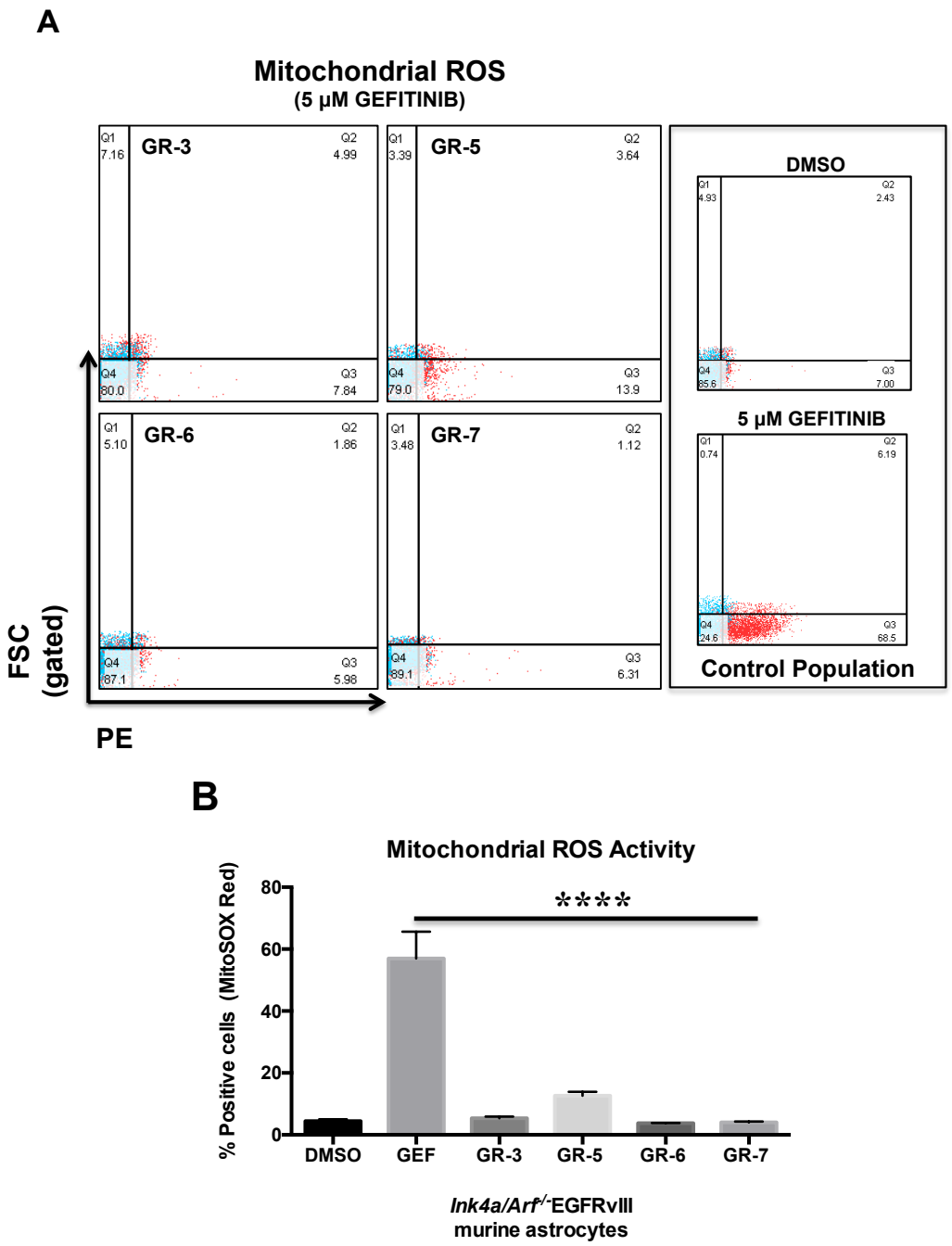


Figure 3.11. Gefitinib resistance is associated with significantly less ROS activity. (A) Representative Flow Cytometry plots depicting mitochondrial ROS activity as measured by the PE-positive population (Q3) for each clone. Control population included as reference. Blue=unlabeled cells; Red=labeled cells; GR=gefitinib resistant (B) Mean percentage of positive cells after background subtraction. Data are means ± SEM ****p<0.0001

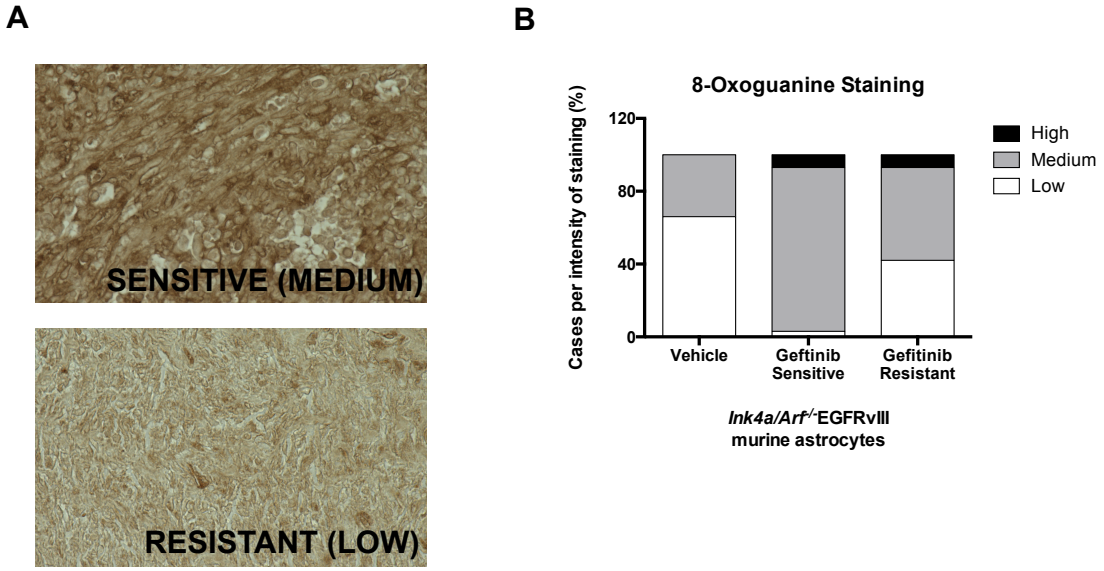


Figure 3.12 Diminished reliance on EGFRvIII receptor function is associated with decreased DNA oxidation. (A) Representative immunohistochemical staining for 8-oxoguanine, in resected tumors (B) Quantification of staining intensity. Data reflect the individual cases and their respective extent of oxidative stress, represented as percentages. Vehicle: low-66%; medium-34%; high-0% vs Gefitinib Sensitive: low-3%; medium-90%; high-7% vs Gefitinib Resistant: low-42%; medium-51%; high-7%

3.6 Summary

In summary, we found that EGFRvIII inhibition alone results in an increase in the persistence of DNA double strand breaks in vivo. This persistence was common to multiple “therapy-sensitive” populations and also correlated with an increase in reactive oxygen species (ROS) activity and oxidative DNA damage in vivo. On the contrary, populations that have escaped the need for EGFRvIII receptor function have significantly less DNA double strand breaks, decreased ROS activity and reduced oxidative DNA damage. Our results suggest that overcoming the effects of ROS-induced DNA damage is one mechanism utilized by GBMs to progressively escape blockade of EGFRvIII.

Increased ROS-induced DNA damage is often characterized as a tumor-promoting phenomenon, suggesting that tumors often capitalize on this damage for the maintenance of tumorigenicity. However, our findings imply that some tumors may be sensitive to ROS-induced DNA damage, necessitating potential adaptive mechanism(s) in order to support tumor survival and subsequent escape. It is known that the tight regulation of intracellular ROS levels is crucial for specific biological outcomes. For instance, constitutive low levels of ROS are necessary to support cell proliferation and differentiation (23). Yet as ROS levels increase, genetic adaptation is required for cell survival, including the transcriptional regulation of genes to antagonize potentially, deleterious oxidation. Based on this, we suspect that genetic changes that are unique to our EGFRvIII-independent populations give rise to their characteristic reduction in oxidative stress. In the next two chapters, we will discuss one of these genetic adaptations, the up-regulation of kelch domain containing 8A (KLHDC8A), and will establish its involvement in this adaptive response.

Chapter 3 contains data that have not been published previously, and are currently being prepared for submission for publication. The work in chapter 3 was completed with the help of Jianhui Ma, M.D, PhD., and Antonia Boyer of the laboratory of Frank Furnari. This work was supported by the NIH R01 NS08939 to (F.B. Furnari).

3.7 Materials and Methods

Cell culture and reagents

U373MG-tetO-EGFRvIII-dependent were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Tet-approved fetal bovine serum (FBS), Penicillin/streptomycin, G418 (2μg/ml), and puromycin (1μg/ml). Escaper cells were cultured in the same media, but with the addition of doxycycline (1μg/ml). Ink4a/arf$^{-/-}$ EGFRvIII murine astrocytes were cultured in DMEM supplemented with 10% heat inactivated FBS, Penicillin/streptomycin, and puromycin (1μg/ml). Gefitinib-resistant Ink4a/arf$^{-/-}$ EGFRvIII murine astrocytes were cultured in the same media, but with the addition of 2μM gefitinib. Gefitinib and lapatanib were purchased from LC Labs. Doxycycline was purchased from Clontech. Trolox, and EUK-134 were purchased from Sigma-Aldrich. Trolox was dissolved in DMSO (20 mg/ml) and EUK-134 (2 mg/ml) was dissolved in molecular grade water.

Xenograft Studies

All animal procedures were approved by the IACUC at the University of California, San Diego. A total of 5 x 10^3 tumor-derived U373MG-tet-O-EGFRvIII cells were injected subcutaneously into the flanks of 6- to 8-week old female athymic mice. Mice were initially fed a regular diet and tumor volume was measured with calipers weekly beginning at day 14 post-injection. When average tumor volume reached approximately 500 mm^3, half of the mice were switched to the doxycycline diet (200 mg/kg; Bio-Serv) for a total of 8 days. At the endpoint, tumors were resected from each group and appropriately prepared for the respective post-analysis experiments.

Quantitative real-time PCR

Subcutaneous tumors were resected from mice and then RNA was isolated from the tissue using the RNeasy Plus Kit (Qiagen). cDNA was synthesized using the RNA to cDNA EcoDry Premix (Clontech). qPCR was performed with a CFX96 Real-Time System (BioRad) using the following program: 95^{o}C for 10 minutes, [95^{o}C for 15 seconds; 58^{o}C for 1 minute] x 40 cycles, 95^{o}C for 1 min and 55^{o}C for 1 min. EGFRvIII and actin gene expression were measured using SYBR Green (BioRad) with internal triplicate determinations for each sample.

Western blotting

Subcutaneous tumors were resected from mice and then tissue was lysed for protein harvesting using RIPA Buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM NaF, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate and 1x Complete protease inhibitor [Roche]) and phosphatase inhibitors). Total protein was quantified using BCA Protein Assay Reagent (Thermo Scientific). Afterwards, 40µg of each protein sample was resolved on a 7.5% to 15% polyacrylamide gel. Proteins were transferred, blocked with 5% milk followed by primary antibody incubation overnight at 4^{o}C. The following antibodies were used at specified concentrations for immunoblots: EGFR clone 13 (1:2500; BD Transduction Labs), EGFR Y1068 (1:1000, Cell Signaling), α-tubulin (1:500, Santa Cruz Technology), GAPDH (1:1000; Cell Signaling). Membranes were washed three times with TBS containing 0.1% Tween-20 and incubated with anti-mouse or anti-rabbit-HRP antibody (Sigma). Chemiluminescence was detected using Amersham ECL Western Blotting Detection Reagent (GE Healthcare) or SuperSignal West Pico Chemiluminescent Substrate

(Thermo Scientific). Images were obtained using ChemiDoc MP imaging system and Image Lab 4.1 software (Bio-Rad).

Immunohistochemistry

Tissue sections were cut from formalin-fixed paraffin-embedded xenograft tissue. Sections were deparaffanized, re-hydrated, and then stained with either anti-53BP1 (1:100; Cell Signaling) or anti-8-oxoguanine (1:1000; Rockland). Heat-induced antigen retrieval was performed for the 53BP1 staining using 10mM sodium citrate, pH 6.0. Antigen retrieval was omitted for 8-oxoguanine staining. Primary antibodies were incubated on slides overnight at 4^{o}C in a humidified chamber. Secondary antibody incubation plus AEC (Scytek) for 53BP1 or plus DAB (Vectastain) for 8-oxoguanine detection, was done according to the manufacturer's instructions. Slides were dehydrated through a series of alcohol and xylene, then coverslips were mounted onto slides. Quantification of positively stained nuclei was conducted using microscopy cell counting software. Stain intensity scoring was determined by establishing an intensity rubric based on the samples displaying the highest, middle, and lowest staining across all samples.

Immunofluorescence

Ink4a/arf$^{-/-}$ EGFRvIII murine astrocytes were seeded on coverslips and allowed to grow for 48 hours. At this point, cells were treated with 5μM of lapatanib for indicated time. Cells were fixed in 4% paraformaldehyde (pH 7.1) for 20 min at room temperature followed by a three PBS washes and permeabilization with 0.4% NP40 buffer for 15 min. After blocking with 5% heat inactivated donor calf serum in HBSS,

cells were incubated with pH2AX antibody (Millipore) followed by FITC-conjugated secondary antibody. Nuclei were counterstained with DAPI simultaneous to mounting coverslips on slides. Images were captured using a fluorescence microscope.

ROS Assays

A total of 50,000 to 80,000 cells were seeded into 6-well plates. After 48 hours, cells were labeled with either MitoSOX Red (superoxide indicator; Life Technologies) or CellROX Orange (general oxidative stress indicator; Life Technologies) according to the manufacturer's instructions. Samples were analyzed for cellular fluorescence on an LSR II (BD Biosciences) and data were analyzed with FlowJo software (Treestar). For the genetic silencing experiments, U373-tetO-EGFRvIII cells were treated with Doxycycline (1 μg/ml) for 6 days prior to labeling and flow cytometry. For the pharmacological inhibition studies, Ink4a/arf$^{-/-}$ EGFRvIII murine astrocytes were treated with 5μM of gefitinib for 48 hours prior to labeling and flow cytometry.

Statistical analysis

All data are presented with mean ± SEM as determined by Prism software (GraphPad). Unpaired Student's t-test or ANOVA was used as appropriate. P values of 0.05 or less were considered significant for all experiments. Data are representative of results obtained in at least two to three independent experiments.

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Chapter 4

Introduction to the Kelch Repeat Superfamily

4.1 Kelch Repeat Superfamily

KLHDC8A is a member of the Kelch repeat superfamily, which is one of the largest evolutionarily conserved gene families, where the presence of the kelch motif that defines this family is found from *Drosophila melanogaster* to *Homo sapiens* (1). The founding member of this family, *kelch*, was first described in *Drosophila melanogaster,* where female homozygous *kelch* mutants produced very small, cup-shaped eggs for which the gene was named; “kelch” in the German language means “cup” (2). Future molecular cloning experiments together with the use of bioinformatics afforded the discovery of the kelch motif as a repeated element in the sequence of the *Drosophila* kelch open reading frame 1 (ORF1) protein (3,4).

The kelch motif is typically 44-56 amino acids in length, occurring in most members as five to seven tandem repeats that collectively fold into a β-propeller tertiary structure (5,6). The crystal structure of galactose oxidase, a converter of alcohol to aldehyde, from the fungus *Dactylium dendroides* was the first of the Kelch superfamily to be solved, becoming the prototype for subsequent analyses used to identify other Kelch superfamily members and to predict their tertiary structures (4,7). On the basis of this crystal structure, each repeat forms a twisted four-stranded antiparallel β-sheet that corresponds to a single blade of the propeller radially arranged around a central axis, and either an amino-terminal (N-terminal) or a carboxyl-terminal (C-terminal) strand closure mechanism links the first and last blades to complete the propeller (5-7). Intra- and inter-blade loops protrude above, below, or adjacent to the β-sheets, constituting the accessible regions of this structure and contribute to the characteristic binding properties of each β-propeller (6).

Multiple sequence alignment and pattern analysis uncovered a group of highly conserved residues now regarded as the consensus sequence of the kelch motif (4). These key residues include a series of four hydrophobic amino acids followed by a double glycine element (GG), and two characteristically spaced aromatic residues, tyrosine (Y) and tryptophan (W) (5,6,8). Aside from these eight residues, the sequence similarity between individual kelch motifs within a given protein is surprisingly low, rendering these residues indispensable to distinguish kelch-containing proteins from those of the similarly structured WD-40 repeat family, which also form β -propellers (4,6).

Within the human genome, the encoding sequences for kelch-repeat proteins are located on all chromosomes except chromosome 21 and the Y chromosome (5). The growing list of Kelch superfamily members currently includes 66 genes, of which sixty-three encode proteins, and three are non-coding (8). Each member is classified into one of three subfamilies: KLHL, KBTBD, and KLHDC, defined by the number and types of protein domains present (5,6,8).

4.2 Kelch Subfamilies and Domain Architecture

The classic molecular architecture of a kelch protein includes one N-terminal BTB/POZ domain, one BACK domain and a series of C-terminal kelch repeats (5,6,8). Members of the largest subfamily, KLHL, contain all of these domains, while the KBTBD members usually lack the BACK domain (5,6,8). Most KLHDC family members lack both the BTB/POZ and BACK domains and consists almost entirely of kelch repeats alone or with other domains such as transmembrane, Glycine rich, Lish or CTLH (CRA C-terminal to Lish) domains (8).

The KLHL subfamily is conserved throughout evolution and to date, 42 KLHL genes have been identified in *H. sapiens* and classified by the HUGO Gene Nomenclature Committee (HGNC) (1,8). The characterization of the BTB/POZ domain found at the N-terminal end of KLHL and KBTBD proteins, but not unique to the Kelch superfamily, was first explored in *Drosophila.* Its name is derived from a highly conserved, 120 amino acid motif that is shared between three *Drosophila* genes*:* bric à brac (*bab*), tramtrack (*ttk*), and Broad-Complex (*BR-C*) (BTB) (9). POZ reflects the concurrent name given to the related motif found in poxvirus that resembled portions of zinc finger proteins (1,10). The structure of this domain is compact and globular, consisting of a cluster of α-helices flanked by short β-sheet structures (11-13). It primarily participates in protein-protein interactions, and though the secondary structures of BTB/POZ proteins are highly similar, the primary sequences are not (8,11,14). This sequence variability permits differential protein-protein interactions, and consequently, diverse functional abilities. Within the Kelch superfamily, the BTB domain typically functions as a substrate adaptor that is required for the formation of Cullin-dependent E3 ubiquitin ligase complexes (8,11).

Other known functions include transcriptional regulation, cytoskeletal organization, and ion conductance, yet given the large number of BTB/POZ proteins that have been identified, the functions of many proteins are still to be determined (6,8,11,12).

The BACK (for BTB and C-terminal Kelch) domain, also found in KLHL subfamily members, is the most conserved domain within the Kelch Superfamily (15). It is ~130 amino acids in length and typically occurs as a single copy between the N-terminal BTB/POZ domain and the C-terminal Kelch repeats (8,15). Its secondary structure is likely to be completely α-helical and its central core, hydrophobic, due to the many non-polar residues that make up its conserved features: an N-terminal Asn-Cys-Leu-Gly-Ile motif, a Val-Arg-[Leu/Met/Phe]-Pro-Leu-Leu motif, two arginine residues, four glutamic acids, and several hydrophobic residues (15). Similar to the BTB/POZ domain, the BACK domain is also found in non-Kelch proteins. However, the majority of BACK domains occur in BTB-Kelch proteins and are predominant among vertebrate species (15).

The predicted secondary structure of a classical BTB-BACK-Kelch (BBK) protein suggests possible structural and functional similarities with the multi-subunit E3 enzymes termed Cullin-Skp1-Cullin-F box (SCF) complexes (15). On the basis of the crystal structure of the Skp1-Cdc4 complex, the BTB domain of a BBK protein would correspond to the N-terminal half of Skp1, which interacts with the respective Cullin protein (15,16). The BACK domain would reflect both the C-terminal half of Skp1 and the subsequent N-terminal region of the F-box protein Cdc4, which are α-helical in structure (15,16). Lastly, Cdc4 recruits target substrates to the SCF via its C-terminal WD40-domain, a structural equivalent to the Kelch domain, which serves as the substrate recognition component of the complex (15,16). Thus, BBK proteins

are potentially able to accomplish the functions of Skp1 and F box proteins as a single polypeptide. The BACK domain is further speculated to mediate substrate orientation in Cullin-based E3 ubiquitin ligase complexes, but the true function for the BACK domain has not been defined (15). Nevertheless, missense mutations within this domain of the BBK proteins, KLHL40 and KLHL41, are associated with the onset of severe nemaline myopathy (a skeletal muscle disorder), substantiating its functional importance in blocking disease (17,18).

4.3 Kelch proteins and biological outcomes

The tertiary structure of the Kelch domain additionally classifies Kelch proteins as members of the β-propeller superfamily of proteins, and this β-propeller structure affords the fulfillment of a number of biological roles. Additionally, many kelch proteins can share a significant amount of sequence identity; yet can differ in subcellular localization and function within the same cell type (6). This highlights the versatility of this domain in the many aspects of cell function.

Several kelch proteins are actin-binding proteins (e.g., Mayven, α-scruin, IPP, AFKin, MRP2) and function in cytoskeletal organization and nervous system development via their β-propeller structures (19,20). Others facilitate germ cell maturation and cell morphology by promoting differentiation, cell spreading and cell polarity (6). In the context of the immune system, the kelch protein RAG-2 is involved in V(D)J recombination of immunoglobulin and T-cell receptors genes, and the glycoprotein attractin functions as a mediator of monocyte spreading (6). Specifically, activated T-cells secrete attractin into the extracellular environment, where it stimulates cell adhesion and migration that are required for the development of T-cell clusters and cell-mediated immunity (21).

Additional biological processes, in which members of the Kelch superfamily are implicated in, include signal transduction, cell division, RNA processing, vesicular trafficking and chemotaxis (6,20,22,23). The critical nature of these molecular functions necessitates tight regulation to impede the onset of disease, and consequently, the aberrant expression and regulation of many kelch repeat proteins are associated with Mendelian disorders and cancer (1). In fact, the elevated secretion of attractin into cerebrospinal fluid supports glioma cell migration and tumor

progression (24). Additional documented cases, whereby the normal function of kelch proteins is usurped to benefit cancer cells, will be discussed below.

4.4 Kelch proteins and cancer

The nuclear-restricted protein/brain (NRP/B or KLHL37), which is also known as ectodermal-neural cortex (ENC-1) gene, is a BTB-Kelch protein that functions as a component of the nuclear matrix (22,25). The nuclear matrix is a three-dimensional insoluble framework of the nucleus, and is involved in modulating gene expression, the cell cycle, and the integrity of the nuclear structure (26). Changes to the nuclear architecture are associated with differentiation and cellular transformation, and consequently, these alterations are prevalent in cancer.

In normal cells, the expression of NRP/B is restricted to neurons, and this specificity has established it as an early marker for neurons during development of the central nervous system (25). However, elevated expression of NRP/B is detected in the non-neuronal cells that make up GBMs and astrocytomas (22). Additionally, NRP/B is normally expressed in the nucleus of neurons, but within brain tumors it is expressed in the cytoplasm (22,26). Thus, a shift in both subcellular localization and cell type-specific expression is associated with the oncogenic version of this protein.

Several brain tumor-specific mutant forms of NRP/B have been identified, with mutations predominantly occurring in the Kelch domain (22). These mutations augment MAPK/ERK-mediated cell proliferation, confer resistance to cisplatin-induced apoptosis, and are sufficient to promote the same tumorigenic properties in other cancer types (22). The NRP/B promoter is activated by p53; and so perhaps the mutant forms of NRP/B are not functionally competent upon p53 recognition and binding, which leads to the cisplatin-resistant phenotype. Additionally, the kelch domain mutations in NRP/B reduce its binding affinity for actin, which would dramatically affect its contribution to the nuclear matrix architecture (22).

Wild-type NRP/B associates with the retinoblastoma (pRb) tumor suppressor protein to promote neuronal differentiation, and this interaction leads to hypophosphorylation of pRb, which induces G1 cell cycle arrest (25). Defects in the pRb pathway are often essential for the development of GBMs, occurring in 70-80% of GBM cases (27). This percentage may in part be due to NRP/B mutations. Hence, the normal functions of NRP/B would suggest that it is a tumor suppressor: however, mutant NRP/B actually contributes to tumorigenesis by promoting cell proliferation, survival and nuclear structure alterations.

The Kelch-like ECH-associated protein 1 (Keap1 or KLHL19) is a six-bladed propeller BTB-Kelch protein that is localized to the cytoplasm (5). It primarily functions as a substrate recognition module within the Cullin3-dependent E3 ubiquitin ligase complex, and through this role, targets the nuclear factor (erythroid 2-related) factor 2 (Nrf2) for proteasome-dependent degradation under normal cellular conditions (28). Specifically, Keap1 negatively controls the transactivation function of Nrf2 by sequestering it in the cytoplasm, but in the presence of stress stimuli, Nrf2 is released from this interaction in its active form and translocates to the nucleus (29). There, it induces the expression of phase II detoxification and oxidative stress response genes, such as heme oxygenase 1 and NADPH dehydrogenase quinone, and one-carbon metabolism enzymes that drive the synthesis of the antioxidant, glutathione (30).

The expression of these genes yields a cellular defense system that neutralizes reactive oxygen species (ROS) and protects against other toxic insults (30). Oxidative/electrophilic stress sensing by KEAP1, initiates this cascade, wherein its cysteine residues act as biosensors for ROS or xenobiotic molecules (31). Upon

cysteine modification, Keap1 undergoes a conformational change that releases bound Nrf2. Such an efficient mechanism of ROS detoxification would present a selective advantage for cancer cells, which are constantly in a pro-oxidant state oxidant, as a consequence of the genetic, metabolic and microenvironment-associated alterations that characterize them (32). Not surprising, the protection function of Nrf2 is often appropriated by cancer cells, to create a pro-survival environment that supports growth and therapeutic resistance (33). Additionally, loss-of-function mutations in Keap1 are also linked to cancer (31).

The majority of the alterations to the Nrf2-Keap1 axis favor a hyperfunctioning Nrf2 protein. Constitutive activation of Nrf2 is associated with poor prognosis in a variety of cancers (28). Additionally, Nrf2-target genes are associated with cancer cell proliferation through the induction of a glucose flux and the generation of purines, which are important for nucleic acid formation and accelerated growth (28). Nrf2-mediated glutathione production is also critical for cell proliferation (34). Furthermore, elevated Nrf2 confers inherent and acquired chemoresistance in many cancer types, and protects against ionizing radiation by decreasing protein oxidative damage (28,35).

A few somatic mutations in Nrf2 have been identified in cancer, which impair Keap1 recognition and binding, resulting in Nrf2 stability, increased nuclear localization and the constitutive activation of cytoprotective genes (28). Keap1 mutations are more frequently encountered, and predominantly occur within the Kelch domain, which is required for binding to Nrf2 (30,31). Epigenetic silencing of Keap1 via hypermethylation of its promoter is also associated with a growth advantage for many cancers, and this mechanism of Keap1 suppression is also associated with

poor prognosis in lung and malignant glioma (36,37). MicroRNA-mediated gene suppression is also employed by cancer cells to eliminate Keap1, and specifically miR-200A acts as a negative regulator of Keap1 stability (38). Thus, the intended tumor suppressor function of the Keap1 is often antagonized by a variety of mechanisms that support cancer cell expansion.

KLHDC8B is one of the only two, seven kelch repeat only-containing proteins within the human genome; the other is KLHDC8A. It is only expressed in mitotic cells and functions as a midbody protein that is essential for proper cytokinesis (39,40). This protein has been studied extensively in the context of classical Hodgkin's lymphoma (cHL), where its deficiency is linked to this disease (40). Classical Hodgkin's lymphoma is unique, in that the cancerous Reed-Sternberg (RS) cells are the minor population within the tumor mass (1 in 100) (39). However, these cells promote the creation of the growing mass by recruiting benign, reactive inflammatory cells, such as eosinophils, fibroblasts, and lymphocytes (40). This disease is primarily associated with Epstein-Barr Virus (EBV) exposure (40% of cases), but other genetic features have been implicated as risk factors (39).

Indeed, the familial risk for cHL is very high, and a screen of several familial cases of cHL identified a common reciprocal translocation between chromosomes 2 and 3 (41). The specific breakpoint mapped to 3p21.31, a region where frequent somatic cytogenetic rearrangements had already been observed for cHL (41). This locus is also where KLHDC8B resides, and in affected individuals, its upstream regulatory elements and exon 1 are eliminated, as a result of the translocation (41). Additionally, a portion of its 5' UTR fused with an intergenic region on chromosome 2q11.2 and a single nucleotide polymorphism (SNP) at a phylogenetically conserved

position within its 5'UTR were also identified in some familial cases (41). The functional consequence of the fusion alteration is abrogated transcription, and the SNP causes attenuated translation of the protein (40,41).

The midbody is a small intracellular structure that functions as the final point of contact between dividing daughter cells before they undergo separation via cytokinesis. Studies that recapitulated the cellular context, whereby KLHDC8B function is disrupted, demonstrated that it protects against a multitude of mitotic errors: multinucleation, aberrant mitoses, centrosomal amplification, micronuclei production, delayed or failed abscission, aneuploidy, multipolar mitotic figures, and asymmetric segregation of daughter cells that lead to the formation of anuclear daughter cells (23). All of these errors are well documented in the pathogenesis of Hodgkin's lymphoma, substantiating the centrality of this protein in maintaining genetic stability. Furthermore, its primary function as a tumor suppressor adheres to the functional theme that appears to be common to this family of proteins.

4.6 KLHDC8A

In 2010, Mukasa *et al*. established the first substantial link between KLHDC8A and tumorigenesis. Gene expression profile analysis conducted on relapse GBM tumors (Escapers) that emerged from a chronic EGFRvIII-negative environment revealed that KLHDC8A was significantly enriched in these populations (42). The pattern of expression was particularly striking due to its absence in EGFRvIII-dependent populations. Furthermore, this study showed that when KLHDC8A is silenced in Escapers, their in vivo growth capacity is significantly impaired, suggesting that this protein is essential in tumor recurrence (42).

KLHDC8A is predicted to be a 7-bladed kelch repeat only-containing protein, based on its primary amino acid sequence (**Figure 4.1A**). Yet, aside from this information, very little is known about the mechanism of its function and the molecular consequences of its dysfunction. Intra-sequence alignment of each repeat highlighted putative residues that may be important for its function and subcellular localization, such as surface residues within the loop regions that can be phosphorylated to initiate signaling cascades or function as redox sensors (**Figure 4.1B**). Additionally, although KLHDC8A is presumably a cytoplasmic protein, a predicted nuclear localization sequence (NLS) exists within its sequence, suggesting that it may be able to shuttle between cellular compartments (**Figure 4.1B**).

Based on the roles of the other Kelch proteins described in this chapter, it is likely that KLHDC8A normally functions as a tumor suppressor; however, none of the functions established for the other members have been attributed to KLHDC8A. Moreover, if its demonstrated involvement in GBM so far is any indication of a “transformed” role, then like the others, it may be a victim of cancer hijacking. In the

next chapter, we will more clearly define a role for KLHDC8A in the context of therapeutic escape in GBM.

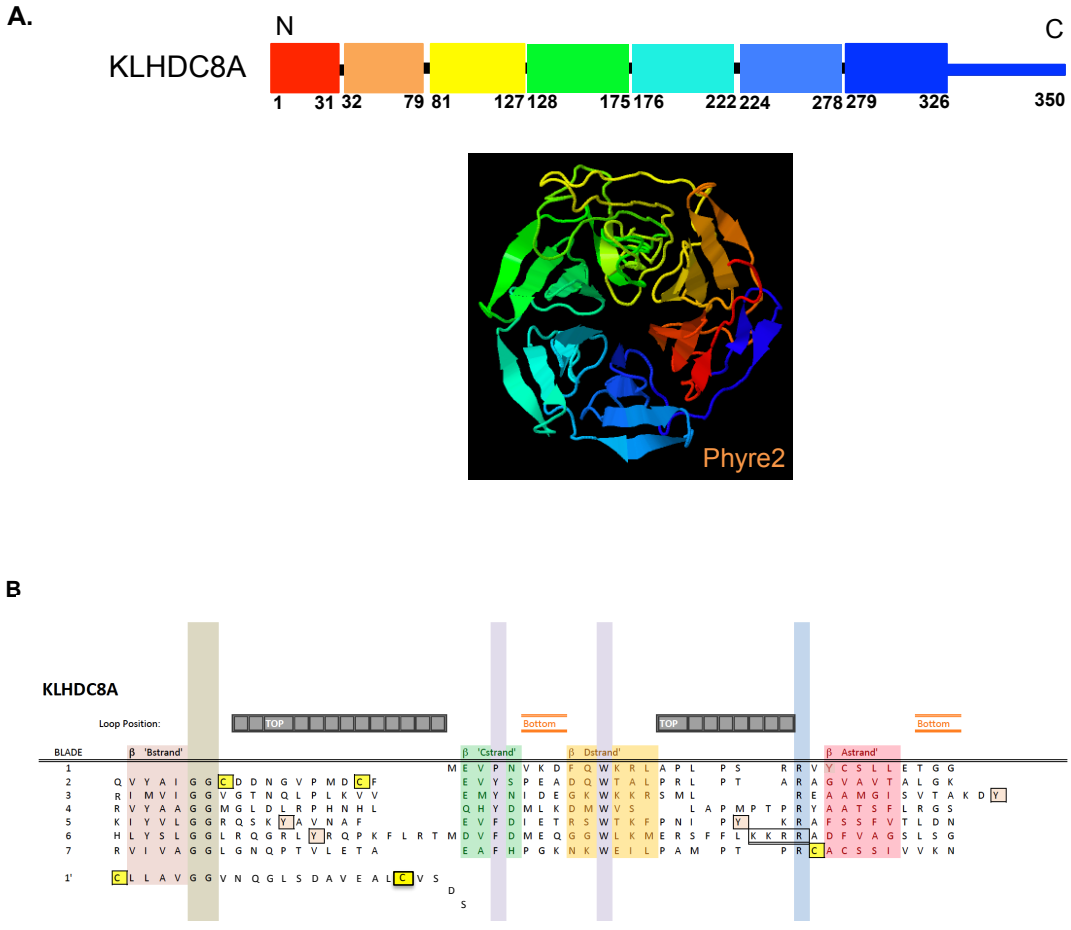

Figure 4.1. KLHDC8A is a 7-bladed β-propeller only containing protein. Schematic diagrams based on the predicted structure of KLHDC8A. (A) Top: linear representation of KLHDC8A depicting the amino acid numbers within each kelch repeat. Bottom: predicted crystal structure using the Phyre2 web portal for protein modeling, prediction and analysis (43), where each blade corresponds to the kelch repeat of the same color in the top linear representation. (B) Intra-sequence alignment for each of the kelch repeats, displaying the specific amino acids that reside in the loop and strand regions and the alignment for the conserved residues that make up the kelch consensus sequence. Note that the C-terminal and N-terminal ends combine to form the first blade. Y-tyrosine (phosphorylation); C-cysteine (redox sensing); KKRR-potential NLS

4.7. References

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Chapter 5

KLHDC8A attenuates the effects of elevated ROS in the absence of EGFRvIII

5.1 Introduction

The earliest evidence for chemoprotective agents that inhibit carcinogenesis arising from chemical exposure dates back to over 85 years ago (1). Since then, the mechanism of their protection has been more clearly defined, whereby these chemoprotectors are known to induce the expression of cellular enzymes that metabolize carcinogens to less reactive forms (2). The inducing activity of electrophilic chemoprotective agents was found to be dependent on the presence of a cis-acting transcriptional enhancer sequence called the antioxidant response element (ARE) within the respective response genes (1). In the previous chapter, we described a primary example of this mechanism: Nrf2 function, which binds the ARE of its respective target genes upon activation by oxidants or electrophiles.

The cellular adaptive response to elevated levels of reactive oxygen species is regulated in multiple ways. This response depends primarily on the transactivation of cellular defense genes, but also on the stability of the transcription factors required for the production of these genes and the oxidative/electrophilic sensing proteins. The stress sensing ability of Keap1 could be describe as the cell's first line of defense against deleterious levels of ROS. Likewise, other proteins within the Kelch superfamily may participate in this process as well. In this chapter, we establish a novel link between KLHDC8A and oxidative stress, based on our hypothesis that KLHDC8A gene up-regulation is a genetic adaptation utilized by GBMs in response to excessive ROS levels.

Results

5.2. Up-regulation of KLHDC8A is consistently observed in GBM models of resistance to EGFRvIII inhibition

KLHDC8A was the second most up-regulated gene within the mRNA profiles of Escaper populations that emerged in our EGFRvIII genetic silencing model. Based on this finding, we first wanted to determine if this genetic change was unique to the U373MG-tetO-EGFRvIII model or if this reflected a common response in different contexts where overcoming blockade of EGFRvIII is observed. To address this question, we first validated the microarray findings for all of the genes that were determined to be associated with "escape" by Mukasa *et al.*, using quantitative polymerase chain reaction (qPCR) analysis (full data not shown). With this approach, we confirmed that KLHDC8A mRNA expression is indeed most expressed in Escapers compared to the EGFRvIII-positive populations and the acute EGFRvIII-negative populations (**Figure 5.1**).

Next, we examined the expression of KLHDC8A mRNA in two established EGFR TKI-resistant models. The first model we examined was the *Ink4a/arf*$^{-/-}$ EGFRvIII murine astrocytes, a model in which both therapeutic sensitivity and acquired resistance to gefitinib phenotypically resembles our genetic model (**Chapter 3**). The second model was the patient derived xenograft (PDX) GBM39 neurosphere model. Resistance in this model is observed following chronic treatment with the EGFR TKI erlotinib in vivo (3). In both models, we found that KLHDC8A mRNA was significantly up-regulated in the resistant populations compared to the sensitive populations (Control) (**Figure 5.1**). Taken together, these data suggest that

KLHDC8A is involved in multiple contexts of acquired resistance, and potentially reflects a common genetic adaptation in response to EGFRvIII inhibition.

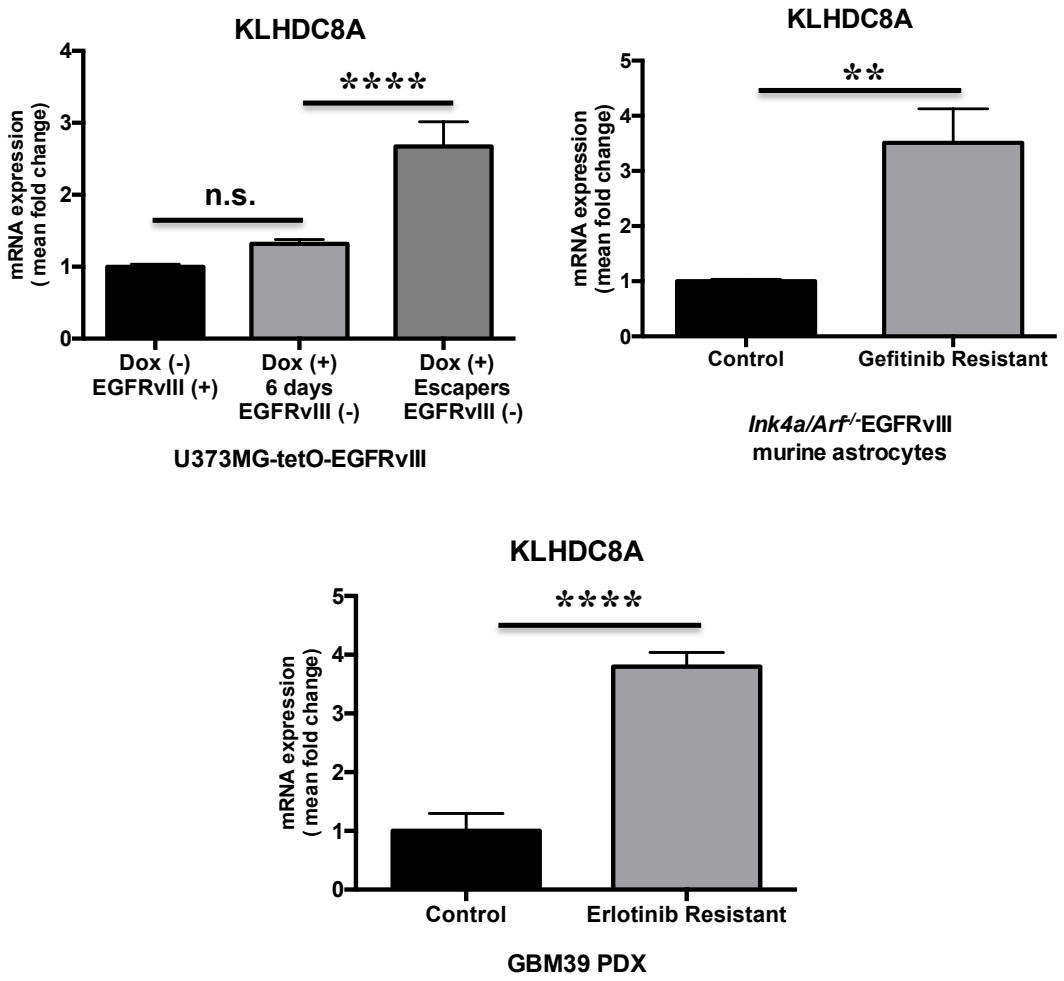


Figure 5.1. Up-regulation of KLHDC8A is common to in several GBM models. Quantitative PCR analysis for KLHDC8A gene expression in three GBM models: (1) U373MG-tetO-EGFRvIII, (2) *Ink4a/arf*$^{-/-}$ EGFRvIII murine astrocytes, (3) GBM39 PDX. Data are means $\pm$ SEM ****$p<0.0001$, **$p<0.01$, n.s. = not significant

5.3. KLHDC8A interacts with the TRiC/CCT chaperonin complex

In order to delineate a functional role for KLHDC8A, we next conducted screening studies to identify putative binding partners. Currently, there is not a reliable KLHDC8A antibody to be used for interaction studies, so for this objective, we employed tagged versions of KLHDC8A. In our first study, we stably transduced HEK293T cells with an engineered C-terminal FLAG-tagged KLHDC8A construct and performed immunoprecipitation followed by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) (**Figure 5.2A**). In this experiment, 39 proteins were identified as putative binding partners (**Figure 5.2C**). We followed up this study with a second interaction approach: proximity-dependent biotin identification (BioID). This technique utilizes a 35 kDa *Escherichia coli* DNA binding biotin ligase called BirA* (R118G) that can be fused to any protein of interest (4). Upon supplementation of culture medium with biotin, BirA biotinylates both direct interacting and vicinal proteins, affording the identification of both transient and permanent interactions (4). Subsequently, the biotinylated proteins can be isolated by streptavidin affinity capture, and then identified by mass spectrometry.

We generated an N-terminal BirA-Myc_KLHDC8A fusion protein and stably transduced HEK293 cells with this construct (**Figure 5.2B**). This time, we identified 163 putative binding partners, and so to triage our extensive list of binding partners, we compared the hits from both approaches to yield a final list of 15 common associations (**Figure 5.2C**). Within our final list, we noticed that there were six proteins that are individual subunits of the TRiC/CCT chaperonin complex. This complex is an ATP-dependent protein folding machine consisting of two stacked rings of eight different but related (CCT1-CCT8) subunits and the apical domain of each

subunit contributes to lid formation that covers the cavity in the form of a cap (5). Approximately, 5-15% of newly synthesized proteins rely on this complex for folding and assembly, and these substrates are processed in a very exclusive fashion, whereby the complex completely encapsulates them during folding (5). Determinants of substrate selection have been established for this complex and they include: 1) proteins that are components of oligomeric complexes, 2) proteins that are ~40-75 kDa in size 3) proteins that are enriched in hydrophobic sequences, and 4) proteins with high β-sheet propensity, particularly long stretches of 35-45 amino acids of β-sheet content (6).

Based on what is currently known about the molecular profile of KLHDC8A, three of the four determinants are fulfilled, and thus, it is likely a TRiC/CCT substrate. We confirmed that this complex directly interacts with KLHDC8A by co-immunoprecipitation. U373MG-tetO-EGFRvIII GBM cells were stably transduced with a C-terminal Myc-tagged KLHDC8A construct, and Myc-tag-associated proteins in the presence and absence of EGFRvIII expression were isolated by affinity capture (**Figure 5.2D**). We analyzed the captured immunoprecipitates by Western Blot, and indeed were able to isolate a substantial amount of the TCP1 subunit in our KLHDC8A_Myc cells, but not in the empty vector (EV) control cells (**Figure 5.2D**). Furthermore, our results demonstrate that this interaction did not require EGFRvIII, which is consistent with the mRNA expression data, whereby a role for KLHDC8A is predicted to be specific to EGFRvIII-independent contexts.

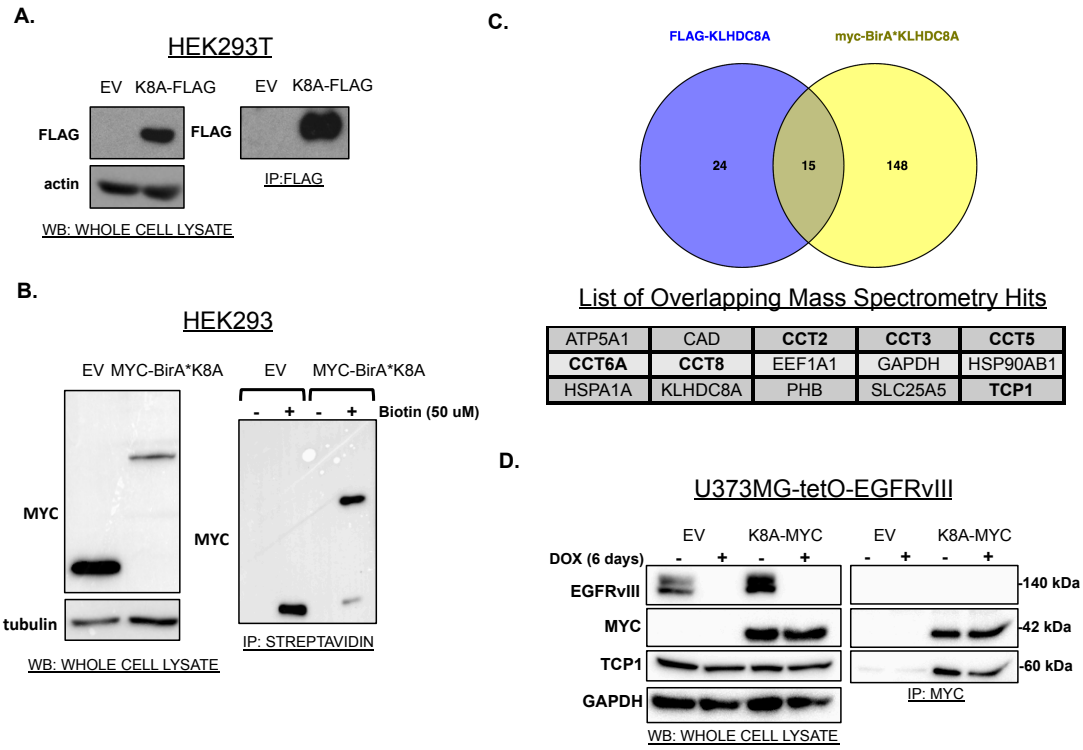


Figure 5.2. KLHDC8A interacts with the TRiC/CCT chaperonin complex. Multiple protein interaction studies revealed proteins commonly associated with KLHDC8A. (A) Co-immunoprecipitation-Mass spectrometry approach using FLAG-tagged KLHDC8A construct in HEK293T cells. (B) Proximity dependent biotin identification-Mass spectrometry approach using the BirA-Myc-KLHDC8A fusion construct in HEK293 cells. (C) Comparative analysis to establish common elements between the two lists identified several subunits of the TRiC/CCT chaperonin complex (in bold) as consistently interacting with KLHDC8A. (D) Immunoprecipitation studies conducted in U373MG-tetO-EGFRvIII GBM cells to validate the direct interaction between KLHDC8A and the TRiC/CCT chaperonin complex.

A great deal of work has been done to characterize how essential the TRiC/CCT complex is to cell viability and also to establish a list of its bonafide substrates. Initially its main substrates were only thought to be actin and tubulin, but a more defined TRiC/CCT interactome has been established through various experimental and computational efforts (6). Other notable TRiC/CCT substrates identified include: the histone deacetylase 3 (HDAC3), the telomerase cofactor TCAB1, the WD40 repeat protein WDR68, the von-Hippel Lindau tumor suppressor protein, and cyclin E (7-11), which have all been linked to oncogenesis.

Interestingly, Gallina *et al.* recently demonstrated that the TRiC/CCT complex is involved in protein quality control and recovery from genotoxic stress. In this work, both the yeast Cmr1 protein and its human orthologue WDR76 were shown to directly interact with components of the TRiC/CCT complex within the context of a novel intranuclear quality control (INQ) compartment (12). This INQ serves as a hub for the establishment of molecular adaptations required for resumption of DNA replication. More importantly, the tertiary structures of Cmr1 and WDR76 are very similar to KLHDC8A, in that they are predominantly made up B-propeller domains. In the absence of these domains, formation of the INQ is impaired, and consequently no recovery from DNA damage is observed (12).

With consideration for these findings, we suspect that the significance of the association between TRiC/CCT and KLHDC8A is linked to cellular response to genotoxic stress that is specifically caused by reactive oxygen species. Thus, we briefly examined the subcellular localization for KLHDC8A, to see if it can also localized to the nucleus, since the involvement of the TRiC/CCT complex to the cell recovery process took place in the nucleus (12). We took our U373MG-tetO-EGFRvIII

cells, stably expressing KLHDC8A-MYC, and performed immunofluorescence staining on these cells using a myc-tag specific antibody as an indicator for KLHDC8A localization. The myc-tag was primarily detected in the nuclear compartments of cells, as reflected in the merge with the DAPI nuclei stain (**Figure 5.3**). Interestingly, in some cases we saw co-localization with nuclear EGFRvIII, but within the same population, we also observed EGFRvIII-positive cells that were negative for KLHDC8A-MYC (**Figure 5.3**). When EGFRvIII was silenced in these cells by doxycycline treatment, we still observed nuclear localization, further supporting a possible EGFRvIII-independent function for KLHDC8A. Additionally, if KLHDC8A does function in cellular recovery from genotoxic stress, then a nuclear role for its involvement would be expected.

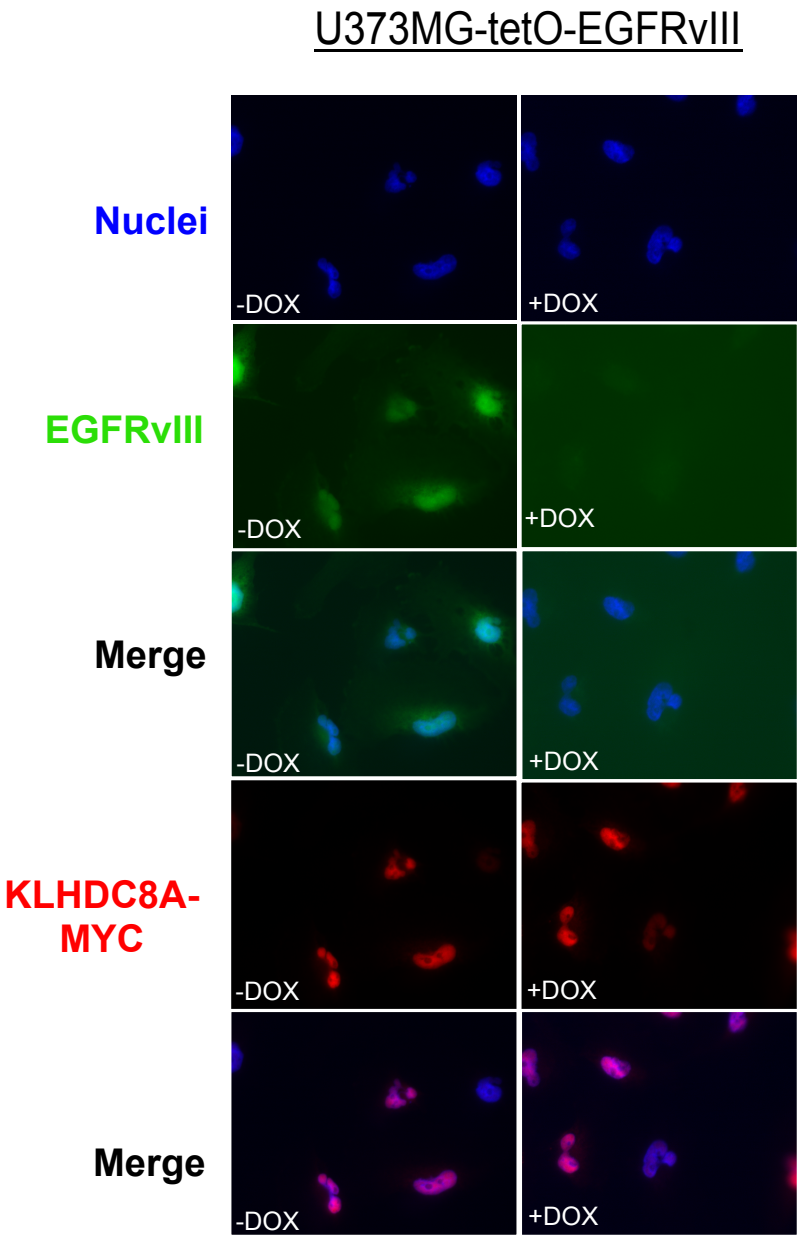


Figure 5.3. KLHDC8A is found in the nucleus independent of EGFRvIII in GBM. Representative immunofluorescence staining for EGFRvIII and KLHDC8A-MYC localization in U373MG-tetO-EGFRvIII cells. Doxycycline treatment was done for 6 days prior to staining. EGFRvIII (FITC-channel); KLHDC8A-MYC (TRITC-channel)

5.4. Loss of KLHDC8A in Escapers leads to an increase in DNA double strand breaks

Our hypothesis is that up-regulation of KLHDC8A represents a genetic adaptation in response to changes in ROS levels. We reasoned then that targeting KLHDC8A should once again restore sensitivity to EGFRvIII inhibition, and this restored sensitivity would be reflected phenotypically. Thus, we investigated the requirement for KLHDC8A in the acquisition of phenotypes that support EGFRvIII independence.

Beginning with two Escapers, whereby loss of KLHDC8A led to impaired in vivo growth (13), we examined the extent of DNA damage burden in these populations by immunohistochemistry. We used 53BP1 positive nuclei as our standard readout for DNA damage, and observed a significant increase in DNA double strand breaks in the KLHDC8A knockdown samples compared to the negative control samples (**Figure 5.4**). This increase was evident in both escapers, suggesting that the role of KLHDC8A in the maintenance of these individual populations is the same. Furthermore, this data represents the first evidence that links KLHDC8A to DNA damage.

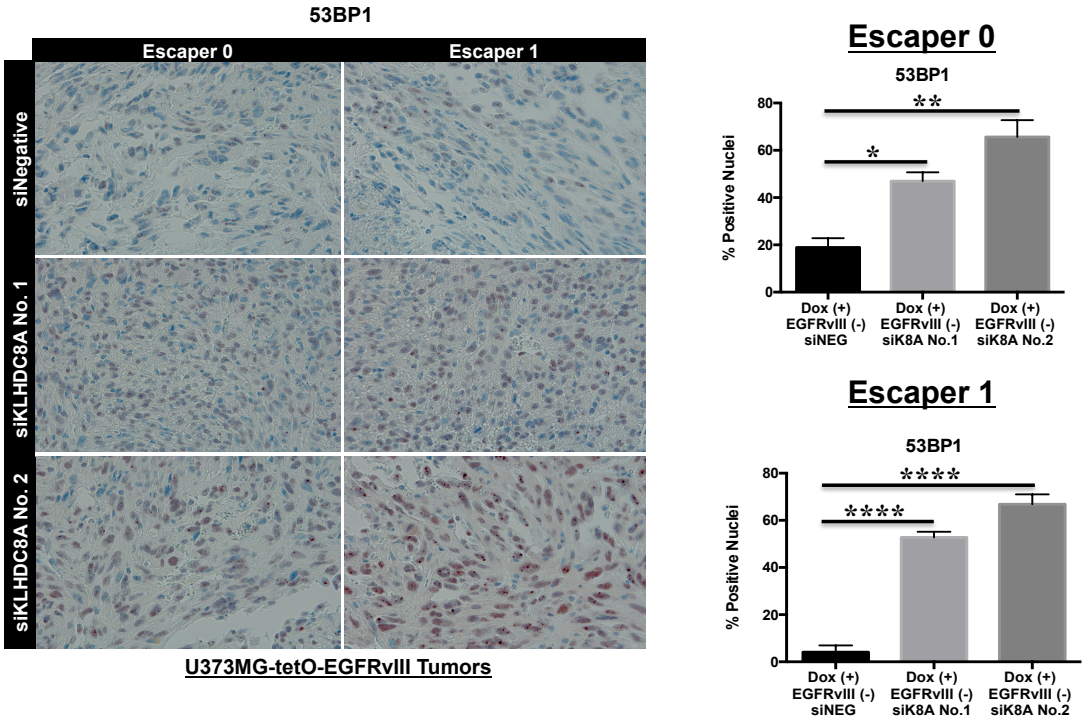


Figure 5.4. siRNA-mediated knockdown of KLHDC8A promotes the persistence of unrepaired DNA double strand breaks in Escapers. Representative immunohistochemical staining for 53BP1 nuclei in Escaper siKLHDC8A populations. Quantification of positively stained nuclei was determined using microscopy cell counting software. Escaper 0 siK8A No.1: (2.5-fold increase), Escaper 0 siK8A No.2: (3.5-fold increase), Escaper 1 siK8A No.1: (12.9-fold increase), Escaper 1 siK8A No.2: (16.3-fold increase) Data are means ± SEM **** $p<0.0001$, ** $p<0.01$, * $p<0.05$

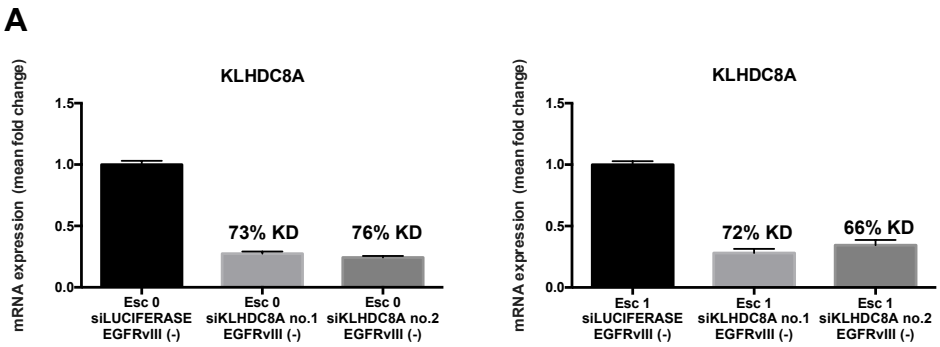
5.5. Combined loss of EGFRvIII and KLHDC8A results in elevated ROS activity and oxidative DNA damage

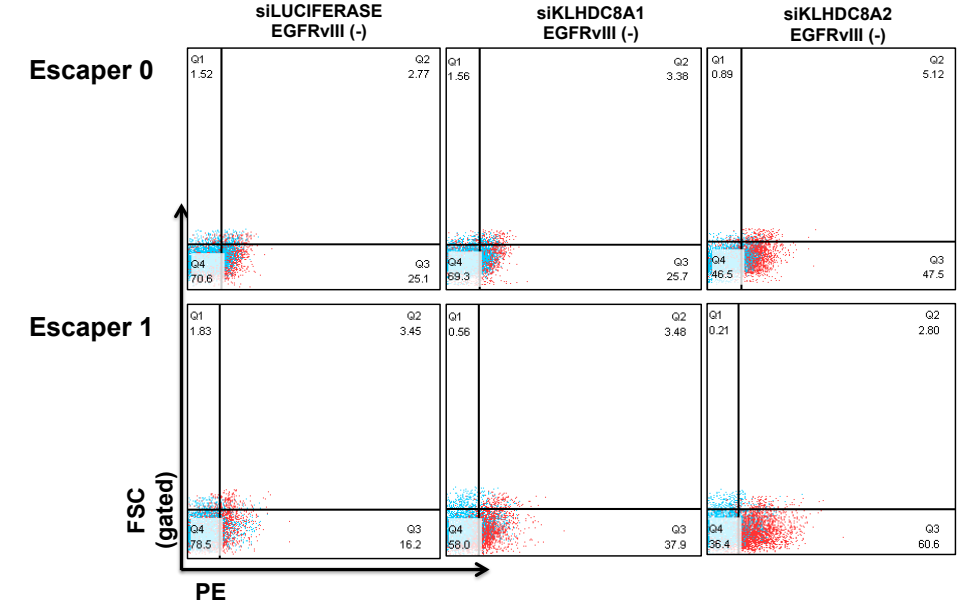
We next moved into examining if reactive oxygen species was the source of the increase in DNA damage observed in KLHDC8A silenced Escaper tumors. To address this question, we took cultured Escaper lines that were generated from the original tumors and stably expressed the two KLHDC8A-speciific siRNAs in these cells. We confirmed the knockdown efficiency by quantitative PCR analysis (**Figure 5.5A**), and then assessed the ROS activity in these cells using our two ROS indicators. Overall, we saw a significant increase in mitochondrial ROS activity when KLHDC8A was inhibited as reflected in the percentage of MitoSOX Red positive cells (**Figure 5.5B-C**). This was observed for one siRNA in Escaper 0 cells and for both siRNAs in Escaper 1 cells.

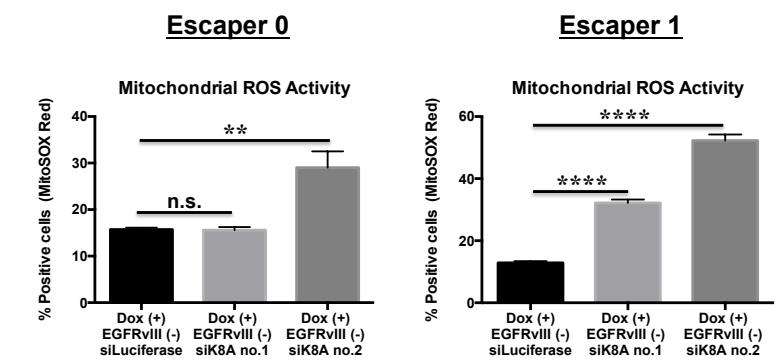
Next, we examined the impact of inhibiting KLHDC8A on cytoplasmic ROS activity. In majority of the cases, we did not observe an appreciable increase in cytoplasmic ROS activity (**Figure 5.6**). The lack of a change in cytoplasmic ROS activity is reminiscent of the findings from the EGFRvIII pharmacological inhibition studies shown in chapter 3. This implies that KLHDC8A can substitute for EGFRvIII, but specifically for the EGFRvIII kinase-dependent tumorigenic activities.

Lastly, we assessed if the impact on mitochondrial ROS activity alone would be sufficient for an observable increase in oxidative DNA damage in vivo. We took tumor tissue from the KLHDC8A knockdown populations and stained for 8-oxoguanine. In every condition, we observed an increase in the detection of 8-oxoguanine (**Figure 5.7**). Taken together, these data suggest that targeting KLHDC8A alone is sufficient to restore sensitivity to EGFRvIII inhibition in Escapers.

Figure 5.5. Inhibition of KLHDC8A restores sensitivity to EGFRvIII inhibition in the form of increased mitochondrial ROS levels. (A) Knockdown efficiency for KLHDC8A mRNA was validated by quantitative PCR in both Escaper cell lines. (B) Representative Flow Cytometry plots depicting ROS activity as measured by the PE-positive population (Q3) for each condition. Blue=unlabeled cells; Red=labeled cells (C) Mean percentage of positive cells after background subtraction. MitoSOX Red is a superoxide indicator. Data are means $\pm$ SEM ****$p<0.0001$,**$p<0.01$


A
KLHDC8A
mRNA expression (mean fold change)
1.5
1.0
0.5
0.0
73% KD
76% KD
Esc 0 siLUCIFERASE EGFRvIII (-)
Esc 0 siKLHDC8A no.1 EGFRvIII (-)
Esc 0 siKLHDC8A no.2 EGFRvIII (-)
KLHDC8A
mRNA expression (mean fold change)
72% KD
66% KD
Esc 1 siLUCIFERASE EGFRvIII (-)
Esc 1 siKLHDC8A no.1 EGFRvIII (-)
Esc 1 siKLHDC8A no.2 EGFRvIII (-)


B
Mitochondrial ROS
siLUCIFERASE EGFRvIII (-)
siKLHDC8A1 EGFRvIII (-)
siKLHDC8A2 EGFRvIII (-)
Escaper 0
Escaper 1
Q1 1.52
Q2 2.77
Q4 70.6
Q3 25.1
Q1 1.56
Q2 3.38
Q4 59.3
Q3 25.7
Q1 0.89
Q2 5.12
Q4 46.5
Q3 47.5
Q1 1.83
Q2 3.45
Q4 78.5
Q3 16.2
Q1 0.56
Q2 3.48
Q4 58.0
Q3 37.9
Q1 0.21
Q2 2.80
Q4 36.4
Q3 60.6
FSC (gated)
PE


C
Escaper 0
Escaper 1
Mitochondrial ROS Activity
% Positive cells (MitoSOX Red)
40
30
20
10
0
**
n.s.
Dox (+) EGFRvIII (-) siLuciferase
Dox (+) EGFRvIII (-) siK8A no.1
Dox (+) EGFRvIII (-) siK8A no.2
Mitochondrial ROS Activity
% Positive cells (MitoSOX Red)
60
40
20
0
****
****
Dox (+) EGFRvIII (-) siLuciferase
Dox (+) EGFRvIII (-) siK8A no.1
Dox (+) EGFRvIII (-) siK8A no.2

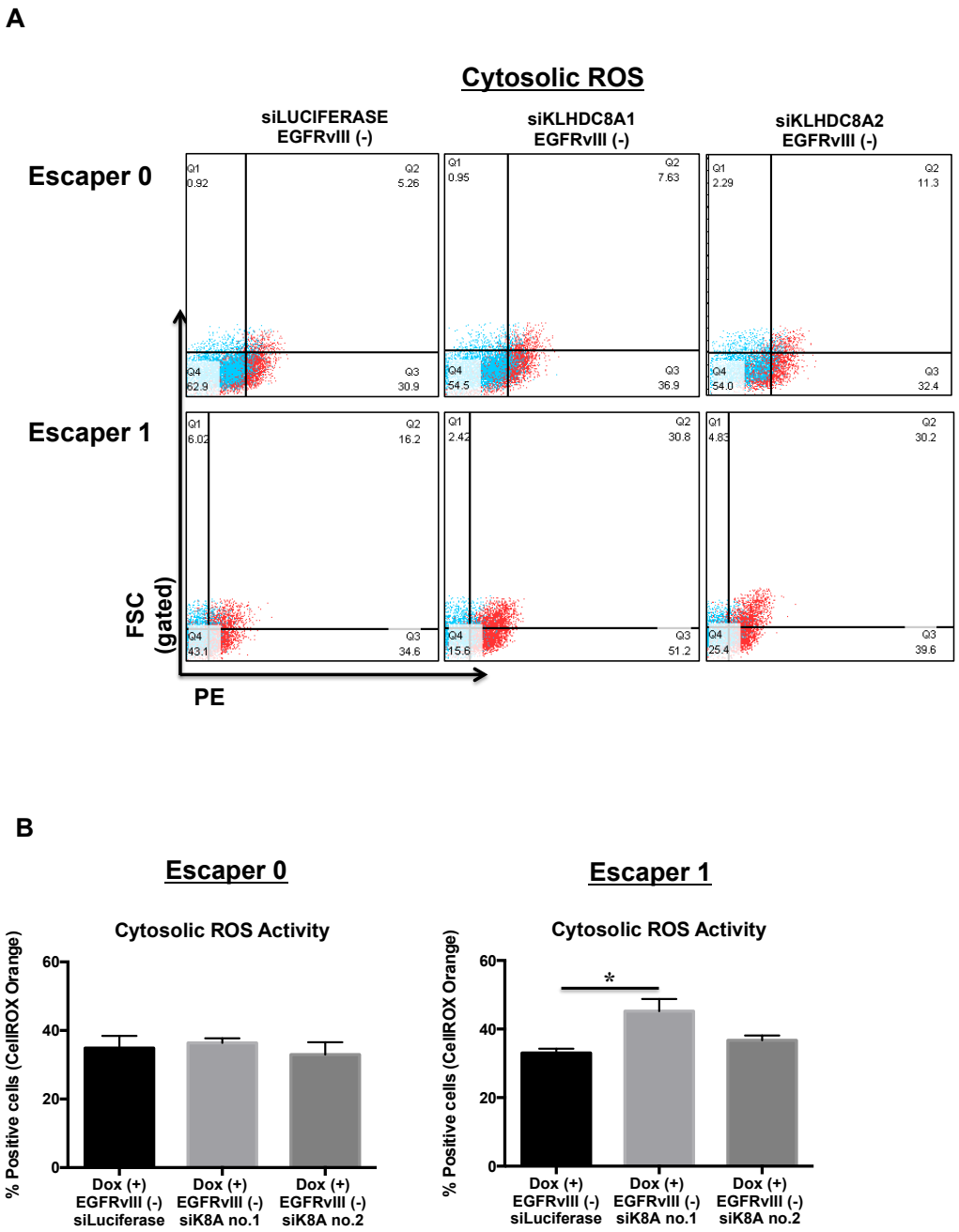


Figure 5.6. Cytoplasmic ROS activity is not affected when KLHDC8A is inhibited. (A) Representative Flow Cytometry plots depicting ROS activity as measured by the PE-positive population (Q3) for each condition. Blue=unlabeled cells; Red=labeled cells (C) Mean percentage of positive cells after background subtraction. Cell ROX Orange is general oxidative stress indicator. Data are means $\pm$ SEM *p<0.05

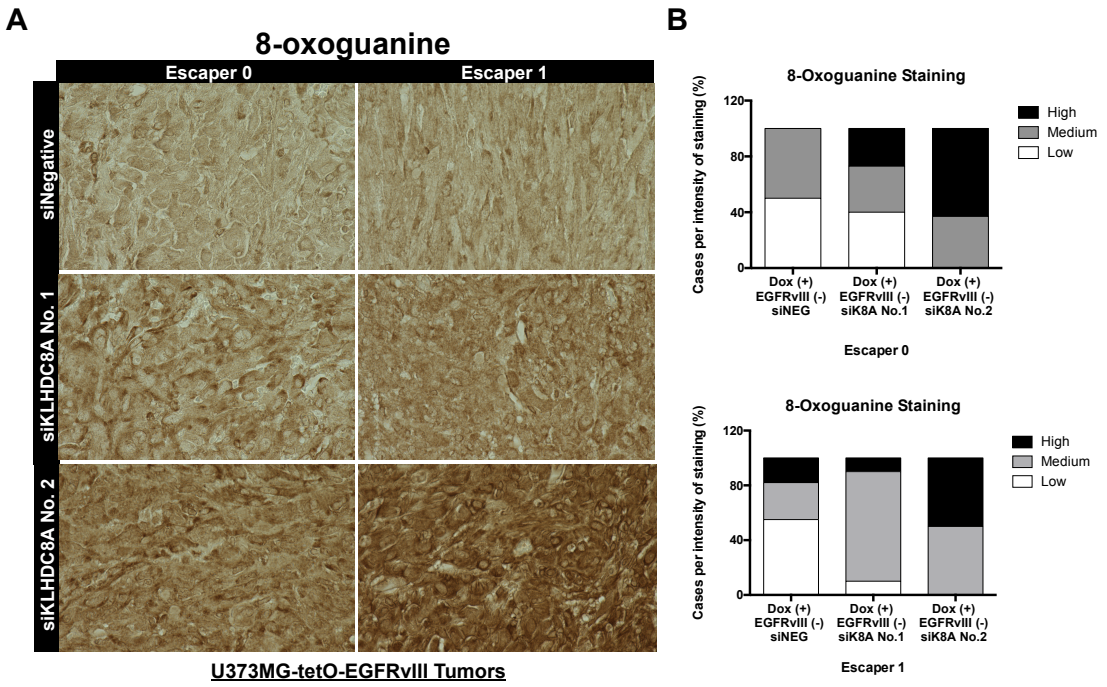


Figure 5.7. Loss of KLHDC8A is associated with an increase in oxidative DNA damage. (A) Representative immunohistochemical staining for 8-oxoguanine, in resected tumors (B) Quantification of staining intensity. Data reflect the individual cases and their respective extent of oxidative stress, represented as percentages. Esc 0 siNEG: low-50%; medium-50%; high-0% vs Esc 0 siK8A No.1: low-40%; medium-33%; high-27% and Esc 0 siK8A No.2: low-0%; medicum-37%; high-63% Esc 1 siNEG: low-55%; medium-27%; high-18% vs Esc 1 siK8A No.1: low-10%; medium-80%; high-10% and Esc 1 siK8A No.2: low-0%; medium-50%, high-50%

5.6 Summary

To date, the only established role for KLHDC8A was for the maintenance of in vivo tumorigenicity in the absence of EGFRvIII in GBM. Here, we extend its role to the context of mediating ROS levels and oxidative DNA damage. KLHDC8A is consistently up-regulated when acquired resistance emerges, and so far our work suggests that the significance of this may be to protect against oxidative stress overload.

KLHDC8A was confirmed to interact with the Group II chaperonin complex, TRiC/CCT. Recently, a role for this complex has extended beyond protein folding and assembly into mediating recovery from oxidative stress as part of a novel intranuclear quality control compartment (INQ). Our preliminary localization studies on KLHDC8A revealed that it localizes in the nucleus in an EGFRvIII-independent fashion. Thus, we suspect that the direct association between KLHDC8A and TRiC/CCT complex may not be simply due to it being a substrate, but also due to their involvement in cellular stress responses.

Similar to EGFRvIII receptor activity, KLHDC8A seems to be required specifically in the context of mitochondrial ROS activity. Although, we did not observe any impact on cytoplasmic ROS activity, we still saw a significant increase in oxidative DNA damage in the respective in vivo samples. This would suggest that mitochondrial ROS activity is the primary determinant for DNA oxidation, but additional studies will be required to establish this claim.

In summary, we have established a novel link between KLHDC8A, ROS and oxidative DNA damage. To our knowledge, this is the first evidence demonstrating such an association and characterization of this protein. We believe that this may be

a common occurrence in multiple contexts of therapeutic resistance to EGFRvIII targeting. The broader implications of this work and the required future studies will be discussed in the next chapter.

Chapter 5 contains data that have not been published previously, and are currently being prepared for submission for publication. The work in chapter 5 was completed with the help of Jill Wykosky, PhD., a former member of the laboratory of Frank Furnari and Jason Liang, a fellow PhD Candidate in the laboratory of Huilin Zhou at the Ludwig Institute for Cancer Research. This work was supported by the NIH F31 NS076343 to (T.E. Taylor) and the NIH R01 NS080939 to (F. B. Furnari).

5.7 Materials and Methods

Cell culture and reagents

HEK 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and Penicillin/streptomycin (1:1000). HEK293 cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS, penicillin/streptomycin (1:1000), and sodium pyruvate (2.2 g/L). U373MG-tetO-EGFRvIII-dependent cells were cultured in (DMEM) supplemented with 10% Tet-approved FBS, Penicillin/streptomycin, G418 (2µg/ml), puromycin (1µg/ml) and blasticidin (2µg/ml). Escaper cells were cultured in the same media, but with the addition of doxycycline (1µg/ml). Tet-approved FBS was purchased from Genesee Scientific. Doxycycline was purchased from Clontech. Blasticidin was purchased from Invitrogen. Biotin was purchased form Sigma-Aldrich.

Construction of Plasmids

The pcDNA3.1mycBioID plasmid was obtained from Addgene. KLHDC8A cDNA was cloned into plasmid backbone between the EcoRI and HindIII restriction sites to generate the final myc-BirA*-KLHDC8A fusion protein. The KLHDC8A-myc cDNA insert was generated by PCR and cloned into the NheI and BamHI restriction sites of the pLV-t2A-Blasticidin lentiviral vector to use for stable transduction. The KLHDC8A-FLAG cDNA insert was generated by PCR and cloned into the SalI and NotI restriction sites of the pDREF lentiviral vector to use for stable transduction. The pSUPER.retro.blasticidin retroviral vector was used to generate stable constructs for knockdown of KLHDC8A. To generate new cells, the same constructs were used as

previously described in (13). The specific siRNA sequences for human KLHDC8A are: No.1: 5′-GCAGCAGCACAATGATTAA-3′; No.2: 5′-AGCGAGAATTGGACATGAA-3′; negative control: 5′-TTCTCTGAACGTGTCACGT-3'; and negative control (siLuciferase): 5'- CTTACGCTGAGTACTTCGA -3'

Stable Cell Line Generation

The HEK 293T packaging cell line was cotransfected with the pSUPER.retro.blast retroviral constructs and pCL10A1 or with the lentirviral construct pLV-t2A-Blast plus pCMV-VSV.G and delta 8.9 using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Lentiviral supernatants were harvested at 48, 72, and 96 hr after transfection, filtered (0.45 µm), then used for overnight infections of U373MG-tet-O-EGFRvIII and Escaper cells in the presence of 8µg/mL polybrene. Cells were allowed to recover in fresh media for 24 hours and were then selected in DMEM media containing 2µg/mL neomycin (G418), 1 µg/mL puromycin, and 4µg/mL blasticidin S for KLHDC8A-MYC and siKLHDC8A stable expression, respectively. HEK293 cells were cotransfected with the pcDNA3.1mycBioID lentiviral constuct and pCMV-VSV.G and delta 8.9 using Lipofectamine 2000, then selected in complete MEM media containing 4µg/mL neomycin (G418) for myc-BirA*-KLHDC8A stable expression. HEK293T were cotransfected with the pDREF lentiviral constuct and pCMV-VSV.G and delta 8.9 using Lipofectamine 2000, then selected in complete DMEM media containing 4 µg/mL hygromycin for FLAG-KLHDC8A stable expression.

Co-Immunoprecipitation

Cells were lysed for protein harvesting using RIPA lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM NaF, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate and 1x Complete protease inhibitor [Roche]) and phosphatase inhibitors) for the Anti-c-Myc approach and lysis buffer (50 mM Tris HCl, pH7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) for the Anti-FLAG approach. Lysates were incubated with either 20µl of Anti-c-Myc or Anti-FLAG agarose based on the respective tag, and incubated overnight at 4°C on end-over-end rotator. Agarose resin was pelleted with a 10-second pulse at 12,000 x g. Resin was washed three times with 500µl of TBST (TBS with 0.05% Tween-20 Detergent), pelleting resin in between washes. Bound proteins were eluted by boiling at 98°C in sample buffer for KLHDC8A-myc, and eluted fractions were analyzed by Western Blotting. Bound protein were eluted using a 3XFLAG peptide for FLAG-KLHDC8A, and eluted fractions were analyzed by mass spectrometry to identify putative binding partners.

Affinity Capture of Biotinylated Proteins

Cells were grown until 90% confluency then incubated for 24 hr in complete media supplemented with 50µM biotin. After one PBS wash, cells were harvested off dish by scraping in 10 ml of 1X PBS on ice. Cells were pelleted by centrifuging at 1200 rpm for 5 min at 4°C. After resuspending in 1 ml of 1X PBS, cells were transferred to a 1.5 ml eppendorf tube, pelleted once more, then lysed at 25°C in 1.5 ml RIPA lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM NaF, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate and 1x Complete protease inhibitor [Roche]) and phosphatase inhibitors) and sonicated on ice (2 sec on/2 sec off for 16 seconds at

10% amplitude). After sonication, lysates were filtered through an Amicon Ultra 3K (2 ml) device to remove free biotin and to concentrate the sample. Lysates were pre-cleared with 250µl Protein G agarose beads, and incubation overnight at 4°C on rotator. Supernatants were transferred to a new eppendorf tube and lysate was incubated again with 250µl agarose beads (MyOne Steptavadin C1; Invitrogen) overnight at 4°C on rotator. Beads were collected and washed twice for 8 min at 25°C (all subsequent steps at 25°C) in 1 ml wash buffer 1 (2% SDS in dH_2O). This was repeated once with wash buffer 2 (0.1% deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA, and 50 mM Hepes, pH 7.5), once with wash buffer 3 (250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, and 10 mM Tris, pH 8.1) and twice with wash buffer 4 (50 mM Tris, pH 7.4, and 50 mM NaCl). 25% of the sample was reserved for Western blot analysis. Bound proteins were removed from the agarose beads with 50µl of 4X SDS-sample buffer, no bromophenol blue, saturated with biotin (10mM) at 98°C. 75% of the sample was analyzed by mass spectrometry.

Western blotting

Cells were lysed for protein for harvesting using RIPA Buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM NaF, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate and 1x Complete protease inhibitor [Roche]) and phosphatase inhibitors). Total protein was quantified using BCA Protein Assay Reagent (Thermo Scientific). Afterwards, 40µg of each protein sample was resolved on a 7.5% to 15% polyacrylamide gel. Proteins were transferred, blocked with 5% milk followed by primary antibody incubation overnight at 4^oC. The following antibodies were used at specified concentrations for immunoblots: EGFR clone 13 (1:2500; BD Transduction

Labs), GAPDH (1:1000; Cell Signaling), α-tubulin (1:500, Santa Cruz Technology), β-actin-HRP (1:25000, Sigma-Aldrich), FLAG-tag (1:2000, OriGene), myc-tag (1:1000, Cell Signaling) and TCP1-α (1:500, Santa Cruz Technology. Membranes were washed three times with TBS containing 0.1% Tween-20 and incubated with anti-mouse or anti-rabbit-HRP antibody (Sigma). Chemiluminescence was detected using Amersham ECL Western Blotting Detection Reagent (GE Healthcare) or SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Images were obtained using ChemiDoc MP imaging system and Image Lab 4.1 software (Bio-Rad).

Protein Identification by Mass Spectrometry

A fraction of the proteins eluted from the streptavidin agarose beads by SDS-sample buffer were reduced and alkylated and separated by SDS-PAGE. Separated proteins were visualized by Silver staining to confirm uniqueness prior to mass spectrometry analysis. Next, on bead tryptic digests were analyzed by 1D LC/MS/MS. Dithiothreitol (DTT) (15 mM) was added to beads suspension mix and proteins were reduced at 42°C for 30 min. Iodoacetamide was added (to 30 mM) and proteins were alkylated at 25°C for 30 min in the dark. Mass spectrometry grade trypsin (Promega) was added (~1:50 ratio) for overnight digestion at 37°C. Agarose beads were removed by centrifugation. Peptides were desalted using a C18 SepPak and lyophilized under vacuum followed by resuspension in 80% acetonitrile/20% water before on-line analysis of peptides by high-resolution, high-accuracy LC-MS/MS, consisting of a TSK gel HILIC, a 15-cm Amide-80 column, a low-flow ADVANCED Michrom MS source, and a LTQ-Orbitrap XL (Thermo Fisher Scientific). A 120-min gradient (20% water, 80% acetonitrile) was used to separate the peptides. Samples were then

analyzed using a LTQ tandem mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Raw MS/MS spectra were searched by SEQUEST on a Sorcerer system for for protein identification against the IPI human protein database v3.80 from EBI (European Bioinformatics Institute), which contains semi-tryptic peptide sequences with the allowance of up to two missed cleavages and precursor mass tolerance of 50.0 ppm. The following search parameters were used: a molecular mass of 57.021465 Da was added to all cysteines to account for alkylation, semi-tryptic, mass tolerance of 1.2 Da for precursor ions, and 226 D on N terminus and lysine for biotinylation. Search results were sorted, filtered, statically analyzed, and displayed using PeptideProphet and ProteinProphet (Institute for Systems Biology). The minimum trans-proteomic pipeline (TPP) probability score for proteins was set to 0.95, to assure TPP error rate of lower than 0.01.

Quantitative real-time PCR

A total of 100,000 transduced cells were seeded in triplicate wells of a 6-well plate. After 24 hours, the cells were put into serum-free DMEM. The following day, RNA was isolated from the cells using the RNeasy Plus Kit (Qiagen). cDNA was synthesized using the RNA to cDNA EcoDry Premix (Clontech). qPCR was performed with a CFX96 Real-Time System (BioRad) using the following program: 95^{o}C for 10 minutes, [95^{o}C for 15 seconds; 58^{o}C for 1 minute] x 40 cycles, 95^{o}C for 1 min and 55^{o}C for 1 min. KLHDC8A and actin gene expression were measured using SYBR Green (BioRad) with internal triplicate determinations for each sample.

Immunohistochemistry

Tissue sections were cut from formalin-fixed paraffin-embedded xenograft tissue. Sections were deparaffanized, re-hydrated, and then stained with either anti-53BP1 (1:100; Cell Signaling) or anti-8-oxoguanine (1:1000; Rockland). Heat-induced antigen retrieval was performed for the 53BP1 staining using 10mM sodium citrate, pH 6.0. Antigen retrieval was omitted for 8-oxoguanine staining. Primary antibodies were incubated on slides overnight at 4^{o}C in a humidified chamber. Secondary antibody incubation plus AEC (Scytek) for 53BP1 or plus DAB (Vectastain) for 8-oxoguanine detection, was done according to the manufacturer's instructions. Slides were dehydrated through a series of alcohol and xylene, then coverslips were mounted onto slides. Quantification of positively stained nuclei was conducted using microscopy cell counting software. Stain intensity scoring was determined by establishing an intensity rubric based on the samples displaying the highest, middle, and lowest staining across all samples.

Immunofluorescence

U373MG-tetO-EGFRvIII-dependent cells were seeded on coverslips and allowed to grow for 48 hours. At this point, cells were fixed in 4% paraformaldehyde (pH 7.1) for 20 min at room temperature followed by a three PBS washes and permeabilization with 0.3% Triton X-100 in PBS buffer for 10 min. After blocking with PBS containing, 2% BSA IgG free, 10% normal goat serum, and 0.3M glycine, cells were incubated with Myc-tag antibody (1:8000, Cell Signaling (9B11)) followed by TRITC-conjugated secondary antibody. Nuclei were counterstained with DAPI simultaneous to mounting coverslips on slides. Images were captured using a fluorescence microscope.

ROS Assays

A total of 50,000 to 80,000 cells were seeded into 6-well plates. After 48 hours, cells were labeled with either MitoSOX Red (superoxide indicator; Life Technologies) or CellROX Orange (general oxidative stress indicator; Life Technologies) according to the manufacturer's instructions. Escaper cells were treated with Doxycycline (1 μg/ml) throughout the entire course of the experiment. Samples were analyzed for cellular fluorescence on an LSR II (BD Biosciences) and data were analyzed with FlowJo software (Treestar).

Statistical analysis

All data are presented with mean ± SEM as determined by Prism software (GraphPad). Unpaired Student's t-test or ANOVA was used as appropriate. P values of 0.05 or less were considered significant for all experiments. Data are representative of results obtained in at least two to three independent experiments.

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Chapter 6

Discussion

6.1 Summary

EGFR and mutant EGFRvIII represent common genetic abnormalities encountered in primary GBM. Over the past several decades, comprehensive roles for their involvement in a number of tumorigenic properties have been established. Based on these tumor-promoting roles, several therapeutic agents have been developed to disrupt their function. However, the efficacy of these agents has been limited by both upfront and acquired resistance. GBM tumors invariably recur, and a great deal of work has been done to characterize new resistance mechanisms that support relapse, in order to inform the development of more durable therapies.

Here, we have shown that another mechanism of acquired resistance to EGFRvIII inhibition involves overcoming oxidative DNA damage. In our primary GBM model, we observed a pattern, whereby the phenotypes that were associated with sensitivity to EGFRvIII inhibition, were overturned upon relapse. These phenotypes included impaired proliferative capacity, increased apoptosis, and an increase in DNA damage burden. The latter phenotype was established in this work, and was found to be the result of elevated ROS activity. On the contrary, GBM populations that have overcome blockade of EGFRvIII displayed both reduced ROS activity and DNA oxidation. Thus, it appears that the very qualities that commonly contribute to tumor initiation and progression (i.e., the tolerance of DNA damage and subsequent mutagenesis) may also threaten the fitness of cancer cells and impede therapeutic escape.

These results imply that a dependence of cancer cells on antioxidant systems is a specific vulnerability that can be exploited to induce targeted cell death. Normal cells are characterized at having lower intracellular ROS levels, and so one way to

achieve this would be to increase oxidative stress above the toxicity threshold in a tumor-specific manner. We have shown that the up-regulation of KLHDC8A is unique to populations that have acquired resistance to therapies targeted against EGFR and EGFRvIII, and that by inhibiting this gene, therapeutic sensitivity is restored. However, it remains to be determined if these induced levels reflect a tumor-specific signature.

KLHDC8A was also found to interact with the TRiC/CCT chaperonin complex, and fulfills many of the determinants of substrate recognition for this complex. The TRiC/CCT interactome encompasses many functional classes of cytoplasmic proteins, including those involved in cytoskeleton organization, DNA replication and repair, cell-cycle progression, RNA processing, and protein trafficking (1,2). Our work demonstrated that KLHDC8A is also found in the nucleus, yet it is still unknown if its interaction with the TRiC/CCT complex occurs in the cytoplasm or the nucleus.

More recently, the TRiC/CCT chaperonin complex, along with other components of the intranuclear quality control (INQ) compartment, have been implicated in cellular capacity to recover from genotoxic stress (3). Furthermore, it has previously been linked to the context of cytoplasmic proteotoxic stress through its interaction with HSF1A, which activates the transcription of HSF1 stress response target genes (4). Given that many of the key regulators of cell growth and differentiation are clients of molecular chaperones, including the TRiC/CCT complex, chaperones are potential mediators of oncogenesis. Thus, it is plausible that the significance of the interaction between TRiC/CCT and KLHDC8A is integral to its role in conferring resistance to EGFRvIII inhibition, even if it simply modulates its folding.

In total, our studies have extended the role for KLHDC8A in gliomagenesis, which was previously only known to be required for in vivo tumor growth in the absence of EGFRvIII (5). We have additionally established that it is required to maintain endogenous ROS levels below the toxic threshold. Consequently, this leads to a decrease in persistent DNA damage and presumably therapeutic escape (**Figure 6.1**). However, there are still remaining questions regarding the exact mechanism for how KLHDC8A protects cancer cells from stress toxicity and stress-induced apoptosis.

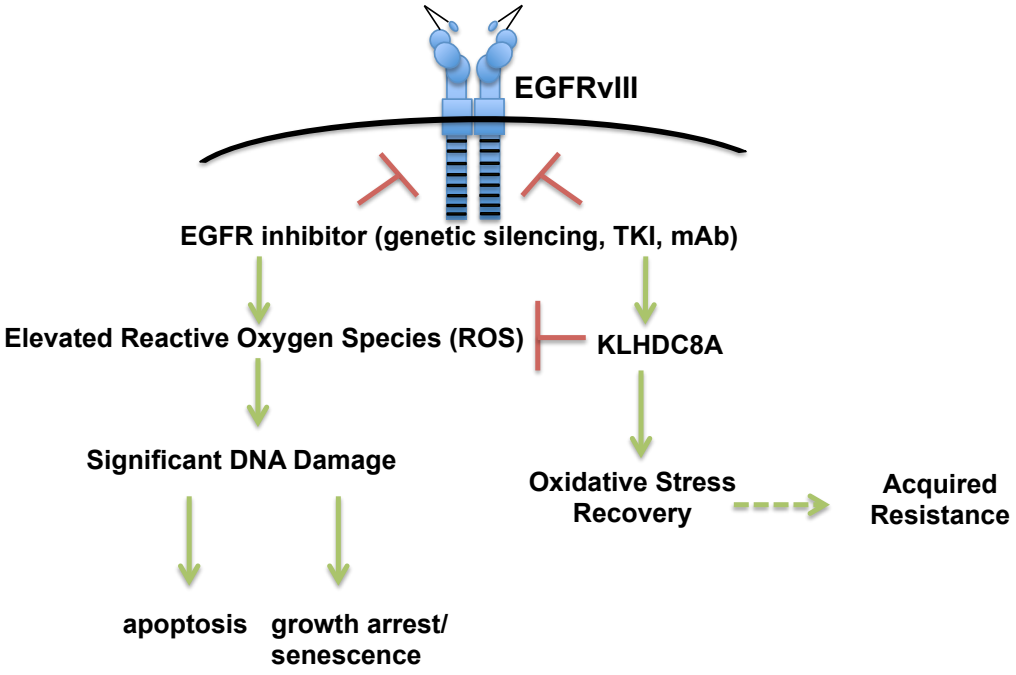

Figure 6.1. Predicted model of KLHDC8A-mediated resistance to EGFRvIII inhibition. In the context of therapeutic sensitivity, EGFRvIII targeting leads to elevated intracellular ROS activity. Unchallenged by antioxidant defenses (e.g., superoxide dismutase, catalase, glutathione), these reactive species can oxidize DNA bases (i.e., guanine) causing double-strand breaks (DSBs). If these breaks are deemed irreparable, then either cell death or growth arrest/senescence results. In the absence of EGFRvIII receptor function, up-regulation of KLHDC8A can serve as an alternative way to regulate ROS levels. The interaction between the TRiC/CCT complex and KLHDC8A may mediate this role. Consequently, cancer cells are able to recover from oxidative stress, which affords the re-initiation of tumor growth (acquired resistance).

6.2 KLHDC8A and the DNA damage burden

EGFR and EGFRvIII have established roles in DNA double-strand break repair. This role is particularly linked to conferring radioresistance in GBM (6-9). Several reports have demonstrated that the primary DNA repair pathway that is employed by these two molecules is the non-homologous end-joining pathway (NHEJ), although a regulatory role in homologous recombination has been observed (10-12). Furthermore, both EGFR nuclear translocation and the activation of the DNA-dependent protein kinase catalytic subunit (DNA-PKCs) repair protein were found to be essential to this process (6,8,13).

Interestingly, within our interaction study using the MycBirA* proximity-dependent biotinylation approach, DNA-PKCs was one of the top five hits determined to interact with KLHDC8A, as established by number of peptides recovered. Moreover, additional components of the stress-induced INQ structure, besides Cmr1/WDR76 and the TRiC/CCT chaperonin complex, included Ku70 and Ku80, which together with DNA-PKCs form the NHEJ-dependent DNA-PK complex (3,14). The INQ was also concluded to be a site of rapid turnover and recycling of phosphorylated, ubiquitinated and sumoylated targets from stalled replication forks, in order to promote an efficient response to DNA replication stress (3).

A predicted functional role for KLHDC8A based on IBA (inferred biological aspect of ancestry) computational analysis (Gene Ontology) is that it is involved in ubiquitin-dependent protein catabolism. This implies that KLHDC8A may indirectly promote the recycling of amino acids for new protein synthesis by facilitating protein turnover. Thus, the role of KLHDC8A may be coupled to the TRiC/CCT complex by way of supplying the building blocks needed for protein synthesis. Furthermore,

chaperones function to impede the accumulation of damaged and misfolded proteins, which would otherwise induce cellular oxidative stress. Therefore, if KLHDC8A stimulates protein quality control mechanisms in an effort to dampen oxidative stress within the cell, this would presumably also decrease the extent of oxidative DNA damage.

A number of WD40 repeat proteins have been shown to directly bind DNA histones at the center of their β-propeller structures (15). A priori, KLHDC8A can alternatively play a more direct role in reducing the DNA damage burden via chromatin binding. Our work demonstrates that KLHDC8A is capable of nuclear entry, and so this would appropriately position it to similarly bind to DNA in order to facilitate DNA repair-promoting modifications. Indeed, nuclear EGFR was shown to drive DNA synthesis and DNA repair by phosphorylating Histone H4 at tyrosine 72, and the disruption of this interaction impaired tumor growth (13). Hence, KLHDC8A could substitute for EGFR in this manner.

Lastly, the fact that an accumulation of DNA damage does not always support tumor progression was quite a surprise to us, given that one of the fundamental features of cancer is genome instability. In fact, another characterized Escaper reflected this feature, displaying no evidence of a significant decrease in DNA damage and thriving tumor growth under these conditions (**Figure 6.2A-C**). Of note, this particular Escaper expresses the lowest levels of KLHDC8A, and so would not be expected to be dependent on its function (**Figure 6.2D**). Thus, some cancer cells are more reliant on an intact DNA repair pathway, which may provide a therapeutic window, whereby the disruption of these pathways could selectively target tumor cells. However, the factors that determine when the accumulation of DNA damage is

a friend or a foe to cancer are still unknown. It would be interesting if the up-regulation of KLHDC8A were one of these tumor-specific determinants.

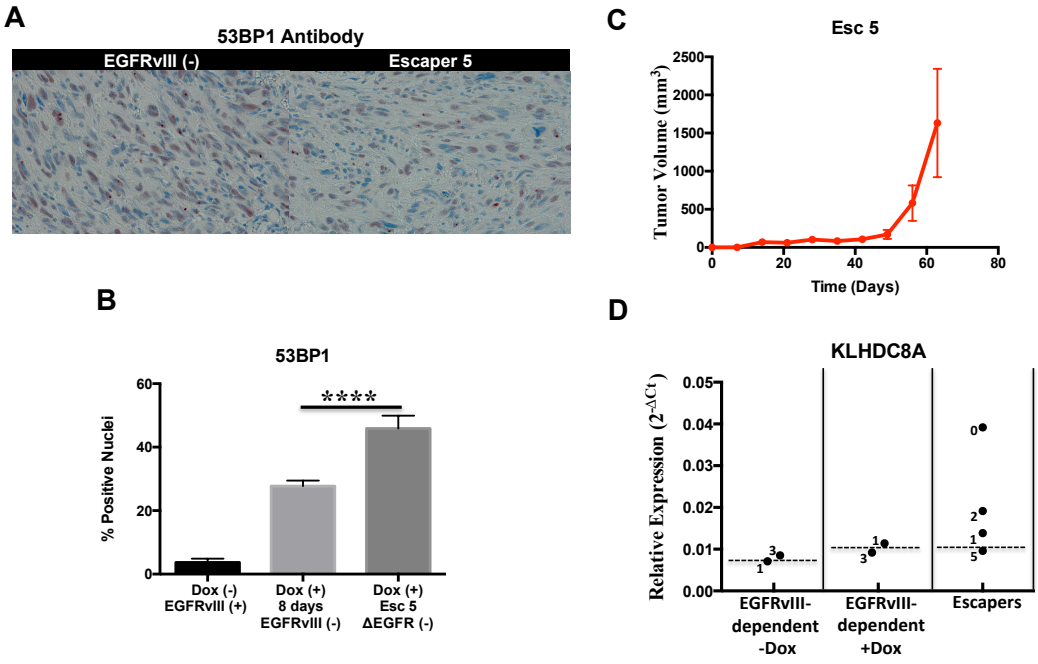

Figure 6.2. Some escapers do not rely on intact DNA repair pathways. (A) Representative immunohistochemical staining for 53BP1 in the EGFRvIII-independent population #5. (B) Quantification of positive nuclei, represented as percentages. (C) In vivo growth curve for this population in the presence of a significant DNA damage burden. (D). qPCR analysis of KLHDC8A mRNA levels in each individual population. Data are ± SEM ****p<0.0001

6.3 KLHDC8A and elevated reactive oxygen species (ROS)

The genetic aberrations that are prevalent in cancer cells promote continuous and excessive production of reactive oxygen species to mediate proliferation (16). However, our work and that of others have shown that ROS can in turn cause DNA damage, triggering growth arrest or apoptosis. Thus, another aspect of oncogene-dependence is reflected in the reliance of the cancer cells on the primary oncogene in order to propagate within such a pro-oxidant environment.

Upon inactivation of the oncogene, cancer cells experience a state of “oncogenic shock”, which is defined by a shift in the levels of pro-survival signals and the pro-apoptotic signals, in favor of cell death (17). This shift could also be perpetuated by the loss of a key modulator of excessive cellular ROS. In our work, we found that blocking the receptor activity of EGFRvIII led to an increase in mitochondrial ROS activity, but cytoplasmic ROS levels remained low. It has been reported that elevated cytoplasmic ROS results in a decrease in lifespan (18). Thus, EGFRvIII kinase activity may regulate mitochondrial ROS levels, while a kinase-independent function may be responsible for maintaining low cytoplasmic ROS levels. Cytoplasmic ROS function as part of signaling pathways; therefore, this could explain why simply blocking EGFRvIII receptor activity is insufficient, since EGFRvIII kinase-independent functions may still interfere with ROS signaling that would decrease cell fitness. Hence, our approach to genetically silence EGFRvIII could result in a more durable response.

Additionally, our work has established a new link between KLHDC8A and mitochondrial ROS homeostasis. Though we have not delineated the mechanism by which KLHDC8A is involved in this process, we suspect that the antioxidant enzyme

systems are recruited. These include: superoxide dismutase 1 and 2, catalase, glutathione peroxidase, peroxiredoxin and thioredoxin. It remains to be determined if loss of KLHDC8A results in a change in either the transcription or translation of these molecules, and how their expression levels align with our current results. Additionally, elevated ROS activity, particularly from peroxynitrite, is known to negatively affect mitochondrial integrity and cause irreversible modifications to proteins, and so it would be interesting to see if these aberrations persist in the absence of KLHDC8A (19). Likewise, it is known that an intact actin cytoskeleton is required for the formation of the juxtanuclear quality control compartment (JUNQ), one of the major cytosolic inclusion bodies that participates in cellular stress response by removing misfolded or aggregated proteins (20). A number of kelch proteins are known to bind actin and function in actin organization (21). Thus, in the event that KLHDC8A does not modulate mitochondrial integrity, it may instead promote the elimination of the resulting damaged proteins by participating in JUNQ architecture.

Based on the significance of amino acid residues within other kelch proteins in the adaptive response to oxidative stress (i.e. Keap1), it is conceivable that functional residues within the accessible loop regions of KLHDC8A may be important in this context. Cysteine residues are predominant targets of redox-linked regulation, and thus can function as sensors of oxidative stress. Moreover, these oxidation modifications can result in changes in conformation, activity, localization and protein-protein interactions. Hence, determining the functional relevance of key amino acid residues, particularly the cysteines, would provide a framework to understand the requirement for KLHDC8A in ROS homeostasis.

6.4 Future Studies

While we have established that there is an association between KLHDC8A and oxidative DNA damage, there are still a number of unanswered questions regarding the specific biology behind this process. Future studies should focus on: (1) Establishing the significance of the interaction between KLHDC8A and the TRiC/CCT complex (2) Determining if within the nucleus, KLHDC8A directly associates with chromatin, and (3) if KLHDC8A possesses post-translational modification activity.

The significance of its interaction with the chaperonin complex would establish more clarity on what appears to be a type of symbiotic relationship. Does KLHDC8A require the TRiC/CCT complex to properly fold, and if so would the TRiC/CCT complex then support tumor progression by folding a client protein that is related to oncogenesis? Additionally, does KLHDC8A partner with the TRiC/CCT complex due to its potential role in protein catabolism, or does the entire interaction operate as a positive feedback loop? Answers to these questions would shed light on the involvement of protein quality control to therapeutic escape.

As it relates to DNA repair, it will be interesting to identify nuclear binding partners of KLHDC8A. Does KLHDC8A bind chromatin, and if so would the disruption of this interaction be sufficient to impact the DNA damage burden. Furthermore, the binding partners would determine if the role for KLHDC8A in the nucleus is different from its role in the cytoplasm. If so, would KLHDC8A then reflect another example, whereby a change in subcellular localization (i.e., NRP/B) results in the impartation of an oncogenic function.

KLHDC8A is a kelch-domain containing only protein, meaning that it lacks the BTB domain. The BTB domain has been substantiated as the structure required for Kelch proteins to function as components of CUL3-RING E3 ubiquitin ligase complexes; establishing a protein-modifying role for these members. KLHDC8B is the most characterized kelch-only containing protein of the two, yet the contribution of its structure to its functional role is not known beyond the ability to bind actin. It would be interesting to see then, if by way of the kelch domains, these two kelch-only containing proteins are also capable of modifying substrates. Studies that would determine this possibility, could uncover the specific mechanisms that are either exploited or disrupted in brain and lymphatic neoplasia, respectively.

In sum, we have found that KLHDC8A promotes the maintenance of EGFRvIII-independent populations by modulating ROS homeostasis, and consequently the extent of DNA damage. Additionally, we have shown that by characterizing therapeutically sensitive phenotypes, one may predict possible escape routes. Interestingly, our work also defined a novel therapeutic window, whereby greater tumor response to radiation may be achievable. We hope that these findings will provoke more efforts toward characterizing tumor sensitivity in addition to identifying novel mechanisms of acquired resistance as a basis for GBM therapeutic development. Future studies that are specific to this project will seek to delineate how the protein structure of KLHDC8A defines its functional role in gliomagenesis.

Chapter 6 contains data that have not been published previously, and are currently being prepared for submission for publication. The dissertation author solely completed the work presented in chapter 6. This work was supported by NIH R01 NS080939 to (F. B. Furnari).

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