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EPIDERMAL GROWTH FACTOR RECEPTOR INDUCES FYN EXPRESSION VIA UP-REGULATION OF P47PHOX IN GLIOBLASTOMA MULTIFORME

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A

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By

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DEDICATION

To my beautiful wife, Rosamond, who has supported me over the course of this very long and arduous educational journey. I am deeply indebted to you for your patience, friendship and love. To my wonderful son, William, your presence is a blessing, and our future as a family has motivated me to complete this work. To my parents, whose support and tireless efforts have afforded me so many opportunities in life. I love you all, and I dedicate this work to you.

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Src family kinases (SFKs) are commonly over-expressed and/or activated in glioblastoma multiforme (GBM), where they serve as key mediators of GBM cell proliferation, survival, invasion and angiogenesis. Mechanisms of allosteric SFK activation are well described; however, the SFK Fyn is commonly up-regulated at the mRNA level in multiple human cancers, including GBM, where the mode of increased expression is poorly understood. Since activating mutations in the epidermal growth factor receptor (EGFR) are commonly occurring in GBM, we examined whether EGFR could induce Fyn expression. Here, we found that wild-type EGFR, and to a greater extent hyper-activating EGFR mutants, EGFRΔIII and R108K, induce a substantial up-regulation of Fyn expression. Furthermore, it was determined that Fyn expression is up-regulated across a panel of patient-derived GBM stem cells (GSCs) relative to normal progenitor controls. Inhibition of Fyn proved to be biologically relevant, as Fyn depletion significantly (p<0.01) reduced cellular proliferation and viability of U87-EGFRΔIII and U87-R108K cells as well as significantly (p<0.001) reducing the sphere forming capacity of GSC 7-2. Mechanistically, Fyn induction was determined to be under the control of early growth response 1 (Egr-1), a previously described redox-responsive transcription factor. Though studies have

previously observed increases in reactive oxygen species (ROS) in GBM, the relationship between EGFR signaling and ROS production remain poorly understood. Using chemical and RNAi-based applications, we demonstrate that EGFR-up-regulates ROS in GBM cells through induction of the NADPH oxidase (Nox)-2 organizer complex, p47phox, which in turn regulates Fyn expression. Therefore, our studies highlight a novel mechanism linking EGFR to the Nox complex and Fyn, providing compelling rationale for redox-targeted strategies in EGFR-expressing GBM.

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Chapter 1

INTRODUCTION

1.1 Malignant Glioma

Originating from glial cells, gliomas account for the majority of intrinsic brain tumors occurring in adults.[1] Gliomas are stratified into different subtypes based upon presumed cell of origin and include astrocytomas, oligodendrogliomas, and ependymomas.[2] Glial tumors, or astrocytomas, represent the most commonly occurring group of gliomas.[3]

On the basis of pathological presentation, namely anaplasia and invasiveness, the World Health Organization (WHO) classifies astrocytomas into four histological grades: grades I-IV.[2] Grade I astrocytic tumors are either pilocytic astrocytomas or subependymal giant cell astrocytomas; grade II tumors include pilomyxoid astrocytomas, diffuse astrocytomas, and pleomorphic xanthoastrocytomas; and grade III tumors are anaplastic astrocytomas.[4] Grade I lesions are considered low-grade, as they are relatively non-aggressive, generally benign and typically curable by resection.[2] In contrast, Grade II and grade III lesions are diffusely infiltrating, may progress to higher-grade tumors and are associated with poorer patient survival and response to therapy.[2] WHO grade IV lesions are the most common and biologically aggressive lesions and include glioblastomas, gliosarcomas and giant cell glioblastoma.[4] High-grade gliomas are exceedingly invasive and confer the worst clinical prognosis as determined by several different factors including lesion grade and Karnofsky performance score.[2,4,5] Among high-grade gliomas, glioblastoma multiforme (GBM) is the most common and lethal type.[6] This generates significant interest in

better understanding the biology of GBM for the purpose of improving clinical outcomes, which will be the focus of the discussion in the following sections.

1.2 GBM

Roughly 3 in 100,000 patients are newly diagnosed with GBM in the United States each year.[7] GBM may develop de novo (primary GBM) or through progression from lower-grade precursor lesions (secondary GBM).[8] Primary GBM are rapidly developing tumors that comprise about 90% of all GBM cases, typically manifesting in elderly patients but also affecting children.[3,7] Secondary GBM arise from lower grade anaplastic astrocytomas and occur far less frequently, affecting patients less than 45 years of age.[7] Though largely indistinguishable by histology, primary and secondary GBM vary significantly in their genetic and epigenetic profiles.[8]

GBM is unique in that it is the most difficult of all human cancers to treat.[9] Despite advances in multimodal therapeutic approaches, patient outcomes have only modestly improved over the past few decades.[1] At the same time, the costs of treatment are substantial, conferring an economic burden of $105,234.00 per per quality-adjusted life-year relative to 2013 US currency rates.[9] Currently, the overall five-year survival rate for GBM patients is 3.4%[10], with the majority of patients succumbing to the disease within 14 months.[5] Nonetheless, as a more comprehensive understanding of the molecular pathology of GBM is attained, the development of targeted therapies will likely transform treatment and quality of life in this disease.[1]

1.2.1 Therapeutic Strategies in GBM

The current standard of care for newly diagnosed GBM patients involves surgical resection followed by fractionated radiation therapy combined with concurrent and adjuvant administration of temozolomide (TMZ).[10] TMZ is an orally available alkylating agent approved for anaplastic astrocytomas and GBM. Structurally, TMZ is a 3-methyl analog of mitozolomide, an antitumor imidazotetrazine developed by Stevens and colleagues circa 1987.[11] In comparison to mitozolomide, TMZ exhibited a more favorable therapeutic index as determined across a variety of in vivo murine tumor models.[12] Mechanistically, TMZ acts to methylate DNA at the O^6 position of guanine.[3] The resulting nucleotide mismatches in complementary DNA give rise to multiple unsuccessful post-replicative attempts at mismatch repair, culminating in an apoptotic response.[13] Detected in roughly 45% of patients, the most salient prognostic indicator for a favorable response to TMZ treatment is the degree of epigenetic silencing of the DNA repair enzyme O^6-methylguanine-DNA-methyltransferase (MGMT) promoter.[14,15] By restoring O^6-alkylated bases caused by chemotherapy, MGMT counteracts the effects of TMZ treatment.[16] Results from a phase III trial have demonstrated the utility of TMZ in the treatment of primary GBM with regard to MGMT promoter status, as improved survival rates were reported for patients whose tumors had methylation of the MGMT promoter.[17] Resistance to TMZ, however, invariably occurs.[9] Factors implicated in the innate resistance of GBM patients to TMZ include: elevated levels of the

aforementioned MGMT activity; loss of the tumor suppressor PTEN, resulting in increased levels of Akt phosphorylation[18]; and augmented base excision repair (BER), capable of rectifying DNA damage induced by TMZ.[18,19]

Despite the advances in surgical resection along with inclusion of TMZ, only modest improvements in survival have been observed.[10] Therefore, studies have sought to better understand the genetic pathology of GBM for the purpose of developing targeted chemotherapies capable of increasing clinical efficacy.[3] Currently, however, a myriad of hurdles impede the responsiveness of GBM to targeted and non-targeted chemotherapy, thus highlighting the clinical challenges presented by GBM. First, incomplete tumor resection particularly often results in tumor recurrence. Secondly, several studies suggest that a population of highly chemo- and radio-resistant tumor-initiating cells, or GBM stem cells (GSCs), is maintained within the bulk of the tumor, giving rise to recurrent lesions that resist further treatment.[20] Thirdly, a highly selective permeability barrier separating the brain from circulating blood, the blood-brain barrier (BBB), precludes delivery of polar compounds of high molecular weight to the central nervous system.[6] Lastly, chemotherapeutics are often actively pumped out of the brain via ABC transporter efflux pumps.[21] As a result, insufficient chemotherapeutic accumulation within the tumor, coupled with the highly heterogeneous composition of GBM, ensure that not all GBM tumor cells receive adequate treatment.[6] In light of these many challenges, it is imperative that new therapies more closely targeting the biology of GBM are developed for the purpose of improving therapeutic outcomes in this devastating disease.

1.2.2 Genetics of GBM

Though the exact cell of origin responsible for primary and secondary GBM remains unclear [3], it has been postulated that primary GBM cells arise from glial progenitors having accrued distinct genetic alterations.[8] Characteristically, primary GBM display amplification, mutation and/or rearrangements of EGFR (57%); mutation or loss of phosphatase and tensin homolog (PTEN) (41%); amplification or overexpression of Cyclin D1/3 and murine double minute 2/4 (MDM2/4) (7.6% and 7.2%, respectively); mutations of tumor protein 53 (TP53) (28%); and loss of heterozygosity (LOH) of chromosome 10 (73%).[8,22,23]

In contrast to primary GBM, secondary GBM have been proposed to originate from progenitor cells harboring mutations of anisocitrate dehydrogenase 1/2 (IDH1/IDH2).[24] IDH1/2 mutations have been catalogued in the majority of WHO Grade II diffuse astrocytomas, where mutation frequency does not correspond with tumor grade.[8] The mutation status of IDH1/2, however, positively correlates with a better prognosis for GBM patients, as patients presenting IDH1/2 mutations are predicted to survive roughly twice as long as patients not bearing an IDH1/2 mutation.[24] Much like primary GBM, diffuse astrocytomas contain TP53 mutations, whereas anaplastic astrocytomas acquire LOH of chromosome 10.[8]

In an effort to broaden the molecular understanding of GBM, The National Cancer Institute (NCI) and The National Human Genome Research Institute (NHGRI) have joined efforts through The Cancer Genome Atlas (TCGA) initiative. Using high-throughput genome analysis technology, the central goal of

the TCGA is to provide the scientific community with valuable genomic information in order to improve diagnosis, treatment and prevention of multiple tumor types, including GBM. Initially, the TCGA published the results of genomic and transcriptomic analysis of 206 GBMs, which included mutational sequencing of 600 genes in 91 tumor samples.[22] The TCGA database has since expanded and now contains molecular and clinical data for a total of 543 GBMs.[25] The current dataset, which is available to the public, includes GBM sequencing of whole genomes, coding exomes and transcriptomes, DNA methylomes as well as targeted proteome profiling. Notably, analysis from the TCGA has uncovered several recurrent and focal alterations not previously detected in GBM, including amplifications in AKT3 and homozygous deletions in PARK2 and NF1.[22] Furthermore, analysis of the TCGA dataset indicates that EGFR alterations are the most commonly occurring among receptor tyrosine kinases (RTKs) in GBM (57%), thus corroborating data from early studies highlighting the essential role of EGFR in GBMs.[26-28]

1.2.3 GBM Subtypes

Using a gene expression-based molecular classification system, GBM has been further stratified into various subtypes. Initial GBM classification studies were performed by Wilson *et al*., in 2006, whereby three GBM subclasses were identified: mesenchymal, proneural and proliferative.[29] The proneural subclass displayed markers indicative of a neural lineage and showed longer survival in patients versus those expressing mesenchymal (angiogenic) or proliferative

markers. Both mesenchymal and proliferative groups exhibited gains or amplification of EGFR as determined by comparative genomic hybridization, which were not present in the proneural GBM subtype. Additionally, the two poor prognosis subgroups expressed increased levels of neural stem cell markers relative to proneural tumors. Interestingly, the results of these studies indicate that recurrent tumors initially displaying proneural or proliferative markers tend to shift towards the mesenchymal phenotype. Recurrent tumors also commonly displayed up-regulation of YKL-40, CD44, STAT3 and vimentin, each of which are markers of the mesenchymal-angiogenic phenotype.[20] As a result of these studies, a set of genes representing both the proneural and mesenchymal GBM subtypes have been identified, allowing for the development of a post-resection clinical test for predicting patient outcome.[14]

More recent high-throughput studies conducted by Verhaak and colleagues[30], expanded on the aforementioned classifications by molecularly stratifying GBM on the basis of genomic profile into four major subtypes: proneural, neural, classical and mesenchymal. DNA copy number and mutation patterns were additionally integrated into their analyses. The proneural subtype displayed aberrations in PDGFRA/IDH1, whereas the mesenchymal and classical subtypes predominantly exhibited NF1 and EGFR aberrations, respectively. Amplification of EGFR was present in upwards of 95% of the classical subtype and in at least 29% of the mesenchymal subtype. With the exception of tumors defined as being proneural, EGFR copy number increases were observed in >86% of all GBM subclasses. These seminal findings could

potentially lead to the identification of prognostic markers indicative of treatment response, allowing for the exploration of optimized therapies for the purpose of individualizing treatment in this deadly disease.[17] Despite this, emerging reports indicate that a population of GBM-derived stem cells significantly contribute to resistant phenotypes in GBM and show no clear correlative patterns with respect to the established classification system. Thus a refined sub-classification integrating GBM-derived stem cell signatures is needed to better utilize molecular and clinical correlates in the treatment of GBM.

1.2.4 GBM Stem Cells

The cancer stem cell hypothesis posits that only a small portion of tumor cells harbor the capacity for self-renewal and tumor-initiation.[31] Accumulating evidence suggests that primary GBM tumors contain such a population of cancer stem cells, referred to as GBM stem cells or GSCs.[32,33] Like neural stem cells, GSCs express CD133 (prominin) and possess the ability to self-renew. In contrast, GSCs are highly refractory to chemotherapy and radiotherapy and therefore culpable in tumor progression and recurrence following conventional GBM therapy.[33] Additionally, recent findings indicate that GSCs more accurately recapitulate the genotype, gene expression profile, and in vivo biology of human GBM when engrafted in nude mice.[34] Interestingly, recent reports indicate that EGFRΔIII is commonly expressed in GSCs, where its expression is associated with enhanced stemness as well as resistance to therapies, including EGFR-targeted modalities.[35,36] These studies determined that EGFRΔIII is preferentially

expressed with CD133 in a subset of human tumor cells. Here, it was demonstrated that EGFRΔIII^{+}/CD133^{+} and EGFRΔIII^{+}/CD133^{-} cells exhibited the greatest potential for self-renewal relative to EGFRΔIII^{-}/CD133^{+} populations. Furthermore, the presence of EGFRΔIII significantly enhanced tumor formation in NOD/SCID mice, highlighting the relevance of EGFRΔIII expression in this setting. These findings, coupled with the frequency of EGFR alterations identified by the TCGA analysis[25], highlight the therapeutic potential of targeting EGFR signaling in GBM and will, therefore, be the focus of the following section.

1.3 EGFR

EGFR, also referred to as HER1/ErbB1, belongs to a larger family of ErbB receptors with tyrosine kinase activity.[37] Other members of the HER family include ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4. EGFR is frequently overexpressed and/or hyper-activated in human malignancies, including GBM, and therefore EGFR-directed therapeutic strategies are often utilized.[16] Increased activation of EGFR can occur through a variety of different mechanisms, both ligand-dependent and ligand-independent.[3,38-40] Among these mechanisms include: aberrant enhancement of ligand production[39]; constitutive receptor activation by multiple exon deletion or missense mutations[40,41]; crosstalk with other receptors[42]; increased receptor protein level via gene amplification[3]; and malfunction in receptor degradation.[38] As shown in Figure 1, EGFR overexpression and activation are known to significantly impact cancer cell hallmark traits, such as increased cell survival, proliferation and invasion.[40,43,44]

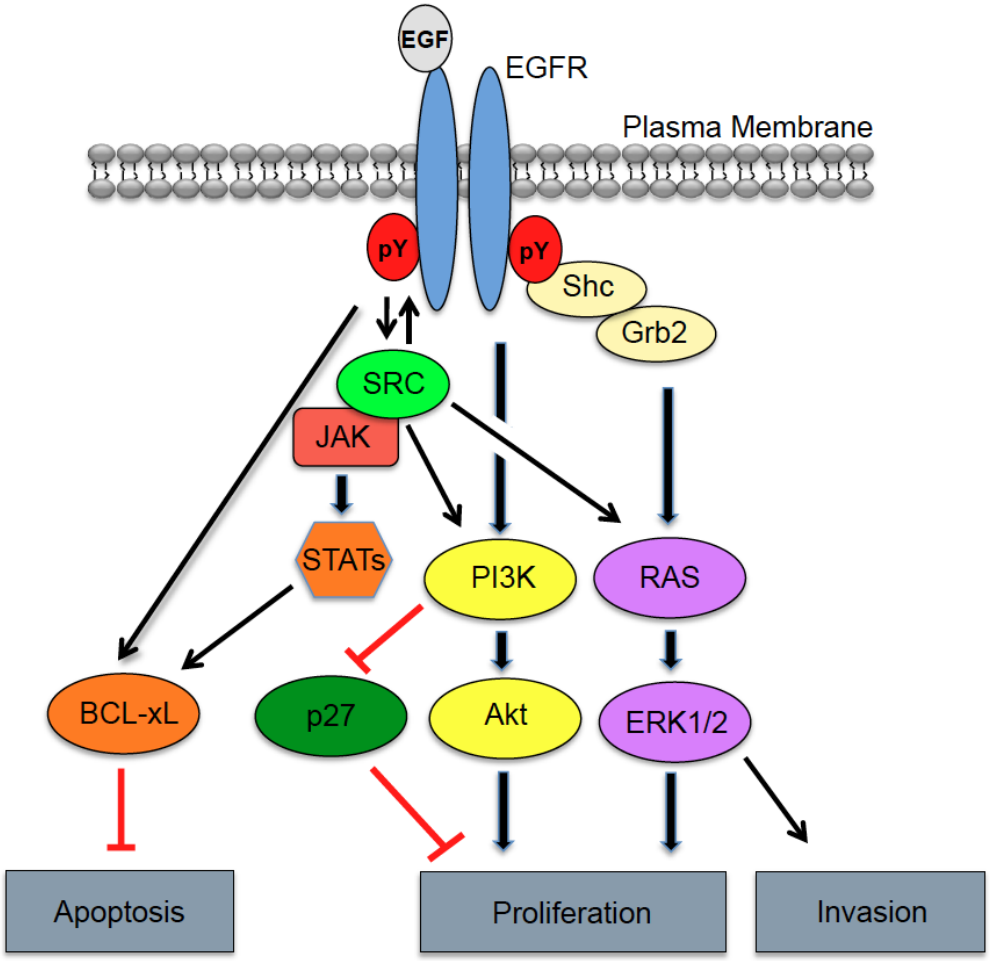

Figure 1. EGFR signaling and related pathways in cancer. Illustration of the gene products involved in transferring signals from the outside of the cell to the nucleus mediated by EGFR, notably through SRC.

1.3.1 Structure

Structurally, EGFR is a 170 KDa glycosylated plasma membrane protein containing three main domains: an extracellular domain, a transmembrane domain and an intracellular domain.[45] The extracellular domain is comprised of four subdomains, where the ligand binding pocket is formed by domains I, II and III, which interact with cognate ligands.[46] The intracellular domain, or tyrosine kinase domain, contains a c-terminal tail with multiple tyrosine residues capable of being autophosphorylated upon receptor dimerization and activation.[47] Canonical activation of EGFR can occur through seven different ligands including: epidermal growth factor (EGF), transforming growth factor-α (TGF- α), heparin-binding EGF-like growth factor (HB- EGF), amphiregulin, epiregulin, betacellulin and epigen.[48] Ligand binding precedes EGFR homo- or heterodimerization with other ErbB family members, dictating autophosphorylation of various preferential tyrosine residues in the cytoplasmic domain.[49,50] Importantly, site-specific tyrosine phosphorylation plays an essential role in determining the selectivity of downstream substrates triggered by EGFR and facilitates binding of adaptor or other signaling molecules.[51] The preferred autophosphorylation site of wild-type EGFR is tyrosine 1173 (Y1173).[3] Tyrosine residues Y1068 and Y1148 are also indicative of receptor activation.[47] The tyrosine residue Y1045 is a c-Cbl binding site, functioning to ubiquitinate and down-regulate the receptor following ligand stimulation.[52] Tyrosine site Y845 is phosphorylated by Src family non-receptor tyrosine kinases (SFKs) following their activation by the EGFR.[53]

1.3.2 EGFR Alterations in GBM

EGFR is overexpressed in approximately 60% of primary GBMs versus only 10% of secondary GBMs and is characteristic of more aggressive GBM phenotypes.[8] In addition to overexpression, several alternative mechanisms account for aberrant induction of EGFR activation in GBM, including enhanced autocrine expression of cognate ligands.[47] Gene amplification and mutation of EGFR also enhance EGFR activation and occur in upwards of 57% of GBMs as determined by the TCGA dataset.[25] From a subtype perspective, classical GBM are synonymous with focal amplification of EGFR (~95%), whereas mesenchymal, neural and proneural GBMs are associated with reduced rates of EGFR amplification at 29%, 67% and 17%, respectively.[30] Mutations of EGFR occur in roughly one-third of all classical tumors and often in mesenchymal, proneural and neural GBMs as well.[30] Of these mutations, extracellular domain EGFR mutations are most commonly observed in GBM.[8]

The most frequently occurring EGFR mutation in GBM, EGFRΔIII, arises from an in-frame deletion of 801 bp in the DNA sequence encoding the extracellular domain, rendering a truncated yet constitutively active form of the receptor.[41,44] EGFRΔIII is a cancer specific mutation, as it not detected in normal tissues, making it an attractive target for therapeutic intervention.[54] Several different studies have indicated that EGFRΔIII is expressed in roughly 50% of GBMs that amplify wild-type EGFR.[54-56] Additionally, data mined from the TCGA indicates that EGFRΔIII is most commonly present in the classical tumors (23%), where EGFR amplification is most prevalent.[30]

Despite being constitutively active, EGFRΔIII sustains a low-level signal capable of evading internalization and down-regulation, which primarily result from inefficient dimerization.[50,57] In contrast, wild-type EGFR is rapidly degraded following acute stimulation with ligand.[58] Though low-level in nature, constitutive signaling downstream of EGFRΔIII leads to increased GBM cell survival in vivo through selective augmentation of various mitogenic factors, namely Akt and repression of apoptosis via enhanced Bcl2 family member expression.[59] EGFRΔIII has also been associated with transformative properties, as INK4A/Arf depleted astrocytes and neural stem cells form high grade tumors *in vivo* when expressing EGFRΔIII.[60] Given this, EGFRΔIII may act as a critical initiating event in tumor development. Not only is EGFRΔIII likely an important factor in gliomagenesis, but the tumorigenic potential of glioma cells *in vivo* are significantly increased by EGFRΔIII expression when compared to xenografts expressing the wild-type EGFR.[61,62] Studies have also shown that EGFRΔIII-expressing GBM cells are highly resistant to both chemotherapy[59] and radiation.[63] Interestingly, recent reports indicate that co-expression of EGFRΔIII and the GSC marker $CD133^{+}$ defines a population of GSCs harboring the greatest tumor-initiating ability, thus further defining its importance in GBM.[35,36] Taken together, it's not surprising that EGFRΔIII expression has been strongly associated with a poor survival prognosis for patients whose tumors amplify EGFR. [56,64]

In addition to EGFRΔIII, sequence analysis of the EGFR coding region in a cohort of 151 GBM tumor and cell lines identified a number of novel

ectodomain missense mutations.[65] Approximately 14% of GBM patient samples and 13% of GBM cell lines displayed this form of mutation. Using missense mutants encoding R108K, T263P, A289V, G598V, and L861Q it was determined that these mutations were 1) hyper-phosphorylated receptor in the absence of ligand; 2) accompanied by an increased EGFR gene dosage; and 3) exhibited a stronger transforming phenotype relative to wild-type EGFR as determined by anchorage-independent growth in NIH-3T3 cells.[65] Importantly, of the missense mutations evaluated, EGFR-R108K shares the greatest degree of signaling and behavioral homology to EGFRΔIII, particularly as it relates to therapeutic resistance.[65,66]

1.3.3 EGFR Therapies in GBM

Overexpression of EGFR has been noted in multiple epithelial tumors, supporting the notion that deregulated EGFR expression and signaling are pivotal events in the origin of human cancers.[67,68] This led to the development of multiple inhibitors of EGFR, including EGFR-targeted monoclonal antibodies (mAB) such as mAB C225 [69] and mAB 528.[69,70] Mechanistically, EGFR-directed mAbs compete with cognate ligands for binding, effectively down-regulating receptor expression and leading to inhibition of cell growth by induction of cell cycle arrest.[71] Initially, mAB C225, dubbed cetuximab, demonstrated promising anti-tumor effects in cell cultures and xenograft models, leading to its implementation as a therapeutic agent.[72] Since, cetuximab has been approved for use in metastatic colorectal cancer (CRC) as well as squamous cell

carcinoma of the head and neck (HNSCC).[73,74] Cetuximab has additionally been under evaluation in progressive non-small cell lung cancer (NSCLS), where activating mutations of EGFR commonly occur.[75] Notably, preclinical studies in GBM cell cultures and mouse models have demonstrated the anti-tumor and radio-sensitizing effects of cetuximab in this setting.[76] Preclinical data also suggests that cetuximab is active against EGFRΔIII, where it binds to and engenders receptor internalization, rendering a reduction in kinase activation.[77] Though cetuximab has displayed promising effects in clinical trials involving CRC, HNSCC and NSCLC, phase I/II trials in patients with recurrent GBM have failed to confer any efficacious advantages over standard of care regimens.[78] Insufficient intratumoral accumulation of cetuximab was cited in the failed inhibition of EGFR autophosphorylation and degradation in these studies.

Small molecule tyrosine kinase inhibitors (TKIs) that competitively target receptor catalytic activity via the EGFR kinase domain adenosine triphosphate (ATP)-binding pocket, present another approach to targeting EGFR.[79] Despite being low in molecular weight and more likely to penetrate the BBB, the specificity of these inhibitors is diminished by the fact that the EGFR ATP-binding pocket shares homology with that of other RTKs, resulting in off-target effects.[80] Three TKIs of EGFR (gefitinib, erlotinib and lapatinib) have previously received regulatory approval for use in NSCLC and breast cancer.[81] In contrast, several phase II clinical trials evaluating gefitinib, erlotinib or lapatinib in newly diagnosed or recurrent GBM have yielded minimal clinical activity as either a monotherapy or in combination regimens.[82-84] These lack of clinical effects were attributed to

insufficient inhibition of Akt activation, which correlated most strongly with EGFRΔIII expression and loss of PTEN. Collectively, these findings highlight the need for novel therapeutic targets capable of improving clinical responses in this deadly disease.

1.3.4 Dysregulated EGFR Signaling Networks in GBM

The EGFR family is a complex system involved in growth factor cellular signaling.[85] Phosphorylation of EGFR at the plasma membrane leads to the recruitment of multiple effector proteins via recognition and binding of Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains to phosphotyrosine motifs on the receptor.[86] Formation of the EGFR signaling complex, in turn, triggers a variety signaling cascades involved in tumor cell proliferation, angiogenesis, motility, differentiation, and survival (Fig. 1).[87] Interestingly, similar substrates are activated downstream of EGFR and EGFRΔIII, but with differing levels of intensity.[50] Among these pathways include the phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription 3 (STAT3) pathways and Src family kinases (SFKs; discussed in 1.4).[50,88,89]

1.3.4.1 PI3K

The class IA PI3Ks form heterodimers that are recruited to activated RTKs and adaptor proteins via their regulatory subunit, of which five isoforms exist: p85a, p55a and p50a, or PIK3R1; p85b or PIKR2; and p55y or PIKR3.[87] p85α

associates with EGFR either through ErbB3 heterodimerization or through phosphorylation of EGFR by the SFK c-Src.[90,91] p85α association with EGFR results in a conformational change in p85α, releasing the inhibition of the catalytic subunit p110 of PI3K. PI3K then localizes to the plasma membrane, where it functions to catalyze the formation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) via the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2). The resulting PIP_3 is a critical activator of Akt, which consequently phosphorylates, or inhibits, numerous target proteins involved in regulating cellular metabolism, motility and protein synthesis.[92,93] Akt activation additionally results in phosphorylation of Bad, a Bcl family member, which when phosphorylated fails to inhibit the survival protein Bcl-Xl, thus precluding apoptotic induction.[94,95] Activation of PI3K can also arise from point mutations, of which roughly 15% have been catalogued in GBM tumors.[25] These mutations occur most commonly in the adaptor-binding domain (ABD) and less frequently in the C2 helical and kinase domains of the catalytic subunit (PIK3CA).[96,97] Though mutations in the regulatory subunit (PI3KR1) are uncommon, prior sequencing analysis from the TCGA indicated the presence of 9 such mutations occurring among a cohort of 91 GBM samples.[22] As a result, aberrant PI3K activation and subsequent activation of Akt is observed in upwards of 85% of GBM samples.[98]

PI3K signaling is negatively regulated by various proteins, most notably PTEN; PTEN, however, is commonly inactivated (~50%) in GBM by either epigenetic silencing or deletion mutation.[99] Loss of PTEN, therefore, disrupts the PI3K:PTEN balance resulting in increased Akt activation and uncontrolled cell

growth. Given the frequency of PI3K pathway aberrations occurring in GBM, inhibition of its signaling components present an attractive target for therapeutic intervention. Based on this, the rapamycin analogs, everolimus (Afinitor) and temsirolimus (Torisel), both of which inhibit mammalian target of rapamycin complex 1 (mTORC1) are regulatory-approved for treatment of advanced renal cell carcinoma and have been evaluated in GBM patients. Unfortunately, the clinical application of rapamycin analogs has yielded infrequent and short-lived responses in GBM.[93-99] Additionally, the PKC/PI3K/AKT inhibitor, enzastaurin, was the first targeted therapy for GBM evaluated in a phase III clinical trial.[100] Regrettably, enrollment of this study was halted as no positive correlations with progression free survival were observed.

1.3.4.2 MAPK

Following EGFR activation, the MAPK signaling pathway is triggered by the growth factor receptor-bound protein 2 (Grb2) binding directly to EGFR via Y1068 and Y1086 or indirectly by SHC binding Y1173 and Y1143.[101] Grb2 also houses two SH3 domains, allowing for interactions with proline-rich sequences, namely those of son of sevenless (SOS).[102] The Grb2/Shc/EGFR interaction precedes recruitment of SOS to the plasma membrane. SOS is a guanine nucleotide exchange factor, which functions to promote the conversion of Ras-GDP to the active Ras-GTP. Subsequently, Ras activates Raf, a serine-threonine protein kinase, which then phosphorylates and activates MEK1/2, resulting in activation of ERK1/2 (MAPK).[103]

1.3.4.3 Signal Transducers and Activators of Transcription

In addition to PI3K and MAPK, Signal transducers and activators of transcription (STAT) proteins are commonly activated downstream of EGFR in GBM.[87] The STAT family of proteins consists of seven members (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6), of which, STAT1, STAT3 and STAT5 are known substrates of oncogenic EGFR signaling.[89] Upon activation, STATs function to increase transcription of proteins involved in cell mitogenesis, survival, and differentiation.[104-107] In contrast to cytokine receptor-mediated activation of STATs, ligand-dependent phosphorylation of STATs by EGFR does not require Janus activated kinases (JAK).[105,108] Interestingly, STAT5b has recently been identified as a preferential substrate of EGFRΔIII signaling in GBM, capable of enhancing GBM cell survival through induction of Bcl-xL.[109] From these studies, it was determined that STAT5b activation by EGFRΔIII was mediated by SFKs, which are commonly activated mediators of dysregulated RTK signaling in GBM and will be the focus of the following section.[110]

1.4 SFKs

The prototypical SFK member, c-Src, was discovered in 1976 as a mammalian homologue of the transforming agent in avian sarcoma virus, v-Src.[111,112] This family of non-receptor protein tyrosine kinases is comprised of nine members, including c-Src, c-Yes, Fyn, Lyn, Lck, Blk, Hck, Fgr and Yrk. While the majority of SFKs are expressed in cells of hematopoietic origin, C-Src,

C-Yes and Fyn, however, display a more ubiquitous pattern of expression.[113] SFKs interact with multiple cell surface receptors, including EGFR, and are rapidly activated upon receptor engagement, serving to promote cell proliferation, viability, motility and invasiveness as depicted in figure 1.[113] Though activating mutations and genomic amplification of SFKs rarely occur, SFKs are commonly activated and/or overexpressed in a variety of cancers, which often correlates with cancer development and progression.[114,115] Consequently, SFKs have emerged as promising targets in cancer therapy, where preclinical and clinical applications of the regulatory approved ATP-binding competitive inhibitor dasatinib have shown promise across several different tumor types.[116-117]

1.4.1 Structure and Activation

Collectively, SFKs exhibit structural homology consisting of an N- terminal membrane localization sequence, a poorly conserved unique domain, an SH3 and SH2 domain, a tyrosine kinase domain and a regulatory sequence.[118] Housed within the c-terminal tyrosine kinase domain are two phosphorylation sites, which serve as critical regulators of protein function: Y527 (Y527 in chicken c-Src; Y530 in human c-Src) and Y416. Phosphorylation on residue Y527 negatively regulates SFK activity, which is imparted by the c-terminal kinase (Csk) family of protein tyrosine kinases.[119-125] The SH2 domain of SFKs bind to Y527 following phosphorylation on this site.[126-127] As a result, this induces binding of the SH3 domain to the linker region between the SH2 and tyrosine kinase domains, precluding ATP from binding which stabilizes the closed conformation

and inactivates the protein.[128-129] Dephosphorylation on residue Y527 is accomplished by several protein tyrosine phosphatases, including CD45, SHP-1, SHP-2, PTP-α, or PTP-λ. Secondly, phosphorylation of Y416 must occur for the protein to be active. Phosphorylation of Y416 is mediated by multiple proteins, including EGFR[114], which results in repositioning of the catalytic domain and subsequent formation of a substrate binding pocket.[128-131] Phosphorylation on Y416 is sufficient to activate c-Src, even in the presence of phosphorylation at residue Y527.[132,133] Hence, dephosphorylation of Y416 is pivotal to the inactivation of SFKs, a function commonly ascribed to the tyrosine phosphatases PTP-α and PTP-λ.[134,135]

1.4.2 SFKs in GBM

Early studies identified elevated expression of SFKs occurring in neuronal cells, specifically c-Src, Fyn and c-Yes, thus generating interest in their potential role in brain and neuronal tumors.[113] Since, multiple studies have reported elevated SFK activity in GBM. Initially, Lyn was identified by immunoblot analysis of p-Src (Y418) as the most commonly activated SFK occurring in GBM patient samples.[136] In contrast, more recent high-throughput phospho-proteomic analyses identified c-Src as the most frequently activated SFKs in this context.[110] These studies additionally showed that treatment with dasatinib significantly lowered T-98 and U87-MG GBM cell proliferation and viability *in vitro*.[110] Using dasatinib resistant mutants (T341I gatekeeper mutation), this study further determined that among SFKs expressed in GBM, only mutant c-Src and Fyn

were capable of rescuing the therapeutic effects of dasatinib.[110] In corroboration with these reports, more recent studies indicated that c-Src and Fyn are preferential effector substrates of EGFRΔIII signaling in GBM cells. Here, it was demonstrated that genetic inhibition of c-Src or Fyn significantly mitigated cell motility *in vitro*. Furthermore, it was reported that dasatinib in combination with the EGFR-targeted antibody (mAB 806) significantly inhibited the growth of EGFRΔIII-expressing subcutaneous tumors in mice.[137] In contrast, additional in vivo investigations using dasatinib in mice bearing orthotopic GBM tumors cited a lack of efficacy attributed to insufficient tumor accumulation.[138] Collectively, these findings reinforce a role for dasatinib in the treatment of GBM, while also highlighting potential limitations in this regard. Regrettably, phase I/II clinical trials in patients with recurrent GBM have exhibited only minimal therapeutic activity when using dasatinib as a monotherapy or in combination with the EGFR kinase inhibitor erlotinib.[139,140] These findings highlight the need for alternative SFK-targeted strategies in GBM.

In addition to being activated, analysis of the TCGA database indicates that Fyn and Lyn mRNA are significantly up-regulated among SFKs in GBM patients samples versus normal brain tissue.[25] Fyn gene expression has also been identified as most significantly correlating with that of EGFR among SFKs in GBM patients.[137] Importantly, increased Fyn expression has additionally been observed in a highly resistant population of GSCs, where dasatinib fails to reduce GSC proliferation and survival.[141] These findings are of great interest, as targeting Fyn expression, as opposed to activation, has previously been

implicated in the inhibition of tumor growth and survival of prostate and breast cancer cells as well as CML cells.[142,143] The role and regulation of increased Fyn expression, however, remains poorly understood in GBM but could provide valuable therapeutic insight into GBM.

1.4.2.1 Fyn

Fyn is a 59-kDa protein comprised of 537 amino acids located on chromosome 6q21.[144] There are three isoforms of Fyn: isoform 1, or FynB, the canonical sequence; isoform 2, or FynT, which tends to be expressed in T-cells and differs from FynB in the linker region; and isoform 3, which is typically found in blood cells and differs from FynB by deletion of sequence 233-287.[144] Fyn, like other SFKs, is comprised of an SH1, SH2 and SH3 domain. Prior reports have linked Fyn to several physiological processes, including cell proliferation and motility.[145] Fyn also functions in pathophysiology, as it was shown to induce transformation and anchorage-independent growth in NIH 3T3 cells.[146]

Increased Fyn expression has been reported in several tumor types, including prostate cancer and CML.[142,143] While the specific function of increased Fyn expression in prostate cancer remains poorly understood, genetic inhibition of Fyn significantly reduced CML cell survival and proliferation as well as enhanced sensitivity to dasatinib.[143] Interestingly, increases in Fyn expression were determined to occur through a reactive oxygen species (ROS)-dependent transcriptional mechanism downstream in CML. Based on observations that GBM is associated with elevated levels of oxidative stress, the potential role for

ROS-dependent transcriptional up-regulation of Fyn provides a plausible hypothesis for evaluation in GBM cells. Thus, the function and mechanistic regulation of cellular oxidative stress will be discussed in the following section.

1.5 Oxidative Stress

Oxidative stress by definition is an imbalance of the cellular pro-oxidant to anti-oxidant ratio. The resulting imbalance is characterized by an increase in reactive oxygen species (ROS). ROS are essential components of the cellular redox system, where they serve multiple roles in both physiological and pathophysiological states.[147] Collectively, ROS are a group of exceedingly reactive oxygen-containing species, which includes singlet oxygen ($^1O^2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl free radical ($^{\cdot}OH$).[148] Cells have developed a variety of ROS defense mechanisms, which include both enzymatic and non-enzymatic antioxidants. Examples of non-enzymatic antioxidants include glutathione (GSH) and thioredoxin[149,150]; among the known enzymatic antioxidants include cytoplasmic, SOD1, the mitochondrial Mn-dependent, SOD2, and the Cu/Zn- dependent, SOD3 (Fig. 3).[151] Once GSH is oxidized, the reduced form can be regenerated via GSH reductase activity (Fig. 4). The resulting balance between GSH and GSSG can be useful in determining the redox state within the cell.

1.5.1 ROS in Cancer Cell Signaling

Steady state increases in oxidative stress have previously been implicated

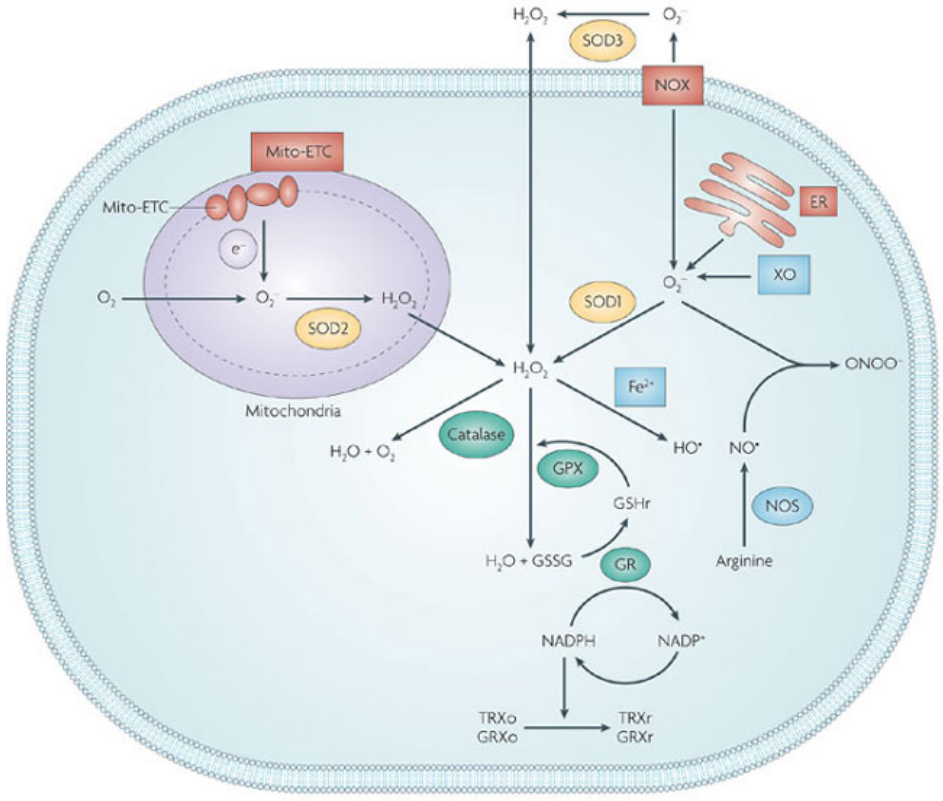

Figure 2. Cellular redox homeostasis. The mitochondrial electron transport chain (Mito-ETC), endoplasmic reticulum (ER) and Nox complex serve as sites of cellular ROS production. GPX, glutathione peroxidase; GR, glutathione reductase; GRXo, glutaredoxin (oxidized); GRXr, glutaredoxin (reduced); GSHr, glutathione (reduced); GSSG, glutathione (oxidized); TRXo, thioredoxin (oxidized); TRXr, thioredoxin (reduced); XO, xanthine oxidase.

Dunyaporn Trachootham, Jerome Alexandre, and Peng Huang. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nature Reviews Drug Discovery* **2009**, *8*, 579-591.
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in the malignant phenotype of cancer.[152] While classically perceived as cytotoxic and mutagenic metabolites, increased ROS have also been implicated as mediators of tumor cell growth and survival through activation of various signal transduction pathways.[152] Specifically, ROS inactivate protein-tyrosine phosphatases via oxidation of the catalytic cysteine, thus impairing kinase de-activation of key signaling intermediates such as MAPK, JAK/STAT and Akt.[153] This, in turn, engenders the induction of various pro-growth and pro-survival transcription factors, including nuclear factor-κB (NF-κB).[154] Recent studies from our lab have identified an additional transcription factor induced by ROS: early growth response-1, or Egr-1.[155] From these studies, Egr-1 was shown to increase CML cell growth and survival through increased transcriptional induction of Fyn.

1.5.1.1 Egr-1

The Egr-1 gene product is a zinc-finger transcription factor of 59 kDa that uniquely activates transcription by binding DNA as a monomer.[156] Based on cellular context, Egr-1 behaves either as a tumor suppressor or oncogene.[157-159] Importantly, increased Egr-1 expression is reported to promote tumor development and progression in both breast and prostate cancer.[159,160] Besides being transcriptionally induced by ROS, Egr-1 up-regulation has previously been determined to occur through a mechanism involving MAPK.[161] Additionally, recent gene expression profiling identified Egr-1 as the most significantly up-regulated gene in EGFRΔIII-overexpressing HEK293 cells versus vector

controls.[162] Furthermore, analysis of the TCGA database reveals that Egr-1 mRNA is significantly up-regulated in GBM versus normal brain tissue.[25] Together, these findings suggest that increased Egr-1 expression could provide a plausible mechanistic explanation for Fyn up-regulation downstream of EGFR and/or ROS-dependent signaling in GBM.

1.5.2 Cellular Sources of ROS

Otto Warburg first hypothesized that increases in cellular oxidative stress stem from alterations in mitochondrial metabolism.[163] Besides the mitochondria, additional cellular sources of ROS include: fatty acyl-CoA oxidase, xanthine oxidase, cyclooxygenases, cytochrome p450, lipoxygenases, the endoplasmic reticulum (ER) and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, or Nox (Fig. 3)[164-167] The Nox complex of enzymes are the most extensively studied ROS producing enzymes in malignancy[168], including GBM, and will be the focus of the following discussion in this regard.

1.5.2.1 Nox Complex

The Nox complex family of enzymes is comprised of seven members: Nox 1–5 as well as two dual oxidases (DUOXs), DUOX 1 and DUOX 2.[169] Each respective enzyme shares highly conserved features: a C-terminal dehydrogenase domain containing binding sites for FAD and NADPH; and an N-terminal transmembrane region comprised of six alpha-helical domains.[170] Enzymatically, Nox family members function solely to produce oxidative bursts at

the expense of NADPH.[169]

The regulation of these enzymes is complex, with several members displaying intricacy with respect to subunit composition, cellular location and tissue-specific expression pattern.[169] Such compositional complexity allows for effective and selective targeting of Nox enzymes in pathology, particularly in cancer where Nox-2 and Nox-4 have been thoroughly described (Fig. 3).[170]

The classical Nox (Nox-2) in phagocytes was the first family member to be described.[171] Structurally, Nox-2 consists of two membrane-bound elements, gp91phox and p22phox as well as four cytosolic proteins, p40phox, p47phox, p67phox and Rac1 (a small G-protein) (Fig. 3).[172] Housed within the C-terminal cytosolic region is a flavoprotein domain, or NADPH binding site.[168,173] In the resting state, the SH3 domains of the integral organizer complex, p47phox, bind the auto-inhibitory region (AIR) in the C-terminal, precluding physical association with p22phox. Upon stimulation, serine residues of p47phox, namely Ser345, are phosphorylated thus associating with p22phox and localization to the cell membrane.[170] Activating phosphorylation of p47phox has previously been described downstream of JAK2 via ERK1/2 in myeloproliferative disorders, where it functions to increase enhance ROS production and cell proliferation.[174] Akt has also been linked to p47phox activation, notably downstream of EGF stimulation, where it enhances colon cancer cell proliferation and survival.[175] Importantly, recent studies in pediatric GBM have shown demonstrated that p47phox is expressed, where its genetic inhibition effectively reduces ROS.[176]

In contrast to Nox-2, Nox-4 activation only requires p22phox, is

constitutively activated and regulated primarily at the transcriptional level. [177,178] Nox-4 is highly expressed in pancreatic cancer, NSCLC and GBM cells, where elevated expression and ROS production have been implicated in increased cell proliferation, survival and migration.[179-181] Though chemical inhibitors of the Nox complex are available, such as apocynin and diphenyleneiodonium (DPI), they generally target all flavonoid proteins.[168] Therefore, more specifically targeting Nox-activation through their unique subunit composition represents a plausible and attractive therapeutic alternative to lowering Nox-induced ROS production.

Multiple reports have identified elevated oxidative stress as a growth and survival-promoting factor in GBM.[180-182] More recent studies have indicated that EGFR further elevates the redox state of GBM.[183,184] Based on prior observations linking EGFR to Nox enzyme expression and activation, studies evaluating their relationship and potential link to increased Fyn expression provide an attractive topic for investigation in GBM.

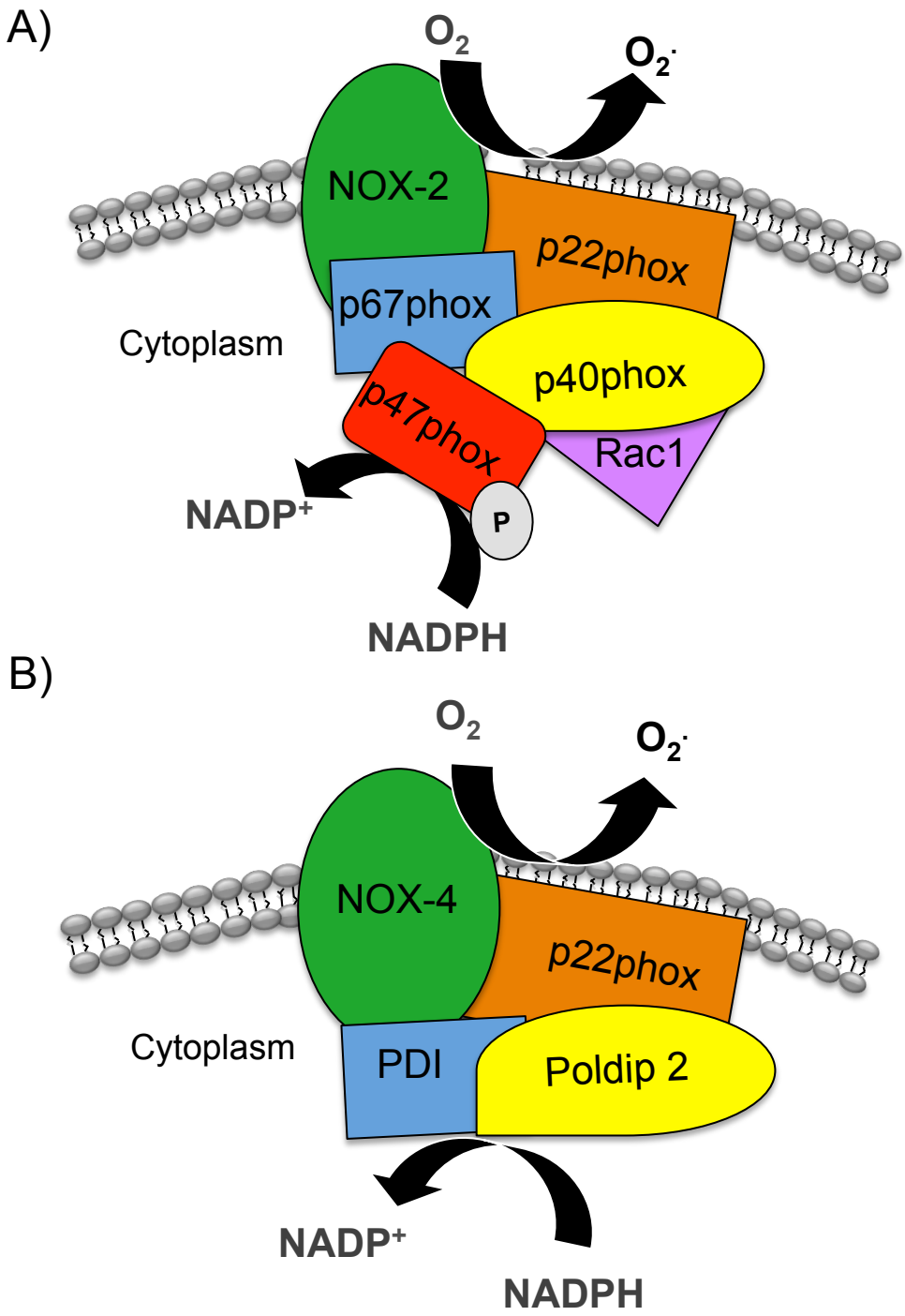


Figure 3. Subunit composition of Nox-2 and Nox-4. (A) Nox-2 is composed of a catalytic core subunit, Nox-2, as well as maturation and stabilization partners (p47phox, p67phox, p22phox) and the small GTPase, Rac1. (B) Nox-4 is far less complex, consisting of the core complex, Nox-4, as well as p22phox, PDI and Poldip2.

1.6 Hypothesis, Rationale and Significance

GBM is the most common and deadly form of primary brain tumor in adults. Amplification and activating mutations of EGFR are commonly detected genetic alterations in GBM.[25] The most frequently occurring EGFR mutation in GBM, EGFRΔIII, has previously been associated with poorer patient survival and higher rates of clinical relapse.[56] This association with poorer clinical outcomes is imparted, in part, by EGFRΔIII-induced pro-proliferative and anti-apoptotic signaling.[59,89,185,186] Regrettably, EGFR-targeted strategies have yielded limited clinical responses in GBM.[187,188] This problem generates significant interest in improving the collective understanding of downstream and/or parallel pathway activation culpable in the failed response to EGFR-targeted therapy.

SFKs are frequently activated in GBM, serving as molecular adaptors of receptor tyrosine kinase growth and survival signaling.[114,115] Interestingly, recent studies have identified Fyn and c-Src as the most relevant dasatinib targets in GBM cells, where they also serve as preferential substrates of EGFRΔIII signaling.[110,137] Though inhibitors of SFKs have enjoyed clinical success against multiple tumor types[189], early-phase clinical trials in GBM have exhibited only minimal therapeutic activity when used alone or in combination with the EGFR kinase inhibitor erlotinib.[139,140] These findings highlight the need for alternative SFK-targeted strategies in GBM. In addition to being activated, analysis of the TCGA database reveals that Fyn expression, but not c-Src, is significantly up-regulated GBM patient samples versus normal brain controls.[25] Individual reports have additionally demonstrated increased Fyn expression to occur in a highly

chemo- and radio-resistant population of GSCs.[141] The role and regulation of Fyn overexpression in GBM, however, remain poorly understood, but are important as prior studies in prostate cancer and CML have identified Fyn overexpression as a pro-proliferative and pro-survival factor.[142,143] Interestingly, the mechanism responsible for increased Fyn expression in CML was determined to involve the transcription factor Egr-1, whose expression is also significantly elevated in GBM patient samples, though its function remains unknown.[25,190] Therefore, a better understanding of the mechanism and contribution of increased Fyn expression may lead to a novel therapeutic strategy in GBM, specifically in tumors with acquired resistance to EGFR and/or SFK inhibitors.

The goal of this Ph.D. dissertation is to investigate the role and regulation of increased Fyn expression with regard to pro-proliferative and pro-survival signaling in EGFR-overexpressing GBM. A less studied but intriguing feature of EGFR is its ability to elevate intracellular ROS through increased expression and activation of Nox enzymes.[170] While classically perceived as cytotoxic and mutagenic metabolites, ROS have also been implicated as mediators of tumor cell growth and survival through activation of various signal transduction pathways.[152] Importantly, Fyn transcription has previously been described as being redox-responsive, particularly downstream of ROS-dependent induction of Egr-1.[155,191] The relationship between EGFR and Nox enzymes, including their potential link to increased Fyn expression, however, remain undetermined in GBM. The hypothesis tested in this dissertation is that **EGFR induces Fyn expression via Nox-mediated redox up-regulation of Egr-1 leading to**

increased GBM cell proliferation and survival. To test this hypothesis, we first determined if EGFR induces Fyn expression in GBM cells and the effects of Fyn inhibition on cell proliferation and survival in this context. These studies were extended to a population of GSCs, where we determined Fyn expression levels and the effect of Fyn inhibition on sphere-forming capacity. Additionally, we evaluated whether the transcription factor Egr-1 was up-regulated by EGFR-overexpression in GBM cells, and if Egr-1 was capable of modulating Fyn expression in this setting. Secondly, we determined if Nox enzymes are involved in the expression of Fyn and the effect of redox-targeted strategies on cell proliferation and survival in EGFR-overexpressing GBM. The work in this dissertation has led to a better understanding of how Fyn expression is regulated in GBM as well as furthered our understanding of Fyn overexpression in EGFR-driven pro-proliferative and survival signaling. The findings presented here have also provided novel insight into role of Fyn expression in a population of refractory GSCs, which are frequently cited in tumor relapse.[32,192] Furthermore, this work has also led to a better mechanistic understanding of how EGFR impacts Nox-mediated ROS production in GBM, thus highlighting the potential for redox-targeted strategies in this deadly disease.

Chapter 2

MATERIALS AND METHODS

2.1 Cell Culture

The human GBM cell line U87-MG stably overexpressing wild-type EGFR, EGFRΔIII or the missense mutation R108K was a kind gift of Dr. Oliver Bogler (UT M.D. Anderson Cancer Center, USA). Cells were cultured in DMEM/F12 containing 10% FBS/2 mM glutamine/100 units/ml penicillin/100 mg/ml streptomycin in 95% air/ 7% CO_2 at 37°C. The cells were routinely maintained in DMEM/F12 growth medium supplemented with 50 μg/ml of Zeocin (Life Technologies, Carlsbad, CA). Patient-derived GSCs were kindly provided by Dr. Frederick Lang (UT M.D. Anderson Cancer Center). GSCs were isolated as previously described[193] and cultured in DMEM/F12 containing 20 ng/mL human recombinant human (hr) EGF (Sigma, St. Louis, MO) and bFGF (Life Technologies).

2.2 Chemicals and Antibodies

N-acetylcysteine (NAC), apocynin, DPI, rotenone and hrEGF were purchased from Sigma. Lapatinib ditosylate was purchased from LC laboratories (Woburn, MA). The antibodies used in this study were obtained from the following sources: Fyn, Egr-1, phospho-Src (Y416) and phospho-EGFR (Tyr1068 and Tyr1173), Cell Signaling Technology (Danvers, MA); EGFR, Lyn and p47phox, Santa Cruz Biotechnology; and β-actin, Sigma. HRP conjugated secondary antibodies (anti-rabbit) were purchased from Sigma.

2.3 Immunoblot Analysis

Protein expression was evaluated in total cell lysates that were prepared using Triton X-100 buffer (PBS with 1% Triton X-100; 25 mM Tris, pH 7.5; and 150 mM NaCl) containing phosphatase and protease inhibitors (Roche, Indianapolis, IN). Resulting lysates were boiled in 5X SDS AT 100° for 5 minutes and subsequently resolved by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel in 1X running buffer for 1 hour at 120 volts. Resolved proteins were then transferred to PVDF membranes for 1 hour at 100 volts (Bio-Rad, Hercules California). Membranes were washed in 1X TBST buffer (0.1% Tween 20, 20 mM Tris base (pH 7.6), 136 mM NaCl and 0.38 mM HCL) and blocked in 5% milk for 1 hour at room temperature. Proteins were detected by immunoblot analysis using 1:1000 concentrations of primary antibodies. Immunoreactive bands were detected using enhanced chemiluminescence (GE Healthcare, Waukesha, WI). Resulting protein levels were quantified by densitometry using ImageJ (National Institutes of Health, Bethesda, MD).

2.4 Real-time Polymerase Chain Reaction

Total RNA was purified using an RNeasy Mini Kit (QIAGEN, Valencia, CA). Reverse transcription reaction was performed for each sample using 1 µg RNA with Omniscript RT kit (QIAGEN) per the manufacturer's protocol. Real-time PCR was carried using the iTaq Universal SYBR Green PCR master mix in a 20 µL total volume containing the following: 1 µL cDNA (1:5 dilution), 10 µL 2x SYBR Green PCR master mix, and 1 µL of 10 µM forward and reverse primers,

respectively. The PCR primer sequences for human Nox-4 were previously described[194] and are as follows: forward 5'- CTCAGCGGAATCAATCAGCTGTG and reverse 5'-AGAGGAACACGACAATCAGCCTTAG; Fyn: forward, 5'-CTGGTCACCAAAGGAAGAGTGC and reverse, 5'-GGTCCTTTTTCAGCAGTGGATC; p47phox as previously described[195]: forward 5'- AGTCCTGACGAGACGAAGA and reverse 5'-GGACGGAAAGTAGCCTGTGA; and β-actin: forward, 5′-CTGTGGCATCCACGAAACTA-3' and reverse 5'-CGCTCAGGAGGAGCAATG-3'. For Fyn, p47phox and β-actin, PCR conditions included 40 cycles with an annealing temperature of 57°C, and for Nox-4 60 cycles with an annealing temperature of 55°C. Relative gene expression was calculated by determination of the cycle threshold (Ct) value and normalizing to actin Ct values. Samples were analyzed in duplicate/triplicate. All experiments were repeated a total of three times.

2.5 Transfection

Transfection with short interfering ribonucleic acids (siRNAs) was performed using RNAiMAX (Life Technologies) according to the manufacturer's instructions. Briefly, U87-EGFR expressing cells were cultured to 60% confluence in 6-well plates and transfected with Fyn, EGFR, Egr-1, p47phox or non-specific scrambled control siRNA at a final concentration of 30 pmol. Fyn EGFR and p47phox siRNA were purchased from Santa Cruz Biotechnology. Egr-1 siRNA was purchased from ThermoScientific (Pittsburgh, PA). Fyn and EGFR

were single siRNA sequences. Egr-1 and p47phox were pools of 3 and 4 siRNA sequences, respectively. Knockdown was evaluated by immunoblotting as previously described.

2.6 Cellular Viability Analysis

Cellular viability was analyzed using the trypan blue exclusion method (1:1 dilution) and counting positive cells by hemocytometry.

2.7 Seahorse Extracellular Flux Analysis

Cellular oxygen consumption rate (OCR) was measured using the Seahorse XF96 Extracellular Flux Analyzer platform (Seahorse Bioscience, Billerica, MA, USA). OCR was measured after sequentially adding oligomycin, FCCP and rotenone, at working concentrations of 1 µg/ml, 1 µM and 1 µM, respectively. All assays were performed using a seeding density of $1x10^3$ cells/well in 200 µL DMEM in a XF96 micro plate (Seahorse Bioscience). OCR was recorded as picomoles per minute.

2.8 EGF Stimulation Conditions

U87 cell lines stably overexpressing wild-type EGFR were seeded in 100 mm plates and cultured in standard conditions. At ~60% confluence, one set of plates was serum starved overnight and the other set of plates was ligand stimulated following serum starvation using hrEGF (10 ng/ml of media for 5 minutes). Cell cultures were then washed with pre-chilled PBS and lysed in Triton

buffer. Protein lysates were obtained and analyzed by immunoblotting as previously described.

2.9 Measurement of intracellular $NADP^+$/NADPH Ratios

NADPH oxidase activity was measured by determination of intracellular $NADP^+$/NADPH ratios using an NADPH assay kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. Briefly, 2.0 X 10^5 cells/mL were treated or untreated with apocynin (apocynin, 24 hours at 100 μM) followed by freeze-thaw lysis (20 min on dry-ice, then 10 min at room temperature) in NADPH extraction buffer. To detect $NADP^+$, portions of samples were subjected to thermal decomposition by application of heat (60°C) for 30 min as per manufacturer's instructions. Samples and standards were quantified using a SpectraMax Gemini EM plate reader (Molecular Devices, Sunnyvale, CA) according to instructions and results presented as NADP/NADPH ratios.

2.10 Intracellular ROS Assessment

Intracellular superoxide levels were measured using the cell permeable dye dihydroethidium (Molecular Probes, Eugene, OR) followed by flow cytometry analysis. Briefly, cells were centrifuged and suspended in 1 mL of PBS containing 330 nM dihydroethidium followed by incubation at 37° for 30 minutes in the dark. Cells were then centrifuged, washed with PBS and re-suspended in 500 μl of PBS. Resulting fluorescence was measured on the FL-3 channel of a

FACSCalibur (BD Biosciences, Palo Alto, CA). Data were analyzed using FlowJo software (Tree Star, Version 7.6.5, Ashland, OR).

2.11 GSC Sphere-Formation Assay

Sphere formation was performed as previously described.[35] Briefly, spheres were dissociated by trypsinization and passaged through a 40 μm nylon mesh (Fisher Scientific), followed by transfection with either Fyn or non-specific scramble control siRNA (30 pmol final concentration) as previously described. Cells were then counted 24 hours later and re-plated at a density of 5,000 cells per well. Resulting spheres $\geq$ 50 μM were counted 10 days later.

2.12 Data Mining and Analysis

The Oncomine database was queried to identify alterations occurring in gene expression. Oncomine 4.5 database analysis tool is available with a subscription at http://www.oncomine.org. Selected data from the TCGA dataset[25] was compared for gene expression levels in primary GBM tumor samples relative to normal brain controls.

2.13 Statistical Analysis

Unless otherwise stated, values listed in figures are expressed as the mean ± SEM of at least three replicates. Statistical comparisons were made using GraphPad Prism 4.0 software (GraphPad Software, Inc., La Jolla, CA) by Student's t-test and one-way ANOVA (Bonferroni correction), where indicated. A

p-value of less than 0.05 was considered significant. Statistical values are as follows: * $p<0.05$; ** $p<0.01$; and *** $p<0.001$.

Chapter 3

RESULTS

EGFR INDUCES FYN EXPRESSION VIA EGR-1 LEADING TO INCREASED GBM CELL PROLIFERATION AND SURVIVAL

3.1 Introduction

Enhanced pro-proliferative and anti-apoptotic signaling are frequently cited events in the failed response to EGFR-targeted therapies in GBM.[80,109] A variety of signaling molecules are known to enhance and sustain the degree of survival signaling often observed in GBM, including SFKs.[109,115,189] In particular, the SFKs Fyn and c-Src serve as effector substrates of EGFRΔIII signaling, which are linked to poorer survival and increased clinical relapse in GBM.[137] Though Fyn and c-Src have additionally been identified as the most relevant dasatinib targets among SFKs in GBM, early-phase clinical application of dasatinib has exhibited very limited clinical efficacy in GBM.[110,139] These findings highlight the need for alternative SFK inhibitory strategies in GBM.

In addition to being activated, Fyn mRNA, but not c-Src, is significantly up-regulated in GBM patients[25], with recent reports indicating that Fyn expression most significantly correlates with that of EGFR among SFKs in GBM patient samples.[137] Furthermore, increased Fyn expression has recently been identified in a population of highly resistant GSCs, where co-expression of EGFRΔIII/CD133^{+} defines a subpopulation of cells with greatest self-renewal potential.[35,36] The role and regulation of increased Fyn expression, however, remain unknown in GBM, but are important as prior studies in prostate cancer and CML have identified Fyn overexpression as a pro-proliferative and pro-survival factor.[142,143] Though unknown in prostate cancer, Fyn induction in CML was determined to occur through a mechanism involving Egr-1: a transcription factor commonly up-regulated at the mRNA level in GBM[25], though its function

remains inadequately addressed.[25,190] Despite being poorly understood in GBM, Egr-1 is reported to promote tumor development and progression in both breast and prostate cancer, notably through a transcriptional mechanism involving EGFR signaling.[159,160] Additionally, recent gene expression profiling identified Egr-1 as the most significantly up-regulated gene in EGFRΔIII-overexpressing HEK293 cells versus vector controls.[162] Thus, an understanding of the role and regulation of increased Fyn and Egr-1 expression may represent novel therapeutic targets in GBM, particularly in tumors with acquired resistance to EGFR and/or SFK inhibitors.

Given the frequency of EGFR alterations in GBM as well as the previously identified correlation between EGFR and Fyn gene expression in GBM patient samples[25,137], we explored if EGFR could induce Fyn expression. We also evaluated Fyn inhibition with regard to cell proliferation and survival in EGFRΔIII and EGFR-R108K-overexpressing GBM, where enhanced and sustained induction of pro-proliferative and anti-apoptotic signaling contributes to EGFR-targeted resistance.[59,109] We extended these studies to include a population of GSCs[32], where we determined Fyn expression levels as well as the effect of Fyn knockdown on sphere-forming capacity. Lastly, we determined whether the transcription factor Egr-1 was regulated by EGFR and if it was capable of modulating Fyn protein expression in this context. Because EGFRΔIII expression is lost in standard cell culture[59], our studies employed an isogenic GBM cell line, U87-MG, engineered to stably overexpress wild-type EGFR (wtEGFR), EGFRΔIII

or EGFR-R108K. The single missense mutation EGFR-R108K was chosen based on its behavioral and signaling likeness to EGFRΔIII.[65]

3.2 Results

3.2.1 EGFR induces Fyn expression in GBM cells

Given the frequency of EGFR alterations in GBM as well as the previously identified correlation between EGFR and Fyn gene expression in GBM patient samples[25,137], we aimed to determine if EGFR could regulate Fyn expression in GBM cells. To test this, we evaluated Fyn expression in GBM cells by examining mRNA and protein levels using real-time PCR and immunoblot analysis in U87-MG cells stably overexpressing vector control, wtEGFR, EGFRΔIII or EGFR-R108K. First, we demonstrated that Fyn mRNA expression was significantly increased in wtEGFR-expressing cells (>2.0-fold), and to a greater extent (>3.0-fold) in EGFRΔIII and EGFR-R108k-overexpressing cells, relative to vector control (Fig. 4). These findings suggest that Fyn induction is imparted, in part, by the kinase activity of EGFR.

To determine if the changes in Fyn mRNA were also occurring at the protein level, we next evaluated Fyn by immunoblotting. Similar to the mRNA expression pattern, Fyn protein levels were increased 2.2-fold in wtEGFR and to a higher degree in EGFRΔIII (3.7-fold) and EGFR-R108K (5.2-fold) overexpressing cells relative to vector control (Fig. 5). To better understand whether the effects of EGFR overexpression were selective for Fyn, we assessed the expression of another SFK identified as significantly up-regulated in the TCGA database, Lyn.[136] EGFR expression did not increase Lyn protein expression (Fig. 5), suggesting a relatively exclusive mechanism for Fyn

induction. These results demonstrate that EGFR-overexpression elevates Fyn mRNA and protein expression, particularly in the context of mutant EGFR, which is commonly associated with therapeutic resistance and poorer clinical outcomes.[59,80] These findings prompted us to further evaluate the impact of EGFR kinase activity in the regulation of Fyn expression.

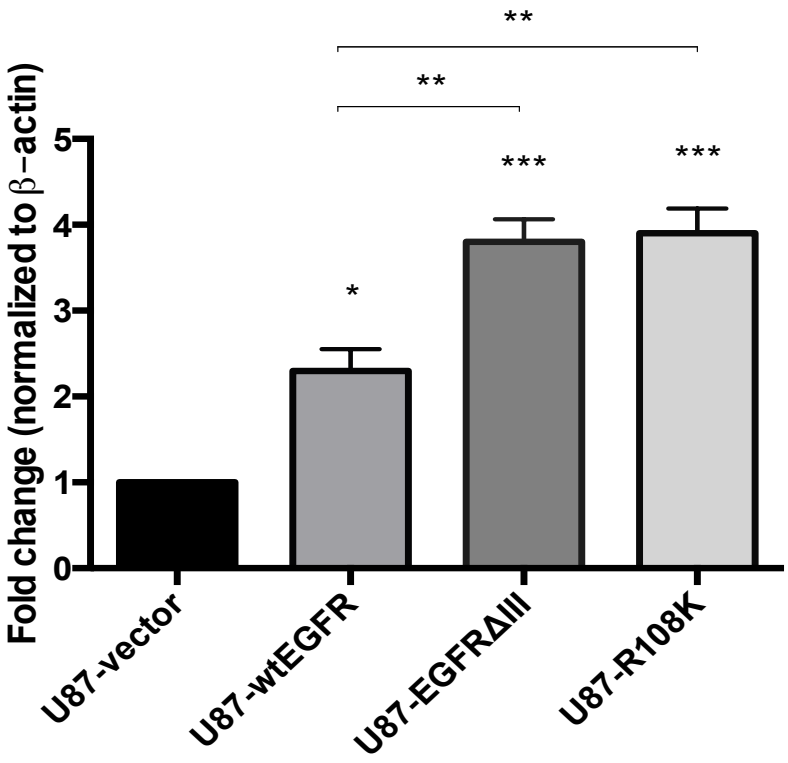


Figure 4. EGFR-overexpression increases Fyn mRNA expression in U87-MG cells. Using real-time PCR, relative Fyn mRNA levels were examined in U87-vector control, U87-wtEGFR, U87-EGFRΔIII and U87-R108K and normalized to the housekeeping gene β-actin. * $p< 0.05$, ** $p<0.01$, *** $p<0.001$; one-way ANOVA with Bonferroni's Multiple Comparison Test. Data are mean ± SEM and are representative of at least three individual experiments.

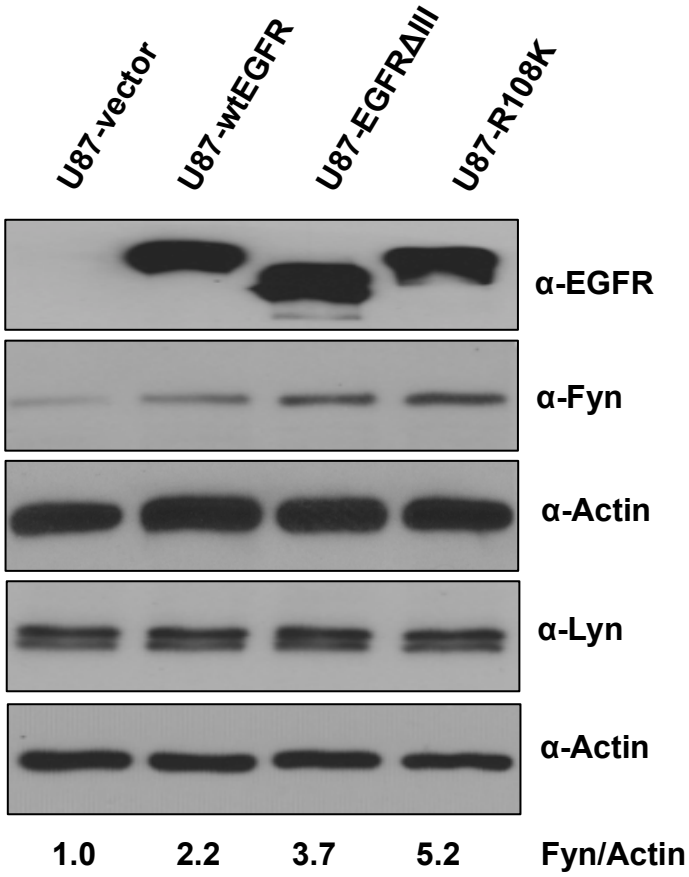


Figure 5. EGFR-overexpression up-regulates Fyn protein expression U87-MG cells. Fyn protein levels were examined in U87-MG stably overexpressing either vector control, wtEGFR, EGFRΔIII or EGFR-R108K by immunoblotting. β-actin was shown as a loading control. Relative protein levels were determined by densitometry using ImageJ. Results are representative of at least three individual experiments.

3.2.2 EGFR kinase inhibition reduces Fyn expression in GBM cells

Based on our finding that Fyn expression was more pronounced in the hyperactive U87-EGFRΔIII and U87-R108K mutants relative to U87-wtEGFR (Figs. 4, 5), we next examined the role of EGFR kinase signaling on Fyn expression. To do so, we measured Fyn protein levels in EGFR-overexpressing GBM cells following 48 hours of treatment with the regulatory approved EGFR kinase inhibitor lapatinib. Inhibition of EGFR kinase activity was validated by immunoblot analysis of EGFR phosphorylation at tyrosine residues 1068 and 1173. Figure 6 shows that lapatinib-mediated inhibition of EGFR activity significantly reduced Fyn protein levels (~50%) in wtEGFR, EGFRΔIII and EGFR-R108K-overexpressing cells.

To examine whether the inhibition of Fyn expression by lapatinib was occurring at the mRNA level, real-time PCR was conducted. Similar to the effects of lapatinib on Fyn protein expression, figure 7 demonstrates that Fyn mRNA levels were significantly reduced in wtEGFR ($p<0.001$), EGFRΔIII ($p<0.01$) and EGFR-R108K-overexpressing cells ($p<0.01$). Like many tyrosine kinase inhibitors, lapatinib exhibits off-target effects.[80] In light of this, we also evaluated the effects of lapatinib treatment in U87-vector controls, which express very low basal levels of EGFR. Fyn expression, however, was not altered in U87-vector controls treated with lapatinib (Fig. 7), thus reinforcing a specific role for EGFR kinase signaling in the regulation of Fyn expression. Together, these results

clearly demonstrate a role for EGFR kinase signaling in the up-regulation of Fyn, irrespective of EGFR mutational status.

To corroborate these findings, we next evaluated effect of EGFR knockdown on Fyn expression in EGFR-overexpressing GBM cells. Here, we demonstrated that EGFR depletion effectively reduced Fyn protein expression, but not Lyn, to a basal level not achievable by kinase inhibition by lapatinib in EGFRΔIII and EGFR-R108K-overexpressing cells (Fig. 8). Collectively, these results indicate that EGFR kinase signaling up-regulates Fyn expression in EGFR-overexpressing GBM cells, which is only partially alleviated by EGFR kinase inhibition in mutant EGFR-overexpressing GBM cells. Therefore, we next wanted to evaluate the relative contribution of Fyn expression in mutant EGFR-overexpressing cells, where cell proliferation and survival are incompletely inhibited by EGFR-directed strategies.[59,196]

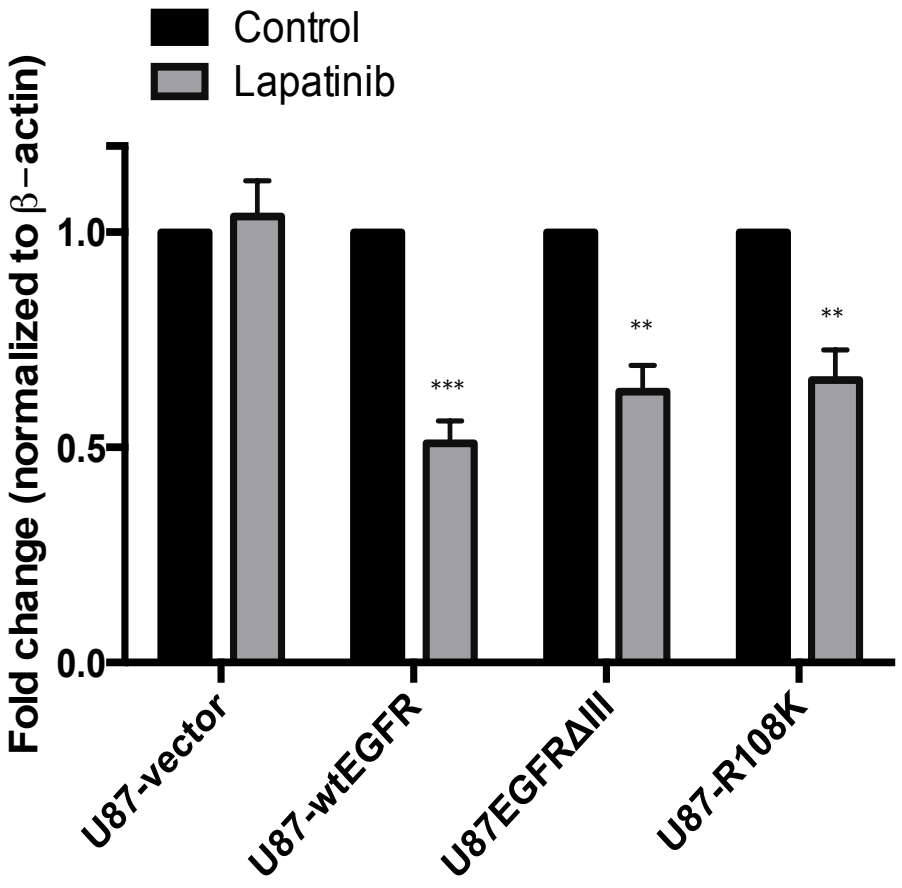


Figure 6. Inhibition of EGFR kinase signaling reduces Fyn mRNA expression in EGFR-overexpressing U87-MG cells. Vector control, wtEGFR, EGFRΔIII and EGFR-R108K-overexpressing U87-MG cells were treated with lapatinib (1 μM; 48 Hrs.) and relative Fyn mRNA levels were examined by real-time PCR and normalized to the housekeeping gene β-actin. ** $p<0.01$, *** $p<0.001$; Student's t-test. Data are mean ± SEM and are representative of at least three individual experiments.

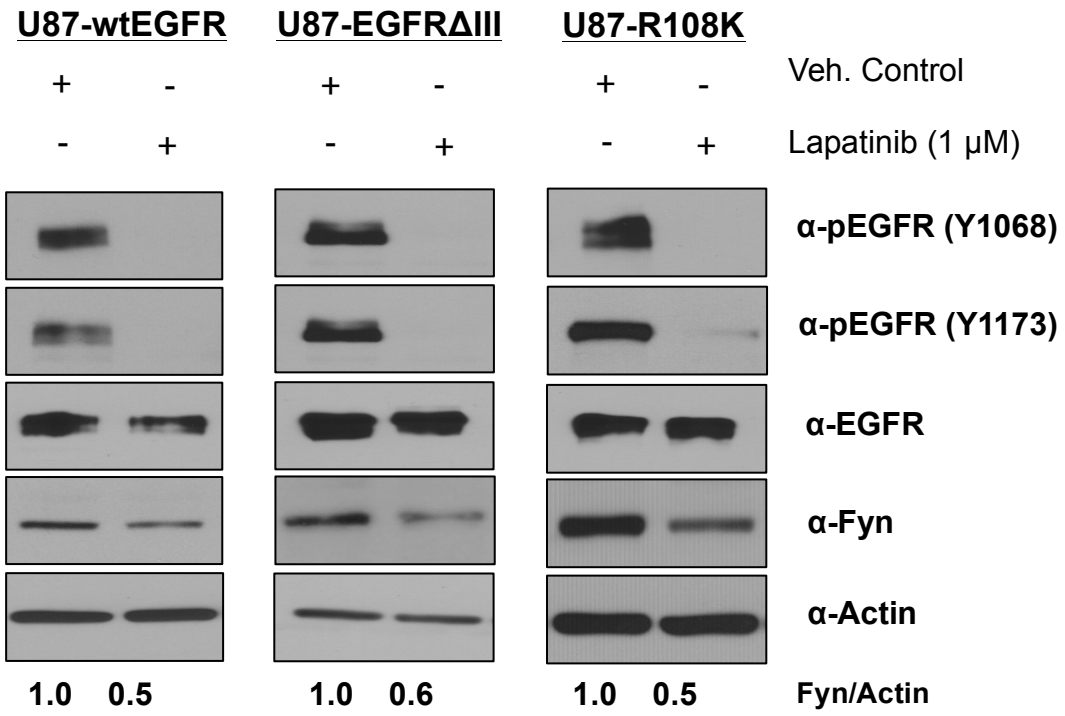


Figure 7. EGFR kinase inhibition reduces Fyn protein expression in EGFR-overexpressing U87-MG cells. Fyn protein levels were examined in U87-wtEGFR, U87-EGFRΔIII and U87-R108K cells following treatment with lapatinib (1 μM; 48 hrs.) by immunoblotting. β-actin was shown as a loading control. Relative protein levels were determined by densitometry using ImageJ. Results are representative of at least three independent experiments.

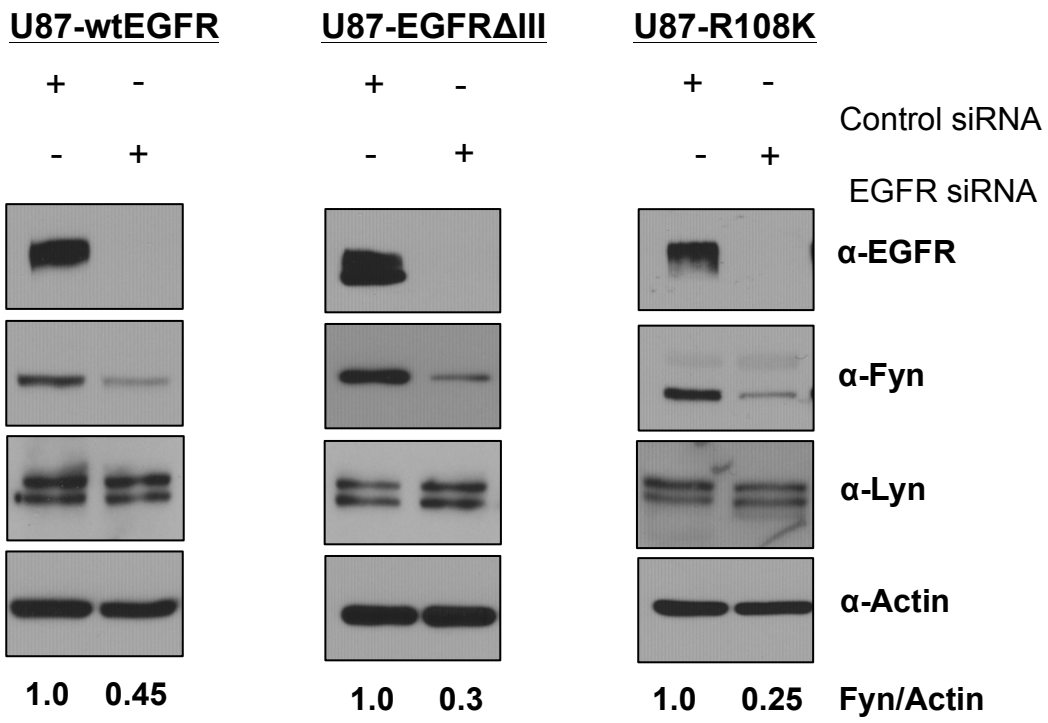


Figure 8. EGFR knockdown reduces Fyn protein expression to a basal level in EGFR-overexpressing U87-MG cells. U87-wtEGFR, U87-EGFRΔIII and U87-R108K cells were transfected with either EGFR-specific or scrambled control siRNA and EGFR, Fyn and Lyn protein levels were evaluated at 48 hours post-transfection by immunoblotting. β-actin was shown as a loading control. Relative protein levels were determined by densitometry using ImageJ. Results are representative of at least three independent experiments.

3.2.3 Fyn knockdown reduces cell proliferation and survival in mutant EGFR-overexpressing GBM cells

SFK activation is well understood to promote GBM cell proliferation and survival.[109,114,143] Moreover, recent studies have demonstrated that Fyn activation increases GBM cell motility in EGFRΔIII-overexpressing GBM cells.[137] We sought to expand on these findings by examining the effect of Fyn knockdown on cell proliferation and survival in U87-EGFRΔIII and U87-R108K, which are associated with therapeutic resistance and poorer clinical outcomes in GBM.[59,109] To deplete Fyn protein levels, we performed RNA interference-based knockdown of Fyn using siRNA. Fyn protein expression was markedly reduced in Fyn siRNA-transfected U87-EGFRΔIII (Fig. 9A) and U87-R108K (Fig. 10A) cells compared to respective scrambled control-transfected cells. Proliferation of Fyn siRNA-transfected cells versus scrambled control-transfected cells was assessed by plating equivalent cell numbers and comparing relative cell counts 48, 72, and 96 hours post-transfection. Both U87-EGFRΔIII (Fig. 9B) and U87-R108K (Fig. 10B) cells transfected with Fyn siRNA grew at a significantly (p<0.01) slower rate relative to scrambled control-transfected cells. Fyn knockdown also significantly (p<0.01) reduced cell viability in U87-EGFRΔIII and U87-R108K-expressing cells relative to scrambled controls as measured by trypan blue exclusion (Figs. 9C and 10C).

We next evaluated whether Fyn inhibition could sensitize otherwise highly resistant U87-EGFRΔIII cells to the EGFR inhibitor lapatinib. Interestingly, our results indicated that the addition of lapatinib conferred only modest effects

(p<0.05) on cell proliferation and survival when combined with Fyn knockdown (Figs. 11 A and B). Collectively, these results suggest that targeting Fyn expression is a plausible therapeutic approach in EGFRΔIII and EGFR-R108K overexpressing cells, which are associated with increased EGFR-resistance and clinical relapse rates.[80,187] To build on these findings, we were eager to evaluate the presence and functional role of Fyn expression in a population of GSCs, which are reported to more faithfully recapitulate the properties of primary tumors observed in humans.[33]

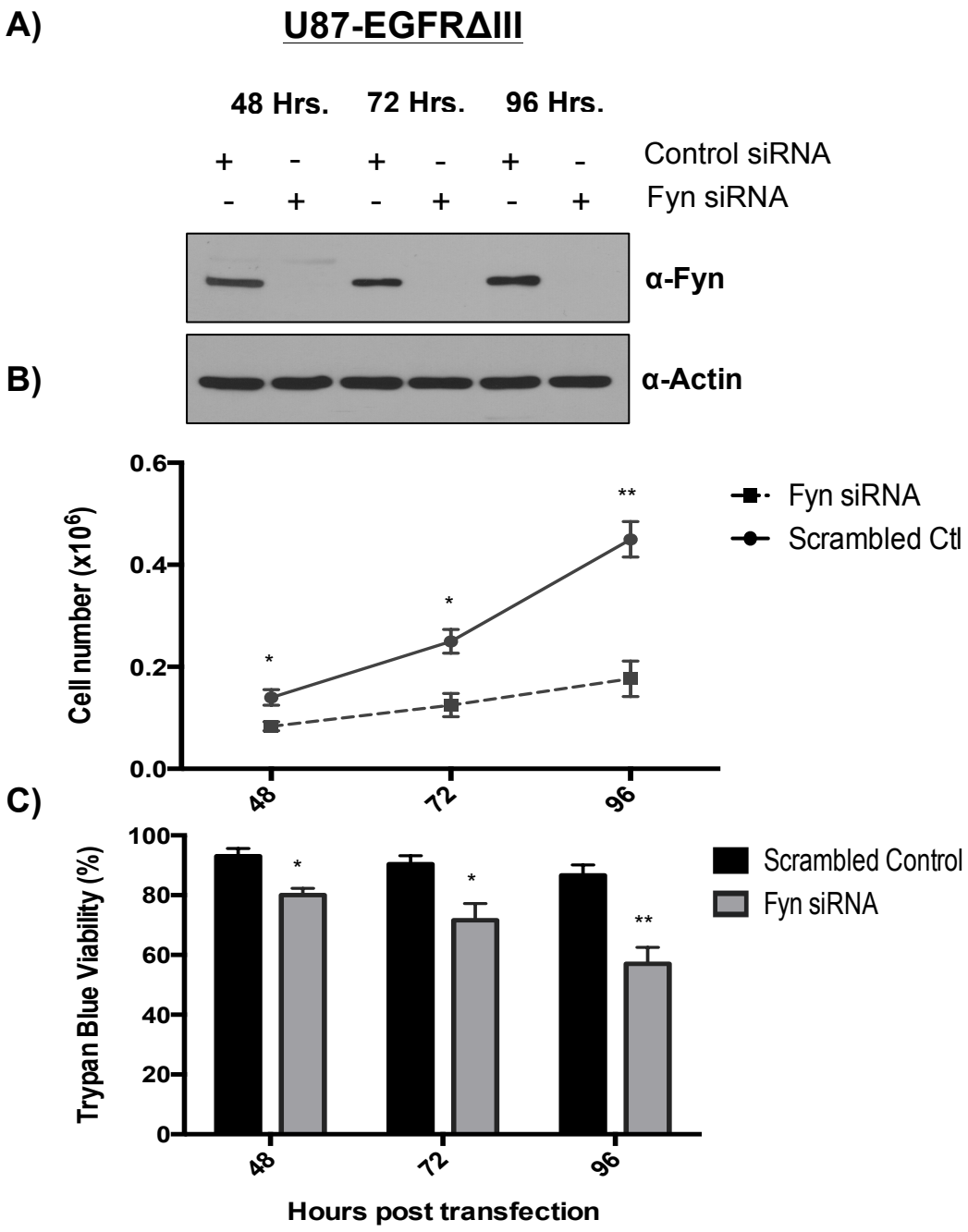


Figure 9. Fyn knockdown reduces cell survival and proliferation in EGFRΔIII-overexpressing U87-MG cells. (A) U87-EGFRΔIII cells were transfected with either Fyn specific or scrambled control siRNA and Fyn protein levels were evaluated by immunoblotting at 48, 72 and 96 hours post-transfection. (B) Resulting cell numbers (x10^6) and (C) viability were determined at 48, 72 and 96 hours by trypan blue exclusion. Percent viability was calculated compared to untreated control. * p<0.05, ** p<0.01; Student's t-test. Data are mean ± SEM and are representative of at least three individual experiments.

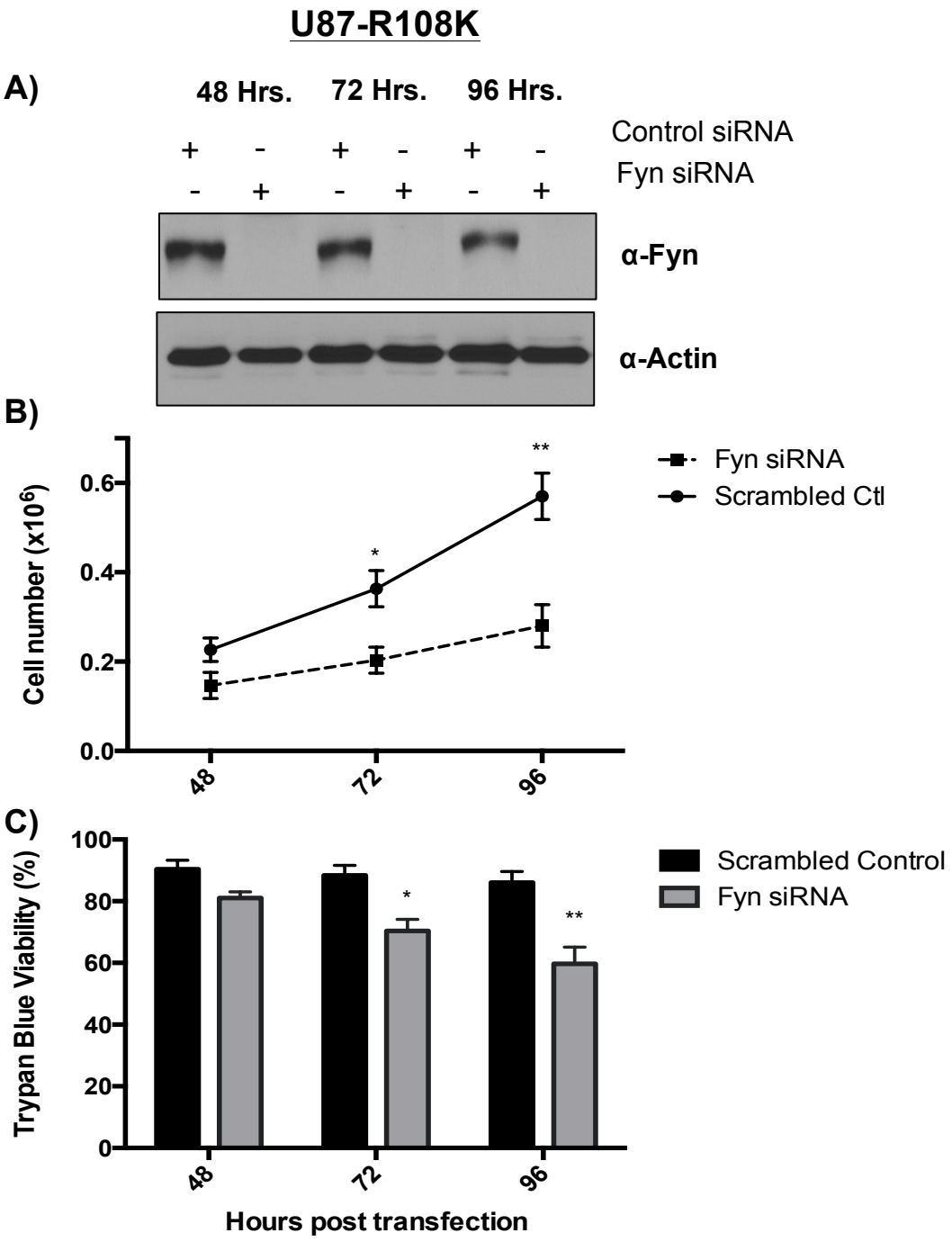


Figure 10. Fyn knockdown reduces cellular survival and proliferation in U87-R108K cells. (A) U87-R108K cells were transfected with either Fyn specific or scrambled control siRNA and Fyn protein levels were evaluated by immunoblotting at 48, 72 and 96 hours post-transfection. (B) Resulting cell numbers (x10^6) and (C) viability were determined at 48, 72 and 96 hours by trypan blue exclusion. Percent viability was calculated compared to untreated control. * p<0.05, ** p<0.01; Student's t-test. Data are mean ± SEM and are representative of at least three individual experiments.

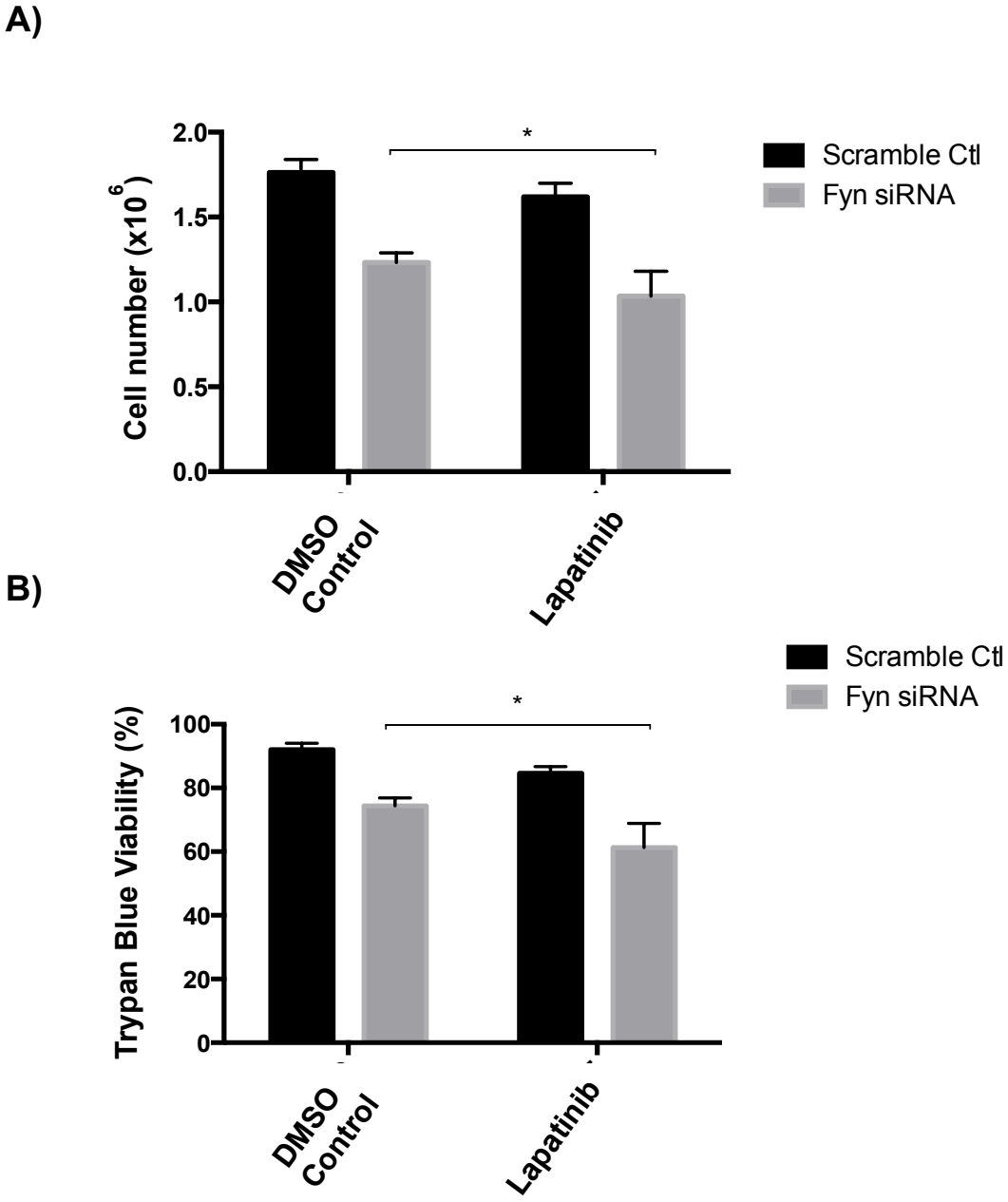


Figure 11. The effect of Fyn knockdown in combination with EGFR kinase inhibition on cell proliferation and survival in U87-EGFRΔIII. (A) U87-EGFRΔIII cells were transfected with Fyn-specific or scrambled control siRNA in the presence of DMSO control or lapatinib (1.0 μM; 48 hrs.) and resulting cell numbers (x10^6) and (B) viability were determined by trypan blue exclusion. Percent viability was calculated compared to untreated control. * indicated $p<0.05$; Student's t-test. Data are mean ± SEM and are representative of at least three individual experiments.

3.2.4 Increased Fyn expression contributes to the sphere-formation capacity of GSCs

Heterogeneity is a hallmark of GBM, which is bolstered by the existence of a highly chemo- and radio-resistant population of GSCs capable of re-populating the tumor following treatment.[32] Importantly, recent reports indicate that EGFRΔIII/CD133^{+} co-expression defines a population of GSCs with the greatest potential for self-renewal.[35,36] Furthermore, increased expression of SFK members, namely Fyn, has been observed in GSCs.[141] These observations, coupled with our previous findings in EGFR-overexpressing GBM cells, led us to examine the presence and potential biological impact of Fyn expression in GSCs. In agreement with previously published findings[141], we showed that Fyn expression was elevated in in the majority (eight of ten) GSC lines relative to normal progenitor control (Fig. 12). To determine the functional relevance of increased Fyn expression in the GSC phenotype, we performed Fyn-directed siRNA in GSC line 7-2 and evaluated resulting sphere formation capacity. Here, we determined that Fyn depletion significantly (p<0.001) reduced sphere formation, suggesting a functional role for Fyn expression in this population of tumor-initiating cells (Fig. 13). Together, these findings indicate that Fyn up-regulation is commonly occurring in a refractory population of GSCs culpable in clinical relapse, where Fyn expression functions to increase sphere-formation capacity.

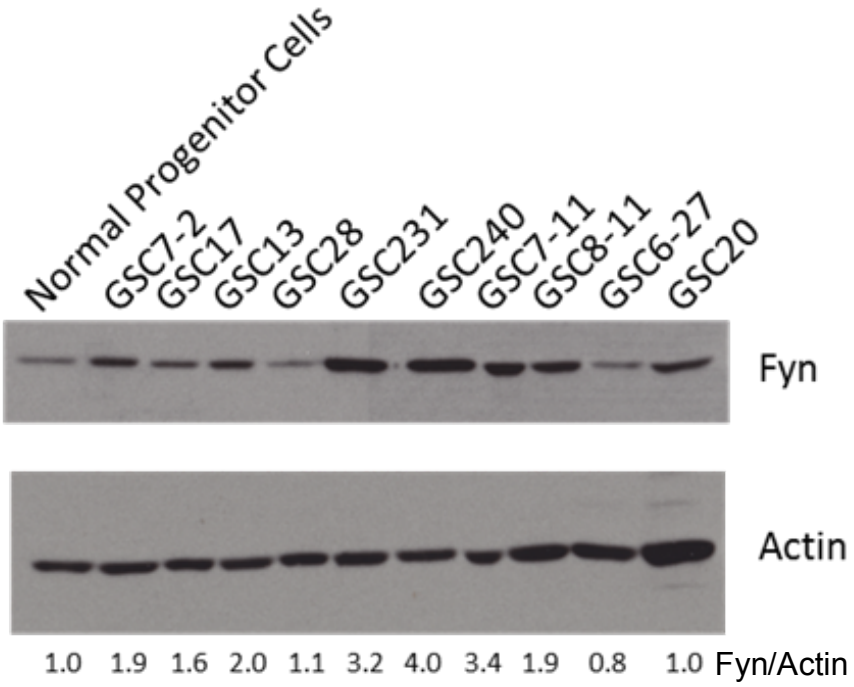


Figure 12. Fyn protein expression is increased in a panel of glioblastoma-derived stem cells. Fyn protein levels were evaluated by immunoblotting in a panel of glioblastoma-derived stem cells relative to normal progenitor counterparts. β-actin was shown as a loading control. Relative protein levels were determined by densitometry using ImageJ.

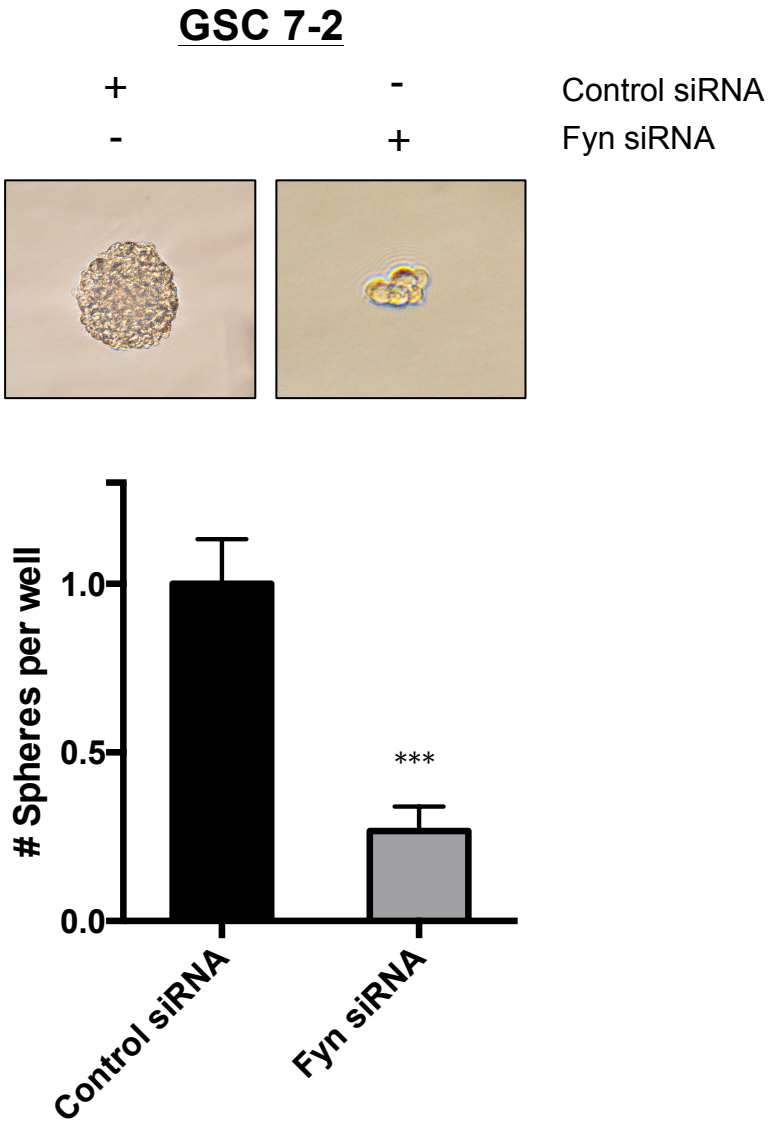


Figure 13. Fyn knockdown reduces sphere-formation in GSC line 7-2. GSC line 7-2 was transfected with either Fyn specific or scrambled control siRNA and sphere-forming capacity was measured as described in materials and methods. Images are shown at 20x magnification. *** indicated $p<0.001$; Student's t-test. Data are mean ± SEM and are representative of at least three individual experiments.

3.2.5 The transcription factor Egr-1 is up-regulated by EGFR and sufficient for induction of Fyn expression in EGFR-overexpressing GBM cells

Given that EGFR-mediated up-regulation of Fyn expression was determined to occur at the mRNA level, we next evaluated the transcription factor responsible for Fyn induction downstream of EGFR. Prior studies from our lab have functionally characterized the Fyn promoter, identifying Sp1 and Egr-1 bindings sites capable of driving Fyn expression.[155] Notably, Egr-1, but not Sp1, is overexpressed in GBM cells and patient samples.[25,190] Interestingly, Egr-1 induction in prostate cancer is EGFR-dependent.[197] In addition, recent microarray analysis identified Egr-1 as the most strongly up-regulated gene downstream of EGFRΔIII-overexpression in HEK293 cells versus vector control.[162] Therefore, we explored the relative contribution of Egr-1 with regard to Fyn up-regulation in EGFR-overexpressing GBM cells. Immunoblot analysis indicated that Egr-1 expression is up-regulated in U87-wtEGFR (4.5-fold), and to a greater degree (>7.0-fold) in U87-EGFRΔIII and U87-R108K cells, relative to vector control (Fig. 14). This finding, much like our Fyn expression result, suggested a role for the kinase activity of EGFR in the regulation of Egr-1 expression. To more closely examine this possibility, we next evaluated Egr-1 expression in U87-wtEGFR cells following acute stimulation with human recombinant hrEGF. Here, we determined that Egr-1 expression was robustly up-regulated in cells stimulated with hrEGF relative to non-stimulated controls (Fig. 15), further indicating a role for EGFR kinase activity in the regulation of Egr-1.

To verify that the increases in Egr-1 expression were EGFR-dependent, EGFR knockdown was performed using EGFR-directed siRNA. Indeed, EGFR knockdown saliently reduced (>90%) Egr-1 protein levels in wtEGFR, EGFRΔIII and EGFR-R108K-overexpressing cells (Fig. 16), providing corroborating evidence for EGFR-mediated regulation of Egr-1 expression in our model system.

Based on these findings, we next wanted to determine whether Egr-1 was responsible for the modulation of Fyn expression downstream of EGFR-overexpression. Here, depletion of Egr-1 by siRNA rendered a significant and concordant level of Fyn protein reduction (~50%) in wtEGFR, EGFRΔIII and EGFR-R108K-overexpressing cells (Fig. 17). These results demonstrate that EGFR-dependent regulation of Fyn expression is imparted, in part, by EGFR-mediated modulation of Egr-1. Together, these findings generate significant interest in better understanding the mechanism of increased Egr-1 expression occurring in EGFR-overexpressing GBM cells as a means of effectively targeting Fyn expression.

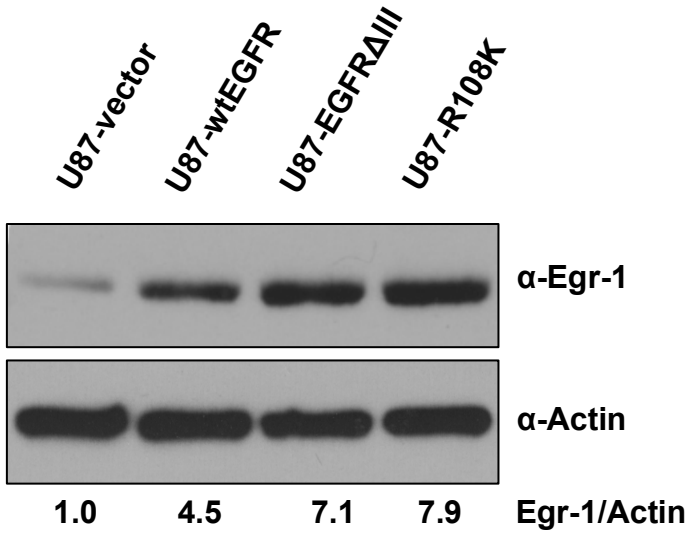


Figure 14. EGFR-overexpression increases Egr-1 expression in U87-MG cells. Egr-1 protein levels were examined in U87-MG stably overexpressing either vector control, wtEGFR, EGFRΔIII or EGFR-R108K by immunoblotting. β-actin was shown as a loading control. Relative protein levels were determined by densitometry using ImageJ. Results are representative of at least three independent experiments.

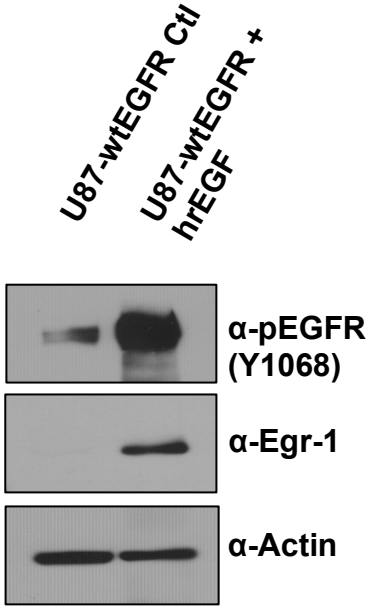


Figure 15. EGF stimulation increases Egr-1 expression in U87-wtEGFR. Egr-1 protein levels were measured in U87-wtEGFR cells acutely stimulated with hrEGF by immunoblot analysis. Total EGFR and phoshporylationed EGFR (Y1068) are shown. Results are representative of at least three independent experiments.

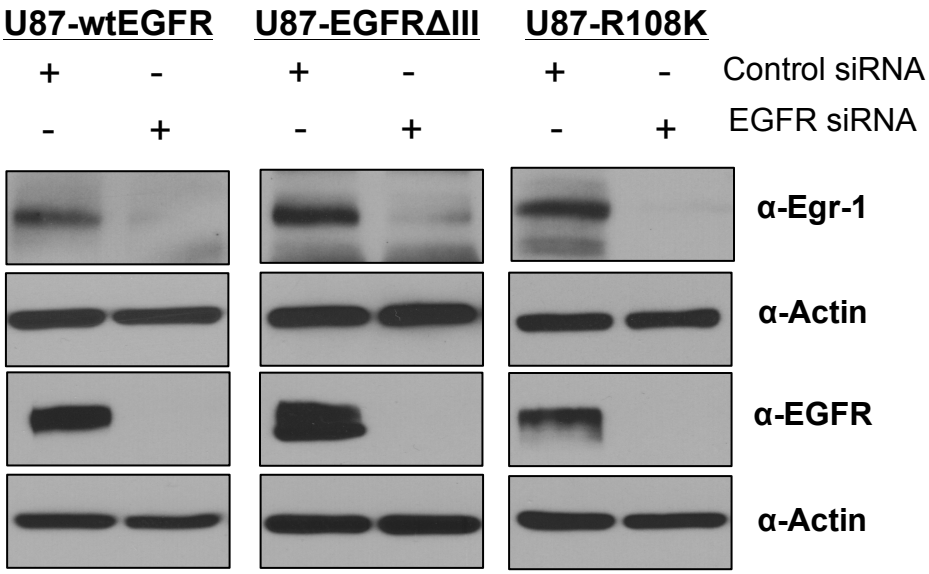


Figure 16. EGFR knockdown reduces Egr-1 expression in EGFR-overexpressing U87-MG cells. U87-wtEGFR, U87-EGFRΔIII and U87-R108K cells were transfected with either EGFR-specific or scrambled control siRNA and EGFR and Egr-1 protein levels were evaluated at 48 hours post-transfection by immunoblotting. β-actin was shown as a loading control. Results are representative of at least three individual experiments.

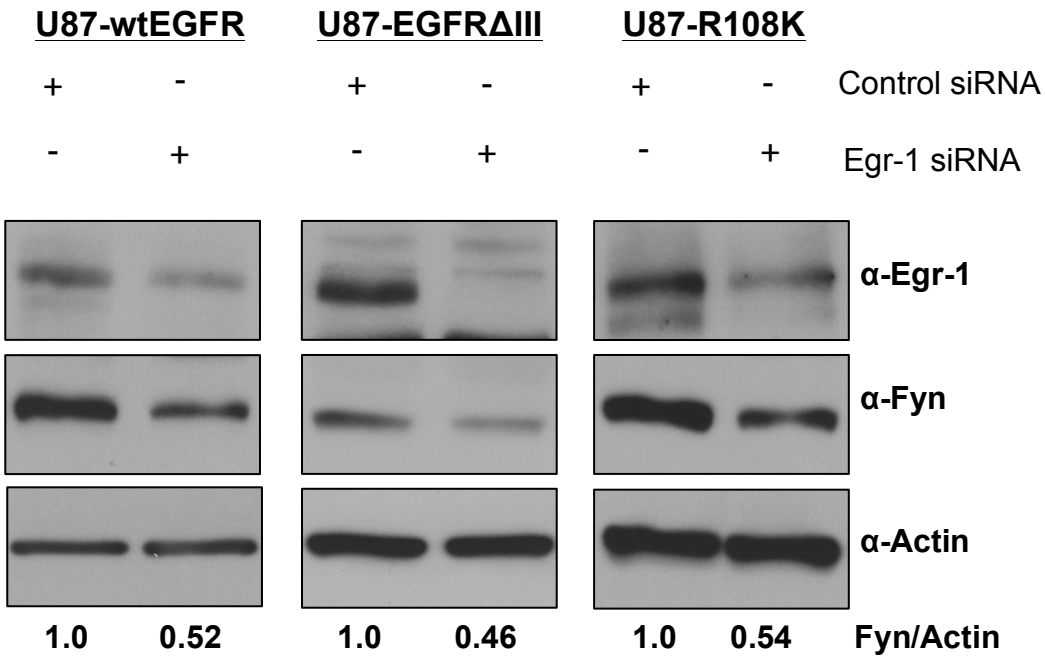


Figure 17. Egr-1 knockdown reduces Fyn expression in EGFR-overexpressing U87-MG cells. U87-wtEGFR, U87-EGFRΔIII and U87-R108K cells were transfected with either Egr-1-specific or scrambled control siRNA and Egr-1 and Fyn protein levels were evaluated at 48 hours post-transfection by immunoblotting. β-actin was shown as a loading control. Relative protein levels were determined by densitometry using ImageJ. Results are representative of at least three individual experiments.

3.2.6 Egr-1 and Fyn expression are redox responsive in EGFRΔIII-overexpressing GBM cells

Currently, there are no clinically available Egr-1-targeted agents. Thus, we explored the mechanism whereby EGFR signaling up-regulates Egr-1 expression in GBM cells. Interestingly, Egr-1 expression has previously been described as being redox-responsive in CML and 293T cells.[155] Therefore, we evaluated the effect of redox alterations on Egr-1 and Fyn expression downstream of EGFRΔIII, which has previously been linked to elevated oxidative stress in GBM.[183,184] To do so, EGFRΔIII-expressing cells were treated for 24 hours with either diluent or a general antioxidant, N-acetylcysteine (NAC). Cells were harvested 48 hours post-treatment and subjected to immunoblot analysis for Egr-1 and Fyn. Our findings demonstrated that treatment with NAC reduced, albeit modestly, both Egr-1 and Fyn protein expression (Fig. 18), suggesting a role for redox-dependent regulation of Fyn via Egr-1 in EGFRΔIII -overexpressing GBM cells.

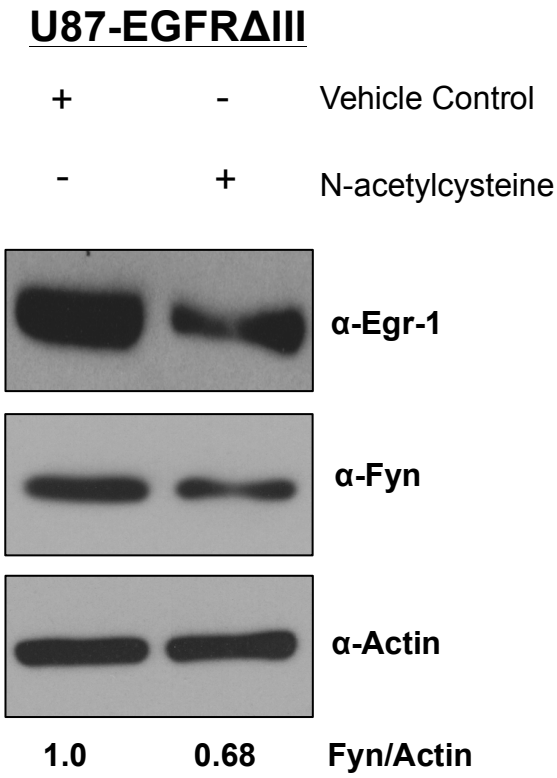


Figure 18. The antioxidant N-acetylcysteine reduces Egr-1 and Fyn protein expression in EGFRΔIII-overexpressing GBM cells. U87-EGFRΔIII cells were treated with either DMSO control or N-acetylcysteine (10 mM; 24 Hrs.) and resulting Egr-1 and Fyn protein levels were analyzed by immunoblotting at 48 hrs. post-treatment. β-actin was shown as a loading control. Relative protein levels were determined by densitometry using ImageJ. Results are representative of at least three individual experiments.

3.3 Discussion and Future Directions

EGFR is commonly dysregulated in GBM, presenting a compelling therapeutic target.[6,80] Regrettably, EGFR-targeted therapies, namely lapatinib, have yielded very modest clinical efficacy, which has been attributed to several factors including: the acquisition of secondary EGFR point mutations as well as co-activation and/or amplification of additional RTKs.[55-57] Thus, a more thorough understanding of signaling intermediates culpable in the failed response to EGFR-directed therapies is needed. In vitro studies have highlighted the therapeutic potential of SFKs in GBM, where inhibition of their enzymatic activity, particularly Fyn and c-Src, reduces cell growth, viability and motility.[110,115,136] Regrettably, early-phase clinical application of the pan-Src inhibitor dasatinib has yielded only marginal therapeutic activity in GBM patients[139,140], highlighting the need for alternative SFK targeted strategies in this setting. Interestingly, in addition to serving as an effector substrate of EGFR signaling in GBM, the SFK member Fyn is commonly overexpressed in GBM patient samples, cell lines and GSCs.[25,137,141,190] Fyn overexpression has previously been identified as a growth promoting factor in breast and prostate cancer and, more recently, in CML where increased expression was imparted via Egr-1, a transcription factor also commonly overexpressed in GBM.[190,142,143,155] However, the regulation and contribution of increased Fyn protein remain poorly understood in GBM. The work presented in this chapter reveals a novel mechanism whereby EGFR induces Fyn expression via the transcription factor Egr-1 in GBM cells. Increases in Fyn expression were also commonly observed across a panel of GSCs.

Furthermore, this chapter uncovered novel biological roles for increased Fyn expression in the proliferation and survival of EGFRΔIII and R108K-overexpressing cells, as well as in the sphere formation potential of GSC line 7-2.

Given the frequency of EGFR alterations in GBM as well as the previously identified correlation between EGFR and Fyn gene expression in GBM patient samples[25,137], we sought to determine the effect of EGFR-overexpression on Fyn expression in GBM cells. Our data demonstrate that Fyn mRNA and protein expression, but not Lyn, are increased in EGFR-overexpressing GBM cells, in a manner concordant with EGFR activation status (Figs. 4, 5). The observed increases in Fyn expression were effectively abrogated by lapatinib in U87-wtEGFR, while on partially blunted in U87-EGFRΔIII and U87-R108K (Figs. 6, 7), suggesting a potential lapatinib escape mechanism involving Fyn expression. These findings were corroborated by EGFR knockdown studies, which effectively repressed Fyn expression to a baseline level not achievable by lapatinib in EGFRΔIII and R108K-expressing cells (Fig. 7). This prompted us to evaluate the contribution of Fyn expression in EGFRΔIII and R108K-overexpressing cell proliferation and survival, where persistent Fyn expression, if tumor promoting, could facilitate resistance to EGFR inhibition. Here, we demonstrated that genetic inhibition of Fyn protein significantly reduced cell proliferation and survival U87-EGFRΔIII and U87-R108K cells (Figs. 9, 10). Importantly, the effects of Fyn depletion in U87-EGFRΔIII were not bolstered by the addition of lapatinib, suggesting that Fyn inhibition alone is sufficient to reduce to U87-EGFRΔIII cell growth whereas lapatinib is not (Figs. 11). These findings are of significant

interest, as enhanced and sustained pro-proliferative and anti-apoptotic signaling are hallmarks of EGFRΔIII and EGFR-R108K signaling, which contribute to therapeutic resistance and clinical relapse in GBM.[59,89,185,186] The heightened resistance of mutant EGFR expressing cells has previously been attributed to increased Bcl-xL expression.[59] Importantly, recent reports indicate that Fyn mediates the activation of Stat5b in GBM cells, which is known to drive expression of Bcl-xL in this context.[109] We did not explore this possibility; future studies should, however, should evaluate potential differences in BCL-xL expression as well as the identity of differentially regulated substrates occurring in the context of Fyn knockdown. These findings would aid the development of the rational therapeutic combination strategies in EGFR-expressing GBM. Together, these observations suggest that insufficient inhibition of Fyn expression could serve as a preferential signaling hub in the maintenance of EGFRΔIII and EGFR-R108K-induced survival signaling.

Though we did not evaluate the effect of Fyn knockdown in wt-EGFR-overexpressing cells, these studies would provide further insight into the functional significance of Fyn expression in GBM as: 1) EGFR amplification is the most commonly occurring alteration in GBM patients (57%)[25]; and 2) cognate ligand production (i.e. TGF-α and EGF) is significantly up-regulated in tumors, resulting in receptor activation.[56] Additionally, though Lyn expression was not altered by EGFR, its potential role cannot be excluded and should be further evaluated in knockdown studies. Nonetheless, these findings suggest a novel role for increased Fyn expression in cell proliferation and survival downstream of

aberrant EGFR signaling in GBM cells, which encouraged us to extend these studies into GSCs.

A growing body of literature suggests that tumor recurrence and therapeutic resistance in GBM are greatly impacted by the presence of GSCs.[32] Interestingly, recent reports indicate that a subpopulation of GSCs co-expressing EGFRΔIII/CD133^{+} maintain the greatest potential for self-renewal.[35,36] Moreover, increased Fyn expression has previously been described in GSCs, where dasatinib treatment failed to suppress GSC growth and viability.[141] Consistent with these findings[141], we determined that Fyn expression was commonly elevated in a panel of GSCs relative to normal neural progenitor controls (Fig.12). Additionally, our studies demonstrate that Fyn knockdown was sufficient to reduce sphere formation in line 7-2 (Fig. 13). Given that sphere formation is an indirect measure of cellular stemness[33], evaluation of stem cell markers such as SOX2, nestin and CD44 should also be examined as a means of delineating a potential role for Fyn expression in this regard. The importance of these findings should be validated in additional GSCs and, furthermore, studies should evaluate whether or not the observed effects are specific for GSCs by examination of Fyn knockdown in normal progenitor controls. Our findings in U87-EGFRΔIII and U87-R108K indicate that Fyn knockdown decreases cell proliferation and viability, which could be contributing to the reductions in sphere formation observed in GSC 7-2. Fyn also, however, also activates focal adhesion kinase (FAK)[142], whose inhibition via Fyn depletion could have a negative impact on cell adhesion, resulting in fewer spheres being formed. The potential contributions of

each aforementioned aspect should be taken into careful consideration moving forward.

Though prior studies have analyzed GSC phenotype in relation to EGFRΔIII, we failed to detect EGFRΔIII expression by immunoblot analysis in our panel of GSCs. The presence of wtEGFR also was not detected in our studies, which can be explained by receptor degradation elicited by hrEGF supplementation in sphere forming media.[31] Due to this, we could not evaluate the potential involvement of EGFR-mediated Fyn regulation in this regard. This, however, alludes to a potential EGFR-independent mechanism of Fyn regulation occurring in GSCs, as Fyn levels were commonly up-regulated in the absence of detectable EGFR protein. Therefore, further studies evaluating the potential mechanism(s) of Fyn regulation in this regard are needed for the purpose of identifying additional mechanisms and therapeutic targets in GSCs. These studies should additionally examine the effects of hrEGF supplementation on Fyn activation and up-regulation. Regardless, our findings indicate that Fyn protein is commonly up-regulated in a panel of GSCs, where it functions to enhance sphere formation in GSC line 7-2.

Since Fyn expression increases were determined to occur at the mRNA level, which corresponds with meta-analysis of the TCGA database [25], we sought to determine the transcription factor responsible for increased Fyn expression. Functional characterization of the Fyn promoter has previously been performed in our lab, identifying Sp1 and Egr-1 bindings sites, capable of driving basal and redox-responsive transcriptional Fyn levels, respectively.[155] Interestingly, Egr-1

but not Sp1, is commonly up-regulated in GBM cells and patient samples[25,190]; however, its role and regulation in GBM are poorly understood. Our findings demonstrated that Egr-1 expression is EGFR-dependent in GBM cells and, much like Fyn, was more pronounced in the setting of mutant EGFR (Figs. 14, 16) In addition, Egr-1 induction was indicated to occur via EGFR kinase signaling, as determined by acute hrEGF stimulation of U87-wtEGFR cells (Fig. 15). These results are in agreement with previously published findings in prostate cancer as well as microarray analysis of EGFRΔIII-overexpressing HEK293 cells, where Egr-1 transcription was imparted by the kinase activity of EGFR.[159,162] We also determined that genetic inhibition of Egr-1 effectively and concordantly reduced Fyn protein expression to an endogenous basal level in U87-wtEGFR, U87-EGFRΔIII and U87-R108K cells (Fig. 17). Further studies are needed to confirm that Egr-1 regulates Fyn transcription in this setting, specifically assessment of Egr-1 association with the Fyn promoter by chromatin immunoprecipitation. Given that lapatinib failed to sufficiently reduce Fyn expression, which was determined to aid cell growth and survival, this finding suggests that targeting Egr-1 is a more effective means of reducing Fyn expression in EGFR-overexpressing GBM cells. However, despite restoring Fyn levels to a baseline in EGFR-overexpressing GBM cells, our Fyn knockdown studies (Figs. 9, 10) imply that complete abrogation of Fyn protein is likely needed to reduce cell growth. Hence, additional studies evaluating the basal transcriptional activator of Fyn, including Sp1 as identified from prior Fyn promoter analysis[155], are needed to more efficiently target Fyn expression in EGFR-overexpressing GBM cells.

As no clinical agents targeting Egr-1 are currently available, we next determined the mechanism whereby EGFR signaling up-regulates Fyn expression in U87-EGFR expressing GBM cells. Previous findings from our lab in CML indicate that Egr-1 expression is responsive to oxidative stress.[155] Thus, we examined Egr-1 and Fyn expression in EGFRΔIII treated with the global antioxidant NAC. Here, we determined that Egr-1 and Fyn expression were modestly reduced by antioxidant treatment, suggesting a role for redox-dependency in the regulation of Egr-1. The modest degree of expression inhibition suggests that more specific ROS inhibitory approaches may be necessary to adequately assess redox-dependent alterations. Interestingly, multiple reports across a variety of tumor types, including GBM, have implicated redox signaling in the growth and survival of tumor cells. [149,153] ROS production in cancer typically occurs downstream of oncogene-induced up-regulation of Nox enzyme expression and/or activation.[153,180,182,198] Therefore, a better understanding of the relationship between EGFR and Nox enzyme expression and activation would enable the development of novel redox-targeted strategies aimed at reducing cell proliferation and survival in EGFR-overexpressing GBM.

In summary, this chapter provides novel evidence whereby EGFR induces Fyn expression via redox-dependent up-regulation of Egr-1, leading to enhanced cell proliferation and survival in U87-EGFRΔIII and U87-R108K. Increased Fyn expression was additionally noted in GSCs, where it served to enhance sphere formation in GSC line 7-2. Though these studies focused on the regulation and functional significance of Fyn expression, the relative contribution of Fyn

enzymatic activity in this regard cannot be excluded and should be further evaluated. Specifically, a kinase-dead mutant of Fyn should be used as a dominant negative approach to better assess whether the pro-proliferative and pro-survival functions of Fyn are mediated in a kinase-dependent or independent manner. Nevertheless, these studies provide compelling rationale for targeting Fyn expression, particularly in tumors with acquired resistance to EGFR and/or SFK inhibitors.

CHAPTER 4

RESULTS

P47PHOX IS A NOVEL REGULATOR OF FYN EXPRESSION IN EGFR-OVEREXPRESSING GBM CELLS

4.1 Introduction

Oncogenes commonly elevate cellular ROS production.[147,199] Though moderate ROS accumulation functions to promote cell differentiation and proliferation, excessive ROS accumulation can give rise to oxidative damage of DNA, proteins, and lipids.[153] Thus, maintenance of ROS homeostasis is vital for standard cell growth and survival, and has generated significant interest in better understanding the role of redox as it pertains to cancer cell signaling.

A less studied but intriguing feature of EGFR is its ability to increase intracellular ROS content, including in GBM.[183,184] Though an understudied feature of GBM, ROS are important as emerging reports indicate that increased ROS production exerts pro-proliferative and pro-survival effects across a variety of different cancer types.[180,182,184] Despite the negative impact of increased ROS in cancer cells, recent studies have highlighted the exploitable nature of this biochemical feature, as genetic and chemical means of ROS inhibition have proven effective in targeting cancer cell growth.[153]

Based on our findings in chapter 3 that inhibition of Egr-1 reduced Fyn expression through redox alterations, we aimed to examine the source of oxidative stress in EGFR-overexpressing GBM as a means of targeting Fyn expression. Though the mitochondria are the most common source of cellular ROS[200], oncogene-induced ROS production, particularly EGFR[175], characteristically involves Nox enzyme up-regulation and/or activation.[170] Because EGFR is known to influence Nox enzymes, we set out to determine the relationship between EGFR and Nox enzymes in GBM and their potential link to

increased Fyn expression. To accomplish this, we employed a series of biochemical analyses aimed at determining basal cellular respiration rates as well as superoxide production in the presence of flavonoid, lipoxygenase and mitochondrial electron transport chain (ETC) inhibitors. The work in this chapter has led to a better understanding of the mechanism responsible for increased ROS accumulation in EGFR-overexpressing GBM cells as well as the redox contributions to cell proliferation and survival therein. Furthermore, the work presented here has broadened our understanding of redox signaling as a therapeutic strategy in GBM via determination of redox-dependent regulation of Fyn expression.

4.2 Results

4.2.1 EGFR-overexpression increases ROS production in GBM cells

Oxidative stress has previously been observed in EGFR-expressing GBM cells.[183,184] The mechanism of elevated ROS production downstream of EGFR in GBM, however, remains unclear. Given our finding that the broad antioxidant NAC reduced Egr-1 and Fyn expression in EGFRΔIII-expressing cells, we wanted to further explore the mechanism of ROS induction imparted by EGFR as a means of modulating Fyn expression. In accordance with previously published findings[184], our data indicate that hyperactive EGFR signaling, via EGFRΔIII and EGFR-108K, significantly elevate ROS levels (p<0.001) relative to wtEGFR- and vector-expressing cells (Fig. 19). To verify that the alterations in ROS were occurring in a kinase-dependent manner, serum starved U87-wtEGFR cells were acutely stimulated with hrEGF and resulting ROS levels were measured. Here, we determined that increased ROS accumulation was indeed imparted by the kinase activity of EGFR, as verified by immunoblot analysis of phosphorylated EGFR at tyrosine residue 1068 and superoxide evaluation (Fig. 20). Having verified that EGFR signaling elevates ROS production we next sought to evaluate the impact of EGFR-overexpression on cellular bioenergetics.

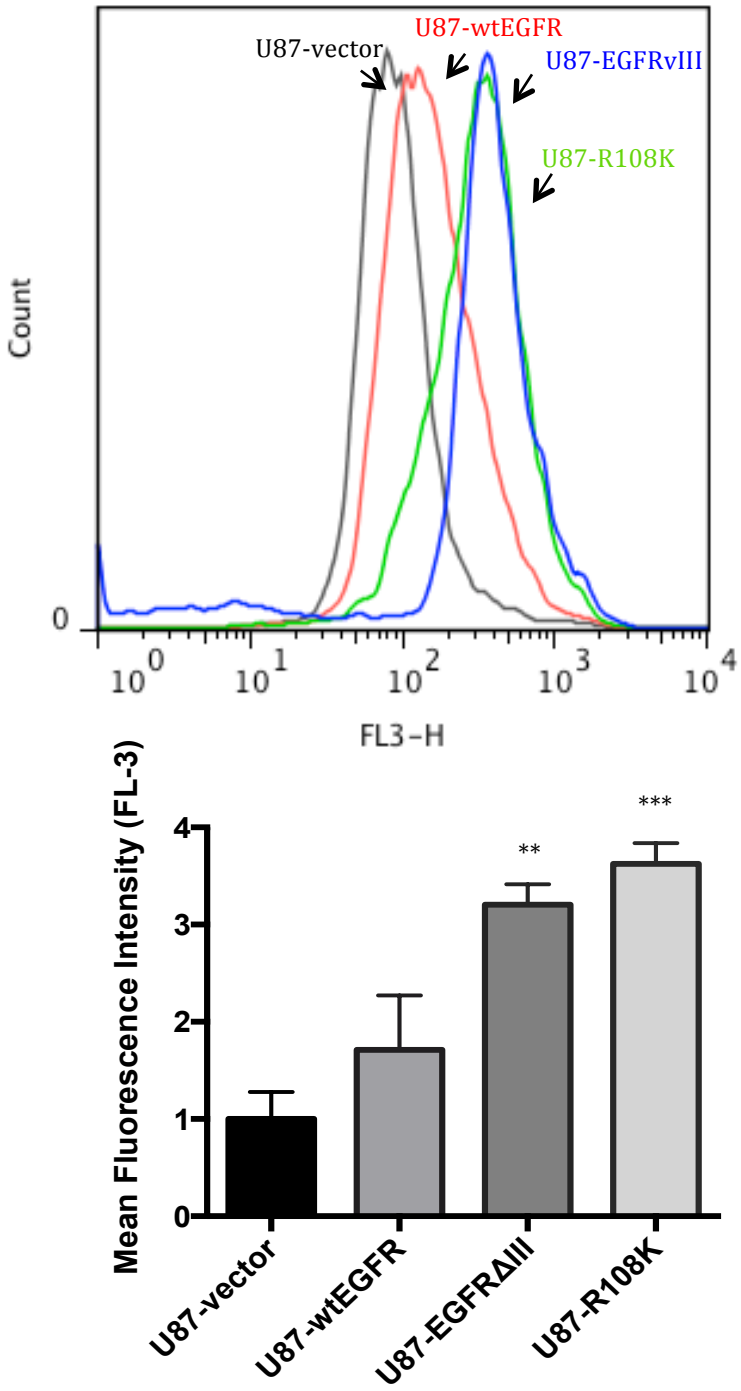
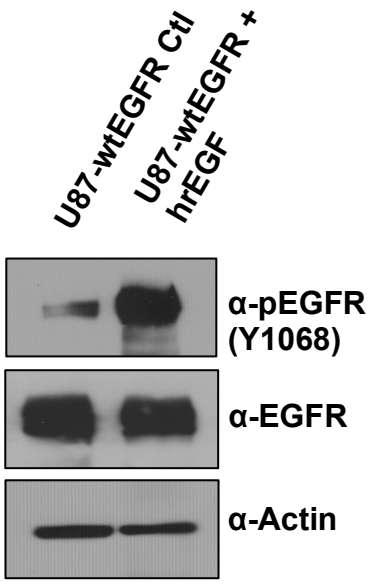


Figure 19. EGFR-overexpression increases superoxide content in GBM cells. (A) Superoxide levels were measured in vector control, U87-wtEGFR, U87-EGFRΔIII and U87-R108K cells by flow cytometry using dihydroethidium staining as described. (B) ** $p<0.01$, *** $p<0.001$; one-way ANOVA with Bonferroni's Multiple Comparison Test. Data are mean ± SEM and are representative of at least three individual experiments.



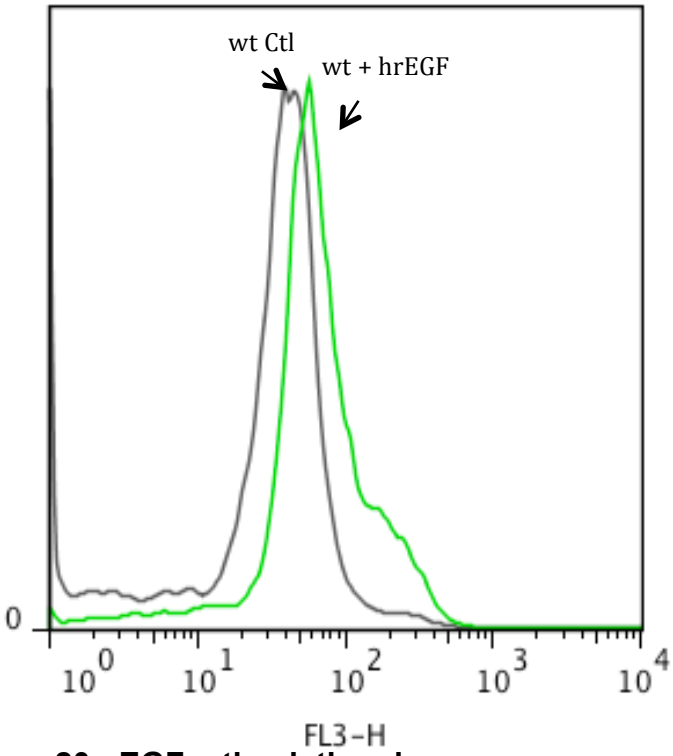


Figure 20. EGF stimulation increases superoxide content in U87-wtEGFR. Superoxide levels were measured in U87-wtEGFR cells acutely stimulated with hrEGF by flow cytometry using dihydroethidium staining as described. Total EGFR and phoshporylationed EGFR (Y1068) are shown. Results are representative of at least three independent experiments.

4.2.2 Mutant EGFR-overexpression augments non-mitochondrial respiration in GBM cells

In many cell types, mitochondria are the primary source of ROS, occurring as undesired byproducts of cellular respiration.[147] Though the relationship between EGFR and Nox enzymes is well described in other systems, oncogenic overexpression can also increase mitochondrial biogenesis.[201] In light of this, we aimed to determine the global effects of EGFR-overexpression on cellular respiration via extracellular flux analysis. From these experiments, it was determined that EGFRΔIII, but not wtEGFR or EGFR-R108K, elevated mitochondrial respiration (Fig. 21). Both EGFRΔIII and EGFR-R108K did, however, elevate levels of non-mitochondrial respiration (Fig. 21). This finding is of great interest, as EGFRΔIII and EGFR-R108K elevate ROS in a pronounced and commensurate manner, whereas non-stimulated wtEGFR confers only modest effects on ROS content (Fig. 19). These findings suggest a similar mechanism of EGFR-induced ROS production stemming from non-mitochondrial sources of respiration, but do not preclude potential mitochondrial involvement. Given this finding, we next sought to determine ROS content following chemical inhibition of both Nox and mitochondrial ROS.

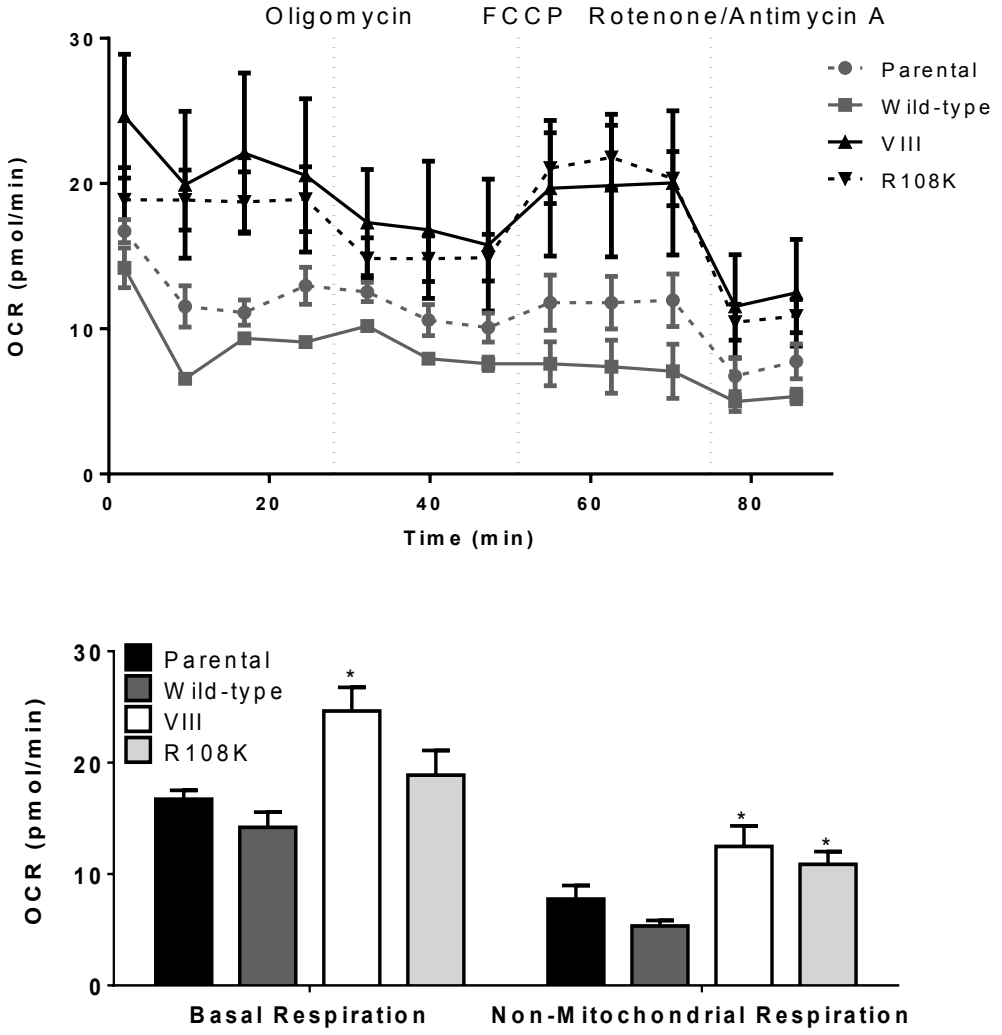


Figure 21. Mutant EGFR-overexpression increases non-mitochondrial respiration in GBM cells. Cellular respiration as depicted by oxygen consumption rates (OCR) were determined over time in wtEGFR, EGFRΔIII, EGFR-R108K-expressing U87-MG. All injections were 1 μM. * indicated $p<0.05$; one-way ANOVA with Bonferroni's Multiple Comparison Test. Data are mean ± SEM and are representative of at least three individual experiments.

4.2.3 Chemical inhibition of the Nox complex reduces ROS in mutant EGFR-overexpressing GBM cells

To more closely examine ROS production, we next evaluated superoxide levels following application of the following chemical inhibitors: an inhibitor of mitochondrial respiration, rotenone; an inhibitor of lipoxygenase-derived ROS, Nordihydroguaiaretic acid (NDGA); two Nox complex inhibitors, diphenelyeneiodonium (DPI) and apocynin; and the global ROS inhibitor, NAC. These studies were performed in cells overexpressing either EGFRΔIII or EGFR-R108K, which harbored the greatest elevations in superoxide content (Fig. 19). Here, DPI and apocynin most significantly (p<0.001) reduced ROS levels, whereas rotenone exhibited only modest effects in this regard (Fig. 22). Thus, these data reinforce a potential role for Nox-mediated ROS production in mutant EGFR-overexpressing cells. It should be noted, however, that apocynin and DPI are reported to inhibit multiple flavoproteins[194], thus requiring a more thorough examination of Nox activity in this regard.

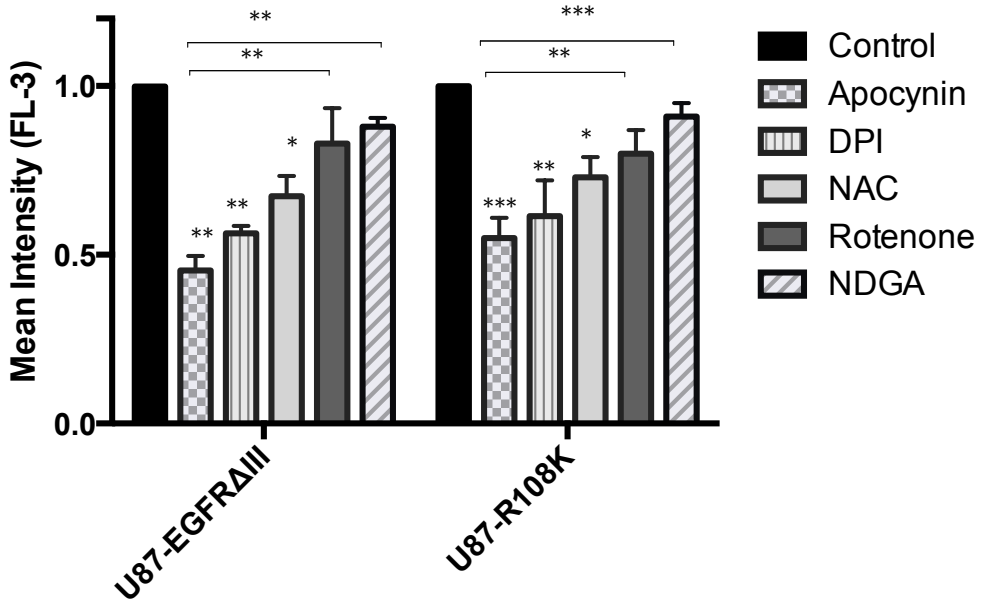


Figure 22. Chemical inhibition of the Nox complex significantly decreases superoxide content in mutant EGFR-overexpressing GBM cells. EGFRΔIII and EGFR-R108K-expressing cells were treated with either NAC (10 mM; 24 hrs.), DPI (5 μM; 4 hrs.) apocynin (100 μM; 24 hrs.) or rotenone (1 μM; 4 hrs). Following treatment, intracellular ROS levels were measured by dihydroethidium staining. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; one-way ANOVA with Bonferroni's Multiple Comparison Test. Data are mean ± SEM and are representative of at least three individual experiments.

4.2.4 EGFRΔIII-overexpression increases Nox activity in GBM cells

The Nox family of enzymes function to produce oxidative bursts via conversion of molecular oxygen to superoxide. This reaction takes place at the expense of NADPH, which is metabolized to $NADP^+$.[169] As such, to further evaluate activation of the NADPH oxidase downstream of EGFR signaling, we measured NADPH consumption (NADP/NADPH ratios) as an indirect readout of Nox activity. Much akin to the effect of EGFR signaling on ROS content and oxygen consumption rates, we determined that the most frequently occurring EGFR mutant, EGFRΔIII, but not wtEGFR, significantly elevated NADPH consumption (Fig. 23). These findings, again, were linked to the kinase activity of EGFR, as lapatinib inhibition of EGFRΔIII restored NADPH consumption to a baseline level (Fig. 23). Collectively, these findings suggest that EGFR-overexpression dictates Nox-mediated ROS production downstream of EGFR signaling.

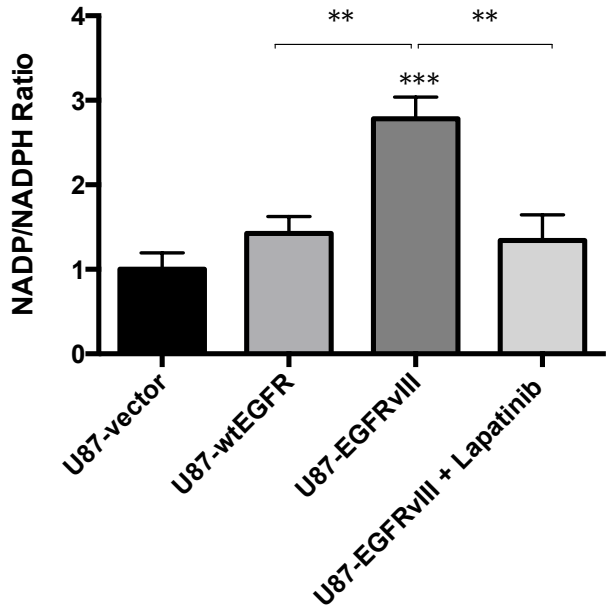


Figure 23. EGFRΔIII-overexpression increases Nox activity in GBM cells. Nox activity was measured in vector control, wtEGFR and EGFRΔIII (+/- lapatinib) and cells as described in materials and methods. ** $p<0.01$, *** $p<0.001$; one-way ANOVA with Bonferroni's Multiple Comparison Test. Data are mean ± SEM and are representative of at least three individual experiments.

4.2.5 p47phox up-regulation contributes to Nox-mediated ROS production downstream of EGFR-overexpression in GBM cells

Increased Nox activity downstream of EGFRΔIII, as well as decreased ROS production following treatment with apocynin and/or DPI, implies involvement of at least one of the known Nox enzymes. Apocynin and DPI, however, reportedly inhibit a variety of flavoproteins.[169] Therefore, we set out to more clearly delineate the specific Nox isoform and/or subunit involved in mutant EGFR-initiated ROS production. Previous studies have identified Nox4 up-regulation and activation as a mediator of ROS production and cell growth in GBM.[180-182] These studies, however, did not evaluate Nox4 status in the context of EGFR. Using real-time PCR, we examined Nox4 expression in EGFR-expressing GBM cells. Nox4 mRNA, however, was not significantly altered by the presence of wtEGFR, EGFRΔIII or EGFR-R108K overexpression (Fig. 24 A). However, in addition to Nox4, clinical evidence suggests a potential role for the Nox-2 organizing complex, p47phox, as it is commonly overexpressed in GBM patient samples compared to normal brain tissue.[190] Furthermore, recent studies have shown p47phox phosphorylation and subsequent Nox activation to be EGF-inducible events contributing ROS production and cell proliferation in human colon cancer cells.[175] Thus, using real-time PCR we determined that p47phox mRNA was significantly up-regulated in wtEGFR (1.5-fold), EGFRΔIII (1.9-fold) and EGFR-R108K (1.7-fold)-overexpressing cells (Figure 24A). The observed increase in p47phox mRNA was determined to occur in a commensurate manner at the protein level by immunoblotting (Fig. 25 B), suggesting a functional role for

its expression. Hence, we sought to determine the effect of p47phox knockdown on superoxide content and Nox activity as previously described. Interestingly, genetic inhibition of p47phox (Fig. 25) significantly reduced both ROS content ($p<0.01$) and Nox activity ($p<0.01$) in EGFRΔIII-expressing cells, providing evidence for p47phox-mediated ROS production via Nox downstream of EGFR signaling in GBM (Fig. 25). To determine the biological significance of p47phox up-regulation, proliferation of p47phox siRNA-transfected U87-EGFRΔIII cells versus scrambled control-transfected U87-EGFRΔIII cells was assessed by plating equivalent cell numbers and comparing relative cell counts 48, 72, and 96 hours post-transfection. Cells transfected with p47phox siRNA grew at a significantly ($p<0.05$) slower rate relative to scrambled control-transfected cells (Fig. 25). U87-EGFRΔIII viability, however, was unaffected by p47phox siRNA as determined by trypan blue exclusion (data not shown).

In summation, these findings reveal a novel mechanism by which EGFR signaling contributes to GBM growth through transcriptional induction of the ROS producing Nox-2 organizer subunit, p47phox. Given this finding we were eager to evaluate the effect of p47phox depletion, as well as chemical inhibition of Nox, as a redox-targeted strategy for regulating Fyn expression.

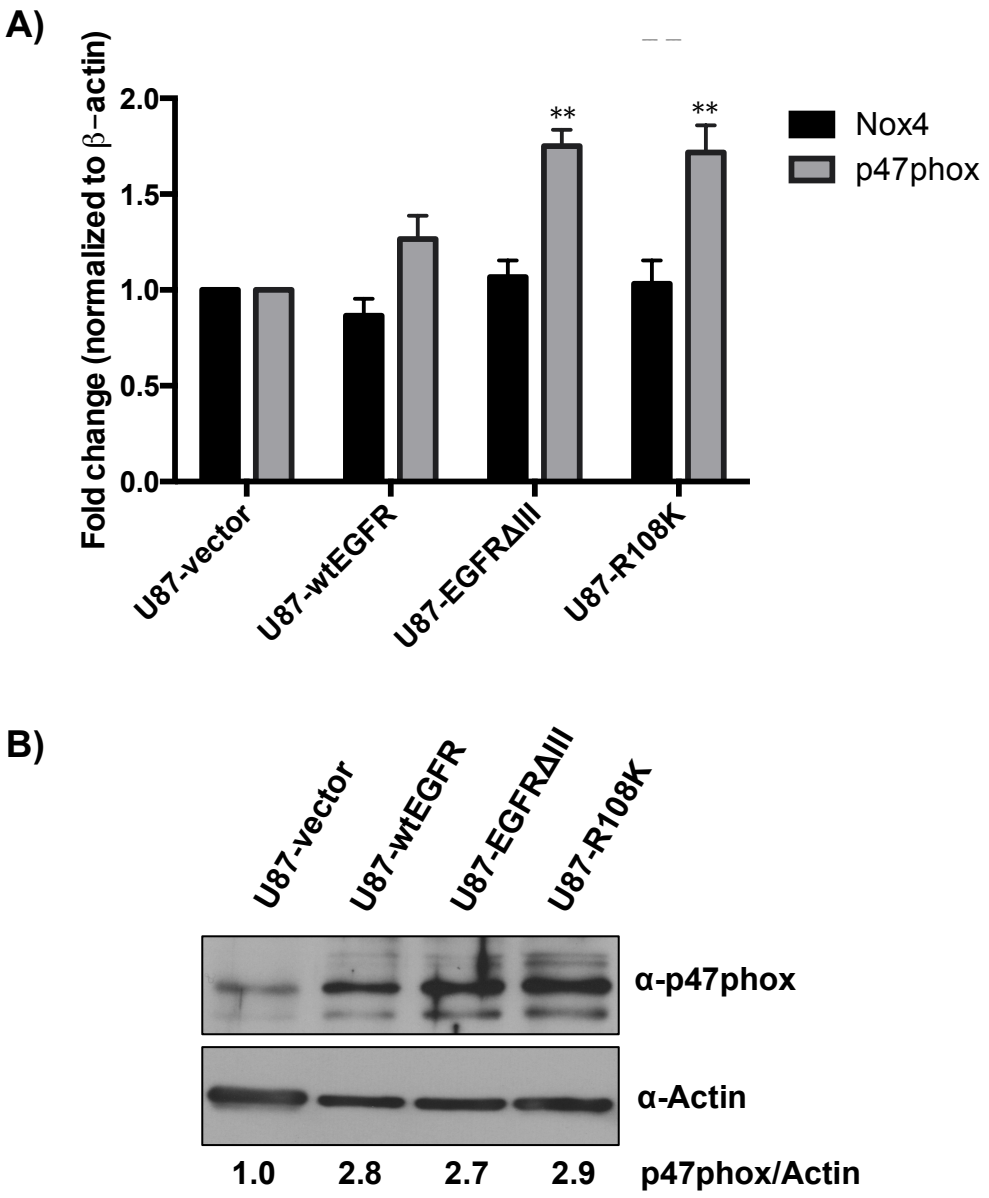


Figure 24. EGFR-overexpression increases p47phox mRNA and protein expression in U87-MG cells. (A) Nox4 and p47phox mRNA levels and (B) p47phox protein levels were examined in U87-MG expressing either wtEGFR, EGFRΔIII, EGFR-R108K or vector control by real-time PCR (housekeeping gene β-actin) and immunoblotting, respectively. β-actin was shown as a loading control. Relative protein levels were determined by densitometry using ImageJ. * $p<0.05$, ** $p<0.01$; one-way ANOVA with Bonferroni's Multiple Comparison Test.

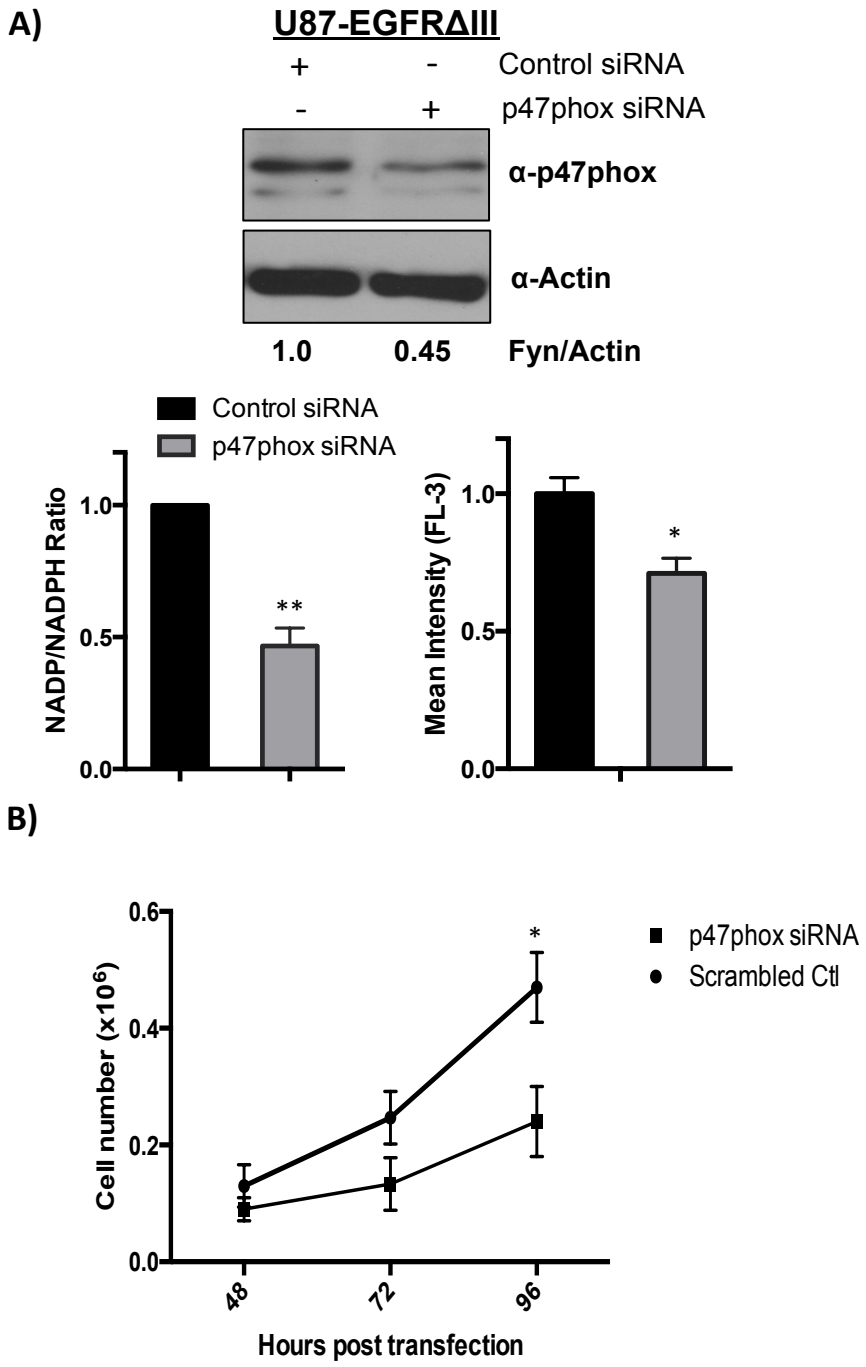


Figure 25. The effect of p47phox knockdown in U87-EGFRΔIII. (A) U87-EGFRΔIII cells were transfected with either p47phox-specific siRNA or scrambled control and p47phox and protein levels were evaluated at 48 hours post-transfection by immunoblotting. Resulting Nox activity and ROS levels were measured as described in materials and methods. (B) Resulting cell numbers (x10^6) were determined at 48, 72 and 96 hours by trypan blue exclusion. ** indicated $p<0.01$; student's t-test. Data are mean ± SEM and are representative of at least three individual experiments.

4.2.6 p47phox contributes to Fyn protein expression in EGFRΔIII-expressing GBM cells

To better understand the relationship between the Nox complex and Fyn expression, we treated U87-EGFRΔIII cells with the Nox inhibitor apocynin and assessed resulting Egr-1 and Fyn protein levels by immunoblotting. Similar to our results obtained with NAC, apocynin reduced, though more effectively (~50%), Egr-1 and Fyn protein expression, providing a direct link between the Nox complex and Fyn expression (Fig. 26 A). To more directly implicate Nox-2 in the induction of Fyn, we next sought to determine the effects of p47phox inhibition on Fyn and Egr-1 expression. Genetic inhibition of p47phox also reduced Egr-1 and Fyn protein in a commensurate manner (Fig. 26 B). These results indicate that Nox, through p47phox, modulates Fyn expression downstream of EGFRΔIII signaling via redox-dependent induction of Egr-1 (Fig. 27) Thus, these findings provide a novel therapeutic means of influencing Fyn expression in EGFRΔIII-overexpressing GBM cells, which is important as Fyn inhibition reduces cell proliferation and survival in EGFRΔIII-overexpressing GBM cells (Fig. 9).

U87-EGFRΔIII

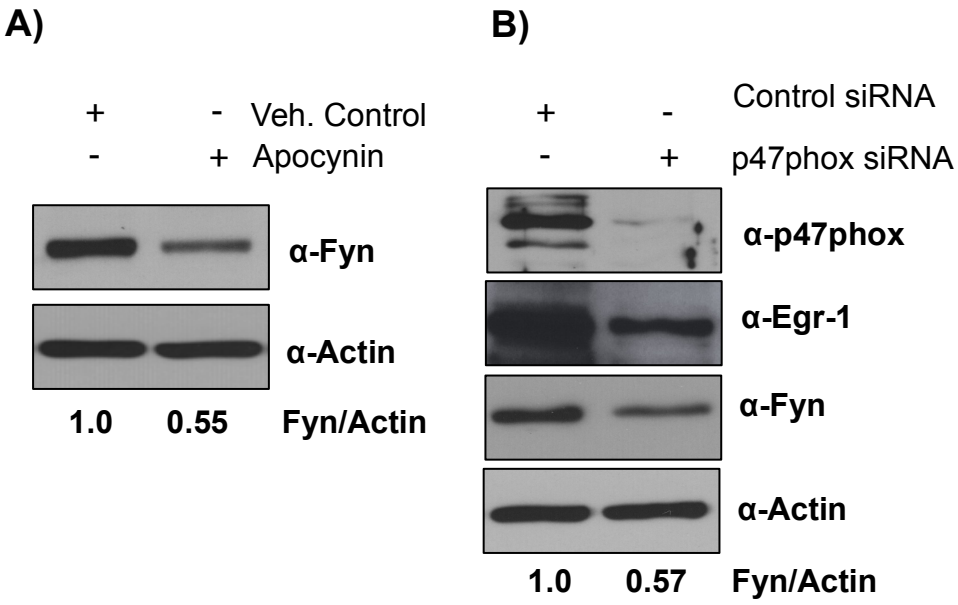


Figure 26. Targeting the NADPH oxidase through p47phox influences Fyn expression in EGFRΔIII-overexpressing GBM cells. (A) U87-EGFRΔIII cells were treated with apocynin (100 μM; 24 hrs.) or (B) transfected with either p47phox-specific siRNA or scrambled control. Resulting p47phox, Egr-1 and Fyn protein levels were evaluated at 48 hours post-transfection by immunoblotting. β-actin was shown as a loading control. Relative protein levels were determined by densitometry using ImageJ. Results are representative of at least three independent experiments.

4.3 Discussion and Future Directions

Elevated ROS content is commonly observed in cancer, particularly in the context of oncogene activation.[198] Importantly, oncogenic-induced ROS production has previously been described as a growth and survival-promoting factor in a variety of cancers, including GBM.[180-182,198] Recent findings have also demonstrated that EGFR activation increases markers of oxidative stress and positively correlates with therapeutic resistance in GBM.[180,183,184] EGFR is known to regulated Nox enzymes in phagocytes colon cancer[170,175]; however, their relationship in GBM remains unclear, but is of clinical value as recent studies have highlighted the potential of redox-targeted strategies in cancer therapy.[153] Given that oncogene overexpression is known to increased mitochondrial biogenesis[201], we first evaluated the impact of EGFR overexpression on respiratory bioenergetics. Here, we determined that both EGFRΔIII and EGFR-R108K elevate non-mitochondrial sources of respiration, but only EGFRΔIII elevated mitochondrial sources of respiration (Fig. 21). This is interesting, as EGFRΔIII and EGFR-R108K elevate ROS in a pronounced and commensurate manner, whereas wtEGFR only modestly elevates ROS (Fig. 19). These findings suggest a similar mechanism of EGFR-induced ROS production involving non-mitochondrial sources of respiration, but do not preclude potential mitochondrial involvement. Inhibitors of the Nox complex, however, more effectively reduced ROS content than rotenone, thus reinforcing a potential role for Nox-mediated ROS production in mutant EGFR-expressing GBM cells (Fig. 22). Notably, EGFRΔIII and EGFR-R108K but not wtEGFR also significantly elevated Nox

activity, which was effectively restored to a baseline by lapatinib in EGFRΔIII-expressing cells (Fig. 23). Collectively, these findings suggest a role for the Nox complex in ROS production downstream of EGFR signaling.

The Nox family of ROS producing complexes includes 5 isoforms, each of which is comprised of a unique set of subunits.[169] Involvement of the Nox complex has previously been reported in GBM, where increased expression, specifically Nox4, augments GBM cell growth and survival.[180-182] These studies, however, did not evaluate Nox status in the context of EGFR. Thus, we aimed to more closely examine the specific Nox isoform and/or subunit involved in EGFR-initiated ROS production. Here, we demonstrate a novel role for p47phox up-regulation in EGFR-overexpressing GBM cells (Fig. 24 A,B). EGFR-overexpression, however, showed no effects on Nox4 expression. Interestingly, these findings are corroborated by the TCGA dataset, which indicates that EGFR and p47phox gene expression show a strong tendency toward co-occurrence, whereas EGFR and Nox4 exhibit no association (data not shown). Though we did not evaluate the mechanism of EGFR-induced p47phox induction, this is important as p47phox knockdown reduces ROS levels, Nox activity and cell proliferation (Fig. 25). Thus, the molecular mechanism(s) of p47phox up-regulation downstream of EGFR should be further evaluated as a means of targeting p47phox expression in this setting.

Given that prior studies have implicated increased ROS in enhanced cell proliferation and survival[153,198], we next sought to determine the functional relevance of increased p47phox expression in this regard. Using EGFRΔIII-

overexpressing cells, our studies indicated that p47phox knockdown effectively reduces ROS content, Nox activity and cell proliferation rates as well as Egr-1 and Fyn expression (Fig. 25 A, B). Notably, our studies did not reveal any changes in cell survival despite reducing cell proliferation, which could be attributed to the robust manner by which EGFRΔIII increases BCL-xL expression.[59] Furthermore, recent reports indicate that oncogene-induced ROS production act primarily to induce aberrant cell proliferation.[198] Regardless, our findings show that p47phox inhibition is sufficient to reduce cell proliferation in U87-EGFRΔIII, which should further be evaluated mechanistically for the purpose of designing rational therapeutic combinations in this setting.

Though these studies placed ROS production downstream of p47phox expression, the impact of p47phox activation was not explored. This, however, is important as prior reports have demonstrated that Akt stimulates p47phox localization/activation via serine phosphorylation (Ser345) in human myeloproliferative disorders.[174] Given the preferential degree of Akt activation occurring in EGFR-overexpressing cells[89], the potential contribution of this mechanism should be further examined. This is important, as EGFR-overexpression, regardless of mutational status, significantly up-regulates p47phox, which could then be serine phosphorylated and activated downstream of various kinases implicated in RTK co-opting mechanisms of therapeutic resistance. Furthermore, though we did not evaluate the effect of Nox4 inhibition of ROS in EGFR-overexpressing cells, these studies are needed to better understand its potential for activation versus mere up-regulation in this context.

Our findings presented in this chapter provide novel evidence for EGFRΔIII-induced Fyn expression downstream of p47phox-mediated redox regulation of Egr-1 (Fig. 27). Collectively, these studies highlight a novel mechanism linking EGFR to the Nox complex and Fyn, providing compelling rationale for novel redox-targeted strategies in EGFR-overexpressing GBM.

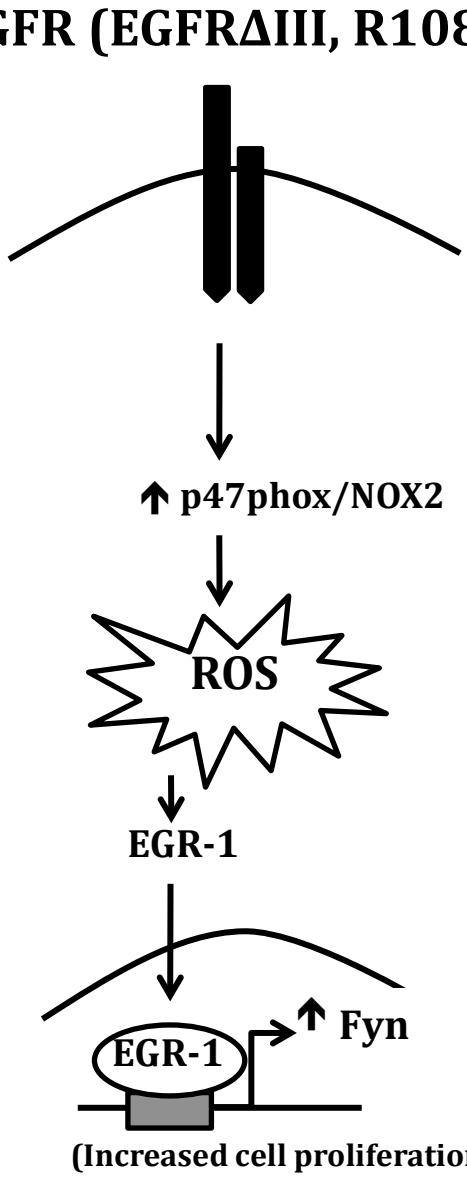


Figure 27. EGFR-overexpression increases Fyn expression via p47phox-mediated induction of Egr-1 in U87-MG cells. Working model indicating that Fyn expression downstream of EGFR is regulated, in part, by increased expression of integral Nox-2 organizer component, p47phox, through induction of the redox-responsive transcription factor Egr-1.

CHAPTER 5

CONCLUSIONS

5.1 Conclusions

Though increases in Fyn expression have previously been noted in GBM, the work presented in this dissertation is the first to connect Fyn expression to EGFR activation, specifically with regard to overexpression and hyper-activating mutation, EGFRΔIII and EGFR-R108K. The resulting increases in Fyn expression were biologically relevant, as Fyn knockdown significantly reduced cell proliferation and viability in EGFRΔIII and EGFR-R108K-overexpressing GBM cells (Figs. 9 & 10). We also demonstrated that Fyn expression is commonly up-regulated in a population of highly chemo- and radio-resistant GSCs (Fig. 12) and, furthermore, showed that siRNA knockdown of Fyn significantly lowered sphere formation in GSC 7-2 (Fig. 13). Therefore targeting Fyn expression, as opposed to activation, may represent a novel therapeutic strategy in EGFR-expressing GBM, as the clinical application of lapatinib and dasatinib exhibit only minimal therapeutic activity in this deadly disease.[7,8,14,15]

The transcription factor Egr-1 has previously been shown to implicated in the up-regulation of Fyn expression in CML[155] and, therefore, targeting Egr-1 expression may be an effective strategy for reducing Fyn expression. From these studies herein, we found that Egr-1 protein expression, much like Fyn, is significantly up-regulated by EGFR-overexpression in GBM cells, specifically in a manner concordant with EGFR kinase activation (Fig. 14). Interestingly, Egr-1 has previously been reported to behave as either a tumor suppressor or an oncogene depending on the cellular context.[157,158,197] Here, we determined that Egr-1 knockdown markedly reduced Fyn protein levels in EGFR-overexpressing

GBM cells (Fig. 17), notably to a degree not achieved by lapatinib in U87-EGFRΔIII and U87-R108K. These findings suggest that increased Egr-1 expression plays an oncogenic role in EGFR-overexpressing GBM, providing rationale for targeting Egr-1 expression in the regulation of Fyn expression in GBM.

The work presented in this dissertation also points to a novel means of regulating Fyn expression via EGFR-dependent induction of the integral Nox-2 organizer complex, p47phox, which up-regulates Egr-1 expression in a redox-responsive manner. Knockdown of p47phox, as well as treatment with the Nox inhibitor, apocynin, significantly reduced ROS content and Nox activity (Fig. 25) as well as Egr-1 and Fyn protein expression (Fig. 26) in U87- EGFRΔIII. Thus, our findings generate compelling interest in better understanding the physiological relevance of p47phox/Egr-1/Fyn pathway inhibition with regard to orthotopic tumor cell growth as well as animal survival in EGFR-expressing GBM. In vivo studies, however, aren't without their limitations, namely insufficient recapitulation of the highly immunoregulatory microenvironment present in human tumors, as immunodeficient mice are employed in xenograft models. Moreover, though the classic Nox inhibitors such as the ones employed in this study, apocynin and DPI, significantly reduce Nox activity, they unfortunately display a non-specific pattern of NOX-targeting consequently limiting their clinical utility.[202] More specific Nox-2 inhibitors, however, are currently under evaluation, including the p47phox binding peptide, Nox2ds-tat[203], providing promise for the clinical application of Nox-2-targeted strategies.[202] Collectively, these findings

indicate that targeting NOX-2 activation downstream of EGFR signaling by inhibition of p47phox represents a plausible means of reducing Fyn expression in EGFR-overexpressing GBM (Fig. 27).

CHAPTER 6

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