Quantitative Analysis of the EGFRvIII Mutant Receptor Signaling Networks in Glioblastoma

by

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II QUANTITATIVE ANALYSIS OF EGFRVIII CELLULAR SIGNALING NETWORKS REVEALS A COMBINATORIAL THERAPEUTIC STRATEGY FOR GLIOBLASTOMA

III QUANTITATIVE ANALYSIS OF SITE-DIRECTED MUTANTS OF EGFRVIII REVEALS A RECEPTOR COMPENSATORY MECHANISM AND A DEPENDENCE ON THE ERK SIGNALING PATHWAY

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ABSTRACT

Glioblastoma multiforme (GBM) is the most aggressive adult brain tumor and remains incurable despite multimodal intensive treatment regimens. EGFRvIII is a truncated extracellular mutant of the EGF receptor (EGFR) that is commonly found in GBMs and confers tumorigenic behavior. Although much work has been done over the past decade to elucidate pathways involved in EGFRvIII receptor signaling, the global map of the signaling networks that it activates remains incomplete, making it difficult to assess downstream components involved in EGFRvIII-mediated transformation. To gain a molecular understanding of the mechanisms by which EGFRvIII acts, we have employed a mass spectrometry-based phosphoproteomic approach to quantitatively map cellular signaling events activated by this receptor.

Using this approach, we have determined the major downstream pathways activated as a function of titrated EGFRvIII receptor levels. This analysis highlighted several aspects of EGFRvIII tumor biology, including crosstalk between EGFRvIII and other receptor tyrosine kinases. Specifically, we have identified the c-Met receptor as a co-target in the treatment of EGFRvIII positive GBM cells, and have shown that an EGFR and c-Met combination inhibitor strategy may be applicable in overcoming the poor efficacy of EGFR kinase inhibitor monotherapy in GBM patients. We then went on to investigate the mechanisms by which signaling networks are regulated in response to site-specific tyrosine mutations on EGFRvIII. This analysis has revealed a receptor compensation mechanism that is capable of restoring network architecture, upon the loss of a major tyrosine phosphorylation site on EGFRvIII. This is, to our knowledge, the first demonstration of signal compensation at the level of receptor phosphorylation and highlights an unexpected level of complexity within the signaling network. Our data also indicates that EGFRvIII fine-tunes the activity of the Erk pathway; some Erk activity is required for growth but excessive pathway activation results in cell death. We believe that the sensitivity to modulation of the Erk pathway may be exploited as a potential means of therapy for EGFRvIII positive tumors.

Taken together, our study highlights the utility of quantitative phosphoproteomic analysis as a tool to gain molecular insights in cancer biology and a means for drug target discovery.

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I INTRODUCTION

A Review of Glioblastoma Tumor Biology,

EGFR Signal Transduction and

Phosphoproteomic Technologies

I.1 GLIOBLASTOMA TUMOR BIOLOGY

I.1.1 Overview

Gliomas are a class of central nervous system tumors that affect 6.4 new patients per 100,000 population per year in the United States [Central brain tumor registry of the United States (CBTRUS): http://www.cbtrus.org]. They are generally classified according to their cell of origin and include astrocytomas, oligodendrogliomas and ependymomas. Gliomas are further subdivided into different grades by the World Health Organization (WHO) based on histological features and extent of malignant behavior (Louis et al. 2007). Grade I tumors such as pilocytic astrocytomas are benign, circumscribed and can be cured by surgical resection of the localized tumor. Grade II tumors consists of low-grade astrocytomas that have undergone some infiltration into the brain. These tumors are generally not curable by surgery and patients may undergo long clinical courses. Grade III tumors are known as anaplastic or malignant gliomas. Patients with grade III tumors have a media overall survival of 2-4 years for anaplastic astrocytomas and 3-5 years for anaplastic oligodendrogliomas (Omuro et al. 2007). Grade IV tumors rank as one of the most aggressive forms of adult cancers and are known as glioblastoma (GBM). This tumor type afflicts 2.96 new patients per 100,000 population per year in the United States [(CBTRUS): http://www.cbtrus.org]. GBMs exhibit the following characteristics: abundant neovascularisation, high proliferation and mitotic activity, necrosis and genetic instability (Furnari et al. 2007). In addition, a hallmark feature of these lesions is

the formation of highly invasive, microscopic infiltrate that penetrate into the surrounding white matter. Due to these characteristics and the unique challenges associated with its location in the central nervous system, GBMs are particularly difficult to treat and almost always result in a poor prognosis. The median overall survival for GBM patients is 12 months with 2-year survival rates of less than 10% (Stupp et al. 2005) .

There are two distinct routes by which GBMs arise in patients (Ohgaki and Kleihues 2007). The majority of patients (>90%) develop *de novo* tumors with no prior clinical history of disease. The average time from the appearance of first symptoms to diagnosis is 6 months (Ohgaki et al. 2004; Ohgaki and Kleihues 2005). This subtype is known as primary GBM and occurs mainly in older patients (mean age of 62 years old) (Ohgaki and Kleihues 2007). A second, less common subtype of GBM arise from pre-existing lower grade astrocytomas within 5 – 10 years of the initial diagnosis (Furnari et al. 2007). This form of the disease is known as secondary GBMs and is primarily observed in patients below the age of 45 years (Ohgaki et al. 2004; Ohgaki and Kleihues 2005). Due to distinct paths taken in the development of disease, it is perhaps not surprising that the genetic makeup of these tumor subtypes is dramatically different. For instance, primary GBMs are prone to amplification and mutation of the epidermal growth factor receptor (EGFR) gene while the p53 tumor suppressor gene is often intact (Watanabe et al. 1996; Ohgaki et al. 2004). Secondary GBMs however, are thought to arise from genetic lesions that accumulate as the tumors progress towards malignancy. These include p53 mutations and platelet-derived

growth factor receptor (PDGFR) amplifications (Hermanson et al. 1992; Hermanson et al. 1996; Watanabe et al. 1997; Ohgaki et al. 2004). In addition, a recent comparative genomic hybridization (aCGH) study has identified many shared common lesions found to be present in both subtypes, such as the loss of the 9p and 10q chromosomal loci where the $p16^{Ink4a}$ and PTEN genes reside respectively (Maher et al. 2006). Intriguingly, this study further classified two genetically distinct subclasses of secondary GBMs which could be distinguished by differences in time to tumor progression. This stratification would suggest that the development of secondary GBMs may progress via two different genetic paths. It is important to note however, that while the molecular pathways by which primary and secondary GBMs arise are dissimilar, the resultant tumors are histologically indistinguishable and the overall median survival (from the time of GBM diagnosis) is statistically identical (Maher et al. 2001). Understanding how these different genetic aberrations modulate cellular pathways and influence tumor outcomes will be critical in the development of the appropriate treatment protocols for each of these distinct subtypes.

I.1.2 Genetic alterations in glioblastoma

Over the past two decades, multiple histopathology studies and classical genetic experiments, together with more recent cancer genome-wide studies, have uncovered a catalog of genes commonly found to be dysregulated in GBM tumors. Table I.1 shows a list commonly dysregulated genes and their frequency in GBMs. In addition, CGH analyses of GBMs have revealed regions of the

chromosome (and the potential oncogenes and tumor suppressors that are localized to these regions) which have recurrent copy number changes (Maher et al. 2006). Many of these genes have since been functionally validated in both culture-based systems and animal models and shown to be critical drivers of GBM tumorigenesis. In this section, I will elaborate on the genetic lesions commonly found in primary GBMs, the focus in my thesis. Specifically, I will discuss alterations in the cell cycle and PI3-K-PTEN pathways and leave the discussion on EGFR alterations in primary GBMs to section I.2 on EGFR signaling.

Gene	Primary GBM Frequency	Secondary GBM Frequency
EGFR overexpression	63%	10%
PTEN mutations	25%	4%
p16 deletion	31%	19%
p53 mutations	28%	65%
RB promoter methylation	14%	43%

Table I.1. Summary of commonly dysregulated genes in glioblastoma. A summary of genes that are commonly dysregulated in primary and secondary glioblastoma, adapted from (Ohgaki and Kleihues 2007).

I.1.2.1 Cell cycle pathways

The predominant cell cycle genetic lesions frequently associated with primary GBMs modulate the cell cycle via dysregulation of the Rb and p53 pathways. While mutations in both Rb and p53 themselves are rare in primary GBMs (Ohgaki and Kleihues 2007), loss of function of these tumor suppressors

is often achieved through functionally equivalent inactivation/amplification of important regulators of the two critical pathways (Figure I.1).

a. The Rb pathway

The retinoblastoma (Rb) protein regulates entry into the cell cycle via its ability to bind to and inactivate the E2F transcription factor. In resting G_0 cells,

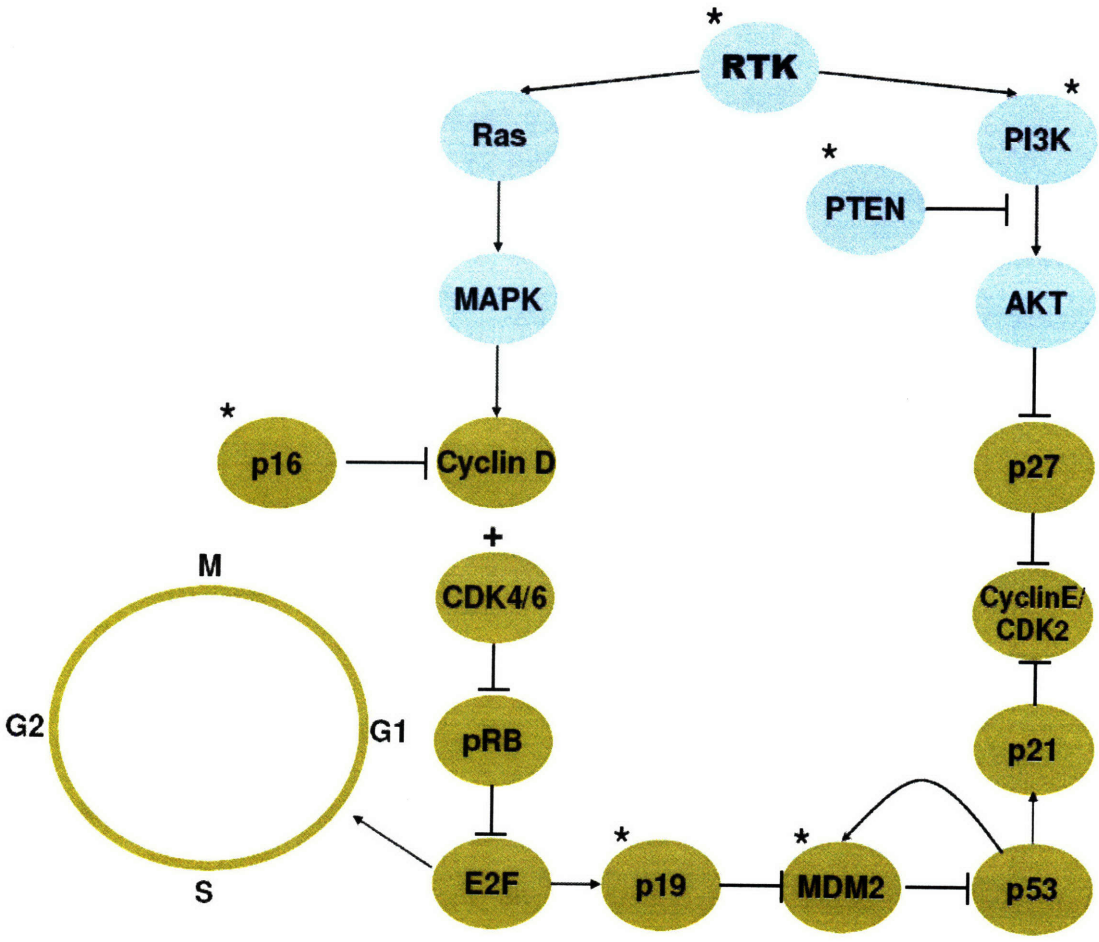

Figure I.1. Pathways Important in GBM tumor biology. Integration between receptor tyrosine kinase (RTK) and cell cycle pathways. RTKs such as EGFR signal through the MAPK and PI3K pathways (blue). This leads to the activation of the cell cycle components (green) via the Rb pathway. Components of this pathway which are commonly mutated in GBM tumors are indicated with an *. Modified from (Maher et al. 2001).

Rb is hypophosphorylated and binds to E2F via its transactivation domain, thus preventing the transcription of genes required for entry into S phase. This complex also directly binds to E2F-responsive promoters and actively represses transcription through the recruitment of histone modifying enzymes (Trimarchi and Lees 2002). Upon receiving mitogenic signals, expression of the D-type cyclins is induced, in response to activation of the Ras/Raf/MEK/Erk pathway. The newly synthesized cyclin D1 then associates and activates the cyclin dependent kinases (CDK) 4 and 6 (Sherr and Roberts 2004). Both the cyclin D1-CDK4/6 and the subsequently activated cyclin E-CDK2 complexes are required for the hyperphosphorylation of Rb in the late G1 phase, releasing E2F for transcription of a large number of genes required for progression into S phase (Trimarchi and Lees 2002). This phosphorylation of Rb is maintained throughout the process of cell division by the activity of cyclin A and B-CDK complexes.

The CDKs are negatively regulated by family of proteins known as the CDK inhibitors (CKIs). CDK4/6 are specifically inhibited by a class of CKIs known as INK4 proteins. These include $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4a}$ and $p19^{Ink4d}$ which directly bind to CDK4/6, preventing association with cyclin D1 (Sherr and Roberts 1999). $p16^{Ink4a}$ is a transcript of the CDKN2A gene and is found to be inactivated in 50-70% of primary GBMs by allelic loss or gene silencing through promoter methylation (Jen et al. 1994; Schmidt et al. 1994; Merlo et al. 1995; Costello et al. 1996). While $p16^{Ink4a}$ loss is important in the progression of disease, it has previously been shown that inactivation of this pathway in itself is not sufficient for transformation to occur. Specifically, $p16^{Ink4a}$ deletion induces

tumor formation in a mouse model of glioma and dedifferentiation in astrocytes only in combination with EGFR activating mutations (Holland et al. 1998; Bachoo et al. 2002). Along these lines, it has recently been discovered in an aCGH study on a panel of primary GBMs, that a subset of tumors harbored concurrent deletion of both CDKN2A and CDKN2C (which encode $p16^{Ink4a}$ and $p18^{Ink4c}$ respectively) (Wiedemeyer et al. 2008). The authors went on to demonstrate that while a single loss of $p16^{Ink4a}$ is insufficient to drive tumorigenesis, the combined loss of $p16^{Ink4a}$ and $p18^{Ink4c}$ cooperate to enhance tumorigenesis in murine xenograft models. Loss of $p16^{Ink4a}$ in immortalized astrocytes also led to an increase in the expression of $p18^{Ink4c}$. This data suggests that the increased $p18^{Ink4c}$ expression could act as a compensatory mechanism to maintain cell cycle regulation upon loss of $p16^{Ink4a}$, and that the deletion of both CKIs is required for cellular transformation.

b. The p53 pathway

The p53 tumor suppressor protein is an important regulator of cellular response to genotoxic stress. Under normal cellular conditions, low levels of p53 are maintained by the binding of MDM2 through two mechanisms. MDM2 binds to the N-terminal transactivation domain of p53, directly preventing transcriptional activation of p53-responsive genes. In addition, MDM2 is an E3 ubiquitin ligase that catalyses the polyubiquitination of both p53 and itself via its RING domain, directing both proteins from the nucleus to the cytoplasm to undergo proteosome-mediated degradation (Coutts and La Thangue 2007; Clegg et al.

2008). Cells subjected to genotoxic stresses such as DNA damage, respond by initiating a hierarchy of signals that propagate from sites of DNA damage to effector proteins that drive diverse cellular processes required to repair the insult. These responses include transient cell cycle arrest to allow time for DNA repair, expression of the appropriate DNA repair enzymes and, if the lesion is irreparable, the decision to commit the cell into apoptosis (Shiloh 2006). In the case of DNA damage, members of the PI3K-like protein kinase (PIKK) family of serine/threonine protein kinases play a particularly important role in the phosphorylation and stabilization of p53 (Shiloh 2006). A series of phosphorylation events on multiple serine and threonine sites on p53 causes the dissociation of MDM2 from p53 (Pluquet and Hainaut 2001; Bakkenist and Kastan 2004; Rodier et al. 2007). These events result in the exposure of multiple lysine residues which subsequently become acetylated, dramatically increasing p53 stability (Lavin and Gueven 2006). The increase in p53 protein levels result in the regulation of ~2500 p53-responsive genes. The gene encoding the MDM2 protein is itself under the regulation of p53, resulting in a negative feedback loop between p53 activity and MDM2 protein levels (Levine et al. 2006).

MDM2 is negatively regulated by the alternative spliced product of the CDKN2A gene, $p14^{ARF}$ (alternative reading frame). $p14^{ARF}$ binds to MDM2 and prevents p53 degradation via sequestering of MDM2 to the nucleolus, and stops p53 from being exported from the nucleus and being degraded in the proteosome (Kamijo et al. 1997; Pomerantz et al. 1998). Although p53 is one of the most commonly mutated proteins found in tumors, mutations in this gene are rarely

found in primary GBMs (Furnari et al. 2007; Ohgaki and Kleihues 2007). Rather, dysregulation of the p53 pathway in GBMs seems to be achieved through the alterations of MDM2 and $p14^{ARF}$. The chromosome region 12q14.3 – q15 that contains the MDM2 gene was found in one study to be amplified in 10% of primary gliomas that concurrently lacked mutations in the p53 gene (Reifenberger et al. 1994). As discussed in the previous section, the CDKN2A gene is lost in 50-70% of gliomas and this occurs either though homozygous deletion of the gene or silencing of the $p14^{ARF}$ via promoter methylation (Nakamura et al. 2001). Similar to the $p16^{Ink4a}$, genetic lesions in the p53 pathway are insufficient to drive transformation alone; $p19^{ARF}$ (mouse ortholog) null mice only form low grade gliomas at low penetrance and may require a second genetic alteration to develop high grade GBMs (Kamijo et al. 1999).

I.1.2.2 PI3K-PTEN phosphoinositide signaling pathway

In response to growth factor signaling, the class Ia phosphoinositide 3-kinases (PI3K) are activated to convert plasma membrane phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$) to phosphatidylinositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$] (Cantley 2002). This class of PI3K enzymes is composed of 2 subunits, a regulatory subunit, p85, and a catalytic subunit, p110. Binding of p85 through the SH2-mediated recognition of the pYXXM motif on growth factor receptors leads to the activation of the p110 subunit (Rordorf-Nikolic et al. 1995). This formation of $PI(3,4,5)P_3$ triggers the recruitment of a number of critical signaling proteins via direct lipid binding to the pleckstrin homology (PH) domain,

including kinases PDK1 and Akt (Cantley 2002). PDK1 phosphorylates Akt on T308. Phosphorylation of Akt on a second site (S473) by the mTORC2 complex is required for full kinase activity (Sarbassov et al. 2005). Akt phosphorylates a large number of substrates at the consensus RxRxxS/T motif (Alessi et al. 1996). This leads to, in a majority of cases, the binding to and inhibition of substrate function by the 14-3-3 family of proteins. These substrates play important roles in the regulation of cell survival (BAD, FOXO transcription factors), growth (TSC2, PRAS40), cell cycle (p27 and p21 CKIs) and metabolism (GSK3) (Manning and Cantley 2007). There are 3 isoforms of p110 encoded by the PIK3CA, PIK3CB and PIK3CD genes (Hawkins et al. 2006). The PIK3CA is found to be mutated in between 5-15% of GBM tumors, some of which have been shown to be oncogenic point mutations (Broderick et al. 2004; Hartmann et al. 2005; Gallia et al. 2006).

The PI3K pathway is negatively regulated by phosphophoinositide phosphatases, including Src-homology 2-containing phosphatases (SHIP-1 and -2) and phosphatase and tensin homolog (PTEN) (Cantley 2002). The former converts $PI(3,4,5)P_3$ to $PI(3,4)P_2$ while the latter converts $PI(3,4,5)P_3$ to $PI(4,5)P_2$. PTEN has also been shown to have protein tyrosine phosphatase activity on the focal adhesion kinase (FAK) protein (Tamura et al. 1998). PTEN is a tumor suppressor and is mutated or deleted in a variety of cancers, including 50% of GBMs (Furnari et al. 2007; Ohgaki and Kleihues 2007). Loss of PTEN upsets the PI3K-PTEN balance and results in elevated $PI(3,4,5)P_3$ and active Akt levels, driving uncontrolled growth and survival. Restoring functional PTEN

expression in PTEN null GBM cells dramatically suppressed the growth of these cells (Furnari et al. 1997). Furthermore, it was determined that growth suppression required the intact phosphoinositide phosphatase activity of PTEN. Inactivation of the protein tyrosine phosphatase activity, however, had no effect on growth (Furnari et al. 1998). The strong dependence of PTEN-null GBM tumors for the PI3K pathway makes targeting this pathway therapeutically attractive. The use of such targeted therapeutics in combination with EGFR kinase inhibitors will be discussed in section I.2.5.

I.1.3 Current therapeutic strategies for glioblastoma

Unlike many of the advances seen in the clinical management of other malignant tumor types such as breast, lung or colon cancer, treatment regiments for GBM have lagged and remained unchanged in the past 20 years. Conventional treatment involves surgical resection of the bulk tumor mass followed by radiation therapy (Omuro et al. 2007). Radiotherapy is usually achieved by administering an accumulated dose of 60Gy using external ion beam irradiation or focused irradiation using stereotactic radiosurgery specifically on the localized region of the tumor (Leibel and Sheline 1987; Loeffler et al. 1992; Shrieve et al. 1999). These treatment protocols have unfortunately not increased the median overall survival of GBM patients past 12 months. The dismal prognosis is due in part to the almost certain recurrence of the tumor after surgical resection, primarily as a result of radio-resistant diffuse infiltrate that remain in the brain following surgery (Furnari et al. 2007).

Chemotherapy may be administered during and after radiation therapy as an adjuvant. A challenge in the development of therapeutics for glioblastomas lies with the inability of many chemotherapeutics to cross the blood-brain-barrier (BBB) and achieve drug levels in the brain required for clinical efficacy. A number of cytotoxics have been evaluated in clinical trials in GBM patients including nitrosourea compounds such as carmustine (Deutsch et al. 1989), as well as combination protocols, with the procarbazine, lomustine (CCNU) and vincristine (PCV) combination being the most common (Levin et al. 1990; Levin et al. 1995). In addition, radiosensitizers such as BrdU have also been evaluated for their ability to enhance the effects of radiotherapy (Groves et al. 1999). While these treatment protocols have been successful in increasing the life expectancy of anaplastic glioma patients (WHO grade III), they have had little survival benefit to GBM patients (Prados et al. 2004; van den Bent et al. 2006).

In 1999, temozolomide (TMZ) was approved by the FDA for the treatment of malignant gliomas (Friedman et al. 2000). TMZ is an oral alkylating agent that is capable of penetrating the BBB (Patel et al. 2003). Its mechanism of action involves the formation of methylated DNA adducts, primarily at the N7 position of guanine, the O3 position of adenine and the O6 position of guanine (Denny et al. 1994). A recently developed protocol (Stupp regimen) in which newly diagnosed GBM patients underwent surgical resection of the tumor and were then treated with either radiation alone or concomitant TMZ with radiotherapy and subsequent administration of TMZ for 6 weeks as an adjuvant (Stupp et al. 2005). The latter group of patients showed an increase in median overall survival from 12 to 15

months and a two-year survival rate from 10% to 27% when compared to treatment with radiation alone. Interestingly, there was a strong correlation between patient response and epigenetic silencing of the O^6-methylguanine–DNA methyltransferase (MGMT) gene promoter, the gene that specifically removes the O6-methyguanine adduct (Hegi et al. 2005; Stupp et al. 2005). This observation indicates that methylation of the MGMT promoter may serve as a biomarker for predicting response to TMZ in GBM patients.

In summary, although the current standard of care has shown a modest clinical benefit, the prognosis of GBM patients remain poor. The dearth of treatment options suggests that this disease may benefit from new approaches to understanding the molecular mechanisms of GBM initiation and progression as well as to guide the selection of new molecular targets for therapeutic development.

I.2 THE EPIDERMAL GROWTH FACTOR RECEPTOR FAMILY

I.2.1 Overview of the EGFR family

The ErbB/EGFR receptor family is the prototypical member (type I subclass) of the large super-family of receptor tyrosine kinases (RTKs) (Hynes and Lane 2005). They were originally named for their sequence homology to a gene product encoded by the avian erythroblastosis tumor virus (*v-erbB*) (Downward et al. 1984). In mammals, the ErbB/EGFR family consists of four members, ErbB1/Her1/EGFR, ErbB2/Her2/neu, ErbB3/Her3 and ErbB4/Her4 that seemed to have diverged from a common ancestral gene (Hynes and Lane 2005; Citri and Yarden 2006) . Together, these four receptors transmit extracellular cues in the form of 13 different ligands into signals that control various components of the intracellular machinery (Citri and Yarden 2006) (Figure I.2). Depending on the nature of the ligand and the cognate receptor to which it binds, members of the ErbB/EGFR family mediate a number of important cellular processes, including cell division, migration, adhesion, differentiation and apoptosis (Yarden and Sliwkowski 2001). Due to the many fundamental cell decision processes that are regulated by the EGFR family of receptors and ligands, dysregulation of these components often leads to disease, with cancer being the most prevalent (Hynes and Lane 2005). In this section, I will give an overview of the EGFR family of receptors and the roles that they play in both physiology and pathology, with a focus on EGFR and GBMs.

I.2.1.1 EGFR family of ligands

There are 13 known ligands for EGFR family that are characterized by the presence of an EGF-like domain, which consists of a consensus sequence of six conserved cysteine residues that come together to form three disulphide-bonded intramolecular loops (Harris et al. 2003; Singh and Harris 2005). This motif is critical for the binding to the EGFR family of proteins. In addition to the EGF-like domains, these ligands contain an N-terminal region, a membrane proximal region, a lipophilic transmembrane region and a cytoplasmic tail (Harris et al. 2003). Two members of the ligand family, heparin binding epidermal growth factor (HB-EGF) and amphiregulin (AR), additionally contain an N-terminal heparin binding domain. In order to engage their cognate receptors, these type I transmembrane glycoproteins are processed into mature soluble factors by the action of a number of matrix metalloproteinases that cleave the protein at multiple sites, in a process known as ectodomain shedding (Fischer et al. 2003; Lee et al. 2003). There are multiple modes by which the EGF ligands mediate receptor binding and this includes, paracrine signaling where ligands are released from one distal cell type to another, autocrine signaling where the same cell secretes its own ligand and juxtacrine signaling, where receptor–ligand complexes form at specific cell-cell contact points (Singh and Harris 2005). Each of these different modes of receptor engagement has been shown to result in distinct signaling and cellular outcomes (Singh and Harris 2005; Joslin et al. 2007).

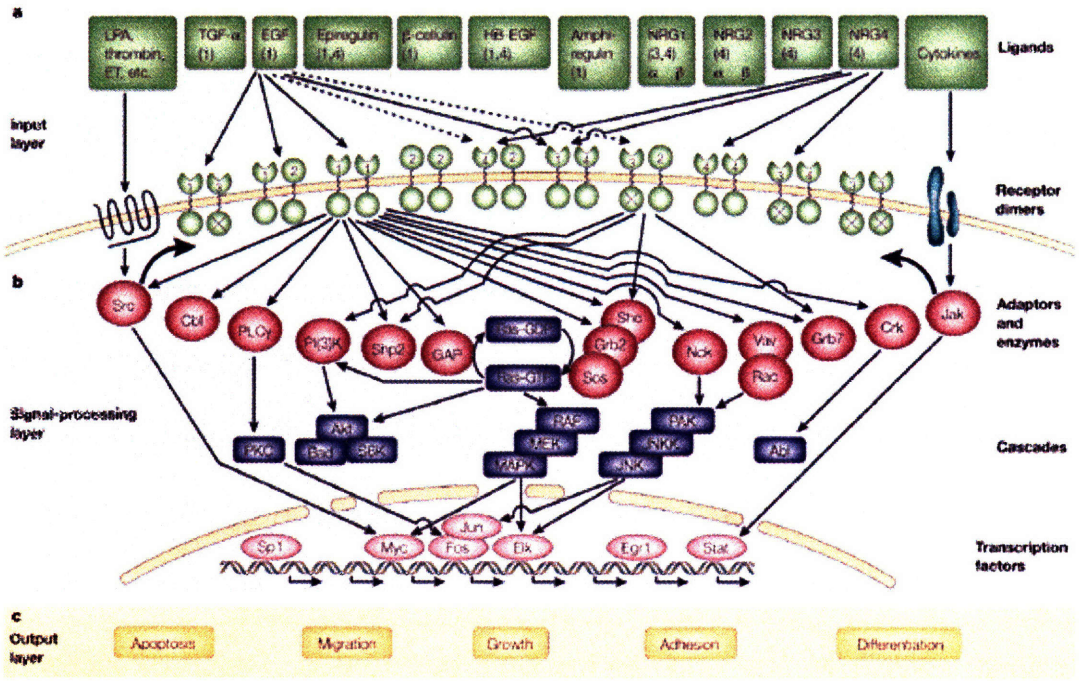

Figure I.2. The ErbB family signaling network. (A) The imput layer is composed of the cues (EGF family of ligands) and the cognate receptors that transduce signals into the cell. Both EGFR and ErbB4 are capable of autonomous signaling and have intact ligand binding and catalytic domains. ErbB2 is unable to bind ligand but is able to form homodimers and heterodimers with the other members of the ErbB family. ErbB3 has an inactive kinase domain and required heterodimer interaction with another member of the ErbB family, most commonly with ErbB2. (B) Signal is transduced and integrated in the signal processing layer via a variety of downstream signaling components. The nature of signaling depends on the type of receptor dimers that form on the cell surface. (C) The ouput layer is composed of cellular phenotypes that are activated in response to upstream signaling pathways in the signal processing layer. (Abl, Abelson murine leukemia viral oncogene homolog; Akt, v-akt murine thymoma viral oncogene homolog; GAP, GTPase activating protein; HB-EGF, heparin-binding EGF; Jak, Janus kinase; PKC, protein kinase C; PLCγ, phospholipase Cγ; shp2, Src homolog domain 2-containing protein tyrosine phosphatase 2; Stat, signal transducer and activator of transcription; RAF-MEK-MAPK cascade regulates the activity of a number of transcription factors). Reprinted with permission from Macmillan Publishers Ltd: (Yarden and Sliwkowski 2001) Nature Reviews Molecular Cell Biology **2**: 127-137. Copyright 2001.

The EGF family of ligands is further classified into 3 categories based on the degrees of promiscuity in ErbB receptor binding. The first group of ligands binds exclusively to EGFR and includes EGF, transforming growth factor α (TGFα), epigen and AR (Harris et al. 2003; Hynes and Lane 2005) (Figure I.2). The second group of ligands binds to both EGFR and ErbB4 and includes HB-

EGF, epiregulin and betacellin (Beerli and Hynes 1996; Elenius et al. 1997). Finally, the neuregulins (NRG) bind to either both ErbB3 and ErbB4 or ErbB4 exclusively and consists of NRG1-4 and toporegulin (Stove and Bracke 2004; Esper et al. 2006). It is thought that there is some possible redundancy between the members of the EGF family of ligand and evidence for this comes from knockout mouse studies. EGF and AR null mice had no apparent phenotype, while TGFα null mice had abnormal skin and hair development (Mann et al. 1993; Luetteke et al. 1999). This result is in contrast to the EGFR null mice which are often embryonically lethal or die at birth with severe developmental defects in multiple organs, such as the lung, pancreas and gastrointestinal tract (Miettinen et al. 1995; Threadgill et al. 1995). It is only in the EGF, AR, and TGFα triple knockout does abnormalities in the gastrointestinal tract start to be apparent (Troyer et al. 2001). Specific differences between the signaling outcomes of these ligands remain to be elucidated and are an active area of research.

I.2.1.2 Receptor structure and mechanisms of action

The EGFR receptor family shares a general domain organization in which an extracellular ligand binding region is linked via a hydrophobic transmembrane domain to a cytoplasmic region that contains both a tyrosine kinase domain and C-terminal tail (Wells 1999). Upon ligand binding, members of the EGFR family undergo receptor dimerization (Ogiso et al. 2002; Ferguson et al. 2003), tyrosine kinase activation and trans-phosphorylation across receptor dimers on multiple

tyrosine residues in the cytoplasmic tail (Wells 1999; Hynes and Lane 2005). In this manner, the EGFR family of proteins can be thought of as allosteric enzymes where the binding of the ligand to one part of the protein leads to a conformational change and activation of catalytic activity in another region of the protein (Bublil and Yarden 2007). In response to receptor phosphorylation, multiple effector proteins are recruited to the receptor via recognition and binding to SH2 and PTB domains (Jones et al. 2006). The formation of this signaling complex results in the propagation of a number of downstream signaling pathways and a multitude of cellular responses (Yarden and Sliwkowski 2001) (Figure I.2). Receptor tyrosine phosphorylation also initiates important negative regulation mechanisms such as receptor internalization and degradation (Dikic 2003). Some of these downstream cellular signaling pathways will be further elaborated upon in section I.2.4.

EGFR and ErbB4 are autonomous in that they have all of the intact domains required to form homodimers upon binding to ligand. ErbB2 and ErbB3 on the other hand, are non-autonomous and require heterodimerization with other members of the ErbB receptor family in order to be functionally active (Bublil and Yarden 2007). The ErbB2 receptor lacks a functional ligand binding domain and relies on heterodimerization with other ligand-bound ErbB family members, most prominently EGFR and ErbB3 (Klapper et al. 1999; Garrett et al. 2003). Conversely, ErbB3 lacks a functional kinase domain and preferentially forms a heterodimer with ErbB2 in order to potentiate downstream signaling pathways (Guy et al. 1994; Sierke et al. 1997). In this manner, the ErbB receptor

system has encoded both diversity and specialization in function where each homo-/hetero-dimer that is formed has a different signaling outcome and biological response. This complexity, layered on to the diversity of ligands available for binding to these receptors, has enabled the ErbB receptor family to mediate a wide variety of cellular functions in a context-dependent manner.

Recent structural information on the ErbB receptor family of proteins has provided insights into the mechanistic basis of receptor dimerization and kinase activation (Figure I.3). The extracellular region of the prototypical member of the ErbB family, EGFR consists of 4 domains (I-IV) (Garrett et al. 2002; Ogiso et al. 2002). Crystal structures of the ectodomains of EGFR, ErbB3 and more recently ErbB4 revealed that EGFR exists in two distinct conformations (Cho and Leahy 2002; Garrett et al. 2002; Ogiso et al. 2002; Ferguson et al. 2003; Bouyain et al. 2005). There is a closed, inactive conformation in which intramolecular interactions between domain II and IV tether each other to prevent domains I and III from coming together to form the ligand-binding site (Cho and Leahy 2002; Bouyain et al. 2005) (Figure 1.3A). This conformation is in equilibrium with an open active state of the receptor (Dawson et al. 2005; Ozcan et al. 2006). In contrast to other RTKs, the ligand binding pocket points away from the dimerization interface. In this open conformation, domains II and IV move away from domains I and III, resulting in not only in the formation of the ligand binding pocket but also exposes the dimerization loop in domain II for interaction with the identical domain of another EGFR molecule to form the homodimer (Garrett et al. 2002; Ogiso et al. 2002) (Figure I.3B). In the absence of ligand, the

equilibrium shifts to favor the closed conformation (95% of the receptor population). However, ligand binding stabilizes the open conformation and shifts the equilibrium, allowing for the accumulation of active homodimers and receptor signaling (Dawson et al. 2005; Ozcan et al. 2006). Crystal structures of the ErbB2 ectodomain indicate that this receptor in locked in the open conformation and is poised to form heterodimers with other ligand-bound ErbB receptor members, despite lacking intrinsic ligand binding capability (Cho et al. 2003; Garrett et al. 2003). It is probably this reason that enables ErbB2 constitutive homodimers to form in ErbB2 overexpressing breast cancer cell lines and mouse models (Hynes and Lane 2005).

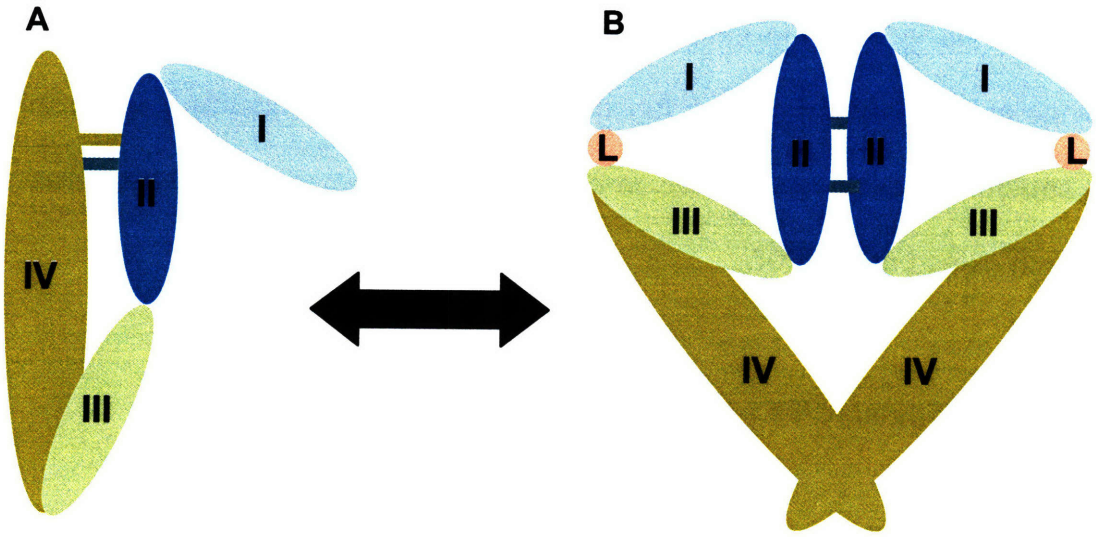

Figure I.3. Domain organization of unliganded and liganded EGFR extracellular domain. (A) Unliganded EGFR extracellular domain with tethering between domains II and IV. (B) Dimerized EGFR extracellular domains in complex with EGF. Apdapted from (Dawson et al. 2005).

X-ray crystallography has also revealed for the first time the mechanism of kinase activation. Similar to the extracellular domain, the kinase domain can exists as an inactive or active form. Comparisons of the crystal structures of both forms demonstrate that similar to the stabilization of CDKs by cyclins, an asymmetrical dimer is formed in the active conformation where the C-terminal lobe of one EGFR monomer kinase domain juxtaposes and stabilizes the active conformation of the other EGFR monomer (Stamos et al. 2002; Zhang et al. 2006). This asymmetrical complex allows the presentation of the cytoplasmic tail of the inactive EGFR monomer to the active kinase domain of the stabilized monomer, exposing the relevant tyrosine sites for transphosphorylation and activation of downstream signaling cascades. As will be discussed in a section I.4, mutations in both the extracellular and cytoplasmic domains of EGFR that impinge on receptor activity and function are prevalent in cancer and establishing the structure-activity relationship in this mutations remains an active area of research (Riese et al. 2007).

I.2.2 Physiological roles of EGFR in the central nervous system

The epidermal growth factor receptor plays an important physiological role in the development of multiple organs in mammals. For the purposes of this thesis, I will give a brief overview of the functions of EGFR in central nervous system (CNS) development. Much of the previous research in the field has focused on the role of the neuregulins and their interaction with ErbB2/ErbB3 in the development and maintenance of the CNS (Murphy et al. 2002). However,

EGFR signaling also plays an important role in CNS development, particularly in astrocytes. While EGFR expression is absent in mature astrocytes (Gomez-Pinilla et al. 1988), EGFR and its ligands are highly expressed in the developing CNS with EGFR expression coinciding with gliomagenesis in the embryonic and perinatal period (Burrows et al. 1997; Kornblum et al. 1997; Tropepe et al. 1999). Specifically, the cell fate of neural progenitor cells appears to be dependent on the extent of EGFR activation. For instance, there is evidence that EGFR signaling is required to maintain neural progenitor cell proliferation and dedifferentiation. In particular, neural progenitor cells that are transplanted into the brain in the presence of exogenously infused EGF remain undifferentiated and continue to proliferate rather than differentiate into astrocytes (Fricker-Gates et al. 2000). This observation has also been made in Ink4A deficient astrocytes which undergo dedifferentiation in the presence of EGFR activation (Bachoo et al. 2002).

There is also a body of evidence that suggests that high EGFR activation drives the differentiation of neural progenitor cells into astrocytes (Burrows et al. 1997). In addition, lineage dependence appears to be driven by EGFR expression levels, where daughter cells that express high EGFR levels commit to become astrocytes while daughter cells expressing low EGFR levels form oligodendrocytes (Sun et al. 2005). The regulation of EGFR activity and levels in the brain is critical for brain and spinal cord development as astrocytes provide a supportive role for the survival of neurons. Along these lines, EGFR null mice that survive postnatally have reduced astrocyte levels and apoptotic neurons

(Kornblum et al. 1998). Furthermore, the *waved 1* and *waved 2* mouse mutants which contain TGFα and EGFR aberrations, respectively, also show decreased levels of astrocytes in the brain (Luetteke et al. 1993; Luetteke et al. 1994). It is likely that EGFR signaling plays a role in both neural progenital cell proliferation and differentiation into astrocytes, with biological outcome depending on the level EGFR activation and the stage of CNS development in which activation occurs.

Upregulation of EGFR in mature astrocytes of the adult CNS has been found to occur in response to pathological conditions such as brain injury, neurodegeneration or in tumor progression such as primary GBMs (Birecree et al. 1988; Ekstrand et al. 1992; Ferrer et al. 1996; Planas et al. 1998; Jin et al. 2002). As described previously, EGFR pathway activation results in a myriad of cellular phenotypes including increased cell proliferation, migration and adhesion. It is not clear if the upregulation of EGFR in response to neural injury is primarily to increase the proliferation and migration of existing adult astrocytes or to revert them to a more dedifferentiated phenotype which may be beneficial in the context of neuronal repair (Liu and Neufeld 2007). Interestingly, these reactive EGFR expressing adult astrocytes were shown to be destructive in a rat model of optic neuropathy where the addition of the EGFR kinase inhibitor, AG1478, led to regeneration of retinal ganglion cells and optic nerve fibers (Liu et al. 2006). This discovery would suggest that several of the EGFR targeted therapeutics that are currently in development for oncology may have potential application for neural regeneration after cerebral injury.

I.2.3 EGFR and primary glioblastoma

I.2.3.1 Overview of EGFR mechanisms of tumorigenesis in GBMs

a. Amplification of the EGFR gene

Dysregulation of EGFR cellular signaling has been associated with poor prognosis in a large number of tumor types including breast cancer, head and neck cancer, prostate cancer, non-small cell lung cancer (NSCLC) and GBMs (Chaffanet et al. 1992; Ekstrand et al. 1992; Turner et al. 1996; Verbeek et al. 1998; Todd and Wong 1999; Sharma et al. 2007). There are multiple mechanisms by which EGFR mediates tumor initiation and progression, all of which have been found to occur in primary gliomas. Upregulation of EGFR levels is common found in primary GBMS and can occur by amplification and/or increased translation of the EGFR gene. 50-70% of primary GBMs have demonstrated amplification of EGFR which is not observed in lower grade astrocytomas (Ekstrand et al. 1992; Ohgaki et al. 2004). This finding suggests that EGFR activation may be a primary driver of tumorigenesis in primary GBMs. All primary glioblastoma with EGFR amplification have concurrent overexpression, but only a subset (70-90%) of tumors bearing EGFR overexpression also show EGFR amplification, indicating that there is a fraction of patients that overexpress the protein in the absence of gene amplification (Ohgaki and Kleihues 2007). In the context of ErbB2-driven breast cancer, the population of patients that overexpress ErbB2 without changes in gene copy

number have a more similar outcome to patients that do not have the ErbB2 alteration rather than the patient population with both ErbB2 overexpression and gene amplification (Pauletti et al. 2000), suggesting that there may be biological and signaling differences between the two different routes of ErbB2 upregulation. It is unclear if the difference in EGFR amplication versus overexpression in GBM tumors has a similar correlation with patient prognosis.

b. Autocrine mechanisms of action

EGFR overexpression in primary GBMs is occasionally accompanied by the increased expression of its cognate ligands, EGF and TGFα. This observation would imply that there may be an autocrine loop that results in unregulated chronic EGFR signaling (Singh and Harris 2005). It has been shown in a small study of 43 brain tumors that GBMs upregulate the ADAM12 metalloproteinase and that this increase resulted in the elevated cleavage and release of HB-EGF (Kodama et al. 2004). Another gene expression study of the U251MG GBM cell line expressing the constitutively active EGFRvIII mutant receptor found that these cells upregulated the expression of TGFα and HB-EGF (Ramnarain et al. 2006). The authors found that an EGFRvIII-HB-EGF-wild type EGFR autocrine loop was at work, in which EGFRvIII stimulates the secretion of HB-EGF that in turns binds to and activates wildtype EGFR (Ramnarain et al. 2006). Such autocrine loops may be particularly important in governing cell migration, as shown by a recent study employing chimeric cleavable EGF ligands in human mammary epithelial cells expressing EGFR (Joslin et al. 2007). In this

study, increases in the dose of chronic autocrine signals resulted in a dose-dependent increase in pErk signals and migration. This migration was significantly higher than that observed by treating the cells with a bolus of exogeneous EGF ligand. Interestingly, proliferation under both stimulation conditions was not statistically different. This result may be particularly important in the context of GBM tumors which are notorious for being highly migratory and have the propensity to form invasive infiltrate deep into the brain (Furnari et al. 2007).

c. EGFR mutations in GBMs

In addition to receptor and ligand overexpression, activating mutations of EGFR have also been found in GBMs. These mutations are summarized in table I.2 and figure I.4, and range from extracellular deletions and point mutations to loss of residues in the cytoplasmic tail of the receptor (Humphrey et al. 1991; Wong et al. 1992; Frederick et al. 2000b; Lee et al. 2006; Zandi et al. 2007). A number of extracellular deletion mutations have been sequenced and found exclusively in GBM. They includes the EGFR type I and type II variant (EGFRvI and vII) mutant receptors (Humphrey et al. 1991; Wong et al. 1992). The gene deletions give rise to truncated proteins that are believed to be oncogenic. The functional and mechanistic basis of transformation remains to be determined. Another class of extracellular domain mutations that were recently revealed in ~14% of GBMs are point mutations that reside primarily at the interfaces of the various domains in the extracellular region of EGFR (Lee et al. 2006). These

mutations include R108K and A289V/D/T at the domain I/II interface and P569L and G598V that occur at the domain II/IV interface. Such mutations are thought to favor the open active conformation of the receptor by preventing tethering of domain II and IV that is the predominant interaction maintaining the closed, inactive form (Garrett et al. 2002; Ogiso et al. 2002). Evidence for this stems from the fact that these mutations result in a receptor that is constitutively active, capable of binding EGF and leads to increased transformation in mouse xenograft models (Lee et al. 2006).

Receptor	Mutation
EGFRvI	N-terminal deletion
EGFRvII	Deletion of exons 14-15
EGFRvIII	Deletion of exons 2-7
EGFRvIV	Deletion of exons 25-27
EGFRvV	Deletions of exons 25-28
Extracellular mutations	R108K and A289V/D/T among others

Table I.2. Summary of EGFR mutations found in glioblastoma. A summary of the mutations found on the EGFR gene in glioblastoma, adapted from (Zandi et al. 2007).

The cytoplasmic tail deletion mutants, EGFRvIV and vV have also been found exclusively in GBMs (Frederick et al. 2000b). These are thought to occur at lower frequency than the EGFRvIII mutation (see section I.4) and may be tumorigenic as a result of defects in receptor internalization and attenuation of downstream signals. In particular, the EGFRvV mutant lacks the c-Cbl binding site at Y1045 which is required for the ubiquitination and degradation of the protein after ligand binding (Peschard and Park 2007). It is interesting to note

that kinase domain mutations that are commonly found in NSCLC are rare in GBM while extracellular mutations that are common in GBMs are rare in NSCLC (Lee et al. 2006; Sharma et al. 2007). This mutual exclusion of receptor mutations and their functional consequences remain an enigma.

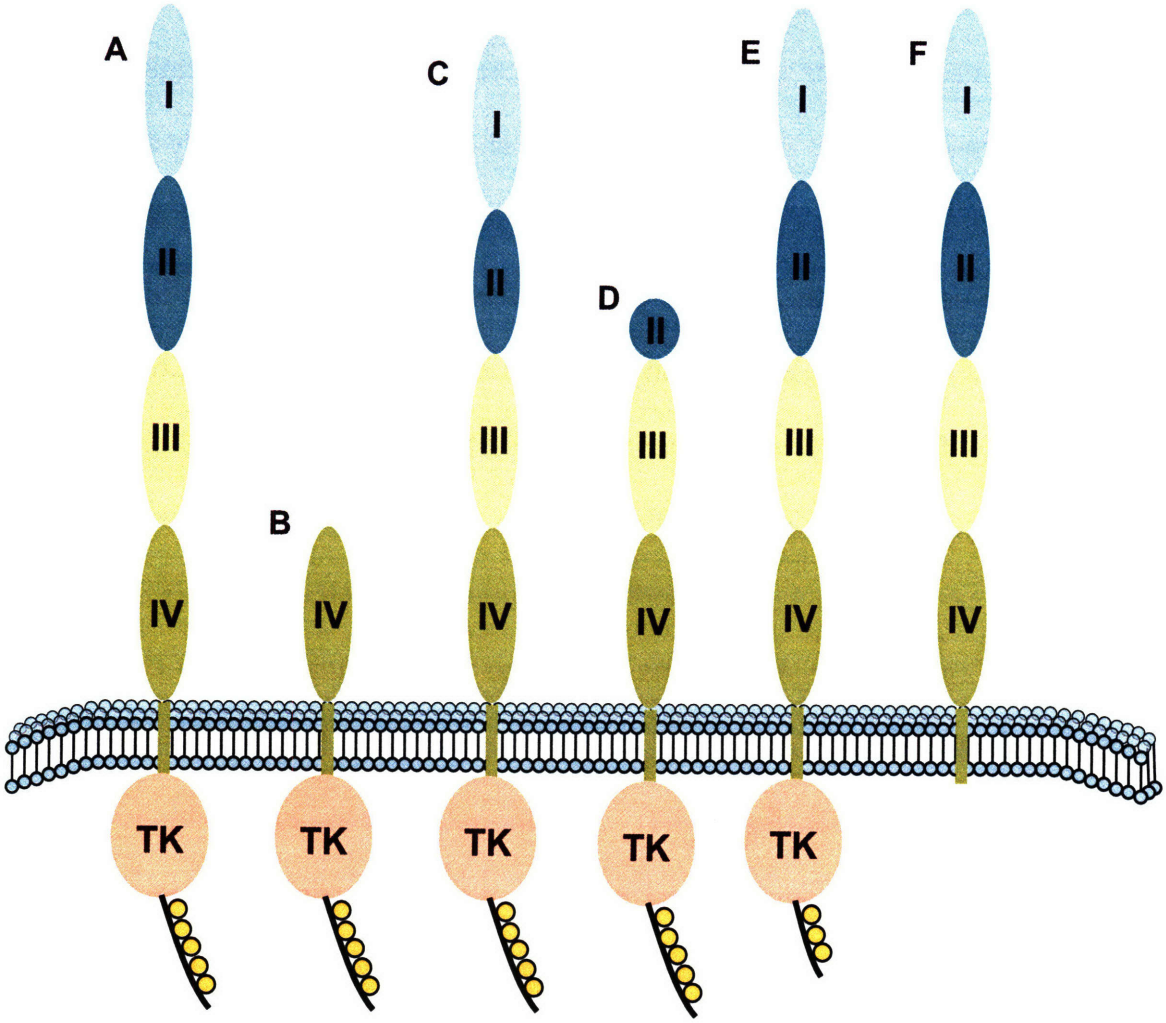

Figure I.4. Schematic depiction of EGFR mutations in glioblastoma. (A) Wildtype EGFR (B) EGFRvI, (C) EGFRvII, (D) EGFRvIII, (E) EGFRvIV and (F) EGFRvV. TK=tyrosine kinase domain. Adapted from (Zandi et al. 2007)

I.2.4 EGFRvIII

I.2.4.1 Overview

The most common EGFR mutation found in GBMs is the type III EGFR variant deletion mutant (EGFRvIII). In addition to GBMs, EGFRvIII has been reported to be detected in non-small cell lung, breast and prostate cancers (Garcia de Palazzo et al. 1993; Moscatello et al. 1995). Approximately 50-60% of GBMs that overexpress wildtype EGFR concurrently express EGFRvIII (20% of all GBMs) (Sugawa et al. 1990; Frederick et al. 2000b). Clinical studies have demonstrated a correlation between EGFRvIII expression and poor prognosis in patients with GBM (4.5 ± 0.6 months compared to 11.2 ± 0.9 months in EGFRvIII-negative patients) (Feldkamp et al. 1999), implying that this genetic lesion may be important in driving transformation in GBMs. EGFRvIII is a truncated mutant which lacks exons 2-7 of the EGFR gene (Wong et al. 1992). This loss of 801 base pairs corresponds to domain I and II of the extracellular region of wildtype EGFR (Figure I.5). It is thought that the loss of the domain II loop prevents this mutant from forming the closed inactive conformation and favors a shift in the equilibrium to the open active conformation. The deletion also results in the formation of a new codon encoding a glycine (Wong et al. 1992). This unique epitope has been the target for the development of multiple EGFRvIII specific antibodies for the treatment of GBMs (Sampson et al. 2000; Mishima et al. 2001) (elaborated in section I.2.5). The mechanisms that drive the formation of this deletion mutant in GBMs is not known, although it has been

proposed that the gene deletion may arise from the recombination event between Alu sequences flanking the deletion junctions in introns 1 and 7 of the EGFR gene (Frederick et al. 2000a).

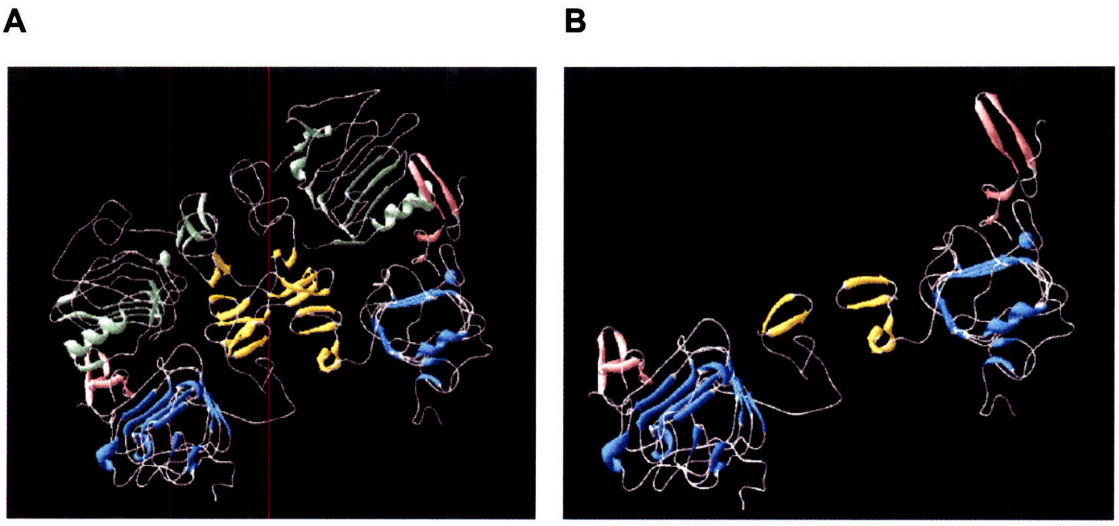

Figure I.5. Modified crystal structure of EGFRvIII. (A) Crystal structure of the extracellular domain of EGFR bound to EGF. (B) modified from (A) to indicate deletion of exons 2-7 resulting in a truncated mutant, EGFRvIII, that is incapable of binding to ligand. Green = domain I, Yellow = domain II, Blue = domain III and truncated domain IV, Pink = EGF ligand

Although EGFRvIII is incapable of binding the EGFR family of ligands, it is reported to be constitutively tyrosine phosphorylated at 10% of the levels of ligand-stimulated EGFR, by mechanisms that are still unknown (Huang et al. 1997). Due to the loss in the domain II dimerization loop, there is some debate as to the whether EGFRvIII has the ability to form functional dimers. Using chemical crosslinkers, it was shown that unlike wildtype EGFR, EGFRvIII does not form homodimers even under incubation at higher temperature conditions (Chu et al. 1997). Conversely, co-immunoprecipitation experiments using EGFRvIII specific antibodies demonstrates that EGFRvIII forms heterodimers with both wildtype EGFR and other members of the ErbB family (Tang et al.

2000; Luwor et al. 2004). It is likely that the types of dimers that form are context-dependent. For instance, in the context of breast cancers, it has been shown that EGFRvIII is co-expressed and forms heterodimers with the ErbB2 receptor which is abundantly expressed in a subset of breast cancer cells (Tang et al. 2000). This heterodimerization event results in the elevated activation of ErbB2 and increased tumorigenicity *in vitro* and *in vivo*.

I.2.4.2 Defects in receptor internalization

Paradoxically, despite being constitutively active, EGFRvIII receptor internalization is defective (Huang et al. 1997). This lack of internalization is in contrast to wildtype EGFR, where receptor activation leads to rapid internalization and termination of the signal. It is thought that failure of EGFRvIII to internalize into the lysosome results in sustained, unattenuated EGFRvIII signaling, contributing to its transforming ability. The evasion of negative feedback regulation has been attributed to the low signal intensity of EGFRvIII which may be below the threshold required for binding to critical proteins involved in ubiquitin-mediated degradation, including Cbl and SETA (Schmidt et al. 2003). EGF-mediated activation of wildtype EGFR results in the recruitment of c-Cbl and SETA via the phosphorylation of Y1045 on the receptor (Soubeyran et al. 2002). It has been shown in multiple studies that EGFRvIII is hypophosphorylated at Y1045 and fails to become polyubiquitinated and degraded (Han et al. 2006; Grandal et al. 2007). A recent report demonstrated that EGFRvIII is internalized but at a much slower rate compared to unstimulated wildtype EGFR and that this

small population of internalized receptors was not transported to the lysosome for degradation but rather recycled back to the surface (Grandal et al. 2007). The authors also showed that while EGFRvIII does not bind to Cbl via its Y1045 site, it does so through an indirect interaction with the Grb2 adaptor protein. However, Cbl binding still fails to ubiquitinate the receptor, resulting in sustained receptor activation levels. Interestingly, overexpression of Cbl in the context of EGFRvIII expression in NIH3T3 cells, leads to receptor internalization and degradation (Davies et al. 2006). Taken together, one interesting hypothesis that remains to be explored is whether increasing EGFRvIII activation, perhaps through imposing receptor dimerization, could increase the levels of receptor phosphorylation beyond the threshold required for Cbl-mediated polyubiquitination and degradation.

I.2.4.3 EGFRvIII-mediated transforming ability

EGFRvIII has been consistently shown to be tumorigenic in a number of different studies. MCF-7 breast cancer cell lines transfected with EGFRvIII exhibited increased tumorigenicity in both *in vitro* colony formation assays as well as *in vivo* xenografts (Tang et al. 2000). Similarly, U87MG GBM cell lines expressing EGFRvIII that were inoculated into nude mice, formed tumors dramatically faster than parental cells or cells expressing wildtype EGFR (Nishikawa et al. 1994). Furthermore, EGFRvIII may drive clonal selection as U87MG-EGFRvIII cells mixed with the parental cell line at 1:10,000 and 1:50,000 ratios in a murine xenograft model, outgrew the parental cell line after 4-5 weeks

by a ratio of 20:1 (Nagane et al. 1996). Analysis of proliferation and apoptosis markers indicated that the increase in tumorigenicity was due to an increase in proliferation and a corresponding reduction in apoptosis (Nagane et al. 1996). NR6 murine fibroblast cells expressing EGFRvIII displayed increased motility while U87MG cells transfected with increasing levels of EGFRvIII showed corresponding increases in cell invasion and migration (Pedersen et al. 2004; Cai et al. 2005). It is noteworthy that the transforming ability of EGFRvIII only manifests itself in the context of other genetic mutations. For example, the expression of EGFRvIII alone was insufficient to form high grade tumors in genetically engineered mouse models (Ding et al. 2003). Only when EGFR was co-expressed in the context of other genetic lesions, such as activated Ras or Ink4A/Arf loss, did tumors form (Holland et al. 1998; Ding et al. 2003). Similarly, reconstitution of PTEN into U87MG cells expressing EGFRvIII resulted in decreased cell proliferation (Wang et al. 2006). This evidence would imply that while EGFRvIII is an important driver of transformation in primary GBMs, it does this exclusively through the cooperation with genetic aberrations that occur in other cellular pathways.

I.2.4.4 Mechanism of tumorigenicity and downstream signaling pathways

The mechanisms by which EGFRvIII drives biological transformation have yet to be fully characterized. In an attempt to understand the biological consequence of EGFRvIII signaling, mutational analysis of tyrosine phosphorylation sites on EGFRvIII have been performed. Mutation of Y1068F,

Y1148F and Y1173F each led to a dramatic decrease in intracranial tumor volume, implicating these sites to be critical for tumorigencity (Huang et al. 1997). This observation is in contrast to wildtype EGFR, where only the combination of all 3 mutations affected transformation and mitogenic potential (Helin et al. 1991). This finding indicates that unlike wildtype EGFR, EGFRvIII phosphorylation levels are sufficiently low that each phosphorylation site is important for its function and compensation of function between the phosphorylation sites do not occur. It is plausible that while the tyrosine sites that are phosphorylated on EGFRvIII are the same as those observed in wildtype EGFR, quantitative differences in the levels of phosphorylation at each individual site may have functional implications on resultant downstream signaling pathways. In this section, I will discuss some of the pathways downstream of EGFRvIII activation.

a. The PI3K pathway

As described in section I.1.2.2, dysregulation of the PI3K-AKT pathway is an important contributor to the incidence of GBM. EGFRvIII has been shown to be a driver of the PI3K-AKT pathway in fibroblasts, GBM cell lines and immortalized astrocytes (Moscatello et al. 1998; Narita et al. 2002). EGFRvIII expressing NIH3T3 cells were found to have an 8-fold increase in PI3K activity compared to parental cells, which was significantly higher than that observed in wildtype EGFR expressing cells (3-fold increase) (Moscatello et al. 1998). Furthermore, consistent with the constitutive activation status of EGFRvIII, this increased PI3K activity was constitutive and independent of EGF levels in the

media. Unlike ErbB3 which has multiple docking sites for the SH2 domain of the p85 PI3K subunit, EGFRvIII likely activates PI3K through indirect binding of the p85 subunit via interaction with the Gab1 adaptor protein. Anti-Gab1 immunoprecipitates of EGFRvIII expressing cells demonstrated a 2 fold increase in PI3K activity compared to EGF stimulated wildtype EGFR cells (Moscatello et al. 1998).

Treatment of cells with AG1478, an EGFRvIII/EGFR inhibitor, or the administration of wortmannin, a PI3K inhibitor, both reduced PI3K activity and inhibited anchorage-independent growth, further emphasizing the importance of this pathway on the transforming activity of EGFRvIII (Moscatello et al. 1998; Narita et al. 2002). The mechanism by which PI3K contributes to EGFRvIII-mediated transformation can be explained in part by the downregulation of the p27 CDK inhibitor by this pathway. The activation of Akt by PI3K, results in the reduction of cellular levels of p27 via phosphorylation and nuclear exclusion of members of the forkhead transcription factor family (Narita et al. 2002). EGFRvIII-U87MG GBM cells have reduced levels of p27 and elevated CDK2-cyclin A activity levels compared to parental cells. This is accompanied by the hypophosphorylation of Rb and lack of G1 arrest under serum starvation conditions. Treatment of cells with LY294002 (a PI3K inhibitor) or dominant negative (DN) Akt led to the restoration of p27 levels and G1 arrest. In addition, DN-Akt decreased tumorigenicity of EGFRvIII-U87MG *in vivo* (Narita et al. 2002). These biochemical studies are complimented by immunohistochemistry studies of human GBMs tumors which show a strong correlation between EGFRvIII

expression and the phosphorylation of Akt, mTOR and forkhead transcription factors (Choe et al. 2003). The therapeutic implications of this pathway will be discussed further in section I.2.5.

b. The Erk1/Erk2 MAPK pathway

The mitogen-activated protein kinase pathway (MAPK) is a critical mediator of the biological effects of wildtype EGFR. Upon ligand binding and receptor dimerization, EGFR becomes phosphorylated at multiple tyrosine sites in its cytoplasmic tail. MAPK pathway activation is triggered by the binding of growth factor receptor-bound protein 2 (Grb2) either directly to the receptor at Y1068 or indirectly through Src homology domain containing adaptor protein C (SHC) binding at Y1173/Y1148 (Schulze et al. 2005). Grb2 recruits the son of sevenless (Sos) guanine nucleotide exchange factor to the receptor complex, which sets off a canonical signaling cascade known as the MAPK cascade: Sos promotes the conversion of the inactive Ras-GDP to the active Ras-GTP. Active Ras then binds to and activates Raf-1 (MAPK kinase kinase), a serine/threonine kinase that phosphorylates MEK (MAPK kinase) at two serine residues. The active MEK then phosphorylates MAP kinases Erk1 and Erk2 at a threonine residue. Full activation of Erk1/2 requires the phosphorylation of both the threonine residue as well as a tyrosine residue, for which the kinase has not been established. Activation of Erk1/2 results in the phosphorylation of a large number of substrates all bearing the PXS/TP motif, including a large number of

latent transcription factors (e.g. c-Fos, Elk-1 and c-Jun) and kinases (RSK and MNK) (Chen et al. 2001).

EGF-mediated MAPK activation is transient and is controlled by a number of negative regulators, most prominently, the MAP kinase phosphatases (MKPs) which are dual specificity threonine/tyrosine phosphatases that dephosphorylate and inactivate Erk1/2 (Amit et al. 2007; Jeffrey et al. 2007). The dynamics of Erk1/2 activation determines cell fate. For instance, it has been demonstrated in the PC12 pheochromocytoma cell line that transient Erk activation results in cell proliferation but sustained Erk activation leads to neuronal differentiation (Marshall 1995).

In the context of EGFRvIII, MAPK cascade activation is sustained, consistent with the constitutive activity of the receptor. Co-immunoprecipitation studies revealed a constitutive association of Shc and Grb2 with EGFRvIII at Y1148 and Y1068 respectively (Montgomery et al. 1995; Moscatello et al. 1996; Prigent et al. 1996; Chu et al. 1997). EGFRvIII-U87MG cells displayed a 2-fold higher Ras activity compared to parental cells, although this observation seemed to be restricted to transformed cells (Prigent et al. 1996). MEK kinase activity is 4 fold higher in EGFRvIII expressing fibroblast cells compared to the corresponding cells expressing wildtype EGFR (Montgomery et al. 1995). The functional contribution of the Erk1/Erk2 MAPK pathways to EGFRvIII-mediated tumorigenesis is less clear. There have been conflicting reports on the activation status of Erk1/2, where EGFRvIII appears to increases the phosphorylation levels of Erk1/2 in some cell lines but not others (Montgomery et al. 1995;

Lorimer and Lavictoire 2001). Taken together, these observations suggest that while EGFRvIII activates the Ras/Raf/MEK pathways, there may be a negative regulation mechanism at the level of MAPK phosphorylation in some cell types. Indeed, treating EGFRvIII expressing cells with sodium vanadate, a tyrosine phosphatase inhibitor, increased the levels of phosphorylated MAPK under steady-state serum free conditions, pointing to the MKPs as possible candidates for Erk dephosphorylation (Montgomery et al. 1995).

c. The STAT pathway

The signal transducer and activator of transcription (STATs) proteins are a family of latent transcription factors that are recruited to ligand-bound EGFR dimers via their SH2 domains. The kinase domain of EGFR phosphorylates the STATs and induces either homo- or heterodimerization via SH2-phosphotyrosine interactions with another STAT molecule (Aaronson and Horvath 2002; Levy and Lee 2002). The dimeric complex translocates to the nucleus where it binds to specific DNA promoter sequences though its transactivating domain, recruits other transcription regulators and activates the transcription of multiple genes important for cell survival. There are 7 members of the STAT family with STAT3 and STAT5 being implicated in EGFR signaling (Song and Grandis 2000; Schulze et al. 2005). STAT3 binds to both Y1068 and Y1086 on the EGFR receptor (Shao et al. 2003). Tyrosine phosphorylation of STAT3 at Y705 has been shown to increase after EGFR activation and is required for protein dimerization and nuclear translocation (Levy and Lee 2002). An additional site at

S727 is phosphorylated by members of the MAPK family of proteins and is thought to enhance transcriptional activity (Decker and Kovarik 2000). STAT5 has been shown in a large scale ErbB family interactome study to bind to two phosphotyrosine sites on EGFR, Y954 and Y974 (Schulze et al. 2005). Similar to STAT3, STAT5 is tyrosine phosphorylated ay Y699. Interestingly, a recent study demonstrated that three other phosphorylation sites on STAT5b (Y725, Y740 and Y743) influenced transcription activity in EGF stimulated breast cancer cells (Weaver and Silva 2006). Examples of genes upregulated by STAT3/5 are cyclin D, BCL_{XL} and the myc oncogene (Gao and Bromberg 2006).

EGFRvIII has been found to interact with STAT3, both in the cytoplasmic fraction and in the nuclear fraction, suggesting that EGFRvIII-STAT3 complexes may exist in the nucleus (de la Iglesia et al. 2008). Knockdown of STAT3 in immortalized astrocytes resulted in a decrease in EGFRvIII expressing cells, implicating it in EGFRvIII-mediated cell proliferation. This observation was also recapitulated *in vivo* using murine xenograft models. Screening a panel of human GBM tumors for the expression of EGFRvIII and STAT3 phosphorylation levels confirmed that a small population of primary tumor cells exhibited localization of EGFRvIII in the nucleus. This study is the first demonstration of the nuclear localization of EGFRvIII in GBMs, and in light of evidence that wildtype EGFR translocates to the nucleus and may play a role in transcription (Wells and Marti 2002), represents an exciting opportunity for the study of the potential transcriptional capability of EGFRvIII in GBMs.

I.2.4.5 Genetically engineered mouse models

Most of the *in vivo* work described above has largely been performed in xenografts of cell lines or immortalized astrocytes stably transfected to express EGFRvIII. The main advantages for using this system are the relative ease in genetic manipulation of cell lines and the rapid progression of large numbers of synchronized tumors. This leads to reproducible incidence, growth rates and survival patterns. However, there are also disadvantages to xenografts such as their failure to accurately model the histological features of malignant gliomas, in particular single-cell infiltration and histological progression (Hu and Holland 2005; Becher and Holland 2006). In addition, many of the cell lines used to generate such xenografts have been cultured *in vitro* for many generations and because of inherent selective pressures under these conditions, are unlikely to be representative of the original tumor. Thus in recent years, there has been a move towards genetically engineered murine models (GEMs, which are better able to recapitulate the genetics and histology of human tumors. Such models will allow us to better study tumorigenesis and tumor maintenance as well as provide a tool for preclinical testing (Hu and Holland 2005; Becher and Holland 2006).

Unfortunately, previous attempts to generate a murine glioma model to study the EGFRvIII system has led to mice that either recapitulates the clinical features of the disease (phenocopy) but not reflect the genetic basis of the disease (not genocopy) or vice versa (Maher et al. 2001). One such model was

generated by Holland and co-workers, where avian retroviral vectors were used to transfer a mutant activated EGFR gene to glial precursors in mice expressing the receptor for these viruses (RCAS/tv-a system). These mice generated highly invasive, intermediate to high grade tumors when expressed in the setting of Ink4a deficiency. However, because the vector system limits gene insertions to a size of 2.6kb or less, the activated EGFR protein that was expressed lacked not only the extracellular domain (as in EGFRvIII) but also its intracellular kinase regulatory domain, making it more similar to the v-erbB protein rather than EGFRvIII (Holland et al. 1998). In another model, Guha and co-workers generated a mouse model expressing the EGFRvIII receptor in an activated H-ras background. However, these mice generated oligodendrocytomas rather than astrocytomas (Ding et al. 2003). While these models have not been faithful in fully recapitulating the genetic lesions observed in human GBMs, they have been informative in generating a list of the types and combinations of mutations critical for tumor formation, as well as the contribution of each mutation to transformation (Furnari et al. 2007). Furthermore, they will provide valuable preclinical information on the pharmacodynamic/pharmacokinetic properties of novel EGFR-based therapeutics, prior to entry into the clinic.

I.2.5 Therapeutic strategies for EGFRvIII positive GBMs

I.2.5.1 Classical chemo- and radiotherapies

The poor prognosis of GBM patients is due in part to the chemo- and radioresistance properties demonstrated by these tumors. EGFRvIII has been shown to confer resistance to classical chemo- and radiotherapy via a number of mechanisms. EGFRvIII expressing GBM cells treated with a DNA damaging agent, cisplatin, showed a 4 fold increase in survival and a 2 fold decrease in apoptosis *in vitro* compared to parental or EGFR overexpressing cells (Nagane et al. 1998). This resistance required the kinase activity of the receptor because the kinase inactive mutant of EGFRvIII did not confer cisplatin resistance. One mechanism by which this resistance is thought to occur is via chemotherapeutic-induced phosphorylation and activation of EGFRvIII. Treatment of U87MG GBM cells with cisplatin, doxorubixin or camptothecin resulted in a dose-dependent elevation in EGFRvIII receptor phosphorylation (Benhar et al. 2002). This increase in receptor phosphorylation is thought to occur through a Src-mediated phosphorylation of Y845 on EGFRvIII. Correspondingly, treatment of EGFRvIII cells with a Src inhibitor PP1, resulted in a decrease in receptor phosphorylation levels (Benhar et al. 2002).

The anti-apoptotic protein Bcl-X_L is another important player in the chemoresistance properties of EGFRvIII. Decreased apoptotic index in EGFRvIII transfected U87MG cells and xenografts correspond to an increase in Bcl-X_L levels (Nagane et al. 1998). BCL-X_L levels remained elevated during cisplatin

treatment of EGFRvIII GBM cells compared to the parental cells. Treatment of cells with AG1478 (an EGFR kinase inhibitor) resulted in a decrease in Bcl-X_L levels (Nagane et al. 1998). These series of experiments indicates that a combination protocol of AG1478 with cisplatin may be used as a strategy to overcome the drug resistance conferred by EGFRvIII. This concept has since been demonstrated in both cell line models and *in vivo* human xenograft models (Nagane et al. 2001).

U373MG GBM cells expressing EGFRvIII showed enhanced survival after exposure to 4Gy of irradiation compared to parental cells. Furthermore, xenografts from these same cells showed accelerated growth when subjected to a treatment protocol consisting of multiple doses of 3Gy irradiation (Lammering et al. 2004a; Lammering et al. 2004b). In the context of multiple cancer cell lines, EGFRvIII has demonstrated radiation-induced receptor phosphorylation at least 3 fold higher than that of wildtype EGFR (Lammering et al. 2003; Lammering et al. 2004b). The increase in receptor phosphorylation coincided with a concomitant increase in downstream AKT and MAPK pathways. This observation suggests that the EGFRvIII may promote radioresistance by elevating pro-survival and cell proliferation pathways through increased receptor activation. This concept is consistent with the clinical observation of accelerated repopulation, where the tumor cells increase their rate of replication during the course of radiation (Schmidt-Ullrich et al. 1997; Chinnaiyan et al. 2005). The mechanism by which radiation induces receptor phosphorylation is unknown but it has been proposed that it could arise from the inactivation of protein tyrosine phosphatases

responsible for regulating EGFR. Irradiation causes an increase in the cellular levels of reactive oxygen/nitrogen species which leads to the covalent inactivation of the catalytic cysteine in these phosphatases (Leach et al. 2001; Leach et al. 2002; Tonks 2005). Similar to the case of cisplatin resistance, inactivation of EGFRvIII by the use of dominant negative EGFR-CD533 restores *in vivo* radiosensitivity (Lammering et al. 2004a).

I.2.5.2 Targeted therapeutics

a. Antibodies

The deletion of exons 2-7 of the EGFR gene results in the formation of a tumor-specific epitope in EGFRvIII. Using a variety of different methods, monoclonal antibodies specific to EGFRvIII and not wildtype EGFR have been generated (Sampson et al. 2000; Mishima et al. 2001). The advantage of generating antibodies that target tumor-specific antigens versus antigens that are merely overexpressed in tumors is that there is little chance of localization to other tissues that may also express high antigen levels. In the case of EGFR, it has previously been shown that EGFR specific antibodies tend to accumulate in organs that have high receptor levels, including the liver and skin (Rettig and Old 1989; Scott et al. 2007).

One EGFRvIII-specifc antibody, Y10, when administered systemically, led to tumor shrinkage in subcutaneous xenografts of melanoma cell lines expressing EGFRvIII (Sampson et al. 2000). However, systemic administration

of this antibody did not have any effect on intracranial tumors, highlighting the need to develop therapeutics that are capable of crossing the blood-brain-barrier (BBB). Another EGFRvIII-specific antibody, mAb806, demonstrated dramatic tumor shrinkage and increased survival (from 13 to 21 days) when administrated systemically to intracranial EGFRvIII-U87MG xenografts (Mishima et al. 2001). mAb806 decreased EGFRvIII expressing tumor volume by 65-95% but did not exhibit any activity in parental tumors, highlighting its specificity. Tumor shrinkage was attributed to its ability to prevent EGFRvIII receptor phosphorylation and activation of downstream signaling pathways, including decreasing Bcl_{XL} levels. Despite the impressive efficacy of this antibody, durable remissions were not achieved and tumors relapsed after a period of drug administration (Mishima et al. 2001). This observation led to the development of combination protocols that involved co-administration of mAb 806 with other small molecule EGFR inhibitors (AG1478) and EGFR antibodies (mAb 528) (Johns et al. 2003; Perera et al. 2005). These treatments showed additive effects on tumor shrinkage in EGFRvIII expressing subcutaneous xenografts. A recent phase 1 study of mAb 806 in 8 patients with tumors at different organ sites demonstrated that this therapeutic was safe and well tolerated (Scott et al. 2007). It was also shown in one patient with anaplastic astrocytoma, that the antibody was able to cross the BBB and localize to the tumor (Scott et al. 2007). While it was not the aim of the trial to show therapeutic efficacy of the unarmed antibody, the ability to localize to the brain tumor holds promise that such EGFRvIII-specific

antibodies may someday be used as therapeutic agents, particularly when conjugated to radioisotopes or toxins.

b. Small molecules kinase inhibitors

An alternative approach to antibodies is the development of small molecules that competitively bind to the ATP-binding pocket in the kinase domain of EGFR and disrupt receptor catalytic activity in a reversible manner. Unlike antibodies, these molecules are far less specific in their action and are known to bind to and inhibit multiple kinases in addition to EGFR (Karaman et al. 2008). Three such molecules (gefitinib, erlotinib and lapatinib) have been approved for use in patients although none of the indications are for the treatment of GBMs (Nyati et al. 2006). EGFRvIII has shown resistance to both gefitinib and erlotinib in GBM cell lines and xenograft models (Learn et al. 2004; Mellinghoff et al. 2005; Pedersen et al. 2005; Ji et al. 2006). AG1478, a tyrphostin-based small molecule inhibitor that showed higher specificity for EGFRvIII over wildtype EGFR *in vitro* also had no efficacy as a single agent in xenograft models (Han et al. 1996; Johns et al. 2003). In one study, administration of an irreversible EGFR kinase inhibitor HKI-272, resulted in tumor volume reduction in a mouse model of EGFRvIII-driven NSCLC (Ji et al. 2006), suggesting that irreversible inhibition of EGFRvIII may be a strategy to overcome EGFR kinase inhibitor resistance.

Several phase II clinical trials employing gefitinib and erlotinib as single agent treatments in newly diagnosed or recurrent glioblastoma have also failed to show a clinical benefit compared previous historical treatment protocols (Rich et

al. 2004; Omuro et al. 2007). While overexpression/amplification of EGFR was not correlated to patient outcome, one retrospective study found a correlation between EGFRvIII and PTEN co-expression and favorable response to treatment with erlotinib/gefitinib (Mellinghoff et al. 2005). This finding was confirmed *in vitro* when the co-expression of EGFRvIII and PTEN in U87MG GBM cells conferred sensitivity to erlotinib. The authors propose that the apparent resistant to EGFR kinase inhibitors is due to the uncoupling of the PI3K-AKT-mTOR pathway from EGFRvIII upstream signals by the loss of PTEN (Mellinghoff et al. 2007).

The results from the retrospective study would support the use of EGFR kinase inhibitors in conjunction with PI3K-AKT-mTOR inhibitors in the clinic for EGFRvIII positive, PTEN null GBM patients which form a significant proportion of all primary GBM patients. *In vitro* experiments in GBM cell lines reinforce this hypothesis. Treatment of EGFRvIII-positive, PTEN null cells with erlotinib and either rapamycin (a small molecule inhibitor of mTOR) or PI-103 (a small molecule inhibitor of PI3K and mTOR) led to a decrease in tumor cell proliferation compared to administration of either drug alone (Wang et al. 2006; Fan et al. 2007). Multiple ongoing clinical trials of EGFR kinase inhibitor together with rapamycin/rapamycin analogs are currently being conducted to determine if this preclinical efficacy materializes in the clinic (Omuro et al. 2007).

I.3 Phosphoproteomics

I.3.1 Overview

Protein phosphorylation is an important mechanism by which cells mediate a large number of regulatory processes. Since its discovery in 1955 (Fischer and Krebs 1955), this post-translational modification has been shown to regulate protein-protein interaction, protein stability and modulation of enzymatic activity. Of the 30% of proteins estimated to be phosphorylated in mammalian cells, the dominant form of phosphorylation occurs on serine and threonine amino acid residues. These modifications comprise ~93% and ~7% of all phosphorylation events in the cell respectively (Cohen 2000). Less than 1% of the global phosphoproteome is modified on tyrosine residues (Hunter and Sefton 1980). Despite its apparent low levels, tyrosine phosphorylation impinges on many critical cellular functions, including communication within and between cells, regulation of gene transcription and expression, cell-cycle, differentiation and locomotion (Hunter 2000). It is thus not surprising that dysfunction in the regulation of tyrosine phosphorylation can result in the development of many diseases such as cancer, diabetes and autoimmune diseases. This observation is most striking in the initiation of cancer where aberrations in cellular signaling result in the dramatic increase of tyrosine phosphorylation levels to 1-2% of the total cellular phosphoproteome (Hunter 2000). The importance of phosphorylation in both physiological and pathological settings has led to an explosion of new studies and techniques to map and elucidate its contribution to

biological processes. In this section, I will elaborate on some of these techniques, with a focus on mass spectrometric (MS)-based approaches to mapping and quantification of the phosphoproteome.

I.3.2 Non-MS based approaches to study the phosphoproteome

Early efforts in the identification of phosphorylation sites on proteins included the use of thin layer chromatography and Edman sequencing. Unfortunately, these methods are low-throughput and have mainly focused on the comprehensive mapping of phosphorylation sites on a single protein. Once these sites have been identified, phospho-specific antibodies can be raised against these phosphorylated proteins and semi-quantitative western blots may be used to obtain relative quantification of phosphorylation sites between different cell states (Schmelzle and White 2006). On a larger scale, this quantitative western blotting approach has now been expanded to analyze up to 200 known protein phosphorylation sites (www.kinexus.ca) and has been employed to monitor the phosphorylation status of 12 cellular signaling components (including kinases and transcription factors) across 4 mammary epithelial cell lines (Lin et al. 2005). However, measuring phosphorylation levels by western blots has the drawback of a limited dynamic range by which accurate quantification by densitometry can be obtained. The generation of phospho-specific antibodies has nevertheless laid the foundation for the development of a number of technologies capable of measuring phosphorylation levels in a multiplexed and high-throughput manner. The most commonly used

technologies are multi-parameter flow cytometry (Irish et al. 2004) and antibody microarrays (Nielsen et al. 2003).

Multi-parameter flow cytometry is an approach in which phosphorylation levels in proteins are measured by labeling cells with fluorophore-conjugated phospho-specific antibodies. These cells are subjected to flow cytometry that quantifies the fluorescence intensity, which is directly proportional to the amount of phospho-antigen in each cell (Perez and Nolan 2006). Current state-of-the-art flow cytometers allow for up to 13 colors to be analyzed, greatly enhancing the ability to measure several parameters simultaneously. Due to the need for cells to be in suspension in the flow cell, this technique has found greatest utility in investigating cells of the blood and hematopoeitic compartments (Irish et al. 2004; Irish et al. 2006b; Irish et al. 2006a; Irish et al. 2007). In a landmark study using this technique, Irish and co-workers stimulated leukemia cells from 30 AML patients with a panel of 6 cytokines and followed the phosphorylation changes in 6 different phosphoproteins important in the JAK/STAT and MAPK pathways (Irish et al. 2004). Subjecting the data to unsupervised hierarchical clustering, the authors demonstrated that tumors from individual cancer patients can be categorized by their molecular response to cytokine inputs, and that this classification can discern genetic mutations and disease outcomes. A major advantage to this approach is that interrogation of phosphorylation occurs at the single cell level and has the potential of isolating subpopulations of cells that have distinct phosphorylation profiles that would otherwise have been lost in averaged cell population data (Irish et al. 2004). An example of this would be

hematopoeitic stem cells which are extremely rare and in the background of a larger heterogeneous population (Rossi et al. 2008). One can envisage the use of antibodies for stem cell identification markers together with antibodies for internal phosphoproteins to simultaneously monitor the phosphorylation profiles in such cells.

By comparison to western blots and flow cytometry, antibody microarrays feature increased throughput and minimal sample/reagent consumption while quantifying tens of phosphorylation sites in a targeted fashion. Several antibody microarray formats have been developed including the sandwich ELISA (enzyme-linked immunosorbant assay) format. In this format, cell lysate is incubated with antibodies conjugated to glass slides. A second fluorescently labeled antibody specific for the phosphorylated epitope on the same protein acts as the detection device and is then used to quantify the amount of the phosphorylated protein (MacBeath 2002). In a proof-of-principle study employing this approach, the phosphorylation state of members of the ErbB receptor family were quantified in 3 cancer cell lines that have distinct EGFR and ErbB2 receptor levels (Nielsen et al. 2003). The authors examined the activation of these receptors as a function of time after stimulation with EGF and were able to account for cellular processes such as receptor activation, internalization and degradation. Furthermore, they demonstrated that antibody microarrays have the capability to monitor the effects of tyrosine kinase inhibitors such as PD153035 on EGFR and ErbB2 phosphorylation states and in doing so, were able to measure the IC_{50} and Ki of the drug (Nielsen et al. 2003).

Unfortunately, the breadth of signaling components measured using this approach is limited by the lack of microarray-compatible matched pairs of antibodies. This limitation has been overcome by eliminating the requirement for a second specific detection antibody. Tempst and colleagues have generated an antibody microarray for protein tyrosine phosphorylation which also uses the sandwich approach (Gembitsky et al. 2004). In this technique, they immobilized antibodies for 35 signaling proteins on a microarray surface and incubated it with lysate from EGF stimulated cells. Instead of using a specific second detection antibody, a fluorophore conjugated pan-specific phosphotyrosine antibody PY-KD1 was used instead. This technique provides information of the tyrosine phosphorylation states of a much larger subset of signaling proteins and has the potential to be expanded for use with other pan-specific phospho-antibodies, such as anti-pSer or anti-pThr antibodies. The disadvantage to this technique is that unlike the previous study, information about the individual phosphorylation sites is lost as overall tyrosine phosphorylation state of the proteins are now being measured.

I.3.3 MS-based approaches to study the phosphoproteome

I.3.3.1 Overview

Phosphoproteomic analysis by mass spectrometry offers several advantages over antibody-based technologies. Although it is not a high-throughput approach, its ability to sequence novel proteins and phosphorylation

sites in an unbiased fashion means that it is not dependant on the availability of phospho-specific antibodies. In addition, using the appropriate phosphoprotein enrichment techniques and MS instrumentation allows for a larger dynamic range and sensitivity in phosphoprotein detection (Schmelzle and White 2006). Finally, recent advances in the development of MS-based isotopically labeled reagents have allowed unprecedented precise and multiplexed quantification of hundreds to thousands of phosphorylation sites in a single experiment (Ong and Mann 2005). Specific examples in the following sections will highlight how MS-based phosphoproteomics has revolutionized the field, in particular in its application to the high resolution mapping of cellular signaling networks.

I.3.3.2 Enrichment strategies

Poor ionization of phosphorylated peptides compared to their non-phosophorylated counterparts in the mass spectrometer necessitates the use of enrichment techniques to isolate the cellular phosphoproteome prior to analysis by mass spectrometry. Enrichment strategies for phosphopeptides include immobilized metal affinity chromatography (IMAC) and strong cation exchange (SCX) (Ficarro et al. 2002; Beausoleil et al. 2004; Brill et al. 2004). IMAC relies on the coordination of negatively charged phosphorylated peptides to immobilized chelated iron. In complex protein mixtures such as cell lysate, there is a propensity for the IMAC to also enrich for negatively charged acidic peptides. This problem can be overcome by chemical derivatization of peptide carboxyl groups to form methyl-esters which reduces non-specific binding during IMAC

enrichment (Ficarro et al. 2002). This approach was first applied to the global phosphoproteome of *S.cerevisiae* (Ficarro et al. 2002) and led to the identification of several hundred phosphorylation sites. An analogous approach using titanium dioxide enrichment has also gained popularity in its application to phosphoproteomics, due to a significant savings in column preparation time (Olsen et al. 2006). SCX has also been extensively applied to the study of the phosphoproteome, often as part of a multi-fractionation approach, either with reverse phase chromatography or IMAC. When performed at pH 2.7, it enriches for phosphopeptides in the early elution fractions due charge differences conferred by the negatively charged phosphate group in phosphopeptides compared to non-phosphorylated peptides (Beausoleil et al. 2004). This technique was applied to study the nuclear phosphoproteome of HeLa cells with the subsequent identification of more than 2000 phosphorylation sites (Beausoleil et al. 2004).

The use of IMAC or SCX as a single step global enrichment of phosphopeptides invariably leads to the identification of the most abundant phosphorylation sites in the cell. These include, phosphorylation sites found on metabolic enzymes, structural proteins and RNA binding proteins (Olsen et al. 2006). However, a large proportion of the low abundance proteins involved in mediating cellular signaling processes are often not isolated in such analyses. In particular, since tyrosine phosphorylation represents less than 1% of the phosphoproteome (Hunter and Sefton 1980), only a small number of such sites are mapped using global enrichment approaches. An alternative, more targeted

approach to study low abundant phosphorylation events is to employ immunoprecipitation (IP) techniques with motif-specific antibodies or protein binding domains that enrich for specific subproteomes of phosphorylated protein substrates (Rush et al. 2005; Matsuoka et al. 2007; Wang et al. 2007). This approach has been particularly successful in the isolation of phosphotyrosine containing peptides where good pan-specific anti-phosphotyrosine antibodies are commercially available (Rush et al. 2005).

IP can be performed at both the protein and peptide level. The advantage of the latter is that since tyrosine phosphorylation is isolated at the peptide level, site-specific information can be obtained rather than the summation of all the phosphorylation sites in a given protein. For instance, a study applying the peptide-IP approach to NIH3T3 cells overexpressing the Src oncogene identified 185 tyrosine phosphorylation sites (Rush et al. 2005). There is an important distinction, especially in the context of mapping and quantifying cellular signaling changes, as it is often the dynamics of individual phosphorylation sites and not the average value of all the phosphorylation sites on a single protein that is important in controlling cellular signaling pathways and responses. However, there is a cost to this site-specific resolution in that information about protein-protein interactions is lost when IP is performed at the peptide level.

I.3.3.3 Quantification approaches

Many of the described approaches for phosphoprotein enrichment have proven useful in the cataloging of phosphorylation sites in multiple systems.

However, these analyses do not provide any quantitative information about the system under study. Along these lines, 2 broad approaches that employ isotopic labeling for MS-based quantification of proteins and have gained wide use by the community in the last 5 years, will be discussed briefly in this section. These techniques rely on the use of non-radioactive stable isotopes (^{13}C, ^{15}N, ^{2}H) to introduce a mass difference between different populations of proteins in the mass spectrometer (Ong and Mann 2005). Comparisons of the signal intensities from co-eluting peptides can then be used to determine the relative amounts of each peptide from multiple conditions/cellular states. The 2 quantification strategies that are commonly used are metabolic incorporation (metabolic labeling) or chemical conjugation of stable isotopes (chemical labeling).

a. Metabolic labeling approaches

Metabolic incorporation of cells using stable isotopes has its roots in pulse-chase experiments and takes advantage of the ability of cultured cells to take up isotopes from the media and incorporate them into their own proteins. In pulse-chase experiments, growing cells in culture are spiked with a brief pulse of radioactively labeled amino acid (E.g. ^{35}S-methionine), which is then incorporated into newly synthesized proteins and followed using radioactive imaging. In mass spectrometry, metabolic labeling has evolved from the use of ^{15}N-ammonium salt labeling in yeast and mammalian cells to completely label the entire proteome of the cell to a now commonly used technique known as stable isotope labeling with

amino acids in cell culture (SILAC) (Oda et al. 1999; Conrads et al. 2001; Ong et al. 2002).

In SILAC, two populations of cells are grown in media containing either 'heavy' or 'light' essential amino acids. Incorporation of amino acids into the whole proteome is allowed to proceed to completion after 6 cell doublings and proteins from these two populations are then combined prior to mass spectrometric analysis (Ong et al. 2002). These 'heavy' essential amino acids (e.g. arginine or lysine) contain ^{13}C or ^{15}N incorporated into their backbone structure such that every 'heavy' and 'light' peptide pair that is analyzed in the mass spectrum, has a mass difference that is introduced by the presence of the stable isotopes. Identification of these peptide pairs of fixed mass difference across an MS analysis allows for the relative quantification of multiple proteins between the two conditions (Ong et al. 2002). An advantage of SILAC is that the proteins from the two conditions can be combined together immediately after cell lysis, minimizing errors that may be introduced as a result of variability in sample preparation steps (Ong and Mann 2005). The main disadvantage of SILAC is that it is restricted to cells in culture. Oda and co-workers have tried to overcome this limitation in metabolic labeling by developing a technique known as culture-derived isotope tags (CDITs). They cultured Neuro2A neuroblastoma cell lines in stable isotope-enriched media and mixed them with mouse brain samples to serve as internal standards. While this allowed the relative quantification of a total of 1,000 proteins (98% of which were expressed in both mouse whole brain

and Neuro2A cells) (Ishihama et al. 2005), a similar analysis performed on the phosphoproteome showed only 20% overlap between the 2 samples (Oda 2006).

b. Chemical labeling approaches

An alternative method for incorporating stable isotopes into proteins is by chemical conjugation to reactive side-chains of amino acids. The first demonstration of this approach was in the development of the isotope-coded affinity tag (ICAT) which was designed to react with the sulfydryl side chains of cysteine (Gygi et al. 1999). The tag consisted of a linker synthesized to include eight ^{2}H to confer a mass difference; enrichment of labeled peptides was performed by purification with a biotin tag attached to the linker. However, cysteine residues are not commonly found in proteins, thus the peptides that are enriched only represent a small subset of proteins in the proteome. The advent of the ICAT technology set the stage for the development of several other chemical approaches based on chemistries that would react with more commonly occurring amino acid residues.

One method is the isobaric tag for relative and absolute quantification (iTRAQ) (Ross et al. 2004) (Figure 1.6). This tag contains an N-hydroxysuccinimide (NHS) reactive group that forms a bond with the primary amines of the lysine side chain or the N-terminal end of peptides. When labeling is performed after enzymatic digestion of the proteome, each peptide that is generated will have at least one label incorporated, dramatically increasing the coverage of the proteome. This tag is also unique in that it is isobaric and only

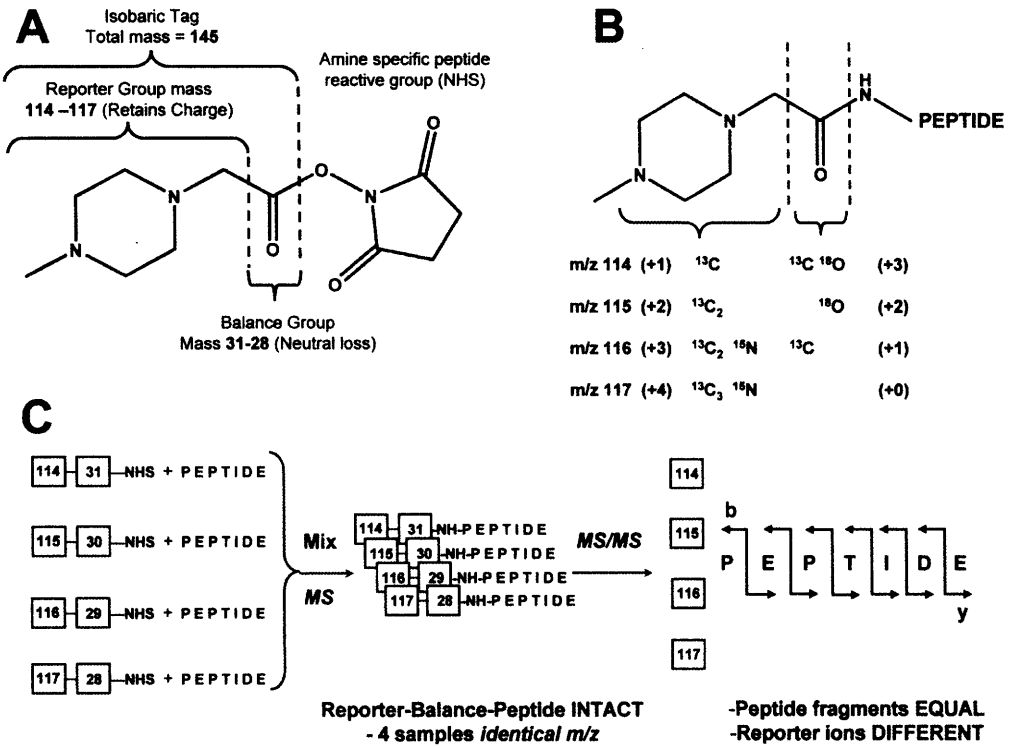

Figure I.6. Diagram of iTRAQ chemistry. (A) diagram showing the components of the multiplexed isobaric tagging chemistry. The complete molecule consists of a reporter group (based on *N*-methylpiperazine), a mass balance group (carbonyl), and a peptide-reactive group (NHS ester). The overall mass of reporter and balance components of the molecule are kept constant using differential isotopic enrichment with ^{13}C, ^{15}N, and ^{18}O atoms (B), thus avoiding problems with chromatographic separation seen with enrichment involving deuterium substitution. The number and position of enriched centers in the ring has no effect on chromatographic or MS behavior. The reporter group ranges in mass from *m/z* 114.1 to 117.1, while the balance group ranges in mass from 28 to 31 Da, such that the combined mass remains constant (145.1 Da) for each of the four reagents. *B*, when reacted with a peptide, the tag forms an amide linkage to any peptide amine (N-terminal or ϵamino group of lysine). These amide linkages fragment in a similar fashion to backbone peptide bonds when subjected to CID. Following fragmentation of the tag amide bond, however, the balance (carbonyl) moiety is lost (neutral loss), while charge is retained by the reporter group fragment. The numbers in parentheses indicate the number of enriched centers in each section of the molecule. (C) Illustration of the isotopic tagging used to arrive at four isobaric combinations with four different reporter group masses. A mixture of four identical peptides each labeled with one member of the multiplex set appears as a single, unresolved precursor ion in MS (identical *m/z*). Following CID, the four reporter group ions appear as distinct masses (114–117 Da). All other sequence-informative fragment ions (b-, y-, etc.) remain isobaric, and their individual ion current signals (signal intensities) are additive. This remains the case even for those tryptic peptides that are labeled at both the N terminus and lysine side chains, and those peptides containing internal lysine residues due to incomplete cleavage with trypsin. The relative concentration of the peptides is thus deduced from the relative intensities of the corresponding reporter ions. In contrast to ICAT and similar mass-difference labeling strategies, quantitation is thus performed at the MS/MS stage rather than in MS. Reprinted with permission from ASBMB Journals: (Ross et al. 2004) Molecular Cellular Proteomics **3**: 1154-1169. Copyright 2004

generates the quantitative signature ions after peptide fragmentation (Figure I.6B). The relative intensities of these signature reporter ions allow one to quantify the same peptide across either 4 or 8 different cellular conditions (Figure I.6C). Since the tag is isobaric and the reporter ions occur only at the peptide fragment spectrum level, the mass spectra acquired are relatively simple in comparison to SILAC. In addition, the ability to quantify 4 or 8 different cell states dramatically increases the multiplexing capability of mass spectrometry which has previously been limited to a maximum of 3 different conditions using SILAC (Ross et al. 2004; Olsen et al. 2006; Ow et al. 2008). Finally, since chemical labeling occurs post cell lysis, this technique is not restricted to cultured cells but can be applied to primary samples including tissues and blood plasma.

I.3.4 Global versus targeted phosphoproteomic analysis of cell signaling networks

The combination of phosphoprotein enrichment approaches and quantitative strategies has transformed our understanding of cell signaling. In particular, the unbiased nature of MS data acquisition has uncovered a previously unappreciated complexity in cellular signaling, in which interrelationships between various signaling pathways result in a multilayered signaling network. In this section, I will highlight specific examples where global and targeted MS analysis of the ErbB family and DNA damage signaling has evolved our understanding from discrete signaling pathways to large-scale signaling networks.

I.3.4.1 Global Approaches

A recent study used an unbiased MS-based approach to study global temporal EGFR signaling dynamics in HeLa cells (Olsen et al. 2006). Sequential phosphopeptide enrichment by SCX and titanium dioxide was performed on SILAC labeled cells treated with EGF over 5 different timepoints prior to analysis by mass spectrometry. This analysis led to the identification of of 6,600 phosphorylation sites, 90% of which were found to be novel. Not surprisingly, due to the unbiased nature of enrichment, 98% of the phosphorylation sites were found on serine and threonine residues. As an added dimensionality to the data set, phosphorylation levels were also quantified as a function of subcellular localization, revealing organelle dynamics for a number of proteins including STAT5. Applying clustering approaches to this dataset, multiple signaling profiles were obtained where the biological function, such as signal initiation or negative regulation, of each of the clusters was inferred from the presence of prominent phosphorylated proteins of known function. As is often the case for discovery based proteomic approaches, a number of proteins identified in this study signaling were previously not associated with growth factor activation, including a series of phosphorylation sites on transcription factors and co-regulators such as DAFT-1 and WBRS9. This study is the most comprehensive phosphoproteomic analysis to date and provides a high density map of the EGFR signaling network. While this heroic effort represents a major advance in the cataloging of cellular phosphorylation sites important in EGF signaling, only a small subset (14%) of all the phosphorylation sites exhibited at least a 2-fold

change upon EGF stimulation. This limited recovery of EGFR-responsive sites highlights the limitation of unbiased global approaches to quantifying cellular phosphorylation.

I.3.4.2 Targeted approaches

A number of different studies have investigated the ErbB phosphotyrosine signaling network using phosphotyrosine IP techniques. Mann and co-workers have used SILAC combined with phosphotyrosine IP at the protein level to study the dynamic EGF and PDGF signaling pathways in mesenchymal stem cells (MSCs) (Kratchmarova et al. 2005). This analysis identified PI3K as an important mediator of bone formation by MSCs. Cells treated with an inhibitor of the PI3K pathway converted PDGF signaling to a bone differentiation signal similar to that observed upon EGF treatment. Our laboratory has also previously developed a technique which combined iTRAQ chemical quantification with phosphotyrosine peptide IP. The immunoprecipitated peptides were further enriched using IMAC prior to analysis by MS. This technique has been applied to study the EGFR signaling network in immortalized human mammary epithelial cells (184A1 HMEC) and yielded quantitative temporal profiles (over 4 time points) for over 100 tyrosine phosphorylation sites, 6 of which were on novel proteins (Zhang et al. 2005).

It is possible to extend the peptide IP approach to the quantitative study of phosphorylation on serine and threonine residues. This approach has recently been applied in 2 studies that focused on the phosphorylation response to DNA

damage after irradiating cells with ionizing radiation (IR). In the first study, human embryonic kidney 293T cells were subjected to IR followed by peptide immunoprecipitation with 68 antibodies recognizing pSQ or pTQ containing motifs (Matsuoka et al. 2007). These motifs are characteristic of substrates recognized by the ATM and ATR DNA damage checkpoint kinases. The authors used mass spectrometry and quantitatively measured 900 phosphorylation sites on 700 proteins that were upregulated at least 4-fold upon DNA damage. These results represent a huge advance over the 25 previously confirmed ATM and ATR substrates (Shiloh 2006). In a second study, the same authors used the BRCT domain of the BRCA1 tumor suppressor protein to enrich for peptides containing the pSXXF motif, again upon irradiation of 293T cells with IR (Wang et al. 2007). Using this approach, they identified a novel BRCA1 protein complex that is required for the DNA damage response to IR.

I.3.5 Intelligent data mining

There exists a large knowledge base of the proteins and genes involved in tumor initiation and progression gleaned from several decades of intensive cancer research. The exponential increase in the knowledge base as a result of large scale phosphoproteomic studies such as those described above, bring about a new set of data mining challenges. For example, in the EGFR study performed in HeLa cells (Olsen et al. 2006), there was minimal mechanistic insight into the functional role of the regulated phosphorylation sites in the large dataset. And phosphorylation site function was assigned through "guilt by

association" clustering with other proteins/phosphorylation sites of known function.

Intelligent data mining offers an alternative by simplifying and assembling large data sets and therefore provides a way to gain mechanistic insight. Statistical modeling approaches designed to correlate signaling network nodes and cancer phenotypes have been developed in an attempt to address these limitations (Janes and Lauffenburger 2006). This approach has been applied to a large scale phosphotyrosine dataset of temporal EGFR and ErbB2 signaling in human mammary epithelial cells (Wolf-Yadlin et al. 2006). From this analysis, it was determined that cellular phenotypic differences can be attributed to specific changes in downstream signaling networks in response to growth factor stimulation. For instance, following EGF stimulation, cells stably transfected to express high ErbB2 levels were found to be highly migratory compared to parental cells. This phenotype was linked to phosphorylation changes associated with decreased cell-cell adhesion and a subset of the known cell migration signaling network, including FAK and p130cas.

Application of partial least squares regression (PLSR) statistical models led to the identification of phosphorylation sites in the EGFR and ErbB2 networks that were highly correlated with cell migration or proliferation, lending functional consequence to phosphoproteomic datasets. Derivation of such network-phenotype relationships represents a rich source of potential targets for biological validation. One such example is the successful use of a small molecule inhibitor of Annexin II, a target found in the study to strongly correlate with migration, for

the inhibition of migration and invasion in cancer cells (Falsey et al. 2006). Further statistical modeling of the data demonstrated that nine phosphorylation sites on six proteins involved in receptor endocytosis and phosphoinositide 3-kinase (PI3K)-mediated pathways were able to fully recapitulate the predictive capability of the PLSR model (Kumar et al. 2007). These nodes correspond to "biomarkers" that may be used to predict the proliferative or migratory potential of EGFR and ErbB2-driven tumors.

I.4 MOTIVATION

Despite significant advances in the understanding of GBM tumor biology, the outlook for patients remains dismal. In particular, while the wealth of knowledge available on the role of the ErbB receptor family in cancer has led to dramatic progress in the clinical management of other tumor types, such as breast and lung cancers, it has not translated into effective therapies for GBM patients. The lack of efficacious agents for this disease would suggest that alternative approaches to elucidating GBM tumor biology may be beneficial in the identification of novel targets for therapeutic development.

Our laboratory has previously developed a quantitative phosphoproteomic approach capable of analyzing cellular signaling networks in a system-wide fashion and has successfully applied it to the study of model systems such as EGFR signaling in human mammary epithelial cells. In this thesis, I have extended the power of this approach to the study of clinically relevant mutations in the EGFR receptor, in particular, the EGFRvIII receptor in the context of glioblastoma.

Although much work has been done to elucidate EGFRvIII-specific signaling pathways in the last decade, the mechanisms by which it contributes to tumor initiation and progression are still not well understood. Furthermore, there are several outstanding questions in which there is a lack of consensus in the field, including how EGFRvIII confers tyrosine kinase inhibitor resistance, the

issue of receptor homo-dimerization and the quantitative nature of dominant signals downstream of the receptor and how they contribute to its tumorigenicity.

The goal of this thesis is two-fold, to employ mass spectrometric-based phosphoproteomic approaches to gain a more complete understanding of the mechanisms by which EGFRvIII exerts it transforming ability and to use this information to identify novel therapeutic targets with the hope of developing new strategies to treat this disease.

The thesis is divided into two sections. The first describes an analysis of the effects of oncogene dose on EGFRvIII downstream signaling networks and the discovery of a therapeutic strategy that exploits receptor tyrosine kinase crosstalk. The second section will focus on a study of how networks are regulated *in vivo,* in response to site-specific tyrosine mutations at the receptor level. This analysis has revealed interesting insights into network modulation and how EGFRvIII fine-tunes the activity of a key downstream signaling node. In the course of this thesis, I have established the power of MS-based quantitative phosphoproteomics as a tool to provide molecular insight into cancer biology and as a means for drug target discovery.

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II QUANTITATIVE ANALYSIS OF EGFRVIII CELLULAR SIGNALING NETWORKS REVEALS A COMBINATORIAL THERAPEUTIC STRATEGY FOR GLIOBLASTOMA

II.1 SUMMARY

While a decade of experiments using classical genetics and molecular biology have provided many insights into the biology of EGFRvIII in the context of glioblastoma (GBM), a comprehensive description of the EGFRvIII cellular signaling network is still lacking. By determining the global map of the signaling networks that are initiated by EGFRvIII, one would be better able to assess the downstream signaling components involved in EGFRvIII-mediated transformation in GBM.

In this chapter, I will describe the use of an unbiased mass spectrometry-based phosphoproteomic approach to quantify tyrosine phosphorylation events that occur in GBM cells upon expression of titrated levels of EGFRvIII (from $1.5x10^6$ to $3x10^6$ receptors). Analysis of EGFRvIII expressing GBM cells resulted in a comprehensive system view of EGFRvIII-mediated tyrosine phosphorylation with the identification and quantification of 99 phosphorylation sites on 69 proteins.

This analysis highlighted several aspects of EGFRvIII biology, including effects of oncogene dose on receptor activation and utilization of downstream biological signaling networks, differential pathway utilization between wildtype EGFR and EGFRvIII, and the identification of crosstalk between EGFRvIII and other receptor tyrosine kinases. For instance, self-organizing map analysis identified the activating phosphorylation site on the c-Met receptor (Y1234) as a node that was highly responsive to EGFRvIII levels, indicating cross-activation of the c-Met receptor tyrosine kinase by EGFRvIII.

To determine the significance of this finding, we devised a combined treatment regimen that used a c-Met kinase inhibitor and either an EGFR kinase inhibitor or cisplatin. This regimen resulted in enhanced cytotoxicity of EGFRvIII expressing cells compared to treatment with either compound alone. These results suggest that the clinical use of c-Met kinase inhibitors in combination with either EGFR inhibitors or standard chemotherapeutics might represent a previously undescribed therapeutic approach to overcome the observed chemoresistance in patients with GBMs expressing EGFRvIII.

This work was performed in collaboration with Frank Furnari and Webster Cavenee at the Ludwig Institute for Cancer Research (San Diego Branch) and has previously been published in the Proceedings of the National Academy of Sciences of the United States of America (PNAS) volume 104 (31), July 2007, pages 12867-12872. PNAS does not required copyright permission requests for reproduction of figures for dissertation purposes. Please refer to the following website for further information, http://www.pnas.org/misc/rightperm.shtml.

II.2 MATERIALS AND METHODS

II.2.1 Cell Culture, Retrovirus Infection, and Transfection

The human glioblastoma cell lines, U87MG and U373MG, and their engineered derivatives were cultured in DMEM with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin in 95% air/5% CO2 atmosphere at 37°C. U87MG cells expressing EGFRvIII or DK receptors were selected in 400 µg/ml G418. For expression of tetracycline regulated EGFRvIII and DK, U373MG glioma cells were transfected with pRev-tet-off (Invitrogen) by the calcium phosphate method (Furnari et al. 1998) and selected in 400 µg/ml G418. Individual tTA-expressing clones were analyzed for GFP expression as expressed from transiently transfected pTRE-GFP, in the presence and absence of 1 µg/ml doxycycline (dox). A clone (c.16) demonstrating robust expression of GFP in the absence of dox was subsequently co-transfected with pBABE-puro and pTRE-EGFRvIII-IRES-GFP or pTRE-DK-IRES-GFP and stable populations were obtained by selection in 1 µg/ml puromycin. Induction of EGFRvIII-IRES-GFP and DK-IRES-GFP was achieved upon growth in dox-free media.

II.2.2 Flow Cytometry

For U87MG cells expressing various levels of EGFRvIII, a bulk population of cells was prepared by retroviral transduction with pLERNL and stained as described (Nishikawa et al. 1994) with anti-EGFR monoclonal antibody Ab-1 (clone 528; Oncogene Science, Cambridge, MA), followed by fluorescein isothiocyanate-conjugated goat anti-mouse Ig antibody (PharMingen, Minneapolis, MN) and sorted for medium (1.5 x 10^6 receptors, U87MG-M), high (2.0 x 10^6 receptors, U87MG-H), and superhigh (3.0 x 10^6 receptors, U87MG-SH) receptor amounts. For this procedure, U87-EGFRvIII cells engineered previously and determined to express 2 x 10^6 receptors per cell were used as a gating control (1). The sorted cells were then maintained in culture and receptor levels were analyzed again by flow cytometry prior to experimental use (Figure II.1). Cells for all the experiments described in this study were performed within 5 cell passages from the time of flow cytometry analysis.

II.2.3 Xenografts

Cells (1 x 10^6) were suspended in 0.1 ml of PBS and injected into the right flanks of nude mice. Tumor volumes were defined as (longest diameter) x (shortest diameter)^2 x 0.5. All of the procedures were approved by the animal care and use committee of the University of California, San Diego.

II.2.4 Cell lysis, Protein digestion and Peptide fractionation

U87MG cells were maintained in DMEM medium supplemented with 10% FBS. $1.5x10^6$ cells per 10cm plate were seeded for 24 hours, then washed with PBS and incubated for 24 hrs in serum-free media. Cells were lysed in 1 ml of 8 M urea. For each of the two biological replicates performed, lysate from three 10cm plates were pooled together. Cells were reduced with 10 mM DTT for 1 hr at 56˚C, alkylated with 55mM iodoacetamide for 45 min at room temperature, and diluted to 12 ml with 100mM ammonium acetate, pH 8.9, prior to digestion with 40 µg of trypsin (Promega). The lysates were digested overnight at room temperature. Digested lysate were acidified to pH 3 with acetic acid and loaded onto a C18 Sep-Pak Plus cartridge (Waters). The peptides were desalted (10ml 0.1% acetic acid) and eluted with 10 ml of a solution of 25% acetonitrile and 0.1% acetic acid. Each sample was divided into 5 aliquots and lyophilized to dryness.

II.2.5 iTRAQ labeling of peptides

Lyophilized peptides were subjected to labeling with the iTRAQ 4-plex reagent (Applied Biosystems). Each aliquot of peptides was dissolved in 30 µl of 0.5 M triethylammonium bicarbonate, pH 8.5 and reacted with two tubes of iTRAQ reagent (dissolved in 70 µl of ethanol each). The reagents for each of the conditions used were, iTRAQ-114 (U87MG-DK), iTRAQ-115 (U87MG-M), iTRAQ-116 (U87MG-H) and iTRAQ-117 (U87MG-SH). The mixture was

incubated at room temperature for 50 mins and then concentrated to 30 µl. The four different isotopically labeled samples were combined and acidified with 360 µl of 0.1% acetic acid and then reduced to dryness.

II.2.6 Peptide immunoprecipitation

The combined sample was reconstituted with 150 µl of IP buffer (100 mM Tris, 100 mM NaCl, 1% NP-40, pH 7.4), 300 µl of water and the pH was adjusted to 7.4. The sample was incubated with 10 µg of protein G Plus-agarose beads (Calbiochem) and 12 µg of anti-phosphotyrosine antibody (PY100 Cell Signaling Technology) for 8 hrs at 4°C. The antibody-bead conjugates were then spun down for 5 mins at 6000 rpm at 4°C and the supernatant was saved. The beads were then washed three times with rinse buffer (100 mM Tris, 100 mM NaCl, pH 7.4) for 5 minutes at 4°C, prior to elution with 70 µl of 100mM glycine pH 2.5 for 30 mins at room temperature.

II.2.7 Immobilized metal affinity chromatography (IMAC) and Mass Spectrometry

Immobilized metal affinity chromatography (IMAC) was performed to enrich for phosphorylated peptides and remove non-specifically retained non-phosphorylated peptides. Eluted peptides were loaded onto a 10 cm self-packed IMAC (20MC, Applied Biosystems) capillary column (200 µm ID, 360 µm OD), and rinsed with organic rinse solution (25% MeCN, 1% HOAc, 100 mM NaCl) for

10 mins at 10 µl/min. The column was then equilibrated with 0.1% HOAc for 10 mins at 10 µl/min and then eluted onto a 10 cm self-packed C18 (YMC-Waters 10 5 µm) precolumn (100 µm ID, 360 µm OD) with 50 µl of 250mM Na_2HPO_4, pH 8.0. After a 10 min rinse with 0.1% HOAc, the precolumn was connected to a 10 cm self-packed C18 (YMC-Waters 5 µm ODS-AQ) analytical capillary column (50 µm ID, 360 µm OD) with an integrated electrospray tip (1 µm orifice). Peptides were eluted with a 125 minute gradient with solvents A (1% HOAC) and B (70% MeCN in 1% OHAc): 10 mins from 0% to 13%, 95 mins from 13% to 42%, 10 min from 42% to 60% and 10 min from 60% to 100%. Eluted peptides were directly electrosprayed into a QqTof mass spectrometer (QSTAR XL Pro, Applied Biosystems). MS/MS spectra of the five most intense peaks with 2 – 5 charge states in the full MS scan were automatically acquired in information-dependent acquisition mode with previously selected peaks excluded for 40 secs.

II.2.8 Phosphopeptide sequencing, quantification and clustering

MS/MS spectra were extracted and searched using ProQuant (Applied Biosystems) and MASCOT (Matrix Science). For ProQuant, an interrogator database was generated by predigesting *in silico*, the human protein database with trypsin and allowing one missed cleavage and up to six modifications on a single peptide (phosphotyrosine ≤ 2, phosphoserine ≤ 1, phosphothreonine ≤ 1, iTRAQ-lysine ≤ 4 and iTRAQ-tyrosine ≤ 4). Mass tolerance was set to 2.2 amu for the precursor ions and 0.15 amu for the fragment ions. For MASCOT, data was searched against the human non-redundant protein database with trypsin

specificity, 2 missed cleavages, precursor mass tolerance of 2.2 amu for the precursor ion and 0.15 for the fragment ion tolerance.

Phosphorylation sites and peptide sequence assignments were validated by manual confirmation of raw MS/MS data. Peak areas of iTRAQ marker ions (m/z 114, 115, 116 and 117) were quantified by ProQuant and normalized with values from the iTRAQ marker ion peak areas of non-phosphorylated peptides in the supernatant of the immunoprecipitation (used as a loading control to account for possible variation in the starting amount of sample for each condition). Each condition was normalized against the U87MG-H cell line to obtain fold changes across all 4 conditions. Normalized data sets were loaded into Spotfire and the self-organizing map algorithm was used to cluster the phosphorylation sites with self-similar profiles.

II.2.9 Immunoblotting Analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium PP_i, 1 mM β-glycerophosphate) containing protease and phosphatase inhibitors after the indicated treatment. Protein concentration of cell lysates was determined using micro bicinchoninic acid assay (Pierce), according to the manufacturer's protocol. 50 µg of protein from the cell lysate was mixed with 4X sample buffer (250 mM Tris-HCl, pH 6.8, 40% glycerol, 0.04% bromophenol blue and 400 mM DTT) and loaded on either 7.5% or 10% SDS-PAGE gels, separated and transferred onto PVDF membranes. After blocking for 1 hr at room temperature with 5% BSA,

membranes were incubated overnight at 4°C in primary antibody and washed 3 times at 10 mins each in TBS-Tween buffer (20mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween-20). Membranes were then incubated at room temperature for 1 hr in secondary antibody and washed 6 times at 10 mins each in TBS-Tween buffer. Blots were developed with the SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce) and scanned on a Kodak Image station 1000. Primary antibodies used were anti-EGFR pY1173, anti-c-Met (Santa Cruz Biotechnology), anti-phosphotyrosine 4G10, anti-c-Met pY1230/1234/1235 (Upstate Biotechnology), anti-EGFR and anti-actin (Cell Signaling Technologies). Secondary antibody used was goat anti-rabbit antibody (Upstate Biotechnology).

II.2.10 Kinase inhibitor treatment

Cells were serum starved for 24 hrs prior to being treated with the indicated dose of either AG1478 (A.G. Scientific) or SU11274 (Calbiochem) for 1 hour. Cells were then lysed as described above for either immunoblotting or mass spectrometric analysis.

II.2.11 Cell viability assays

4,000 cells were seeded per well in a 96 well plate. 24 hrs later, the cells were serum starved for 24 hrs prior to addition of 100 µl fresh serum free media containing AG1478, SU11274, PHA665752 (A kind gift from Eli Lilly) or cisplatin (Sigma-Aldrich) at the indicated doses and combinations. After 72 hrs, cell

viability was measured using the WST-1 reagent (Roche Applied Sciences). 10 µl WST-1 reagent was added to each well and incubated at 37°C for two hours, prior to measuring absorbance at 450nm in a spectrophotometer.

II.2.12 Apoptosis assay

10,000 cells were seeded per well in a 96 well plate. 24 hrs later, the cells were serum starved for 24 hours prior to addition of 100 µl fresh serum free media containing AG1478 and SU11274 at the indicated dose and combinations. After 24 hrs of drug treatment, caspase 3/7 activity was measured using Apo-ONE Homogeneous Caspase-3/7 Assay (Promega), following manufacturer's recommendations. Briefly, 100 µl of Apo-ONE Caspase-3/7 reagent with fluorescent substrate was added to each well, incubated for 12 hrs, prior to measuring fluorescence at $485_{Ex}/527_{Em}$

II.2.13 Hepatocyte growth factor ELISA

1.5×10^6 cells were serum-starved for 24 hrs before removal of media for measurement. Secreted HGF levels were measured using HGF Elisa kit (BioSource International, Camarillo, CA) according to the manufacturer's recommendations. After removal of media, cells were counted, and all HGF measurements were normalized to cell number.

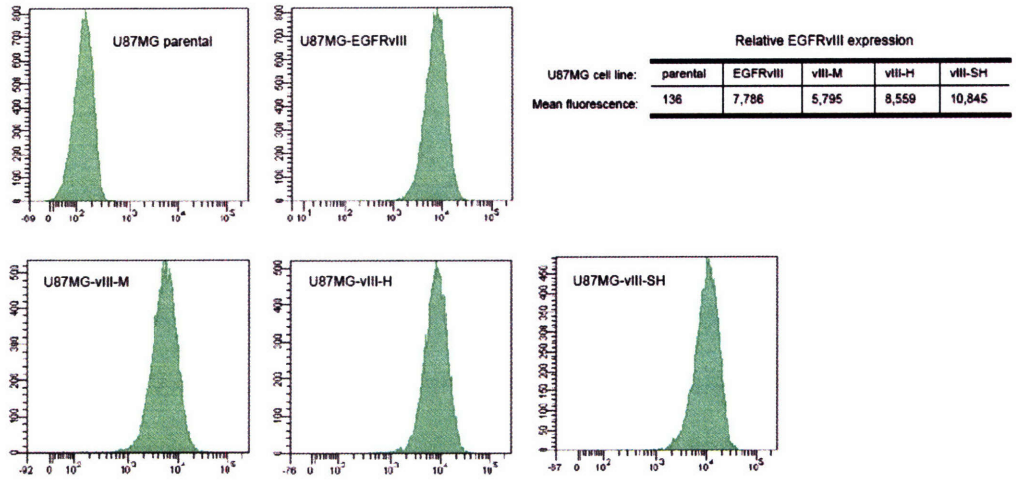
II.2.14 Anti-HGF treatment

U87-H cells were serum-starved for 24 hrs before treatment with either 5μg/ml anti-HGF (R&D Systems, Minneapolis, MN) or 5 μg/ml control IgG (Sigma-Aldrich, St. Louis, MO) for 30 min. As a positive control, U87H cells were stimulated with 50 ng/ml HGF (R&D Systems) for 5 min after 30 min treatment with either anti-HGF or control IgG. Cells were then lysed as described above for immunoblotting analysis.

II.3 RESULTS

II.3.1 Cell lines and experimental strategy

To determine the effect that EGFRvIII receptor expression levels has on phosphotyrosine-mediated cellular signaling networks, U87MG glioblastoma cell lines expressing differential levels of EGFRvIII were isolated by FACS sorting of transduced populations, maintained in culture and subsequently analyzed by mass spectrometry to identify and quantify tyrosine phosphorylation sites on cellular signaling proteins. Flow cytometry and western blot analysis of these cells prior to mass spectrometric analysis were performed to confirm the titrated expression levels of EGFRvIII and quantify relative levels of tyrosine phosphorylation across the 3 cell lines (Figure II.1A and Figure II.2B). In addition, a previously derived U87MG cell line expressing 2 million copies of a kinase dead EGFRvIII receptor was used as a control (Huang et al. 1997); It has also been previously shown that tumorigenic potential increases with increased EGFRvIII receptor levels (Johns et al. 2007). Flow cytometry analysis of the sorted U87MG cells that were maintained in culture for more than 20 passages showed a drift in mean receptor levels, resulting in U87-M with 1.5 x 10^6 receptors, U87-H with 2.2 x 10^6 receptors and U87-SH with 2.8 x 10^6 receptors (Figures I and IIA). To minimize possible variation that may be caused by further drifts in mean receptor levels of the population, all experiments described in this study were performed within 5 passages of flow cytometry analysis. We also



Relative EGFRvIII expression

U87MG cell line:	parental	EGFRvIII	vIII-M	vIII-H	vIII-SH
Mean fluorescence:	136	7,786	5,795	8,559	10,845

Figure II.1. Flow cytometry analysis of receptor levels. Flow cytometry analysis of EGFRvIII levels expressed in sorted U87MG cells was performed as described in Section II.2.2 prior to experimental use. Histograms show relative levels of membrane-expressed receptors as determined by FITC-conjugated antibody staining fluorescence intensity. Fluorescence for U87MG parental cells was arbitrarily set to 100. U87MG-EGFRvIII correspond to cells previously characterized to express 2 x 10^6 receptors (Nishikawa et al. 1994).

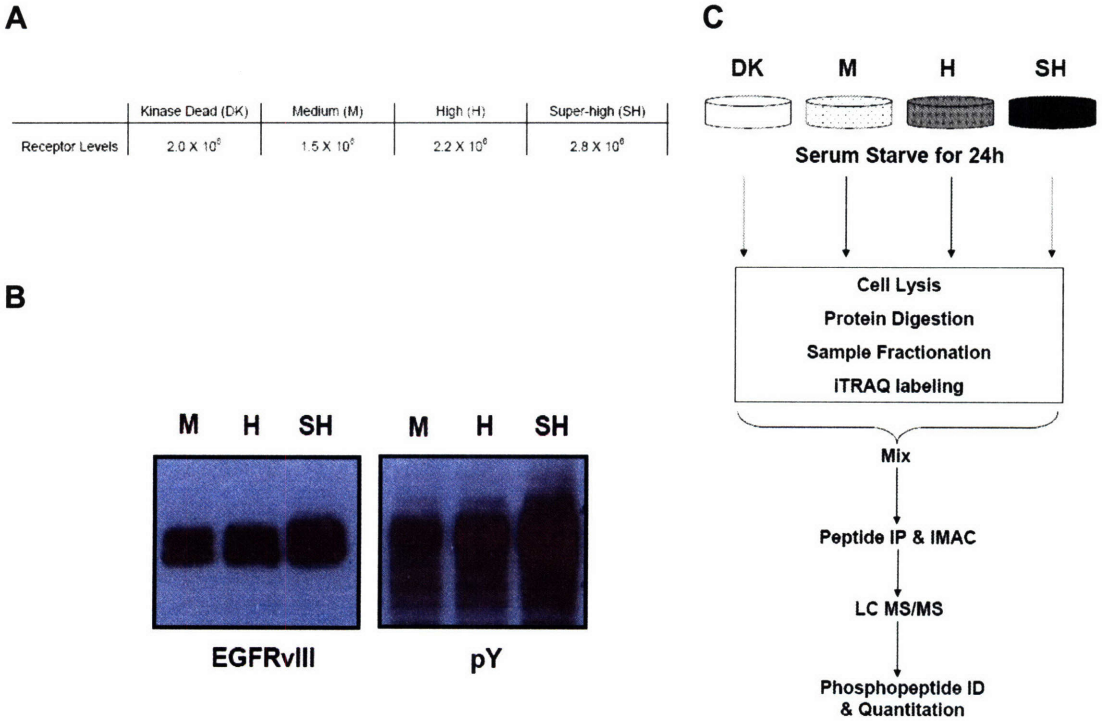


Figure II.2. Cell lines and experimental strategy. (A) EGFRvIII expression levels in retrovirally transfected U87MG cell lines as calculated from flow cytometry analysis. U87MG-DK cell lines were previously derived in (Huang et al. 1997) (B) Western blot of U87MG cell lines expressing titrated levels of EGFRvIII. Cells were serum-starved for 36 h, lysed, and probed for EGFRvIII or phosphotyrosine levels. (C) Outline of MS-based experimental strategy.

observe that the distribution of receptor levels for the cell lines as indicated by the flow cytometry histograms (Figure II.1) is broad with a 75% overlap between the U87-M and U87-H populations and a 83% overlap between the U87-H and U87-SH populations.

To identify the signaling pathways that are constitutively activated downstream of the EGFRvIII receptor while minimizing confounding signaling associated with serum and cell culture media, the four cell lines were serum starved for 24 hours prior to cell lysis and sample preparation. Peptides from the four samples were stable-isotope labeled, mixed, and tyrosine phosphorylated peptides were immunoprecipitated with a pan-specific anti-phosphotyrosine antibody (Figure II.2C). Following immunoprecipitation, phosphorylated peptideswere further enriched by immobilized metal affinity chromatography (IMAC) and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). In total, quantitative phosphorylation profiles were generated for 99 phosphorylation sites on 69 proteins across the 4 cell lines. Two biological replicates were performed with an average SD of 15% from the mean for tyrosine phosphorylated peptides that appear in both analyses. Due to the broad distributions of receptor levels within each cell population, we recognize that the standard deviation in the mass spectrometric data may be larger than what we observe experimentally.

II.3.2 Quantitative effects of titrated EGFRvIII levels on receptor phosphorylation and major downstream signaling pathways

We identified eight phosphorylation sites on EGFRvIII and determined their quantitative phosphorylation profiles as a function of increasing EGFRvIII receptor levels (Figure II.3). Interestingly, phosphorylation of Y974, Y1068, Y1114, Y1148 and Y1173 increased greater than two-fold from the U87-M (1.5 million copies EGFRvIII/cell) to U87-H (2.2 million copies EGFRvIII/cell) cells. This increase in EGFRvIII autophosphorylation levels would suggest the presence of a threshold of EGFRvIII expression: once EGFRvIII exceeds this threshold expression level, activation of the receptor and resulting autophosphorylation increases significantly. For these sites, increasing receptor expression beyond 2.2 million copies/cell did not increase the level of phosphorylation, suggesting a saturation point. Due to the broad distributions found between the different cell populations (Figure II.1), it is plausible that the lack of an increase in receptor phosphorylation above 2.2 million copies/cell may be the result of the large overlapping fraction of cells expressing similar receptor levels between the U87-H and U87-SH cells. However, it is also worthy to note that the phosphorylation levels on several proteins downstream from the receptor continue to increase past this saturation point (figure II.5A), suggesting that the lack of increase in EGFR autophosphorylation in the U87-SH cells may also be due to negative feedback mechanisms acting on the EGFRvIII receptor.

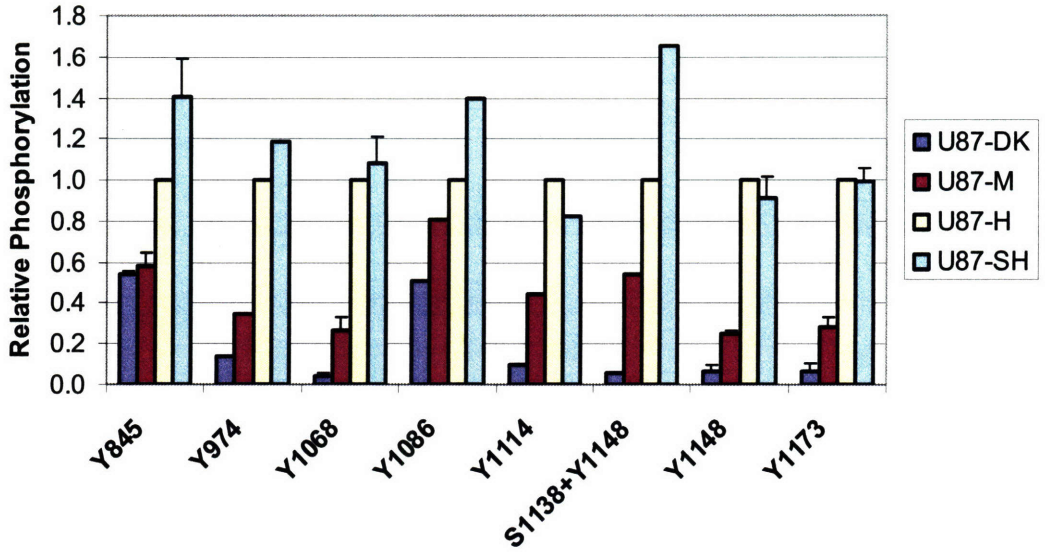


Figure II.3. Relative quantification of EGFRvIII phosphorylation sites across the four cell lines. Phosphorylation levels were normalized relative to that of the U87-H cell line with error bars representing phosphorylation sites that appeared in both biological replicate analyses.

Mapping the phosphorylation data to canonical EGFR signaling cascades (Figure II.4), indicates that EGFRvIII favors the utilization of different downstream pathways compared to wild-type EGFR. It has been previously demonstrated that EGF stimulation of human mammary epithelial cells expressing wild-type EGFR led to a dramatic increase in the active form of Erk1, Erk 2 and STAT3 within 5 minutes of stimulation (Wolf-Yadlin et al. 2007). In contrast, increasing EGFRvIII receptor expression levels had little effect on the phosphorylation levels of these proteins. Moreover, temporal analysis of wild-type EGFR signaling indicated that activation of this receptor led to a modest increase in the tyrosine phosphorylation levels on PI3K and its upstream adaptor protein GAB1 (Wolf-Yadlin et al. 2007). However, increasing EGFRvIII receptor levels dramatically increased the phosphorylation levels on these proteins by more than three-fold, indicating that the PI3K pathway is highly active in EGFRvIII overexpressing

cells. These data are consistent with previous studies in which EGFRvIII has been found to activate the PI3K pathway (Moscatello et al. 1998). Preferential activation of this pathway by EGFRvIII (in addition to its constitutive activation) may be a reason for its observed tumorigenic properties *in vivo,* since PI3K signaling has been implicated in promoting cell proliferation, survival and migration.

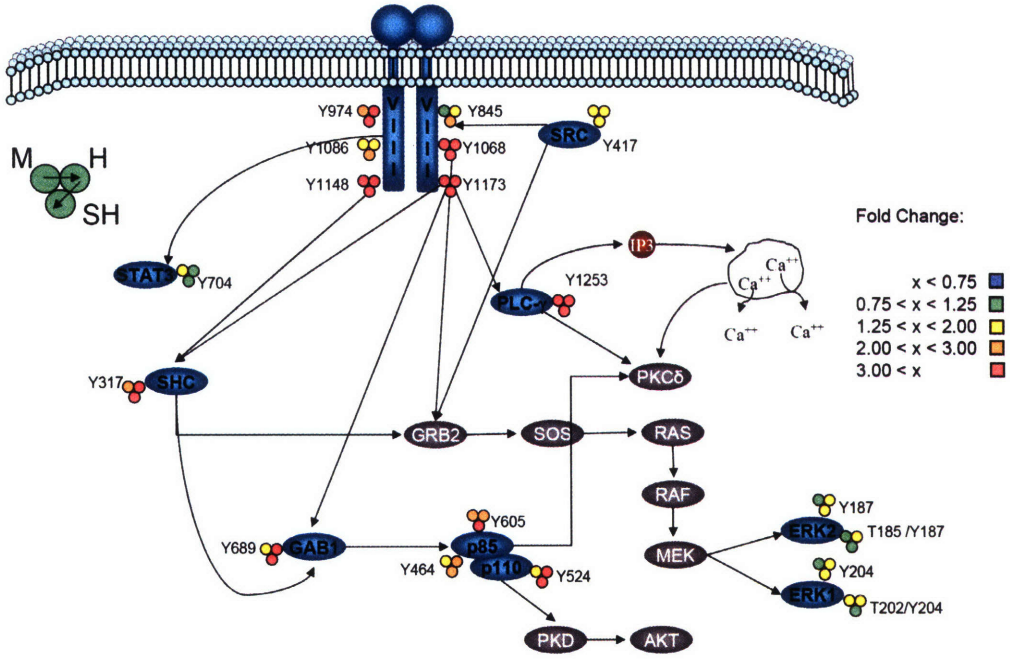


Figure II.4. Effect of increased EGFRvIII expression levels on phophorylation sites within the EGFR network. Visualization of the fold change in phosphorylation levels in the canonical EGFR signaling cascades as a function of titrated EGFRvIII levels provides a network view of the mechanistic effects of EGFRvIII levels. Phosphorylation levels are normalized relative to that of the DK cell line.

II.3.3 c-Met receptor tyrosine kinase activation is highly responsive to EGFRvIII receptor levels

To characterize relationships within the dataset, a self-organizing map was used to identify clusters of tyrosine phosphorylation sites with similar profiles

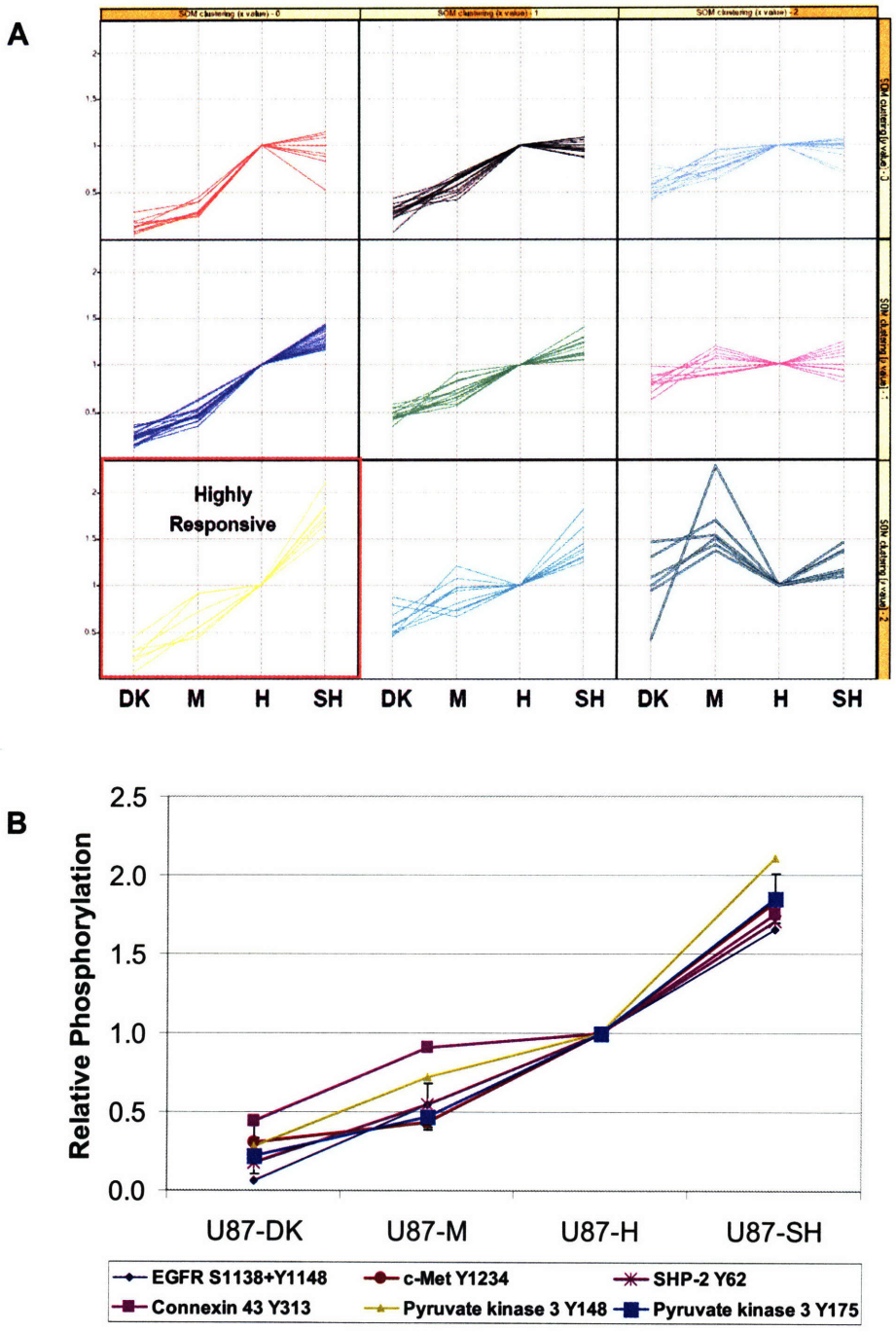


Figure II.5. Clustering analysis of phosphotyrosine data set using self-organizing maps. (A) Each column within the matrix components represent the relative phosphorylation level in the -DK, -M, -H, and -SH U87MG cell lines normalized against the U87H cell line. Optimal SOM architecture was a 3 x 3 matrix, because smaller matrices tended to cluster dissimilar phosphorylation profiles. (B) A subset of protein phosphorylation sites found within the highly responsive cluster with error bars representing phosphorylation sites that appeared in both biological replicate analysis.

(Figure II.5A). One such cluster ('highly responsive cluster') consisted of phosphorylation sites that increased as a function of increasing EGFRvIII expression levels. Sites in the cluster include Y1234, the activating phosphorylation site on the catalytic loop of the c-Met receptor tyrosine kinase (6 fold increase relative to U87-DK) and an uncharacterized phosphorylation site (Y62) on the tyrosine phosphatase SHP-2 (9 fold increase relative to U87-DK), a protein known to be downstream of the c-Met receptor (Figure II.5B).

Phosphorylation of the c-Met receptor was confirmed by western blot analysis of the 4 cell lines both in vitro and *in vivo* (Figures II.6A and B respectively). To ensure that c-Met phosphorylation was not unique to U87MG cells, tet-inducible EGFRvIII expressing U373MG glioblastoma cell lines were tested. EGFRvIII-mediated activation of the c-Met receptor was also observed in these cells (Figure II.6C).

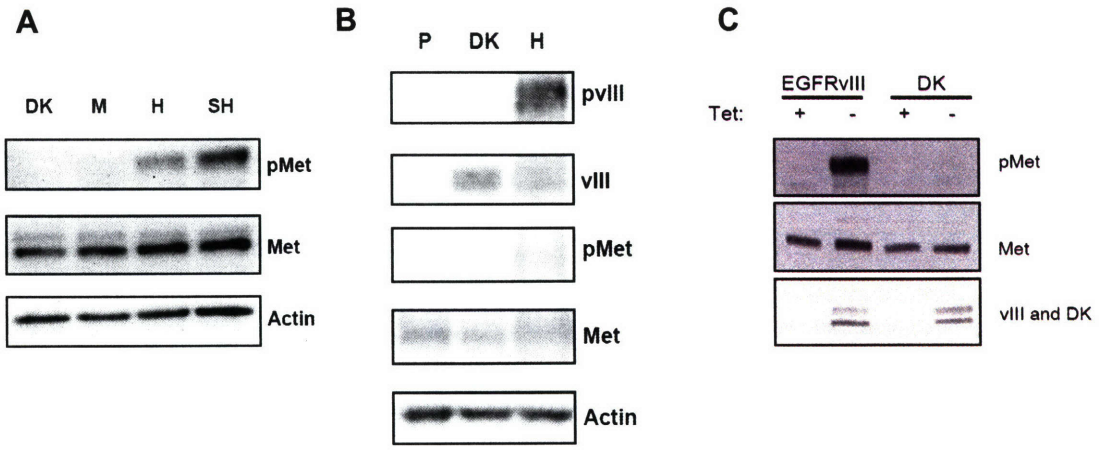


Figure II.6. Western blot confirmation of c-Met receptor phosphoryaltion. (A) Western blot of specific phosphorylation sites on the c-Met receptor (Y1230/Y1234/Y1235) across the four different U87MG cell lines *in vitro* after 24 hrs serum starvation. (B) Western blot of c-Met receptor phosphorylation levels of *in vivo* parental (P), DK, or EGFRvIII High-expressing U87MG-derived xenografts. (C) Western blot of c-Met receptor phosphorylation after 36 hrs serum starvation in tet-inducible U373MG cell lines expressing either EGFRvIII or the kinase-dead (DK) version of the EGFRvIII.

Increased phosphorylation of the c-Met activation site led us to postulate that the EGFRvIII receptor was constitutively activating the c-Met receptor pathway. Mapping of our phosphoproteomic data to previously described c-Met signaling pathways (Birchmeier et al. 2003) confirmed that many of the known components downstream of the c-Met receptor were activated at least three-fold as a function of increasing EGFRvIII expression levels (Figure II.7). Many of the activated downstream components of the c-Met receptor overlap with the downstream targets of EGFRvIII (Figure II.4). It is well established that receptor tyrosine kinases share common downstream signaling components. The activation of a common set of downstream signaling events may represent an

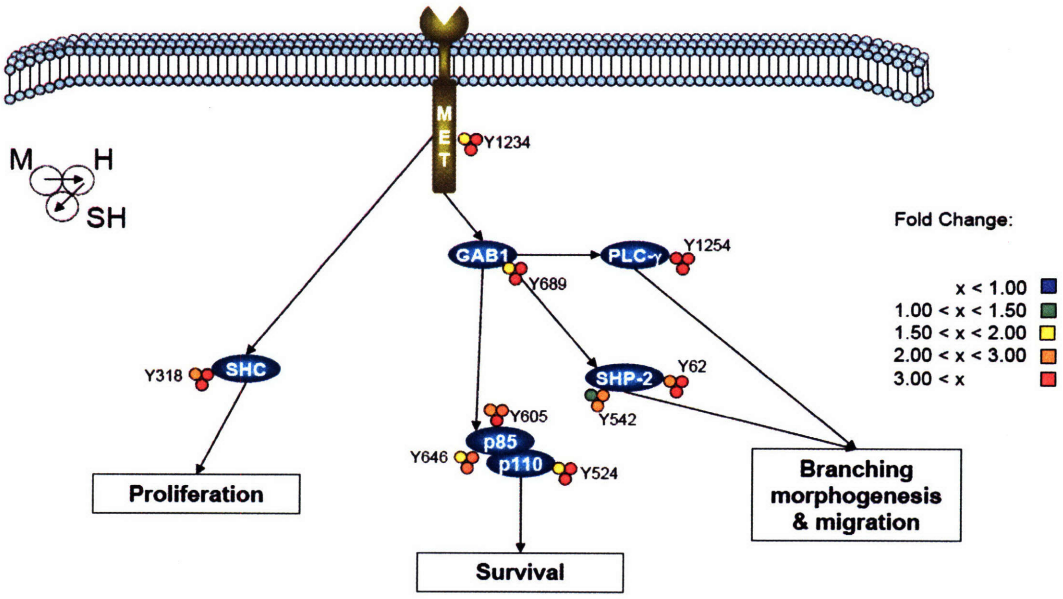


Figure II.7. Effect of increased EGFRvIII expression levels on phophorylation sites within the c-Met receptor network. Visualization of the fold change in phosphorylation levels in the canonical c-Met signaling pathways as a function of titrated EGFRvIII levels provides a network view of the mechanistic effects of downstream components of c-Met. Phosphorylation levels are normalized relative to that of the DK cell line.

integrated signaling cascade resulting from the co-activation of both the c-Met and EGFRvIII receptors.

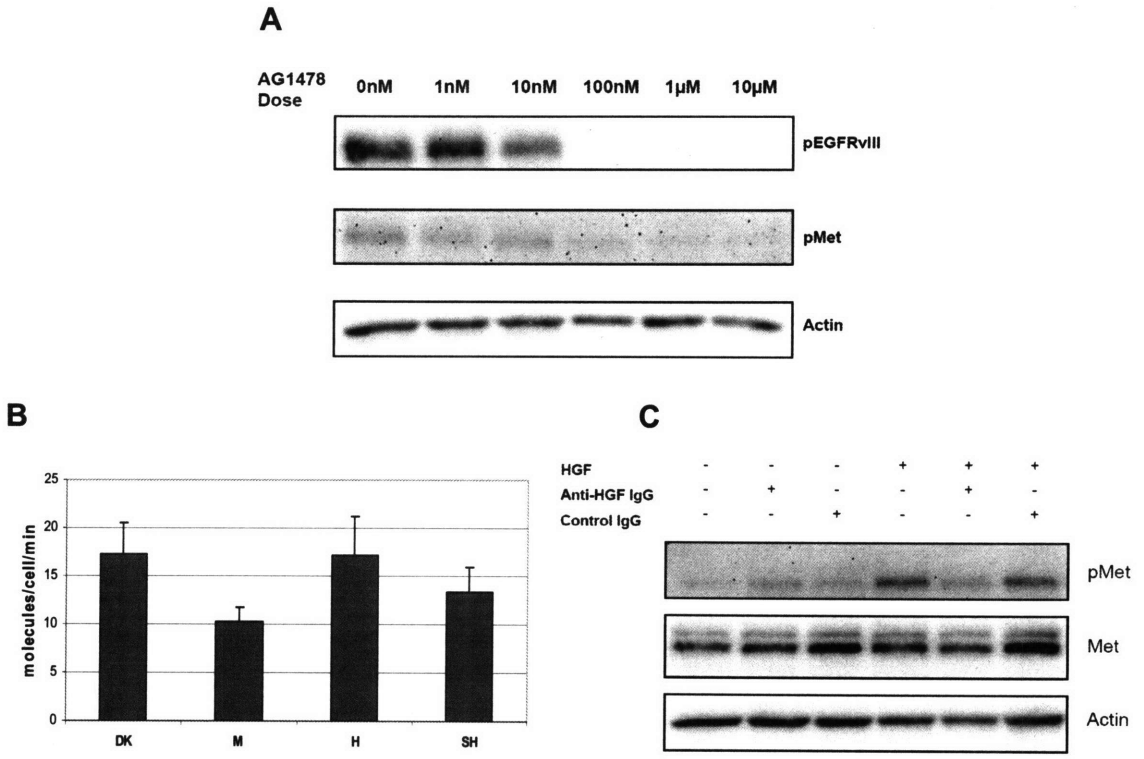


Figure II.8. c-Met mechanism of activation. (A) Western blot of U87-H cell line subjected to 1 hr AG1478 dose escalation after 24 hr serum starvation. (B) Measurement of HGF secreted into the media after 24-h serum starvation by Anti-HGF ELISA. Data were normalized to the total cell number. (C) Western blot of phosphorylation on the c-Met receptor (Y1230/Y1234/Y1235) on the U87-H cell line after 24 hr serum starvation and treatment with either 5 mg/ml anti-HGF or goat control IgG; 50 ng/ml HGF treatment was used as a positive control.

As a complementary approach to demonstrate that c-Met receptor activation was a direct consequence of EGFRvIII receptor activation, we treated U87-H cells with AG1478, an EGFR kinase inhibitor that has some preference for EGFRvIII (Han et al. 1996). Western blot analysis revealed a dose-dependent decrease in EGFRvIII phosphorylation levels accompanied by a concomitant decrease in the phosphorylation status of c-Met (Figure II.8A). Since the U87MG cell line has previously been shown to express the c-Met ligand HGF

(Chattopadhyay et al. 2001), we sought to determine if this EGFRvIII-mediated c-Met activation was ligand dependent. HGF levels secreted into the media by the 4 cell lines were determined by ELISA (Figure II.8B). The U87MG cell lines secreted HGF at a rate of between 10-18 molecules/cell/min. Previous analysis of EGF/EGFR autocrine loops in a mouse fibroblast cell-line and human mammary epithelial cell lines demonstrated that levels of EGF or TGFα in the media were only measurable when a significant fraction of EGFR receptors were occupied (greater than 30-40%) (DeWitt et al. 2001). Our ability to measure HGF in the media indicates that a proportion of c-Met receptors in the U87MG cells are bound to HGF and is consistent with the observation that the c-Met receptor is basally phosphorylated at Y1234 in the U87-DK cell line (Figure II.5B). However, receptor occupancy is not at saturation because addition of exogeneous HGF resulted in further increases in c-Met receptor phoshorylation (Figure II.8C). While treating the U87-H cells with an anti-HGF antibody did not completely abolish c-Met phosphorylation levels (Figure II.8C), the lack of a commercially available anti-c-Met blocking antibody prevents us for eliminating the possibility that c-Met activation by EGFRvIII is ligand-mediated. Further studies are required to fully elucidate the mechanism of EGFRvIII-mediated activation of c-Met.

II.3.4 Combined inhibition of the EGFRvIII and c-Met receptors enhanced cytotoxic effects of an EGFR kinase inhibitor

To determine the biological consequence of the c-Met activation, we treated U87-H cells with the c-Met kinase inhibitor, SU11274 (Sattler et al. 2003). Quantitative mass spectrometric analysis of the c-Met receptor Y1234 phosphorylation site confirmed a decrease in c-Met receptor phosphorylation upon treatment of the U87-H cell line with SU11274 (Figure II.9).

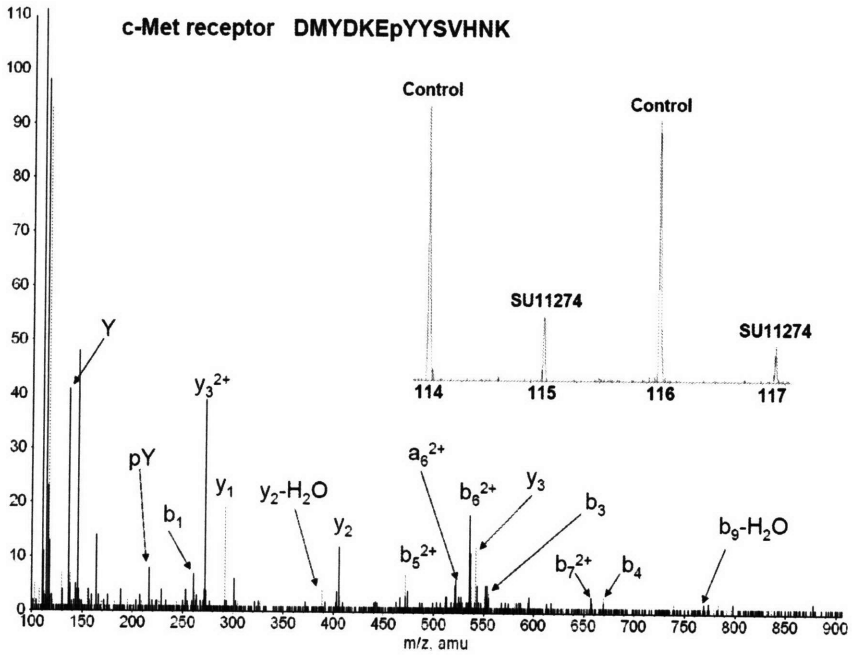


Figure II.9. Mass spectrum of c-Met Y1234 upon kinase inhibition. Comparison of the quantification of the phosphorylation levels for c-Met Y1234 upon treatment with either DMSO (control) or 10 μM c-Met kinase inhibitor SU11274 for 1 h after 24-h serum starvation. Two biological replicates were performed and peak areas for iTRAQ marker ions enable quantification of phosphorylation for each condition.

Due to the observed co-activation of EGFRvIII and c-Met receptors, we hypothesized that co-treatment of EGFRvIII-expressing cells with EGFR and c-Met kinase inhibitors may have an additive effect on cell viability and death. Treatment of U87-H cells with either AG1478 or SU11274 followed a similar

profile in which cell viability was decreased only at high inhibitor doses. In contrast, the combination of a constant dose of 5μM AG1478 with an increasing dose of SU11274 led to a significant decrease in cell viability and increase in cell death compared to the 5μM AG1478 monotherapy alone (Figure II.10A and 10B). To test for the possibility that off-target effects of SU11274 may have been responsible for decreased cell viability, we performed similar analyses with another c-Met inhibitor, PHA665752 (Christensen et al. 2003), and found that it

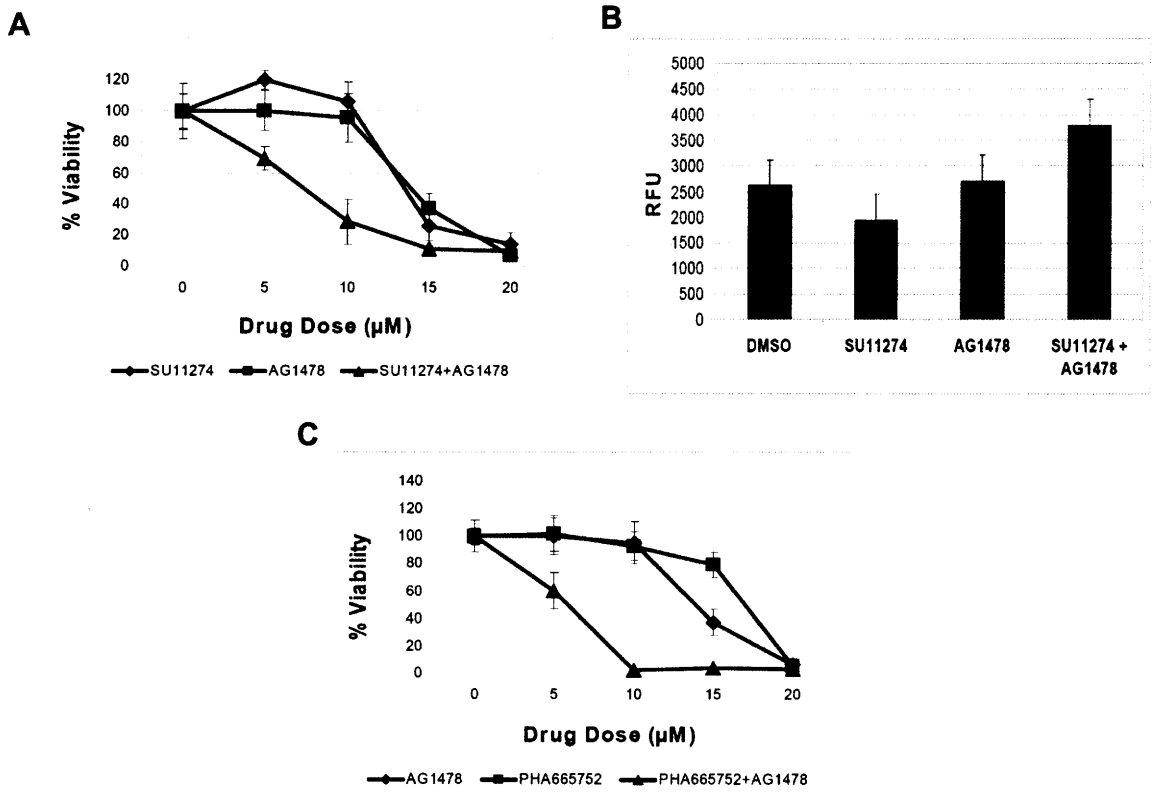


Figure II.10. Dose–response of U87-H cell line upon treatment with c-Met kinase inhibitors. (A) Dose–response of U87-H cells to AG1478, SU11274, or a combination of SU11274 and 5 μM AG1478 over 72 hrs after 24 hrs serum starvation. Viability was measured by using the metabolic dye WST-1. Combination treatment significantly enhanced cytotoxicity at 10 μM SU11274 ($P < 0.001$). (B) Apoptosis measured by caspase 3/7 cleavage upon drug treatment over 24 hrs after 24 hrs serum starvation. Concentration of drugs used was 10 μM SU11274, 10 μM AG1478, or a combination of 10 μM SU11274 and 5 μM AG1478. Combination treatment significantly enhanced apoptosis ($P < 0.01$). (C) Dose–response of U87-H to AG1478, PHA665752, or a combination of PHA665752 and 5 μM AG1478 over 72 hrs after 24 hrs serum starvation. Combination treatment significantly enhanced cytotoxicity at 10 μM PHA665752 ($P < 0.0001$).

also demonstrated similar enhanced effects upon co-treatment of the U87-H with AG1478 (Figure II.10C). These results suggest that the biological responses to the c-Met inhibitors were driven by on-target effects against c-Met itself or, perhaps, c-Met and another kinase which is a shared off-target molecule.

II.3.5 c-Met kinase inhibition overcomes chemoresistance conferred by EGFRvIII

EGFRvIII confers chemoresistance to classical chemotherapeutics such as cisplatin through modulation of Bcl-X_L and caspase 3 expression levels. Correspondingly, human glioblastoma xenografts expressing EGFRvIII are resistant to cisplatin unless cisplatin is co-administered with the EGFR kinase inhibitor, AG1478 (Nagane et al. 1998; Nagane et al. 2001). Activation of the c-Met receptor has also previously been shown to confer resistance to a wide variety of chemotherapeutics (Bowers et al. 2000). We hypothesized that the observed chemoresistance of EGFRvIII-expressing tumors may be due in part to EGFRvIII-mediated activation of the c-Met receptor. In order to test this hypothesis, we treated U87-H cells with increasing dose of the c-Met kinase inhibitor SU11274 in combination with a constant 10μg/ml dose of cisplatin. As demonstrated in Figure II.11B, combination treatment resulted in a dramatic decrease in cell viability when compared to treatment with cisplatin alone (Figure II.11A). This result suggests that c-Met may play a significant role in the chemoresistance of EGFRvIII-positive tumors.

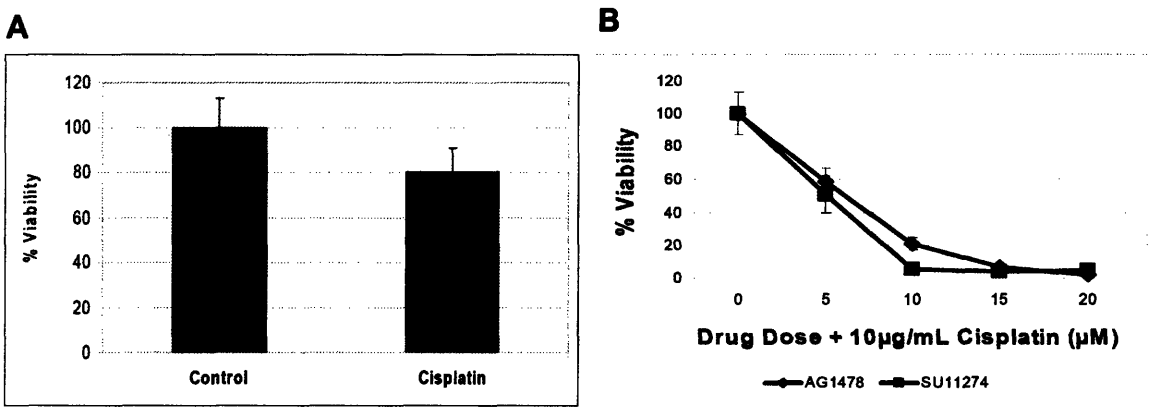


Figure II.11. Dose–response of U87-H cell line upon treatment with cisplatin. (A) U87H cells are resistant to treatment with cisplatin. Response of U87H to 10 □g/ml of cisplatin treatment over 72 hrs after 24 hrs serum starvation. (B) Viability of U87-H cells in a dose response to a combination treatment of 10 µg/ml cisplatin with either AG1478 or SU11274. Viability was measured using the metabolic dye WST-1.

II.4 DISCUSSION

Cancer is a heterogeneous disease in which oncogene levels vary both within the tumor in individual patients and between tumors in different patients. Although previous studies have estimated that the EGFRvIII receptor may be expressed at several million copies per cell in GBM tumors (Wikstrand et al. 1997), this value may vary significantly within the tumor and across patients. Moreover, it has previously been shown in a xenograft model that tumorigenicity is directly proportional to EGFRvIII receptor load (Johns et al. 2007). Here we report the first large-scale analysis of phosphotyrosine-mediated signaling pathways downstream of the EGFRvIII receptor as a function of receptor load. In this analysis, 99 phosphorylation sites on 69 proteins were identified and quantified, including one serine and seven tyrosine phosphorylation sites on the EGFRvIII receptor.

II.4.1 Oncogene dose effects

Although the tyrosine sites observed to be phosphorylated on EGFRvIII are the same as those seen in wildtype EGFR, quantitative differences in the levels of phosphorylation at each individual site may have functional implications on resultant downstream signaling pathways and biological responses. EGFRvIII phosphorylation appears to have both a threshold and a saturation level. Between the threshold and saturation levels, EGFRvIII autophosphorylation

increases disproportionately to changes in EGFRvIII expression, while above the saturation level, autophosphorylation is largely unaffected by further increases in receptor expression. These data highlight the need for additional functional analysis by site-directed mutagenesis to uncover the biological consequence of altered phosphorylation levels of these sites in the context of different EGFRvIII expression levels. It is tempting to speculate that regulation of activation at the level of the receptor may be the consequence of receptor dimerization. Unlike wildtype EGFR, where ligand binding catalyses the receptor dimerization event, the lack of ligand binding potential in EGFRvIII may mean that a critical receptor level is required before homo-dimerization can occur. Although EGFRvIII autophosphorylation levels appear to saturate, many downstream protein phosphorylation sites continue to increase in the U87-SH cells relative to the U87-H cells, suggesting that negative feedback mechanisms (e.g. phosphatases) may decrease receptor phosphorylation when EGFRvIII expression exceeds a critical saturating level.

Additionally, all eight phosphorylation sites on EGFRvIII were differentially phosphorylated as a function of titrated receptor levels, suggesting independent regulation of each site, and that each site may perform a different function in the propagation of downstream signal transduction pathways and the resulting tumor phenotype. Clustering of the data by self-organizing maps revealed that the three phosphorylation sites with the most similar profiles were Y1173, Y1148 and Y1068. Previous site directed mutagenesis studies have indicated that each of these 3 phosphorylation sites were critical for tumorigenesis *in vivo* (Huang et al.

1997). The biological consequence of the other phosphorylation sites remains unknown; these sites therefore represent obvious targets for future mutagenesis studies.

These observations raise clinically important issues for the use of EGFR-targeted drugs in the treatment of EGFRvIII-expressing tumors. For instance, it may not be necessary to use high doses of inhibitors to completely shut down the EGFRvIII receptor; instead it may be sufficient to simply decrease activation below the threshold level. Also, treatment with EGFR inhibitors might not affect all phosphorylation sites equally, potentially leading to residual phosphorylation of differentially regulated sites, which may account for the drug resistance observed in patients. Lastly, these data suggest that dimerization inhibitors may be as effective as ATP-analog inhibitors in treating EGFRvIII-expressing tumors.

Our study also provides the first systematic demonstration of the importance of oncogene dosage in the selection and propagation of downstream cellular signaling pathways. There is a modulation of downstream signal transduction networks as receptor levels are increased. Although we did not ascertain the absolute amounts of signaling proteins downstream of EGFRvIII, relative quantification of the phosphorylation status of STAT3, MAP kinase (MAPK), and the PI3K pathway indicate that these proteins are activated to a similar extent relative to the control cells in the U87-M cells expressing 1.5 million copies of the receptor. However, as EGFRvIII levels increase, pathway utilization appears to favor the PI3K pathway. This result provides a possible mechanistic basis for the success of PI3K and mTOR small molecule inhibitors in

combination with EGFR kinase inhibitors in the treatment of EGFRvIII expressing cells and xenografts (Fan et al. 2006; Wang et al. 2006). Our data also suggest that therapeutic approaches targeting the MAP kinase and STAT3 pathways for the treatment of tumors that express high levels of EGFRvIII would be predicted to be ineffective. Quantitative determination of such functional threshold limits for other cancer genes may represent a means to determine the relative order and dominance of oncogenes and their resultant cellular signaling pathways in human tumors.

Our data underscores the need to quantify oncogene levels when performing pair-wise comparisons between controls and oncogene-expressing cells/tumors. In addition, it implies that therapies combining receptor inhibitors with pathway blockade may benefit from monitoring phosphorylation level changes in receptor and downstream components after administering treatment, which may influence subsequent follow-up inhibitor selection. A recent study focusing on titrated levels of Ras in a mouse model of breast cancer also reinforces the importance of oncogene levels in the development of tumors (Sarkisian et al. 2007). In that study, low Ras expression led to increased proliferation but not transformation while high Ras expression induced cellular senescence *in vivo*. Thus, in order for tumorigenesis to occur in Ras-driven tumors, the senescence checkpoints in the cell must be overcome. Although the signaling mechanisms underlying these contrasting outcomes remain to be elucidated, these studies suggest that quantification of activated oncogene levels in tumors may aid in the design of treatment protocols targeting dominant

signaling pathways that are preferentially activated as a function of oncogene dose.

II.4.2 Differential pathway utilization of EGFRvIII versus wildtype EGFR

It has been shown previously in a xenograft model that EGFRvIII is more tumorigenic than wildtype EGFR (wtEGFR) (Huang et al. 1997). Paradoxically, although EGFRvIII activation is sustained, EGFRvIII phosphorylation levels are only 10% that of activated wtEGFR (Huang et al. 1997). This apparent contradiction in tumor phenotype and signaling may be explained in part by differential pathway utilization by the two receptors. Our laboratory has recently performed a temporal phosphoproteomic analysis of human mammary epithelial cells (HMECs) stimulated by EGF (Wolf-Yadlin et al. 2007). This study showed a dramatic increase in the phosphorylation of both MAPK and STAT3 within 1 minute of stimulation of wtEGFR. This activation is transient, degrades over time and is consistent with a recent study in which several negative regulators present in the delayed early gene cluster after EGF stimulation of HeLa cells were shown to regulate the immediate early signaling components (Amit et al. 2007). These negative regulators include the dual specificity phosphatases (DUSPs) which downregulate phosphorylation of active MAPK.

Our analysis of EGFRvIII signaling demonstrates that the expression of high levels of EGFRvIII favors the activation of the PI3K pathway over the MAPK and STAT3 pathways. Since transient wtEGFR activation is a balance of immediate early signaling components and subsequent delayed negative

regulation (Amit et al. 2007), it would be interesting to see if the EGFRvIII gene transcription network represents a hybrid of this model, in which certain negative regulators such as the DUSPs are constantly activated due to the sustained signaling of the EGFRvIII receptor while alternative immediate early signaling components such as PI3K remain active due to the lack of downstream negative regulation. It should be noted that other constitutively active oncogenic receptor tyrosine kinases such as Tpr-Met and Fig-Ros also favor the utilization of the PI3K pathway over the MAPK pathways (Kamikura et al. 2000; Charest et al. 2006). It is tempting to speculate that constitutively activated receptors may share a common signaling profile, or at least common network utilization.

The differential pathway activation of EGFRvIII and wtEGFR also suggests that a therapeutic approach toward tumors expressing these two distinct receptors should be fundamentally different. Consistent with our data on PI3K pathway activation by EGFRvIII, there has been some success in the combination of EGFR kinase inhibitors with PI3K/mTOR inhibitors in xenograft models (Fan et al. 2006; Wang et al. 2006). Our data would suggest that therapeutic approaches that combine EGFR kinase inhibitors with MEK inhibitors may be more successful in the treatment of wtEGFR expressing tumors as compared to EGFRvIII expressing tumors. Phosphoproteomic-based systems level signaling network analysis thus provides a means for informed target selection for therapeutic intervention.

II.4.3 Receptor tyrosine kinase crosstalk

Cluster analysis of our phosphoproteomic data revealed that phosphorylation of the activation site on c-Met increased as a function of EGFRvIII expression level. Constitutive activation of c-Met by EGFRvIII is reminiscent of the constitutively active Tpr-Met fusion mutant of the c-Met receptor which exhibits a more potent signaling potential than transient receptor activation stimulated by HGF ligand binding (Peschard and Park 2007). c-Met has been implicated in the progression of a wide variety of cancers including glioma, lung and breast cancer. An analysis of 32 glioma tumors showed that all the tumors examined were positive for c-Met expression (Koochekpour et al. 1997). Furthermore, glioma cell lines expressing c-Met were highly proliferative, motile and invasive upon treatment with HGF, suggesting that c-Met may play an important role in glioma tumorigenicity. Amplification of c-Met and HGF has been associated with highly invasive and metastatic tumors , as well as poor prognosis in lung cancer.

Overexpression of c-Met has also recently been linked to the development of gefitinib-resistant lung cancer (Engelman et al. 2007). In cell lines derived from these tumors, a correlation between c-Met overexpression and ErbB3 activation has been demonstrated along with enhanced cytotoxicity, following combined treatment with an EGFR kinase inhibitor and a c-Met kinase inhibitor. Although we have also observed enhanced effects of combining EGFR and c-Met kinase inhibitors, it is important to note that the underlying mechanisms appear to be different. For instance, gefitinib-resistant lung cancer relies on the

overexpression of c-Met to activate HER3, whereas EGFRvIII overexpression drives c-Met phosphorylation and activation regardless of c-Met expression levels in GBM cell lines. In addition, GBM tumors have been shown to express very low levels of HER3, indicating that the sensitivity of EGFRvIII expressing cells to c-Met kinase inhibition is not due to the proposed reduction in c-Met-mediated activation of the HER3/PI3K/Akt pathway observed in the gefitinib-resistant cell lines (Schlegel et al. 1994; Andersson et al. 2004). However, it is possible that EGFRvIII activates the c-Met receptor via an intermediary signaling component. It has previously been shown in a human bladder carcinoma cell line that autocrine stimulation of wtEGFR activates the c-Met receptor via the proto-oncogene Src (Figure 1); a similar mechanism may be applicable to EGFRvIII (Yamamoto et al. 2006).

The activation of c-Met by EGFRvIII represents a paradigm shift in the fundamentals of receptor-mediated signaling. Whereas in the past, activation of a single receptor by a single ligand was thought to activate a specific set of pathways, we have now shown that the activation of a single receptor leads to the activation of other surface components, each of which has its own set of pathways. Data from systems-wide studies focusing on pair-wise activation of receptors suggests that the co-activation of multiple receptors probably leads to an integrated downstream signal, different from that resulting from the independent activation of each contributing receptor (Janes et al. 2005; Natarajan et al. 2006). The specificity of receptor crosstalk is also context dependent. In addition to GBM, EGFRvIII is found to be expressed in breast,

ovarian, prostate and head and neck cancers. It is likely that crosstalk mechanisms observed in these tumor types are different from that seen in GBM. For instance, in the context of breast cancer, it has been shown that EGFRvIII activates the ErbB2 receptor which is abundantly expressed in a subset of breast cancer cells (Tang et al. 2000). Quantitative comparison of EGFRvIII-mediated signaling networks in these various tumors will identify differential aspects of the signaling networks as well as common nodes which may serve as targets for future multi-functional therapeutics.

II.4.4 Therapeutic consequences of EGFRvIII-c-Met crosstalk

There are a wide variety of approaches to regulate c-Met receptor activation, including the use of anti-HGF monoclonal antibodies and c-Met small molecule kinase inhibitors. Our data indicate that constitutive c-Met activation in EGFRvIII overexpressing cells is not abolished by the use of anti-HGF antibodies. Due to the lack of a commercially available anti-c-Met receptor blocking antibody, it is not possible to eliminate the possibility that the activation of the c-Met receptor by EGFRvIII is mediated by autocrine mechanisms and it does not preclude the utilization of anti-c-Met antibodies as a means to suppress c-Met activation levels in EGFRvIII expressing tumors.

We have demonstrated that c-Met kinase inhibitors provide enhanced cytotoxicity when combined with EGFRvIII kinase inhibitors in EGFRvIII expressing glioblastoma cells. Since most kinase inhibitors are known to inhibit multiple kinases, it is therefore possible that enhanced cell killing is associated

with off-target effects in addition to inhibition of c-Met. However, SU11274 and PHA665752 have different structures and should have different specificities, thereby decreasing the likelihood of similar off-target effects. It is important to note that our observations were made in the U87MG cell line which contains secondary genetic lesions commonly found to occur in human GBM patients, namely loss of PTEN and Ink4A/Arf. PTEN is a tumor suppressor protein with both phosphoinositide and phosphotyrosine phosphatase activities and is commonly mutated in many advanced cancers including lung and prostate carcinomas. Correspondingly, it has been previously demonstrated that clinical response to EGFR inhibitors such as erlotinib and gefitinib in human glioblastoma patients was significantly associated with the co-expression of PTEN and EGFRvIII in tumors and *in vitro* in U87MG cell lines transfected to co-express both EGFRvIII and PTEN (Mellinghoff et al. 2005). Since PTEN mutation is seen in 30%-44% of high-grade gliomas (Maher et al. 2001), a large proportion of GBM patients are refractory to EGFR kinase inhibitor therapy. Our *in vitro* data suggests that co-treatment of EGFRvIII overexpressing tumors with both EGFR and c-Met kinase inhibitors may overcome this chemoresistance even in PTEN null tumors. Assaying for the expression/activation of EGFRvIII and c-Met in human gliomas may guide the combined use of these inhibitors in the clinic. It is also interesting to note that The U87MG cells expressing EGFRvIII are resistant to c-Met kinase inhibitor monotherapy. Further epidemiological data would be required to determine if the loss of PTEN in GBM

tumors also results in resistance to c-Met kinase inhibitor monotherapy in the clinic.

Chemoresistance of diffuse lesions in glioblastoma patients results in recurrence after surgical resection for almost all patients (Maher et al. 2001). Here we have demonstrated that co-treatment of U87-H cells with cisplatin and a c-Met kinase inhibitor led to a dose-dependent decrease in cell viability similar to co-treatment with cisplatin and AG1478, an EGFR kinase inhibitor. This result raises the possibility that c-Met activation may account for a significant proportion of EGFRvIII-mediated chemoresistance. In fact, it is plausible to suspect that many of the tumor-associated phenotypes previously attributed to the EGFRvIII receptor may be due in part to cross-activation of c-Met or other RTKs. Activation of multiple RTKs by EGFRvIII may thus potentiate a multitude of additional tumorigenic properties, arising from an integrated signal downstream of co-activated RTKs.

EGFRvIII-mediated phosphorylation and activation of c-Met was uncovered through network analysis of EGFRvIII signaling pathways in U87MG cell lines by mass spectrometry. Co-treatment with c-Met kinase inhibitors and cisplatin or c-Met kinase inhibitors and EGFR kinase inhibitors demonstrated enhanced cytotoxicity in U87-H cells. It is important to extend these studies to murine xenograft models and eventually to other clinical models to evaluate the efficacy of this co-treatment in treating tumors *in vivo*. Our results highlight the potential of using unbiased network analysis to identify novel therapeutic targets in signaling networks. Further analyses of other GBM cell lines and human

tumors will highlight additional proteins and key signaling nodes which may serve as targets to treat this devastating disease.

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III QUANTITATIVE ANALYSIS OF SITE-DIRECTED MUTANTS OF EGFRVIII REVEALS A RECEPTOR COMPENSATORY MECHANISM AND A DEPENDENCE ON THE ERK SIGNALING PATHWAY

III.1 SUMMARY

The functional role of site-specific EGFR tyrosine phosphorylation has previously been predicted based on *in vitro* binding measurements of protein or domains to phosphopeptides representing known receptor phosphorylation sites. However, by performing *in vitro* assays, these studies have largely neglected the important cellular context of the ErbB receptor system, including positive and negative feedback from the downstream signaling network. This loss in the cellular context also extends to compensatory mechanisms that may function upon loss of a particular phosphorylation site.

To address this deficiency, in this chapter, I will describe the use of the constitutively active mutant receptor EGFRvIII as a model system for probing the effects of site-specific phosphorylation on intracellular signaling networks. We have employed an unbiased mass spectrometric approach to determine the global network effects of six loss-of-function (tyrosine to phenylalanine) mutations on the EGFRvIII receptor, with the aim of mapping *in vivo* changes and identifying distinct signaling components controlled by specific receptor tyrosine phosphorylation sites. Analysis of the EGFRvIII site-direct mutants resulted in the identification and quantification of 149 phosphorylation sites across 112 proteins, including 9 sites on EGFRvIII.

Contrary to pre-existing views, rather than a decrease in receptor tyrosine phosphorylation levels, a single mutation at Y1173F resulted in an increased (up to 1.5-fold) in the levels on 8 other phosphorylation sites on the receptor relative

to intact EGFRvIII. Increased phosphorylation of these sites was functionally significant, as indicated by the ability of the upregulated EGFRvIII phosphorylation sites to compensate for the loss of Y1173 and maintain similar levels in tyrosine phosphorylation of downstream signaling network components, including the PI3K and STAT3 pathways. This is, to our knowledge, the first demonstration of signal compensation at the level of receptor phosphorylation and highlights an unexpected level of complexity within the signaling network. This compensatory mechanism may represent a common theme found in the other ErbB family members.

Phenotypic endpoint measurements of cells expressing these mutants indicate that all the mutants are deficient in growth, consistent with previous *in vivo* xenograft studies. Taken together, these observations suggest that in some of the mutant cell lines, cell proliferation is independent of receptor phosphorylation levels and may rather be the consequence of an orthogonal signaling component in the cells. Principle component analysis of the phosphoproteomic and phenotypic data sets highlights a strong anti-correlation between Erk1/2 phosphorylation activity and cell proliferation.

To determine the functional importance of this observation, we have generated U87MG-EGFRvIII cell lines that stably express either constitutively active (CA) or dominant negative (DN) MEK, the upstream activation kinase of Erk1/2. U87MG-EGFRvIII cells expressing CA or DN MEK mutants showed a 25% decrease in cell proliferation compared to the control cells. Additionally, the cells expressing the CA MEK mutant had also undergone a 2 fold increase in

apoptosis. Our data suggests that EGFRvIII fine-tunes the activity of the Erk pathway; some activity of the pathway is required for growth but excessive activation of the pathway results in apoptosis. We believe that the sensitivity to modulation of the Erk pathway may be exploited as a potential means of therapy for EGFRvIII expressing tumors.

This work was performed in collaboration with Emily Miraldi in the White lab, who performed the PCA analysis, as well as with Frank Furnari and Webster Cavenee at the Ludwig Institute for Cancer Research (San Diego Branch).

III.2 MATERIALS AND METHODS

III.2.1 Cell Culture, Retrovirus Infection, and Transfection

The human glioblastoma cell line U87MG and their engineered derivatives were obtained from Frank Furnari and are described in (Huang et al. 1997). They were cultured in DMEM with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin in 95% air/5% CO2 atmosphere at 37°C. U87MG cells expressing EGFRvIII or engineered mutant receptors were selected in 400 µg/ml G418. For the expression of MEK mutants (a kind gift from Christopher Marshall, ICR), U87MG cell expressing EGFRvIII and mutant receptors were transfected with the retroviral vectors containing, control pBABE-puro, constitutively active MEK (CA) pBABE-MANE or dominant negative MEK (DN) pBABE-LIDA. Viruses were produced by seeding 293-GP cells at $3x10^6$ cells per 10cm plate and transfected with FuGene 6 transfection reagent with 10 µg of pBABE retroviral plasmid and 5 µg of VSV-G plasmid. After 16 hours, cells were washed with PBS and replaced with 10 ml of fresh media. Viruses were harvested from the media 48 hours later, filtered with a 0.45 µm SFCA filter and used to infect the U87MG cell lines. Stable populations were obtained by selection in 2.5 µg/ml puromycin and expression of MEK mutants was confirmed by immunoblotting.

III.2.2 Cell lysis, Protein digestion and Peptide fractionation

U87MG cells were maintained in DMEM medium supplemented with 10% FBS. $1.5x10^6$ cells per 10cm plate were seeded for 24 hours, then washed with PBS and incubated for 24 hrs in serum-free media. Cells were lysed in 1 ml of 8 M urea. For each of the two biological replicates performed, lysate from three 10cm plates were pooled together. Cells were reduced with 10 mM DTT for 1 hr at 56°C, alkylated with 55mM iodoacetamide for 45 min at room temperature, and diluted to 12 ml with 100mM ammonium acetate, pH 8.9, prior to digestion with 40 µg of trypsin (Promega). The lysates were digested overnight at room temperature. Digested lysate were acidified to pH 3 with acetic acid and loaded onto a C18 Sep-Pak Plus cartridge (Waters). The peptides were desalted (10ml 0.1% acetic acid) and eluted with 10 ml of a solution of 25% acetonitrile and 0.1% acetic acid. Each sample was divided into 5 aliquots and lyophilized to dryness.

III.2.3 iTRAQ labeling of peptides

Lyophilized peptides were subjected to labeling with the iTRAQ 4-plex reagent (Applied Biosystems). Each aliquot of peptides was dissolved in 30 µl of 0.5 M triethylammonium bicarbonate, pH 8.5 and reacted with two tubes of iTRAQ reagent (dissolved in 70 µl of ethanol each). The reagents for each of the conditions used were, iTRAQ-114 (U87MG-EGFRvIII), iTRAQ-115 (U87MG-Y1068F and U87MG-Y1173F), iTRAQ-116 (U87MG-Y1148F and U87MG-DY2)

and iTRAQ-117 (U87MG-845F and U87MG-DY3). The mixture was incubated at room temperature for 50 mins and the concentrated to 30 µl. The four different isotopically labeled samples from each set (outlined in Figure III.3.1B) were combined and acidified with 360 µl of 0.1% acetic acid and then reduced to dryness.

III.2.4 Peptide immunoprecipitation

The combined sample is reconstituted with 150 µl of IP buffer (100 mM Tris, 100 mM NaCl, 1% NP-40, pH 7.4), 300 µl of water and the pH was adjusted to 7.4. The sample was incubated with 10 µg of protein G Plus-agarose beads (Calbiochem) and 12 µg of anti-phosphotyrosine antibody (PY100 Cell Signaling Technology) for 8 hrs at 4°C. The antibody-bead conjugates were then spun down for 5 mins at 6000 rpm at 4°C and the supernatant was saved. The beads were then washed three times with rinse buffer (100 mM Tris, 100 mM NaCl, pH 7.4) for 5 minutes at 4°C, prior to elution with 70 µl of 100mM glycine pH 2.5 for 30 mins at room temperature.

III.2.5 Immobilized metal affinity chromatography (IMAC) and Mass Spectrometry

Immobilized metal affinity chromatography (IMAC) was performed to enrich for phosphorylated peptides and remove non-specifically retained non-phosphorylated peptides. Eluted peptides were loaded onto a 10 cm self-packed

IMAC (20MC, Applied Biosystems) capillary column (200 µm ID, 360 µm OD), and rinsed with organic rinse solution (25% MeCN, 1% HOAc, 100 mM NaCl) for 10 mins at 10 µl/min. The column was then equilibrated with 0.1% HOAc for 10 mins at 10 µl/min and then eluted onto a 10 cm self-packed C18 (YMC-Waters 10 5 µm) precolumn (100 µm ID, 360 µm OD) with 50 µl of 250mM Na_2HPO_4, pH 8.0. After a 10 mins rinse with 0.1% HOAc, the precolumn was connected to a 10 cm self-packed C18 (YMC-Waters 5 µm ODS-AQ) analytical capillary column (50 µm ID, 360 µm OD) with an integrated electrospray tip (1 µm orifice). Peptides were eluted with a 125 minute gradient with solvents A (1% HOAC) and B (70% MeCN in 1% OHAc): 10 mins from 0% to 13%, 95 mins from 13% to 42%, 10 min from 42% to 60% and 10 min from 60% to 100%. Eluted peptides were directly electrosprayed into a QqTof mass spectrometer (QSTAR Elite, Applied Biosystems). MS/MS spectra of the five most intense peaks with 2 – 5 charge states in the full MS scan were automatically acquired in information-dependent acquisition.

III.2.6 Phosphopeptide sequencing and quantification

MS/MS spectra were extracted and searched using MASCOT (Matrix Science). For MASCOT, data was searched against the human non-redundant protein database with trypsin specificity, 2 missed cleavages, precursor mass tolerance of 2.2 amu for the precursor ion and 0.15 for the fragment ion tolerance.

Phosphorylation sites and peptide sequence assignments were validated and quantified by manual confirmation of raw MS/MS data. Peak areas of iTRAQ marker ions (m/z 114, 115, 116 and 117) were obtained and corrected according to manufacturer's instructions to account for isotopic overlap. The quantified data was then normalized with values from the iTRAQ marker ion peak areas of non-phosphorylated peptides in the supernatant of the immunoprecipitation (used as a loading control to account for possible variation in the starting amount of sample for each condition). Each condition was normalized against the U87MG-EGFRvIII (114 label) cell line to obtain fold changes across all 7 conditions.

III.2.7 Immunoblotting Analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium PP_i, 1 mM β-glycerophosphate) containing protease and phosphatase inhibitors after the indicated treatment. Protein concentration of cell lysates was determined using micro bicinchoninic acid assay (Pierce), according to the manufacturer's protocol. 50 µg of protein from the cell lysate was mixed with 4X sample buffer (250 mM Tris-HCl, pH 6.8, 40% glycerol, 0.04% bromophenol blue and 400 mM DTT) and loaded on either 7.5% or 10% SDS-PAGE gels, separated and transferred onto PVDF membranes. After blocking for 1 hr at room temperature with 5% BSA, membranes were incubated overnight at 4°C in primary antibody and washed 3 times at 10 mins each in TBS-Tween buffer (20mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween-20). Membranes were then incubated at room temperature

for 1 hr in secondary antibody and washed 6 times at 10 mins each in TBS-Tween buffer. Blots were developed with the SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce) and scanned on a Kodak Image station 1000. Primary antibodies used were anti-phopho-Erk1/2 and anti-tubulin (Cell Signaling Technologies). Secondary antibody used was goat anti-rabbit antibody (Jackson Immunoresearch).

III.2.8 Cell proliferation measurement

For cell proliferation measurement of mutant cell lines, 5 x 10^5 cells were plated on 10 cm plates. 24 hours later, the cells were serum starved prior to being counted at 12 hour intervals using the Nexcelom cellometer system for a total of 60 hours. 4 replicates were performed and cell counts were normalized to the number of cells at the start of serum starvation (0 hr time-point). Statistical significance was calculated using unpaired student t-test for the growth difference between each mutant cell line and the U87MG-EGFRvIII control. For MEK mutant cell counts, 1 x 10^5 cells were plated per well in a 6-well plate. 24 hours later, the cells were serum starved. Cells were counted at 72 hrs after serum starvation using the Vi-cell cytometer system. Similarly, for the EGFR kinase inhibitor treatment, 2 x 10^5 cells were plated per well in a 6-well plate. 24 hours later, cells were serum starved for 24 hrs prior to being treated with the indicated dose of either AG1478 (A.G. Scientific) or gefitinib (a gift from Astra Zeneca) for 72 hours. Cells were counted at 72 hrs using the Vi-cell cytometer system using the trypan blue exclusion method. Statistical significance was

calculated using paired student t-test for the difference between control DMSO treatment and kinase inhibition for each mutant cell line.

III.2.9 Cell viability assays

4,000 cells were seeded per well in a 96 well plate. 24 hrs later, the cells were serum starved for 24 hrs prior to addition of 100 µl fresh serum free media containing U0126 (Promega) or phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich) at the indicated doses. After 72 hrs, cell viability was measured using the WST-1 reagent (Roche Applied Sciences). 10 µl WST-1 reagent was added to each well and incubated at 37°C for two hours, prior to measuring absorbance at 450nm in a spectrophotometer. Statistical significance was calculated using paired student t-test for the difference between control DMSO treatment and drug treatment for each mutant cell line.

III.2.10 Apoptosis assay

10,000 cells were seeded per well in a 96 well plate. 24 hrs later, the cells were serum starved for 24 hours prior to addition of 100 µl fresh serum free media. At 24 hr intervals after serum starvation, caspase 3/7 activity was measured using Apo-ONE Homogeneous Caspase-3/7 Assay (Promega), following manufacturer's recommendations. Briefly, 100 µl of Apo-ONE Caspase-3/7 reagent with fluorescent substrate was added to each well, incubated for 12 hrs and prior to measuring fluorescence at $485_{Ex}/527_{Em}$.

Statistical significance was calculated using paired student t-test for the difference in response between control pBABE-puro cell line and MEK mutant transfectants for each EGFR mutant cell line.

III.2.11 Principle component analysis

Data was arranged into an N-by-M matrix, where each of the N rows corresponded to a cell line and each of the M columns corresponded to either a phosphosite or phenotypic measurement for each of the cell lines. This matrix was converted into a matrix of z-scores; each column was transformed to have zero mean and a variance of one. Principle components analysis was performed on this matrix using the Statisitcs Toolbox in Matlab 7.4.0. The first principle component, constructed to maximally capture variance in the data, was largely a weighted average of all phosphosites and phenotypes. The second principle component captured variance that was not well represented by the first component and helped us visualize correlations between specific phosphosites and growth measurements.

III.3 RESULTS

III.3.1 Cell lines and experimental strategy

To examine how cellular phosphotyrosine-mediated networks are modulated upon tyrosine site-specific changes at the receptor level, we utilized a series of U87MG GBM cell lines that have previously been engineered to express 2 x 10^6 tyrosine (Y) to phenylalanine (F) site-directed EGFRvIII mutants (Huang et al. 1997). These mutants are depicted in figure III.1A and consists of four cell lines expressing single mutations at Y845F, Y1068F, Y1148F and Y1173F, one cell line expressing a double mutation at Y1068F and Y1173F (denoted as DY2) and a final cell line expressing a triple mutation at Y1068F, Y1148F and Y1173F (denoted as DY3). As a control, a previously described cell line expressing the intact EGFRvIII receptor at 2.0 x 10^6 copies (U87MG-EGFRvIII) was employed as a control (Huang et al. 2007). The tyrosine to phenylalanine EGFRvIII mutant cell lines were engineered 11 years ago and have been maintained in culture for multiple passages. It is possible that these cell lines may have undergone a drift in receptor levels. Further experimental controls such as ELISA measurements of EGFRvIII levels will need to be performed in future to ascertain if receptor levels between the different engineered cell lines have changed.

In order to minimize confounding cell signaling events associated with serum in cell culture media, the 7 cell lines were serum starved for 24 hours prior

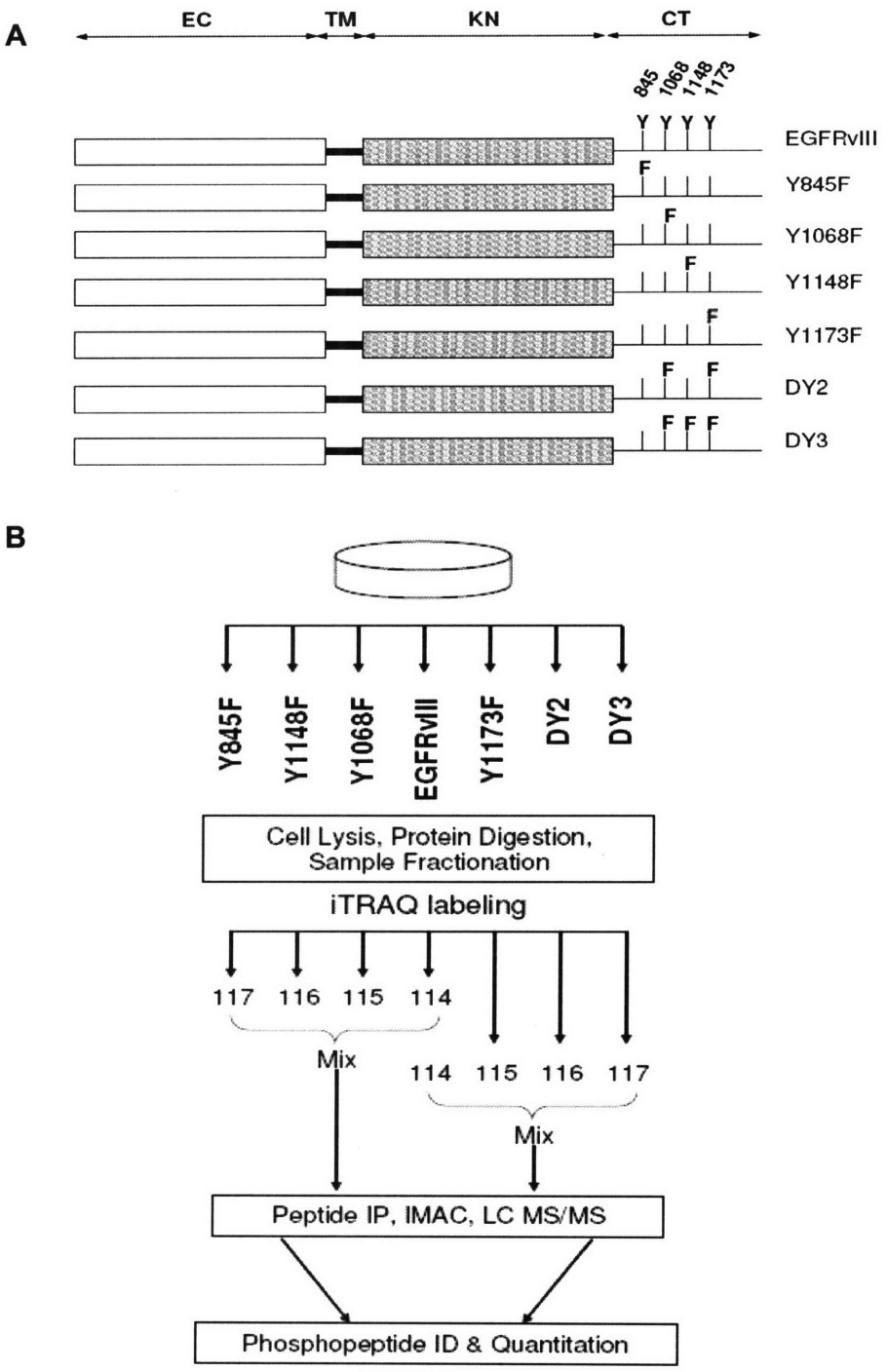

Figure III.1. Mutant EGFRvIII receptors and experimental strategy. (A) Six site-directed mutants of EGFRvIII were examined in this analysis. Y = tyrosine residue and F = mutation of an existing tyrosine residue to phenylalanine. EC = extracellular region, TM = transmembrane domain, KN = kinase domain and CT = cytoplasmic tail. (B) Outline of MS-based experimental strategy. Samples were labeled with iTRAQ isobaric reagent in two sets each consisting of 3 mutant cell lines and U87MG-EGFRvIII. U87MG-EGFRvIII (label 114) was then used as a control to integrate both data sets together.

to cell lysis and sample preparation. As depicted in figure III.1B, the 7 cell lines were labeled with stable isotopes in 2 different sets. Each set consists of 4 cell lines with one cell line (U87MG-EGFRvIII) being used as overlapping control for the integration of the two datasets. Peptides from the seven samples were stable isotope labeled, mixed, and tyrosine phosphorylated peptides were immunoprecipitated with a pan-specific anti-phosphotyrosine antibody (Figure III.1B). Following immunoprecipitation, phosphorylated peptides were further enriched by immobilized metal affinity chromatography (IMAC) and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). In total, quantitative phosphorylation profiles were generated for two biological replicates with a total of 149 phosphorylation sites on 112 proteins across the 7 cell lines.

III.3.2 Quantitative effects of EGFRvIII tyrosine site mutations on receptor phosphorylation

In this analysis, we identified 9 phosphorylation sites of the EGFRvIII receptor, 8 on tyrosine residues and 1 on a serine residue (Figure III.2). Single mutations at Y845, Y1068, Y1148 and the triple mutant DY3 showed an at least 2-fold decrease in tyrosine phosphorylation in all the non-mutated tyrosine/serine residues compared to intact EGFRvIII. This observation suggests that receptor–mediated phosphorylation at these sites may be required for the optimal phosphorylation of EGFRvIII. Interestingly, the Y1173 single mutant resulted in increased (at least 1.5 fold) phosphorylation levels in 8 of 9 sites that were mapped. This increase of receptor phosphorylation levels was also found to

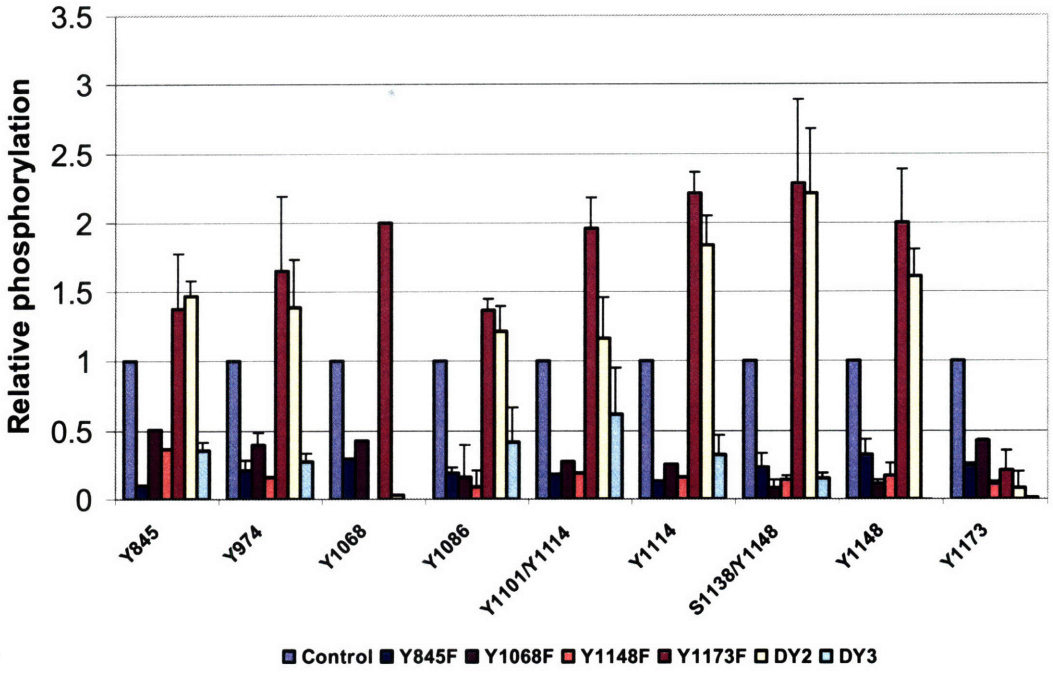

Figure III.2. Relative quantification of EGFRvIII phosphorylation sites across the mutant cell lines. Phosphorylation levels were normalized relative to that of the U87-EGFRvIII (control) cell line with error bars representing phosphorylation sites that appeared in both biological replicate analyses.

occur to a lesser extent in the DY2 double mutant. We recognize that the observed changes in receptor autophosphorylation levels in the EGFRvIII mutants may be attributed to differences in total EGFRvIII levels that could occur as a result of receptor drift after FACS sorting. Future work will include the normalization of the phosphoproteomic data with measured receptor levels by ELISA. This will provide a more accurate representation of the signaling changes in response to site specific mutations of the EGFRvIII receptor. We have also observed low levels of phosphorylation at tyrosine residues that were mutated in the engineered receptors. This residual phosphorylation is likely to be due to the endogenous levels of wildtype EGFR that are expressed at 2×10^5

receptor levels in the U87MG cells in response to autocrine secretion of the EGF family ligands (Ramnarain et al. 2006).

III.3.3 Effects of EGFRvIII tyrosine site mutations on major downstream signaling pathways

To determine how tyrosine site-directed mutants of EGFRvIII modulated downstream signaling pathways, we mapped the phosphoproteomic data onto the canonical EGFR signaling network. (Figure III.3). Similar to the trend in receptor phosphorylation levels, we observed that signaling nodes important for EGFR function in the Y845F, Y1068F, Y1148F and DY3 mutants were decreased compared to intact EGFRvIII. These nodes include, the activation

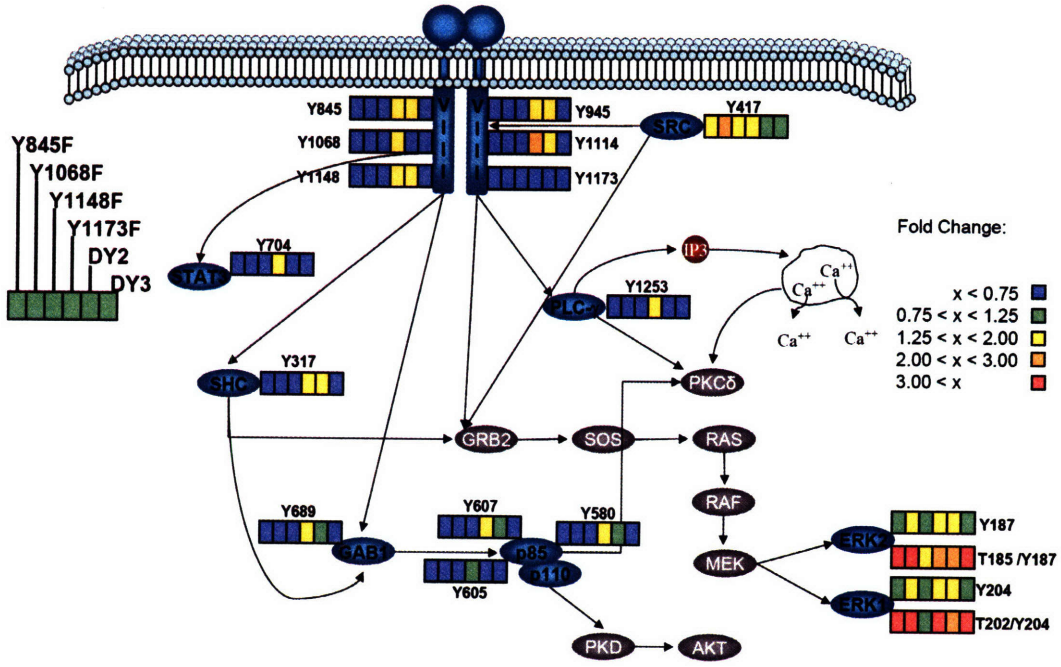

Figure III.3. Effect of site-specific EGFRvIII mutations on phosphorylation sites within the EGFR network. Visualization of the fold change in phosphorylation levels in the canonical EGFR signaling network as a function of EGFRvIII site-specific tyrosine residue mutants. Phosphorylation levels are normalized to that of the U87MG-EGFRvIII control cell line.

sites on STAT3 (Y704), SHC (Y317), and PLC-γ (Y1253), a docking site on GAB-1 (Y689) and multiple tyrosine sites on the p85 regulatory subunit of PI3K (Y605, Y607 and Y590).

Our data also demonstrates that increases in the phosphorylation levels of 8 other sites on the EGFRvIII receptor were sufficient to compensate for the loss in the Y1173F mutation and to a lesser extent in the DY2 mutant. This is reflected in the maintenance of at least similar (if not higher) tyrosine phosphorylation levels of downstream signaling components of the EGFR network, for instance, in the STAT3 and SHC-GAB1-PI3K pathways compared to the U87MG-EGFRvIII control cells. Our data suggests that the upregulated tyrosine phosphorylation sites on the Y1173F EGFRvIII mutant may have overlapping functions with Y1173 and can thus compensate for its function.

There were also proteins that had distinct phosphorylation profiles from the majority of the canonical EGFR signaling nodes. These proteins include the tyrosine phosphorylation site (Y417) on the Src family of proteins and the MAP kinase pathway components Erk1 and Erk1. In a previous phosphoproteomic analysis of EGFRvIII signaling networks as a function of titrated receptor levels, we showed that increasing EGFRvIII levels did not increase the phosphorylation levels of the double phosphorylated active form of Erk1 and Erk2 (Huang et al. 2007). Taken together, our current data would suggest that EGFRvIII actively represses Erk1/2 protein phosphorylation and mutation of tyrosine phosphorylation sites on the receptor relieves this repression, resulting in an

increase in Erk1 (up to 4 fold) and Erk2 (up to 5 fold) phosphorylation levels (Figure III.4).

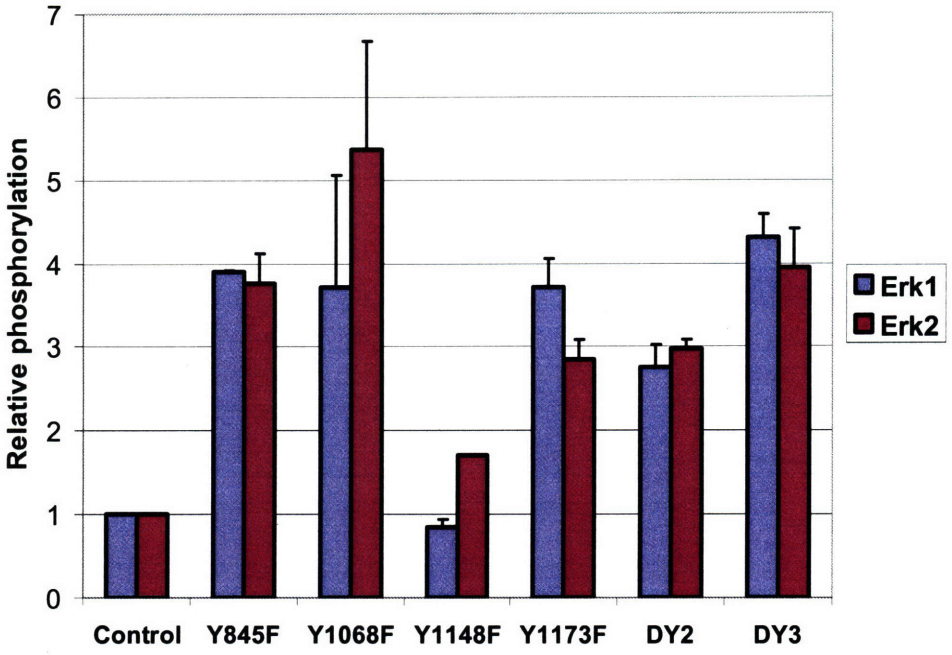

Figure III.4. Effect of site-specific EGFRvIII mutations on Erk1/2 phosphorylation. Visualization of the fold change in phosphorylation levels the double phosphorylated form of Erk1 (T202/Y204) and Erk2 (T185/Y197) as a function of EGFRvIII-site specific tyrosine residue mutations. Phosphorylation levels are normalized relative to that of the U87MG-EGFRvIII cell line with error bars representing results from biological duplicates.

III.3.4 Cell proliferation and EGFR kinase inhibition responses of EGFRvIII mutants are distinct from intact EGFRvIII cells

In order to correlate the signaling network data with cellular responses, we quantified the cell proliferation of the 7 cell lines under serum starvation over the course of 60 hours. Similar to previously reported *in vivo* data, the mutant cell lines were deficient in growth compared to control cells expressing intact EGFRvIII (figure III.5A). The growth defect was most prominent between 24 to

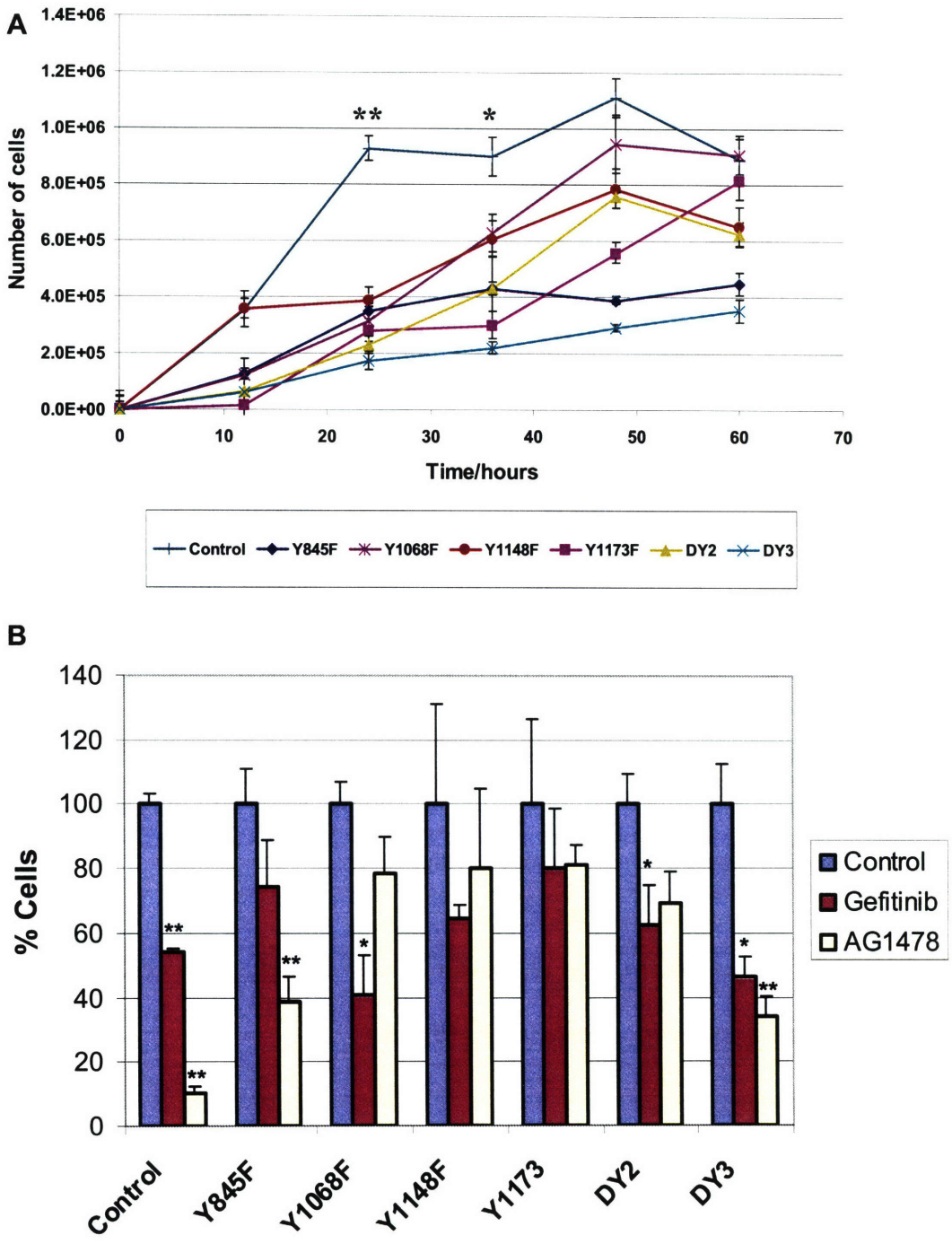

Figure III.5. Effect of site-specific EGFRvIII mutations on cell growth and tyrosine kinase inhibitor response. (A) Growth profiles of mutant cell lines under serum starvation over 60 hours. Experiments were performed in 4 replicates and with significant growth difference of *p<0.01, **p<0.001 was observed at 24 and 36 hours of serum starvation. (B) Response of mutant cell line to treatment with EGFR kinase inhibitors gefitinib and AG1478 at 15 μM. Cells were treated under serum starvation conditions for 72 hours prior to counting viable cells by trypan blue exclusion method. Experiments were performed in 4 replicates normalized to DMSO treatment with a statistical significance of *p<0.01, **p<0.001.

48 hours of serum starvation, after which the U87MG-EGFRvIII expressing cells undergo neurosphere formation and decrease the rate of cell proliferation.

In addition to cell proliferation, we were interested in determining if the mutant cell lines maintained a survival dependence on the EGFRvIII pathway. To test this, we treated the 6 mutant cell lines with 2 structurally different EGFR kinase inhibitors, AG1478 and gefitinib, at a dose (15 μM) that has been reported to decrease U87MG-EGFRvIII cell viability (Pedersen et al. 2005; Huang et al. 2007). As shown in figure III.5B, AG1478 and gefitinib treatment decreased the percentage of viable U87MG-EGFRvIII cells by 90% and 50% respectively. All the mutant lines demonstrated some resistance to AG1478 compared to U87MG-EGFRvIII, with the drug having almost no effect on 3 of the 6 mutant cell lines (Y1068F, Y1148F and Y1173F). Similarly, gefitinib only induced a significant response in 3 of the 6 cell lines (Y1148F, DY2 and DY3). Interestingly, the Y1148F mutant was more sensitive to gefitinib treatment than AG1478 treatment. Our data indicates that all the site-directed mutants examined in this study had some degree of resistance to AG1478 and in all but 3 cases, resistance to gefitinib.

III.3.5 Principle component analysis reveals an anti-correlation between ppErk1/2 and cell proliferation

In contrast to the striking differences in receptor phosphorylation levels (figure III.2) between Y1173F, DY2 and the other mutant cell lines, the growth

rates across all the mutant lines were similarly defective (Figure III.5A). This data implies that in some of the mutant cell lines, cell proliferation is independent of receptor phosphorylation levels and may rather be the result of an orthogonal signaling component.

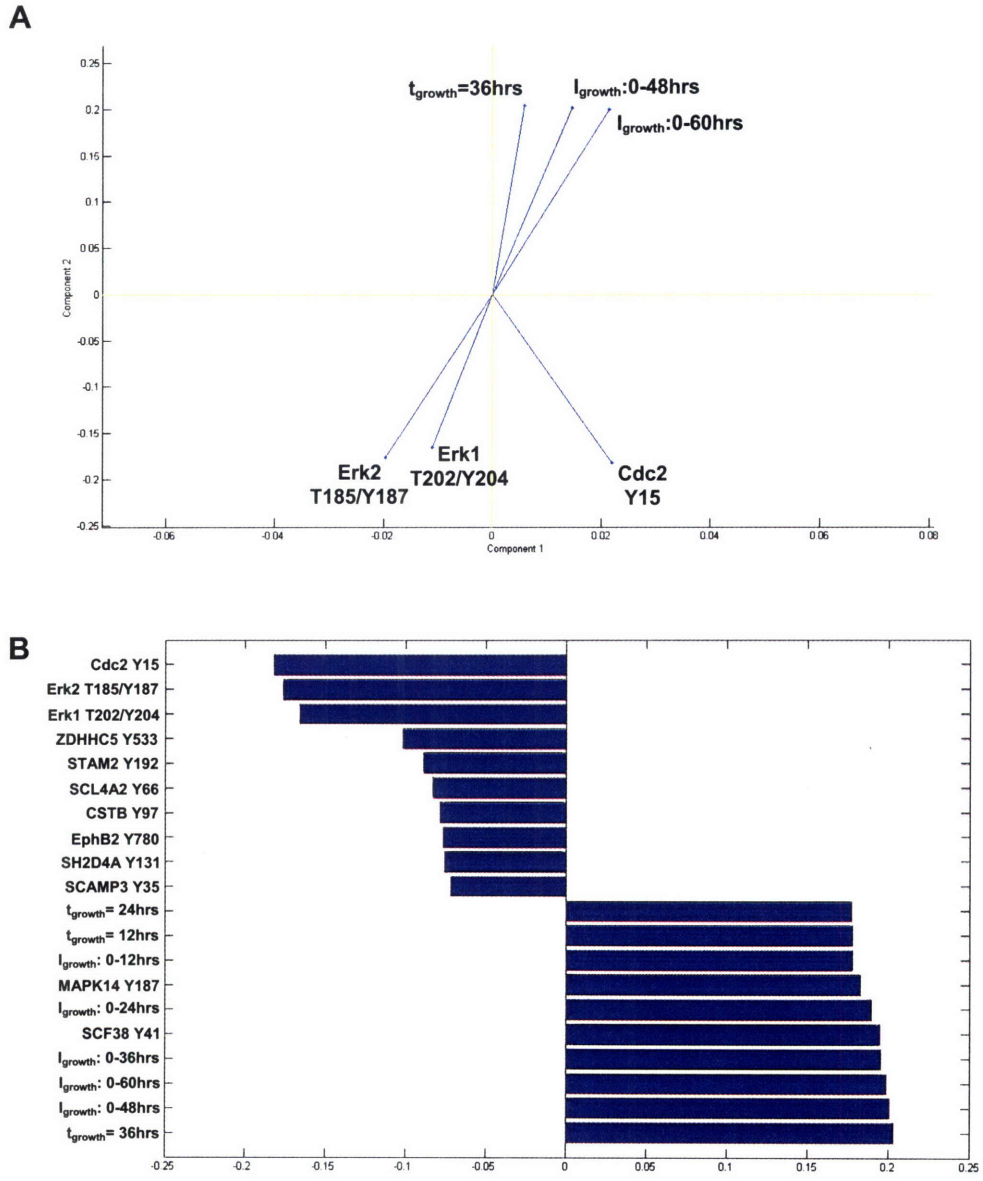

Figure III.6. Principle component analysis of phosphoproteomic and phenotypic data. (A) 2D Bi-plot of principle components 1 and 2, highlighting the top 3 positive and negative contributors to the second principle component. (B) Pareto diagram indicating the top 10 positive and negative contributors to the second principle component. t=time of growth, I=integral of growth.

To determine if a relationship between the signaling nodes in the EGFRvIII network and phenotypes such as cell proliferation exists, principle component analysis (PCA) was performed on the phosphoproteomic and the phenotypic datasets. As shown in figure III.6, the growth of mutant lines at multiple time-points (at 36 hours or the integral of 0-48 and 0-60 hours) were anti-correlative to the phosphorylation levels of Cdc2, Erk1 and Erk2 in the second principle component. The phosphorylation site on Cdc2 (Y15) has previously been shown to have an inhibitory effect on Cdc2 enzymatic activity and contributes to cell cycle arrest (Atherton-Fessler et al. 1993). We thus hypothesized that the double phosphorylated active form of Erk1 and Erk2 may also be inhibitory to the growth of U87-EGFRvIII cells.

III.3.6 Activation of Erk1/2 in U87-EGFRvIII cells decreases the percentage of viable cells and induces apoptosis

To test whether hyperactivation of Erk1/2 affected cell proliferation, U87MG-EGFRvIII cells were stably transfected with constitutively active (CA) mutant MEK, in which mutation of two serine residues into aspartate converts MEK into a constitutively active protein (Cowley et al. 1994). MEK is the upstream kinase activator of Erk1/2 and overexpression of CA-MEK in the U87MG-EGFRvIII cells resulted in an upregulation of ppErk1/2 (Figure III.7A). When grown under serum-starvation conditions for 72 hours, CA-MEK expressing cells showed a 25% decrease in the number of viable cells compared to the control cell line (Figure III.7B). A similar result was obtained when

EGFRvIII cells were treated with phorbol-12-myristate-13-acetate (PMA), a known activator of MEK (Figure III.8) (Marquardt et al. 1994). We then measured the effect of hyperactivation of Erk1/2 on apoptosis. When grown under serum-starvation conditions, CA-MEK expressing cells showed a 2-fold increase in apoptosis at multiple time-points after serum starvation (Figure III.7C).

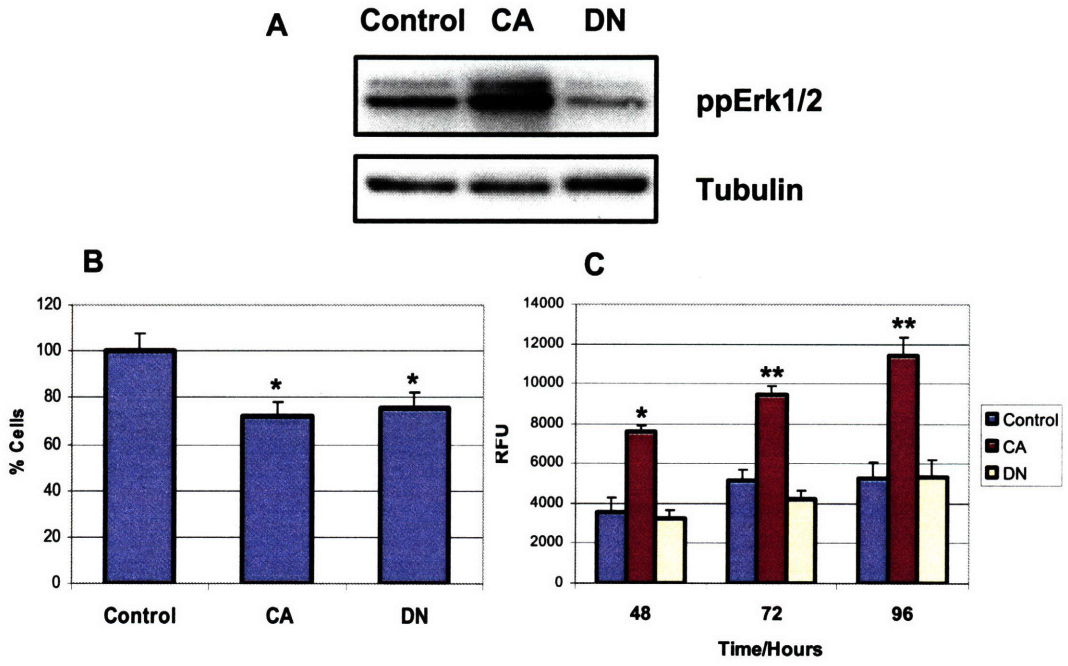

Figure III.7. Growth and apoptotic response of hyperactivation or inhibition of Erk1/2 in U87MG-EGFRvIII cells. (A) Western blot of double phosphorylated Erk1/2 after transfection of U87MG-EGFRvIII cells with either pBabe-puro (Control), constitutively activate MEK (CA) or dominant negative MEK (DN). (B) Viability response of U87MG-EGFRvIII cells upon modulation of the Erk pathway. Transfected cells were subjected to serum depravation conditions for 72 hours prior to counting viable cells by trypan blue exclusion method. Experiments were performed in 6 replicates normalized to control cells. *$p<0.01$. (C) Transfected cells were subjected to serum depravation over 4 days with measurements being performed from 48 hours at 24 hour intervals. Apoptosis was measured by caspase 3/7 cleavage with 4 biological replicates being performed. Significance between different MEK mutants were calculated using paired student t-test, *$p<0.01$, **$p<0.01$. RFU=relative fluorescence units

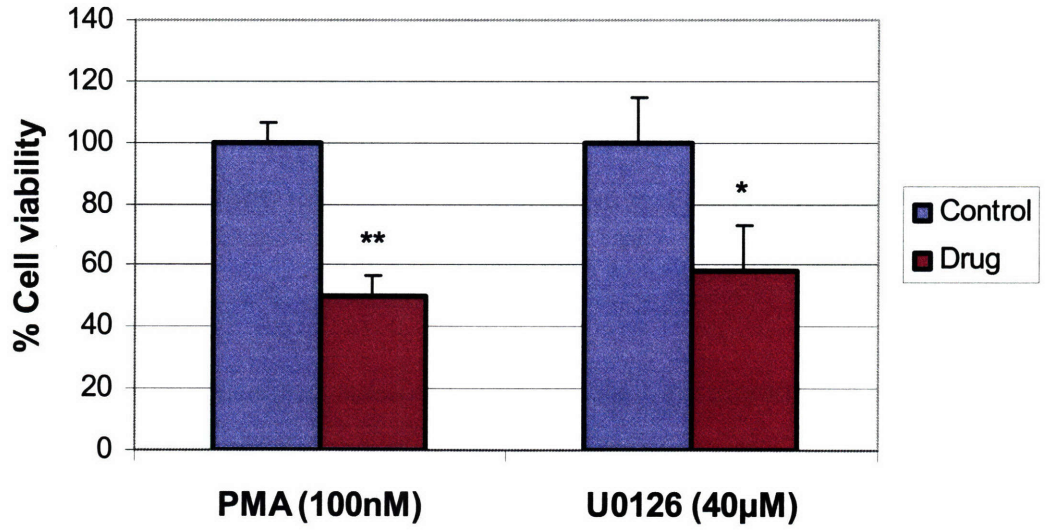

Figure III.8. Dose–response of U87-EGFRvIII cell line upon treatment with PMA or U0126. Dose–response of U87-EGFRvIII cells to PMA (100nM) or U0126 (40µM) 72 hrs after 24 hrs serum starvation. Viability was measured by using the metabolic dye WST-1. *p<0.01, **p<0.001.

III.3.7 Inhibition of Erk1/2 in U87-EGFRvIII cells inhibits the percentage of viable cells and but has no effect on apoptosis

We also wanted to determine if the U87MG-EGFRvIII cells required the Erk pathway for growth. To test this, U87MG-EGFRvIII cells were stably transfected with dominant-negative (DN) mutant MEK, in which mutation of two serine residues into alanines inactivates the protein (Cowley et al. 1994). Overexpression of DN-MEK in the U87MG-EGFRvIII cells resulted in the inhibition of ppErk1/2 (Figure III.7A). When grown under serum-starvation conditions for 72 hours, DN-MEK expressing cells showed a ~20% decrease in the number of viable cells compared to the control cell line (Figure III.7B). Pharmacological inhibition of MEK by U0126 also resulted in a decrease in cell

viability (Figure III.8). However, inhibition of the ppErk did not have an effect on apoptosis of the cell under serum-starvation conditions. (Figure III.7C).

III.3.8 Experimental validation of principle component analysis generated hypothesis

We sought to experimentally validate the hypothesis generated by the principle component analysis: that the phosphorylation of Erk1/2 correlates with a decrease in cell proliferation. In order to do this, we generated stably-transfected CA-MEK and DN-MEK mutants in 5 of the 6 mutant cell lines (Y1068F, Y1148F, Y1173F, DY2 and DY3). Western blot analysis indicates that, as expected, transfection of CA-MEK and DN-MEK hyperactivated and inhibited the phosphorylation of Erk1/2, respectively (Figure III.9A). When grown under serum-starvation conditions for 72 hours, CA-MEK mutant cells demonstrated varying viability profiles, with some lines having no significant response to hyperactivation of Erk1/2 (Y1173F, DY2, DY3) while others showing sensitivity to increased Erk1/2 phosphorylation (Y1068F and Y1148F). In contrast, the percentage of viable cells decreased when Erk1/2 phosphorylation was inhibited by DN-MEK for all the mutant cell lines, albeit with varying levels of sensitivity.

To better characterize the manner in which decrease in cell viability was achieved, we determined the proportion of cells undergoing apoptosis. CA-MEK and DN-MEK mutant lines were subjected to serum starvation for 48 and 72 hours prior to measurement of apoptosis levels (Figure III.10A and 10B). Similar to the U87-EGFRvIII-CA-MEK mutant, two fold increases in apoptosis were

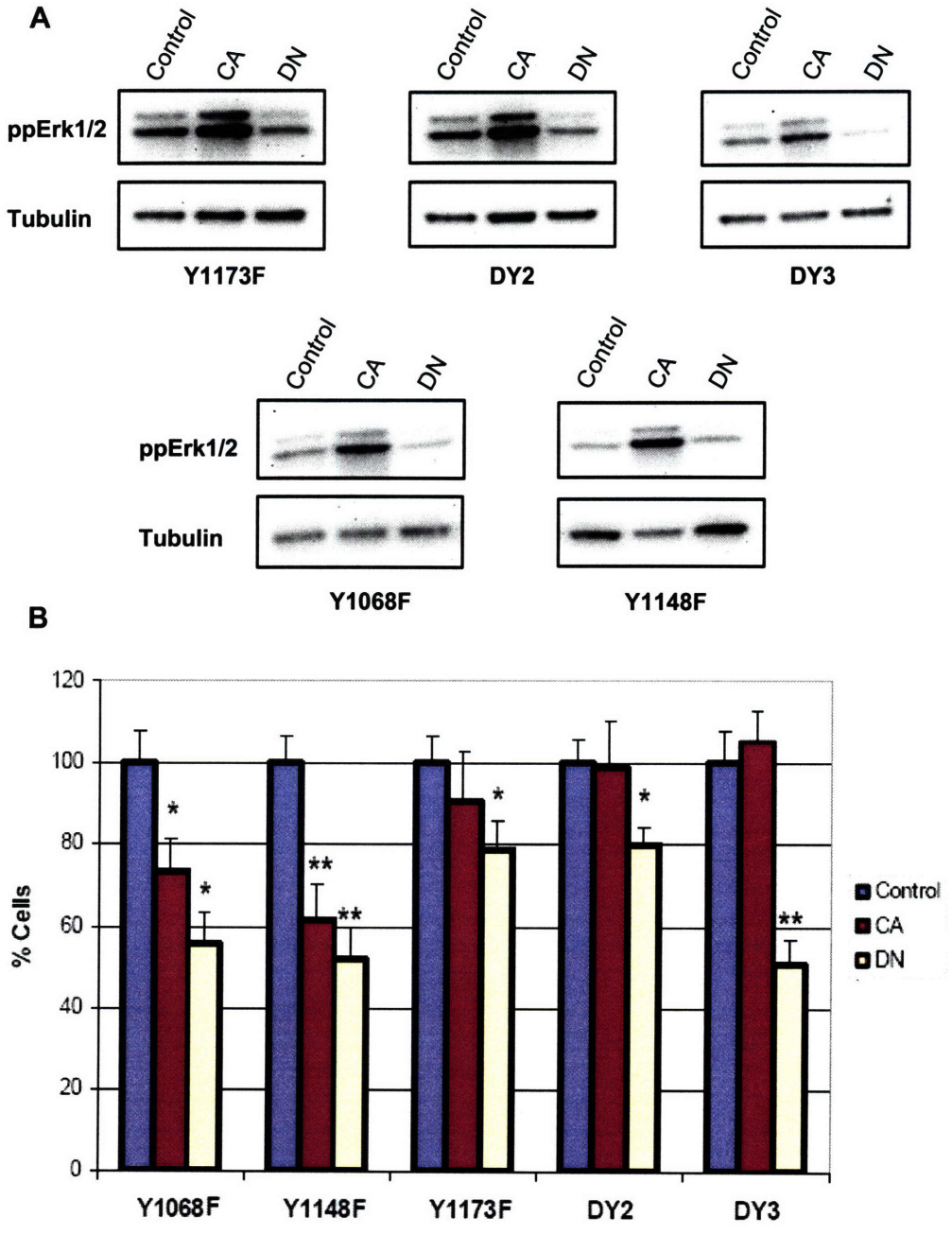

Figure III.9. Growth response to hyperactivation or inhibition of Erk1/2 on EGFRvIII site-directed mutant cell lines. (A) Western blot of double phosphorylated Erk1/2 after transfection of EGFRvIII site-directed mutant cells with either pBabe-puro (Control), constitutively activate MEK(CA) or dominant negative MEK (DN). (B) Pecentage of viable EGFRvIII mutant cells upon modulation of the Erk pathway. Transfected cells were subjected to serum depravation conditions for 72 hours prior to counting viable cells by trypan blue exclusion method. Experiments were performed in 6 replicates normalized to control cells. Significance between CA- and DN-MEK mutant response compared to control cells were calculated using paired student t-test, *$p<0.01$, **$p<0.001$.

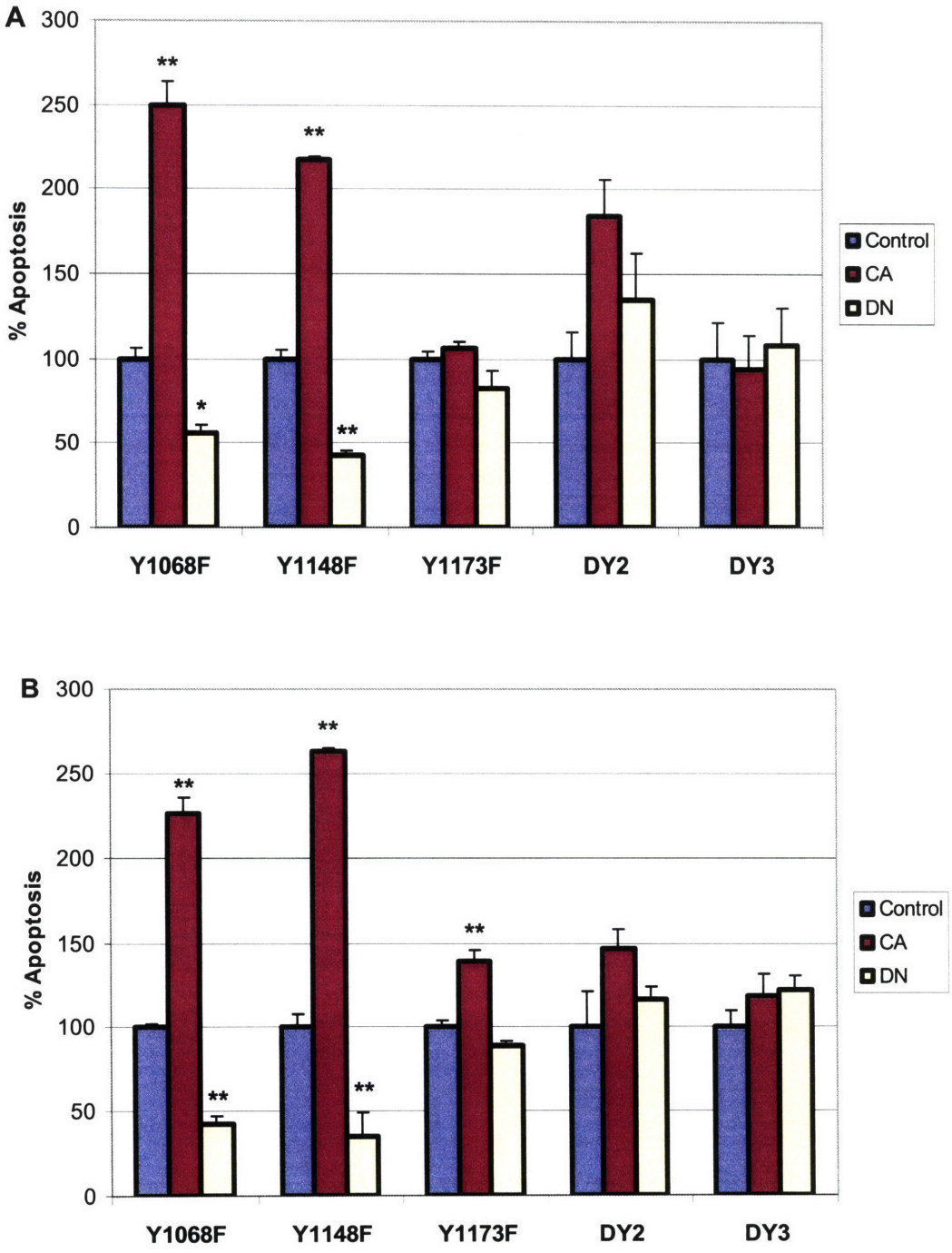

Figure III.10. Apoptotic response to hyperactivation or inhibition of Erk1/2 on EGFRvIII site-directed mutant cell lines. Transfected cells were subjected to serum depravation with measurements being performed at (a) 48 hours or (B) 72 hours. Apoptosis was measured by caspase 3/7 cleavage with 4 biological replicates performed and data normalized to pBabe-puro control. Significance between different MEK mutants were calculated using paired student t-test, *$p<0.01$, **$p<0.01$.

observed in the Y1068F and Y1148F mutants when CA-MEK was overexpressed and this corresponded to significant decreases in the percentage of viable cells (Figure III.9B). Interestingly, inhibition of Erk1/2 phosphorylation resulted in either no change (Y1173F, DY2 and DY3) or a decrease in apoptosis fraction (Y1068F and Y1148F). Taken together with the cell viability data, this apoptosis data would suggest that inhibition of Erk1/2 led to a general decrease in cell growth without an increase in apoptosis.

The cell viability and the apoptosis data of the CA-MEK and DN-MEK mutant and control (U87-EGFRvIII) lines were then subjected to principle component analysis together with the phosphoproteomic and previous phenotypic data (figure III.5). Consistent with the earlier PCA analysis, growth of mutant lines at multiple time points (36 hours or the integral of 0-48 and 0-60 hours) was anti-correlative to the phosphorylation levels of Cdc2, Erk1 and Erk2 in the second principle component (Figure III.11). As predicted by the previous PCA analysis, the experimentally measured cell viability/growth data (percentage of viable cells after transfection compared to control cells) of the mutant cells with hyperphosphorylated Erk1/2 (CA-MEK, figure III.9B) clustered with the measured phosphorylation levels of Erk1/2 (figure III.4). This observation experimentally validates the PCA analysis in its ability to generate hypotheses relating phosphorylation of signaling nodes and the growth characteristics in the U87MG-EGFRvIII cells.

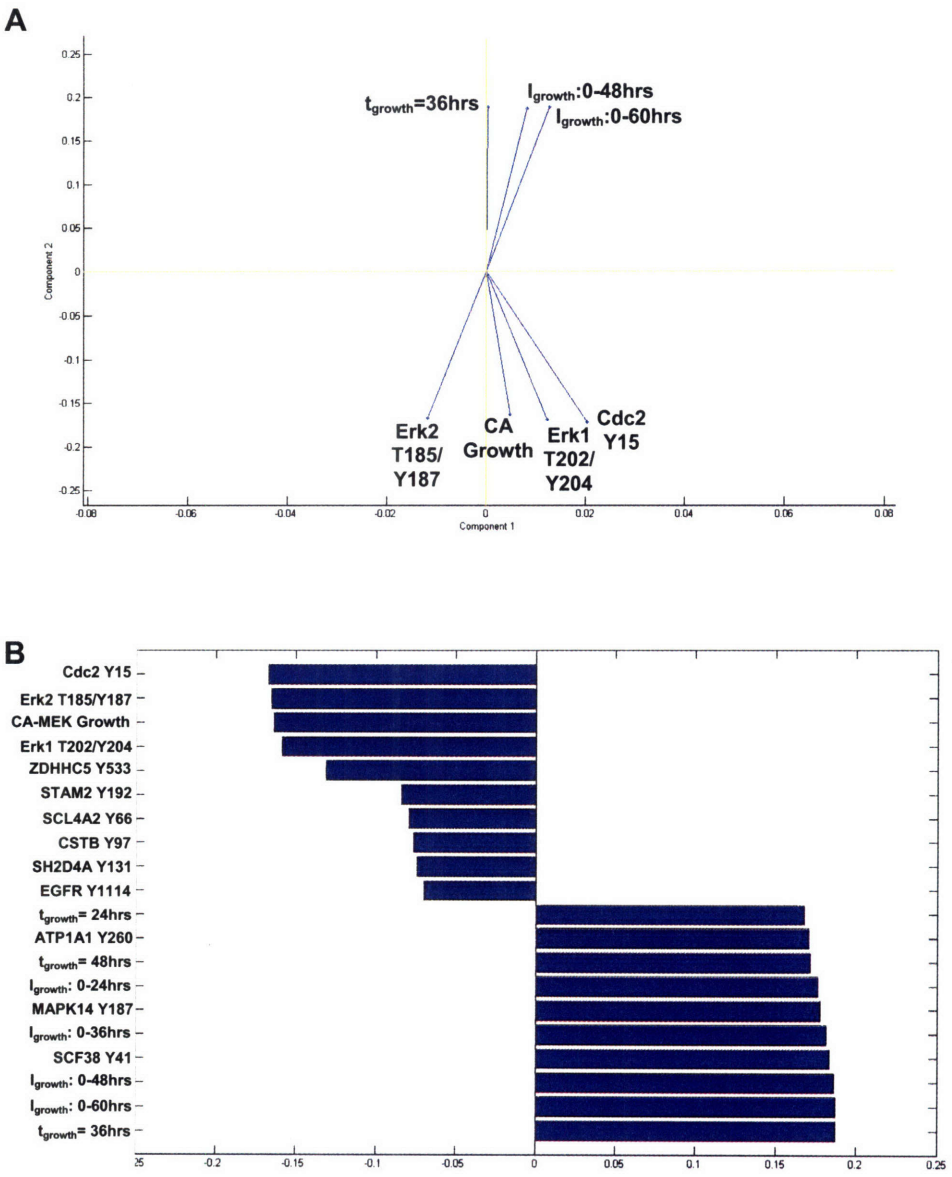

Figure III.11. Principle component analysis of phosphoproteomic and phenotypic data, including phenotypic data of the MEK mutant cell lines. (A) 2D Bi-plot of principle component 1 and 2, highlighting the top 3 positive and top 4 negative contributors to the second principle component. CA growth indicates the percentage of viable cells (compared to the control cell line) of all CA-MEK transfected mutant cell lines after serum depravation for 72 hours. (B) Pareto diagram indicating the top 10 positive and negative contributors to the second principle component. t=time of growth, I=integral of growth.

III.4 DISCUSSION

The functional role of tyrosine phosphorylation sites on the EGFR receptor has previously been studied using *in vitro* methods that rely on measuring interactions between phosphorylated peptides representing known phosphorylation sites on EGFR and potential downstream substrates. These analyses can either be performed on specific protein/domains, cell lysates or more recently, large scale protein domain binding experiments (Songyang et al. 1994; Schulze et al. 2005; Jones et al. 2006). Such approaches provide the opportunity to not only identify site-specific protein interactions but also allows for the measurement of important biophysical parameters such as binding constants (Jones et al. 2006). However, since these experiments are done *in vitro*, the network information encoded in the cellular context is often lost. This network information includes, regulatory loops that occur downstream of receptor activation, protein localization effects and pathway compensation. In order to overcome these deficiencies, we have performed a phosphoproteomic analysis of six EGFRvIII tyrosine site mutants in the background of the U87MG glioblastoma cell line (Huang et al. 1997). This analysis has provided us with a systems view of signaling network modulation that occurs upon loss of specific tyrosine phosphorylation sites *in vivo*.

III.4.1 Receptor phosphorylation in response to EGFRvIII site-specific mutations

At the receptor level, we observed that phosphorylation outcomes broadly fit into 2 categories, based on their responses relative to intact EGFRvIII. The mutations either led to a general decrease in the phosphorylation levels of the non-mutated tyrosine sites on EGFRvIII (Y845F, Y1068F, Y1148F and DY3) or resulted in a compensatory increase in the phosphorylation sites on EGFRvIII (Y1173F and DY2).

It has previously been shown that the Src proto-oncogene phosphorylates wildtype EGFR on Y845. This phosphorylation is critical for cell proliferation as cell expressing the Y845F EGFR mutant does not undergo DNA synthesis upon EGF stimulation (Maa et al. 1995; Boerner et al. 2005). This observation indicates that Src-mediated phosphorylation of EGFR is required for its downstream signaling. Our data sheds some light on the mechanism by which such growth arrest could occur. Abrogation of the Y845 site on EGFRvIII led to a ~2.5 fold decrease in cell growth after 24 hours of serum deprivation compared to intact EGFRvIII. The growth defect is accompanied by a decrease in the levels of all 8 phosphorylation sites that were identified on EGFRvIII receptor, resulting in a concurrent drop in the phosphorylation levels of downstream signaling components in the EGFR network. Consistent with our data, a recent report indicates that overexpression of dominant negative Src protein in gefitinib-resistant cells led to a decrease in the phosphorylation levels at Y845, Y992 and Y1086 on the EGFR receptor (Mueller et al. 2008). The authors did not observe

any change in the phosphorylation levels on Y1045, Y1068, Y1148 and Y1173 upon inhibition of Src but this was shown to be due to the cross-activation of EGFR by the c-Met receptor (Mueller et al. 2008). Intriguingly, we found that the Y845F mutation led to an increase in the activation site (Y417) on Src, which may suggest the presence of a negative feedback loop in which phosphorylation of Y845 in EGFRvIII downregulates Src activation levels. Our phosphorylation data would support the clinical use of Src inhibitors as a means of reducing EGFRvIII activation levels in glioblastoma patients, either as a therapeutic or as an adjuvant to overcome previously reported radio- or chemotherapeutic induced EGFRvIII receptor activation (Benhar et al. 2002; Lammering et al. 2004).

Of the three remaining mutant lines that showed a reduction in EGFRvIII phosphorylation levels, it is not known why a decrease in the phosphorylation levels of the other 8 non-mutated tyrosine residues on EGFRvIII occurred as a consequence of single mutations at Y1068F and Y1148F. However, it highlights the importance of studying the function of receptor tyrosine phosphorylation sites within cellular context. Analysis of the functional roles of post-translational modifications under *in vitro* conditions does not fully recapitulate the biological milieu in which receptors normally function, and would have otherwise obscured the apparent relationship between Y1068/Y1148 and the other receptor phosphorylation sites that we have uncovered. It is also important to note that this effect on Y1068 and Y1148 is specific to EGFRvIII. Previous analysis of the function of these sites on wildtype EGFR revealed that single mutations at these sites had no effect on receptor activation or transformation efficiency, and only

the combined mutations of Y1068F, Y1148F and Y1173F resulted in receptor inactivation (Honegger et al. 1988; Helin et al. 1991). The authors conclude that in the wildtype EGFR receptors with single site-direct mutations, compensating pathways activated as a result of other intact phosphorylation sites may counteract the loss of Y1068 and Y1148. In line with our previous findings (Huang et al. 2007), the difference in receptor phosphorylation response in the EGFRvIII mutants versus the wildtype EGFR mutants would indicate that these two receptors have quantitative differences in receptor activation and pathway utilization.

The second category of EGFRvIII mutants showed a compensatory increase in receptor phosphorylation in response to either the Y1173F mutation or the Y1173F/Y1068F double mutation. This is, to our knowledge, the first demonstration of compensation at the receptor phosphorylation level as a means to overcome loss in receptor signaling potential. It has previously been shown that mutation at these sites in EGFRvIII and wildtype EGFR does not affect receptor kinase activity (Honegger et al. 1988; Helin et al. 1991; Huang et al. 1997). The increase in receptor phosphorylation that we have observed could potentially arise from an elevated level of alternative tyrosine substrates available for EGFRvIII kinase phosphorylation upon removal of the preferential tyrosine substrate, Y1173. However, this hypothesis does not explain the decrease in receptor phosphorylation levels in the DY3 triple mutant which also has intact kinase activity (Huang et al. 1997).

An alternative hypothesis is that the Y1173F results in the loss of function of a negative regulator of receptor phosphorylation, for instance, protein tyrosine phosphatases. A number of protein tyrosine phosphatases have been shown to regulate tyrosine phosphorylation levels of wildtype EGFR, including PTP1B (Haj et al. 2003), TCPTP (Tiganis et al. 1998), SHP-1 (Keilhack et al. 1998), SHP-2 (Zhang et al. 2002), LAR (Kulas et al. 1996), DEP-1 (Berset et al. 2005) and Cdc25 (Wang et al. 2002). Of these, TCPTP and SHP-2 influence the phosphorylation levels of EGFRvIII in glioblastoma cells (Klingler-Hoffmann et al. 2001; Zhan and O'Rourke 2004). Overexpression of TCPTP in EGFRvIII expressing cells reduced receptor phosphorylation levels and led to a decrease in tumor growth *in vivo* (Klingler-Hoffmann et al. 2001). SHP-2, on the other hand, regulates EGFRvIII phosphorylation levels indirectly through Erk pathway activation in glioblastoma cells. Overexpression of catalytically inactive mutants of SHP-2 in the U87MG-EGFRvIII cells led to a decrease in Erk pathway activity and a subsequent decrease in receptor phosphorylation (Zhan and O'Rourke 2004). It is unlikely that this mechanism plays a role in receptor compensation in our system, since the Erk1/2 pathway is highly upregulated in almost all the EGFRvIII mutants. SHP-2 also directly dephosphorylates Y992 on wildtype EGFR, a site that is known to recruit Ras GTPase-activating protein (RasGAP) to the plasma membrane, leading to Ras-MAPK pathway activation (Agazie and Hayman 2003). However, we did not observe this phosphorylation site in our analysis and cannot ascertain if the EGFRvIII mutants display compensatory phosphorylation at Y992 as a result of SHP-2 inactivation.

A more plausible candidate phosphatase is the SHP-1 protein tyrosine phosphatase. SHP-1 is a phosphatase that is highly expressed in hematopoietic cells and a closely related isoform of the protein is expressed in epithelial cells (Banville et al. 1995). A previous report has shown that this protein binds to wildtype EGFR via the Y1173 site and mediates dephosphorylation of the receptor (Keilhack et al. 1998). The authors demonstrated that the Y1173F EGFR mutation reduces SHP-1-mediated receptor dephosphorylation (Keilhack et al. 1998). It is thus tempting to speculate that the loss of Y1173 on the EGFRvIII prevents SHP-1 binding and thus enhances the phosphorylation levels of other sites on the receptor. Further studies are required to fully elucidate the mechanism of EGFRvIII receptor compensation.

The increase in the levels of the other phosphorylation sites in the Y1173F mutant (and to a lesser extent, the DY2 mutant) was able to compensate for the loss of Y1173 and maintain similar (if not higher) levels of tyrosine phosphorylation on downstream EGFR canonical signaling network components. These include components of the SHC-GAB-1-PI3K pathway, STAT3 and PLCγ. Y1173 is a major phosphorylation site on EGFRvIII and EGFR, important for the activation of multiple downstream signaling components through the binding of adaptor proteins such as SHC and Grb-2. Our data would suggest the EGFRvIII receptor has evolved some redundancy in the activation of downstream signaling pathways, such that multiple tyrosine phosphorylation sites recruit similar adaptor proteins, albeit with different binding affinities (Jones et al. 2006). Such overlap in adaptor binding has also been reported in *in vitro* phosphopeptide interaction

experiments. For instance, in addition to Y1173, the adaptor protein SHC can also bind to Y974, Y1086, Y1114 and Y1148, all of which were shown to increase in our phosphorylation study (Schulze et al. 2005). These redundant interactions may account for the maintenance of the SHC-GAB-1-PI3K pathway activation upon the loss of Y1173. The compensatory mechanism that we have observed may also explain why cytoplasmic mutants of EGFR found in GBM patients (EGFRvIV) confer tumorigenicity despite the loss of a number of tyrosine phosphorylation sites in its cytoplasmic tail (Frederick et al. 2000). A better understanding of the mechanisms of receptor compensation would allow us to devise better strategies to treat GBM tumors driven by these mutations.

III.4.2 Sensitivity of U87MG-EGFRvIII cells to the Erk1/2 activation levels

In a recent study of EGFRvIII signaling networks, we have observed that the activation of Erk1/2 does not vary as a consequence of increasing EGFRvIII receptor levels (Huang et al. 2007). This finding is in contrast to the activation of wildtype EGFR, where the addition of EGF leads to a high, transient activation of the Erk1/2 within 1 minute of EGFR activation (Wolf-Yadlin et al. 2007). The dynamics of Erk1/2 activation has important consequences on biological outcome. For instance, PC12 pheochromocytoma cells proliferate upon transient Erk activation by EGF but undergo differentiation into neurons when sustained Erk activation is achieved through the action of nerve growth factor (NGF) (Marshall 1995). Transient activation of Erk1/2 is maintained by a series of negative regulation modules including the upregulation of the MAPK

phosphatases (MKPs) / dual specificity phosphatases (DUSPs) in response to the MAPK activation (Amit et al. 2007). The DUSPs are a family of protein phosphatases that inactivate Erk1/2 through the dephosphorylation of the pTXpY consensus sequences found on the MAPK activation loop (Farooq and Zhou 2004).

EGFRvIII constitutively interacts with the adaptor proteins Grb-2 and SHC via Y1068 and Y1148 respectively (Montgomery et al. 1995; Moscatello et al. 1996; Prigent et al. 1996; Chu et al. 1997). This interaction results in a 2-fold contitutive upregulation of Ras activity and a subsequent four-fold increase in MEK activity (Montgomery et al. 1995; Prigent et al. 1996). Consistant with our own observations (Huang et al. 2007), this increase in MEK activity has no effect on its downstream substrate Erk1/2 (Montgomery et al. 1995). The authors went on to show that upon treatment of EGFRvIII-expressing cells with sodium vanadate (a tyrosine phosphatase inhibitor), Erk1/2 phosphorylation levels increase, suggesting that the failure to activate Erk1/2 is due to the action of tyrosine phosphatases (Montgomery et al. 1995).

Despite the importance of the Erk pathway in mediating multiple biological processes in response to wildtype EGFR activation, we have previously suggested that the lack of Erk1/2 activation by EGFRvIII indicates that the EGFRvIII receptor mediates its tumorigenic effect through the preferential utilization of other upregulated pathways (e.g. the PI3K pathway) over the MAPK pathway (Huang et al. 2007). In this study, we have shown that, in addition to the preferential utilization of alternative signaling pathways, EGFRvIII mediates

its tumorigenic activity in part by actively repressing the phosphorylation of Erk1/2, presumably through the activation of specific DUSPs. Mutation of specific tyrosine phosphorylation sites on EGFRvIII (with the exception of Y1148F) relieves this repression, resulting in increased phosphorylation levels of Erk1/2. Furthermore, this active repression of the Erk1/2 pathway has functional consequences, as hyperactivation of this pathway in the context of EGFRvIII expression results in a two-fold increase in apoptosis.

A similar finding was made in mutant K-Ras^{G12D}-driven colon tumors where MEK activity is upregulated while phosphorylated Erk levels are attenuated by the expression of DUSP6 (Haigis et al. 2008). The authors in this study suggested that tumorigenicity is potentially driven by Erk-independent MEK effects, although Erk is the only known downstream substrate of MEK. This seemingly contradictory result may be reconciled by our observations that hyperphosphorylation of Erk1/2 led to increased apoptosis in the presence of a constitutively active oncogene. This report, together with reports of Erk1/2 attenuation in other constitutively active tyrosine kinases (Kamikura et al. 2000; Charest et al. 2006), would imply that the phenomenon of Erk1/2 repression may be a broader survival mechanism by which constitutively active oncogenes avoid cell death.

The mechanism by which the upregulation of Erk1/2 activity results in apoptosis is unclear. It has previously been reported that phosphorylation of EGFR at T699 induces receptor internalization and signal attenuation (Heisermann and Gill 1988; Countaway et al. 1989; Heisermann et al. 1990).

This site is embedded in a PXTP motif which is the consensus motif for MAPK substrate phosphorylation. The transforming activity of EGFRvIII is due in part to its inability to become ubiquitinated and internalized for degradation, resulting in aberrant localization at the plasma membrane (Huang et al. 1997). It is plausible that hyperactivation of the Erk pathway increases receptor phosphorylation at T699, leading to receptor internalization and subsequent degradation. Since tumor cells expressing EGFRvIII exhibit oncogene dependence for the receptor, such a downregulation would lead to cell cycle arrest and apoptosis (Fan and Weiss 2005).

Hyperactivation of Erk1/2 has also been linked to the upregulation of the $p21^{WAF1/CIP1}$ protein. p21 is a member of the cyclin-dependent kinase inhibitor (CKIs) protein family and regulates entry into the cell cycle by interacting and preventing the activation of the cyclin dependent kinases (Cdks). It has previously been shown that p21 expression is induced due through the activation of the Erk signaling pathway upon growth factor stimulation (Liu et al. 1996; Kivinen and Laiho 1999). Under physiological conditions, growth factor-induced p21 expression is required to promote the assembly of cyclin-cdk-proliferating cell nuclear antigen complexes, allowing the cell cycle to proceed (Zhang et al. 1994). However, sustained Erk activation results in high p21 expression levels causing cell cycle arrest and ultimately apoptosis. It is possible that EGFRvIII actively suppresses the activation of the Erk1/2 pathway so as to prevent accumulation of cellular p21 levels.

We have also determined that some activation of the Erk pathway is required for EGFRvIII-mediated tumor cell growth as inhibition of Erk phosphorylation by DN-MEK and U0126 treatment results in a decrease in cell growth. This is again consistent with the K-Ras^{G12D} study where treating mice with CI-1040 (an oral inhibitor of MEK) suppressed proliferation in the colonic epithelia (Haigis et al. 2008). We propose a model in which EGFRvIII fine-tunes the activation of the Erk1/2 pathway. Some activation of this pathway is required for growth but if Erk1/2 phosphorylation exceeds a particular threshold, cells undergo apoptosis. Our data would also imply that the sensitivity of EGFRvIII expressing tumor cells to threshold levels of Erk1/2 activation may be exploited for therapeutic purposes. Glioblastoma patients with PTEN-null, EGFRvIII-positive tumors are refractory to treatment with EGFR kinase inhibitors (Mellinghoff et al. 2005). This poor efficacy plagues a significant population of GBM patients, since ~ 40% of GBM patients lack the expression of functional PTEN (Maher et al. 2001). All of our experiments were performed in the U87MG cell line which is PTEN-null (Furnari et al. 1997). Hyperactivation of Erk, through the inhibition of negative regulators such as DUSPs or Erk inhibition via the use of MEK inhibitors may be alternative treatment strategies for EGFRvIII positive, PTEN null patients.

III.4.3. Principle component analysis

Principle component analysis (PCA) was used as a tool to identify signaling nodes in our phosphoproteomic data set that may influence phenotypic

endpoints. In this study, we experimentally validated the PCA-generated observation that the phosphorylation status of Erk1/2 was anti-correlative to cell proliferation in the U87MG-EGFRvIII cells. In addition to Erk1/2 and Cdc2, several other proteins were found to also correlate/anti-correlate with cell proliferation. For example, although to a lesser extent than Erk1/2, the STAM2 (signal transducing adaptor molecule (SH3 domain and ITAM motif) 2) protein was also predicted to have an inhibitory effect on cell proliferation. A recent study has shown that STAM2, in a complex with Rin1, plays an important role in the endocytosis and degradation of wildtype EGFR following EGF stimulation (Kong et al. 2007). Loss of this the STAM1/Rin1 interaction either by the deletion of the SH3 domain of STAM2 or the proline-rich domain of Rin1 resulted in the loss in EGFR receptor degradation. It is thus possible that upregulation of STAM2 in the U87MG-EGFRvIII cells would result in the degradation of EGFRvIII and subsequent cell cycle arrest (Fan and Weiss 2005).

The phosphorylation of another protein, SCF38 (solute carrier protein 38) was found to strongly correlate with cell growth. SCF38 is a member of a family of System A transporters that mediate the transport of amino acids. In response to growth factors and insulin, it regulates sodium-coupled uptake of amino acids into the cell. When stimulated with insulin, SCF38 undergoes exocytosis from an endosomal compartment into the plasma membrane in a PI3K-dependent manner (Hyde et al. 2002). A recent partial least squares regression (PLSR) analysis identified this phosphorylation site on SCF38 as one of nine sites that were able to fully recapitulate the cell proliferation and migration behavior of

human mammary epithelial cells in response to EGF and heregulin treatment (Kumar et al. 2007). It is plausible that SCF38 plays a similar role in regulation cell proliferation in EGFRvIII expressing cells. It would also be interesting to expand on the analysis of this data set using the PLSR approach in order to establish a set of signaling metrics that correlate with cell proliferation or drug response (e.g. to AG1478 or U0126), as well as to predict cellular responses to combination protocols of EGFR kinase inhibitors and MEK agonists/antagonists.

In summary, phosphoproteomic analysis of EGFRvIII site-specific mutants has led to the discovery of a novel receptor compensation mechanism. This compensation is able to functionally overcome the loss of Y1173 and maintain signaling through the canonical components of the EGFR pathway. We have also established that EGFRvIII fine-tunes the activity of the Erk1/2 pathway such that threshold levels of Erk activation are not exceeded. Both the hyperactivation and inhibition of the Erk pathway result in a decrease in the viability of U87-EGFRvIII cells. Extending this cell-based approach to the study of clinical tumor samples will enable us to determine the exact nature of the Erk activation thresholds in tumors and design better Erk-based therapeutic strategies to treat EGFRvIII-positive glioblastoma patients.

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IV CONCLUSION

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Through a series of related experiments in this thesis, I have identified a number of molecular mechanisms by which EGFRvIII mediates its tumorigenic properties. These characteristic properties are key drivers for the inability to effectively treat glioblastoma patients and include, rampant tumor cell proliferation, chemo-resistance to classical cytotoxics and targeted therapeutics, and anti-apoptotic features. The work that has been described in this thesis can be divided into 2 major sections.

In the first section, we quantitatively mapped the major downstream pathways activated by EGFRvIII in a GBM cell line and demonstrated that the networks undergo significant modulation as a function of oncogene load. This finding has important implications with regards to the clinical use of targeted therapeutics. Tumors are heterogeneous in nature and consist of multiple cell populations expressing varying receptor levels. Our data indicates that care should be taken in the choice of therapeutic intervention as distinct pathways appear to dominant in response to different EGFRvIII receptor levels. Targeting poorly activated signaling pathways in tumors could result in the sparing of tumor cells that may be capable of repopulating the bulk tumor in a recurrent manner. Applying molecular profiling approaches, such as flow cytometry analysis or gene microarray technology to determine receptor levels and identify suitable patient populations, prior to administration of targeted therapeutics would reduce the likelihood of tumor recurrence after chemotherapy in GBM patients.

In this study, we have also identified the c-Met receptor tyrosine kinase as an EGFRvIII-responsive element. c-Met appears to play a role in conferring resistance to both classical chemotherapeutics and EGFR kinase inhibitors in EGFRvIII cell lines. Since our discovery, others have also reported similar findings, both in the context of EGFRvIII and wildtype EGFR (Stommel et al. 2007; Mueller et al. 2008). In particular, a recent study has shown that EGFR and c-Met are co-activated in multiple human GBM tumors (Stommel et al. 2007). Taken together, these recent data would suggest that an EGFR and c-Met combination inhibitor strategy may be broadly applicable in overcoming the poor efficacy of EGFR kinase inhibitor monotherapy in both EGFR and EGFRvIII overexpressing GBM tumors. Furthermore, this combination approach will allow us to leverage on the availability of receptor tyrosine kinase inhibitors, that have either already been approved for clinical use or are currently in clinical trials, for rapid implementation in the clinic.

In the second section, we established that the signaling response of receptor tyrosine phosphorylation sites *in vivo* cannot be extrapolated *a priori* from *in vitro* receptor binding measurements. For instance, tyrosine site mutations in the EGFRvIII receptor result in a knock-on effect on the levels of other receptor tyrosine phosphorylation sites, rather than just ablating the specific pathway attributed to the mutated phosphorylation site. Particularly interesting, was the observation that alternative tyrosine phosphorylation sites can be upregulated in response to the Y1173F mutation, in a manner that is able to compensate for the loss in downstream signaling potential. The exact

mechanisms by which these compensation responses occur are not clear but it does indicate that the EGFR receptor may have evolved to encode redundancy in receptor function through the activation of multiple phosphorylation sites that bind similar adaptor proteins. In addition, this finding may provide an explanation as to why naturally-occurring cytoplasmic mutants of EGFR (such as EGFRvIV) are tumorigenic in GBMs even with the loss of multiple tyrosine phosphorylation residues in the cytoplasmic tail. It would also be important to extend this study to the other ErbB receptors to determine if receptor compensation represents a common theme in the ErbB family.

We have shown that EGFRvIII normally represses the Erk1/2 MAP kinase pathway in glioblastoma cells (through a still unresolved mechanism) and mutations of specific tyrosine phosphorylation sites on the receptor are able to relieve this repression. This suppression appears to be part of a mechanism in which EGFRvIII fine-tunes the signaling thresholds of the Erk1/2 pathway in tumor cells. A minimum level of Erk activity is required for the optimal proliferation of tumor cells but exceeding the upper limit of this threshold results in apoptosis. There is some evidence that the suppression of the Erk pathway is also found in the context of other constitutively active oncogenes (Kamikura et al. 2000; Charest et al. 2006; Haigis et al. 2008), and may represent a broader survival mechanism employed by tumors expressing such oncogenes. We believe that exploiting the sensitivity of EGFRvIII GBM cells to threshold levels of Erk activation has the potential to be developed into a therapeutic approach for the treatment of GBM patients.

In conclusion, we have utilized quantitative mass spectrometry to obtain molecular insights into the tumor biology of EGFRvIII. We identified the c-Met receptor tyrosine kinase and the Erk1/2 signaling pathway as potential therapeutic targets for the treatment EGFRvIII-positive GBM patients that are currently refractory to treatment with EGFR antagonists. Extend these cell-based studies to murine xenograft and other clinical models to evaluate the efficacy of these treatment protocols *in vivo* will bring us one step closer to developing better strategies for the clinical management of this devastating disease.

IV.2 REFERENCES

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V. FUTURE PERSPECTIVES

V.1 FUTURE PERSPECTIVES

In this thesis, I have explored the utility of phosphoproteomics as a tool for understanding EGFRvIII signaling in GBM cells. Several outstanding questions have arisen from this work, and in this section I will offer my perspectives on addressing some of these questions and how to translate our discoveries from the bench into the clinic.

V.1.1 Beyond in vitro cell signaling

All of the work described in this thesis has primarily been limited to *in vitro* cell-based systems. Tumors are heterogeneous in nature and represent a mixture of multiple cells types with distinct genetic backgrounds. In addition, changes in the tumor microenvironment have been shown to have a critical influence in the initiation and progression of tumors. These important characteristics are ultimately lost when cellular signaling is studied *in vitro*. Although some of these limitations can be overcome by growing cancer cells in 3D cell culture systems (Debnath and Brugge 2005), it still represents an artificial system for studying signaling networks pertinent to cancer progression. It is thus important to extend our phosphoproteomic analysis to the study of tumors, both from murine xenograft models and genetically engineered mouse models of GBM as well as from human clinical GBM samples. These analyses will provide the molecular basis of the pathophysiological hallmarks of GBMs, including the tremendous proliferative and invasive potential of these tumors (Furnari et al.

2007). It will also provide an insight into the contribution of the tumor microenvironment to tumor signaling networks, in particular the development of angiogenesis (Jain et al. 2007). Unlike gene microarray analysis which only gives a global view of gene expression levels, phosphoproteomics provides a quantitative characterization of the activation states of proteins, the primary targets in the drug development process. It is envisioned that phosphoproteomic analyses of GBM tumors will not only uncover clinically relevant therapeutic targets but will also identify important biomarkers for disease prognosis, thereby predicting response to targeted therapeutics.

V.1.2 Developing a mechanistic understanding of receptor tyrosine kinase co-activation

While the research in this thesis has provided some insight of the contribution of receptor tyrosine kinase (RTK) co-activation on one facet of tumor biology (that of tyrosine kinase inhibitor (TKI) kinase inhibitor resistance), the extent to which the signaling networks downstream of RTK co-activation are responsible for tumor progression are still poorly understood. Candidate RTKs that have been shown to be simultaneously co-activated in human gliomas include EGFR, c-Met, Axl and PDGFR (Huang et al. 2007; Stommel et al. 2007). Previous and ongoing phosphoproteomic studies suggest that there appears to be a differential promiscuity in the manner in which RTK co-activation occurs, where the activation of different initiator RTKs result in the co-activation of distinct RTK combinations ((Zhang et al. 2005; Wolf-Yadlin et al. 2006) and

unpublished observations). This promiscuity leads to a hierarchy of RTK signaling events where the stimulation of one RTK sets off a cascade of multiple RTK activation events, each governed by its own activation/termination kinetics. It is plausible that some of the clinical features (e.g. tumor chemoresistance) observed in patients may be attributed to shared RTK co-activation signaling nodes while others (e.g. tumor invasion) may be due to the action of specific RTKs, for example c-Met. Additionally, it would be interesting to establish the "combination index" of RTK co-activation and determine if the signaling and phenotypic outcomes resulting from RTK co-activation is additive or synergistic compared to individual RTKs. Further research into these possibilities will provide a mechanistic basis in the decision for which combinations of RTKs to co-inhibit to achieve maximum efficacy. Understanding the signaling nodes that influence cellular decision processes in gliomas will allow one to better tailor treatment protocols for optimal responses in tumors driven by different RTK co-activation combinations.

V.1.3 Other EGFR mutations in GBM

In addition to EGFRvIII, there is a spectrum of EGFR mutations that are commonly found in glioma patients. These include deletions in the cytoplasmic region of the gene (such as EGFRvIV and vV found in 15% of glioma patients) as well as the recently discovered extracellular domain single point mutations (Frederick et al. 2000; Lee et al. 2006; Zandi et al. 2007). The cancer genome sequencing efforts of the cancer genome atlas (TCGA) consortium, for which

GBM has been selected as one of the 3 tumor types in the pilot study, will undoubtedly reveal further mutations in the EGFR gene (Heng 2007). In chapter II, I have shown that EGFRvIII preferentially utilizes differential pathways compared to wildtype EGFR, it is also likely that pathway utilization between the different EGFR mutants found in human GBMs are also distinct from wildtype EGFR signaling. Unlike EGFRvIII, the other EGFR mutations reported in the literature possess an intact extracellular ligand binding domain and are responsive to EGF stimulation (Frederick et al. 2000; Lee et al. 2006; Zandi et al. 2007). It will thus be important not only to determine the steady state activation profile of these mutants, but also elucidate how such mutations modulate signal response upon ligand binding.

Using unbiased quantitative MS approaches to probe signaling networks has the added advantage of not just uncovering quantitative signaling differences between EGFR mutants, but also to discover novel network nodes. Determining both the common and distinct pathways employed by these EGFR mutants would allow one to determine the mechanisms by which these mutants contribute to tumorigenicity. Shared effector signaling pathways in which utilization is highly conserved between different activating mutants may reveal critical nodes required for tumor progression and maintenance. Conversely, establishing the distinct pathways specific to individual EGFR mutants will shed light on the basis of observed differences in tumor phenotype and outcomes between the different mutants. In the absence of structural data, these mutant receptor signaling profiles will provide a network view of how mutations modulate downstream

signaling pathways and impinge on phenotypes both distal and proximal to the receptor, such as EGFR TKI response. These profiles may then serve as molecular signatures for screening clinical samples using more targeted approaches such as antibody microarrays.

V.1.4 Integration of MS-based phosphoproteomics with other proteomic tools

Phosphoproteomic technologies can be broadly classified into discovery and targeted approaches. Discovery techniques such as quantitative MS generate unbiased cancer signaling maps with high density coverage, while targeted technologies such as protein microarrays provide high-throughput datasets with less network detail (Schmelzle and White 2006). Each technique has independently provided insights into disease biology, and I believe that the integration of multiple approaches, including both "discovery" and "targeted" technologies, as shown in figure V.1 will provide a more complete picture of cancer networks and improve the translation of such discoveries into clinical benefit.

The need for integration can be illustrated using our phosphoproteomic study described in chapter II and another protein microarray study of receptor tyrosine kinase (RTK) co-activation in GBM (Huang et al. 2007; Stommel et al. 2007). Each study utilized a different approach (large scale quantitative MS versus RTK antibody microarrays), yet both demonstrated that simultaneous co-activation of multiple RTKs contributes to resistance of GBM to EGFR tyrosine

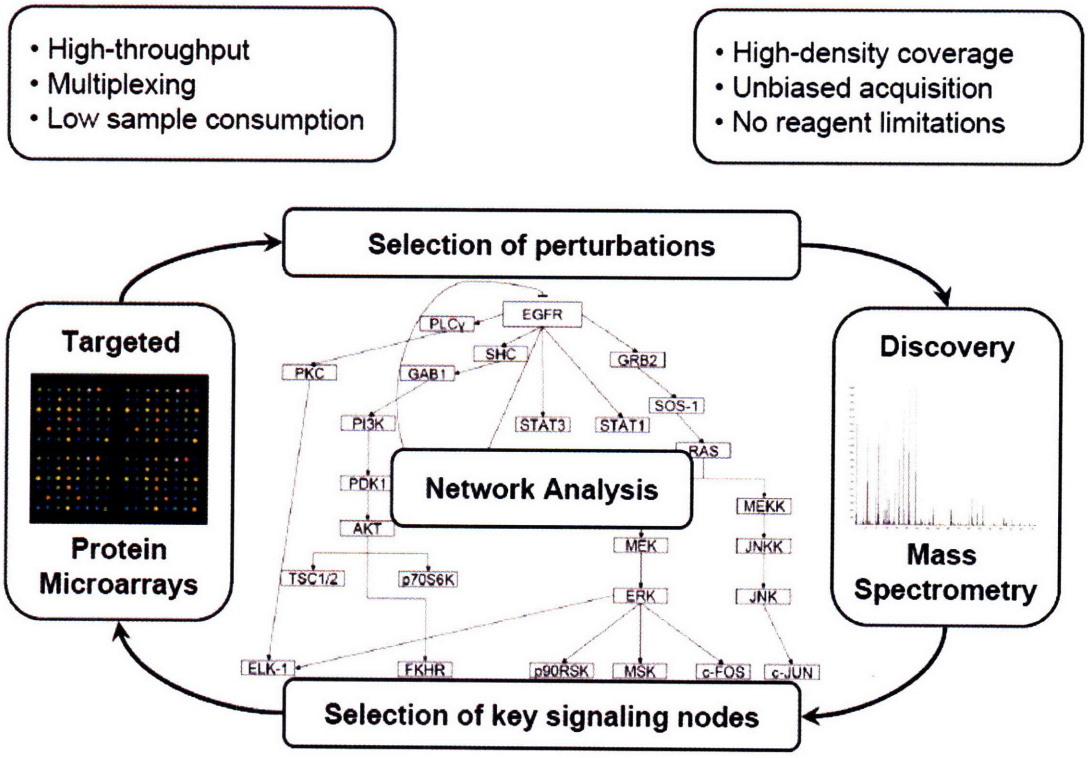

Figure V.1. Integration of discovery and targeted phosphoproteomic technologies. Integration of these two broad classes of proteomic technologies will capitalize on their individual strengths. Discovery-based approaches such as quantitative mass spectrometry can be used to generate high-density maps of cancer networks. Nodes which are most descriptive of the network are then selected for higher-throughput screening of multiple conditions (e.g. human tumors) to identify molecular signatures (e.g. susceptibility to specific therapeutics). Targeted technologies may also reveal specific conditions/ perturbations which may benefit from more in-depth mechanistic network coverage using discovery phosphoproteomics. This iterative process results in a more complete system-wide view of cancer signaling networks.

kinase inhibitors (TKIs). Although both studies also found that inhibition of EGFRvIII and c-Met may be required to overcome this resistance, the microarray studies were extended to multiple cell lines and clinical samples, while the MS-based study provided a quantitative analysis of the signaling networks associated with EGFRvIII activation in a single GBM cell line. In the future, one can envisage a workflow in which discovery MS is employed to initially determine the signaling profiles characteristic of EGFR TKI resistance (Figure V.1). Representative signature nodes from these profiles can then be used in a protein

microarray format for a targeted screen of a large number of human gliomas. Patients with the appropriate profile can subsequently be considered as candidates for treatment with selected small molecule kinase inhibitors. Conversely, information gleaned from higher throughput targeted screening can isolate clinical conditions which may benefit from further mechanistic insight by discovery approaches. For instance, if a targeted screen uncovered a subset of patients that demonstrated clinical hypersensitivity to TKIs, discovery-based MS could then be used to probe the potential mechanisms of action.

Such an integrated approach, should allow one to refine the signaling nodes that result in drug resistance/susceptibility. Incorporating both discovery and targeted based phosphoproteomic technologies will be critical in understanding the pathophysiology of cancer; this information will ultimately lead to the identification of additional targets and novel intervention strategies for the clinical management of GBM.

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INDEX OF TABLES AND FIGURES

I. INTRODUCTION

II QUANTITATIVE ANALYSIS OF EGFRVIII CELLULAR SIGNALING NETWORKS REVEALS A COMBINATORIAL THERAPEUTIC STRATEGY FOR GLIOBLASTOMA

III QUANTITATIVE ANALYSIS OF SITE-DIRECTED MUTANTS OF EGFRVIII REVEALS A RECEPTOR COMPENSATORY MECHANISM AND A DEPENDENCE ON THE ERK SIGNALING PATHWAY

V FUTURE PERSPECTIVES

ABBREVIATIONS

EGFR:	Epidermal Growth Factor Receptor
LC/MS/MS:	Liquid chromatography tandem mass spectrometry
pY:	Phosphotyrosine
IMAC:	Immobilized metal affinity chromatography
GBM:	Glioblastoma
RTK:	Receptor tyrosine kinase

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