Molecular Characterization of EGFR and EGFRvIII Signaling Networks in Human Glioblastoma Tumor Xenografts*§

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Glioblastoma multiforme (GBM) is a malignant primary brain tumor with a mean survival of 15 months with the current standard of care. Genetic profiling efforts have identified the amplification, overexpression, and mutation of the wild-type (wt) epidermal growth factor receptor tyrosine kinase (EGFR) in ~50% of GBM patients. The genetic aberration of wtEGFR is frequently accompanied by the overexpression of a mutant EGFR known as EGFR variant III (EGFRvIII, de2–7EGFR, ΔEGFR), which is expressed in 30% of GBM tumors. The molecular mechanisms of tumorigenesis driven by EGFRvIII overexpression in human tumors have not been fully elucidated. To identify specific therapeutic targets for EGFRvIII driven tumors, it is important to gather a broad understanding of EGFRvIII specific signaling. Here, we have characterized signaling through the quantitative analysis of protein expression and tyrosine phosphorylation across a panel of glioblastoma tumor xenografts established from patient surgical specimens expressing wtEGFR or overexpressing wtEGFR (wtEGFR+) or EGFRvIII (EGFRvIII+). S100A10 (p11), major vault protein, guanylate-binding protein 1(GBP1), and carbonic anhydrase III (CAIII) were identified to have significantly increased expression in EGFRvIII expressing xenograft tumors relative to wtEGFR xenograft tumors. Increased expression of these four individual proteins was found to be correlated with poor survival in patients with GBM; the combination of these four proteins represents a prognostic signature for poor survival in gliomas. Integration of protein expression and phosphorylation data has uncovered significant heterogeneity among the various tumors and has highlighted several novel pathways, related to EGFR trafficking, activated in glioblastoma. The pathways and proteins identified in these tumor xenografts represent potential therapeutic targets for this disease. Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.019984, 1724–1740, 2012.

Glioblastoma multiforme (GBM)¹ is the most frequent and aggressive form of primary brain tumor (1). The current standard of care for GBM consists of surgical removal, radiotherapy, and adjuvant chemotherapy (typically temozolomide) (1). However, despite these interventions the prognosis is still poor, with mean survival time at ~15 months following diagnosis (2). Genetic profiling of GBM tumors has been used to identify multiple distinct genetic aberrations across a diverse array of genes such as the deletion of phosphatase and tensin homolog (PTEN), p16 deletion, and mutation of TP53 (3, 4). Additionally, amplification, overexpression, and/or mutation of the wild-type (wt) epidermal growth factor receptor tyrosine kinase (EGFR) has been identified to be a key genetic alteration in ~50% of GBM patients (5). EGFR amplification is often accompanied by the overexpression of a mutant EGFR known as EGFR variant III (EGFRvIII, de2–7EGFR, ΔEGFR), which is expressed in 30% of GBM tumors (6–8). EGFRvIII is characterized by the deletion of exon 2–7, resulting in an in-frame deletion of 267 amino acid residues from the extracellular domain. This deletion generates a receptor which is unable to bind ligand yet is constitutively, but weakly, active (9). Continuous low level activation leads to impaired internalization and degradation of the receptor, causing prolonged signaling (10). Expression of EGFRvIII in the absence of wtEGFR leads to the transformation of cells in vivo, drives cell proliferation in vitro, and expression of EGFRvIII correlates with poor prog-

¹ The abbreviations used are: GBM, Glioblastoma multiforme; BIC, Bayesian information criterion; CAIII, carbonic anhydrase III; EGFR, Epidermal growth factor receptor; EGFRvIII, Epidermal growth factor receptor variant III; EMT, Epithelial to mesenchymal transition; Eps15, Epidermal growth factor receptor substrate 15; Gab1, GRB2-associated-binding protein 1; GBP1, Guanylate-binding protein 1; GO, Gene ontology; IEF, Isoelectric fractionation; IMAC, Immobilized metal affinity chromatography; IP, Immunoprecipitation; LC-MS/MS, Liquid chromatography tandem mass spectrometry; MVP, Major vault protein; NTRK1, Neurotrophic receptor tyrosine kinase; PANTHER, Protein Analysis Through Evolutionary Relationships; PDGFR, Platelet derived growth factor receptor; PIP₃, Phosphatidylinositol(3,4,5)-trisphosphate; PLC-γ, Phospholipase C gamma; PTEN, Phosphatase and tensin homolog; PTM, Post-translational modification; REMBRANDT, Repository for Molecular Brain Neoplasia Data; RIPA, Radioimmunoprecipitation assay; TCGA, The Cancer Genome Atlas; Wt, Wild type.
nosis in the clinic (6, 11, 12). EGFRvIII has been identified in GBM, lung, ovarian, and breast cancers, but has never been identified in normal tissue (13, 14). Because of the absence of this mutant receptor in normal tissue, EGFRvIII is an attractive therapeutic target. Although EGFR inhibitors, such as erlotinib and gefitinib, inhibit EGFR, EGFRvIII bearing xenograft models and cell lines are resistant to these inhibitors (15, 16). Therapeutic agents directly targeting EGFRvIII in murine GBM xenografts initially resulted in reduced tumor volume and a modest increase in survival (17). However, tumor recurrence was inevitable because of resistance by uncharacterized evasion mechanisms and adaptations (17). We propose that an improved understanding of the system-wide changes in protein expression and signaling caused by EGFRvIII expression should provide insight into specific therapeutic targets for EGFRvIII driven tumors.

It is thought that EGFRvIII enhances tumorigenicity by differential utilization (e.g. altered amplitude and kinetics and potentially novel components or pathways) of signal transduction pathways compared with ligand activated wtEGFR. Quantitative mass spectrometry has previously been applied to the identification of EGFRvIII specific phosphotyrosine signaling across four GBM cell lines expressing titrated levels of EGFRvIII relative to cells expressing the kinase-dead control (18). Cross-activation of EGFRvIII and the c-Met receptor tyrosine kinase is prevalent within these EGFRvIII overexpressing cell lines, revealing an attractive therapeutic strategy (18), which was later extended to include cross-activation of PDGFR (platelet-derived growth factor receptor) (19).

Although EGFRvIII signaling has been extensively studied in GBM cell lines, the molecular mechanisms of increased tumorigenesis driven by EGFRvIII overexpression in human tumors have not been fully elucidated (20, 21). In addition, tissue culture conditions dramatically change the genetic and molecular characteristics found in primary human tumors. In particular, EGFRvIII expression is rapidly lost during generation of primary culture cells from GBM tumors. Most of the EGFRvIII-expressing cell lines are a result of stable transfection, rather than endogenous expression, of the mutant receptor (22). Additionally, the micro-environment and cellular heterogeneity of the tumor have a significant impact on the response to therapeutics, yet are poorly reflected in cell culture. As a consequence, quantification of signaling networks in glioblastoma cell lines provide a limited understanding of the signaling networks in GBM tumor samples.

To overcome this limitation, the James and Sarkaria labs have generated, from patient surgical specimens, a panel of glioblastoma tumor xenografts that are maintained through serial passaging as subcutaneous xenografts in nude mice (22, 23). Maintenance of GBM tumors in this in vivo setting preserves the genetic features and phenotypes crucial to the tumorigenicity of the primary human tumors (23). With these tumor xenografts it is possible to analyze in vivo signaling networks, predict optimal therapeutic strategies based on these data, and test these predictions in a physiologically relevant system.

To quantify signaling networks activated in glioblastoma tumor xenografts and determine the effect of wtEGFR or EGFRvIII expression on these networks, we applied quantitative mass spectrometry to eight human GBM xenografts expressing wtEGFR (wt) or overexpressing wtEGFR (wtEGFR+) or EGFRvIII (EGFRvIII+) implanted into the flanks of nude mice. This analysis led to the identification and quantification of 1588 proteins (across two or more biological replicates) and 225 tyrosine phosphorylation sites on 168 proteins across eight tumor xenografts. Integration of quantitative phosphotyrosine data into protein expression profiles have uncovered the differential regulation of novel proteins and phosphotyrosine sites, which relate to the mode of action of wtEGFR and EGFRvIII overexpression in vivo. Quantification of tyrosine phosphorylation networks revealed specific to each tumor xenograft. These data provide evidence for a significant amount of variation across the eight xenografts, and suggests that optimal therapeutic strategies might be specific to each tumor.

**EXPERIMENTAL PROCEDURES**

**Generation of GBM Xenografts**—Human GBM xenografts were established with the ectopic injection of 100–200 μl of tumor homogenate mixed 1:1 with matrigel into the flanks of nude mice (Fig. 1A). Each of the eight xenografts used in this study (GBM6, GBM8, GBM10, GBM12, GBM15, GBM26, GBM39, and GBM59) were derived from primary tumors from different patients undergoing surgical treatment and serially passaged in mice before this study (23). Four tumor xenografts were generated for each GBM tumor except for GBM6 where two tumor xenografts were generated. Tumor xenografts were resected from mice and immediately flash frozen and stored in liquid nitrogen prior to tissue homogenization.

**Tissue Homogenization**—Tumor xenograft tissues were homogenized (Polytron) in ice-cold 8 M urea, 1 mM sodium orthovanadate, 0.1% Nonidet P-40, and protease and phosphatase inhibitor mixture tablets (Roche) for mass spectrometric analyses. For immunoblotting, ice-cold radioimmunoprecipitation assay (RIPA) buffer plus 1 mM sodium orthovanadate, 0.1% Nonidet P-40, and protease and phosphatase inhibitor mixture tables (Roche) was used for homogenization. Samples were homogenized on ice, with 6 × 10 s pulses (full speed), separated by 10 s intervals to prevent heating of the sample. Once homogenization was complete tumor lysates were left to settle on ice for 2 min. A 50 μl aliquot was taken for BCA protein quantification and the total homogenate was stored at −80 °C.

**Immunoblotting**—Homogenized tumor lysates (generated as described above) were separated on a 7.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose (Bio-Rad). Blocking buffers were made in TBS-T (150 mM NaCl, 0.1% Tween 20, 50 mM Tris, pH 8.0) and contained 5% nonfat dry milk or 3% bovine serum albumin. Antibodies used are as follows; Anti-EGFR (BD Biosciences), Anti-GAPDH (CST, Danvers, MA), Anti-phosphotyrosine (4G10, Millipore), Anti-EGFR (BD Biosciences), Anti-GEF11 (CST), Anti-Hrs (Enzo Life Sciences, Farmingdale, NY), Anti-Shp2 (CST), Anti-EGFR pY1173 (CST), Anti-EGFR pY1045 (CST), Anti-STAT3 (CST), Anti-STAT3 pY705 (CST), Anti-PLCγ (BD Biosciences), Anti-Gal1 (Millipore), and Anti-Gal1 pY627 (Millipore). Appropriate antibodies were diluted in blocking buffer and incubated with nitrocellulose overnight at 4 °C. Secondary antibodies (either goat anti-rabbit or goat anti-mouse
conjugated to horseradish peroxidase) were diluted 1/10,000 in TBS-T and incubated at room temperature for 1 h. Antibody binding was detected using the enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL).

Reduction, Alkylation and Tryptic Digestion—Proteins were quantified using BCA assay (Pierce) and proteins were reduced (10 mM dithiothreitol, 56 °C for 45 min), alkylated (50 mM iodoacetamide, room temperature in the dark for 1 h), and excess iodoacetamide was quenched with dithiothreitol to a final concentration of 25 mM. Protein was subsequently digested with trypsin (sequencing grade, Promega, Madison, WI), at an enzyme/substrate ratio of 1:100, at room temperature overnight in 100 mM ammonium acetate pH 8.9. Trypsin activity was quenched by adding formic acid to a final concentration of 5%. Urea was removed from the samples by reverse phase desalting using a C18 cartridge (Waters, Milford, MA) and peptides were lyophilized and stored at −80 °C.

iTRAQ Labeling—Peptide labeling with iTRAQ 8plex (AB Sciex) was performed as previously described (24). Briefly, for each analysis, −8 mg (wet weight) tumor (equivalent to 800 µg peptide before desalting and processing) for each of the eight tumors was labeled with two tubes of iTRAQ 8plex reagent. GBM xenografts were labeled using the iTRAQ 8plex channels as follows: 113-GBM6; 114-GBM8; 115-GBM10; 116-GBM12; 117-GBM15; 118-GBM26; 119-GBM39; and 121-GBM59 throughout all four biological replicates. GBM6 biological replicate 1 and biological replicate 4 were analyzed in the place of biological replicates 2 and 3 respectively. Lyophilized samples were dissolved in 60 µL of 500 mM triethylammonium bicarbonate, pH 8.5, and the iTRAQ reagent was dissolved in 70 µL of isopropanol. The solution containing peptides and iTRAQ reagent was vortexed, incubated at room temperature for 2 h and concentrated to 40 µL. Samples labeled with different isotopic iTRAQ reagents were combined and concentrated to completion. Peptides were then dissolved in 400 µL of IP buffer (100 mM Tris, 100 mM NaCl, and 1% Nonidet P-40, pH 7.4) and the pH was adjusted to 7.4 before phosphotyrosine immunoprecipitation (IP).

Phosphotyrosine Enrichment—Protein G agarose (80 µL, EMD) was incubated with three phosphotyrosine antibodies; 12 µg PT66 (Sigma-Aldrich), 12 µg pY100 (CST), and 12 µg 4G10 (Millipore) and 200 µL of IP buffer (100 mM Tris, 1% Nonidet P-40, pH 7.4) was added and the mixture was incubated for 8 h at 4 °C with rotation. Antibody conjugated protein G was then rinsed and iTRAQ 8plex labeled peptides re-suspended in the IP buffer, added to the conjugated protein G and incubated overnight at 4 °C with rotation. Conjugated protein G agarose was rinsed with 400 µL of IP buffer and 4 × 400 µL of rinse buffer (100 mM Tris, pH 7.4), and peptides were eluted into 70 µL of 100 mM glycine pH 2. Phosphotyrosine peptides were further enriched using an offline immobilized metal affinity chromatography (IMAC) column (24). Peptides were loaded onto the pre-column and were subsequently separated by reverse phase HPLC (Agilent, Santa Clara, CA) over a 150 min gradient before nano-electrospray into an Orbitrap XL mass spectrometer (Thermo scientific) for phosphotyrosine analyses. To correct for slight variations in the amount of sample in each of the iTRAQ channels, the mean iTRAQ ratios for all proteins identified in each biological replicate analysis was used to normalize the data. The mass spectrometer was operated in data-dependent mode with a full scan MS spectrum followed by MS/MS (collision-induced dissociation (CID) was set at 35% energy for sequence information and higher-energy c-trap dissociation (HCD) at 75% energy for iTRAQ quantitation for Orbitrap XL) for the top 10 precursor ions in each cycle. Ion trap injection time was set to 100 ms and FTMS injection time was set to 1000 ms with a resolution of 60,000 across m/z 400–2000. For FT and FT-MS/MS scans, fragmentation was carried out on ions above a threshold of 500 counts and an FTMS resolution of 7500.
Protein Expression Data Analysis—Relative quantification and protein identification were performed with the ProteinPilot™ software (version 2.0: AB Sciex) using the Paragon™ algorithm as the search engine (26). MS/MS spectra were searched against human protein sequence database (NCBI, released May 2011, downloaded from ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/protein/). The search parameters allowed for carbamidomethylation of cysteines by iodoacetamide and a standard extensive list of biological modifications that were programmed in the algorithm. Identified proteins were grouped by the ProGroup algorithm (AB Sciex) to minimize redundancy. The peptide and protein selection criteria for relative quantitation were performed as follows. Only peptides unique for a given protein were considered for relative quantitation, excluding those common to other isoforms or proteins of the same family. Proteins were identified on the basis of having at least two peptides with an ion score above 95% confidence. Identified peptides were further excluded from quantitative analyses if 1) the peaks corresponding to the iTRAQ labels were not detected, 2) the same peptide sequence was shared by more multiple proteins, or 3) the peptide sequence was discordant. The protein sequence coverage was calculated for proteins by the percentage of matching amino acids from the identified peptides having confidence greater than or equal to 95% divided by the total number of amino acids in the sequence. The total list of peptides and proteins identified and quantified can be found in supplemental Tables S3 and S4.

A decoy database search strategy was used to estimate the false discovery rate (FDR), defined as the percentage of decoy proteins identified against the total protein identification. The FDR was calculated by searching the spectra against the NCBI nonredundant Homo sapiens decoy database. Before filtering the protein expression data (explained above), the protein level FDR was calculated at 1%, corresponding to 2357, 2122, 2434, and 2222 proteins in biological replicates 1, 2, 3, and 4 respectively. After application of the above filter criteria, the estimated FDR value was <1% (at the protein level) for each of the biological replicates analyzed, indicating a high reliability in the proteins identified. Peptide summaries were exported from ProteinPilot™ and isotope correction and relative quantification was calculated in Excel. Based on the biological variation, observed proteins with iTRAQ ratios below 0.75 were considered to be reduced in expression and proteins with iTRAQ ratios above 1.25 were considered to be increased in expression. These values were also applied to the phosphoryrosine analyses (the number of proteins shown to be changing based on this criteria can be found in supplemental Table S5).

Affinity Propagation Clustering Analysis—Quantitative phosphorylation site profiles across all eight xenografts were clustered using the affinity propagation algorithm proposed by Frey and Dueck (27). Euclidean distance was used as the similarity metric. We constructed a Gaussian mixture model based on the exemplar clustering solution and used a Bayesian information criterion (BIC) scoring metric with a model complexity penalty to select the most appropriate clustering solution. Self-similarity values were varied, and BIC scores were calculated for each clustering solution at that self-similarity value. The highest scoring model was selected.

Kaplan Meier Survival Curves—The Repository for Molecular Brain Neoplasia Data (REMBRANDT) gene expression data from clinical trials involving patients suffering from Gliomas (https://caintegrator.nci.nih.gov/rembrandt/) was used to identify the proteins with the most significant effect on survival in the top 11 up-regulated proteins in the EGFRVIII tumors. The survival curve was based on Kaplan-Meier estimates and a Log rank (Mantel-Cox) test was carried out to indicate differences in survival (significance is expressed by p value). GraphPad Prism 5 was used for all calculations and to plot the Kaplan-Meier survival curves.

Gene Ontology Annotation—Gene ontology (GO) annotations were identified by uploading gene lists to the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system (http://www.pantherdb.org/).

RESULTS

Characterization of GMB Xenograft Tumors—Initial characterization of the four tumor xenografts generated for each GBM tumor (resulting in a total of 32 tumor xenografts) was performed, with the goal of providing an estimate of the variance across different mice and different tumors (Fig. 1). A summary of previously characterized information, including molecular features and the status of genetic mutations commonly found in GBM, for each tumor is provided in Table I (23). To confirm EGFR expression, xenograft tumors were homogenized and immunoblotting was performed using an anti-EGFR antibody (Fig. 1B). EGFR expression was quantified by densitometry, and average EGFR expression levels for the four biological replicates are displayed at the bottom panel of Fig. 1B. Based on the immunoblotting data, each tumor was subsequently classified as either wtEGFR (wt; 10 and 12), wtEGFR amplified (wtEGFR++; 8, 15, and 26), or EGFRVIII amplified (EGFRVIII+; 6, 39, and 59) (Table I). Total tyrosine phosphorylation in each of the samples was visualized by immunoblotting each of the four biological replicates of each tumor with a total phosphoryrosine antibody (4G10) (Fig. 1C). Based on these data, the biological replicates of each tumor xenograft were more similar to each other, at the total tyrosine phosphoryrosine level and EGFR expression levels, than the distinct differences across different tumor xenografts. Biological variation across the four replicates of each tumor is to be expected given that each of the tumors was obtained from a different mouse and thus had a distinct microenvironment. Although these immunoblot can provide estimates of the total protein or tyrosine phosphorylation, to gain a molecular-level view of the various xenografts we applied quantitative mass spectrometry.

Protein Expression Profiling of wtEGFR and EGFRVIII Overexpressing GBM Xenografts—To gather a broad understanding of the effects of wtEGFR and EGFRVIII overexpression, we characterized protein expression profiles for the eight GBM xenograft tumors. Protein expression was quantified through iTRAQ 8plex-based labeling of the peptides from each tumor xenograft followed by LC-MS/MS analysis using a triple- time of flight (ToF) 5600 instrument (AB Sciex) (Fig. 2). To reduce sample complexity, the iTRAQ 8plex labeled peptide samples were separated into 5 fractions ranging from pH3 to pH10 by isoelectric focusing (IEF) (Fig. 2). From these analyses, we identified and quantified a total of 1947 proteins (with each protein being identified by two or more distinct peptides) across the eight tumor xenografts. After discarding proteins that were only identified in one biological replicate, a total of 1588 proteins were identified and quantified across the eight tumor xenografts in two or more biological replicates; this set...
A. Human tumor homogenate 1:1 matrigel

30-60 days

B. Table I

| GBM tumor number | EGFR amplification | EGFR status | PTEN status | p53 Mutation | p16 Deletion |
|------------------|-------------------|-------------|-------------|--------------|--------------|
| 6                | YES, vIII         | vIII        | wt          | 273:arg>cys  | YES          |
| 8                | YES, wt           | wt          | D           | None         | YES          |
| 10               | NO                | wt          | None        | YES          |
| 12               | NO                | wt          | 5,3 Osplice | YES          |
| 15               | YES, wt           | wt          | None        | YES          |
| 26               | YES, wt           | wt          | D           | None         |
| 39               | YES, vIII         | vIII        | wt          | None         |
| 59               | YES, vIII         | vIII        | D           | None         |

Previously identified mutations shown in this table were extracted from (23). wt; Wild Type, vIII; EGF receptor Variant III, D; Deleted.

C. Fig. 1. Characterization of human GBM xenografts differentially expressing wtEGFR and EGFRvIII. A, GBM xenografts were generated from 100–200 μl of tumor homogenate, derived from primary tumors from different patients undergoing surgical treatment and serially passaged in mice before this study, mixed 1:1 with matrigel and subcutaneously injected into the flanks of nude mice. Tumors were grown for 30–60 days at which time mice were sacrificed and subcutaneous tumors were harvested. B and C, For all blots the upper panel indicates either EGFR or phosphotyrosine and the lower panel indicates the GAPDH loading control blot. B, EGFR immunoblotting across eight human GBM xenograft lines (four biological replicates). Tumor lines GBM6, 8, 10, 12, 15, 26, 39, and 59 are indicated by numbers. There are two biological replicates for GBM6. Biological replicate 1 (61) and biological replicate 4 (64) were analyzed in the place of biological replicate 2 and 3 respectively. For all analyses there are four biological replicates for the seven remaining GBM xenograft lines. wtEGFR is indicated by the higher MW band and EGFRvIII is indicated by the lower MW band. Bottom panel: immunoblot band intensities were quantified across all four biological replicates, EGFR expression was normalized to GAPDH, then normalized to GBM8. C, Total phosphotyrosine immunoblots of 8 human GBM xenograft lines (four biological replicates).

quantitative changes were apparent and the average percentage of proteins that increase (>1.25-fold) or decrease (<0.75-fold) relative to the mean across the eight GBM tumors was 10 and 12% (supplemental Table S5). To visualize the quantitative profiles for the 1588 proteins across the various tumor xenografts, affinity propagation was
used to organize the proteins into clusters, which are displayed in a heat map in Fig. 4A. The quantitative profiles of all eight GBM xenografts were distinct, with each GBM tumor displaying specific dominant clusters of proteins (Fig. 4A). This result is not necessarily surprising, given that GBM is a heterogeneous disease and the eight tumor xenografts were from different patients whose tumors display different genetic backgrounds (Table I). To characterize the types and functions of proteins that were analyzed in this study, GO annotation was performed on the 1588 proteins. The biological processes and protein classes identified from this analysis are represented in Figs. 4B and 4C, respectively. Many of the...
proteins identified were involved in metabolic processes (33.3%), cellular processes (15.2%), transport (9.6%), cell communication (12.5%), and developmental process (5.7%), indicating that we have captured a wide variety of protein functions within the cell (Fig. 4B). Furthermore, we looked at protein classes and identified that 2.8% of proteins identified were transcription factors whereas 2.4% are signaling molecules and 2.3%, 1.6%, and 1.4% are kinases, receptors, and phosphatases (Fig. 4C). The presence of these typically low expression level protein classes indicates a reasonable depth of coverage for this protein expression profiling experiment. Furthermore, these classes of proteins are critically important for transducing the effects of increased expression and/or activation of wtEGFR and EGFRvIII in these tumor xenograft samples (28).

Given the well-established role of EGFR in glioblastoma, we extracted quantitative data for 44 peptides from EGFR, spanning from amino acid 14 to 1186 (Fig. 4D). Identification of

**Fig. 4.** Proteins quantified across eight GBM xenograft lines differentially overexpressing wtEGFR and EGFRvIII. A, Heat map of the 1588 proteins quantified across eight GBM xenograft lines expressing wtEGFR, overexpressing wtEGFR (wt/EGFR+) or overexpressing EGFRvIII (EGFRvIII+/H11001+) indicated at the top of the heat map. The heat map is a representation of iTRAQ8plex fold changes normalized to the mean of all channels and Log 2 transformed. Proteins were clustered using affinity propagation to reveal groups of proteins that show similar expression profiles (see experimental procedures). B, Panther GO biological processes annotation analyses of all 1588 proteins identified and quantified. C, Panther GO protein classes’ annotation analyses of all 1588 proteins identified and quantified. D, Compilation of the 44 EGFR peptides identified and quantified across the eight GBM xenografts. The amino acid position in the full length is indicated on the x axis. The amino acid sequence that is specific to the wtEGFR is indicated alongside the amino acid sequence specific to both wtEGFR and the truncated EGFRvIII. wtEGFR (wt) tumors are shown in gray, wtEGFR overexpressing tumors (wt+/H11001+) are shown in blue, and EGFRvIII overexpressing tumors (vIII+/H11001+) are shown in red.
proteins may effectively comprise a signature for poor prognosis in glioblastoma patients.

**Phosphotyrosine Profiling of wtEGFR and EGFRvIII Overexpressing GBM Xenografts**—To identify activated signaling pathways and networks in the glioblastoma tumor xenografts and determine the effect of wtEGFR and EGFRvIII expression on these networks, iTRAQ labeled phosphotyrosine peptides were isolated by peptide IP and analyzed by reverse phase LC-MS/MS (Fig. 2). This analysis led to the identification and quantification of 225 unique tyrosine phosphorylation peptides on 168 proteins across four biological replicates (complete phosphotyrosine quantification can be found in supplemental Tables S1 and S2 and manually validated, annotated mass spectra for each of the 225 identified phosphotyrosine peptides can be found in supplemental Fig. S1). Of the identified phosphorylation sites, 100 were identified in at least two replicates, and 21 of the sites were identified in all four biological replicates (Fig. 3B) (note that these proportions are similar to those obtained from replicate analysis of similar samples) (30).

**Activated Signaling Networks in GBM Tumor Xenografts**—The quantitative phosphotyrosine profiles of all 225 phosphotyrosine sites on 168 proteins across the eight human GBM xenografts are summarized in a heat map in Fig. 6A. Despite similar EGFR expression levels, either wtEGFR or EGFRvIII, across the two groups of overexpressing tumors; distinct phosphotyrosine profiles were found within each individual GBM tumor xenograft (Fig. 6A). To identify classes of proteins quantified in this study, we carried out GO annotation of protein classes (Fig. 6B). A variety of protein classes were encapsulated in this study, including transglutaminases, kinases, receptors, phosphatases, and signaling molecules among the most predominant classes quantified (Fig. 6B).

To gain insight into the sets of tyrosine phosphorylation sites that are most highly phosphorylated in individual tumors, and to identify pathways and modules of phosphorylation sites based on their similar profiles, affinity propagation was
also used to cluster groups of phosphorylation sites that share similar quantitative profiles. Nine of the 10 resulting clusters represent profiles where the phosphotyrosine signal was significantly higher in one tumor (Fig. 6C). To identify dominant pathways and kinases responsible for propagating signal within these GBM tumors, we rank-ordered the 20 most highly phosphorylated proteins for each tumor (supplemental Table S7). From this analysis, it is clear that although GBM10 and 12 both have low-level expression of wtEGFR, they have distinctly different activated signaling networks. For instance, the phosphorylation sites that feature the greatest increase in GBM10 relative to the other xenografts were vimentin pY117, FIG. 5.

**Fig. 5**. Proteins significantly increased in EGFRvIII expressing tumors. A, Heat map of the 63 protein significantly differentially expressed in the EGFRvIII tumors when compared with the wtEGFR tumors. The heat map is a representation of iTRAQ8plex fold changes normalized to the mean of all eight channels and Log2 transformed. * indicates the proteins depicted in section C. B, GO protein class annotation for the 63 proteins differentially expressed in the EGFRvIII tumors. C, Kaplan meier survival plots of the four proteins with the most significant effect on survival across all gliomas in REMBRANDT. n refers to the number of human patients and the p value shown is associated with the survival curves for the specified up-regulated gene expression (>2 fold) compared with intermediate expression calculated with a log rank (Mantel-Cox) test. D, Kaplan meier survival curve for the simultaneous up-regulation of all five of the most significant genes shown in panel C. E, The bar charts shown are representative of quantification of immunoblotting images shown in supplemental Fig. S2. The wtEGFR, wtEGFR+, and EGFRvIII+ band intensities were averaged together and the mean value across all four biological replicates are shown in the bar charts for S100A10, MVP, CAIII, and GBP1. The standard error across four biological replicates is shown.
prune homolog 2 pY1738, neurotrophic tyrosine receptor kinase 1 (NTRK1) pY681, and phospholipase C gamma (PLC-γ) pY481. However, phosphorylation sites on multiple other kinases also appear in the 20 sites that were most strongly increased in this tumor; the combination of multiple activated kinases may be responsible for proliferation in this tumor. Furthermore, from these lists of signaling molecules it is clear that EGFR expression does not correlate with the predominant signaling in these eight tumor xenografts.

EGFR Signaling—Multiple proteins are known to interact with tyrosine phosphorylated EGFR, such as SHC (31), PLC-γ (32), STAT3 (33), Gab1 (34), and Cbl (35). Phosphorylation sites on these interacting proteins were quantified in this study; these data are displayed in Figs. 7A and 7B. Of the seven EGFR tyrosine phosphorylation sites quantified, pY1148, pY1173, and the doubly phosphorylated pY1045 and pY1068 peptide have almost identical profiles. To confirm the relative quantification of EGFR pY1045 and EGFR pY1173 we carried out validation immunoblotting (Fig. 7B). The trends seen here were the same as those identified in the iTRAQ MS data set.

Phosphorylation sites on the interacting proteins in Fig. 7A displayed significantly different profiles across the different tumors, and sometimes across the different sites on the given protein. For instance, four tyrosine phosphorylation sites were quantified on PLC-γ, each with distinct profiles across the eight tumor xenografts; it is worth noting that none of these sites correlate well with EGFR pY992, the putative binding
site for PLC-γ (36). Of the PLC-γ sites, phosphorylation of Y783 is responsible for activation of PLC-γ (37), and was relatively constant across seven of the eight tumors, with a twofold increase in GBM39 compared with the other tumors. Phosphorylation of Y481 was fourfold higher in GBM10 compared with the other tumors, even though this tumor had minimal EGFR expression. The other two sites, pY771 and pY1253, were increased in the EGFRvIII-expressing GBMs 6, 39, and 59, and in GBM15 (Fig. 7A). We carried out immunoblotting for PLC-γ and Gab1 to complement the phosphotyrosine data and saw contrasting trends between tyrosine phosphorylation levels and protein expression levels (Fig. 7B). These trends indicate that regulation is occurring at the protein level and at the signaling level, as there is altered

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**Fig. 7.** Quantification of EGFR phosphorylation sites and known downstream signaling molecules. A, Seven EGFR phosphorylation sites are depicted along with their fold change relative to the mean. Specific downstream signaling molecules SHC, PLC-γ, STAT3, Gab1, and Cbl are shown. The y axis on all graphs indicates fold changes relative to the mean, and x axis indicates the tumor in which the signal was identified and quantified. Error bars represent standard deviations. B, Validation Western blotting of phosphotyrosine sites on EGFR and directly downstream. EGFR protein expression, EGFR pY1173 and pY1045, STAT3 protein expression and STAT3 pY705, PLC-γ protein expression and Gab1 protein expression and Gab1 py627 across all four biological replicates β-tubulin is the loading control.
phosphorylation stoichiometry on each of the tumor xenografts. Phosphorylation of the transcription factor STAT3 was significantly increased on the activation site pY705 across all wtEGFR+ and EGFRvIII-expressing tumors (Fig. 7A). This increase in tyrosine phosphorylation across the wtEGFR and EGFRvIII tumors was also confirmed by immunoblotting and shows that the levels of phosphorylation are high in the EGFR overexpressing tumors despite lower protein expression levels (Fig. 7B).

Increased Signaling in EGFRvIII Overexpressing Human GBM Xenografts—To identify phosphorylation sites that are differentially regulated with wtEGFR and EGFRvIII amplification, we carried out student t-tests to compare the wtEGFR to the wtEGFR+ tumors, the wtEGFR to the EGFRvIII+, and the wtEGFR to the EGFRvIII+ tumors. Eight phosphorylation sites on seven proteins were found to be significantly (p < 0.05) differentially regulated (Fig. 8). Among these sites, SHP-2 phosphorylation on Y62 was increased specifically in EGFRvIII+ tumor xenografts and SHIP2 Y1135 phosphorylation was also significantly increased in the EGFRvIII+ tumors relative to the wtEGFR or wtEGFR+ tumors. Two sites (pY141 and pY371) on Cbl, a protein that regulates the ubiquitylation, internalization, and degradation of EGFR, were also significantly increased in the EGFRvIII+ tumors (38). Phosphorylation of Gab3 (GRB2-associated binder 3) at pY560 was significantly increased in the EGFRvIII+ tumors when compared with the wtEGFR+ tumors. Although this protein has not yet been associated with EGFR receptor internalization, its binding partner Grb2 is involved in Cbl interactions with EGFR (39). We also identified significantly increased phosphorylation of Epidermal growth factor receptor substrate 15 (Eps15) at pY849 in EGFRvIII+ cells. Eps15 (EGFR pathway substrate 15) plays an important role in clathrin-coated vesicle formation at the plasma membrane and at the Golgi in EGFR endocytosis (40). Another protein integral to the internalization and processing of EGFR is the ubiquitin binding protein Hrs (41), which also demonstrated increased phosphorylation on Y216 in EGFRvIII+ tumors when compared with wtEGFR (p = 0.05) (Fig. 8A). Beyond the phosphotyrosine data for these phosphorylation sites we carried out validation immunoblotting to identify the protein expression levels for Cbl, GEF-H1, Hrs, and SHP2 and found that despite the increased level of tyrosine phosphorylation, the protein levels were not increased in the EGFRvIII+ tumors (Fig. 8B).

DISCUSSION

To identify the mechanisms by which wtEGFR and EGFRvIII drive glioblastoma tumor progression, we have quantified protein expression profiles and tyrosine phosphorylation signaling networks across a panel of eight GBM human tumor xenografts, including three that express high levels of wtEGFR and three that express high levels of EGFRvIII. The identification of protein expression profiles and tyrosine phosphorylation has enabled us to identify multiple proteins that are correlated with the overexpression of EGFRvIII and poor survival in patients with gliomas. Furthermore, tyrosine phosphorylation profiling has allowed us to further understand differences in wtEGFR and EGFRvIII signaling. These data give strong motivation for the identification of activated downstream signaling networks in GBM tumor tissues, to supplement genomic and transcriptomic experiments that are being conducted in human tumors by the Cancer Genome Atlas (TCGA) and other groups (5). Specifically, our data indicate diverse downstream signaling networks in vivo that do not correlate with EGFR or EGFRvIII expression. Together, proteins with altered expression and phosphorylation states in EGFRvIII tumor xenografts compared with wtEGFR or wtEGFR+ tumor xenografts have provided some insight into the mechanisms by which EGFRvIII drives increased tumor progression (Fig. 9).

EGFRvIII+ expressing GBM cell lines are highly resistant to chemotherapies, although the underlying mechanism has not been established (29). Furthermore, attempts to target EGFRvIII directly have also resulted in relapse (15). Although we do not know the basis for the heterogeneity among the different tumor xenografts observed in this study, this variation might underlie the failure of targeted therapies; additional potential drivers in each tumor may lead to compensation for the loss of EGFR (in any given cell), or selective proliferation (if the drivers are in different cells), in the presence of anti-EGFR therapeutics. Despite the heterogeneity among the different tumors, there were several proteins whose expression was significantly altered in the EGFRvIII-expressing tumors relative to those that expressed either low or high levels of wtEGFR. Of particular interest are four proteins: S100A10, MVP, GBP1, and CA11, all found to be significantly correlated with poor survival in patients with gliomas. Furthermore, simultaneous up-regulation of all four proteins is associated with a further decline in patient survival in glioma patients. S100A10 belongs to a family of 20 low molecular weight calcium-binding proteins that have been implicated in cancer progression in breast (42) and bladder cancers (43), yet the functional role of the S100 family of proteins in cancer is not well understood. MVP is a multimeric vault particle (also known as lung resistance-related protein (LRP)) that consists of a telomerase-associated protein, a vault-poly (ADP-ribose) polymerase, and a small un-translated RNA. MVP has been suggested to contribute to resistance toward chemotherapeutic agents in lung cancer, as the name suggests (44, 45). MVP expression is significantly increased across malignant brain tumors when compared with nonmalignant brain tissues (46), and MVP expression correlates with EGFR inhibitor (gefitinib) resistance (45), suggesting that increased expression of MVP may contribute to therapeutic resistance in EGFRvIII expressing tumors. GBP1 is a large GTP-binding interferon-inducible protein belonging to the dynamin family (47). A recent study has identified GBP1 as a gene regulated by EGFR activation through gene expression array analysis (48). Increased GBP1
expression is correlated with increased EGFR expression in GBM and mediates MMP1 expression and cell invasion in glioma cells (48). These findings corroborate the data we present here and, because of its role in GBM invasiveness, highlight GBP1 as a potential therapeutic target in GBM. CAIII belongs to the carbonic anhydrase family of metalloenzymes that catalyze the reversible hydration of carbon dioxide for pH homeostasis (49). CAIII overexpression enhances anchorage

**Fig. 8.** Proteins that are significantly differentially tyrosine phosphorylated in EGFRvIII expressing tumors. *A.* Eight tyrosine phosphorylation sites were found to be significantly ($p < 0.05$) differentially phosphorylated in the five wtEGFR tumors relative to the three EGFRvIII tumors. Average phosphorylation levels in the wtEGFR, wtEGFR+, and EGFRvIII expressing tumors are represented. ** indicates proteins where $p < 0.01$ and * indicates $p < 0.05$. Error bars indicate standard deviations. *B.* Western blotting analyses to identify protein expression of Cbl, GEFH1, Hrs, and Shp2, and β-tubulin is the loading control across all four biological replicates.
independent growth, cell motility, and invasion in hepatocellular carcinoma cell lines through an unknown mechanism (50). Furthermore, CAIII is unique in the carbonic anhydrase family as it has phosphatase activity (with a preference for tyrosine phosphorylation) (49). These findings indicate a potential role for CAIII in EGFRvIII cancer progression, although the role of CAIII in cancers is not well characterized. In this study we identify the individual and combined prognostic value of S100A10, MVP, GBP1, and CAIII. Although the roles of the four proteins are not fully elucidated, the known roles of GBP1 in growth and invasion suggest important roles of S100A10, CAIII, and MVP in EGFRvIII driven GBM tumor biology.

Throughout the tyrosine phosphorylation analyses we identified that SHP-2 was increased in EGFRvIII GBM in agreement with the previous finding that SHP-2 is required for oncogenic transformation of EGFRvIII in vitro (51). SHIP2 was also increased in EGFRvIII data set; this protein dephosphorylates the 5' position of phosphatidylinositol 3,4,5-phosphate (PIP3) to generate phosphatidylinositol 3,4 phosphate (PIP2), and phosphorylation of this site increases phosphatase activity (52). These data suggest that PIP3-mediated activation of the Akt pathway should be decreased in the EGFRvIII+ tumor xenografts, contrary to previous results linking EGFRvIII with increased signaling through the PI3K/Akt pathway (18). Furthermore, previous publications have found that EGFRvIII has higher surface lifetime relative to wtEGFR; this long-lived signaling is speculated to enhance the tumorigenic effect of EGFRvIII (39). Reduced down-regulation of EGFRvIII may be caused by inefficient internalization caused by a lack of ubiquitinylation and impaired sorting to lysosomes, thought to be brought about by hypo-phosphorylation of Y1045 (39, 53). Here, EGFRvIII+ tumors demonstrated increased phosphorylation of Cbl Y371, which regulates E3 activity and induces conformational changes critical for EGFR ubiquitination in vitro (54). The phosphorylation of Cbl Y141 was also increased in the EGFRvIII+ cells, but the functional role of this site has not been elucidated. Increased phosphorylation on multiple proteins associated with EGFR trafficking in the EGFRvIII+ tumor xenografts indicates a potential mechanism for differential signal propagation of the mutant receptor.

More broadly, we detected increased STAT3 Y705 phosphorylation relative to EGFR phosphorylation across the wtEGFR and EGFRvIII-expressing tumor xenografts. STAT3 can specifically interact with pY1068 and/or pY1086 and can be directly phosphorylated by the receptor (55). Previous studies have highlighted a role for STAT3 in EGFRvIII expressing astrocytes, and it is thought that EGFRvIII forms a complex with STAT3, thereby enhancing its oncogenic activity through an unknown mechanism (56). We also highlight vimentin and neurotrophic receptor tyrosine kinase (NTRK1) as highly phosphorylated in tumors not overexpressing EGFR. Although vimentin is not a kinase, it is often overexpressed in cancer and is a recognized marker for the epithelial to mesenchymal transition (EMT) (57). Vimentin expression also correlates well with tumor growth, invasion, and poor prognosis.,
yet the role of pY117 phosphorylation has not been elucidated. The phosphorylation of NTRK1 was a dominant event in GBM10, and is possibly associated with the strong increase in PLC-γ Y681 phosphorylation in these tumors as PLC-γ is a well known downstream substrate of NTRK1 (58). Our results suggest a mechanism where tumor progression is because of activation of multiple pathways that act together toward proliferation and invasive migration of human GBM tumors (Fig. 9). It is interesting to see whether some of these altered proteins and their associated pathways might be shared among other constitutively active oncogenes, or across aggressive, difficult-to-treat tumor subtypes. The distinct signaling networks in each GBM tumor xenograft indicate that other factors, such as microenvironment differences or additional genetic lesions, significantly outweigh the effects of wtEGFR or EGFRvIII expression on the signaling networks in these xenografts.

The data generated in this study highlight increased phosphorylation, in different tumors, of numerous previously identified pathways known to regulate glioblastoma oncogenesis and tumor progression. Importantly, in addition to these previously characterized proteins and pathways, we have also identified increased phosphorylation of multiple proteins and phosphorylation sites that have not been previously associated with GBM progression (Fig. 9). In the near future, application of this approach to immediately frozen tumor samples should enable the identification of critical proproliferation or proinvasion signaling nodes in each tumor, allowing for personalized therapy based on direct detection of signaling networks, rather than implied signaling network alterations based on genomic alterations. Through comparison of human tumor specimens and their associated tumor xenografts, it will be possible to not only identify key therapeutic targets in each tumor, but also to develop optimal therapeutic strategies in mice before therapeutic intervention in human patients.

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