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EGFRvIII-Stat5 Signaling Enhances Glioblastoma Cell Migration and Survival

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Abstract

Glioblastoma multiforme (GBM) is the most common brain malignancies in adults. Most GBM patients succumb to the disease less than one year post-diagnosis due to the highly invasive nature of the tumor, which prevents complete surgical resection and gives rise to tumor recurrence. The invasive phenotype also confers radio- and chemo-resistant properties to the tumor cells; therefore, there is a critical need to develop new therapeutics that target drivers of GBM invasion. Amplification of EGFR is observed in over 50% of GBM tumors, of which half concurrently overexpress the variant EGFRvIII, and expression of both receptors confers a worse prognosis. EGFR and EGFRvIII cooperate to promote tumor progression and invasion, in part, through activation of the Stat signaling pathway. Here it is reported that EGFRvIII activates Stat5 and GBM invasion by inducing the expression of a previously established mediator of glioma cell invasion and survival: fibroblast growth factor-inducible 14 (Fn14). EGFRvIII-mediated induction of Fn14 expression is Stat5-dependent and requires activation of Src, whereas EGFR regulation of Fn14 is dependent upon Src-MEK/ERK-Stat3 activation. Notably, treatment of EGFRvIII-expressing GBM cells with the FDA-approved Stat5 inhibitor pimozone blocked Stat5 phosphorylation, Fn14 expression, and cell migration and survival. Since EGFR inhibitors display limited therapeutic efficacy in GBM patients, the EGFRvIII-Stat5-Fn14 signaling pathway represents a node of vulnerability in the invasive GBM cell populations.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Keywords

Glioblastoma; EGFR; Stat5; Fn14; Invasion

Introduction

GBM is the most common malignant brain tumor in adults(1). Until the recent survival benefits afforded by tumor treating fields, the treatment regimen and the overall survival had remained unaltered for nearly three decades(2,3). GBM is characterized by a high degree of tumor heterogeneity and aggressive infiltration into the surrounding brain parenchyma, which contribute to the clinical evasiveness of this tumor(4). Since cell invasion is a universal property of GBM, studies that focus on the development of therapies targeting this cell population are greatly needed in order to significantly improve the survival of GBM patients.

Genomic and epigenomic interrogation of GBM tumors has identified frequent alterations in receptor tyrosine kinase, p53, and retinoblastoma signaling pathways(5,6). One key genetic alteration seen in about half of GBM patients is amplification or overexpression of the epidermal growth factor receptor (*EGFR*) gene, which is frequently accompanied by various *EGFR* mutations(6). In 30% of cases with *EGFR* amplification/overexpression, deletions of exons 2–7 results in expression of the mutant isoform EGFRvIII, which has an in-frame deletion of 801 base-pairs in the extracellular domain(7). This deletion renders the mutant receptor insensitive to EGF stimulation and lysosomal degradation, which results in constitutive downstream signaling(8–10). Expression of EGFRvIII confers a tumorigenic phenotype and correlates with poor clinical prognosis in GBM patients(7,9,11–14). Compared to EGF-stimulated EGFR, EGFRvIII signals at a lower amplitude and utilizes unique signaling components(15). EGFRvIII initiates a pleiotrophic phospho-cascade, including the activation of the Src family of kinases, the mitogen-activated protein kinase (MAPK) pathway, and signal transducer and activator of transcription (Stat) transcription factors(9,13,16–19). Stats can be activated by both receptor and non-receptor tyrosine kinases, and Stat activation in response to EGF is potentiated by Src(20). The Stat family consists of seven members that are activated by growth factors and cytokines, but only Stat1, Stat3, Stat5a, and Stat5b have been implicated in tumorigenesis(21). Stat transcription factors drive the expression of multiple EGFR and EGFRvIII target genes(13,16,18,21). EGFRvIII participates in a feed-forward loop with the cytokine receptor oncostatin M (OSMR) to activate Stat3(22). Moreover, EGFRvIII activates Stat3 and Stat5 to drive pro-tumorigenic phenotypes in GBM cells and Stat3 small molecule inhibitors reduced target gene expression in EGFR-driven NSCLC(16,23,24). Phosphorylation of Stat5 correlates with EGFR expression, cell invasion, and poor prognosis in GBM(13,25). Due to its tumor specific expression, EGFRvIII is an attractive therapeutic target. However, tyrosine kinase inhibitors that have clinical efficacy in non-CNS solid tumors expressing activating EGFR mutations are ineffective in the treatment of EGFRvIII expressing GBM(26–30). Thus, novel therapeutics targeting EGFR and/or the EGFR intracellular signaling pathway are being investigated(30).

In this study, we examined the signaling mechanism by which EGFR and EGFRvIII drive GBM invasion and survival. We show that Stat5 is active in the invasive population of GBM cells *in situ* and induces Fn14 expression to induce cell invasion and survival. We demonstrate that EGFRvIII-induced Fn14 expression is dependent upon Stat5 and requires Src activation, whereas EGFR regulation of Fn14 is dependent upon MEK/ERK-Stat3 activation. Ablating the expression of Stat5 or Fn14 enhances chemosensitivity and reduces invasion in GBM cells. Notably, treatment of EGFRvIII- expressing GBM cells with pimozone, a reported Stat5 inhibitor, blocks Stat5 phosphorylation and Fn14 expression downstream of EGFRvIII signaling and positions Stat5 as a therapeutic target for treatment of invasive GBM cells.

Materials and Methods

Expression Profile Dataset of Stat3 and Stat5 Target Signature Genes in Human Gliomas

The expression microarray database of laser capture microdissected GBM cells collected from 19 paired patient GBM tumor core and invading rim (GES12689) regions was previously described (33). Gene expression differences were deemed statistically significant using parametric tests where variances were not assumed equal (Welch ANOVA). Supervised clustering heatmaps were generated using R *ggplot2* package and row z-score transformation was performed prior to the clustering.

Antibodies and reagents

Phospho-EGFR (3777, 2231), EGFR (4267), phospho-Src (6943), Src (2109), phospho-p44/42 (4370), p44/42 (9102), phospho-Stat3 (9145), Stat3 (4904), phospho-Stat5 (4322), Stat5 (9363), Fn14 (4403), Cleaved Caspase 3 (9661), γ H2AX (9718), HA (2367), and GAPDH (2118) were from Cell Signaling Technology. Antibodies to α -tubulin and β -actin were from Millipore.

Human recombinant EGF was purchased from PeproTech. Temozolomide and Pimozide (P1793) were obtained from Sigma. U0126 (9903) was purchased from Cell Signaling Technology. Erlotinib (S7786), Gefitinib (S1025), and Saracatinib (S1006) were purchased from Selleck Chem.

Cell culture

The U373 WT, EGFRvIII, and EGFRvIII KD human GBM cell lines were a kind gift from Dr. Frank Furnari (UCSD) and were passaged in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Tet-free FBS (Clontech)(11). When indicated, cells were serum starved by replacing the culture medium with DMEM supplemented with 0.1% bovine serum albumin (BSA). For doxycycline treatment, cells were maintained in serum starvation media with doxycycline (1mg/mL) for the indicated times. The primary GBM PDX lines 8, 12, 39, and 59 were established from a patient surgical sample and maintained as a flank xenograft in immunodeficient mice(53,54). GBM 8, 12, 39, and 59 flank tumors were resected, brought to single cell suspension via mechanical dissociation, and maintained in neurosphere media (DMEM/F12 supplemented with B-27, N-2, EGF, and FGF).

Transfection and small interfering RNA

The siRNA specific for Fn14 (siRNA #4; CGC CCA CTC ATC ATT CAT TCA) was purchased from Qiagen. siRNAs specific for Stat3, Stat5a, and Stat5b are as followed: [Stat3, GCA CCU UCC UGC UAA GAU Utt (Ambion); Stat3-7, (CAG CCT CTC TGC AGA ATT CAA (Qiagen); Stat3-8, CAG GCT GGT AAT TTA TAT AAT (Qiagen); Stat5a, GCG CUU UAG UGA CUC AGA Att (Ambion); Stat5a-2, AGC GGT CGT GTT GTG AGT TTA (Qiagen); Stat5a-3, AAC CTT GTC GAC AAA GAG GTA (Qiagen); Stat5b, CCU UCA UCA GAU GCA AGC GUU AUA U (Invitrogen); Stat5b-2, CCG AGC GAG ATT GTA AAC CAT (Qiagen); Stat5b-3, CCG CTT GGG AGA CTT GAA TTA (Qiagen)]. Transient transfection of siRNA (10nM) was performed using Lipofectamine RNAiMax following the manufacturer's protocol.

Expression constructs

A bacterial plasmid containing the coding sequence of human STAT5A (Clone ID: HsCD00043806) was obtained from the DNASU plasmid repository (55). The coding sequence was amplified by PCR and subcloned into pcDNA3 in frame with a C-terminal 3× HA epitope. A constitutively active STAT5A (STAT CA) containing the point mutation N642H (56) was generated using the QuikChange II Site-Directed Mutagenesis Kit (Agilent). A 3× HA epitope-tagged dominant negative variant of STAT5 (STAT DN) was generated by truncation of STAT5A after Y683 (57). All alterations were confirmed by DNA sequencing.

Western blot analysis

Immunoblot analysis and protein determination experiments were performed as previously described(58). Briefly, monolayers of cells were washed in phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonylfluoride and 1 mM sodium orthovanadate and then lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations were determined using the BCA Assay (Pierce). Forty micrograms of total protein was loaded per lane and separated by SDS-PAGE. After transfer, the nitrocellulose membrane (Invitrogen) was blocked with either 5% nonfat-milk or 5% BSA in TBST before addition of primary antibodies and followed with peroxidase-conjugated secondary antibody (Promega). Protein bands were detected using SuperSignal Chemiluminescent Substrate (Pierce) with a UVP BioSpectrum 500 Imaging System.

Colony formation assay

A clonogenic assay was used to assess cell survival after radiation and TMZ treatment as described previously (59). Cells (5.0×10^5) were seeded in 100-mm diameter culture dishes and incubated overnight at 37 °C. For pimozone studies, cells were pretreated with 10uM pimozone for one hour. Subsequently, cells were either treated with 25 μM TMZ for 24 hours or exposed to 2 Gy radiation dose using a RS 2000 X-ray irradiator. Following treatment, cells were trypsinized, counted, and plated in a 6-well culture dish at densities of 500 cells per well in triplicate. Cells were incubated for 14 days and then fixed, stained with 0.5 % crystal violet solution, and counted manually by blinded observers.

Transwell migration and invasion assays

Glioma cells were seeded in 100-mm diameter culture dishes and incubated overnight at 37 °C. Subsequently, cells were serum starved for 16 h at 37 °C. For pimozone studies, cells were pretreated with pimozone for one hour. Cells were then harvested and added in triplicate to collagen (Advanced BioMatrix)-coated transwell chambers (migration) or matrigel (Corning)-coated transwell chambers (invasion) according to manufacturer's protocols and allowed to migrate in presence of 10% FBS. After incubation for 4 hours at 37 °C, non-migrated cells were scrapped off the upper side of the membrane and cells migrated to the other side of the membrane were fixed with 4% paraformaldehyde (PFA) (Affymetrix) and stained with DAPI (Invitrogen). Nuclei of migrated cells were counted in five high power fields (HPF) with a 10× objective. Data represents the average of triplicate transwells.

RNA isolation and quantitative reverse transcriptase-PCR

Total RNA was isolated using the Qiagen RNeasy kit. cDNA was synthesized from total RNA in a 20 µL reaction volume using the SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen) for 50 minutes at 50°C, followed by 85°C for 5 minutes. qPCR analysis was performed with primers specific for: Fn14 (sense 5'-CCA AGC TCC TCC AAC CAC AA-3; anti-sense 5'-TGG GGC CTA GTG TCA AGT CT-3), Stat3 (sense 5'-CAG CAG CTT GAC ACA CGG TA-3; anti-sense 5'-AAA CAC CAA AGT GGC ATG TGA-3), GAPDH (sense 5'-CTG CAC CAC CAA CTG CTT AG-3; anti-sense 5'-GTC TTC TGG GTG GCA GTG AT), and histone H3.3 (sense: 5'- CCA CTG AAC TTC TGA TTC GC-3'; antisense: 5'-GCG TGC TAG CTG GAT GTC TT-3'). qPCR primers for Stat5a and Stat5b were purchased from Qiagen. mRNA levels were quantified using SYBR green (Roche) fluorescence for detection of amplification after each cycle with the Quantstudio 6. The relative mRNA expression was calculated with the 2^{-CT} method.

Immunohistochemistry

A glioma invasion tumor microarray (TMA) containing representative punches of tumor core, edge, and invasive rim from 44 clinically annotated cases of WHO grade IV GBM specimens from 10 institutes was previously described(60). Five-micrometer thick sections from the TMA were processed for immunohistochemistry (IHC) staining. IHC staining for Stat5 (ab32043, Abcam, Cambridge, MA) and Phospho-Stat3 (#9145, Cell Signaling Technology, Boston, MA) was performed using the Leica Bond™ RXm automated IHC stainer (Leica Biosystems, Buffalo Grove, IL) Antigen retrieval was performed using Bond™ Epitope Retrieval 2 and developed using the Bond™ Refine Detection system (Leica Biosystems Buffalo Grove, IL). Stained slides were cleared and coverslipped using routine procedures.

Statistics

For IHC staining, statistical analysis was performed using the Fisher's exact test. For the migration and invasion assay, significance was measured by Student's t-test. P-values <0.05 were considered significant. All tests were performed using GraphPad Prism (LaJolla, CA) or R3.2.2 (R Foundation for Statistical Computing; Vienna, Austria) software.

Results

Stat5 is activated in GBM invasive rim cells

Proliferation and invasion are mutually exclusive processes in GBM and cells in the proliferative core have a distinct transcriptional profile compared to cells in the invasive rim(31,32). Stat transcription factors have been implicated in the pathogenesis of GBM, but their role in mediating proliferation or invasion has yet to be fully established(25). Using gene signatures specific to Stat3 and Stat5, we investigated which Stat family members were activated in the GBM core and rim cells based on their ability to regulate differentially expressed genes. We noticed that the Stat5 signature was expressed higher in the rim, while expression of the Stat3 signature was higher in the proliferative core (Figure 1A). This data suggests a Stat3/5 signaling dichotomy that may dictate GBM cell proliferation versus invasion. We next assessed the clinical relevance of differential intratumoral localization of the Stat transcription factors by measuring levels of activated Stat3 and Stat5 on a GBM invasion TMA(33). Detection of Stat3 activation was performed using a phospho-specific Stat3 antibody, whereas detection of Stat5 activation was assessed by examination of Stat5 nuclear localization. We found that activated Stat3 was significantly elevated in the tumor core compared to the rim whereas activated Stat5 had the opposite distribution (Figure 1B).

EGFRvIII-induced glioma cell invasion and survival is dependent upon Stat5

Expression of EGFRvIII confers poor prognosis and enhances invasion in GBM and EGFR and EGFRvIII activate Stat3 and Stat5 in GBM(14,16). We utilized immunoblot analysis to probe for Stat activation in EGFR- or EGFRvIII-expressing GBM PDX tumor tissue and GBM cells (Figure 2A, Supplemental Figure 1A). We observed that Stat3 and Stat5 phosphorylation was enhanced in EGFRvIII-expressing GBM PDX tumors compared to EGFR expressing samples (Figure 2A). To investigate if EGFRvIII is necessary for sustained Stat activation, we utilized the U373 cell line expressing a doxycycline-regulated EGFRvIII protein(11). The addition of doxycycline repressed the expression of EGFRvIII and significantly decreased Stat phosphorylation (Figure 2A). Since we observed higher Stat5 activation in the GBM rim cells, we next investigated the role of Stat5 in the regulation of GBM migration. We tested three different siRNAs targeting each of the Stat isoforms and chose the siRNAs displaying the highest specific mRNA depletion for functional studies (Supplementary Figure 2A). U373 EGFRvIII cells were transfected with a non-targeting siRNA or siRNAs targeting Stat5a or Stat5b for 24 hours, serum starved, and then plated for transwell migration assays. Knockdown of Stat5 mRNA was confirmed by qPCR analysis (Supplemental Figure 2A). We observed a significant decrease in migration in Stat5-depleted cells (Figure 2B). Additionally, expression of a Stat5 dominant negative vector significantly decreased cell migration (Figure 2B). Pimozide is a FDA-approved drug that is used for the treatment of neurologic syndromes, including Tourette syndrome(34) and has been shown to target Stat5 activity(34). To test if pharmacological inhibition of Stat5 mitigates GBM migration, we pretreated U373 EGFRvIII cells and GBM39 PDX neurospheres with pimozide and then performed a transwell migration assay. Treatment with pimozide decreased Stat5 activation in EGFRvIII-expressing glioma cells. In addition, pimozide treatment suppressed cell migration in U373 EGFRvIII and GBM39 cells (Figure 2C). Since migratory GBM cells are also chemoresistant(35), we tested if pimozide would

sensitize GBM cells to TMZ. We pretreated U373 EGFRvIII cells with pimozone and then treated the cells with TMZ. We noticed that pimozone sensitized the cells to TMZ and decreased cell survival (Figure 2D). Pimozone decreased cell survival, in part, through sensitizing cells to TMZ-induced apoptosis, which is demonstrated by enhanced markers of apoptosis including cleaved caspase 3 and γ H2A.X (Figure 2E). These data demonstrate that inhibiting Stat5 decreases cell migration and sensitizes GBM cells to chemotherapy.

Stat5 mediates migration, in part, through up-regulating Fn14 gene expression

Through gene expression analysis on GBM patient tumors harboring a wide set of genetic aberrations, we have reported that expression of the fibroblast growth factor-inducible 14 (Fn14) protein, a member of the TNFR superfamily, is low in normal brain tissue but is highly expressed by infiltrating glioma cells(36). Increased Fn14-mediated signaling increases GBM cell migration/invasion and survival *in vitro* while knockdown of Fn14 expression increases sensitivity to TMZ in an intracranial xenograft model, which substantiates its potential as a target to inhibit GBM cell invasion and decrease therapeutic resistance(36,37). Using MatInspector and TRANSFAC 7.0 databases, we identified a couple of putative Stat5 binding sites in the Fn14 gene promoter region (Chr16; position: 3023089-3023099 and 3078111-3078135), and it has been reported that Fn14 is a downstream target of Stat3 during tissue wound repair(23). Therefore, we investigated Stat-dependent regulation of Fn14 in GBM PDX tissue and cell lines. Since Stats are constitutively activated by EGFRvIII (Figure 2A), we first compared Fn14 expression in EGFR- or EGFRvIII-expressing GBM cells and PDX tissue. U373 cells display a low basal level of Fn14 expression that is robustly induced after approximately 4 hours of EGF-stimulation (Figure 3A). Conversely, U373 EGFRvIII cells express high basal levels of Fn14 that is not influenced by EGF treatment (Figure 3A). We validated this data in PDXs expressing either EGFR WT (GBM8 and GBM12) or EGFRvIII (GBM39 and GBM59) (Figure 3A). The correlation between activated Stat transcription factors and expression of Fn14 in EGFRvIII-expressing cell lines and GBM PDX tumors implicate Stats as potential regulators of Fn14 expression. To investigate the role of specific Stat transcription factors in the regulation of Fn14 expression, we transfected U373 EGFRvIII cells with a non-targeting siRNA or siRNAs targeting Stat3, Stat5a, or Stat5b for 48 hours, and then isolated total protein and RNA. Knockdown of Stat mRNA by siRNA was confirmed by qPCR (Supplemental Figure 2A). While we did not observe a significant decrease in Fn14 mRNA or protein upon knockdown or inhibition of Stat3, we noticed a significant decrease in Fn14 mRNA and protein in cells with Stat5 depleted, in particular, with Stat5a depletion (Figure 3B, Supplemental Figure 1B). Likewise, expression of dominant negative Stat5 repressed Fn14 expression (Figure 3B). Treatment of U373 EGFRvIII cells with pimozone decreased the phosphorylation of Stat5 and Fn14 expression (Figure 3C). In EGF-stimulated, EGFR-expressing cells, we noticed that depletion of Stat3 or Stat5 both reduced Fn14 expression (Figure 3D, Supplemental Figure 1B). Expression of a constitutive active Stat5 was not sufficient to induce the expression of Fn14, which suggests both Stat3 and Stat5 are required for Fn14 expression (Figure 3D). These data establish a role for Stat5 in EGFR-upregulation of Fn14 and reveal a dichotomy in transcription factor utilization between EGFR and EGFRvIII in GBM.

EGFRvIII activates Stat5 in a Src-dependent manner

EGFRvIII can activate Stat transcription factors directly or indirectly(13,19,38). We investigated if the kinase activity of EGFRvIII was necessary for activation of Stat5 and Fn14 up-regulation using two small molecule inhibitors of EGFR tyrosine kinase activity: erlotinib and gefitinib. We serum starved U373 EGFRvIII cells in the presence of the erlotinib or gefitinib for 24 hours and then isolated protein and RNA. We observed a decrease in the phosphorylation of Stat5 and expression level of Fn14 in the cells treated with the EGFR inhibitors compared to untreated controls (Figure 4A, Supplemental Figure 1C). We also cultured GBM12 and GBM39 neurospheres in the presence of erlotinib or gefitinib for 24 hours and then isolated protein and RNA. We observed a decrease in Fn14 protein expression and activated Stat5 in the neurospheres treated with the EGFR inhibitors compared to untreated controls (Figure 4A, Supplemental Figure 1C). These data establish a role for the kinase activity of EGFR in Stat5 activation and Fn14 expression in GBM cells. EGFR signaling induces Src family kinase (SFK) and mitogen-activated protein kinase (MAPK) pathways to activate Stats(21,39). SFKs are known activators of Stats and mediate EGFRvIII-driven invasion in GBM(40). In response to activation of EGFR, Src phosphorylates Stats at a unique site, tyrosine 694(21). Therefore, we tested whether inhibiting Src would block EGFR/Stat-dependent Fn14 expression. We treated U373 and U373 EGFRvIII cells with the SFK inhibitor saracatinib and noticed a decrease in activated Stat5 and the Fn14 protein expression level (Figure 4B). These data reveal that Src is an important effector of EGFR/Stat5-dependent activation of Fn14 gene expression in GBM.

We next investigated the role of MAPK signaling in EGFRvIII/Stat5 regulation of Fn14 levels by treating U373 and U373 EGFRvIII cells as well as GBM39 and GBM12 neurospheres with the MEK inhibitor U0126. We did not observe a significant decrease in Fn14 expression or Stat5 activation after MEK inhibition in EGFRvIII-expressing U373 or GBM39 cells (Figure 4C). However, U0126 treatment of EGFR-expressing U373 cells or GBM12 neurospheres resulted in a decrease in Fn14 protein expression (Figure 4C). Taken together, these data demonstrate that EGFRvIII-mediated induction of Fn14 expression is dependent upon Stat5 and requires activation of Src, whereas EGFR regulation of Fn14 expression is dependent upon MEK/ERK-Stat3 activation.

Fn14 depletion reduces EGFR-and EGFRvIII-mediated U373 cell migratory capacity

We have previously shown that Fn14 expression and signaling confers invasive and chemoresistance properties to GBM cells (41–43). Here, we assessed if reducing the expression of Fn14 would inhibit the chemoresistant and invasive properties conferred by the expression of oncogenic EGFRvIII. We generated stable EGFRvIII cell lines expressing a non-targeting control (ctl shRNA) or shRNA targeting Fn14 (shFn14) and assayed for migratory properties using a Transwell assay. We observed a significant decrease in migration in the shFn14 cells (Figure 5A). Fn14 also regulated EGF-induced cell migration in U373 cells (Figure 5B). Notably, EGFRvIII-expressing U373 cells showed increased invasion as compared to U373 cells, and depletion of Fn14 expression by siRNA suppressed both EGF- and EGFRvIII-mediated cell invasion (Figure 5C). Moreover, when compared to U373 EGFRvIII cells expressing a control shRNA, expressing cells, shFn14-expressing cells were more sensitive to both TMZ and radiation therapy (Figure 5D), as displayed by a

significant decrease in survival. These data implicate a role for Fn14 in the pro-tumorigenic properties conferred by EGFRvIII-Src-Stat5 signaling (Figure 6).

Discussion

Transcriptome profiling of tumors has uncovered therapeutic targets for the treatment of patients with GBM. Transcription factors act as the central node between cues from the extracellular and intracellular environment and gene expression changes. Targeting master regulators of gene expression is an attractive approach to control the prevalent heterogeneity in GBM. We previously demonstrated that transcriptional regulation is distinct in invasive cells in comparison to cells in the proliferative core(31). Here, we investigated the activity of Stat transcription factors in GBM clinical samples, specifically Stat3 and Stat5, and their role in migration. We show distinct regional Stat transcriptional signatures exist in GBM, with Stat5 being more active in the rim and Stat3 more active in the core. Stat3 has long been identified as a putative target for GBM and preclinical studies have tested small molecule inhibition of Stat3 as a therapeutic strategy(44,45). Based on our data, inhibiting Stat3 would affect the biology of the tumor core, while Stat5 inhibition would limit local invasion and render the GBM cells sensitive to standard of care. Since local invasion limits complete clinical control of this deadly disease, Stat5 inhibitors could significantly improve patient survival.

The regional differences in Stat activation could be attributed to local micro-environmental differences. Rapid proliferation in the tumor core results in low vascularity, which creates a hypoxic environment and a high degree of necrosis(46). In other solid tumors, including breast and ovarian cancer, hypoxia activates Stat3 and confers chemoresistant properties(47,48). Thus, the hypoxic environment in the tumor core may maintain Stat3 activity. Once GBM cells migrate from the tumor core into the normal brain, the cells encounter multiple normal brain, vascular cells, and immune cells, including the resident brain immune cells, microglia(49). Microglia secrete growth factors, cytokines, and chemokines that are known facilitators of GBM invasion(50). Thus, further investigations into microenvironmental stimuli that activate Stat3 and Stat5 are warranted to understand driving factors of this unique transcriptional dichotomy.

Mutations resulting in amplified or constitutively active EGFR are frequently identified in NSCLC and GBM. While treatment with TKIs enhances progression-free survival in patients with EGFR-driven NSCLC, targeting GBM cells with active EGFR has failed clinically(27,30,51,52). Another novel observation in this study is the differential pathway utilization between EGFRwt and EGFRvIII, which may complicate therapeutic control of tumors expressing both EGFR isoforms. Our data shows that EGFRvIII preferentially activates the Src-Stat5 pathway, while EGFR signals through the MEK-Stat3 pathway. Analysis of the Fn14 promoter reveals a Stat5a consensus site, but not a Stat3 consensus site. Thus, a Stat5 homodimer may regulate Fn14 in the EGFRvIII background, while a Stat3/Stat5 heterodimer may regulate Fn14 downstream of EGF-EGFR. Future investigations will address this interesting question.

In conclusion, our study is the first to document the regional activation of Stat3 and Stat5 in GBM tumors, with Stat5 being highly active in cells in the invasive rim. We demonstrate that Stat5 drives cell migration and chemotherapeutic resistance, in part, through up-regulation of Fn14 gene expression. Finally, we uncovered a novel pathway bifurcation between EGFRwt and EGFRvIII, where EGFRwt signals through the MAPK-Stat3 pathway and EGFRvIII preferentially signals through the Src-Stat5 pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

GBM	Glioblastoma multiforme
Fn14	fibroblast growth factor-inducible 14
NF-κB	the nuclear factor kappa B
EGFR	the epidermal growth factor receptor
Stat	signal transducer and activator of transcription

References

1. Ostrom QT, Gittleman H, Fulop J, Liu M, Blanda R, Kromer C, et al. CBTRUS Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2008–2012. *Neuro Oncol.* 2015; 17(Suppl 4):iv1–iv62. DOI: 10.1093/neuonc/nov189 [PubMed: 26511214]
2. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med.* 2005; 352(10):987–96. DOI: 10.1056/NEJMoa043330 [PubMed: 15758009]
3. Stupp R, Taillibert S, Kanner AA, Kesari S, Steinberg DM, Toms SA, et al. Maintenance Therapy With Tumor-Treating Fields Plus Temozolomide vs Temozolomide Alone for Glioblastoma: A Randomized Clinical Trial. *JAMA.* 2015; 314(23):2535–43. DOI: 10.1001/jama.2015.16669 [PubMed: 26670971]
4. Xie Q, Mittal S, Berens ME. Targeting adaptive glioblastoma: an overview of proliferation and invasion. *Neuro Oncol.* 2014; 16(12):1575–84. DOI: 10.1093/neuonc/nou147 [PubMed: 25082799]
5. Brennan CW, Verhaak RG, McKenna A, Campos B, Nounshmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. *Cell.* 2013; 155(2):462–77. DOI: 10.1016/j.cell.2013.09.034 [PubMed: 24120142]
6. Cancer Genome Atlas Research N. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* 2008; 455(7216):1061–8. DOI: 10.1038/nature07385 [PubMed: 18772890]
7. Nishikawa R, Ji XD, Harmon RC, Lazar CS, Gill GN, Cavenee WK, et al. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc Natl Acad Sci U S A.* 1994; 91(16):7727–31. [PubMed: 8052651]

8. Grandal MV, Zandi R, Pedersen MW, Willumsen BM, van Deurs B, Poulsen HS. EGFRvIII escapes down-regulation due to impaired internalization and sorting to lysosomes. *Carcinogenesis*. 2007; 28(7):1408–17. DOI: 10.1093/carcin/bgm058 [PubMed: 17372273]
9. Huang HS, Nagane M, Klingbeil CK, Lin H, Nishikawa R, Ji XD, et al. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J Biol Chem*. 1997; 272(5):2927–35. [PubMed: 9006938]
10. Batra SK, Castellino-Prabhu S, Wikstrand CJ, Zhu X, Humphrey PA, Friedman HS, et al. Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene. *Cell Growth Differ*. 1995; 6(10):1251–9. [PubMed: 8845302]
11. Mukasa A, Wykosky J, Ligon KL, Chin L, Cavenee WK, Furnari F. Mutant EGFR is required for maintenance of glioma growth in vivo, and its ablation leads to escape from receptor dependence. *Proc Natl Acad Sci U S A*. 2010; 107(6):2616–21. DOI: 10.1073/pnas.0914356107 [PubMed: 20133782]
12. Schmidt MH, Furnari FB, Cavenee WK, Bogler O. Epidermal growth factor receptor signaling intensity determines intracellular protein interactions, ubiquitination, and internalization. *Proc Natl Acad Sci U S A*. 2003; 100(11):6505–10. DOI: 10.1073/pnas.1031790100 [PubMed: 12734385]
13. Latha K, Li M, Chumbalkar V, Gururaj A, Hwang Y, Dakeng S, et al. Nuclear EGFRvIII-STAT5b complex contributes to glioblastoma cell survival by direct activation of the Bcl-XL promoter. *Int J Cancer*. 2013; 132(3):509–20. DOI: 10.1002/ijc.27690 [PubMed: 22729867]
14. Keller S, Schmidt MHH. EGFR and EGFRvIII Promote Angiogenesis and Cell Invasion in Glioblastoma: Combination Therapies for an Effective Treatment. *Int J Mol Sci*. 2017; 18(6)doi: 10.3390/ijms18061295
15. Huang PH, Xu AM, White FM. Oncogenic EGFR signaling networks in glioma. *Sci Signal*. 2009; 2(87):re6.doi: 10.1126/scisignal.287re6 [PubMed: 19738203]
16. Fan QW, Cheng CK, Gustafson WC, Charron E, Zipper P, Wong RA, et al. EGFR phosphorylates tumor-derived EGFRvIII driving STAT3/5 and progression in glioblastoma. *Cancer Cell*. 2013; 24(4):438–49. DOI: 10.1016/j.ccr.2013.09.004 [PubMed: 24135280]
17. Huang PH, Mukasa A, Bonavia R, Flynn RA, Brewer ZE, Cavenee WK, et al. Quantitative analysis of EGFRvIII cellular signaling networks reveals a combinatorial therapeutic strategy for glioblastoma. *Proc Natl Acad Sci U S A*. 2007; 104(31):12867–72. DOI: 10.1073/pnas.0705158104 [PubMed: 17646646]
18. Hung LY, Tseng JT, Lee YC, Xia W, Wang YN, Wu ML, et al. Nuclear epidermal growth factor receptor (EGFR) interacts with signal transducer and activator of transcription 5 (STAT5) in activating Aurora-A gene expression. *Nucleic Acids Res*. 2008; 36(13):4337–51. DOI: 10.1093/nar/gkn417 [PubMed: 18586824]
19. Zheng Q, Han L, Dong Y, Tian J, Huang W, Liu Z, et al. JAK2/STAT3 targeted therapy suppresses tumor invasion via disruption of the EGFRvIII/JAK2/STAT3 axis and associated focal adhesion in EGFRvIII-expressing glioblastoma. *Neuro Oncol*. 2014; 16(9):1229–43. DOI: 10.1093/neuonc/nou046 [PubMed: 24861878]
20. Olayioye MA, Beuvink I, Horsch K, Daly JM, Hynes NE. ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. *J Biol Chem*. 1999; 274(24):17209–18. [PubMed: 10358079]
21. Quesnelle KM, Boehm AL, Grandis JR. STAT-mediated EGFR signaling in cancer. *J Cell Biochem*. 2007; 102(2):311–9. DOI: 10.1002/jcb.21475 [PubMed: 17661350]
22. Jahani-Asl A, Yin H, Soleimani VD, Haque T, Luchman HA, Chang NC, et al. Control of glioblastoma tumorigenesis by feed-forward cytokine signaling. *Nat Neurosci*. 2016; 19(6):798–806. DOI: 10.1038/nn.4295 [PubMed: 27110918]
23. Dauer DJ, Ferraro B, Song L, Yu B, Mora L, Buettner R, et al. Stat3 regulates genes common to both wound healing and cancer. *Oncogene*. 2005; 24(21):3397–408. DOI: 10.1038/sj.onc.1208469 [PubMed: 15735721]

24. Cheng E, Whitsett TG, Tran NL, Winkles JA. The TWEAK Receptor Fn14 Is an Src-Inducible Protein and a Positive Regulator of Src-Driven Cell Invasion. *Mol Cancer Res.* 2015; 13(3):575–83. DOI: 10.1158/1541-7786.MCR-14-0411 [PubMed: 25392346]
25. Cao S, Wang C, Zheng Q, Qiao Y, Xu K, Jiang T, et al. STAT5 regulates glioma cell invasion by pathways dependent and independent of STAT5 DNA binding. *Neurosci Lett.* 2011; 487(2):228–33. DOI: 10.1016/j.neulet.2010.10.028 [PubMed: 20969921]
26. Heimberger AB, Learn CA, Archer GE, McLendon RE, Chewning TA, Tuck FL, et al. Brain tumors in mice are susceptible to blockade of epidermal growth factor receptor (EGFR) with the oral, specific, EGFR-tyrosine kinase inhibitor ZD1839 (iressa). *Clin Cancer Res.* 2002; 8(11):3496–502. [PubMed: 12429640]
27. Gazdar AF. Activating and resistance mutations of EGFR in non-small-cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors. *Oncogene.* 2009; 28(Suppl 1):S24–31. DOI: 10.1038/onc.2009.198 [PubMed: 19680293]
28. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med.* 2004; 350(21):2129–39. DOI: 10.1056/NEJMoa040938 [PubMed: 15118073]
29. Learn CA, Hartzell TL, Wikstrand CJ, Archer GE, Rich JN, Friedman AH, et al. Resistance to tyrosine kinase inhibition by mutant epidermal growth factor receptor variant III contributes to the neoplastic phenotype of glioblastoma multiforme. *Clin Cancer Res.* 2004; 10(9):3216–24. [PubMed: 15131063]
30. Thorne AH, Zanca C, Furnari F. Epidermal growth factor receptor targeting and challenges in glioblastoma. *Neuro Oncol.* 2016; 18(7):914–8. DOI: 10.1093/neuonc/nov319 [PubMed: 26755074]
31. Dhruv HD, McDonough Winslow WS, Armstrong B, Tuncali S, Eschbacher J, Kislin K, et al. Reciprocal activation of transcription factors underlies the dichotomy between proliferation and invasion of glioma cells. *PLoS One.* 2013; 8(8):e72134.doi: 10.1371/journal.pone.0072134 [PubMed: 23967279]
32. Hoelzinger DB, Mariani L, Weis J, Woyke T, Berens TJ, McDonough WS, et al. Gene expression profile of glioblastoma multiforme invasive phenotype points to new therapeutic targets. *Neoplasia.* 2005; 7(1):7–16. DOI: 10.1593/neo.04535 [PubMed: 15720813]
33. Kislin KL, McDonough WS, Eschbacher JM, Armstrong BA, Berens ME. NHERF-1: modulator of glioblastoma cell migration and invasion. *Neoplasia.* 2009; 11(4):377–87. [PubMed: 19308292]
34. Nelson EA, Walker SR, Weisberg E, Bar-Natan M, Barrett R, Gashin LB, et al. The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors. *Blood.* 2011; 117(12):3421–9. DOI: 10.1182/blood-2009-11-255232 [PubMed: 21233313]
35. Giese A, Bjerkvig R, Berens ME, Westphal M. Cost of migration: invasion of malignant gliomas and implications for treatment. *J Clin Oncol.* 2003; 21(8):1624–36. DOI: 10.1200/JCO.2003.05.063 [PubMed: 12697889]
36. Winkles JA. The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. *Nat Rev Drug Discov.* 2008; 7(5):411–25. DOI: 10.1038/nrd2488 [PubMed: 18404150]
37. Roos A, Dhruv HD, Mathews IT, Inge LJ, Tuncali S, Hartman LK, et al. Identification of aurintricarboxylic acid as a selective inhibitor of the TWEAK-Fn14 signaling pathway in glioblastoma cells. *Oncotarget.* 2017; doi: 10.18632/oncotarget.14685
38. Chumbalkar V, Latha K, Hwang Y, Maywald R, Hawley L, Sawaya R, et al. Analysis of phosphotyrosine signaling in glioblastoma identifies STAT5 as a novel downstream target of DeltaEGFR. *J Proteome Res.* 2011; 10(3):1343–52. DOI: 10.1021/pr101075e [PubMed: 21214269]
39. Padfield E, Ellis HP, Kurian KM. Current Therapeutic Advances Targeting EGFR and EGFRvIII in Glioblastoma. *Front Oncol.* 2015; 5:5.doi: 10.3389/fonc.2015.00005 [PubMed: 25688333]
40. Lu KV, Zhu S, Cvrljevic A, Huang TT, Sarkaria S, Ahkavan D, et al. Fyn and SRC are effectors of oncogenic epidermal growth factor receptor signaling in glioblastoma patients. *Cancer Res.* 2009; 69(17):6889–98. DOI: 10.1158/0008-5472.CAN-09-0347 [PubMed: 19690143]

41. Tran NL, McDonough WS, Donohue PJ, Winkles JA, Berens TJ, Ross KR, et al. The human Fn14 receptor gene is up-regulated in migrating glioma cells in vitro and overexpressed in advanced glial tumors. *Am J Pathol.* 2003; 162(4):1313–21. DOI: 10.1016/S0002-9440(10)63927-2 [PubMed: 12651623]
42. Tran NL, McDonough WS, Savitch BA, Fortin SP, Winkles JA, Symons M, et al. Increased fibroblast growth factor-inducible 14 expression levels promote glioma cell invasion via Rac1 and nuclear factor-kappaB and correlate with poor patient outcome. *Cancer Res.* 2006; 66(19):9535–42. DOI: 10.1158/0008-5472.CAN-06-0418 [PubMed: 17018610]
43. Tran NL, McDonough WS, Savitch BA, Sawyer TF, Winkles JA, Berens ME. The tumor necrosis factor-like weak inducer of apoptosis (TWEAK)-fibroblast growth factor-inducible 14 (Fn14) signaling system regulates glioma cell survival via NFkappaB pathway activation and BCL-XL/BCL-W expression. *J Biol Chem.* 2005; 280(5):3483–92. DOI: 10.1074/jbc.M409906200 [PubMed: 15611130]
44. de la Iglesia N, Puram SV, Bonni A. STAT3 regulation of glioblastoma pathogenesis. *Curr Mol Med.* 2009; 9(5):580–90. [PubMed: 19601808]
45. McFarland BC, Ma JY, Langford CP, Gillespie GY, Yu H, Zheng Y, et al. Therapeutic potential of AZD1480 for the treatment of human glioblastoma. *Mol Cancer Ther.* 2011; 10(12):2384–93. DOI: 10.1158/1535-7163.MCT-11-0480 [PubMed: 22027691]
46. Jensen RL. Brain tumor hypoxia: tumorigenesis, angiogenesis, imaging, pseudoprogression, and as a therapeutic target. *J Neurooncol.* 2009; 92(3):317–35. DOI: 10.1007/s11060-009-9827-2 [PubMed: 19357959]
47. Lee MY, Joung YH, Lim EJ, Park JH, Ye SK, Park T, et al. Phosphorylation and activation of STAT proteins by hypoxia in breast cancer cells. *Breast.* 2006; 15(2):187–95. DOI: 10.1016/j.breast.2005.05.005 [PubMed: 16084091]
48. Selvendiran K, Bratasz A, Kuppusamy ML, Tazi MF, Rivera BK, Kuppusamy P. Hypoxia induces chemoresistance in ovarian cancer cells by activation of signal transducer and activator of transcription 3. *Int J Cancer.* 2009; 125(9):2198–204. DOI: 10.1002/ijc.24601 [PubMed: 19623660]
49. Roos A, Ding Z, Loftus JC, Tran NL. Molecular and Microenvironmental Determinants of Glioma Stem-Like Cell Survival and Invasion. *Front Oncol.* 2017; 7:120.doi: 10.3389/fonc.2017.00120 [PubMed: 28670569]
50. Hambardzumyan D, Gutmann DH, Kettenmann H. The role of microglia and macrophages in glioma maintenance and progression. *Nat Neurosci.* 2016; 19(1):20–7. DOI: 10.1038/nn.4185 [PubMed: 26713745]
51. Russo A, Franchina T, Ricciardi GR, Picone A, Ferraro G, Zanghi M, et al. A decade of EGFR inhibition in EGFR-mutated non small cell lung cancer (NSCLC): Old successes and future perspectives. *Oncotarget.* 2015; 6(29):26814–25. DOI: 10.18632/oncotarget.4254 [PubMed: 26308162]
52. De Witt Hamer PC. Small molecule kinase inhibitors in glioblastoma: a systematic review of clinical studies. *Neuro Oncol.* 2010; 12(3):304–16. DOI: 10.1093/neuonc/nop068 [PubMed: 20167819]
53. Giannini C, Sarkaria JN, Saito A, Uhm JH, Galanis E, Carlson BL, et al. Patient tumor EGFR and PDGFRA gene amplifications retained in an invasive intracranial xenograft model of glioblastoma multiforme. *Neuro Oncol.* 2005; 7(2):164–76. DOI: 10.1215/S1152851704000821 [PubMed: 15831234]
54. Sarkaria JN, Yang L, Grogan PT, Kitange GJ, Carlson BL, Schroeder MA, et al. Identification of molecular characteristics correlated with glioblastoma sensitivity to EGFR kinase inhibition through use of an intracranial xenograft test panel. *Mol Cancer Ther.* 2007; 6(3):1167–74. DOI: 10.1158/1535-7163.MCT-06-0691 [PubMed: 17363510]
55. Seiler CY, Park JG, Sharma A, Hunter P, Surapaneni P, Sedillo C, et al. DNASU plasmid and PSI: Biology-Materials repositories: resources to accelerate biological research. *Nucleic Acids Res.* 2014; 42(Database issue):D1253–60. DOI: 10.1093/nar/gkt1060 [PubMed: 24225319]
56. Ariyoshi K, Nosaka T, Yamada K, Onishi M, Oka Y, Miyajima A, et al. Constitutive activation of STAT5 by a point mutation in the SH2 domain. *J Biol Chem.* 2000; 275(32):24407–13. DOI: 10.1074/jbc.M909771199 [PubMed: 10823841]

57. Mui AL, Wakao H, Kinoshita T, Kitamura T, Miyajima A. Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. *EMBO J.* 1996; 15(10):2425–33. [PubMed: 8665850]
58. McDonough WS, Tran NL, Berens ME. Regulation of glioma cell migration by serine-phosphorylated P311. *Neoplasia.* 2005; 7(9):862–72. [PubMed: 16229809]
59. Ensign SP, Roos A, Mathews IT, Dhruv HD, Tuncali S, Sarkaria JN, et al. SGEF Is Regulated via TWEAK/Fn14/NF-kappaB Signaling and Promotes Survival by Modulation of the DNA Repair Response to Temozolomide. *Mol Cancer Res.* 2016; 14(3):302–12. DOI: 10.1158/1541-7786.MCR-15-0183 [PubMed: 26764186]
60. Fortin SP, Ennis MJ, Savitch BA, Carpentieri D, McDonough WS, Winkles JA, et al. Tumor necrosis factor-like weak inducer of apoptosis stimulation of glioma cell survival is dependent on Akt2 function. *Mol Cancer Res.* 2009; 7(11):1871–81. DOI: 10.1158/1541-7786.MCR-09-0194 [PubMed: 19861406]

Implications

Targeting critical effectors in the EGFRvIII-Stat5-Fn14 pathway may limit GBM tumor dispersion, mitigate therapeutic resistance, and increase survival.

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Figure 1. Differential activation of Stat3 and Stat5 in the core and invasive rim region of GBM tumors

(A) Gene expression analysis for Stat5 and Stat3 signatures in the matched rim and core samples from 19 GBM clinical specimens (GSE 12689). Stat5 gene signature is increased in the invading glioma cells (rim), whereas Stat3 gene signature was high in the tumor core.

(B) IHC staining and comparative analysis of matched GBM core and rim samples from a glioma invasion-specific tissue microarray. Detection of Stat3 activation was performed using a phospho-specific Stat3 antibody, whereas detection of Stat5 activation was assessed by examination of Stat5 nuclear localization. A representative GBM case with increased Stat3 activation in the tumor core and increased Stat5 activation in the invasive cells at the tumor edge is shown.

Figure 2. Stat5 is required for EGFRvIII-mediated GBM cell migration

(A) Stat activation in GBM PDX tumors and U373 cells. Total protein was isolated from EGFR WT (GBM8, 12) and EGFRvIII (GBM39, 59) expressing tumors. U373 EGFRvIII glioma cells were treated with doxycycline (dox) for 4 days, serum starved for 18 hours and total protein was isolated. Western blot analysis was performed using the specified antibodies. Tubulin was used as a loading control. (B) U373 EGFRvIII cells were transfected with a non-targeting siRNA (siCtrl) or Stat5a siRNA (siStat5a), or a Stat5b siRNA (siStat5b) (left) or with a Stat5 dominant negative (DN) vector (right). Migration was assayed over 4 hours utilizing a Transwell migration assay, ** $p < 0.01$. (C) U373 EGFRvIII cells were serum starved for 18 hours and then pretreated with pimozide for 1 hour. Migration was assayed over 4 hours utilizing a Transwell migration assay, ** $p < 0.01$,

*** $p < 0.001$. GBM39 neurospheres were pretreated with different concentrations of pimozone for 1 hour. Migration was assayed over 4 hours utilizing a Transwell migration assay. **(D)** U373 EGFRvIII cells were pretreated with 10 μM pimozone for 1 hour and then treated with two doses of TMZ for 24 hours. Cells were plated at 500 cells/ well in triplicate in 35 mm dish and allowed to form colonies. At the end of the assay, cells were fixed in PFA and stained with crystal violet, and the number of colonies were counted and presented as bar graph. Values are mean $\pm$ standard deviation of three separate measurements, * $p < 0.05$. **(E)** U373 EGFRvIII cells were pretreated with 10 μM pimozone for 1 hour and then treated with 25 μM TMZ for 24 hours. Total protein was isolated and Western blot analysis was performed using the specified antibodies.

Figure 3. Stat5 and EGFRvIII kinase activity are required for upregulation of Fn14 gene expression

(A) U373 and U373 EGFRvIII cells were serum starved for 18 hours and then stimulated with EGF (50 ng/mL) for the indicated time. Total protein was isolated from EGFR WT (GBM8, 12) or EGFRvIII (GBM39, 59) expressing tumors. Western blot analysis was performed using the specified antibodies. Tubulin was used as a loading control (B) U373 EGFRvIII cells were transfected with a non-targeting siRNA (siCtrl), Stat3 siRNA (siStat3), Stat5a siRNA (siStat5a), or a Stat5b siRNA (siStat5b) for 24 hours, serum starved for 18 hours, and then protein was isolated (left). U373 EGFRvIII cells were transfected with Stat5 dominant negative (DN) vector, serum starved for 18 hours, and then total protein was isolated (right). Protein lysates were analyzed by Western blot analysis with the specified antibodies. Tubulin was used as a loading control. (C) U373 EGFRvIII cells were serum starved for 18 hours and then pretreated with pimozide for 4 hours. Total protein was isolated and protein lysates were analyzed by Western blot analysis with the specified antibodies. Tubulin was used as a loading control. (D) U373 cells were transfected with the siCtrl, siStat3, siStat5a or siStat5b for 24 hours, serum starved for 18 hours, and then stimulated with EGF (50 ng/mL) for 4 hours (left) or transfected with a plasmid encoding constitutively active (CA) Stat5 (right). Total protein was isolated and protein lysates were

analyzed by Western blot analysis with the specified antibodies. Tubulin was used as a loading control.

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Figure 4. Src signaling mediates EGFRvIII-dependent Stat5 activation

(A) U373 EGFRvIII cells were treated with EGFR tyrosine kinase inhibitors erlotinib (1 μ M) and gefitinib (1 μ M) for 24 hours in serum-free conditions and then total protein was isolated. GBM39 and GBM12 neurospheres were treated with DMSO or treated with erlotinib (1 μ M) and gefitinib (1 μ M) for 24 hours. Protein lysates were analyzed by Western blot analysis with the indicated antibodies. Tubulin was used as a loading control. (B) U373 and U373 EGFRvIII cells were treated with the Src kinase inhibitor Saracatinib (1 μ M) for 24 hours in serum-free conditions. U373 cells were stimulated with EGF (50 ng/mL) for 4 hours, total protein was isolated, and Western blot analysis was performed with the indicated antibodies. Tubulin was used as a loading control. (C) U373 and U373 EGFRvIII cells and GBM39 and GBM12 neurospheres were treated with the MEK inhibitor, U0216 (1 μ M) for 24 hours. U373 cells were stimulated with EGF (50 ng/mL) for 4 hours, and protein lysates were analyzed by Western blot analysis with the indicated antibodies. Tubulin was used as a loading control.

Figure 5. Fn14 depletion in EGFRvIII U373 cells decreases EGFRvIII-driven migration, invasion and survival after TMZ exposure or radiation treatment

(A) U373 EGFRvIII cells were stably transduced with a non-specific (ctl shRNA) or Fn14 shRNA (shFn14) lentivirus, serum starved, and migration was assayed over 4 hours utilizing a Transwell migration assay, * $p < 0.05$. (B) U373 cells were transfected with a non-specific (siCtrl) or Fn14 siRNA (siFn14), serum starved, and migration was assayed over 4 hours utilizing a Transwell migration assay, * $p < 0.05$. (C) U373 and U373vIII cells were transfected with a non-specific (siCtrl) or Fn14 siRNA (siFn14), serum starved, and invasion was assayed over 4 hours utilizing a matrigel-coated Transwell migration assay, * $p < 0.05$. (D) U373 EGFRvIII ctrl shRNA and shFn14 cells were treated with TMZ (250 μ M) or IR (2 Gy). For TMZ treatment, cells were trypsinized 24 hours after drug treatment and cells were seeded in triplicates in 35 mm dishes and allowed to form colonies. For IR treatment, cells were treated with 2 Gy irradiation, trypsinized, and seeded in triplicates in 35 mm dishes and allowed to form colonies. At the end of the assay, cells were fixed in PFA and stained with crystal violet, and the number of colonies were counted and presented as bar graph. Values are mean $\pm$ standard deviation of three separate measurements, *** $p < 0.001$.

Figure 6. Schematic of EGFR-Src-Mek-Stat3 and EGFRvIII-Src-Stat5 pathway activation in GBM cells

A schematic pathway bifurcation between EGFR and EGFRvIII, where EGFR signals through the MAPK-Stat3 pathway and EGFRvIII preferentially signals through the Src-Stat5 pathway to drive Fn14 expression and GBM migration and survival.