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# Identification of an EGFRvIII-JNK2-HGF/c-Met signaling axis required for intercellular crosstalk and GBM cell invasion

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**Running Title:** JNK2 mediates EGFRvIII-driven HGF production.

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**List of nonstandard abbreviations:**

BME, basement membrane extract; CM, conditioned media; EGFR, epidermal growth factor receptor; EGFRvIII, epidermal growth factor receptor variant III; EGFRwt, epidermal growth factor receptor wild type; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GBM, glioblastoma multiforme; HGF, hepatocyte growth factor; IGFBP5, insulin-like growth factor binding protein 5; IPA, ingenuity pathway analysis; JNK2, c-jun-N-terminal kinase isoform 2; LIF, leukemia inhibitory factor; MMP7, matrix metallopeptidase 7; PDGFRA, platelet derived growth factor receptor alpha; RTK, receptor tyrosine kinase; STAT5A, signal transducer and activator of transcription 5A; TBST, tris-buffered saline with 0.1% Tween-20; VEGFR, vascular endothelial growth factor receptor.

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## Abstract

Glioblastoma Multiforme (GBM) is the most aggressive and common form of adult brain cancer. Current therapeutic strategies include surgical resection followed by radiotherapy and chemotherapy. Despite such aggressive multi-modal therapy, prognosis remains poor with a median patient survival of 14 months. A proper understanding of the molecular drivers responsible for GBM progression are therefore necessary to instruct the development of novel targeted agents and to enable design of effective treatment strategies. Activation of the c-jun-N-terminal kinase isoform 2 (JNK2) is reported in primary brain cancers where it associates with histological grade and amplification of the epidermal growth factor receptor (EGFR). In this manuscript, we demonstrate an important role for JNK2 in the tumor promoting and invasive capacity of EGFR variant III (EGFRvIII), a constitutively active mutant form of the receptor commonly found in GBM. Expression of EGFRvIII induces transactivation of JNK2 in GBM cells that is required for a tumorigenic phenotype *in vivo*. Furthermore, JNK2 expression and activity is required to promote increased cellular invasion through stimulation of an HGF-c-Met signaling circuit whereby secretion of this extracellular ligand activates the RTK in both a cell autonomous and non-autonomous manner. Collectively, these findings demonstrate the co-operative and parallel activation of multiple RTKs in GBM and suggests that the development of selective JNK2 inhibitors could be therapeutically beneficial either as single agents or in combination with inhibitors of EGFR and/or c-Met.

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## Introduction

Glioblastoma is the most malignant central nervous system cancer and accounts for the majority of primary brain cancer-related deaths (Porter et al., 2010). Despite advances in multimodality therapies, such as surgery, radiotherapy and chemotherapy, the outcome for patients remains extremely poor with an average post-diagnostic survival of just over 14 months (Behin et al., 2003; Louis et al., 2007; Stupp et al., 2005). The high mortality rate results from the universal resurgence of tumors post-treatment which occurs due to infiltrating tumor cells that escape initial surgery and exhibit profound resistance to irradiation and current chemotherapy treatments (Claes et al., 2007). Thus, identification of novel tractable targets for improved therapeutics is desperately needed.

Genomic and proteomic analyses have identified a number of key oncogenic drivers of GBM tumorigenesis and therapeutic resistance, including receptor tyrosine kinases (RTKs) (Beroukhim et al., 2007; Huang et al., 2007a; Huang et al., 2007b). In particular, amplification of the epidermal growth factor receptor (EGFR) is present in approximately half of all GBMs (Hurtt et al., 1992; Jaros et al., 1992) with a large proportion also expressing activating mutations, such as deletion of exons 2-7, which results in a ligand-independent, constitutively active mutant commonly referred to as EGFRvIII (Batra et al., 1995; Huang et al., 1997; Nishikawa et al., 1994). While it is unclear how EGFRvIII mutations are generated, it appears to occur late during tumor progression and its co-expression with the wild type receptor (EGFRwt) have been reported to confer poor prognosis and a shorter patient survival time (Heimberger et al.,

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2005; Shinojima et al., 2003). This phenomenon is also observed in orthotopic xenograft mouse studies whereby expression of EGFRvIII in human GBM cell lines leads to an overwhelming enhancement in tumor growth, invasion and resistance to radiation and chemotherapies (Huang et al., 1997; Nagane et al., 1996).

The c-Jun NH2–terminal family of kinases (JNK) consists of three isoforms, JNK1 JNK2 and JNK3, which are activated by a variety of stimuli including UV light, cytokines and growth factor signaling (Davis, 2000). JNKs have many cellular substrates and play diverse cellular roles from induction of apoptosis to proliferation, depending upon the cell type and initial stressor (Kennedy and Davis, 2003). In GBM, constitutive JNK activation is observed in up to 86% of human glioblastomas, where activation strongly correlates with both histological grade and expression of EGFR (Antonyak et al., 2002; Cui et al., 2006; Li et al., 2008). JNKs 1/2 are further known to regulate GBM stem cell-like characteristics and tumor initiating potential. Most notably, genetic knockdown of JNK2 impairs intracranial tumor formation and extends survival in GBM mouse models, suggesting that this particular isoform is important for the pathology of the disease (Matsuda et al., 2012; Yoon et al., 2012).

Here, we have investigated the involvement of the JNK2 isoform in EGFRvIII driven GBM tumorigenesis. We demonstrate that JNK2 is necessary for the growth of aggressive EGFRvIII positive tumors *in vivo* and invasiveness *ex vivo*. Our mechanistic studies identify a critical role for the EGFRvIII-JNK2 signaling axis in the regulation of HGF production and c-Met activation. Importantly, JNK2 dependent secretion of HGF is sufficient to activate c-Met both in a cell intrinsic and extrinsic manner where conditioned media from EGFRvIII expressing cells promotes c-Met activation in cells

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which do not express the mutated receptor. Finally, we demonstrate functional importance of this signaling circuit in the promotion of cellular invasion; addition of exogenous HGF is sufficient to partially restore invasion of JNK2 depleted cells. Together these data define a critical role for JNK2 in activation of a HGF/c-Met signaling downstream of EGFRvIII in GBM.

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## Materials and Methods

### Cells Lines and Reagents

U87 and T98G human glioblastoma cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). DK-MG cells were purchased from Leibniz-Institut DSMZ (Braunschweig, Germany). Modified U87 cell lines were a kind gift from Dr. Frank Furnari (Ludwig Institute for Cancer Research, University of California at San Diego). GBM6 cells were a kind gift from Dr. Jann N. Sarkaria (Department of Radiation Oncology, Mayo Clinic, MN). GBM6 cells were maintained by serial passage in the flanks of nude mice and cultured short term as described previously (Carlson et al., 2011). Cell lines were maintained in Dulbecco's Minimum Essential Medium with glutamax (DMEM; GIBCO by Life Technologies, Grand Isle, NY) supplemented with 10% FBS (Sigma, St. Louis, MO). Cells were incubated at 37°C, 5% $CO_2$, 95% humidity. U87vIII and U87vIII-KD cells were maintained in media containing G418 (Life Technologies). To study the effects of conditioned media (CM), cells were seeded for 24 hours and subsequently serum starved for an additional 24 hours. CM was obtained from cells in serum-free media and clarified by centrifugation.

### Lentiviral transduction

Sequence specific shRNA pGIPZ vectors for the inhibition of JNK2 or non-silencing control were purchased from Open Biosystems (Thermo Fischer Scientific, Pittsburg PA). ShRNA JNK2 sequences were (1) 5'-CTAGCAACATTGTTGTGAA-3'; (2) 5'-CTTCTGAAGTTATCTCTTA-3' (3) 5'-GCATTAAAGCAGCGTATC-3'. Lentiviral particles

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were generated using the Trans-Lentiviral shRNA packaging kit (TLP5912, Thermo Fischer Scientific, Pittsburg PA) as per manufacturer's recommendations. U87vIII cells ($1 \times 10^6$) were seeded in $10cm^2$ dishes and transduced with shRNA lentiviruses for a period of 72 hours. Transduced cells were selected by addition of 1 µg/ml of puromycin (Sigma, St. Louis, MO). Stable cell lines were maintained at 37 °C in a humidified 5% CO2 atmosphere in DMEM with glutamax (DMEM; GIBCO by Life Technologies, Grand Isle, NY) supplemented with 10% FBS.

**Western Blot Analysis**

GBM cell lines were seeded into 6-well plates at a concentration of $2.5 \times 10^5$ cells / well, cell media was removed and cells were washed in ice-cold PBS and pelleted. Cells were lysed in 100 µL of ice-cold RIPA buffer (Boston BioProducts, Ashland, MA) containing protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IN), and centrifuged at 14,000 g for 15 min at 4 °C. Protein concentrations were determined using the Pierce BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL). Samples were analyzed using NuPAGE® Novex® Bis-Tris polyacrylamide gel electrophoresis (Life Technologies, Carlsbad, CA). Gels containing the separated protein were transferred to nitrocellulose membranes using standard protocols. Membranes were probed with the following antibodies: anti-EGFR, anti-phospho-EGFR (Y1068), anti-c-Met, anti-phospho-c-Met (Y1234/1235), anti-phospho-c-Met (Y1349), anti-phospho-Gab1 (Y307), anti- JNK2, anti-GAPDH or anti α-tubulin antibodies. All primary antibodies were purchased from Cell Signaling Technology and used at a 1:1000 dilution. After repeated washes with TBST (20 mM Tris, pH 7.6, 140 mM NaCl,

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and 0.1% Tween-20) membranes were incubated with the appropriate IRDye-conjugated secondary antibody (LI-COR Biosciences, Lincoln, NE) in a 1:10,000 dilution. Membranes were imaged using the LI-COR Odyssey infrared imaging system (LI-COR, Lincoln, NE). Human HGF polyclonal antibody was purchased from R&D Systems (Minneapolis, MN).

**ELISA**

Cells ($2.5x10^5$) were plated in 6-well plates for 24 hours and subsequently serum starved for an additional 24 hours. Human hepatocyte growth factor levels were determined using a Human HGF ELISA kit as per manufacturer's recommendation (Invitrogen by Life Technologies, Grand Isle, NY).

**Invasion Assays**

Invasion assays were performed using the CultureCoat 24-well Basement Membrane Extract (BME)-Coated Cell Invasion Assay (Trevigen Inc., Gaithersburg, MD). The assay consist of an 8µm pore sized modified Boyden chamber coated with a thin layer of BME. Cells were cultured to 80% confluence and subsequently serum starved for 4-6 hours, resuspended in serum free media and added to the top of each chamber as per manufacturer's recommendations. Recombinant human HGF (40ng/ml) was added to the cells (R&D Systems, Minneapolis, MN). DMEM with glutamax supplemented with 10%FBS was added to the bottom of each chamber (500µl). Invasive cells on the underside of the membranes were dissociated in a solution containing calcein-AM. The

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plates were analyzed using a SpectraMax (Molecular Devices, Sunnyvale, CA) plate reader with fixed excitation and emission wavelengths (485nm/520nm).

**Microarray Analysis**

Total mRNA was extracted from GBM cell lines using the RNeasy plus mini kit (Qiagen Sciences, Maryland). Using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), RNA (10μg) was reverse transcribed into single-stranded cDNA as per manufacturer's recommendations. Samples were heated to 95ºC for ~1min to denature RNA/cDNA hybrids followed by treatment with RNaseA for 30 minutes at 37ºC and DNA was purified using a QIAquick PCR Purification Kit (Qiagen Sciences, Maryland), as per manufacturer's recommendation. DNA concentrations were calculated using the NanoDrop 1000 spectrophotometer (Thermo Scientific). Hybridization of the cDNA to the Human Gene Expression 12x135K Array was performed at the FSU NimbleGen Microarray Facility. Differentially expressed genes were identified using the following criteria; Fold change>2 in both direction, unpaired t-test p-value<0.05. 360 probes were identified as the "JNK2 signature" and imported into IPA (Ingenuity Pathway Analysis Tool) for pathway and upstream regulator analysis.

***In vivo* Tumor Xenograft**

Male Nu/Nu mice were obtained from Charles River, received food and water *ad libitum* and were kept in a controlled environment at ambient temperature on a 12-hour light and dark cycle. Experiments were performed when animals reached 5 – 6 weeks of age, upon which mice were subcutaneously inoculated in the flank with $7.5 \times 10^5$ GBM

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cells. Tumors were measured using calipers and tumor volumes were determined using methodologies where width, length and height are used [eg. v=(π/6)x(LxWxH)] (Tomayko and Reynolds, 1989)). All animal studies were approved by the Scripps-Florida Institutional Animal Care and Use Committee and care and maintenance were in accordance with the principles described in the Guide for Care and Use of Laboratory Animals (NIH Publication 85-23, 1985).

**Statistical Analysis**

All values in figures are presented as means ± standard deviation of at least three independent experiments except for the *in vivo* study where values are presented as means ± standard error of the mean. All the experiments were analyzed using a student's t-test where significant p-values were presented as * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001.

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## Results

### JNK2 is required for EGFRvIII driven GBM tumorigenicity

To investigate a role of JNK2 in EGFRvIII mediated GBM tumorigeneis we first examined JNK2 pathway activation in GBM cells expressing EGFRvIII. U87 cells were engineered to express the mutated receptor and analyzed for activated signaling by measuring phosphorylation of the downstream transcription factor c-Jun (p-c-Jun). Using this approach we identified that EGFRvIII expression in both U87 cells, as well as in the patient derived line GBM6, resulted in the activation of JNK2. In contrast, p-c-Jun was not detected in U87 cells in the absence of the mutant EGFR and silencing of JNK2 was sufficient to impair activation of c-Jun (Figure 1A). These findings suggest an important role for the JNK2 isoform in activation of signaling downstream of EGFRvIII. Since previous studies have identified a role for JNK2 in the promotion of a tumorigenic phenotype in GBM (Antonyak et al., 2002; Cui et al., 2006), we examined the effects of JNK2 shRNAs on the growth of U87vIII cells *in vivo*. JNK2 knockdown resulted in a significant reduction in tumor growth over time, when compared to either non-transduced cells or those expressing non-targeting shRNAs (Figure 1B).

### JNK2 mediates EGFRvIII-induced cellular invasion

To elucidate the molecular mechanisms by which JNK2 confers tumorigenicity to U87vIII, cells expressing either non-targeting or JNK2 shRNAs were subjected to microarray analysis. Using this approach we identified 360 differentially expressed genes (p=<0.05, >2 fold change), with the majority of transcripts showing decreased expression upon JNK2 knockdown (Figure 1C). Ingenuity Pathway Analysis (IPA)

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further revealed key canonical pathways regulated by JNK2 that included categories such as cancer, tissue development, cellular growth and proliferation and inflammation (Figure 1D). Notably, the most significantly enriched category was cellular movement with 68 genes related to this pathway showing differential expression upon JNK2 knockdown (Supplemental Table 1). These observations are consistent with a large body of evidence showing that EGFRvIII mediates invasiveness of GBM tumors (Dunn et al., 2012) (Zhu et al., 2009) and implicates a role for JNK2 in this phenotype. We therefore examined the effects of JNK2 knockdown on cellular invasion. Since JNK2 is implicated in GBM cell proliferation and to distinguish this effect from invasive phenotype, all the experiments were performed in a short-term period after modulation of JNK2 expression where the proliferation was not modulated. As expected, EGFRvIII expressing U87 cells showed almost 2 fold increase in invasive capacity using a Boyden chamber assay. Further, silencing of JNK2 in U87 and DK-MG EGFRvIII expressing cells resulted in a significant reduction in invasion through the basement membrane coated transwell (Figure 1E and F), suggesting that JNK2 is indeed an important mediator of GBM invasiveness in EGFRvIII expressing cells.

**JNK2 is required for HGF production in EGFRvIII expressing GBM cells**

To identify key effectors of JNK2 mediated invasiveness we performed further pathway analyses on our microarray data. Using the IPA software we evaluated known gene interactions within the signature list (Figure 2A and B). A number of genes whose products are known to be associated with cellular movement were identified as either direct or indirect signaling mediators including HGF, IGFBP5, MMP7, PDGFRA and

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STAT5A (Figure 2A and Supplemental Table 1). Importantly, HGF was identified as the most significantly predicted upstream regulator of the gene signature associated with JNK2 knockdown (Figure 2B) (IPA overlap p-value =5.72E-05). HGF is the only known activating ligand for the receptor tyrosine kinase (RTK) c-Met (Lai et al., 2009) and is known to have tumor promoting roles in GBM, including; promotion of cell proliferation, cell migration and maintenance of self-renewal leading to therapeutic resistance of glioma stem cells (Joo et al., 2012).

Like JNK2, increased HGF expression and c-Met activation is associated with advanced tumor grade and poor prognosis in patients with GBM (Lamszus et al., 1999; Laterra et al., 1997). Accordingly, the treatment of GBM xenografts with either neutralizing anti-HGF antibody or c-Met kinase inhibitor (Crizotinib) impairs glial tumor growth ((Kim et al., 2006; Li et al., 2005; Martens et al., 2006; Rath et al., 2013). Moreover, studies indicate that constitutive EGFR/EGFRvIII signaling results in transactivation of additional RTK's such as platelet derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR) as well as c-Met itself (Huang et al., 2007b; Stommel et al., 2007). Based on these findings we hypothesized that JNK2 is required for the activation of HGF/c-MET signaling in EGFRvIII expressing GBM cells. To investigate this, we first examined HGF production in U87 cells, U87 cells expressing EGFRvIII and in those with a kinase dead version of the mutant receptor. Notably, EGFRvIII kinase activity resulted in an induction of HGF in these cells, as measured by ELISA (Figure 2C). Further, activation of c-Met (p-c-Met) and its downstream adaptor molecule GRB2-associated-binding protein 1 (p-Gab1) was observed upon expression of active EGFRvIII in U87 and T98G cells (Figure 2D and E)

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(Li et al., 2013). In the case of the kinase dead mutant we observed a slight increase in p-c-Met (Y1234/1235) levels, but no increase in p-c-Met (Y1345) or p-Gab levels and HGF levels were significantly lower compared to active EGFRvIII. Kinase independent roles for EGFR have been observed and may explain the slight increase in p-c-Met (Y1234/1235) levels (Zhu et al., 2010; Tan et al., 2015). We next investigated the requirement for JNK2 in this process by studying the effects of JNK2 shRNAs on both HGF production and activation of c-Met signaling. In confirmation of our gene expression data, knockdown of JNK2 reduced HGF expression in U87 cells expressing EGFRvIII (Figure 2F). Moreover, JNK2 loss resulted in impaired activation of both c-Met and Gab1 in these cells as well as in DK-MG EGFRvIII expressing cells, demonstrating a critical role for JNK2 in EGFRvIII mediated activation of c-Met (Figure 2G and H).

**EGFRvIII/JNK2 Induced HGF expression mediates cellular crosstalk**

Expression of EGFRvIII in glioblastoma is heterogeneous and is usually observed in a subpopulation of tumor cells (Nishikawa et al., 2004). Indeed, EGFRvIII expressing tumors rarely arise independently of amplified EGFR and experimental models imitating the human condition have revealed that tumors containing a small fraction of EGFRvIII expressing cells significantly enhance tumor growth reducing overall survival (Inda et al., 2010; Nagane et al., 1996). IL6 and leukemia inhibitory factor (LIF), for example, were identified as EGFRvIII-induced paracrine factors that stimulated tumorigenicity (Inda et al., 2010). To this end, we hypothesized that JNK2 mediated production of HGF by EGFRvIII expressing GBM cells may serve to transactivate c-Met signaling in non EGFRvIII expressing tumor cells. To test this,

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conditioned media (CM) derived from U87 cells expressing either EGFRvIII or the kinase dead mutant was first added to U87 cells and activation of c-Met examined. CM derived from EGFRvIII expressing cells significantly induced phospho-c-Met, in contrast to U87 cells grown in normal media. Further, phospho-c-Met level was markedly reduced in cells incubated with CM derived from EGFRvIII-Kinase dead expressing cells (Figure 3A and B). To confirm that HGF is the active component of the EGFRvIII CM, we utilized a neutralizing antibody which efficiently blocks activation of c-Met in T98G cells treated with recombinant human HGF (Supplemental Figure 1). Pretreatment of U87vIII CM with the HGF antibody impaired activation of c-Met in T98G target cells (Figure 3C). Consistent with this, transfection of U87vIII cells with siRNAs directed against HGF prior to collection of CM also abrogated activation of c-Met in T98G cells demonstrating a critical role for HGF in the activation of c-Met by EGFRvIII (Figure 3D) (Huang et al., 2007b).

We next assessed whether inhibition of JNK2 can block the HGF/c-Met paracrine signaling in GBM cells. Conditioned media was isolated from cultures of U87, U87vIII, U87vIII-shNT or U87vIII-shJNK2 cells and incubated with T98G cells. Cell treated with CM derived from JNK2 knockdown cells resulted in a significant reduction in phospho-c-Met levels compared to those treated with CM of cells expressing a non-targeting shRNA (Figure 4A). Moreover, exposure of these cells to CM derived from U87vIII cells pretreated with the pan-JNK inhibitor SP600125 (due to a lack of isoform specific inhibitor) significantly reduced the activation of c-Met (Figure 4B). Finally, we addressed whether the role of JNK2 in cellular invasion is dependent on the EGFRvIII-HGF signaling circuit. Addition of recombinant human HGF (rhuHGF) to invading cultures

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was able to partially restore the invasive capacity of U87 EGFRvIII-JNK2 knockdown cells (Figure 4C). Moreover, the addition of recombinant human HGF did not rescue the growth inhibition of the U87 EGFRvIII shJNK2 cells (Supplemental Figure 2). This suggests that while the EGFRvIII-JNK2 axis plays a role in GBM cell proliferation this is driven through a pathway independent of HGF/c-Met. Collectively our findings elucidate critical signaling interactions between EGFRvIII-JNK2 and the HGF-c-Met pathway in GBM cell invasion (Figure 5).

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## Discussion

Major clinical challenges in the treatment of GBM include the ability of infiltrating tumor cells to disperse into distant brain tissue, and the refractory nature of these cells to current therapies (Claes et al., 2007; Lefranc et al., 2005). Accordingly, identification of oncogenic signaling pathways driving GBM invasiveness and disease progression is required to develop targeted therapies leading to prolonged overall survival. GBMs are however, known to be highly heterogeneous in terms of their molecular profiling with complex signaling interactions governing differential responses to therapeutic intervention. For example, several pro-tumorigenic mechanisms have been linked to signaling via EGFRvIII, the constitutively active EGFR deletion mutant common to GBM (Bonavia et al., 2012; Huang et al., 2007b; Stommel et al., 2007). EGFR signaling is a known driver of GBM pathogenesis where it has been shown to promote tumor initiation and development as well as infiltration of surrounding brain tissue and resistance to therapy. Importantly, EGFRvIII is able to transactivate multiple RTK's within the same tumor, resulting in overlapping and redundant signaling pathways (Huang et al., 2007b; Stommel et al., 2007). For example, c-Met is activated by EGFRvIII in GBM where it is associated with advanced tumor grade and poor prognosis (Lamszus et al., 1999; Laterra et al., 1997). Importantly, such complexity is thought to account for the lack of significant efficacy of EGFR targeted agents in the clinic (Taylor et al., 2012), with recent studies demonstrating that combined inhibition of EGFRvIII and c-Met synergistically impairs tumor cell growth in mouse models of GBM and lung cancer (Huang et al., 2007b; Lai et al., 2009; Lal et al., 2009; Pillay et al., 2009). These findings suggest that an understanding of the complex signaling interactions in human cancers

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are required for the rational design of combined and effective targeted therapeutic strategies.

Amplification of c-Met and HGF has been associated with highly invasive and metastatic tumors and poor prognosis in multiple tumors types. In GBM, EGFRvIII overexpression is known to drive activation of c-Met via a previously uncharacterized mechanism (Huang et al., 2007b). In this study we define a role for the JNK2 as a central mediator of EGFRvIII-HGF/c-Met signaling circuit in GBM. Specifically, EGFRvIII dependent activation of JNK2 is required for increased cellular invasion through transcriptional up-regulation of a cast of genes involved in tumor cell movement, including HGF. We show that JNK2 dependent secretion of HGF leads to activation of c-Met in EGFRvIII expressing cells, both in a paracrine and autocrine dependent manner. Phospho-c-Met is observed not only in GBM cells expressing the mutated receptor but also in cells that lack EGFRvIII expression following incubation with conditioned media isolated from EGFRvIII expressing cells. Confirmation of a role for secreted HGF ligand EGFRvIII cellular crosstalk was achieved using neutralizing antibodies and siRNAs targeted against HGF. Finally, we established that JNK2-HGF signaling is important for GBM cellular invasion and recombinant HGF rescues the inhibitory effects of JNK2 knockdown. Collectively our findings elucidate critical signaling interactions in GBM cell invasiveness and cellular crosstalk, both important pathological features of GBM. Further, our findings suggest that selective inhibitors of JNK2 could represent potential therapeutics for use in GBM, particularly when combined with additional RTK inhibitors, including those targeted against EGFR and c-Met.

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## Authorship Contributions

*Participated in research design:* Saunders, Lafitte, Adrados, Quereda, Feurstein and Duckett.

*Conducted experiments:* Saunders, Lafitte, Adrados, Quereda and Ling.

*Contributed analytical tools:* Fallahi.

*Performed data analysis:* Saunders, Lafitte, Fallahi, Adrados, Quereda, Ling, Rosenberg and Duckett.

*Wrote or contributed to the writing of the manuscript:* Saunders, Lafitte, Rosenberg, Adrados, Quereda, Fallahi, and Duckett.

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## Footnotes

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## Figure Legends

**Figure 1**: JNK2 is required for the tumorigenicity and invasiveness of EGFRvIII expressing GBM cells. (A) Western blot analysis of EGFR and c-Jun phosphorylation in lysates from U87, U87vIII, U87vIII-JNK2shRNA cells and a primary (GBM6) cell line. (B) Growth of U87vIII subcutaneous flank tumors expressing indicated shRNAs. (C) Heat map generated from microarray data showing differentially expressed genes ($p<0.05$, >2 fold change) in JNK2 knockdown vs. non-targeting knockdown (shNT) U87vIII cells. (D) Canonical pathways significantly modulated by JNK2, as identified by Ingenuity Pathway Analysis (IPA) of microarray data. (E) Invasive capacity of U87 cells vs. U87vIII expressing either JNK2 or non-targeting shRNAs over 24 hours (***$p\leq0.001$). (F) Invasive capacity of DK-MG EGFRvIII expressing cells with either JNK2 or non-targeting shRNAs over 24 hours (***$p\leq0.001$).

**Figure 2**: JNK2 is required for activated HGF/c-Met signaling in EGFRvIII expressing GBM cells. (A) IPA reveals multiple direct and indirect interactions between genes involved in cell movement. Interactions between signature genes (differentially expressed) based on The Ingenuity Knowledge Base and (B) direct downstream targets of HGF (*Green*: down-regulated in JNK2 knockdown cells; *Red*: Up-regulated in JNK2 knockdown cells). (C) ELISA analysis of HGF production in supernatants derived from U87 cells or U87 cells expressing EGFRvIII vs. a kinase dead version of the receptor (KD) (**$p\leq0.01$). Immunoblots showing the effects of EGFRvIII activity on activation of c-Met and Gab-1 in (D) U87 cells and (E) T98G. (F) ELISA analysis of HGF levels in

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media derived from U87 cells and in U87vIII cells expressing indicated shRNAs (***p≤0.001). (G) Immunoblots showing the effects of EGFRvIII expression and JNK2 knockdown on c-Met and Gab-1 phosphorylation status in (G) U87 cells and (H) DK-MG EGFRvIII expressing cells.

**Figure 3**: Paracrine activation of c-Met by EGFRvIII requires HGF secretion. (A) Phosphorylation status of c-Met in lysates derived from U87 cells treated with conditioned media from either U87vIII or U87vIII-KD cells. (B) Quantification of p-c-Met levels shown in A (**p≤0.01; ***p≤0.001). (C) p-c-Met levels in T98G lysates treated with conditioned media from parental U87 or EGFRvIII expressing cultures pretreated with anti-HGF (2µg/ml) polyclonal antibodies. (D) Activation of c-Met in T98G cells treated with conditioned media from U87vIII cells transfected with either non-targeting siRNA's or those directed against HGF (E) HGF production by ELISA in U87vIII cells transfected with siRNAs against HGF vs. a non-targeting control (*p<0.05).

**Figure 4**: Inhibition of JNK2 is sufficient to impair EGFRvIII induced c-Met signaling. (A) c-Met phosphorylation in lysates from T98G cells exposed to conditioned media from U87vIII-shJNK2 vs. U87vIII-shNT cells. (B) T98G cells pretreated with either SP6000125 or vehicle control (-) and incubated with conditioned media derived from either U87vIII-shJNK2 vs. U87vIII-shNT cells were analyzed for p-c-Met levels. (C) Effect of JNK2 knockdown on invasion capacity of U87vIII cells (**p<0.01). Addition of recombinant human HGF (rhuHGF) partially restores the invasive capacity of U87 EGFRvIII-JNK2 knockdown cells (*p<0.05).

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**Figure 5**: Proposed model for the signaling interactions between EGFRvIII-JNK2 and the HGF-c-Met pathway in glioblastoma. EGFRvIII mediated activation of JNK2 induces secretion of HGF leading to the activation of c-Met both in a paracrine and an autocrine dependent manner. HGF mediated intercellular crosstalk will enhance neighboring non-EGFRvIII cell tumorigenicity.

**Figure 1**

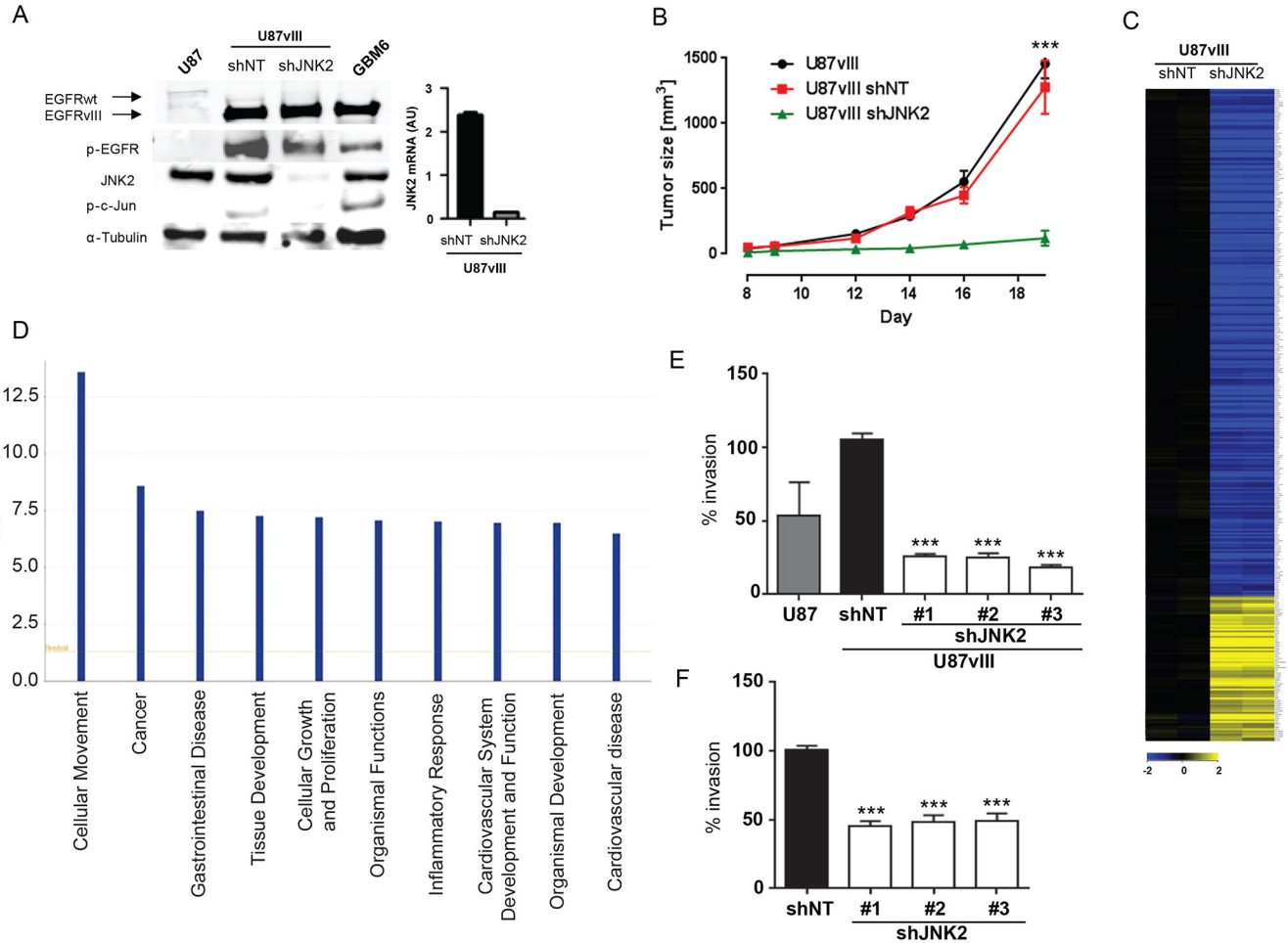

A
U87
U87vIII
shNT
shJNK2
GBM6
EGFRwt
EGFRvIII
p-EGFR
JNK2
p-c-Jun
α-Tubulin
JNK2 mRNA (AU)
shNT
shJNK2
U87vIII
B
U87vIII
U87vIII shNT
U87vIII shJNK2
Tumor size [mm3]
Day
***
C
U87vIII
shNT
shJNK2
D
-log(p-value)
Cellular Movement
Cancer
Gastrointestinal Disease
Tissue Development
Cellular Growth and Proliferation
Organismal Functions
Inflammatory Response
Cardiovascular System Development and Function
Organismal Development
Cardiovascular disease
E
% invasion
U87
shNT
#1
#2
#3
shJNK2
U87vIII
F
% invasion
shNT
#1
#2
#3
shJNK2

Figure 2

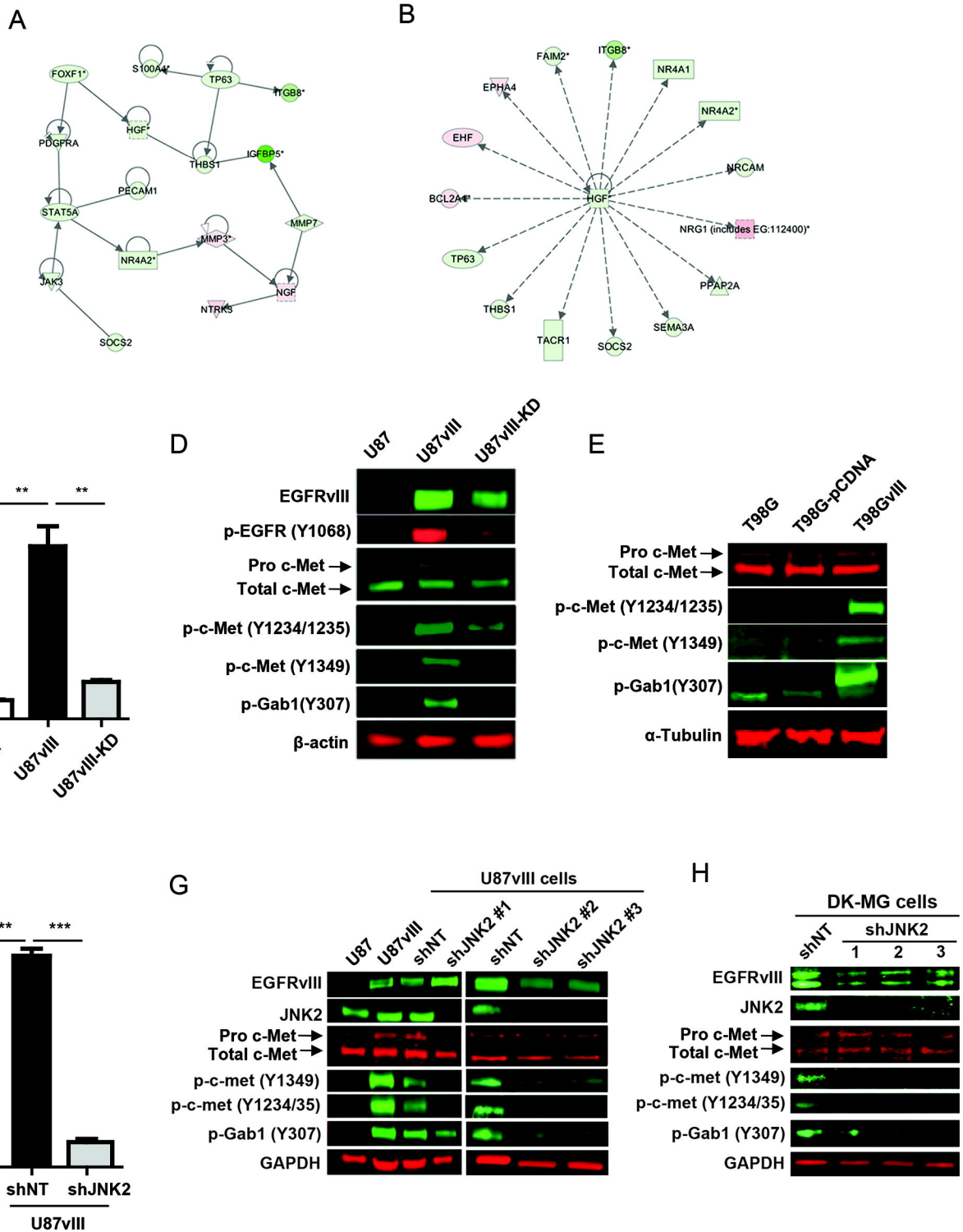

A
FOXF1*
S100A4*
TP63
ITGB8*
PDGFRA
HGF*
THBS1
IGFBP5*
PECAM1
STAT5A
MMP7
MMP3*
NR4A2*
JAK3
NGF
NTRK3
SOCS2
B
FAIM2*
ITGB8*
NR4A1
EPHA4
NR4A2*
EHF
NRCAM
BCL2A1*
HGF
NRG1 (includes EG:112400)*
TP63
PPAP2A
THBS1
SEMA3A
TACR1
SOCS2
C
HGF (pg/ml)
3000
2000
1000
0
**
**
U87
U87vIII
U87vIII-KD
D
U87
U87vIII
U87vIII-KD
EGFRvIII
p-EGFR (Y1068)
Pro c-Met
Total c-Met
p-c-Met (Y1234/1235)
p-c-Met (Y1349)
p-Gab1(Y307)
β-actin
E
T98G
T98G-pCDNA
T98GvIII
Pro c-Met
Total c-Met
p-c-Met (Y1234/1235)
p-c-Met (Y1349)
p-Gab1(Y307)
α-Tubulin
F
HGF (pg/ml)
3000
2000
1000
0
***
***
U87
shNT
shJNK2
U87vIII
G
U87vIII cells
U87
U87vIII
shNT
shJNK2 #1
shNT
shJNK2 #2
shJNK2 #3
EGFRvIII
JNK2
Pro c-Met
Total c-Met
p-c-met (Y1349)
p-c-met (Y1234/35)
p-Gab1 (Y307)
GAPDH
H
DK-MG cells
shNT
shJNK2
1
2
3
EGFRvIII
JNK2
Pro c-Met
Total c-Met
p-c-met (Y1349)
p-c-met (Y1234/35)
p-Gab1 (Y307)
GAPDH

Figure 3

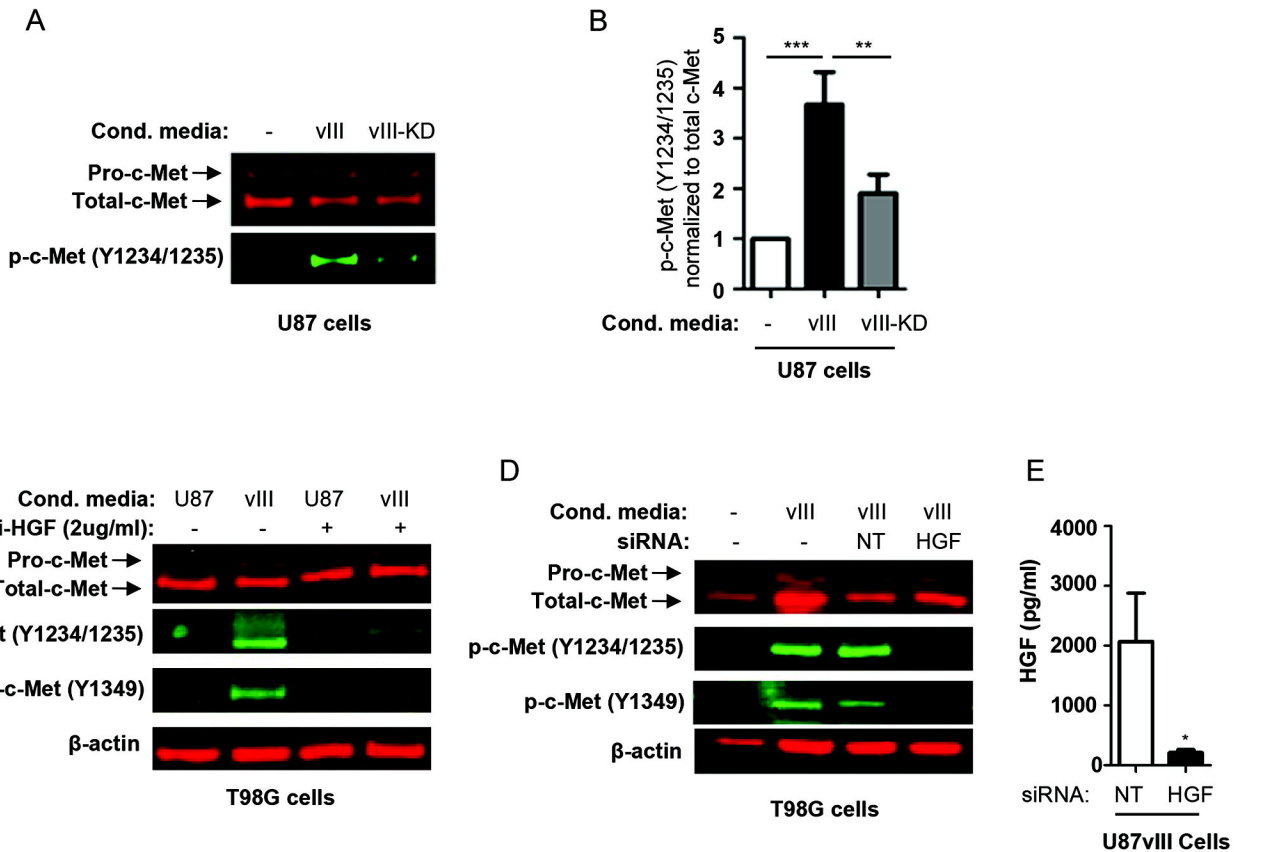
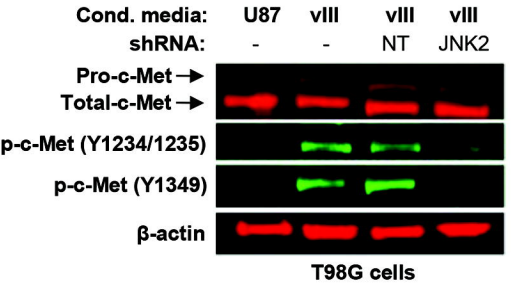

A
Cond. media: - vIII vIII-KD
Pro-c-Met
Total-c-Met
p-c-Met (Y1234/1235)
U87 cells
B
p-c-Met (Y1234/1235) normalized to total c-Met
***
**
Cond. media: - vIII vIII-KD
U87 cells
C
Cond. media: U87 vIII U87 vIII
Anti-HGF (2ug/ml): - - + +
Pro-c-Met
Total-c-Met
p-c-Met (Y1234/1235)
p-c-Met (Y1349)
β-actin
T98G cells
D
Cond. media: - vIII vIII vIII
siRNA: - - NT HGF
Pro-c-Met
Total-c-Met
p-c-Met (Y1234/1235)
p-c-Met (Y1349)
β-actin
T98G cells
E
HGF (pg/ml)
siRNA: NT HGF
U87vIII Cells

Figure 4

A


Cond. media: U87 vIII vIII vIII
shRNA: - - NT JNK2
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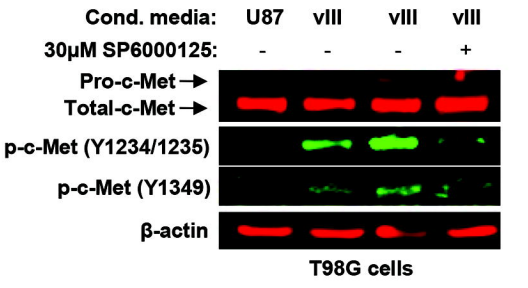
Total-c-Met
p-c-Met (Y1234/1235)
p-c-Met (Y1349)
β-actin
T98G cells

B


Cond. media: U87 vIII vIII vIII
30μM SP6000125: - - - +
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Total-c-Met
p-c-Met (Y1234/1235)
p-c-Met (Y1349)
β-actin
T98G cells

C

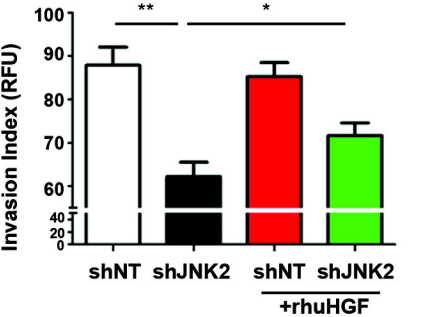
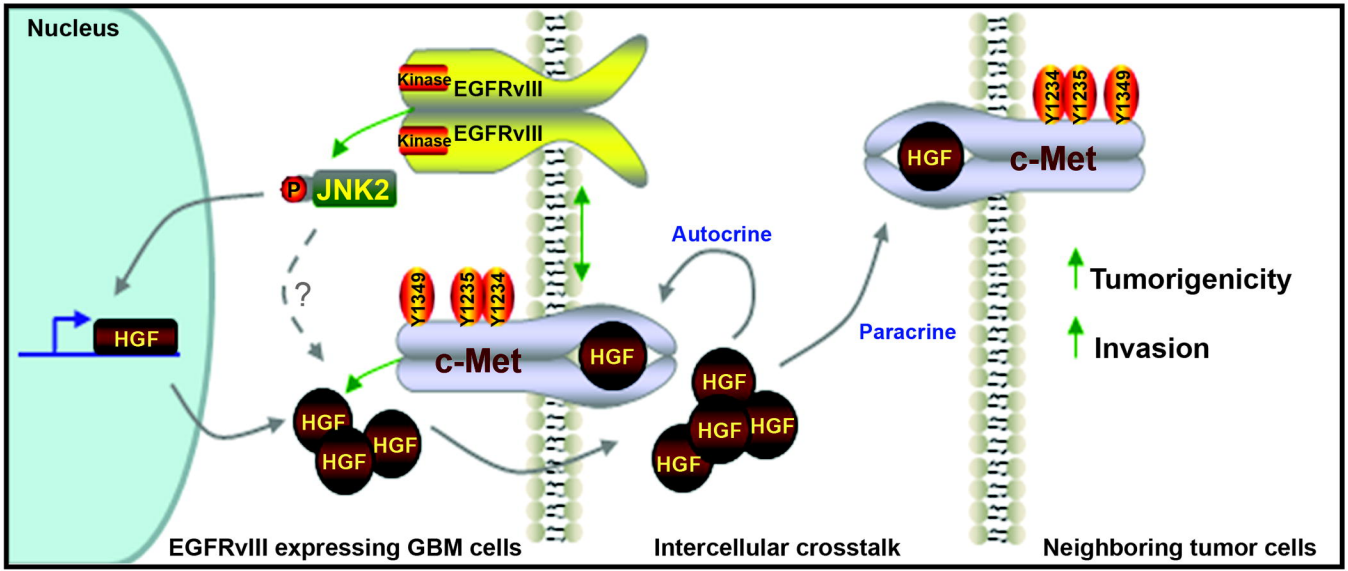

Invasion Index (RFU)
100
90
80
70
60
40
20
0
**
*
shNT
shJNK2
shNT
shJNK2
+rhuHGF

Figure 5


Nucleus
Kinase EGFRvIII
Kinase EGFRvIII
P JNK2
?
HGF
Y1349
Y1235
Y1234
c-Met
HGF
HGF
HGF
HGF
Autocrine
Paracrine
HGF
HGF HGF
HGF
Y1234
Y1235
Y1349
HGF
c-Met
Tumorigenicity
Invasion
EGFRvIII expressing GBM cells
Intercellular crosstalk
Neighboring tumor cells