5-2015

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Yingxuan Chua

Recommended Citation

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By

Yingxuan Chua, M.S.

APPROVED:

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Wei Zhang, Ph.D., Advisory Professor

_____

Gregory N. Fuller, M.D., Ph.D.

_____

Oliver Bogler, Ph.D.

_____

Paul J. Chiao, Ph.D.

_____

Frederick F. Lang Jr, M.D.

_____

Zhimin Lu, M.D., Ph.D.

APPROVED:

_____
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

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A

DISSERTATION

Presented to the faculty of

The University of Texas Health Science Center at Houston

And

The University of Texas

M.D. Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR of PHILOSOPHY

BY

Yingxuan Chua

Houston, TX

May, 2015

To those who have succumbed to

and to those who have survived

this devastating disease.

ACKNOWLEDGEMENTS

IGFBP2 POTENTIATES EGFR-STAT3 SIGNALING IN GLIOMA

Yingxuan Chua, M.S.

Advisory professor: Wei Zhang, Ph.D.

Gliomas are clinically challenging brain tumors with dismal survival rates due to its infiltrative nature and ineffective standard therapy. Insulin-like growth factor binding protein 2 (IGFBP2) is a pleiotropic oncogenic protein that has both extracellular and intracellular functions. Despite a clear causal role in cancer development, the contributions of intracellular IGFBP2 to tumor development and progression are poorly understood. Here we present evidence that both exogenous IGFBP2 treatment and cellular IGFBP2 overexpression lead to aberrant activation of EGFR, which subsequently activates STAT3 signaling. Furthermore, we demonstrate that IGFBP2 augments the nuclear accumulation of EGFR to potentiate STAT3 transactivation activities, via activation of the nuclear EGFR signaling pathway. Nuclear IGFBP2 directly influences the invasive and migratory capacities of human glioma cells, providing a direct link between intracellular (and particularly nuclear) IGFBP2 and cancer hallmarks. These activities are also consistent with the strong association between IGFBP2 and STAT3-activated genes derived from the TCGA database for human glioma. A high level of all 3 proteins (IGFBP2, EGFR and STAT3) was strongly correlated with poorer survival in an independent patient dataset. These results identify a novel tumor-promoting function for IGFBP2 of activating EGFR/STAT3 signaling and facilitating EGFR accumulation in the nucleus, thereby deregulating EGFR signaling by 2 distinct mechanisms. As targeting EGFR in glioma has been relatively unsuccessful, this study suggests that IGFBP2 may be a novel therapeutic target.

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CHAPTER ONE: INTRODUCTION

Gliomas

     Introduction. Gliomas are the most common adult primary malignant brain tumor, representing 70% of adult primary brain tumors [1,2]. Gliomas are thought to arise from glial cells or their precursors and occur in the central nervous system (CNS), the brain and spinal cord [3]. The CNS is comprised of many cells types, including glial cells, neurons and vascular cells [4]. Glial cells include astrocytes, oligodendrocytes and ependymal cells. Astrocytes actively sense control synaptic transmissions, while oligodendrocytes wrap myelin sheaths around axons, and ependymal cells line the ventricle walls and regulate transfer of ions and proteins from the cerebral spinal fluid into the brain [5-7]. Neurons function to process and transmit electrical signals whereas the vascular network in the brain is responsible for oxygen and nutrient delivery [6,8].

     Gliomas are classified as World Health Organization Grades I to IV based on histopathological appearance, including predominant cell type, nuclear aytpia, mitotic figures, necrosis and microvascular proliferation [9] . Low-grade gliomas (LGG) consist of grade I tumors (such as pilocytic astrocytoma), which are considered non-malignant, and grade II tumors (diffuse astrocytoma, oligodendroglioma). High-grade gliomas (HGG) consist of grade III tumors (anaplastic astrocytomas, anaplastic oligodendrogliomas) and grade IV tumors (glioblastomas - GBMs). Approximately 55% of malignant gliomas are GBMs [10]. Grade I tumors are benign and generally have a favorable prognosis after surgical resection [11]. High-grade gliomas are diffusive and infiltrate into normal brain parenchyma, rendering surgical resection alone insufficient; thus standard therapy in these cases include adjuvant chemoradiotherapy, which can prolong median survival [12]. Extracranial GBM metastasis occurs in about 0.4% - 0.5% cases [13-17]. Even though GBMs rarely metastasizes, it remains one of the most lethal cancers, with a median survival of 12-15 months [1,18].

The prognosis of glioma patients varies depending on many factors, including tumor size and location, treatment, age, Karnofsky performance score (KPS), histology of tumor, and molecular genetic factors [19]. Gliomas typically occur in the cerebral hemisphere (86%), most frequently in the frontal and temporal lobe; however tumor size and exact location limits the extent of surgical resection, as tumors often infiltrate deep into the cerebrum [20]. Advanced age and postoperative survival are inversely correlated; glioblastoma patients younger than age 40 years have a 5-year survival rate of 34%, compared with 6% for patients 40 years old and older [21]. Studies reported higher KPS, which measures the functional status of patients, correlates with improved patient outcome [19]. Several risk factors for glioma have been suggested, including occupation and environmental carcinogens; however these studies remain controversial [22]. To date, the only established risk factors for gliomas are hereditary syndromes and exposure to ionizing radiation [2]. Hereditary syndromes such as neurofibromatosis types 1 and 2 and the Li−Fraumeni syndrome may predispose individuals to glioma, mainly astrocytomas, which accounts for 5% of all glioma cases. Therapeutic cranial irradiation for patients with non-related cancers such as acute lymphoblastic leukemia was also highly associated with increased risk of developing gliomas.

The Cancer Genome Atlas (TCGA) consortia along with other researchers have performed comprehensive large-scale genomic and epigenomic profiling of glioma and uncovered distinctive recurring genetic and epigenetic aberrations [18,23-25]. This accumulative research resulted in a molecular classification of distinct glioma subgroups with diagnostic and predictive significance. Furthermore, Nutt et al demonstrated that prediction modeling using molecular profiling of histologically ambiguous gliomas better correlated with survival outcome than standard pathology [26]. Thus, there is ongoing effort to refine the WHO classification system by incorporating clinically relevant molecular signatures of glioma [27] , as the genetic profiles can aid in diagnosis and prognosis prediction [28].

Genetic alterations in glioma. Decades of extensive research have characterized the key genetic events in gliomas, including alteration of receptor tyrosine kinases, PI3K pathway activation, and p53 and retinoblastoma (Rb) tumor suppressor pathways inactivation [24,29]. Overall, the genetic and epigenetic alterations in glioma contribute to cancer hallmarks, leading to tumorigenesis and progression [30,31]. The main molecular alteration in grade I pilocytic astrocytoma is BRAF alteration, typically occurring in children [32,33]. Oligodendrogliomas typically have loss of both chromosomal arms 1p (short arm of chromosome 1) and 19q (long arm of chromosome 19); whereby this 1p/19q codeletion has a better prognosis than gliomas without codeletion [34-36]. HGGs generally are characterized by loss of heterozygosity at chromosome arm 10q (about 60%), PTEN (for Phosphatase and tensin homolog) loss or alterations, EGFR amplifications, and INK4a/ARF loss [37]. Primary GBM arises *de novo* without detectable precursor diseases, but from acquisition of multiple genetic alterations. Meanwhile progression into secondary GBM is thought to involve stepwise development of genetic events that involves loss of tumor suppressor genes and oncogene overexpression, arising from LGG (grade II) [38].

Receptor tyrosine kinases in glioma. In GBMS, the two most significantly mutated or amplified RTKs are EGFR (57%) and PDGFRA (10%), followed by FGFR (3.2%) and MET (1.6%) [39]. EGFR and PDGFRA can be simultaneously activated in primary GBM [40]. 40% of amplified *PDGFRA* harbor an intragenic deletion (termed PDGFRAΔ8,9) in which exon8-9 is deleted resulting in a truncated extracellular domain but constitutively active protein [38,41]. *MET* amplification occurs in about 5% of GBM, overexpression in 29% GBM, and is rarely mutated [42]. EGFR can interact with and activate c-MET in the absence of HGF ligand [43]. FGFR3-TACC3 (for transforming acidic coiled-coil 3) genes oncogenic fusion genes occur in 3-7% GBMs [44,45]. Our group reported that FGFR3-TACC3 expression is mutually exclusive with *EGFR, PDGFRA*, and *MET* amplifications [45]. We further demonstrate that FGFR3-

TACC3 fusion is formed by tandem duplication, resulting in at least three different variants that produce functional oncogenic in-frame fusion proteins.

PI3K/PTEN/AKT pathway. According to analysis of TCGA GBM database, PI3K (for Phosphatidylinositol-4,5-bisphosphate 3-kinase) and PTEN (for Phosphatase and tensin homolog) mutations are mutually exclusive, with 25.1% of GBM harboring PI3K mutations and 41% with PTEN loss or mutation[39]. PTEN loss correlated with increased AKT (also known as Protein kinase B) activity, whereas PI3K mutant specimens express lower AKT activity than specimens lacking PI3K mutations [39]. AKT activation due to PTEN loss has been demonstrated to contribute to EGFR kinase inhibitor insensitivity in GBMs [46]. Recently, our group identified AKT3 isoform as the main isoform in mediating glioma progression, primarily through activation of DNA repair pathways, which leads to radiation and temozolomide resistance [47].

TP53. The TP53 pathway (p53, MDM2 (for Murine Double Minute 2), MDM4) dysregulation occurs in 86% of GBMs, but is particularly prevalent in the development of secondary glioblastoma [39,48]. The p53 protein is a tumor suppressive transcription factor important for regulating cell growth, DNA repair, and apoptosis through protein-protein interaction or gene regulation. According to TCGA analysis of GBM, p53 alterations through mutation or deletion occurred in 28% of GBMs, MDM2 alterations occurred in 7.6% of GBMs, and MDM4 alterations occurred in 7.2% of GBMs [39]. More than 90% of secondary GBM have p53 mutations compared to less than 35% in primary GBM [49]. The prognostic impact of p53 in glioma remains inconclusive. The diversity of p53 alterations affects the response to therapy: inactivating p53 mutation in GBM cell lines [50] or in patients [51] led to increased chemoradiotherapy sensitivity, while others report p53 status of GBM patients did not affect sensitivity to radiotherapy [52]. Some studies report p53 mutation or expression correlated with

better survival [53-55], whereas other reports no significant impact on survival in patients with p53 mutation [56-58].

In physiological conditions, p53 is degraded or inhibited upon binding of MDM2 ubiquitinase [59]. MDM2 binds to the p53 transactivation domain and suppresses p53 transcriptional regulatory mechanisms, or promotes p53 proteasomal degradation through its ubiquitin ligase functions. Thus even in gliomas expressing wild-type p53, p53 may remain dysfunctional due to MDM2 overexpression. MDM2 and MDM4 cooperate to modulate p53 activity: MDM2 primarily prevents p53 protein accumulation, whereas MDM4 regulates p53 transcriptional activity.

RB (for retinoblastoma 1). In GBMs, RB1 mutation or deletion occurs in 7.6% cases, and Rb downstream signaling is impeded mainly through *CDKN2* family deletion [39]. RB1 tumor suppressor protein regulates cell cycle progression from G1 to S phase and its signaling effectors include CDKs (for cyclin dependent kinases), CCNDs (for cyclins) and CDKN2A (for cyclin-dependent kinase inhibitor 2A) [48]. *CDKN2A* encodes 2 proteins, p16INK4a and p14ARF; P16INK4a prevents phosphorylation of RB1, and thus prevents G1/S cell cycle transition, whereas p14ARF binds to MDM2, which stabilize p53 and prevents cell cycle progression. Furthermore, Rb downstream signaling is also impeded through amplification of CDK4 (14%), CDK6 (1.6%), and CCNDs (2%).

IDH1/2 (isocitrate dehydrogenase 1/2) mutations. *IDH1* or *IDH2* mutations are early genetic events that occur in 50%–80% grade II and III gliomas, and is a marker of secondary GBMs, but are rare in primary glioblastomas (5%) [60-62]. Patients harboring *IDH1* or *IDH2* mutation have a median overall survival of 31 months, significantly longer than in patients with wild-type IDH (15-month survival)[62]. The role of *IDH* mutations in glioma has yet to be clearly elucidated. However, it is known that *IDH1/2* mutations produce 2-hydroxyglutarate (2-HG),

which functions as an oncometabolite that contributes to glioma progression through aberrant HIF1a stabilization and induction of histone demethylation [61,62].

DNA methylation. Cancer-specific DNA methylation is the aberrant addition of a methyl group at CpG (cytosine-phosphodiester-guanine) dinucleotides located in CpG islands within the promoter of a gene. TCGA network identified a glioma CpG island methylation phenotype (G-CIMP) that represents a subtype of the proneural tumors, which is associated with grade II and III gliomas, with frequent IDH1 mutations and longer overall survival [18]. The *IDH1* mutation can induce the G-CIMP glioma phenotype, which frequently has *MGMT* methylation [63]. *MGMT* methylation frequently occurs in gliomas [64,65], particularly LGGs. MGMT is involved in DNA repair; thus the epigenetic silencing of *MGMT* by promoter methylation can confer sensitivity to temozolomide [64].

GBM subtypes. TCGA consortia classified GBMs into 4 subtypes based on genomic characteristics, namely classical, proneural, mesenchymal, and neural subtypes [25]. In addition to the gene expression-based molecular classification, these subtypes are defined by patient age, survival rates and treatment response. The classical subtype harbors the most genomic alternations in GBM: 93% have chromosome 7 amplifications and 10 deletions, and 95% express EGFR amplification [25]. Classical subtype also distinctively lacks alterations of TP53, NF1, PDGFRA or IDH1 [25]. Clinically this highly proliferative classical subtype has poor prognosis; however, of all the subtypes, aggressive treatment causes the most reduction in mortality in the classical subtype [25]. Proneural subtype is characterized by PDGFRA mutations, which occur almost exclusively in this subtype [25]. Proneural subtype also has the most frequent IDH1 mutations of all the subtypes and TP53 is frequently mutated in this subtype [25]. Secondary GBMs are generally classified as proneural [25]. Almost all G-CIMP positive tumors have been shown to display the proneural gene expression profile [18]. Proneural subtype grade II and III gliomas with IDH1 mutation and G-CIMP positivity, is generally

associated with better survival [66]. NF1 mutations most frequently occur in the mesenchymal subtype, which also harbors mutations and/or loss of TP53, PTEN, and CDKN2A, and express mesenchymal markers MET and YKL40 [25]. Tumor necrosis factor family pathway and NF-κB pathway are also overexpressed in the mesenchymal subtype. The mesenchymal subtype is typically associated with poor prognosis [67]. Genetic alterations in the neural subtype include gene types that are expressed in normal brain or differentially expressed by neurons, including *NEFL, GABRA1, SYT1* and *SLC12A5 [25]*. The neural subtype typically consists of LGGs or grade III gliomas [25]. Overall, the comprehensive molecular signature classification of GBMs provides better insights into the complex signaling pathways of GBMs and can ultimately lead to personalized therapy for GBM patients.
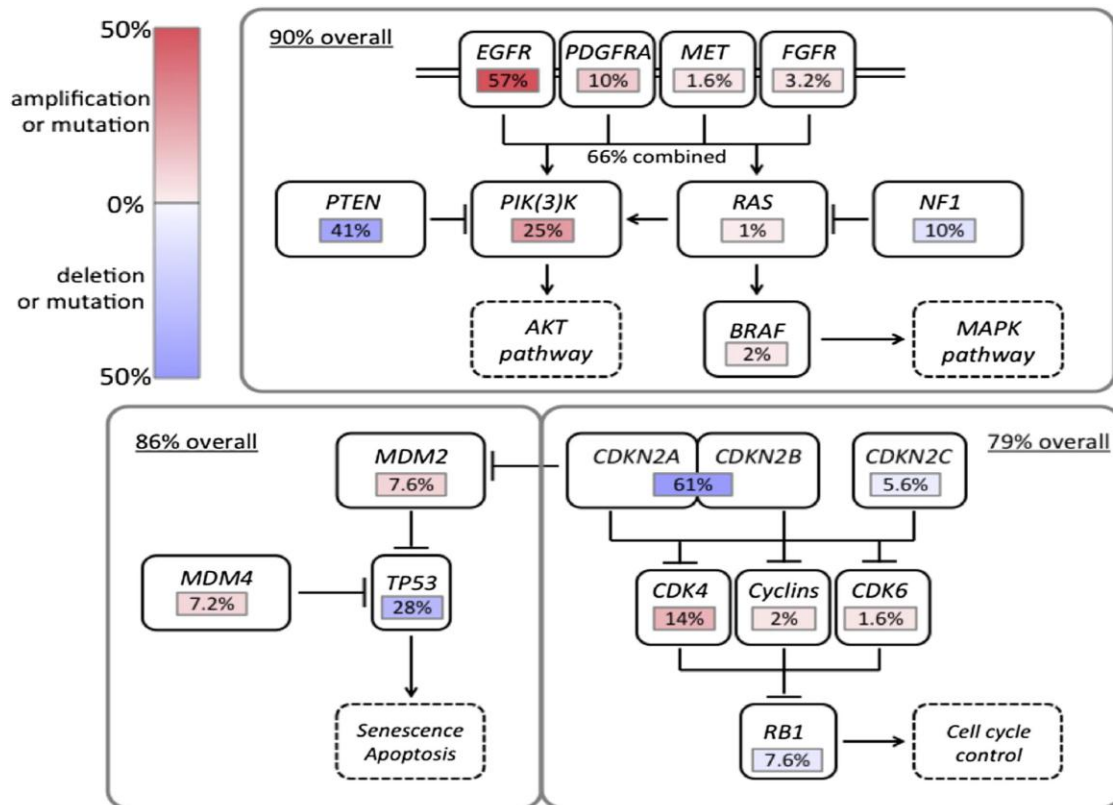
Figure 1. Overall alterations rates of most common signal transduction and tumor suppressive pathways in GBM. Used with permission and originally published by Brennan et al.The somatic genomic landscape of glioblastoma. Cell. 2013;155(2):462-477. [39].

Therapeutics. Comprehensive genomic and epigenomic profiling of gliomas indicate that heterogeneities within tumor cells play a dominant role in the progression, development of resistance to therapy and recurrence. Despite the increasing knowledge of molecular aberrations involved in gliomas, the standard therapy remains to be primarily surgical resection, radiotherapy and chemotherapy. Anaplastic gliomas with codeletion of 1p/19q are more sensitive to chemotherapy with procarbazine, lomustine, and vincristine, compared to those without deletions [1]. A study has shown that TMZ treatment of GBM patients with MGMT methylation had a median survival of 21.7 months, compared to 12.7 months in GBM patients without MGMT methylation [64]. Thus even though chemotherapy is the standard, it is not beneficial to all patients. Therefore it is important to appreciate the heterogeneity of gliomas and utilize the existing knowledge of signature molecular biomarkers so that patients can prospectively be treated with the appropriate therapy.

Two independent Phase II clinical trial studies for recurrent GBMs, using erlotinib or gefinitib, which are EGFR small molecule tyrosine kinase inhibitors (TKIs), demonstrated EGFR inhibitors alone failed to improve prognosis[68]. Other studies with newly diagnose GBMs utilized erlotinib or gefitinib in combination with TMZ and/or radiation therapy also demonstrated no significant improvements [68]. This suggests that even though EGFR is a major oncogene in glioma, targeting one pathway alone is insufficient. Some lung cancer patients harboring EGFR kinase domain mutations respond better to gefitinib, thus it is possible that EGFR mutations in glioma may have an impact on response to EGFR inhibitors. In line with that, Mellinghoff et al reported positive albeit modest response of GBM patients with coexpression of EGFRvIII and PTEN to erlotinib and gefitinib [69]. Imatinib (STI571/Gleevec/ Glivec; Novartis), which inhibits the PDGFR, BCR-ABL and c-KIT pathways and has success in leukemia and gastrointestinal tumors, demonstrated no clinical benefits in GBM patients [68].

Besides TKIs, monoclonal antibodies that recognize epitopes with high selectivity and affinity are actively being researched in gliomas. Bevacizumab is a FDA approved anti-VEGF humanized monoclonal antibody that binds and inhibits VEGF activity and has been used in combination with chemotherapy in many cancers [70]. Several Phase II clinical trials of combination bevacizumab and irnitoecan (a toposisomerase inhibitor) in recurrent GBMs were effective and thus lead to FDA approval for recurrent GBMs despite severe toxicity of treatment [68]. Cetuximab, a chimeric monoclonal antibody that binds to EGFR ligand binding domain and inhibits downstream signaling, was also tested in a Phase II trial in patients with recurrent GBM [71]. Cetuximab in combination with bevacizumab and irinotecan demonstrated a positive response rate; however the response was not notably different than single agent or bevacizumab and irinotecan treatment [71]. However, xenograft mouse model data demonstrated reduced tumorigenenicity in GBM tumors with exon 27 deletion mutation in EGFR C-terminal [72].

Antitumor vaccines such as ΔEGFR-specific vaccines are directed against EGFRvIII mutations, or wtEGFr vaccines are currently undergoing clinical testing [73-75]. The premise is that these peptide-based vaccines will be captured by antigen presenting cells and brought to the lymph node where circulating cytotoxic T-lymphocytes will be activated to recognize and kill cells expressing EGFRvIII or wtEGFR. Other type of GBM-specific vaccines, such as tumor lysate vaccine and cancer stem cell vaccine are also currently undergoing early clinical trials [75].

Despite intensive research, prognosis remains poor for HGG patients. Thus better understanding of the molecular mechanisms involved in tumor progression, along with novel treatment regimens including target therapy or combination therapy may potentially improve the clinical outcome of GBM patients.

Insulin-like growth factor binding protein 2

Introduction. Insulin-like growth factor (IGF) binding protein 2 (IGFBP2) is a member of the IGFBP family of secreted proteins consisting of IGFBP1-6 [76]. Human *IGFBP2* is located on chromosome 2q33-q34 and predominantly expressed in fetal tissues [77,78]. IGFBP2 consists of three domains, the N-terminal, linker, and C-terminal. The cysteine-rich N- and C-terminal are important for IGFI and IGFII binding, while interaction with extracellular matrix and integrins occur at the heparin binding domain (HBD) in the linker domain and C-terminal [79].

IGFBP2 is the major IGFBP expressed in cerebral spinal fluid and during brain development [80-82]. Postnatal IGFBP2 expression is significantly decreases and is limited to hematopoietic stem cells and in liver and spleen progenitor cell populations [83-85]. However, IGFBP2 is upregulated in a pathological conditions such as liver cirrhosis, renal failure [86], and in cancers [87]. IGFBP2 functions can be IGF-dependent or IGF-independent, depending on cell type and microenvironment.

IGFBP2 functions. As a secreted protein, IGFBP2 binds IGFI and IGFII mitogens with high affinity in the extracellular environment. IGFBP2 can act as a carrier protein to transport IGFs and regulate interaction with their receptors or to prolong their stability [88,89]. IGFBP2 binds to IGFII with higher affinity than IGFI [90]. IGFBP2 binding to IGFs is predominantly inhibitory to IGF mitogenic signaling [91-93], however IGFBP2 may also transport IGFs to their receptors to potentiate IGF mitogenic signaling [94,95]. In vascular smooth muscle cells, IGFBP2 potentiates IGF-1 mediated proliferation [95], while in osteoblasts IGFBP2 potentiates IGF-II-stimulated differentiation [96]. On the contrary, IGFBP2 was demonstrated to bind and suppress IGF-1-mediated proliferation in a breast cancer study [97].

In addition to its functions as a secreted protein, IGFBP2 can interact with other proteins at the cell surface (such as integrins) or intracellularly, independent of IGF binding [98-100].

Independent of its IGF binding role, Periera et al demonstrated that IGFBP2 binds to integrin α5β3 to inhibit breast cancer migration and growth [101]. Upon interaction with extracellular matrix or integrin binding, or upon proteolysis by serine proteases or a distintegrin metalloproteases (ADAMs), IGFBP2 releases IGF and permits free IGF to bind to its receptors [102]. There are four major protease cleavage sites in IGFBP2, located between Tyr103 and Gly104, Leu152 and Ala153, Arg156 and Glu157, and Gln165 and Met166 in the linker domain [102]. Using a protease-resistant and non-ECM-binding IGFBP2 mutant, Soh et al demonstrated that this mutant inhibited IGF-stimulated *in vitro* cell proliferation and *in vivo* tumor growth of MCF-7 breast tumor xenograft mice [79]. This study suggests that protease cleavage of IGFBP2 to release IGF (which presumably allows freed IGF to bind to its receptor) is required to facilitate tumor cell proliferation and growth. In another study, Russo et al demonstrated that IGF1-stimulated cell proliferation was inhibited upon addition of IGFBP2 in neuroblastoma cells [94]. However when IGFBP2 was overexpressed, cell proliferation was significantly enhanced in the presence of IGFI. Further investigation revealed that ECM-binding was required to mediate this proliferative advantage, indicating that pericellular-localized IGFBP2 (through ECM binding) releases IGFs in close proximity to its receptors, thus facilitating enhanced proliferation, migration and invasion.

IGFBP2 is involved in response to CNS injuries such as cerebral contusion or stroke [103,104]. In response to CNS injuries, IGFBP2 is the only IGFBP expressed in activated microglia [105] and the major IGFBP expressed in neurons and proliferating (reactive) astrocytes [104,106]. Neurons are terminally differentiated and have limited proliferative capacity, whereas astrocytes are highly proliferative and can convert from resting (differentiated) states to reactive (proliferative) states upon stimulation [106]. *IGFBP2* gene expression is similar in both resting and reactive astrocytes; however compared to resting astrocytes, reactive astrocytes had increased IGFBP2 protein levels [106]. Interestingly, there is

a markedly lower IGFBP2 level in the culture media of reactive astrocytes, compared with resting astrocytes. This possibly suggests that reactive astrocytes rely on the intracellular functions of IGFBP2 rather than secreted IGFBP2 for proliferative activity. Alternatively, because the culture media of reactive astrocytes contained proteolytically cleaved IGFBP2, it is possible that cleavage releases IGFs from IGFBP2 inhibition and permits IGF-mediated proliferation.

IGFBP2 in cancer. IGFBP2 is overexpressed in various cancers, including gliomas [84,107-109], ovarian cancer [110,111], prostate cancer [112,113], breast cancer [114], lung cancer [115,116], leukemia [117], and *PAX/FKHR* translocation negative rhabdomyosarcomas [118]. Elevated IGFBP2 expression typically correlates with higher tumor grade, poor survival, increased tumor recurrence, and increased drug resistance [108-110,119-122]. Elevated IGFBP2 is also observed in the cerebral spinal fluid of patients with central nervous system tumors [80]. IGFBP2 is involved in tumorigenesis [123,124], invasion and metastasis [94,125,126], angiogenesis [124], cancer stem cell expansion [127] and drug resistance [121,122]. IGFBP2 expression typically decreases upon cancer remission [80].

IGFBP2 is an oncogenic factor that has many roles in cancer. IGFBP2 regulates key genes involved in tumorigenesis, including proliferation, cell cycle progression, migration, and invasion. IGFBP2 silencing in rhadomyosarcoma cells resulted in decreased *CCND1* (cyclin D1), *MMP2* (matrix metalloproteinase 2), and *MCM* (minichromosome maintenance protein) gene expression, which are genes involved in cell cycle progression, migration and invasion [118]. Research using neuroblastoma cells showed that IGFBP2 can regulate genes involved in migration, proliferation or angiogenesis-enhancing genes, including *MMP2*, *STAT3* and *VEGF* [124].

In accordance to its role in regulating genes involved in tumorigenesis, IGFBP2 is involved in mediating tumorigenic activities in cancer cells. IGFBP2 inhibits growth of normal prostate epithelial cells, but stimulates growth of prostate cancer cells; the stimulatory growth effect on prostate cancer cells are androgen-dependent and partially mediated through MAPK and PI3K signaling [128]. In ovarian cancer cells, IGFBP2 enhances invasion capacity [129] and stimulates cell growth and proliferation through ERK1/2 and JNK pathway [130]. IGFBP2 is highly expressed in acute myeloid leukemia (AML) and is a critical for promoting the survival and migration of AML cells, through PTEN/AKT and STAT3 signaling [117]. IGFBP2 is also linked to drug resistance, which is a major clinical obstacle. In AML, high IGFBP2 expression is associated with higher risk of relapse after stem cell transplant and resistance to chemotherapy [121,131]. Through interaction with integrin β1, IGFBP2 drives resistance to docetaxel by inactivating PTEN in prostate cancer [122]. High IGFBP2 levels in non-small cell lung cancer are associated with resistance to dasatinib, a small molecule inhibitor of SRC family tyrosine kinases and receptor tyrosine kinases including EGFR [132]. Furthermore, IGFBP2 was recently identified as a biomarker of metastasis and a pro-angiogenic gene [126]. Metastasis is the cause of 90% of cancer deaths [133], while angiogenesis is an important cancer hallmark [31]. Using highly metastatic breast cancer cell lines, Png et al demonstrated that IGFBP2 is secreted by metastatic cells to recruit endothelial cells via the IGFI-dependent activation of IGF1R [126]. Research in neuroblastoma demonstrated that IGFBP2 enhanced VEGF transcription and protein level, and subsequently promoted angiogenesis [124].

IGFBP2 in glioma. Our group's discovery that IGFBP2 is aberrantly reactivated and overexpressed in GBMs [84] was followed by validation from other groups [108,119,134]. As high plasma levels are predictive of clinical outcome, IGFBP2 is proposed as a prognostic and predictive factor in gliomas [109,134-137]. G-CIMP positive glioma (which is associated with longer patient survival) has decreased IGFBP2 and COX2 expression compared to G-CIMP

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negative glioma [39]. Moreover, low IGFBP2 is associated with global hypermethylation, which is also linked to longer patient survival [138]. Furthermore, analysis of the TCGA GBM database demonstrated that *IGFBP2* and *STAT3* are among the 12 upregulated genes predictive of decreased survival [137].

IGFBP2 can exert its oncogenic functions through cooperation with other oncogenic factors. Research from our group demonstrated that IGFBP2 in combination with PDGFB expression can promote gliomagenesis and progression to HGG [123]. Glioma cells exhibit increased migration and invasion capacities, which are cancer hallmarks that contribute to the aggressive phenotype of HGGs. Independent of IGF binding, IGFBP2 enhances glioma cell migration and invasion through its interaction with integrin α5 [139], to stimulate JNK [140] or integrin β1 activation [141]. Furthermore, IGFBP2-mediated activation of integrin β1 leads to the activation of migration- and invasion-related pathways, ILK (for integrin-linked kinase) and NFKB (for nuclear factor kappa B), in gliomas [141]. Besides the integrin β1/ILK/NFKB pathway, IGFBP2 also promotes glioma cell proliferation, invasion and chemoresistance through integrin β1-mediated ERK activation [125].

Microarray analysis of IGFBP2-overexpressing glioma cells demonstrated upregulation of genes involved in enhancing cell motility and invasion, including MMP2 and integrins [142]. IGFBP2 depletion lead to decreased glioma cell invasion *in vitro* through reduction of *CD24*, an invasion-related gene, and reduced tumorigenecity in nude mice [143]. In addition to its role in enhancing cell motility and invasion-related genes, analysis of glioma patient samples revealed that *IGFBP2* is coexpressed and correlated with angiogenesis-related genes, specifically *VEGF* [144]. Angiogenesis is one of the key features of GBM, and IGFBP2 expression is associated with angiogenesis in GBM [145]. In addition to angiogenesis, IGFBP2 can also facilitate cell cycle progression by promoting S-phase and G2/M entry in glioma [125].

IGFBP2 is inversely linked to tumor suppressor genes, such as PTEN and p16/INK4. IGFBP2 and PTEN are inversely correlated in gliomas: PTEN negatively regulates IGFBP2 and thus elevated IGFBP2 can be attributed to PTEN loss, which is common in gliomas, however the underlying mechanism of regulation remains unknown [146,147]. Furthermore, IGFBP2 expression can be induced by PI3K/Akt signaling [146], and IGFBP2 can induce AKT activation [127] in a positive feedback loop. In addition to PTEN loss, loss of *INK4A-ARF* (which encodes for p16INK4a and p14ARF tumor suppressors) also frequently occurs in HGGs. Our group demonstrated using human glioma samples and glioma mouse model that IGFBP2 is inversely correlated with p16INK4a and ARF [148]. A mouse model study demonstrated *Ink4a-Arf* loss resulted in increased glioma incidence [149]. In addition to establishing IGFBP2 overexpression as a marker for *Ink4a/ARF* deletion, our group demonstrated that IGFBP2 inhibition using antisense oligonucleotide in a glioma mouse model with *Ink4a/ARF−/−* background resulted in prolonged survival.

IGFBP2 is negatively regulated by MIIP (for migration and invasion inhibitory protein), the protein product of tumor suppressive gene *IIP45* (for invasion inhibitory protein 45). Using *in vitro* and *in vivo* mouse xenograft studies, Song et al demonstrated that MIIP binds to IGFBP2 and inhibits glioma cell invasion [150]. However, *IIP45* is frequently downregulated in GBM [150]. IGFBP2 is also negatively regulated by mircoRNA-491 (MIR491) [151]. IGFBP2 is one of the targets of *MIR-491* gene product, miR-491-3p. However, *MIR-491* tumor suppressive gene is frequently deleted in GBM [151]. Furthermore, the deletion of *MIR491* in GBMs contributes to aberrant proliferation, invasion and glioma stem cell expansion [151].

Cancer stem cells are thought to be cells responsible for repopulating the tumor bulk and contribute to cancer relapse and drug resistance. In a study using glioma stem cells (GSCs) isolated from GBM patients, Hsieh et al demonstrated that IGFBP2 is overexpressed in glioma stem cells [127]. They showed that IGFBP2 regulates GSC self-renewal and promotes GSC

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expansion and survival through AKT activation [127]. When IGFBP2 was inhibited, stem cell related genes, *BMI, NES, CD133*, and *SOX2* were markedly decreased. Furthermore, IGFBP2 was found to promote G1/S-phase progression by regulating cell cycle related genes, *CCND1, CDC2, CDK4*, and *CCNE1* in GSCs [127]*.*

IGFBP2 localization. Even though it is a secreted protein, IGFBP2 can localize on the cell surface, in the cytoplasm and in the nucleus and its functions differ based on its localization. In pulmonary alveolar type 2 cells (stem cells of the lung which can proliferate during lung development or repair), increased IGFBP2 secretion is associated with proliferation inhibition [152]. In another study, hyperoxia exposure (prolonged exposure to 95% oxygen) of these type 2 cells demonstrated concomitant inhibition of proliferation and increased *IGFBP2* gene and accumulation of IGFBP2 protein in the media [153]. In human alveolar lung epithelial cells, hyperoxia led to decreased secreted IGFBP2, and accumulation of intracellular IGFBP2, particularly nuclear [154]. Consequently, cell proliferation was inhibited and apoptosis was induced. These studies demonstrate that in these two types of lung cells, both extracellular and intracellular IGFBP2 functions to inhibit proliferation.

IGFBP2 binds to the cell membrane through proteoglycans as demonstrated in postnatal rat olfactory bulb [155]. IGFBP2 has also been demonstrated to interact with extracellular matrix (ECM) components (laminin, fibronectin, vitronectin, collagen type IV, and proteoglycans) via its heparin binding domain (HBD) located at 179PKKLRP184 in the linker domain in neuroblastoma cells [94]. This interaction resulted in neuroblastoma proliferation, invasion and angiogenesis. Besides ECM interaction, IGFBP2 can also localize to the cell surface by binding to integrin α5β1 through its Arginine-Glycine-Aspartic (RGD) acid sequence in the C-terminal independent of IGF binding. In Ewing's sarcoma cell, IGFBP2 interaction with integrin α5β1 leads to cellular deadhesion and decreased cell proliferation [156]. On the contrary, IGFBP2 interaction with integrin α5β1 in glioma results in increased oncogenic activities, including enhanced cell motility

and invasion [139,141]. Independent of its RGD sequence, IGFBP2 can also localize to the cell surface by interacting with integrin αvβ3, which results in inhibition of IGF-mediated breast cancer cell migration and reduction in tumor growth [101]. IGFBP2 is overexpressed and secreted in similar amounts in both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC); however membrane-associated IGFBP2 is predominant in SCLC, while in NSCLC relies on secreted IGFBP2 to regulate IGF actions [92].

In addition to cell surface localization, IGFBP2 can also localize intracellularly. Research using a CMV-IGFBP2 transgenic mouse model demonstrated peri-nuclear IGFBP2 localization [157]. Peri-nuclear localization of IGFBP2 was also detected in rhabdomyosarcoma cells [118]. No studies to date have established the role of peri-nuclear IGFBP2. Besides peri-nuclear localization, IGFBP2 is also detected in the nucleus. IGFBP2 is localized to the nucleus in normal astrocytes [104]. Research using mouse lung epithelial cells demonstrated that IGFBP2 binds to $p21^{CIP1/WAF1}$ and co-localizes in the cytoplasm and the nucleus, leading to growth inhibition [158]. Terrien et al demonstrated that serum deprivation induced IGFBP2 expression increase, along with IGFBP2 nuclear localization and $p21^{CIP1/WAF1}$ induction, thus resulting in growth inhibition. In lung adenocarcinoma, IGFBP2 was detected in the nucleus upon exposure to oxidants [154]. In prostate cancer, IGFBP2 was demonstrated to co-localize and bind with PAPA-1, a transcription factor related to growth inhibition, in the nucleus [159]. Recent studies by Azar et al identified a monopartite nuclear localization signal (NLS) at 179PKKLRP184 of IGFBP2, which overlaps with the HBD [160]. Azar et al demonstrated that importin α interacts with IGFBP2 at the NLS to shuttle IGFBP2 into the nucleus [160]. Furthermore, nuclear localization of IGFBP2 in neuroblastoma cells lead to enhanced *VEGF* promoter activity and subsequently, angiogenesis [124]. Besides promoting VEGF activation, the tumorigenic functions of nuclear IGFBP2 have yet to be elucidated.
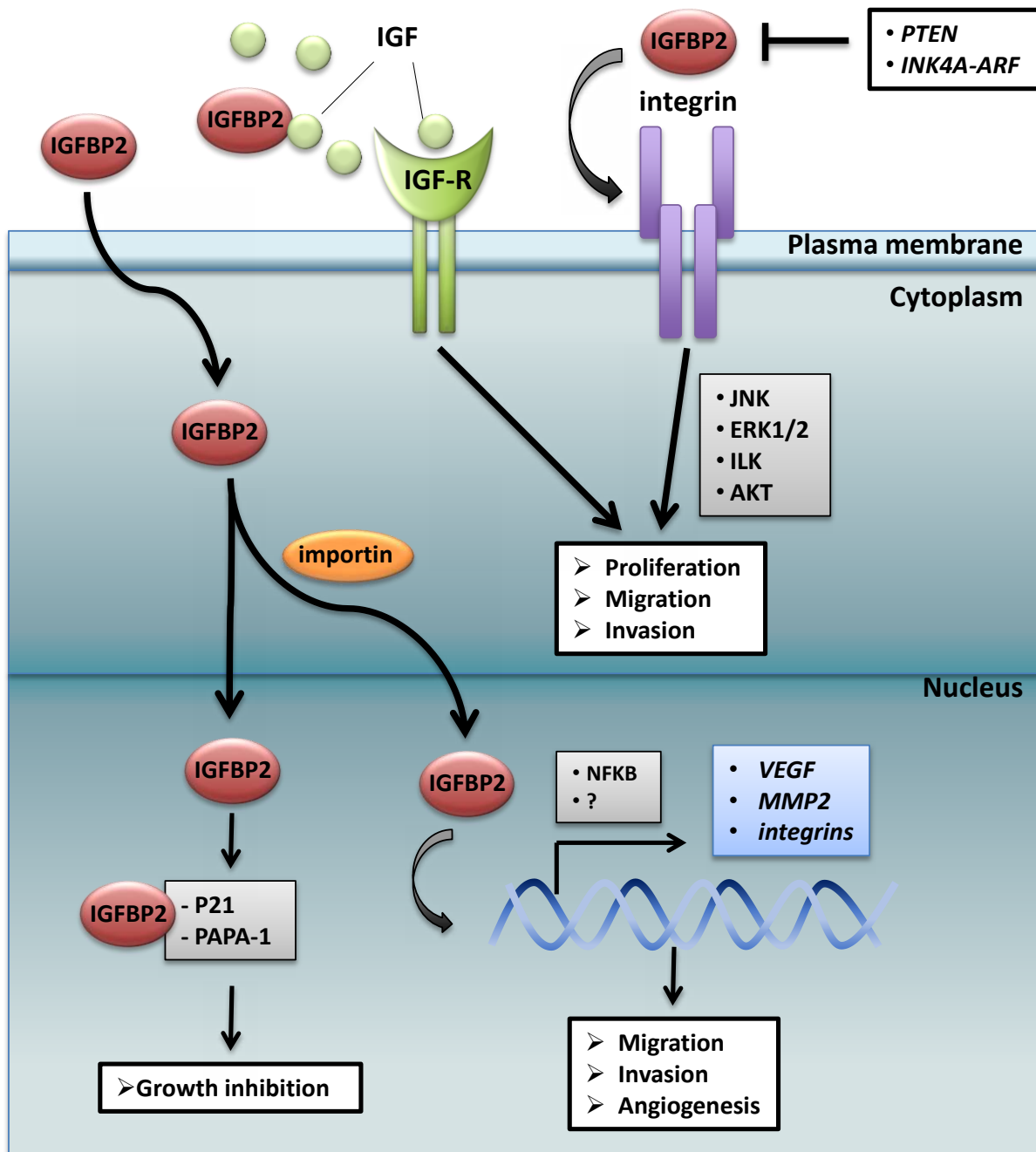
Figure 2. IGFBP2 functions in the cell. IGFBP2 is a pleiotropic protein with extracellular and intracellular functions. Extracellular IGFBP2 binding to IGF, can either inhibit IGF-mediated mitogenic signaling by preventing binding to its receptor, or potentiate IGF-mediated signaling by transporting IGF to its receptor. IGFBP2 binding to integrin can also activate JNK, ERK1/2, ILK, AKT, and NFKB, leading to enhanced proliferation, migration and invasion. IGFBP2 through its nuclear localization signal can bind importin and translocate into the nucleus. Nuclear IGFBP2 can bind to p21 or PAPA-1 to inhibit growth. However, nuclear IGFBP2 is also known to activate transcription of *VEGF*, *MMPs* and integrins. Other than that, there is limited knowledge on the tumor-promoting functions of nuclear IGFBP2.

Epidermal growth factor receptor

Introduction. The epidermal growth factor receptor (EGFR)/HER1/ErbB1 is a cell-surface receptor tyrosine kinase (RTK) belonging to the ErbB family. Other members of the ErbB family are HER2 (ErbB2/neu), HER3 (ErbB3), and HER4 (ErbB4). EGFR is the first RTK to be discovered, characterized as a ligand-binding cell surface protein with tyrosine kinase activity, and linked to human cancers [161,162]. EGFR family members have an extracellular ligand-binding domain, a single pass transmembrane domain, and a conserved cytoplasmic protein tyrosine kinase domain. EGFR members can form homo- and heterodimers that are activated by different ligands. HER2, an orphan receptor with no known ligand, is the preferred binding partner of other EGFR family members [163], while HER3 kinase domain is catalytically inactive and relies on heterodimerization with other EGFR members for signal transduction [164].

EGFR and its ligands. EGFR can be canonically activated by binding of ligands: EGF, transforming growth factor α (TGFα), heparin-binding EGF-like growth factor, amphiregulin, betacellulin, epiregulin, and epigen [165]. These ligands have a conserved EGF motif (CX7 CX4–5 CX10–13 CXCX8 C) [166], although recently identified non-canonical EGFR ligands, prolidase C and CCN2 lack this motif [167,168]. EGF ligands are anchored on the plasma membrane and require proteolytic cleavage by metalloproteases to release mature, soluble ligands, a process termed ectodomain or ligand shedding [166]. Metalloproteases (or "sheddases) involved in EGFR ligand shedding include a disintegrin and metalloproteases (ADAMs) or matrix metalloproteases (MMPs) [165]. Ligand shedding of EGFR ligands allows for mainly paracrine and autocrine EGFR signaling, although it is reported that ADAM17 (also known as tumor necrosis factor (TNF) alpha converting enzyme (TACE)) is required for juxtacrine signaling of TGFα/EGFR [169]. ADAM10 and ADAM17 are the main ADAMs for

EGFR ligands shedding [170]. ADAM17 has a key role in activating EGFR signaling [169]. Mice lacking ADAM17 display similar phenotype to mice lacking EGFR, TGFα, and HB-EGF [170].

EGFR and its ligands are important for skin, hair follicle, bone and female reproductive system development [166]. EGFR expression in the brain is high during embryonic development and peaks peri-natally; in adult brain, expression is markedly diminished and limited to the neural progenitors cells in the subventricular zone [171]. EGFR ligands, EGF, TGFa and HB-EGF, are constitutively expressed throughout the developing and adult brain [171,172]. In the adult brain HB-EGF and TGFα expression are ubiquitous, with HB-EGF more abundantly expressed than TGF-a; while EGF is lowly expressed in comparison, and expression is limited to discrete areas in the brain [172-174]. EGF can stimulate neural stem cell division and differentiation, while EGFR activation is required for neural stem cell expansion and survival [171]. EGF, HB-EGF and TGFa are key mitogenic and pro-survival factors for glial cells and astrocytes, and key maintenance factors for neurons [175]. Similar to IGFBP2, in response to brain injury, EGFR and its ligands are upregulated specifically in reactive astrocytes, microglial cells and neurons to aid in brain repair [171].

EGFR signaling pathways. Ligand binding at the extracellular domain of EGFR causes a conformational change to the extended stabilized form, inducing dimerization [176]. Dimerization then triggers the activation of intrinsic tyrosine kinase to auto-phosphorylate tyrosine residues on the cytoplasmic tail by catalyzing the transfer of the γ-phosphate of bound ATP to the other tyrosine residues [177]. Phosphorylated (activated) EGFR provides docking sites for recruiting effector proteins with Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains, thereby initiating activation of several downstream signaling pathways [177,178]. The docking sites on EGFR of which includes tyrosine (Y) residues Y920, Y992, Y1068, Y1086, and Y1173, can mediate activation of different signaling pathways [177,179]. Adaptors Grb2 (docking site at Y1068 and Y1086) and Shc (Y1148 and Y1173) mediate MAPK

signaling, Grb2 and docking protein Gab1 (Y1068 and Y1086) mediate P13K/AKT signaling, while Phospholipase-gamma C directly binds at Y992 and Y1173 [177,180]. Signal transducer and activator of transcription 3 (STAT3) via its SH2 domain binds directly to EGFR at Y1068 and Y1086 [181,182], leading to phosphorylation of STAT3 at Y705 (pSTAT3 Y705). E3 ubiquitin ligase Cbl (for Casitas B-lineage lymphoma proto-oncogene) binding negatively regulates EGFR through receptor internalization and subsequent lysosomal degradation [183,184]. Cbl can directly bind to phosphorylated EGFR at Y1045 or indirectly through association with Grb2.

EGFR activation results in signal transduction that leads to cell migration, proliferation and survival [177]. In normal tissues, EGFR and its ligands availability is tightly regulated and only activated to regulate tissue development and maintain homeostasis [185]. Aberrant activation of EGFR pathway ultimately leads to oncogenesis. Many *in vitro* studies demonstrated that overexpression of EGFR along with its ligands, EGF or TGFa can induce neoplastic transformation of fibroblasts and mammary epithelial cells [186].

EGFR in cancer. EGFR is one of the most commonly amplified and overexpressed receptor tyrosine kinase in human cancers [187]. EGFR is overexpressed in various cancers including head and neck cancer, breast cancer, renal cell carcinoma, non-small cell lung cancer (NSCLC), colon cancer, ovarian cancer, and gliomas [176]. In NSCLC, EGFR is overexpressed in about 60% cases and correlates with poor prognosis, and the tyrosine kinase domain is frequently mutated, resulting in constitutively active EGFR signaling [188].

EGFR in gliomas. EGFR is a signature oncogene of high-grade glioma; a recent study by the TCGA GBM Analysis Working Group reported 57% patients have *EGFR* alterations (including amplification and mutation), which is also associated with significant upregulation of EGFR protein expression and phosphorylation [39]. EGFR amplification and overexpression is

more commonly detected in primary GBM, and rarely in secondary GBM [48]. EGFR amplicons are frequently mutated, with EGFR variant III (EGFRvIII) mutation being the most prevalent form [48]. EGFRvIII is an in-frame deletion of exons 2-7 (which codes for a portion of the ligand binding domain) that results in constitutive activation of EGFR signaling [189]. EGFRvIII, also known as termed ΔEGFR, is prevalent in 50% of HGGs with EGFR amplification or overexpression [183,190]. A study by Fan et al demonstrated that some GBM cells co-express wildtype EGFR (wtEGFR) and EGFRvIII; in these cells, EGFR phosphorylates EGFRvIII and together subsequently activates STAT3 [191]. They also demonstrated that co-expression of wtEGFR and EGFRvIII synergistically enhanced cellular transformation *in vitro* and promoted tumor growth in nude mice, more potently than expression of the receptors alone. This EGFR-EGFRvIII-STAT3 signaling axis confers a more aggressive phenotype in GBM. Other EGFR deletion mutations include EGFRvI and EGFRvII mutations occur in the N-terminal, whereas EGFRvIV and EGFRvV mutations are in the C-terminal [183]. In 14% of GBMs, EGFR point mutations occur in the C-terminal locking the receptor in an open and active conformation [183].

Besides activating mutations, EGFR can be aberrantly activated by ligand stimulation. EGFR ligands (EGF, TGF-α, and HB-EGF) which stimulate the activation of EGFR, are increased concurrently with EGFR or EGFRvIII overexpression in gliomas [175,183,192,193]. ADAM17, the primary EGFR ligand sheddase involved in EGFR transactivation, is overexpressed in GBM and promotes GBM progression by enhancing proliferation, invasion, and angiogenesis [194]. ADAM17 also promotes GSC migration and invasion potential through activation of EGFR/AKT or EGFR/ERK signaling [195].

EGFR activation leads to the recruitment of PI3K to the cell membrane, which then activates AKT (Protein kinase B) or mTOR (mammalian target of rapamycin) signaling. EGFR/PI3K/AKT or mTOR signaling increases glioma cell proliferation and survival [183]. PTEN can suppress this signaling axis, however in GBMs, PTEN is mutated in 15 to 40% cases,

resulting in inactivation and loss of function [196]. GBM patients with concurrent EGFR and p53 alterations are significantly associated with worse survival [197]. Another major downstream pathway of EGFR activation is STAT3. EGFR is frequently expressed concurrently with constitutively activated STAT3 in HGGs [198]. Furthermore, a study of glioma patient samples demonstrated that STAT3 and AKT activation positively correlated with EGFR expression in high-grade gliomas [199]. Both EGFR and EGFRvIII can interact with STAT3 in the cytoplasm to mediate signal transduction, or in the nucleus to regulate gene transcription (nuclear EGFR activities will be discussed in the following section).

EGFR localization. Upon activation, EGFR is internalized and trafficked into endosomes via clathrin-mediated or clathrin-independent endocytosis [176]. In the endosomes, EGFR is recycled back to the membrane or to the lysosome for degradation, depending on the ligand. EGF stimulation generally leads to lysosomal degradation while TGF-α stimulation typically leads to receptor recycling [200]. Although controversial, there is accumulating evidence that endosomal EGFR can mediate signaling. Endosomal EGF-EGFR complex maintains its phosphorylated state and remains bound to adaptor proteins such as Grb2 and Shc [201]. An *in vitro* study using MDCK (canine kidney epithelial cells) and BT20 (breast cancer) cells showed that upon EGF stimulation, endosomal EGFR, which remained activated for 2 hours, activated Ras, ERK, and Akt, leading to enhanced cell survival [202]. Conversely, endocytosis can negatively regulate EGFR signaling; endosomal EGFR was also demonstrated to induce apoptosis, a protective feedback mechanism to restrict uncontrolled proliferation and maintain homeostasis [203]. Furthermore, EGF stimulation or apoptotic stimuli can also stimulate internalized EGFR (and EGFRvIII) to translocate the mitochondria and interact with cytochrome c oxidase (Cox) subunit II, leading to enhanced cell survival [204-206].

Alternatively, endosomal EGFR can also be shuttled into the nucleus in cooperation with nuclear transport receptors, importin α and importin β1 [207,208]. EGFR has a tripartite nuclear

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localization signal ^{645}*RRR*HIV*RKR*TL*RR*^{657} within the juxtamembrane region that interacts with importins [209]. Nuclear EGFR was first detected in hepatocytes during liver regeneration and in human adrenocortical carcinomas [210]. EGF and TGF-a were also found to be localized in the nucleus of proliferating hepatocytes [207]. Later, nuclear EGFR was reported in other highly proliferative cells and tissues including placenta, uterus of pregnant mice, and basal cells of normal mouth mucosa [211]. Nuclear translocation of EGFR can be mediated by ligand stimulation, irradiation, heat shock, H2O2 and cisplatin treatment [207]. In tumors, nuclear EGFR has been detected in thyroid follicular carcinoma [212], breast carcinoma [213], esophageal squamous cell carcinoma [214], ovarian cancer [215], non-small cell lung cancer [216] and glioma [217], and correlates with poor survival. In the nucleus, EGFR functions as a transcriptional co-activator with other transcription factors, as a nuclear tyrosine kinase to phosphorylate nuclear proteins, and also as a modulator of DNA repair [218].

Despite the lack of DNA binding domain, nuclear EGFR can promote gene transcription through its transactivation domain in its C-terminus and interaction with DNA-binding transcription factors [211]. Nuclear EGFR forms a complex with RNA helicase A (RHA) to bind to AT-rich sequences (ATRS) in the promoter of target genes, such as *CCND1* (cyclin D1) and *INOS* (inducible nitric oxide synthase; iNOS) [219]. Additionally, nuclear EGFR can also partner with STAT3 transcription factor to promote gene transcription of *COX2* (cyclooxygenase 2) in gliomas [220], *INOS* in breast cancer[221] and *CMYC* in pancreatic cancer[222]. Nuclear complex containing EGFR and STAT5 can bind the ATRS and activate gene transcription of Aurora-A, a serine/threonine kinase important for cell cycle progression, survival and neoplastic transformation [223]. Both constitutively activated EGFR and overexpression of Aurora A are known to be involved in chromosomal instability, thus the discovery of EGFR-STAT5-Aurora A axis provides insights into the mechanism. Furthermore, nuclear EGFRvIII-STAT5a/STAT5b complex can activate *Aurora-A* transcription, while nuclear EGFRvIII-STAT5b can activate *Bcl-*

xL transcription [224]. Nuclear EGFRvIII-cMyc complex were also discovered in the nucleus and contributed to oncogenic phenotypes [225].

Acting as a tyrosine kinase in the nucleus, EGFR can bind and phosphorylate chromatin-bound proliferating cell nuclear antigen (PCNA), leading to PCNA stabilization and DNA damage repair, and cell proliferation [226]. In addition to association with PCNA to facilitate DNA repair, EGFR translocates into the nucleus and interacts with DNA-protein kinase (DNA-PK) upon ionizing radiation to initiate repair of DNA-strand break repair [227]. Anti-EGFR monoclonal antibody called C225 or cetuximab, triggers interaction between EGFR and DNA-PK, but decreases nuclear DNA-PK activity [228]. Another study showed that cetuximab can inhibit radiation-induced EGFR nuclear transport and subsequent DNA-PK activity resulting in impaired DNA repair [229]. Overall, nuclear EGFR is demonstrated to be involved in resistance to cetuximab, gefitinib, radiation and chemotherapy [209].

Increasing evidence demonstrate the tumor-promoting functions of nuclear EGFR, however the mechanisms that regulate EGFR nuclear localization and functions remain elusive. In breast cancer, MUC1 (mucin-1) was demonstrated to interact with EGFR and regulate EGFR trafficking to the nucleus [230]. However the mechanisms that regulate nuclear transport of EGFR in other cancers, including gliomas, have yet to be elucidated. The presence of nuclear EGFR correlates with poor survival of cancer patients, therefore it is important to understand the mechanisms that regulate aberrant EGFR nuclear trafficking and nuclear functions.
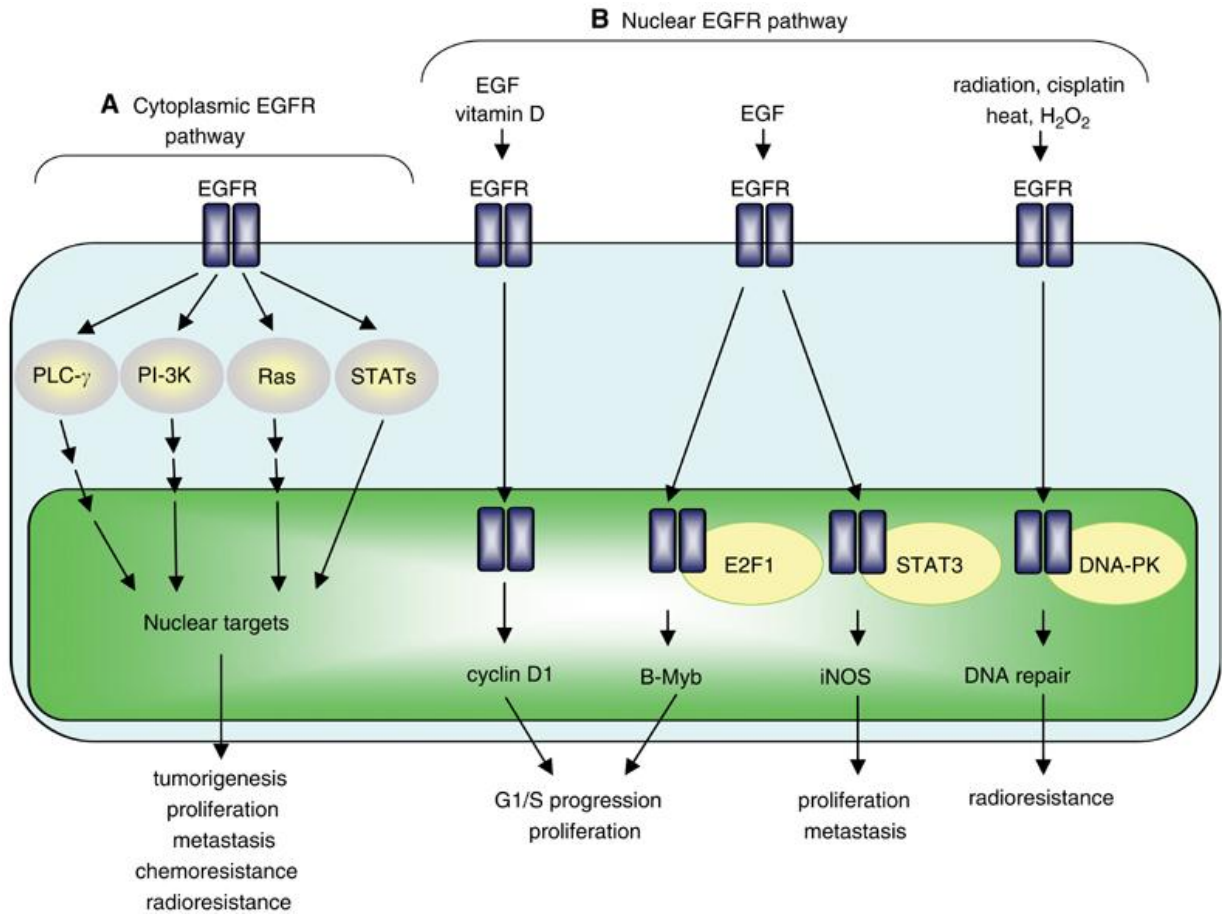
Figure 3. Cytoplasmic and nuclear EGFR signaling. Originally published by HW Lo and MC Hung. Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival. Br J Cancer. 2006;94(2):184-188. [207]. Used with permission.

Signal transducer and activator of transcription

Introduction. Signal transducer and activator of transcription (STAT) is a family of latent transcription factor consisting of 7 members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 [231]. STATs primarily mediate signaling downstream of cytokine and growth factor receptors, by transducing signals through the cytoplasm and transcriptional regulation in the nucleus. STAT protein structure consists of the N-terminus, followed by the coiled-coiled domain (for protein-protein interaction), DNA-binding domain, linker domain (unknown function), SH2 domain (for dimerization with other STATs) and the transactivation domain (contains binding sites for transcriptional co-regulators), and the C-terminus [231].

Among the STATs, STAT3 is the most thoroughly studied in development and oncogenesis, because it is crucial for regulating the transcription of genes involved in cell proliferation, apoptosis, angiogenesis, immune responses, invasion and metastasis [232]. Mouse model studies demonstrated that unlike all the other *stat* genes, only targeted deletion of stat3 causes early embryonic lethality, indicating the importance of STAT3 in normal development [233,234]. STAT3 is key for maintaining tissue homeostasis in the intestine, skin, thymus, and is also important for wound healing and mammary development [235,236]. STAT3 is expressed in the developing CNS [237]. In the developing brain, STAT3 is crucial for maintenance of neural stem cells, neuronal cell survival, and glial and astrocytic differentiation [231,237-239]. STAT3 activation also occurs in reactive astrocytes, activated microglia, and neurons in neurodegenerative diseases such as Alzheimer's disease or in response to CNS injury [238,240-242].
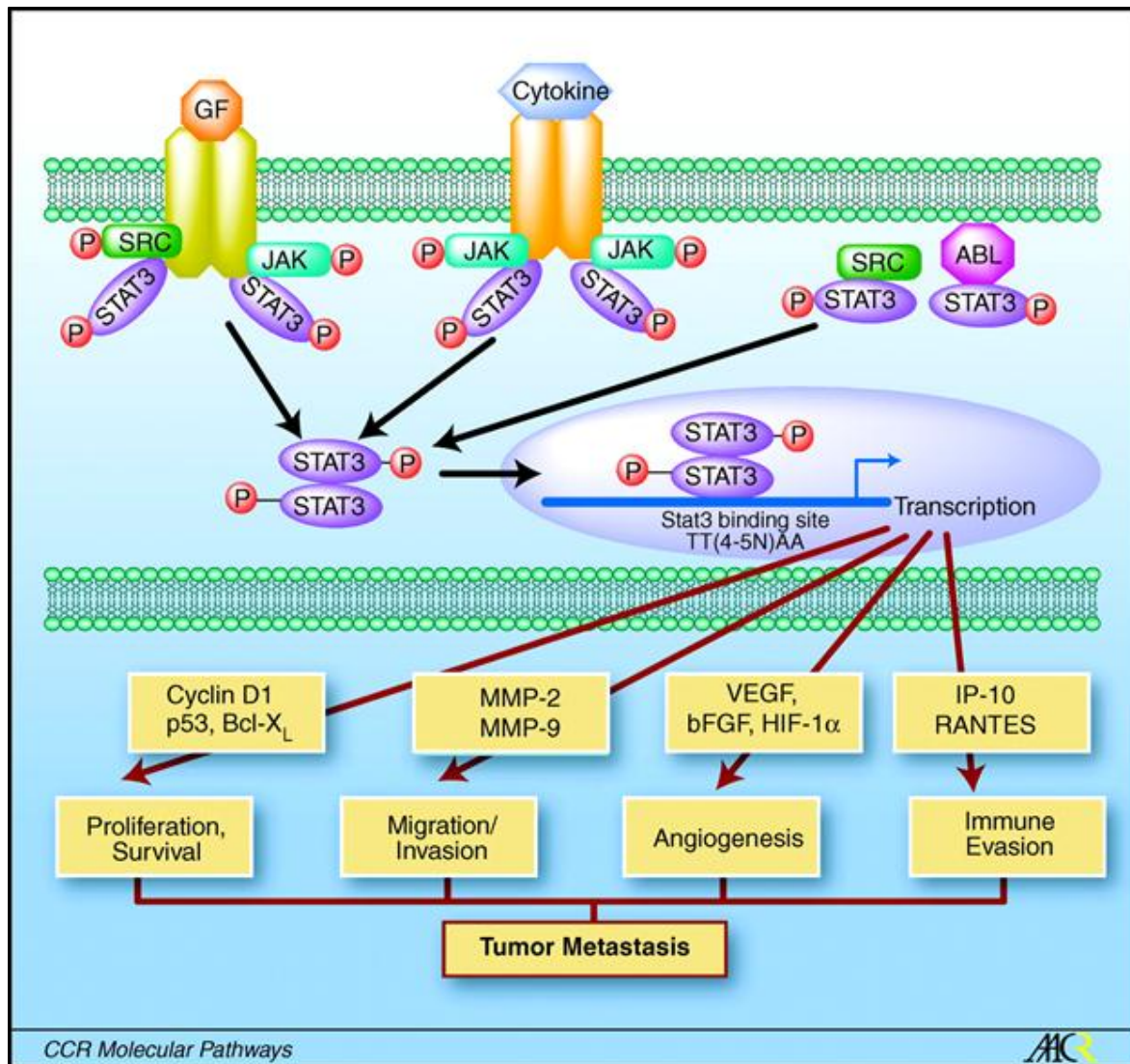
Figure 4. STAT3 signaling pathway in cancer. Originally published by SY Huang. Regulation of metastases by signal transducer and activator of transcription 3 signaling pathway: clinical implications. Clin Cancer Res. 2007;13(5):1362-1366. [152]. Published with permission.

Mechanisms of STAT3 activation. Cytokine receptors, Janus kinases (JAKs), and receptor tyrosine kinases are the main proteins that lead to activation of STATs by phosphorylation. One major mechanism of STAT3 activation is by way of cytokines: interleukin (IL) cytokines signal through a dual receptor system comprising IL-receptor (IL-R), which lacks intrinsic kinase activity, and the signal transducing component, gp130 (also known as CD130) [243]. IL binding to the IL-R/gp130 complex recruits JAK to gp130, resulting in JAK phosphorylation. Activated JAK in turn phosphorylates tyrosine kinase residues in gp130 C-terminus, which become docking sites for STAT3, leading to STAT3 phosphorylation. STAT3 can also be activated through receptor tyrosine kinases (RTKs), including epidermal growth factor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR), and non-receptor tyrosine kinases, src and abl. In addition to directly binding to RTKs such as in the case of EGFR (as mentioned previously), STAT3 can be activated by binding to JAK or src, which is bound on RTK [244].

STAT3 activation primarily occurs by phosphorylation of tyrosine residue 705 (Y705) located in the SH2 domain, which is required for STAT3 dimerization and DNA-binding function [232]. Serine/threonine kinases such as MAPK (p38MAPK, ERK, JNK), PKCδ, and mTOR, can phosphorylate STAT3 at serine position 727 (S727) located in the transactivational domain [234]. Environmental stress or inflammatory cytokines can also induce phosphorylation of S727, but not Y705 [245]. The functions of S727 remain controversial; most research established a positive role of S727, demonstrating that it is necessary for STAT3 enhanced transcriptional activity [234]. On the contrary, some studies suggest that S727 phosphorylation inhibits Y705 phosphorylation, leading to inhibition of STAT3 DNA binding and transcriptional activation [245,246]. Thus, S727 can have either a positive or negative regulatory role depending on cell-type or stimulus. Phosphorylation of Y705 and S727 differentially regulate mouse embryonic stem cell (mESC) fates: leukemia inhibitory factor (LIF)-mediated STAT3 phosphorylation at

Y705 is indispensable for mESC self-renewal, while Fgf/Erk-mediated phosphorylation at S727 is essential for promoting mESC differentiation and neuronal commitment [247]. Conversely in human ESC, LIF/STAT3 signaling was insufficient to maintain self-renewal and pluripotency state despite induction of STAT3 phosphorylation at Y705 and modest induction of Y727 [248].

Non-canonical STAT3 activity can also occur independent of phosphorylation and direct DNA binding [249]. Yang et al demonstrated using STAT3 Y705F (Tyr705 → Phe705) mutant that cannot be phosphorylated at Y705, unphosphorylated STAT3 in response to IL6 stimulation can cooperatively bind to NFKB and mediate gene transcription of *RANTES*, an important mediator of inflammation [250]. Unphosphorylated STAT3 can also bind to the promoter of several pro-apoptotic genes including *FOS*, to suppress transcription in tumor cells [251]. In a separate study, STAT3 was demonstrated to localize in the mitochondria independent of tyrosine phosphorylation, SH2 domain and DNA-binding domain, to support Ras-mediated malignant transformation [252]. However, this mitochondrial STAT3 localization remains controversial [249].

STAT3 can transcriptionally induce its own inhibitor, the suppressor of cytokine signaling 3 (SOCS3) of the SOCS family [253]. Besides binding to gp130 and promoting the degradation of gp130 complex, SOCS3 through its SH2 domain can bind to the kinase inhibitory region of JAK and inhibit JAK-mediated STAT3 activation[234,253]. Furthermore, SOCS3 can also compete with STAT3 SH2 domains for binding to receptor [234]. Protein tyrosine phosphatases (PTPs) and protein inhibitors of activated STATs (PIAS) can also deactivate STAT3. PTPs dephosphorylate STAT3 while PIAS block DNA-binding activity or recruit transcriptional corepressors [234].

STAT3 target genes. Upon STAT3 tyrosine phosphorylation (thereby activation), STAT3 forms homo- or hetero- dimers through reciprocal phosphotyrosine–SH2 interactions

and translocate to the nucleus to regulate gene transcription [243]. Unphopshorylated STAT3 has also been frequently demonstrated to localize in the nucleus, although its role is not well established yet [254]. STAT3 nuclear import occurs through NLS located in the coiled-coiled domain and importin-α3/importin-β1-Ran-mediated active transport, independent of tyrosine phosphorylation [249,254].

In the nucleus, although STAT3 primarily dimerizes, it can also cooperate with C/EBPβ, NFKB, activator protein 1, and glucocorticoid receptor to regulate genes [243]. STAT3 activates genes involved in cell cycle regulation (cyclin D1, c-Myc), angiogenesis (vascular endothelial growth factor/VEGF), migration and invasion (MMP-2 and MMP-9), and anti-apoptosis (survivin, Mcl-1, and Bcl-XL) [255-257]. STAT3 is also a key mediator of cancer inflammation, as it upregulates crucial inflammatory genes [258].

STAT3 directly binds to the promoter region of *VEGF*, a critical neovascularization factor that promotes angiogenesis [255,259]. Accordingly, STAT3 activation and VEGF expression were significantly correlated in breast cancer, head and neck carcinoma, melanoma and pancreatic cancer [260]. STAT3-VEGF signaling axis is not limited to cancer cells, as it was also observed in endothelial cells of the tumor microenvironment, ultimately leading to angiogenesis and metastasis [234,260,261]. MMPs upregulated by STAT3 activation promote cancer cell migration and invasion by degradation of various extracellular matrix proteins, thereby facilitating metastasis [257,260].

STAT3 in cancer. Among the STAT proteins, constitutively activated STAT3 is most frequently detected in majority of human cancers [234]. To investigate the role STAT3 in oncogenesis, Bromberg et al created a constitutively activated STAT3 (STAT3-C) plasmid by mutating two key cysteine residues (A661C and N663C) to allow formation of disulfide bridges in SH2 domains, thus generating spontaneous STAT3 homodimers independent of stimulation

[262]. This study led to the discovery of STAT3 as an oncogene, because STAT3 by itself could induce malignant transformation of fibroblasts in soft colony formation assay and mediate tumor formation in nude mice tumor xenografts [262].

Aberrant activation of STAT3 is detected in many cancers, including but not limited to, lymphoma, leukemia, brain, prostate, breast, lung, and colon cancers [263-265]. There is a positive correlation between elevated EGFR activation and STAT3 activation in many cancers [266,267]. EGFR/STAT3 signaling is constitutively activated in approximately 50% of early stage NSCLC resulting in suppressed apoptosis and enhanced tumor growth and survival [266]. Studies in lung adenocarcinoma demonstrated activated STAT3 (pY705) expression is positively correlated with the presence of somatic-activating EGFR mutations [268]. Somatic constitutively active STAT3 mutations have been detected in leukemia, lymphomas and also hepatocellular carcinoma [260]. These mutations occur in the SH2 domain, inducing amino acid changes that confer higher hydrophobicity to STAT3 dimerization surface, thus activating STAT3 by potentially facilitating phosphorylation at Y705 [260].

Depending on the genetic background of the cancer cells, STAT3 can either have an oncogenic or tumor-suppressive function. Although STAT3 is primarily linked to oncogenic functions, a few studies have demonstrated the tumor suppressive functions of STAT3 [260]. Loss of PTEN induces malignant transformation, however, STAT3 activation is able to suppress PTEN-loss induced transformation [269]. In contrast, STAT3 in cooperation with nuclear EGFRvIII in PTEN-deficient astrocytes can mediate malignant transformation [269]. Thus EGFRvIII acts as a switch to convert STAT3 from a tumor-suppressive to oncogenic protein. Furthermore, in p19ARF knockout background, STAT3 or STAT3C dramatically suppressed tumorigenecity in a SCID mouse xenograft model. Conversely, in p19ARF-positive Ras-transformed hepatocytes, STAT3 exhibited tumor-promoting functions by increased tumor formation in SCID mice [270]. In gastrointestinal cancers, STAT3 in cooperation with NFKB is

responsible for maintaining a pro-carcinogenic inflammatory microenvironment and inflammation-associated tumorigenesis [268]. However, NFKB also mediates activation of target genes involved in anti-tumor immune response, which is inhibited by STAT3 [268].

STAT3 in gliomas. STAT3 is constitutively active in 28.6% of LGGs and 60% of HGGs, while co-expression with EGFR occurs in 27% of HGGs [198]. STAT3 is a master regulator of the mesenchymal transformation of glioblastoma, whereby coexpression with C/EBPβ can reprogram neural stem cells towards an aberrant mesenchymal phenotype similar to HGG [271].

STAT3 is constitutively activated in GSCs and is important for proliferation, survival and maintenance of GSCs [272,273]. Enhancer of Zeste homolog 2 (EZH2), a lysine methyl transferase and a critical regulator for GSC maintenance, can bind and activate STAT3 via lysine methylation at K180 of STAT3 [274]. It is speculated that STAT3 methylation at K180 protects phosphorylated Y705 from dephosphorylation, and thus enhances its oncogenic activity. BMX (bone marrow X-linked), a nonreceptor tyrosine kinase, activates STAT3 signaling to maintain GSC self-renewal and proliferative potential and to upregulate essential GSC transcription factors [275]. An alternative route of STAT3 activation is through cytokine signaling: GBM cells and GSCs expressing EGFRvIII secrete elevated levels of IL-6 family cytokines, which then activates gp130 in neighboring tumors cells with wild-type EGFR, thus sustaining high STAT3 activation and thereby accelerating tumor growth [276]. IL6, a key modulator of STAT3, was demonstrated to be amplified, along with increased protein expression in GBM, compared to LGG [277].

STAT3 is negatively regulated by endogenous inhibitors. However, these endogenous inhibitors are frequently inactivated in gliomas. Expression of PIAS3, an inhibitor of STAT3, is reduced in GBM and is correlated with increased STAT3 activation and consequently, cell

proliferation [278] . Protein tyrosine phosphatase receptor delta (PTPRD), a STAT3 inhibitor, negatively correlated with STAT3-mediated tumorigenicity in GBM [277]. PTPRD is a tumor suppressor that is frequently mutated or inactivated by deletion or epigenetic silencing through CpG island hypermethylation in more than 50% of GBM tumors [279]. PTPRD knockdown using shRNA resulted in increased cell growth in immortalized primary human astrocytes in tumor xenografts on SCID mice [279].

Many *in vitro* and *in vivo* studies have demonstrated the oncogenic functions of STAT3 in glioma, whereby inhibition of STAT3 activation induces apoptosis and suppresses tumor proliferation and growth [280-282]. Interestingly, i*n vitro* inhibition of STAT3 resulted in sensitization of HGGs to anti-EGFR therapy or chemotherapy [198]. GBM cells are resistant to temozolomide-induced DNA damage due to elevated expression of the DNA repair enzyme, O6-methylguanine-DNA methyltransferase (MGMT) with concomitant upregulation of STAT3 [283]. Analysis of temozolomide-treated GBM patient samples revealed that MGMT and pSTAT3 (Y705) are positively correlated. Further investigation using small molecule STAT3 inhibitor VI to inhibit STAT3 dimerization and DNA-binding and shRNA to deplete STAT3 expression, led to downregulation of MGMT expression, indicating MGMT expression is STAT3-dependent in GBM. In this study, Kohsaka et al demonstrated that STAT3 inhibition potentiates temozolomide efficacy in temozolomide-resistant glioma cell lines, suggesting that STAT3 is a potential target for TMZ-resistant GBMs.

STAT3 activation is associated with upregulation of tumor-promoting factors in glioma [284] and inhibition of STAT3 results in suppressed proliferation and survival of glioma cells through decreased levels of proteins such as anti-apoptotic protein Bcl-xL [281]. *MMP2* and *VEGF* are known STAT3 target genes that are significantly and positively correlated with *IGFBP2* in GBM [285]. The VEGF signaling pathway is associated with IGFBP2 in glioma cells [141], and stable IGFBP2 overexpression in a GBM cell line also demonstrated increased

MMP2 expression [142]. An *in vitro* and *in vivo* study using glioma cell lines and human GBM tissues demonstrated that co-expression of EGFR and EGFRvIII is associated with increased pSTAT3(Y705) [191]. EGFR/EGFRvIII co-expressing GBM cells enhanced both nuclear transport of EGFRvIII, and phosphorylation of nuclear STAT3 [191]. Furthermore, this study demonstrated that both EGFR and EGFRvIII were able to form a complex with STAT3 in the nucleus. EGFR/STAT3 nuclear complex can activate gene transcription of COX2, iNOS and cMYC, resulting in enhanced oncogenic activity.

As STAT3 is constitutively activated in gliomas, it is important to understand the mechanisms that regulate STAT3 activation to better understand glioma pathogenesis and develop improved therapeutics.

SUMMARY

IGFBP2 is overexpressed in gliomas and is associated with poor clinical outcome. Despite the wealth of research linking IGFBP2 to glioma progression and poor prognosis, detailed mechanisms of the tumor-promoting functions are still poorly understood. While typically considered a secreted factor, IGFBP2 is pleiotropic and can be localized intracellularly, and its functions differ based on its localization. The intracellular mechanism of actions of IGFBP2, specifically nuclear functions, remains unclear. Similarly, EGFR, which is traditionally considered a cell surface receptor, is a dynamic protein with intracellular functions, particularly in the nucleus. In addition to functioning as an upstream activator of STAT3, EGFR can form a complex with STAT3 in the nucleus and activate the transcription of oncogenic genes. The significance of nuclear EGFR in tumor biology is increasingly evident, specifically the presence of a nuclear EGFR/STAT3 complex in glioma which promotes its aggressiveness, but regulators of this pathway have yet to be identified. **I hypothesize that IGFBP2 enhances EGFR and STAT3 signaling to promote tumorigenic activity in gliomas.** This hypothesis will be tested in 2 aims: 1) elucidate the link between IGFBP2, EGFR and STAT3, and 2) determine the functions of nuclear IGFBP2 in relation to nuclear EGFR and STAT3.
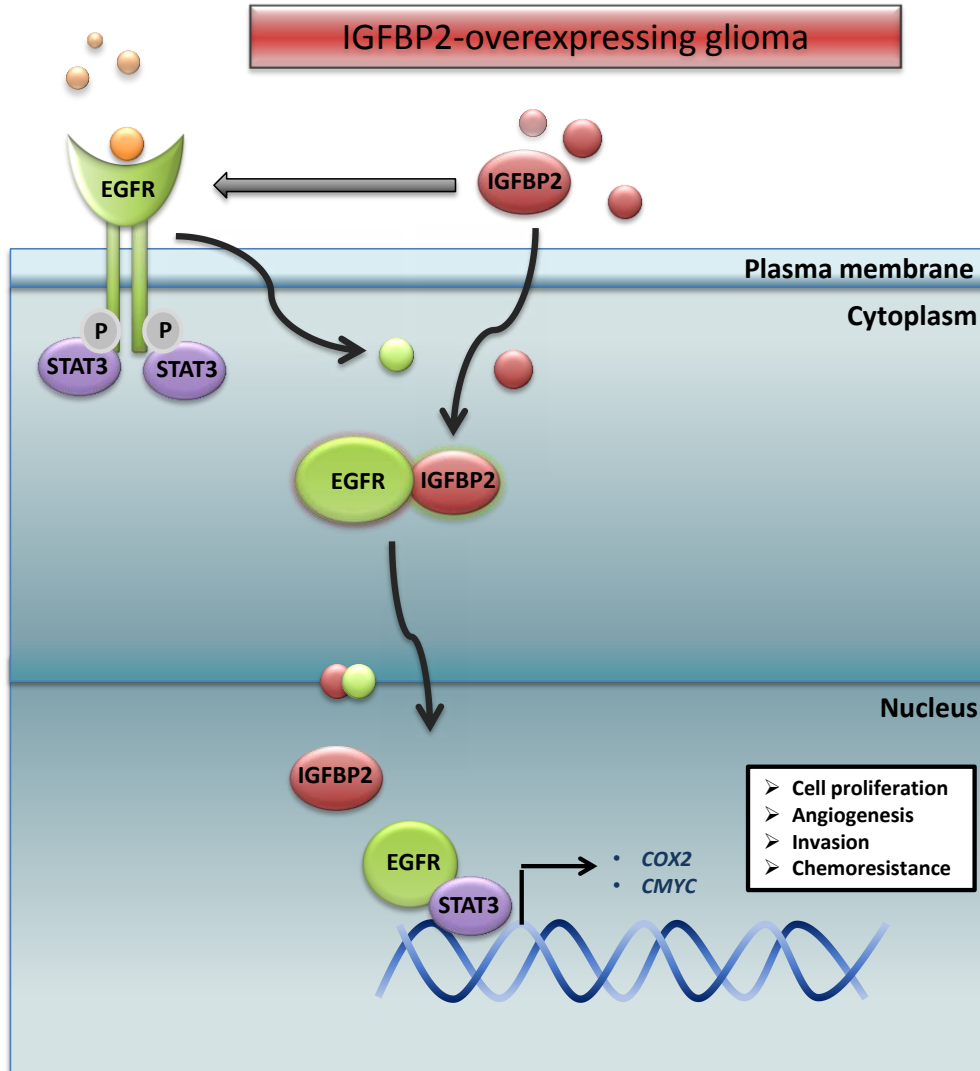
Figure 5. Proposed mechanism of IGFBP2-mediated EGFR/STAT3 signaling activation.
IGFBP2 regulates two mechanisms of EGFR signaling: activates intracellular EGFR-STAT3 signal transduction, and facilitates EGFR nuclear accumulation resulting in elevated nuclear EGFR/STAT3 activity. IGFBP2-mediated activation of cytoplasmic and nuclear EGFR-STAT3 signaling leads to enhanced oncogenic activity.

CHAPTER 2: MATERIALS AND METHODS

This chapter is based upon: Chua CY, Liu Y, Granberg KJ, Hu L, Haapasalo H, Annala MJ, Cogdell DE, Verploegen M, Moore LM, Fuller GN, Nykter M, Cavenee WK, Zhang W. IGFBP2 potentiates nuclear EGFR-STAT3 signaling. Oncogene. 2015. doi: 10.1038/onc.2015.131 [286].

Cell culture, treatments, plasmids and transfections

SNB19, U87 and T98G cells were obtained from ATCC (Manassas, VA). Cells were cultured in Dulbecco modified essential/F12 50:50 medium supplemented with 10% fetal bovine serum (FBS) and 5% penicillin/streptomycin in an incubator with 5% CO2 at 37□C. SNB19.EV (empty vector) and SNB19.BP2 WT (IGFBP2 wild-type) cells were created as previously described [50]. To generate BP2ΔNLS (IGFBP2 mutation at the nuclear localization signal), amino acid residues 179PKKLRPP185 of the IGFBP2 nuclear localization signal were mutated to 179PNNLAPP185 using the Quikchange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. A stable SNB19.BP2ΔNLS cell line was created by transfection of pcDNA3.1.IGFBP2ΔNLS plasmid via FuGENE HD (Promega, Fitchburg, WI) according to the manufacturer's protocol, followed by G418 selection for 3 weeks.

IGFBP2 stimulation experiments were performed by using recombinant IGFBP2 (ab63223; Abcam, Cambridge, MA) with cells starved of serum overnight. Depletion of IGFBP2 and EGFR was achieved via transfection of Lipofectamine RNAiMAX (Life Technologies, Grand Island, NY) according to the manufacturer's protocol with 2 different pools of siRNA from Mission siRNA (Sigma, St Louis, MO) for 48 hours. Some cells were treated with a broad-spectrum ADAM inhibitor, TAPI-2 (#14695; Cayman Chemical, Ann Arbor, MI) or marimatstat (#M2699; Sigma), at 20□M for 2 hours. Depletion of ADAM17 siRNA was achieved via transfection of Lipofectamine RNAiMAX according to the manufacturer's protocol with 2 different pools of siRNA from Life Technologies (#s13718 and #s13719).

Cell viability assay

Cells were seeded at 2000 cells/well (WP1066, Erlotinib) or 600 cells/well (TMZ) in quadruplicate in 96 well plates and allowed to attach overnight. Cells were subjected to treatment for 72 hours (WP1066, Erlotinib) or 5 days (TMZ). Cell viability was measured after 2 hour incubation with 0.5mg/mL MTT reagent and lysed with DMSO. Plates were read at 590nm using Tecan SpectraFluor Microplate Reader (Tecan Group Ltd.)

Bioinformatics analysis

GSEA. A total of 268 LGG samples obtained from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) were subjected to RNA sequencing. The gene expression data were median-centered and then transformed to log2 space. We calculated the correlation of IGFBP2 gene expression with all other genes in the genome and ranked the genes in descending order based on the correlation coefficients. Using the gene expression correlation as the ranking metric, GSEA was then used to calculate the score for the degree of enrichment of the genes with higher correlation coefficients among genes involved in the STAT3 signaling pathway [287].

TCGA RPPA analysis. In a similar manner to GSEA, the correlation of IGFBP2 or STAT3 protein expression with proteins in the TCGA TMA was calculated for 257 LGG samples for which reverse phase protein array (RPPA) data were available. Proteins that had higher correlation coefficients with both IGFBP2 and STAT3 proteins were considered the most likely candidates to represent molecular mechanisms underlying the association of IGFBP2 and the STAT3 signaling pathway.

Ingenuity Pathway Analysis. The interaction network feature of Ingenuity Pathway Analysis was used to determine direct downstream targets of STAT3. Interactions were filtered on the basis of their confidence level so that only interactions experimentally observed in

humans were included in the table of results. Interactions were also filtered by relationship type so that only interactions of type "expression" or "transcription" were included.

Hierarchical clustering. Hierarchical clustering of 157 experimentally validated STAT3 target genes were performed across all samples in the NCI Rembrandt public data repository (http://rembrandt.nci.nih.gov) [288], which has 329 tumors: 59 oligodendrogliomas, 102 astrocytomas, and 178 glioblastomas.

Immunoprecipitation, immunoblotting and cellular fractionation

For immunoprecipitation (IP), cells were subjected to lysis in NP-40 buffer with 0.1% phosphatase inhibitor cocktail (Pierce Biotechnology, Thermo Fisher Scientific, Waltham, MA). After preclearing for 1 hour at 4□C with Protein G agarose beads (SC#2002; Santa Cruz Biotechnology, Santa Cruz, CA) and appropriate species normal IgG, lysates were immunoprecipitated overnight at 4□C with Protein G agarose beads using antibodies to IGFBP2 (#SC-6001; Santa Cruz Biotechnology; 1:100) and EGFR (#2256; Cell Signaling Technology, Beverly, MA; 1:100). Beads were washed with NP-40 buffer 3 times and boiled in Laemmli buffer. Proteins from the IP experiment or extracted from cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) in running buffer and transferred onto an Immobilon TM-PVDF membrane (Millipore, Billerica, MA) for 1 hour at 100V in transfer buffer (24 mM Tris base, 191 mM glycine and 20% [v/v] methanol). Membranes were blocked for 1 hour at room temperature with 5% (w/v) non-fat milk powder in phosphate-buffered saline solution (PBS) with 0.1% Tween-20 (PBST) and incubated overnight at 4□C with primary antibody: IGFBP2 (#SC-6001; 1:500); EGFR (#4267; Cell Signaling Technology; 1:1000), EGFR-Y1068 (#3777; Cell Signaling Technology; 1:1000), beta-tubulin (#2128; Cell Signaling Technology; 1:1000), PARP (#9542; Cell Signaling Technology; 1:1000), STAT3 (#9139; Cell Signaling Technology; 1:1000), STAT3-Y705 (#9145; Cell Signaling Technology; 1:1000), Bcl-

xL (#2764; Cell Signaling Technology; 1:1000), cyclin D1 (#2978; Cell Signaling Technology; 1:1000), c-MYC (#SC-40; Santa Cruz Biotechnology; 1:1000), COX-2 (#160112; Cayman Chemical; 1:250), or ADAM17 (#T5442; Sigma; 1:500) in blocking solution. After washing in PBST, blots were incubated for 1 hour at room temperature in PBST with secondary antibodies (anti-goat IgG, anti-rabbit IgG, or anti-mouse IgG; Santa Cruz Biotechnology; 1:5000) couple to horseradish peroxidase (HRP). Immunoblots were incubated with enhanced chemiluminescence SuperSignal West Pico or Femto solution (Pierce Biotechnology). Cellular fractionation was performed by using the NE-PER nuclear and cytoplasmic kit (Pierce Biotechnology) according to the manufacturer's protocol. Densitometric analysis of immunoblot bands were quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, MD).

Confocal imaging

Cell on chamber slides were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and incubated with primary antibody to EGFR (#4267; 1:100) and IGFBP2 (#SC-6001; 1:100) at 4°C overnight. They were then incubated with secondary antibody (Life Technologies [Alexa Fluor]; 1:500) for 1 hour at room temperature in 1% bovine serum albumin/PBS buffer. They were mounted in Vectashield (Vector Laboratories, Burlingame, CA), and nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole, dihydrochloride). Immunofluorescence images were acquired by using an Olympus FV1000 Laser Confocal Microscope at 40x/NA 1.3 objective (stacking from basement membrane to apical site at 1μM intervals).

Tissue microarray construction and immunohistochemical analysis

Tumor samples were collected and the TMA comprising formalin-fixed, paraffin-embedded astrocytoma tissues was processed at Tampere University Hospital as described

previously [108]. Briefly, histologically representative tumor regions were selected by a neuropathologist (HH), and samples from these areas were placed in TMA blocks using a custom-built instrument (Beecher Instruments, Sun Prairie, WI). The diameter of the tissue cores in the microarray block was 1 mm. Altogether, 222 diffusely infiltrating astrocytomas (167 glioblastomas, 17 grade 3 astrocytomas, and 38 grade 2 astrocytomas) were included in the immunohistochemical analysis. For staining, 5-µm sections from TMA blocks were deparaffinized in xylene or hexane and rehydrated through an ethanol dilution series. Immunohistochemical staining was performed with goat antibodies against human IGFBP2 (#SC-6001; 1:300), phosphorylated STAT3 (#9145; 1:100), and EGFR (GR-01, Calbiochem, San Diego, CA; 1:50), together with the HRP-diaminobenzidine (DAB)–based Cell and Tissue Staining Kit (R&D Systems, Minneapolis, MN) or the Envision+System HRP-DAB kit (Dako, Carpenteria, CA).

Intensity of cytosolic expression levels of the proteins in tumor cells was manually quantified by using a scoring system from 0 to 3 (0 = no signal, 1 = weak signal, 2 = moderate signal and 3 = strong signal). The proportion of the cells with nuclear protein localization was manually classified into 4 categories: 0%, <10%, 10-30% and ≥30%. Intensity of nuclear expression levels in tumor cells was manually quantified by using a scoring system from 0 to 2 (0 = no signal, 1 = weak signal, 2 = strong signal). The TMA samples were examined and scored by 2 neuropathologists who were blinded to the clinical data. A survival association analysis of the patients from whom these samples were taken compared survival in patients with nuclear co-localization of all 3 proteins—IGFBP2, EGFR and phosphorylated STAT3 (≥1% cells with nuclear staining)—with survival of all the other patients. The survival data were analyzed by the log-rank test and visualized with a Kaplan-Meier plot. Statistical analyses were run with SPSS 20.0 software for Windows (SPSS Inc., Chicago, IL). The statistical significance of associations was evaluated by using the Pearson chi-square test.

Invasion and migration assays

The cell invasion assay was performed in triplicate in Matrigel-coated transwell chambers (8-μm pore size; (BD Biosciences, San Jose, CA). The cells were plated in 500 μL of serum-free medium (4×10^4 cells per transwell) and allowed to invade toward a medium containing 10% FBS for 16 hours. Cells that invaded into the underside of the filter were fixed and stained with HEMA-DIFF solution (Thermo Fisher Scientific). The numbers of invaded cells from 5 randomly chosen fields from each membrane were counted. The cell migration assay was performed the same way as the invasion assay, using transwell chambers (8-μm pore size, BD Biosciences) and the cells were allowed to migrate for 4 hours.

Statistical analysis

GraphPrism 6 (GraphPad, La Jolla, CA, USA) and SPSS 20.0 software for Windows (SPSS Inc., Chicago, IL) were used for statistical analysis and graphing. The Spearman correlation test was used to examine correlation between protein or phosphoprotein expression in the TCGA RPPA data set. The survival data were analyzed by the log-rank test and visualized with a Kaplan-Meier plot. The statistical significance of protein associations in the TMA data set was evaluated by using the Pearson chi-square test. Statistical test on GSEA was estimated as previously described [287]. Student t-tests were used for paired comparisons where variances were estimated to be similar. Except for one-side test for the GSEA analysis [287], all other tests were two-sided with $P<0.05$ as the threshold for statistical significance in all tests. Indicated annotations correspond to the following P-values: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$.

CHAPTER 3: RESULTS

Introduction

Secreted proteins such as growth factors and hormones exert their function by binding to the extracellular domain of membrane receptors. Secreted factors can also enter the cell through receptor-mediated endocytosis [289-292]. Once internalized, these proteins can regulate intracellular cytoplasmic signal transduction and transcriptional activity in the nucleus [211,293-298]. Insulin-like growth factor (IGF) binding protein 2 (IGFBP2) is a secreted protein that was initially characterized as binding and modulating the activity of IGF-I and -II [299-301]. IGFBP2 can also function independently of IGF binding, and its versatility as a secreted or cytoplasmic signaling effector has been widely characterized. IGFBP2 can bind integrins [101,139,141] and activate PI3K/AKT [146], NF☐B [141] and ERK [302]. Recently, a classic nuclear localization signal sequence that is responsible for nuclear entry has been identified in *IGFBP2* [124]. However, the functional and clinical significance of nuclear IGFBP2 has not been clearly elucidated [154,157-159].

In mammals, IGFBP2 is expressed at high levels in embryonic tissues, but the expression is drastically decreased after birth. Postnatally, IGFBP2 expression is observed in limited cell populations: hematopoietic stem cells and liver and spleen progenitor cells [77,78,82-84,303]. *IGFBP2* is reactivated during progression of a wide spectrum of cancer types, including glioma and prostate, breast and lung cancers [84,114,146,304]. IGFBP2 plays an oncogenic role in tumor initiation and progression to high-grade glioma [123] and is a signature gene associated with poor clinical outcome in high-grade glioma [305]. Furthermore,

IGFBP2 mediates cell expansion and survival of glioma stem cells [127]. Despite the clear role for IGFBP2 in tumorigenesis, the mechanisms underlying the contribution of intracellular IGFBP2 (particularly nuclear) to the tumorigenic program remain unknown.

EGFR/IGFBP2 and EGFR/STAT3 [198,306] are concurrently co-expressed in glioma. EGFR is activated in 30-50% of high-grade gliomas through amplification, overexpression or mutation [307-309]. EGFR signal transduction can be mediated by STAT3. STAT3 interacts with EGFR at 2 autophosphorylation sites in the cytoplasmic domain, tyrosine 1068 or tyrosine 1086 [181], and is activated by phosphorylation at tyrosine 705 (Y705) [310]. In addition to this cytoplasmic interaction, EGFR and STAT3, after translocation into the nucleus, can form a complex to activate transcription of genes such as *COX2* [220]*, iNOS* [221] and *c-MYC* [222]. Nuclear EGFR expression in glioma and other cancers, such as breast carcinoma [213], esophageal squamous cell carcinoma [214] and ovarian cancer [215], is associated with poor survival and linked to an aggressive tumor phenotype [207]. Furthermore, IGFBP2 regulates expression of the *VEGF, MMP2, TIMP1, TWIST, BCL2* and *HIF1A* genes [124,142], which are known transcriptional targets of STAT3. Recent research implicated nuclear IGFPB2 in angiogenesis through activation of *VEGF*, a STAT3 target gene [124]. These observations suggest that there is a functional connection between IGFBP2, EGFR and STAT3 in glioma. Here we tested this hypothesis and provide evidence that IGFBP2 mediates the tumorigenic program through a tightly linked IGFBP2-EGFR-STAT3 regulatory signaling network.

IGFBP2 activates the STAT3 signaling pathway via an EGFR-dependent mechanism

To explore the functional interaction between IGFBP2, EGFR and STAT3, we stimulated SNB19 parental (SNB19.par) glioma cells, which had been serum-starved overnight, with increasing amounts of exogenous IGFBP2 protein. Immunoblotting analysis demonstrated increased expression of both total EGFR and EGFR activated via phosphorylation at tyrosine

Y1068, or pEGFR(Y1068), in parallel with IGFBP2 stimulation (Fig. 6A). STAT3 activation via

phosphorylation at tyrosine 705, designated pSTAT3(Y705), and expression of STAT3

transcriptional targets Bcl-xL, cyclin D1 and c-MYC also increased in response to IGFBP2

stimulation.
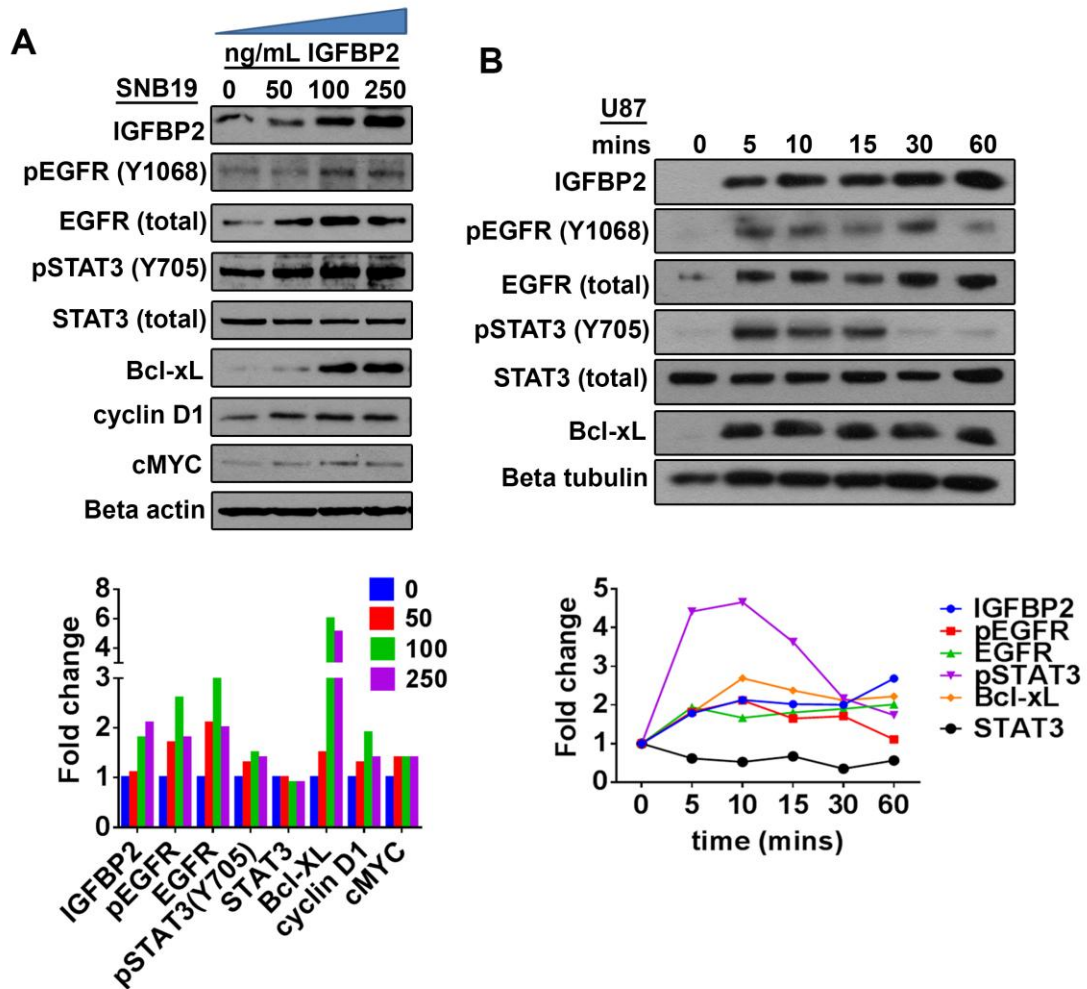
Figure 6. IGFBP2 activates EGFR-STAT3 signaling pathway. (**A**) Immunoblot analysis of SNB19 cells starved of serum overnight then stimulated with exogenous IGFBP2 protein at the indicated dosages (0, 50, 100, 250 ng/mL) for 60 minutes. Densitometric analysis shown below the immunoblot indicates fold-change relative to unstimulated control cells (normalized to beta-actin loading control or total protein for phosphorylated proteins). (**B**) Immunoblot analysis of U87 cells starved of serum overnight then stimulated with exogenous IGFBP2 (100ng/mL) for the indicated time points (0, 5, 10, 15, 30, 60 minutes). Densitometric analysis shown below the immunoblot indicates fold-change relative to unstimulated control cells (normalized to loading control or total protein for phosphorylated proteins).

Next, we performed a time-course experiment in which U87 glioma cells, which lack endogenous IGFBP2 expression, were stimulated with exogenous IGFBP2 after overnight serum-starvation. Immunoblotting analysis revealed induction of EGFR, STAT3 and Bcl-xL expression as early as 5 minutes following addition of exogenous IGFBP2 (Fig. 6B).

Furthermore, immunoblotting analysis of SNB19 cells stably overexpressing IGFBP2 (SNB19.BP2) demonstrated that, compared to SNB19 cells stably transfected with empty vector (SNB19.EV), IGFBP2 overexpression resulted in increased expression of EGFR and phosphorylated STAT3, along with Bcl-xL, cyclin D1 and c-MYC (Fig. 7A). To examine the involvement of EGFR in IGFBP2-mediated STAT3 activation, we depleted EGFR by using 2 different pools of small interfering RNA (siRNA) in SNB19.BP2 cells and observed decreases in STAT3 activation (Fig. 7B), supporting the hypothesis that IGFBP2 mediates STAT3 activation through EGFR. To rule out the possibility of off-target effects of EGFR siRNA-mediated knockdown, we knocked down EGFR in SNB19.BP2 cells and stimulated the cells with recombinant IL6. We observed STAT3 phosphorylation in these cells, confirming that EGFR knockdown impairs STAT3 activation by IGFBP2 without compromising alternate STAT3 activation pathways (Fig 8).

EGFR can be indirectly activated through transactivation, which involves a disintegrin and metalloproteinases (ADAMs) [169]. To determine whether ADAMs are involved in IGFBP2-mediated EGFR activation, we inhibited ADAMs by treatment with 2 different ADAM inhibitors, TAPI2 and marimastat [311,312]. U87 cells serum-starved overnight were pretreated with 20µM TAPI-2 or marimastat, then stimulated with exogenous IGFBP2 for 5 minutes (Fig. 9A). Immunoblotting analysis demonstrated that exogenous IGFBP2 stimulated EGFR and STAT3 activation despite ADAMs inhibition. Furthermore, because ADAM17 is essential to regulation of EGFR transactivation [169], we knocked down ADAM17 using 2 different pools of siRNA to evaluate whether IGFBP2-mediated EGFR activation involves ADAM17 (Fig. 9B).

Immunoblotting analysis showed that ADAM17 knockdown did not affect EGFR and STAT3

activation in SNB19.BP2 cells. These data demonstrate that ADAMs are not involved in

IGFBP2-mediated EGFR/STAT3 activation.
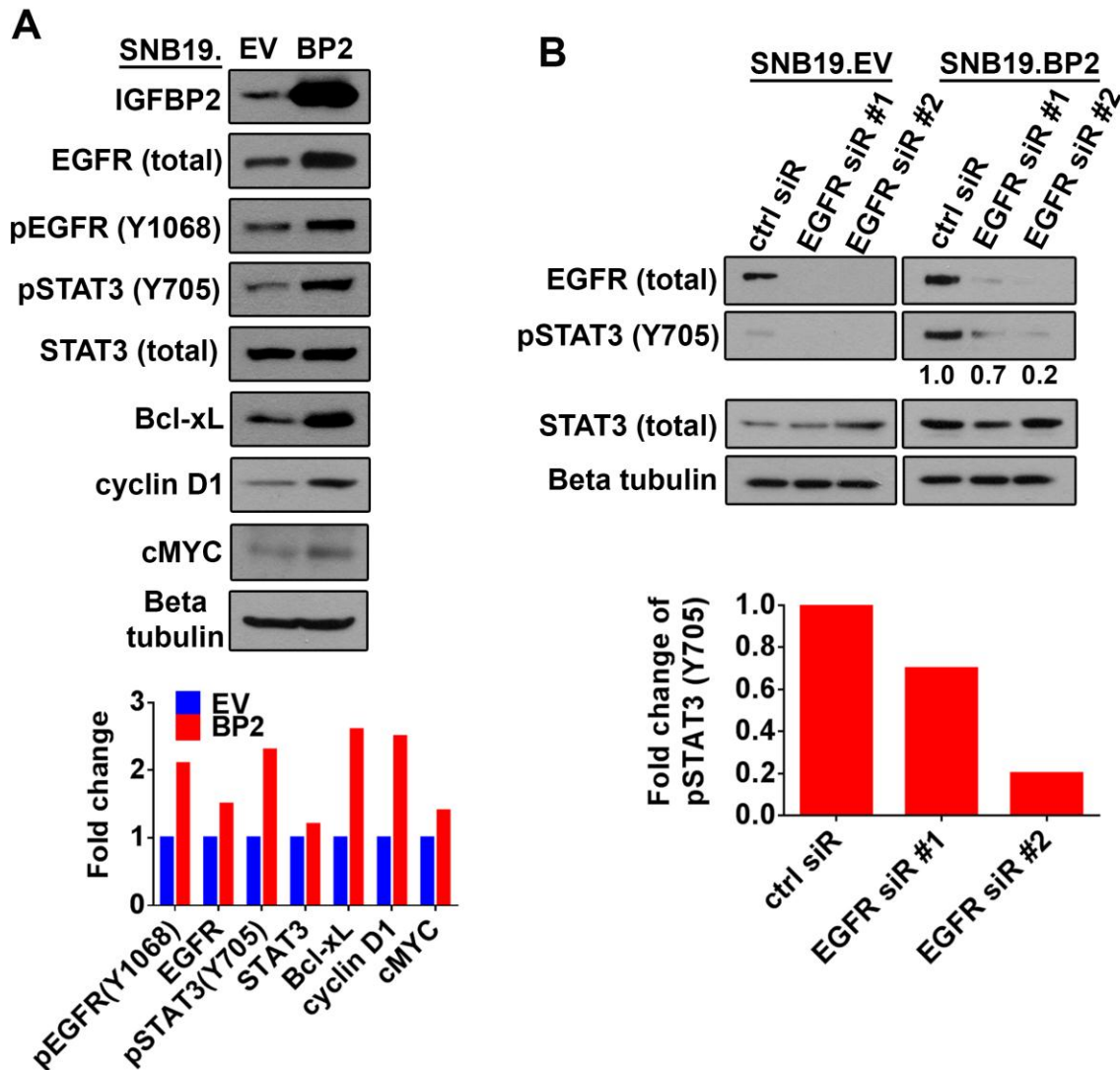
Figure 7. IGFBP2 activates STAT3 through EGFR. (A) Immunoblot analysis comparing stable
SNB19 empty vector cells (SNB19.EV) to SNB19 cells stably overexpressing IGFBP2
(SNB19.BP2). Densitometric analysis shown below the immunoblot indicates fold-change
relative to SNB19.EV after normalization to beta-tubulin loading control (or total protein for
phosphorylated proteins). (**B**) Immunoblot analysis comparing SNB19.EV and SNB19.BP2 cells
depleted of EGFR via 2 independent pools of EGFR siRNA (EGFR sir#1, EGFR sir#2) to cells
transfected with scrambled negative control siRNA (ctrl siR). The intensity of pSTAT3(Y705),
quantified by densitometry, is shown below the immunoblot as fold-change relative to control
siRNA, normalized to total STAT3.
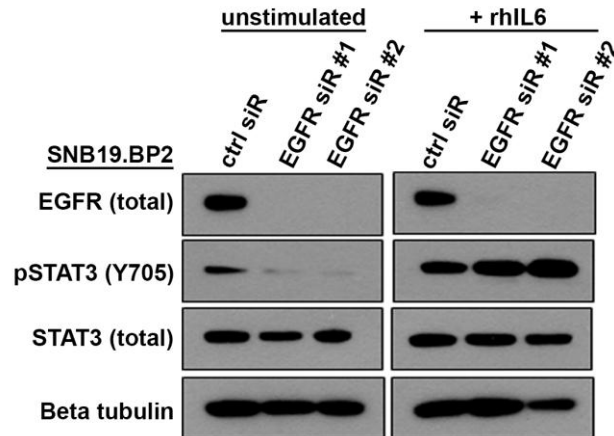
Figure 8. IGFBP2-induced STAT3 activation is mediated through EGFR. Immunoblot analysis of SNB19.BP2 cells depleted of EGFR by using 2 independent pools of EGFR siRNA (EGFR sir#1, EGFR sir#2) or scrambled negative control siRNA (ctrl siR), followed by overnight serum starvation and stimulation with 100ng/mL recombinant human IL6 (rhIL6) for 15 minutes.
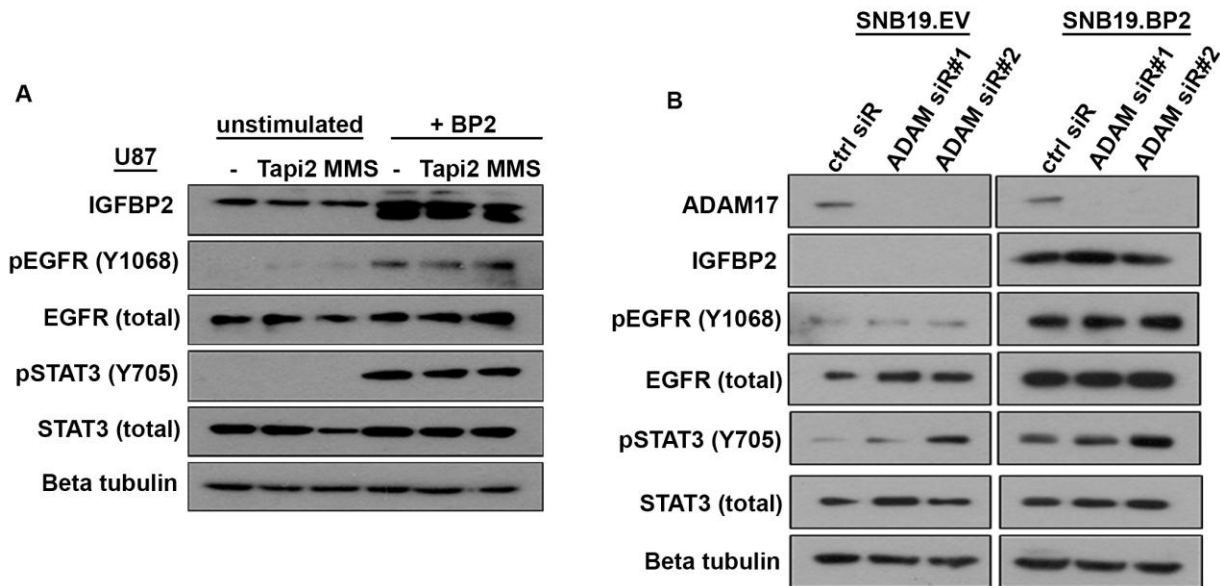
Figure 9. Inhibition of ADAMs do not affect IGFBP2-mediated EGFR signaling activation.
(A) U87 glioma cells were serum-starved overnight, then pretreated with an ADAMs inhibitor, TAPI-2 or marimastat (MMS) (20μM), for 2 hours. After pretreatment, cells were stimulated with 100ng/mL IGFBP2 in serum-free medium with fresh TAPI-2 or marimastat (20μM) for 5 minutes. Whole-cell lysates were collected for immunoblotting. (**B**) SNB19.EV and SNB19.BP2 cells were transfected with ADAM17 siRNA or scramble negative control siRNA for 48 hours and serum-starved overnight, then treated with 100ng/mL IGFBP2 for 5 minutes. Whole-cell lysates were collected for immunoblotting.

To evaluate the importance of STAT3 activity for cell viability of IGFBP2 overexpressing glioma cells, we used a preclinical STAT3 inhibitor, WP1066 [38] to treat SNB19.BP2 cells, and SNB19.EV cells as a control. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay demonstrated that SNB19.BP2 cell viability was significantly decreased with STAT3 inhibition compared to SNB19.EV cells, indicating that STAT3 activation is a vital downstream pathway of IGFBP2 in glioma (Fig. 10A). Consistent with reports of STAT3-mediated temozolomide (TMZ) resistance in glioma [39, 40], SNB19.BP2 cells were less sensitive to TMZ treatment compared to SNB19.EV as demonstrated by MTT assay (Fig. 10B). To investigate sensitivity of IGFBP2 overexpressing cells to EGFR inhibitor, we assessed the drug response to erlotinib via MTT assay. Erlotinib is a small molecule tyrosine kinase inhibitor that binds and inhibits EGFR activation via phosphorylation. There was no difference between SNB19.EV and SNB19.BP2 response to Erlotinib (Fig. 10C), consistent with clinical reports of insensitivity of glioma to EGFR inhibition.
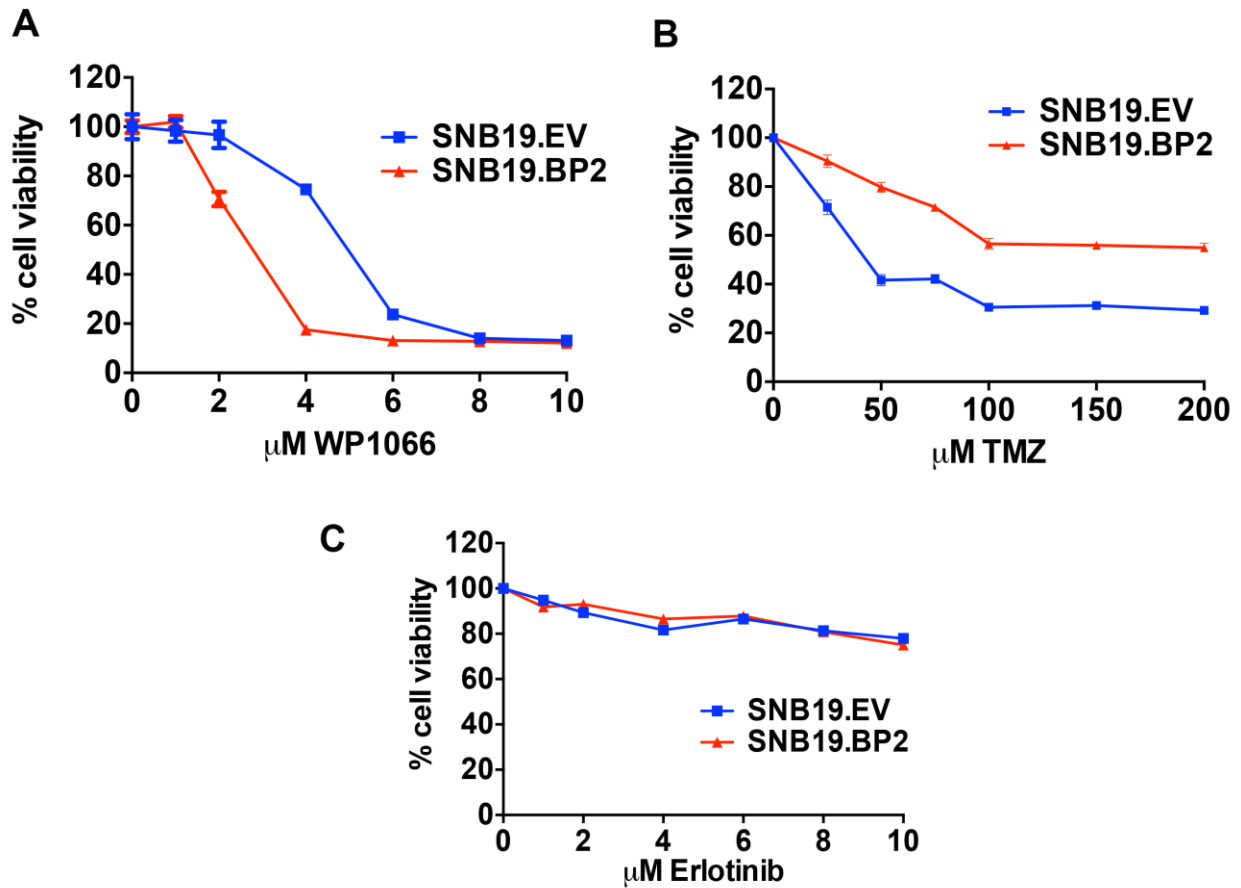
Figure 10. Cell viability assays of SNB19.EV and SNB19.BP2 cells. (A) Cell viability of - SNB19.EV and SNB19.BP2 cells were determined via MTT assay after treatment with increasing concentration of WP1066 for 72 hours. (B) Cell viability of SNB19.EV and SNB19.BP2 cells were determined via MTT assay after treatment with increasing concentration of TMZ for 5 days. (C) Cell viability of SNB19.EV and SNB19.BP2 cells were determined via MTT assay after treatment with increasing concentration of Erlotinib for 72 hours. All MTT experiments were performed in quadruplicate (n=4, mean ± s.d.)

IGFBP2 is significantly correlated with STAT3 pathway activation in glioma

Previous studies showed that IGFBP2 regulates the expression of many STAT3 target

genes [124,142], and our results demonstrate that IGFBP2 can stimulate STAT3 activation

through EGFR. To gain a comprehensive view of the relationship between IGFBP2 and STAT3

signaling, we analyzed the whole-genome gene expression profiling data from The Cancer Genome Atlas (TCGA) low-grade glioma (LGG) database. To assess whether STAT3 pathway genes are enriched in samples with *IGFBP2* expression, we performed a gene set enrichment analysis (GSEA) using STAT3 pathway gene set derived from the Ingenuity Pathway Analysis (IPA). GSEA revealed that STAT3-activated genes were significantly correlated with *IGFBP2* (P<0.001; Fig. 11), suggesting that IGFBP2 expression is associated with STAT3 pathway activation. To further substantiate the IGFBP2-STAT3 link, we performed hierarchical clustering on the 157 experimentally validated STAT3 target genes across all samples in the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) dataset. Two distinct clusters were formed, associated with tumor grade and *IGFBP2* and *STAT3* expression but not with other transcription factors such as beta-catenin (*CTNNB1*) or Forkhead box protein M1 (*FOXM1)* (Fig. 12). Thus, using 2 independent glioma datasets (TCGA and REMBRANDT), we further validate that *IGFBP2* and *STAT3* expression are tightly linked.

Next we postulated that the most functionally important of the correlated genes would likely be associated with STAT3 activity (as measured by phosphorylation) in the reverse-phase protein array (RPPA) data of the same TCGA cohort. In this proteomic analysis, we identified the 7 proteins (Fig. 13A, 13B) that were most significantly and strongly correlated with both IGFBP2 and pSTAT3(Y705) (correlation coefficients >0.2). Of these 7 strongly correlated proteins, 5 are closely related to the STAT3 signaling pathway, namely plasminogen activator inhibitor-1 (PAI-1), fibronectin, cyclin B1, pHER2(Y1248), and, notably, pEGFR(Y1068). HER2 is a member of the EGFR family and an upstream regulator of STAT3, however it has not been shown to have clinical significance in glioma [313-317]. These results from patient samples are consistent with the results of our *in vitro* cell line–based studies, and together these results illustrate the potential importance of the IGFBP2-EGFR-STAT3 signaling axis in glioma.

STAT3 signaling pathway enrichment
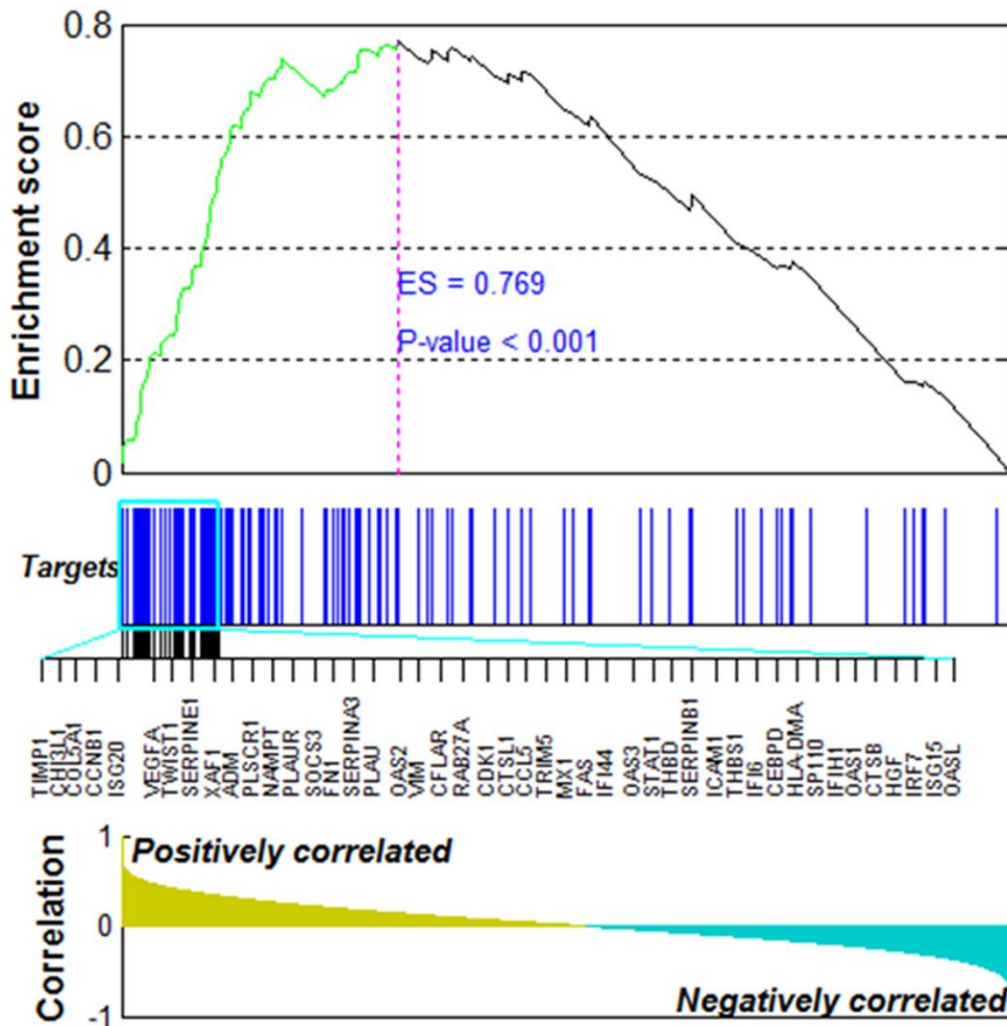
Figure 11. IGFBP2 is strongly and significantly correlated with STAT3 pathway genes. (**A**) GSEA demonstrated enrichment for STAT3 target genes based on correlation with *IGFBP2* expression in the TCGA low-grade glioma database. The top of the panel shows the enrichment score (ES) for genes associated with STAT3 signaling pathway targets. The blue lines indicate where the STAT3 target genes appear in the ranked gene list, and the black lines represent the top 45 highly correlated targets. The bottom of the panel shows the ranking scores (correlation of all genes associated with the STAT3 signaling pathway targets with *IGFBP2*). *This work was performed by Yuexin Liu.*
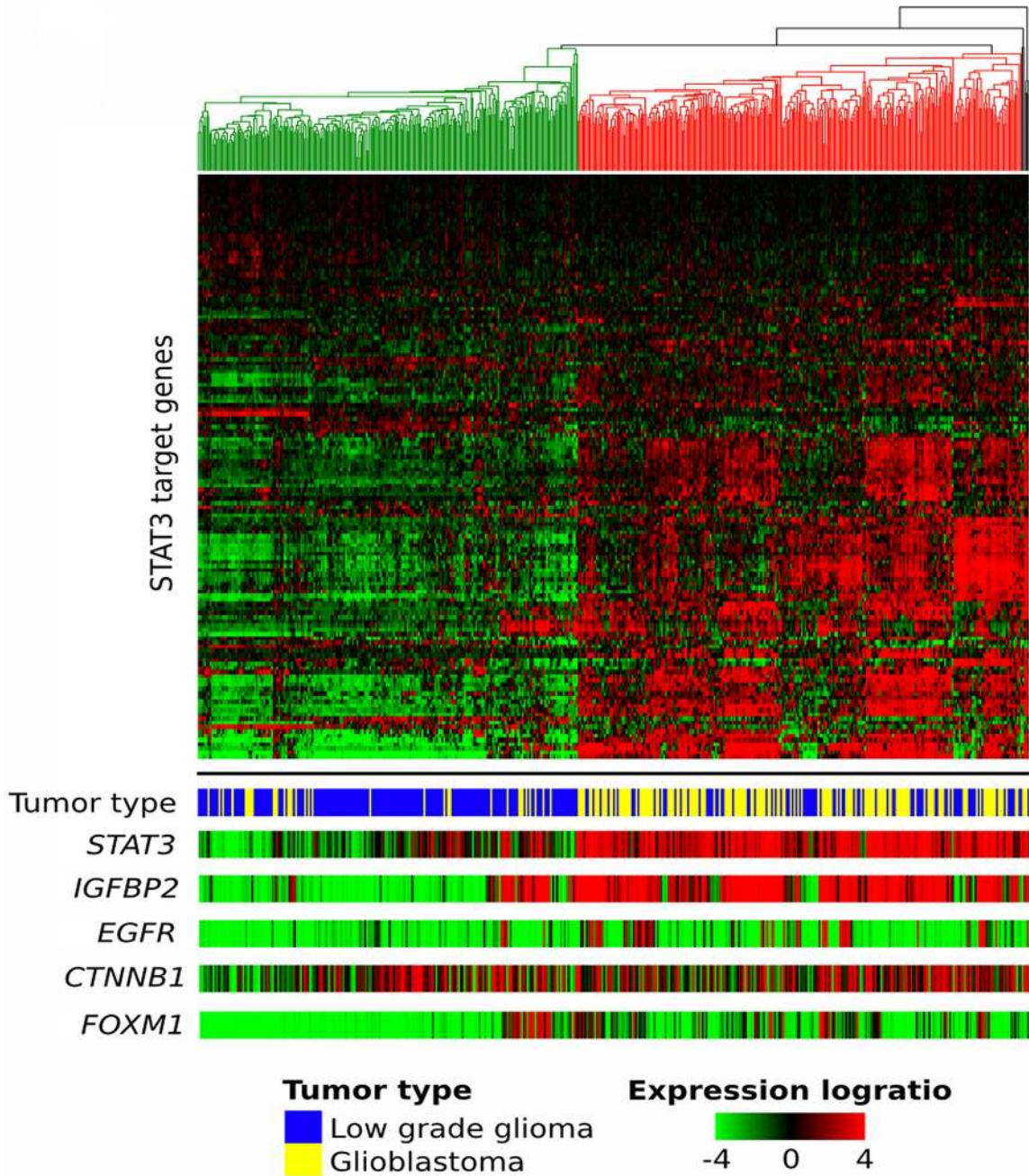
Figure 12. Hierarchical clustering of 157 experimentally validated STAT3 target genes from Ingenuity Pathway Analysis across all samples in the Rembrandt glioma dataset. Two distinct clusters formed and associated with tumor grade and IGFBP2 and STAT3 expression, but not with CTNN1B or FOXM1 expression. EGFR expression was elevated in a subset of glioblastomas. Blue bar represents low-grade glioma, and yellow bar represents high-grade glioma. *This work was performed by Matti Annala.*
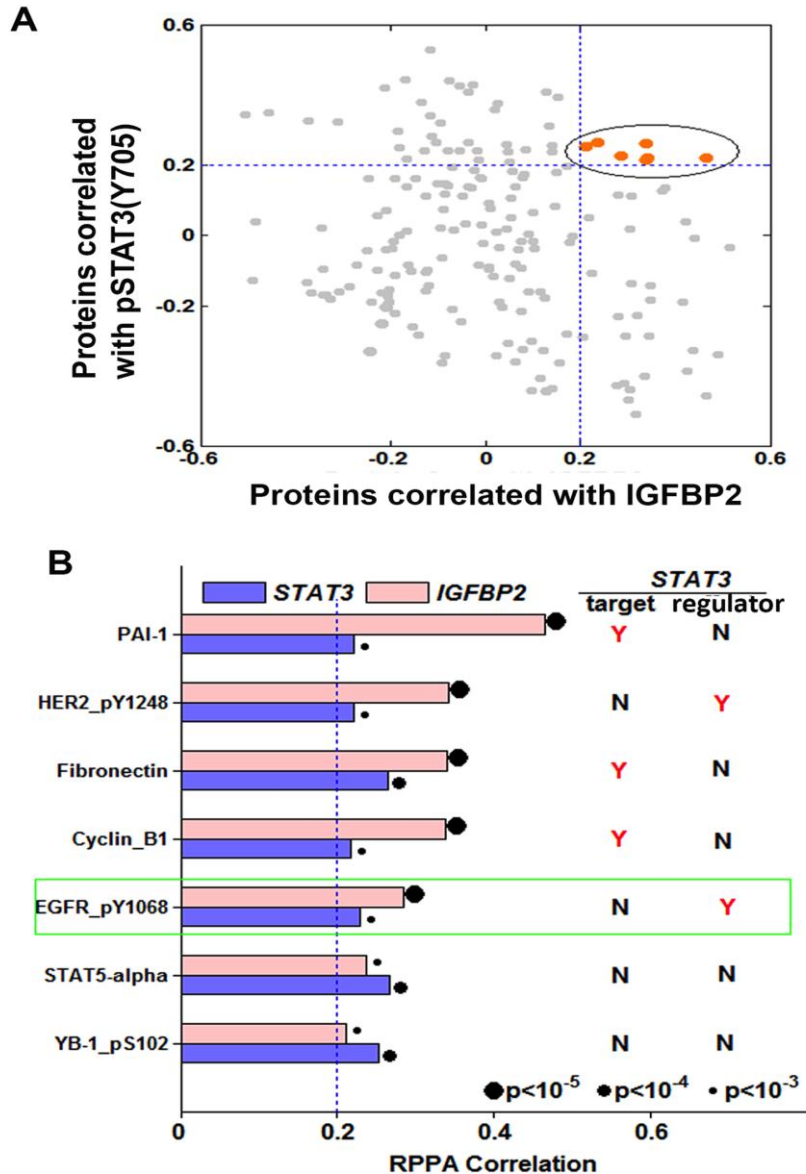
Figure 13. IGFBP2 is significantly correlated with pSTAT3(Y705)-correlated proteins. (**A**) Correlation of expression of proteins in the TCGA RPPA data with IGFBP2 (x-axis) and pSTAT3(Y705) (y-axis). Each dot represents a protein. Proteins with correlation coefficients greater than 0.2 are highlighted in orange. (**B**) Correlation of the 7 proteins with the highest correlation coefficients with both IGFBP2 and STAT3. Also shown is the relationship of each protein with STAT3 ("target" = STAT3 transcriptional target; "regulator" = STAT3 upstream regulator). Y = yes, a known target or upstream regulator of STAT3; N = not a known target or upstream regulator of STAT3. *This work was performed by Yuexin Liu.*

IGFBP2 co-precipitates and co-localizes with EGFR

To further evaluate the functional relationship between IGFBP2 and EGFR, we performed

reciprocal immunoprecipitation (IP) studies followed by immunoblotting comparing IGFBP2-

overexpressing SNB19 cells and empty vector control cells. Co-IP experiments revealed co-

precipitation of IGFBP2 and EGFR (Fig. 14A). We next treated U87 cells, which had been

serum-starved overnight, with 2 different doses of exogenous IGFBP2 followed by IP analysis

and immunoblotting. The results showed a dose-dependent increase of IGFBP2 co-precipitated

with EGFR (Fig. 14B). Confocal imaging analysis of SNB19.BP2 cells demonstrated clear co-

localization of IGFBP2 and EGFR proteins on the cell membrane and in the cytoplasm and

nucleus (Fig. 15). Co-localization of IGFBP2 and EGFR provides further evidence of a complex

containing IGFBP2 and EGFR.
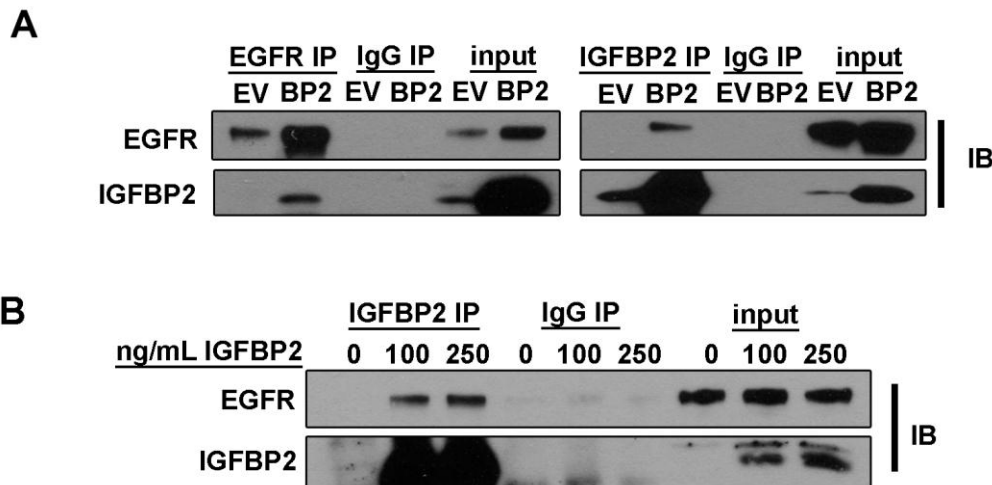
Figure 14. IGFBP2 co-precipitates with EGFR. (**A**) Co-immunoprecipitation (IP) of IGFBP2 and EGFR in SNB19.EV control cells versus SNB19.BP2 cells analyzed by immunoblot (IB). (**B**) Immunoprecipitation of IGFBP2 in U87 cells starved of serum overnight then stimulated with 2 different doses of IGFBP2 for 30 minutes, analyzed by immunoblotting.
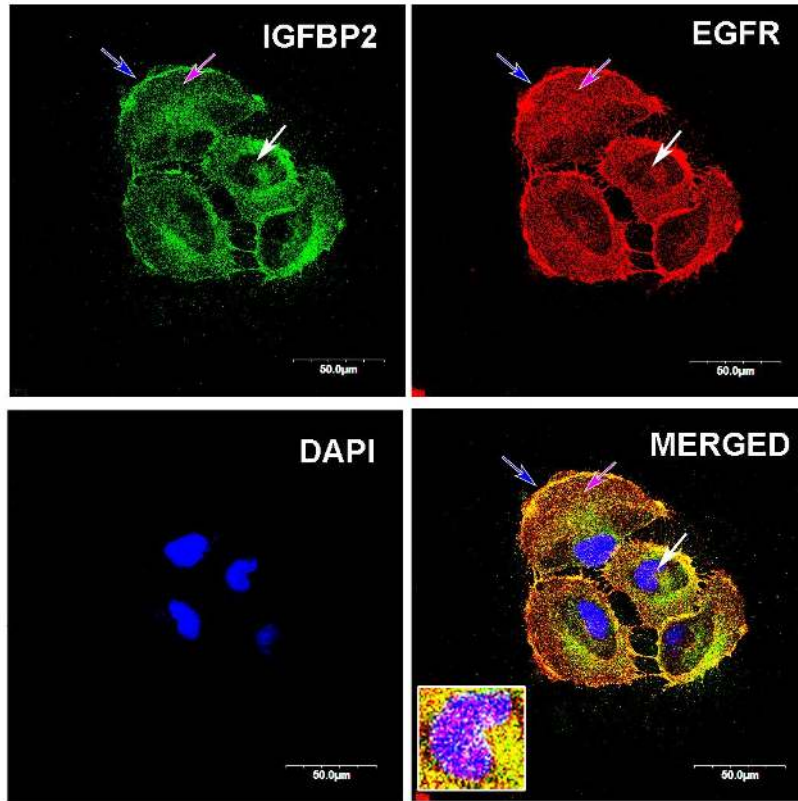
Figure 15. IGFBP2 co-localizes with EGFR. Confocal microscopy images of immunofluorescence staining for IGFBP2 (green), EGFR (red) and DAPI (blue) in SNB19.BP2 cells show IGFBP2 and EGFR co-localization; blue arrow = cell membrane; purple arrow = cytoplasm; white arrow = nucleus. *This work was performed in collaboration with Limei Hu.*

IGFBP2 facilitates EGFR nuclear accumulation

Because we observed IGFBP2 and EGFR co-localization in the cytoplasm and nucleus, we investigated whether nuclear IGFBP2 is interacts with nuclear EGFR and whether this complex augments STAT3 transcriptional activation. We first fractionated SNB19.BP2 and SNB19.EV cells into cytoplasmic and nuclear fractions and performed immunoblotting to detect IGFBP2, EGFR and STAT3. Our results revealed that a substantial proportion of IGFBP2 and EGFR localized to the nucleus in SNB19.BP2 cells (Fig. 16A). We then determined the ratio of nuclear to cytoplasmic EGFR via densitometric analysis and found that SNB19.BP2 cells had more than twice as much nuclear EGFR as SNB19.EV cells.

To investigate whether IGFBP2 facilitates EGFR nuclear accumulation, we stimulated SNB19.par cells (which had been serum-starved overnight) with exogenous IGFBP2 protein and then visualized EGFR protein localization by confocal imaging. IGFBP2 stimulation of SNB19.par cells resulted in EGFR accumulation in the nucleus (Fig. 16B). A time-course study with the same cells demonstrated that IGFBP2 nuclear accumulation paralleled EGFR nuclear accumulation in a time-dependent manner (Fig. 16C). To validate that EGFR nuclear accumulation is mediated through IGFBP2, we knocked down IGFBP2 using 2 different pools of siRNA in SNB19.BP2 cells and performed immunoblotting analysis on the fractionated cells. IGFBP2 depletion led to impaired EGFR nuclear localization with coordinate cytoplasmic accumulation of EGFR, whereas control knockdown did not affect EGFR nuclear accumulation (Fig. 16D). These results suggest that IGFBP2 plays a role in promoting EGFR nuclear accumulation.
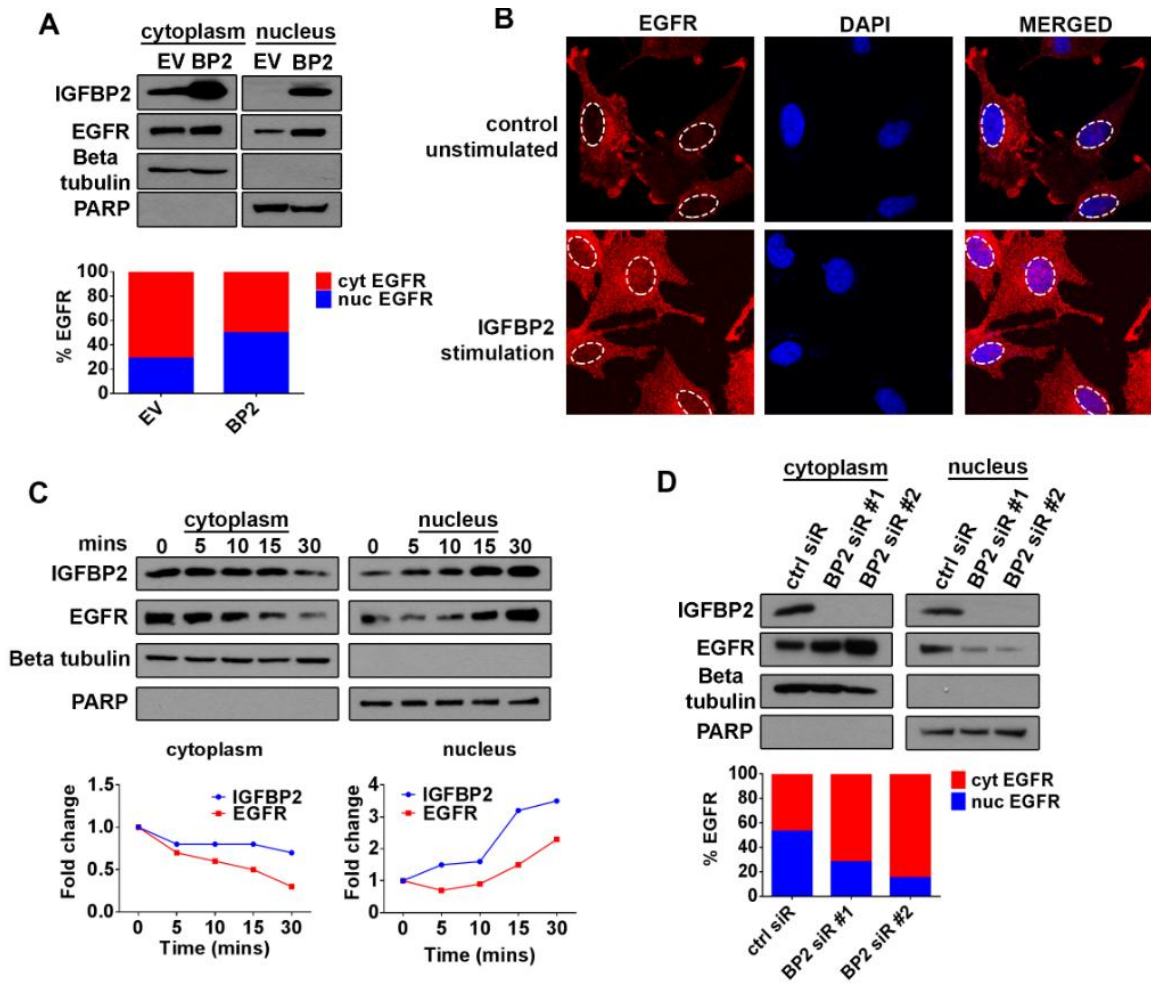
Figure 16. IGFBP2 drives EGFR nuclear accumulation. (**A**) Immunoblot analysis of cytoplasmic (cyt) and nuclear (nuc) fractions of SNB19.EV and SNB19.BP2 cells. Beta-tubulin represents a loading control for the cytoplasmic fraction, and PARP represents a loading control for the nuclear fraction. Densitometric analysis represented by the bar graph, demonstrates percentage of cytoplasmic or nuclear EGFR. (**B**) Confocal images of SNB19 parental cells and SNB19 parental cells stimulated with exogenous IGFBP2 protein (250ng/mL for 30 minutes). Cells were stained for EGFR (red) and the nuclei stained with DAPI (blue). (**C**) Immunoblot analysis of cytoplasmic and nuclear fractions of SNB19 parental cells stimulated with exogenous IGFBP2 (250ng/mL for indicated times). The graph represents fold-change of cytoplasmic or nuclear IGFBP2 and EGFR calculated from densitometric analysis of the immunoblot bands. (**D**) Immunoblot analysis comparing cytoplasmic and nuclear fractions of SNB19.BP2 cells depleted of IGFBP2 via 2 independent pools of IGFBP2 siRNA (BP2 siR #1, #2) to cells transfected with scrambled negative control siRNA (ctrl siR). Densitometric analysis represented by the bar graph, demonstrates percentage of cytoplasmic or nuclear EGFR. *Panel B was performed in collaboration with Limei Hu.*

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Nuclear translocation of IGFBP2 is required for IGFBP2-mediated EGFR nuclear accumulation

To better understand the mechanism of nuclear IGFBP2–mediated EGFR nuclear accumulation, we generated an IGFBP2 construct with a mutant nuclear localization signal [160] (BP2ΔNLS; Fig 17). Transient transfection of BP2ΔNLS plasmid into SNB19.par cells resulted in the expected compromise of IGFBP2 nuclear entry and also impaired EGFR nuclear accumulation (Fig. 18A). Next, we created a stable BP2ΔNLS-overexpressing cell line (SNB19.BP2ΔNLS). Compared to SNB19.BP2 WT (wild-type IGFBP2), impaired EGFR nuclear accumulation in fractionated stable SNB19.BP2ΔNLS cells resulted in decreased nuclear expression of COX2 and cMYC, which are known downstream targets of nuclear EGFR/STAT3 complex (Fig. 18B). These results were replicated in another glioma cell line, T98G (Fig. 19A, 19B). To determine whether BP2ΔNLS can bind to EGFR, we transiently transfected U87 cells with BP2 WT or BP2ΔNLS plasmid and performed IP followed by immunoblotting (Fig. 20). The results showed that mutation of IGFBP2 NLS does not affect binding to EGFR, demonstrating that nuclear translocation of IGFBP2 is important for mediating EGFR nuclear accumulation. Because IGFBP2 is involved in glioma cell migration and invasion [141,142], we then performed migration and invasion assays using the SNB19.EV, SNB19.BP2 WT, and SNB19.BP2ΔNLS cell lines. Migration and invasion potential were significantly impaired in the SNB19.BP2ΔNLS cells compared to SNB19.BP2 WT (Fig. 21A, 21B), indicating that nuclear IGFBP2 is important for the invasive phenotype of glioma cells, plausibly through regulation of nuclear EGFR-STAT3 activity.
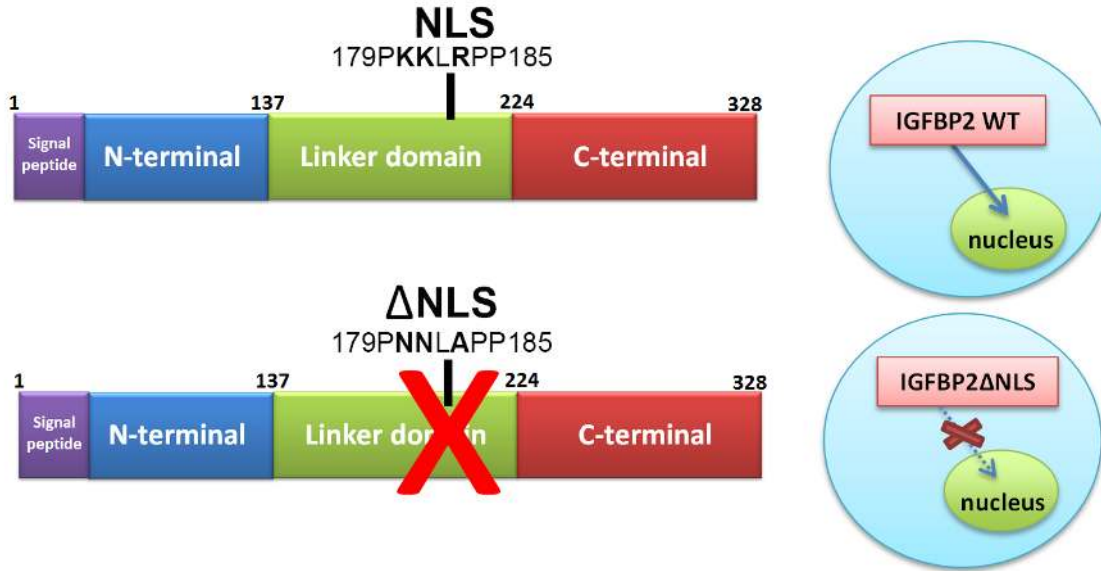
Figure 17. Diagram of IGFBP2 domains and nuclear localization signal (NLS).
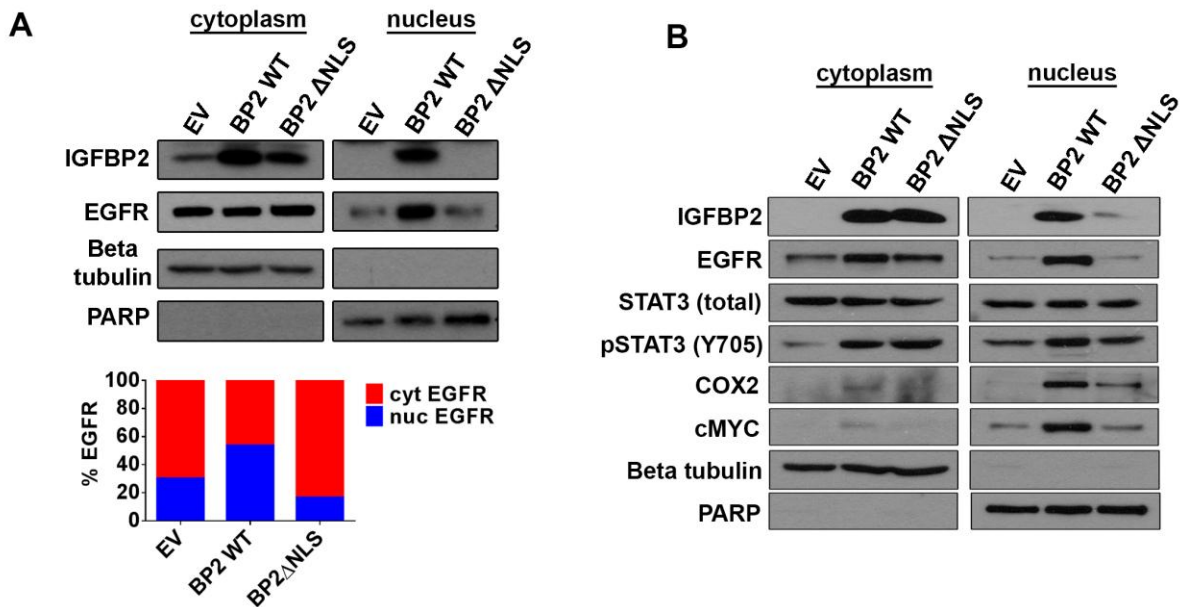
Figure 18. BP2ΔNLS impairs nuclear EGFR accumulation. (A) Immunoblot analysis of cytoplasmic and nuclear fractions of transiently transfected SNB19.EV, SNB19.BP2 wild type (BP2 WT) and SNB19 with a mutated IGFBP2 nuclear localization signal (BP2ΔNLS). Densitometric analysis represented by the bar graph, demonstrates percentage of cytoplasmic or nuclear EGFR. (**B**) Immunoblot analysis of cytoplasmic and nuclear proteins in stable SNB19.EV, SNB19.BP2 WT and SNB19.BP2ΔNLS cells.
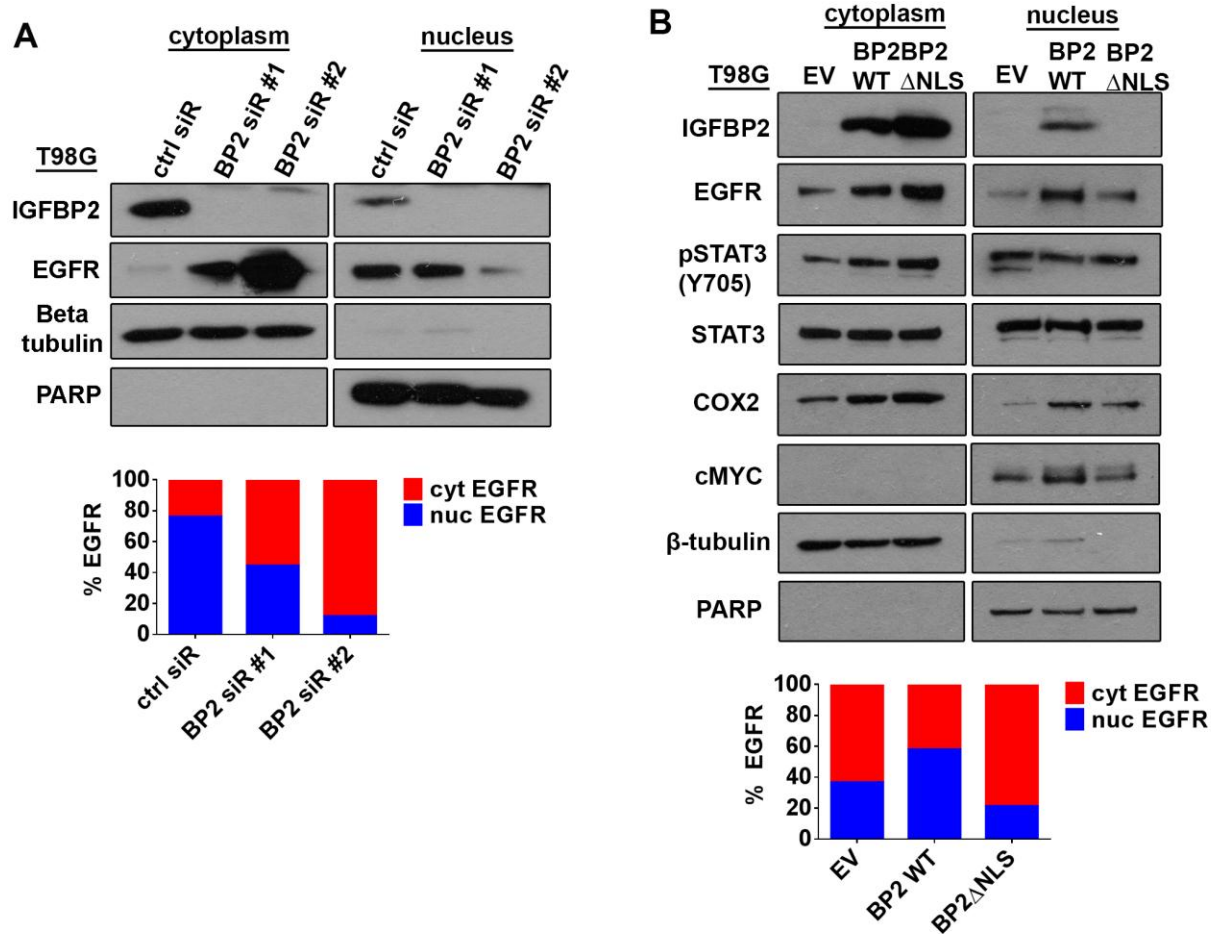
Figure 19. IGFBP2 promotes nuclear EGFR accumulation in T98G glioma cells. (**A**) Immunoblot analysis comparing cytoplasmic and nuclear fractions of T98G cells depleted of IGFBP2 via 2 independent pools of IGFBP2 siRNA (BP2 siR #1, #2) for 48 hours to those treated with scrambled negative control siRNA (ctrl siR). Densitometric analysis of EGFR represented by the bar graph, demonstrates percentage of cytoplasmic (cyt) or nuclear (nuc) EGFR. (**B**) Immunoblot analysis of cytoplasmic and nuclear proteins in T98G cells transiently transfected with EV, BP2 WT and BP2ΔNLS. Densitometric analysis of EGFR represented by the bar graph, demonstrates percentage of cytoplasmic (cyt) or nuclear (nuc) EGFR.
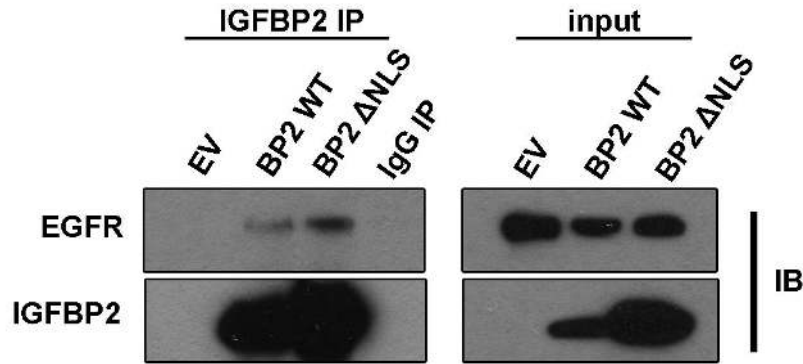
Figure 20. Mutation of the IGFBP2 NLS does not affect binding to EGFR. U87 glioma cells were transiently transfected with EV, BP2 WT or BP2ΔNLS plasmid followed by immunoprecipitation (IP) for IGFBP2 and immunoblotting (IB).
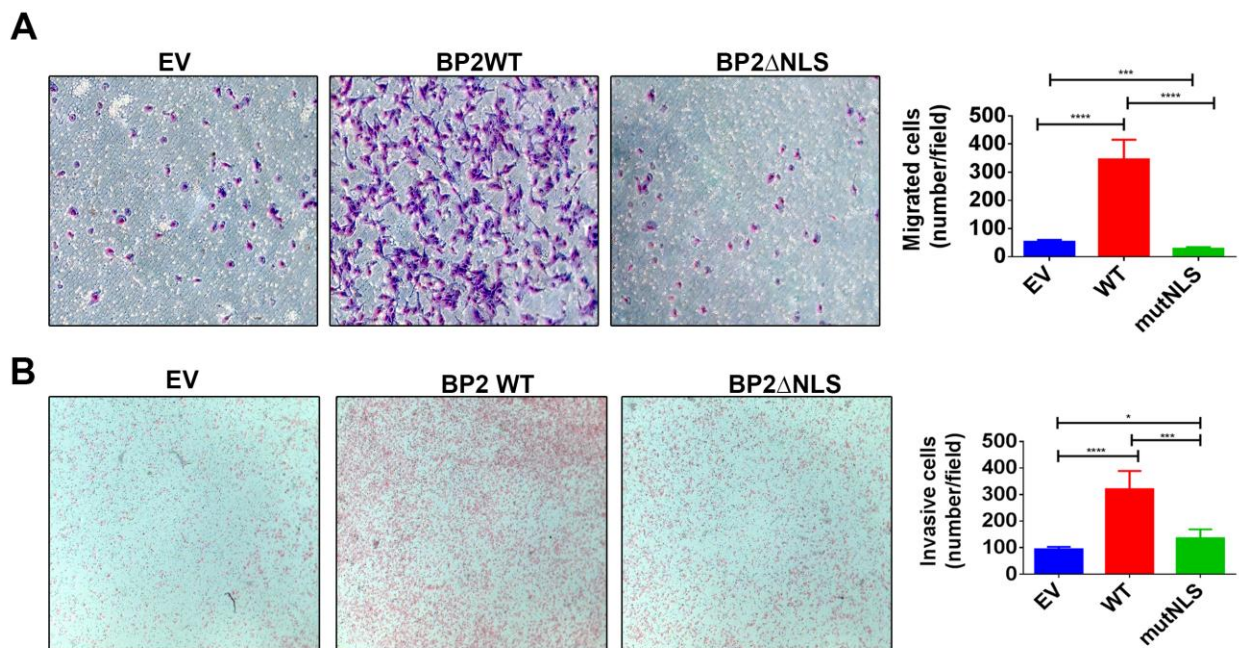
Figure 21. Migration and invasion potential were significantly impaired in the SNB19.BP2ΔNLS cells. (**A**) A migration assay was performed on SNB19.EV (empty vector), SNB19.BP2 WT (wild type) and SNB19.BP2 with a mutant NLS (SNB19.BP2ΔNLS or mutNLS) cells using a transwell migration chamber. (Left) Cells were fixed and stained after incubation for 4 hours. (Right) Bar graph represents the mean number of migrated cells in 5 random view fields (mean ± s.e.m.). (**B**) An invasion assay was performed on SNB19.EV, SNB19.BP2 WT and SNB19.BP2ΔNLS cells using a transwell invasion chamber. (Left) Cells were fixed and stained after incubation for 16 hours. (Right) Bar graph represents the mean number of invaded cells in 5 random view fields (mean ± s.e.m.). Indicated annotations correspond to the following P-values: *P<0.05, ***P<0.005, and ****P<0.0001. *This work was performed in collaboration with Limei Hu.*

Levels of nuclear EGFR, nuclear IGFBP2 and pSTAT3 are significantly correlated in glioma

The RPPA LGG data from TCGA revealed a close relationship between IGFBP2, activated EGFR and activated STAT3, but did not provide spatial information. To further investigate localization of these proteins, we performed immunohistochemical analysis to determine the association between IGFBP2, EGFR and pSTAT3(Y705) in a clinical glioma tissue microarray (TMA) comprising 222 samples of grade 2-4 gliomas. We observed both cytosolic and nuclear localization of IGFBP2, both of which were strongly associated with STAT3 phosphorylation in these gliomas (Fig. 22A, 22B, Table 1). Both cytosolic and nuclear IGFBP2 expression positively correlated with increased fraction and degree of phosphorylation of STAT3 ($p=0.023$ and $p=0.018$, respectively), suggesting a functional link between IGFBP2 expression and STAT3 phosphorylation.

We observed nuclear co-localization of IGFBP2 and EGFR in the clinical samples (Fig. 22C, 22D, and Table 1). Cytosolic IGFBP2 did not correlate with nuclear EGFR and nuclear IGFBP2 did not correlate with cytosolic EGFR. However, nuclear IGFBP2 positively associated with nuclear EGFR localization ($p=0.011$). Furthermore, clinical samples that were triple positive for nuclear accumulation of IGFBP2, phosphorylated STAT3 and EGFR were strongly associated with poor survival (Fig. 23).
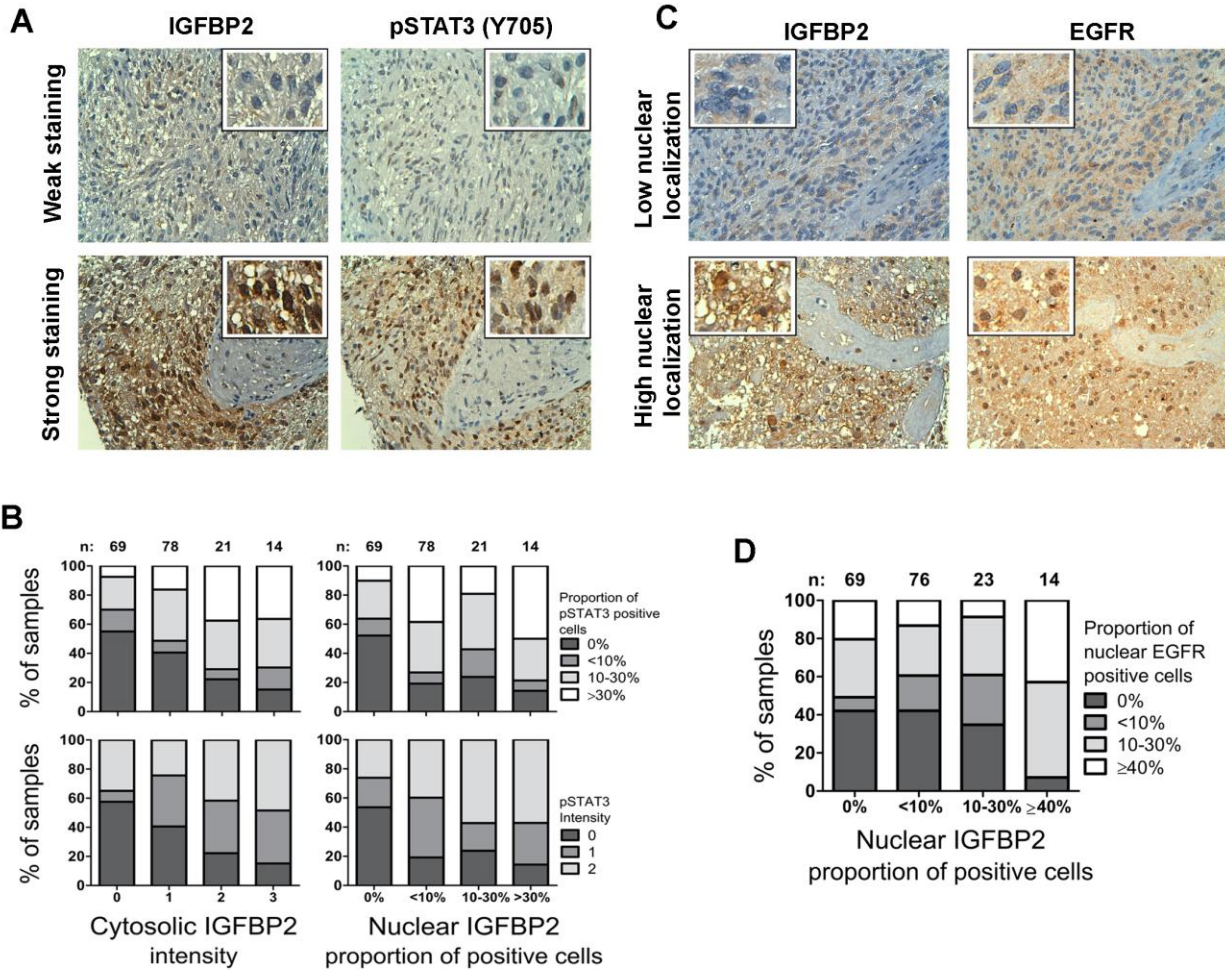
Figure 22. IGFBP2 correlates with STAT3 activation and nuclear EGFR localization in clinical samples. Expression and localization of IGFBP2, pSTAT3(Y705) and EGFR were detected with immunohistochemistry from a TMA that included 222 human grade 2-4 gliomas. (**A**) TMA immunostaining images (magnification 40×) representing weak and strong staining of IGFBP2 and pSTAT3(Y705). (**B**) Cytosolic and nuclear IGFBP2 expression associated with the percentage of cells positive for pSTAT3 and with pSTAT3 staining intensity. Bar graphs illustrate the increasing fractions of pSTAT3-positive cells and pSTAT3 intensity upon increasing IGFBP2 intensity or nuclear accumulation. (**C**) TMA immunostaining images (magnification 40×) representing low and high nuclear localization of IGFBP2 and EGFR. (**D**) Nuclear IGFBP2 associated with nuclear EGFR. The bar graph illustrates the fraction of samples with increasing nuclear EGFR localization upon increasing nuclear accumulation of IGFBP2. *This work was performed by Kirsi Granberg and Hannu Haapasalo.*

Nuclear IGFBP2/pSTAT3/EGFR triple positive
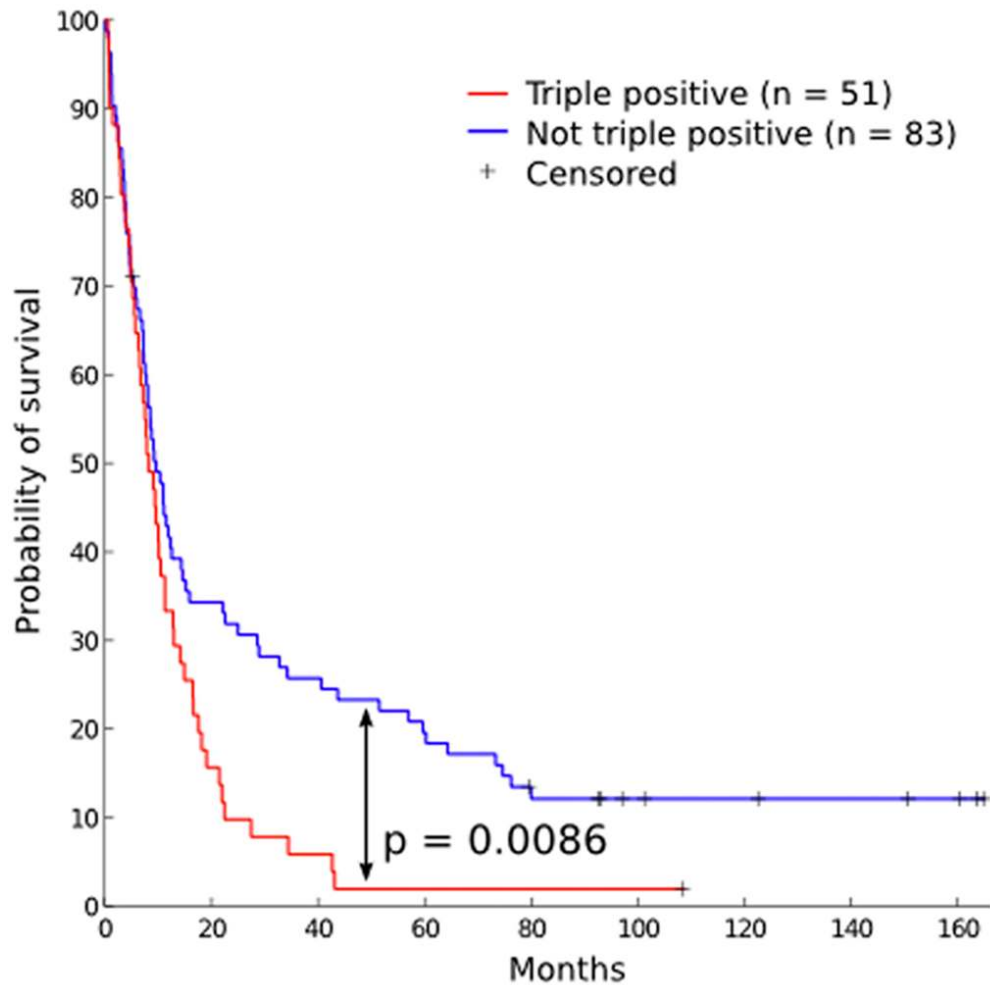
Figure 23. Nuclear co-localization of IGFBP2, EGFR and phosphorylated STAT3 predicted poor survival among patients with human grade 2-4 glioma. Patients were stratified into 2 cohorts based on the nuclear staining of all 3 proteins: triple positives (≥1% of cells with nuclear expression, n=51, red line) and all other cases (n=83, blue line). Survival rates were visualized by using a Kaplan-Meier survival plot (p=0.0086). *This work was performed by Matti Annala.*

Table 1. Analysis of IGFBP2, pSTAT3 (Y705) and EGFR in human glioma TMA. Levels of IGFBP2, pSTAT3(Y705) and EGFR in a TMA of glioma samples from patients were evaluated using immunohistochemical analysis. Correlation levels were calculated by using the Pearson chi-square analysis. *This work was performed by Kirsi Granberg and Hannu Haapasalo.*

| | | pSTAT3 intensity | | | | | | Row total | | Pearson Chi-Square |
| | | 0 | | 1 | | 2 | | | | |
		n	(%)	n	(%)	n	(%)	n	%	p-value
IGFBP2 intensity	0	23	(39.0)	3	(5.6)	14	(20.3)	40	22.0	
	1	15	(25.4)	13	(24.1)	9	(13.0)	37	20.3	<0.001
	2	16	(27.1)	26	(48.1)	30	(43.5)	72	39.6	
	3	5	(8.5)	12	(22.2)	16	(23.2)	33	18.1	
	Column total	59	(32.4)	54	(29.7)	69	(37.9)	182	100	
Nuclear IGFBP2	0 %	23	(39.0)	3	(5.6)	14	(20.3)	40	22.0	
	<10%	15	(25.4)	13	(24.1)	9	(13.0)	37	20.3	<0.001
	10-30%	16	(27.1)	26	(48.1)	30	(43.5)	72	39.6	
	≥40%	5	(8.5)	12	(22.2)	16	(23.2)	33	18.1	
	Column total	59	(32.4)	54	(29.6)	69	(37.9)	182	100	

| | | Nuclear pSTAT3 | | | | | | | | Row total | | Pearson Chi-Square |
| | | 0 % | | <10% | | 10-30% | | ≥40% | | | | |
		n	(%)	n	(%)	n	(%)	n	(%)	n	%	p-value
Cytosolic IGFBP2 intensity	0	22	(37.9)	6	(31.6)	9	(15.8)	3	(6.3)	40	22.0	
	1	15	(25.9)	3	(15.8)	13	(22.8)	6	(12.5)	37	20.3	0.001
	2	16	(27.6)	5	(26.3)	24	(42.1)	27	(56.3)	72	39.6	
	3	5	(8.6)	5	(26.3)	11	(19.3)	12	(25.0)	33	18.1	
	Column total	58	(31.9)	19	(10.4)	57	(31.3)	48	(26.4)	182	100	
Nuclear IGFBP2	0 %	36	(62.1)	8	(42.1)	18	(31.6)	7	(14.6)	69	37.9	
	<10%	15	(25.9)	6	(31.6)	27	(47.4)	30	(62.5)	78	42.9	<0.001
	10-30%	5	(8.6)	4	(21.1)	8	(14.0)	4	(8.3)	21	11.5	
	≥40%	2	(3.4)	1	(5.3)	4	(7.0)	7	(14.6)	14	7.7	
	Column total	58	(31.9)	19	(10.4)	57	(31.3)	48	(26.4)	182	100	

| | | Nuclear IGFBP2 | | | | | | | | Row total | | Pearson Chi-Square |
| | | 0 % | | <10% | | 10-30% | | ≥40% | | | | |
		n	(%)	n	(%)	n	(%)	n	(%)	n	%	p-value
Nuclear EGFR	0 %	29	(42.0)	32	(42.1)	8	(34.8)	1	(7.1)	70	38.5	
	<10%	5	(7.2)	14	(18.4)	6	(26.1)	0	(0.0)	25	13.7	0.01
	10-30%	21	(30.4)	20	(26.3)	7	(30.4)	7	(50.0)	55	30.2	
	≥40%	14	(20.3)	10	(13.2)	2	(8.7)	6	(42.9)	32	17.6	
	Column total	69	(37.9)	76	(41.8)	23	(12.6)	14	(7.7)	182	100	

CHAPTER 4: DISCUSSION

SUMMARY. Gliomas remain one of the most lethal cancers despite decades of research. The standard of care therapy fails to improve clinical outcome due to the deeply infiltrative and drug resistant nature of the tumors. The complexity of oncogenic signaling in gliomas further intensifies the aggressive phenotype. Thus it is crucial to identify the key oncogenic signaling network(s) in glioma in order to develop novel therapeutic targets. IGFBP2 is an important oncogene for promoting glioma progression. The tumor-promoting mechanisms of IGFBP2 in glioma remains poorly elucidated as it is highly dynamic and can be secreted from the cell, or localize in the cytosol or nucleus, or bind different proteins. In this study, I combined genomics and functional analyses to examine the functional interaction of IGFBP2 with 2 other proteins highly activated in gliomas, EGFR and STAT3. My findings demonstrate that IGFBP2 is the key signaling activator of both the signal transduction activity and nuclear activity of EGFR and STAT3 (Fig. 24). Therefore IGFBP2 may be a potentially effective target for treating glioma.

IGFBP2 in EGFR/STAT3 signaling

Receptor tyrosine kinases activate intracellular signaling pathways to transmit signals from the extracellular environment into the nucleus where cellular activity is coordinated and controlled primarily through gene transcription. In physiological conditions, RTK signaling is tightly regulated to maintain homeostasis. In cancer, tumor cells acquire mutations that alter RTK leading to dysregulated signaling, resulting in uncontrolled growth and proliferation capacities. RTK signaling is altered in 88% gliomas [39], and the resultant acquired oncogenic capacities allow glioma cells to infiltrate into the brain parenchyma. As a result, maximal surgical resection while preserving normal brain function is hindered by infiltrative and poorly defined lesions. The residual lesions are typically resistant to adjuvant chemoradiotherapy and thus continue to proliferate and inevitably cause recurrence. Therefore, identifying critical oncogenic

signaling mediators in gliomas is pivotal for effective targeted treatment.

EGFR is a signature glioma oncogenic RTK predominantly altered by amplification, overexpression, and activating mutations. Depending on the type of alteration, EGFR is aberrantly activated in a ligand-dependent or –independent manner. Aberrantly activated EGFR constitutively initiates a signaling cascade that activates transcription factors which then translocate into the nucleus to activate gene transcription. One of the main EGFR downstream transcription factors is STAT3 in gliomas. Activated STAT3 subsequently translocates to the nucleus to activate transcription of proliferation, migration, invasion, and angiogenesis related genes. Thus it is important to understand the regulatory factors involved in EGFR-STAT3 pathway activation in gliomas. Our study expands the understanding of this network by demonstrating that IGFBP2 plays a role in the activation of EGFR-STAT3 and downstream pathways.

Our group and others have found that IGFBP2 upregulates transcription of migration, invasion and angiogenesis-related genes, particularly *STAT3, MMP2* and *VEGF* [124,142]. Because IGFBP2/EGFR and EGFR/STAT3 are concurrently expressed in gliomas[198,306], I hypothesize that IGFBP2, EGFR and STAT3 are functionally connected. In my study, I discovered that IGFBP2 activates STAT3 pathway through an EGFR-dependent mechanism. Forced overexpression of IGFBP2 or exogenous stimulation with recombinant IGFBP2, resulted in activated EGFR-STAT3 pathway and the downstream targets in glioma cells. I report that IGFBP2-mediated EGFR-STAT3 signaling is independent of ADAMs activity, suggesting that increased EGFR ligand shedding is not involved. Although I cannot eliminate the possibility that IGFBP2 may stimulate increased production of EGFR ligands to facilitate uncontrolled overstimulation of EGFR. Additionally, even though EGFR depletion inhibits STAT3 activation, cancerous cells may utilize alternative routes to activate STAT3 as I demonstrate using cytokine IL6.

My discovery of the IGFBP2-EGFR-STAT3 signaling axis is further validated through bioinformatics analyses of two independent databases, TCGA and REMBRANDT. Because IGFBP2 expression varies in LGGs, analysis of human LGG samples from the TCGA LGG dataset allows for a broader dynamic range and hence a more wide-ranging view of *IGFBP2* in relation to *STAT3*. While both TGCA LGG and REMBRANDT analysis substantiated the tight link between IGFBP2 and STAT3, REMBRANDT analysis demonstrated correlation with tumor grade. The availability of TGCA LGG RPPA data further validated the correlative link between IGFBP2, activated EGFR (by way of phosphorylation at tyrosine residue 1068;pY1068) and activated STAT3 (pY705). Activated EGFR at Y1068 is one of the major docking sites for STAT3; thus the detection of pEGFR-Y1068 among the top 7 correlated proteins validates our findings. Overall, using *in vitro* and bioinformatics analysis, I confirmed evidence of IGFBP2-EGFR-STAT3 signaling axis in gliomas.

This discovery of IGFBP2-mediated EGFR activation is not without precedent; in breast cancer cells, IGFBP2 can mediate signaling activation of ErbB2, a member of EGFR family, in a time and dose-dependent manner [318]. However, the exact mechanism of IGFBP2-mediated EGFR family signaling activation has up to now not been elucidated. Trastuzumab, a humanized monoclonal antibody targeting ErbB2 extracellular domain, induces ErbB2 receptor internalization and degradation. In contrast, as reported by Dokmanovic et al, IGFBP2 does not induce ErbB2 receptor degradation [318]. Therefore it is possible that IGFBP2 can stabilize ErbB2 either by preventing internalization or degradation. My immunoblot analysis demonstrates increased total EGFR protein upon IGFBP2 stimulation or overexpression. In my studies, the presence of IGFBP2 does not affect EGFR internalization since EGFR is detected in the cytoplasm and the nucleus. Thus it is plausible that IGFBP2 may affect EGFR degradation, perhaps through Cdc42-mediated modulation of c-Cbl, a known mediator of EGFR degradation. Cdc42 sequesters c-Cbl and prevents EGFR degradation [319]. Conventionally

Cdc42 is associated with actin cytoskeletion regulation; however accumulating evidence implicates Cdc42 in malignant transformation and invasion [319]. Our previous studies demonstrated that IGFBP2 overexpression activates Cdc42 signaling, whereas IGFBP2 inhibition through MIIP overexpression resulted in decreased CDC42 [141,150]. Further studies are required to investigate the role of Cdc42 in IGFBP2-mediated EGFR signaling.

The observed interaction and co-localization of IGFBP2 and EGFR demonstrates an additional mechanism for the pleiotropic functions of IGFBP2. It is currently unclear where this interaction occurs or whether it is direct or indirect. Studies using vascular smooth muscle cells demonstrated that IGFBP2 binds to the extracellular domain of receptor protein tyrosine phosphatase β via its heparin binding domain (HBD) in an IGF-dependent manner [95]. Because IGFBP2 NLS is located in the HBD, we used mutated IGFBP2 NLS to demonstrate that IGFBP2 binding to EGFR is independent of the NLS or HBD domain. Even though IGFBP2 lacks EGF-like motifs, I cannot exclude the possibility of binding to EGFR extracellular domain. Other studies have demonstrated EGFR ligand-like functions of prolidase C [167] and connective tissue growth factor [168], which both lack EGF motifs but can bind to EGFR extracellular domain and activate EGFR signaling.

Another plausible mechanism of binding may be indirectly through integrins. In cells with high EGFR expression, integrin α5β1 can interact with EGFR and form a complex on the cell surface [320]. Furthermore, integrin α5β1 association stimulates EGFR tyrosine phosphorylation independent of EGFR ligands, although the addition of ligands can increase EGFR activation levels. Constitutive elevation of urokinase plasminogen activator receptor (uPAR) can induce activation and association of EGFR with integrin α5β1 in a study performed using human hepatoma and fibrosarcoma cell lines [321]. Accordingly, our lab's previous studies demonstrated that IGFBP2 binds and activates integrin α5β1 in gliomas. Therefore, IGFBP2-EGFR interaction may potentially indirectly occur through mutual association with integrin α5β1.

Nuclear functions of IGFBP2

To add to the complexity of oncogenic pathways, signaling molecules are spatiotemporally dynamic [60-65]. In addition to cell surface–initiated signaling, EGFR can also mediate signaling in the nucleus after internalization[209]. Analogously, in addition to acting as a secreted or cytoplasmic signaling effector, IGFBP2 can translocate to the nucleus, albeit the functions remain poorly defined.

My findings indicate that nuclear IGFBP2 is important for mediating glioma cell migration and invasion. Furthermore, our study also highlights IGFBP2 as a non-canonical pathway of promoting EGFR nuclear accumulation. EGF ligand not only can activate EGFR signaling cascade in the cytoplasm, but also stimulate EGFR nuclear translocation. Similarly, IGFBP2 is a dynamic secreted protein with intracellular functions that can mediate intrinsic or extrinsic signaling depending on the context. The mechanisms of IGFBP2-induced EGFR nuclear accumulation are currently unknown although our study ruled out the involvement of NLS or HBD domain in IGFBP2-mediated EGFR nuclear accumulation.

In a study using breast cancer cells, IGFBP3 can associate with EGFR in the cytoplasm, but upon etoposide treatment, IGFBP3, EGFR and DNA-PKs (DNA repair enzyme) can form a complex in the nucleus [322]. EGFR kinase inhibition using gefitinib inhibited the etoposide-induced nuclear increase of both EGFR and IGFBP-3, indicating that EGFR kinase activity is required for both EGFR and IGFBP3 nuclear translocation in response to DNA damage by etoposide. As with my study, the mechanisms of IGFBP3-induced EGFR nuclear translocation remain unknown. It is possible that IGFBP2 may promote EGFR internalization and both proteins interact with importins that mediate nuclear translocation. Furthermore as mentioned earlier, IGFBP2 interaction with EGFR may stabilize EGFR, prevent degradation or recycling, and instead shuttle EGFR to the nuclear import route.

My investigation revealed that nuclear IGFBP2 induced increased EGFR/STAT3 activity as measured by downstream target expression, COX2 and cMYC. COX2 expression in gliomas is primarily regulated by EGFR either through activation of signaling cascade [323,324], or in cooperation with STAT3 in the nucleus [220]. COX2 overexpression correlates with glioma grade and is associated with poor survival [325]. In addition to increasing glioma cell migration, invasion and angiogenesis *in vitro*, COX2 increases tumor growth in xenograft glioma mouse models [325]. cMYC is required for glioma stem cell maintenance and correlated with HGGs [326-328]. This highlights the potential of targeting IGFBP2 to disrupt EGFR/STAT3 transcriptional activity in glioma. IGFBP2 induces DNA-PKs expression in HGGs in an IGF-independent manner [329]. In addition to cytosolic interaction, EGFR binds and activates DNA-PK in the nucleus to regulate DNA repair [227,330], resulting in radiation and chemotherapy resistance. Though it remains to be investigated, IGFBP2-mediated EGFR nuclear accumulation may also induce DNA-PK expression in glioma, leading to DNA repair and consequently chemoradioresistance.

Therapeutic implications.

STAT3 overexpression is attributed to resistance to TMZ, the first-line chemotherapy in high-grade gliomas [283]. Consistent with this, we showed that IGFBP2 overexpressing cells were more resistant to TMZ treatment whereas the upregulated expression of activated STAT3 in IGFBP2 overexpressing cells sensitized the cells to STAT3 inhibitor, WP1066. Furthermore, treatment with EGFR inhibitors or radiation can induce EGFR nuclear translocation [207]. It is possible that in IGFBP2 overexpressing glioma, EGFR is actively being shuttled into the nucleus by IGFBP2, rendering the cells resistant to EGFR-targeted therapies such as erlotinib that targets the kinase activity of EGFR. Thus our results may explain the lack of response to EGFR inhibitors in IGFBP2-overexpressing glioma patients.

EGFR crosstalks with STAT3 through 2 routes: tyrosine kinase–mediated activation of

STAT3 [181,182] and nuclear cooperation as transcriptional cofactors to activate iNOS, COX2 and cMYC [220-222]. Our study identifies IGFBP2 as a key activator of two fundamental functions of EGFR-STAT3, signal transduction and nuclear activity in glioma. Canonical EGFR activation mechanism primarily involves ligand binding. Because EGFR ligands including EGF, TGFα and HB-EGF are constitutively expressed in normal adult brain [331-334], therapeutic targeting of these ligands is not feasible without compromising normal brain function. IGFBP2 is expressed in fetal brain and gliomas, but not in normal adult brain [78,84], thus making IGFBP2 a more attractive therapeutic target in gliomas.

Thus far, EGFR inhibition has limited patient response in glioma while STAT3 targeted therapy also has limited success in clinical trials in other cancers. By identifying the pivotal role of IGFBP2 in perpetuating nuclear crosstalk of EGFR/STAT3, our study uncovers the importance of exploiting IGFBP2 as a target for glioma therapy. In addition, Celecoxib can induce radiosensitivity in tumor cells by inhibiting radiation-induced nuclear EGFR transport [335]. Omomyc, which is a dominant negative form of cMYC, can inhibit glioma cell proliferation and survival, and also suppress tumor growth in orthotopic mouse xenograft [336]. Combination targeted therapy of IGFBP2, COX2, and cMYC may be a beneficial alternative to EGFR or STAT3 targeted therapy. Overall, our discovery can be extended to other tumor types with known IGFBP2/EGFR/STAT3 alterations, such as lung and breast cancer.
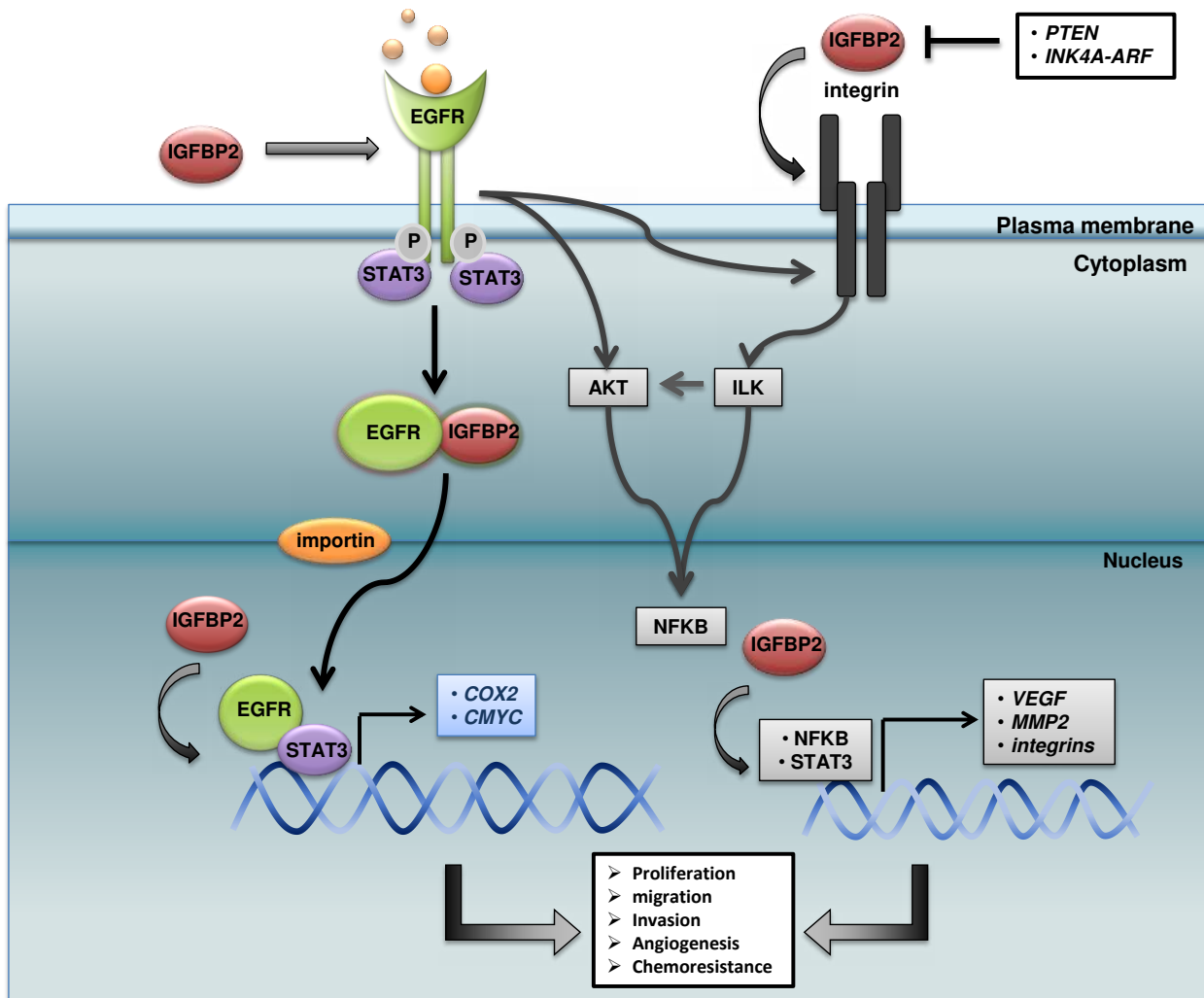
Figure 24. IGFBP2 functions in glioma. IGFBP2 through activation of integrin α5β1 can activate ILK-NFKB pathway. EGFR can also crosstalk with integrins to activate AKT-NFKB pathway. This project highlights a novel role of IGFBP2 in activating two functions of EGFR, intracellular signal transduction by way of STAT3 activation, and nuclear EGFR-STAT3 signaling. Ultimately, IGFBP2 activation of these oncogenic pathways lead to tumorigenic events.

CHAPTER 5: FUTURE DIRECTIONS

IGFBP2-EGFR-STAT3 mechanism of activation

As mentioned in the discussion, IGFBP2 interaction with EGFR should be further investigated to determine the mechanisms involved in EGFR-STAT3 activation (Fig. 25A, B). One possibility is that IGFBP2 may over-stimulate the production of EGFR ligands. Furthermore, as novel EGFR extracellular domain binding proteins have been reported to activate EGFR signaling, it is important to determine whether IGFBP2 can bind to the extracellular domain to activate EGFR. The possibility that IGFBP2 binding to EGFR confers EGFR to a constitutively activated state should also be investigated. Moreover, because IGFBP2 binds to integrin α5β1 and integrin α5β1 can activate EGFR, an IGFBP2-integrin α5β1-EGFR route of activation should be assessed (Fig. 25C).

IGFBP2 may also stabilize EGFR by preventing degradation possibly by upregulated Cdc42 to interfere with Cbl ubiquitinase. Because we observed increased nuclear EGFR accumulation with IGFBP2 expression, we should investigate whether IGFBP2 plays a role in actively transporting EGFR into the nucleus by cooperating with importins, or that IGFBP2 interferes with nuclear export mechanisms of EGFR (Fig. 25D).

Bild et al demonstrated that receptor-mediated endocytosis is required for STAT3 translocation from the cytoplasm into the perinuclear region [337]. STAT3 colocalizes with EGF-EGFR complex in endocytic vesicles and this complex is then transported from the cytosol to the perinuclear region. It is suggested that EGF is released from the complex at the perinuclear region, and that allows STAT3 to subsequently be released and imported into the nucleus. It is conceivable that IGFBP2-EGFR-STAT3 may also exist in a complex in glioma cells. Phenylarsine oxide (PAO), a pharmacological endocytosis inhibitor, blocks EGFR endocytosis, resulting in the loss of nuclear Stat3 DNA-binding activity. PAO can be used to assess whether

IGFBP2 is endocytosed into the cell via receptor-mediated endocytosis, particularly EGFR.

Therapeutic IGFBP2 inhibition

IGFBP2 inhibition has mainly been tested *in vitro* using neutralizing antibody (Ab) or antisense oligonucleotide (ASO). Neutralizing antibody blocks the functions of IGFBP2, but in general does not alter its expression. Antisense IGFBP2 is complementary to IGFBP2 mRNA and by base pairing with IGFBP2 mRNA, can inhibit physically inhibit translation of IGFBP2 [338]. In a study of metastasis, IGFBP2 inhibition using neutralizing antibody inhibited endothelial cell recruitment by metastatic cells [126]. IGFBP2 inhibition using neutralizing antibody suppressed angiogenic activity in melanoma cells [339]. In prostate cancer cells, neutralizing IGFBP2 antibody or antisense IGFBP2 can inhibit IGFBP2-mediated cell proliferation [340]. IGFBP2 can stimulate adult neural stem cells to differentiate into neurons whereas blocking of IGFBP2 by neutralizing antibody can inhibit neuronal differentiation [341].

Our group introduced antisense IGFBP2 into a PDGF-driven glioma mouse model, and demonstrated that in addition to reduced IGFBP2 expression, survival was prolonged compared to control mice [148]. OGX-225 is a second-generation antisense preclinical drug that inhibits both IGFBP2 and IGFBP5. So et al demonstrated that OGX-225 downregulates IGFBP2 expression in both IGFBP2-overexpressing or endogenous IGFBP2 expressing breast cancer cells [342]. OGX-225 also decreased IGFBP2-mediated cell growth *in vitro* and tumor growth in xenograft mice. Furthermore, IGFBP2 overexpression results in resistant to paclitaxel-induced growth inhibition whereas OGX-225 chemosensitized these cells to paclitaxel.

Because of the effectiveness of IGFBP2 inhibition *in vitro* and *in vivo*, future studies to test the efficacy of IGFBP2 inhibition with neutralizing antibody or OGX-225 using the RCAS glioma mouse model should be performed (Fig. 25E). Furthermore, targeted therapy of EGFR, STAT3, COX2, or cMYC in combination with IGFBP2 should also be investigated (Fig. 25F).
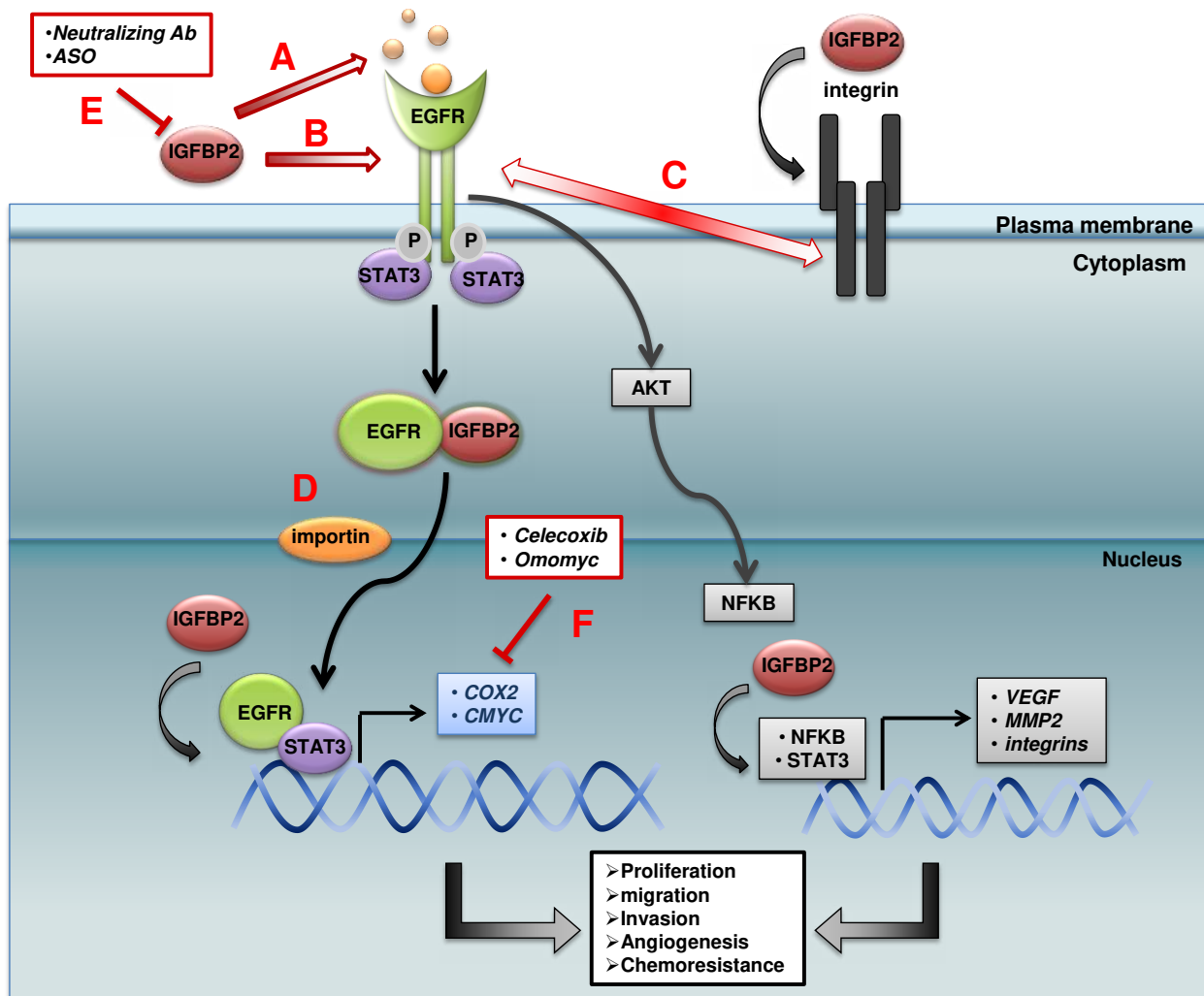
Figure 25. Proposed future research plans for IGFBP2 in glioma. Determine whether IGFBP2 activates EGFR through (**A**) increasing EGFR ligand production, or (**B**) stabilization of EGFR, or (**C**) crosstalk with integrin a5b1. (**D**) The role of IGFBP2 in EGFR nuclear import or export should also be investigated. Efficacy of therapeutic inhibition using (**E**) IGFBP2 neutralizing antibody (Ab) or antisense oligonucleotide (ASO), and/or combination therapy with (**F**) celecoxib or omomyc should be assessed.

CHAPTER 6: BIBLIOGRAPHY

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Vita

Yingxuan (Corrine) Chua was born in Kuala Lumpur, Malaysia on April 28, 1984, to Chua Lock

Wah and Tneh Mooi Hong. She graduated from SMK IJC in Malacca, Malaysia in 2001. She

pursued her bachelor's degree at the University of Louisiana at Lafayette and graduated *magna*

cum laude in May 2006 with a microbiology degree. She then pursued a master's degree at the

University of Texas Health Science Center at San Antonio and graduated in December 2009. In

January 2010, she entered the University of Texas Health Science Center at Houston Graduate

School of Biomedical Sciences. After completing laboratory rotations in the labs of Drs. Dihua

Yu, Wei Zhang and Edward Jackson, she joined Dr Wei Zhang's lab in August 2010. She is a

co-author on the following publication: Holmes KM, Annala M, Chua YX, Dunlap SM, Liu X,

Hugen N, Moore LM, Cogdell DE, Hu L, Nykter M, Hess K, Fuller GN, and Zhang W. IGFBP2-

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ILK, and NF-κB. Proc Natl Acad Sci U S A. 2012 Feb 28;109(9):3475-80. Her work on IGFBP2

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