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ORIGINAL ARTICLE

IGFBP2 potentiates nuclear EGFR–STAT3 signaling

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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 tumor-promoting mechanisms of IGFBP2 are poorly understood. The contributions of intracellular IGFBP2 to tumor development and progression are also unclear. Here we present evidence that both exogenous IGFBP2 treatment and cellular IGFBP2 overexpression lead to aberrant activation of epidermal growth factor receptor (EGFR), which subsequently activates signal transducer and activator of transcription factor 3 (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 glioblastoma 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 Cancer Genome Atlas database for human glioma. A high level of all three proteins (IGFBP2, EGFR and STAT3) was strongly correlated with poorer survival in an independent patient data set. 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 two 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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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.[1–4] Once internalized, these proteins can regulate intracellular cytoplasmic signal transduction and transcriptional activity in the nucleus.[5–11] 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.[12–14] 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[15–17] and activate phosphatidylinositol 3′-kinase/AKT,[18] nuclear factor-κB[15] and extracellular signal–regulated kinase.[19] Recently, a classic nuclear localization signal sequence that is responsible for nuclear entry has been identified in *IGFBP2*.[20] However, the functional and clinical significance of nuclear IGFBP2 has not been clearly elucidated.[21–24]

In mammals, IGFBP2 is expressed at high levels in embryonic tissues, but the expression is drastically decreased after birth. However, IGFBP2 expression has been observed postnatally in hematopoietic stem cells and in the liver and spleen progenitor cell populations.[25–30] *IGFBP2* is reactivated during progression of a wide spectrum of cancer types, including glioma and prostate, breast and lung cancers.[18,30–32] IGFBP2 has an oncogenic role in tumor initiation and progression to high-grade glioma[33] and is reported as one of the nine genes in a signature associated with poor clinical outcome in high-grade glioma.[34] IGFBP2 mediates cell expansion and survival of glioma stem cells.[35,36] Despite the clear role for IGFBP2 in tumorigenesis, the mechanisms underlying nuclear IGFBP2's contribution to the tumorigenic program remain unknown.

EGFR/IGFBP2 and EGFR/STAT3[37,38] are concurrently co-expressed in glioma. EGFR, a cell surface tyrosine kinase receptor, is activated in 30–50% of high-grade gliomas through amplification, overexpression or mutation.[39–41] EGFR signal transduction can be mediated by STAT3. STAT3 interacts with EGFR at two autophosphorylation sites in the cytoplasmic domain, tyrosine 1068 or tyrosine 1086[42] and is activated by phosphorylation at tyrosine 705 (Y705).[43] 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 cyclooxygenase 2 (*COX2*),[44] *iNOS*[45] and *c-MYC*.[46] Nuclear EGFR expression in glioma and other cancers, such as breast carcinoma,[47] esophageal squamous cell carcinoma [48] and ovarian cancer,[49] is associated with poor survival and linked to an aggressive tumor phenotype.[50] Furthermore, IGFBP2 regulates the expression of *VEGF*, *MMP2*, *TIMP1*, *TWIST*, *BCL2* and *HIF1A* genes,[20,51] which are known transcriptional targets of STAT3. Recent research implicated nuclear IGFPB2 in angiogenesis through activation of *VEGF*, a STAT3 target gene.[20] 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

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IGFBP2 mediates the tumorigenic program through a tightly linked IGFBP2–EGFR–STAT3 regulatory signaling network.

RESULTS

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 uptake into the cell (Figure 1a). STAT3 activation via phosphorylation at tyrosine 705, designated pSTAT3 (Y705), and the expression of STAT3 transcriptional targets Bcl-xL, cyclin D1 and c-MYC also increased in response to IGFBP2 stimulation.

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 min following addition of exogenous IGFBP2 (Figure 1b). In comparison with EGF (EGFR ligand) stimulation, IGFBP2 stimulation of EGFR activation was substantially less than that produced by EGF (data not shown). Furthermore, immunoblotting analysis of SNB19 cells stably overexpressing IGFBP2 (SNB19.BP2) demonstrated that, compared with SNB19 cells stably transfected with empty vector (SNB19.EV), IGFBP2 overexpression resulted in the increased expression of EGFR and phosphorylated STAT3, along with Bcl-xL, cyclin D1 and c-MYC (Figure 1c).

To examine the involvement of EGFR in IGFBP2-mediated STAT3 activation, we depleted EGFR by using two different pools of small interfering RNA (siRNA) in SNB19.BP2 cells and observed decreases in STAT3 activation (Figure 1d), 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 interleukin-6. We observed STAT3 phosphorylation in these cells, confirming that EGFR knockdown impairs STAT3 activation by IGFBP2 without compromising alternate STAT3 activation pathways (Supplementary Figure S1).

EGFR can be indirectly activated through transactivation, which involves a disintegrin and metalloproteinases (ADAMs).[52] To determine whether ADAMs are involved in IGFBP2-mediated EGFR activation, we inhibited ADAMs by treatment with two different ADAM inhibitors, TAPI2 and marimastat.[53,54] U87 cells serum-starved overnight were pretreated with 20 μM TAPI-2 or marimastat and then stimulated with exogenous IGFBP2 for 5 min (Supplementary Figure S2A). Immunoblotting analysis demonstrated that exogenous IGFBP2 stimulated EGFR and STAT3 activation despite ADAM inhibition. Furthermore, because ADAM17 is essential to regulation of EGFR transactivation,[52] we knocked down ADAM17 using two different pools of siRNA to evaluate whether IGFBP2-mediated EGFR activation involves ADAM17 (Supplementary Figure S2B). 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.

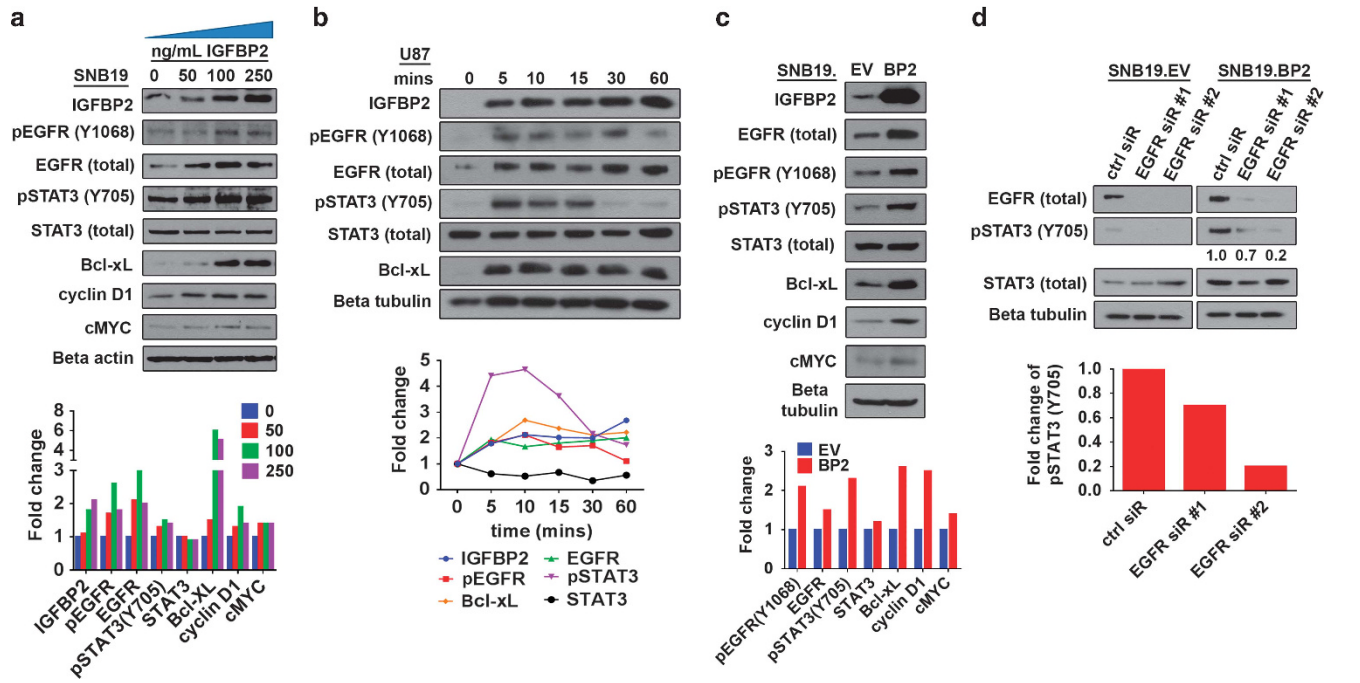


Figure 1. IGFBP2 activates STAT3 through EGFR. (**a**) Immunoblot analysis of SNB19 cells starved of serum overnight and then stimulated with exogenous IGFBP2 protein at the indicated dosages (0, 50, 100, 250 ng/ml) for 60 min. Densitometric analysis shown below the immunoblot indicates fold-change relative 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 and then stimulated with exogenous IGFBP2 (100 ng/ml) for the indicated time points (0, 5, 10, 15, 30, 60 min). Densitometric analysis shown below the immunoblot indicates fold-change relative to unstimulated control cells (normalized to loading control or total protein for phosphorylated proteins). (**c**) 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). (**d**) Immunoblot analysis comparing SNB19.EV and SNB19.BP2 cells depleted of EGFR via two 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.

IGFBP2 is significantly correlated with STAT3 pathway activation in glioma

Previous studies showed that IGFBP2 regulates the expression of many STAT3 target genes,[20,51] 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. Gene set enrichment analysis (GSEA) revealed that STAT3-activated genes were significantly and positively correlated with *IGFBP2* (Figure 2a), suggesting that IGFBP2 expression is associated with STAT3 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) data set. 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)* (Supplementary Figure S3). This finding further validates that the expression of *IGFBP2* and *STAT3* 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 seven proteins (Figures 2b and c) that were most significantly and strongly correlated with both IGFBP2 and pSTAT3 (Y705) (correlation coefficients >0.2). Of these seven strongly correlated proteins, five are closely related to the STAT3 signaling pathway, namely plasminogen activator inhibitor-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.[55–59] Thus 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.

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

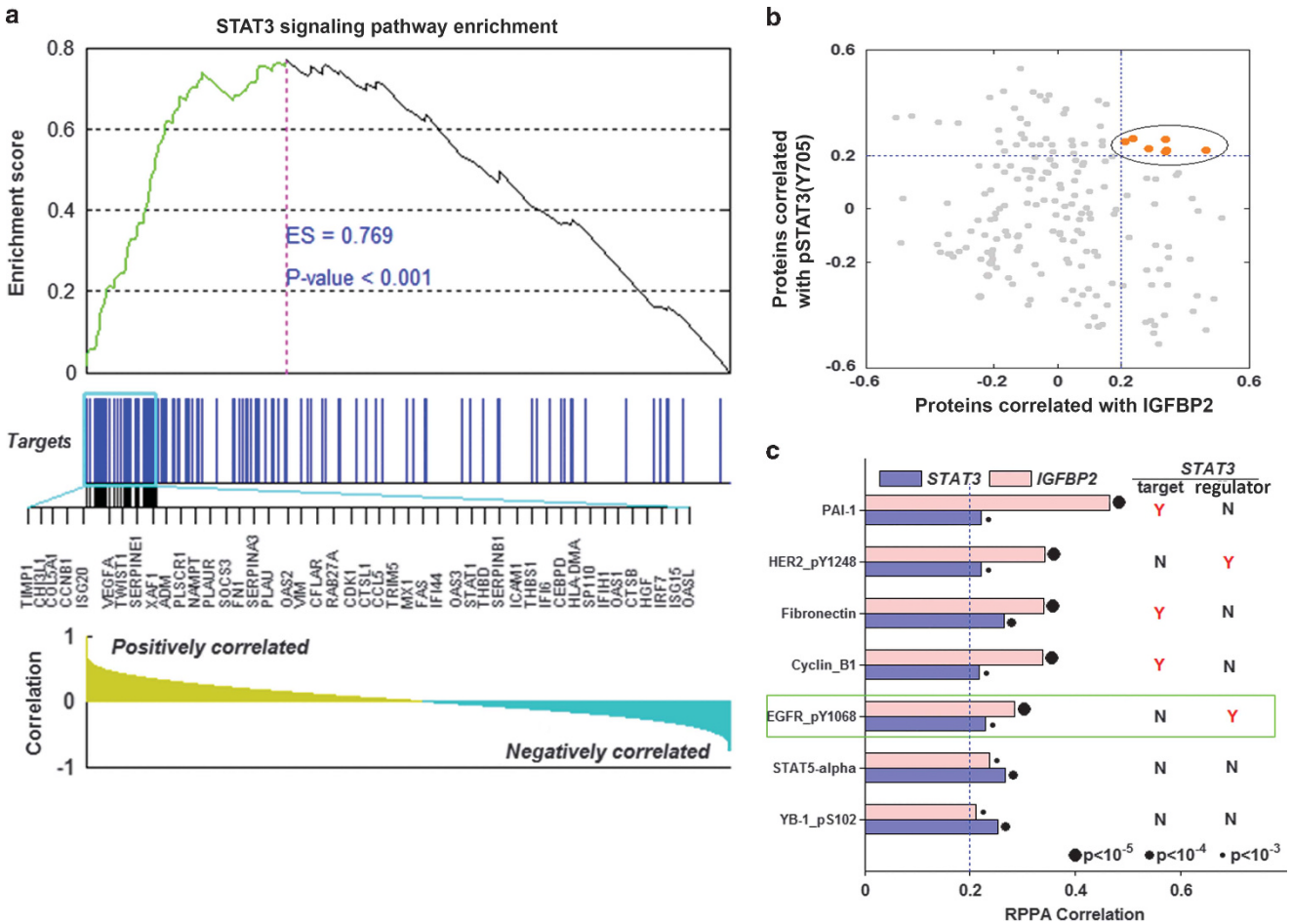


Figure 2. IGFBP2 is strongly and significantly correlated with STAT3 pathway genes. (**a**) GSEA demonstrated enrichment for STAT3 target genes based on correlation with *IGFBP2* expression in the TCGA low-grade glioma database. The top of the panel shows the enrichment score (ES) for genes associated 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*). (**b**) 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 >0.2 are highlighted in orange. (**c**) Correlation of the seven 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.

SNB19 cells and empty vector control cells. Co-IP experiments revealed co-precipitation of IGFBP2 and EGFR (Figure 3a). We next treated U87 cells, which had been serum-starved overnight, with two different doses of exogenous IGFBP2 followed by IP analysis and immunoblotting. The results showed a dose-dependent increase of IGFBP2 co-precipitated with EGFR (Figure 3b). 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 (Figure 3c). Co-localization of IGFBP2 and EGFR provides further evidence of a complex containing IGFBP2 and EGFR.

IGFBP2 facilitates EGFR nuclear accumulation

Because we observed IGFBP2 and EGFR co-localization in the cytoplasm and nucleus, we investigated whether nuclear IGFBP2 is closely linked to 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 (Figure 4a). 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 (Figure 4b). A time-course study with the same cells demonstrated that IGFBP2 nuclear accumulation paralleled EGFR nuclear accumulation in a time-dependent manner (Figure 4c). To validate that EGFR nuclear accumulation is mediated through IGFBP2, we knocked down IGFBP2 using two 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 (Figure 4d). These results suggest that IGFBP2 has a role in promoting EGFR nuclear accumulation.

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[60] (BP2ΔNLS; Supplementary Figure S4A). Transient transfection of BP2ΔNLS plasmid into SNB19.par cells resulted in the expected compromise of IGFBP2 nuclear entry and also impaired EGFR nuclear accumulation (Figure 4e). Next, we created a stable BP2ΔNLS-overexpressing cell line (SNB19.BP2ΔNLS). Impaired EGFR nuclear accumulation in fractionated stable SNB19.BP2ΔNLS cells, compared with SNB19.BP2 WT (wild-type IGFBP2), resulted in decreased nuclear expression of COX2 and c-MYC, which are known downstream targets of nuclear EGFR/STAT3 complex (Figure 4f). These results were replicated in another glioma cell line, T98G (Supplementary Figures S5A and B). 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 (Supplementary Figure S6). 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,[15,51] 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 with SNB19.BP2 WT (Supplementary Figures S4B and C), indicating that nuclear IGFBP2

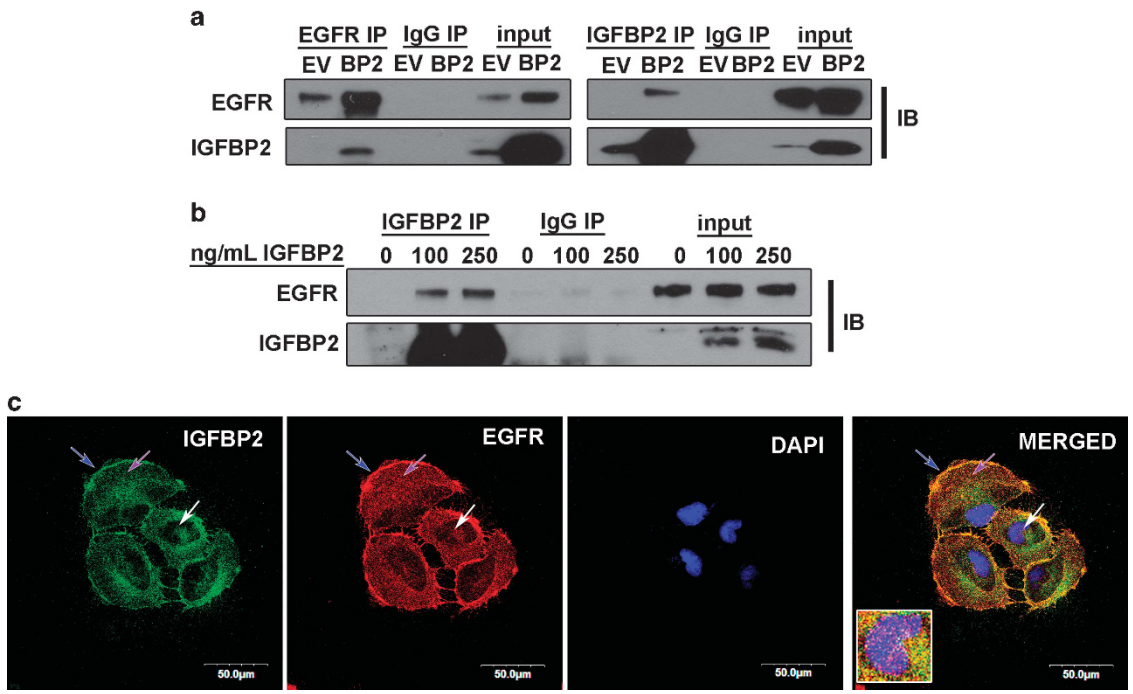


Figure 3. IGFBP2 co-precipitates and co-localizes with EGFR. (**a**) Co-IP of IGFBP2 and EGFR in SNB19.EV control cells versus SNB19.BP2 cells analyzed by immunoblotting (IB). (**b**) IP of IGFBP2 in U87 cells starved of serum overnight and then stimulated with two different doses of IGFBP2 for 30 min, analyzed by IB. (**c**) Confocal microscopic 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.

is important for the invasive phenotype of glioma cells, plausibly through regulation of nuclear EGFR–STAT3 activity.

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 (Figures 5a and b, Supplementary Table S1). 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 (Figures 5c and d, and Supplementary Table S1). 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

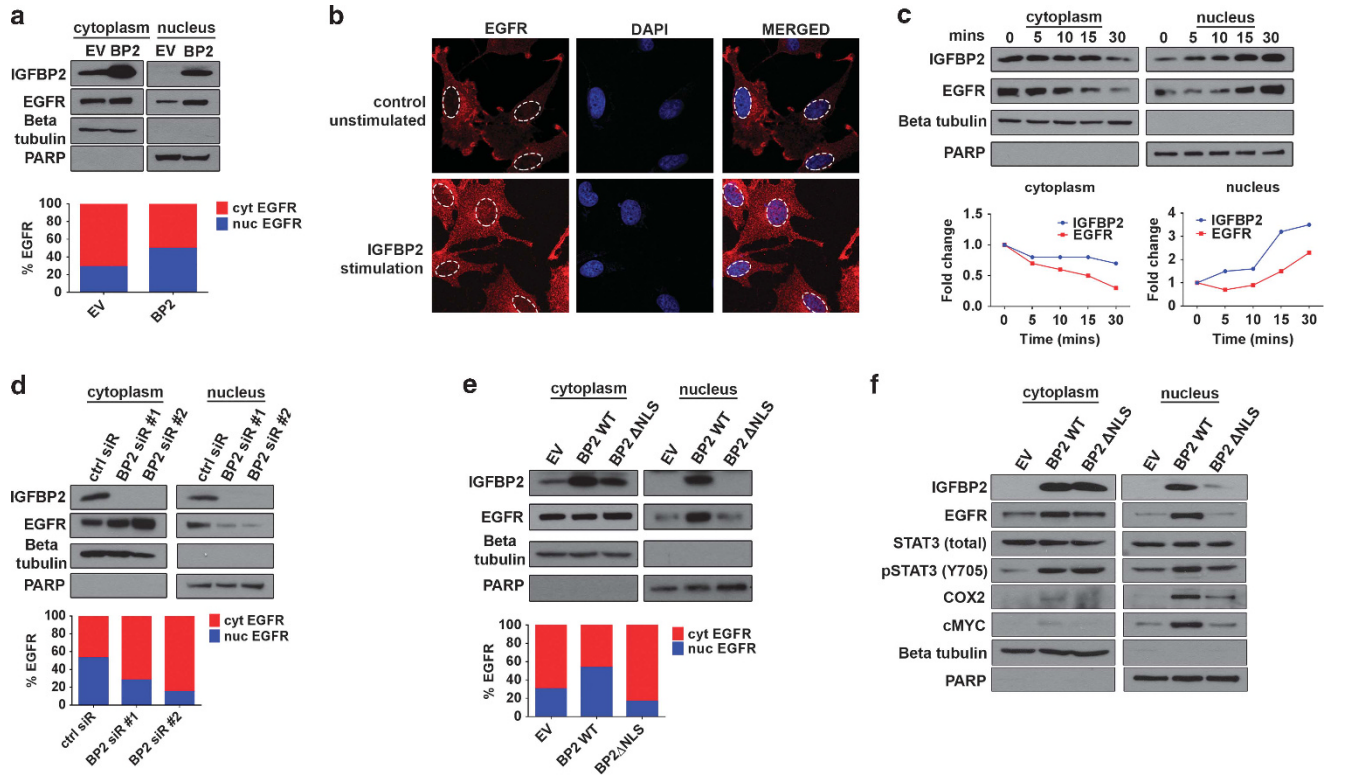


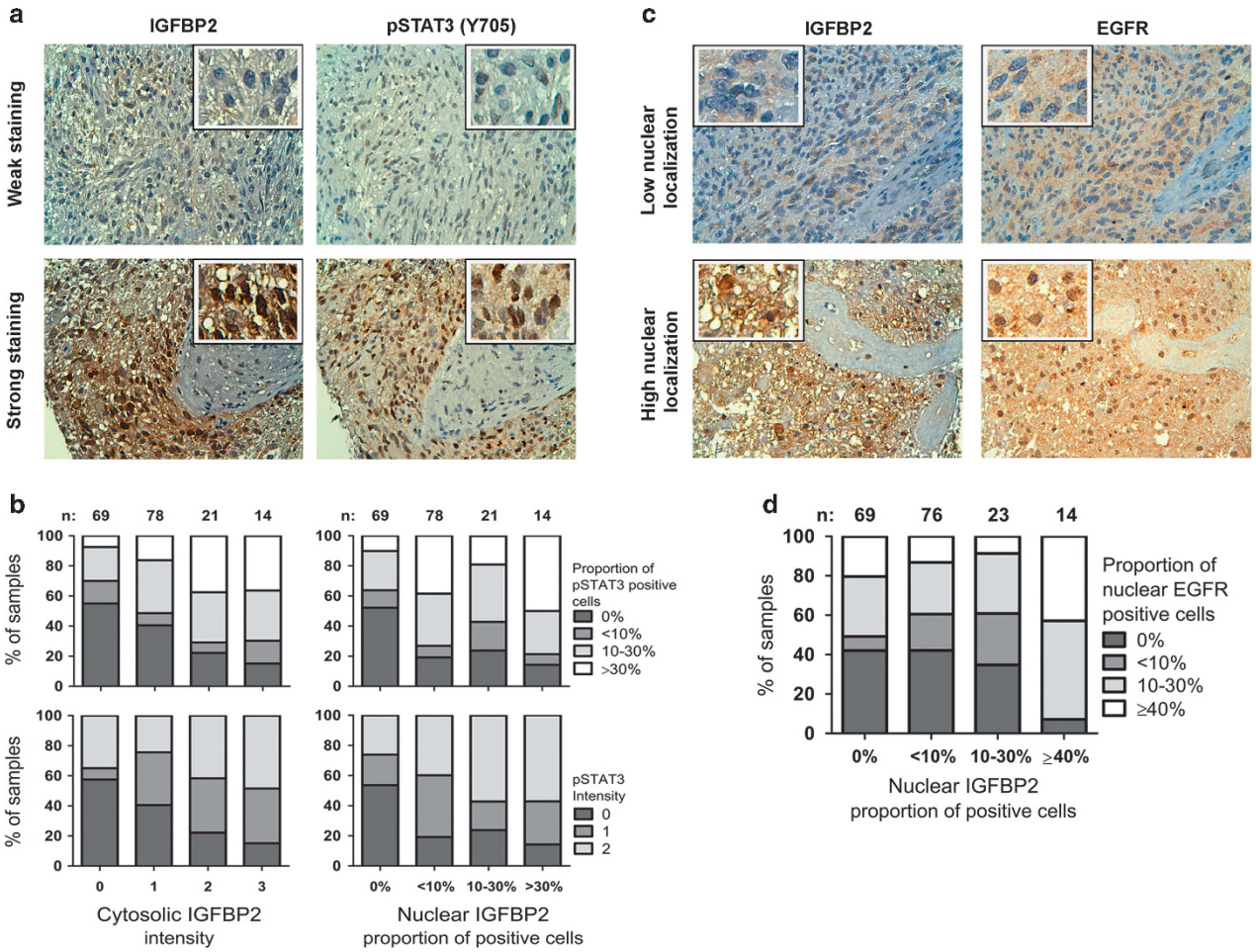
Figure 4. IGFBP2 drives EGFR nuclear accumulation. (**a**) Immunoblot analysis of cytoplasmic (cyt) and nuclear (nuc) fractions of SNB19.EV and SNB19.BP2 cells. Beta-tubulin represents a loading control for the cytoplasmic fraction, and PARP represents a loading control for the nuclear fraction. Densitometric analysis represented by the bar graph demonstrates the percentage of cytoplasmic or nuclear EGFR. (**b**) Confocal images of SNB19 parental cells and SNB19 parental cells stimulated with exogenous IGFBP2 protein (250 ng/ml for 30 min). Cells were stained for EGFR (red) and the nuclei were stained with DAPI (blue). (**c**) Immunoblot analysis of cytoplasmic and nuclear fractions of SNB19 parental cells stimulated with exogenous IGFBP2 (250 ng/ml for the 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 two 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 the percentage of cytoplasmic or nuclear EGFR. (**e**) 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 the percentage of cytoplasmic or nuclear EGFR. (**f**) Immunoblot analysis of cytoplasmic and nuclear proteins in stable SNB19.EV, SNB19.BP2 WT and SNB19.BP2ΔNLS cells.

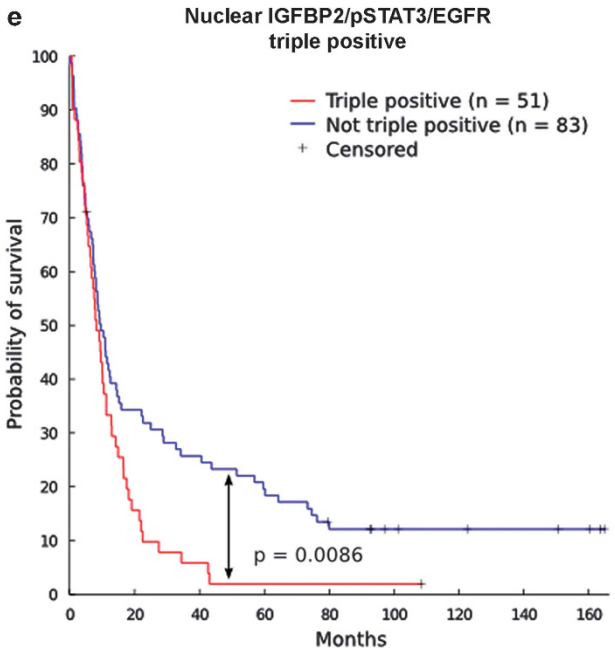
Figure 5. IGFBP2 correlates with STAT3 activation and nuclear EGFR localization in clinical samples. Expression and localization of IGFBP2, pSTAT3(Y705) and EGFR were detected with immunohistochemistry from a TMA that included 222 human grade 2–4 gliomas. (**a**) TMA immunostaining images (magnification ×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. (**e**) Nuclear co-localization of IGFBP2, EGFR and phosphorylated STAT3 predicted poor survival among patients with human grade 2–4 gliomas. Patients were stratified into two cohorts based on the nuclear staining of all three 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 ($P=0.0086$).

($P = 0.011$). Furthermore, clinical samples that were triply positive for nuclear accumulation of IGFBP2, phosphorylated STAT3 and EGFR were strongly associated with poor survival (Figure 5e).

DISCUSSION

Conventionally, proteins are categorized as secreted, membrane-bound or intracellular. However, accumulating evidence





demonstrates that signaling molecules are spatiotemporally dynamic;[61–66]
in addition to cell surface–initiated signaling, upon internalization, EGFR can mediate signaling in the endosome,[67–70] nucleus[71] or mitochondria.[72,73] EGFR crosstalks with STAT3 through two levels: tyrosine kinase–mediated activation of STAT3[42,43,74] and nuclear cooperation as transcriptional cofactors.[44–46] Our results significantly expand our understanding of this network by demonstrating that IGFBP2 stimulation or overexpression contributes to activation of EGFR–STAT3 and downstream pathways. IGFBP2 forms a complex containing EGFR and facilitates the nuclear accumulation of EGFR to potentiate nuclear EGFR–STAT3 activity. Thus IGFBP2 controls two fundamental functions of EGFR, cytoplasmic signal transduction and nuclear accumulation.

These results also elucidate newly identified oncogenic functions for nuclear IGFBP2. By facilitating nuclear EGFR accumulation, nuclear IGFBP2 activates an EGFR–STAT3-mediated transcriptional program, which promotes transcription of *iNOS*, *c-MYC* and *COX2*.[44–46] The resulting aberrant activation of these genes leads to uncontrolled cell proliferation, survival and metastasis and hence poor prognosis. COX2 is upregulated and correlated with poor survival in glioma,[75–77] iNOS is critical for glioma stem cell survival and tumorigenesis[78–81] and c-MYC is correlated with high-grade glioma and important for glioma stem cell maintenance.[82–84] We also demonstrated a role for nuclear IGFBP2 in promoting cancer cell migration and invasion, a key hallmark of cancer and a major aspect of glioma aggressiveness and treatment response.

The dynamic nature of these oncogenic signaling molecules may contribute to the ineffectiveness of EGFR-targeted therapy in glioma, perhaps because EGFR is being actively translocated into the nucleus by IGFBP2, rendering the cells resistant to therapies targeting membrane EGFR. Thus IGFBP2 may serve as an escape mechanism for glioma cells from EGFR-targeted therapy. EGFR can be activated by eight different ligands,[85,86] including EGF, transforming growth factor α and heparin-binding EGF. EGF, transforming growth factor α and heparin-binding EGF are constitutively expressed in the developing and normal adult brain.[87–90] Therefore, therapeutic targeting of these ligands is not feasible. Conversely, IGFBP2 is highly expressed in the fetal brain and glioma but not in the normal adult brain,[26,27,30,91,92] making it a better therapeutic target than the known EGF ligands. EGFR amplification and activation are among the most common oncogenic events in human cancer,[93,94] and IGFBP2 overexpression is also a frequent event in glioma and other cancers. Our analysis of three independent clinical data sets revealed strong correlation between IGFBP2 and EGFR in terms of both gene expression and protein localization in the cell. The development of therapeutic strategies that target both molecules may represent a rational approach for cancer therapeutics.

MATERIALS AND METHODS

Cell culture, treatments, plasmids and transfections

SNB19, U87 and T98G cells were obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco modified essential/F12 50:50 medium supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin in an incubator with 5% CO_2 at 37 °C. SNB19.EV (empty vector) and SNB19.BP2 WT (IGFBP2 wild type) cells were created as previously described.[51] 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, USA) 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, USA) 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, USA) with cells starved of serum overnight. Depletion of IGFBP2 and EGFR was achieved via transfection of Lipofectamine RNAiMAX (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol with two different pools of siRNA from Mission siRNA (Sigma, St Louis, MO, USA) for 48 h. Some cells were treated with a broad-spectrum ADAM inhibitor, TAPI-2 (no. 14695; Cayman Chemical, Ann Arbor, MI, USA) or marimatstat (no. M2699; Sigma) at 20 μM for 2 h. Depletion of ADAM17 siRNA was achieved via transfection of Lipofectamine RNAiMAX according to the manufacturer's protocol with two different pools of siRNA from Life Technologies (nos. s13718 and s13719).

Gene set enrichment analysis

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.[95]

In a similar manner, the correlation of IGFBP2 or STAT3 protein expression with proteins in the TCGA TMA was calculated for 257 LGG samples for which 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.

IP, immunoblotting and cellular fractionation

For IP, cells were subjected to lysis in NP-40 buffer with 0.1% phosphatase inhibitor cocktail (Pierce Biotechnology, Thermo Fisher Scientific, Waltham, MA, USA). After preclearing for 1 h at 4 °C with Protein G agarose beads (SC no. 2002; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and appropriate species normal immnuoglobulin G (IgG), lysates were immunoprecipitated overnight at 4 °C with Protein G agarose beads using antibodies to IGFBP2 (no. SC-6001; Santa Cruz Biotechnology; 1:100) and EGFR (no. 2256; Cell Signaling Technology, Beverly, MA, USA; 1:100). Beads were washed with NP-40 buffer three 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, USA) for 1 h at 100 V in transfer buffer (24 mM Tris base, 191 mM glycine and 20% (v/v) methanol). Membranes were blocked for 1 h 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 (no. SC-6001; 1:500); EGFR (no. 4267; Cell Signaling Technology; 1:1000), EGFR-Y1068 (no. 3777; Cell Signaling Technology; 1:1000), beta-tubulin (no. 2128; Cell Signaling Technology; 1:1000), PARP (poly ADP-ribose polymerase; no. 9542; Cell Signaling Technology; 1:1000), STAT3 (no. 9139; Cell Signaling Technology; 1:1000), STAT3-Y705 (no. 9145; Cell Signaling Technology; 1:1000), Bcl-xL (no. 2764; Cell Signaling Technology; 1:1000), cyclin D1 (no. 2978; Cell Signaling Technology; 1:1000), c-MYC (no. SC-40; Santa Cruz Biotechnology; 1:1000), COX-2 (no. 160112; Cayman Chemical; 1:250), or ADAM17 (no. T5442; Sigma; 1:500) in blocking solution. After washing in PBST, blots were incubated for 1 h at room temperature in PBST with secondary antibodies (anti-goat IgG, anti-rabbit IgG, or anti-mouse IgG; Santa Cruz Biotechnology; 1:5000) coupled 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 the ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

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 (no. 4267; 1:100) and IGFBP2 (no. SC-6001; 1:100) at 4 °C overnight. They were then incubated with secondary antibody (Life Technologies (Alexa Fluor); 1:500) for 1 h at room temperature in 1% bovine serum albumin/PBS buffer. They were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA), 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 ×40/NA 1.3 objective (stacking from basement membrane to apical site at 1-μm intervals).

TMA 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.[96] 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, USA). 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 (no. SC-6001; 1:300), phosphorylated STAT3 (no. 9145; 1:100) and EGFR (GR-01, Calbiochem, San Diego, CA, USA; 1:50), together with the HRP-diaminobenzidine (DAB)–based Cell and Tissue Staining Kit (R&D Systems, Minneapolis, MN, USA) or the Envision +System HRP-DAB kit (Dako, Carpenteria, CA, USA).

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 four categories: 0, <10, 10–30 and $\geqslant 30\%$. 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 two 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 three proteins—IGFBP2, EGFR and phosphorylated STAT3 ($\geqslant$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 the SPSS 20.0 software for Windows (SPSS Inc., Chicago, IL, USA). 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, USA). 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% fetal bovine serum for 16 h. 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 five 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 h. The data were expressed as means ± s.e.m. and analyzed by Student's *t*-test for difference between the two groups.

Statistical analysis

Experiments were performed at least three times. GraphPrism 6 (GraphPad, La Jolla, CA, USA) and SPSS 20.0 software for Windows (SPSS Inc.) 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.[95] Student's *t*-tests were used for paired comparisons where variances were estimated to be similar. Except for one-side test for the GSEA analysis,[95] all other tests were two-sided, with $P<0.05$ as the threshold for statistical significance in all the tests. Indicated annotations correspond to the following *P*-values: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)