Guanylate binding protein 1 is a novel effector of EGFR-driven invasion in glioblastoma

Ming Li,[1] Akitake Mukasa,[1] Maria del-Mar Inda,[1] Jianhua Zhang,[2] Lynda Chin,[2] Webster Cavenee,[1] and Frank Furnari[1]

[1]Ludwig Institute for Cancer Research, School of Medicine, University of California, San Diego, La Jolla, CA 92093
[2]Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA 02215

Although GBP1 (guanylate binding protein 1) was among the first interferon-inducible proteins identified, its function is still largely unknown. Epidermal growth factor receptor (EGFR) activation by amplification or mutation is one of the most frequent genetic lesions in a variety of human tumors. These include glioblastoma multiforme (GBM), which is characterized by independent but interrelated features of extensive invasion into normal brain parenchyma, rapid growth, necrosis, and angiogenesis. In this study, we show that EGFR activation promoted GBP1 expression in GBM cell lines through a signaling pathway involving Src and p38 mitogen-activated protein kinase. Moreover, we identified YY1 (Yin Yang 1) as the downstream transcriptional regulator regulating EGFR-driven GBP1 expression. GBP1 was required for EGFR-mediated MMP1 (matrix metalloproteinase 1) expression and glioma cell invasion in vitro. Although deregulation of GBP1 expression did not affect glioma cell proliferation, overexpression of GBP1 enhanced glioma cell invasion through MMP1 induction, which required its C-terminal helical domain and was independent of its GTPase activity. Reducing GBP1 levels by RNA interference in invasive GBM cells also markedly inhibited their ability to infiltrate the brain parenchyma of mice. GBP1 expression was high and positively correlated with EGFR expression in human GBM tumors and cell lines, particularly those of the neural subtype. Together, these findings establish GBP1 as a previously unknown link between EGFR activity and MMP1 expression and nominate it as a novel potential therapeutic target for inhibiting GBM invasion.

CORRESPONDENCE
Frank Furnari:
ffurnari@ucsd.edu

Abbreviation used: AD, actinomycin D; ChIP, chromatin immunoprecipitation; CHX, cycloheximide; EGF, epidermal growth factor; EGFR, EGF receptor; EMSA, electrophoretic mobility shift assay; GBM, glioblastoma multiforme; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; PI3K, phosphatidylinositol 3-kinase; shRNA, short hairpin RNA; siRNA, small interfering RNA.

Glioblastoma multiforme (GBM) is the most common and deadly primary malignant brain tumor primarily because of its rapid growth, neovascularization, and invasiveness throughout the brain (Furnari et al., 2007). Its ability to migrate into brain parenchyma makes it resistant to multimodal therapies combining surgical resection, radiotherapy, and chemotherapy and results in a median survival time for patients of ~12–16 mo (Stupp et al., 2005). This demands identification of pathways controlling GBM cell invasion, which complement those affecting its growth and angiogenesis, as an important investigative endeavor in the search for therapies that improve patient survival.

Attempts to understand the biology of malignant gliomas have focused on genetic and molecular alterations of tumor cells. The most common genetic alteration associated with GBM is amplification and/or mutation of the epidermal growth factor (EGF) receptor (EGFR) gene, a transmembrane receptor tyrosine kinase which has been detected in 40–60% of patients with GBM (Libermann et al., 1985; Wong et al., 1987, 1992). We previously demonstrated that mutant EGFR dramatically enhances the tumorigenicity of glioma cells in a pleiotropic manner by increasing proliferation and resistance to apoptosis (Nishikawa et al., 1994; Nagane et al., 1996; Huang et al., 1997; Narita et al., 2002). However, the effect of this oncogenic driver in the diffuse invasiveness that also characterizes GBM and the downstream pathways and effector molecules that might mediate this remain largely unknown.

© 2011 Li et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 208 No. 13 2657-2673
www.jem.org/cgi/doi/10.1084/jem.20111102

The Journal of Experimental Medicine

To identify the target genes regulated by EGFR activation, we performed expression array analysis and found that the most commonly altered expression was from a gene module normally associated with IFN stimulation and Stat function. This included *GBP1* (*guanylate binding protein 1*), initially identified as a type I and II IFN-induced gene, that encodes a 67-kD protein belonging to a large GTPase family, which includes dynamins and Mx proteins (Prakash et al., 2000a,b). In endothelial cells, GBP1 can be induced by IL-1β, TNF, and IFN-γ in vitro (Guenzi et al., 2001, 2003), and it is expressed in these cells in vivo during an inflammatory response (Lubeseder-Martellato et al., 2002) to interfere with angiogenesis and to inhibit the expression of MMP1 (matrix metalloproteinase 1), a collagenase necessary for cell migration through the extracellular matrix (Guenzi et al., 2003). A more recent study has demonstrated that GBP1 overexpression is associated with paclitaxel drug resistance in ovarian cancer cells (Duan et al., 2006). However, how GBP1 directs any of these actions is not clear.

In this study, we report a novel EGFR–Src–p38–YY1 (Yin Yang 1) signaling cascade that directly induces GBP1 expression in GBM cell lines and is distinct from the IFN-γ–stimulated GBP1 expression occurring through Stat1. Furthermore, we show that GBP1 is up-regulated in GBM tumor samples compared with the adjacent normal brain tissues and, consistent with our findings, is co-overexpressed with EGFR in GBM tumors and cell lines. Finally, in contrast to its function in endothelial cells, we show that GBP1 is required for EGFR signaling–mediated MMP1 expression and cell invasion in glioma cells, suggesting that GBP1 may represent a novel target for GBM therapeutics.

RESULTS

EGFR activity promotes GBP1 expression in glioblastoma cells

As a first step toward identifying the target genes of EGFR activity in glioma cells, genome-wide expression analyses were performed using the Affymetrix GeneChip Human Genome U133A array, which allows for the simultaneous study of ~14,500 characterized human genes. To accomplish this, messenger RNA (mRNA) expression levels of these genes were measured in the glioblastoma cell lines, U87 and U178, engineered with EGFR by retrovirus transduction (termed U87-EGFR and U178-EGFR, respectively), with or without 20 ng/ml EGF treatment for 3 h. The increased ratios (fold change) after the stimulation of EGF in these cells were calculated. The number of probe sets whose increased ratio showed more than twofold and more than threefold were 205 and 82 in U87-EGFR and 334 and 140 in U178-EGFR, respectively. To further identify genes that could be reproducibly induced by EGF stimulation in both U87-EGFR and U178-EGFR glioblastoma cell lines, the 13 probe sets (12 genes) whose expression were up-regulated more than threefold in all four sets of experiments were extracted (Table 1). Interestingly, many of these genes could be grouped into an IFN-γ response gene module including *SOCS1* and *SOCS3* (*suppressor of cytokines signaling 1 and 3*), *IRF1* (*IFN regulatory factor 1*), *IL-6*, *MCP1* and *MCP2* (*monocyte chemotactic protein 1* and *2*), and *GBP1*. We focused on GBP1 because it was one of the most highly up-regulated genes in this group and its function in cancer is largely unknown.

Table 1. Primary genes induced by EGFR activation

Gene name	Gene symbol	Accession number	Affymetrix probe set	Fold change (EGF plus/minus)			
				U87-EGFR		U178-EGFR	
				1st	2nd	1st	2nd
Chemokine (C-C motif) ligand 2/MCP-1	**CCL2*	NM_002982	216598_s_at	21.1	147.0	14.9	4.9
Chemokine (C-C motif) ligand 8/MCP-2	**CCL8*	NM_005623	214038_at	8.0	21.1	45.3	24.3
Guanine nucleotide binding protein (G protein), alpha 14	*GNA14*	NM_004297	220108_at	4.6	3.2	5.7	3.0
Guanylate binding protein 1	**GBP1*	NM_002053	202269_x_at	13.9	16.0	8.6	7.0
Guanylate binding protein 1	**GBP1*	NM_002053	202270_at	9.8	13.9	6.1	8.0
Interleukin 6	**IL6*	NM_000600	205207_at	16.0	6.1	5.7	8.0
Interferon-induced protein with tetratricopeptide repeats 3	**IFIT3*	NM_001031683	204747_at	3.7	3.7	5.3	3.5
Interferon regulatory factor 1	**IRF1*	NM_002198	202531_at	9.8	5.7	13.9	14.9
Interleukin 24	*IL24*	NM_006850	206569_at	12.1	6.5	10.6	3.7
Nuclear receptor subfamily 4, group A, member 3	*NR4A3*	NM_006981	209959_at	52.0	12.1	8.6	16.0
Suppressor of cytokine signaling 1	**SOCS1*	NM_003745	209999_x_at	4.0	5.3	6.5	8.6
Suppressor of cytokine signaling 3	**SOCS3*	NM_003955	206359_at	5.7	3.2	55.7	48.5
Tenascin C	*TNC*	NM_002160	216005_at	9.8	5.3	7.5	11.3

The human HG-U133A Affymetrix arrays were performed twice in U87-EGFR and U178-EGFR cells with or without EGF stimulation. The genes whose expression was up-regulated more than threefold in all four sets of experiments are listed. The genes that could be grouped into an IFN-γ response gene module are indicated by an asterisk. Accession numbers are from GenBank/EMBL/DDBJ.

GBP1 is up-regulated and positively correlated with EGFR expression in glioblastoma

Next, we validated the expression array data by RT-PCR and Western blot analyses. We found that EGF induced GBP1 expression in U87-EGFR in a time- and dose-dependent manner at both the mRNA and protein levels (Fig. 1, A and B). To determine whether the EGFR signaling–induced GBP1 expression was directly regulated at the transcriptional level, U87-EGFR cells were treated with 5 μg/ml actinomycin D (AD; a transcription inhibitor) or 100 nM cycloheximide (CHX; a protein synthesis inhibitor) for 1 h followed by EGF treatment. Fig. 1 C shows that AD prevented the EGF-mediated up-regulation of GBP1 in U87-EGFR cells, whereas no effect was observed in CHX-treated cells. These results suggest that EGFR signaling enhanced GBP1 expression at the level of transcription and was independent of de novo protein synthesis.

To determine whether the induction of GBP1 is a general response to EGFR activation in glioma, we also investigated U178-EGFR, U373-EGFR, and the GBM serial xenograft line, GBM26, which harbors amplification of EGFR (Sarkaria et al., 2007). Similar to the aforementioned results with U87-EGFR, it was determined that EGF also induced GBP1 expression in each of these three additional cell lines (Fig. 1 D).

To further investigate the relationship between GBP1 and EGFR, we analyzed their mRNA expression levels in 19 GBM tumor tissues by quantitative RT-PCR (RT-qPCR). Compared with normal brain tissue, GBP1 expression was increased in 12 of 19 (63%) GBM samples and, importantly, displayed a positive correlation with EGFR expression ($R = 0.624$; $P < 0.05$; Fig. 2 A). Consistent with these qPCR results, mining the cancer profiling database Oncomine (Rhodes et al., 2004; Sun et al., 2006) showed significantly elevated expression of GBP1 in GBM (Fig. 2 B). We then sought to expand our analysis of GBP1 expression in GBM tissues through The Cancer Genome Atlas (TCGA), a publicly available repository which has accumulated comparative genomic hybridization, gene expression, and DNA methylation analyses for 301 GBM samples (Cancer Genome Atlas Research Network, 2008). Using this database, coexpression of EGFR and GBP1 in GBM tumor samples was confirmed ($R = 0.375$; $P < 0.001$) with GBP1 expression appearing to correlate with GBM neural, classical, and mesenchymal subtypes (Verhaak et al., 2010), with the neural subtype presenting the strongest correlation ($R = 0.683$; $P < 0.001$) and the proneural subtype reliably not correlated ($R = 0.112$; $P = 0.33$; Fig. 2 C). Altogether, these data are consistent with the expression array results and indicate that EGFR signaling induces GBP1 expression in GBM.

EGFR signaling–stimulated GBP1 expression is Src–p38 mitogen-activated protein kinase (MAPK) dependent, whereas IFN-γ–induced GBP1 expression does not require Src or p38 MAPK

In response to EGF stimulation, EGFR triggers several downstream signaling cascades, including the MAPK, phosphatidylinositol 3-kinase (PI3K)/AKT, and Stat pathways (Wells, 1999). Therefore, we determined the downstream signaling pathway from EGFR that leads to GBP1 up-regulation by using both pharmacological (using a series of specific chemical inhibitors) and genetic (using small interfering RNA [siRNA]) approaches to target known EGFR effector pathways. When U87-EGFR cells were pretreated with an EGFR inhibitor (AG1478), MEK1/2 inhibitor (U0126 or PD98059), p38 inhibitor (SB203580), JNK1/2 inhibitor (SP600125), or PI3K inhibitor (LY294002) for 1 h followed by EGF treatment for 24 h, we observed that only the EGFR or p38 inhibitor abrogated EGF-induced GBP1 expression (Fig. 3 A), suggesting that GBP1 induction employs an EGFR–p38 signaling cascade. To eliminate

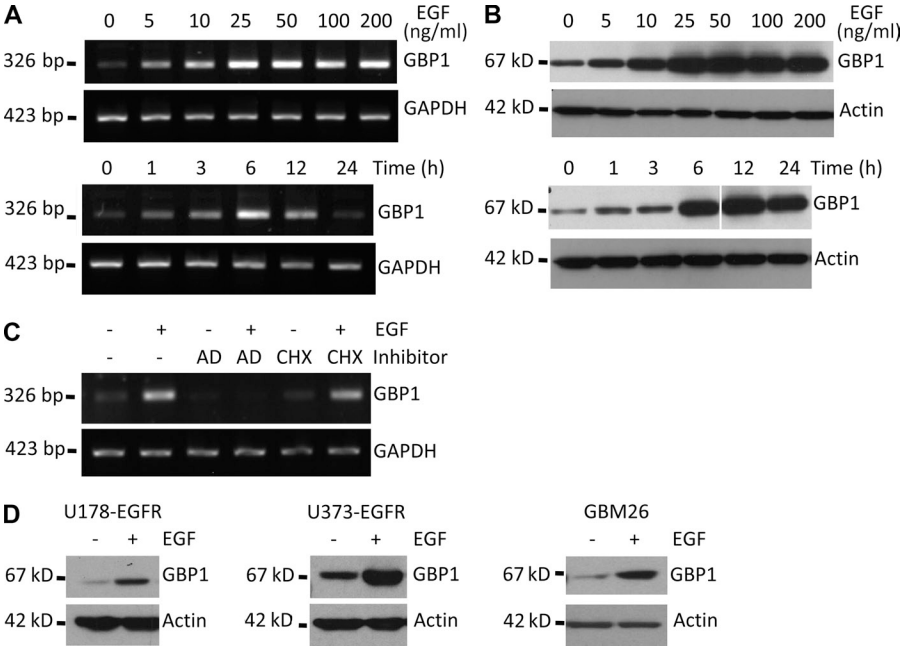

Figure 1. EGFR activity promotes GBP1 expression in GBM cells. (A and B) U87-EGFR cells were serum starved for 24 h and then stimulated with the indicated amount of EGF for 6 h (top) or with 20 ng/ml EGF treatment for the indicated time period (bottom). The expression of GBP1 was analyzed by semi-quantitative RT-PCR (A) and Western blot (B). (C) After 24 h of serum starvation, U87-EGFR cells were pretreated with AD or CHX for 1 h followed by 20 ng/ml EGF treatment for 6 h. GBP1 mRNA was measured by RT-PCR. (D) Western blot analysis of GBP1 induction by EGF in three other GBM cell lines. Cells were serum starved and treated with 20 ng/ml EGF for 24 h. Data are representative of at least two independent experiments.

any off-target effects of the p38 inhibitors, we also used siRNA to knockdown p38 in U87-EGFR cells. As shown in Fig. 3 B, depletion of p38 by treatment with 10 nM siRNA significantly prevented GBP1 induction by EGF. Consistent with this, the p38 inhibitor blocked EGF/EGFR-mediated p38 activation, and AG1478 inhibited both EGFR and p38 phosphorylation (Fig. 3 C).

Because Src family kinases are associated with MAPK activation in response to receptor tyrosine kinase activation (Ishizawar and Parsons, 2004; Summy et al., 2005; Lieskovska et al., 2006), we determined whether Src family kinases are involved in p38 activation and GBP1 induction in response to EGFR activation. Exposure of U87-EGFR cells to EGF induced activation of EGFR, c-Src, and p38 (Fig. 3 C). Inhibition of c-Src activation by pretreatment of cells with PP2 or dasatinib, two independent Src family kinase inhibitors, decreased EGF-induced p38 MAPK activation (Fig. 3 C and not depicted). In contrast, p38 inhibition was unable to block Src activation, suggesting that EGFR signaling activates p38 through Src (Fig. 3 C). Importantly, like p38 inhibition, PP2 or dasatinib dramatically reduced the GBP1 induction by EGF treatment in the three independent glioma cells lines (U87-EGFR, U178-EGFR, and U373-EGFR) tested (Fig. 3 D and not depicted).

Because IFN-γ is a well-known inducer of GBP1 expression in other cell types (Naschberger et al., 2004), we determined whether IFN-γ utilizes Src–p38 MAPK to induce GBP1 expression in GBM cell lines. As shown in Fig. 3 E,

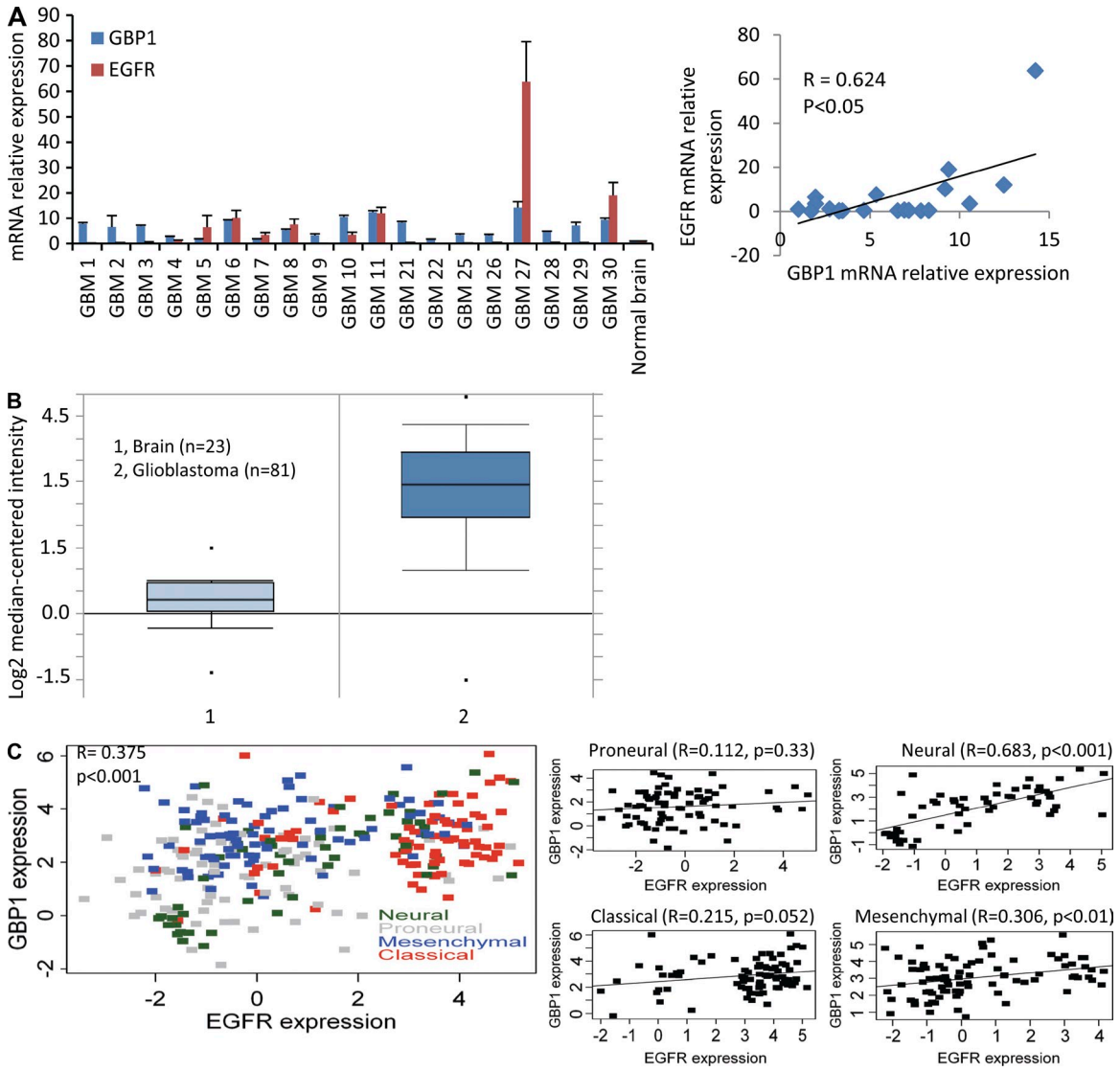

Figure 2. GBP1 is co-overexpressed with EGFR in GBM. (A) GBP1 and EGFR expression in GBM patients was measured by qPCR analysis. Normal denotes normal brain tissue. (right) Pearson r test. (B) Analysis of GBP1 gene expression in glioblastoma using the Oncomine database. (C) TCGA analysis of GBP1 and EGFR correlation in 301 GBM patient samples. The correlation is also shown in the four indicated subtypes of GBM (Verhaak et al., 2010). Classical, $n = 82$; mesenchymal, $n = 88$; neural, $n = 55$; proneural, $n = 76$. Data are representative of at least two independent experiments. Error bars represent SD.

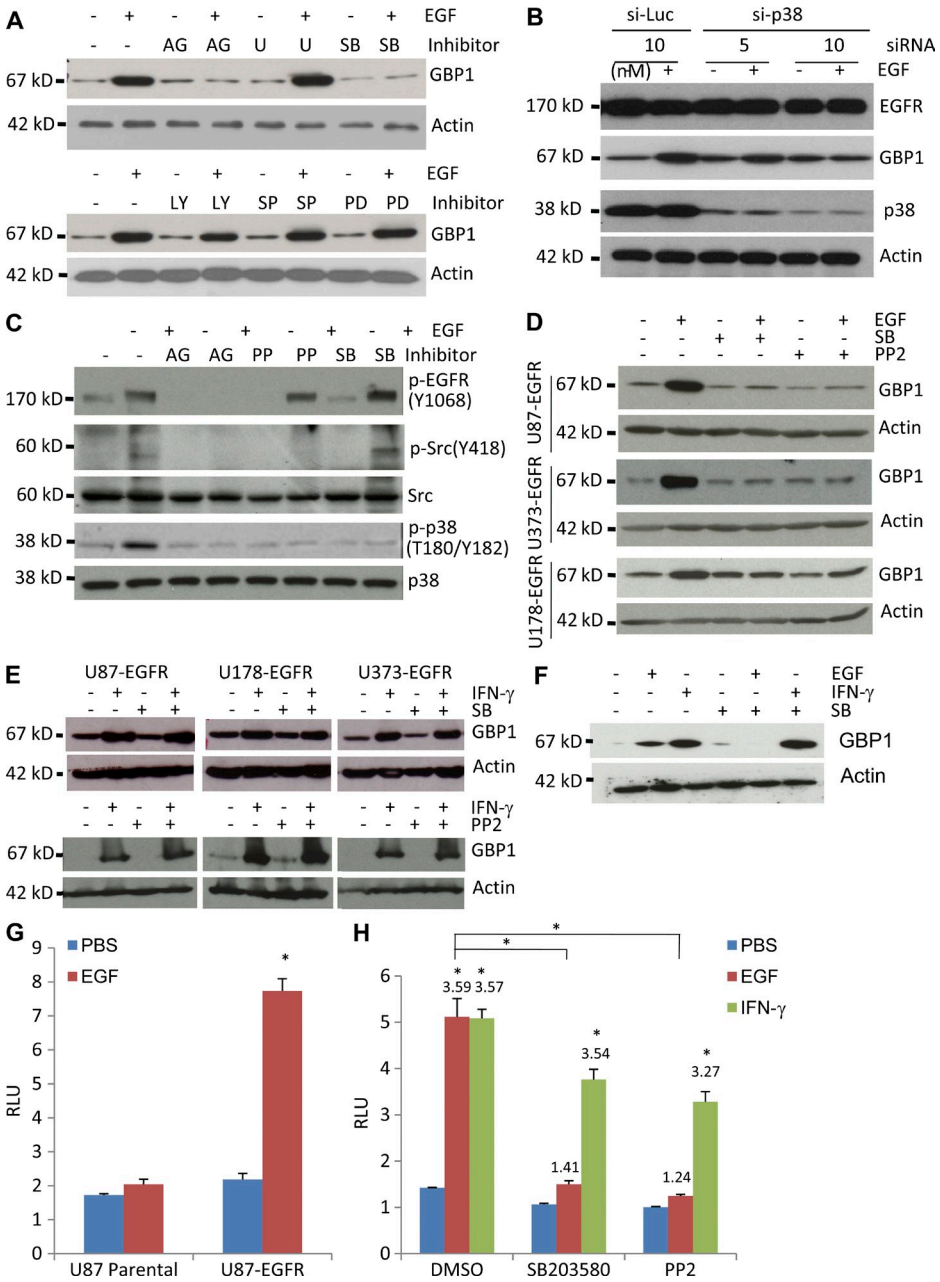

Figure 3. EGFR signaling–stimulated GBP1 expression is Src and p38 MAPK dependent, whereas IFN-γ–induced GBP1 expression is not. (A) After 24 h of serum starvation, U87-EGFR cells were treated with DMSO (−), 10 µM of the EGFR tyrosine kinase inhibitor AG1478 (AG), 20 µM of the MEK inhibitors U0126 (U) or PD980589 (PD), 20 µM of the p38 inhibitor SB203580 (SB), 10 µM of the PI3K inhibitor LY294002 (LY), or 20 µM of the JNK inhibitor SP600125 (SP) for 1 h followed by 20 ng/ml EGF treatment for 24 h before Western blot analysis. (B) U87-EGFR cells were transfected with the indicated concentration of p38 siRNA (si-p38) or control siRNA (si-Luc) for 24 h and then serum starved for 24 h before 20 ng/ml EGF treatment for an additional 24 h followed by Western blot analysis. (C) U87-EGFR cells were pretreated with DMSO (−), AG1478, SB203580, or PP2 (PP) for 1 h and then treated with 100 ng/ml EGF for 30 min before Western blot analysis. Total Src and p38 were used as loading controls. (D) U87-EGFR, U373-EGFR, and U178-EGFR cells were treated with DMSO (−), PP2, or SB203580 for 1 h before 20 ng/ml EGF treatment for 24 h. Cells were analyzed by Western blot. (E) U87-EGFR, U373-EGFR, and U178-EGFR cells were treated with DMSO (−), PP2, or SB203580 for 1 h before 20 ng/ml IFN-γ treatment for 24 h before Western blot analysis. (F) GBM26 cells were treated with DMSO (−) or SB203580 for 1 h before 20 ng/ml EGF or 20 ng/ml IFN-γ treatment for 24 h before Western blot analysis. All data are representative of at least two independent experiments. (G) U87 parental or U87-EGFR cells were transfected with pGL3-237 and pRL-TK for 24 h and then serum starved for 24 h followed by PBS or 20 ng/ml EGF treatment for an additional 6 h. Firefly and Renilla luciferase activities were measured, and promoter activity is presented as the fold induction of RLU (values of firefly luciferase unit/values of Renilla) as compared with the control. This result is expressed as the mean of three independent experiments ± SD. *, $P < 0.01$. (H) U87-EGFR cells were transfected with pGL3-237 and pRL-TK for 24 h and then serum starved for 24 h. The starved cells were pretreated with DMSO, 10 µM PP2, or 20 µM SB203580 for 1 h and then exposed to PBS, 20 ng/ml EGF, or 20 ng/ml IFN-γ for 6 h before reporter assay. This result is expressed as the mean of three independent experiments ± SD. *, $P < 0.01$.

Src or p38 inhibition failed to block IFN-γ–mediated GBP1 induction in U87-EGFR, U178-EGFR, and U373-EGFR cells. Consistent with these results, p38 inhibition abrogated EGF but not IFN-γ–induced GBP1 expression in GBM26 cells (Fig. 3 F). Collectively, these data demonstrate that in GBM cells, IFN-γ signaling–stimulated GBP1 expression is independent of the Src–p38 MAPK pathway, whereas the EGFR signaling–induced GBP1 expression requires it.

EGFR activates the GBP1 promoter through Src–p38 MAPK

The aforementioned results suggest that EGFR mediates GBP1 expression through Src–p38 MAPK. To further validate this, we cloned the GBP1 proximal promoter (237 bp; −218/19 bp) as described previously (Lew et al., 1991; Naschberger et al., 2004) and tested whether EGFR signaling is able to activate it. Upon EGF treatment, EGFR significantly stimulated GBP1 promoter activity in U87-EGFR cells (3.59-fold; $P < 0.01$), whereas EGF had no effect on GBP1 promoter activation in U87 parental cells (Fig. 3 G). Moreover, although p38 or Src inhibition prevented GBP1 promoter activation by EGF stimulation ($P < 0.01$; 3.59- vs. 1.41- or 1.21-fold), it failed to block its activation by IFN-γ (3.57- vs. 3.54- or 3.27-fold; Fig. 3 H). These data further confirm that EGFR–Src–p38 signaling up-regulates GBP1 expression in glioma cells at the transcriptional level.

Stat1 is not required for EGFR signaling–induced GBP1 expression but is necessary for IFN-γ–mediated GBP1 induction

Stat1 has previously been shown to be a transcription factor that mediates cytokine and growth factor–induced signals downstream of p38 MAPK (Wells, 1999; Battle and Frank, 2002). Indeed, we found that both EGF and IFN-γ strongly activated Stat1 phosphorylation at both Ser727 and Tyr701 in U87-EGFR cells. However, EGF but not IFN-γ led to p38 phosphorylation. Consistently, p38 inhibition blocked EGF-mediated but only had a partial effect on IFN-γ–mediated Stat1 phosphorylation at both phosphorylation sites (Fig. 4 A).

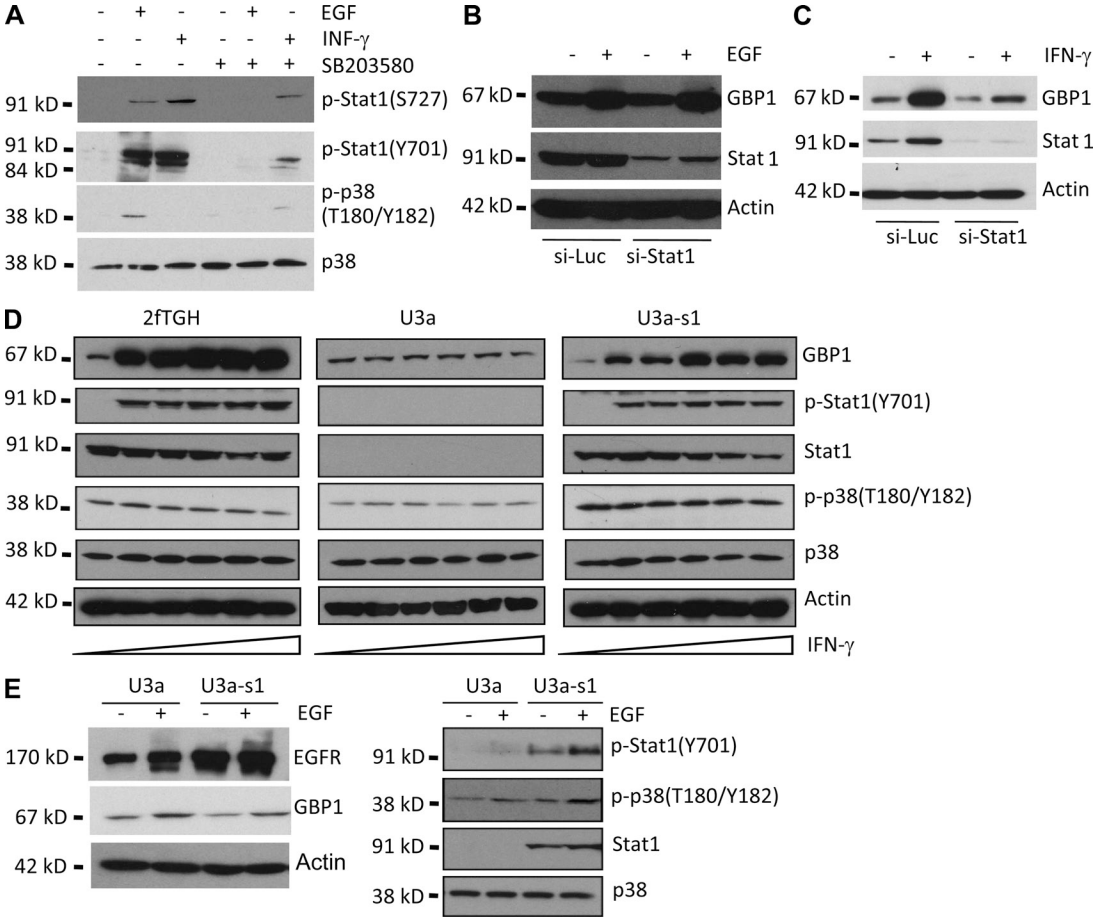

Figure 4. Stat1 is not required for EGFR-mediated GBP1 expression. (A) U87-EGFR cells were pretreated with 20 μM SB203580 for 1 h and then exposed to 100 ng/ml EGF or 100 ng/ml IFN-γ for 30 min before Western blot analysis. Total p38 is shown as a loading control. (B and C) U87-EGFR cells were transfected with Stat1 siRNA (si-stat1) or the control siRNA (si-Luc) before 20 ng/ml EGF (B) or 20 ng/ml IFN-γ treatment (C) for 24 h, and GBP1 expression was analyzed by Western blot. Data are representative of two independent experiments. (D) Stat1-null U3a cells are derived from parental 2fTGH cells. U3a-S1 cells are U3a cells reconstituted for Stat1. The cells were treated with 0, 5, 10, 20, 50, or 100 ng/ml IFN-γ for 24 h before Western blot analysis. (E) U3a and U3a-s1 cells transduced with EGFR were treated with 20 ng/ml EGF for 24 h before Western blot analysis. Data are representative of two independent experiments.

We next examined whether Stat1 is involved in EGFR signaling–mediated GBP1 induction. Although Stat1 knockdown dramatically attenuated IFN-γ–induced GBP1 expression in U87-EGFR cells, it had no effect on GBP1 induction by EGF stimulation (Fig. 4, B and C).

To further study the role of Stat1 in GBP1 induction, we also used the isogenic human fibrosarcoma cell lines 2fTGH, U3a (2fTGH-derived Stat1-null cell), and U3a-S1 (U3a reconstituted for Stat1). In the Stat1-deficient U3a cells, IFN-γ was unable to induce GBP1 expression, while being able to do so in parental 2fTGH and reconstituted U3a-S1 cells in which Stat1 was activated (Fig. 4 D). Interestingly, we observed that IFN-γ activated Stat1 in 2fTGH and U3a-f1 cells but failed to activate p38 phosphorylation in each of the three lines, which is consistent with the aforementioned results obtained in glioma cells (Fig. 4 D). As these three cell lines express a very low level of EGFR (Leaman et al., 1996), we stably expressed full-length human EGFR in U3a and U3a-S1 cells. In the Stat1-deficient U3a cells expressing EGFR, EGF was now able to induce GBP1 expression, whereas no further enhancement

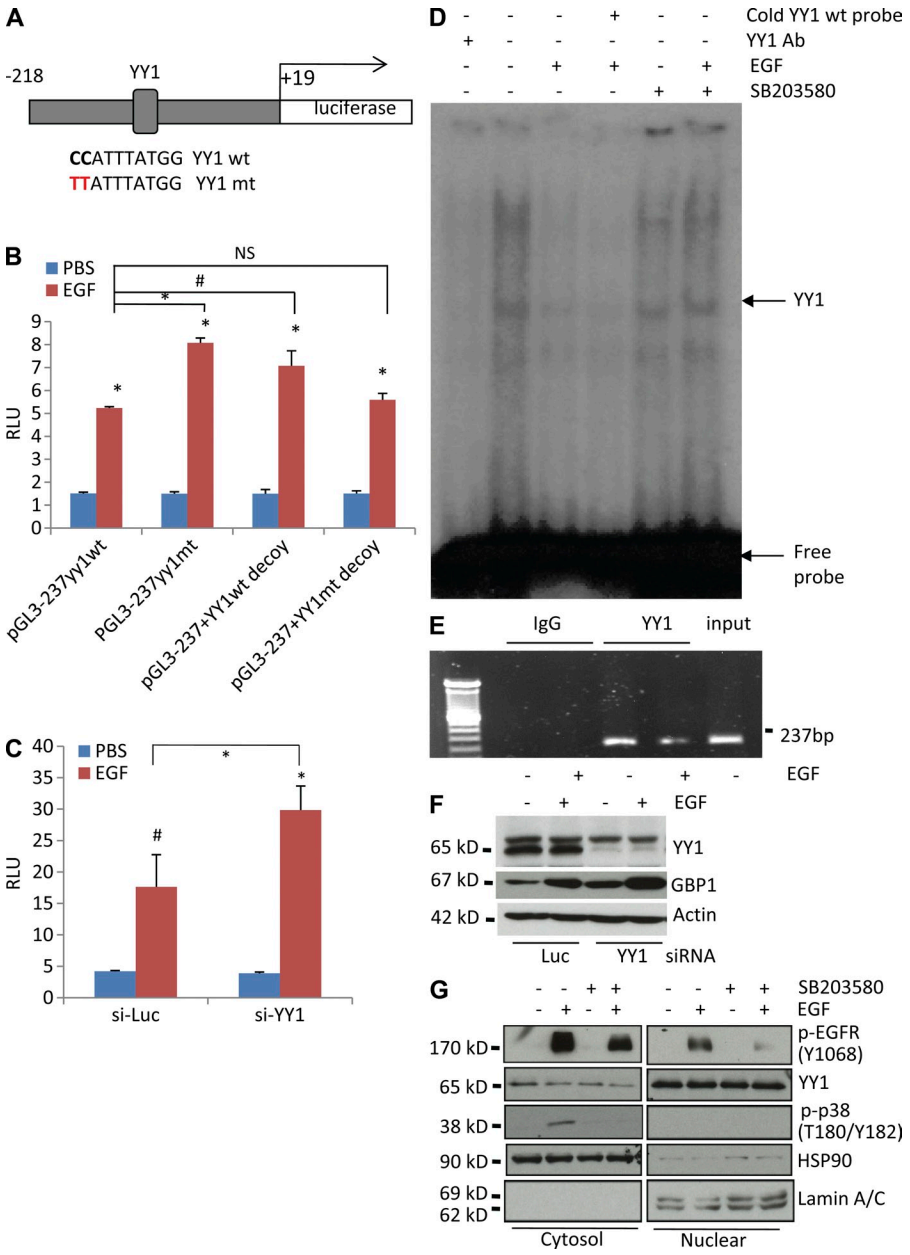

Figure 5. YY1 is involved in regulation of EGFR-mediated GBP1 expression. (A) Schematic representation of cis intact (WT) and mutant (mt) YY1 binding motif in the GBP1 proximal promoter. (B) U87-EGFR cells were transfected with the GBP1 wild-type promoter pGL3-237 and the internal control pRL-TK with or without 200-fold excess of YY1 wild-type or mutant decoy or the YY1 deactivated GBP1 promoter pGL3-237-yy1mt and pRL-TK for 24 h and then serum starved for 24 h before 20 ng/ml EGF treatment for 6 h. Firefly and Renilla luciferase activities were measured, and promoter activity was presented as the fold induction of RLU (values of firefly luciferase unit/values of Renilla luciferase unit) as compared with the control. This result is expressed as the mean of three independent experiments ± SD. #, $P < 0.05$; *, $P < 0.01$. (C) U87-EGFR cells were transfected with YY1 or Luc-specific siRNA for 24 h and then transfected with pGL3-237/pRL-TK for 24 h. The cells were serum starved for an additional 24 h before 20 ng/ml EGF or PBS treatment for 6 h. Promoter activity was presented as the fold induction of RLU as compared with the control. The result is presented as mean ± SD of three independent experiments. #, $P < 0.05$; *, $P < 0.01$. (D) EMSA analysis. Double-strand YY1 DNA probe was labeled with γ-[^{32}P]ATP and bound to the nuclear extracts of EGF- and/or SB203580-treated U87-EGFR cells with or without preincubation with a 100-fold excess of YY1 probe or YY1-specific antibody. (E) ChIP analysis of YY1 element from untreated and EGF-treated (100 ng/ml, 30 min) U87-EGFR cells using an antibody specific for YY1 or rabbit IgG control. Input chromatin is presented. PCR was performed to amplify the proximal GBP1 promoter (237 bp). This experiment was repeated twice, yielding identical results. (F) U87-EGFR cells were transfected with YY1 or control siRNA for 24 h and then serum starved for 24 h before 20 ng/ml EGF stimulation for 24 h. GBP1 and YY1 expression were analyzed by Western blot. Data are representative of at least two independent experiments. (G) After 24 h of serum starvation, U87-EGFR cells were treated with DMSO vehicle or 20 μM of p38 inhibitor SB203580 for 1 h followed by 100 ng/ml EGF treatment for 30 min before cell fractionation and Western blot analysis. Data are representative of two independent experiments.

was detected in the U3a-S1 cells. As expected, EGFR activation by EGF stimulated p38 phosphorylation in both cells and Stat1 phosphorylation only in U3a-s1 cells (Fig. 4 E). Collectively, these data strongly suggest that although Stat1 is essential for GBP1 induction by IFN-γ, it is dispensable for GBP1 induction by EGFR signaling.

YY1 is an important regulator for GBP1 induction by EGFR signaling

To identify the transcription factor that mediates GBP1 mRNA induction by EGFR signaling, we analyzed the GBP1 proximal promoter in silico using TFSEARCH software. In addition to a GAS (IFN-γ–activated sequence) element that binds to Stat1 (Lew et al., 1991; Naschberger et al., 2004), the proximal promoter of the GBP1 gene also contains a consensus YY1 motif CCATTT (CCATTTATGG; −167/−158 bp; Fig. 5 A). To examine whether YY1 is involved in GBP1 promoter activation by EGFR signaling, the YY1 binding site was mutated, resulting in an unexpected increase in GBP1 promoter activity compared with the intact wild-type GBP1 promoter in response to EGF stimulation in U87-EGFR cells (5.6- vs. 3.5-fold; $P < 0.01$; Fig. 5 B). Furthermore, decoy experiments were performed to delineate further the involvement of YY1 in EGF-induced enhancement of GBP1 gene transcription through the 237-bp promoter. Fig. 5 B demonstrated that the wild-type but not the mutant YY1 decoy oligonucleotides further increased EGF-induced GBP1 transcription (4.7- vs. 3.5-fold; $P < 0.05$; Fig. 5 B), implicating the YY1 motif as a potential repressor element. This notion was supported in experiments using siRNA knockdown of YY1 that demonstrated enhanced GBP1 promoter activity ($P < 0.01$; Fig. 5 C).

To test the binding capacity of the GBP1 promoter YY1 motif, electrophoretic mobility shift assays (EMSAs) were performed. Double-stranded oligonucleotides containing the YY1 motif (−176/−142 bp) were radiolabeled and used as probes of nuclear extracts from U87-EGFR cells with or without 100 ng/ml EGF treatment for 30 min as a source of YY1. Unlabeled 100-fold excess of YY1 probe was used as a specific competitor. As would be predicted from the foregoing, EGF stimulation decreased YY1 DNA binding activity in U87-EGFR cells (Fig. 5 D, third lane vs. second lane), and the specific band was completely blocked by unlabeled competitor (Fig. 5 D, fourth lane). Importantly, p38 inhibition blocked the DNA binding decrease induced by EGF (Fig. 5 D, sixth lane vs. third lane). To confirm that YY1 is indeed a component of the DNA–protein complex in EMSA, the effect of the anti-YY1–specific antibody on EMSA was tested. Although the DNA–protein complex was not super-shifted by the addition of specific antibody, the antibody disrupted the complex, suggesting that YY1 is indeed at least a component of the DNA binding component detected in the nuclear extracts (Fig. 5 D, first lane vs. second lane).

To substantiate the activity of YY1 in vivo, we performed a chromatin immunoprecipitation (ChIP) assay by using a specific antibody against YY1. DNA associated with these immunoprecipitates was then amplified by PCR with primers specific for the 237-bp proximal GBP1 promoter (−218/19 bp). As expected, no DNA fragments were detected when normal rabbit IgG was used (Fig. 5 E, first and second lanes). In contrast, DNA fragments with the expected size (237 bp) were detected using the anti-YY1 antibody in U87-EGFR cells (Fig. 5 E, third through fifth lanes). In agreement with the EMSA results, EGF stimulation decreased the amount of GBP1 promoter DNA immunoprecipitated with YY1 (Fig. 5 E, fourth lane vs. fifth lane).

To further verify the role of YY1 in GBP1 induction, we depleted YY1 by siRNA in U87-EGFR cells. As shown in Fig. 5 F, Western blot analysis demonstrated that knockdown of YY1 can increase both the basal GBP1 protein level and GBP1 induction by EGF in U87-EGFR cells. These findings suggested that YY1 functions as a negative regulator of GBP1 expression and that EGFR signaling acts to relieve this repression.

GBP1 is required for EGFR-mediated MMP1 expression in GBM

A previous study demonstrated that GBP1 inhibits endothelial cell invasion and tube formation by down-regulating MMP1 expression (Guenzi et al., 2003). This led us to analyze the expression profiles of GBP1, MMP1, and EGFR in a collection of GBM tumors and cell lines. Surprisingly, it appears that these three proteins were overexpressed in a positive correlation pattern in four of eight patients and 4 of 10 cell lines (U178, T98G, LN308, and A1207 cells; Fig. 6, A and B). To further validate the relationship between these three proteins, we compared their expression levels in U178-EGFR cells with or without EGF stimulation using a loss of function strategy because these cells express relatively high levels of both GBP1 and MMP1. We found that EGFR activation by EGF stimulated both GBP1 and MMP1 expression. In contrast, knockdown of GBP1 by siRNA largely blocked EGF-mediated MMP1 induction in U178-EGFR cells (Fig. 6 C, left). To examine how GBP1 controls MMP1 expression in glioma cells, we first determined whether EGFR-induced MMP1 expression in U178-EGFR was at the transcriptional or translational level. Pretreating these cells with AD or CHX completely blocked EGFR-mediated MMP1 mRNA expression (Fig. 6 C, right; $P < 0.01$), indicating that MMP1 induction by EGFR activation is at the transcriptional level and requires de novo protein synthesis. As shown in Fig. 6 E, the protein levels of basal and induced MMP1 were increased when the si-Luc–transfected U178-EGFR cells were posttreated with MG132, a specific proteasome inhibitor; however, MG132 was still able to increase MMP1 expression in the si-GBP1–transfected cells, suggesting that GBP1 had no effect on MMP1 stability. Additionally, we found that reduction of GBP1 by siRNA significantly decreased both the basal and the EGFR-induced MMP1 mRNA expression in U178-EGFR cells (Fig. 6 D, left and middle; $P < 0.001$). To confirm this, we cloned the human MMP1 promoter (2,942 bp) into the pGL3-basic vector

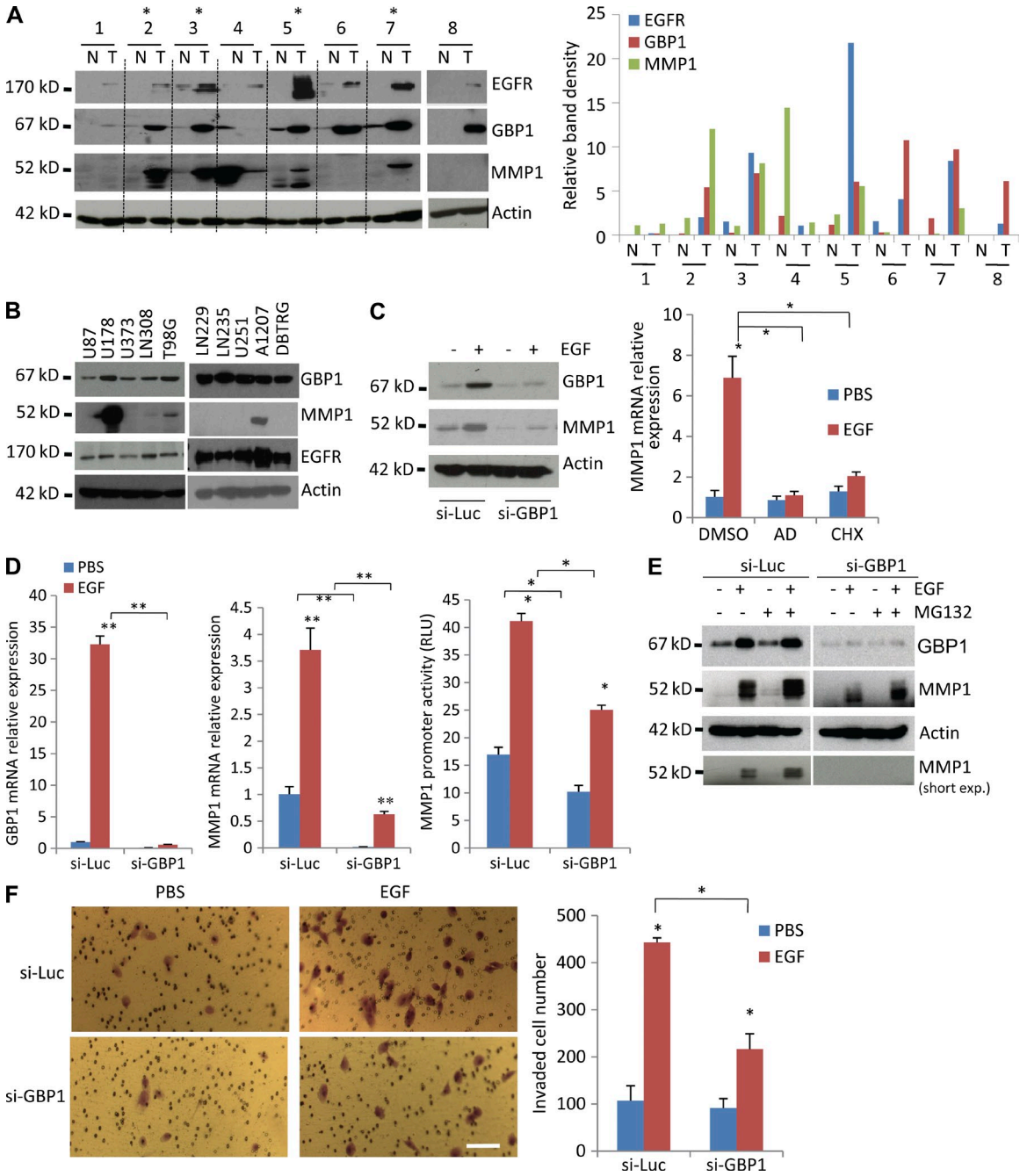

Figure 6. GBP1 is required for EGFR-mediated MMP1 expression and invasion. (A) Expression profiles of EGFR, GBP1, and MMP1 in GBM patient samples were analyzed by Western blot (left). The band density was analyzed by ImageJ software (National Institutes of Health; right). T and N denote tumor and normal brain, respectively. Asterisks denote coexpression of the three proteins in the indicated samples. (B) Western blot analysis of expression of EGFR, GBP1, and MMP1 in GBM cell line cultures. (C, left) U178-EGFR cells were transfected with GBP1 siRNA (si-GBP1) or control siRNA (si-Luc) for 24 h and then serum starved for 24 h before EGF stimulation for an additional 24 h. The expression of GBP1 and MMP1 was analyzed by Western blot. (right) RT-qPCR analysis of MMP1 expression. Serum-starved U178-EGFR cells were pretreated with AD or CHX for 1 h followed by 20 ng/ml EGF treatment for 6 h. *, $P < 0.01$. (D) RT-qPCR analysis of GBP1 (left) and MMP1 (middle) mRNA expression was performed in U87-EGFR cells transfected with si-Luc or si-GBP1 in the presence or absence of 20 ng/ml EGF for 6 h. Human MMP1 promoter activity was determined by cotransfecting pGL3-MMP1 (2,942 bp) and pRL-TK (internal control) with siRNA targeting GBP1 (si-GBP1) or luciferase control (si-Luc) into U87-EGFR cells with or without 20 ng/ml EGF treatment for 6 h. (right) Firefly and Renilla luciferase activities were measured, and promoter activity is presented as the fold induction of RLU as compared with the control. This result is expressed as the mean of three independent experiments ± SD. *, $P < 0.01$; **, $P < 0.001$. (E) U178-EGFR/si-Luc and U178-EGFR/si-GBP1 cells were treated with or without 20 ng/ml EGF for 24 h followed by 10 µM MG132 treatment for 6 h before Western blot analysis. (F) U178-EGFR/si-Luc and U178-EGFR/si-GBP1 cells were treated with or without 20 ng/ml EGF for 24 h followed by analysis of cell invasion using BioCoat Matrigel invasion chambers. *, $P < 0.05$. Data are representative of three independent experiments. Error bars represent SD. Bar, 100 µm.

(Armstrong et al., 2009). The reporter assay showed that knockdown of GBP1 dramatically reduced the basal and EGF-induced MMP1 promoter activation (Fig. 6 D, right; $P < 0.01$). Similar data were obtained in another glioma cell line, U87-EGFR, in which MMP1 was mainly secreted in the culture medium (not depicted). Collectively, these data demonstrate that GBP1 contributes to the basal and EGFR-mediated MMP1 transcription in glioma cells.

GBP1 enhances glioma cell invasion through MMP1 induction in vitro and in vivo

Given the important role that MMP1 plays in glioma progression and invasion and its correlation with GBP1 expression (Stojic et al., 2008), we examined the potential role of GBP1 in glioma cell invasion. We first examined whether GBP1 is involved in EGF-mediated glioma cell invasion. Knockdown of GBP1 significantly inhibited EGF-mediated U178-EGFR and U87-EGFR cell invasion through extracellular matrix (Fig. 6 F and not depicted). Conversely, overexpression of GBP1 in A1207 cells dramatically increased MMP1 expression at the transcriptional level (Fig. 7, A and B) and enhanced cell invasiveness (Fig. 7 D), whereas in contrast, siRNA knockdown of MMP1 abolished GBP1-mediated cell invasion (Fig. 7, C and D). Together, these data suggest that GBP1 is involved in EGFR-mediated glioma cell invasion through modulation of MMP1 expression.

GBP1 is a member of a large GTPase family (Prakash et al., 2000a,b; Guenzi et al., 2001). To assess whether the GTPase activity of GBP1 is involved in MMP1 induction, we created two different dominant-negative mutants of GBP1, D184N, and R48P, which exhibit no detectable GTP binding or hydrolysis (Guenzi et al., 2003; Praefcke et al., 2004). As shown in Fig. 7 (E and F), both the wild-type and GTPase mutant alleles of GBP1 increased MMP1 protein expression and protease activity. Similarly, we observed that overexpression of GBP1 and these two mutant alleles also increased MMP1 expression in U178 cell lines (not depicted). Next, we characterized the role of the individual domains of GBP1 in MMP1 induction. We cloned the truncated N-terminal globular domain (Glo; 1–290 aa), which harbors GTPase activity, and the C-terminal helical domain (Hel; 288–592 aa) of GBP1 into the pBABE-puro vector to allow for retroviral expression (Fig. 7 G). We found that the C-terminal helical domain but not the globular domain could induce MMP1 induction (Fig. 7 H). Furthermore, we observed that the helical domain alone could induce cell invasiveness (Fig. 7 I), which further suggests that the GTPase activity of GBP1 is not required for MMP1 expression and glioma cell invasion.

The invasive behavior of glioma cells in vitro may not completely reflect their infiltrative phenotype in the brain because of the differences in extracellular components (Bellail et al., 2004). To determine whether the in vitro effects we observed extended to the in vivo situation, we targeted GBP1 expression (and GFP as a control) by lentiviral short hairpin RNA (shRNA) transduction in the invasive glioblastoma cell line, SNB19 (Fig. 8 A). Because SNB19 cells express a relatively low level of EGFR, EGF is unable to induce GBP1 expression (which needs a high level of EGFR in glioma cells, as shown in Fig. 3 G). In contrast, EGF is able to induce MMP1 expression that is mainly localized in the conditioned medium (Fig. 8 A). Importantly, knockdown of GBP1 significantly reduced both the basal and the EGF-induced MMP1 expression at both mRNA and protein level (Fig. 8, A and B), similar to our results obtained with U87 and U178 EGFR-overexpressing cell lines. Consistently, we showed that GBP1 reduction by siRNA dramatically inhibited the human MMP1 basal promoter activity and EGF-stimulated promoter activation (Fig. 8 B, right).

We then stereotactically implanted SNB19-shGFP and SNB19-shGBP1 cells into the brains of immunocompromised mice. Microscopic analysis of brain sections showed that the mice implanted with control SNB19-shGFP cells developed diffuse tumors, whereas mice implanted with SNB19-shGBP1 cells developed circumscribed tumors (Fig. 8 C). The control SNB19-shGFP tumor-bearing mice revealed focal and invasive lesions, often in periventricular regions of the brain, as expected for this highly invasive GBM cell line. In contrast, SNB19-shGBP1 cells formed tumors with smooth tumor–parenchyma interfaces with an associated decrease in glioma cell infiltration and associated infiltrative tumor masses (Fig. 8, C and E). In addition, consistent with in vitro results showing that GBP1 mediates MMP1 expression in glioma cells (Fig. 8 A), we observed a dramatic decrease in GBP1 and MMP1 expression, which are mainly localized in the cytoplasm of the cancer cells, in SNB19-shGBP1 tumor tissues (Fig. 8 C). Interestingly, large perivascular infiltrations were observed in the SNB19-shGFP but not the SNB19-shGBP1 tumor-bearing mice (Fig. 8 D). The functional specificity of GBP1 action in tumor cell invasion is underscored by the minimal effects it had on cellular proliferation, apoptosis, and angiogenesis in vivo (Fig. 9 A), consistent with results showing that deregulation of GBP1 had no effect on glioma cell proliferation in vitro (Fig. 9 B). These results strongly support the conclusion that GBP1 is specifically involved in glioblastoma invasion both in vitro and in vivo.

DISCUSSION

A previous study has shown that GBP1 is the most abundant protein induced by IFNs, with IFN-γ being the most effective (Cheng et al., 1983). In this study, we show for the first time that EGFR can also induce GBP1 expression in GBM cell lines through an Src–p38 MAPK–YY1 signaling pathway. Significantly, we found that GBP1 is coexpressed with EGFR in GBMs and that GBP1 could increase GBM cell invasion by modulating MMP1 expression through its C-terminal helical domain in a GTPase-independent manner. This ability to enhance GBM cell invasion establishes GBP1 as a novel target for anti-GBM invasion therapy. In contrast to our finding of Src–p38 MAPK induction of GBP1 in glioma cells, this pathway is not required for IFN-γ–induced

GBP1 expression, as IFN-γ was unable to activate p38, a result which is consistent with a previous finding that cell stress, such as bacterial LPS, UV irradiation, and TNF, causes activation of p38 MAPK–dependent target gene transcription, whereas p38 inhibition does not affect IFN-γ–mediated transcription (Kovarik et al., 1999).

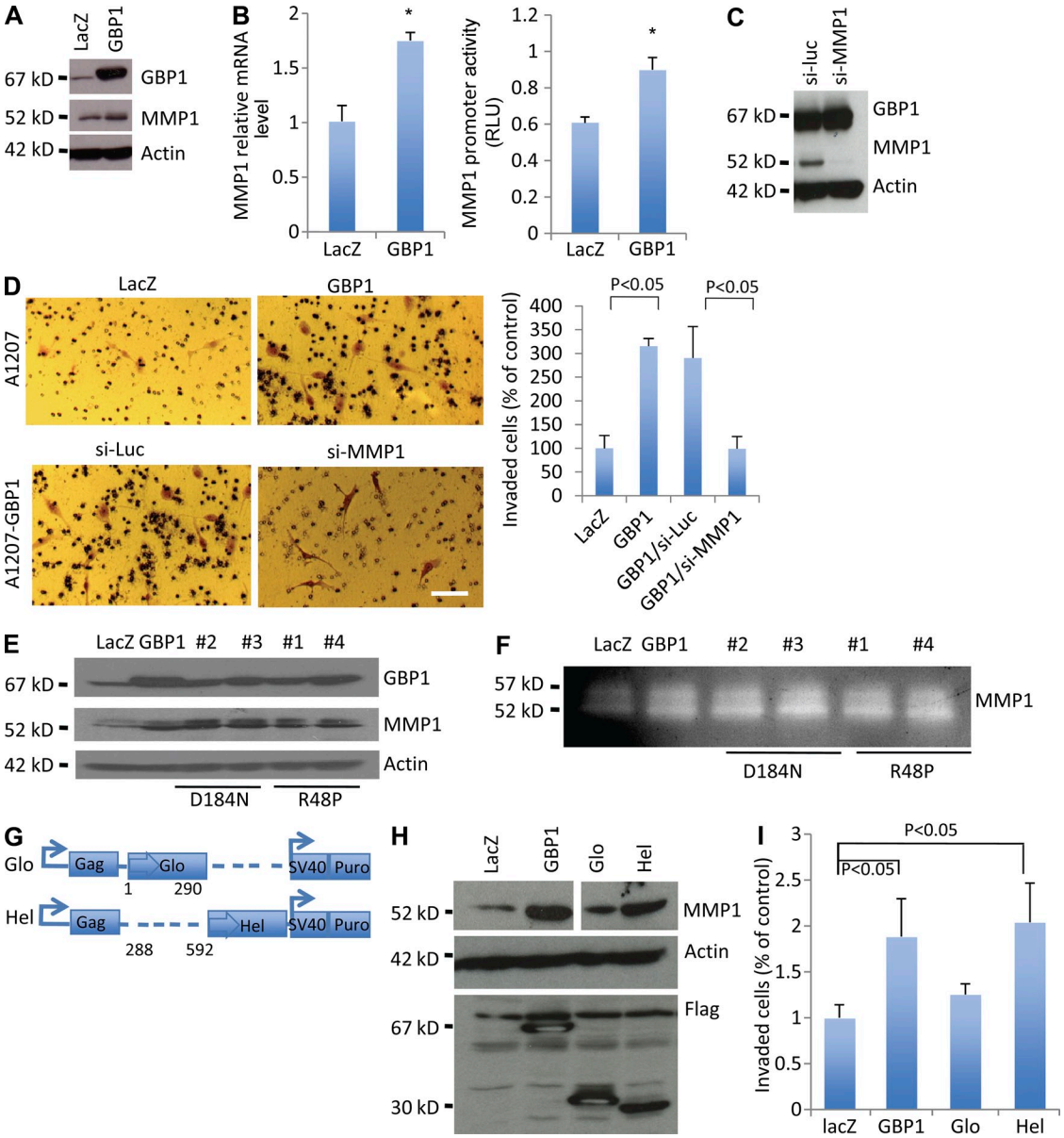

Figure 7. The helical domain of GBP1 is essential for MMP1 induction and GBM cell invasion. (A) The expression of the protein was analyzed by Western blot in A1207-LacZ and A1207-GBP1 cells. (B) RT-qPCR analysis of MMP1 mRNA expression was performed in A1207-LacZ and A1207-GBP1 cells (left). Reporter assays were performed to analyze the effect of GBP1 expression on MMP1 promoter activation in the A1207 cells (right). The cells were transfected with pGL3-MMP1 (2,942 bp) and pRL-TK for 48 h. Firefly and Renilla luciferase activities were measured, and promoter activity is presented as the fold induction of RLU as compared with the control. This result is expressed as the mean of three independent experiments ± SD. *, $P < 0.01$. (C) The expression of the protein was analyzed by Western blot in siRNA-Luc (control)– and siRNA-MMP1–transfected A1207-GBP1 cells. (D) Cell invasion was analyzed by BioCoat Matrigel invasion chambers. A1207-lacZ, A1207-GBP1, A1207-GBP1/siRNA-Luc, or A1207-GBP1/siRNA-MMP1 cells were plated at a density of 2.0×10^4 per insert. Medium with 10% FBS was in the lower chamber as a chemoattractant. After 22 h, invasive cells on the lower surface were counted after fixing and staining. Shown are representative data of at least two independent experiments. Bar, 100 µm. (E) The expression of MMP1, GBP1, and dominant-negative mutants (D184N and R48P) of GBP1 in A1207 cells was analyzed by Western blot. (F) Zymography analysis of the MMP1 activity in A1207 cells expressing GBP1 or its mutants D184N and R48P. (G) Schematic representation of the retroviral expression vector pBABE-puro encoding individual domains of GBP1: N-terminal globular domain (Glo-GBP1; Glo) and C-terminal helical domain (Hel-GBP1; Hel). The numbers refer to the positions of the amino acids in GBP1. (H) Effect of Flag-tagged GBP1 and truncated Glo and Hel on the expression of MMP1 in A1207 cells were analyzed by Western blot. (I) Quantitative results for the invasive ability of A1207 cells expressing GBP1 or two different truncated domains Glo and Hel as assessed by BioCoat Matrigel invasion chambers as described in D. Data are representative of three independent experiments. Error bars represent SD.

Both EGF and IFN-γ are able to cause phosphorylation of Stat1 at both Tyr701 and Ser727 for its full activation. Phosphorylation of Stat1 at Tyr701 induces Stat1 dimerization, nuclear translocation, and DNA binding (Ihle et al., 1994), whereas Stat1 Ser727 phosphorylation strongly increases its transactivation activity (Wen et al., 1995). Although EGF and IFN-γ caused phosphorylation of Stat1 at both sites, only EGF activated p38 phosphorylation. Consistent with this, p38 inhibition blocked EGF- but not IFN-γ–stimulated Stat1 phosphorylation and GBP1 promoter activity and expression as well. This is in agreement with earlier findings that p38 activity is dispensable for the IFN-γ–induced Stat1 phosphorylation (Kovarik et al., 1999; Ramsauer et al., 2002). In addition, it has been reported that LPS-, UV irradiation–, or TNF stress–stimulated Stat1 Ser727 phosphorylation occurred through a signaling pathway sensitive to the p38 MAPK inhibitor SB203580, whereas IFN-γ–mediated Stat1 Ser727 phosphorylation was not inhibited by this inhibitor (Kovarik et al., 1999). Therefore, we speculate that EGFR may use a signaling pathway similar to that used under stress to induce GBP1 expression.

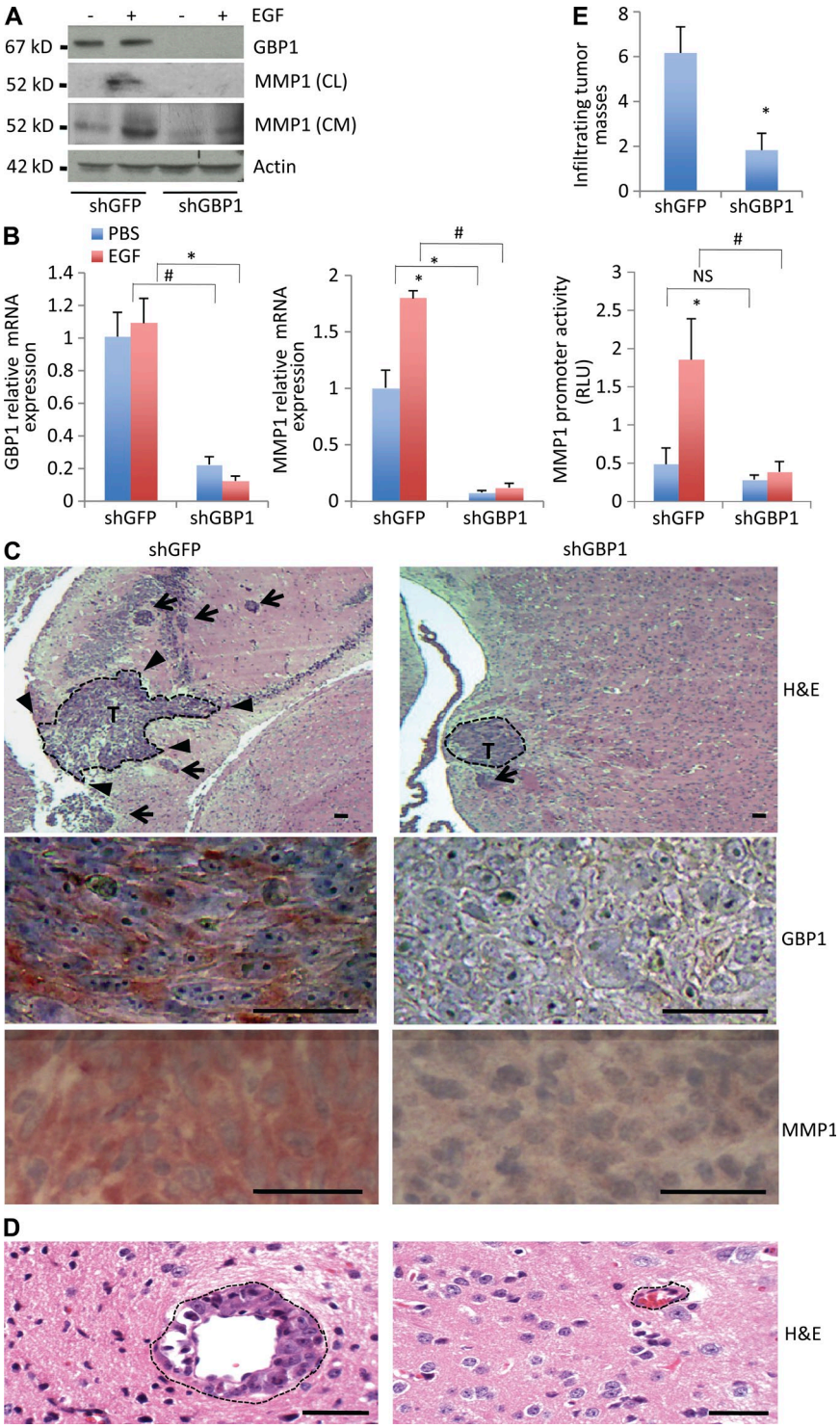

Figure 8. Targeting GBP1 inhibits glioblastoma invasion in vivo. (A) Western blot analysis of MMP1 and GBP1 in cell lysates (CL) and of MMP1 in conditioned medium (CM) of SNB19-shGFP and -shGBP1 cells with or without 20 ng/ml EGF stimulation for 24 h. (B) RT-qPCR analysis of GBP1 (left) and MMP1 (middle) mRNA expression in SNB19-shGFP and SNB19–shGBP1 cells with or without PBS or 20 ng/ml EGF stimulation for 6 h. (right) The human MMP1 promoter activity was determined by transfecting with pGL3-MMP1 (2,942 bp) and pRL-TK (internal control) in the shRNA-transfected SNB19 cells with or without PBS or 20 ng/ml EGF treatment for 6 h. Firefly and Renilla luciferase activities were measured, and promoter activity is presented as the fold induction of RLU as compared with the control. This result is expressed as the mean of three independent experiments ± SD. *, $P < 0.05$; #, $P < 0.01$. (C) H&E, GBP1, and MMP1 staining of brain sections on day 20 after intracranial inoculation of SNB19-shGFP (left) or SNB19-shGBP1 (right; 1×10^6 cells/mouse). Shown are representative brain slices from tumor-bearing mice. Tumor margins are delineated using a dotted line. Arrowheads denote invasive extensions from tumor mass (T). Arrows indicate invasive tumor cells and disseminated tumor clusters away from the tumor mass. The animal experiments were performed two independent times with 10 mice per group with similar results. (D) Representative image showing perivascular infiltrations in SNB19-shGFP (left) but not SNB19-shGBP1 (right) tumor-bearing mice. Bars, 50 µm. (E) Quantification of the infiltrating tumor masses observed in SNB19-shGFP and SNB19-shGBP1 tumor-bearing mice on day 20 ($n = 10$; *, $P < 0.05$). Data are representative of two independent experiments. Error bars represent SD.

We identified YY1, a ubiquitous and dual-functional GLI-Krüppel zinc finger transcription factor, as a potential transcriptional repressor for GBP1 induction in GBM cells. Depending on cell type–specific factors and its relative concentration, the promoter sequences surrounding the YY1 binding sites, and its cellular environment, YY1 can function as a transcriptional activator or repressor (Yao et al., 2001). The present study showed that EGFR signaling attenuated YY1 binding to the GBP1 promoter mediated by p38 MAPK. We also showed by cell fractionation that EGF stimulation has no effect on YY1 translocation with or without p38 inhibition (Fig. 5 G). It is possible that EGFR–Src–p38 signaling may affect YY1 activation or its interaction with other transcriptional factors, which in turn affects the transcriptional activity of the GBP1 promoter. In agreement with this possibility, a recent study has demonstrated that TNF-activated p38α kinase promotes the interaction between YY1 and PRC2, leading to the formation of repressive chromatin on the Pax7 promoter (Palacios et al., 2010).

Given that EGFR kinase activity can up-regulate GBP1 expression in vitro, we compared the expression profile of EGFR and GBP1 in a panel of GBM tumor samples and cell lines. Together with TCGA analysis, the data suggest that both EGFR and GBP1 are highly expressed and show the strongest correlation with the neural subclass of GBM, which is in agreement with the idea that EGFR amplification is one of the signatures for this subclass (Verhaak et al., 2010). Our results are also consistent with a recent study showing that GBP1 is overexpressed in all gliomas with EGFR amplification (Ducray et al., 2008). Interestingly, we observed that EGFR, GBP1, and MMP1 expression appears to be positively correlated with each other in GBM patients and cell lines. More importantly, GBP1 is required for EGFR-mediated MMP1 transcription, and GBP1 mediates MMP1 expression at the transcriptional level in glioma cells. Thus, this may explain why these proteins display such concordance of expression in GBM.

Overexpression of EGFR has been shown to promote glioma cell motility and invasion (Pedersen et al., 2004; Cai et al., 2005), and the Src and p38 pathway have been implicated in glioma invasion previously (Park et al., 2002; Angers-Loustau et al., 2004; Demuth et al., 2007; Lu et al., 2009). However, the basis for initiation and maintenance of the aberrant motility is still not known. Numerous studies suggest that EGFR activation causes MMP1 expression, which is associated with invasion and metastasis of cancer cells (Nutt and Lunec, 1996; Nutt et al., 1998; Itoh et al., 2006; Cury et al., 2007). As GBP1 can also be induced by inflammatory cytokines and down-regulates MMP1 expression in endothelial cells (Guenzi et al., 2001, 2003), we investigated the functional relationship between these three proteins in GBM cell lines. We found that GBP1 is induced by the EGFR–Src–p38 cascade and is sufficient for EGFR-mediated MMP1 expression and cell invasion. Moreover, overexpression of GBP1 alone enhanced GBM cell invasion by up-regulating MMP1. Our finding that in GBM cells GBP1 acts as a positive regulator of MMP1

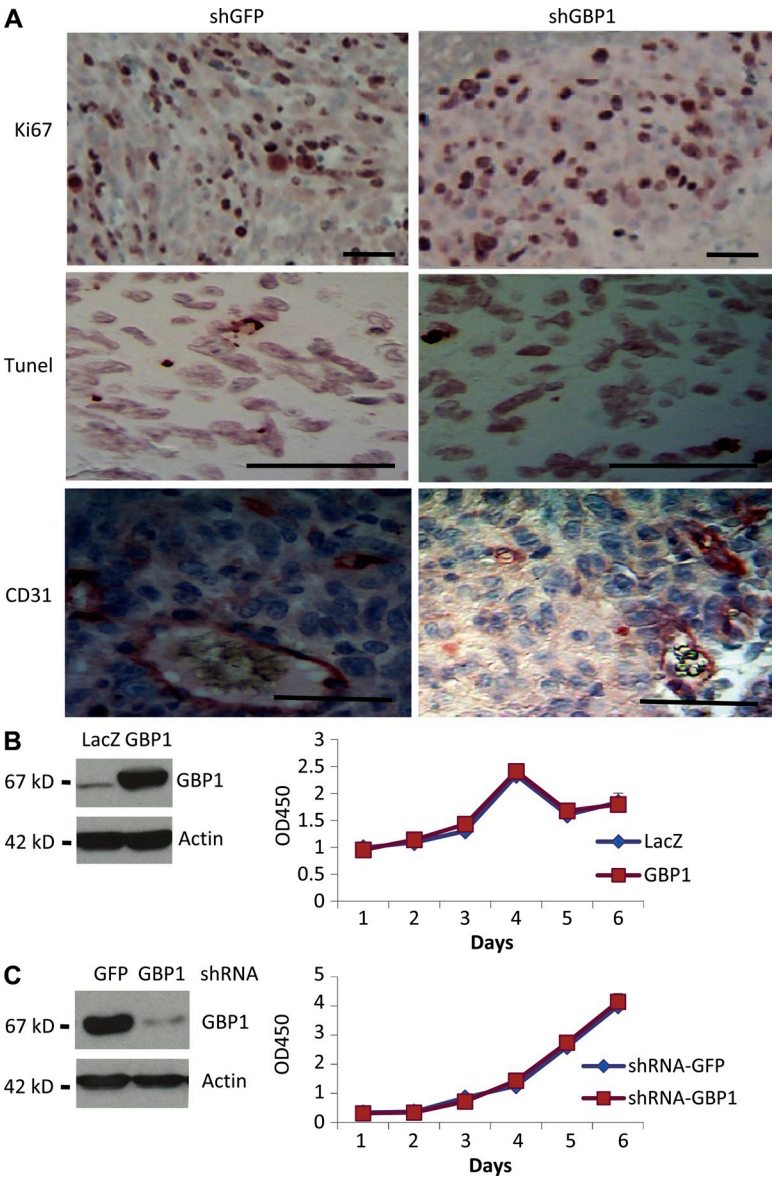

Figure 9. Effect of GBP1 on glioblastoma cell growth in vivo and in vitro. (A) Shown is the representative Ki67, Tunel, and CD31 staining image of the tumor mass of SNB19-shGFP (left) and SNB19-shGBP1 (right) on day 20 after intracranial inoculation. Data are representative of two independent experiments. Bars, 50 µm. (B and C) WST-1 assay was performed to examine the effect of overexpression of GBP1 on A1207 cell proliferation (B) and the effect of knockdown of GBP1 by shRNA on U178 cell proliferation (C). Data are representative of three independent experiments. Error bars represent SD.

expression instead of MMP2 and MMP14 while in endothelial cells it acts in the opposite fashion (Guenzi et al., 2003; unpublished data) is particularly intriguing. It is possible that this switch in function is caused by additional molecular aberrations specific to the tumor cells. Alternatively, this difference may be tissue specific and caused by the differential expression of transcription factor networks in the brain versus the endothelium or by differences between the normal and transformed state of the cell. We are actively investigating these possibilities.

In summary, this study demonstrates that GBP1 expression in GBM cells is up-regulated by EGFR through a unique p38 MAPK–dependent relief of transcriptional repression mediated by YY1. In addition, GBP1 is required for EGFR-induced MMP1 expression and glioma cell invasion. Our identification of a novel signaling pathway controlling glioma invasion may have significant clinical implications because it uncovers several key molecules, the targeting of which may serve to inhibit glioma invasion, one of the key obstacles to achieving effective management of this disease.

MATERIALS AND METHODS

Reagents and plasmids. Pan Src inhibitor PP2, dasatinib, p38 inhibitor SB203580, Mek1/2 inhibitors U0126 and PD98059, JNK1/2 inhibitor SP600125, PI3K inhibitor LY294002, EGFR inhibitor AG1478, and MG132 were obtained from EMD. N-terminal Flag-tagged GBP1 and its mutants GBP1 D184N, R48P, Glo, and Hel were cloned as described previously (Guenzi et al., 2001) and inserted into retrovirus vector pBABE-puro. pLKO.1-shRNA-GFP and -GBP1 constructs were obtained through the RNAi Consortium shRNA Library (Broad Institute of Massachusetts Institute of Technology and Harvard). The human GBP1 promoter 237 bp (−218/19 bp) was cloned by PCR using specific primers sense (5′-AGCTTCTGGTTGAGAAATCTTTAAACC-3′) and antisense (5′-TGGCTTCTAGCACTTCTGTGTCTCTC-3′) and inserted into the firefly luciferase vector, pGL3 basic (Promega). To generate a construct with mutation of the YY1-binding elements, the QuikChange site-directed mutagenesis kit (Agilent Technologies) was used to change the potential YY1 binding motif from CCATTT to TTATTT.

Cell culture and gene transfection. All GBM cell lines were cultured in DME with 10% FBS. SNB19 cells were a gift from W. Debinski (Wake Forest University, Winston-Salem, NC). GBM xenografts procedures have previously been described (Sarkaria et al., 2007). GBM26 xenograft cells were cultured as nonadherent neurospheres in neural stem cell medium to maintain EGFR expression. 2fTGH, U3a, and U3a-s1 cells were provide by G. Stark (Cleveland Clinic Foundation Research Institute, Cleveland, OH) and cultured in DME/10% FBS medium.

Virus production and infection. To produce retrovirus, 293T cells were transfected with pBABE-puro–LacZ, GBP1, or its mutants together with pCL10A1 using Lipofectamine 2000 (Invitrogen). To produce lentiviruses, 293FT cells were cotransfected by pLKO.1-shRNA-GFP (5′-CAAGCTGACCCTGAAGTTCAT-3′) or -GBP1 (5′-CGGAAATTCTTCCCAAAGAAA-3′) with pCMVDR8.91 and pMD.G-VSV-G using Lipofectamine 2000. Viral supernatants were harvested and filtered (0.45 μm) at 48 and 72 h after transfection. Glioma cells were infected overnight in the presence of 8 μg/ml polybrene and then selected for 5 d in growth medium containing 1 μg/ml puromycin. The stable clones were verified by Western blot.

Clinical samples. GBM samples, provided by R. Nishikawa (Saitama Medical University, Hidaka-shi, Saitama, Japan), were obtained at the time of surgery after written informed consent. Frozen tumor samples were ground in liquid nitrogen and lysed in RIPA buffer for Western blot analysis. Total RNA was isolated from frozen tumor samples using the RNeasy kit (QIAGEN) according to the manufacturer's instructions.

Microarray probe preparation and Affymetrix GeneChip hybridization. U87 and U178 cells engineered to express EGFR were serum starved for 24 h and stimulated with or without 20 ng/ml of recombinant EGF for 3 h before sample collection. The experiment was replicated twice for each U87 and U178 cells for microarray analyses. Transcriptional profiling was performed on human HG-U133A arrays (Affymetrix), which contain 22,283 probe sets representing ~14,500 human genes and expressed sequence tags, according to the manufacturer's protocol. Biotin-labeled cRNA was synthesized from 10-μg aliquots of total RNA from each sample, and hybridization, washing, and detection of signals were performed. Microarray Analysis Suite 5.0 software (Affymetrix) was used to calculate and compare the gene expression levels. Microarray data were deposited in GEO DataSets under accession no. GSE33442.

Western blots and antibodies. Cells were lysed in RIPA buffer (150 mM NaCl/1.0% Triton X-100/0.5% Na deoxycholate/0.1% SDS/50 mM Tris, pH 8.0/complete protease inhibitor; Roche). The conditioned media was collected and centrifuged at 1,000 *g* to remove cell debris. The supernatant was concentrated using Amicon centrifugal filters (Millipore). Primary antibodies used were anti-YY1 (c20), anti-p38 (Santa Cruz Biotechnology, Inc.), anti-EGFR (c13; BD), anti–p-EGFR (Y1068), anti-Stat1, anti–p-Stat1 (Y701), anti-Hsp27, anti–p-Hsp27 (S82), anti–β-actin (Cell Signaling Technology), anti–p-Stat1 (S727; Biosource International, Inc.), anti-GBP1 (MBL International), and anti-MMP1 (R&D Systems).

RT-PCR and real-time qPCR. Total RNA was harvested by TRIZOL reagent (Invitrogen) and reverse transcribed (SuperScript II First Strand kit; Invitrogen). Semiquantitative RT-PCR conditions were as follows: 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C for 26 cycles. The primer pairs for GBP1 were sense, 5′-TGGAACGTGTGAAAGCTGAG-3′; and antisense, 5′-TGACAGGAAGGCTCTGGTCT-3′; for EGFR were sense, 5′-GAGAGGAGAACTGCCAGAA-3′; and antisense, 5′-GTAGCATTTATGGAGAGTG-3′; and for GAPDH were sense, 5′-TGCCTCCTGCACCACCAACT-3′; and antisense, 5′-CCCGTTCAGCTCAGGGATGA-3′.

qPCR was performed with 2 μl of diluted cDNA on an iCycler IQ using IQ Syber Green (Bio-Rad Laboratories) according to the manufacturer's instructions. All reactions were performed in duplicate and repeated at least three times. Relative quantification was performed for each sample and normalized with GAPDH or β-actin expression for comparison. Primers used for real-time PCR were EGFR (104 bp): sense, 5′-TTTGCCAAGGCACGAGTAACA-3′; and antisense, 5′-ATTCCCAAGGACCACCTCACA-3′; GBP1 (197 bp): sense, 5′-AACGACAGGGTCCAGTTGCTGAAAG-3′; and antisense, 5′-TAGGGGTGACAGGAAGGCTCTGG-3′; GAPDH (131 bp): sense, 5′-CCACATGGCCTCCAAGGAGTAAGAC-3′; and antisense, 5′-AGGAGGGGAGATTCAGTGTGGTGGG-3′; β-actin (141 bp): sense, 5′-AGAAGGAGATCACTGCCCTGGCACC-3′; and antisense, 5′-CCTGCTTGCTGATCCACATCTGCTG-3′; and MMP1 (234 bp): sense, 5′-ATGCTGAAACCCTGAAGGTG-3′; and antisense, 5′-CTGCTTGACCCTCAGAGACC-3′.

Nuclear protein extraction. Cells were resuspended in buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A), lysed with 0.625% Nonidet P-40, and centrifuged at 3,000 rpm for 5 min at 4°C. The supernatant was collected and used as the cytoplasmic extracts. The nuclei pellet was washed twice with buffer A and resuspended in 40 μl buffer B (20 mM Hepes, pH 7.9, containing 1.5 mM $MgCl_2$, 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A) and agitated for 60 min at 4°C, and the nuclear debris was spun down at 20,000 *g* for 15 min. The supernatant (nuclear extract) was collected and stored at −80°C until ready for analysis.

EMSA. Double-strand DNA probes (−175/−142 bp of GBP1 promoter) were labeled with 5 μCi γ-[^{32}P]ATP (PerkinElmer) using T4 polynucleotide kinase (Promega). The labeled oligonucleotides were purified from the free γ-[^{32}P]ATP using a quick spin column (Roche) according to the manufacturer's instructions. For EMSA, total reaction mixtures containing 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 50 mM NaCl, 4% glycerol, and 50 μg poly(dI-dC)-poly(dI-C)/ml were incubated with 10 μg of nuclear extracts and unlabeled competing oligonucleotides for 10 min at room temperature, followed by the addition of 1 μl (0.5–2 × 10^5 cpm) of ^{32}P-end–labeled oligonucleotides. Reactions were separated on 6% nondenaturing polyacrylamide gels that were dried and subjected to autoradiography.

Reporter assays. U87-EGFR cells were cotransfected with pGL3-237 or pGL3-237yy1mt with the Renilla luciferase plasmid pRL-TK (Promega). 24 h after transfection, the cells were starved in serum-free medium for 24 h and then were stimulated with 20 ng/ml EGF or 20 ng/ml IFN-γ for 6 h. To further validate the role of YY1 in regulating GBP1 promoter activity, 200-fold excess of the double-stranded YY1 decoy (forward strand, 5′-CCACTAATTCCATTTATGGTAGTGGAGTCAGTG-3′) or mutant YY1 control decoy (forward strand with mutation underlined, 5′-CCACTAATTTTATTTATGGTAGTGGAGTCAGTG-3′) was cotransfected with pGL3-237 and pRL-TK (Promega) in U87-EGFR cells followed by serum starvation and EGF treatment. The Dual-Luciferase Reporter Assay (Promega) was performed according to the manufacturer's instructions, and values were read on a GENios Pro (Tecan). To study the role of GBP1 on MMP1 induction, EGFR-overexpressing U87 and U178 cells and SNB19 or A1207 cells were transfected with pGL3-MMP1 (2,942 bp; a gift of M. Vincenti, Dartmouth Medical School, Hanover, NH; Armstrong et al., 2009), with or without EGF stimulation.

ChIP. The assay was modified from the chip-on-chip assay described by Kim et al. (2007). In brief, cells were treated with 1% formaldehyde to cross-link proteins to DNA. The cell pellets were resuspended in lysis buffer and sonicated to yield a mean DNA size of 500 bp. Sonicated extracts were subsequently clarified by centrifugation and diluted with ChIP dilution buffer. 20 μl of the diluted lysates was left as the input control. Other lysates were precleared with sheep anti–rabbit Dynal beads (Invitrogen) and then divided into two fractions and incubated with 1 μg of normal rabbit IgG or rabbit anti-YY1 antibody each and rotated overnight at 4°C. After thoroughly washing, the cross-linked DNA–protein complexes were reversed by heating at 65°C. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Recovery of the DNA from the GBP1 promoter regions was determined by PCR using the same specific primers for GBP1 promoter cloning.

siRNA transfection. The GBP1 siRNA (sense strand; siRNA, 5′-GAAUGAGGUUGAGGAUUCATT-3′) and siRNA targeting luciferase (5′-ACAUCACGUACGCGGAAUACUUCGA-3′) were synthesized by Integrated DNA Technologies, Inc. siRNAs against p38, YY1, or MMP1 were obtained from Santa Cruz Biotechnology, Inc. The cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Cell invasion assay. The invasion assay was performed as described by Li et al. (2006) by using BioCoat Matrigel invasion chambers (BD). In brief, cells were plated at a density of 2.0 × 10^4 per insert. Medium with 10% FBS was in the lower chamber as a chemoattractant. After 22 h, cells on the upper surface of the membrane were removed. Invasive cells on the lower surface were fixed with 100% methanol and stained with 1% crystal violet (Sigma-Aldrich) before counting under an inverted microscope (Leica). In all experiments, data were collected from triplicate chambers.

Zymography assay. To measure MMP1 proteolytic activity, conditioned media was assayed according to the Novex Zymogram Gels (Casein) protocol (Invitrogen). In brief, after electrophoresis, the gel was incubated in renaturing buffer with gentle agitation. The gel was subsequently equilibrated and developed in developing buffer and stained in SimplyBlue SafeStain (Invitrogen).

Intracranial xenograft model and histological analysis. 1 × 10^6 of SNB19-shGFP or -shGBP1 cells in 5 μl PBS were injected intracranially into 4–5-wk-old athymic nude mice using a guide screw system as described by Lal et al. (2000). After 14–20 d, mice were euthanized, and their brains were removed and embedded in paraffin. All animal experiments conformed to ethical principles and guidelines approved by the University of California, San Diego Institutional Animal Care and Use Committee. Brain tissues were sectioned and stained with CD31 (Abcam), Ki67 (Abcam), Tunel (Abcam), GBP1 1B1 (MBL International), MMP1 Ab-6 (Thermo Fisher Scientific), and hematoxylin and eosin (H&E) staining (University of California, San Diego Histology core).

Statistical analysis. Correlation analysis between GBP1 and EGFR expression in human glioma samples was analyzed for significance using Prism 5.0 software (GraphPad Software) with Pearson r test, where $P < 0.05$ was considered statistically significant. For other experiments, results are expressed as the mean ± SD. Statistical analyses were performed by Student's *t* test. $P < 0.05$ was considered statistically significant.

We thank Dr. Nissi Varki for expert histology advice and Drs. George Stark, Waldemar Debinski, and Matthew Vincenti for gifts of reagents. We are grateful to Drs. Tim Fenton, German Gomez, and Ciro Zanca for critical reading and discussion of this manuscript.

This work was supported by an award from the Goldhirsh Foundation (to F. Furnari) and National Institutes of Health grant P01-CA95616 (to W. Cavenee, F. Furnari, and L. Chin). W. Cavenee is a fellow of the National Foundation for Cancer Research. M. Li is partially supported by an American Brain Tumor Association Fellowship in honor of Richard Schaffner.

The authors have no conflicting financial interests.

Submitted: 31 May 2011
Accepted: 14 November 2011

REFERENCES

Angers-Loustau, A., R. Hering, T.E. Werbowetski, D.R. Kaplan, and R.F. Del Maestro. 2004. SRC regulates actin dynamics and invasion of malignant glial cells in three dimensions. *Mol. Cancer Res.* 2:595–605.

Armstrong, D.A., L.N. Phelps, and M.P. Vincenti. 2009. CCAAT enhancer binding protein-beta regulates matrix metalloproteinase-1 expression in interleukin-1beta-stimulated A549 lung carcinoma cells. *Mol. Cancer Res.* 7:1517–1524. http://dx.doi.org/10.1158/1541-7786.MCR-09-0082

Battle, T.E., and D.A. Frank. 2002. The role of STATs in apoptosis. *Curr. Mol. Med.* 2:381–392. http://dx.doi.org/10.2174/1566524023362456

Bellail, A.C., S.B. Hunter, D.J. Brat, C. Tan, and E.G. Van Meir. 2004. Microregional extracellular matrix heterogeneity in brain modulates glioma cell invasion. *Int. J. Biochem. Cell Biol.* 36:1046–1069. http://dx.doi.org/10.1016/j.biocel.2004.01.013

Cai, X.M., B.B. Tao, L.Y. Wang, Y.L. Liang, J.W. Jin, Y. Yang, Y.L. Hu, and X.L. Zha. 2005. Protein phosphatase activity of PTEN inhibited the invasion of glioma cells with epidermal growth factor receptor mutation type III expression. *Int. J. Cancer.* 117:905–912. http://dx.doi.org/10.1002/ijc.21251

Cancer Genome Atlas Research Network. 2008. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* 455:1061–1068. http://dx.doi.org/10.1038/nature07385

Cheng, Y.S., R.J. Colonno, and F.H. Yin. 1983. Interferon induction of fibroblast proteins with guanylate binding activity. *J. Biol. Chem.* 258:7746–7750.

Cury, P.R., V.C. de Araújo, F. Canavez, C. Furuse, K.R. Leite, and N.S. de Araújo. 2007. The effect of epidermal growth factor on matrix metalloproteinases and tissue inhibitors of metalloproteinase gene expression in cultured human gingival fibroblasts. *Arch. Oral Biol.* 52:585–590. http://dx.doi.org/10.1016/j.archoralbio.2006.11.006

Demuth, T., L.B. Reavie, J.L. Rennert, M. Nakada, S. Nakada, D.B. Hoelzinger, C.E. Beaudry, A.N. Henrichs, E.M. Anderson, and M.E.

Berens. 2007. MAP-ing glioma invasion: mitogen-activated protein kinase kinase 3 and p38 drive glioma invasion and progression and predict patient survival. *Mol. Cancer Ther.* 6:1212–1222. http://dx.doi.org/10.1158/1535-7163.MCT-06-0711

Duan, Z., R. Foster, K.A. Brakora, R.Z. Yusuf, and M.V. Seiden. 2006. GBP1 overexpression is associated with a paclitaxel resistance phenotype. *Cancer Chemother. Pharmacol.* 57:25–33. http://dx.doi.org/10.1007/s00280-005-0026-3

Ducray, F., A. Idbaih, A. de Reyniès, I. Bièche, J. Thillet, K. Mokhtari, S. Lair, Y. Marie, S. Paris, M. Vidaud, et al. 2008. Anaplastic oligodendrogliomas with 1p19q codeletion have a proneural gene expression profile. *Mol. Cancer.* 7:41. http://dx.doi.org/10.1186/1476-4598-7-41

Furnari, F.B., T. Fenton, R.M. Bachoo, A. Mukasa, J.M. Stommel, A. Stegh, W.C. Hahn, K.L. Ligon, D.N. Louis, C. Brennan, et al. 2007. Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev.* 21:2683–2710. http://dx.doi.org/10.1101/gad.1596707

Guenzi, E., K. Töpolt, E. Cornali, C. Lubeseder-Martellato, A. Jörg, K. Matzen, C. Zietz, E. Kremmer, F. Nappi, M. Schwemmle, et al. 2001. The helical domain of GBP-1 mediates the inhibition of endothelial cell proliferation by inflammatory cytokines. *EMBO J.* 20:5568–5577. http://dx.doi.org/10.1093/emboj/20.20.5568

Guenzi, E., K. Töpolt, C. Lubeseder-Martellato, A. Jörg, E. Naschberger, R. Benelli, A. Albini, and M. Stürzl. 2003. The guanylate binding protein-1 GTPase controls the invasive and angiogenic capability of endothelial cells through inhibition of MMP-1 expression. *EMBO J.* 22:3772–3782. http://dx.doi.org/10.1093/emboj/cdg382

Huang, H.S., M. Nagane, C.K. Klingbeil, H. Lin, R. Nishikawa, X.D. Ji, C.M. Huang, G.N. Gill, H.S. Wiley, and W.K. Cavenee. 1997. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J. Biol. Chem.* 272:2927–2935. http://dx.doi.org/10.1074/jbc.272.5.2927

Ihle, J.N., B.A. Witthuhn, F.W. Quelle, K. Yamamoto, W.E. Thierfelder, B. Kreider, and O. Silvennoinen. 1994. Signaling by the cytokine receptor superfamily: JAKs and STATs. *Trends Biochem. Sci.* 19:222–227. http://dx.doi.org/10.1016/0968-0004(94)90026-4

Ishizawar, R., and S.J. Parsons. 2004. c-Src and cooperating partners in human cancer. *Cancer Cell.* 6:209–214. http://dx.doi.org/10.1016/j.ccr.2004.09.001

Itoh, M., T. Murata, T. Suzuki, M. Shindoh, K. Nakajima, K. Imai, and K. Yoshida. 2006. Requirement of STAT3 activation for maximal collagenase-1 (MMP-1) induction by epidermal growth factor and malignant characteristics in T24 bladder cancer cells. *Oncogene.* 25:1195–1204. http://dx.doi.org/10.1038/sj.onc.1209149

Kim, T.H., L.O. Barrera, and B. Ren. 2007. ChIP-chip for genome-wide analysis of protein binding in mammalian cells. *Curr. Protoc. Mol. Biol.* Chapter 21:Unit 21.13.

Kovarik, P., D. Stoiber, P.A. Eyers, R. Menghini, A. Neininger, M. Gaestel, P. Cohen, and T. Decker. 1999. Stress-induced phosphorylation of STAT1 at Ser727 requires p38 mitogen-activated protein kinase whereas IFN-gamma uses a different signaling pathway. *Proc. Natl. Acad. Sci. USA.* 96:13956–13961. http://dx.doi.org/10.1073/pnas.96.24.13956

Lal, S., M. Lacroix, P. Tofilon, G.N. Fuller, R. Sawaya, and F.F. Lang. 2000. An implantable guide-screw system for brain tumor studies in small animals. *J. Neurosurg.* 92:326–333. http://dx.doi.org/10.3171/jns.2000.92.2.0326

Leaman, D.W., S. Pisharody, T.W. Flickinger, M.A. Commane, J. Schlessinger, I.M. Kerr, D.E. Levy, and G.R. Stark. 1996. Roles of JAKs in activation of STATs and stimulation of c-fos gene expression by epidermal growth factor. *Mol. Cell. Biol.* 16:369–375.

Lew, D.J., T. Decker, I. Strehlow, and J.E. Darnell. 1991. Overlapping elements in the guanylate-binding protein gene promoter mediate transcriptional induction by α and γ interferons. *Mol. Cell. Biol.* 11:182–191.

Li, M., S.S. Ng, J. Wang, L. Lai, S.Y. Leung, M. Franco, Y. Peng, M.L. He, H.F. Kung, and M.C. Lin. 2006. EFA6A enhances glioma cell invasion through ADP ribosylation factor 6/extracellular signal-regulated kinase signaling. *Cancer Res.* 66:1583–1590. http://dx.doi.org/10.1158/0008-5472.CAN-05-2424

Libermann, T.A., H.R. Nusbaum, N. Razon, R. Kris, I. Lax, H. Soreq, N. Whittle, M.D. Waterfield, A. Ullrich, and J. Schlessinger. 1985. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature.* 313:144–147. http://dx.doi.org/10.1038/313144a0

Lieskovska, J., Y. Ling, J. Badley-Clarke, and D.R. Clemmons. 2006. The role of Src kinase in insulin-like growth factor-dependent mitogenic signaling in vascular smooth muscle cells. *J. Biol. Chem.* 281:25041–25053. http://dx.doi.org/10.1074/jbc.M602866200

Lu, K.V., S. Zhu, A. Cvrljevic, T.T. Huang, S. Sarkaria, D. Ahkavan, J. Dang, E.B. Dinca, S.B. Plaisier, I. Oderberg, et al. 2009. Fyn and SRC are effectors of oncogenic epidermal growth factor receptor signaling in glioblastoma patients. *Cancer Res.* 69:6889–6898. http://dx.doi.org/10.1158/0008-5472.CAN-09-0347

Lubeseder-Martellato, C., E. Guenzi, A. Jörg, K. Töpolt, E. Naschberger, E. Kremmer, C. Zietz, E. Tschachler, P. Hutzler, M. Schwemmle, et al. 2002. Guanylate-binding protein-1 expression is selectively induced by inflammatory cytokines and is an activation marker of endothelial cells during inflammatory diseases. *Am. J. Pathol.* 161:1749–1759. http://dx.doi.org/10.1016/S0002-9440(10)64452-5

Nagane, M., F. Coufal, H. Lin, O. Bögler, W.K. Cavenee, and H.J. Huang. 1996. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. *Cancer Res.* 56:5079–5086.

Narita, Y., M. Nagane, K. Mishima, H.J. Huang, F.B. Furnari, and W.K. Cavenee. 2002. Mutant epidermal growth factor receptor signaling down-regulates p27 through activation of the phosphatidylinositol 3-kinase/Akt pathway in glioblastomas. *Cancer Res.* 62:6764–6769.

Naschberger, E., T. Werner, A.B. Vicente, E. Guenzi, K. Töpolt, R. Leubert, C. Lubeseder-Martellato, P.J. Nelson, and M. Stürzl. 2004. Nuclear factor-kappaB motif and interferon-alpha-stimulated response element co-operate in the activation of guanylate-binding protein-1 expression by inflammatory cytokines in endothelial cells. *Biochem. J.* 379:409–420. http://dx.doi.org/10.1042/BJ20031873

Nishikawa, R., X.D. Ji, R.C. Harmon, C.S. Lazar, G.N. Gill, W.K. Cavenee, and H.J. Huang. 1994. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc. Natl. Acad. Sci. USA.* 91:7727–7731. http://dx.doi.org/10.1073/pnas.91.16.7727

Nutt, J.E., and J. Lunec. 1996. Induction of metalloproteinase (MMP1) expression by epidermal growth factor (EGF) receptor stimulation and serum deprivation in human breast tumour cells. *Eur. J. Cancer.* 32:2127–2135. http://dx.doi.org/10.1016/S0959-8049(96)00261-4

Nutt, J.E., J.K. Mellon, K. Qureshi, and J. Lunec. 1998. Matrix metalloproteinase-1 is induced by epidermal growth factor in human bladder tumour cell lines and is detectable in urine of patients with bladder tumours. *Br. J. Cancer.* 78:215–220. http://dx.doi.org/10.1038/bjc.1998.467

Palacios, D., C. Mozzetta, S. Consalvi, G. Caretti, V. Saccone, V. Proserpio, V.E. Marquez, S. Valente, A. Mai, S.V. Forcales, et al. 2010. TNF/p38α/polycomb signaling to Pax7 locus in satellite cells links inflammation to the epigenetic control of muscle regeneration. *Cell Stem Cell.* 7:455–469. http://dx.doi.org/10.1016/j.stem.2010.08.013

Park, M.J., I.C. Park, J.H. Hur, M.S. Kim, H.C. Lee, S.H. Woo, K.H. Lee, C.H. Rhee, S.I. Hong, and S.H. Lee. 2002. Modulation of phorbol ester-induced regulation of matrix metalloproteinases and tissue inhibitors of metalloproteinases by SB203580, a specific inhibitor of p38 mitogen-activated protein kinase. *J. Neurosurg.* 97:112–118. http://dx.doi.org/10.3171/jns.2002.97.1.0112

Pedersen, M.W., V. Tkach, N. Pedersen, V. Berezin, and H.S. Poulsen. 2004. Expression of a naturally occurring constitutively active variant of the epidermal growth factor receptor in mouse fibroblasts increases motility. *Int. J. Cancer.* 108:643–653. http://dx.doi.org/10.1002/ijc.11566

Praefcke, G.J., S. Kloep, U. Benscheid, H. Lilie, B. Prakash, and C. Herrmann. 2004. Identification of residues in the human guanylate-binding protein 1 critical for nucleotide binding and cooperative GTP hydrolysis. *J. Mol. Biol.* 344:257–269. http://dx.doi.org/10.1016/j.jmb.2004.09.026

Prakash, B., G.J. Praefcke, L. Renault, A. Wittinghofer, and C. Herrmann. 2000a. Structure of human guanylate-binding protein 1 representing a unique class of GTP-binding proteins. *Nature.* 403:567–571. http://dx.doi.org/10.1038/35000617

Prakash, B., L. Renault, G.J. Praefcke, C. Herrmann, and A. Wittinghofer. 2000b. Triphosphate structure of guanylate-binding protein 1 and implications for nucleotide binding and GTPase mechanism. *EMBO J.* 19:4555–4564. http://dx.doi.org/10.1093/emboj/19.17.4555

Ramsauer, K., I. Sadzak, A. Porras, A. Pilz, A.R. Nebreda, T. Decker, and P. Kovarik. 2002. p38 MAPK enhances STAT1-dependent transcription independently of Ser-727 phosphorylation. *Proc. Natl. Acad. Sci. USA.* 99:12859–12864. http://dx.doi.org/10.1073/pnas.192264999

Rhodes, D.R., J. Yu, K. Shanker, N. Deshpande, R. Varambally, D. Ghosh, T. Barrette, A. Pandey, and A.M. Chinnaiyan. 2004. ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia.* 6:1–6.

Sarkaria, J.N., L. Yang, P.T. Grogan, G.J. Kitange, B.L. Carlson, M.A. Schroeder, E. Galanis, C. Giannini, W. Wu, E.B. Dinca, and C.D. James. 2007. Identification of molecular characteristics correlated with glioblastoma sensitivity to EGFR kinase inhibition through use of an intracranial xenograft test panel. *Mol. Cancer Ther.* 6:1167–1174. http://dx.doi.org/10.1158/1535-7163.MCT-06-0691

Stojic, J., C. Hagemann, S. Haas, C. Herbold, S. Kühnel, S. Gerngras, W. Roggendorf, K. Roosen, and G.H. Vince. 2008. Expression of matrix metalloproteinases MMP-1, MMP-11 and MMP-19 is correlated with the WHO-grading of human malignant gliomas. *Neurosci. Res.* 60:40–49. http://dx.doi.org/10.1016/j.neures.2007.09.009

Stupp, R., W.P. Mason, M.J. van den Bent, M. Weller, B. Fisher, M.J. Taphoorn, K. Belanger, A.A. Brandes, C. Marosi, U. Bogdahn, et al; European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group. 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N. Engl. J. Med.* 352:987–996. http://dx.doi.org/10.1056/NEJMoa043330

Summy, J.M., J.G. Trevino, C.H. Baker, and G.E. Gallick. 2005. c-Src regulates constitutive and EGF-mediated VEGF expression in pancreatic tumor cells through activation of phosphatidyl inositol-3 kinase and p38 MAPK. *Pancreas.* 31:263–274. http://dx.doi.org/10.1097/01.mpa.0000178280.50534.0c

Sun, L., A.M. Hui, Q. Su, A. Vortmeyer, Y. Kotliarov, S. Pastorino, A. Passaniti, J. Menon, J. Walling, R. Bailey, et al. 2006. Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain. *Cancer Cell.* 9:287–300. http://dx.doi.org/10.1016/j.ccr.2006.03.003

Verhaak, R.G., K.A. Hoadley, E. Purdom, V. Wang, Y. Qi, M.D. Wilkerson, C.R. Miller, L. Ding, T. Golub, J.P. Mesirov, et al; Cancer Genome Atlas Research Network. 2010. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell.* 17:98–110. http://dx.doi.org/10.1016/j.ccr.2009.12.020

Wells, A. 1999. EGF receptor. *Int. J. Biochem. Cell Biol.* 31:637–643. http://dx.doi.org/10.1016/S1357-2725(99)00015-1

Wen, Z., Z. Zhong, and J.E. Darnell Jr. 1995. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell.* 82:241–250. http://dx.doi.org/10.1016/0092-8674(95)90311-9

Wong, A.J., S.H. Bigner, D.D. Bigner, K.W. Kinzler, S.R. Hamilton, and B. Vogelstein. 1987. Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. *Proc. Natl. Acad. Sci. USA.* 84:6899–6903. http://dx.doi.org/10.1073/pnas.84.19.6899

Wong, A.J., J.M. Ruppert, S.H. Bigner, C.H. Grzeschik, P.A. Humphrey, D.S. Bigner, and B. Vogelstein. 1992. Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc. Natl. Acad. Sci. USA.* 89:2965–2969. http://dx.doi.org/10.1073/pnas.89.7.2965

Yao, Y.L., W.M. Yang, and E. Seto. 2001. Regulation of transcription factor YY1 by acetylation and deacetylation. *Mol. Cell. Biol.* 21:5979–5991. http://dx.doi.org/10.1128/MCB.21.17.5979-5991.2001